THESIS

COMPLEX METAL MIXTURE REDUCES APPARENT PROTEIN CARBONYLATION IN A TOLERANT AQUATIC MACROINVERTEBRATE, *ARCTOPSYCHE GRANDIS*

Submitted by

Sam B. Duggan

Department of Environmental and Radiological Health Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2015

Master's Committee:

Advisor: Howard Ramsdell

William Clements
Marie Legare

ABSTRACT

COMPLEX METAL MIXTURE REDUCES APPARENT PROTEIN CARBONYLATION IN A TOLERANT AQUATIC MACROINVERTEBRATE, *ARCTOPSYCHE GRANDIS*

Mining is widespread and an economically important industry. Unfortunately, acid mine drainage (AMD) can pollute ecosystems with a cocktail of contaminants too complex for accurately forecasting its health consequences. However, through quantification of fundamental toxic events, the effects of complex mixtures can be observed. This project explored two potentially insightful and convenient endpoints. First, oxygen consumption (MO_2), a well-established and sensitive indicator of respiratory impairment was utilized. Second, protein carbonyl content (PCC), an experimental ecological biomarker widely lauded in biomedical circles as a highly conserved indicator of health status was assessed for its utility in a metal tolerant aquatic macroinvertebrate, *Arctopsyche grandis. A. grandis* were exposed to eight environmentally relevant target concentrations (in duplicate) of AMD for eight days at a temperature controlled greenhouse containing artificial flow-through streams. As expected, MO_2 was inversely related to treatment concentration (R^2=0.35, p=0.015). Protein carbonyl content, however, diverged from predictions. Protein carbonyl content analysis detected significantly more oxidative protein injury in control treatments than in metal-rich AMD treatments ($p<0.001$). Moreover, there was not a significant difference in PCC between different AMD concentrations. Protein carbonyl content's departure from anticipated results likely is the consequence of dynamic interactions between direct and indirect effects at the chemical, biochemical, physiologic and behavioral levels. The results of this project illustrate flaws of

utilizing a single biochemical marker to observe effects of a toxic mixture. Rather, a broad suite of biomarkers should be assayed to determine sublethal toxicity. These results also illustrates how multiple stressors can yield unanticipated outcomes.

ACKNOWLEDGEMENTS

Thank you to my funding sources, National Institute of Environmental Health Sciences (Grant #: 1R01ES020917-01) which paid for water chemistry analysis and the entire exposure portion of this project. Colorado Parks and Wildlife for bravely purchasing the first protein carbonyl assay kit used in this project. Dr. Howard Ramsdell and Colorado State University's Environmental and Radiologic Health program for purchasing important laboratory supplies. Also to the contributors of the crowdfunding campaign that allowed for completion of the laboratory portion of this project. I will always be grateful for your generosity: Lisa Schaeffer, Skip Hamill, Joanne Goldfarb, Randy Cole, Donna Duggan, Ben Duggan, Chis Celani, Dave Carstensen, Dr. Jennifer Ruh, Casey Branchini, Dr. Antony Auerbach, Jax Nguyen, Bryan Kean, Meaghan Engel, Shannon Engel, Sandy Lapham, Faris Al-Jarboa and Katie Campos.

I would like to thank my friends who contributed invaluable ideas, time and labor with sample collection, and various experimental procedures: Brandon Durand, Chis Tullos, Elliot Martin, Sammy Katz, Brittanie Dabney, Yuichi Iwasaki and members of the aquatic ecotoxicology group. Also to collaborators at Colorado School of Mines for conducting water chemistry analysis.

To my advisor and committee members without whom I stood no chance of success; Dr. William Clements, Dr. Marie Legare and Dr. Howard Ramsdell thank you for helping me when I needed the help and also giving me the freedom to help myself. Thank you for your knowledge, wisdom and creativity. To Dr. Greg Dooley and Dr. Danica Degroot for guiding me through experimental procedures during the initial phases of this project. To Pete Cadmus for allowing

me to piggy-back on his project, for his optimism and strategic pessimism, his skill and mentorship.

Thank you to Ben, Annie and Donna Duggan, John and Corinna Ceballos and Joanne Goldfarb for allowing me to vent frustrations and failures. You made me feel like I was capable of overcoming any obstacle. To Dr. Anthony Auerbach for being a straight-shooter and advising me to putting one foot in front of the other when I got bogged down in the weeds. Thank you to my friends, family and the unnamed who molded and inspired me. Thank you to the many scientists who laid the foundation for this project

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

AMD = Acid Mine Drainage
BLM = Biotic Ligand Model
DNPH = 2,4-Dinitrophenylhydrazine
GIE = Gregory Incline Effluent
ICP-OES = Inductively Coupled Plasma Optical Emission Spectrometry
MCO = Metal Catalyzed Oxidation
NFCC = North Fork of Clear Creek
PCC = Protein Carbonyl Content
RS = Reactive Species
SRL = Stream Research Laboratory

INTRODUCTION

Mining is globally widespread and an economically important industry, particularly in regions with complex geomorphology such as Colorado's Rocky Mountains. Colorado was founded, in large part, due to mining driven expansion stimulated by discovery of rich mineral and precious metal deposits during the mid to late 19th century. Mining remains an important historic and economic feature of the state. However, consequences of its legacy continue to impact health of regional ecosystems, especially aquatic habitats. There are seven hard rock mining Superfund sites in Colorado, including the Central City/Clear Creek Superfund site which is used as the model for this project. Although, every site impacted by mining disturbances is chemically and physically unique, all contribute source metals to the environment altering natural balances, shifting the community structure of organisms, affecting health and behavior.

Mining related metal contamination typically occurs when water and oxygen react with metallic minerals of pyrite (FeS_2), sphalerite (ZnS) and chalcopyrite ($CuFeS_2$) (EPA 2004). These reactions produce labile metals and sulfuric acid. The overall reaction between water and pyrite presented below is fundamental to common mining related metal contamination:

$$2FeS_2(s) + 7O_2(g) + 2H_2O(l) \rightarrow 2Fe^{2+}(aq) + 4SO_4^{2-}(aq) + 4H^+(aq)$$

Importantly, the sulfuric acid produced lowers pH of water. The net effect is accelerated aqueous metal production through acid mediated metal dissolution. Therefore, two mechanisms co-occur to free geologically sequestered metals. The resulting metal rich effluent is commonly referred to as 'acid mine drainage' (AMD). Acid mine drainage is often a complex mixture consisting of numerous constituents and is the primary origin of metal contamination at most mining sites.

Predicting toxicity of mixtures is a daunting prospect. Yet, predictions are considered crucial to risk assessment and resource allocation. The Biotic Ligand Model (BLM) was developed for this purpose, although to date the BLM is generally capable of predicting toxicity associated with a sole metal constituent in simple mixtures (Di Toro et al. 2001; Balistrieri and Mebane 2014). Even in simple mixtures toxicity often diverges from expectations predicted by the BLM and this divergence intensifies with mixture complexity (Kamo and Nagai 2008). Shortcomings of the BLM result from dynamic interactions among mixture constituents, the environment, and organisms. Moreover, when one considers how organisms shift behavior, physiology, and biochemical defenses during contaminant exposures, forecasting mixture toxicity becomes even more problematic.

Unfortunately, in many AMD contaminated ecosystems metals occur in complex mixtures that complicate forecasting toxic effects. However, through quantification of fundamental toxic events, the toxic effects of mixtures can be inferred. In doing so, exposures can be related to organism impairment, potentially assessing mixture effects during ongoing contamination disturbances, without need for assessing specific mixture interactions.

In this project, sub-lethal toxicity of a complex metal mixture is explored using an aquatic macroinvertebrate species, *Arctopsyche grandis*. *A. grandis* is a large net-spinning caddisfly (Order: Trichoptera) with a wide distribution among lotic waterways in Western North America (Hauer and Stanford 1981). Aquatic macroinvertebrates are particularly useful for observing ecosystem health in disturbed streams (Clements 1991, Clements et al. 2010). They are easy to collect, found in nearly all aquatic habitats and are a critical link between primary producers and top predators (Resh 1992). Aquatic macroinvertebrates are crucial to bioavailability and biomagnification of contaminants due to their low to intermediate trophic

positions and high abundance (Cain et al. 2004; Barata et al. 2005). Moreover, in many aquatic ecosystems, macroinvertebrates maintain ecosystem services and thus water quality (Cheimonopoulou et al. 2011).

Here, an attempt is made to simplify the issue of predicting mixture toxicity by directly observing fundamental physiological and biochemical responses that are well established indicators of health status. Oxygen consumption (MO_2) during strenuous activity was used to demonstrate the cumulative effects of a complex metal mixture at the physiologic level. Then oxidative protein damage was observed through quantification of protein carbonyl content (PCC). Similar PCC methods are currently being exploited by biomedical and pharmaceutical researchers to elucidate human health status for a variety of pathologies, but they have not been adequately scrutinized in an ecological context. As protein carbonylation correlates with complex human pathologies, this assay may also prove useful for monitoring health status of *A. grandis* in mining impacted aquatic ecosystems.

Metal Toxicity in Aquatic Organisms

Organisms require certain metals at low physiologic concentrations. For example, zinc, copper and manganese are critical structural components of various enzymes. Iron or copper are required for oxygen binding and transport. Cobalt is needed for vitamin synthesis. Many others (eg., lead, cadmium, aluminum) have no known biological function and are potent toxins, though all metals have toxic potential.

Metal bioavailability varies widely in accordance with interactions between dissolved materials, particulates, sediment, and the water column. These interactions are governed by

physiochemical factors including temperature, salinity, pH, environmental redox status, competition for metal binding sites and the presence of complexing agents (Rand et al. 1995; Lushchak 2011). Only labile metals are bioavailable to aquatic organisms and exposure occurs predominantly through respiratory pathways via receptor mediated metal uptake from sediment and the water column (Rand et al. 1995). In general, dietary inputs are a minor contribution to metal loading compared with diffusion across gill membranes (Barata et al. 2002). However, the relative importance of respiratory versus dietary exposures is fiercely debated and likely varies by species (Xie and Buchwalter 2011). Because individual species predominantly occupy specific microhabitats within an aquatic ecosystem, organisms are exposed to metals at various concentrations and bioavailabilities depending on their habitat preferences. In general, benthic organisms closely associated with sediment and interstitial spaces have the greatest likelihood of high exposures (Rand et al. 1995).

Metals elicit toxic effect through numerous modes of action. Inhibition of calcium and sodium ion regulation is particularly relevant to aquatic organisms. Many metals, either directly or indirectly, disrupt ion channels at gill surfaces. (Niyogi and Wood 2004). Copper is a sodium uptake inhibitor, whereas cobalt, zinc and cadmium block calcium uptake (Kamo and Nagai 2008). Some studies also point to copper as a calcium uptake inhibitor (McGeer et al. 2000). Regardless, the result is osmoregulatory impairment which can be fatal itself, or in less severe exposures cause mucus secretion, cell injury and widening diffusion distances for gas and ion exchange in gill tissue (Spicer and Weber 1991). Reduced lamina surface area accompanies osmoregulatory dysfunction, and cell death follows severe or chronic exposures (Lushchak 2011). Hypoxia often results from metal induced gill injury, resulting in further tissue damage and organism death (Skidmore 1970; Skidmore and Tovell 1972).

In contrast, nickel and iron are not thought to be direct ion channel disruptants. However, osomoregulatory dysfunction and respiratory distress also results from exposure. Accumulation of nickel and iron at gill surfaces causes irritation, mucus secretion and epithelial hypertrophy (Dalzell and Macfarlane 1999; Pane et al. 2004; Tkatcheva et al. 2004). Consequently, the net effect of metal exposure to gill tissue is similar between metals even though the modes of toxicity are different.

To determine the net effect of metals acting as respiratory toxicants MO_2 has been utilized as an indicator of gill impairment. In fact, Spicer and Weber (1991) demonstrated that although zinc and copper caused pathological changes in aquatic organisms, MO_2 was a more sensitive marker of organism distress. In rainbow trout (*Oncorhynchus mykiss*), nickel exposures lead to reduced MO_2 during strenuous activity, and this effect persisted after exposures ended (Pane et al. 2004). Pane (2004) concluded that depressed MO_2 results in reduced fitness by negatively influencing predator avoidance and prey capture. Similar conclusions were reached when *O. mykiss* were exposed to aluminum (Wilson et al. 1994). In *Hydropsyche angustipennis*, copper exposure resulted in significantly reduced ventilation rates and respiratory activity (Van der Geest et al. 1999). Moreover, communities of aquatic macroinvertebrates exposed to metals resulted in reduced community MO_2 (Clements 2004).

Gill tissue is thought to be the initial target for many metals in aquatic systems, but it is not the sole target. After first interacting with gill tissue or being consumed through dietary exposures, metals distribute throughout an organism causing pathological damage to various structures. Liver pathologies, tubular necrosis of kidney nephrons, muscle fiber vacuolization and congestion, gastrointestinal brush border atrophy and irregular cardiac morphology are observed in aquatic species exposed to toxic metals (Oliva et al 2009; Marchand et al. 2009;

Lushchak 2011; Maharajan et al. 2012). Moreover, in mammalian systems toxic metals are known to cause osteoporosis-like conditions, neurological toxicity, immunosuppression, endocrine disruption and play roles in carcinogenesis (Kacmar et al. 1999; Toyokuni 2002; Borthiry 2007; Prousek 2007; Rivera-Mancía et al. 2010; Abbas et al. 2013). There is little reason to assume these effects are limited to mammals.

The previously discussed studies predominantly evaluated effects of metals in simple mixtures without additional perturbations by other stressors. However, in natural systems metal toxicity is more nuanced. For example, at low concentrations (2-20 µg/L) copper is a potent olfactory toxicant in fishes (Tierney et al. 2010; McIntyre et al 2012). Although not directly lethal in itself, olfactory disruption results in reduced predator avoidance behaviors, altering predator and prey dynamics. Moreover, metal exposures have been linked to reduced predator avoidance behaviors in aquatic macroinvertebrates (Mogren and Trumble 2010). Negative influences on predatory foraging have also been observed in metal exposed aquatic macroinvertebrates (Riddell et al. 2005).

Studies have even shown the existence of cumulative effects between metal toxicity and seemingly unrelated stressors. Metal-adapted benthic macroinvertebrate communities are more susceptible to UV-B radiation than communities without a history of metal exposure (Kashian et al. 2007). Metal contamination can also disproportionately affect species with important ecosystem services such organic matter decomposition (Carlisle and Clements 2005). Therefore, metal contamination can have indirect ecosystem level effects by disrupting energy transfer.

Using insect communities, researchers have demonstrated cumulative direct and indirect effects of metal toxicity. Elevated metal concentrations are associated with reduced insect abundance and species richness (Clements 2004, Clements et al. 2010; Schmidt et al. 2012).

Additionally, studies on insect communities demonstrated habitat avoidance behavior during metal exposure. Benthic macroinvertebrate drift, an escape response to exogenous stressors, was directly correlated with metal exposure (Clements 1999, Clements 2004). However, hydropsychids specifically, decreased their drift response (Leland 1985). It is worth noting that taxis responses, like drift, increases vulnerability to predation.

Altered ecosystem health and organism fitness are a consequence of direct and indirect effects of toxic metals. Moreover, toxic outcomes are complicated by metals co-occurring in complex and dynamic mixtures. When contaminants occur in mixtures toxicity often diverges from predicted responses. In fact, toxicity associated with identical metal mixtures demonstrated antagonistic, additive or synergistic effects depending on the test species selected (Eaton 1973; Finlayson and Verrue 1982). In a review of metal mixture toxicity studies on aquatic organisms, Norwood (2003) concluded there was no obvious rule governing additivity of metal mixtures. Rather, effects of complex mixtures in natural systems often yield 'ecological surprises' (Paine et al. 1998).

Ecotoxicological and environmental literature have focused on metals acting predominantly in gill tissue to elicit lethal effects on aquatic organisms; however, medical literature has long described metals as catalysts capable of producing harmful free radicals. For example, toxic mechanisms catalyzed by free radical generating transition metals (eg., iron, copper, manganese, cobalt, chromium), are thought to play fundamental and secondary roles in the etiology of numerous disease states (Prousek 2007).

Free Radicals and Protein Oxidation

Free radicals are defined as a reactive molecule or atom containing an unpaired electron in its outermost valence shell (Halliwell and Gutteridge 1986). Free radicals are composed of diverse frameworks including lipids, other organic structures and reactive oxygen, nitrogen, and sulfur species. In addition, there are other molecules, such as singlet oxygen, that can act like free radicals. Although singlet oxygen has paired electrons in its outermost valence shell, the electrons occupy high energy orbitals. Therefore, singlet oxygen is unstable and will react with nearby molecules in a similar manner as free radicals. Hydrogen peroxide, hypochlorous acid, and ozone are other high energy molecules (Prousek 2007). For these reasons this thesis refers to all such high energy molecules and atoms as reactive species (RS)

The consequence of RS reactions in biological systems are complex and multifaceted, but a general characteristic is electron abstraction from macromolecules resulting in chemical oxidation (Joyner-Matos et al. 2006). To counteract the oxidative effects of RS, organisms have developed defense systems that repair RS injury, directly scavenge RS or remove oxidized molecules. Collectively, these systems are known as antioxidants. In healthy organisms, antioxidants and oxidants are balanced. However, oxidative stress occurs when that balance shifts towards oxidants (Halliwell and Gutteridge 1999; Dalle-Donne et al. 2003a; Fedorova et al. 2013). During oxidative stress, RS generation rates accelerate exacerbating cellular injury (Dalle-Donne et al. 2003a; Dalle-Donne et al. 2003b; Moller et al. 2011; Fedorova et al. 2013).

There are several endogenous process that form RS. Aerobic metabolism in eukaryotic cells is thought to be the single largest contributor of RS under normal conditions (Turrens 2003; Orrenius et al. 2007). Between 0.1% and 3% of oxygen consumed during mitochondrial

respiration is uncoupled from the electron transport chain and converted to superoxide anion radical ($\cdot O_2^-$) (Boveris and Chance 1973; Fridovich, 2004). Superoxide anion radical, the precursor of most other cellular RS, can then proceed through transformation steps in chain reactions to yield additional RS.

The immune system is another significant source of endogenous RS generation (Oliver 1987). Macrophages and neutrophils form RS as a direct consequence of their phagocytic immune function (Prousek 2007; Fedorova et al. 2014). Various oxidases and reductases such as the cytochrome p450 family contribute to RS generation as well (Prousek 2007). Additionally, neurotransmitters of the catecholamine family can produce RS, particularly during times of stress (McAnulty et al. 2003). Exogenous contributors to RS generation include ionizing radiation, organic and inorganic toxins including transition metals.

Although there is wide range of mechanisms for RS macromolecule damage, some of which are not perfectly understood, RS are capable of harming DNA, carbohydrates, lipids and proteins (Kohen & Nyska 2002; Halliwell 2007a; Prousek 2007). Interestingly, not all macromolecule oxidation is harmful at the organismal level, rather some oxidative processes are critically important for normal cellular function and homeostasis. Reactive species (RS) and related oxidative changes have important regulatory roles in signal transduction, hormonal responses and regulation of transcription factors (Wu and Weiss 1991; Fedorova et al. 2014). Basal levels of macromolecule oxidation serve integral and beneficial functions for organisms. It is only when oxidation proceeds at elevated rates that RS are injurious at the organismal level, and this injurious state is known as oxidative stress.

Oxidative stress related pathologies have been well explored in mammalian systems and are considered highly relevant to numerous diseases and impairments. Human and veterinary

medical literature is replete with examples relating RS oxidation to an organism's health status. Much debate exists over the causes of disease and whether RS oxidation is an effect or fundamental to disease etiology (Dalle-Donne et al. 2003b). Although oxidative stress is not always causative, over 200 diseases have been linked to it (Lushchak 2007; Fedorova et al. 2014). Reactive species (RS) have been observed as damage inducing agents in cancer, heart failure, endothelial dysfunction, cardiovascular disorders, inflammation, intestinal tract diseases, neurological disorders, diabetes, eye diseases, ischemia and senescence (Levine, 1993; Conrad et al. 2000; Barnham et al 2004; Ferreira et al. 2004; Shao et al. 2006; Halliwell, 2007b; Prousek 2007; Butterfield and Sultana 2008; Senoglu et al. 2008; Amir et al. 2009; Eleuteri et al. 2009; Mattaliano et al. 2009; Reddy et al. 2009; Selle et al. 2009; Tripathy and Grammas, 2009).

> *"Oxidative damage to proteins, lipids or DNA may all be seriously deleterious and may be concomitant. However, proteins are possibly the most immediate vehicle for inflicting oxidative damage on cells because they are often catalysts rather than stoichiometric mediators; hence the effect of damaging one molecule is greater than stoichiometric"* (Dalle-Donne et al. 2003a).

There is general consensus that protein oxidation is a relevant marker of oxidative stress and is particularly suitable for monitoring an organism's health status or disease progression (Lushchak 2007). Although all oxidized macromolecules leave hallmarks of RS oxidation, proteins are preferable markers compared with lipid, carbohydrate or nucleic acid oxidations for several reasons. Because a protein's function is completely dependent on its structure and protein oxidation causes structural changes, protein oxidation is linked with protein dysfunction. Proteins also have specific functions, many of which are well understood. Therefore, protein oxidation can often be linked to cellular mechanisms. Products of protein oxidation form earlier and are less transient than other RS markers (Dalle-Donne et al. 2003b). Often protein

oxidations are irreversible and stable, thus protein oxidation is convenient for analytical quantification in both fresh and preserved tissue.

Antioxidant concentration and regulation have been utilized to monitor organism health status as proximate markers of oxidative stress. However, antioxidants are not consistent between species, individuals or tissue types (Lushchak 2007). Therefore, direct observations of protein oxidations are preferable to indirect measurements like enzyme regulation. Protein oxidation is also preferable to direct observations of RS themselves. The intrinsic reactivity of RS leads to an ephemeral existence. Ironically, it is the lack of stability that is critical to RS modes of action that also limits their practical use as a monitoring tool.

Protein carbonyls are the most commonly utilized and well-studied marker of protein oxidation (Dalle-Donne et al. 2003b; Moller et al. 2011; Fedorova et al. 2014). Protein carbonyls are aldehyde or ketone products of amino acid oxidation by RS. They form during diverse sets of oxidative conditions and from diverse RS sources. Therefore, protein carbonyls are considered a very general indicator of oxidative stress (Dalle-Donne et al. 2003b).

As with other forms of protein oxidation, protein carbonylation is generally considered irreversible, although cysteine and methionine can be reduced back to their original form through the action of the thioredoxin reductase system and methionine sulfoxide reductases, respectively (Breusing et al. 2008). Cysteine and methionine carbonylations are thought to be crucial to numerous signal transduction pathways and are important mediators of normal physiologic processes, whereas irreversible protein carbonylation is a mediator of cellular injury (Moller et al. 2011).

Carbonyl adducts cause alterations to a protein's tertiary structure resulting in partial, or complete dysfunction (Fedorova et al. 2014). The consequence of protein carbonylation varies

based on the location of oxidation, the duration of insult and the rate of protein turnover (Davies 1987; Breusing et al. 2008). In mammalian systems, accumulation of protein carbonyls cause protein aggregations, inflammatory and autoimmune responses, apoptosis and increased energy expenditures associated with proteasome mediated protein degradation (Foell et al. 2007; Miller et al. 2011; Curtis et al. 2012; Fedorova et al. 2013). Irreversibly carbonylated proteins must be degraded in order to maintain cellular hemostasis (Friguet et al. 1994a; Friguet et al. 1994b).

Proteins are carbonylated through interactions with lipid peroxidation products, reactive carbohydrates, and peptide backbone cleavage (Swallow 1960; Garrison 1987; Mullarkey et al. 1990; Friguet et al. 1994). However, metal-catalyzed oxidation (MCO) is a notably important mechanism for cells exposed to elevated concentrations of transition metals (Stadtman and Berlett 1991). In MCO, a Fenton reaction occurs between a reduced transition metal and hydrogen peroxide forming a highly unstable hydroxyl radical (Haber and Weiss 1932). All macromolecules are susceptible to oxidation by hydroxyl radicals; thus a variety of oxidation products including protein carbonyls are produced as a result of a Fenton reaction (Stadtman and Levine 2003). A similar reaction between reduced transition metals and alkyl peroxides forming an alkoxyl radical (·RO) can also take place. An example of MCO with iron (II) is presented below:

$$H_2O_2 + LFe(II) \rightarrow HO\cdot + OH^- + LFe\ (III)$$

$$ROOH + LFe(II) \rightarrow RO\cdot + OH^- + LFe(III)$$

Here, LFe refers to the transition metal bound to a biological ligand such as a protein. Metal catalyzed oxidation (MCO) of proteins commonly occurs when reduced transition metals bind to the metal binding sites of cysteine, methionine, glutamic acid, aspartic acid, histidine or tyrosine (Bertini and Turano 2007). Moller (2011) reported that these amino acids represent

approximately 21% of all residues in eukaryotes, therefore an extraordinarily large number of potential MCO sites exist. However, not all metal binding sites allow for a Fenton reaction. If all coordination sites are occupied then, no reaction will occur as hydrogen or alkyl peroxides cannot bind (Lushchak 2007).

Metal catalyzed oxidation is characterized as a 'caged' reaction because protein oxidation occurs very near the site of radical formation (Stadtman and Oliver 1991). Hydroxyl and alkoxyl radicals often react with the first macromolecule they encounter, consequently protein oxidation develops within a few nanometers of metal binding sites on adjacent amino acids (Moller et al. 2011). Moreover, MCO derived protein carbonyls are elevated in proteins containing more metal binding sites than in proteins with fewer (Yuan et al. 2007). Maisonneuve (2009) defined protein carbonyl 'hot-spots' as three or four consecutive amino acids consisting of arginine, lysine, proline, or threonine with the addition of a nearby metal binding site. Proline, histidine, lysine and arginine are the most commonly carbonylated amino acids as they are often located near metal binding sites and are easily oxidized (Climent et al. 1989).

Because protein carbonyls are a general marker of oxidative stress, PCC has been successfully used as a marker of senescence, disease, various stressors and toxins (Lushchak 2007). Studies demonstrate, Alzheimer's and Parkinson's disease, diabetes, rheumatoid arthritis, muscular dystrophy, cataracts, cancers, amyotrophic lateral sclerosis, chronic alcohol consumption, carbon tetrachloride exposure, transition metal exposure, aromatic hydrocarbon exposure, all correlate with PCC (Garland et al. 1988; Chapman et al. 1989; Murphy and Kehrer 1989; Hensley et al. 1994; Hensley et al. 1995; Uchida et al. 1995; Kong et al. 1996; Grattagliano et al. 1996; Alam et al 1997; Sundari et al. 1997; Floor and Wetzel 1998; Smith et al 1998; Baynes and Thorpe 1999; Lushchak 2011). Furthermore, PCC is elevated during

sickness and diminishes with health improvement (Lushchak 2007). Dalle-Donne (2003a) asserts that PCC is not simply an indicator of oxidative stress, rather it is a marker of "disease-derived protein dysfunction".

In addition to mammalian systems, protein carbonylation has been explored in several aquatic species. Protein carbonyl content was elevated in goldfish, *Carassius auratus*, brain tissue, but not liver or kidney after exposure to trivalent chromium (Lushchak et al. 2009a; Lushchak et al. 2009b). Protein carbonyls in the mussel *Mytilus galloprovincialis* were elevated when they were exposed to metals and polycyclic aromatic hydrocarbons (Kaloyianni et al. 2009). Similar results were observed in the scallop *Chlamys farreri* (Liu et al. 2012; Xiu 2014). In field studies, protein carbonyls were elevated in sea snail *Echinolittorina peruviana* and corkwing wrasse *Symphodus melops* at aquatic sites heavily impacted by mining (Almroth et al. 2008; Jara et al. 2014). Protein carbonyl content was also elevated in mussels *Dreissena polymorpha* from exposure to a complex mixture of organic pollutants at heavily impacted sites (Parolini et al. 2013). Antioxidant responses (e.g., superoxide dismutase, catalase and glutathione-S-transferases) have been observed in insects exposed to transition metals; however, PCC was not directly measured (Xie et al. 2009; Barata et al. 2005; Wang and Wang 2009; Xie and Buchwalter 2011).

Although relatively little work has explored the previously discussed effects in aquatic macroinvertebrates, RS generation and Fenton reactions are not specific to mammalian systems. Rather RS are capable of causing injury in all taxa (Livingstone 2001; Lushchak 2011). Prousek (2007) contends that MCO was potentially the earliest mechanism for free radical production in life. Reactive species generation through Fenton and Fenton-like reactions are as relevant to disease etiology in aquatic organisms as they are in mammals (Livingstone 2001). Lushchak

(2011) reiterated the idea that all responses to RS in terrestrial animals are shared with their aquatic counterparts. Interestingly, many pathologies known to occur from metal mixture exposure also occur as effects of RS injury. In ecological studies, toxic mechanisms are rarely determined. However, there is reason to pursue the possibility of MCO as a potential cause of toxicity in aquatic organisms.

The objective of this project is to explore changes in *A. grandis* health status as affected by exposure to a complex metal mixture. A well-established physiological marker, MO_2, will be examined to validate organism distress. Then, an experimental ecological biochemical marker, PCC, will be assayed to determine its utility as a biochemical marker of oxidative stress. I hypothesize that PCC will have a positive relationship with metal exposure concentration, and MO_2 will have a negative relationship.

METHODS

Central City/Clear Creek Superfund Site

The Central City/Clear Creek Superfund Site is located in Clear Creek, Jefferson and Gilpin Counties, west of Denver, Colorado in the Rocky Mountains. Considered a 'mega site' within the Superfund program, numerous inactive precious metal mines are dispersed across a 1000-square kilometer drainage basin. Placer and hard rock mining activity date back to the mid-nineteenth century, however few mines are currently active. Despite inactivity, impacts from historical mining continue to produce substantial metal contamination in the region, resulting in elevated metal loading with a corresponding reduction in biodiversity and abundance of aquatic organisms (EPA 2004). Contaminated water originating from the Superfund site drains into Clear Creek's mainstem. Clear Creek's mainstem serves as a water source for the Denver metropolitan area, and also supports recreational fishing, gold panning, whitewater, and other popular outdoor activities.

Several tributaries and numerous contaminant sources comprise the Central City/Clear Creek Superfund Site. However, this project focused on a single point-source, Gregory Incline. Gregory Incline drains flooded underground mines and discharges directly into the North Fork of Clear Creek (NFCC). Effluent flow rate of Gregory Incline is relatively consistent and is the largest contaminant source for NFCC during low-flow conditions (EPA 2004). Pure Gregory Incline effluent (GIE) is expected to be acutely toxic to trout and macroinvertebrates due to its acidity and high concentrations of toxic metals. (EPA 2004).

Test Organism Collection

The headwaters of the Cache La Poudre River are located in and near Rocky Mountain National Park in Northern Colorado, East of the Continental Divide. The upper reaches of the Cache La Poudre are minimally impacted by contaminants including metals (Clements 2004). *Acrtopsyche grandis* collected from this section of the Cache La Poudre River were considered naïve with negligible contaminant exposure history. Naïve organisms were selected because historic metal exposure can alter metal accumulation processes and influence toxic effects (Cain et al. 2006).

A. grandis, and related hydropsychids, have been previously studied as test species due to their ubiquitous distribution and relatively high metal tolerance (Cuffney and Minshall 1981; Cain et al. 2004; Mogren and Trumble 2010). Therefore, *A. grandis* was selected as the test organism because they were thought to be an environmentally relevant species predicted to survive exposure to Gregory Incline effluent. Secondly, this species' minimal chitinous exoskeleton as well as their relative large size (mean wet weight=79 mg, sd=2.0**)** aided in homogenization and biochemical procedures. *A. grandis'* large size and conspicuous nets also allowed for targeted collection.

All *A. grandis* used in this experiment were collected from one site (40.68 N, -105.76 W) upstream of recent fire disturbances on a single sampling occasion (August 6, 2013). *A. grandis* nymphs (n=252) were collected by hand from the Cache La Poudre reference site. Highest population densities were found in swift water secured to basketball-sized rocks with rough, asymmetrical surfaces rather than small rocks with smooth surfaces in slow moving water. *A. grandis* were stored in insulated coolers filled with river water aerated by air pumps and air

stones. Frequent water changes limited temperature deviations from river conditions during storage. Ridged teflon mesh was used to simulate structure minimizing density dependent aggression and related stress during transport to Colorado State University's Stream Research Laboratory (SRL). Collected *A. grandis* were housed in coolers for a maximum of ten hours before transplant into the SRL's artificial streams.

Acclimation and Exposures

The SRL is contained within a translucent, temperature controlled, polycarbonate greenhouse**.** Horsetooth Reservoir, a mesotrophic reservoir with similar water chemistry as the Cache La Poudre reference site, served as a the SRL's reference water source (Clements et al. 2013). *A. grandis* (N=14 organisms per stream) were distributed among sixteen artificial streams randomly assigned to eight target concentration of GIE, in duplicate. For each respective artificial stream, all *A. grandis* were confined within a single covered mesh basket **(**11.0 cm diameter, 12.7 cm long, 600 µm mesh, nylon monofilament**)**. Current was produced by a paddlewheel maintaining flow rates near 0.35 m/s. All artificial streams were set up as flow-through systems with complete water turnover occurring every 20 minutes. Reference water from Horsetooth Reservoir was dispensed from a headbox at 1.0 L/min, and the volume of each artificial stream was maintained at 20L by a standpipe.

A. grandis were acclimated for 48-hours to SRL conditions prior to GIE exposure. Immediately preceding exposures, 'day-zero' samples were preserved by flash freezing in liquid nitrogen and then stored at -80˚C for subsequent biochemical analysis. During exposures, approximately three-hundred gallons of effluent collected directly from Gregory Incline adit

were transported to the SRL. Effluent collection was repeated every 48-hours to limit metal oxidation during effluent storage.

To achieve various environmentally relevant GIE concentrations, effluent was continuously pumped by peristaltic pumps into each respective artificial stream. *A. grandis* were exposed for eight days to eight target GIE concentrations, in duplicate, consisting of 0, 6, 12, 25, 40, 80, 100 and 200 percent of NFCC's metals concentrations measured during summer base flow conditions**.** For example, the target concentration of 100% was intended to mimic the observed metals concentration in NFCC directly downstream of the Gregory Incline adit. The 200% target concentration was approximately twice the observed metals concentration, and 25% was approximately one-quarter the observed metals concentration in NFCC directly downstream of the Gregory Incline adit. All target concentrations were designed to achieve environmentally relevant metal exposures while also bracketing current exposures at the NFCC. However, these target concentrations were not used as an independent variable in statistical procedures. Instead, observed concentrations of zinc (a metal constituent of GIE) were used to relate GIE concentrations to response variables.

Peristaltic pumps were calibrated twice daily to precisely control effluent flow rates and thus exposure concentrations. Temperature, pH, conductivity and dissolved oxygen were measured on five occasions using calibrated YSI 63 and 550a (YSI Inc. Yellow Springs, Ohio) hand held meters. Water hardness was calculated based on inductively coupled plasma-optical emission spectrometry (ICP-OES) determined concentrations of dissolved Sr, Al, Fe, Zn, Mg, Ca and Mn (Wetzel et al. 2000). Alkalinity was determined by HACH Model AL-DT Alkalinity Test Kit (Hach Co. Loveland, Colorado). Additionally, filtered (0.45um) and unfiltered water chemistry samples were collected on four occasions. These samples were acidified with trace

metal grade nitric acid before being analyzed for major elements. Analysis was conducted by collaborators at Colorado School of Mines through ICP-OES with a Perkin Elmer Optima 5300 DV (Perkin Elmer. Waltham, Massachusetts) using EPA method 200.7. Collaborators at Colorado School of Mines also determined total and dissolved organic carbon in a representative subset of unfiltered and filtered water samples, respectively. Analysis was conducted using persulfate oxidation/UV irradiation with a Sievers 5310C TOC analyzer (GE Analytical Instruments. Boulder, CO).

During acclimation and exposures, mesh baskets were brushed daily with a soft bristled toothbrush to prevent diminished current resulting from algae accumulation on mesh baskets in less concentrated treatments and accumulation of precipitated metals in more concentrated treatments. Care was taken to prevent alterations in basket position during cleaning, thereby limiting stress associated with changes to *A. grandis* net orientation.

Oxygen Consumption

A subset of three *A. grandis* from each artificial stream were measured for oxygen consumption immediately following exposures. Three *A. grandis* were confined, as a group, within a glass tube hermetically joined to a YSI 550A dissolved oxygen probe. Reference water within the respiration chamber was mixed with a stir rod separated from the organisms by Teflon mesh and washers. Changes in water temperature within the chamber were limited by a water bath resting on the stir plate. Dissolved oxygen concentrations were recorded every 30 seconds for 10 minutes.

Individual *A. grandis* were not measured for oxygen consumption because individual oxygen consumption rates were not significantly different from blanks (data not shown). To overcome the limited sensitivity of the YSI 550A dissolved oxygen probe three organisms were confined simultaneously within the respiration chamber. As a result, organisms interacted in an aggressive manner. Therefore, all oxygen consumption rates were a measure of active respiration. No individuals subjected to oxygen consumption measurements were subsequently utilized in any biochemical procedures. Stress associated with oxygen consumption measurements and associated aggressive behavior were thought to potentially influence and confound protein carbonylation results (Lushchak 2007; Mogren and Trumble 2010; Lushchak 2011).

Sample Preservation

All *A. grandis* were gently dried with Delicate Task Wipers (Kimberly-Clark. Fullerton, CA) and weighed to the nearest 100 µg. Immediately following, organisms were placed in 2mL polypropylene cryotubes then flash frozen in liquid nitrogen. Cryotubes were preserved at -80˚C for biochemical analysis.

Determination of Protein Carbonyl Content

A. grandis were washed in a cold 100mM EDTA solution to remove surface bound metals. Surface bound metals were not expected to significantly contribute to oxidative toxicity, but could influence the protein carbonyl analysis**.** Washed organisms were homogenized in cold

phosphate buffered saline (pH 7.4) containing Halt™ protease inhibitor cocktail (Pierce Biotechnology Co. Rockford, Illinois) and a general antioxidant, butylated hydroxytoluene, (Sigma-Aldrich Co. St. Louis, Missouri) with a Teflon pestle homogenizer (Thomas Scientific. Swedesboro, New Jersey) and an electric hand drill. Cell membranes were lysed by sonication using a Sonifier® Cell Disruptor (Heat Systems Co.). Great care was taken during homogenization and sonication to maintain all tools, materials, reagents and homogenates at ice-cold temperatures. Lipids were removed by passing homogenates through cotton. Nucleic acids were removed by precipitation with 1% streptomycin sulfate (Sigma-Aldrich Co. St. Louis, Missouri). The protein concentration of homogenates was diluted with PBS to 10µg protein/mL as determined by the Bradford dye binding assay (Bradford 1976). Protein carbonyl content was measured using a commercially available Oxiselect™ Protein Carbonyl ELISA kit (Cell Biolabs Inc. San Diego, California). Briefly, protein carbonyls were derivatized by reaction with 2,4-dinitrophenylhydrazine (DNPH) forming a DNP hydrazone product. Anti-DNP antibodies and secondary horseradish peroxidase conjugated antibodies allowed for protein carbonyl quantification using a Versa Max tunable microplate reader (Molecular Devices LLC. Sunnyvale, California) reading at 450nm. Standards were created using carbonylated bovine serum albumin (Cell Biolabs Inc. San Diego, California). Standards were plated in triplicate (sd=0.445) and unknowns plated in quadruplicate (sd=0.349).

Data Analysis

As mentioned earlier, target GIE concentrations (0, 6, 12, 25, 40, 80, 100 and 200%) were used to bracket metal concentrations observed in NFCC directly downstream of Gregory

Incline adit and also to calibrate GIE flow rate into the artificial streams. However, target GIE concentrations were not used to statistically relate toxic effects to metal concentrations. Rather, mean observed total zinc concentrations, determined by ICP-OES, were used to relate exposure to response variables. Although GIE is a complex mixture consisting of numerous metal and non-metal constituents, zinc was selected as a tracer metal for GIE because it responded in a highly predictable manner to changes in GIE concentration (Figure 1). Moreover, low concentrations of zinc in reference water from Horsetooth Reservoir and the toxicological importance of zinc to aquatic organisms made it an ideal tracer for GIE concentration. Therefore, mean total zinc concentration was used as the independent variable for nearly all statistical procedures even though GIE contains many meaningful contaminants in addition to zinc.

All water chemistry values, including element concentrations were subjected to descriptive statistical analysis. Respective means and standard deviations were then analyzed for correlation to zinc concentrations by linear regression with Excel software (Microsoft Co. Redmond, Washington). Respiration data was plotted as dissolved oxygen concentration vs. time for each treatment. Trendlines for each respective treatment were determined by Excel software (Microsoft Co. Redmond, Washington). The slope of each resulting trendline represented a dissolved oxygen consumption rate within the respiration chamber. The slope of a blank was subtracted from this trendline and then oxygen consumption rates were normalized to the total mass for all *A. grandis* individuals within the respective respiration chambers in order to compare mass-normalized oxygen consumption rates (MO_2) between treatments. Mass-normalized oxygen consumption rates were plotted against zinc concentrations and analyzed by

linear regression. Coefficients of determination and p-values were calculated using the Proc Reg function on SAS™ 9.4 (SAS Institute Inc. Cary, North Carolina).

For analysis of PCC, standard curves were created using four-parameter curve fitting software Soft Max Pro (Molecular Devices LLC. Sunnyvale, California). Relative PCC was determined by dividing absolute PCC of day-8 unknowns by the mean of day-0 unknowns from a given ELISA plate. Correlations between zinc concentration and PCC were determined by linear regression. Coefficients of determination and p-values were calculated using the Proc Reg function on SAS™ 9.4 (SAS Institute Inc. Cary, North Carolina). Categorical analysis was conducted using analysis of variance (ANOVA) with post hoc multiple comparisons by Proc GLM function on SAS™ 9.4 (SAS Institute Inc. Cary, North Carolina). To create categories the mean total zinc concentration was determined for both duplicates from the 0, 40, 100, and 200% GIE target concentrations. This produced the categories of 0.004, 0.082, 0.223, and 0.462 mg/L zinc, respectively. Relative PCC was analyzed for significant differences ($P < 0.05$) between these categories by the Proc GLM procedure discussed above.

RESULTS

Survivorship

Mortality was observed in all but two artificial streams. However, no definitive trend was observed across treatments (R^2<0.01). Survivorship ranged from 100% to 50%, mean survivorship was 81% (Figure 2). Because *A. grandis* constructed and resided within silk retreats during exposures, it was not practical to determine a temporal component for mortality without causing undue stress to the organisms. Therefore, mortality was only assessed after exposures were complete. Partly consumed *A. grandis* were observed in numerous treatments; whole deceased organisms were not observed. It was unclear what caused mortality. It was also unclear whether observed cannibalism was predatory or opportunistic scavenging of deceased individuals by surviving *A. grandis*. Moreover, in surviving individuals, iron and other metals were noticeably deposited at gill surfaces. This deposition displayed an obvious staining on gills. *A. grandis* gills were deeply reddish/orange in concentrated GIE treatments compared with light tan in less concentrated GIE treatments.

Physicochemical Parameters

Mean total organic carbon was 3.19 ppm (sd=0.21). Mean dissolved organic carbon was 3.26 ppm (sd=0.49). Across all treatments, temperature fluctuated between 10.0 °C and 12.3 °C. Temperature was directly related to effluent concentration (R^2=0.66) (Figure 3). Mean temperatures ranged from 10.92 °C (sd=0.51) to 11.88 °C (sd=0.41) (Table 1). pH was inversely related to effluent concentration (R^2=0.89) (Figure 3). Mean pH ranged from 7.67 (sd=0.13) to

6.81 (sd=0.06) (Table 1). Both dissolved oxygen concentration and oxygen saturation were inversely related to effluent concentration (R^2=0.72 and 0.48 respectively) (Figure 4). Mean dissolved oxygen concentration ranged from 8.25mg/L (sd=0.09) to 7.46 mg/L (sd=0.14) (Table 1). Mean dissolved oxygen saturation ranged from 75.22% (sd=1.92) to 68.86% (sd=0.98) (Table 1).

Specific conductivity was directly related to and highly correlated with treatment concentration (R^2=0.99) (Figure 5). Mean specific conductivity ranged from 55.70 µS (sd=9.49) to 313.98 µS (sd=16.50) (Table 1). Water hardness was also directly related to and highly correlated with treatment concentration (R^2=0.99) (Figure 6). Mean water hardness ranged from 30.18 mg/L to 158.34 mg/L (Table 1). No standard deviations for water hardness were calculated because mean metal concentrations, rather than metal concentrations from individual sampling events, were used to calculate mean water hardness. Alkalinity was inversely related to treatment concentration (R^2=0.43) (Figure 6). Alkalinity ranged from 26.0 mg/L (sd=2.58) to 11.6 mg/L (sd=2.39) (Table 1).

ICP-OES Analysis

The mean dissolved concentration for iron ranged from 0.048 mg/L (sd=0.005) to 14.115 mg/L (sd=5.631) (Table 2). The mean dissolved concentration for manganese ranged from 0.002 mg/L (sd=0.001) to 2.581 mg/L (sd=0.148) (Table 2). The mean dissolved concentration for copper ranged from 0.0016 mg/L (sd=0.0009) to 0.0057 mg/L (sd=0.0027) (Table 2). The mean dissolved concentration for nickel ranged from 0.0009 mg/L (sd=0.0019) to 0.0191 mg/L (sd=0.0009) (Table 2). The mean dissolved concentration for zinc ranged from 0.0016 mg/L

(sd=0.013) to 0.493 mg/L (sd=0.073) (Table 2). Mean dissolved sulfur concentrations ranged from 1.953 mg/L (sd=0.100) to 51.343 mg/L (sd=2.958) (Table 2). All dissolved element concentrations discussed here directly correlated with total zinc concentrations (R^2>0.67) indicating that target exposure concentrations were achieved, particularly with respect to each other (Figure 7).

Total metal concentrations for all metals were similar to dissolved concentrations indicating limited metal precipitation and non-target binding. However, iron and copper had the largest discrepancies between dissolved and total concentrations. Total iron was the most abundant transition metal measured. Its mean concentration ranged from 0.145 mg/L (sd=0.009) to 18.005 mg/L (sd=5.845) (Table 3). The mean total concentration for copper ranged from 0.0014 mg/L (sd=0.0009) to 0.152mg/L (sd=0.0056) (Table 3). All total element concentrations discussed here directly correlated with total zinc concentrations (R^2>0.91) indicating that target exposure concentrations were achieved, particularly with respect to each other (Figure 8). The total concentrations for other metal and non-metal constituents of the GIE exposures are located in table 3 and dissolved concentrations are located in table 2.

Protein Carbonyl Content

Relative PCC was determined by dividing the absolute PCC of exposed individual *A. grandis* by the absolute PCC for 'day-0' unexposed individual *A. grandis*. This procedure was conducted to normalize PCC data to inter-plate analytic variations making comparisons of PCC between assay plates more robust. Relative PCC was inversely related to treatment concentration (R^2=0.24, $p<0.01$) (Figure 9). Although PCC correlated with treatment

concentration, simple linear regression analysis failed to fully describe PCC's relationship to GIE exposure. Rather than a continuous concentration-dependent trend, PCC results appeared to have a binary relationship with GIE consisting of two parts. One effect was evident in control organisms and a separate distinct effect in GIE exposed organisms regardless of GIE concentration. This thesis refers to this relationship as a binary effect.

As a result of regression analysis appearing to display a binary effect for relative PCC versus zinc concentration, an ANOVA with multiple comparison analysis was conducted on a subset of treatments. Mean relative PCC was 3.53 for 0.004 mg/L Zn, 2.17 for 0.082 mg/L Zn, 2.03 for 0.223 mg/L Zn and 1.82 for 0.462 mg/L Zn after eight days of exposure (Figure 10). To put the relative PCC values in context, a relative PCC of 1 would be equivalent to 'day-0' PCC and a relative PCC of 2 would be twice the PCC of 'day-0' organisms. All mean relative PCC values were elevated compared with 'day-0' values. ANOVA revealed a significant difference between GIE treatments ($F_{3,20}$=11.07, $p<0.001$) (Figure 10). Furthermore, multiple comparison analysis demonstrated relative PCC was significantly elevated in control treatments compared with other treatments ($p<0.001$). However, no significant difference was observed in relative PCC between non-control treatments.

Oxygen consumption

Oxygen consumption rate (MO_2) was inversely related to treatment concentration (R^2=0.35, P=0.015) (Figure 11). *A. grandis* consumed more oxygen during strenuous activity, in treatments with lower concentrations of GIE than in more highly concentrated treatments.

Additionally, during oxygen consumption measurements, *A. grandis* from concentrated treatments were noticeably less active than control *A. grandis.*

Relative Protein Carbonyl Content versus Oxygen Consumption

Relative protein carbonyl content was not well correlated with MO_2 ($R^2 = 0.09$, $P = 0.4672$) (Figure 10). Although there was a nominal positive relationship between the two endpoints, a definitive trend was not observed. Interestingly, data points representing the more concentrated treatments (0.082, 0.223 and 0.462 mg/L Zn) were closely clustered, suggesting some similarity between these treatments. Data points representing the less concentrated treatments (0.004 and 0.018 mg/L Zn) were dissimilar from each other as well as all other treatments.

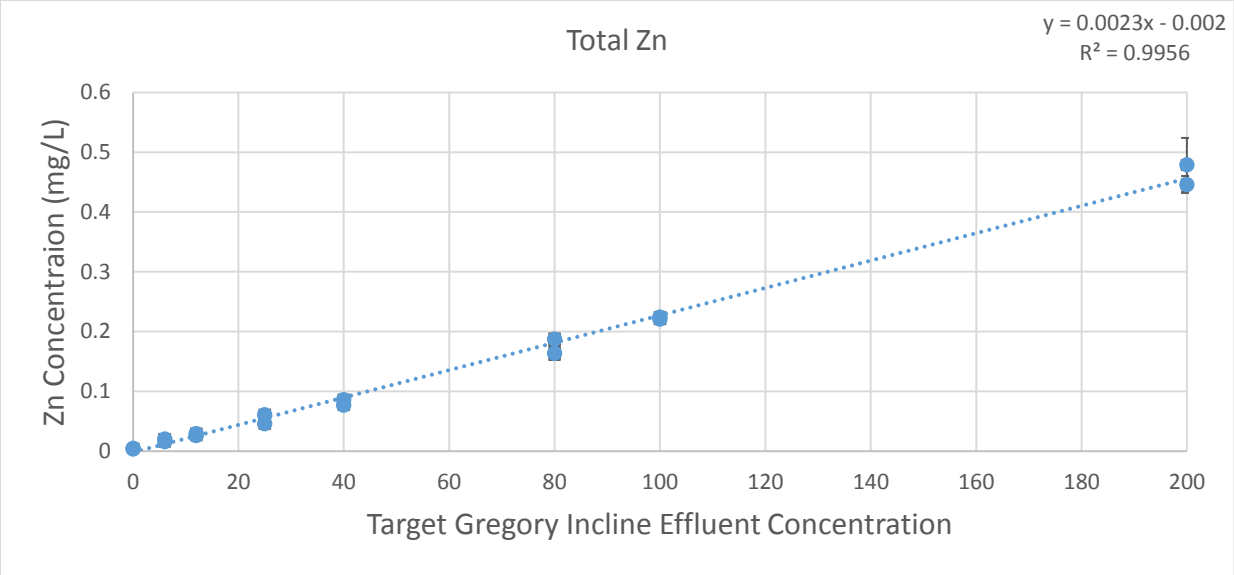


Figure 1: Metal concentrations were determined through collaboration with Colorado School of Mines by ICP-OES. Total Zn concentrations were plotted against eight target Gregory Incline effluent exposure concentrations (in duplicate). Target Gregory Incline effluent concentrations were intended to bracket the actually metal concentrations observed in the North Fork of Clear Creek directly downstream of Gregory Incline adit during summer base flows. Each point represents observations of a single artificial stream. Data are expressed as means +/- standard deviation of four sampling events.

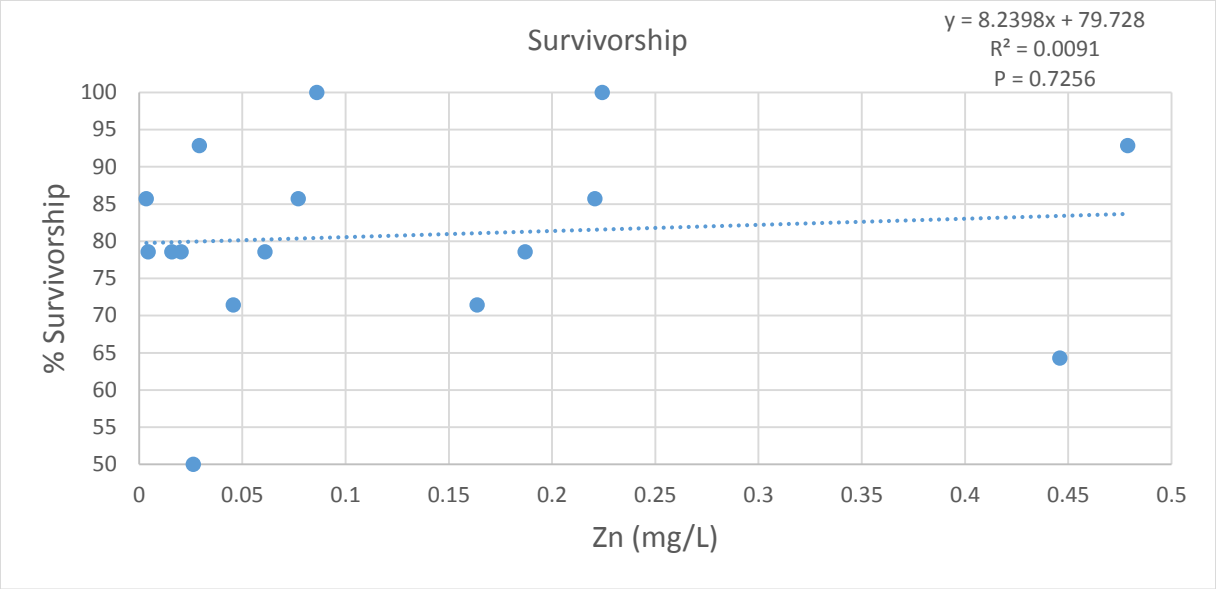


Figure 2: Effects of Gregory Incline effluent on *Arctopsyche grandis* survival (N=14 per artificial stream) after eight days of exposure. Each point represents the survivorship for a single artificial stream. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

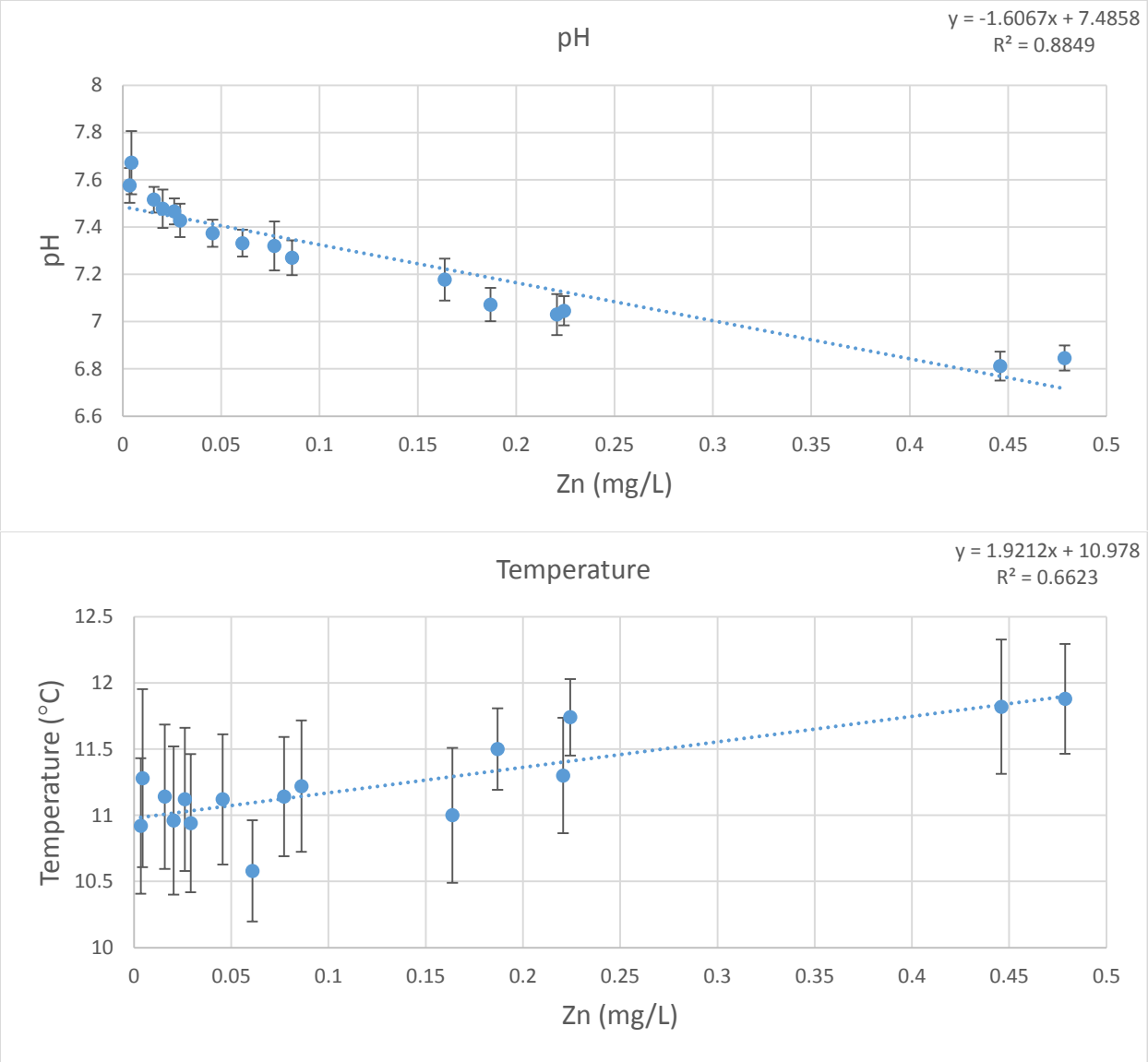


Figure 3: Effects of Gregory Incline effluent on pH and temperature. Each point represents observations of a single artificial stream. Data are expressed as means +/- standard deviation of five sampling events. Measurements were conducted using a YSI 63 hand held meter. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

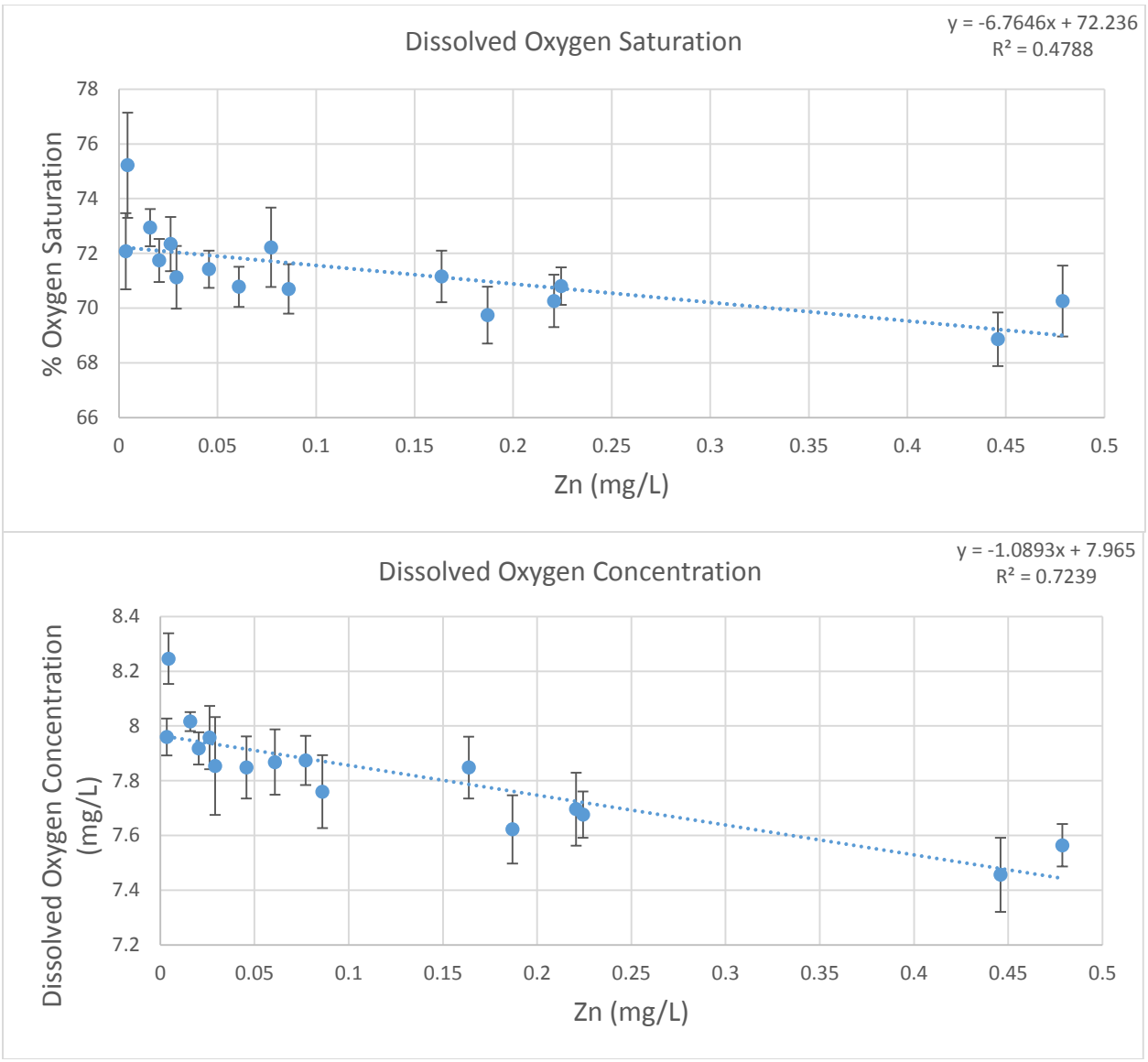


Figure 4: Effects of Gregory Incline effluent on dissolved oxygen concentration and dissolved oxygen saturation. Each point represents observations of a single artificial stream. Data are expressed as means +/- standard deviation of five sampling events. Measurements were conducted using a YSI 550a hand held meter. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

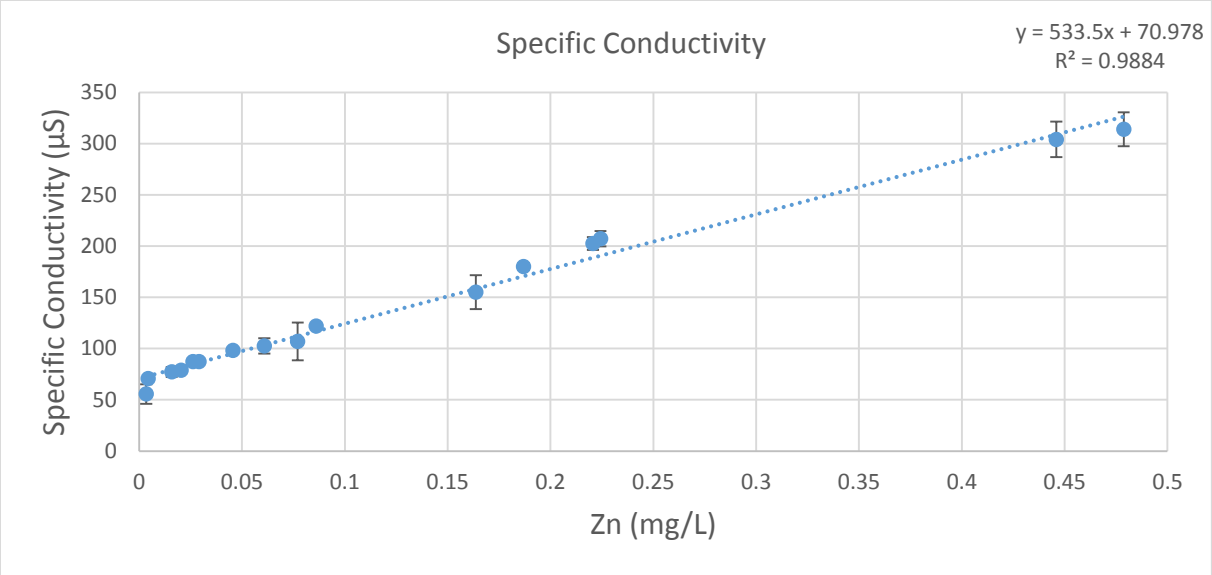
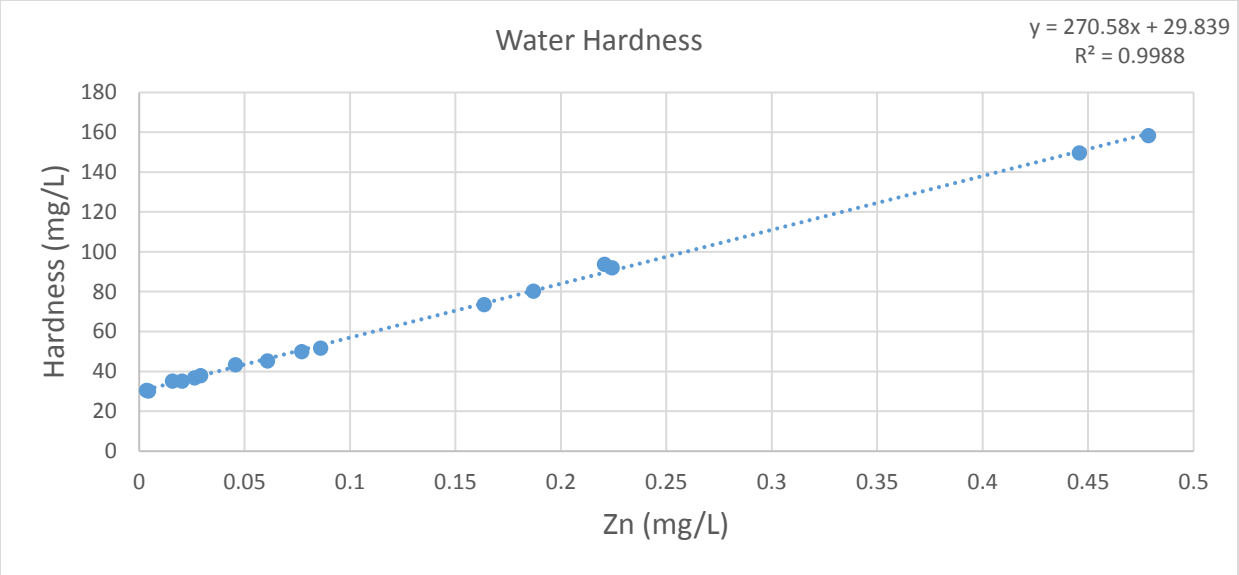


Figure 5: Effects of Gregory Incline effluent on specific conductivity. Each point represents observations of a single artificial stream. Data are expressed as means +/- standard deviation of five sampling events. Measurements were conducted using a YSI 63 hand held meter. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.



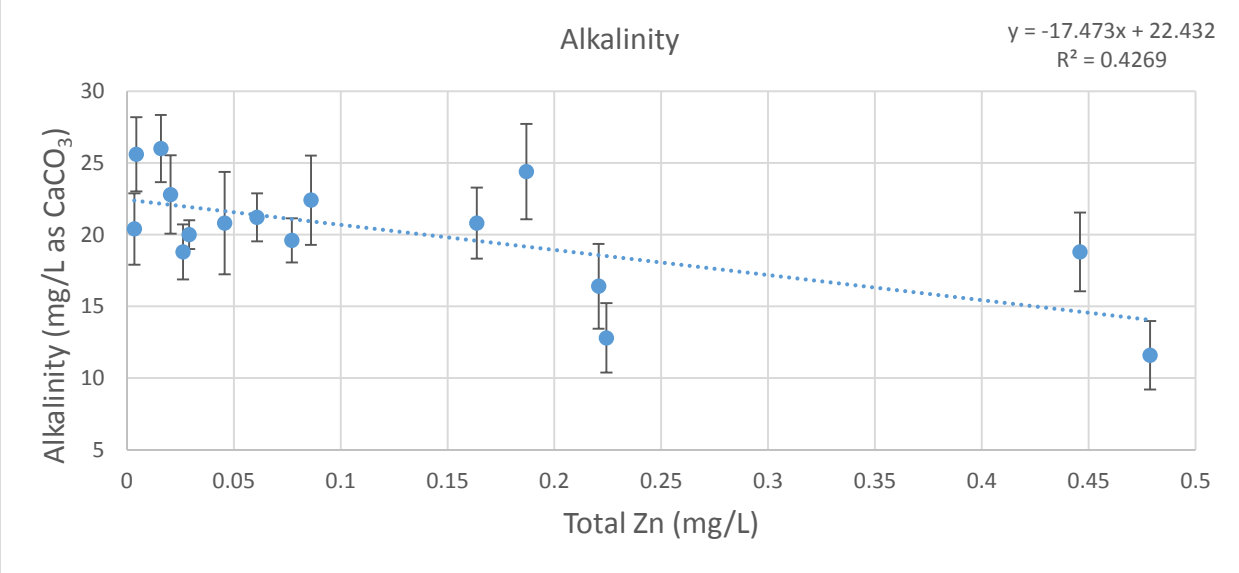


Figure 6: Effects of Gregory Incline effluent on alkinity and water hardness. Each point represents observations of a single artificial stream. Hardness was calculated based on mean ICP-OES determined concentrations of dissolved Sr, Al, Fe, Zn, Mg, Ca and Mn (Wetzel et al. 2000). Alkalinity was determined by HACH Model AL-DT Alkalinity Test Kit. Alkalinity data are expressed as means +/- standard deviation of four sampling events. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

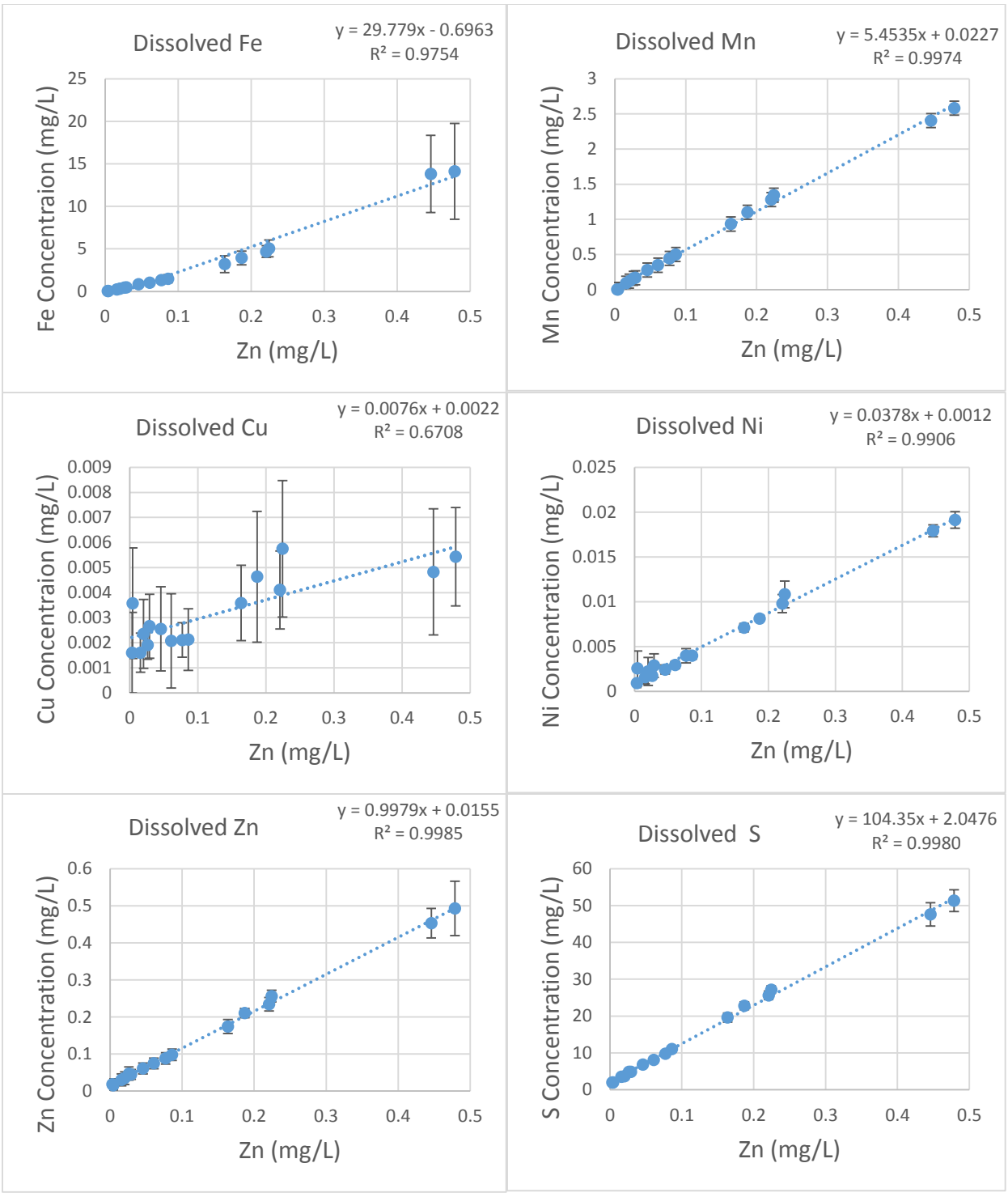


Figure 7: Metal concentrations were determined through collaboration with Colorado School of Mines by ICP-OES. Each point represents observations of a single artificial stream. Data are expressed as means +/- standard deviation of four sampling events. Samples were filtered through a 0.45um filter during sample collection. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

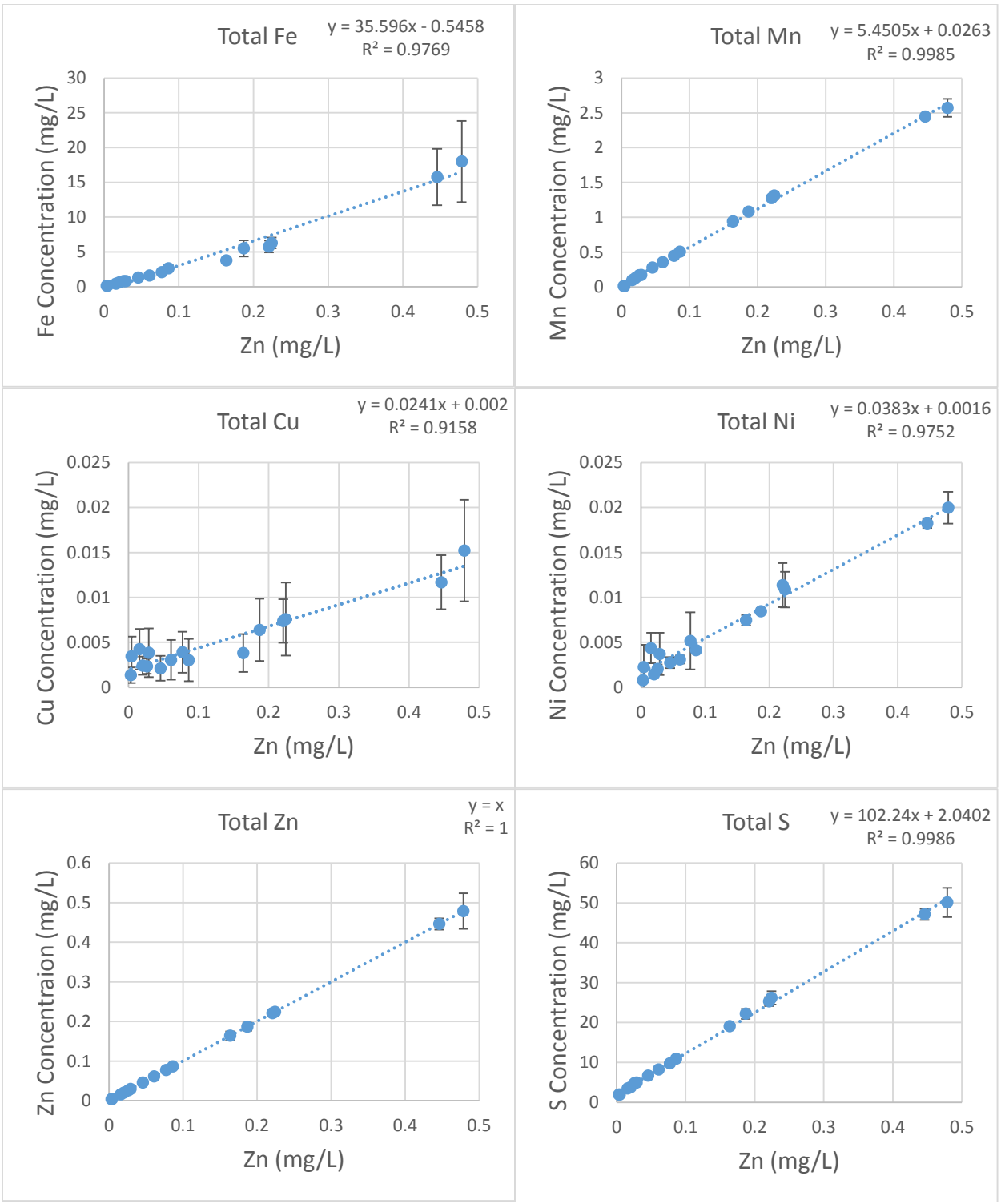


Figure 8: Metal concentrations were determined through collaboration with Colorado School of Mines by ICP-OES. Each point represents observations of a single artificial stream. Data are expressed as means +/- standard deviation of four sampling events. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

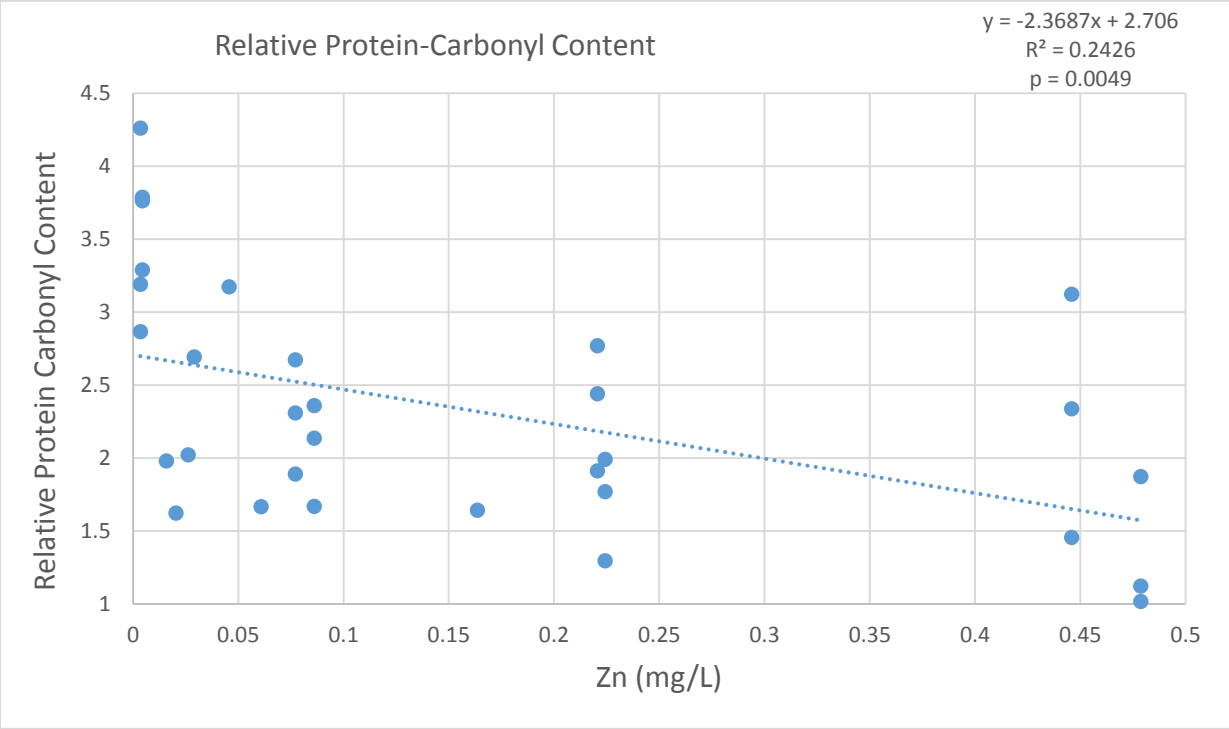


Figure 9: Effects of Gregory Incline effluent on *Arctopsyche grandis* relative protein-carbonyl content (PCC) after eight days of exposure. Relative PCC was determined by dividing the absolute PCC of exposed individual *A. grandis* by the mean absolute PCC for 'day-0' unexposed *A. grandis*. Each point represents the relative protein-carbonyl content of an individual organism. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

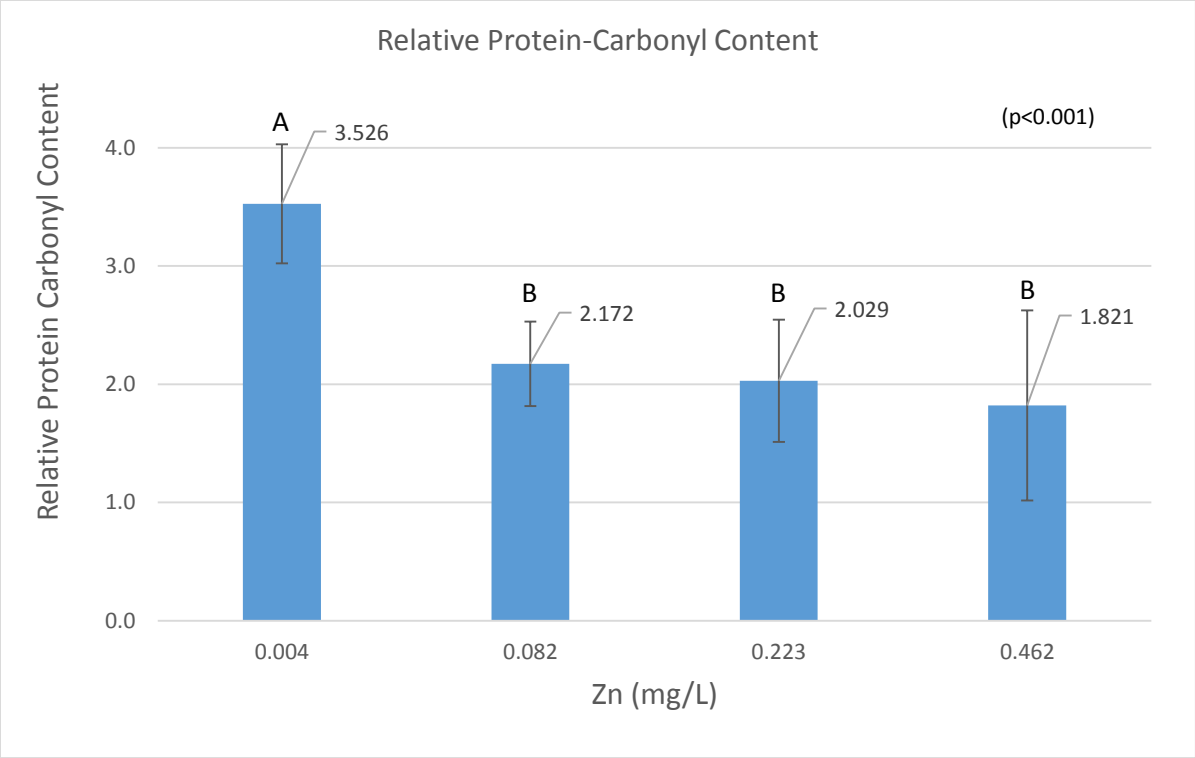


Figure 10: Effects of Gregory Incline effluent on *Arctopsyche grandis* relative protein-carbonyl content (PCC) after eight days of exposure. Relative PCC was determined by dividing the absolute PCC of exposed individual *A. grandis* by the mean absolute PCC for 'day-0' unexposed *A. grandis.* Relative PCC results from duplicate target exposure concentrations (0, 40, 100 and 200% Gregory Incline effluent) were respectively pooled to permit categorically analysis across target concentrations. Categories are the mean observed total Zn concentration for each respective target concentration. Relative PCC data are expressed as means +/- standard deviation of six individual organisms per category. Letters above histograms indicate significant differences between categories determined by ANOVA with multiple comparison analysis ($p<0.001$).

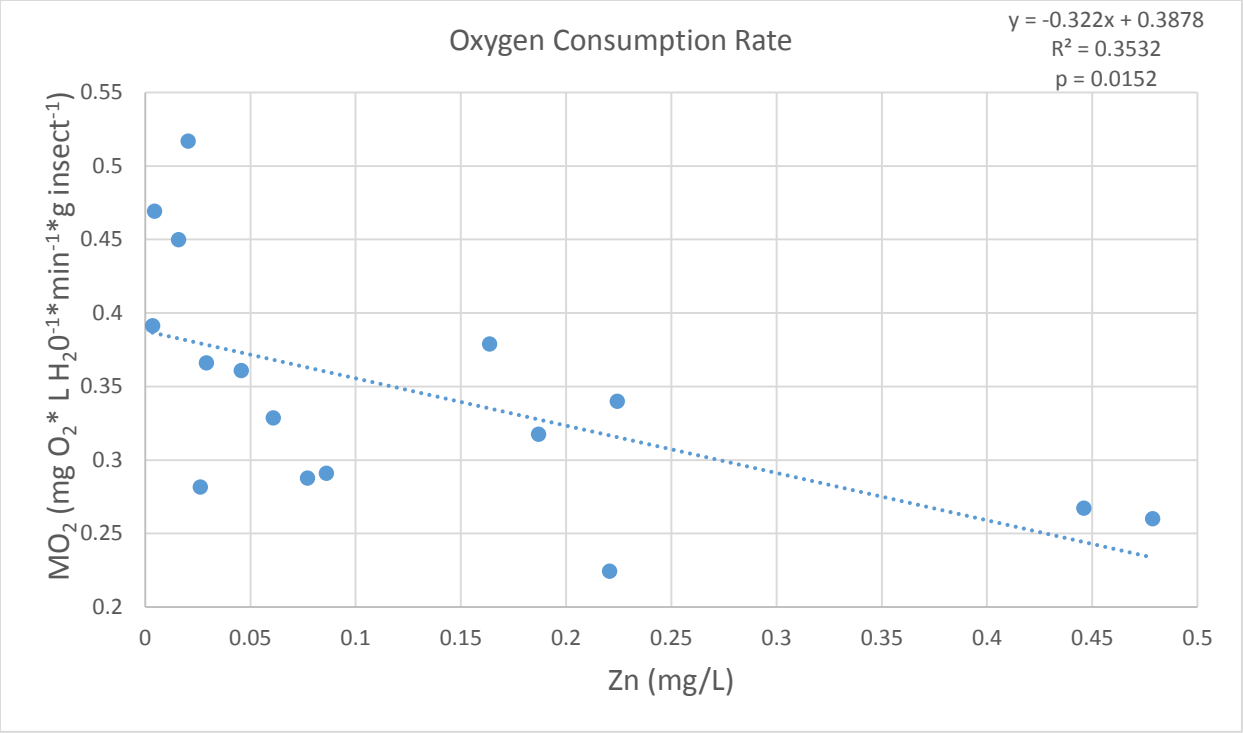


Figure 11: Effects of Gregory Incline effluent on *Arctopsyche grandis* oxygen consumption (MO_2) after eight-day exposures. Each point represents a mass normalized, pooled oxygen consumption rate for three organisms from a single artificial stream during strenuous activity. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

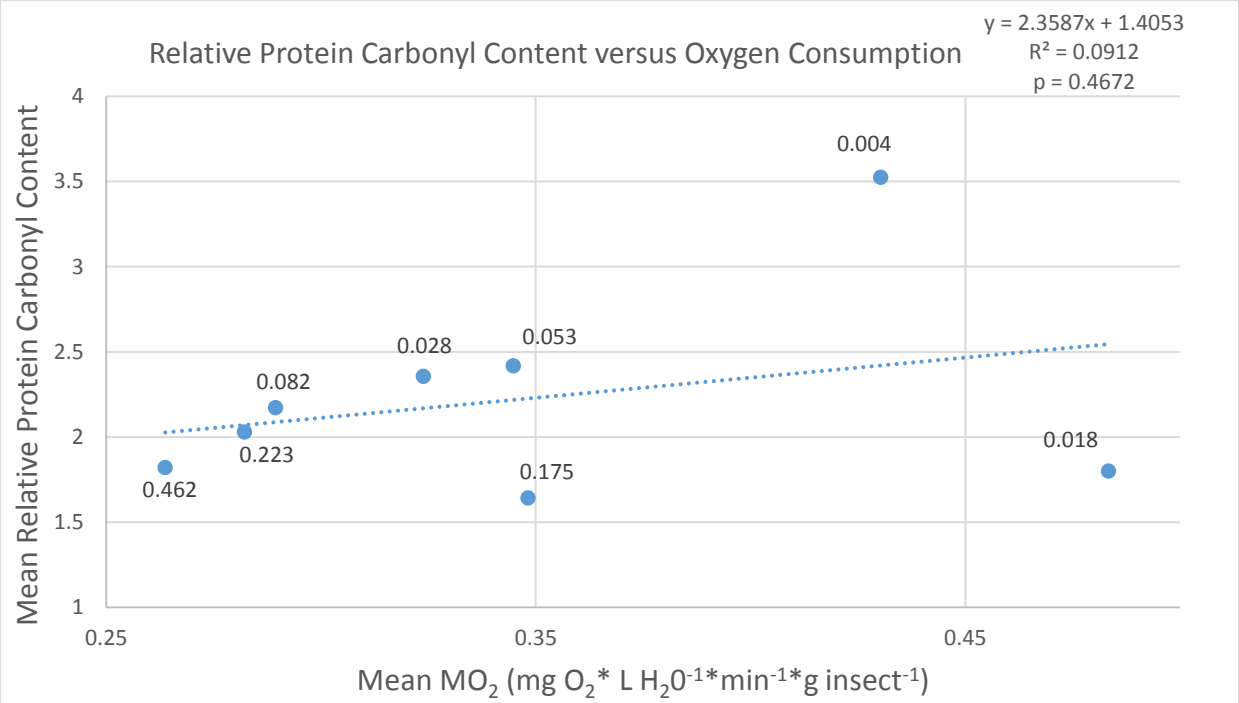


Figure 12: *Arctopsyche grandis* relative protein carbonyl content plotted against oxygen consumption after eight-days of exposure to various Gregory Incline effluent concentrations. Each point represents the mean relative protein carbonyl content and mean MO_2 for a particular target Gregory Incline effluent concentration. Data point values indicate a pooled mean total Zn concentration for both duplicates of a given target Gregory Incline effluent concentration.

Table 1: Phyisicochemical parameters for sixteen artificial streams were sampled on four to five occasions with a YSI 63 and 550a handheld meters. Each data point is a mean with standard deviations in respective parentheses. Hardness was calculated based on ICP-OES determined concentrations of dissolved Sr, Al, Fe, Zn, Mg, Ca and Mn. (Wetzel et al. 2000). Alkalinity was determined by HACH Model AL-DT Alkalinity Test Kit. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

Stream (#)	Zn Tracer (mg/L)	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Oxygen Saturation (%)
3	0.004	11.28 (sd=0.67)	7.67 (sd=0.13)	8.25 (sd=0.09)	75.22 (sd=1.92)
14	0.003	10.92 (sd=0.51)	7.58 (sd=0.07)	7.96(sd=0.07)	72.08 (sd=1.39)
1	0.016	11.14 (sd=0.55)	7.51 (sd=0.05)	8.02 (sd=0.04)	72.94 (sd=0.68)
12	0.020	10.96 (sd=0.56)	7.48 (sd=0.08)	7.92 (sd=0.06)	71.74 (sd=0.78)
2	0.026	11.12 (sd=0.54)	7.47 (sd=0.06)	7.96 (sd=0.13)	72.34 (sd=0.99)
11	0.029	10.94 (sd=0.52)	7.43 (sd=0.07)	7.85 (sd=0.18)	71.12 (sd=1.15)
4	0.046	11.12 (sd=0.49)	7.37 (sd=0.06)	7.85 (sd=0.11)	71.42 (sd=0.68)
10	0.061	10.58 (sd=0.38)	7.33 (sd=0.06)	7.87 (sd=0.12)	70.78 (sd=0.73)
8	0.077	11.14 (sd=0.45)	7.32 (sd=0.10)	7.87 (sd=0.09)	72.22 (sd=1.45)
15	0.086	11.22 (sd=0.50)	7.27 (sd=0.07)	7.76 (sd=0.13)	70.70 (sd=0.91)
9	0.164	11.00 (sd=0.51)	7.18 (sd=0.09)	7.85 (sd=0.11)	71.16 (sd=0.94)
17	0.187	11.50 (sd=0.31)	7.07 (sd=0.07)	7.62 (sd=0.12)	69.74 (sd=1.04)
6	0.221	11.30 (sd=0.44)	7.03 (sd=0.09)	7.70 (sd=0.13)	70.26 (sd=0.96)
16	0.224	11.74 (sd=0.29)	7.05 (sd=0.06)	7.68 (sd=0.08)	70.80 (sd=0.69)
5	0.446	11.82 (sd=0.51)	6.81 (sd=0.06)	7.46 (sd=0.14)	68.86 (sd=0.98)
18	0.479	11.88 (sd=0.42)	6.85 (sd=0.05)	7.56 (sd=0.08)	70.26 (sd=1.30)

Stream (#)	Zn Tracer (mg/L)	Conductivity (µS)	Specific Conductivity (µS)	Hardness (mg/L)	Alkalinity (mg/L as $CaCO_3$)
3	0.004	52.18 (sd=1.21)	70.66 (sd=0.55)	30.18	25.6 (sd=2.58)
14	0.003	42.20 (sd=6.14)	55.70 (sd=9.49)	30.50	20.4 (sd=2.49)
1	0.016	55.60 (sd=6.39)	77.30 (sd=4.85)	35.17	26.0 (sd=2.34)
12	0.020	57.40 (sd=1.89)	78.96 (sd=1.86)	35.05	22.8 (sd=2.74)
2	0.026	64.24 (sd=1.32)	87.44 (sd=0.72)	36.79	18.8 (sd=1.92)
11	0.029	63.64 (sd=2.14)	87.16 (sd=1.93)	37.78	20.0 (sd=1.00)
4	0.046	72.04 (sd=1.46)	98.30 (sd=1.20)	43.22	20.8 (sd=3.57)
10	0.061	74.56 (sd=4.63)	102.54 (sd=7.42)	45.21	21.2 (sd=1.68)
8	0.077	78.46 (sd=14.01)	107.08 (sd=18.42)	49.86	19.6 (sd=1.55)
15	0.086	89.96 (sd=2.42)	122.04 (sd=1.94)	51.70	22.4 (sd=3.12)
9	0.164	114.56 (sd=12.62)	155.22 (sd=16.55)	73.46	20.8 (sd=2.48)
17	0.187	133.38 (sd=3.30)	180.02 (sd=3.44)	80.25	24.4 (sd=3.32)
6	0.221	143.80 (sd=16.79)	202.60 (sd=6.26)	93.75	16.4 (sd=2.96)
16	0.224	154.40 (sd=5.82)	207.28 (sd=7.46)	91.88	12.8 (sd=2.42)
5	0.446	226.26 (sd=14.35)	304.06 (sd=17.30)	149.63	18.8 (sd=2.74)
18	0.479	234.22 (sd=15.97)	313.98 (sd=16.50)	158.34	11.6 (sd=2.39)

Table 2: Dissolved element concentrations were determined through collaboration with Colorado School of Mines by ICP-OES. Filtered (0.45um) water samples were collected on four occasions. Each data point is a mean with standard deviations in respective parentheses. No standard deviation was calculated if two or more measurements were below detection limits. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

Stream (#)	Zn Tracer (mg/L)	K (mg/L)	Na (mg/L)	Mg (mg/L)	Ca (mg/L)	S (mg/L)
3	0.004	0.835(sd=0.155)	3.042 (sd=0.237)	1.675(sd=0.063)	9.088(sd=0.323)	1.953 (sd=0.100)
14	0.003	0.758(sd=0.083)	2.830 (sd=0.032)	1.687(sd=0.072)	9.217(sd=0.450)	1.964 (sd=0.084)
1	0.016	0.815(sd=0.090)	3.146 (sd=0.334)	2.024(sd=0.159)	10.319(sd=0.690)	3.555 (sd=0.270)
12	0.020	0.765(sd=0.064)	3.012 (sd=0.172)	1.998(sd=0.075)	10.214(sd=0.360)	3.674 (sd=0.147)
2	0.026	0.777(sd=0.051)	3.121 (sd=0.253)	2.149(sd=0.140)	10.566(sd=0.639)	4.857 (sd=0.308)
11	0.029	0.797(sd=0.055)	3.138 (sd=0.103)	2.197(sd=0.099)	10.831(sd=0.563)	4.889 (sd=0.185)
4	0.046	0.842(sd=0.047)	3.271 (sd=0.019)	2.563(sd=0.111)	12.024(sd=0.581)	6.832 (sd=0.246)
10	0.061	0.828(sd=0.064)	3.291 (sd=0.112)	2.713(sd=0.089)	12.415(sd=0.506)	8.092 (sd=0.210)
8	0.077	0.896(sd=0.040)	3.395 (sd=0.148)	3.021(sd=0.143)	13.483(sd=0.525)	9.814 (sd=0.500)
15	0.086	0.897(sd=0.051)	3.442 (sd=0.128)	3.158(sd=0.044)	13.859(sd=0.308)	11.059 (sd=0.688)
9	0.164	1.055(sd=0.039)	4.096 (sd=0.409)	4.637(sd=0.174)	18.408(sd=0.757)	19.595 (sd=1.253)
17	0.187	1.082(sd=0.027)	4.193 (sd=0.117)	4.998(sd=0.056)	19.896(sd=0.388)	22.775 (sd=1.054)
6	0.221	1.252(sd=0.203)	4.820 (sd=0.593)	5.986(sd=0.683)	23.033(sd=2.690)	25.630 (sd=1.172)
16	0.224	1.169(sd=0.052)	4.655 (sd=0.118)	5.777(sd=0.190)	22.287(sd=0.682)	27.162 (sd=1.025)
5	0.446	1.473(sd=0.161)	5.805 (sd=0.549)	9.106(sd=0.918)	32.770(sd=3.264)	47.607 (sd=3.172)
18	0.479	1.518(sd=0.139)	6.486 (sd=0.617)	9.672(sd=0.270)	34.942(sd=1.183)	51.343 (sd=2.958)

Stream (#)	Zn Tracer (mg/L)	B (mg/L)	Co (mg/L)	Si (mg/L)	Sr (mg/L)	Al (mg/L)	Cd (mg/L)
3	0.004	0.029	0.0016(sd=0.0014)	2.465(sd=0.142)	0.048(sd=0.008)	0.076(sd=0.018)	0.0014
14	0.003	0.025	0.0009	2.629(sd=0.144)	0.044(sd=0.002)	0.068(sd=0.018)	0.0002
1	0.016	0.033	0.0005(sd=0.0003)	2.641(sd=0.097)	0.053(sd=0.004)	0.072(sd=0.018)	0.0006
12	0.020	0.029	0.0005(sd=0.0002)	2.725(sd=0.074)	0.051(sd=0.002)	0.074(sd=0.013)	BDL
2	0.026	0.024	0.0008(sd=0.0003)	2.67(sd=0.094)	0.055(sd=0.003)	0.063(sd=0.020)	0.0002
11	0.029	0.04	0.0013(sd=0.0005)	2.747(sd=0.048)	0.059(sd=0.005)	0.070(sd=0.010)	0.0007(sd=0.0007)
4	0.046	0.021	0.0014(sd=0.0006)	2.844(sd=0.135)	0.065(sd=0.003)	0.083(sd=0.025)	0.0002(sd=0.0001)
10	0.061	0.025	0.0015(sd=0.0003)	2.823(sd=0.08)	0.068(sd=0.002)	0.065(sd=0.014)	BDL
8	0.077	0.039	0.0021(sd=0.0005)	2.864(sd=0.061)	0.076(sd=0.004)	0.060(sd=0.008)	0.0004
15	0.086	0.030	0.0023(sd=0.0005)	2.858(sd=0.071)	0.079(sd=0.001)	0.051(sd=0.015)	0.0003
9	0.164	0.042	0.0045(sd=0.0005)	3.198(sd=0.244)	0.114(sd=0.003)	0.100(sd=0.076)	0.0003(sd=0.0002)
17	0.187	0.038	0.0052(sd=0.0002)	3.26(sd=0.092)	0.123(sd=0.003)	0.085(sd=0.028)	0.0004(sd=0.0001)
6	0.221	0.069	0.0062(sd=0.0004)	3.266(sd=0.094)	0.149(sd=0.018)	0.062(sd=0.021)	0.0007(sd=0.0004)
16	0.224	0.048	0.0067(sd=0.0005)	3.367(sd=0.136)	0.144(sd=0.004)	0.069(sd=0.021)	0.0009(sd=0.0006)
5	0.446	0.061	0.0117(sd=0.0004)	3.775(sd=0.246)	0.224(sd=0.020)	0.041(sd=0.024)	0.0012(sd=0.0003)
18	0.479	0.086	0.0126(sd=0.0006)	3.925(sd=0.244)	0.237(sd=0.010)	0.046(sd=0.029)	0.0013(sd=0.0003)

Stream (#)	Zn Tracer (mg/L)	Fe (mg/L)	Mn (mg/L)	Cu (mg/L)	Ni (mg/L)	Zn (mg/L)
3	0.004	0.048(sd=0.005)	0.002(sd=0.001)	0.0036(sd=0.0022)	0.0026(sd=0.0019)	0.016(sd=0.013)
14	0.003	0.048(sd=0.008)	0.002	0.0016(sd=0.0016)	0.0009(sd=0.0005)	0.018(sd=0.015)
1	0.016	0.224(sd=0.025)	0.091(sd=0.006)	0.0016(sd=0.0008)	0.0016(sd=0.0008)	0.030(sd=0.016)
12	0.020	0.326(sd=0.131)	0.119(sd=0.034)	0.0024(sd=0.0014)	0.0022(sd=0.0016)	0.035(sd=0.017)
2	0.026	0.436(sd=0.057)	0.161(sd=0.007)	0.0019(sd=0.0006)	0.0018(sd=0.0004)	0.047(sd=0.018)
11	0.029	0.483(sd=0.068)	0.167(sd=0.003)	0.0027(sd=0.0013)	0.0029(sd=0.0013)	0.045(sd=0.013)
4	0.046	0.841(sd=0.133)	0.280(sd=0.008)	0.0026(sd=0.0017)	0.0024(sd=0.0005)	0.061(sd=0.015)
10	0.061	1.037(sd=0.198)	0.347(sd=0.018)	0.0021(sd=0.0019)	0.0030(sd=0.0004)	0.075(sd=0.014)
8	0.077	1.335(sd=0.324)	0.445(sd=0.014)	0.0021(sd=0.0007)	0.0040(sd=0.0008)	0.088(sd=0.015)
15	0.086	1.489(sd=0.476)	0.499(sd=0.009)	0.0021(sd=0.0012)	0.0040(sd=0.0004)	0.098(sd=0.015)
9	0.164	3.208(sd=0.997)	0.933(sd=0.008)	0.0036(sd=0.0015)	0.0071(sd=0.0005)	0.174(sd=0.019)
17	0.187	3.939(sd=0.809)	1.102(sd=0.058)	0.0046(sd=0.0026)	0.0081(sd=0.0002)	0.210(sd=0.013)
6	0.221	4.682(sd=0.686)	1.282(sd=0.020)	0.0041(sd=0.0016)	0.0098(sd=0.0010)	0.234(sd=0.018)
16	0.224	5.055(sd=0.988)	1.343(sd=0.046)	0.0057(sd=0.0027)	0.0108(sd=0.0015)	0.256(sd=0.016)
5	0.446	13.816(sd=4.543)	2.405(sd=0.103)	0.0048(sd=0.0025)	0.0179(sd=0.0007)	0.453(sd=0.040)
18	0.479	14.115(sd=5.631)	2.581(sd=0.148)	0.0054(sd=0.002)	0.0191(sd=0.0009)	0.493(sd=0.073)

Table 3. Total metal concentrations were determined through collaboration with Colorado School of Mines by ICP-OES. Unfiltered water samples were collected on four occasions. Each data point is a mean with standard deviations in respective parentheses. No standard deviation was calculated if two or more measurements were below detection limits. Mean total zinc concentration of four sampling events is used as a tracer for the complex Gregory Incline effluent mixture.

Stream (#)	Zn Tracer (mg/L)	K (mg/L)	Na (mg/L)	Mg (mg/L)	Ca (mg/L)	S (mg/L)
3	0.004	0.854(sd=0.070)	2.829 (sd=0.159)	1.735(sd=0.112)	9.287(sd=0.578)	1.878(sd=0.116)
14	0.003	0.792(sd=0.059)	2.713 (sd=0.113)	1.722(sd=0.078)	9.285(sd=0.416)	1.93 (sd=0.110)
1	0.016	0.856(sd=0.033)	2.973 (sd=0.124)	1.966(sd=0.068)	9.886(sd=0.329)	3.462(sd=0.271)
12	0.020	0.829(sd=0.045)	2.823 (sd=0.088)	2.002(sd=0.069)	10.147(sd=0.351)	3.764(sd=0.199)
2	0.026	0.873(sd=0.043)	2.93 (sd=0.076)	2.186(sd=0.048)	10.643(sd=0.270)	4.789(sd=0.219)
11	0.029	0.853(sd=0.064)	2.981 (sd=0.193)	2.194(sd=0.058)	10.692(sd=0.303)	4.94 (sd=0.268)
4	0.046	0.922(sd=0.091)	3.134 (sd=0.225)	2.589(sd=0.186)	12.027(sd=0.873)	6.646(sd=0.299)
10	0.061	0.924(sd=0.041)	3.174 (sd=0.092)	2.813(sd=0.061)	12.728(sd=0.364)	8.224(sd=0.389)
8	0.077	0.961(sd=0.058)	3.297 (sd=0.082)	3.044(sd=0.046)	13.584(sd=0.257)	9.759(sd=0.444)
15	0.086	0.955(sd=0.089)	3.273 (sd=0.140)	3.215(sd=0.160)	14.041(sd=0.656)	10.874(sd=0.827)
9	0.164	1.084(sd=0.023)	3.879 (sd=0.196)	4.540(sd=0.131)	18.395(sd=0.316)	19.050(sd=0.673)
17	0.187	1.133(sd=0.041)	3.963 (sd=0.060)	4.987(sd=0.016)	19.770(sd=0.193)	22.205(sd=1.246)
6	0.221	1.234(sd=0.053)	4.411 (sd=0.074)	5.664(sd=0.289)	21.786(sd=0.922)	25.279(sd=1.123)
16	0.224	1.201(sd=0.065)	4.283 (sd=0.128)	5.651(sd=0.155)	21.836(sd=0.367)	26.216(sd=1.679)
5	0.446	1.529(sd=0.105)	5.694 (sd=0.270)	9.147(sd=0.518)	32.895(sd=1.865)	47.136(sd=1.370)
18	0.479	1.577(sd=0.103)	5.881 (sd=0.242)	9.589(sd=0.371)	34.474(sd=1.204)	50.115(sd=3.661)

Stream (#)	Zn Tracer (mg/L)	B (mg/L)	Co (mg/L)	Si (mg/L)	Sr (mg/L)	Al (mg/L)	Cd (mg/L)
3	0.004	0.038	0.0016(sd=0.0015)	2.902(sd=0.166)	0.052(sd=0.014)	0.259(sd=0.045)	0.0014
14	0.003	0.022	0.0008	3.022(sd=0.103)	0.044(sd=0.002)	0.238(sd=0.043)	BDL
1	0.016	0.033	0.0021(sd=0.001)	3.069(sd=0.128)	0.061(sd=0.007)	0.226(sd=0.034)	0.0022(sd=0.0003)
12	0.020	0.025	0.0007(sd=0.0003)	3.219(sd=0.130)	0.051(sd=0.001)	0.258(sd=0.031)	0.0003(sd=0.0002)
2	0.026	0.017	0.0008(sd=0.0004)	3.139(sd=0.073)	0.056(sd=0.001)	0.257(sd=0.019)	0.0002
11	0.029	0.046	0.0016(sd=0.0013)	3.201(sd=0.145)	0.063(sd=0.010)	0.239(sd=0.049)	0.0023
4	0.046	0.021	0.0014(sd=0.0002)	3.223(sd=0.166)	0.065(sd=0.004)	0.261(sd=0.032)	0.0002
10	0.061	0.020	0.0018(sd=0.0004)	3.304(sd=0.166)	0.070(sd=0.002)	0.256(sd=0.050)	0.0002(sd=0.0001)
8	0.077	0.048	0.0028(sd=0.0013)	3.309(sd=0.057)	0.082(sd=0.011)	0.254(sd=0.017)	0.0012(sd=0.0017)
15	0.086	0.027	0.0024(sd=0.0004)	3.313(sd=0.130)	0.079(sd=0.003)	0.247(sd=0.036)	0.0003(sd=0.0001)
9	0.164	0.037	0.0045(sd=0.0003)	3.397(sd=0.262)	0.112(sd=0.004)	0.205(sd=0.108)	0.0003(sd=0.0001)
17	0.187	0.037	0.0052(sd=0.0003)	3.588(sd=0.100)	0.123(sd=0.002)	0.253(sd=0.046)	0.0005(sd=0.0001)
6	0.221	0.063	0.0069(sd=0.0012)	3.717(sd=0.086)	0.147(sd=0.003)	0.257(sd=0.030)	0.0015(sd=0.0012)
16	0.224	0.061	0.0067(sd=0.0009)	3.610(sd=0.126)	0.143(sd=0.008)	0.223(sd=0.031)	0.0011(sd=0.0011)
5	0.446	0.062	0.0120(sd=0.0002)	3.996(sd=0.087)	0.226(sd=0.009)	0.213(sd=0.014)	0.0014(sd=0.0002)
18	0.479	0.079	0.0129(sd=0.0010)	4.150(sd=0.196)	0.241(sd=0.017)	0.228(sd=0.046)	0.0020(sd=0.0008)

Stream (#)	Zn Tracer (mg/L)	Fe (mg/L)	Mn (mg/L)	Cu (mg/L)	Ni (mg/L)	Zn (mg/L)
3	0.004	0.145(sd=0.009)	0.011(sd=0.003)	0.0034(sd=0.0022)	0.0022(sd=0.0025)	0.004(sd=0.002)
14	0.003	0.148(sd=0.005)	0.013(sd=0.002)	0.0014(sd=0.0009)	0.0008(sd=0.0002)	0.003(sd=0.001)
1	0.016	0.435(sd=0.029)	0.097(sd=0.004)	0.0042(sd=0.0023)	0.0044(sd=0.0017)	0.016
12	0.020	0.607(sd=0.305)	0.128(sd=0.041)	0.0024(sd=0.0010)	0.0014(sd=0.0002)	0.020(sd=0.008)
2	0.026	0.783(sd=0.044)	0.168(sd=0.004)	0.0023(sd=0.0008)	0.0021(sd=0.0005)	0.026(sd=0.001)
11	0.029	0.802(sd=0.185)	0.173(sd=0.012)	0.0038(sd=0.0027)	0.0037(sd=0.0024)	0.029(sd=0.002)
4	0.046	1.297(sd=0.127)	0.280(sd=0.013)	0.0021(sd=0.0014)	0.0027(sd=0.0006)	0.046(sd=0.002)
10	0.061	1.621(sd=0.303)	0.356(sd=0.025)	0.0031(sd=0.0022)	0.0031(sd=0.0004)	0.061(sd=0.005)
8	0.077	2.099(sd=0.276)	0.448(sd=0.015)	0.0039(sd=0.0023)	0.0052(sd=0.0032)	0.077(sd=0.004)
15	0.086	2.630(sd=0.480)	0.505(sd=0.017)	0.0030(sd=0.0024)	0.0041(sd=0.0002)	0.086(sd=0.004)
9	0.164	3.778(sd=0.282)	0.940(sd=0.019)	0.0038(sd=0.0021)	0.0075(sd=0.0006)	0.164(sd=0.011)
17	0.187	5.498(sd=1.151)	1.082(sd=0.039)	0.0064(sd=0.0035)	0.0085(sd=0.0003)	0.187(sd=0.010)
6	0.221	5.761(sd=0.846)	1.277(sd=0.026)	0.0074(sd=0.0024)	0.0114(sd=0.0025)	0.221(sd=0.007)
16	0.224	6.287(sd=0.783)	1.313(sd=0.051)	0.0076(sd=0.0041)	0.0109(sd=0.0020)	0.224(sd=0.006)
5	0.446	15.746(sd=4.053)	2.446(sd=0.024)	0.0117(sd=0.0030)	0.0182(sd=0.0005)	0.446(sd=0.014)
18	0.479	18.005(sd=5.845)	2.572(sd=0.128)	0.0152(sd=0.0056)	0.0200(sd=0.0018)	0.479(sd=0.045)

DISCUSSION

Paine (1998) coined the term 'ecological surprises' to describe effects of complex perturbations on organisms. There is consensus that the cumulative effects of multiple stressors stray from additivity (Paine 1998). According to numerous investigators, protein carbonyl content (PCC) and oxygen consumption (MO_2) are powerful indicators of organism distress (Spicer and Weber 1991; Dalle-Donne 2003; Clements 2004; Pane et al. 2004; Lushchak 2007; Fedorova et al. 2013). Both are known to correlate with organism health status and were expected to correlate with exposure to metal constituents of Gregory Incline effluent (GIE). However, in this experiment the directionality of some response variables diverged from predictions.

Oxygen consumption observations supported this experiment's hypothesis, whereas PCC notably did not. Protein carbonyl content was expected to increase with GIE concentration, and MO_2 was predicted to decrease. Protein dysfunction, expressed through PCC, was anticipated to reflect concentration-dependent exposure to reactive species (RS) generating transition metals. Reduced MO_2 was anticipated to accompany protein dysfunction, as gill tissues were damaged by metal ions. While MO_2 was diminished across treatments, PCC was also negatively associated with GIE concentration, and neither PCC nor MO_2 correlated well with one another. (Figures 10, 11 and 12).

Interestingly, a prominent increase in PCC for control *A. grandis* was observed after eight days of treatment, and this level was significantly greater than PCC in all GIE treatments (Figure 10). Moreover, PCC was not significantly different between GIE concentrations (Figure 10). These results suggest that over the eight-day exposure some event or stressor led to oxidative

protein injury in the control organisms. In fact, mean PCC was approximately 3.5 times higher in control organisms than in unexposed day-0 organisms (Figures 9 and 10). Stadtman and Levine (2001), seminal researchers in oxidative stress and PCC responses, claim that severe states of oxidative stress are denoted by threefold increases in PCC. Lushchak (2007) maintains PCC fold changes are remarkably well conserved among species. Thus, *A. grandis* exposed to eight days of control water, with similar physiochemical characteristics as the *A. grandis* collection site in the Cache la Poudre River, were likely experiencing elevated rates of RS production.

Other studies exploring PCC in aquatic species demonstrated that oxidative contaminants contributed to protein injury in a positive concentration-dependent manner (Almroth et al. 2008; Lushchak et al. 2009b; Kaloyianni et al. 2009; Liu et al. 2012; Xiu 2014; Jara et al. 2014). However, PCC was approximately 75% greater in controls than in treatments mimicking contamination levels at the GIE point-source and GIE exposed *A. grandis* had PCC approximately two times greater than day-0 observations (Figure 10). Interestingly, there was not a clear difference between any of the GIE treatments indicating a binary or threshold effect at some GIE concentration below those used in this experiment (Figures 9 and 10).

It is important to note that all PCC responses were elevated compared to day-0 organisms. This result demonstrates the importance of evaluating day-0 organisms during laboratory toxicology experiments. Without day-0 observations, it would appear that GIE exposure led to overall reductions in PCC, when in reality GIE exposed *A. grandis* had approximately twice as much PCC as day-0 organisms.

Oxygen consumption and PCC results suggest at least two partly independent mechanisms of injury occurred. One mechanism caused PCC to increase in *A. grandis* between

day-0 and day-8 regardless of treatment, although the effect is more pronounced in controls. This mechanism of oxidative injury did not correlate well with MO_2 and appeared to have a binary relationship with GIE (Figures 9,10, and 12). Simultaneously, a different mechanism of toxicity was demonstrated through MO_2 observations. Oxygen consumption displayed a concentration-dependent negative relationship with GIE. Therefore, GIE was shown to induce respiratory changes in exposed *A. grandis* potentially independent from PCC. Moreover, linear regression analysis indicated *A. grandis* survivorship did not correlate with GIE exposures (Figure 2). If traditional methods for determining toxicity by lethal concentration were utilized, no effects would have been observed at environmentally relevant GIE concentrations.

Oxygen Consumption versus Gregory Incline Effluent

Oxygen consumption diminished in a concentration-dependent manner with GIE exposure (Figure 11). This result is in agreement with numerous studies (Spicer and Weber 1991; Clements 2004; Pane et al. 2004; Mogren and Trumble 2010). Moreover, because respiration chambers were transparent, a noticeable reduction in *A. grandis* activity was observed in organisms exposed to concentrated GIE. *A. grandis* exposed to control treatments fought vigorously with each other during MO_2 measurements. In contrast, those exposed to higher concentrations of GIE were lethargic.

Respiration rates diminished as GIE concentration increased indicating respiration changes were related to GIE concentration. There are several modes of action that could explain reduced MO_2 from metal exposure: physical deposition of metals at gill surfaces impeding gas exchange, gill osmoregulatory dysfunction, reduced cellular oxygen transport, reduced

ventilation rates and direct inhibition of mitochondrial respiration (Spicer and Weber 1991). Assuming that gills are the principal site of ion exchange and respiration in *A. grandis*, like other gilled aquatic organisms, any damage to gills could interfere with MO_2.

Spicer and Weber (1991) suggest that essential metals affect respiration at the surface of gill tissues by disrupting osmoregulation and gas exchange, whereas non-essential metals may disrupt mitochondrial respiration itself. In this experiment, essential metals (eg., iron, zinc and manganese) comprised the vast majority of GIE constituents compared with non-essential metals (eg., aluminum and cadmium) (Tables 2 and 3). Therefore, gill tissue dysfunction may have been particularly germane to the GIE mode of action. Moreover, iron and other metals were noticeably deposited at gill surfaces. This deposition displayed an obvious staining on gills. *A. grandis* gills were deeply reddish/orange in concentrated GIE treatments compared with light tan in less concentrated GIE treatments. Whether or not mitochondrial respiration, ventilation rates or oxygen transport were influenced by GIE, metals clearly stained gill surfaces suggesting they acted as a physical barrier at gill-water interfaces.

As mentioned earlier, activity levels in the respiration chamber were noticeably reduced in concentrated GIE treatments. This could be the direct result of gill dysfunction, or it could signify a GIE induced hypometabolic state. Some organisms are known to enter hypometabolic states when environmental conditions are not conducive to life (Hand and Hardewig 1996; Mogren and Trumble 2010). Importantly, a GIE induced hypometabolic state could explain both reduced MO_2 across treatments and reduced PCC in GIE treatments compared with controls.

Elevated Protein Carbonyl Content in Control Treatments

In order to understand the seemingly counterintuitive negative relationship between PCC and GIE concentration, we need to consider potential reasons for PCC elevation in control treatments. One potential explanation is density-dependent stress. During acclimation and exposures, individual *A. grandis* (N=14) were confined within mesh baskets forcing them into high densities without means for escape. Although *A. grandis* commonly occupy habitat in densities even greater than those imposed by this experiment, in nature *A. grandis* nearly always have the ability to escape competitors or predators (Hauer and Stanford 1981; Voelz and Ward 1992). In this experiment, there was no escape for threatened individuals. During times of stress, neurotransmitters of the catecholamine family are released producing a 'fight or flight' response and RS are generated as a byproduct (McAnulty et al. 2003). Therefore, it is possible that density-dependent stress influenced PCC in controls and GIE treatments, although the effect was more pronounced in controls for reasons discussed later. Density-dependent aggression was evident by observations of cannibalism within basket enclosures. It was observed in all treatments where mortality occurred. However, it is unclear if cannibalism was the cause of death or simply opportunistic feeding on deceased individuals.

A. grandis also build funnel-like retreats that are carefully orientated upstream channeling food and oxygen-rich water to the organism (Voelz and Ward 1992). Net orientation and water flow rate are critically important for both food and oxygen consumption. However, maintaining adequate flow within baskets enclosures was a consistent challenge during this experiment. Depending on GIE concentration either algae or precipitated metal accumulation significantly reduced flow rates within mesh baskets. Baskets were thus removed from artificial streams daily

and brushed with a soft-bristled toothbrush to remove accumulated matter and restore flow. Care was taken to return the baskets to their original orientation, although slight alterations in net orientation may have occurred leading to drastic changes in net functionally. This may have caused additional stress to organisms as well as contributed to starvation and hypoxia. Furthermore, if food was not sufficiently available during exposures, starvation itself could have caused an increase in oxidative stress (Postma et al. 1994; Beketov and Liess 2005). It is possible that the effects of starvation or hypoxia were greater in controls than in treatments due to greater metabolic rates in controls. This could explain elevated PCC in controls.

Silk retreats were observed in all treatments. Unfortunately, the quality and quantity of retreats were not determined. This information may have provided valuable information related to *A. grandis* fitness. Several studies have attempted to evaluate effects of metals on hydropsychid silk retreat construction and functionality, however conclusions were inconsistent between studies (Mogren and Trumble 2010).

Protein Carbonyl Content versus Gregory Incline Effluent

There are numerous explanations for PCC's relationship with GIE concentration. Unintended exposures are one possibility. However, linear regression analysis of metal concentrations demonstrated positive linear trends with high coefficients of determination (Figures 7 and 8). Therefore, metal concentrations responded as expected across GIE treatments. Another potential explanation is that *A. grandis* were exposed to an unidentified contaminant. This unidentified contaminant could have induced protein oxidation in controls, but then lost its toxicity due to interactions with metals during GIE exposures. Alternatively, PCC results may

be intrinsic to the complex composition of GIE. Gregory Incline effluent is composed of several metals known to induce protein oxidation as individual constituents. Perhaps interactions among GIE constituents created an antagonistic mixture less toxic than the sum of its parts. However, these explanations are unlikely given the negative relationship between MO_2 and GIE treatments, as well as elevated PCC in all GIE exposed organisms compared with day-0s.

In this experiment, water hardness positively correlated with exposure concentration due to the presence of divalent cations in GIE (Figure 6). Water hardness buffers metal toxicity by competing with toxic metals for uptake through respiratory pathways (Di Toro et al. 2001). It is possible that increasing water hardness limited PCC by limiting metal accumulation into tissues. However, hardness is unlikely to be the sole reason for PCC's relationship with GIE. If hardness was the sole reason for observed PCC effects, then one would expect PCC to have a more linear relationship with GIE. Moreover, metal concentration increases in concert with hardness, thereby minimizing the net buffering effect of hardness on toxic metal bioavailability. Additionally, increases in hardness would not prevent metal deposition at gill surfaces.

Temperature is another physiochemical characteristic that has potential to explain effects observed in this experiment. Temperature is widely known to influence metabolic rates in organisms by affecting kinetic energies that govern chemical reactions (Lushchak 2007). Also, oxygen becomes less soluble in water as temperature rises (Figure 3). In this experiment, there was a positive correlation between exposure concentration and temperature (Figure 3). This relationship was likely caused as a consequence of our dosing procedure. Gregory Incline effluent was continuously pumped from carboys (at room temperature) into artificial streams (at 11-12°C). Exposure concentration was dictated by the flow rate of GIE into the artificial streams. Therefore, more concentrated treatments received a larger input of room temperature

effluent than less concentrated treatments and controls. This resulted in a positive relationship between GIE concentration and temperature. Additionally, a conspicuous gradient in color was observed in artificial streams; concentrated treatments were darker than less concentrated treatments, presumably due to GIE metal deposition. Color differences between streams may have results in an albedo effect that caused darker stained artificial streams to absorb more solar energy than relatively clear ones. All else being equal, PCC would be expected to increase with increasing temperature as metabolism and biochemical reaction rates speed up. Despite this, PCC decreased with increasing temperature (Figures 3, 9 and 10).

Both dissolved oxygen concentration and oxygen saturation negatively correlated with GIE concentration (Figure 4). While dissolved oxygen concentration can be explained by increasing temperatures; oxygen saturation, an altitude and temperature adjusted measure of dissolved oxygen cannot be fully explained by temperature. It is possible that reductions in oxygen saturation reflect increases in metal oxidation in more concentrated exposures. It also possible that there was diminished autotrophic production of oxygen in more concentrated treatments. Less oxygen available for respiration could theoretically explain diminished MO_2 observed in this experiment. A causal relationship is unlikely, however. Dissolved oxygen and oxygen saturation were reduced by approximately 10% across all treatments (Figure 3). In contrast, MO_2 was reduced by nearly 50% (Figure 9). At most, dissolved oxygen concentrations and oxygen saturations were partly responsible for reduced MO_2.

The picture becomes more complicated when considering oxygen's relationship to PCC. Reactive oxygen species, a causative agent for protein carbonyl formation, have been repeatedly demonstrated to increase with increasing cellular and extracellular oxygen levels (Barata et al. 2005; Joyner-Matos et al. 2006). In this experiment, dissolved oxygen and MO_2 diminished as

exposure concentration increased. Therefore, it is logical that reduced oxygen availability and consumption resulted in reduced PCC. However, there are several flaws with this logic. Reactive oxygen species are one of several RS that influence protein carbonyl formation. Reactive nitrogen, sulfur and organic species also comprise relevant RS. Nitrogen and organic species were not directly measured in this experiment but were expected to be consistent between treatments, while sulfur was positively correlated with GIE concentration (Figures 7 and 8). In fact, sulfur increased with GIE concentration at a faster rate than oxygen diminished (Figures 4, 7 and 8). Therefore, even if reactive oxygen species were curtailed with increasing GIE concentrations, increased reactive sulfur species and RS catalyzing transition metals would temper the effects of reduced oxygen. It is also worth noting that under certain conditions sulfur has strong affinity for and will bind with metals forming unavailable, insoluble metal sulfides. However, experimental conditions were not suitable for metal sulfide production and modest differences observed between dissolved and total metals concentrations support this conclusion (Figures 7 and 8). Another flaw in this hypothesis is that MO_2 and dissolved oxygen measurements demonstrated linear responses to exposure concentration. However, PCC's response was more binary. If PCC was fully explained by MO_2, then PCC should have a more linear response to GIE concentration.

To protect cells from RS, eukaryotic organisms upregulate antioxidants in response to exogenous pro-oxidants such as transition metals. (Livingstone 2001; Prousek 2007; Lushchak 2007, Lushchak 2011). Numerous studies in diverse taxa have shown metal insults inducing biochemical defense systems (Dalle-Donne et al. 2003; Lushchak 2007, Lushchak 2011, Fedorova et al. 2014). Although antioxidants were not measured, upregulation in superoxide dismutase, catalase, glutathione-S-transferases have previously been observed in aquatic insect

species exposed to metals (Xie et al. 2009; Barata et al. 2005; Wang and Wang 2009; Xie and Buchwalter 2011). Antioxidant upregulation is a conserved feature among oxidatively stressed organisms, but particular antioxidants and degree of responses vary between species (Livingstone 2001; Prousek 2007; Lushchak 2007, Lushchak 2011). It is likely that GIE exposed *A. grandis* also upregulated antioxidants to abate effects of metal catalyzed oxidation (MCO). This effect may have been more pronounced at higher GIE concentrations leading to reduced PCC in treatments versus controls. Unfortunately, increased antioxidant protection mechanisms cannot be determined by data obtained in this study.

Accelerated protein turnover is another relevant defense system that can occur during metal exposures. Irreversibly oxidized proteins require proteolysis to protect cells from harmful protein aggregations (Friguet et al. 1994a; Friguet et al. 1994b). If metal exposure stimulated proteolysis in GIE treatments compared with controls, then PCC could appear diminished even though protein carbonylation was occurring at elevated rates in GIE treatments. Additionally, metals can induce expression of metal binding enzymes, including frataxin, ferritin and metallothionein, capable of sequestering and reducing bioavailability or facilitating clearance (Moller et al. 2011). Therefore, differential stimulation of cellular defense and detoxification systems may have veiled the effects of severe oxidative stress in GIE exposed *A. grandis.* It is also likely that controls lacked metal stimulus to upregulate defense systems.

It is important to note that detoxifying contaminants and upregulation of compensatory systems require energy. When one considers that oxygen consumption is directly related to ATP production; then also considers the negative relationship between GIE and MO_2 observed in this experiment, it is possible that GIE exposed *A. grandis* were rapidly depleting their energy reserves. As a result, GIE exposed organisms may have reduced fitness and increased

susceptibility to additional stressors. Moreover, increases in energy requirements necessitated by upregulating biochemical defenses would have buffered MO_2 observations. Therefore, without oxidative insults the negative relationship between GIE and MO_2 could have been stronger.

Future Studies

In order to understand the effects of metals, and other stressors associated with mines on *A. grandis* PCC, several experimental additions would be beneficial. One major limitation of this study was a lack of *A. grandis* metal accumulation data. It is possible that metals were not bioaccumulated at sufficient levels to induce oxidative stress by MCO. Additionally, metals could have been accumulated but were sequestered into non-toxic compartments. Sub-cellular fractionation with ICP-OES analysis would help to determine if GIE metals were accumulated at sufficiently toxic concentrations and whether they were bioavailable. A temporal component would have also been beneficial. Protein carbonyls have been shown to develop quickly after oxidative insults and diminish over time as cellular defense systems clear oxidized proteins and antioxidant systems are upregulated (Parvez and Raisuddin 2005). Therefore, the relationship between exposure and PCC may be a consequence of this experiment's exposure duration. Sample collection over a time-course would more accurately reflect system dynamics than collections at day-0 and day-8 only. Alterations in *A. grandis* mesh basket enclosures would also be necessary to prevent density-dependent stress and maximize water flow rates.

Adding a broad suite of endpoints would help resolve proteolysis, redox status, oxidative damage, antioxidant systems and repair of oxidative damage and energy reserves. Cellular redox status determined by ratios of glutathione to glutathione disulfides would help elucidate the

severity of oxidative stress. Markers of oxidative damage such as lipid peroxidation products, DNA strand breaks, and PCC would demonstrate how oxidative stress was manifesting as cellular injury. Measuring antioxidants such as superoxide dismutase, catalase, glutathione-S-transferase and metallothionein would help determine levels of cellular defense mechanisms. Proteolytic enzymes and ubiquitin, a marker of protein recycling, could be assayed to determine protein turnover. Energy reserves should also be determined by measuring lipid content or ATP to ADP ratios. Additionally, a subset of organisms preserved in formalin would allow histological analysis. With histological techniques morphological changes, inflammatory and immune responses could be observed, as well as cell death.

The results of this project illustrate the potential limitations of utilizing a single biochemical marker to demonstrate the effects of a toxic mixture. Here, a metal mixture is shown to reduce apparent PCC, potentially limiting protein dysfunction. This information could easily be misinterpreted that GIE improved *A. grandis* health status, when in fact it did not. Diminished MO_2 demonstrated the adverse impact of GIE, confirming that GIE was deleterious. However, a broad suite of biomarkers would have been valuable for describing the effects of GIE and PCC on *A. grandis* health status. In a biomonitoring setting, markers of cellular injury, like PCC, should always be measured in conjunction with markers of cellular defenses as well as costs of increased energy expenditures.

CONCLUSION

Water chemistry is routinely utilized as a principle indicator of ecosystem health in polluted environments. Often natural resource managers and lawmakers use chemistry data as the sole justification for conservation plans and regulation, respectively. Chemistry alone, however, is an insufficient indicator of toxic effects of contaminants. Because contaminants often occur in mixtures, toxicity frequently diverges from chemistry based expectations. In response to the limitations of chemical testing, sophisticated bio-monitoring efforts use chemistry data in conjunction with species richness and abundances assessments or with sublethal endpoints. The combination of these indicators are enormously useful for determining health status of impacted ecosystems, but current biotic integrity indices require substantial time, labor and expertise. Developing rapid, universal and standard tools for determining mixture toxicity is needed to keep pace with our increasingly complex understanding of current environmental health problems.

This project explored two potentially insightful and convenient endpoints for determining toxic effects of a complex metal mixture. First, oxygen consumption, a well-established and sensitive indicator of organism impairment was utilized. Second, protein carbonyl content, an experimental ecological biomarker widely lauded in biomedical circles as a marker of "disease-derived protein dysfunction" was analyzed for its utility in a metal tolerant aquatic macroinvertebrate species *A. grandis* (Dalle-Donne 2003).

Here, further evidence for MO_2 as an insightful and concentration-dependent indicator of metal exposure is contributed. Moreover, this project described an inexpensive and user-friendly method for determining MO_2 in aquatic macroinvertebrates. Conclusions related to PCC,

however, were less certain. In fact, results diverged from predictions, and from current scientific literature describing PCC's response to oxidative contaminants like transition metals (Almroth et al. 2008; Lushchak et al 2009a; Lushchak et al 2009b; Kaloyianni et al. 2009; Liu et al. 2012; Xiu 2014; Jara et al. 2014). Protein carbonyl content alone does not appear to be an appropriate biomonitoring tool for *A. grandis* exposed to complex metal mixtures. Rather, a broad suite of endpoints measuring metal bioaccumulation, energy reserves, redox status, oxidative injury and defense systems should be collectively quantified to characterize metal mixture induced oxidative stress.

Protein carbonyl content's departure from anticipated results likely is the consequence of complex interactions between direct and indirect effects at the chemical, biochemical, physiologic and behavioral levels. Mixture interactions, direct effects and indirect effects are each well known to occur in nature. Although toxicologists rarely account for the intricacies of natural systems in controlled laboratory studies, these studies are influential for determining environmental regulatory standards. It is possible that controlled laboratory studies, the foundation for mechanistic determinations and cause/effect relationships, are inherently disparate from nature. Egler (1970) declared: "Nature is not only more complex than we think. It is more complex than we can think." If true, we must consider the relevance of rigorously controlled studies to natural systems and also consider new ways of thinking about predicting toxicity. Perhaps, in the near-term, predicting toxicity of complex mixtures in natural systems is not practical. Rather, direct observations of toxic effects should be used to regulate ecosystem health.

REFERENCES

Abbas, S., et al. "Developmental Exposure to as, Cd, and Pb Mixture Diminishes Skeletal Growth and Causes Osteopenia at Maturity Via Osteoblast and Chondrocyte Malfunctioning in Female Rats." *Toxicological Sciences* 134.1 (2013): 207-20. Print.

Alam, Z. I., et al. "A generalised increase in protein carbonyls in the brain in Parkinson's but not incidental Lewy body disease." *Journal of neurochemistry* 69.3 (1997): 1326-1329. Print.

Almroth, B. C., et al. "Protein Carbonyls and Antioxidant Defenses in Corkwing Wrasse (Symphodus Melops) from a Heavy Metal Polluted and a Pah Polluted Site." *Marine Environmental Research* 66.2 (2008): 271-77. Print.

Amir, O., et al. "Serum Oxidative Stress Level Correlates with Clinical Parameters in Chronic Systolic Heart Failure Patients." *Clinical Cardiology* 32.4 (2009): 199-203. Print.

Balistrieri, L. S., and C. A. Mebane. "Predicting the Toxicity of Metal Mixtures." *Science of the Total Environment* 466 (2014): 788-99. Print.

Barata, C., et al. "The Relative Importance of Water and Food as Cadmium Sources to Daphnia Magna Straus." *Aquatic Toxicology* 61.3-4 (2002): 143-54. Print.

Barata, C., et al. "Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the tricoptera larvae Hydropsyche exocellata from the Llobregat river basin (NE Spain)." *Aquatic Toxicology* 74.1 (2005): 3-19. Print.

Barnham, K. J., C. L. Masters, and A. I. Bush. "Neurodegenerative Diseases and Oxidative Stress." *Nature Reviews Drug Discovery* 3.3 (2004): 205-14. Print.

Baynes, J. W., and S. R. Thorpe. "Role of Oxidative Stress in Diabetic Complications - a New Perspective on an Old Paradigm." *Diabetes* 48.1 (1999): 1-9. Print.

Beketov, M. A., and M. Liess. "Acute Contamination with Esfenvalerate and Food Limitation: Chronic Effects on the Mayfly, Cloeon Dipterum." *Environmental Toxicology and Chemistry* 24.5 (2005): 1281-86. Print.

Bertini, I., and P. Turano. "Metal Ions and Proteins: Binding, Stability, and Folding." *Biological Inorganic Chemistry Structure and Reactivity*, University Science Books, Sausalito, CA. (2007): 31–41. Print.

Borthiry, G. R., et al. "Generation of Reactive Chromium and Oxygen Species in Chromate-Treated Human Lung Epithelial Cells." *Free Radical Biology and Medicine* 43 (2007): S24-S24. Print.

Boveris, A., and B. Chance. "Mitochondrial Generation of Hydrogen-Peroxide - General Properties and Effect of Hyperbaric-Oxygen." *Biochemical Journal* 134.3 (1973): 707-16. Print.

Bradford, M. M. "Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding." *Analytical Biochemistry* 72.1-2 (1976): 248-54. Print.

Breusing, N., and T. Grune. "Regulation of Proteasome-Mediated Protein Degradation During Oxidative Stress and Aging." *Biological Chemistry* 389.3 (2008): 203-09. Print.

Butterfield, D. A., and R. Sultana. "Redox Proteomics: Understanding Oxidative Stress in the Progression of Age-Related Neurodegenerative Disorders." *Expert Review of Proteomics* 5.2 (2008): 157-60. Print.

Cain, D. J., D. B. Buchwalter, and S. N. Luoma. "Influence of Metal Exposure History on the Bioaccumulation and Subcellular Distribution of Aqueous Cadmium in the Insect Hydropsyche Californica." *Environmental Toxicology and Chemistry* 25.4 (2006): 1042-49. Print.

Cain, D. J., S. N. Luoma, and W. G. Wallace. "Linking Metal Bioaccumulation of Aquatic Insects to Their Distribution Patterns in a Mining-Impacted River." *Environmental Toxicology and Chemistry* 23.6 (2004): 1463-73. Print.

Carlisle, D. M., and W. H. Clements. "Leaf Litter Breakdown, Microbial Respiration and Shredder Production in Metal-Polluted Streams." *Freshwater Biology* 50.2 (2005): 380-90. Print.

Chapman, M. L., B. R. Rubin, and R. W. Gracy. "Increased carbonyl content of proteins in synovial fluid from patients with rheumatoid arthritis." *The Journal of rheumatology* 16.1 (1989): 15-18. Print.

Cheimonopoulou, M. T., et al. "Assessing Ecological Water Quality with Macroinvertebrates and Fish: A Case Study from a Small Mediterranean River." *Environmental Management* 47.2 (2011): 279-90. Print.

Clements, W. H. "Community responses of stream organisms to heavy metals: a review of observational and experimental approaches." *Metal ecotoxicology: concepts and applications. Lewis Publishers, Chelsea, Michigan* (1991): 363-391.

Clements, W. H. "Metal Tolerance and Predator-Prey Interactions in Benthic Macroinvertebrate Stream Communities." *Ecological Applications* 9.3 (1999): 1073-84. Print.

Clements, W. H. "Small-Scale Experiments Support Causal Relationships between Metal Contamination and Macroinvertebrate Community Responses." *Ecological Applications* 14.3 (2004): 954-67. Print.

Clements, W. H., N. K. M. Vieira, and S. E. Church. "Quantifying Restoration Success and Recovery in a Metal-Polluted Stream: A 17-Year Assessment of Physicochemical and Biological Responses." *Journal of Applied Ecology* 47.4 (2010): 899-910. Print.

Climent, I., L. Tsai, and R. L. Levine. "Derivatization of Gamma-Glutamyl-Transferase Semialdehyde Residues in Oxidized Proteins by Fluoresceinamine." *Analytical Biochemistry* 182.2 (1989): 226-32. Print.

Conrad, C. C., et al. "Oxidized Proteins in Alzheimer's Plasma." *Biochemical and Biophysical Research Communications* 275.2 (2000): 678-81. Print.

Cuffney, T. F., and G. W. Minshall. "Life-History and Bionomics of Arctopsyche Grandis (Trichoptera) in a Central Idaho Stream." *Holarctic Ecology* 4.4 (1981): 252-62. Print.

Curtis, J. M., et al. "Protein Carbonylation and Metabolic Control Systems." *Trends in Endocrinology and Metabolism* 23.8 (2012): 399-406. Print.

Dalle-Donne, I., et al. "Protein Carbonylation in Human Diseases." *Trends in Molecular Medicine* 9.4 (2003a): 169-76. Print.

Dalle-Donne, Isabella, et al. "Protein Carbonyl Groups as Biomarkers of Oxidative Stress." *Clinica Chimica Acta* 329.1-2 (2003b): 23-38. Print.

Dalzell, D. J. B., and N. A. A. Macfarlane. "The Toxicity of Iron to Brown Trout and Effects on the Gills: A Comparison of Two Grades of Iron Sulphate." *Journal of Fish Biology* 55.2 (1999): 301-15. Print.

Davies, K. J. A. "Protein Damage and Degradation by Oxygen Radicals .1. General-Aspects." *Journal of Biological Chemistry* 262.20 (1987): 9895-901. Print.

Di Toro, D. M., et al. "Biotic Ligand Model of the Acute Toxicity of Metals. 1. Technical Basis." *Environmental Toxicology and Chemistry* 20.10 (2001): 2383-96. Print.

Eaton, J. G. "Chronic toxicity of a copper, cadmium and zinc mixture to the fathead minnow (Pimephales promelas Rafinesque)." *Water Research* 7.11 (1973): 1723-1736. Print

Eleuteri, E., et al. "Role of Oxidative and Nitrosative Stress Biomarkers in Chronic Heart Failure." *Frontiers in Bioscience* 14 (2009): 2230-37. Print.

Egler, F. E. "The way of science: a philosophy of ecology for the layman". *Hafner Publishing Company.* (1970). Print.

Fedorova, M., R. C. Bollineni, and R. Hoffmann. "Protein Carbonylation as a Major Hallmark of Oxidative Damage: Update of Analytical Strategies." *Mass Spectrometry Reviews* 33.2 (2014): 79-97. Print.

Ferreira, S. M., et al. "Oxidative Stress Markers in Aqueous Humor of Glaucoma Patients." *American Journal of Ophthalmology* 137.1 (2004): 62-69. Print.

Finlayson, B. J., and K. M. Verrue. "Toxicities of Copper, Zinc, and Cadmium Mixtures to Juvenile Chinook Salmon." *Transactions of the American Fisheries Society* 111.5 (1982): 645-50. Print.

Floor, E., and M. G. Wetzel. "Increased Protein Oxidation in Human Substantia Nigra Pars Compacta in Comparison with Basal Ganglia and Prefrontal Cortex Measured with an Improved Dinitrophenylhydrazine Assay." *Journal of Neurochemistry* 70.1 (1998): 268-75. Print.

Foell, D., H. Wittkowski, and J. Roth. "Mechanisms of Disease: A 'Damp' View of Inflammatory Arthritis." *Nature Clinical Practice Rheumatology* 3.7 (2007): 382-90. Print.

Fridovich, I. "Mitochondria: Are They the Seat of Senescence?" *Aging Cell* 3.1 (2004): 13-16. Print.

Friguet, B., E. R. Stadtman, and L. I. Szweda. "Modification of Glucose-6-Phosphate-Dehydrogenase by 4-Hydroxy-2-Nonenal - Formation of Cross-Linked Protein That Inhibits the Multicatalytic Protease." *Journal of Biological Chemistry* 269.34 (1994): 21639-43. Print.

Friguet, B., L. I. Szweda, and E. R. Stadtman. "Susceptibility of Glucose-6-Phosphate-Dehydrogenase Modified by 4-Hydroxy-2-Nonenal and Metal-Catalyzed Oxidation to Proteolysis by the Multicatalytic Protease." *Archives of Biochemistry and Biophysics* 311.1 (1994): 168-73. Print.

Garland, D., P. Russell, J. S., and Zigler Jr, J. S.. The oxidative modification of lens proteins. *Oxygen Radicals in Biology and Medicine.* Springer US. (1988): 347-352. Print.

Garrison, W. M. "Reaction-Mechanisms in the Radiolysis of Peptides, Polypeptides, and Proteins." *Chemical Reviews* 87.2 (1987): 381-98. Print.

Grattagliano, I., et al. "Oxidation of Circulating Proteins in Alcoholics: Role of Acetaldehyde and Xanthine Oxidase." *Journal of Hepatology* 25.1 (1996): 28-36. Print.

Haber F., Weiss J. "Über die Katalyse des Hydroperoxydes (On the catalysis of hydroperoxide)". Naturwissenschaften 20.51 (1932): 948–950. Print.

Halliwell, B. "Oxidative Stress and Cancer: Have We Moved Forward?" *Biochemical Journal* 401 (2007): 1-11. Print.

Halliwell, B., and J. M. C. Gutteridge. "Oxygen Free-Radicals and Iron in Relation to Biology and Medicine - Some Problems and Concepts." *Archives of Biochemistry and Biophysics* 246.2 (1986): 501-14. Print.

Hand, S. C., and I. Hardewig. "Downregulation of Cellular Metabolism During Environmental Stress: Mechanisms and Implications." *Annual Review of Physiology* 58 (1996): 539-63. Print.

Hauer, F. R., and J. A. Stanford. "Larval Specialization and Phenotypic Variation in Arctopsyche-grandis (Trichoptera, Hydropsychidae)." *Ecology* 62.3 (1981): 645-53. Print.

Hensley, K., et al. "A Model for Beta-Amyloid Aggregation and Neurotoxicity Based on Free-Radical Generation by the Peptide - Relevance to Alzheimer-Disease." *Proceedings of the National Academy of Sciences of the United States of America* 91.8 (1994): 3270-74. Print.

Hensley, Kenneth, et al."Brain Regional Correspondence between Alzheimers-Disease Histopathology and Biomarkers of Protein Oxidation." *Journal of Neurochemistry* 65.5 (1995): 2146-56. Print.

Jara, C., et al. "Oxidative Stress in the Mollusk Echinolittorina Peruviana (Gasteropoda: Littorinidae, Lamarck, 1822) and Trace Metals in Coastal Sectors with Mining Activity." *Ecotoxicology* 23.6 (2014): 1099-108. Print.

Joyner-Matos, J., C. A. Downs, and D. Julian. "Increased Expression of Stress Proteins in the Surf Clam Donax Variabilis Following Hydrogen Sulfide Exposure." *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* 145.2 (2006): 245-57. Print.

Kacmar, P., J. Pistl, and I. Mikula. "Immunotoxicology and Veterinary Medicine." *Acta Veterinaria Brno* 68.1 (1999): 57-79. Print.

Kaloyianni, M., et al. "Oxidative Effects of Inorganic and Organic Contaminants on Haemolymph of Mussels." *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* 149.4 (2009): 631-39. Print.

Kamo, M., and T. Nagai. "An Application of the Biotic Ligand Model to Predict the Toxic Effects of Metal Mixtures." *Environmental Toxicology and Chemistry* 27.7 (2008): 1479-87. Print.

Kashian, D. R., et al. "The Cost of Tolerance: Sensitivity of Stream Benthic Communities to Uv-B and Metals." *Ecological Applications* 17.2 (2007): 365-75. Print.

Kong, S. K., et al. "Peroxynitrite Disables the Tyrosine Phosphorylation Regulatory Mechanism: Lymphocyte-Specific Tyrosine Kinase Fails to Phosphorylate Nitrated Cdc2(6-20)Nh2 Peptide." *Proceedings of the National Academy of Sciences of the United States of America* 93.8 (1996): 3377-82. Print.

Leland, H. V. "Drift response of aquatic insects to copper." *Verhandlung Internationale Vereinigung Limnologie* 22.4 (1985). Print.

Levine, R. L. "Ischemia - from Acidosis to Oxidation." *Faseb Journal* 7.13 (1993): 1242-46. Print.

Levine, R. L., and E. R. Stadtman. "Oxidative Modification of Proteins During Aging." *Experimental Gerontology* 36.9 (2001): 1495-502. Print.

Liu, N., et al. "Application of the Biomarker Responses in Scallop (Chlamys Farreri) to Assess Metals and Pahs Pollution in Jiaozhou Bay, China." *Marine Environmental Research* 80 (2012): 38-45. Print.

Livingstone, D. R. "Contaminant-Stimulated Reactive Oxygen Species Production and Oxidative Damage in Aquatic Organisms." *Marine Pollution Bulletin* 42.8 (2001): 656-66. Print.

Luoma, S.N., P.S. Rainbow. "Metal Contamination in Aquatic Environments: Science and Lateral Management." *Cambridge University Press*, Cambridge, UK. (2008).

Lushchak, O. V. "Environmentally induced oxidative stress in aquatic animals." *Aquatic Toxicology* 101.1 (2011): 13-30. Print

Lushchak, O. V., et al. "Chromium(III) Induces Oxidative Stress in Goldfish Liver and Kidney." *Aquatic Toxicology* 93.1 (2009): 45-52. Print.

Lushchak, V., et al. "Trivalent Chromium Induces Oxidative Stress in Goldfish Brain." *Chemosphere* 75.1 (2009): 56-62. Print.

Lushchak, V. I. "Free Radical Oxidation of Proteins and Its Relationship with Functional State of Organisms." *Biochemistry-Moscow* 72.8 (2007): 809-27. Print.

Maharajan, A., et al. "Sublethal Effect of Copper Toxicity against Histopathological Changes in the Spiny Lobster, Panulirus Homarus (Linnaeus, 1758)." *Biological Trace Element Research* 145.2 (2012): 201-10. Print.

Maisonneuve, E., et al. "Rules Governing Selective Protein Carbonylation." *Plos One* 4.10 (2009). Print.

Marchand, M. J., et al. "Histopathological Alterations in the Liver of the Sharptooth Catfish Clarias Gariepinus from Polluted Aquatic Systems in South Africa." *Environmental Toxicology* 24.2 (2009): 133-47. Print.

Mattaliano, M. D., et al. "Lox-1-Dependent Transcriptional Regulation in Response to Oxidized Ldl Treatment of Human Aortic Endothelial Cells." *American Journal of Physiology-Cell Physiology* 296.6 (2009): C1329-C37. Print.

McAnulty, S. R., et al. "Influence of Carbohydrate Ingestion on Oxidative Stress and Plasma Antioxidant Potential Following a 3h Run." *Free Radical Research* 37.8 (2003): 835-40. Print.

McGeer, J. C., et al. "Effects of Chronic Sublethal Exposure to Waterborne Cu, Cd or Zn in Rainbow Trout. 1: Iono-Regulatory Disturbance and Metabolic Costs." *Aquatic Toxicology* 50.3 (2000): 231-43. Print.

McIntyre, J. K., et al. "Low-Level Copper Exposures Increase Visibility and Vulnerability of Juvenile Coho Salmon to Cutthroat Trout Predators." *Ecological Applications* 22.5 (2012): 1460-71. Print.

Miller, Y. I., et al. "Oxidation-Specific Epitopes Are Danger-Associated Molecular Patterns Recognized by Pattern Recognition Receptors of Innate Immunity." *Circulation Research* 108.2 (2011): 235-48. Print.

Mogren, C. L., and J. T. Trumble. "The Impacts of Metals and Metalloids on Insect Behavior." *Entomologia Experimentalis Et Applicata* 135.1 (2010): 1-17. Print.

Moller, I. M., A. Rogowska-Wrzesinska, and R. S. P. Rao. "Protein Carbonylation and Metal-Catalyzed Protein Oxidation in a Cellular Perspective." *Journal of Proteomics* 74.11 (2011): 2228-42. Print.

Mullarkey, C. J., D. Edelstein, and M. Brownlee. "Free-Radical Generation by Early Glycation Products - a Mechanism for Accelerated Atherogenesis in Diabetes." *Biochemical and Biophysical Research Communications* 173.3 (1990): 932-39. Print.

Murphy, M. E., and J. P. Kehrer. "Oxidation-State of Tissue Thiol-Groups and Content of Protein Carbonyl Groups in Chickens with Inherited Muscular-Dystrophy." *Biochemical Journal* 260.2 (1989): 359-64. Print.

Niyogi, S., and C. M. Wood. "Biotic Ligand Model, a Flexible Tool for Developing Site-Specific Water Quality Guidelines for Metals." *Environmental Science & Technology* 38.23 (2004): 6177-92. Print.

Nyska, A., and R. Kohen. "Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for Their Quantification." *Toxicologic Pathology* 30.6 (2002): 620-50. Print.

Oliva, M., et al. "Sublethal and Lethal Toxicity in Juvenile Senegal Sole (Solea Senegalensis) Exposed to Copper: A Preliminary Toxicity Range-Finding Test." *Experimental and Toxicologic Pathology* 61.2 (2009): 113-21. Print.

Oliver, C. N. "Inactivation of Enzymes and Oxidative Modification of Proteins by Stimulated Neutrophils." *Archives of Biochemistry and Biophysics* 253.1 (1987): 62-72. Print.

Orrenius, S., A. Gogvadze, and B. Zhivotovsky. "Mitochondrial Oxidative Stress: Implications for Cell Death." *Annual Review of Pharmacology and Toxicology* 47 (2007): 143-83. Print.

Paine, R. T., M. J. Tegner, and E. A. Johnson. "Compounded Perturbations Yield Ecological Surprises." *Ecosystems* 1.6 (1998): 535-45. Print.

Pane, E. F., et al. "The Physiological Consequences of Exposure to Chronic, Sublethal Waterborne Nickel in Rainbow Trout (Oncorhynchus Mykiss): Exercise Vs Resting Physiology." *Journal of Experimental Biology* 207.7 (2004): 1249-61. Print.

Parolini, M., A. Pedriali, and A. Binelli. "Chemical and Biomarker Responses for Site-Specific Quality Assessment of the Lake Maggiore (Northern Italy)." *Environmental Science and Pollution Research* 20.8 (2013): 5545-57. Print.

Parvez, S., and S. Raisuddin. "Protein Carbonyls: Novel Biomarkers of Exposure to Oxidative Stress-Inducing Pesticides in Freshwater Fish Channa Punctata (Bloch)." *Environmental Toxicology and Pharmacology* 20.1 (2005): 112-17. Print.

Postma, J. F., et al. "Chronic Toxicity of Cadmium to Chironomus-Riparius (Diptera, Chironomidae) at Different Food Levels." *Archives of Environmental Contamination and Toxicology* 26.2 (1994): 143-48. Print.

Prousek, J. "Fenton Chemistry in Biology and Medicine." *Pure and Applied Chemistry* 79.12 (2007): 2325-38. Print.

Rand, G. M., P. G. Wells, and L. S. McCarty. "Introduction to aquatic toxicology." *Fundamentals of aquatic toxicology effects, environmental fate, and risk assessment. Taylor and Francis Publishers, North Palm Beach, Florida, USA* (1995): 3-67.

Reddy, V. P., et al. "Oxidative Stress in Diabetes and Alzheimer's Disease." *Journal of Alzheimers Disease* 16.4 (2009): 763-74. Print.

Resh, V. H. "Recent trends in the use of Trichoptera in water quality monitoring." *Proceedings of the 7th International Symposium on Trichoptera. Backhuys Publishers, Leiden, The Netherlands*. (1993).

Riddell, D. J., J. M. Culp, and D. J. Baird. "Behavioral Responses to Sublethal Cadmium Exposure within an Experimental Aquatic Food Web." *Environmental Toxicology and Chemistry* 24.2 (2005): 431-41. Print.

Rivera-Mancia, S., et al. "The Transition Metals Copper and Iron in Neurodegenerative Diseases." *Chemico-Biological Interactions* 186.2 (2010): 184-99. Print.

Schmidt, T. S., W. H. Clements, and B. S. Cade. "Estimating Risks to Aquatic Life Using Quantile Regression." *Freshwater Science* 31.3 (2012): 709-23. Print.

Selle, Tina, et al. "Reviewing peripartum cardiomyopathy: current state of knowledge." (2009): 175-89. Print.

Senoglu, N., et al. "Protective Effects of N-Acetylcysteine and Beta-Glucan Pretreatment on Oxidative Stress in Cecal Ligation and Puncture Model of Sepsis." *Journal of Investigative Surgery* 21.5 (2008): 237-43. Print.

Shacter, E. "Quantification and Significance of Protein Oxidation in Biological Samples." *Drug Metabolism Reviews* 32.3-4 (2000): 307-26. Print.

Shao, B., et al. "Pathways for Oxidation of High-Density Lipoprotein in Human Cardiovascular Disease." *Current Opinion in Molecular Therapeutics* 8.3 (2006): 198-205. Print.

Skidmore, J. F. "Respiration and Osmoregulation in Rainbow Trout with Gills Damaged by Zinc Sulphate." *Journal of Experimental Biology* 52.2 (1970): 481-&. Print.

Skidmore, J. F., and P. W. A. Tovell. "Toxic effects of zinc sulphate on the gills of rainbow trout." *Water Research* 6.3 (1972). Print.

Smith, M. A., et al. "Cytochemical Demonstration of Oxidative Damage in Alzheimer Disease by Immunochemical Enhancement of the Carbonyl Reaction with 2,4-Dinitrophenylhydrazine." *Journal of Histochemistry & Cytochemistry* 46.6 (1998): 731-35. Print.

Spicer, J. I., and R. E. Weber. "Respiratory Impairment in Crustaceans and Mollusks Due to Exposure to Heavy-Metals." *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* 100.3 (1991): 339-42. Print.

Stadtman, E. R., and B. S. Berlett. "Fenton Chemistry - Amino-Acid Oxidation." *Journal of Biological Chemistry* 266.26 (1991): 17201-11. Print.

Stadtman, E. R., and R. L. Levine. "Free Radical-Mediated Oxidation of Free Amino Acids and Amino Acid Residues in Proteins." *Amino Acids* 25.3-4 (2003): 207-18. Print.

Stadtman, E. R., and C. N. Oliver. "Metal-Catalyzed Oxidation of Proteins - Physiological Consequences." *Journal of Biological Chemistry* 266.4 (1991): 2005-08. Print.

Sundari, P. N., W. G, and B. Ramakrishna. "Does Oxidative Protein Damage Play a Role in the Pathogenesis of Carbon Tetrachloride-Induced Liver Injury in the Rat?" *Biochimica Et Biophysica Acta-Molecular Basis of Disease* 1362.2-3 (1997): 169-76. Print.

Swallow, A. J. Effect of ionizing radiation on proteins, RCO groups, peptide bond cleavage, inactivation, -SH oxidation. *Radiation Chemistry of Organic Compounds*. John Wiley & Sons. New York, NY. (1960): 211–224. Print.

Tierney, K. B., et al. "Olfactory Toxicity in Fishes." *Aquatic Toxicology* 96.1 (2010): 2-26. Print.

Tkatcheva, V., et al. "Toxic Effects of Mining Effluents on Fish Gills in a Subarctic Lake System in Nw Russia." *Ecotoxicology and Environmental Safety* 57.3 (2004): 278-89. Print.

Toyokuni, S., et al. "Target Genes in Fenton Reaction-Induced Carcinogenisis: A Novel Concept." *Free Radical Biology and Medicine* 33 (2002): S177-S77. Print.

Tripathy, D., and P. Grammas. "Acetaminophen Inhibits Neuronal Inflammation and Protects Neurons from Oxidative Stress." *Journal of Neuroinflammation* 6 (2009). Print.

Turrens, J. F. "Mitochondrial Formation of Reactive Oxygen Species." *Journal of Physiology-London* 552.2 (2003): 335-44. Print.

Uchida, K., et al. "A Renal Carcinogen Ferric Nitrilotriacetate Mediates a Temporary Accumulation of Aldehyde-Modified Proteins within Cytosolic Compartment of Rat-Kidney." *Archives of Biochemistry and Biophysics* 317.2 (1995): 405-11. Print.

Van der Geest, H. G., et al. "Survival and Behavioral Responses of Larvae of the Caddisfly Hydropsyche Angustipennis to Copper and Diazinon." *Environmental Toxicology and Chemistry* 18.9 (1999): 1965-71. Print.

Voelz, N. J., and J. V. Ward. "Feeding-Habits and Food Resources of Filter-Feeding Trichoptera in a Regulated Mountain Stream." *Hydrobiologia* 231.3 (1992): 187-96. Print.

Wang, M. H., and G. Z. Wang. "Biochemical Response of the Copepod Tigriopus Japonicus Mori Experimentally Exposed to Cadmium." *Archives of Environmental Contamination and Toxicology* 57.4 (2009): 707-17. Print.

Wetzel, R. G., and G. E. Likens. "Limnological Analyses." *Springer Science.* New York, NY." (1991): 125. Print.

Wilson, R. W., H. L. Bergman, and C. M. Wood. "Metabolic Costs and Physiological Consequences of Acclimation to Aluminum in Juvenile Rainbow-Trout (Oncorhynchus-Mykiss) .2. Gill Morphology, Swimming Performance, and Aerobic Scope." *Canadian Journal of Fisheries and Aquatic Sciences* 51.3 (1994): 536-44. Print.

Wu, J., and B. Weiss. "2 Divergently Transcribed Genes, Soxr and Soxs, Control a Superoxide Response Regulon of Escherichia-Coli." *Journal of Bacteriology* 173.9 (1991): 2864-71. Print.

Xie, L., et al. "Mercury(Ii) Bioaccumulation and Antioxidant Physiology in Four Aquatic Insects." *Environmental Science & Technology* 43.3 (2009): 934-40. Print.

Xie, L. T., and D. B. Buchwalter. "Cadmium Exposure Route Affects Antioxidant Responses in the Mayfly Centroptilum Triangulifer." *Aquatic Toxicology* 105.3-4 (2011): 199-205. Print.

Xiu, M., L. Q. Pan, and Q. Jin. "Bioaccumulation and Oxidative Damage in Juvenile Scallop Chlamys Farreri Exposed to Benzo a Pyrene, Benzo B Fluoranthene and Chrysene." *Ecotoxicology and Environmental Safety* 107 (2014): 103-10. Print.

Yan, Liang-Jun. "Analysis of oxidative modification of proteins." *Current Protocols in Protein Science* (2009): 14-4. Print

Yuan, Q., X. C. Zhu, and L. M. Sayre. "Chemical Nature of Stochastic Generation of Protein-Based Carbonyls: Metal-Catalyzed Oxidation Versus Modification by Products of Lipid Oxidation." *Chemical Research in Toxicology* 20.1 (2007): 129-39. Print.

APPENDIX

Biochemical Procedures

In all steps, except where otherwise noted, great care should be taken to keep samples as cold as possible while also avoiding repeated freeze/thaw cycles.

Organism Homogenization:

1) Place organism in 1.5 mL microcentrifuge tube. Add approximately 1mL of cold 100mM EDTA wash solution. Vortex for five seconds and discard liquid. Repeat two more times.
2) Add approximately 1mL of cold 1X PBS. Vortex for five seconds and discard liquid. Repeat two more times.
3) Transfer washed sample to pre-chilled glass mortar cylinder and add 1.35mL of PBS homogenization solution. Using a Teflon homogenizer connected to a power drill, homogenize, on ice, with five separate pulses of five seconds each. Allow homogenate to cool for ten seconds between each pulse.
4) Transfer homogenate to a pre-chilled 15 mL conical tube.
5) Sonicate homogenate, at power level 3 using a Sonifier® Cell Disruptor (Heat Systems Co.), on ice, with five separate pulses of five seconds each. Allow homogenate to cool for ten seconds between each pulse.
6) Store homogenate on ice and repeat steps 1-4 until all samples have been homogenized. Wash all tools thoroughly with EDTA wash solution and PBS to prevent cross contamination between samples.

7) Transfer homogenates to pre-chilled 1.5 mL microcentrifuge tubes. Centrifuge for ten minutes at 12,000 G at 4˚C.
8) Carefully pour off supernatant to create a clarified homogenate containing the soluble protein fraction for use in subsequent purification steps. Store clarified homogenate on ice.

Notes: A) If pellet is loosely bound or disturbed in step 7, repeat steps 6-7.

Lipid Removal:

1) Created a column by tightly packing a small quantity of pure cotton into a glass pasteur pipette.
2) Wash with 1X PBS three times. A pipette bulb may necessary to force PBS through the column.
3) Transfer clarified homogenate to the column. Slowly (1-2 drops per second) force homogenate through column using a pipette bulb and retain in a 1.5 mL microcentrifuge tube.
4) Store on ice.
5) Repeat steps 1-4 for each sample.

Notes: A) If white flakes are observed in the sample after passing through the cotton column repeat steps 1-3.

Nucleic Acid Removal:

1) To each sample, add 10X streptomycin sulfate stock solution to achieve a 1% streptomycin sulfate/homogenate solution.

2) Incubate for 30 minutes at room temperature with periodic vortexing.
3) Centrifuge for ten minutes at 6,000 G at 4˚C.
4) Transfer supernatants to 1.5 mL microcentrifuge tubes. Aliquot purified protein homogenates. Save one aliquot for subsequent protein carbonyl analysis and store at 4˚C for a maximum of two days. Store other aliquots at -80˚C for long-term preservation.

Notes: A) Pellet may be difficult to see.

Protein normalization:

1) Following manufacturer's instructions, determine protein concentration for each purified protein homogenate by Bradford Assay (Thermo Fisher Scientific). http://www.piercenet.com/instructions/2160129.pdf. Dilutions may be necessary to ensure unknown protein concentrations are within the bounds of the standard curve.
2) Dilute each sample to 20 µg/mL protein with 1X PBS.
3) Following manufacturer's instructions, repeat protein concentration determinations. Dilute each sample to 10µg/mL protein with 1X PBS.

Notes: A) Keep samples on ice at all times unless specified by the manufacturer. B) Limit time low protein concentration samples are stored in microcentrifuge tubes as it risks protein binding to plastic.

Protein Carbonyl ELISA:

1) Closely follow manufacturer's instructions (Oxiselect™ Protein Carbonyl ELISA kit, Cell Biolabs). http://www.cellbiolabs.com/sites/default/files/STA-310-protein-carbonyl-elisa-kit.pdf.

Notes: A) Avoid freeze/thaw cycles with protein carbonyl standards. B) Plate standards in non-consecutive columns to help determine the plate reader's measurement uniformity from one side of the plate to the other. C) Overnight incubation for protein-ELISA plate binding at 4˚C is preferred. D) Extra wash steps circumvent need for aspiration.

Recipes

100 mM Ethylenediaminetetraacetic acid (EDTA) Wash Solution

- 400 mL type 1 H_2O
- 14.61g EDTA (100 mM)
- Add NaOH as needed to dissolve EDTA
- QS to 500mL with type 1 H_2O
- pH to 7.4 with HCL, or NaOH

1X Phosphate buffer saline (PBS)

- 4.00 g NaCl (0.14 M)
- 0.10 g KCl (0.0027M)
- 0.71 g $NaHPO_4$ (0.010M)
- 0.14 g KH_2PO_4 (0.0020M)
- pH to 7.4 with HCL, or NaOH
- QS to 0.5L with type 1 H_2O

5% Butylated Hydroxytoluene (BHT) Stock Solution

- 0.5 g BHT (5% w/v)

- QS to 10mL with methanol

PBS Homogenization Solution

- 25mL 1X PBS

- 30uL 5% BHT stock solution (0.005% w/v)

- 400uL 100X Halt™ protease inhibitor cocktail (1.33X)

- 43.8mg EDTA (5mM)

- PBS must be heated, stirring vigorously, and 5% BHT stock solution incorporated as slowly as possible to prevent BHT from falling out of solution. Cool PBS/BHT solution on ice while stirring vigorously before adding protease inhibitor cocktail.

- QS to 30mL with 1X PBS

- Prepare shortly before use and store at 4˚C

10X Streptomycin Sulfate Stock Solution

1.0g streptomycin sulfate (10% w/v)

QS to 10 mL with 1X PBS