Water Pollution Studies

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The results of the research investigations contained in this report represent work of the authors and may or may not have been implemented as Colorado Parks 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 and 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 Medal 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 ten 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 2020-2021 activities

Research

Effects Of Zinc 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 2019-2020 and in 2020 to 2021 conducted long term (four to nine months) experiments using *Paratanitarsus sp.*. 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 in an effort to avoid density dependent effects. Preserved samples are being enumerated. Water chemistry data has been analyzed and compiled. Statistical analysis is pending but EC_{20} values for each experiment appear to fall at 11.5 µg/l Zn. (Note: the genus *Paratanitarsus* is referred to as the taxonomic tribe "Tanitarsini" in previous CPW reports). CPW's culture was adopted by the USEPA Cincinnati Water Quality Laboratory as a semi-permanent or possibly permanent culture. This culture was collected by Pete Cadmus and Steven Brinkman in 2007 from a Colorado stream. It was sent to EPA in 2018 and 2019. The availability and the standard operating procedures enabled by the USEPA encourages use of this invertebrate outside of CPW functions and is far more pertinent to Colorado watersheds than common laboratory cultured invertebrates.

Effects Of Copper, Molybdenum And Herbicides On Algal Colonization And Competition - After extensive fabrication and method development the first multispecies algal colonization trial was conducted in 2019. 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. The CPW Aquatic Toxicology Laboratory plans to recreate this study with other toxicants as resources allow. The CPW Water Unit has requested that we prioritize assessment of molybdenum over herbicides and this will be conducted early 2022. Our laboratory is fielding many calls about the findings printed in the 2020 annual report on this topic. We hope to begin characterizing a suite of herbicides in the fall of 2022.

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) last fiscal year. A trophic transfer model was calculated for Colorado sample sites and compared to USEPA's model. The last year was spent organizing these data into a suitable database. Currently CPW and colleagues are examining the results in a mixed effects model.

Sample Processing And Laboratory Maintenance - 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 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. Floors, plumbing, and some ventilation were serviced.

Scientific Communication - Three manuscripts were submitted for formal peer review:

Clift, A. K., A. M. Malmlov, C. L. Wells, P. Cadmus, and P. A. Schaffer. *In Review* Branchitis and Mortality in Rainbow Trout *Oncorhynchus mykiss* (Walbaum) exposed to Iron Oxidizing Bacteria: Diagnostics and Management in a Colorado Hatchery.

Iwasaki, Y., P. Cadmus, J. F. Ranville, and W. H. Clements. *In Review*. Stream mesocosm experiments show no protective effects of calcium on copper toxicity to macroinvertebrates.

Cadmus, P., R. Friebertshauser, S.F. Brinkman, N. Rhein, and W. H. Clements. *In Review.* Subcellular accumulation and depuration of zinc in periphytic algae during episodic and continuous exposures.

Technical Support

On-site Assessment Of Rotenone - 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), Wolf Creek (Wolf Creek Pass, CO) and Williams Gulch (Red Feather, CO).

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

River Watch Technical Support - 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 analytical services. Infrastructure improvements to the building were researched, planned and facilitated. Technical review of new inductively coupled plasma mass spectrometers (ICP-MS) was conducted to assist CPW water unit.

Effects of Copper In Low Hardness Waters - CPW Aquatic Toxicology Laboratory staff helped the University of Alaska Fairbanks and the Alaska Fisheries Research Co-op Unit build gravity fed serial diluters. This research focuses on a salmon and trout species important to Colorado and examines toxicity in low hardness and low dissolved organic carbon, similar chemistry to Colorado's headwater rivers and reservoirs.

Technical Support And Scientific Contributions As Needed - Ecotoxicological support and expertise was provided to CPW managers, Colorado universities, and natural resource management agencies as requested. 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. CPW Aquatic Toxicology Laboratory staff peer reviewed internal and externally published scientific literature when those papers were pertinent to the unique taxa or unique chemistries of Colorado.

Spill Response - CPW Aquatic Toxicology Laboratory staff responded to numerous spills and assessments. This includes response as the sole responder and as technical support for CPW's Water Unit and the Colorado Attorney General's Office.

On-going Projects – Activities scheduled for the early 2021-2022 fiscal year.

Chloride, Sulfate And Ammonia Studies - Effects of chloride, sulfate, and ammonia on a suite of fish species has already begun and is being supported by Colorado State University (CSU) professors and students. These studies will include dissolved oxygen tolerance, thermal tolerance, competition, chemical avoidance 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. These studies were delayed due to COVID closures.

Benthic Macroinvertebrate Sample Identification, Enumeration And Sorting - Quantitative subsampling, enumeration, and identification of aquatic macroinvertebrate samples from 2015 to 2022 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. Experiments using *Paratanitarsus sp.* are also being processed. Use of the Federal Work-Study program has allowed CPW to process samples at significant cost savings while hosting learning experiences for university level students in the biological and chemical sciences.

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

As Needed Support Of CPW Fishery Biologists And Hatcheries - Continued analytical chemistry support of CPW chemical habitat reclamations will be conducted in late summer and early fall. Milt extender will be delivered to hatchery and biologist staff in late winter of 2022. Research and development in water treatment systems to convert ferrous iron to ferric iron at CPW's Poudre River Fish Hatchery will continue and prototypes are scheduled to be installed during spring of 2022.

Interagency Support And Collaboration - Continued support of CPW Water Unit goals, CDPHE water quality efforts, River Watch, Colorado universities and researchers.

CPW Aquatic Toxicology Laboratory Maintenance - Infrastructure improvements, analytical equipment improvements and improvements to our mobile laboratory will be made as time allows in 2022.

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RESEARCH PRIORITY

Reduced Predator Evasion Following a Sub-Acute (24 Hour) Sub-Lethal Exposure of Diesel Fuel to Plains Minnow (*Hybognathus placitus*)

ABSTRACT

Standardized methodologies for deriving species or mean acute threshold values has historically only considered mortality of organisms in acute toxicity experiments (Acute: first four days of exposure or first two days for some small organisms). The argument against consideration of sub-lethal endpoints is the organism in nature is sure to recover after the toxic exposure. It is assumed that changes in behavior, loss of olfactory function, reproduction, loss of fitness, up-regulation of genes, and any other sublethal endpoint has full likelihood 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 Plains Minnow (*Hybognathus placitus*) to sublethal levels of diesel (75 mg/l nominal diesel fuel. 2.0 mg/l observed total petroleum hydrocarbons C10-C28) for 24 hours. In non-contaminated conditions we then conducted predator avoidance trials in which a Plains Minnow from a control group (0 mg/l diesel fuel) and exposure group competed to avoid predation by a Green Sunfish (*Lepomis cyanellus*). Diesel exposure at 2.0 mg/l for twenty-four hours significantly reduced predator avoidance of the prey species.

INTRODUCTION

Consumption and transportation of diesel fuel is growing in Colorado and with this growth in use is a growing risk of hazardous material spills (Alternative Fuels Data Center 2021). Colorado highways are often co-located next to rivers so diesel or gasoline is the most frequent hazardous material in spills and accident reports associated with traffic collisions. According to the Center for Western Priorities, a nonpartisan conservation and advocacy organization, 20% of >340 oil and gas (petroleum) spills that occurred in Colorado in the past year (2020) were within 500 feet of surface water (McIntosh 2021). Historically, Colorado's response and characterization of resource damage after petroleum spills was limited to counting dead fish on stream banks. A more extensive understanding of the potential effects on freshwater fish within Colorado waters can help better predict and evaluate the ecosystem effects of a diesel fuel spill in nature. Gaining a better understanding of the lethal and sublethal effects amongst freshwater fish after a diesel spill event can serve to help better inform managers of appropriate information gathering and mitigation efforts in a spill event, beyond immediate mortality counts downstream of a traffic accident.

Previous Colorado Parks and Wildlife laboratory studies and observations following spill events looked at the toxic thresholds of various freshwater fish species, periphyton, and aquatic invertebrates following acute exposures to diesel fuels. Drift or chemical avoidance was found to happen immediately when aquatic invertebrates were exposed to diesel. Similar behavioral alterations are likely in fish. Behavioral responses have potential to extirpate species from spill sites especially when fish passage precludes return when toxicant levels subside. Such behavioral endpoints are rarely characterized or considered in the development of standards, criteria or policy to protect Colorado's fish from petroleum spills. Behavior changes at sub-lethal levels can manifest into mortality including a lowered ability to avoid predation.

Parks and Wildlife studies in 2014 and 2015 investigated survival of trout to acute diesel exposure comparable laboratory conditions. Six-month old (18.1 g +/- 0.44; 122.8 mm +/- 1.1) Mt. Shasta strain Rainbow Trout (*Onchorinchus mykiss),* and Greenback Cutthroat Trout (*Oncorhynchus clarkii stomias*) fry exhibited full survival at or below pulse exposures 75 mg/L or 100 mg/L respectively. These exposures were a single dose spike followed by four days of static exposure before replacing water. Based on chromatography assessments, most of the diesel was lost from the water column in the first couple hours of exposure. These exposure values were nominal (what was delivered) and do not perfectly reflect the true concentrations of the many chemicals in the water column. In both field and laboratory studies, petroleum hydrocarbons are quickly lost from the water column. This makes study of diesel and petroleum spills difficult. When diesel fuel enters surface water the petroleum hydrocarbon compound is believed to sit on top of the solution due to the greater density of water. The fuel breaks down in the water near the surface where one of the most toxic components within diesel fuel, the PAH's, become more bioavailable for aquatic biota and are excited in the presence of ultra violet light increasing the potential harm to aquatic life (Duggan 2013, Hussein I. Abdel-Shafy, Mona S.M. 2016). Despite the belief that diesel initially remains on top of surface waters, CPW scientists have frequently observed the release of a sheen of oil or organics when walking on fine substrate. This occurred even long after a reach was tested by private and public entities and found to be below detection limits (P. Cadmus and M. May personal observations 2014-2021).

Often this was observed immediately below a spill or too proximal to allow for the needed time to be dissolved. This suggests mixing of lotic environments may be greater than predicted in the above paradigm or suggests the surface area of the benthic zone is rapidly accumulating PAH's (Duggan 2013). The concentration, duration, loss and reactivity can be widely variable dependent on stream flows (volume, gradient, velocity), light exposure, sediment load, habitat structure and total volume of spilled toxicant. Challenges exist in measuring the true exposure of fish to diesel in the field. For the same reason, accurately dosing fish in laboratory toxicity trials is a barrier to devising effective standards, toxic thresholds or criteria to the protection of Colorado fish. Standardized methodologies for deriving species or mean acute threshold values has historically only considered mortality of organisms in acute toxicity experiments (Acute: first four days of exposure or first two days for some small organisms). The argument against consideration of sub-lethal endpoints is the organism in nature is sure to recover after the toxic exposure. It was assumed that changes in behavior, loss of olfactory function, reproduction, loss of fitness, up-regulation of genes, and any other sublethal endpoint has full likelihood to recover after the pulse stress event is ameliorated. Plains Minnow (*Hybognathus placitus)* were exposed to sublethal levels of diesel (75 mg/l nominal diesel fuel. 2.0 mg/l observed total petroleum hydrocarbons C10-C28) for twenty-four hours. In non-contaminated conditions we then conducted predator avoidance trials in which a Plains Minnow from a control group (0 mg/l diesel fuel) and exposure group (2.0 mg/l) competed to avoid predation by a Green Sunfish (*Lepomis cyanellus*).

METHODS

Plains Minnow (*Hybognathus placitus)* were obtained from the Colorado Parks and Wildlife Native Aquatic Species Restoration Facility (Alamosa, CO, USA). Fish were 35mm +/- 7mm in standard length at the time of toxicant exposure. Fish were marked with Visual Implant Elastomer (VIE) tags. These colors are visible by the naked eye with or without the florescence of an ultra-violet (UV) lamp. This allowed for identification of individuals during predator avoidance trials detailed below. Fish were exposed to MS-222 per label to anesthetize them during tagging. One of three colors was injected epidurally at the base of the dorsal fin. Fish were then promptly returned to a recovery tank after tagging and held for four weeks. After four weeks no mortality from aestivation or tagging was observed.

Six liter stainless steel hotel pans served as exposure vessels. Each pan was filled with three liters of water that had been mixed to the appropriate concentration of diesel fuel to create nominal levels of 0, 75 and 150 mg/L fuel. A subsample of fish from each color (Red, Green and Blue VIE tag) group was then randomly assigned to each pan with a density of seven Plains Minnows (0.264 g +/- 0.24, 35 mm +/- 7) per vessel. Exposures were staggered temporally to allow for exactly twenty-four hours exposure plus one hour recovery from exposure before examining the predator avoidance. Each pan was aerated with an airline connected to a sodium silicate Pasteur pipette possibly removing light volatile organics. Every six hours a 50% static renewal of the water was conducted extracting and replacing 1.5 liters of water mixed in order to the maintain the designated concentrations. A 16:8 hour light cycle using wide spectrum and ultraviolet UVA/UVB bulbs hung directly over the pans was used to simulate the natural UV regime. This has been found to photo-activate toxic compounds within petroleum but was orders of magnitude less intense than natural sunlight. Water samples were taken for assessment of total petroleum hydrocarbons C10-C28 by gas chromatograph immediately before and after the second six hour replenishment ("before" represents what is likely the lowest level of toxicant and "after" represents a fresh replenishment). Full mortality (100%) was observed after twenty-four hour exposure to the 150 mg/l nominal fuel concentration. After twenty-four hours of exposure, fish receiving 75 mg/l nominal fuel concentration showed no signs of morbidity, responded to visual stimulus, and vigorously attempted to escape palpation with a fine paint brush. Standardized toxicity trials following ASTM and EPA guidelines would characterize these fish as healthy and consider the nominal 75 mg/l concentration unable to illicit a toxic response.

Field collected Green Sunfish (*Lepomis cyanellus*) 80 mm to 150 mm in length were used for the predator species due to their larger gape limit. The native range of both predator and prey species co-occur in the Arkansas River in southeast Colorado. Predators were unfed for two days prior to running the predator avoidance trials.

Numerous circular black plastic 204 L tanks (KMB 101. Tuff Stuff Products, Terra Bella, California, USA. Figure A1) were used for predator avoidance trials. Each tank was 50% full of oxygenated reference (no toxicants) water at 14 -17° C. Technicians were hidden behind blackout curtains to limit disturbance or stress to fish. One exposed and one non-exposed Plains Minnow, of different color VIE tag, were assigned to a bottomless cylindrical stainless steel cage made of wire mesh (175 mm diameter) within the tub (Figure A2). This cylindrical cage prevented predation prior to the trial and allowed all organisms to acclimate to the tank for 30 minutes. A single Green Sunfish was then added to each tank and all fish acclimated an additional 30 minutes prior to starting each predator avoidance trial. The mesh cylindrical cage protecting Plains Minnow was lifted from the water column by pulling a cord from behind the curtain. The fish within the tub were then censused every three minutes by careful examination from behind the curtains. If a fish had been eaten, the trial was stopped and both a UV and visible light were used to identify the surviving fish. Each surviving fish was then weighed and measured and euthanized. Following each predation experiment trial each vessel (tub) was fully drained, scrubbed, rinsed, and refilled before use in a subsequent predator avoidance trial. If no predation occurred after three hours the trial was ended, fish euthanized, not measured and not considered for statistical analysis of predator avoidance.

RESULTS

Observed toxicant exposure concentrations were well below the predicted or nominal concentrations. In-house assessments found most c10-c28 mass petroleum hydrocarbons were rapidly lost to the vessel or to volatilization. This was confirmed by an external (private sector) state and national certified analytical laboratory. This is a common problem for almost any laboratory exposing using static renewal techniques, especially petroleum hydrocarbons. For the nominal 150 mg/l diesel fuel exposure solutions the hydrocarbons C10-C28 values immediately before and after the six hour replenishments averaged 15.55 (SD=1.7) mg/l and 7.3 (SD=1.06) mg/l total petroleum hydrocarbons C10-C28. Nominal treatment levels of 75 mg/l were observed at 2.0 mg/l (SD=0.7) total petroleum hydrocarbons C10-C28.

A total of twenty-three predator evasion trials were conducted. Three of the trials resulted in neither the exposed nor control fish being eaten after a total of three hours. These were excluded from statistical analysis. Of the twenty remaining successful trials the diesel exposed Plains Minnow was eaten before the non-exposed Plains Minnow nineteen of the twenty times. This is significantly ($p = 0.00002$) greater than what would be expected (ten of twenty trials) if diesel fuel had no effect on predator avoidance.

IMPLICATIONS

The results of the bioassay and the predator evasion trials indicate that even though the Plains Minnow appear uneffected and healthy by traditional standardized assessments, a twenty-four hour exposure to 2.0 mg/l of diesel fuel alters behavior important for survival. Well below the twenty-four hour toxic threshold (likely between 15.55 and 2.0 mg/l total petroleum hydrocarbons C10-C28). Acute standards and criteria historically have considered only lethal tests. During the formation of this policy industry and regulatory agencies assumed that organisms will recover from all sublethal acute (four day or less) exposures. In this trial a sublethal compromise in behavior manifested into 95% mortality at a concentration that traditional acute toxicity trials would have considered non-effect. Fish did not recover from the pollutant exposure despite removing the fish from the toxicant. Consumed fish did not recover from being consumed. As review of new chemical classes and the triannual (mandated every three years by Clean Water Act) review of existing standards are considered, inclusion of sublethal response variables for acute exposures should be considered if there is a chance that the measured function reduces survival in nature.

Figure A1: The 204 L predator cage after initiating predator avoidance trial. (cage and air stone was removed for photo).

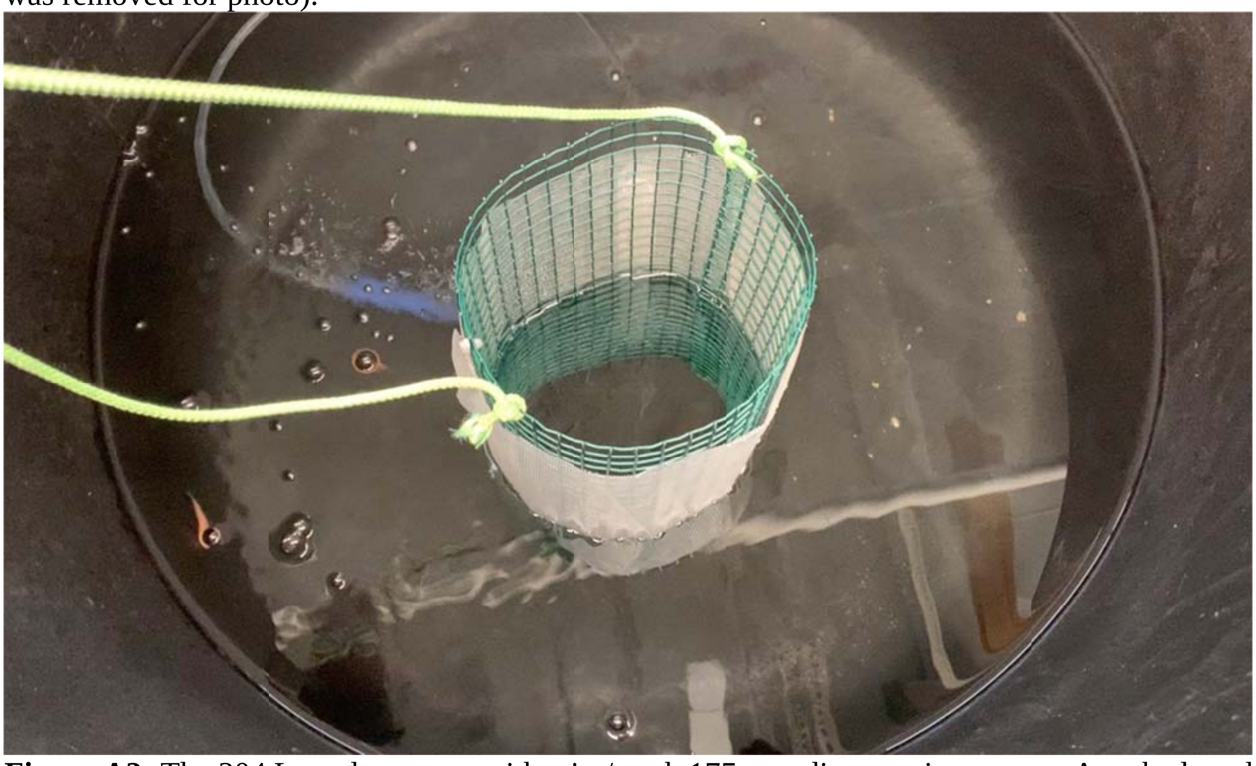

Figure A2: The 204 L predator cage with wire/mesh 175 mm diameter inner cage. Attached cord allows trial to be started by technician from behind curtains.

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RESEARCH PRIORITY

Reduced thermal tolerance in the Greenback Cutthroat Trout (*Oncorhynchus clarkii*) after acute exposure to magnesium chloride at the national recommended acute chloride limit

OBJECTIVE

Characterize thermal tolerance in Cutthroat Trout after acute exposure to chloride.

INTRODUCTION

Temperature tolerance of Colorado salmonids is reduced after acute aqueous copper (Cu) exposure (Cadmus 2014, 2015). The fisheries of Colorado will face unnaturally high temperature events given the predictions of climate change and the diversion of Colorado's water from rivers to agricultural and municipal uses. Magnesium chloride ($MgCl_2$) is a commonly applied deicer in Colorado and its application to roads co-occurs with the habitat of Colorado's native threatened Greenback Cutthroat Trout (*Oncorhynchus clarkii*). To assess the risk of interaction between $MgCl_2$ and warm temperature, Greenback Cutthroat Trout were exposed to magnesium chloride at the national recommended chronic and acute chloride standards (USEPA 1988) for one hour, one day and four days. Then critical thermal maxima (CTmax) and critical dissolved oxygen minima (CDOmin) were assessed as a measure of thermal tolerance and tolerance of low dissolved oxygen (DO). Acute (96 hr) pre-exposure to $MgCl_2$ at the national standard caused a significant reduction in thermal tolerance.

METHODS

Greenback Cutthroat Trout (*Oncorhynchus clarkii*) were obtained from the Colorado Parks and Wildlife Bellvue Fish Hatchery (Fort Collins, CO, USA) and were kept at a temperature of 12^{o} Celsius (+/-1). Fish 23 to 39 mm total length and 0.12 to 0.37 g mass were randomly assigned to 75 liter aquaria. Life support, aquaculture and flow-through delivery of toxicants was administered per Cadmus (2014, 2015) however a separate peristaltic pump and stock solution was used for each treatment level to avoid the geometric dilution ratio of the gravity fed serial diluter.

Flow through supply of water and $MgCl_2$ (40 ml/min to each experimental unit) was assigned at target chloride (excluding Mg) concentrations of 860 mg/l (μ=860 SD=100), 230 mg/l (μ=215 mg/l, SD=78), and control (Below Detection Limits). These concentrations were assigned to simulate the acute and chronic national limits advised by the US EPA (EPA 1988); 860 mg/L and 230 mg/L respectively. Physical and chemical properties were assessed and water samples were taken twice daily. Concentrations of chloride were analyzed by flow-through injection using a Lachet Quikchem 8500 series (Lachet Instruments, Loveland, CO USA. Method 10-117-07-1-C) using ISO traceable external standards.

The initiation of exposure to each experimental block was staggered temporally by one hour to allow for staggered assessment of CDOmin and CTmax and ensure exactly one hour, twenty-four hour and 96 hour exposure for each experimental unit. During the 96 hour exposure eight fish from each exposure level were removed for assessment of CTmax after one, twenty-four and 96 hours. Six organisms from each treatment level were removed for CDOmin assessment after 96 hours. To conform to assumptions of statistical replication, the two fish from each experimental unit were assessed separately in test chambers (Figure B1) but values were averaged prior to statistical analysis. A systematic Latin squares assignment of organisms to testing chambers ensured no bias. Critical Dissolved Oxygen Minima trials and Critical Temperature Maxima trials were performed per Cadmus (2014, 2015). Temperatures were assessed with new traceable thermometers to 0.1° C. After ensuring homogeneity of variance Dunnet's Multiple Comparison Test for comparison to the control was conducted within One-way Analysis of Variance (ANOVA) at each duration (alpha=0.05).

RESULTS

Temperature, Dissolved Oxygen (DO) and pH were consistent across treatments (Table B1). Hardness of incoming water was 38 mg/l $CaCO_3$ equivalents. As predicted, all four replicates (n=4) of each of the three treatment levels saw no mortality. After 96 hours of exposure to the national recommended maximum acute chloride limit (860 mg/L), the CTmax values were significantly (p = 0.00503) reduced relative to controls (Figure B2, Table B2). This was unexpected given this concentration is generally considered protective of fish. No trend (Table B3) or statistical difference in CTmax was detected after one hour and twenty-four hours of exposure. Nor was a significant difference observed in CTmax values between controls and national chronic standard of 230 mg/L. Critical dissolved oxygen minimum values also were not statistically different (Figure B3).

IMPLICATIONS

No derived chloride standard has been accepted for the state of Colorado. The standardized methods for deriving water quality standards fail to incorporate multiple stressors. Industry and regulators assumed such synergistic risks were negligible, too cost prohibitive to study or were deemed unlikely and could be prevented by addition of a safety factor (divide by two). Counterintuitively, great effort and investment has been made to consider ameliorating chemical analytes and physical properties for criteria such as copper, zinc, lead, and aluminum. Acute standards rarely incorporate ecologically relevant endpoints aside from mortality. This experiment and similar studies with copper (Cadmus 2014, 2015) suggest that additional protections are needed for the fisheries of Colorado in areas where potential for thermal stress co-occurs with copper or chloride pollution. Acute standards in the USA are historically calculated using only experiments that consider lethality as the response variable. Assumptions of industry and regulators at the time were that organisms could recover from any sub-lethal endpoint after an acute exposure. The diversion of water from Colorado rivers for agricultural and municipal needs increased the risk of high temperature or rapid temperature changes. This is further exacerbated by predictions of increased stream temperatures in the face of anthropogenic climate change in the Headwater State. The ability to deal with temperature stress events will become ever present for aquatic species. Risk of interactions between thermal stress and chemical stress will increase.

Policy dictating the derivation of acceptable chemical and thermal standards should be reconsidered to better reduce the risk of additive and synergistic interactions. In this study we demonstrate effects at the national criteria for chloride. Rainbow trout, a member of this genus (*Oncorhynchus* was formerly *Salmo*) were included in the 1988 derivation and were found to exhibit a species mean acute value of 6,743 mg/l. Even after consideration of many other, more sensitive, species and even after application of the universal safety factor (divide by 2) the ability of Cutthroat Trout to tolerate heat was compromised at the acute standard. As Colorado or the nation moves towards triannual review of the chloride standard a more contemporary consideration of suitable data should be adopted. Studies looking at ecologically relevant endpoints beyond mortality should be included.

This study shows that stresses associated with anthropogenic climate change are likely to compromise the protections currently in place for Colorado's fisheries and aquatic life. Furthermore, amelioration of thermal stress events associated with climate change, as well as the dilution of some pollution sources, can be obtained by reducing the amount of water removed from natural systems for agriculture and municipal use.

Table B1: Observed Water Chemistry

Exposure Level	Temperature (C)	DO (mg/L)	pH	Chloride (mg/L)
Control	12.6 (0.11)	120.5 (2.27)	7.89 (0.26)	36 (1.9)
Chronic (230 mg/L)	12.5 (0.16)	120.7 (2.16)	7.45 (0.28)	215 (78)
Acute (860 mg/L)	12.6 (0.24)	120.3 (2.31)	7.27 (0.29)	860 (100)

Table B2: Analysis of variance of critical thermal maxima (CTmax, ^{o}C) and Dissolved oxygen minima (DOmin, % Saturation n) for Greenback Cutthroat Trout at three treatment levels. Bolded values indicate significance

Trials	Source of Variation	Degrees of Freedom	Sum Squares	Mean Squares	F value	P value
1-hour CTmax	ANOVA	2	0.966	0.4829	1.225	0.318
24-hour CTmax	ANOVA	2	0.951	0.4754	1.625	0.223
4-day CTmax	ANOVA	2	1.501	0.7504	5.81	**0.00981**
4-day DOmin	ANOVA	2	7.109	3.555	1.822	0.201

Table B3: Mean Critical Thermal Maxima (CTmax, ^{o}C) and Dissolved Oxygen Minima (DOmin, percent saturation) for three exposure levels

Trial	Acute Mean (SD)	Chronic Mean (SD)	Control Mean (SD)
1-hour CTmtax	27.6 (0.50)	27.9 (0.46)	27.5 (0.89)
24-hour CTmax	27.5 (0.64)	27.6 (0.40)	27.6 (0.51)
4-day CTmax	27.6 (0.27)	27.8 (0.46)	28.2 (0.33)
4-day DOmin	10.1 (2.89)	13.1 (2.95)	10.73 (4.50)

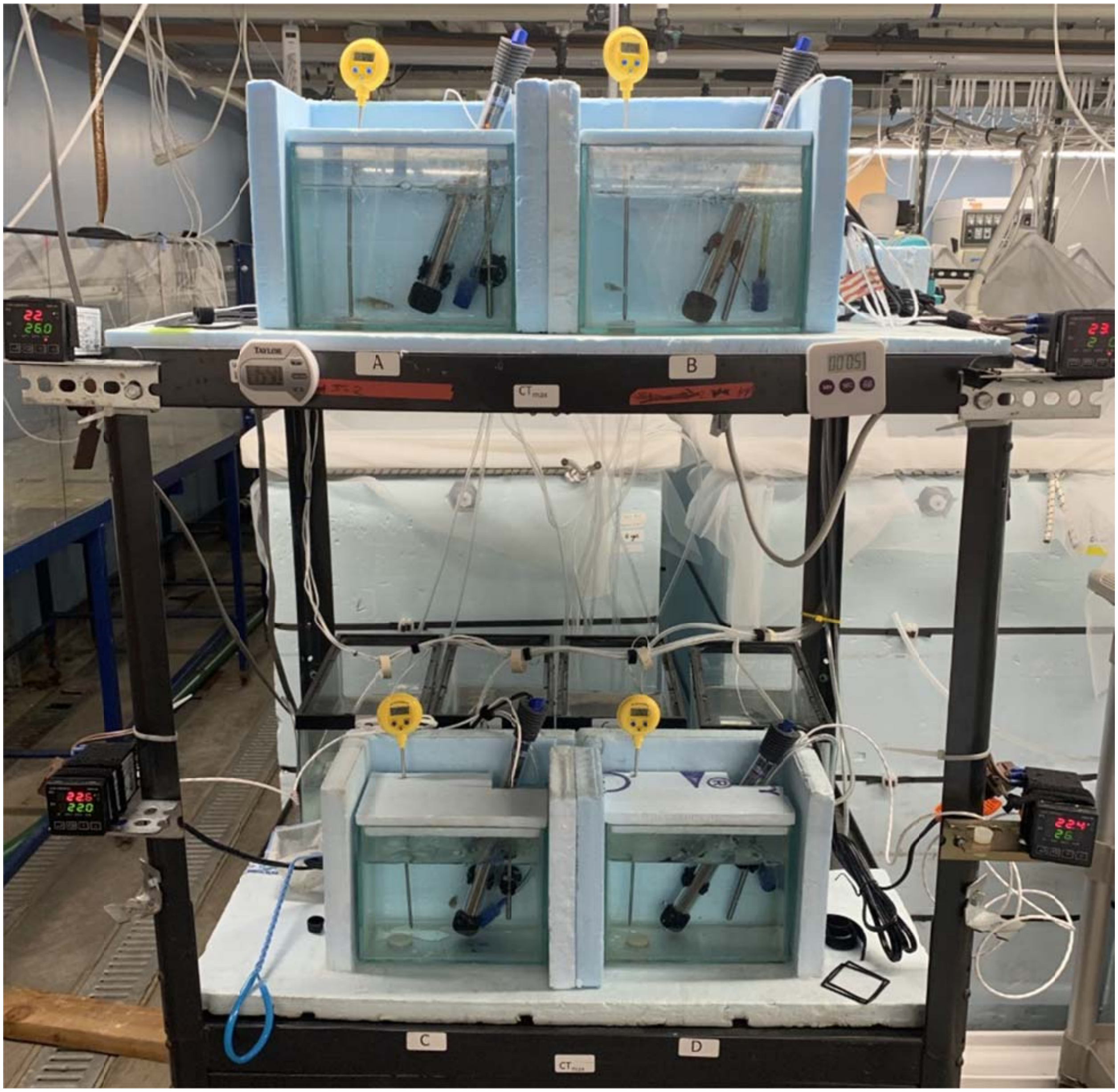
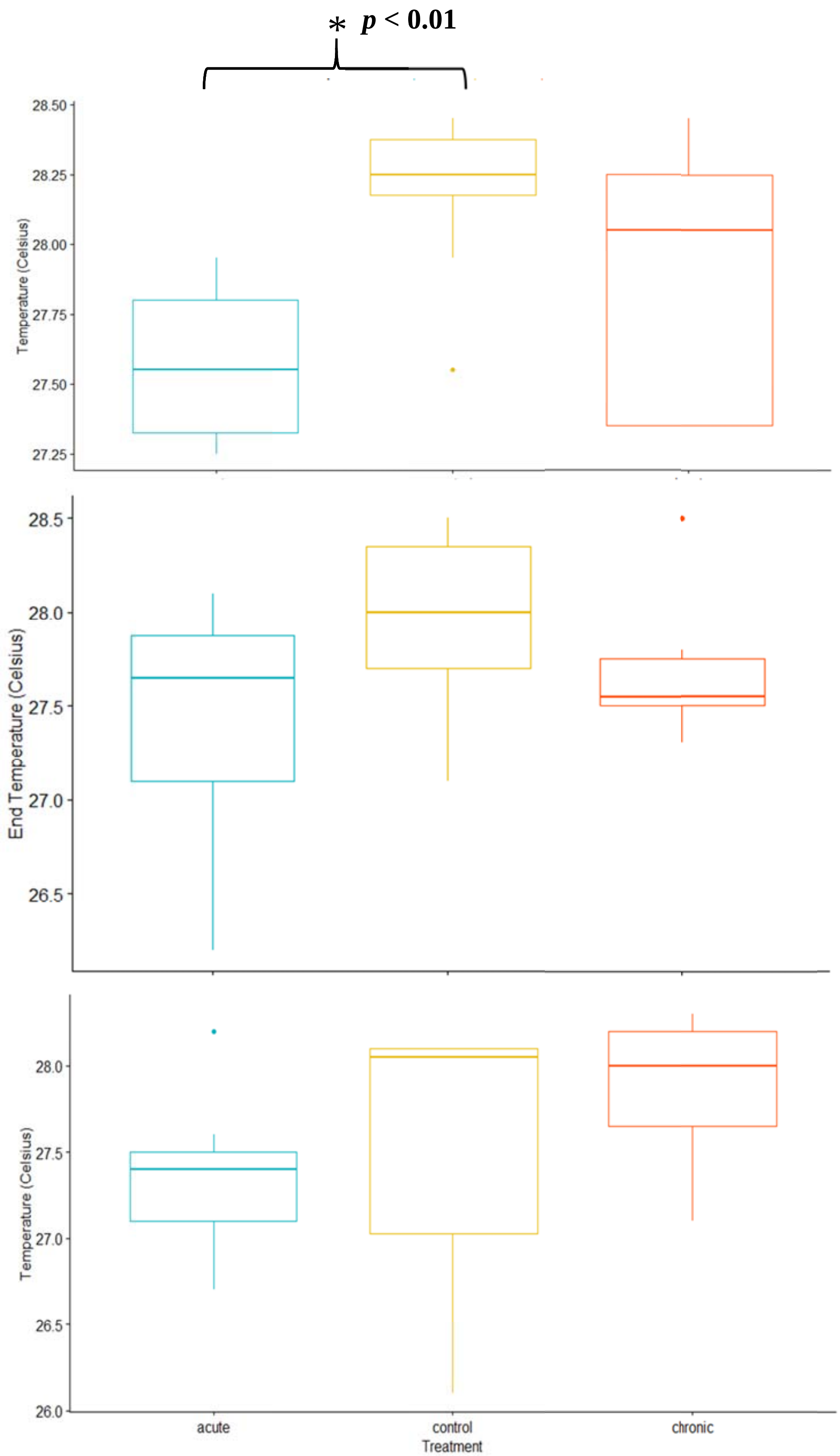


Figure B1: Photo of critical thermal maxima testing chambers



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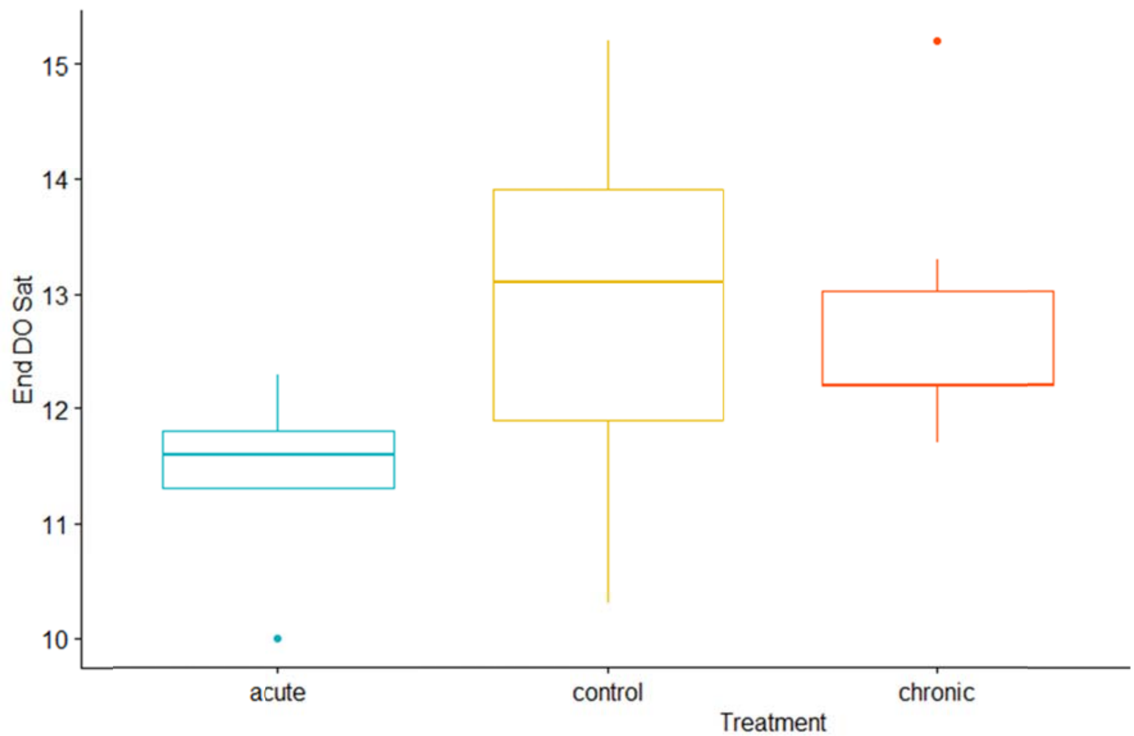

Figure B3: Four-day dissolved oxygen minima results. The box shows the interquartile range from first quartile (Q1) to the third quartile (Q3), while the middle hinge indicates the median. The whisker bar ends indicate the maximum and minimum values not standard error or deviation.

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