Water Pollution Studies

Pete Cadmus
Aquatic Research Scientist

Margaret J. Spangler
Colorado Parks and Wildlife Technician
Alexander S. Townsend
Colorado State University, MS Candidate

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

Alexandria Austermann, Librarian

Prepared by: _____
Pete Cadmus, Aquatic Research Scientist

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

Date: 18th of December, 2020

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 and Wildlife policy by the Director or the Wildlife Commission.

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Need and Objective

Prior to mining and westward expansion, Colorado had pristine headwaters supporting dense and mature trout populations. By the 1900s most of Colorado's headwater rivers could not support fish due to mining pollution. For this reason, Colorado was the first state in the nation to adopt water quality standards to protect aquatic life, preceding the United States Environmental Protection Agency (USEPA) by a decade. The research conducted by the then "Colorado Game & Fish" became the backbone of Colorado surface water standards and later became a majority of data used in many national standards in the late 1970s. Additionally, water chemistry assessments and laboratory experiments informed management decisions, determined what age classes could be stocked below mines, and what mine "clean-up" was most needed to improve fisheries. It was Colorado Parks and Wildlife (CPW)'s heavy metal research and Colorado Department of Public Health and Environment (CDPHE)'s regulations that converted rivers deemed "dead" by managers into Gold Metal Trout Streams such as the Animas River (below Durango) and the Arkansas River (between Leadville and Salida). This research and service to managers continues to this day.

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 USEPA and 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 solicitations 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.

Functions of the CPW Aquatic Toxicology Laboratory have historically included:

1- Assess toxicity of emerging contaminants pertinent to Colorado surface waters by conducting toxicity trials on fish, aquatic macroinvertebrates, algae and other fish forage species.

2- Improve state and national water quality standards to ensure they are protective of the aquatic life of 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 triennial reevaluation of toxicant standards by USEPA and CDPHE. Experiments should:

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

b) When possible expose rare or sensitive taxa, not laboratory cultured organisms. Expose for environmentally relevant durations, not only 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), consider phenology, species interaction, multi-generational effects, and exposure regimes unique to Colorado.

c) Consider ecologically relevant sublethal endpoints as technology and infrastructure become available to CPW aquatic toxicology laboratory (*e.g.* predator avoidance, olfactory function, fecundity, thermal tolerance, apoptosis, protein carbonyl content, histopathology, blood chemistry).

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

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

f) Consider multiple stressors simultaneously, not limited to interactions between numerous toxicants, interactions between toxicants and

temperature or interactions between toxicants and disease (*e.g.* whirling disease).

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

Water quality characteristics and pollution effect fish health and the viability of Colorado's 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 local fisheries. Efforts to restore Colorado's endangered fish species often require precise use of piscicides 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 topics that concern CPW's fish and wildlife. By collaborating with these researchers and agencies and by sharing equipment/infrastructure, these projects often produce better data that is more useful to CPW's mission. Technical support conducted by the CPW Aquatic Toxicology Laboratory includes, but is not limited to:

1- 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.

2- Assist in the investigation of fish kills.

3- Conduct short or long term experiments to produce toxicity data, or develop site-specific field studies to address local management decisions or local site-specific variances, when such data in the literature are lacking or inadequate.

4- Collect and analyze water and/or fish tissues to assess water quality problems as requested.

5- Analyze rotenone (and other piscicides) in water samples as part of Colorado Parks and Wildlife reclamation projects.

6- Publish and review results of experiments and water quality investigations in peer-reviewed journals for consideration in policy making by other agencies.

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Overview of 2019-2020 activities

Research

● Effects of Zn on *Paratanitarsus – Paratanitarsus sp.* is a small chironomid ubiquitous across the United States. USEPA experiments from the 1970s with this organism suggested this species was sensitive at low levels of Zinc (Zn). Results from the USEPA trials were excluded from several site specific standards in Colorado for being "insufficient duration of exposure" or less than 30 d. CPW's Aquatic Toxicology Laboratory cultured this insect and in 2019 conducted the first of several long term (4-9 months) experiments using *Paratanitarsus*. Emergence rates and photo analysis were used to estimate populations of larvae at many points in time to determine the optimal duration for future experiments. Preserved samples are being enumerated during the fall and winter of 2020-2021, so statistical analysis is pending. (Note: *Paratanitarsus* is referred to as the taxonomic tribe "Tanitarsini" in previous CPW reports)

● Effects of Cu and herbicides on algal colonization and competition - After extensive fabrication and method development the first multispecies algal colonization trial was conducted. This trial demonstrated that trace levels of copper prevent colonization of diatoms, the algae group that is most palatable and nutritious to fish. Also, green and diatom species of algae outcompeted cyanobacteria in control and low (1.3 µg/l) copper levels but in higher treatments (2.5-20 µg/l) this natural deterrence of toxic algal blooms was lost. (see "Algal colonization rates and community structure changes in the presence of trace levels of copper" below)

● Dietary exposure of insecticides to algivorous fish - Efforts to characterize dietary exposure of chlorpyrifos to eastern plains fish species were imperfect and this experiment was abandoned at the start of COVID-19 closure. CPW's organic instrumentation (HPLC) was unable to provide a low enough detection limit for chlorpyrifos. Private sector laboratories in Colorado also had poor (overly high) detection limits for this analyte. Dietary exposure of algivorous species was limited in static renewal systems, which could not maintain the target level of toxicant as would be observed in nature. A flow through system with water treatment will be devised.

● Selenium trophic transfer model - Final report of sampling methods and raw data was compiled by Pete Cadmus and Alexander Townsend and submitted to CDPHE (sole funder). A trophic transfer model was calculated for Colorado sample sites and compared to USEPA's model. Currently CPW, CDPHE, and Colorado State University (CSU) are analyzing these data for trends explaining accumulation or toxic effects. (see "Selenium concentrations and trophic transfer rates for Brown Trout (*Salmo trutta*) and White Sucker (*Catostomus commersonii*) populations within Colorado" below)

● Petroleum hydrocarbon assessments - As part of a series of diesel fuel and petroleum hydrocarbon studies initiated after the West Creek tanker spill settlement, we examined sub-acute (24-hr) exposure of the threatened Common Plains Minnow to diesel fuel at sublethal levels. Plains minnow were then assigned to tanks with predators. In nineteen of twenty trials the predator consumed the exposed prey preferentially to the control prey. (See "Sub-acute (24 hr) exposure to sub-lethal levels of diesel fuel decrease predator avoidance in Common Plains Minnow (*Hybognathus placitus)*" below)

● During laboratory closures and reduced staffing associated with COVID-19 restrictions, aquatic benthic macroinvertebrate samples from numerous field studies were quantitatively subsampled or enumerated.

● During laboratory closures and reduced staffing associated with COVID-19 restrictions, CPW Aquatic Toxicology Laboratory staff began combining River Watch records, fish stocking records and fish sampling records. Quantile regression analysis exploring water chemistries limiting fish survival will be conducted in the near future.

● During laboratory closures and reduced staffing associated with COVID-19 restrictions, CPW Aquatic Toxicology Laboratory staff attended to maintenance that is not possible when life support of fish must be maintained. Fume hoods and emergency showers were serviced, glassware was acid-washed and reorganized, and storage spaces were reorganized. The hard-water well was serviced and descaled. Laboratory electrical systems were modernized to allow better use of emergency generator capacity and to increase worker safety in wet environments.

● The manuscript "Before-After Control-Impact field surveys and novel experimental approaches provide valuable insights for characterizing stream recovery from acid mine drainage" (describing biomonitoring and experiments on the North Fork of Clear Creek) was submitted for peer review and is currently in the first round of revisions.

●"Morbidity and mortality in *Danio rerio* and *Pimephales promelas* exposed to antilipidemic drug mixtures (fibrates and statins) during embryogenesis: Comprehensive assessment via ante and post mortem endpoints" was accepted in the journal Chemosphere.

● Four additional manuscripts were written and are being prepared for submission with colleagues and collaborators internal and external to CPW.

Technical Support

● The CPW Aquatic Toxicology Laboratory conducted on-site assessment of rotenone during chemical reclamation projects to restore Native Cutthroat Trout habitat. This included but was

not limited to: George-Cornelius Creek Reclamation Project (Red Feather, CO), Rock Creek-Black Canyon Reclamation project (Jefferson, CO), Hermosa Creek (Purgatory, CO) and Beaver Creek Reclamation Project (Powderhorn, CO).

● Milt extender was produced for federal and state natural resource management agencies across the country.

● CPW Aquatic Toxicology Laboratory provided advising and support for Colorado River Watch, a non-profit that provides Colorado Parks and Wildlife and other state and federal agencies water quality monitoring and instrumentation.

● CPW Aquatic Toxicology Laboratory collaborated with Colorado School of Mines and Colorado State University on field experiments and long-term biomonitoring of algae, aquatic macroinvertebrates and fish at mine restoration efforts in the North Fork of Clear Creek (Blackhawk, CO). This drainage was once void of life and now has aquatic insects and fish occupying reaches downstream of historical mine activity.
● Ecotoxicological support and expertise was provided to CPW managers, Colorado universities, and natural resource management agencies as requested.

● CPW Aquatic Toxicology Laboratory staff peer reviewed internal and externally published scientific literature.

● CPW Aquatic Toxicology Laboratory staff repeatedly provided expert opinions and problem solving for CPW managers and Colorado municipalities concerned about fish health, habitat, and management practices.

On-going Projects – Activities scheduled in the early 2020-2021 fiscal year.
● Effects of chloride, sulfate, and ammonia on a suite of fish species has already begun and is being supported by CSU professors and students. These studies will include dissolved oxygen tolerance, thermal tolerance, competition, behavior and other endpoints pioneered at the CPW Aquatic Toxicology Laboratory and will make use of the markedly improved detection limits of Colorado River Watch.

● Quantitative subsampling, enumeration, and identification of aquatic macroinvertebrate samples from 2015 to 2020 will be completed. These include many from field experiments that simulated flood events before and after the North Fork of Clear Creek (Blackhawk, CO) mine effluent treatment facility was built. This field study and an in-stream fish cage experiment (2016) will be published as predictive tools for prioritizing and predicting effects of mine restoration efforts.

● If COVID-19 work restrictions allow, algal community colonization experiments will be repeated using molybdenum and/or common herbicides.

● If COVID-19 work restrictions allow, *Paratanitarsus* mesocosm studies will be repeated using Cu and/or other metals and/or nutrients.

● Continued support of CPW fishery biologists and hatcheries: On-site Rotenone Analysis, production of milt extender, assistance as requested.

● Continued support of CPW Water Unit goals, CDPHE water quality efforts, River Watch, Colorado universities and researchers.

● Infrastructure improvements, analytical equipment improvements and improvements to our mobile laboratory will be made as time allows.

Preliminary Report A: Algal colonization rates and community structure changes in the presence of trace levels of copper (Cu)

Personal: Pete Cadmus. Margaret J. Spangler. Mathew R. Bolerjack

Many of Colorado's threatened species, endangered species, species of special concern and sport fish co-occur with anthropogenic pollution. Safe levels of pollution are regulated by water quality standards that were derived using standardized experimental methods based almost entirely on direct aqueous exposure of organisms via the gills (ASTM 1997; EPA 1985). Only recently have such standards incorporated dietary exposure, effects on ecosystem function, or effects on obligate forage species such as algae, fungus, meiofauna and microbes. Laboratory tests regularly underestimate toxicity of pollutants when compared to field studies (Brix et al 2011; Clements et al 2013; Cairns 1983). This may be due to increased environmental realism and duration. Such studies include intra- and inter-specific competition, full life cycles or important life cycle events (colonization, reproduction, molting, emergence, etc). Additionally these studies often examine more ecological effects. Historically, the endpoints (also known as "measured results" "toxic effects" or "response variables") considered in toxicity tests rarely extend beyond mortality even when sublethal effects can manifest to mortality, population loss or reduced ecosystem function. This is especially true for freshwater algae which is often the sole primary producer supporting Colorado's aquatic ecosystems. Single species trials measuring only survival of cells failed to consider physiological health, photosynthetic aptitude, competition, growth rate, and more meaningful ecotoxicological measures of sensitivity.

More than half the aquatic species on the Colorado threatened and endangered list consume algae (Table A1). Brassy Minnow (*Hybognathus hankinsoni*), Mountain Sucker (*Catostomus playtrhynchus*), Northern Redbelly Dace (*Phoxinus eos*), Plains Minnow (*Hybognathus placitus*), Rio Grande Sucker (*Catostomus plebeius*), Razorback Sucker (*Xyrauchen texanus*), Southern Redbelly Dace (*Chrosomus erythrogaster*), Rocky Mountain Capshell (*Acroloxus coloradensis*), and Cylindrical Papershell (*Anodontoides ferussacianus*) use algae as a primary food source for the majority of their lives. Additionally, Bony Tail Chub (*Gila elegans*), Colorado Roundtail Chub (*Gila robusta*), Common Shiner (*Luxilus cornutus*), Lake Chub (*Couesius plumbeus*), Rio Grande Chub (*Gila pandora*), Boreal Toad (*Bufo boreas*), Couch's Spadefoot (*Scaphiopus couchii*), Great Plains Narrowmouth Toad *(Gastrophryne olivacea*), Northern Cricket Frog (*Acris crepitans*), Northern Leopard Frog (*Rana pipiens*), Plains Leopard Frog (*Rana blairi*), and Wood Frog (*Rana sylvatica*) consume algae during early life stages or to supplement an omnivorous diet. Colorado's lentic and lotic sport fish species are largely supported by foraging on macroinvertebrates. Aside from limited allochthonous resources such as fallen leaves, macroinvertebrate communities that sustain Colorado's sport fish are supported almost exclusively by algae growth.

In this study we examined colonization rates and growth rates of three major groups of algae; Diatom, Green and Cyanobacteria. For algae, colonization is a more ecologically relevant endpoint than survival. Cyanobacteria (also known as Blue-Green Algae) are responsible for toxic algal blooms, which are becoming common in Colorado's reservoirs, including numerous state parks. Cyanobacteria are generally not nutritious to fish species and many taxa have potential to kill both mammals and fish. Diatoms are rich in fatty oils and are major sources of food for fish and the aquatic insect communities that fish and wildlife consume. Green Algae are consumed by aquatic life but are generally less nutritious to fish and macroinvertebrates than Diatom species but are consumed and play an important role in ecosystem function. Competition and colonization rates between these three groups in the early spring, or after a disturbance event, have potential to drive community structure to those algae that cause toxic algae blooms or to those algae that provide food for fish and forage species.

Rather than single species studies, we expose members of the three major groups of algae simultaneously. This multiple species approach is superior to single species trials because it allows competition between taxonomic groups. Traditional microscopy of algae cells allows for great taxonomic resolution, however, such lethal (consumptive) assessments can only be conducted once in each experiment. We employed pulse amplitude modulated fluorimetry assessments of chlorophyll, which can be conducted repeatedly on the same periphyton mat. These technologies can assess density of chlorophyll, assess the taxonomic group from which chlorophyll was associated, and determine the physiological health of the algae (Cornet 2011).

We predicted that trace levels of pollution could stress palatable algae (Diatoms and Green) and give competitive advantage to Cyanobacteria. This would explain why toxic algae blooms occur inconsistently, and sometimes irrelevant to nutrient availability. It would also explain the loss of algivorous fish species in ecosystems with trace levels of herbicides, algaecides, or other forms of pollution.

Copper (II) sulfate is a common algaecide applied to surface waters to control algal blooms. Unlike peroxide or chlorine based algaecides, copper (Cu) ions are persistent in aquatic environments. Toxic effects of Cu are heavily studied and regulated by state (CDPHE) and national (USEPA) water quality standards. Colorado's headwaters are commonly described as oligotrophic, with low dissolved organic carbon and hardness of 30 to 100 mg/l $CaCO_3$ equivalents. This hardness range typically results in acute Cu limits of 4.3-13.4 µg/l and chronic limits of 3.2-8.9 µg/l, however, many site-specific standards have relaxed this pollution limit. Our study used a novel experimental system that allowed algal competition and colonization to be examined at Cu concentrations between 0 and 20 µg/l (50 mg/l $CaCO_3$ equivalents hardness) to determine if drastic community composition changes could occur in Colorado's aquatic ecosystems at Cu levels well below regulatory limits.

Methods

Three periphyton-covered cobble (20-40 cm diameter) were collected from 27 sample sites on the Saint Vrain and Cache La Poudre Rivers between 3,140 and 1,364 m elevation. Rocks were held in dechlorinated water in chilled coolers during transport (<2 hours travel time) to the Colorado Parks and Wildlife Aquatic Toxicology Laboratory (Fort Collins, CO, USA). Periphyton-covered rocks were randomly assigned to locations within a chilled periphyton culturing system which consisted of two 140 liter recirculating tanks (Figure A1) that provided lentic and lotic microhabitat, and wide spectrum light. Flow-through dechlorinated tap water provided 95% replacement every 18 hours. Micronutrients were provided prior to the experiment (90 ml F2 Guillard growth media and 0.6 g sodium metasilicate per 280 L). Algae were held twenty-four hours before start of the experiment. Twenty porcelain tiles (25.9 cm^2) were added to the culture tanks every 3 to 5 days to support those pioneer algal species that first colonize substrate.

Gravity serial diluters (Figure A2), constructed of marine grade stainless steel, delivered chilled dechlorinated municipal tap water (Fort Collins, CO, USA) to experimental units at 40 ml/min. Experimental units (Figure A3 and A4) consisted of marine grade stainless steel troughs 10 x 10 x 65.5 cm. A slow lotic flow (3.0 cm deep) was created by recirculating water with bubble pumps (also known as "bubble lifters") through a 190 cm loop of 1.5 cm internal diameter stainless steel pipe. Air from a regenerative blower was fed through borosilicate glass tubes (5 mm internal diameter) into the lifting pipe of the "U" shaped bubble lifter. Eight unglazed porcelain tiles (Daltile® Mohawk Industries Inc. Calhoun, Georgia, USA. Keystone® series) were positioned in troughs and served as substrate for colonizing algae. Wide spectrum halogen grow lights were positioned 60 cm above each of the twenty four experimental streams. The five treatment levels and control were assigned randomly within four stratified blocks. Plumbing for each trough was submerged in a chilled water bath. Experimental streams and algal culturing systems received 16:8 hour light cycles.

Prior to the experiment, all experimental streams were washed with nitric acid, distilled water, sodium hypochlorite and then thoroughly rinsed with dechlorinated municipal tap water to ensure no residue remained. To ensure all experimental streams had equal potential to be colonized by algae, an array of four peristaltic pumps delivered 75 ml/min water from numerous locations in the algal culturing system to the head tank that supplied constant chilled water flow to the serial toxicant diluter.

Copper sulfate stock solution was delivered to the serial diluter which provided 0, 1.3, 2.5, 5, 10, and 20 µg/l Cu to experimental streams. Water chemistry was sampled five times throughout the experiment. Cu, Ca, K, Na, Mg and dissolved organic carbon (DOC) samples were filtered through a 0.45 micron filter prior to preservation. Inductively Coupled Plasma

Spectroscopy using optical emission and mass spectroscopy (for lowest treatment levels) was used to validate concentrations.

After four, seven, ten and twelve days of exposure a BenthoTorch (bbe Moldaenke GmbH, Schwentinental, Germany) fluorimeter was used to assess density of Diatom, Green and Cyanobacteria Algae. After fifteen and twenty-two days a Walz PAM-2500 (Heinz Walz GmbH, Effeltrich, Germany) was used to assess light adapted photosynthetic efficiency of each experimental unit. Measurement of an experimental unit at any point in time included assessment of every tile. Values of each tile in a single experimental stream were averaged prior to statistical analysis in observance of assumptions of statistical independence. To ensure pulse amplitude modulated fluorimetry assessments did not disturb periphyton an adapter was devised to suspend the lenses of both instruments at a uniform and repeatable distance without disturbing more than the edge of each tile (Figure A5).

At the end of the experiment two tiles from each experimental stream were preserved in Lugol's solution (2%). Tiles were scraped with stainless steel razor blades. Suspended cells were allowed to settle in Utermöhl sedimentation chambers and were systematically sub-sampled and algae were identified to genus.

Preliminary Results

Temperature, pH, conductivity, hardness, alkalinity, and dissolved oxygen were consistent throughout the twelve days. Copper concentrations were within 2.5% of target. Dissolved organic carbon was low but consistent with Colorado's surface waters. Microscopy assessments of community composition at the end of the experiment were limited to Green and Diatom species (Table A2). *Sellaphora* sp. dominated algal composition. Several euglenoid species were also found in each treatment level.

Chlorophyll associated with each of the three major groups changed drastically across treatment levels at numerous points in time (Figure A6). Both total chlorophyll and photosynthetic efficiency was negatively correlated with Cu (Figures A6 and A7). As is often the case in nature, Cyanobacteria (Figure A8) and Green Algae (Figure A9) species colonized substrate first (7-Jan-2020, Day 4) at very low algal density(~0.03 µg Cu total chlorophyll per cm^2) before Diatom species colonized (Figure A10). After only 96 hours of exposure a statistically significant reduction in Green Algae colonization was observed between the control and the 2.5 µg/l (p=0.02) treatment level, as well as all higher treatment levels (5 µg/l Cu:p<0.001, 10 µg/l Cu:p=0.01, 20 µg/l Cu:p<0.001). After seven days of exposure, Diatoms (Figures A10 and A6) began to colonize substrate in the control and the lowest treatment level but were absent in Cu levels of 2.5 µg/l Cu and above. Despite Cyanobacteria showing no reduction in colonization rate in even the high (20 µg/l) treatment, a clean dose response in total

chlorophyll was present. This trend was driven by reduction in Green Algae species which were significantly reduced from controls in all treatment levels greater than 1.3 µg/l Cu (p<0.001 for all; 10-Jan-2020, Day 7). After 10 days of colonization and competition (13-Jan-2020, Day 10), healthy Green and Diatom communities appeared to out-compete Cyanobacteria in the control and low (1.3 µg/l) treatment levels. Chlorophyll from Cyanobacteria was greater than controls for all treatment levels above 1.3 µg/l Cu (p<0.001 for all). Chlorophyll associated with Cyanobacteria had remained unaffected by even the highest treatment 20 µg/l Cu well into the final day of assessment (15-Jan-2020, Day 12) suggesting these reductions in Cyanobacteria in the lower treatment levels were the result of competition with Green and Diatom species. This trend became more pronounced in the days that followed.

On the twelfth day of exposure we observed signs that communities were becoming space limited as total chlorophyll for all communities approached a maximum for the experimental streams (~0.2 ug/cm^2). In the laboratory setting our mesocosms became space limited in only ~15 days. When projected to the scale of a large lake, space limitations of growth are likely negligible for many days or weeks. Assessments of community composition in our first ten to twenty days could be analogous to what larger lakes and streams would observe through the spring and summer months before seasonal algae senescence. Smaller lentic ecosystems or localized segments of larger lakes might progress through this succession in a much shorter duration than would a large reservoir or river. At the end of our observations (15-Jan-2020, Day 12) Diatom colonization was significantly reduced from controls in the 2.5 µg/l treatment level (p=0.03) and above (p<0.001 for all), as was Green Algae (p<0.001 for all). The ability of Green and Diatom Algae to out-compete Cyanobacteria was reduced above the level of 1.3 µg/l Cu during this non-space limited phase (2.5 µg/l Cu p=0.03, 5 µg/l Cu and above p<0.001).

Discussion

After 96 hours, these studies demonstrated clear reduction of Green Algae below the Acute state standard for Cu, and showed reduction of total chlorophyll, Green Algae, and Diatoms at ten and twelve days well below the Chronic Cu standard. The threshold of these responses occurred between our low (1.3 µg/l Cu) and mid-low (2.5 µg/l Cu) treatments. This was only discovered when laboratory infrastructure was designed to simulate the colonization and competition of algae, rather than survival. Even at 20 µg/l Cu the cells of Cyanobacteria, Diatoms and Green Algae can likely "survive" for the standardized duration of four or thirty days if we determine survival using microscopy of stained slides. However this endpoint of survival fails to capture population growth rate, competition with nuisance taxa, photosynthetic health and other ecologically important endpoints.

The biological significance of using a standardized 96 hour toxicity trial was best described by Ronald Eisler (USFWS); "Acute toxicity laboratory bioassays rarely exceed 96 hours; this interval permits investigator and technicians alike to enjoy an uninterrupted

weekend." (Eisler 1970) The minimum of 30 days duration for chronic studies has similar biological significance. Standardized methods encourage longer duration studies but these are rare in the literature. A 30 d trial characterizes only 1.2 to 1.6% of a typical salmonid's life, while short lived species can reproduce multiple times in several days. Single species or multiple species tests with algae do not fit the standardized exposure durations and standardized toxic endpoints. Here we employed pulse amplitude modulated fluorimetry to assess biomass. This non-consumptive and non-intrusive measure allowed repeated assessment of competing species over time. Without repeated non-consumptive assessment the ideal time to take measurements would not be obvious and the risk of overestimating a toxic threshold would be likely. The paradigm for laboratory trials using species with reproductive cycles greater than the duration of an experiment is that longer duration typically means more mortality leading to lower calculated effect concentrations (e.g. EC10, EC20, LC50). This is not true with organism whose life cycle is less than the duration of an experiment. In this experiment we examined colonies of growing single cellular organisms whose growth is density dependent and influenced by competitors. Excessive exposure duration risks overestimating a safe threshold as the differences between the lowest effect concentration group and the no-effect concentration group decreases as the NOEC nears carrying capacity of the artificial laboratory vessel. The duration at which a control experimental unit reaches natural carrying capacity is a function of space (size of vessel or mesocosm), food, reproductive rate, and reproductive age of the organism.

Our experimental system held very little surface area and water volume relative to a Colorado stream or reservoir. We predict the exponential growth phase observed between four and twelve days would last numerous weeks in a large reservoir. The patterns observed here could very well explain how large community shifts occur between palatable algae (Green and Diatom) and toxic algae (Cyanobacteria).

Figure A1. Algal culture system. Two chilled water baths received flow-through dechlorinated municipal water at a 95% replenishment rate of 18 hours. Additionally baths recirculated into each other, had drippers and misters that kept cobble hydrated. Surfaces were disturbed daily and additional substrate (unglazed tiles) were added daily to ensure pioneer species were not out-competed during the experiment.

Figure A2. Gravity-fed serial diluter.

Figure A3. Algal culture systems (left) delivered a slow supply to the serial toxicant diluter (right) which delivered toxicants and dechlorinated water to the twenty-four experiment streams in a chilled water bath (center).

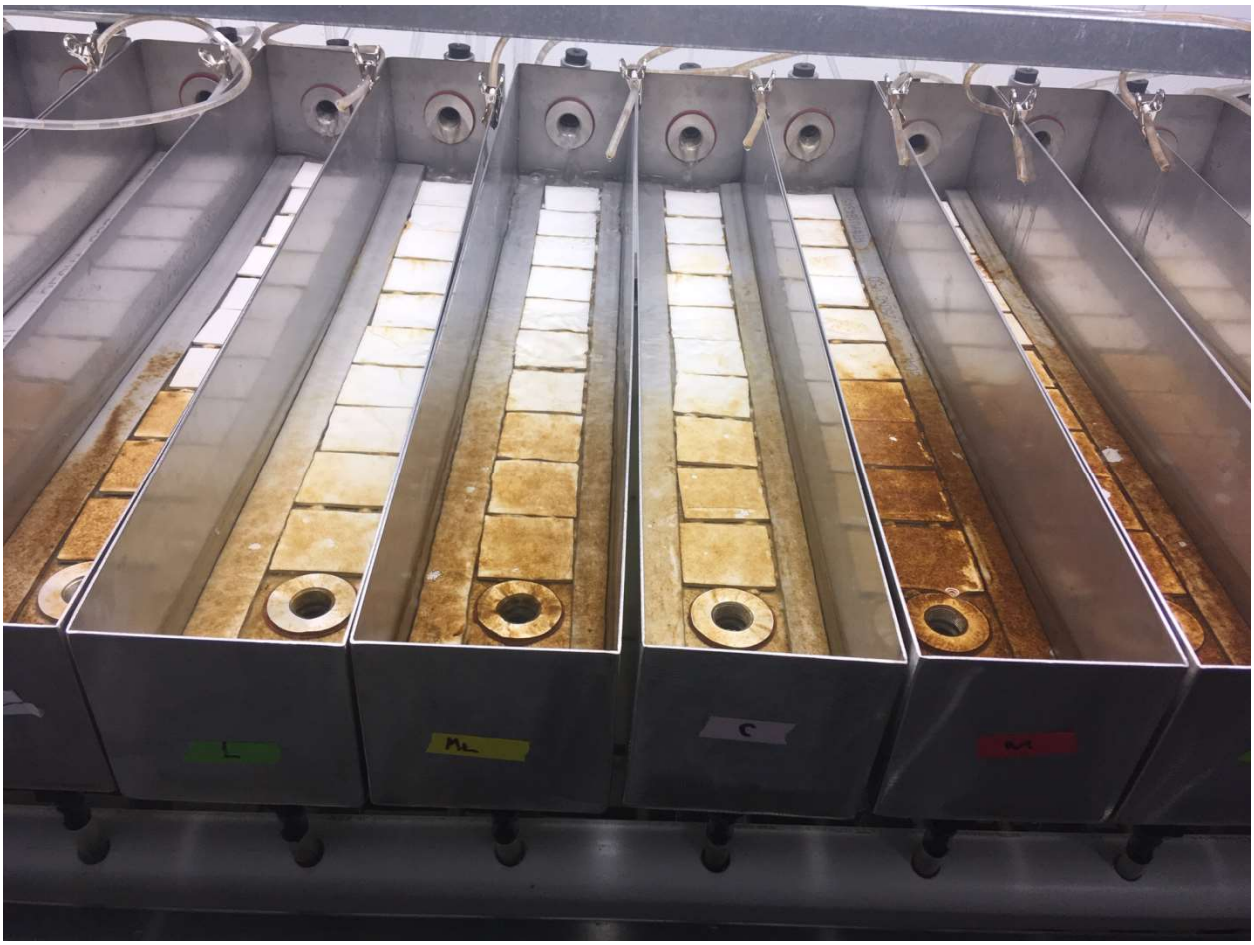

Figure A4. Experimental streams with periphyton covered tiles. Streams shown here were from research and development and are not part of the experiment reported here within.

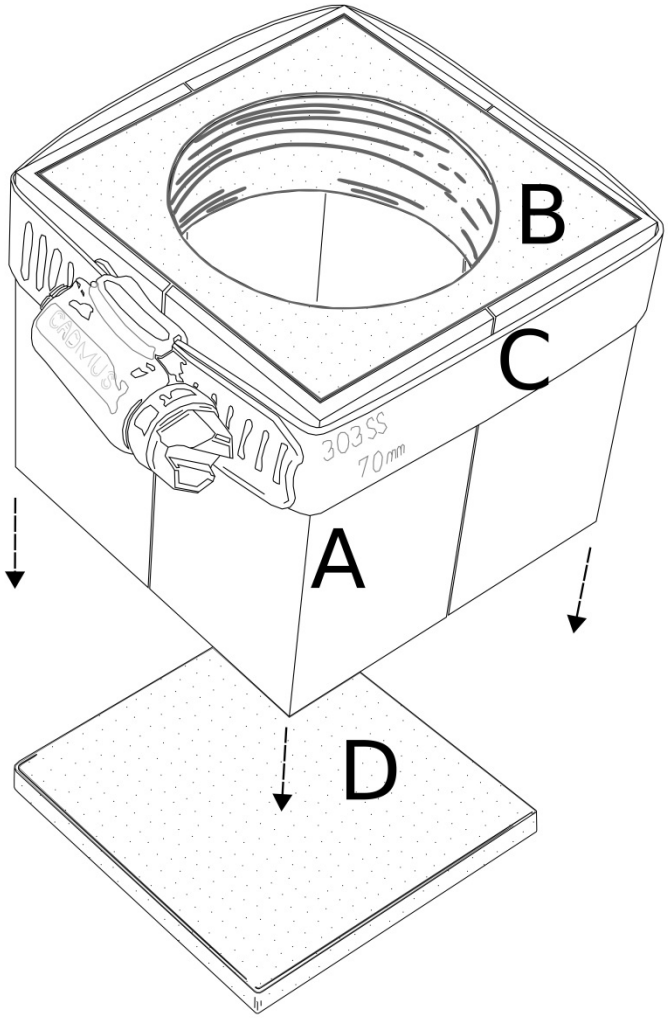

Figure A5. BenthoTorch (left) shown with a tile adapter in place of the manufacturer's foam endpiece. The adapter (right) was made of four pieces of angle (90 deg.) stock (25.4 x 25.4 mm, 1.6 mm thick. 49 mm long. A) clamped onto a block of black high-density polyethylene (48x48x16 mm. B) using a stainless-steel hose clamp (C). A 36 mm hole in the center of the plastic block accepted the lens of the BenthoTorch. Only the edges of the 48x48 mm tile were disturbed by the adapter allowing repeated measurement of the same periphyton without disturbance.

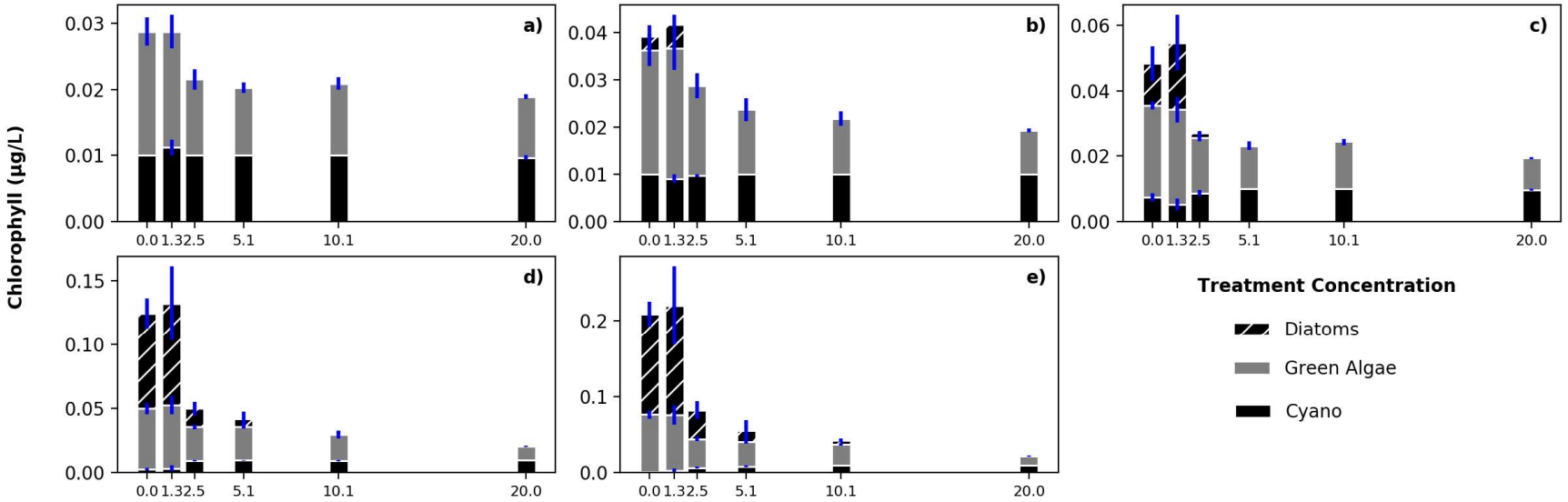

Figure A6. Algal community composition as a function of Cu (µg/l) at five observation dates. The average Cyanobacteria, Green Algae, and Diatoms biomass as measured by chlorophyll florescence (µg/cm^2). Measurements are shown for a: Day 4 (7-Jan-2020) , b: Day 6 (9-Jan-2020) c: Day 7 (10-Jan-2020), e: Day 12 (15-Jan-2020). Day 6 (9-Jan-2020) observations were excluded from Figures A8-A10 and statistical evaluations because the observations were nearly identical to Day 7.

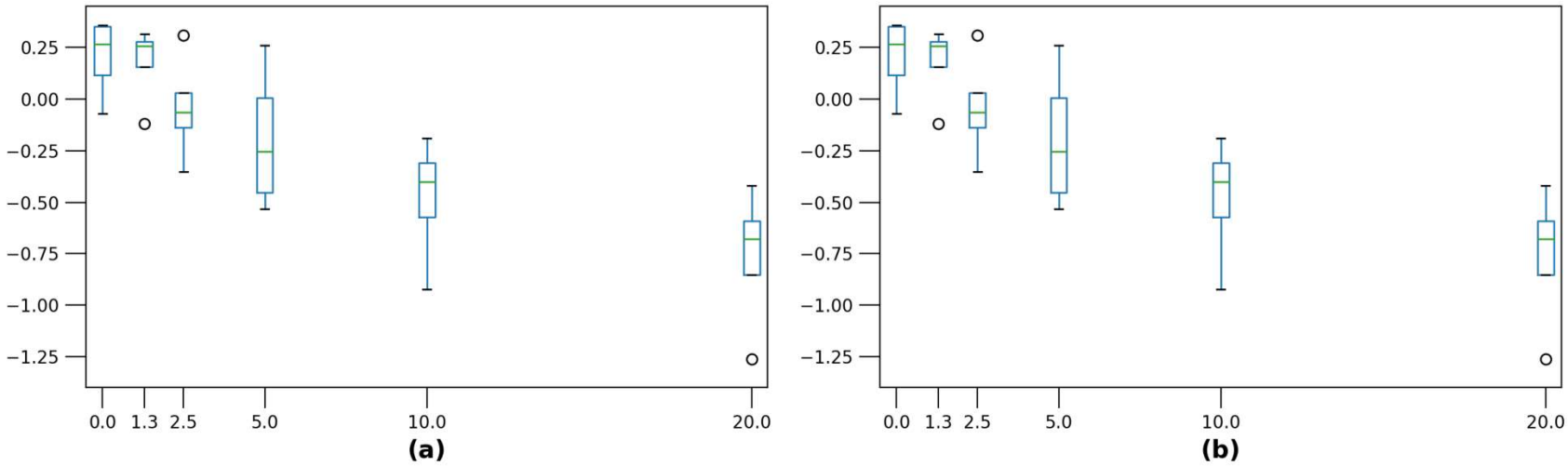

Figure A7. Algal photosynthetic efficiency. Light adapted photosynthetic efficiency (a unitless ratio) was inversely correlated with Cu (µg/l) after fifteen (a) and twenty-two (b) days of exposure.

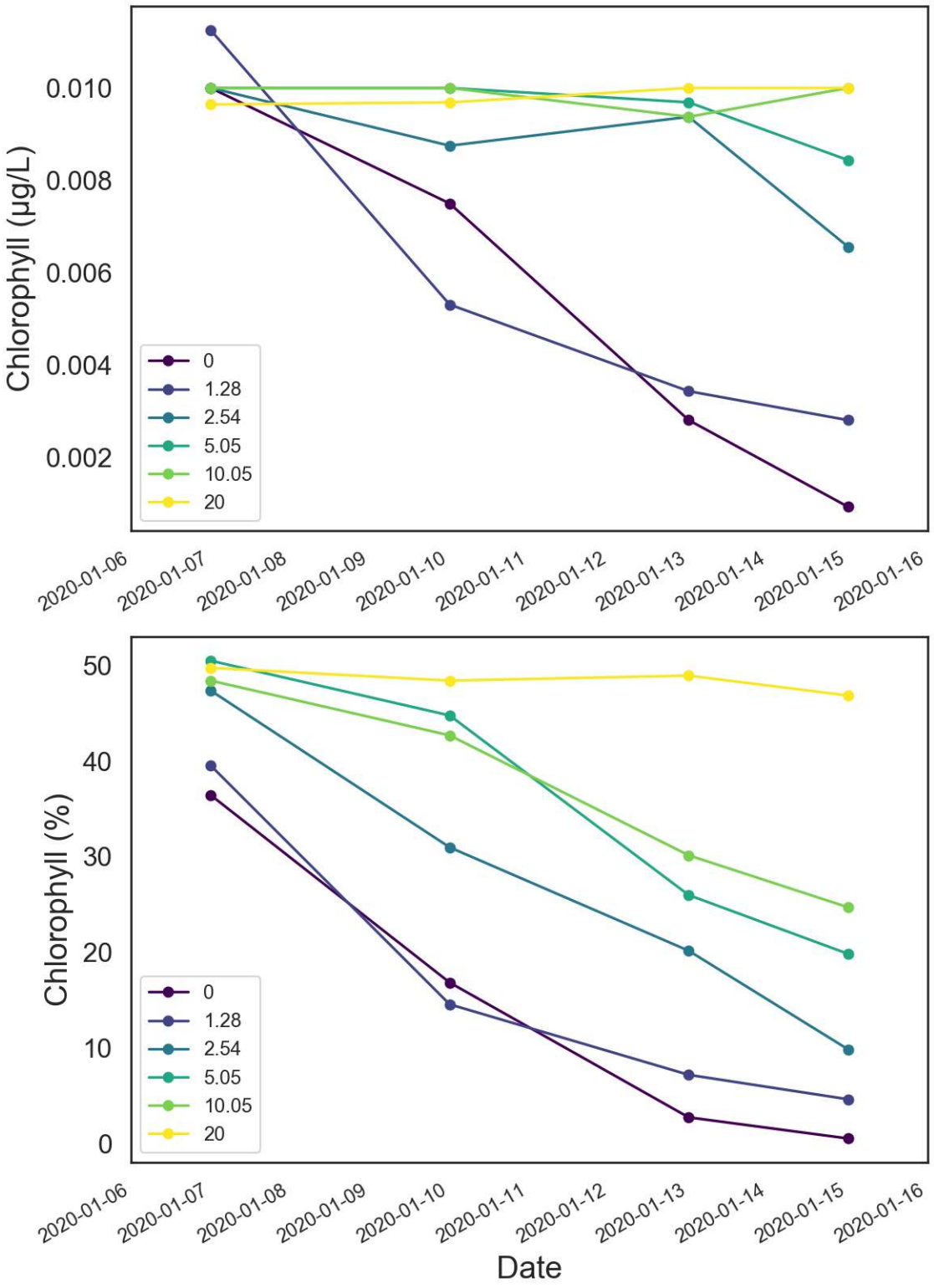

Figure A8. Cyanobacteria – Biomass as estimated by chlorophyll florescence (µg/cm^2) over time (above). Proportion (%) of the total algal community biomass associated with Cyanobacteria species (below) over time. Shown for each of the six copper treatment groups (µg/l Cu).

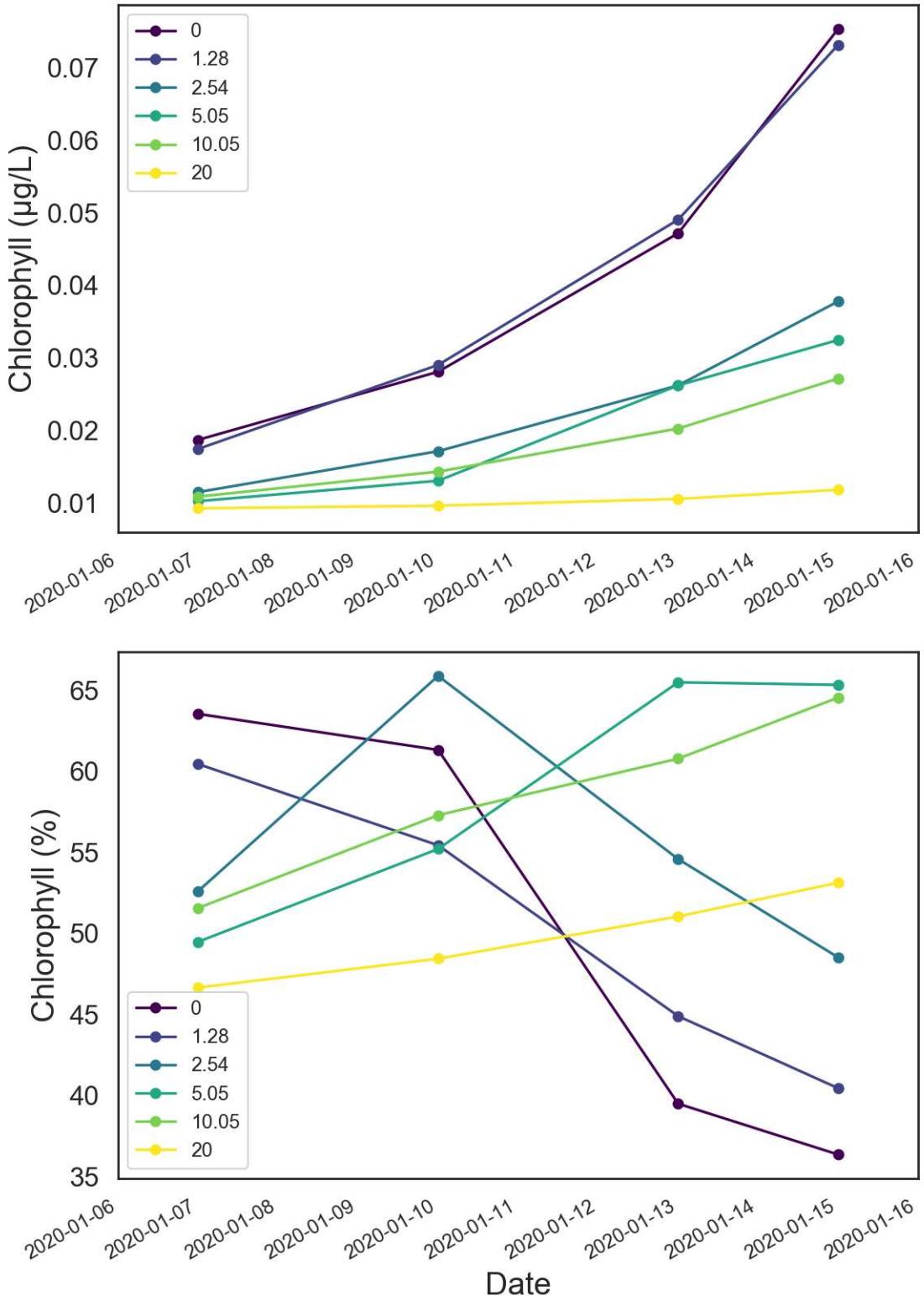

Figure A9. Green Algae –Biomass as estimated by chlorophyll florescence (µg/cm^2) over time (above). Proportion (%) of the total algal community biomass associated with Green Algae species (below) over time. Shown for each of the six copper treatment groups (µg/l Cu).

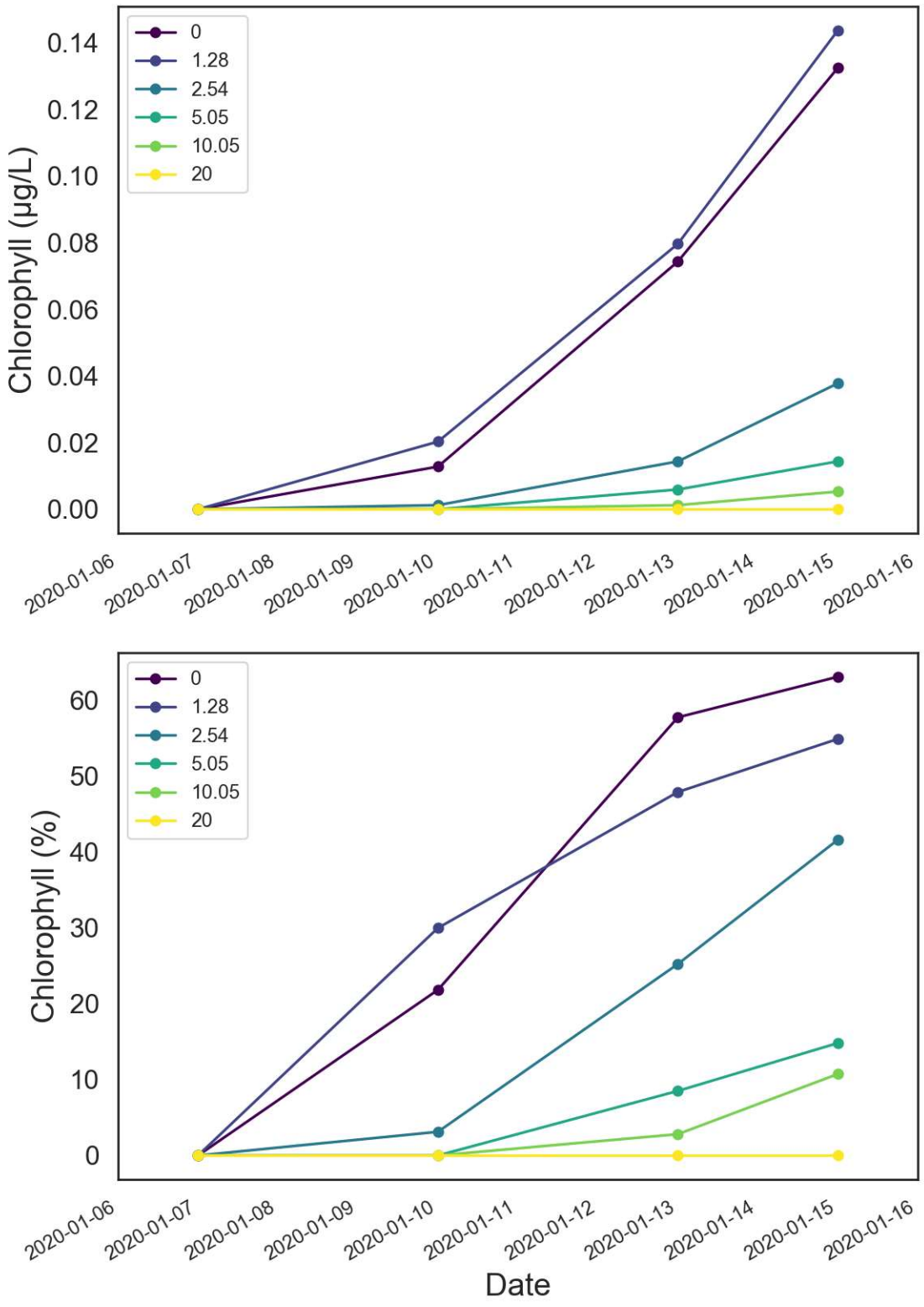

Figure A10. Diatom Algae –Biomass as estimated by chlorophyll florescence (µg/cm^2) over time (above). Proportion (%) of the total algal community biomass associated with Diatom species (below) over time. Shown for each of the six copper treatment groups (µg/l Cu).

Table A1 –Algivorous aquatic organisms considered threatened, endangered or species of concern in the state of Colorado

Species	likelihood of co-occurrence with toxicants	Diet
Amphibians		
Boreal toad	metal: high	algae and detritus*
Couch's Spadefoot	agricultural: high	algae*
Great Plains Narrowmouth Toad	agricultural: high, urban: low	algae*
Northern Cricket Frog	agricultural: high, urban: moderate	algae*
Northern Leopard Frog	urban, agricultural and metal: moderate	algae and aquatic plants*
Plains Leopard Frog	urban and agricultural: high	algae*
Wood Frog	metal: moderate, urban and agricultural: low	algae and aquatic plants*
Fish		
Bonytail Chub	unknown	algae, aquatic plants, small fish and terrestrial invertebrates
Brassy Minnow	urban and agricultural: high, metal: low	algae
Colorado Roundtail Chub	unknown	aquatic and terrestrial invertebrates, amphibians, fish, sometimes algae
Common Shiner	urban, agricultural and metal: high	aquatic and terrestrial invertebrates, sometimes algae
Lake Chub	urban and metal: high, agricultural: low	algae, aquatic invertebrates, zooplankton and some small fish
Mountain Sucker	agricultural and metal: high, urban: low	algae and some aquatic invertebrates
Northern Redbelly Dace	urban and metal: high, agricultural: low	algae and some zooplankton and aquatic invertebrates
Plains Minnow	agricultural and metal: high, urban: low	algae, detritus and some aquatic invertebrates
Rio Grande Chub	agricultural: high, metal: moderate	algae, aquatic and terrestrial invertebrates, some small fish
Rio Grande Sucker	agricultural: high, metal: moderate	algae, aquatic invertebrates, small fish and detritus
Razorback Sucker	metal: low	algae, aquatic invertebrates and detritus
South. Redbelly Dace	agricultural: high	algae and some aquatic invertebrates
Mollusks		
Rocky Mt. Capshell	metal: low	algae and detritus
Cylindrical Papershell	urban and agricultural: moderate	algae, bacteria, protozoans and detritus

Diet determined from literature review. Risk of pollution sources determined from co-location of species historical distribution and recent water quality observations. *=*tadpole diet*

Table A2 - Genera of algae identified in by microscopy

Algal Genera
Sellaphora, Fragilaria, Stephanodiscus, Nitzschia, Navicula, Pediastrum, Scenedesmus, Cymbella, Closterium, Staurosira, Gomphonema

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Preliminary Report B: Selenium concentrations and trophic transfer rates for Brown Trout (*Salmo trutta*) and White Sucker (*Catostomus commersonii*) populations within Colorado

Personal: Alexander S. Townsend. Pete Cadmus.

Abstract

From 2016 to 2019 Colorado Parks and Wildlife (CPW), with financial support from Colorado Department of Public Health and Environment (CDPHE), conducted extensive field sampling of selenium concentrations of fish ovary and egg masses, fish muscle tissue, substrate, periphyton, invertebrate tissue and water column measurements. This continued preliminary work conducted by CPW in 2015. Pooling CDPHE's sampling history, CPW's fish surveys and the advice of local CPW biologists, over 60 warm and cold water habitats/locations were investigated or successfully sampled. Target fish species and gravid females of target fish species however, were found at only a subset of these locations. This report describes successful sampling locations, sampling results, sampling methods, and interpretation of results from sampling, including an investigation of the relationships regarding selenium uptake and transfer between environmental matrices.

Introduction

Selenium's effects on aquatic and riparian species have become increasingly important topics of toxicological research and a global contaminant issue (Lemly 2002, 2014). Chronic selenium exposure has negatively influenced fish reproductive success and physiological health for many aquatic systems influenced by coal-fired power generation (Lemly 2002) and mining related effluent (McDonald et al. 2010; Muscatello and Janz 2009). Inputs from natural sources, such as cretaceous shale formations, can also contribute large amounts of selenium into aquatic systems (Butler et al. 1996; Morrison et al. 2012). This process can be influenced by anthropogenic disturbances of these formations through land development, agriculture and irrigation (Sieler et al. 2003).

Selenium inputs vary greatly by region based upon anthropogenic activity (fossil fuel production, oil refining and various industrial processes) and from underlying geological formations subject to land disturbance via agriculture, mining and development (Maher et al. 2010; Presser et al. 1990; Muscatello and Janz 2009). Many of Colorado's watersheds receive selenium inputs from the underlying Mancos Shale formations during weathering events, runoff through non-irrigated soils and irrigated canal systems (Mast et al. 2014; Mills et al. 2016; Morrison et al. 2012). Selenium availability is often increased by artificial weathering accelerated by anthropogenic disturbance of selenium-bearing materials and can be transported

considerable distances by hydrologic processes (Young et al. 2010). Much of southwest Colorado between Grand Junction and Durango is geologically tied to the Mancos Shale formation which contributes selenium to groundwater in concentrations that often exceed 50 µg/L (Morrison et al. 2012). Intensive irrigation of alluvial soils and agricultural land disturbance have been primary drivers of selenium contamination in the Arkansas River drainage (Gates et al. 2009), Lower Gunnison River basin (Butler and Leib 2002), and Uncompahgre River drainage (Butler et al. 1996) in Colorado.

Selenium is present in many forms; however, transfer to surface water and groundwater in Colorado is driven by dissolution of selenium-bearing salts, gypsum and other highly soluble sources (Mast et al. 2014; Tuttle et al. 2014). The oxidation state in which selenium exists has a great influence on solubility and movement in the food web (Martens 2003). Selenite and selenate are two soluble inorganic selenium species that are abundant in selenium laden aquatic ecosystems with selenate being believed to be more bioavailable to fish. Selenite and selenate concentrations in aquatic ecosystems are determined by pH, oxidation-reduction status, and microbial activity (Martens 2003). Selenite is not as stable as selenate in oxidizing conditions (Geering et al. 1968). Selenite has a higher affinity for organic particles and common minerals in acidic conditions (Goldberg and Glaubig 1988) and can be selectively assimilated by microbial communities over selenate (Losi and Frankenberger 1997). Soils containing selenium-bearing geologic formations are more likely to mobilize selenate species (Seiler et al. 2003). Previous research has demonstrated that organic Se forms such as selenomethionine (SeMet) are more toxic to aquatic organisms when compared to inorganic selenium species. Zebrafish *Danio rerio* fed SeMet spiked diets demonstrated maternal transfer of selenium and the subsequent juvenile fish exhibited significantly increased mortality over controls at egg selenium concentrations of 9.6 µg/g dry weight (dw) (Thomas and Janz 2014). Significant reduction in Bluegill *Lepomis macrochirus* larval survival was demonstrated in an experiment where parental fish were exposed to 10 µg/L aqueous and 33.3 µg/g dietary SeMet (Coyle et al. 1993). As selenium is transferred through trophic levels, its speciation can change into more toxic and biologically available organic forms. Conversion of selenium from inorganic to organic forms primarily occurs at the microbial level in benthos and periphyton. Selenium uptake by biofilm and periphyton is a critical step in this conversion and in these complexes large concentrations of SeMet can exist (Janz et al. 2014).

Selenium is an essential element for fish and dietary requirements range from 0.15 to 0.5 mg/kg dw of diet (Watanabe et al. 1997). In some reaches and surface waters, excessive inputs from natural and anthropogenic sources have resulted in concentrations in fish diets that are above safe levels. For fish species the risks of selenium exposure are predominantly related to dietary transfer in the food web and aqueous exposure has limited natural applicability (Young et al. 2010). Selenium concentrations of 13 to 15 mg/kg dw of food can exhibit toxic effects such as reduced growth rate, sub-optimal feeding rate and mortality in Rainbow Trout *Oncorhynchus*

mykiss and Channel Catfish *Ictalurus punctatus* (Gatlin and Wilson 1984; Hilton et al. 1980). Selenium biomagnifies in aquatic food webs and the effects on invertebrates usually occur at higher concentrations than those that affect fish (United States Environmental Protection Agency 2016). The oligochaete *Lumbriculus variegatus* demonstrated reduced worm densities in sediment concentrations of 20 µg/g selenite and complete mortality in sediment concentrations of 20 µg/g SeMet (Xie et al. 2016). Ingersoll et al. (1990) conducted a long-term exposure study with *Daphnia magna* and found that a 6:1 mixture of selenate to selenite at concentrations of 156 µg/L caused reduced growth and reproduction rate. In predatory fish, selenium bioaccumulation is highly dependent on food web dynamics and variability in trophic transfer functions (TTF) at consumer levels are important in evaluating the amounts of selenium different predators accumulate (Luoma and Presser 2009; Stewart et al. 2004). Selenium biomagnification, at 1.5 to 6 fold increase in successive trophic levels, was found between primary producers, invertebrates, and Spottail Shiners *Notropis hudsonius* (Muscatello et al. 2008). Rhea et al. (2013) reported consistently increasing concentrations of selenium from biofilm to macroinvertebrates and fish in the Yankee Fork of the Salmon River in Idaho.

A large volume of recent selenium research has focused on maternal transfer and selenium's effects on larval fish development and survival (Conley et al. 2014; Greely et al. 2016; Thomas and Janz 2014). Assessing the reproductive and teratogenic effects on offspring is of critical importance because there is a close relationship between teratogenic deformity and reproductive failure which has implications for effects on larval fish abundance, recruitment, age structure and population growth. Associating selenium toxicity with population level effects can be difficult, although methods are available for estimating selenium's effects on population metrics. Outside of lethal effects (egg and larval mortality) the most prominent sublethal indicator of selenium toxicosis is teratogenic deformity, and methods for assessing the impacts of selenium on fish populations based upon this symptom have been developed by Lemly (1997). The teratogenic deformities evaluated in most selenium studies are craniofacial, skeletal, fin and edema related.

Selenium's effects on reproduction and larval development vary by fish species and location. Macdonald et Al. (2010) found an effective concentration for 10 percent of the test population (EC10) of 54 mg/kg dw, for egg tissue, with larval deformity in Dolly Varden Char *Salvelinus malma* exposed to high levels of dietary selenium in British Columbia, Canada. Holm et al. (2005) evaluated teratogenic deformity in larval Rainbow Trout *Oncorhynchus mykiss* and found an EC10 of 9.5 mg/kg egg wet weight (ww) (converted to 24.5 mg/kg egg dw) for edema. In Rainbow Trout, an EC10 of 24.5 mg/kg dw was established for skeletal deformities (Holm et al. 2003) and an EC10 of 24.7 mg/kg dw was found for Westslope Cutthroat Trout *Oncorhynchus clarki lewisi* alevin mortality (Rudolph et al. 2008). A study conducted by Formation Environmental (2011) on Brown Trout *Salmo trutta* found EC10 values of 19.33 mg/kg egg dw for total deformity frequency and 17.68 mg/kg egg dw for post hatch survival. This study found

sizeable correlations between selenium in eggs and adult fish tissues and utilized this relationship to derive an EC10 value of 12.9 mg/kg whole body dw.

In 2016, the United States Environmental Protection Agency (USEPA) released a finalized Ambient Water Quality Criterion for selenium (United States Environmental Protection Agency 2016). The purpose of the criterion is to ensure that all states adopting water quality standards for selenium under the Clean Water Act (CWA) have scientifically based recommendations for ambient selenium concentrations (United States Environmental Protection Agency 2016). The proposed selenium standard is based on four elements; egg/ovary tissues (15.1 mg Se/kg dw), whole body (8.5 mg Se/kg dw) or muscle tissue (11.3 mg Se/kg dw), monthly average water column exposure (1.5 µg/L in lentic systems and 3.1 µg/L in lotic systems), and an intermittent water column exposure calculated by the formula: $WQC_{int}=(WQC_{30day}-C_{background}\ (1-f_{int}))/f_{int,}$ where WQC_{30day} is the monthly water column element, $C_{background}$ is the average background selenium concentration and f_{int} is the fraction of the 30 day period with elevated selenium concentrations (United States Environmental Protection Agency 2016). The EPA criterion specifies that the egg/ovary element supersedes all other elements if egg/ovary concentrations are measured with the other elements.

It is in Colorado's best interest to adopt water quality standards that are protective of all aquatic life present in the state. Adopting the USEPA water quality criterion is constrained by limited data on many fish species present in Colorado. One of the lowest EC10 values for salmonids (survival hatch to swim-up) used in the 2016 USEPA selenium criterion (21.0 mg/kg egg dw) was derived from a 2011 Brown Trout *Salmo trutta* selenium study (Formation Environmental 2011; United States Environmental Protection Agency 2016). Brown Trout are an important sport fish to Colorado as they constitute a large proportion of salmonids in the state and are highly sought after by anglers. Other maternal transfer toxicity studies included in the USEPA criterion utilized species including White Sturgeon *Acipenser transmontanus*, Desert Pupfish *Cyprinodon macularius* and Dolly Varden Char *Salvelinus malma* that do not exist in Colorado or never have (United States Environmental Protection Agency 2016). Our state aquatic standards would benefit from inclusion of Se studies with native Colorado species. Our native species are largely underrepresented in selenium literature and their lack of representation in Se literature should raise concern.

Many analytes are well regulated by aqueous standards, although selenium has been shown to quickly bioaccumulate in aquatic ecosystems via dietary pathways. This tendency to bioaccumulate makes tissue based standards more relevant than aqueous standards for this analyte. Accumulation of Se is largely a result of dietary exposure and maternal transfer via egg yolk proteins. Laboratory Se exposure trials are difficult to conduct and reproduce with environmental realism. Thus, field based observational studies have become the primary study method used to evaluate Se and aquatic ecosystems.

The primary objective of the study was to evaluate the transfer of selenium through several environmental compartments and trophic levels. Due to the costly nature of selenium analysis, broad trophic levels of analysis were defined as periphyton/algae, invertebrates and fish in different feeding guilds. The environmental sources were defined as selenium in sediment and total/dissolved aqueous selenium.

The secondary objective of the study was to assess effects of maternal transfer of selenium on larval deformity, survival, hatch success and larval fitness.

Methods and Materials

Species Selection

A chronic EC10 fry survival value of 21.0 µg Se/g dry weight (dw) in egg/ovary tissue was derived in a maternal selenium transfer study with Brown Trout *Salmo trutta* (Formation Environmental 2011). Cutthroat Trout *Oncorhynchus clarkii* were also studied and an EC10 for fry survival of 17 µg Se/g dw egg tissue was reported (Rudolph et al. 2008). Holm et al. 2005 found EC10 values for larval deformity of Brook Trout *Salvelinus fontinalis* (20 µg Se/g dw egg tissue (EC06)) and Rainbow Trout *Oncorhynchus mykiss* (26 µg Se/g dw egg tissue). Bluegill *Lepomis macrochirus* are also found to be sensitive to maternally transferred Se toxicity. A larval survival EC10 of 22 µg Se/g dw egg tissue (Coyle et al. 1993) and larval deformity EC10 of 21 µg Se/g dw egg tissue (Doroshov et al. 1992) were published for Bluegill. These studies were used to help set the standards for the EPA's 2016 Se water quality criterion. These species represent a significant population of fish managed by Colorado Parks and Wildlife. Several valuable sport fish in Colorado are sensitive to the effects of maternally transferred selenium (Se) toxicity. Sport fish such as Brown Trout are economically and ecologically important to fisheries managers. For certain species in some aquatic ecosystems, removal of gravid females for Se assessment would have negative effects on the reproductive success of the population.

Brown Trout are one of the most abundant and widespread salmonid species in Colorado. They represent a large financial investment by Colorado Parks and Wildlife's aquatic biologists and hatchery managers. In watersheds influenced by legacy mine pollution, Colorado's native Cutthroat Trout are often not able to survive. Lethal sampling of native species would have been unsustainable. Brown Trout are used to fill the niche of native trout to ensure ecosystems are functional and fisheries are maintained. Unlike Brook and Rainbow Trout, Browns cannot hybridize with Cutthroat Trout. This makes reintroduction of native fish easier in the decades to come, where mine pollution reduction efforts prove effective. These valuable sport fish also attract recreational fishing related spending to the State. Their ubiquitous presence in mountain and transition streams throughout the state as well as the ability to perform field spawning and

lethal sampling operations with these fish, made them ideal candidates for a study species. In Colorado, certain Brown Trout fisheries are at risk of being out of compliance with the 2016 USEPA Se criterion, which sets a standard of 15.1 mg/kg dw egg tissue. Brood stocks of Brown Trout such as those in North Delaney Lake in Jackson County, Colorado have exhibited selenium concentrations that exceeded the 2016 USEPA criterion for egg/ovary tissues. This issue is of concern for fisheries managers because North Delaney Lake represents the largest brood stock of Brown Trout in the state and supplies hundreds of thousands of eggs to Colorado hatcheries. The Brown Trout spawn conducted at North Delaney Lake by CPW biologists targets healthy reproductively active individuals, fish that do not meet these standards are returned.

Larval White Sucker *Catostomus commersonii* have been shown to exhibit toxicity to maternal transfer of Se, resulting in larval deformities at EC13 values of 26 µg/g dw egg tissue (De Rosemond et al. 2005). Muscatello and Janz 2009 found elevated levels of edema related deformity when comparing Se impaired streams to reference streams. White Suckers are present in many cold and warm water streams of the eastern and western slopes of Colorado and are an ideal species on which to base a tissue standard. White Suckers not as sensitive as salmonid species. This makes White Sucker ideal because the species is more likely to be present in polluted ecosystems from which other fish species have been extirpated. They are considered an invasive species on the west slope of Colorado where they are not native and present a risk for hybridization with native suckers. Most local fisheries biologists predict the White Sucker is unlikely to ever be reduced in density or range by even intensive lethal sampling. This represents an opportunity to study a ubiquitous and economically unimportant species that is ideal for tissue sampling. If a strong correlation between the Se tissue level of White Sucker and the Se tissue level of ecologically and economically important fish species is found, It allows local biologists and toxicologists an alternative to unsustainable sampling gravid female trout.

Site Selection

Selecting sample sites for the Se study was based on many variables. Optimal sites needed to contain a large population of the target species to facilitate a viable wild spawn take operation. Of the sample locations with reproductive populations of the target species, we selected sites that represented a gradient of selenium concentrations. Historical selenium concentrations in Colorado Department of Public Health and Environment (CDPHE), USEPA and CPW datasets were used to evaluate potential sample locations. Because routes of selenium bioconcentration will differ in lakes and streams, a mixture of lentic and lotic systems were sampled to compare selenium in the same species from different ecosystems.

CPW fisheries managers provided valuable insight and advice on potential spawning locations and spawn timing for our target species. These managers have a unique knowledge of the waters in their management boundaries and were crucial in finding populations of fish to

spawn. Some sample locations were not successful at producing spawning fish and subsequently were abandoned in the study.

Brown Trout were spawned at North Delaney Butte Lake, the Poudre River in Poudre Canyon, Barker Reservoir, Middle Fork of the South Platte River at Tomahawk SWA, Antero Reservoir, Lake Fork of The Gunnison River at high bridge, the Uncompahgre River at N Grand Ave and W 9th St in Montrose, the Arkansas River at Hwy 24 and CR 55 and Colorado River at Elk Creek. Unsuccessful sampling trips were made on the South Platte River downstream of Cheesman Canyon, the Poudre River downstream of the Bellevue/Watson hatchery, the Lake Fork of the Gunnison River at red bridge campground, the Arkansas River near Hayden Meadows Reservoir, the Middle Fork of the South Platte River at upper Buffalo Peaks, and the Uncompahgre River at the Montrose Watersports Park.

White Suckers were spawned at Dillon Reservoir, Willow Creek Reservoir, Wolford Mountain Reservoir, Deep Ward Lake, Alexander Lake, Watson Lake, Chatfield Reservoir, Spring Creek at Edora Park, Parvin Lake, The Colorado River in De Beque Canyon and The Gunnison River at Delta. Unsuccessful sampling trips were made at three locations on Fountain Creek (two sites in Clear Spring Ranch and one site north of Pueblo).

Field collections of viable eggs from sites with historical records of Se concentrations above the proposed EPA standards was difficult. Sites historically considered out of compliance yielded no or limited gravid females above the standard suggesting that fish above federal threshold were absent or undersampled. This study largely relied on the belief that many healthy populations of White Sucker and Brown trout were reproducing at locations that historically would have been out of compliance with the newly released (2016) USEPA selenium standards. Sampling bias may exist because the lack of reproductively active fish with Se tissue concentrations above the proposed limits results in fewer tissue samples that would be out of compliance. This may lead to an abundance of tissue samples under the proposed limits and few samples over.

Collection, Spawning and Rearing of Fish

Wild spawning and lethal sampling of Brown Trout and White sucker were attempted at the various aforementioned locations throughout the state in 2015, 2016, 2017 and 2018 (Figure B1). Wild spawning and lethal sampling operations of trout and suckers are difficult due to the many environmental variables and logistics involved. Most every sample location was revisited multiple times per spawning season in an effort to capture sufficient numbers of fish at optimal reproductive conditions. A population was considered reproductively optimal for sampling when a majority of females were freely expressing ripe eggs and males were freely expressing milt, as detailed below. The reproductive timing of the male and female fish are generally aligned,

although many observations were made of ripe male fish with no gravid female fish found. At sites listed above, field observations were made every 7 to 14 days starting three weeks prior to dates gravid females or spawning behavior were observed by area biologists in year prior.

Rivers and streams were sampled using various combinations of backpack, bank, barge, and raft electrofishing equipment. Gill nets and trap/fyke nets were used to capture fish in lake and reservoir sampling. Electrofishing equipment, nets, boats and spawning/fish handling equipment was provided by Colorado State University (CSU) Foothills Fish Research Laboratory, Colorado Parks and Wildlife (CPW) aquatic biologists (local to sampling locations) and The CPW Aquatic Toxicology Laboratory. When feasible, fish were collected by CPW biologists and technicians during their routine sampling, spawning and disease studies to reduce risk of nuisance species spread. To ensure compliance with state regulations and CSU Institutional Animal Care and Use Committee (IACUC), collection permits were obtained from the state, and detailed IACUC protocols were be submitted to the university and approved before any sampling event. In all cases, efforts were made to capture as near 100% of target species in a reach or sample location. Fish were then sorted for gender and reproductive states. Sites that lacked any target fish species were not resampled and are not reported herewithin.

Sampling events were planned to coincide with peak spawning activity at a given sample location. Brown Trout are fall spawning fish and we concentrated our sampling efforts for them from late September to early December. White Suckers spawn during spring and early summer and sampling ran from early April to late June. There was large variation in the peak reproductive timing for each species based upon the sample location and the water temperature. Regional CPW aquatic biologists provided up to date information on the spawning patterns of fish in their areas, when possible.

Male fish were identified as sexually mature when they freely expressed milt. Applying gentle pressure with the thumb and index finger on the ventral surface behind the pelvic fins was an effective method to express milt from sexually mature male fish. Reproductively active male Brown Trout can also develop kype jaws and have enhanced coloration. The reproductive status of female fish was classified as green, ripe or spent. Green fish may have been gravid with eggs yet still had firm ovaries not expressing any eggs. Ripe female fish will generally have softer ventral surfaces and will freely express eggs when pressure is applied to the ventral surface in front of the pelvic fins. The ovipositor on green female fish will not be as prominent as that of a ripe female fish. The female fish was classified as spent if it was void of most or all eggs. Fish that did not exhibit characteristics of sexually mature or gravid female fish were discarded from sampling because egg tissue would be unavailable. Male fish were not sampled for tissue concentrations of Se. Aging these fish (otolith analysis) and/or sampling muscle tissue of these fish for selenium levels would have been informative, but budgetary restraints limited our ability.

All target species collected were immediately sexed and assessed for reproductive state, then released or sorted in live wells and net pens. Fish collected in the field were spawned on site. Female fish to be spawned were placed in an anesthetic solution of MS-222 (25 mg/L buffered to a pH of 7.0-7.5) until sufficiently anesthetized to reduce stress during handling. Male fish that were not euthanized for tissues were not anesthetized during spawning to prevent accidental human consumption of MS-222 in fish tissues after release. Weight in grams (g) and total length in millimeters (mm) were measured before spawning. The spawning procedures followed standardized CPW methods for Brown Trout and Walleye. Eggs were stripped from individual females into a clean and dry spawning pan and the egg mass was weighed. Milt was stripped from 2 - 3 male fish and added to the pan along with 500 ml of filtered water. For White Sucker eggs a mixture of 500 ml filtered water and 20g of powdered bentonite clay was added to the pan to reduce egg wall adhesion and reduce egg clumping. The eggs, milt and water were gently mixed with a feather for two minutes until the mixture was completely homogenized. The mixture was decanted with filtered water for two minutes until the water was clear and free of any milt or clay. Bad eggs and visceral masses were removed from the egg mixtures. These were not considered mortality and were a negligible amount to affect fecundity or survival. It is typical in all spawning to have a limited number of unsuccessful eggs. Fertilized eggs were placed in two-liter coolers with 500 ml fresh filtered water and then placed in a large cooler. The temperature in the large cooler was regulated with ice and water to keep temperatures equal to water in the spawn location. The temperature of the water in the egg coolers was checked every few hours during long transports to ensure consistency.

After the female fish were spawned, they were euthanized in a solution of MS-222 (250 mg/L buffered to a pH of 7.0-7.5). A muscle tissue fillet was taken from one side of the fish and placed in a labeled zippered top polyvinyl bag. Unfertilized egg tissue samples (~20 ml) were taken from each egg batch and placed in 50-mL centrifuge tubes. In the EPA Se criterion, egg and ovary tissues are regarded as synonymous. The remains of fish was placed on ice for transport and then transferred to a freezer until needed for future tissue extraction.

Eggs were transported directly to the CPW Aquatic Toxicology Laboratory in Fort Collins, CO. After the eggs were disinfected and acclimated to the temperature in the egg rearing bays they were placed in specialized egg rearing cups (Figure B2). Egg cups received temperature controlled flow-through dechlorinated tap water at 50 ml per minute. Water was delivered from low pressure 3-5 cm adjustable head nozzles located approximately 10 cm above the water level. The temperature of the water in the egg transport coolers was slowly acclimated over one hour to the constant 10°C (Brown Trout)/12°C (White Sucker) of the bath in the egg rearing bay. The eggs and sac fry were incubated at these temperatures for the remainder of the experiment. These rearing temperatures were selected based on the optimal rearing temperatures for each species and the chilling capacity of the laboratory (Realis-Doyelle et al. 2017; Najafpour et al. 2019;McCormick et al. 1977). To help prevent the development of fungus on the eggs, a

disinfecting formalin bath of 1600 ppm for 15 minutes was administered before transfer to the egg cups. This bath was repeated weekly in the rearing bay until hatching was observed. Wide spectrum florescent lights on timers provided 16 hour on/8 hour off photoperiod.

The egg cup design ensured that all eggs are submerged in five cm of water. Approximately 100 eggs were placed in each cup so that a single layer of eggs formed on the mesh bottom. In the first 24 hours, eggs were evaluated and any unfertilized eggs were removed from the cup before an initial picture and egg count was taken. Egg counts were verified with photographs and adjusted. The number of eggs per cup was limited to 100 due to the risk of fungal outbreaks and to reduce difficulty in egg maintenance. The eggs from a single female fish were separated into two egg cups and placed randomly in the bay to account for small differences in temperature and light exposure throughout the bay. Hatch success and other fitness measures for a female were reported as an average if eggs were split to multiple egg rearing baskets. All eggs not utilized in rearing were used for measurements and frozen for future tissue analysis. Average egg diameters and weights were recorded from a sample of approximately 50 eggs laid end to end and positioned in a straight line along a ruler and then weighed on an Ohaus analytical balance (0.00001g +/-).

Daily egg care consisted of removing any eggs that showed signs of fungal growth or mortality. Egg and fry mortalities were placed in Davidson's fixative solution. Temperature was taken daily and any physiological changes in the eggs such as eye-up and movement were noted. Water quality measurements of pH, alkalinity, hardness, dissolved oxygen and conductivity were measured randomly in three cups on a weekly basis. Numbers of successfully hatched fry were recorded daily until complete hatch. Once all sac-fry hatched they were transferred to the 500 ml Tripour® section of the egg cup and the hatching basket was removed. The fry were observed daily for progress of yolk sac absorption and transition to the swim-up (exogenous feeding) stage.

The end of the yolk sac absorption stage is the most critical period for the appearance of selenium related teratogenic issues in the larval fish as selenium rich vitellogenin, an egg yolk precursor protein and primary food source, is consumed as the yolk sac is depleted (Kunz 2004). As the fish start to exhibit exogenous feeding the potential for teratogenic effects rapidly declines as exogenous food sources are generally low in Se concentration in comparison to vitellogenin in the yolk sac (Lemly 1997). Half of the fish in each egg cup were euthanized and preserved in Davidson's solution for deformity analysis. The second half of the larval fish were used immediately for fitness endpoint testing.

Sediment, Periphyton, Invertebrate and Water Sampling

If sampling conditions would allow, at each of the field locations where fish tissues and eggs were collected four (replicate) samples of sediment, algae/periphyton, invertebrates, total aqueous selenium and dissolved aqueous selenium were taken. These four samples were taken at different points throughout the same reach or location sampled for fish. Samples were taken for all sites in the spring and the fall to evaluate changes in selenium concentrations due to seasonal changes in flows, water chemistry, bioconcentration and bioaccumulation at different trophic levels. If funding permits in the years to come, evaluation of lower trophic levels of Selenium should be assessed at field locations void of fish and sites unable to produce gravid females. Such assessments were not possible given the temporal, budgetary, and staffing limitations of this study.

Bed sediment samples were taken using spoon, scoop or spatula methods prescribed by Shelton and Capel (1994). Depositional areas were targeted in certain streams due to lack of fine sediment. A minimum sample of 30 ml was placed in a 50 ml centrifuge tube. Sediment samples were placed on ice during transport and stored in a freezer until needed for analysis.

Periphyton in lentic and lotic systems was collected as growth on rocks, large wood, substrate, or free floating algal masses. A single edge stainless steel razor blade was used to scrape periphyton into 50 ml centrifuge tubes. Large rocks and wood structures were targeted. All samples of algae and periphyton were placed in large plastic trays and mixed with a small amount of water from the site. All visible invertebrates were removed from the sample using tweezers and micropipettes. The remaining water was decanted off the sample and a 30 mL sample was taken and preserved as described above.

Invertebrates were collected with a variety of methods including kick nets, D-frame nets, Surber samplers, trawls and seines. Each sampling method targets a particular type of habitat. Kick and D-frame nets are limited to high gradient lotic systems. The contents of the nets and samplers were rinsed with site water into large white plastic trays. All invertebrates were picked out by hand with tweezers and transfer pipettes and placed in 50 ml centrifuge tubes. As many taxa as possible were collected and placed into a composite sample. To meet minimum analytical requirements we attempted to collect 5g total invertebrate samples. A majority of the water was decanted off the samples and they were processed as described above.

Water samples of total and dissolved selenium were taken using standardized USEPA methods (United States Environmental Protection Agency 1983). All dissolved samples were run through a 0.45 micron filter. A minimum of 250 ml was necessary to meet minimum analytical requirements. Water samples were placed in pre-acidified 250 ml clear plastic Nalgene® polypropylene bottles supplied by ACZ laboratories in Steamboat, Colorado. The water samples

were acidified with nitric acid (HNO3) to a pH no greater than two. A secondary batch of total and dissolved water samples was taken in the spring and summer of 2018 and sent to Colorado Department of Public Health and Environment (CDPHE) labs for analysis. These tissue and water samples were processed and analyzed using the methods listed in Table 1.

Water Quality, Nutrient and Benthotorch Sampling

Water quality parameters were taken at each sample location including dissolved oxygen (percentage and mg/L), pH, conductivity (µS/cm) and temperature (°C). A YSI ProODO meter was used to measure dissolved oxygen. Conductivity and pH were measured with an Oakton pH/CON 300 meter. Temperature was measured with a YSI temperature probe calibrated to a secondary YSI temperature probe used in the egg rearing bays in the CPW laboratory.

In 2018 water samples were taken at each sample site for nutrient analysis and sent to Colorado Analytical Laboratories in Commerce City, Colorado. The nutrients analyzed were ammonia nitrogen (mg/L), chloride (mg/L), dissolved organic carbon (mg/L), nitrate nitrogen (mg/L), nitrate/nitrite nitrogen (mg/L), nitrite nitrogen (mg/L), phosphate ortho as P (mg/L), phosphate ortho as PO4 (mg/L), phosphorus total (mg/L), sulfate (mg/L), total Kjeldahl nitrogen (mg/L), total nitrogen (mg/L), calcium (mg/L), magnesium (mg/L), potassium (mg/L) and sodium (mg/L). The samples were placed in plastic Nalgene® bottles provided by Colorado Analytical Labs and immediately placed in iced coolers for transport and delivery to the laboratory. Methods used for the nutrient analysis are listed in Table 1.

In the summer of 2018, Benthotorch sampling was used to determine ratios of Green Algae, Cyanobacteria and Diatoms. Benthotorch readings were taken in pelagic and littoral zones in lentic systems and along margins and center channels in lotic systems. Discrepancy between species counts, HPLC analysis, and fluorimetry (such as used here) are often a byproduct of substrate and thickness of periphyton. Benthotorch observations limit assessment to the surface of periphyton mats that is permeable by light. Three littoral readings in lentic systems or three margin readings in rivers were taken between water's edge and the maximum depth of the instrument (10 -48 cm). Three pelagic lake readings or three river main channel readings were taken from 200 ml and 100 ml water samples filtered through filter paper. Temperature (°C), pH, conductivity (µS/cm) and dissolved oxygen (% saturation) were recorded during each sampling event.

Experimental Endpoints

Recent selenium studies involving maternal transfer of selenium have used larval deformity, survival to swim-up life stage and hatch success as viable study endpoints (Muscatello and Janz 2009; McDonald et al. 2010; Formation Environmental 2011; Formation Environmental and

Habitech 2012; Thomas and Janz 2014). These traditional endpoints were included in our field assessments by rearing eggs collected as part of state-wide sampling. Additionally, a suite of endpoints were included to assess the survival and fitness of progeny (fast start response, bolt speed, thermal tolerance and low dissolved oxygen tolerance. Increased water temperatures and low dissolved oxygen levels predicted in Colorado as a byproduct of anthropogenic climate change present a huge risk to Colorado's fish. To address climate change risks, this study assessed thermal tolerance (CTM or CT_{max}) and tolerance to low Dissolved Oxygen (CDOM or Critical $DO_{minimum}$).

Survival and Hatch Success

Hatch success and survival was evaluated for each female's egg clutch. Hatch success was defined as the proportion of larval fish that has shed its entire egg casing and survived the entire hatching process. Survival was defined as the proportion of fish reaching the swim-up stage either from original egg count or hatched fish count.

Fast-start Response

The ability to escape from predator-prey encounters is essential for larval fish survival and these survival mechanisms can be slowed or weakened by contaminants in the environment. Tudorache (2008) found that Brown Trout acutely exposed to elevated concentrations of ammonia (1 mg/L) experienced reduced fast-start velocity. Juvenile *Spinibarbus sinensis* showed significantly reduced linear acceleration when exposed to 0.8-mg/L perfluorooctane sulfonate (Xia et al. 2015). Kimmel et al. (1980) irradiated larval Zebrafish *Danio rerio* and observed reduction or absence of the Mauthner neurons, due to irradiation, caused reduced fast-start performance.

To assess possible selenium-related reduced fast-start response behaviors in post swim-up larval fish, high speed recordings were taken of fast-start responses after an introduced stimulus. If numbers of swim-up fish were available, 20 fish from each egg cup and a total of 40 fish from each female were used in the fast-start tests.

The fish were placed into the startle response tank and held at 10°C (Figure B3). The startle response tank measured 15 cm wide x 30 cm deep x 30 cm long and had a 1 cm grid under the glass bottom to aid in photo analysis. To keep the temperature consistent throughout the test the tank was placed in a cold water bath fed by a water chiller. The dissolved oxygen in the tank was saturated to 100% with an air stone before a fish was added. The air stone was removed prior to adding a fish to the tank. The water level in the tank was held at a depth of 5 cm to limit vertical movement in the startle response. The tank was surrounded by curtains to ensure no outside stimulus. On one side of the tank a 5 cm by 5 cm hole was cut in the curtain to allow for a

window where the stimulus was to be introduced. On the top of the tank two holes were cut in a 1.5 cm thick foam sheet, taped to the curtain, which fit tightly around the lenses of the two cameras used. A tethered tennis ball was mounted so that it could be released remotely from a standardized height and allowed to swing, with a pendulous motion, into the side of the tank making contact at the window. A bright incandescent bulb in a flood light fixture was mounted underneath the chilling bath and tank to provide enough light on the 1 cm square grid to clearly evaluate fish movement in the videos. A box fan was used to ventilate the area under the tank to prevent rapid heating of the tank water from the light fixture. Fluorescent bulbs mounted under the tank were found to provide sufficient light, however under the playback of the high speed video the strobe effect from the fluorescent bulb made evaluation of the videos very difficult. To start the test the fish were given a minimum of 15 minutes acclimation. The stimulus was introduced after the acclimation period only if the fish was located in the half of the tank closest to the window and the anterior end of the fish was oriented within a 180° view of the window. If the fish failed to meet these criteria at 45 minutes, a secondary fish was selected and the processes repeated.

Fast start trials were captured using a dual video camera system. A 720p Logitech webcam and a high speed Casio Exilim EX-F1 (recording at 400 fps) were mounted above the tank and the focus was adjusted to capture the entire grid on the bottom of the tank. The webcam was used to monitor the fish over the acclimation period and ensure that the fish was correctly oriented before releasing the tennis ball. In order to keep the video file size manageable the high speed recording was started right before the tennis ball was released. The high speed camera was able to capture the formation of a C or S fast-start shape and the resulting movement. The videos were analyzed using Media Player Classic version 1.7.13. A time to initial movement response from stimulus, time to formation of full C or S shape, resulting movement after C or S shape formation and bolt speed were recorded.

After the startle response was recorded, the fish was immediately placed into an individual recovery net pen held at 10°C for 24 hours and survival was recorded. The recovery pens were fully saturated with dissolved oxygen to help reduce stress response. After recovery, the fish were euthanized in a MS-222 solution (250 mg/L buffered to a pH of 7.0-7.5) and measured for total length, standard length and wet weight then preserved in Davidson's fixative.

Critical Thermal Maxima

If numbers of swim-up fish were available, 10 fish from each egg cup and a total of 20 fish from each female were used in the tests of critical thermal maxima (CTM). These tests were based on methods recommended by Becker and Genoway (1979). A CTM testing apparatus was designed to test up to four larval fish at the same time (Figure B4).

The critical thermal maximum tests were conducted in a series of four rectangular glass tanks (18 x 9 x 12 cm). Individual fry were transferred to the tanks containing 1.75 L of water at rearing cup temperature. A temperature controller/programmer (B-series Love Controls Division, Model number: 16b) was wired to a submersible Aqueon 100w aquarium heater and a titanium temperature probe. After an acclamation period of five minutes the water was heated at a rate of 0.3 °C/min. Vigorous aeration of the tank with a small air stone maintained saturated dissolved oxygen levels and generated enough current in the tank to ensure a homogeneous temperature throughout the tank. Water temperatures were increased until the fish displayed loss of equilibrium (LOE) for 10 seconds. LOE was defined as failure to maintain a dorsal-ventral vertical orientation. Once a fish lost its ability to maintain equilibrium, the temperature of the water was recorded and the fish was removed from the experimental tank and placed into a 2.5 gallon recovery tank containing 2000 ml water at the acclimation temperature. Each fish was monitored for twenty minutes in order to ensure immediate test survival and return of equilibrium. The fish was then transferred to an individual recovery net pen held at 10°C for 24 hours and survival was recorded. The recovery pens were fully saturated with dissolved oxygen to help reduce stress response. After recovery, the fish were euthanized in a MS-222 solution (250 mg/L buffered to a pH of 7.0-7.5) and measured for total length, standard length and wet weight then preserved in Davidson's fixative.

Critical Dissolved Oxygen Minima

If numbers of swim-up fish were available, 10 fish from each egg cup and a total of 20 fish from each female were used in the tests of critical dissolved oxygen minima (CDOM). A CDOM testing apparatus was designed to test up to four larval fish at the same time (Figure B5).

The CDOM tests were conducted in rectangular glass tanks (18 x 9 x 12 cm). Water in the tanks was kept saturated with dissolved oxygen (DO) at the start of the test by a small air stone that was shut off after an acclimation period. Nitrogen gas bubbled through wood air stones was used to displace the oxygen in the water. Wood air stones produce finer bubbles than traditional air stones and increase gas diffusion rates. Dissolved oxygen was removed at a rate of 1.0 L/min/hr. To keep oxygen level and temperature homogenized in each tank insulation surrounded 5/8 of the aquaria and the water was homogenized with a stir bar. The temperature in each tank was held constant with a titanium heat sink circulated with chilled water (5° C) from 22 L insulated cooler filled with ice water. The temperature in each test tank was regulated by four temperature controllers (Model: 16b. Love Controls Division. Dwyer Instruments Inc. Michigan City, Indiana, USA) wired to a titanium temperature probe in the test tank and individual aquarium pumps in the 22 L ice bath. Individual fry were transferred to the tank containing 2.0 L of oxygen saturated water at the acclimation temperature for five minutes after which the flow of nitrogen gas was started and the air bubbler was shut off. A calibrated YSI proDO digital dissolved oxygen meter was used to monitor saturation percentage and dissolved

oxygen concentrations (mg/L) in each tank. The test was run until the fish exhibited LOE as defined in the CTM testing. At this time the DO percentage, concentration and testing time were recorded and the fish was immediately placed into a 9.4 L recovery tank containing 2000 ml water at the acclimation temperature fully saturated with DO. Each fish was monitored for twenty minutes in order to ensure immediate test survival and return of equilibrium. The fish was then transferred to an individual recovery net pen held at 10°C for 24 hours and survival was recorded. The recovery pens were fully saturated with dissolved oxygen to help reduce stress response. After recovery, the fish were euthanized in a MS-222 solution (250 mg/L buffered to a pH of 7.0-7.5) and measured for total length, standard length and wet weight then preserved in Davidson's fixative.

Larval Deformity Assessment

All of the fish assessed for CTM and CDOM along with the fish assessed for fast-start response were analyzed for larval deformity. If numbers of swim-up fish were available, 20 additional swim-up fish from each egg batch were analyzed for deformity. The deformity analysis was modeled after Dr. Kevin Bestgen's (Colorado State University, Larval Fish Laboratory) method for assessing deformities of physical features along a graduated (four-level) graduated severity index (GSI) (Formation Environmental 2011). These methods use the same basic structure as Holm et al. (2003) and Holm et al. (2005) and focused on craniofacial deformity, skeletal deformity, fin deformity and edematous deformity. A scoring criterion was developed for each of these four main deformity categories and their sub categories (Figure B6).

Data Analysis and Processing

Results and trends included here within are preliminary or precursory. A more holistic statistical analysis will be conducted in the 2020-2021 fiscal year including predictor variables outlined in Table 5.

Selenium Trophic Transfer Models

All of the trophic level data collected were used to model the movement of Se through the aquatic food webs of the sample sites. To evaluate the concentrations of Se in these food webs, bioaccumulation factors (BAF) were calculated from each food web transfer step ([Se organism]/[Se food Source]). Bioconcentration factors (BCF) were calculated from the dissolved Se concentrations in water to Se concentrations in invertebrates, fish muscle and fish eggs ([Se organism]/[Se dissolved water]). Enrichment factors (EF) were calculated as [Se periphyton] or [Se sediment]/[Se dissolved water]. Egg to muscle conversion factors (CF [egg]/[muscle]) were calculated for Brown Trout sites and White Sucker sites. These steady state models were created for trout and suckers across all individual species sample sites. The Se concentrations were

calculated as averages of all samples taken over the course of the study. This was a preliminary assessment of the food web data and further assessment needs to incorporate habitat data and other covariates. A preliminary comparison of the BAF and EF results to data used in the USEPA model was conducted.

Results

We compared the Se concentrations observed in fish eggs/muscle and water to the 2016 USEPA Criterion standards (egg = 15.1 mg/kg dw, muscle filet = 11.3 mg/kg dw, lentic water = 1.5 µg/L and lotic water = 3.1 µg/L). From all data we collected, only eight exceedances of the USEPA Se criterion were found. In 2015, three egg tissue samples taken from individual female Brown Trout at North Delaney Lake yielded Se concentrations (mg/kg dw) of 19.3, 17.6 and 16.9. In 2018, one female White Sucker muscle filet from the Gunnison River in Delta, CO had a Se concentration of 14.19 mg/kg dw. The highest dissolved water Se concentrations observed were from the Gunnison River in Delta, CO and the Colorado River at Main Elk Creek in Newcastle, CO (Figures B11 and B12). Two dissolved Se water samples from the Colorado River at Main Elk Creek (4.68 µg/L and 4.42 µg/L) and two dissolved Se water samples from the Gunnison River in Delta, CO (3.61 µg/L and 3.46 µg/L) were above the lotic standard. Although the USEPA criterion is based on dissolved Se water concentrations, it is worth noting that total Se water samples from the Gunnison River in Delta, CO (5.33 µg/L and 3.68 µg/L), the Colorado River at Main Elk Creek (3.51 µg/L and 3.44 µg/L), Wolford Mountain Reservoir (2.12 µg/L and 1.77 µg/L), Watson Lake (1.7 µg/L) and Chatfield Reservoir (1.5 µg/L) were recorded. It is important to consider that a priori, CPW and CDPHE believed ample parents (gravid females) would be in exceedance of the proposed USEPA standard given the history of high tissue concentrations from sites such as North Delaney Butte Lake. It is possible the four years of this study co-occurred with low Se levels. It is also possible that fish above the USEPA tissue standard were not fertile/gravid enough for spawning and therefore were not collected. Randomization and non-bias sampling of fish is difficult when sample size is limited, and bias is introduced when only gravid females are collected. Assessing tissue levels of the population at large were beyond the scope of this study which aimed to establish TTFs for Colorado, build baseline tissue concentrations of ovary-egg masses for Colorado, and find correlation between tissue burden of parents and sublethal or lethal effects of offspring.

On average the concentrations of Se in North Delaney Brown Trout eggs (10.15 mg/kg dw) were higher and had a greater range in values (5.43 mg/kg dw to 19.33 mg/kg dw) than any other Brown Trout or White Sucker sample site (Figures B7 and B8). The egg Se concentrations at the White Sucker sample sites were generally lower than at the Brown Trout sites (Figures B7 and B8). The egg Se concentrations from White Sucker at Wolford Mountain Reservoir were significantly higher than any other White Sucker sample site (Figure B8). Figures B7 and B8

show higher amounts of variation in the egg Se concentrations for Brown Trout sites versus White Sucker sites.

Muscle tissue Se concentrations were more similar among Brown Trout and White Sucker sample sites than the egg Se concentrations (Figures B9 and B10). The largest range in muscle tissue concentrations was observed for White Sucker in the Gunnison River in Delta, CO (2.69 mg/kg dw to 14.19 mg/kg dw). The highest average muscle Se concentration was recorded at North Delaney Lake (6.73 mg/kg dw).

Table B2 shows BAF and BCF calculations for all White Sucker and Brown Trout sample sites as well as average values for all sample sites of each species. Brown Trout have positive BAF values for transfer from invertebrate to fish egg (2.77) and invertebrate to fish muscle (1.47) (Table B2), showing a tendency towards bioaccumulation in the last step of Se trophic transfer to Brown Trout. The opposite is seen for White Sucker BAF values of transfer from invertebrate to fish egg (0.726) and transfer from invertebrate to fish muscle (0.623) (Table B2).

Average periphyton Se concentrations at White Sucker and Brown Trout sample sites were elevated compared to concentrations in water, sediment, invertebrates, fish muscle and fish egg (Figures B13 and B14). A BAF of 0.33 for accumulation between periphyton and invertebrates in Brown Trout sites indicates negative accumulation in this step, however a BAF of 2.77 for accumulation between invertebrates and fish egg tissue in trout sites indicates a large accumulation in the last step of the trophic transfer model (Figure B15). The White Sucker sample sites had a similar BAF of 0.35 for accumulation between periphyton and invertebrates (Figure B16). The BAF of 0.73 for accumulation between invertebrates and fish egg tissue in White Sucker sites differs from Brown Trout sites and indicates a loss of total Se in this final step of the trophic transfer model (Figure B16).

Brown Trout sample sites had a mean EF (dissolved Se in water to Se in periphyton) value of 12.81 L/g (Figure B15) and White Sucker sample sites had a mean EF value of 16.55 L/g. Mean EF values of 1.03 L/g (Brown Trout) and 0.94 L/g (White Sucker) were calculated from the transfer of dissolved Se in water to sediment (Table B3). The EF sediment values were much lower than the EF periphyton values at sample sites where both species were collected. Trophic transfer functions (TTF) were calculated for the transfer of Se from invertebrate to fish tissues (average of all fish egg and muscle tissue Se concentrations) from our data (TTF Trophic Level 3) (Table B3). Data from this study indicates Brown Trout had over triple the TTF Trophic Level 3 (2.15) of White Sucker (0.66) (Table 3). A composite TTF was calculated as the product of TTF Trophic Level 3 and TTF Trophic Level 2 (TTF of Se transfer from periphyton to invertebrates) (Table B3). Again the TTF composite value for Brown Trout (0.71) was over

triple the value for White Sucker (0.23) (Table 3). Table 3 also shows the conversion factors (CF) calculated as average egg concentration divided by average muscle concentration.

Table B3 also highlights these same metrics used in the 2016 USEPA selenium criterion. CF values calculated from this study (1.88 for Brown Trout and 1.16 for White Sucker) are close to the median CF values from the USEPA criterion (1.81 for Brown Trout and 1.0 for White Sucker) (Table B3). Table 3 shows the difference between the EPA TTF Trophic Level 3 (1.38 for Brown Trout and 1.11 for White Sucker) and our TTF Trophic Level 3 values (2.15 for Brown Trout and 0.66 for White Sucker). For both Brown Trout and White Sucker the USEPA's TTF composite values (2.78 and 1.58) were much larger than calculated values in this study (0.71 and 0.23). The EF periphyton values from this study (12.18 for Brown Trout and 16.55 for White Sucker) were much larger than the average EF from studies used in the USEPA model (0.88 for Brown Trout and 0.851 for White Sucker) (Table B3). The USEPA values were more aligned with our EF sediment values (1.03 for Brown Trout and 0.94 for White Sucker) (Table B3).

Discussion

Data collected in this observational study provides a large set of predictor variables such as selenium concentrations, environmental factors, habitat types, parent fitness, water chemistry and isotope ratios (Table B5). Ultimately this data will be used in multiple-regression analysis or creation and selection of large mixed models to better inform and predict various response variables (Table B4). The analysis provided in this report was precursory.

The trophic transfer metrics calculated in this study were compared to the values calculated in the USEPA selenium criterion. Egg Se to muscle Se CF ratios derived from this study (Brown Trout 1.88 and White Sucker 1.16) were similar to values from the USEPA criterion (Brown Trout 1.81 and White Sucker 1.0) (Table B3). For this preliminary analysis we calculated CF egg to muscle as the mean of all fish egg tissues divided by the mean of all fish muscle tissues from all Brown Trout or White Sucker sites. The USEPA document states that their CF ratios are median ratios from matched pairs of measurements from the studies used in the standard (United States Environmental Protection Agency 2016). This result suggests that the ratio of egg to muscle selenium we are seeing for these two Colorado fish is comparable to fish from studies across the country. We found this similarity in egg to muscle CF striking due to geographic disparities in selenium concentrations for these species. Compared to the United Sates as a whole, Colorado Se data comes from systems that are more oligotrophic in nature.

The USEPA derived their composite TTF parameter from numerous trophic transfer models each with various levels and complexities of the food web (see Figure 3.4 of United States Environmental Protection Agency 2016). To best compare the USEPA parameters to our

calculations our data was fitted to the simple three trophic level USEPA model. The second step in this model is designated TTF^{TL2} (periphyton to invertebrates) and the third step in the model is designated TTF^{TL3} (invertebrates to fish). The composite TTF was the product of TTF^{TL2} and TTF^{TL3}. The TTF^{TL3} value for Brown Trout in this study (2.15) was larger than the USEPA value (1.38) suggesting that bioaccumulation for Brown Trout in the last step of this model was increased compared to fish in studies used by the USEPA (Table B3). The composite TTF calculated from this study (0.71) was much lower than the USEPA value (2.78) (Table B3). This difference may be partly explained by the larger average Se concentrations measured in periphyton (Figure B15). Based on the USEPA model the high periphyton concentrations measured in our data caused lower composite TTF values.

White Sucker TTF^{TL3} values from this study (0.66) were lower than the value of the USEPA criterion (1.11) (Table B3). The composite TTF calculated for White Suckers in this study (0.23) was lower than the value of the USEPA criterion (1.58) (Table B3). Similar to the Brown Trout in this study the difference in TTF values may be explained by the larger average Se concentrations measured in periphyton at White Sucker sites (Figure B16).

The 2016 USEPA selenium standard used "particulate" Se concentrations for their EF values. The field particulate measurements the USEPA included were a collection of sediment, periphyton and detrital samples which were then correlated to water column measurements taken within one year of the "particulate" sampling event. The USEPA used median particulate matter ratios for any site that had more than one type of particulate measurement (periphyton, sediment and detritus). The USEPA used data from all three particulate types in their EF calculations. Table 3 shows the averages of all the EF values reported for White Sucker or Brown Trout in the USEPA criterion (Appendix I in United States Environmental Protection Agency 2016). From the studies listed in the Appendix I table of the USEPA criterion a mean EF for Brown Trout (0.88) and a mean EF for White Sucker (0.85) were calculated (Table B3). These values are close to the EF (sediment) values in Table 3 calculated for this study (1.03 for Brown Trout and 0.94 for White Sucker). The EF (periphyton) values calculated from data in this study (12.18 for Brown Trout and 16.55 for White Sucker) were much higher than the USEPA values or the sediment values from this study. These results are surprising and suggest that the periphyton Se concentrations seen in Colorado are much higher than the concentrations seen in the studies used in the USEPA criterion. Even if we were to take the median value of all our particulate Se measurements (periphyton and sediment) it would still be a great deal higher than the values of the USEPA standard.

Prior to the initiation of this study, it was long believed that populations of Brown Trout with muscle tissue concentrations well above proposed (2014 and 2016) USEPA standard were present in some of CPW's most productive brood stock. In hopes of bracketing a toxic threshold value for fitness, survival, or deformity endpoints, this study made extensive efforts to include

gravid females of White Sucker and Brown Trout that were above the USEPA tissue based criterion value. Despite this effort, few organisms were found with tissue concentrations above the criterion value. This suggests populations above the standard might be absent from our waters or that gravid females were absent from our field sites or were missed by our sampling methods as highlighted above. This may also suggest that our sampling methodology excluded fish that may have been of reproductive age yet had poor reproductive condition. This is important to note for numerous reasons. 1) If a tissue-based standard is to be adopted, Colorado should have a non-biased way to sample all fish present, not just those that appear to be female because of morphology indicative of egg bearing gravid females or female fish with substantially higher quality egg clutches. Because the egg based standard is considered optimal, targeting only those fish with obvious egg development could lead to sampling bias and a falsely low value of selenium might be obtained. 2) Precursory relationships presented here within show limited correlation perhaps not just because covariants (Table B5) are not yet included in multiple-regression analysis, but because a vast majority of fish sampled were below the toxic threshold. 3) The toxic threshold for Colorado's waters could be a close fit to the proposed USEPA criterion value prescribed to the nation. If this is true, little margin of safety is afforded.

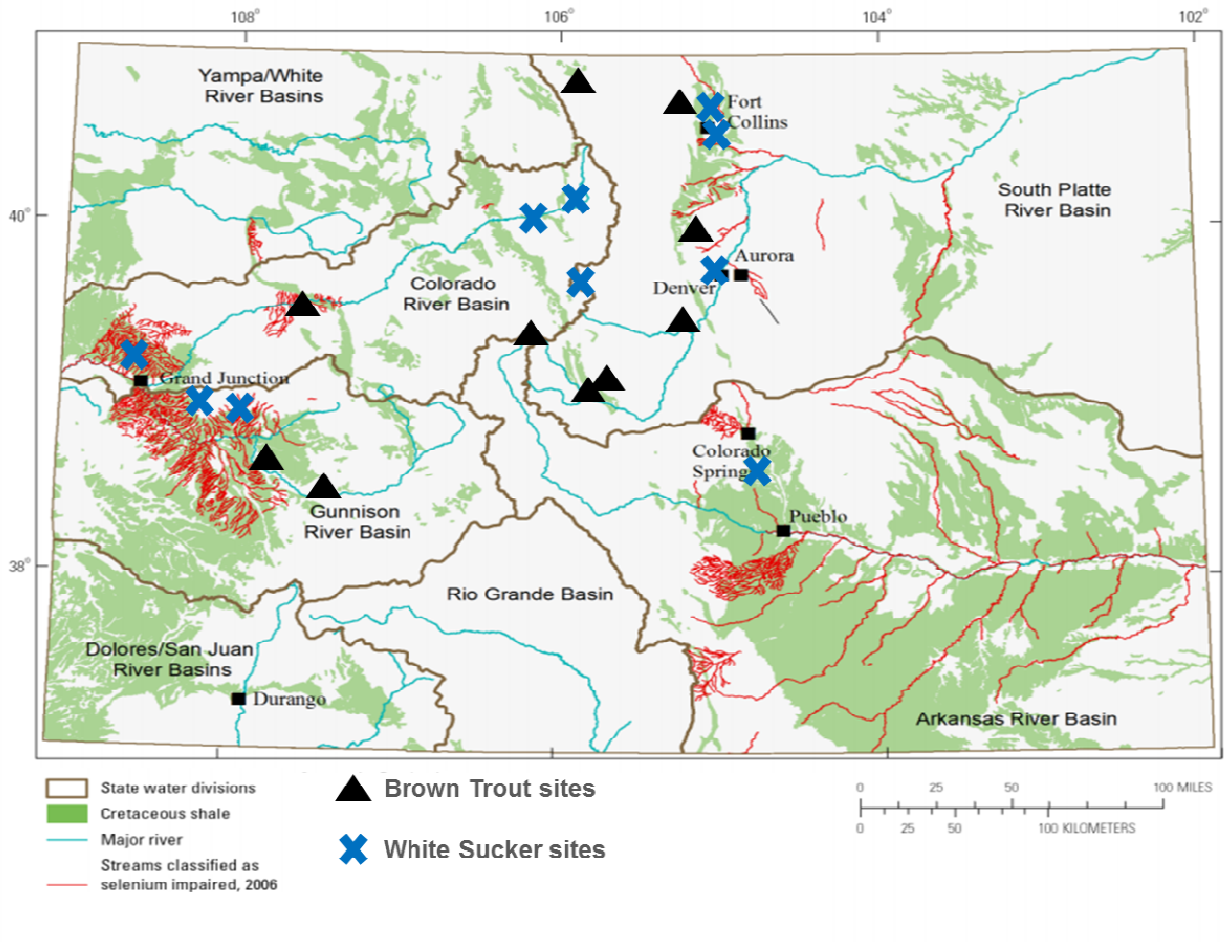

Figure B1. Sampling locations for Brown Trout (black triangle) and White Sucker (blue X) across the state of Colorado. Green areas signify an underlying geology that includes cretaceous shale formations and red lines denote streams classified as selenium impaired as of 2006. The brown lines denote state watershed divisions. Brown Trout sites: Antero Reservoir, Arkansas River at CR55, Barker Reservoir, Colorado River at Elk Creek, Lake Fork of Gunnison River at High Bridge, Middle Fork South Platte River at Tomahawk SWA, North Delaney Lake, Poudre River in Poudre Canyon, Uncompahgre River in Montrose. Brown Trout collection attempted at South Platte River (Deckers, CO). White Sucker Sites: Alexander Lake, Chatfield Reservoir, Colorado River (De Beque, CO), Deep Ward Lake, Dillon Reservoir, Gunnison River (Delta, CO), Parvin Lake, Watson Lake, Willow Creek Reservoir and Wolford Mountain Reservoir. White Sucker tissue collection attempted at Fountain Creek (Fountain, CO). The map has been modified from Paschke et al. 2014.

Figure B2. Egg rearing cups are constructed out of a 50 mm internal diameter PVC coupling approximately 7 cm long with a 500 micron mesh glued to the bottom. Two plastic dowels are glued to the top of the egg cup and act as supports holding the cup in a 500 ml Tripour® disposable beaker. Each Tripour® beaker had a 3.0 cm hole drilled near the top of the Tripour®, covered with a small piece of 500 micron mesh. The bottom of the Tripour® discharge sits 3 cm above the water level in the bay to prevent backflow. Water is delivered to each cup via a spray bar with adjustable jets at a rate of 50 ml/min.

Figure B3. Fast-start response testing apparatus. A 1 cm grid was imposed on the bottom of the test tank and the test tank was placed in a chilled water bath maintaining temperature (10°C) throughout the test. A 200w incandescent bulb was used illuminate the underside of the testing apparatus. To elicit a startle response the tennis ball was remotely triggered to fall from a standardized height striking the tank in the small curtain opening. Tests recorded at 400fps with Casio Exilim EX-F1 and monitored with Logitech HD 720p webcam.

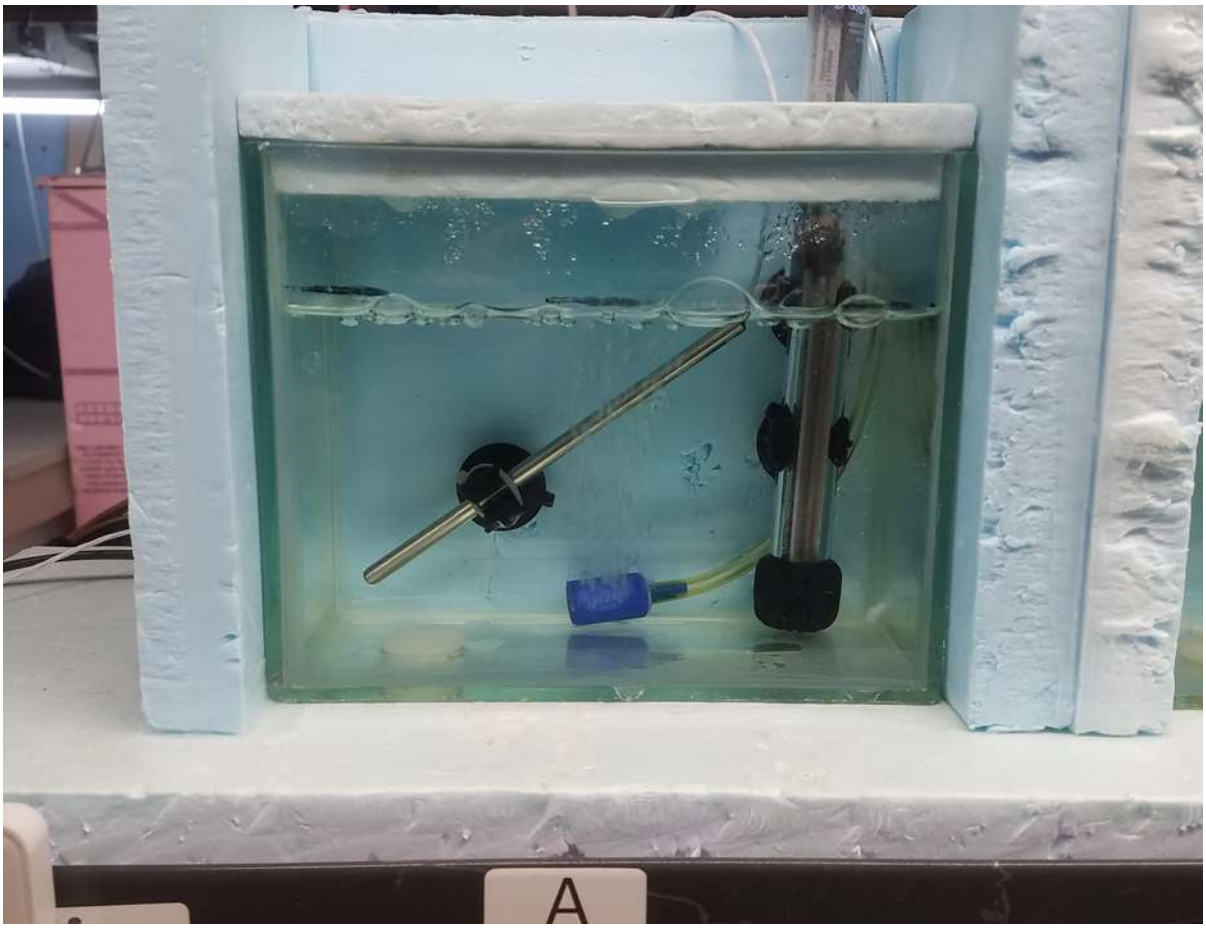

Figure B3. Critical thermal maxima (CTM) test tank. Fish were placed in the test tanks at their holding temperature (10°C). After an acclimation period the temperature was increased 0.3 °C/min until the fish displayed sustained loss of equilibrium. Love Controls series 16b temperature controllers fitted with titanium heat probes and 100w aquarium heaters were programmed to adjust the temperature. Dissolved oxygen levels were held constant throughout the test with an adjustable air stone.

Figure B5. Critical dissolved oxygen minima (CDOM) test tank. Fish were placed in the test tanks at their holding temperature (10°C). Temperature was held constant throughout the test and regulated by a Love Controls series 16b temperature controller. The controller was hooked to a titanium heat probe and a submersible aquarium pump which was placed in an ice bath. The controller was programmed to run chilled water from the ice bath through a titanium tube in the test tank. Oxygen levels and temperature were homogenized throughout the tank with a 2 cm magnetic stir bar and stir plate mounted under the test tank. Nitrogen was bubbled in the test tank through a fine wood air stone to displace the oxygen at a rate of 1.0 L/min/hr until the fish displayed sustained loss of equilibrium.

Sample #: _____

Date: _____

Initials: _____

Parental Unit: _____

Egg Cup #: _____

Endpoint (DEF, CTM, DOM, STA): _____

Fish #: _____

Left Side	Score	Ventral	Score	Right Side	Score	Dorsa
Skeletal		**Skeletal**		**Skeletal**		**Skelet**
Lordosis		Scoliosis		Lordosis		Scolio
Kyphosis		Constriction		Kyphosis		Const
Constriction		**Craniofacial**		Constriction		**Craniо**
Bend		Jaw shortened		Bend		Eye bl
Craniofacial		Blunt snout		**Craniofacial**		Eye pi
Eye blind		Skull bulbous		Eye blind		Eye de
Eye pigment				Eye pigment		Eye bu
Eye develop				Eye develop		Blunt
Blunt snout				Blunt snout		Skull b
Skull bulbous				Skull bulbous		
Fin				**Fin**		
Smaller				Smaller		
Missing				Missing		
Bend				Bend		
Incomplet ray				Incomplet ray		
Edema				**Edema**		
Abdomin swell				Abdomin swell		
Displaced gut				Displaced gut		

Notes:

Figure B6. The scoring matrix used for deformity analysis of the four main deformity categories (craniofacial, skeletal, fin and edema) and their subcategories. Scores ranged from zero to three, with zero representing no deformity and three representing very deformed.

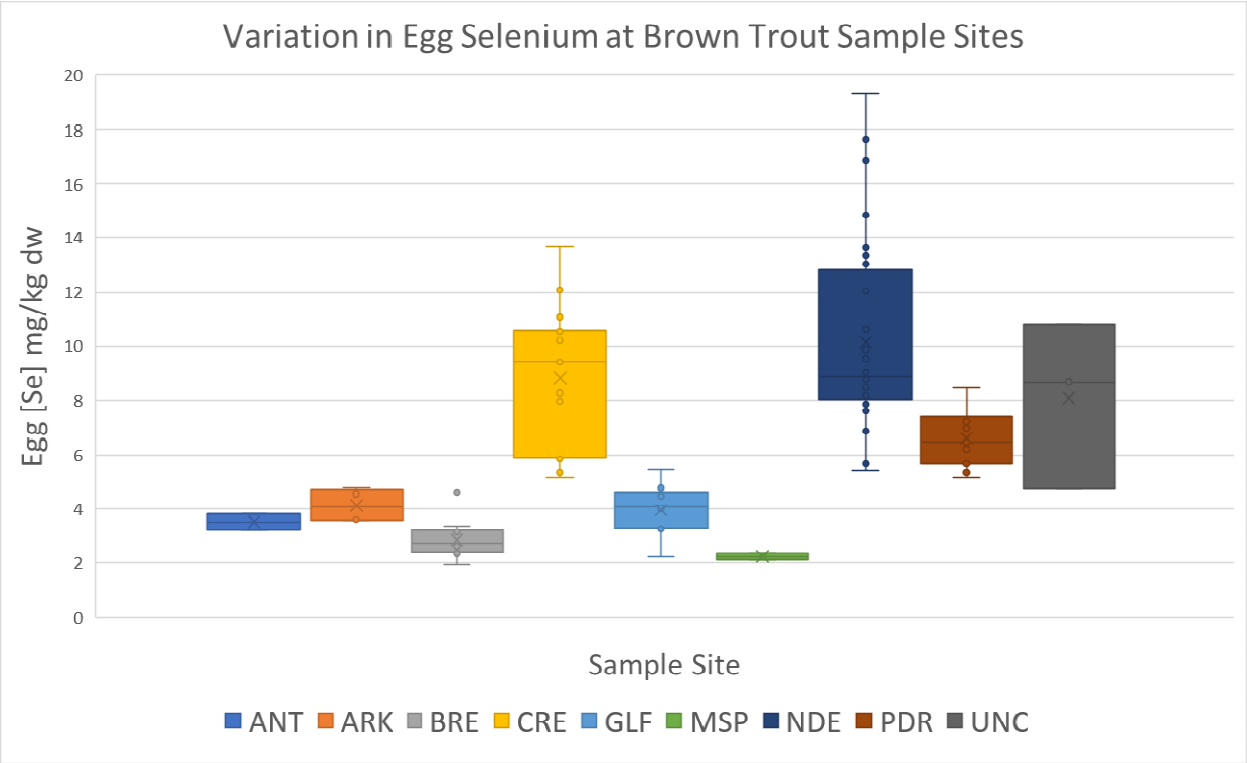

Figure B7. A box plot of egg selenium (Se) concentrations in milligrams per kilogram of dry weight (mg/kg dw) of all Brown Trout sample sites where egg tissues were available for collection. The sample site codes are ANT (Antero Reservoir), ARK (Arkansas River at CR55), BRE (Barker Reservoir), CRE (Colorado River at Elk Creek), GLF (Lake Fork of Gunnison River at high bridge), MSP (Middle Fork South Platte River at Tomahawk SWA), NDE (North Delaney Lake), PDR (Poudre River in Poudre Canyon) and UNC (Uncompahgre River in Montrose).

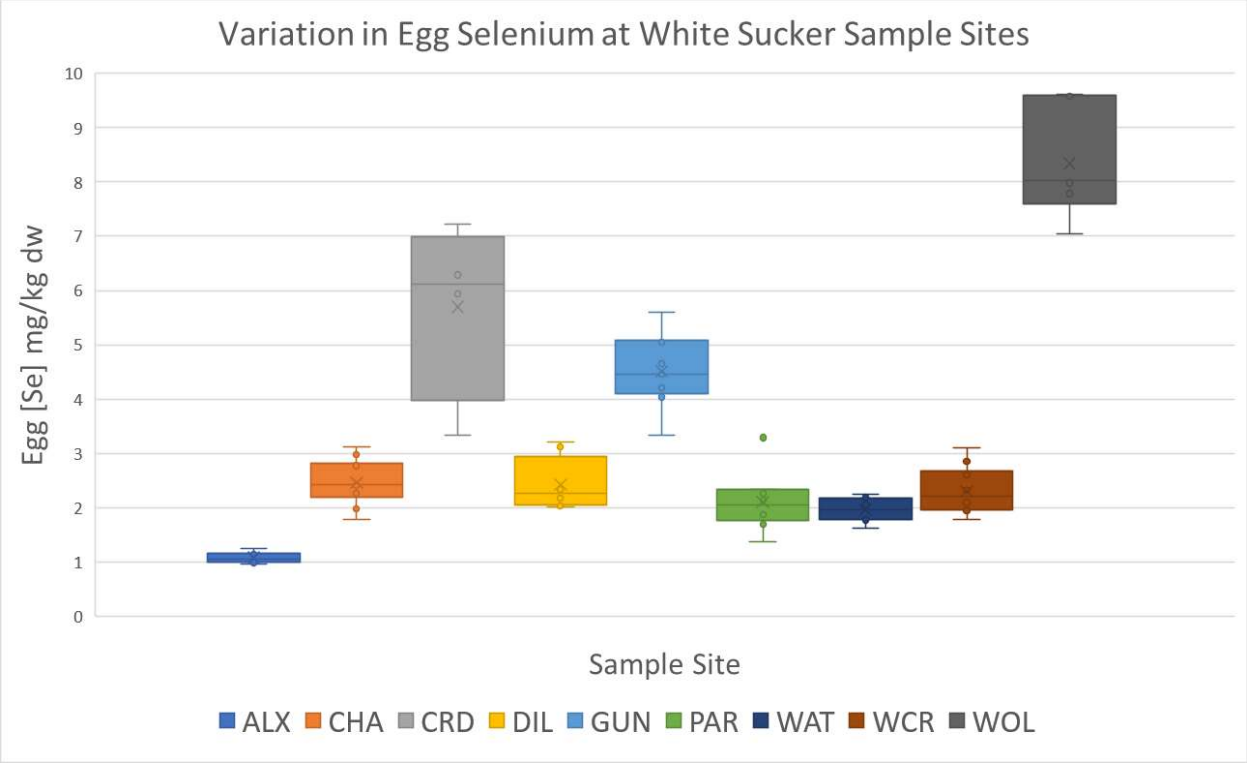

Figure B8. A box plot of egg selenium (Se) concentrations in milligrams per kilogram of dry weight (mg/kg dw) of all White Sucker sample sites where egg tissues were available for collection. The sample site codes are ALX (Alexander Lake), CHA (Chatfield Reservoir), CRD (Colorado River at De Beque, CO), DIL (Dillon Reservoir), GUN (Gunnison River at Delta, CO), PAR (Parvin Lake), WAT (Watson Lake), WCR (Willow Creek Reservoir) and WOL (Wolford Mountain Reservoir).

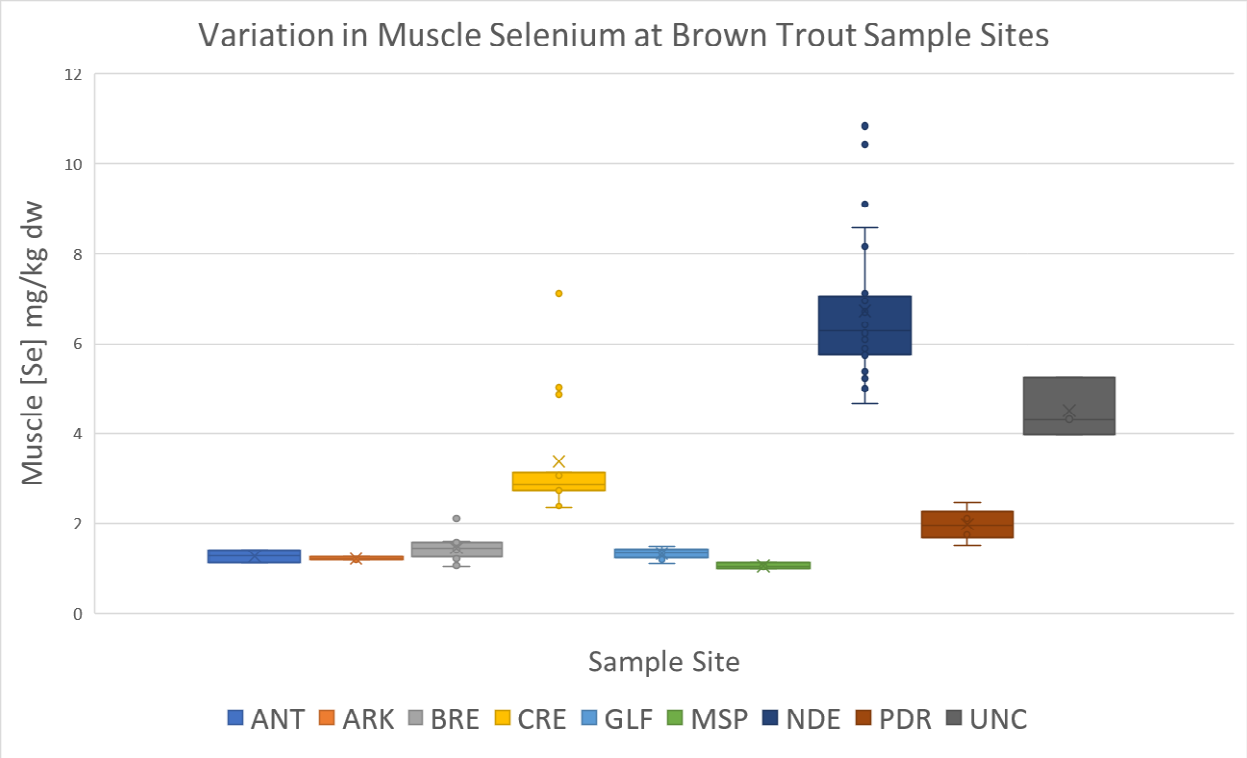

Figure B9. A box plot of muscle selenium (Se) concentrations in milligrams per kilogram of dry weight (mg/kg dw) of all Brown Trout sample sites where fish were available for collection. The sample site codes are ANT (Antero Reservoir), ARK (Arkansas River at CR55), BRE (Barker Reservoir), CRE (Colorado River at Elk Creek), GLF (Lake Fork of Gunnison River at high bridge), MSP (Middle Fork South Platte River at Tomahawk SWA), NDE (North Delaney Lake), PDR (Poudre River in Poudre Canyon) and UNC (Uncompahgre River in Montrose).

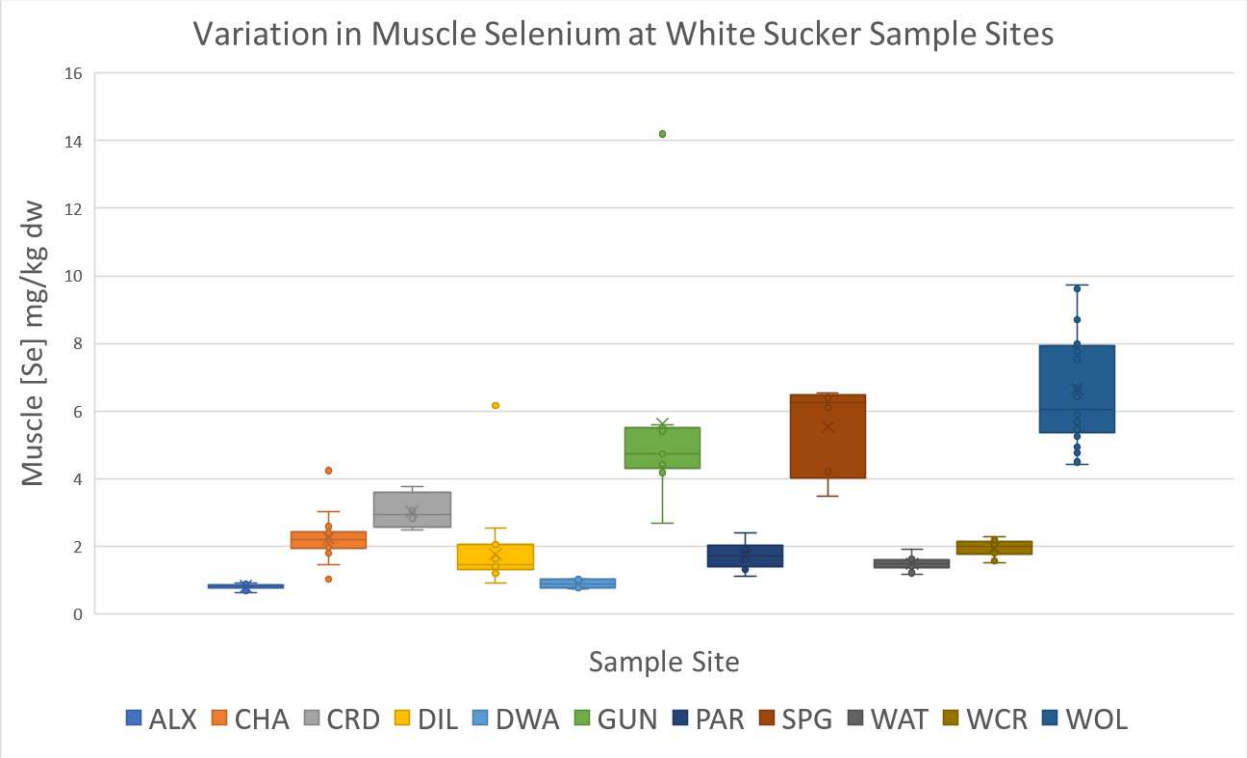

Figure B10. A box plot of egg selenium (Se) concentrations in milligrams per kilogram of dry weight (mg/kg dw) of all White Sucker sample sites where fish were available for collection. The sample site codes are ALX (Alexander Lake), CHA (Chatfield Reservoir), CRD (Colorado River at De Beque, CO), DIL (Dillon Reservoir), DWA (Deep Ward Lake), GUN (Gunnison River at Delta, CO), PAR (Parvin Lake), SPG (Spring Creek at Edora Park), WAT (Watson Lake), WCR (Willow Creek Reservoir) and WOL (Wolford Mountain Reservoir).

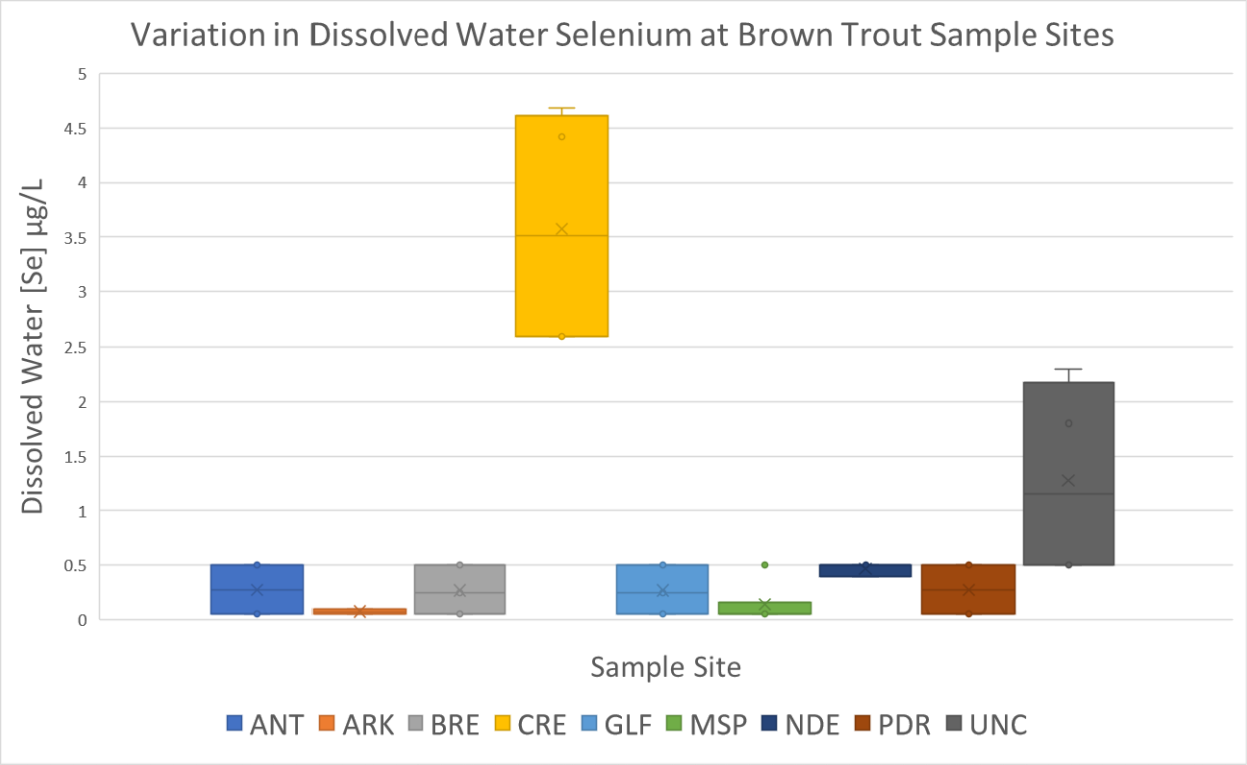

Figure B11. A box plot of dissolved water selenium (Se) concentrations in micrograms per liter (µg/L) of all Brown Trout sample sites. The sample site codes are ANT (Antero Reservoir), ARK (Arkansas River at CR55), BRE (Barker Reservoir), CRE (Colorado River at Elk Creek), GLF (Lake Fork of Gunnison River at high bridge), MSP (Middle Fork South Platte River at Tomahawk SWA), NDE (North Delaney Lake), PDR (Poudre River in Poudre Canyon) and UNC (Uncompahgre River in Montrose).

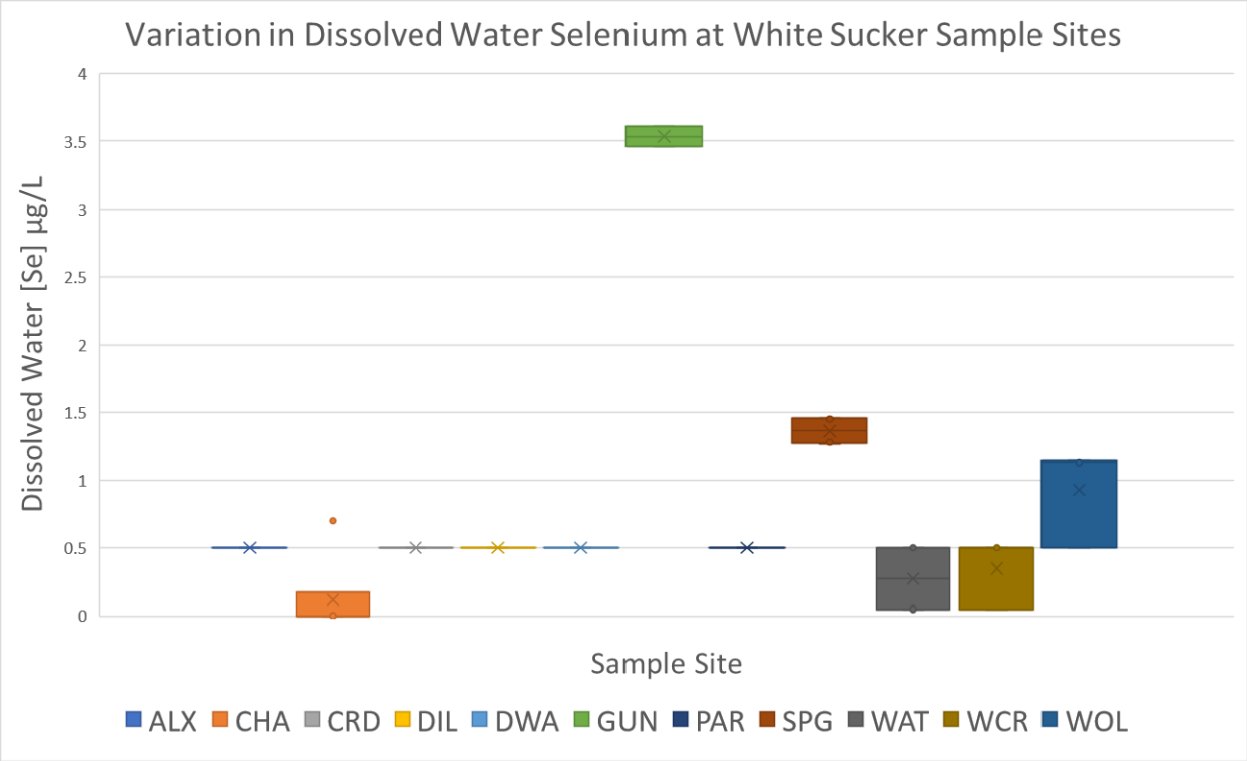

Figure B12. A box and whisker plot of dissolved water selenium (Se) concentrations in micrograms per liter (µg/L) of all White Sucker sample sites. The sample site codes are ALX (Alexander Lake), CHA (Chatfield Reservoir), CRD (Colorado River at De Beque, CO), DIL (Dillon Reservoir), DWA (Deep Ward Lake), GUN (Gunnison River at Delta, CO), PAR (Parvin Lake), SPG (Spring Creek at Edora Park), WAT (Watson Lake), WCR (Willow Creek Reservoir) and WOL (Wolford Mountain Reservoir).

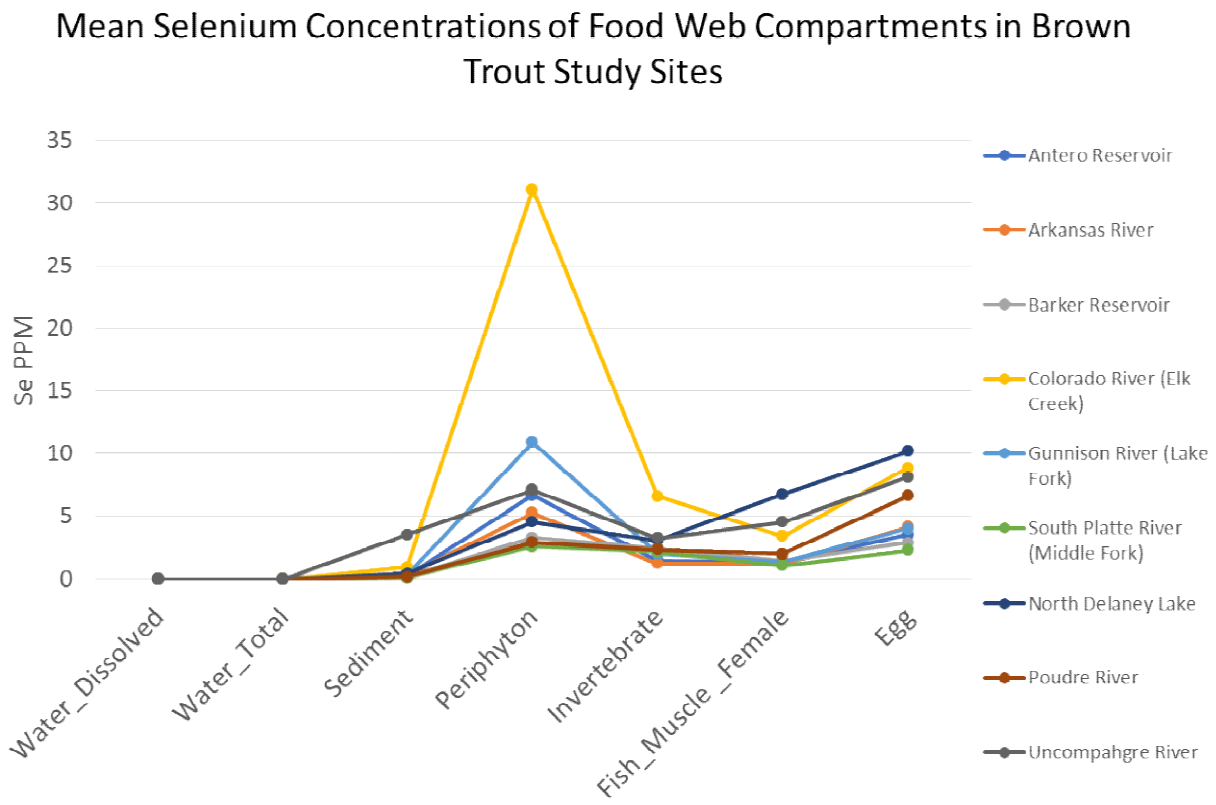

Figure B13. A line graph of average Selenium (Se) concentrations in parts per million (PPM) for all food web compartments analyzed in Brown Trout study sites.

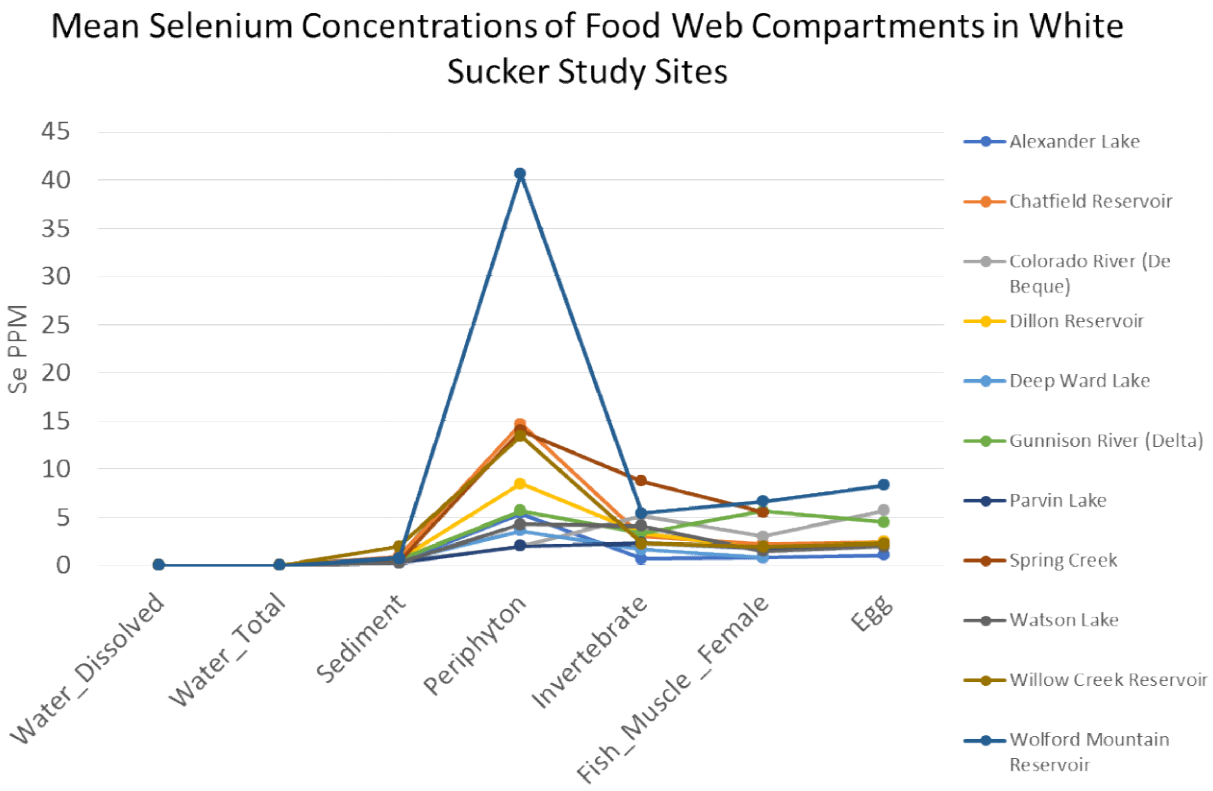

Figure B14. A line graph of average Selenium (Se) concentrations in parts per million (PPM) for all food web compartments analyzed in White Sucker study sites.

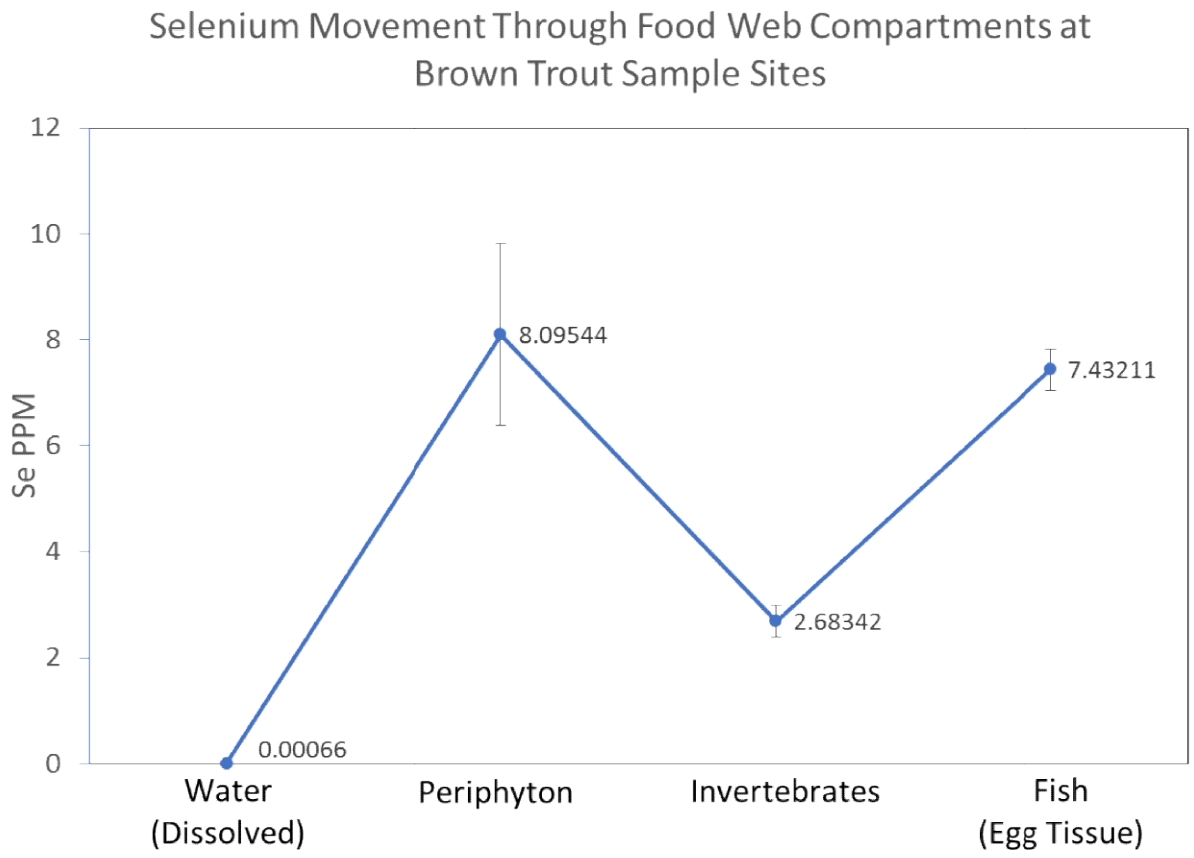

Figure B15. A line graph of mean Selenium (Se) concentrations for all Brown Trout (*Salmo trutta)* sample locations. Mean bioaccumulation Factors (BAF) were calculated as: [Se] organism/[Se] food. The enrichment factor (EF) was calculated as: [Se] periphyton/[Se] water. The BAF and EF models assume the food web to be at a steady state. Samples were collected from Antero Reservoir, Arkansas River at CR55, Barker Reservoir, Colorado River at Main Elk Creek, Lake Fork of Gunnison River, North Delaney Lake, Poudre River and Uncompahgre River (2015-2018).

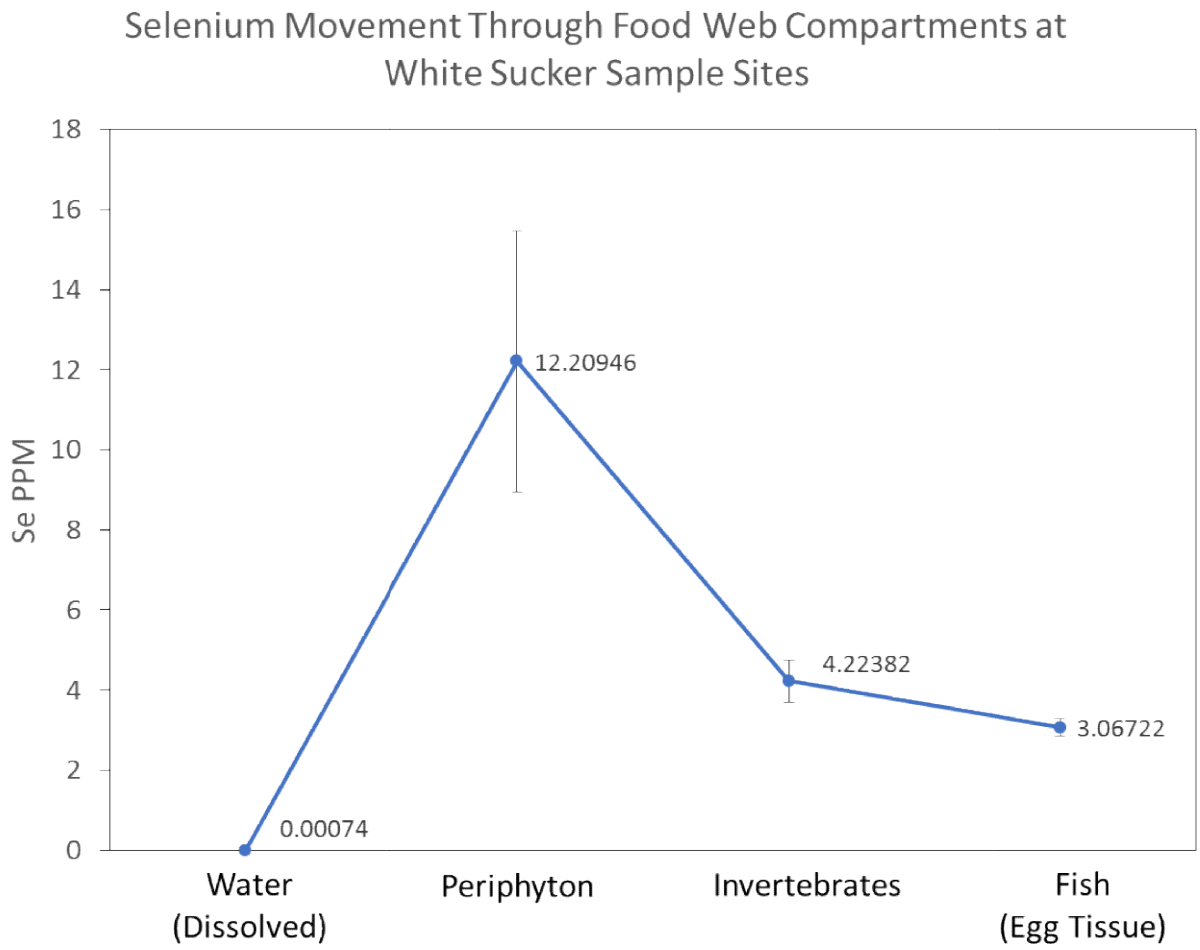

Figure B16. A line graph of mean Selenium (Se) concentrations for all White Sucker (*Catostomus commersonii)* sample locations. Mean bioaccumulation Factors (BAF) were calculated as: [Se] organism/[Se] food. The enrichment factor (EF) was calculated as: [Se] periphyton/[Se] water. The BAF and EF models assume the food web to be at a steady state. Samples were collected from Alexander Lake, Chatfield Reservoir, Colorado River (De Beque, CO), Dillon Reservoir, Gunnison River (Delta, CO), Parvin Lake, Spring Creek (Fort Collins, CO), Watson Lake, Willow Creek Reservoir and Wolford Mountain Reservoir (2017-2018).

Table B1. Standardized methods employed by contract laboratories. Units: milligrams per liter (mg/L), milligrams per kilogram wet weight (mg/kg ww), percent (%) and parts per thousand (‰)

Analyte	Lab Used	Method Used	Units	Minimum Detection Limit
[Se] Water Total	ACZ Laboratories	M200.8 ICP-MS	mg/L	0.0001
[Se] Water Dissolved	ACZ Laboratories	M200.8 ICP-MS	mg/L	0.0001
[Se] Sediment	ACZ Laboratories	M6020 ICP-MS	mg/kg ww	0.05
[Se] Periphyton	ACZ Laboratories	M6020 ICP-MS	mg/kg ww	0.05
[Se] Invertebrates	ACZ Laboratories	M6020 ICP-MS	mg/kg ww	0.02
[Se] Fish Muscle	ACZ Laboratories	M6020 ICP-MS	mg/kg ww	0.02
[Se] Fish Egg	ACZ Laboratories	M6020 ICP-MS	mg/kg ww	0.02
Moisture Content	ACZ Laboratories	D2216-80	%	0.1
Percent Solids	ACZ Laboratories	D2216-80	%	0.1
[Se] Water Total	CDPHE Laboratory Services Division	EPA 200.8	mg/L	0.001
[Se] Water Dissolved	CDPHE Laboratory Services Division	EPA 200.8	mg/L	0.001
Ammonia Nitrogen	Colorado Analytical Laboratories	SM 4500-NH3-G	mg/L	0.03
Chloride	Colorado Analytical Laboratories	EPA 300.0	mg/L	0.01
Dissolved Organic Carbon	Colorado Analytical Laboratories	SM 5310-C	mg/L	0.5
Nitrate Nitrogen	Colorado Analytical Laboratories	EPA 300.0	mg/L	0.05
Nitrate/Nitrite Nitrogen	Colorado Analytical Laboratories	Calculation	mg/L	0.05
Nitrite Nitrogen	Colorado Analytical Laboratories	EPA 300.0	mg/L	0.03
Phosphate - Ortho (as P)	Colorado Analytical Laboratories	EPA 300.0	mg/L	0.01
Phosphate - Ortho (as PO4)	Colorado Analytical Laboratories	EPA 300.0	mg/L	0.01
Phosphorus - Total	Colorado Analytical Laboratories	EPA 365.1	mg/L	0.01
Sulfate	Colorado Analytical Laboratories	EPA 300.0	mg/L	0.01
Total Kjeldahl Nitrogen	Colorado Analytical Laboratories	SM 4500-Norg-B	mg/L	0.1
Total Nitrogen	Colorado Analytical Laboratories	Calculation	mg/L	0.1
Calcium	Colorado Analytical Laboratories	EPA 200.7	mg/L	0.1
Magnesium	Colorado Analytical Laboratories	EPA 200.7	mg/L	0.02
Potassium	Colorado Analytical Laboratories	EPA 200.7	mg/L	0.1
Sodium	Colorado Analytical Laboratories	EPA 200.7	mg/L	0.1
% Nitrogen	University of California Riverside FIRMS	Costech EA - Delta V IRMS	%	0-100% CHNS-O
δ 15N vs Air-N2	University of California Riverside FIRMS	Costech EA - Delta V IRMS	‰	800 molecules per CO2 ion
% Carbon	University of California Riverside FIRMS	Costech EA - Delta V IRMS	%	0-100% CHNS-O
δ 13C vs VPDB	University of California Riverside FIRMS	Costech EA - Delta V IRMS	‰	800 molecules per CO2 ion

Table B2. A summary of bioaccumulation factors (BAF) and bioconcentration factors (BCF) calculated for Brown Trout and White Sucker sample sites. BAF is defined as the concentration of Selenium (Se) in an organism divided by the concentration of Se in the organism's food source. BCF is defined as the concentration of Se in an organism divided by the concentration of Se in dissolved water.

Sample Site	BAF Invertebrate to Fish Egg	BAF Invertebrate to Fish Muscle	BAF Periphyton to Invertebrate	BAF Sediment to Invertebrate	BCF Dissolved Water to Fish Egg	BCF Dissolved Water to Fish Muscle	BCF Dissolved Water to Invertebrate
All Brown Trout	2.770	1.470	0.331	3.911	11182.253	5933.609	4037.434
All White Sucker	0.726	0.623	0.346	6.097	4157.379	3567.205	5725.062
All Sites	1.597	0.886	0.341	5.072	8079.712	4483.337	5058.478
Alexander Lake	1.489	1.131	0.134	1.727	2165.879	1645.225	1454.812
Antero Reservoir	2.444	0.877	0.216	7.332	12797.004	4592.088	5235.617
Arkansas River (CR55)	3.410	0.998	0.230	3.724	55057.626	16118.394	16147.571
Barker Reservoir	1.172	0.591	0.748	16.317	8912.300	4489.883	7602.588
Chatfield Reservoir	0.801	0.731	0.209	3.174	3209.772	2929.856	4008.970
Colorado River (De Beque)	1.105	0.589	2.581	13.214	5274.228	2812.003	4773.514
Colorado River (Elk Creek)	1.343	0.512	0.212	7.261	2884.077	1100.178	2147.944
Dillon Reservoir	0.723	0.527	0.396	5.049	4860.087	3541.770	6725.566
Deep Ward Lake		0.532	0.462	3.462		1779.648	3345.960
Gunnison River (Lake Fork)	2.035	0.679	0.179	5.935	14722.764	4911.894	7235.514
Gunnison River (Delta)	1.338	1.666	0.590	5.242	1002.363	1248.449	749.172
South Platte River (Middle Fork)	1.057	0.494	0.830	19.370	16011.738	7477.435	15146.733
North Delaney Lake	3.326	2.203	0.675	6.976	12087.597	8007.230	3634.281
Parvin Lake	0.901	0.741	1.172	8.222	4224.180	3470.316	4686.275
Poudre River	2.849	0.850	0.799	11.700	24006.641	7164.030	8425.563
Spring Creek (Fort Collins)		0.631	0.626	23.902		2412.659	3825.227
Uncompahgre River	2.505	1.398	0.455	0.924	6217.405	3470.794	2482.014
Watson Lake	0.479	0.363	0.965	15.225	3822.200	2894.037	7977.305
Willow Creek Reservoir	1.000	0.854	0.172	1.179	6288.307	5366.822	6285.302
Wolford Mountain Reservoir	1.530	1.220	0.134	6.561	5697.835	4544.113	3723.962

Table B3. Conversion factors (CF), trophic transfer functions (TTF) and enrichment functions (EF) derived from the 2016 USEPA Selenium Criterion and this study. USEPA CF and TTF values were derived from median values of studies used in the criterion and the values from this study are averaged across all sample sites for each species. USEPA EF values are calculated as the geometric mean of periphyton, sediment and detritus ratios. The EF values from this study are shown as the EF of dissolved water to periphyton and the EF of dissolved water to sediment.

Source of Value	CF ([Egg]/[Muscle])	TTF (Trophic Level 3)	TTF (Composite)	Enrichment Function (L/g) ([Mean Particulate]/[Dissolved Water])	Enrichment Function (L/g) ([Periphyton]/[Dissolved Water])	Enrichment Function (L/g) ([Sediment]/[Dissolved Water])
Median Value from USEPA Brown Trout Studies	1.81	1.38	2.78	0.88	NA	NA
Values for Brown Trout From Current Study	1.88	2.15	0.71	NA	12.18	1.03
Median Value from USEPA White Sucker Studies	1	1.11	1.58	0.851	NA	NA
Values for White Sucker From Current Study	1.16	0.66	0.23	NA	16.55	0.94

Table B4. A list of response variables that will be used in the creation of selenium toxicity models in 2020 and 2021

Response Variables for CSU Biodynamic and Toxicity Models

-Deformities (craniofacial, skeletal, fin and edema)
-Bolt speed
-Reaction time
-Sensitivity to low dissolved oxygen
-Sensitivity to high temperatures
-Survival rate to swim-up
-Hatch success
-Fecundity of parent (measured by number, egg diameter, total mass of eggs)
-Body size of larva at swim-up
-Sustainable populations of fishes as recorded in the CPW ADAMAS database (not funded at this time)
-Bioaccumulation factors across trophic levels (AKA Trophic Transfer Functions)
-[Se]egg of target species when those species are not present

Table 5. Predictor variables that will likely inform models of the response variables listed in Table 4

Predictor Variables Likely to Inform Models of Response Variables

-Site
-Date/Season
-Elevation, Aspect, Exposure
-Gradient (slope)
-Parent Material (geology)
-Temperature, pH, Conductivity, DO, Hardness, Alkalinity
-Sulfate, Chloride, Ammonia, Nitrate-nitrite, Phosphates
-Habitat Type (Lentic, Lotic)
-Substrate type (cobble, gravel, sand, silt, detritus)
-[Se]water
-[Se]parent muscle
-[Se]parent ovary and eggs
-[Se]substrate
-[Se]invertebrates
-[Se]Muscle
-[Se]Parent Egg
-[Se]invertebrates
-[Se]invertebrates
- $^{15}N/^{14}N$ ratios for parent muscle tissue
- $^{13}C/^{12}C$ ratios for parent muscle tissue
- $^{15}N/^{14}N$ ratios for parent eggs tissue
- $^{13}C/^{12}C$ ratios for parent eggs tissue
-[Se] of other fish species egg and muscle tissues at the same site
-Parent length
-Parent Mass
-Parent Mass/Length
-Fecundity of parent (measured by number, egg diameter, total mass of eggs)
-Algae community composition (ratios of Cyano:Green:Diatom)

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Preliminary Report: Sub-acute (24 hr) exposure to sub-lethal levels of diesel decreases predator avoidance in Common Plains Minnow (*Hybognathus placitus)*

Personal: Pete Cadmus. Mathew R. Bolerjack

Background

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

Standardized methodologies and public policy has historically only considered mortality of organisms in the first four days of exposure (two days for some small organisms). The argument against consideration of sub-lethal endpoints is the acute duration informs policy surrounding a short term pulse of pollution not a chronic event. It is assumed that changes in behavior, loss of olfactory function, reproduction, loss of fitness, up-regulation of genes, and all other sublethal endpoints have the potential to recover after the pulse stress event is ameliorated. It is irresponsible to claim that all sub-lethal endpoints are temporary and therefore do not have population effects in acute spill events. To showcase this, we conducted a study looking at a behavior endpoint that is sub-lethal but immediately leads to mortality.

As part of a series of experiments characterizing petroleum hydrocarbons we exposed Common Plains Minnow (*Hybognathus placitus)* to sublethal levels of diesel (75mg/l) for 24 hours. We then facilitated numerous predator avoidance trials in which Plains Minnow from a control group (0 mg/l diesel fuel) and exposure group (75 mg/l) competed to avoid a Spotted Sunfish (*Lepomis punctatus*).

Methods

Common Plains Minnow were obtained from the CPW Native Aquatic Species Restoration Facility (Alamosa, CO, USA). Preliminary 24-hr exposures of Plains Minnow showed 100% mortality at nominal exposure of 150mg/l and near 100% survival at nominal exposure of 75mg/l. In similar studies we observed measured concentrations to be significantly lower than target concentrations. Subdural color-coded visual identification tags were applied three months prior to exposure. Seven Plains Minnows were randomly assigned to each of the 5.9 L stainless steel hotel pans containing 3.0 L of dechlorinated municipal tap water (Fort Collins, CO, USA) at target concentrations of 0 and 75 mg/L diesel fuel. Concentrations were maintained using 50% static renewal four times per day. Exposure vessels were maintained in a recirculating temperature controlled water bath in a negative pressure ventilation hood. Aeration

was provided thorough glass Pasteur pipettes. Wide spectrum and ultraviolet A/B fluorescent lights hung directly above treatment vessels provided 16:8 light cycle.

Spotted Sunfish were starved for 48 hours prior to predator avoidance trials. Sunfish were retained in a fine mesh cylindrical cage with an open base in the center of 60 L black plastic circular tanks. One control and one treatment Plains Minnow were assigned to each of the twenty three circular tanks to ensure color-coded visual identification tags allowed proper differentiation of control and exposed Plains Minnows. Curtains allowed observation of tanks without disturbing predators or prey. Fish were allowed to acclimate to the cages for 1 hour, after which the cage retaining the sunfish was lifted using a string. Every 5 minutes tanks were assessed for consumed fish. If a predation event was not observed after 3 hours, the trial was ended, results were not considered and fish were not reused.

Preliminary Results

There were a total of 23 predator evasion tests conducted. In three of the tests neither the control nor the exposed fish was eaten after three hours of predator avoidance. These three trials were removed from consideration per methods determined *a priori*. In nineteen of the remaining twenty trials the Plains Minnow exposed to diesel were eaten before the Plains Minnow exposed to only control water. This is significantly ($p = 0.00002$) greater than what would be expected if diesel fuel had no effect on predator avoidance.

Discussion

Acute (96 hr) exposure of rainbow trout using nearly identical methods produced an LC50 of 1,042 mg/l and negligible mortality was observed at 175 mg/l (2016). After only 24 hours of exposure, Common Plains Minnow appeared healthy and responsive by traditional measures. Despite this, fish that were exposed to diesel fuel were far more susceptible to predation. Proponents of inclusion of only lethal endpoints in the derivation of acute standards would argue that behavior such as predator avoidance is temporary, and organisms will recover. In the case of decreased predator avoidance behavior, an organism exposed to diesel fuel cannot recover from exposures if it failed to avoid a predator. When a sub-lethal exposure to a toxicant occurs in lotic systems those populations are lost from the local food chain. Risk assessment based on only mortality may be inappropriate when assessing effects of petroleum hydrocarbons on fish.

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