The impacts of metal and salts similar in composition to Oil sands processes affected water (OSPW) on Rainbow trout respirometry, gill structure, and gill enzyme dynamics

By

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Abstract

Remediation of Northern Athabasca Oil sands areas has become an essential goal for engineers, biologists and industry stakeholders. Tailing ponds containing oil sand process-affected water (OSPW) have been growing in number as a byproduct of bitumen extraction activity. Treatment of OSPW can reduce some of the toxic organic components, but the inorganic constituents cannot be remediated or removed. The organic fraction of OSPW has been examined in recent years, but few studies have studied the inorganic fraction. Metals and salts (M&S) mixtures similar to OSPW were created and rainbow trout (*Oncorhynchus mykiss*) were exposed to these mixtures to investigate how these solutions would impact gill and swimming physiology. Medium M&S exposure caused a 25±3% decline in mean U_{crit}; however, metabolic rates were primarily unaffected. An examination of gill structure and morphometrics revealed that gills were not impacted by exposure. The activities of carbonic anhydrase were similar between the control and M&S exposures, but my Cu exposure (Cu positive control) was substantially higher than the rest of the exposures. The activity of sodium potassium ATPase in fish exposed to the medium M&S mixture did not differ from control, but when compared to NaCl exposure (salt control), medium M&S exposure may have caused some ionoregulatory dysfunction. Given that treated OSPW is likely to be discharged in future, the importance of assessing the impacts of the metals and salts found in OSPW on surrounding aquatic communities should not be ignored. Future work should evaluate the implications of acute discharge of OSPW and the merits of treatment technology.

Preface

This thesis is an original work by Zachary Mueller. This project is part of research that has received research ethics approved from the University of Alberta Research Ethics Board, Assessment of Fish Swimming Performance, AUP0000022, April 13, 2018.

Dedication

To my Mom and Dad.
None of this would be possible without your help and guidance.

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Abbreviation list

Oil sands process affected water (OSPW)

Naphthenic acids (NA)

Polycyclic aromatic hydrocarbons (PAH)

BTEX (benzene, toluene, ethyl benzene, and xylenes)

Canadian Council of Minister of the Environment (CCME)

Dissolved organic carbon (DOM)

Total dissolved solids (TDS)

Biotic ligand model (BLM)

Lethal accumulation (LA50%)

Sodium (Na)

Magnesium (Mg)

Calcium (Ca)

Chloride (Cl)

Aluminium (Al)

Copper (Cu)

Potassium (K)

Molybdemum (Mo)

Nickel (Ni)

Zinc (Zn)

Voltage regulated ATPase (V-type ATPase)

Sodium potassium ATPase (NKA)

Carbonic anhydrase (CA)

Mitochondria rich cells (MRCs)

Cost of transport (COT)

Animal Support Services (SASS)

Reactive oxygen species (ROS)

Committee on the status of endangered wildlife in Canada (COSEWIC)

Chapter 1: Introduction to issues surrounds oil sands process affected water and background to metals of concern in OSPW

1.0. Oils sands process affected water

Understanding the toxicity of oil sands process-affected water (OSPW) is an essential endeavor to government, industry and scientists [1,2]. OSPW, a by-product of oil extraction, has been accumulating in tailing ponds and settling basins in the Alberta Athabasca region [3]. The possible toxicity of OSPW is a serious concern; thus a moratorium on OSPW discharge has been in place [3,4]. OSPW is chemically complex and diverse. Furthermore, it has been difficult to determine the toxicity of OSPW constituents [1]. How animals are affected by OSPW and what those effects are have been the subject of numerous investigations in previous decades [4–7].

OSPW is composed of metals, salts and cyclic carboxylic acids [1]. The composition of OSPW varies based on analysis method and source [1]. It is difficult to compare lab and field studies of OSPW in part due to OSPW composition [2]. Naphthenic acids (NA) are the most prevalent cyclic carboxylic acids in OSPW and are thought to be the primary source of toxicity [2]. OSPW also contains polycyclic aromatic hydrocarbons (PAH), BTEX (benzene, toluene, ethylbenzene and xylenes), and phenols; however, these compounds are less abundant than NAs [1]. Many studies have investigated the toxicity the organic fraction in OSPW while few studies have investigated the inorganic fraction that contains various salts and metals.

Many studies by scientists and engineers have endeavored to provide methods to decrease the toxicity of OSPW. Through physical and chemical processes, OSPW's chemical composition can be altered. As a result of treatment, OSPW has been shown to be less toxic than the raw OSPW. Treatment techniques such as ozonation [8–10] and activated charcoal [10–12], which cancan break carbon bonds or sequester hydrocarbons, and thus lower the toxicity of NAs. For example, treated OSPW caused less mortality, spontaneous movement, pericardial edema, spinal malformation and reactive oxygen species (ROS) in larval fish [10]. Furthermore, natural processes such as photolysis and microbial degradation can reduce some of the organics in OSPW [11,13]. Hopefully, with sufficient aging and the application treatment technology, most of the organics present in OSPW tailing ponds can be removed. Unfortunately, there are no economically feasible remediation strategies for the inorganic fraction of OSPW.

The salts and metals found in OSPW may be a persistent threat to aquatic life owing to their non-degradability. Studies have shown that treatment and aging attenuate some toxicological endpoints; however, the research is far from exhaustive [8,10]. Metals and organic hydrocarbons tend to target different facets of physiology. Potentially, studies have underexplored the possible contribution to the toxicity of the inorganic fraction of OSPW [14]. Studies in which the organic fraction of OSPW has been removed have only examined lethality, developmental impairment, and endocrine disruption [8,9,12,15]. To date, only plants and algae studies have investigated the specific toxicity of the inorganic fraction of OSPW [14].

Trace amounts of metals and salts are ubiquitous in aquatic environments [16]. However, when present in higher concentrations, metals and salts can be toxic to aquatic life. Interestingly, other physical properties of water play an integral role in determining toxicity. Factors such as pH, hardness, alkalinity, total dissolved solids (TDS), and composition/amount of dissolved organic carbon (DOM), can play essential roles in the toxicity of some metals [16]. To predict the toxicity of salts and metals the following are required: the identity and chemical form of an ion, the ions concentration in solution, the route of exposure, and the exposure time [16]. Toxicologists use this information along with standardized protocols and dose-dependent responses to predict the toxicity of a particular compound [16]. Risk assessment models such as the biotic ligand model (BLM) examine how various water parameters affect the risk of exposure to various concentrations of a specific metal to specific aquatic organisms [17]. This model predicts the amount of a metal required to accumulate on/in the gills to cause mortality in 50% of the population (LA50%) [17]. Stakeholder and regulatory bodies have used this model and others like it to help monitor/regulate the environment. The Canadian Council of Minister of the Environment (CCME) is a regulatory organization that sets guidelines for environmental contaminants in Canada. According to these guidelines, OSPW contains 14 metals that are considered to be above acceptable levels for aquatic life [18]. These guidelines do not include criteria for mixtures of metals, and models produce varying results [16].

Despite many investigations into the toxicity of whole and organic fractions of OSPW and the extensive knowledge about the toxicity of individual metal, there appear to be knowledge gaps about the possible effects of the inorganic fraction of OSPW on fish [13]. Although risk assessment models and bioassay experiments have examined the inorganic fraction of OSWP, few, nonembryonic animal-based experiments exist [14]. Many constituents of the inorganic

fraction of OPSW are considered toxic, but little is known about how this specific combination of metals and salts might affect fish [14,18].

1.1. Thesis organization

This thesis is presented in three chapters. Chapter 1 includes background and introduction. Chapter 2 is the first data chapter, which details the effects of metals and salts mixtures similar to OSPW on fish swimming performance and metabolism. Chapter 3 compares and contrasts water chemistry with gill structure and ionoregulatory enzymes. The last chapter also includes a general discussion of integrating findings of Chapters 2 and 3.

1.2. Rainbow trout

The species used in a toxicological investigation can have a significant impact on the results of the experiments. Investigations require that organisms be obtainable, culturable and testable [19]. Each organism has its costs and benefits. Using laboratory model organisms lowers the variation between investigators, and it also reduces the complexity of any given experiment [19]. However, using animals not endemic to the environment in which the toxicant is likely to be exposed is not ideal [19]. Model organisms tend to be relatively robust, which benefits rearing them in labs, but it also means they may be relatively insensitive to some stressors [19]. Rainbow trout are anadromous euryhaline fish that is adept at dealing with various ionic/osmotic environments. From egg to fry, rainbow trout grow in freshwater streams and rivers and then may transition to the ocean. rainbow trout are historically distributed between Northeastern Siberia and western North America [20]. There is one native population of rainbow trout found in Athabasca rivers headwaters. Unlike some rainbow trout populations, Athabaskan rainbow trout do not migrate to the ocean [20] . This population inhabits headwater streams which are cold and unproductive, but the fish have few competitors [20]. Compared to aquacultured populations, this population has a lower thermal optimum, grows slower, matures at a smaller size, and spawns in late spring [20]. Furthermore, this population is considered a species at risk by the committee on the status of endangered wildlife in Canada (COSEWIC) [20]. I chose to use a laboratory strain of rainbow trout in my investigation. Rainbow trout are the primary model

organism for investigating the effects of wastewater and effluent, even in seawater [19]. The bioavailability and specific binding affinity for a metal strongly influence the toxicity for any given species of fish [21]. Rainbow trout exposed to OSPW appear to be very sensitive to the organic fraction (mortality 3 %v/vs.OSPW), but how rainbow trout will respond to the inorganic fraction is unknown [1]. Since rainbow trout is often used in toxicity investigations and is endemic to the Athabasca headwaters, I believe it is a reasonable model to investigate the possible effects of metals and salts similar to OSPW on fish.

1.3. Inorganic fraction literature review

I will review the literature on the toxic effects of the constituent ions found in OPSW. I am restricting this review to ions, which are well studied, and were shown to change metabolism and swimming ability in fishes. Other inorganic compounds found in OSPW that may influence behavior, reproduction, immune function, endocrine signaling were not covered in this review. The information below was used to inform me on what I would use to make a mixture of salts and metals to investigate the possible effects of the inorganic fraction of OSPW on rainbow trout.

1.4. Toxicity of Sodium, Magnesium, Calcium, and Chloride

Ions such as sodium (Na), magnesium (Mg), calcium (Ca) and chloride (Cl) in OSPW are often not considered as a source of toxicity. All of these metals are found in high concentrations in OSPW (Na: 6.92-36.7; Ca: 0.05-2.57; Mg: 0.08-1.42; Cl: 1.18-24.4 mmol/l) as well as the environment (Na: 0.78-4.09; Ca: 0.57-0.99; Mg: 0.36-0.91; Cl: 0.13-1.58 mmol/l) [18]. Sodium, Mg, Ca, and Cl are all essential metals for physiological function [18,22,23]. Toxicity could occur at high and low concentrations of these ions [24]. Although the latter is doubtful, high salt concentrations can be destructive [23]. Some fish lack the physiological tools to osmoregulate in certain salinities. In freshwater, fish can lose salts and gain water, which causes hypertonicity. The absorption and toxicity of these ions (Na, Ca, Mg, and Cl) are strongly dose dependent [2]. Although rainbow trout are capable of tolerating salts, for the purpose of comparison, a NaCl salt control was produced for comparative purposes. BLM predicts that these salts are a high risk to

many invertebrates [14]. However, various fish species including rainbow trout are often very tolerant to the Na, Mg, Ca and Cl present in effluents [15,22]. Despite this, Na, Mg, Ca and Cl cannot be ignored in this investigation because they may strongly influence the toxicity of other metals present in OSPW [14,17].

1.5. Toxicity of Aluminum

Aluminium (Al) is an non-essential metal and is often the most prevalent metal in OSPW (2.6-3670 μmol/l) [4,18]. In fish, Al causes ionoregulatory, osmoregulatory and respiratory dysfunction [25]. Specifically, Al causes a loss of plasma ions, a decline in regulatory enzyme activity (sodium-potassium ATPase (NKA), Mg-ATPase, and carbonic anhydrase (CA)), gill morphology deformations (inflammation, oedema, swelling, increase number of mucus and mitochondria-rich cells (MRCs)), and causes polycythemia [26]. In gills, the mechanism of absorption is unclear, but Ca and protons appear to compete with Al for absorption [26]. Aluminum toxicity appears to interfere with ionoregulation and causes oxidative stress in freshwater fish [26]. However, Al requires low pH (4.6-5.3) and Ca (0.01-0.04 mmol/l) for toxicity to occur [26]. OSPW has a very high pH (7.2-8.9) and Ca (0.05-2.57 mmol/l) concentrations, which would suggest that there is a low possibility that Al will be able to enter the gills [18]. However, Al has rarely been assessed as part of a complex mixture and in such high concentrations. Given the high concentration of Al in OSPW, I believe it should be included in my investigation.

1.6. Toxicity of Copper

Copper (Cu) is a metal that has been well-studied for its toxic effects on aquatic organisms and is found in high concentrations (0.03-13.2 μmol/l) in OSPW [18,27]. This metal is prevalent in many freshwater environments and is an essential metal [28]. Copper has the following sublethal effects: behavioral alterations (e.g., loss of aggression and risk aversion), loss of olfaction, impairment of swim performance and pH imbalance [17,29]. Exposure to Cu can perturb physiology and behaviour sufficiently that fish are no longer able to deal with typical environmental threats (including predators and starvation) [30]. Water parameters can alter the

toxicity of copper by changing Cu's valent state, as well as by limiting its absorption [17]. Absorption of Cu appears to occur through apical Na channels [27]. High pH and Ca concentrations appear to mitigate Cu absorption by changing Cu speciation and out-competing Cu for ligand binding through Ca channels [31]. Interestingly, Cu inhibits voltage regulated ATPase (V-type ATPase) in pavement cells, which may cause differences in membrane potential between pavement cells and MCRs [31]. This could disrupt paracellular transport of ions. Copper also causes Na imbalance within blood plasma and this could be mediated though V-type ATPase [31]. Once absorbed, Cu acts as a noncompetitive and mixed inhibitor to proteins containing methionine, histidine and cysteine functional groups [27,32,33]. This can cause rapid generation of ROS in mitochondria in conjunction with copper redux properties [27]. Thus, copper causes enzymatic dysfunction as well an apoptosis through ROS [27]. Also, NKA and CA activity are sometimes used as biomarkers of Cu exposure [27].

Given that various water parameters can influence the toxicity of various metals, including Cu, models have been produced to predict the relative risk of copper to organisms [16]. According to the BLM, Cu in OSPW has a low risk to aquatic health [14]. The low risk is likely due to high water hardness (13.2-399 ppm) and pH (7.2-8.9) of OSPW minimizing the speciation and absorption of Cu thus limiting Cu bioavailability [18]. However, the BLM assesses risk based on the LA50% of copper, which is much higher than the concentrations required to cause sublethal effects. Therefore, Cu should still be investigated as a metal in OSPW that may impact fish health [16]. The mechanism by which Cu causes declines in swim performance and disrupts ionoregulation is among the most studied of the metals in this review. If salts and other metals play a possible role in the mitigating the effects of the decline in swim performance, then a positive control Cu treatment should be investigated.

1.7. **Toxicity of Molybdenum**

Molybdenum (Mo) is a rarely investigated essential metal in the environment but is present in high concentration in OSPW (0.02-11.7 μmol/l) [18]. Aquatic organisms appear relatively resistant to Mo. However, few studies have investigated the effects of Mo [18]. The mechanism of absorption is not well understood, but Mo is prevalent in many enzymes [18]. Over 50 enzymes have Mo as their catalytic center, and high concentrations of Mo (>2.61

mmol/l) have caused gill lamellae fusion, increased mucus production, increased ventilation frequency, post-exercise loss of equilibrium and exercised-induced mortality [33,34]. However, rainbow trout exposed to 0.02 mmol/l of Mo showed no difference in plasma cortisol, blood glucose, and hematocrit [35]. Thus at rest, Mo did not cause significant physiological or cellular stress responses in rainbow trout [35]. The exacerbating and mitigating factors of Mo on the toxicity of other metals and salts have been largely unexplored. Furthermore, BLM suggests that Mo is a reasonable concern for toxicity on aquatic animals and thus I believe it should be investigated as a metal in OSPW that may impact fish health [14].

1.8. **Toxicity of Nickel**

Nickel (Ni) is a micronutrient prevalent in OSPW (0.24-4.60 μmol/l) and, when compared to the other metals discussed above, has unique toxic effects [36]. This metal may cause blood acidosis, lamellar fusion, secondary epithelia cell lifting, gill epithelium hypertrophy, gill necrosis, increased cardiac output, decreased locomotor activity, altered immunoregulation, and reduced growth [37–40]. This metal may also cause respiratory issues without affecting ionoregulation in fish gills [38]. Epithelia and pillar cells appear to be the first impacted by Ni pathology and cause fusion of the secondary lamellae [41]. The mechanism by which this occurs is not fully understood, but Ni appear to antagonize Mg reabsorption [41,42]. Nickel exposure can cause a build-up of carbon dioxide and lactic acid due to respiratory dysfunction and this often results in blood acidosis [38]. Calcium appears to mitigate Ni absorption by out competing Ni for Ca channels for entry; however, the exact method of absorption is not well understood [41]. Despite this, BLM compiled by White (2017) suggests that Ni in OPSW is a risk to aquatic life and thus I believe should be investigated [14].

1.9. **Toxicity of Zinc**

Zinc (Zn), as with many other essential trace metals, is a micronutrient prevalent in aquatic environments and OSPW (0.02-19.0 μmol/l) [1]. In fish, Zn exposure may cause excess mucus production, gill epithelium lifting, slower growth, increased respiration rate, and decreased metabolic rate and swim performance [43,44]. The mechanism of absorption of Zn is

not fully understood, but Ca channels on the apical surface are possible candidates [45]. Unlike other trace metals, water hardness appears to be the primary mitigating factor affecting Zn absorption in the gills [45]. The presence of positively charged cations (Ca^{2+}, Na^{+}, NH^{4+} and Mg^{2+}) decreases Zn toxicity [45]. Calcium has a most potent mitigating effect on Zn toxicity because Ca competes with Zn for absorption in Ca channels; however, Zn-specific channels have been found in both the apical and basolateral surfaces of fish [45,46]. The kinetics of ion absorption also favor high charge and molecular weight particles, such as Ca [45]. Above a certain threshold, Zn strongly inhibits Ca-ATPase activity in the basolateral membrane of gills and impedes Ca reabsorption [45]. Thus, Ca mitigates Zn toxicity, but Zn can affect internal Ca concentrations and cause hypocalcemia [45]. This condition impacts cardiac and skeletal muscle function by limiting endoplasmic reticulum and mitochondria stores of Ca [45]. The BLM suggests that Zn in OSPW (0.02-19.0 μmol/l) has a low risk to aquatic organisms [14,18]. Despite this, I believe Zn should be investigated in OSPW because Zn has the potential of synergistically or additively enhancing the toxicity of other metals [47,48].

1.10. **Project rational and research objectives**

The inorganic fraction of OSPW has the potential to cause sublethal effects on rainbow trout. Here I will independently assess metals and ions contributions to changes in rainbow trout physiology. However, it is difficult to obtain sufficient quantities of OSPW to conduct animal-based studies on large organisms such as juvenile rainbow trout. Furthermore, removing the organic fraction as well as conducting analytical chemistry on OSPW is costly and time-consuming. As a compromise, I have attempted to simulate the inorganic fraction of OSPW by selecting specific salts and metals of interest in concentrations similar to OSPW. The specific research hypotheses were as follow:

1) A metal and salt mixture will decrease swimming performance because of the energetic debt associated with metal and salt exposure, or exposure will cause physiological changes that will limit oxygen delivery at high swimming activity.

2) Exposure will decrease aerobic scope due to the energetic debt accrued over exposure. This energetic debt should be symptomatic of ionoregulatory and/or respiratory dysfunction.

3) Exposure will disrupt gill morphology because metals will alter epithelium integrity and induce oxidative stress. Metal and salts absorption will interfere with lipid and protein function, which will cause visible structural damage in MCRs, pavement and accessory cells.

4) CA and NKA activity in fish gills will be decreased in metals and salts exposed fish since metals bind to functional groups in proteins, inhibiting proper functionality.

2.0 Chapter 2: Effects of metals and salts on metabolism and swimming ability in rainbow trout

OSPW has received considerable research attention in recent years. Various studies have quantified physiological effects of OSPW on larval and adult fish [10,13,15]. Most of these investigations have focused on NAs since they are considered to be the most toxic component [2]. However, physical and chemical methods exist that can remove or decrease the concentration of the organic components [8–12]. The amount of tailings is growing consistently and discharging pond waters is inevitable [14,49,50]. The current regulatory framework (tailings management framework) may allow for the discharge of OSPW if specific criteria are met [49,50]. The organic fraction can be removed or reduced, but the inorganic fraction cannot be. Evaluating whether metals and salts found in OSPW can have a negative impact on the environment should be a priority [4,49,50]. The inorganic fraction is not generally evaluated as a potential source of toxicity to fish. Although other studies have shown that the inorganic fraction is not overtly toxic, sublethal effects have not been explored [5]. Models, such as the BLM, have been used to predict the possible risk associated with trace metals and salts in OPSW to invertebrates [14]. Although invertebrates may be more sensitive than fish, these studies fail to examine possible sublethal effects [16]. Models and regulatory guidelines suggest that OSPW contains 14 metals that are considered a risk to aquatic life [1]. However, these guidelines fail to assess how these metals may interact in a mixture similar to OSPW [16]. The possible physiological effects of a mixture of the metals and salts in concentrations similar to OSPW needs to be assessed.

2.1 Complexity of for eco-physiological investigations

Toxicants are investigated to evaluate their lethality or their ability to affect homeostasis [51]. Proving evidence for the former is relatively easy, but the latter is not. It is not always true that a shift in physiology will eventually lead to death since the regulation of homeostasis often incorporates many redundancies [52]. Also, biologically significant changes in physiology and statistically significant changes in a biological endpoint are not always equivalent [52].

Conversely, organisms in the environment are sometimes more sensitive to stressors than they are in a lab [53]. Ideally, scientists should design experiments that make results in the lab congruent with circumstances in the environment.

2.2 Swim performance

Swimming is an essential behavior shared by almost all fish [54]. The ability to swim is integral for foraging, avoiding predators and finding mates [54]. Physiologists experimentally subdivide swimming into two categories: anaerobic (fatiguing exercise) and aerobic (sustained exercise) [54]. Anaerobic swimming activities can be used to capture prey and to avoid predators, i.e., high acceleration, low duration activities [55]. Aerobic swimming best describes basal activity and migration, i.e., slow acceleration, prolonged duration activities [54]. Brett (1964) created an incremental step test (U_{crit}) in a swim tunnel flume that evaluates aerobic swimming ability in salmon [56]. Since then, U_{crit} has been used to predict factors that impact salmon aerobic swimming and therefore migration to spawning grounds [54,56–59].

The physiological processes driving locomotion are complex. In rainbow trout, red (aerobic) and white (anaerobic) muscle work in tandem to propel a fish through the water [60,61]. These muscles groups partition the work of locomotion under different conditions [60,61]. In a flume, fish fatigue occurs in one of two ways: either muscles are unable to produce enough force to match the work ($\omega=F\times D$) required to swim, or there is insufficient energy to fuel the muscles for continued swimming [62,63]. The former is governed by fish size, muscle composition, neuron-electrochemistry, and hydrodynamics; while a later are governed by a host of cardiovascular and metabolic processes [62–64]. In rainbow trout, the metabolic rate often scales with aerobic swimming speeds [62,63]. Under aerobic conditions, oxygen and glycogens/fatty acids are wholly hydrolyzed into ATP and creatine-phosphate to power locomotion [62,63]. The rate of oxygen consumption (MO_2) is often synonymous with whole body metabolic activity since the rate of oxygen consumption is primarily correlated with the sum of all metabolic pathways, although this may not always be the case [65]. In flumes at low speeds, metabolites in muscles are plentiful and MO_2 closely relates to the energy consumption [65]. If sufficient quantities of oxygen are not transported to the muscles, heart, and brain, then fatigue will set in, and the fish will stop swimming. In addition to MO_2, other processes involved

in the transport of metabolites or processes within locomotor muscles can lead to fatigue and halt locomotion within a flume.

The muscle composition (relative amount of red and white muscle) under certain conditions can be the rate-limiting factor and can contribute to fatigue. As a fish approaches U_{crit}, a higher proportion of white muscles are used in propulsion however below 80% U_{crit} the muscles involved in propulsion are almost exclusively red muscles [61,62]. Red muscles are less capable of producing high forces due to the slower enzyme kinetics (V_{max}) and lower fiber density [63,66]. White muscle contraction cannot be sustained over long periods and eventual fatigue will occur. Unlike red muscles, white muscle does not completely metabolize glycogen or ketones into CO_2 [63]. The end product of metabolism in white muscle is lactic acid (lactate and H_{met}^{+}) [63]. Although lactate is mostly constrained to white muscle, some of it can be circulated [67]. The majority of lactate stays localized in white muscle until 8-24 h after fatigue; this occurs because lactate is likely converted back into glycogen stores locally by red muscle [67]. Lactate accumulation results in acidosis of the blood and muscle tissue [68]. Acidosis impairs muscle contraction by inhibiting enzymes and channels required for muscle contraction [66]. Acidosis may also cause red blood cells (RBCs) to decrease their binding affinity of oxygen, referred to as the 'root effect'. The root effect could significantly deplete the oxygen available downstream for muscles and more significantly, the heart. However, to reduce the binding affinity of hemoglobin for oxygen, the RBCs pH has to decline, and some studies have found the opposite during burst exercise fish [69]. Catecholamine has been shown to be responsible for RBCs swelling (pH increase) as well as a rise in RBC concentrations (H_{ct}) and may play an important role in prolonging swimming as the white muscle starts to become an essential factor in locomotion [69].

Maximum cardiac output could also be a significant component in limiting aerobic exercise [70]. At the transition between aerobic and anaerobic swimming (~70% U_{crit}), muscle oxygen partial pressure does not appear to change as fatigue approaches [70]. However, blood circulation is on a single closed loop, and the heart receives the most deoxygenated blood. Increased white muscle involvement in swimming may be a way of adjusting body oxygen budget to prevent heart hypoxia and atrophy [70]. Cardiac muscles are increasingly vulnerable to becoming hypoxic as locomotor activity intensifies [70]. Experiments have consistently found that maximum metabolic rate, cardiac output, and gate transition all occur around the same time

[71]. However, the physiological and behavioral processes governing when, how and where fatigue occurs are still not well understood [67]. In all of these rate-limiting processes, fish often cease locomotion before complete exhaustion, which reduces the recovery period [67].

In freshwater environments, loss of electrolytes occurs during exercise due to the increase in perfusion of blood across the gills [68]. The loss of plasma osmolality is a cost of increasing cardiac output from exercise. The loss of plasma osmolality can cause profound osmotic stress on various cardiovascular and muscle fiber enzyme kinetics [68]. Glomerular filtration rate in the kidney often increases to compensate for the increased water uptake across the gills, but the process is often unable to keep up with hydromineral flux (sum of osmotic and ionic flux) [68]. Thus, increases in electrolyte loss and water absorption can exacerbate various locomotor processes and can be rate limiting.

As previously discussed, metals and salts can impair various physiological processes in fish, and if homeostasis is impaired substantially, swimming ability may also be affected. However, there is also a high degree of redundancy in processes modulating swim performance. Thus only large stressors may have the potential to decrease U_{crit} [70]. Metabolic and ionoregulatory processes play a pivotal role in both swim performance and metal exposure [62]. Although never directly validated, a small decrease in swim performance in rainbow trout could prevent them from being able to swim to spawning grounds or migrate to new areas [72,73]. Therefore, metal exposure could significantly affect fitness without causing lethality [74]. However, other examples of where changes in swimming ability directly impact fitness are scarce [72]. Little is known about how differences in U_{crit} may impact foraging or avoiding predators in the environment [72,75].

2.3 Metabolic scope

Metabolic processes within fish have received considerable focus. Despite the vast diversity of physiological, cellular and biomolecular processes within animals, whole organism metabolic rate is highly conserved [76]. Regardless of life history or ancestor, metabolic rates are scalable across all Teleosts based on mass and temperature [76]. The aerobic scope of a fish defines the range of metabolic rates available to them. Factors that decreased aerobic scope can have substantial implications for aerobic activities (movement, feeding, growth, and

reproduction) [77]. Stressors (high temperature, hypoxia, salinity, and toxicants) either marginalize aerobic scope by adding an energetic debt on top of the regular physiological function or by limiting the transport of metabolites [57,62].

Osmoregulation is an incredibly expensive process for Teleosts. Depending on the salinity, osmoregulation across the gills can constitute between 4-20% of whole-body metabolic rate [78,79]. Although salmon have adaptive strategies to deal with changes in salinity, there are two possible metabolic consequences of coping with salinity. Firstly, fish may increase the expression of proteins used in osmoregulation; these processes require energy to maintain and can constitute a substantial metabolic cost [24,62,78]. This metabolic cost could cause an increase in metabolic rate under low swimming activity. Alternatively, the permeability of the gills could decrease to limit the movement of ions and water, but this would also decrease gas diffusion [24,62]. An increase in gill boundary layer may not change MO_2 at low activity but at high activity, may decrease MO_2. Which of these consequences are likely to occur are usually dose and species dependent [24,79]. An alternative strategy to these are that fish could tolerate the changes by adjusting their internal osmotic and ionic environment [24,79]. Although this would not directly impact MO_2, it may impact the kinetics of osmotically-sensitive enzyme pathways and associated physiological processes [24,79]. The energetic costs associated with adapting to changing osmotic environments can be considerable and can alter metabolic demand [80].

Various metals such as Al, Cu, Ni, and Zn also may negativity affect aerobic scope [81–84]. These metals may cause an energetic debt, which raises metabolic rate under specific physical and chemical conditions [62]. These metabolic effects can be transient or permanent, dose and exposure period dependent, and are often species-specific [62,85].

2.4 Cost of Transport

Locomotion has a high energetic cost, but swimming is a surprisingly efficient mode of locomotion [86]. The physical work done in locomotion can be described as a force carried out over a given distance ($\omega = F \times D$). Muscles must both contract and relax to displace a fish forward. As water speeds increase, the work required increases disproportionally due to drag [87,88]. As a result, the energy required to continue exercise also increases [87,88]. At low swimming speeds,

red muscles and other physiological processes share oxygen relatively evenly since exercise is mild [88,89]. Thus, locomotor work (as described as the cost of transport) is not very efficient at low speeds. As swimming intensity increases, blood perfusion over red muscles increases and blood is shunted away from other organs [88,89]. Therefore, the efficiency of energy transfer into forwarding motion increases [88,89]. At the transition from aerobic to anaerobic energy, efficiency decreases since red muscles primarily use pyruvate, while white uses glycogenolysis [89]. Thus, the cost of transport (COT) often has a parabolic shape in respirometry experiments [87,90]. Factors such as temperature have been shown to change both the optimal speed and COT [87]. As expected, this should have implications for migration in salmon because optimizing energy reserves is paramount since salmon do not feed during migration [91]. Although not validated, it theoretically suggests that the cost of transport should pay a pivotal role in the movement and distribution of fish within an ecosystem [87,91].

2.5 Relevance

Many individual metals in OSPW are in concentrations above CCME guidelines; however, lab mixtures as complex as OSPW are rarely examined. If tailing ponds are to be discharged into local waters, we must have a strong understanding of the toxic potential of the inorganic fraction of OSPW. Discharging OSPW will likely occur intermittently from spring until late summer. It is crucial to determine how acute exposure of OSPW will influence salmonids, given their importance to the ecosystem and local communities. I chose to investigate whether metal and salt mixtures similar to those found in OSPW decreased swim performance and impacted aerobic scope in rainbow trout.

2.6 Methods

Animal housing

Juvenile rainbow trout (48-329g; 14-29.4 cm) were housed and cared for by Science Animal Support Services (SASS) at the University of Alberta aquatics facility and hatchery technicians at Sam Livingston Hatchery. Fish were housed in 40L tanks held at 13 ± 1 C^{o} during 96 hr exposure with 14hr light: 10 hr dark photoperiod. Fish were fed once daily with 3 mm

floating fish pellets (Skretting; Canada) when not being experimentally manipulated, and treatment trials and fish selection were made randomly. Tank water was refreshed 48 hr into exposures. Fish were fasted for 24 hr prior to exposure and fish that were housed together during exposure that engaged in conspecific aggression were excluded from analysis (n=11).

Metal and salt exposure

Metal and salt mixture compositions are shown in Table 1. Stock solutions of aluminium chloride, calcium chloride dihydrate, copper sulfate, nickel chloride, magnesium chloride heptahydrate, sodium chloride and zinc sulfate (Sigma; Ontario) were dissolved in 1 L of milli-Q water. These stock solutions were diluted in 40 L of dechlorinated city water (University of Alberta aquatics facility) or well water (Sam Livingston Hatchery). The ingredients for metal and salt (M&S) exposures groups were: aluminium chloride, calcium chloride dihydrate, copper sulfate, nickel chloride, magnesium chloride heptahydrate, sodium chloride and zinc sulfate. The only ingredient added to the Cu control was copper sulfate, and similarly, the only ingredient added to the NaCl control was sodium chloride. Lastly, an aerobic positive control, pentachlorophenol (PCP; 10 mg/l), was made in a tinted 1 L bottle with sodium hydroxide (2 g/l). Once diluted, exposure solutions were given 24 hr to equilibrate before fish were exposed.

Respirometry procedure

Fish were weighed ($\pm$1g), then placed in a 10 L swim tunnel respirometer ('flume'; Loligo; Denmark). Fish's body length, depth, and width were measured for subsequent solid-blocking correction. A recirculating tank was used to supply fresh exposure water for the duration of the swim tests. The respirometer water jacket was continuously supplied with water to maintain temperature (13$\pm$1 C^{o}). AutoResp software (Loligo) was responsible for powering and refreshing the flume. A Witrox 1 probe (Loligo) was used to measure oxygen concentrations and temperature in the flume. The AutoResp program was used to calculate metabolic rate (mg O_2/kg hr), as well as maintain flume temperature (13$\pm$1 C^{o}) and oxygen saturation (>80% air saturation). Fish were swam in their respective exposures solutions. In brief, metabolic rate was calculated according to equation (1), were $[O_2]t_o$ was the oxygen concentration at beginning of

measurement, $[O_2]t_1$ was the oxygen concentration at the end of measurement, BW represents mass of fish, V represented the volume of water of flume, and t represented time of measurement. For determining the fish volume of the respirometer, it was assumed that the density of fish tissue was $1 g/cm^3$. A solid-blocking correction was used to correct the water speed according to Bell and Terhune (1970). This correction was calculated according to equation (2), where L represents fish length, r represents fish radius, FSA represents the fish squared area, and C represented the cross areas of swim tunnels working section.

Fish were allowed to acclimate in the flume at low speeds (0.3 body lengths (bl/s)) for at least 30 min. The first swim test trial consisted of a ramp U_{crit} test (as described by Jain et al, (1997) [92]) and was administered with step heights of 0.3 bl/s. The equation for U_{crit} is described in equation (3), where U_i is the last fully completed step of the test, U_f is the speed at which the fish fatigued, T_i is the step length (time) of the test, and T_f the length of time from start of last step till fish fatigue. In brief, step heights increased every 5 min for the first six steps, while all proceeding steps were 20 min until fatigue. Fatigue was defined as 10 s of unmotivated swimming despite 5V electrical stimulation on the rear gate. After fatigue, fish were allowed to recover at low water speed (0.4 bl/s). After 30 min of recovery, a second ramp U_{crit} test was administered with a step height of 0.4 bl/s. After fatigue, fish were allowed 15 min to recover at which point fish were taken out of flume and put into the exposure tank. The fractional aerobic scope was calculated by dividing the maximum MO_2 during the swim by minimum MO_2. The equation for cost of transport (COT mgO_2/kg m) as defined in equation 4, MO_{2i} is the MO_2 at any given time i in swim test and U, is the water speed (cm/s) at any given time I in the swim test. The optimum swimming speed (U_{opt}) was calculated to find the corresponding water speed where COT was at its minimum.

Approximately 96 hr after being housed in the exposure tanks, fish were reweighed and put into the flume. Swim performance procedures were repeated (swim trials 3 and 4). Afterward, fish were euthanized in TMS (2 g/l buffered 1:1 sodium bicarbonate), weighed and dissected. The second gill arch on the right-hand side was extracted and stored in 10% formalin solution for gill histology. Small hematocrit (Hct) tubes were dipped in severed arches to extract blood. Tubes were centrifuged for 15 min at 1000 rpm, and the red blood cell layer was considered as a percent of the total blood volume in the tube.

Gill filaments, liver and muscle tissues were removed and flash-frozen in liquid nitrogen (University of Alberta) or dry ice (Sam Livingston Hatchery) for later processing. For swim trials 1 and 2 at 0 hr, and swim trials 3 and 4 at 96hr, U_{crit}, MO_2 minimum, MO_2 maximum, U_{opt}, and COT minimum was collected for each fish and determined.

Equation (1): $MO_2=([O_2]t_o-[O_2]t_1)\ V/(t\ BW)$

Equation (2): $Fractional\ error = 0.8 \times 0.5(F/r) \times (FSA/TS) \times {}^{3}/_{2}$

Equation (3): $Ucrit = Ui + \left[Uf\ ({}^{tf}/_{ti})\right]$

Equation (3): $COT = \left[{}^{MO_{2i}}/_{Ui}\right] * 36$

Statistical analysis

Data was analyzed in SigmaPlot (Systat; California), using a one-factor (swim trial) repeated measures analysis of variance (ANOVA) to compare each of the four endpoints: U_{crit}, aerobic scope, $COT_{min,}$ and U_{opt}. Statistical analysis was divided into two groups, one with my control and the three M&S exposures and the other with my control as well as the PCP, NaCl and Cu exposure groups (positive controls). An additional exposure group, low Cu, was excluded from analysis due to low n value (n=3). A Holm Sidak post-hoc test was used to determine differences between exposures, swim trial, and the interaction between these factors. A Pearson product test was used to determine the correlation between U_{crit}/U_{opt} and fish length as well as COT_{min} and aerobic scope and weight to determine if length and weight were important covariates. An alpha level of 0.05 was used for all tests.

2.7 Results

There were differences in swim performance after exposure in some of my exposure groups. The difference in swim performance did not owe to fish size as body length (cm), and body mass did not differ between exposure groups. A Pearson product analysis revealed that none of the factors investigated (U_{crit}, aerobic scope, COT_{min}, and U_{opt}) co-varied significantly with weight and length ($p>0.05$), nor did the residuals of U_{crit}, aerobic scope, COT_{min}, and U_{opt}

against weight and length change the results of repeated measure ANOVA. Also, blood Hct was similar between all exposure groups (H=2.160, df=6,p=0.904, fig 1). Even though I found that there were differences in U_{crit} between swim trials, it was not the same for all of the exposures ($F_{9,72}$=4.063, p<0.001;fig 2). The medium M&S exposure reduced swim performance (swim trial one vs.4 t=4.308, p<0.001; swim trial one vs.3 t=2.753, p=0.022; swim trial two vs.3 t=3.353, p=0.005; swim trial two vs.4 t=4.966, p<0.001; fig 2). There were differences in repeated swim trials the U_{crit} of positive control exposure groups comparison but not due to exposure ($F_{3,63}$=3.808, p=0.021; $F_{9,63}$=1.341, p=0.235; fig 3). However, a post-hoc test could not determine differences in repeated trials (swim trial one vs.2 t=0.0546, p=0.957,swim trial one vs.4 t=1.903, p<0.062; swim trial one vs.3 t=2.399, p=0.019; swim trial two vs.3 t=2.245, p=0.028; swim trial two vs.4 t=1.820, p<0.074; swim trial three vs.4 t=0.189, p=0.850 fig 3).

As with swim performance, there the was an interaction between exposure and repeated swim performance ($F_{9,67}$=2.112, p=0.040). The post hoc test revealed that for control and high M&S exposure, their first swim trial was slower than the fourth swim trial (t=2.738, p=0.047;t=2.726, p=0.048, fig 4).

The aerobic scope increased by 34±0.5% and 37±1.2% for control and high M&S exposures between the first and last swim. There were differences in repeated swim trials in the aerobic scope of positive control exposure groups, but it was not due to exposure ($F_{3,60}$=5.658, p=0.002; $F_{9,60}$=232, p=0874; fig 5). The first swim trial had a smaller aerobic scope than swim trial two, and swim trial four was larger than swim trial one (swim trial one vs.2 t=3.573, p<0.001, swim trial one vs.4 t=2.771, p<0.007; fig 5).

The COT_{min} was not different between exposures ($F_{3,70}$=2.381, p=0.087), but there was a difference in COT_{min} between repeated swim trials ($F_{3,70}$=5.101, p=0.003). The post-hoc test revealed that swim trial one was larger than swim trial three and 4 (t=3.534,p=0.004; t=2,957, p=0.021;fig 6). Swim trial three and 4 were 15±1% and 17±1% lower than swim trial one. For the positive control exposure comparison, there was a change COT_{min} in the repeated swim trial and an interaction between repeated swim trials and exposure group ($F_{3,62}$=4.868, p=0.007; $F_{9,62}$=3.652, p=0.001; fig 7). Specifically, the control exposure group control trial 1 and 2 were both higher than trial 3 (swim trial one vs.3 t=2.893, p=0.005; swim trial two vs.3 t=3.282, p=0.002;fig 7). Also, the NaCl exposure group swim trial one and 2 were higher than swim trial

three and 4 (swim trial one vs.4 $t=3.602$, $p<0.001$; swim trial one vs.3 $t=3.326$, $p=0.001$; swim trial two vs.4 $t=2.594$, $p=0.012$; fig 7).

The U_{opt} changed in swim trials ($F_{3,70}=3.199$, $p=0.029$), but none of these changes were due the exposure ($F_{3,70}=0.840$,$p=0.482$). The post hoc test revealed that the fourth swim trial U_{opt} was lower than first swim trial ($t=3.054$, $p=0.019$; fig 8). The fourth swim trial was 16±1% lower than the first swim trial. The positive control exposure groups did not differ in U_{opt} ($F_{3,62}=0.810$, $p=0.497$) or swim trials ($F_{3,62}=1.232$, $p=0.306$; fig 9).

2.8 Discussion

The toxicity of the inorganic fraction of OSPW could be in part due to the high concentrations of various metals. However, salts played an essential role in mitigating toxicity. Since rainbow trout are highly tolerant to salts and salts often limit metal absorption, and valent state [16,80], both low and high M&S exposures did appear to affect the fish. The medium M&S exposure caused the most substantial change in swim performance. Although the inorganic fraction of OSPW is less toxic than the organic fraction to fish, there may be some effects due to exposure [2]. However, mass spectrometry revealed that the exposure groups did not contain the concentrations of metals and salts I initially intended (table 1).

Overall, exposures were more dilute than intended. Also, aluminum and zinc concentrations present in medium M&S were lower than low M&S exposure, thus metal and salt mixtures were not increments of the stock solution. The total amount of salts (the concentration of Na, Mg, and Ca together) present in low, medium, and high M&S exposure groups were approximately equivalent to 44±1.0 %, 74±14%, and 202±6.1%% of the maximum total salt concentrations in OSPW. The total amount of metals (the concentration of Al, Cu, Ni, Mo, and Zn) present in low, medium and high M&S exposure groups were approximately equivalent to 23±1.0 %, 44±2.5%, and 104±1.4 % the maximum metal concentrations measured in OSPW. However the total amount of Ni, Zn, and Cu present in low, medium, and high M&S exposure groups were approximately equivalent to 2.8±1.0 %, 3.0±0.4%, and 6.4±0.9 % the maximum concentrations of those metals measured in OSPW. Overall, the proportion of salts in mixtures was elevated. Metals present in low concentration in control and NaCl exposures were likely due to cross contamination due in part to the experimental plastic tanks. Despite these issues, the

water chemistry indicated the mixtures were substantially different from one another and therefore could have caused differing physiological effects.

Scientists have used swim performance as a tool to test the toxicity of many compounds. However, few studies have reported changes in swim performance in rainbow trout [71]. In this study, a 25±3% decline in mean swim performance occurred as a result of medium M&S exposure (fig 2). However, the other exposure groups were unchanged (fig 3). NaCl and the low M&S mixture had similar amounts of Na, Mg, Ca, Cu, TDS and pH, but low M&S had 28× the amount of total metals (table 1). Despite this, there was no change in swim performance in either of these exposure groups. The low M&S mixture had approximately half the total salts and metals as medium M&S, but low M&S mixture was not associated with declines in swim performance. Elevated concentrations of Al, Cu, Ni, Mo, and Zn can impair swim performance as long as other mitigating parameters are absent. The medium M&S mixture contained 12 times of the total salts and 112 times the amount of total metals present in control exposure water. Whereas, OSPW contained 1.7 times the amount of total salts and 183 times the amount of total metals present in medium M&S exposure water (table 1). Furthermore, the high M&S exposure had 40 times the total amount of salts and 296 times the total amount of metal that of control water (table 1). It appears that sufficiently high concentration of salts might have mitigated any toxic effects. Thus, dose-dependence was not demonstrated due to the specific chemical makeup of metal and salt solutions. Time constraints prevented the evaluation of the NaCl control from medium M&S mixtures, and thus the interaction between salts and metals in medium M&S cannot be adequately assessed. A solution with an osmolarity of 59.8±8.94 mOsm containing Al (1.17±1.06 µmol/l), Cu (0.28±0.03 µmol/l), Mo (20.3±1.13 µmol/l), Ni (0.56±0.02 µmol/l), and Zn (0.28±0.10 µmol/l) was able to decrease swim performance in rainbow trout while other solutions including PCP were not.

Previous work has shown that attributing specific metal constituents to declines in swim performance is difficult [63,93]. Another study found that chronic copper exposure (75 µg/l) caused a 12% decrease in swim performance [94]. Metals such as Ni may have also reduced swim performance in rainbow trout (42%), but over a much more extended exposure period (12 days) and at 30× the concentrations found in OSPW [82]. Other toxicants such as ammonia (4.9 mg/l) can reduce swim performance by 27% [95]. Since fish were housed in static-renewal design at a relatively high density (>5 g/l), it is possible ammonia may have played a role in

reducing swim performance. OPSW also has high in ammonia (1-65 mg/l) and could play a possible role in its toxicity. However, ammonia was below detection limits in water samples, except in Cu exposure (table 1) [18]. Brackish water (salinity 30%) alone failed to change swim performance in rainbow trout as well, underscoring the high osmotic tolerance of the species [96,97]. Despite these complications, exposures were able to detect a decrease swim performance.

PCP was intended to be used as a way to decrease swim performance but was unable to change swim performance effectively. Perhaps the low solubility of PCP in hard water and its high lethality (LC50%) caused high variability in results. The protocol outlined by Farrell et al. (1998) was used, but it caused mortality [98]. Therefore, the solution was diluted to 10 µg/l, but the exposed fish did not have an impaired swim performance. The low solubility of PCP in hard water, as well as its photoactivity, may have been responsible the lack of an effect of this toxicant. High copper in isolation from other metals and salts found in OSPW was also intended to be used as a positive control. Although the average swim performance declined by 16±0.05% (9.0±0.4 cm slower), low statistical power prevents a strong assertion. The amount of copper present in the Cu exposure was similar to medium M&S exposure, but again, the predicted concentration of metal is rarely identical to measured concentrations.

The metabolic data accompanying swim performance generally follows trends in swim performance; however; the lack of change in aerobic scope in medium M&S exposure was unanticipated. Conversely, aerobic scope increased in control and high M&S exposure between the initial and last swim experimented. It would appear that training could have a substantial effect on the aerobic scope. Other studies have found that training chinook salmon (*Oncorhynchus tshawytscha*) increased fractional aerobic scope by 28% without increasing U_{crit} [97]. In the present study, the average aerobic scope increased by 53% in control exposure, but this was mainly due to declines in routine MO_2 after training, while Gallaugher et al. (2001) found a 50% increase in the maximum MO_2, while routine MO_2 was unchanged between trained and untrained fish [97].

It was unusual to find that aerobic scope increased the most in high M&S treated fish and that this increase only appeared after the second swim post-exposure. Typically immersion in solutions that are more osmotically similar to blood plasma should allow for the greater scope of metabolic activity [99,100]. Due to experimental limitations, plasma osmolality could not be

assessed after exposure, but the literature suggests that the concentration of solutes in rainbow trout plasma is typically around 280-340mOsm/kg [101,102]. The total osmolality of high M&S exposure is probably most similar to the osmolality of rainbow trout (table 1). The positive controls all appear to be similar, swim trials two and four had higher aerobic scope than swim trial one. Again, this was probably due to exercise training. This apparent increase in scope could also be an artifact of elevated routine metabolic rates during acclimation in the first swim trial [91,103]. The range of metabolic activities available to these fish was unchanged by exposure; however, this was curious because medium M&S exposure group had a substantial decrease in swim performance.

Studies have shown that a decrease in aerobic scope often corresponded with a decrease in swim performance [62]. An approximate 30% reduction in gill area decreased swim performance 24±4%, highlighting the importance of oxygen uptake [104]. A connection between swim performance and aerobic scope is assumed, and metals are known to limit oxygen delivery (e.g., via gill deformation) or add energetic debt which could limit exercise performance [29,62,82,83]. Furthermore, rainbow trout change their swimming activity to compensate for dietary copper exposure [105]. However, the oxygen debt/delivery paradigm may be incomplete.

In a study by Beaumont, Butler, and Taylor (2003), copper exposed brown trout had similar metabolic rates and cardiac output as control fish during any given activity level in a swim test. In their study, the U_{crit} values of copper exposed brown trout (*Salmo trutta*) was 1/3 that of the control group, thus the maximum MO_2 was less, but whether declining aerobic scope was a contributing factor or if swim performance was responsible for the decreased aerobic scope was unclear [29]. The study suggested that hyperammonaemia caused by copper induced loss of osmoregulation of Na and Cl, and this may be responsible for the reduction in U_{crit} [29]. Cu exposure in my study was associated with an elevated concentration of ammonia, but no other exposure group had concentrations of ammonia above the detection limit (table 1).

Few studies have investigated the effects of stressors on the COT in fish. In control and high M&S exposure, the COT_{min} decreased by 0.77 calories and 0.75 calories between swim trial one and three and between swim trial one and four. Thus, repeated exercise appears to result in more efficient swimming, and fish were not adversely affected by high M&S exposure (for COT). When comparing the control exposures to the positive controls, however, NaCl treated fish's COT_{min} increased by 0.97 calories from swim trial one to four. It would appear that a loss

of work efficiency may be due to an energetic debt incurred during exposure to an osmotic stressor (20 mmol/l; table 1) [29,31]. Conversely, Rao (1971) found that rainbow trout exposed to approximately 3.5% seawater (35 mmol/l) had a lower COT_{min} (0.35 calories less than control) than fish exposed to freshwater. Previous work has found that swimming efficiency may correspond with differences in osmotic gradients between the fish and the environment [96,99,100]. The increase in the COT in NaCl-exposed fish was likely due to the cost associated to acclimating to a new osmotic environment, while fish in Rao (1971) had sufficient time to acclimate before they entered the respirometer. Although NaCl exposure did not affect U_{crit}, it appears that there was some metabolic cost associated with salt exposure. Exposure did decrease the U_{opt} between the first and last swim trial for M&S and control fish, while no change occurred in positive controls. The decline in U_{opt} appears to be related to exercise training. A reduction in metabolic rate in the middle of a swim trial would shift the U_{opt} to a lower water speed. Whether U_{opt} corresponds with swimming in the environment has not been investigated for practical reasons [87].

Several possible mechanisms could explain the observed effects in medium M&S that are unrelated to exposure. Sampling bias and conspecific aggression could be confounding variables. Hyperammonemia has been shown to cause a decline in swim performance in rainbow trout [81]. Given that fish had high experimental housing density (10.1±0.7 g/l), ammonia accumulation in static-renewal tanks could have occurred. However, despite complications, I am confident that the medium M&S exposure caused a significant decrease in swim performance in rainbow trout.

Although metals and salts can decrease swim performance, the mechanism of action is far from fully understood. It is possible that sodium loss could impede cardiovascular function during exercise medium M&S exposure [91,106,107]. Various metals can impact ionoregulation in rainbow trout, and it follows that low plasma sodium could impact U_{crit} independently of oxygen delivery. Other studies have shown that Cu can cause a decline in swim performance without impacting aerobic scope [108]. The loss of plasma Na, K and Ca from disrupted ionoregulation and the subsequent rise in plasma proteins and hemoglobin could impact the viscosity of the blood and impair oxygen transport to red muscle [109]. Impaired oxygen delivery can interfere with muscle and nerve function, which would cause a lower contractile force of skeletal muscle [110]. Elevated concentrations of ammonia were found in Cu exposure groups, but not the medium M&S exposure group (table 1). Aside from Hct, no other

hematological data was collected. As a result, an osmoregulatory-related dysfunction in swim performance observed in the medium M&S treated fish is speculative. Rainbow trout are tolerant to salts, but other fish species endemic to the Athabasca region may not be [20,111]. It is clear that further investigation into the possible toxicity of the inorganic fraction of OSPW is needed.

An essential question for this investigation is whether my mixture of salts and metals was a good approximation for the inorganic fraction of OSPW [63,112]. In general, the ability to discern differences between effluents and lab created approximations should be dependent upon the sensitivity and precision of the physiological measurement [85]. Even moderately sick, as defined by the Environmental Protection Agency (EPA) guidelines for qualitatively assessing fish health, salmon have been shown to have repeatable U_{crit} in various environmental conditions [57]. I cannot claim that fish given OSPW exposure will behave the same as my M&S treated fish, but given that metals and salts found in my mixtures exist in similar concentrations to those documented at various tailing ponds sites, it is possible that discharging treated OSPW may cause physiological effects on exposed fish.

2.9 Conclusions

I have demonstrated that salts and metals similar to OSPW can alter swim performance and aspects of metabolism in rainbow trout. Fish exposed to a diluted concentration of metals and salts similar to constituents found in OSPW did not behave differently than an approximately equal concentration of sodium chloride. Conversely, specific mixtures of salts and metals can decrease swim performance in rainbow trout. Studies have shown that concentrations of metals and salts tend to fluctuate from year to year in tailing ponds [14]. These fluctuation in water parameters significantly increase the probability that metal may exceed its hazard quotients [14]. As a result, it is not recommended that undiluted OSPW be discharged into the environment [14]. Baseline migration and inter-population movement of all fish in Athabasca river are not fully described but, concentrated treated OPSW could significantly hamper salmonid migration in certain seasons [113].

OSPW remediation poses an interesting toxic question. Currently tailing ponds prevent any appreciable toxic impacts of OSPW on the environment; however, the ponds cannot expand indefinitely. Remediation of the oil sands and being able to discharge OSPW, and/or the creation of potential end pit lakes, offers possible solutions to some of this issue. However, I believe that stakeholders in academia, industry and the government should have a good understanding of the effects of OPSW on the environment. We should be able to make predictions and plan for the effects of OSPW discharge.

3.0 Chapter 3: Effects of metals and salts on gill structure and enzyme dynamics

3.1. Ionic regulation in fish

One of the primary roles of homeostasis is maintaining a constant internal osmotic and ionic gradient between the animal and the environment. However, when the external environmental osmotic and ionic gradient change this may disrupt homeostasis. Regarding fish, some species can deal with a wide range of ionic environments (euryhaline fish), while others cannot (stenohaline fish). In freshwater ecosystems, fish must actively reabsorb salts, while in marine environments fish must excrete salts. Interestingly, marine and freshwater fish try to maintain similar osmotic concentrations, ~300 mOsm/l [24]. If OSPW is discharged, it could pose a substantial osmotic stress to aquatic organisms and could alter their internal osmolarity. The total dissolved solids in OSPW alone may be more than some species can withstand (e.g., 1340 mg/l) [114]. However, some euryhaline fish may possess a physiological mechanism to adapt to this osmotic disturbance. Understanding the cost associated with maintaining ionic balance within euryhaline fish, such as rainbow trout, could provide useful insight into the possible risks associated with discharging OSPW in local tributaries.

3.2. Gill structure and morphology

The gills are a highly vascularized respiratory and excretory tissue that is essential in regulating ion homeostasis. Gills have a high surface area, allowing for active gas and ion exchange between the blood and the environment [112]. Lamellae, large evaginations of respiratory tissue along the gill arch, are the site of gas and ion transport in gills [112]. These lamellae are a recursive structure, which is composed of primary lamellae which branch off gill arch and secondary lamellae which are branched off primary lamellae. Blood moves through the secondary lamellae in a counter-current exchange to maximize gas and ion exchange [112]. Ions and water diffuse between (paracellular) and across (transcellular) the epithelium in gills [79,115]. Tight junctions between epithelial cells can halt the movement of ions but are far from impenetrable [79,115]. In fish gills, there are four cell types important in the maintains of ionic homeostasis: pillar cells, accessory cells, pavement cells and mitochondria-rich cells (MRCs) [22,79,116]. Pillar cells modulate the capillary sinus from the overlying epithelium on the

secondary lamellae [116]. Accessory cells secrete mucus to prevent epithelial damage as well as protect against pathogens [116]. Pavement cells hold the structure of gill together as well as maintain a barrier between the gill and the surrounding environment [116]. High numbers of mitochondria within MRCs mediate the transport various compounds in and out of the gills [116].

Gills are very morphologically and cellularly complex and dynamic. Gills are often examined in effluent exposure experiments to measure how toxicants impact gill morphology and morphometrics[5,6,117]. OSPW has been shown to affect gills structure and prevalence of lesions [6]. These structural changes and lesions may disrupt respiratory and ionic homeostasis, but only a few studies have examined these effects beyond histology [5,7]. Additionally, determining whether changes in gill structure are permanent and transient is not well understood [118].

Gills constitute the majority of the surface area in contact with water [119]. This organ is re-modeled in response to environmental changes to maintain gas, ion, and excretory homeostasis, and can be impacted by toxic effluents [120]. Various effluents have been known to cause lesions such as epithelial lifting of lamellae, necrosis of pavement and chloride cells, epithelial swelling, aneurysm and epithelial ruptures, and lamellar fusion [22]. These anatomical pathologies can be used to help diagnose toxicity since these observations can be an indicator of impaired cell functionality [23]. These pathologies can be used to identify cellular processes that metals exposure may impact. Distinguishing between toxicity and acclimation is difficult from a histological perspective [121]. Changes in gill anatomy and structure (morphometrics) have been used to investigate the effects OPSW on fish [5,6]. Gill morphometrics could be a useful tool to determine the effects of complex inorganic effluents, like the inorganic fraction of OSPW, on fish respiration and ion regulation.

3.3. Enzyme dynamics

Sodium potassium ATPase (NKA) and carbonic anhydrase (CA) are essential proteins in secondary active transport in various types MCRs, accessory cells and pavement cells [79,116,122]. In gills, NKA is prevalent in the basolateral membrane of MCRs and pavement cells [79]. NKA uses ATP to transport sodium (Na) extracellularly and potassium (K) into the

cytosol, the resulting ionic gradient facilitates the transport across and between cells [116]. CA is found in the cytosol and epithelium in the gills and catalyzes the interconversion of carbon dioxide and water into a proton and bicarbonate [122,123]. The products of this reaction facilitate the electroneutral transport of specific ions across the cell membrane [116]. Ion channels are present in the apical and basolateral membrane to allow iontransport. Although many ion channels are involved in transcellular transportation, they are often difficult to quantify in gill tissue and vary between species [124]. Studies have correlated changes in expression of NKA and CA with salt and fresh water adaptation [79,116]. Since these proteins are readily measurable and central to ion transport, they are often used as biomarkers of salt water exposure [125].

3.4. Connection between gills and exercise physiology

Investigating how metals and salts cause gill dysfunction may give us a better understanding of the effects observed in the previous chapter. My previous experiments have indicated that certain concentrations of metals and salts (medium M&S treatment) may impede swim performance in rainbow trout [62]. The gills are a primary target of investigation and any alteration observed in gill functioning could be associated with a decline in swim performance In the previous chapter, a normal dose-response in respirometry data was not evident thus an in-depth analysis of water chemistry was required to ensure that treatment groups were accurate. There are gaps in knowledge that must be investigated if we are to understand the possible toxic effects of OSPW inorganic fraction-based mixtures.

3.5. Methods

Gill histology

Gill tissues were dehydrated in 70%, 90% 100% ethanol, toluene, then fixed in wax. Gills were sectioned on Leica RM2125 RTS micrometer (Leica; United States) in 5 µm sections and stained with hematoxylin eosin stain at the University of Alberta Advanced microscopy facility. Photos were taken of sections at 10× magnification on Zeiss AX10 microscope (Zeiss; Germany) with an Optronics camera (Optronics; United States). ImageJ was used to measure interlamellae

distance (ID), secondary lamellae length (SSL), basal epithelial thickness (BET), and interlamellar cell mass height (ICMH). The proportion of the secondary lamellae available for gas exchange (PAGE) was calculated using equation 4 (below). Gill histopathological alterations were evaluated using a scoring framework developed by Bernet et al. (1999) [121]. In brief, observed morphological alterations (MA_{gill}) were scored on a scale of 0 to 6, and scores were defined as follows: 1 was 10-20%,… 5 was 51-60%, and 6 was >61% prevalence of morphological alternation. The prevalence of chloride cell proliferation, necrosis, epithelial lifting, epithelial atrophy, lamellar tip fusion, mucus cell hypertrophy, aneurysm, oedema, hypertrophy, and hyperplasia were evaluated with examples provided in figure 10. Each morphological alteration had an importance factor (IF) ranging (from 1-3). Hypertrophy, oedema, aneurysm mucous cell hypertrophy, epithelial lifting had IF of 1. Epithelium atrophy, lamellar tip fusion, and epithelium hyperplasia had IF of 2. Lastly, necrosis had an IF of 3, and the pathological index (PI) is described in equation 5. Morphological alternations were identified according to a paper by Mallatt (1985) [22].

Equation (4) PAGE (%) = 100 X (mean SLL / (mean BET + mean SLL))

Equation (5) PI = $\sum MA_{gill} \times IF$

NKA activity

The NKA activity was measured in gill homogenate following a method adapted from Blewett et al., (2015). In brief, gill in buffer (125mM sucrose, 5mM EGTA, 2.4mM sodium deoxycholate) were homogenized with a micro-pestle (Fisher; Ontario).The raw homogenate was centrifuged at 10,000 g for 15 min, and the supernatant was extracted. The difference in absorption (at 340 nm) between ouabain solution and assay solution was used to determine NKA activity. Ouabain solution contained 2.8mM phospho(enol) pyruvic acid, 3.5mM ATP, 0.22mM NADH, 4U/ml L-lactate dehydrogenase, 5U/ml pyruvate kinase, 189mM NaCl,10.5mM magnesium chloride hexahydrate, 42mM potassium chloride, 50mM imidazole, and 0.65mM ouabain. Assay solution contained 2.8mM phospho(enol) pyruvic acid, 3.5mM ATP, 0.22mM NADH, 4U/ml L-lactate dehydrogenase, 5U/ml pyruvate kinase, 189mM NaCl,10.5mM magnesium chloride hexahydrate, 42mM potassium chloride, and 50mM imidazole. Samples,

ouabain, and assay solution were added to 96 well plate, and absorption was measured over 10 minutes. Protein concentration was measured using the Branford method (Sigma; ON) in a plate reader (at 595 nm). Activity was measured as µmol ADP/mg protein/hr, and 4mM ADP standard was used to quantify the amount of ATP samples NKA consumed.

CA activity

The CA activity was measured from gill homogenate following methods adapted from Henry (1991) [123]. Supernatant samples from NKA were put in a scrint bottle in a 4ºC water bath with 7.5ml of CA assay solution along with 1ml of CO_2 saturated water. The CA assay solution contained 225mM mannitol, 75mM sucrose, 10 mM Tris buffer, and 10mM sodium phosphate monobasic. The change in pH was measured over 35 s after the CO_2 solution was added to the scrint bottle. Activity was measured as the change in the pH/mg protein.

Water Chemistry and analysis

Three water samples were collected from each of the water exposures, except for control (n=1) and high M&S exposure (n=4). The water's temperature, specific conductivity (uS/cm ºC), and ORP (mv) were measured with YSI probe (Xylem; Ohio). Alkalinity was calculated by titrating to pH 4.5 with 0.1 N HCl using pH probe (Fisher). Mass spectrometry was collected for Na, Ca, Mg, K, Al, Cu, Ni, Zn and Mo by University of Alberta Canadian Centre for isotopic microanalysis using inductively coupled plasma mass spectrometry (ICP-MS). Water samples were also filtered (4 µm), and NH_4, Cl^-, SO_4^{-2}, NO_2^{-2}, and NO_3^- were analyzed using ion chromatography (IC) in the Gamal El Din lab at the University of Alberta. Visual MINTEQ was used to determine the speciation, and free ion state of metals. Since dissolved organic carbon (DOC) could not be collected from samples and that water either came from a freshwater well or from city water treatment plant DOC was ignored from Visual MINTEQ speciation equilibrium.

Statistical analysis

Data were analyzed in SigmaPlot using a one-way ANOVA to compare histological and enzyme activity data. Data were square root transformed to meet assumptions of equal variance and homogeneity. If these assumptions were not met, an ANOVA on ranks was performed. Dunn's and Holms-Sidak post hoc tests were used for ANOVA on ranks and one-way ANOVA, respectively.

3.6. Results

The gill histology of exposed fish showed that some lesions were present but that there were no differences between treatments. Morphological abnormalities were not different between exposure groups ($F_{6,43}$=1.083, p=0.387, fig 11). The surface areas available for gas exchange, PAGE, was similar between all fish (H=7.558, df=6, p=0.272, fig 12). However low M&S, medium M&S, and high M&S mixtures had thicker ICMH than control, NaCl, Cu, and PCP exposures (H=71.798, df=6, p<0.001; low M&S vs control Q=5.513, p<0.05; medium M&S vs control Q=5.586, p<0.05; high M&S vs control Q=4.895, p<0.05; fig 13). The average ICMH was 6, 7, and 9 μm thicker than control for low M&S, medium M&S, and high M&S treated fish respectively (fig 13).

Carbonic anhydrase activity in Cu exposure was higher than all other exposure groups ($F_{8,57}$=29.133, p<0.001; control vs Cu t=13.710, p<0.001, fig 14). The CA activity in gills of fish in Cu exposure group was 2.5× higher than of fish in the control exposure group. Sodium potassium ATPase was higher in high M&S and NaCl than all other exposure groups ($F_{6,51}$=5.049, p<0.001; control vs high M&S t=3.171, p=0.003; control vs NaCl t=2.942, p=0.005; fig 15). The NKA activity in gills of fish in NaCl and high M&S were nearly twice that of fish in control exposure.

3.7. Discussion

Gills are responsible for various physiological functions and toxicants can impede some functions while not affecting others. In the previous chapter, I found that specific metal and salt mixtures could decrease swim performance but not change aerobic scope. Exposure may have

impacted fish, how this exposure caused the decline in swim performance was not immediately evident. Gills are the primary target of metal and salt exposures. Understanding how these exposures affect gills is a crucial initial step in understanding the mechanism by which metals affect the whole organism.

Gills had few defects and did not differ from unexposed fish at Sam Livingston Hatchery. Also, the gill surface area was not different between exposure groups. Previous work has found that decreasing 30% of gill area by cauterization was required to reduce swim performance in rainbow trout [126]. Thus, substantial changes in gill area are necessary to limit aerobic exercise in rainbow trout. High concentrations of Al in acidic water has been shown to act in this way [83]. A 5 days exposure to 38 µg/l of Ni in acidic water (pH 5.2) decreased gill surface area enough that swim performance was impaired [83]. However, metals such as copper (105 µg/l) have been shown to reduce swim performance without affecting gill area for gas exchange [127]. Thus, if gills are integral to any change in swim performance, then processes related to ionoregulation and waste excretion may be essential aspects to investigate in the future [30]. Overall, my exposures did not cause substantial structural changes in gills, but minor changes were present.

I found that ICMH was higher in metal and salt mixture-exposed fish compared to all other exposures. Similar results were also found in yellow perch (*Perca flavescens*), and goldfish (*Carassius auratus*) exposed to OPSW [5]. In rainbow trout, the proliferation rate of epithelial cells is relatively low and takes approximately eight days for cells to migrate from progenitor at the base of secondary lamellae to the tip [128]. Therefore, the increase in cell height is likely not due to cell proliferation during the 96 hr exposure. Artic grayling (*Thymallus arcticus*) have been shown to be much more sensitive salt stresses in acute exposures (96 hr) [117]. These fish did exhibit a cell proliferation, and given their distribution in the Athabasca river, ICMH may be a useful biomarker of salt exposure in these fish [117]. MCRs and pavement cells are prevalent in the ICMH, and cell swelling could disrupt cell function. Further investigation into the disruption ionoregulation in M&S exposure is recommended.

CA activity is integral in ionoregulation in fish gills. High copper exposure had a strong influence on CA activity. Metal complexing (Zn>Co>Cu>Ni) anions can competitively inhibit CA [129]. Although other exposure groups had a higher dissolved copper concentration (low, medium, and high M&S exposures), all of them had substantially more cations competing with

copper for absorption (fig 16-19). Dissolved Ni^{+2} and Zn^{+2} ions are also capable of inhibiting CA, but only trace amounts were present in Cu exposure group (fig 16-19). Relative to other studies, Cu concentrations were low and the exposure period was short (96 hr). It is possible that the high protein turnover rate in gill epithelia (1.27 mg/hr) was able to elevate CA concentrations during exposure to maintain homeostasis during exposure [130]. However, Ni has been shown to slow protein turnover rate, which may suggest that there was more than one unaccounted for factor causing the rise in CA activity in Cu exposure group [131]. Since CA activity was 2.5× that of control fish, it was likely that gills were engaging in more acid and/or base excretion, which may be why an evaluated ammonia concentration was found in Cu exposure water [122]. Furthermore, relatively high in CA exposure may have occurred to facilitate transcellular transport of ammonia [29,132]. The role of other trace metals such as Zn and Ni cannot be discounted [27]. Typically high concentrations of dissolved copper (0.29-0.36 μmol/l) have been shown to reduce CA activity in both freshwater (0 ppt) and saltwater (25 ppt) fish (*Poecilia vivipara*) [133]. However, Cd, Cu, and Zn have been shown to cause biphasic changes in CA activity in estuarine crab (*Chasmagnathus granulate*) [134]. Given that rainbow trout are very sensitive to Cu and that the dissolved Cu in the Cu exposure was between 10 and 20× less concentrated than the ones used by Zimmer (2012), it is possible the increase in CA activity may have offset the other effects of Cu exposure [134]. Since gill tissue was not washed before freezing possible contamination from blood CA could be an issue [123]. However, multiple aliquots of gill tissue were sampled from the same individual, and the trend likely represents a shift in CA activity due to exposure. If Cu treated fish have difficulties ionoregulating, it was not significant enough to cause declines in swim performance.

Other exposures with higher Cu concentrations (low, medium, high M&S exposures) may have had a lower demand for CA. The Na^{2+} and Cl^{-} gradients in fish in low, medium, high, and NaCl exposure groups were lower than they were in Cu and control exposures groups (table 1). Thus, electroneutral transfer of Na^{2+} and Cl^{-} could have been lower. Also, environmental Na concentrations higher than one mmol/l were previously shown to reduce the rate of Cu uptake across the gills [27]. Thus the absorption of copper in Cu treatment (Na 0.34mmol/l) was likely unaffected by environmental sodium, but in low (Na 15 mmol/l), medium (Na 27 mmol/l) and high(Na 77 mmol/l) copper absorption was likely impeded by environmental sodium I did not determine the metal and salt uptake rates from any of my exposure groups. Nevertheless, it

appears that CA activity in fish exposed to metal and salt mixtures similar to OSPW were not different from control.

NKA activity in NaCl and high M&S exposure groups were higher than those of control, which may suggest that these fish had higher osmoregulatory demand [24]. Although these results are consistent with the literature, it was unusual to see an increase in NKA activity at such low salinity [102]. High M&S exposure had less NKA activity than the NaCl treatment despite having over four times the total osmolarity (160±3.44 mOsm and 38.5±0.50 mOsm). Furthermore, low M&S (35.4±0.57 mOsm) and medium M&S (59.8±8.94 mOsm) exposure NKA activity were similar to control (2.89 mOsm), despite having similar or higher osmolarity to NaCl exposure (38.5±0.52 mOsm). Many of the metals (Al, Cu, and Zn) in my M&S exposures have been shown to inhibit NKA activity, but the quantity of salts in my M&S exposures may also have limited the bioavailability of those metals and thus may have slowed their uptake [27,135,136]. Conversely, salts present in NaCl and high M&S exposure may have increased NKA activity to combat the osmotic stress. Although NKA activity was not elevated relative to the osmolarity of the water in low and medium M&S exposure groups, there may have been an osmoregulatory challenge. It is possible that there was insufficient NKA activity in low and medium M&S treated fish to maintain ionic and osmotic homeostasis. However, without a measurement of plasma or body osmolarity, this cannot be confirmed.

General discussion

The metal and salt mixtures used in this study did not generally impact metabolic rate and swimming performance as I had hypothesized. I did find some specific mixtures of metals and salts could impact swim performance, but the physiological endpoints I examined did not provide me with a specific mechanism. As previously mentioned, there are a wide array of factors that could limit swim performance and many possible mechanisms by which salts or metals could cause a decline in swimming ability. However, I believe most of these mechanisms can be excluded given the assortment of other phenomena observed.

Firstly, it was unlikely that absorption and delivery of oxygen from the environment to the muscle was responsible for the decline. Respiratory dysfunction rarely occurs in fish outside of extreme Ni and hypoxia exposure [41,131]. Swimming and histological examinations were congruent in that most metabolic activities of these fish were not impacted by any of the exposures, including the one that was designed to do so (PCP exposure).

For the fish that did experience reduced swimming performance, it is possible that the M&S exposure resulted in the accumulation of metals in white muscle. Metals in muscle could inhibit enzyme kinetics enough that under high intensity exercise, white muscle was impaired after the switch from red to white muscle was altered.

Metal and salt exposures can cause ROS production and thus increases in ROS could have caused a decline in performance in medium M&S exposure. In this experiment, the COT_{min} typically declined between the first and the last swim but COT_{min} in the third swim trial Cu and medium M&S exposures were elevated (fig 6-7). Blood acidosis from H^{+}_{met} and ROS may be responsible for increased COT by disrupting blood homeostasis [29]. However, Cu exposure did not have reduced swim performance, and COT_{min} in the third swim trial was not higher than first swim trial due to low statistical power. Also, I would have expected that substantial ROS production would have impacted the Hct or repeatability of swim performance after exposure, but it did not.

The most probable mechanism causing the decline in swim performance in medium M&S exposure was likely the loss of ionoregulatory function due to exposure. An inability to reabsorb ions, possibly Ca, could results in cardiovascular and muscle impairment under high-intensity exercise [29,36,68,93,131]. This impairment would appear to be congruent with most of my

results. However, I would have to confirm the decline in plasma osmolytes as well as replicate and measure the accumulation of metals in gills. Since most of my metal could conceivably cause this decline swim performance, it is important to consider other physiological systems in determining how metal mixtures may impact swim performance.

Field studies in Sudbury, Ontario, Canada, have shown that mixtures of metals did not decrease swim performance in a dose- and metal-specific manner. Yellow perch acquired from lakes had a concerning concentration of aluminum, cadmium, copper, nickel, and zinc in liver and muscle tissue [93]. Interestingly, the accumulation of those metals in liver and muscle tissue did not always equate to the concentration of metals in the water, nor did fish from lakes with the most amount of total metals have lowest swim performance [93]. The uptake, exposure duration and affinity of metals for enzymes are vital to discerning in the mechanism underlying a change in swim performance [137]. Furthermore, changes in the metabolic condition of exposed fish could substantially change how much of any metal is absorbed across the gills [14].

Studies that exposed yellow perch to OSPW (19-22 days) found that both PAGE and gill pathological indices were different from the control [5]. Thus, exposure period would likely influence the extent of gill dysfunction in my exposures [6]. Both salts ($NaSO_4$) and NAs were capable of causing these changes in PAGE and gill pathology index [6]. The implications of treated OSPW discharge is likely strong exposure period dependent.

Species have varying sensitivities to individual toxicants, not to mention toxicants in complex mixtures such as OSPW [16,17]. Lab created solutions have varying toxicity to an array of organisms, however, treated OSPW may be tolerable for some fish species [1]. OSPW has a high proportion of metals compared to the salts used in my M&S exposures, so the observations obtained from my mixtures could be underreporting the possible toxicity of OSPW on rainbow trout. Many metals are underrepresented in my exposures. The investigation here hopefully underlines the importance of exploring the possible effects of the OSPW inorganic fraction on metabolism and ionoregulation in fish. Specific mixtures of metals and salts may negativity perturb some aspects of physiology temporarily, but whether the changes in physiology assessed herein constitute a threat to fish health remains unknown. Other, more local species may not be as tolerant as rainbow trout [10,17]. Also, multiple exposures may yield substantially different results than single exposure [138,139].

Future directions

Futures studies should determine whether OSPW can disrupt ionoregulation in local species, as well as determine the uptake rate and latency for effects of acute exposure. Most of my mixtures had an emphasis on metal cations, and anions, such as sulfates were underrepresented and may play an essential role in either toxicity or mitigating toxicity. Moreover, a better understanding of acute OPSW discharge on stream communities. The evidence herein suggests that limited pilot studies into the discharging of treated OSPW should proceed given that there is limited initial effects of metals and salt mixtures on the metabolism of fish and that bioaccumulation of metals and salts from OSPW is likely to be minimal [140]. Furthermore, the susceptibility of endemic stenohaline fish has mostly been ignored. The effectiveness of dilution should match the salt tolerance of the local communities and most sensitive species, not the most tolerant (yellow perch and rainbow trout).

Conclusions

I found that rainbow trout were generally able to tolerate the exposure to the inorganic fraction of OSPW, but specific mixtures of metals and salts impacted fish physiology. Particular concentrations of metals and salts had negative impacts on swim performance in rainbow trout. The gills appeared mostly unaffected by acute exposure, but exposure may have impaired the gills ability to effectivity osmoregulate. Likewise, NKA and CA activity of M&S-exposed fish appeared similar to control, but when compared to NaCl treatment NKA and CA activity may be insufficient. Whether NKA and CA activity in M&S-exposed fish highlights osmoregulatory dysfunction or tolerance remains unclear. More research is required to assess how the metals and salts in OSPW impact fish health.

Table

Table 1. A) water parameters ORP, TDS, pH and ion chromotography (IC) of ammonium, chloride, nitrite, sulfate, nitrate of exposure groups. B) total metals (ICP-MS) of exposure groups with a summary of metals and salts found in OSPW outlined by Li, et al. (2014)[a] and White (2017)[b].

Exposure A)	ORP (mv)	TDS (mg/l)	pH	Ammonium (mmol/l)	Chloride (mmol/l)	Nitrite (mmol/l)	Sulfate (mmol/l)	Nitrate (mmol/l)
Control	272	68	7.82	0	0.20±0.01	0.68±0.01	0.01±0.00	0.02±0.00
Low M&S	285±18.8	852±17.6	7.09±0.18	0	17.0±0.13	1.10±0.15	0	0
Medium M&S	283±26.9	1140±128	7.27±0.14	0	29.0±2.90	0.75±0.26	0.43±0.01	0.39±0.00
High M&S	337±30.5	2827±236	7.40±0.23	0	78.0±0.60	0.70±0.22	0.45±0.01	0
Cu	247±7.65	117±12.5	7.51±0.06	0.33±0.00	0.22±0.01	0.46±0.10	0.36±0.00	0.05±0.01
NaCl	301±31.0	1166±164	7.52±0.10	0	18.0±0.15	0.62±0.02	0	0
OSPW	na	200-2600[a]	7.2-8.9[a]	<0.01-3.83[a]	1.18-24.4[a]	na	0.23-4.89[a]	<0.01-0.04[b]

B)	Na (mmol/l)	Mg (mmol/l)	Ca (mmol/l)	Al (µmol/l)	Cu (µmol/l)	Mo (µmol/l)	Ni (µmol/l)	Zn (µmol/l)
Control	0.32	0.64	1.03	0	0.01	0	0.03	0.14
Low M&S	15.6±0.17	0.78±0.01	1.57±0.05	1.42±0.48	0.19±0.01	10.2±0.08	0.31±0.01	0.52±0.36
Medium M&S	26.7±5.55	0.95±0.01	2.40±0.16	1.17±1.06	0.28±0.03	20.3±1.13	0.56±0.02	0.28±0.10
High M&S	76.8±1.82	1.35±0.25	4.08±0.53	4.56±0.99	0.74±0.03	48.3±0.60	1.14±0.09	0.48±0.21
Cu	0.34±0.02	0.65±0.01	1.10±0.03	0	0.31±0.02	0.06±0.03	0.03±0.00	0.07±0.02
NaCl	18.5±0.21	0.64±0.01	1.07±0.04	0	0.17±0.01	0.21±0.08	0.03±0.00	0.03±0.03
OSPW	10.3-36.7[a]	0.08-1.36[a]	0.05-2.57[a]	2.60-3670[a]	0.03-13.2[a]	0.02-11.7[a]	0.24-4.60[a]	0.02-19.0[a]

Figures

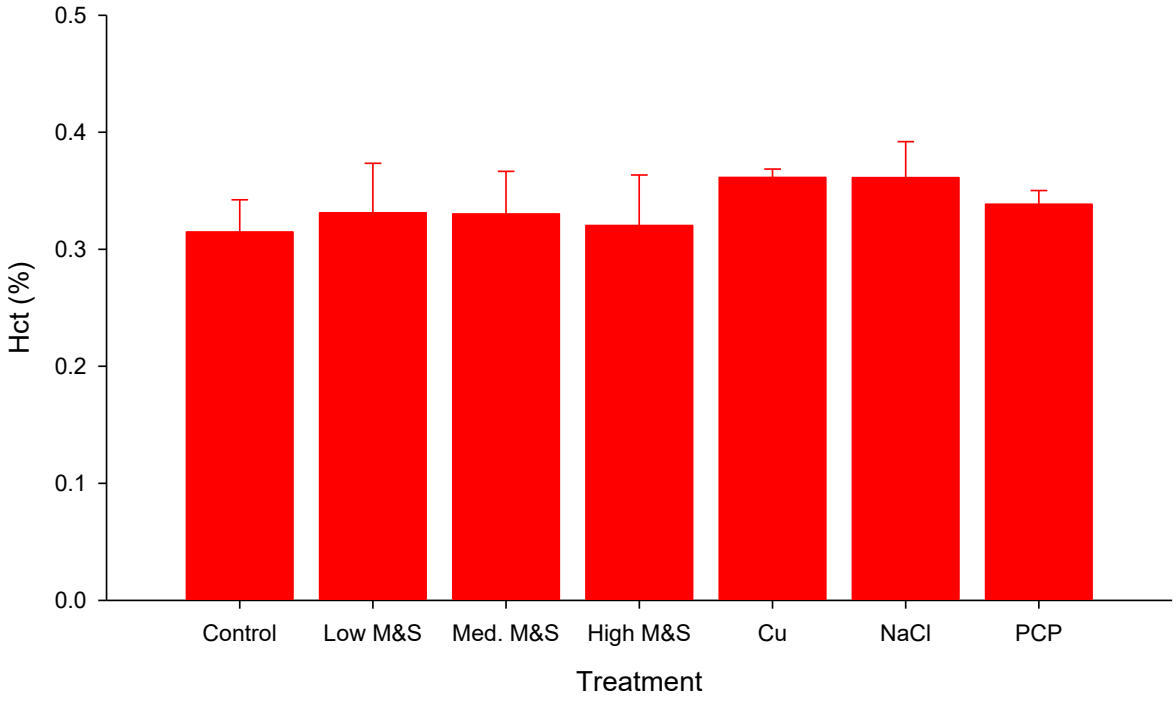

Figure 1. Packed red cell volume (hematocrit; Hct) of rainbow trout exposed to 96 hr static refresh exposure of three metal and salt (M&S) mixture exposures (n=7, 7, and 7 for low, medium, and high respectively) as well control (n=9), Cu (n=5), NaCl (n=6), and pentachlorophenol (PCP) control (n=8) exposure groups. Hct was expressed as a proportion of red blood cells to total blood column.

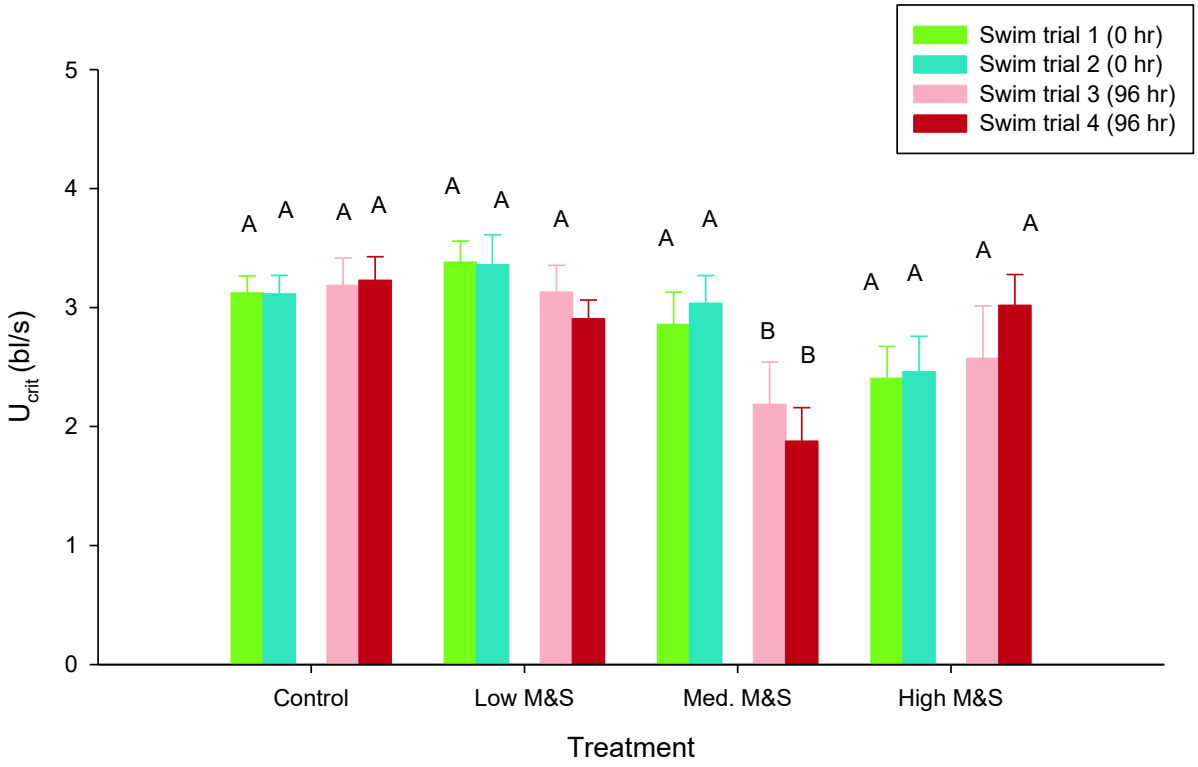

Figure 2. Critical swimming speed (ramp U_{crit}) of rainbow trout exposed to 96 hr static refresh exposure of three metal and salt (M&S) mixtures (n=7, 5, and 6 for low medium and high respectively) and control (n=8) exposures. Fish were repeatedly swam a total of four times. Values are expressed as means+S.E.M and letters A and B denote statistically significant differences in ramp U_{crit} (repeated measures ANOVA followed by Holm-Sidak post hoc test). Swim speed were converted into body lengths/s (bl/s)

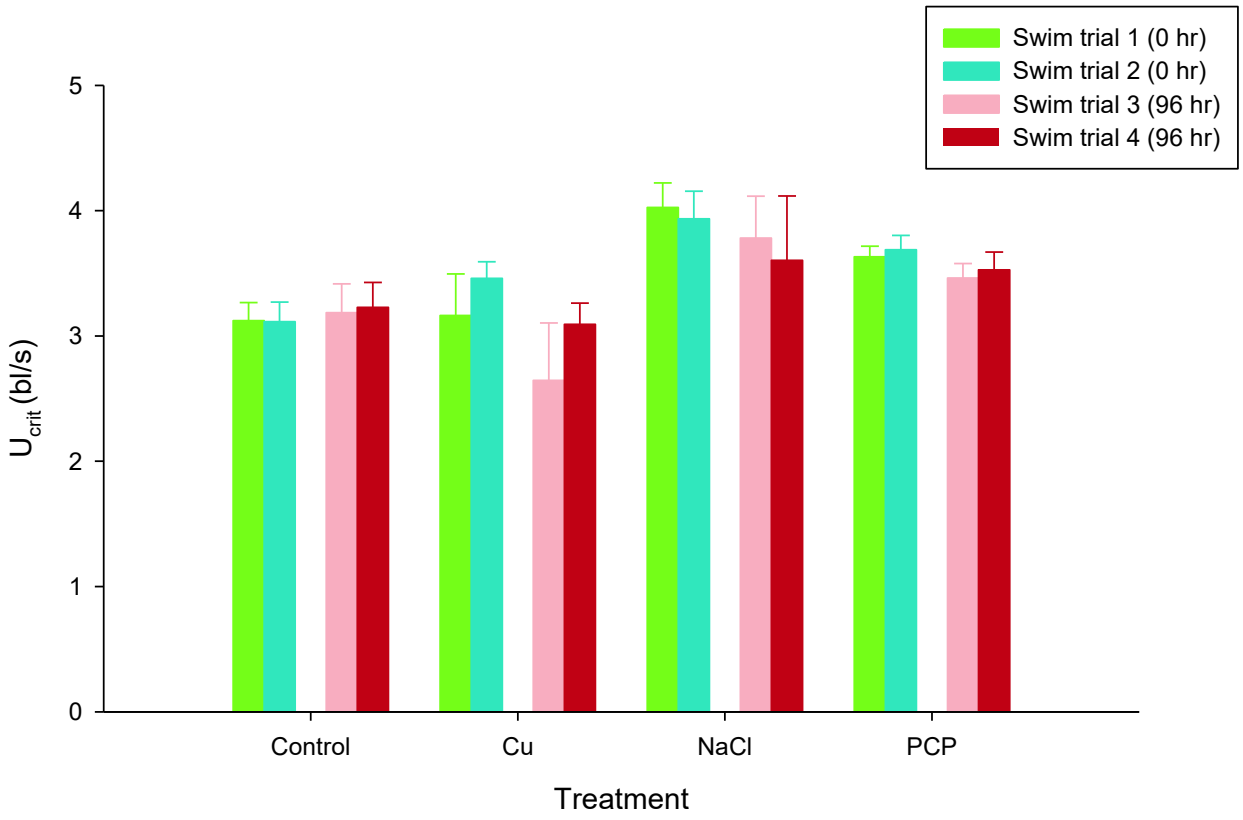

Figure 3. Critical swimming speed (ramp U_{crit}) of rainbow trout exposed to 96 hr static refresh exposure Control (n=8), Cu (n=4), NaCl (n=6), and pentachlorophenol (PCP) control (n=7) exposure groups. Fish were repeated swam a total of four times. Values are expressed as means+S.E.M and letters A and B denote statistically significant differences in ramp U_{crit} (repeated measures ANOVA followed by Holm-Sidak post hoc test). Swim speeds were converted into body lengths/s (bl/s)

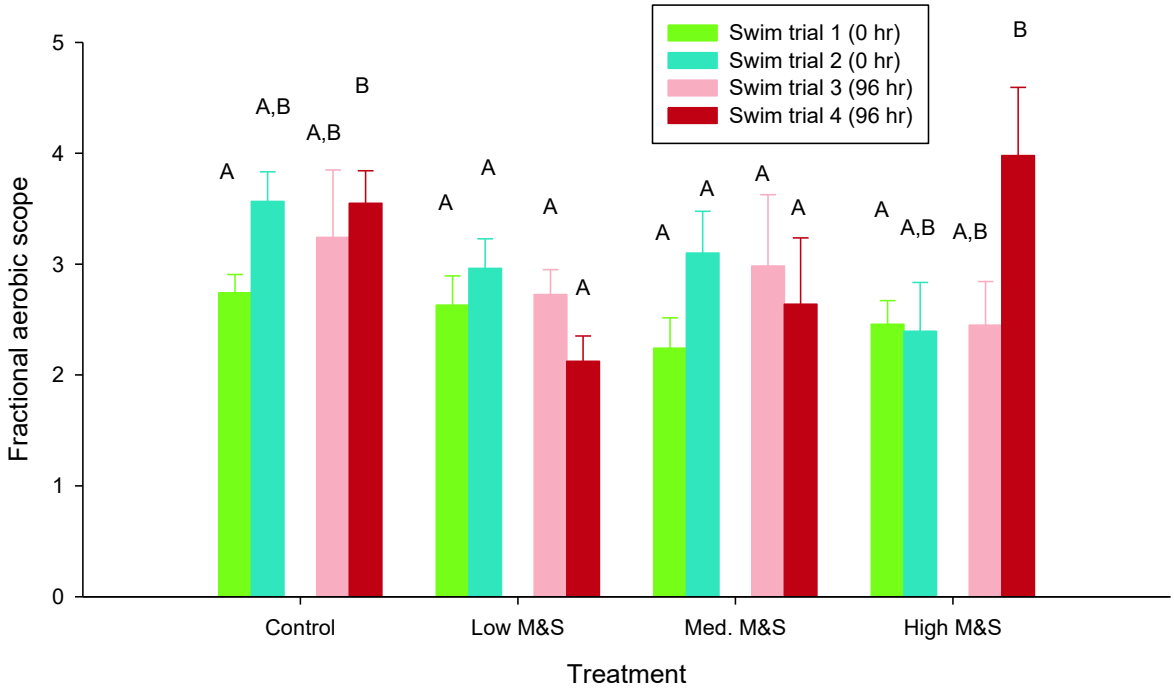

Figure 4. Fractional aerobic scope (maximum MO_2 / minimum MO_2) of rainbow trout exposed to 96 hr static refresh exposure of three metal and salt (M&S) mixtures (n=7, 5, and 6 for low medium and high respectively) and control (n=8) exposures. Fish were repeatedly swam a total of four times. Values are expressed as means+S.E.M and letters A and B denote statistically significant differences in Fractional aerobic scope (repeated measures ANOVA followed by Holm-Sidka post hoc test). MO_2 values with $R^2<0.90$ were excluded as possible maximum or minimum MO_2.

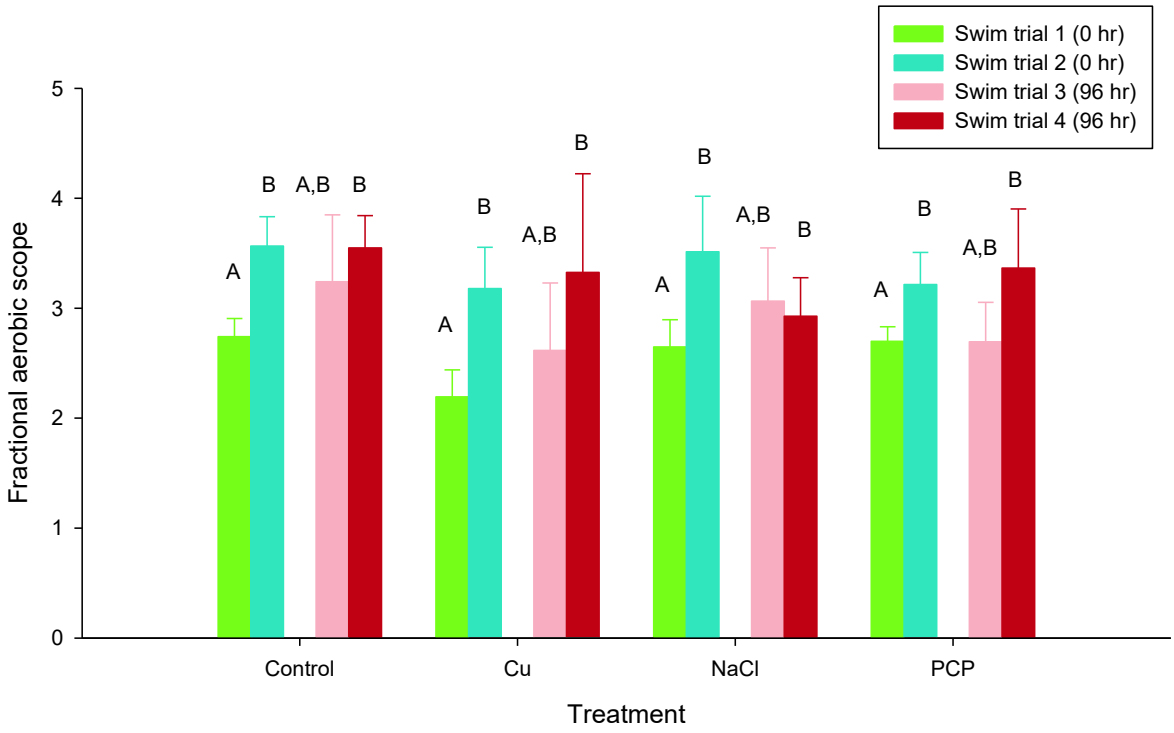

Figure 5. Fractional aerobic scope (maximum MO_2 / minimum MO_2) of rainbow trout exposed to 96 hr static refresh exposure to control (n=8), Cu (n=4), NaCl (n=6), and pentachlorophenol (PCP) control (n=7) exposure groups. Fish were repeatedly swam a total of four times. Values are expressed as means+S.E.M and letters A and B denote statistically significant differences in Fractional aerobic scope (repeated measures ANOVA followed by Holm-Sidak post hoc test). MO_2 values with $R^2<0.90$ were excluded as possible maximum or minimum MO_2.

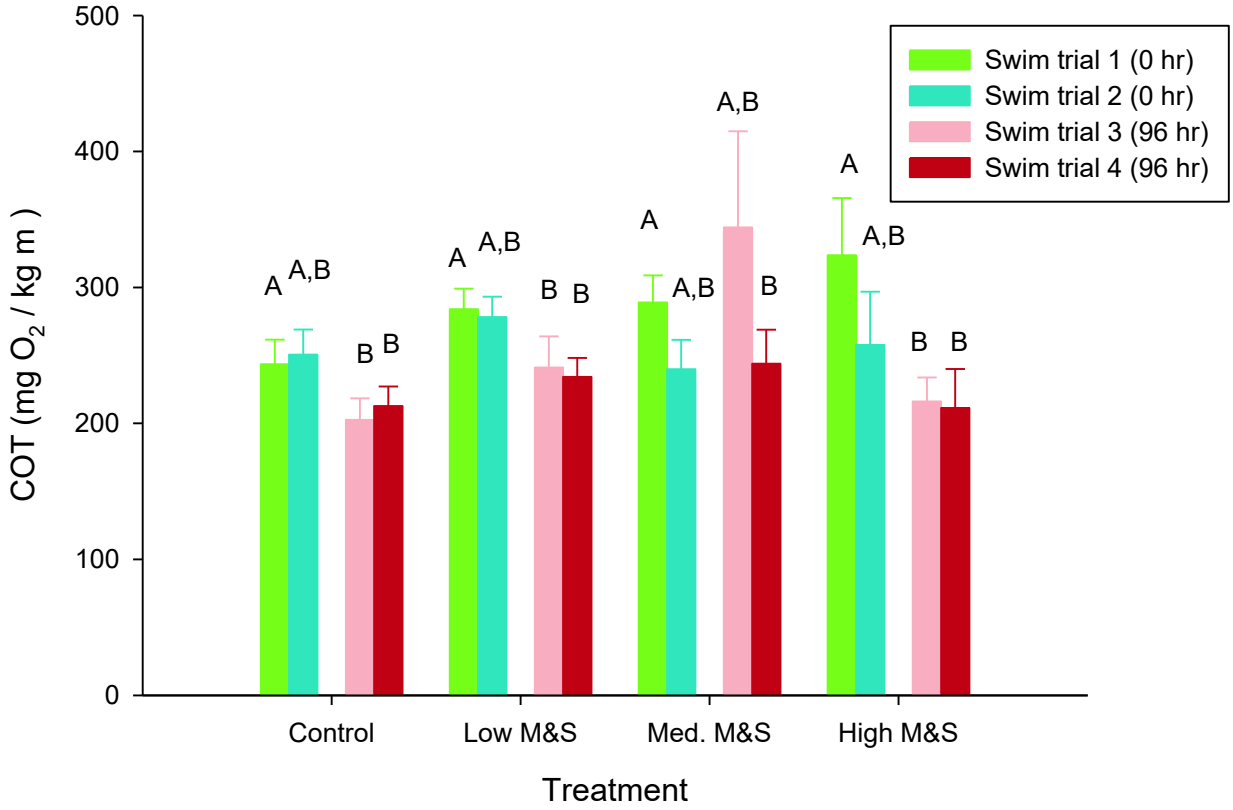

Figure 6. The minimum cost of transport (COT) of rainbow trout exposed to 96 hr static refresh exposure of three metal and salt (M&S) mixture exposures (n=7, 5, and 6 for low medium and high respectively) and control (n=8) exposures. Fish were repeated swam a total of four times. Values are expressed as means+S.E.M and letters A and B denote statistically significant differences in COT (repeated measures ANOVA followed by Holm-Sidak post hoc test). MO_2 values with $R^2<0.90$ were excluded from analysis. Exposure groups without letters are not statistically different from one another.

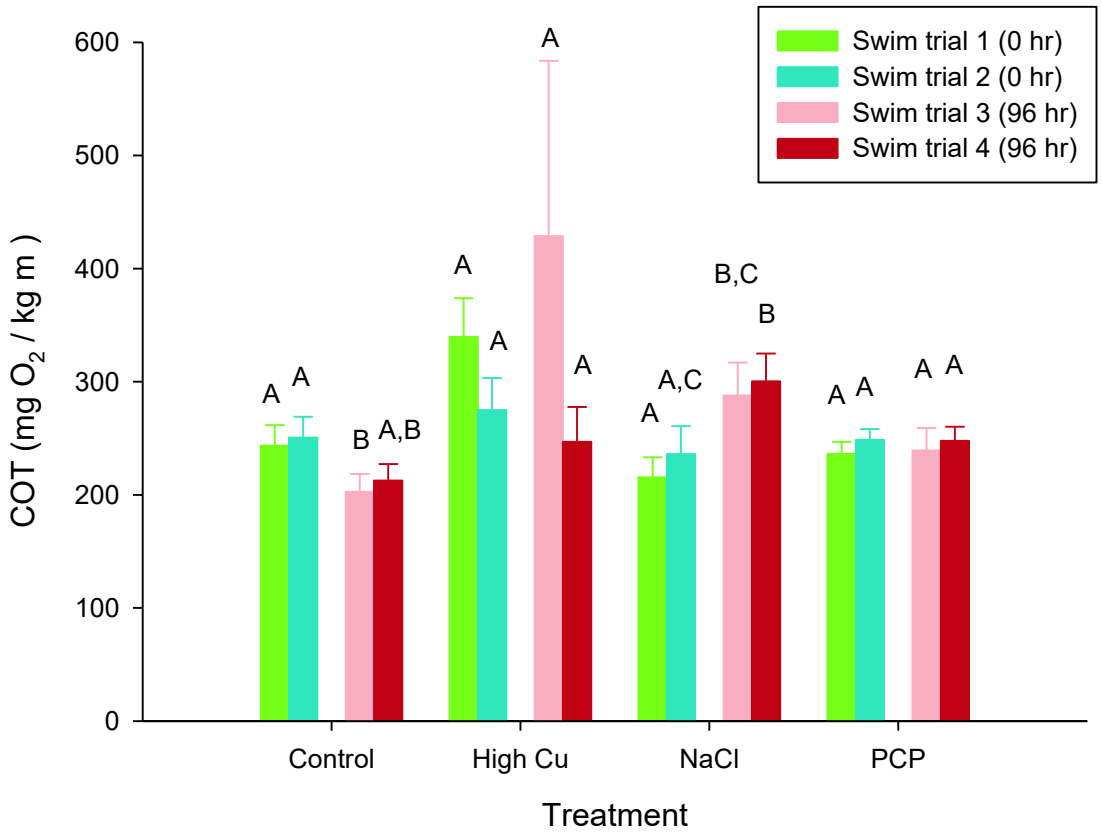

Figure 7. Cost of transport (COT) of rainbow trout exposed to 96 hr static refresh exposure to control (n=8), Cu (n=4), NaCl (n=6), and pentachlorophenol (PCP) control (n=7) exposure groups. Fish were repeatedly swam a total of four times. Values expressed as means+S.E.M and letters A, B, and C denote statistically significant differences in COT (repeated measures ANOVA followed by Holm-Sidak post hoc test). MO_2 values with $R^2<0.90$ were excluded from analysis. Exposure groups without letters are not statistically different from one another

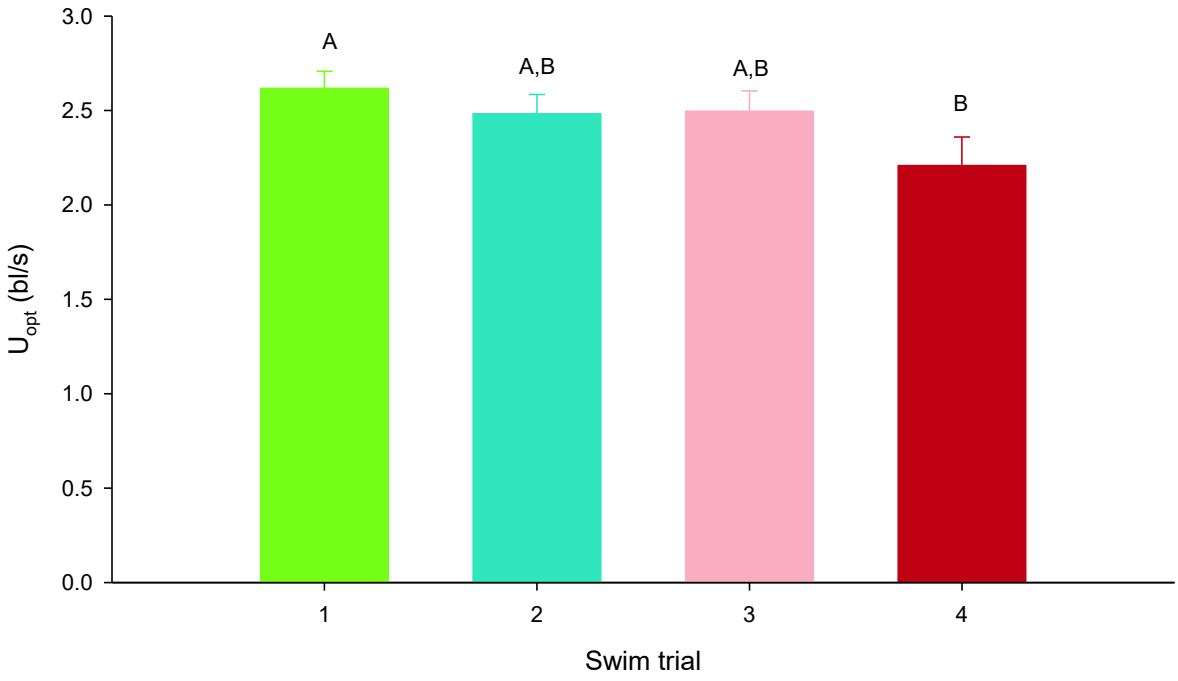

Figure 8. Mean U_{opt} (bl/s) of rainbow trout exposed to 96 hr static refresh exposure of three metal and salt (M&S) mixture exposures (n=7, 5, and 6 for low medium and high respectively) and control (n=8) exposures. Fish were repeatedly swam a total of four times. Values are expressed as means+S.E.M and shared letter (A and B) denotes exposures with statistical differences (repeated measures ANOVA followed by Holm-Sidak post hoc test). MO_2 values with $R^2<0.90$ were excluded from analysis. Exposure groups without letters are not statistically different from one another

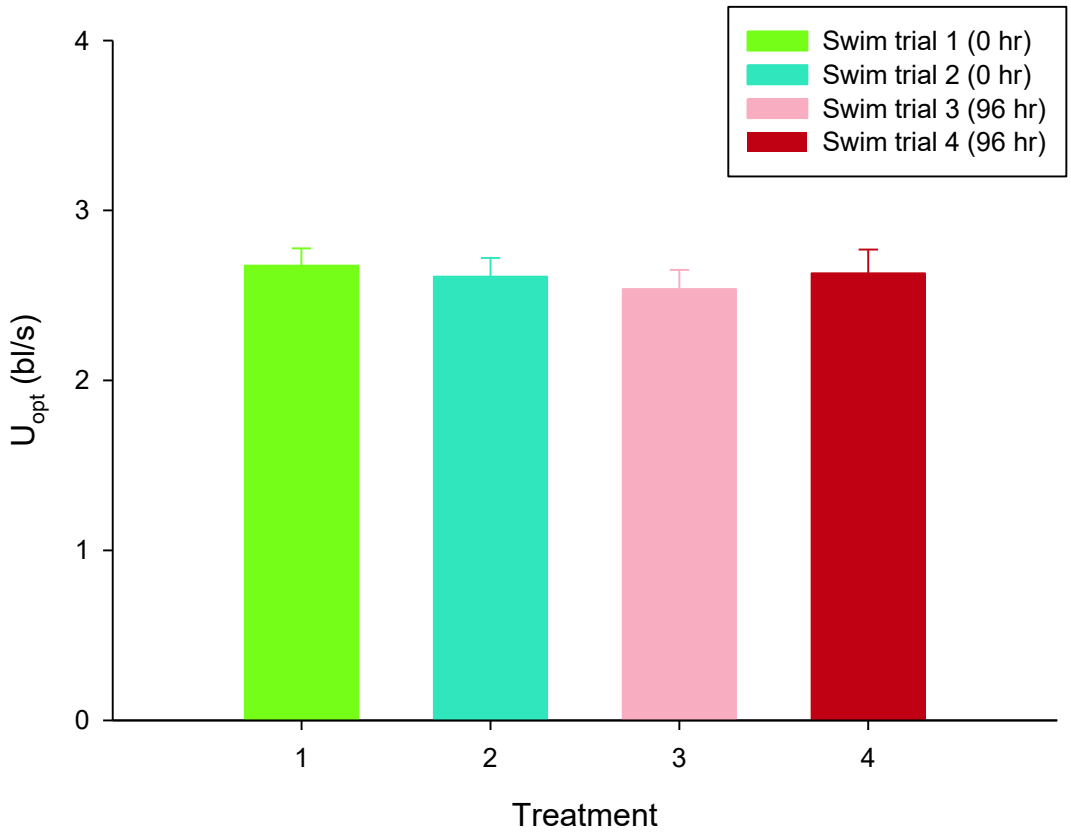

Figure 9. Mean U_{opt} (bl/s) of rainbow trout exposed to 96 hr static refresh exposure of control (n=8), Cu (n=4), NaCl (n=6), and pentachlorophenol (PCP) control (n=7) exposure groups. Fish were repeated swam at total of four times. Values expressed as means+S.E.M. MO_2 values with R^2<0.90 were excluded from analysis. Exposure groups without letters are not statistically different from one another

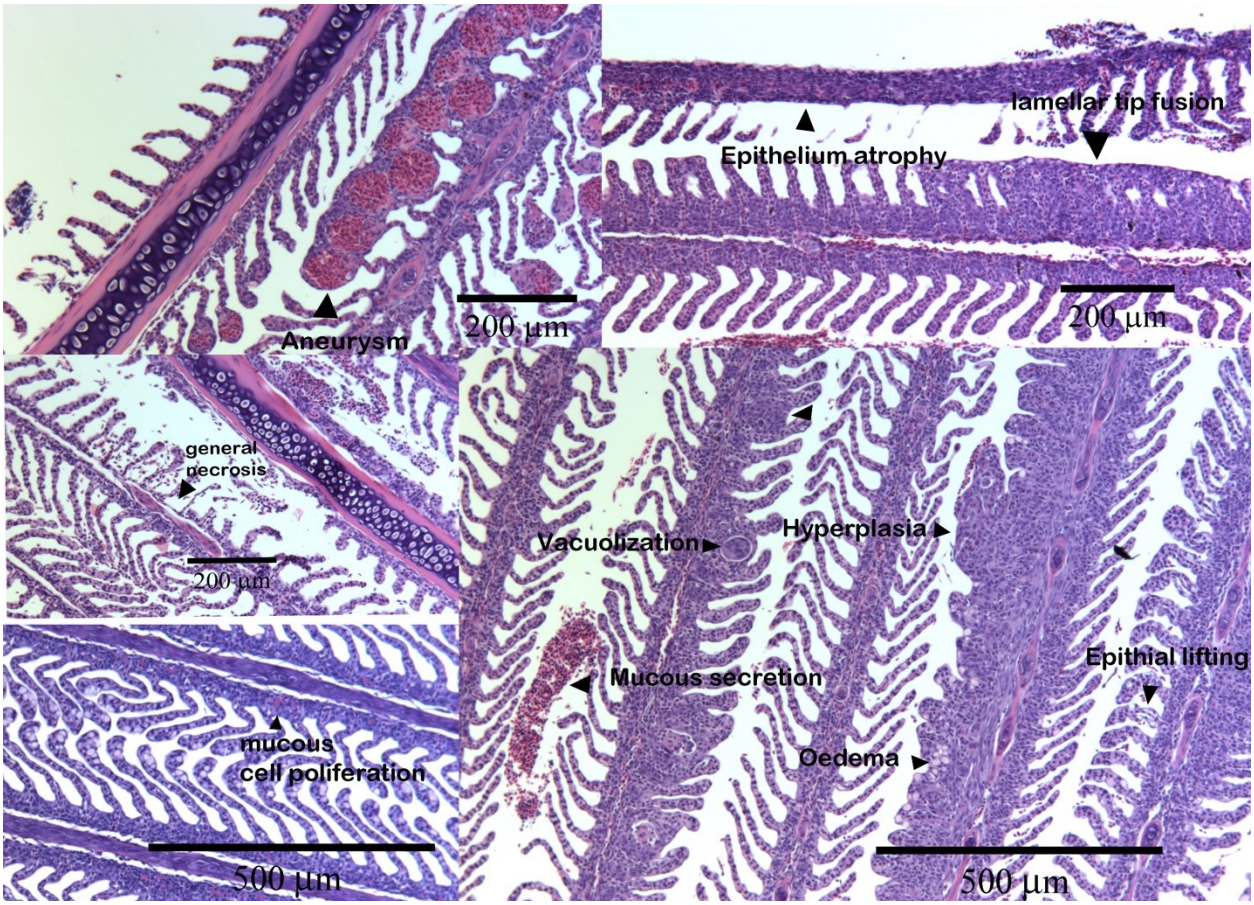

Figure 10. Light microscopy image showing example of gill malformation used in pathology index. Photos are gill longitudinal section (5µm) and were stained with haematoxylin and eosin from second gill lamellae of various rainbow trout.

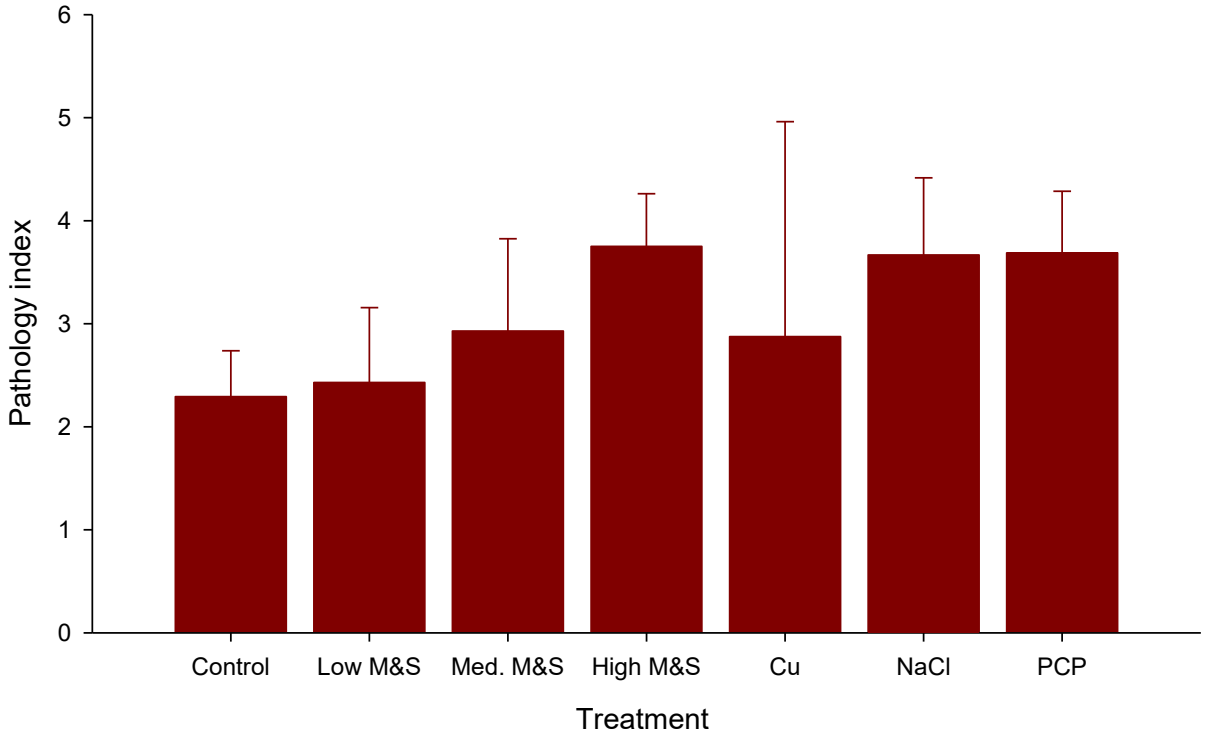

Figure 11. Pathology index (mean +S.E.M) of rainbow trout exposed to 96 hr static refresh exposure of three metal and salt (M&S) mixture exposures (n=7, 7, and 6 for low, medium, and high respectively) as well control (n=12), Cu (n=4), NaCl (n=6), and pentachlorophenol (PCP) control (n=8) exposure groups. Gill longitudinal section (5µm) was stained (hemotoxin eosin) and was examined blind by two assessor at 10X magnification. Pathology index was calculated according to Bernet et al. (1999). Exposure groups without letters are not statistically different from one another.

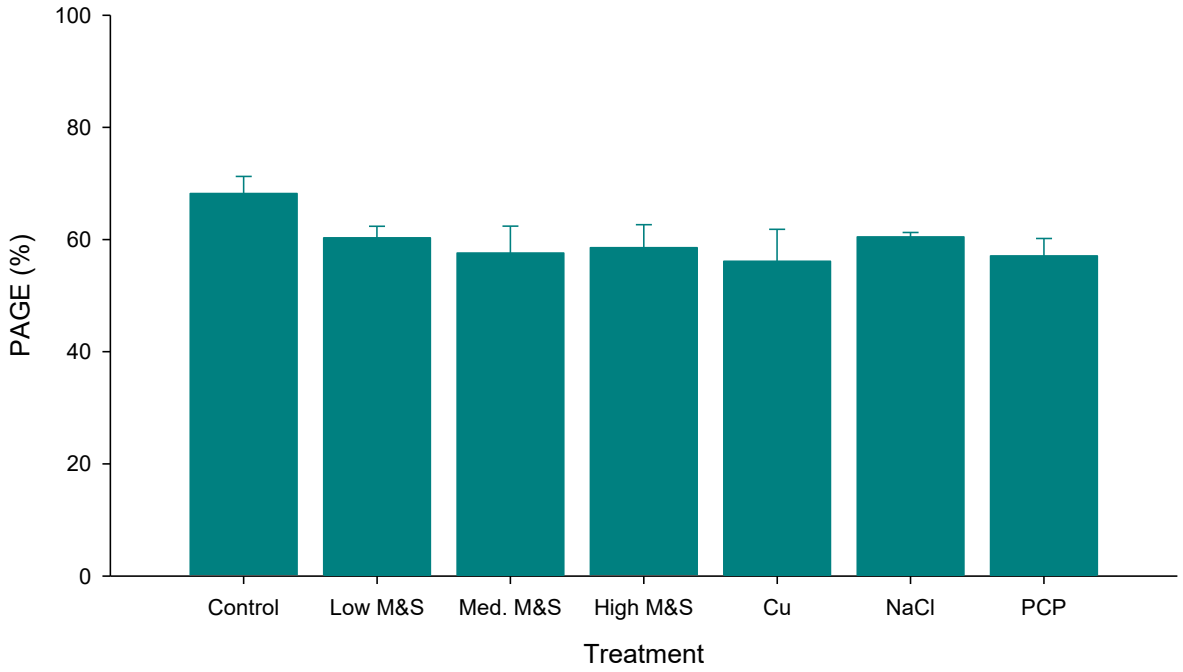

Figure 12. The proportion of area of the secondary lamellae available for gas exchange (PAGE) (%) (mean +S.E.M) of rainbow trout exposed to 96 hr static refresh exposure to three metal and salt (M&S) mixture exposures (n=7, 7, and 6 for low, medium, and high respectively) as well control (n=12), Cu (n=4), NaCl (n=6), and pentachlorophenol (PCP) control (n=8) exposure groups. Gill longitudinal section (5µm) was stained (hemotoxin eosin) and were measured using ImageJ.

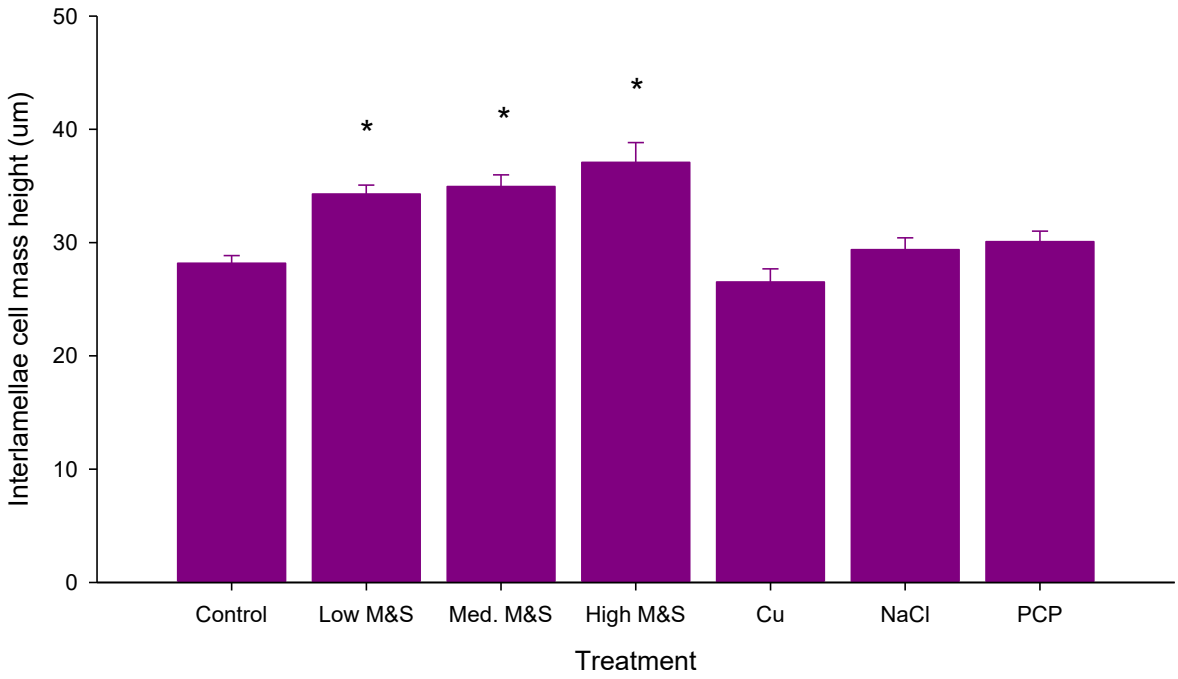

Figure 13. interlamellar cell mass height (IMCH) (µm) (mean +S.E.M) of rainbow trout exposed to 96 hr static refresh to three metal and salt (M&S) mixture exposures (n=7, 7, and 6 for low, medium, and high respectively) as well control (n=12), Cu (n=4), NaCl (n=6), and pentachlorophenol (PCP) control (n=8) exposure groups. Gill longitudinal section (5µm) was stained (hematoxylin and eosin) and were measured using ImageJ. The asterisk denotes a statistically significant from control (ANOVA on ranks followed by Dunn post hoc test.

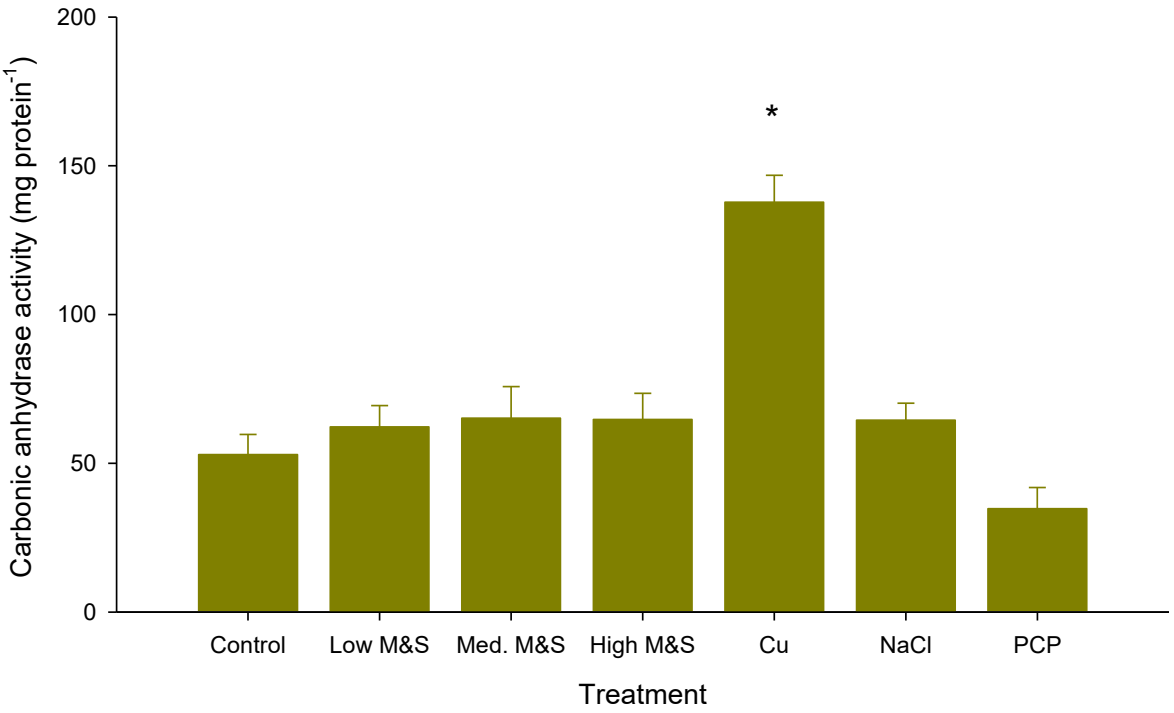

Figure 14. Carbonic anhydrase activity (1/mg protein) of rainbow trout exposed to 96 hr static refresh to three metal and salt (M&S) mixture exposures (n=3, 5, and 5 for low, medium, and high respectively) as well control (n=7), Cu (n=5), NaCl (n=5), and pentachlorophenol (PCP) control (n=6) exposure groups. Values are expressed as mean plus S.E.M and asterisk denotes statistical difference from control (one way ANOVA followed by Holm Sidak post hoc test).

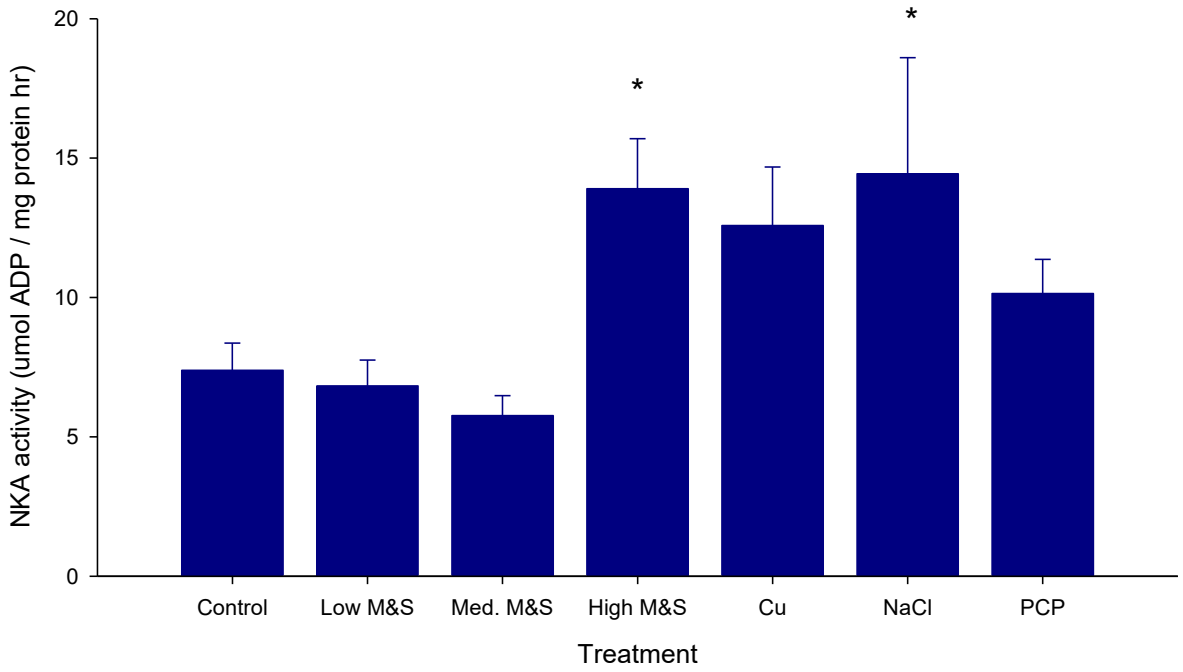

Figure 15. Sodium potassium ATPase (NKA µmol ADP/ mg protein hr) of rainbow trout exposed to 96 hr static refresh to three metal and salt (M&S) mixture exposures (n=6, 7, and 6 for low, medium, and high respectively) as well control (n=14), Cu (n=4), NaCl (n=6), and pentachlorophenol (PCP) control (n=6) exposure groups. Values are expressed as mean plus S.E.M and asterisk denotes statistical difference from control (one way ANOVA followed by Holm Sidka post hoc test.

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Appendix

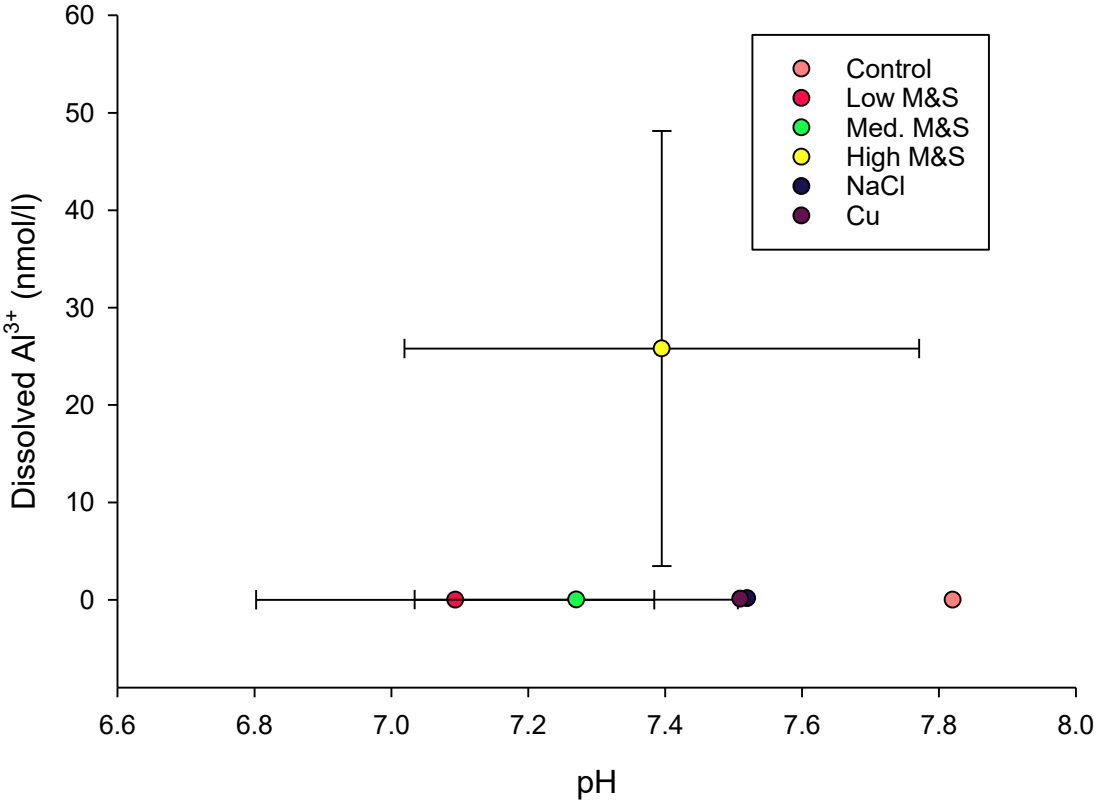

Figure 16. Scatterplot of mean±S.E.M dissolved Al^{3+} (nmol/l) against pH of water samples collected from exposure groups data. Water sample Al was measured with ICP-MS and dissolved Al^{3+} was calculated with Visual MINTEQ.

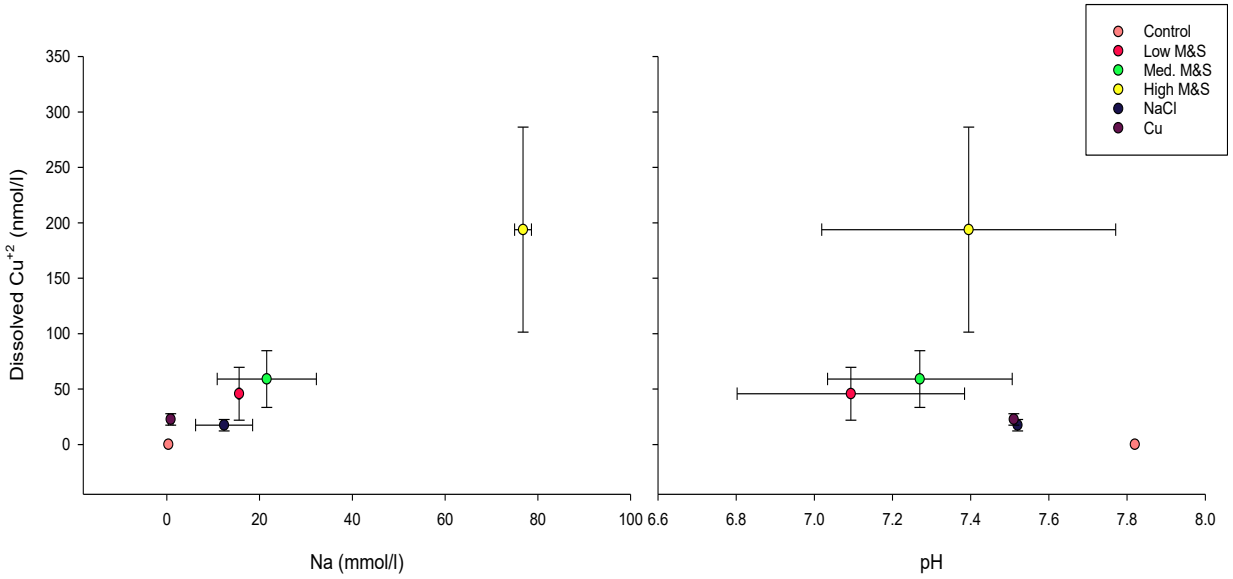

Figure 17. Scatterplot of mean±S.E.M dissolve Cu^{2+} (nmol/l) against Sodium (mmol/l) and pH of water samples collected from exposure groups data. Water samples Cu was measured with ICP-MS and dissolved Cu^{2+} was calculated with Visual MINTEQ.

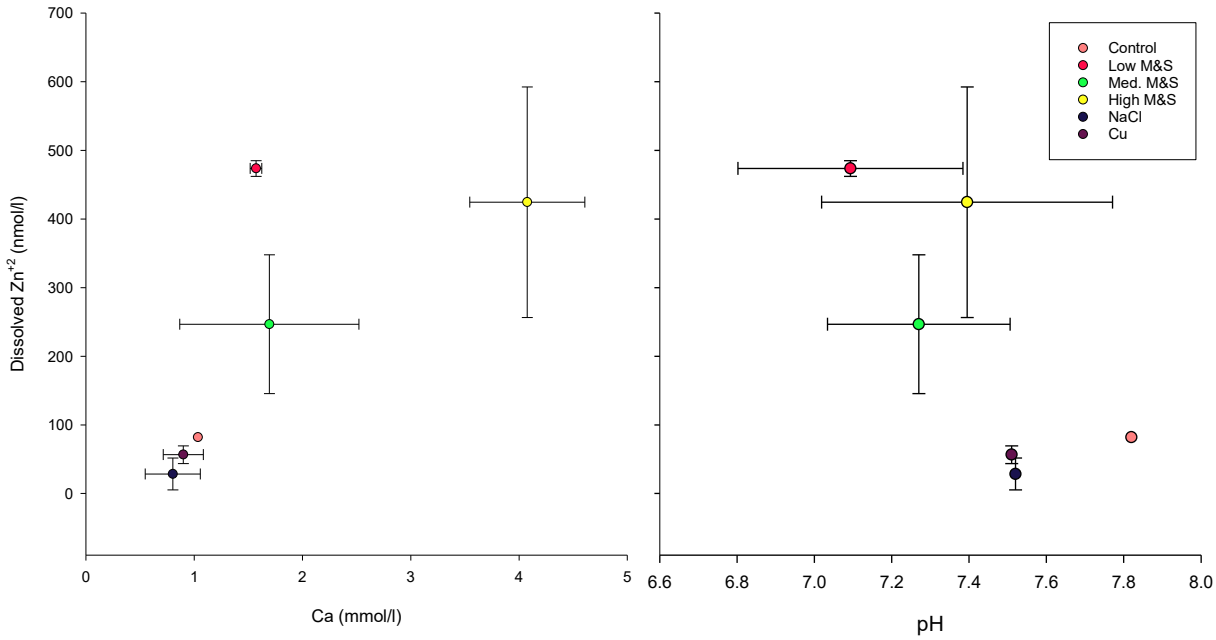

Figure 18. Scatterplot of mean$\pm S.E.M$ dissolved Zn^{2+} ([32]/l) against Calcium (mmol/l) and pH of water samples collected from exposure groups. Water samples Zn was measured with ICP-MS and dissolved Zn^{2+} was calculated with Visual MINTEQ.

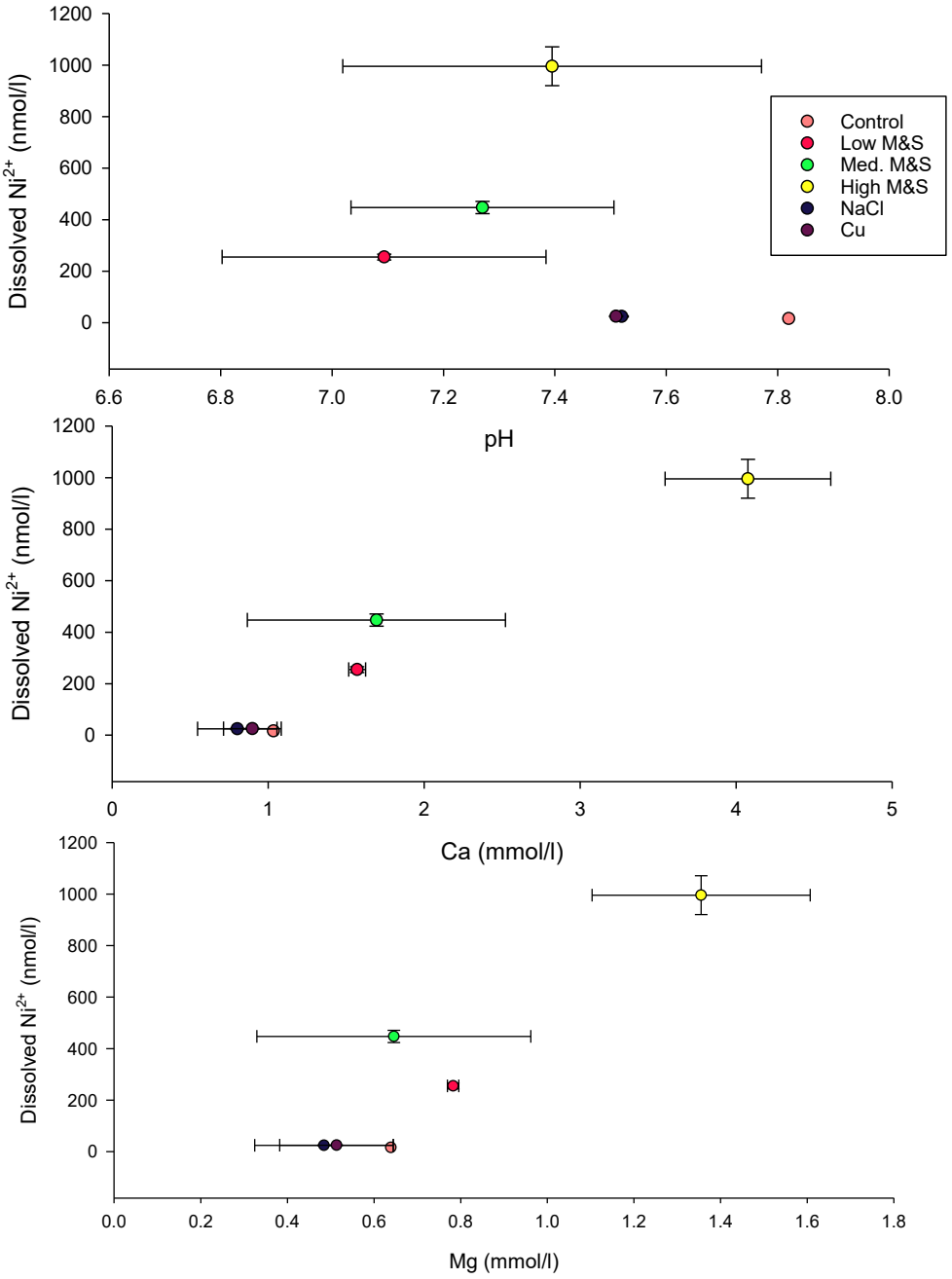

Figure 19. Scatterplot of mean$\pm S.E.M$ dissolved Ni^{2+} (nmol/l) against pH, calcium (mmol/l), magnesium (mmol/l) of water samples collected from exposure groups. Water samples Ni was measured with ICP-MS and dissolved Ni^{2+} was calculated with Visual MINTEQ.

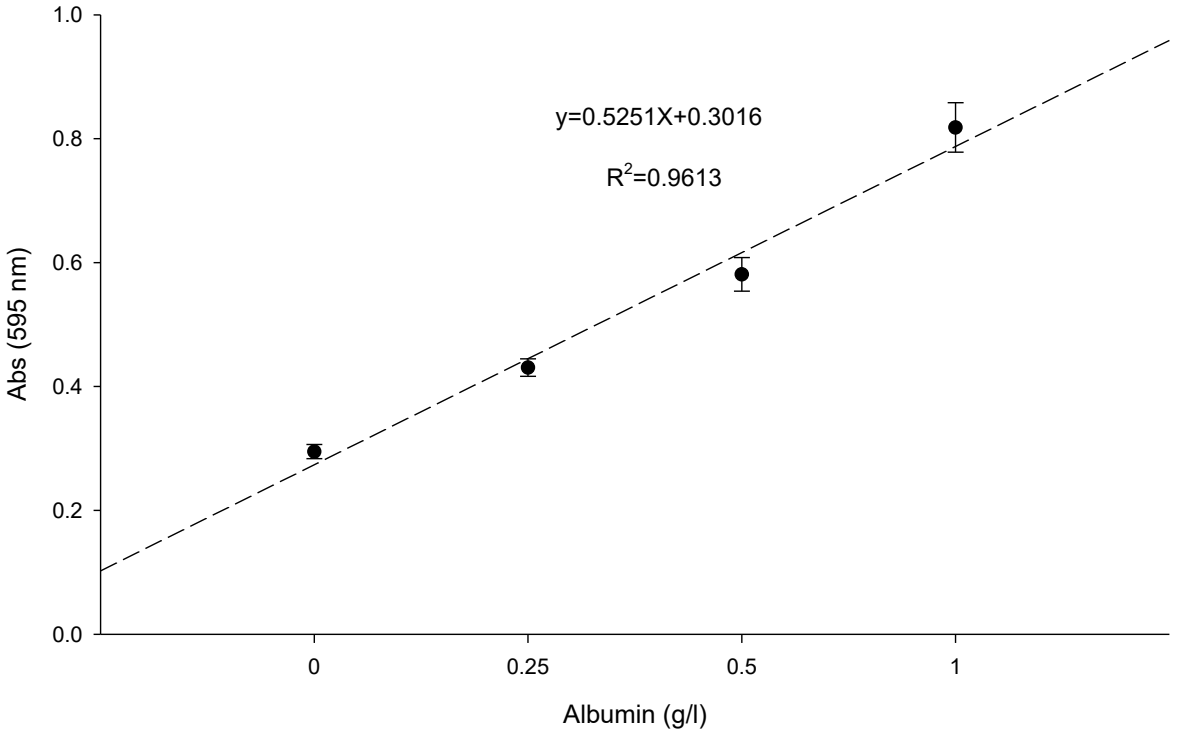

Figure 20. Standard curve for Bradford assay of blank 0.25,0.5 and 1 g/l of albumin stock solution were absorption (abs) was measured at 595nm. Error bars represent 95% CI of mean and dashed line represents linear regression were equation and R^2 are presented on the figure.

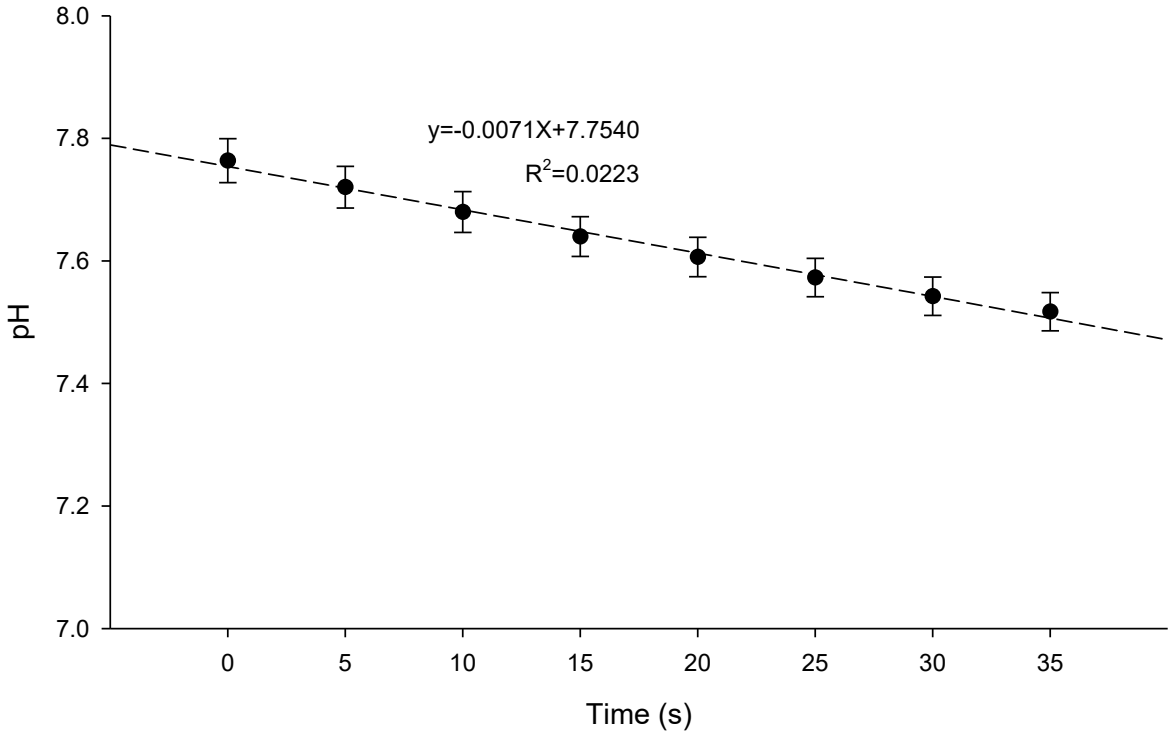

Figure 21. Blank run of carbonic anhydrase assay containing 7.5 ml of assay solution and 1ml of CO_2 saturated dH_2O in 4°C water bath. Error bars represent 95% CI of mean of pH after 1ml of CO_2 saturated dH_2O was added to assay solution. Dashed line represents linear regression were equation and R^2 are presented on the figure.

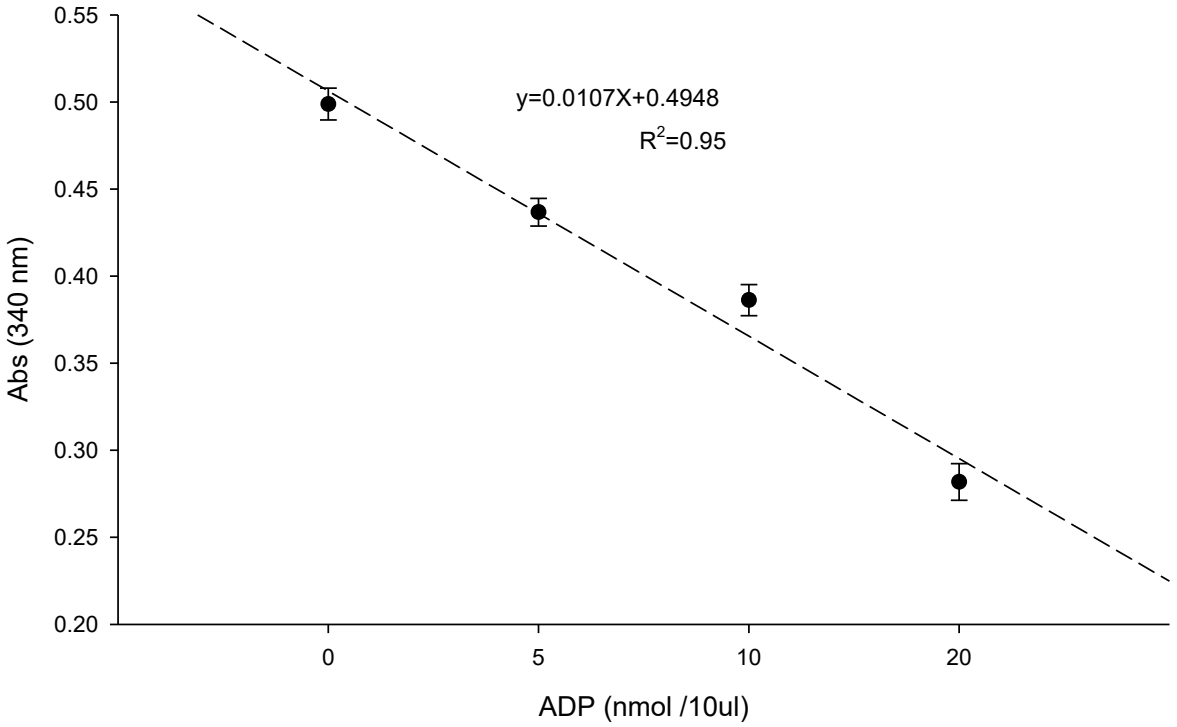

Figure 22. ADP Standard curve for NKA assay after 10 minutes of recording on kinetic curve in spectrometer measuring absorption (Abs) at 340nm. Plate wells contain 0, 5, 10, and 20 nmol of ADP in 200 µl of NKA assay solution. Error bars represent 95% CI and dashed line represents linear regression were equation and R^2 are presented on the figure.

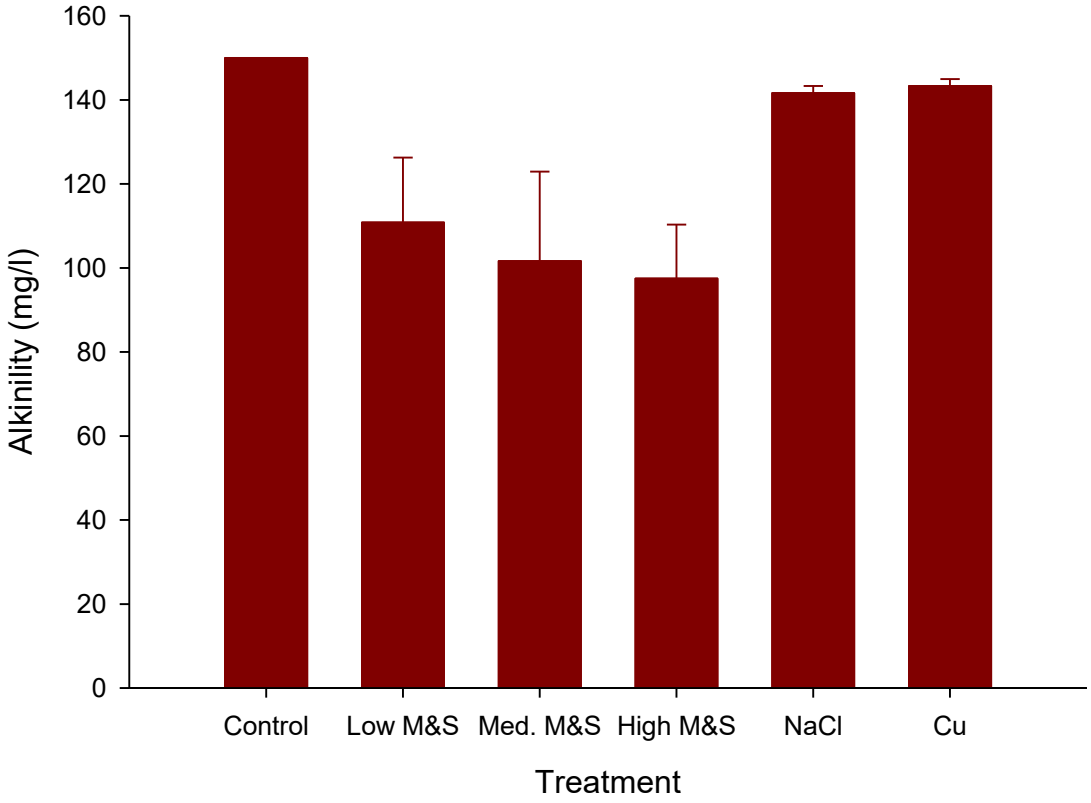

Figure 23. Alkalinity (mg/l) of water samples collected from control, low M&S, medium M&S, high M&S, NaCl, and Cu exposure groups values are expressed as mean plus S.E.M. Alkalinity was calculated through titration with 0.1 N HCl.