THESIS

EFFECTS OF CHRONIC, SUBLETHAL FERRIC IRON EXPOSURE ON THE CRITICAL

SWIM SPEED OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND CRITICAL

THERMAL MAXIMUM OF CUTTHROAT TROUT (*ONCORHYNCHUS CLARKII)*

Submitted by

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ABSTRACT

EFFECTS OF CHRONIC, SUBLETHAL FERRIC IRON EXPOSURE ON THE CRITICAL
SWIM SPEED OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND CRITICAL
THERMAL MAXIMUM OF CUTTHROAT TROUT (*ONCORHYNCHUS CLARKII)*

Two experiments were performed to aid in establishing a new Colorado chronic
water quality criterion for total iron. Although the effects of dissolved ferrous iron have been
well documented, limited data are available regarding ferric iron specific toxicity in aquatic
ecosystems. Juvenile rainbow trout (*Oncorhynchus mykiss*) critical swim speed (U_{crit}) was
measured to establish if there was a relationship between chronic sublethal ferric iron exposure
and changes in U_{crit}. The gills were examined for histological changes and fish growth was
measured as endpoints. No significant changes in U_{crit} growth or gill histology were found in
the first experiment, although suggestive trends were noted. The U_{crit} experiment was
challenging on multiple levels, in part due to the diminutive size of the fish used in the
experiment. A second study was performed on juvenile cutthroat trout chronically exposed to
sublethal concentrations of ferric iron. The critical thermal maximum (CT_{max}) and changes in
weight were tested. There were no significant changes in CT_{max} or weight measured in the
second experiment.

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I. INTRODUCTION

Toxic metal contamination of Colorado's surface water primarily comes from sulfide containing minerals reacting with oxygen and water (Singer and Stumm 1970; Lewis et al. 1992). Iron is a common toxic metal found in Colorado's surface waters. Colorado has approximately 1085 km of rivers and streams and 55 hectares of lakes, reservoirs and ponds impaired by iron (USEPA, 2015). Although natural sources such as hydrothermally-altered rocks contribute to iron and other toxic metal contamination, abandoned mine sites in Colorado also contribute to the contamination through acid mine drainage (Sares et al. 2000). A literature review found few experiments that investigated the specific effects of ferric iron (Fe^{3+}) on individual fish species or fish populations. The main focus of past experiments has been ferrous iron (Fe^{2+}) or ferric iron in combination with other dissolved metals. The current United States Environmental Protection Agency (USEPA) and Colorado State chronic iron standard for total recoverable iron for protection of aquatic life is 1.0 mg/L and 0.3 mg/L for dissolved iron (USEPA 1976; 5CCR 1002-31, 2012). Total recoverable iron is comprised of dissolved (Fe^{2+}) and precipitated (Fe^{3+}) forms of iron.

Challenges to Colorado's chronic iron standard have lobbied for a standard that solely regulates Fe^{2+}. The basis for this argument stems primarily from the differences in bio-availability of the two forms of iron. The argument for this challenge is that Fe^{3+} is less bioavailable than Fe^{2+}, therefore, exposure to Fe^{3+} will have fewer negative effects on aquatic organisms than exposure to Fe^{2+} and there is less need to regulate ferric iron. The bioavailability of ferrous iron in aquatic organisms is in part due to its ability to be transported across the gill lamella and in the intestines by divalent metal transporters (Gunshin et al. 1997;

Dorschner and Phillis 1999; Bury et al. 2001; Bury et al. 2003). Excess iron in living organisms is toxic, generating reactive oxygen species via the Fenton reaction (Crichton et al. 2002).

In the oxygenated, circumneutral pH aqueous environment that supports aquatic life, Fe^{3+} is predominately found as insoluble colloidal and as oxide-hydrate precipitates that are in a form that is slow to reduce to Fe^{2+}, and not readily bioavailable to aquatic vertebrates (Hoffmann 2005). Although Fe^{3+} must be first reduced before it can be directly transported across the cell wall, Gerhardt (1992) observed that ferric precipitates cause physical stress in the aquatic insect larva, *Leptophlebia- marginata*. Survival and reproduction of fathead minnows, brook trout and coho salmon have also been found to be altered by iron precipitates (Smith et al.1973; Smith & Sykora 1976).

Two studies were conducted to investigate effects of chronic, sublethal ferric iron exposure on rainbow and cutthroat trout. The objective of the studies was to determine if Fe^{3+} had deleterious effects on fish gills resulting in decreased fitness, growth and ability to sustain prolonged aerobic swimming. The first study measured the effects of chronic, sublethal ferric iron exposure on critical swim speed (U_{crit}), length, weight and changes to gill morphology. The second study looked at the effects sublethal, chronic ferric iron exposure has on thermal maximum (CT_{max}) and weight of cutthroat trout. The ability of a species to cope with combined unique stressors such as ferric iron exposure and increased water temperature can impact the long-term fitness of that species.

II. LITERATURE REVIEW

2.1. Iron in the Aquatic Environment

Iron, a transitional metal, is the fourth most abundant element in the Earth's crust. In addition to its elemental state, iron has five oxidation states (Fe^{2+}-Fe^{6+}). The most common states for iron in aquatic environments are Fe^{2+} and Fe^{3+} (Figure 1). Iron is an essential mineral for living organisms in part due it its ability to undergo univalent redox reactions, making it an essential component in a broad range of cellular functions including respiration, photosynthesis and metabolism.
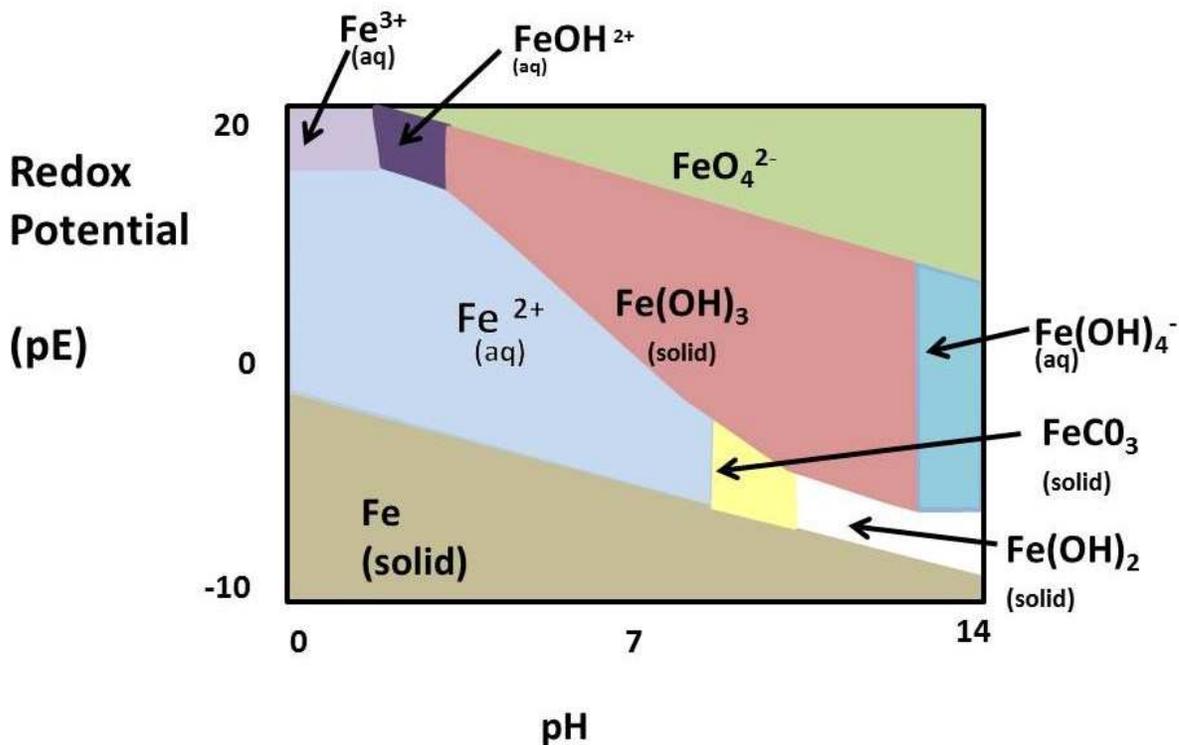

Figure 1. Speciation of iron in surface water.

In the aerobic conditions of an organism's internal environment, excess iron is toxic, catalyzing a chain-reaction that produces reactive oxygen species (ROS) in the form of hydroxyl radicals and hydroxide ions (Bishu 2006; Salgado 2013) (Figure 2). The ROS interact with lipid molecules, resulting in lipid peroxidation and formation of longer-lived, highly reactive and cytotoxic aldehydes, including malondialdehyde (MDA). An electrophile, MDA attacks proteins and other macromolecules, forming glycation end-products (Esterbauer et al. 1991; Girotti 1998; Crichton et al. 2002).
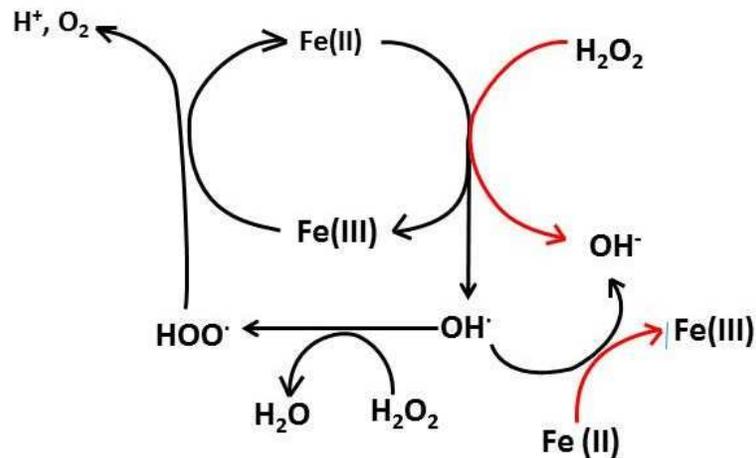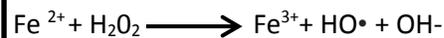

Figure 2. Haber-Weiss Cycle/Fenton Reaction. Free radical production via the Fenton reaction.

Fenton Reaction

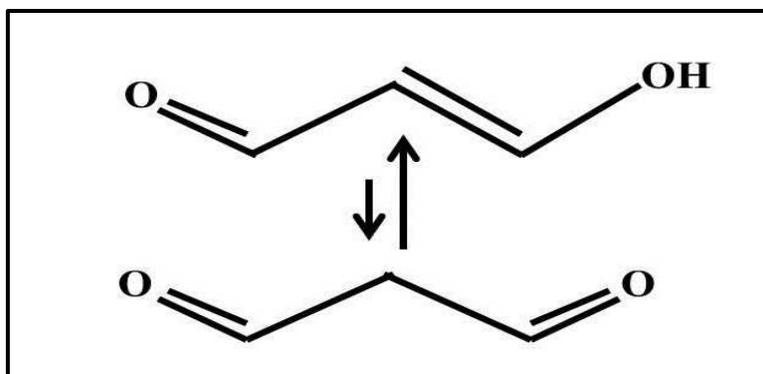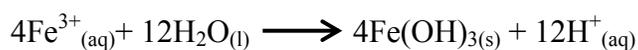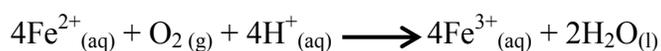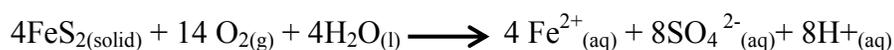$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^\bullet + OH^-$$

Figure 3. Malondialdehyde (MDA).

The behavior of iron in aquatic ecosystems is dynamic and influenced by a complex set of environmental conditions, including light, dissolved oxygen content, pH and dissolved organic matter (Theis 1973; Hazen et al. 2002; Hoffmann 2005). Of the two oxidation states most commonly found in aquatic environments, the reduced Fe^{2+} form is the more stable form at lower pH and the oxidized Fe^{3+} form is the more stable form of iron in neutral and alkaline environments (Broshears et al. 1996). Iron is present in the environment in naturally occurring pyrite. When pyrite ($4FeS_2$) is exposed to water and oxygen, it forms ferrous irons. If exposed to surface conditions where oxygen is found at a higher concentration, the ferrous iron reacts with oxygen forming ferric iron. Ferric iron is hydrolyzed and precipitates out as ferric hydroxide, also commonly referred to as "yellowboy".

Reactions that produces ferric hydroxide:

$$4FeS_{2(solid)} + 14\ O_{2(g)} + 4H_2O_{(l)} \longrightarrow 4\ Fe^{2+}_{(aq)} + 8SO_4^{\ 2-}_{(aq)} + 8H+_{(aq)}$$

$$4Fe^{2+}_{(aq)} + O_{2\ (g)} + 4H^+_{(aq)} \longrightarrow 4Fe^{3+}_{(aq)} + 2H_2O_{(l)}$$

$$4Fe^{3+}_{(aq)} + 12H_2O_{(l)} \longrightarrow 4Fe(OH)_{3(s)} + 12H^+_{(aq)}$$

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Of the two forms, Fe^{2+} is more bioavailable and is readily taken up into aquatic organisms through divalent metal transporters found in the gills and the intestines (Gunshin et al. 1997; Dorschner and Phillis 1999; Bury et al. 2001; Bury et al. 2003).

2.2. The Fish Gill

Fish gills are utilized for respiration, excretion acid-base regulation and osmoregulation and are in constant and direct contact with the aqueous environment. Gills tissue responds quickly to alterations in water chemistry and quality with changes to tissue architecture (Hughes 1984; Nero et al. 2006; Ogundiran et al. 2009). Normal fish gills have a large surface area, minimized diffusion distance and counter-current gas exchange that allows the gills to be effective respiration organs. Teleost fish gills are comprised of the outer operculum, gill rakers and gill filaments. Gill filaments are broken down into the primary and secondary lamellae (Figure 4). The primary lamellae have a thick epithelium that contain specialized cells for mucous production (mucous cells) and ion exchange (chloride cells). The secondary lamellae are found on the lateral side of the primary lamella and are the area where the majority of gas exchange occurs (Figure 4). The surface of the secondary lamellae is composed of elongated squamous epithelial cells. The secondary lamellae are supplied blood by lamellar blood capillaries, separated by pillar cells (Figure 4). Counter-current flow of blood and water makes it possible for uptake of up to 80% of oxygen from the water that comes in contact with the gills (Randall and Daxboec 1984). Under hypoxic conditions the heart beats in phase with gill oscillations. When this occurs, the gill capillaries can be fully filled with deoxygenated blood through an increased cardiac stroke volume that approaches the volume of the gills. The change

in heartbeat rate and stroke volume slows the flow of the blood through the gills. Under high water flow conditions this process results in increased gas diffusion through the gills (Randall and Smith 1967; Randall and Daxboeck 1984). Histological changes in sensitive fish tissues such as the gills have been used as a bioindicator for monitoring chemicals in the aquatic environment (Velisek et al. 2009). Gill tissue alterations often include adaptive barrier mechanisms that reduce the surface area and increase diffusion distance and are nonspecific changes the gill undergoes when exposed to toxicants and irritants (Mallatt 1985). Gill architectural alterations in response to toxins and irritants include, epithelial hypertrophy, hyperplasia, lamellar fusion, intraepithelial edema, epithelial lifting, excessive mucus secretion and, in extreme cases, aneurysms and necrotic lesions (Leino et al. 1987; Poleksic and Mitrovic-Tutundzic 1994; Ortiz et al. 2003; Flores-Lopez and Thomaz 2010). Gill alterations can lead to increased diffusion distance; such alterations to the secondary lamellae have particular impacts to the ability of a fish to efficiently diffuse oxygen into the blood. Tuurala (1983) found that morphologic changes in rainbow trout gills exposed to zinc corresponded with decreased ability to oxygenate the blood effectively.
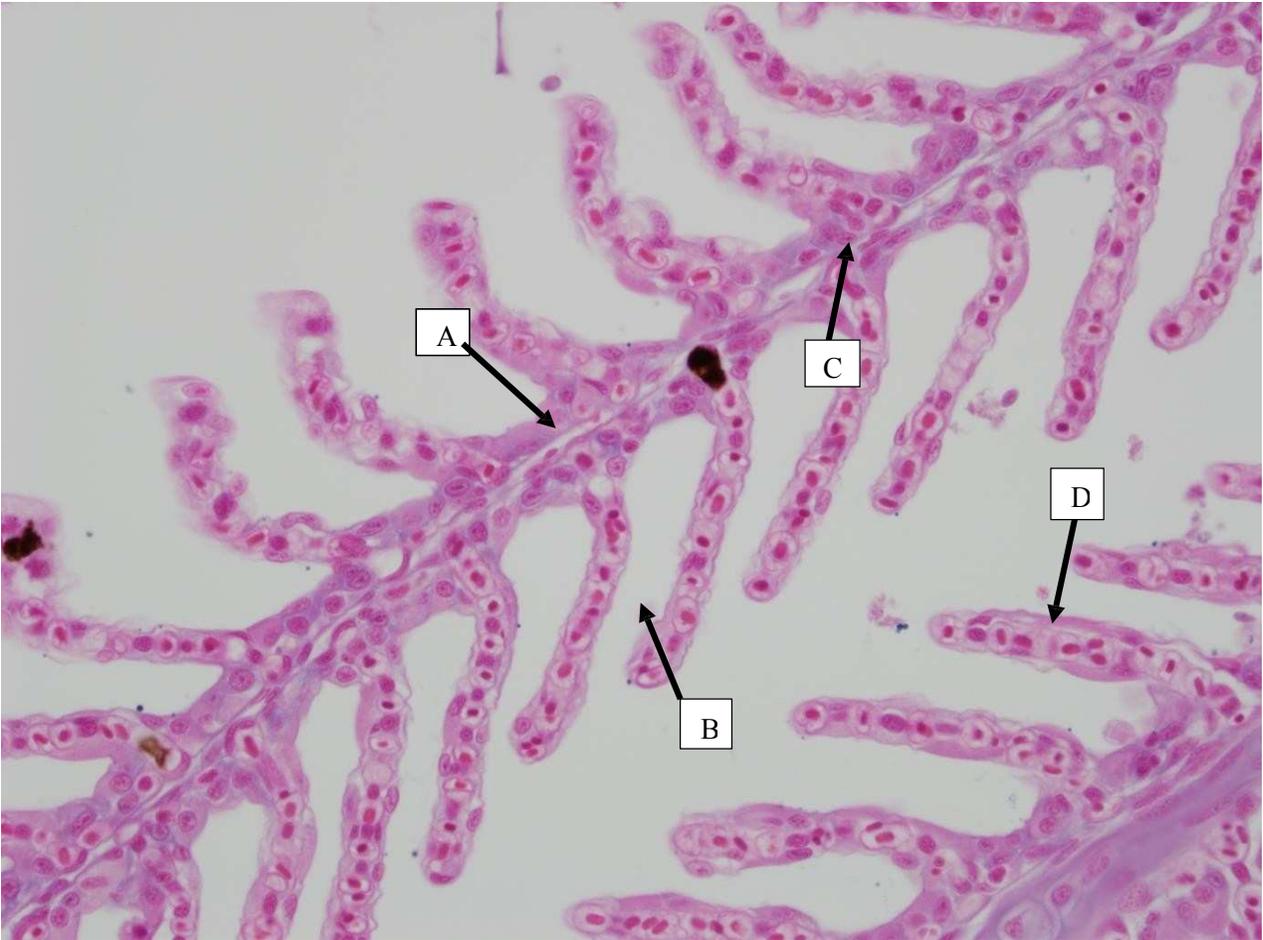
Figure 4. Gill filament sagittal section A. primary lamellae, B. secondary lamellae, C. chloride cell, D. erythrocyte within capillary lumen.

2.3. Effects of Metal Exposure on Fish Gills

Previous fish studies using whitefish (*Coregonus lavaretus)* and brown trout (*Salmo trutta*) that focused on Fe^{2+}, found that exposure to iron resulted in direct physical damage and obstruction of the gills (Dalzell and Macfarlane 1999; Lappivaara and Marttinen 2005). Osman and Werner (2010) found that, of eight metals investigated, iron accumulated on the gills of African Catfish (*Clarias gariepinus*) at higher rates than zinc, manganese, lead, chromium, copper, cadmium, mercury. Korai et al. (2010) also detected high levels of iron accumulation on cyprinid gills exposed to a combination of toxic metals found in Keenjhar Lake. Epithelial

lifting, vacuole formation, hypertrophy, epithelial cell necrosis, lamellar rupture, lamellar

fusion and the formation of subepithelial spaces have been observed in gill tissue exposed to

Fe^{3+} precipitates (Peuranen et al. 1994; Dalzell and Macfarlane 1999). This type of gill damage

results in decreased surface area for gas exchange as well as increased diffusion distance.

Decreased surface area and increased diffusion distance reduces the ability of the gills to

function and decrease the rate of oxygen diffusion into the blood (Tuurala and Soivio 1983;

Yasser and Naser 2011).

2.4. Effects of Toxic Metals on Fish Behavior

Sublethal exposure to inorganic metals has been observed to elicit behavioral changes

in fish at concentrations where physiological changes are not detected (Little and Finger 1990).

Alterations to fish behavior include avoidance of aquatic contaminants, impaired feeding,

impaired olfaction, changes in activity level, altered orientation and abnormal respiratory

responses such as coughing and abnormal ventilation behavior (Giattina and Garton 1983;

Atchison et al. 1987; Hartwell et al. 1987; Woodward et al. 1997; Hansen et al. 1999).

Avoidance behaviors observed in fish at sublethal concentrations may not be seen when higher

metal concentrations are encountered (Giattina and Garton 1983; Hartwell et al. 1987).

Swimming performance and altered migration have also been observed in salmonids exposed

to metals (Lorz et al. 1978; Waiwood and Beamish 1978).

2.5. Physiological Indicators of Contaminant Stress in Fish

Oxygen consumption rates can increase 5 to 20 fold over resting in a fish swimming at maximal levels of sustained exercise (Brett 1964; Randall and Daxboeck 1984). Due in part to this increased consumption of oxygen, changes in aerobic swimming ability in fish exposed to a contaminant is of interests. The maximum sustainable swimming speed, or U_{crit}, has been found to be a viable measure of aerobic swimming ability (Farrell et al. 1996; Gregory and Wood 1998; Brauner et al. 2000; Plaut. 2001; MacNutt et al. 2004; MacNutt et al. 2006). Exposure to environmental stressors such as elevated temperature, low pH, aquatic toxicants and toxic metals has been found to result in decreases in U_{crit} that may contribute to decreased survival in the wild (Waiwood and Beamish 1978; Butler et al.1992; Wilson and Wood 1992; Nikl and Farrell 1993; Beaumont et al. 1995; Dalzell and Macfarlane 1999). Decreases in U_{crit} can be indicative of physiological changes within the fish that may result in negative effects on fitness.

In ectotherms, such as fish, the critical thermal maximum (CT_{max}) has been widely utilized to test the upper thermal tolerance (Becker and Genoway 1979; Lutterschmidt and Hutchison 1997). Previous experiments have found that CT_{max} in cutthroat and rainbow trout decreases as the oxygen carrying capacity of hemoglobin decreases (Beers and Sidell 2011). Precipitated toxic metals such as ferric iron oxide (Fe_2O_3) can coat fish gills and cause gill damage, resulting in increased diffusion distance and a decreased rate of gas diffusion across the gills (Peuranen et al. 1994; Lappivaara et al.; Teien et al. 2008; Fish 2009). The reduction in diffusion rate negatively impacts the rate of oxygen uptake by blood hemoglobin (Tuurala and Soivio 1983; Peuranen et al. 1994; Yasser and Naser 2011).

The potential effects of precipitated metals such a ferric iron on a fish specie's CT_{max} is significant due in part to Colorado's 1085 km of rivers and streams impacted by iron (USEPA 2015). Precipitated iron adds to the total solids (TS) in water, increasing the total suspended solids in water (TSS). Increased TSS results in increased water temperature in streams and rivers and reduced dissolved oxygen. Elevated water temperature in combination with physical effects of precipitated iron on gill tissue increases the energy costs for gill ventilation (Roberts 2012). Decreased dissolved oxygen content in warmer water, reduced oxygen diffusion rate and the added energy required to ventilate the gills can result in reduced fitness as well as decreased ability to compensate when exposed to additional stressors. Reduced fitness combined with climate change can negatively impact the habitat range of a sensitive fish species. Other effects can include increased susceptibility to predation, increased susceptibility to interspecific competition and displacement by a more thermally tolerant fish species (Bear et al. 2007; Pörtner et al. 2007; Somero 2009).

The optimal temperature range of fish species varies by species. Regional differences in some fish species' CT_{max} have also been noted (Carline and James 2011). The optimal temperature range of cutthroat trout is between 13.6°C and 16.4°C, although they are able to tolerate a wider range of temperatures (Vigg and Koch 1980; Bell 1986; Bear et al. 2007; Myrick 2008; Todd et al. 2008; Brandt 2009). The incipient lethal temperature for cutthroat trout is 19.6 °C (Bear et al. 2007). Previous studies have found that smaller sized trout have higher thermal tolerance than larger trout (Selong et al. 2001; Bear et al. 2007; Underwood et al. 2012).

III. MATERIALS AND METHODS

3.1. Experiment One: Critical Maximum Swim Speed of Juvenile Rainbow Trout

Rainbow trout (*Oncorhynchus mykiss*) eggs were obtained from the Colorado Department of Parks and Wildlife's Bellvue-Watson fish hatchery in Bellvue, Colorado. Upon arrival at the Colorado Division of Parks and Wildlife Aquatic Toxicology Laboratory in Fort Collins, Colorado, the eggs were treated with 1600.0 mg/L formalin for fifteen minutes (Piper et al. 1982). The eggs were then placed in holding tanks maintained at a mean temperature of 15°C (+/- 1°C) with a continuous flow of dechlorinated Fort Collin's municipal tap water. Fry were fed starter trout chow and reared up until they were approximately 4 cm long.

Juvenile rainbow trout were exposed to three concentrations of Fe^{3+}: 1.0 mg/L, 3.0 mg/L, 9.0 mg/L and a control of 0.0 mg/L. The 10.0 mg/L iron stock solutions were prepared by dissolving ferric chloride hexahydrate ($FeCl_3$ $6H_2O$, Mallinckrodt™ analytical reagent grade) in 20 L of H_2O with NaOH (1:3 stoichiometry or $FeCl_3 + H_2O -> 3Fe(OH)^3 + 3$ HCl). An airstone was placed into the carboy with the stock solution in an effort to keep the precipitated iron in the water column. Twenty-four 9.5 L round replicate tanks that housed ten fish each were randomly assigned to one of the three iron concentrations or as the control group. A 10.0 mg/L Iron ($Fe(OH)_3$) stock solution was pumped at a rate of 2 mL/min into a continuous-flow serial diluter. An airstone was placed into the serial diluter to mix the $Fe(OH)_3$ evenly into the water column. Dechlorinated Fort Collin's municipal tap water flowed into the diluter at the rate of 90 mL/min (Benoit et al. 1982). The iron/water mix was diluted to the appropriate $Fe(OH)_3$

concentration in the serial diluter, and then delivered to each experimental tank via Nalgene™ food-grade vinyl tubing. Each round 9.5 L treatment tank had a moderate circular current generated by an airstone. The airstone also helped to suspend the $Fe(OH)_3$ in the water column. The 24 experimental tanks were randomly assigned treatments in six blocks of four tanks, each consisting of one 0.0 mg/L control tank and three treatment tanks that received 1.0 mg/L, 3.0 mg/L, or 9.0 mg/L $Fe(OH)_3$. The temperature for the duration of the experiment was maintained at approximately 15°C (+/- 1.5°C) via a chilled water bath using a recirculating chiller (VWR ™ model 1175MD). Fish were fed trout chow once a day at a rate of 4% of body weight per day. Lighting was provided by ambient fluorescent lights for 16 hours a day with an 8 hour darkness period.

Water quality parameters tests were conducted weekly for alkalinity, dissolved oxygen (DO), conductivity and pH. An electronic Oakton™ Model 300 meter, calibrated prior to each test was used to measure pH and DO. A YSI™ model 35 conductance meter was used to test conductivity. VWR Scientific Products™ sulfuric acid and a Brinkman™ Digital Bottle-Top Buret (50.0 mL) were used to determine alkalinity via titration.

Grab samples of filtered (0.45 μ syringe filter) and unfiltered water samples were collected weekly to determine measurable Fe^{2+} and Fe^{3+} concentration in each experimental tank. All samples were collected in 2 oz HDPE bottles (Nalgene™) and preserved with high purity nitric acid (Avantor, Center Valley, PA). Sample splits were collected each week for reproducibility and recovery quality assurance. All water samples were analyzed using a calibrated Instrumentation Laboratory™ Video 22 atomic absorption spectrometer (Allied

Analytical Systems, Franklin, MA) with an air-acetylene flame and Smith-Hieftje background correction.

At one and three weeks of exposure, all fish were fasted for 24 hours prior to critical swim speed (U_{crit}) trials. Two randomly selected fish from each experimental tank were placed singularly into one of four swim chambers sitting in a tank of 15°C (+/- 1°C) chilled and de-chlorinated Fort Collin's municipal tap water. Water temperature in the swimming tank was maintained with recirculating chiller (Delta Star™ water heater pump model DSHP-7). An airstone was placed in the corner of the swimming tank to oxygenate the water. Swim chambers were designed by Steve Brinkman. Each swim chamber's water velocity was created with a Lifeguard Aquatics™ Quiet One 4000 powerhead. The water velocity was determined using a calibrated Swoffer ™ water velocity meter (model 2100). The fish were allowed to acclimate for 10 minutes in the swim chambers. Prior to the beginning of the experiment, the mean body length of the fish was determined and used to establish a logical starting water velocity and to determine what the incremental water velocity rate would be.

The swimming velocity tests were conducted for a period of 20 minutes at each velocity. The velocity was steadily increased incrementally until the fish was unable to maintain its position in the water column. The U_{crit} of the fish was the velocity prior to the final velocity when the fish became fatigued, stuck on the screen for over ten seconds and was unable to free itself when the water current was momentarily decreased (Lee et al. 2003). Once the U_{crit} for a fish was reached, the fish was removed and placed into a recovery tank for 20 minutes (Plaut 2001). The equation U_{crit} (cm/s) = U_{fin} + (t_{fail}/t_{int})U_i, was used to establish the U_{crit} of each fish, where, U_{fin} is the final interval water velocity at which steady swimming

could be maintained, t_{fail} is the time spent at the final, failed interval (min) and t_{int} is the time

interval between speed increments (20 min) and U_i is the velocity increment (Brett 1965).

This is a sublethal experiment; the data from fish that failed to recover and expired

during the experiment or during the post U_{crit} recovery period were not used. After the recovery

period, the fish were euthanized in tricaine methanesulfonate (MS-222). The length and weight

of each fish was recorded and the fish were preserved in Bouin solution. Histological sections

were prepared from fish gills and stained with hematoxylin and eosin (H&E). Two of the H&E

slides were later restained in an attempt to better define the chloride cells. Two additional

slides were stained with Prussian blue for evidence of precipitated iron on the gills, and two

slides were stained with periodic acid-Schiff (PAS) stain to examine the number of mucous

cells and mucous present. The slides were examined for histological changes and diffusion

distances from inner capillary endothelium to outer cell epithelium were measured on the

primary and secondary lamellae using an Olympus™ U-TV0.65XC microscope camera. Ten

measurements from inner capillary wall to outer epithelial cell wall were taken at random

points on the primary and secondary lamellae on each slide. The mean distance was used to

test for significant changes to the diffusion distances in primary and secondary lamellae. The

samples were randomly assigned numbers and the treatment associated with the sample was

unknown until the slides had all been read and the results were recorded to eliminate bias. The

slides were examined for observable histological changes in the control fish and the fish

exposed to iron.

3.2. Experiment Two: Critical Thermal Maximum of Juvenile Cutthroat Trout

Cutthroat trout (*Oncorhynchus clarkii)* eggs were obtained from Bellvue-Watson fish hatchery (Bellvue, Colorado). Upon arrival at the Colorado Division of Parks and Wildlife Aquatic Toxicology Laboratory in Fort Collins, Colorado, the eggs were treated with 1600.0 mg/L formalin for fifteen minutes (Piper et al. 1982). The eggs were then placed in holding tanks maintained at a mean temperature of 13°C (+/-1°C) with a continuous flow of dechlorinated Fort Collin's municipal tap water. Fry were fed starter trout chow and reared up until they were approximately 3 cm long.

Juvenile cutthroat trout were exposed to two nominal concentrations of Fe^{3+}: 1.0 mg/L, 10.0 mg/L and a control of 0.0 mg/L. Iron stock solutions were prepared (20 mg/L) by dissolving ferric chloride hexahydrate ($FeCl_3$ $6H_20$, Mallinckrodt™ analytical reagent grade) with addition of NaOH (1:3 stoichiometry or $FeCl_3 + H_2O \rightarrow 3Fe(OH)^3 + 3\ HCl$). Peristaltic pumps were set at a rate of 2 mL/min of the stock $Fe(OH)_3$ solution. A continuous flow of approximately 70.0 mL/min of chilled, dechlorinated Fort Collin's municipal tap water maintained at approximately 13°C (+/- 0.6°C) was delivered to the 10.0 mg/L Fe^{3+} experimental tank. A continuous flow of approximately 90.0 mL/min of chilled, dechlorinated Fort Collin's municipal tap water maintained at approximately 13°C (+/- 0.6°C) was delivered to the 1.0 mg/L Fe^{3+} experimental tank. A continuous flow of approximately 90.0 mL/min of chilled, dechlorinated Fort Collin's municipal tap water maintained at approximately 13°C (+/- 0.6°C) was delivered to the 0.0 mg/L Fe^{3+} experimental tank. Iron concentration, water flow rate and temperature were monitored daily. The experimental tanks and the carboys with the stock solutions were aerated to suspend iron precipitates in the water

column. The three 75 L experimental tanks were initially stocked with 25 fish per tank. Fish were fed trout chow once a day at a rate of 2% of body weight per day. Lighting was provided by ambient fluorescent lights 16 hours a day with an 8 hour darkness period.

Weekly water quality parameters test were conducted, measuring dissolved oxygen (DO), conductivity and pH. The pH and DO were taken using an electronic Oakton™ Model 300 meter, calibrated prior to each test. A YSI™ model 35 conductance meter was used to test conductivity.

Grab samples of filtered (0.45 µ syringe filter) and unfiltered water samples were taken weekly to determine measurable Fe^{2+} and Fe^{3+} concentration in each tank. All samples were collected in 2 oz HDPE bottles (Nalgene™) and preserved with Ultrex™ nitric acid (Avantor, Center Valley, PA). Sample splits were collected each week for reproducibility and recovery quality assurance. All water samples were analyzed using a calibrated Instrumentation Laboratory™ Video 22 atomic absorption spectrometer (Allied Analytical Systems, Franklin, MA) with an air-acetylene flame and Smith- Hieftje background correction.

At one and two weeks of exposure, the fish were fasted for 24 hours prior to the critical thermal maximum (CT_{max}) trials. Ten randomly selected fish per treatment were tested at each of the exposure time periods (ten at one week and ten at two weeks of exposure) to determine each fish's CT_{max}, with a total of 20 fish from each treatment tank tested. The test fish were place singly in an insulated glass aquarium CT_{max} test tank holding 2 L of dechlorinated water chilled to 13°C. Each CT_{max} test tank was equipped with a Love Controls™ temperature controller that controlled an Aqueon™ 100 watt heater. A Traceable™ thermometer (-50°C to

150°C) was used to measure the temperature of the water. Each tank had an airstone that was used to circulate the water in the CT_{max} test tanks. The water temperature in each tank was increased by 0.3°C per minute. Fish behavior and tank temperature was continuously monitored during the CT_{max} testing. Fish were closely observed for signs indicating that the fish had reached its critical maximum thermal tolerance. The CT_{max} is defined as the temperature at which the fish cannot remain swimming upright, or loss of righting response (LRR) (Beers and Sidell 2011).

Once the LRR endpoint was reached, the fish was promptly removed from the thermal test tank, placed into a recovery tank with 13°C water and allowed to recover over 20 minutes. This experiment was designed as a sublethal test of maximum thermal tolerance, the recovery period was to ensure that the fish would recover. After the recovery period, the fish were euthanized in tricaine methanesulfonate (MS- 222). Weight data were recorded for each fish and the fish was preserved in Bouin solution.

3.3. Statistical Analysis

Experiment One: Critical Maximum Swim Speed of Juvenile Rainbow Trout

Statistical analyses of data was conducted using SAS™ 9.3 and Microsoft Excel™ 2010 software. Two way analysis of variance (mixed ANOVA) was used to test if there was a relationship between ferric iron exposure and the sublethal endpoints of U_{crit}, gill diffusion distance and fish weight. T-tests were run to compare the mean U_{crit} and diffusion distance from fish exposed to one and three weeks of ferric iron. Differences between mean U_{crit}, fish

weight, fish length and gill diffusion distance of the exposed fish and control fish will be considered significant at $P < 0.05$.

Experiment Two Critical Maximum Temperature of Juvenile Cutthroat Trout

Statistical analyses of data was conducted using SAS™ 9.3 and Microsoft Excel™ 2010 software. Two way analysis of variance (ANOVA) was used to test if there was a relationship between ferric iron exposure and the sublethal endpoints of CT_{max} and changes in fish weight. A T-test was run to compare mean CT_{max} from fish exposed to one and two weeks of ferric iron. Differences between mean CT_{max} of fish exposed to one and two weeks of ferric iron and the control fish will be considered significant at $P < 0.05$.

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IV. RESULTS

The results of the swimming speed (U_{crit}) and thermal tolerance (CT_{max}) studies examined effects from chronic sublethal exposure to ferric iron on the endpoints of U_{crit}, CT_{max}, weight, length and changes in gill diffusion distance. The U_{crit} study was conducted over a three week period with swim trials at one and three weeks of exposure. The CT_{max} study was conducted over a two week period with CT_{max} tested at one and two weeks of exposure. The results from the U_{crit} and CT_{max} studies will be pooled with results from additional studies conducted by the CDPW to aid in creating a new iron water quality standard.

4. 1. Experiment One: Critical Maximum Swim Speed of Juvenile Rainbow Trout

There were no significant effects from chronic Fe^{3+} exposure on juvenile rainbow trout U_{crit}, weight, length or mortality observed at the ferric iron exposure concentrations tested in this experiment. There were no significant differences between the control and exposed fishes' gill diffusion distance in the gill histopathology slides. There were no significant differences between the results from one and three weeks of ferric iron exposure for the measured endpoints of U_{crit} or diffusion distance. Survival rate overall in the experimental tanks was 97.9%. Survival rate during the U_{crit} swim trials was 100%, with all of the fish recovering during the 20 minute post-experiment recovery period.

Filtered and unfiltered water samples were used to assess ferric and ferrous iron concentration using flame atomic absorption spectrometry. There was no detectable iron

measured in the filtered water samples, indicating the absence of the more soluble ferrous form of iron. The results for each exposure concentration from the unfiltered water samples are listed in Table 1. The results from the unfiltered water samples indicated that the concentration of the control was closest to the target concentration, with a background measurement of 0.02 mg/L Fe^{3+}. The water samples from the 1.0 mg/L treatment tank had a measured Fe^{3+} concentration 36% higher than the target Fe^{3+} concentration. In the 3.0 mg/L and 9.0 mg/L target treatment tanks, the mean measured Fe^{3+} was 32% higher than the target concentrations. The control iron concentration had a relative standard deviation (RSD) of 50%. The RDS of the 1.0 mg/L target treatment was 67% and the 3.0 mg/L target treatment concentration had a RSD of 13%. The sample from the 9.0 mg/L target treatment tank had a RSD of 23%.

Table 1. Measured mean Fe^{3+} mg/L concentration.

Target [Fe^{3+}]mg/L	0.0	1.0	3.0	9.0
Measured [Fe^{3+}] mg/L	0.02[a] (0.01)	1.36 (0.91)	3.95 (0.51)	11.87 (2.78)
Sample Size	8	7	8	9

[a.] measured mean concentration (standard deviation)

The mean U_{crit}, length, weight and sample size for juvenile rainbow trout from the first and second set of swim trials for each treatment tank are shown in Table 2. The control fish from the first week's swim trials had the highest measured U_{crit} (26.43 cm s^{-1}/6.03 BL s^{-1}), length (43.20 mm) and weight (0.75 gm). For the second week of swim trials, although not significantly different from the ferric iron exposed fish, the control fish had the fastest

measured U_{crit} (29.78 cm s^{-1}/5.90 BL s^{-1}). Although not statistically significant, the greatest

mean length (51.70 mm) and weight (1.48 g) were measured in the fish exposed to three weeks

of 1.0 mg/L ferric iron. In the initial experimental design, the U_{crit} swim trials were scheduled

to be completed after one and two weeks of exposure. Due to technical difficulties with one of

the powerhead water pumps used to generate the current in the swim chamber, the U_{crit} swim

trials at two weeks of exposure were aborted. The malfunctioning powerhead water pump was

replaced and the U_{crit} swim trial was conducted the following week.

Table 2. Mean U_{crit}, length, weight and sample size of juvenile rainbow trout.

$[Fe^{3+}]$ (mg /L)	U_{crit} (cm s^{-1})[b]	U_{crit} (BL s^{-1})[c]	Total Length (mm)	Weight (dg)	Sample size
1st Week of Exposure					
0.0	26.43[a] (10.23)	6.03 (1.86)	43.20 (4.08)	0.75 (0.30)	6
1.0	23.87 (8.46)	5.55 (1.67)	42.40 (2.59)	0.68 (0.13)	5
3.0	19.07 (3.76)	4.57 (1.23)	42.40 (3.91)	0.71 (0.22)	6
9.0	23.28 (3.52)	5.44 (0.99)	43.00 (1.91)	0.64 (0.08)	5
3rd Week of Exposure					
0.0	29.78 (6.62)	5.90 (1.20)	50.40 (4.04)	1.37 (0.49)	6
1.0	26.08 (4.84)	5.10 (1.19)	51.70 (4.72)	1.48 (0.317)	6
3.0	24.68 (2.12)	5.20 (0.54)	47.70 (4.20)	1.10 (0.28)	6
9.0	22.93 (3.74)	4.46 (0.76)	51.60 (3.76)	1.40 (0.36)	6

[a.]Mean (standard deviation)
[b.]Critical swim speed in cms^{-1}
[c.]Critical swim speed in total body length per second
[d.]Total length is from the tip of snout to tip of the longest lobe of the compressed caudal fin

The mean U_{crit} (cm s^{-1}) of juvenile rainbow trout from the first and second swim trials did not show significant effects from ferric iron exposure (Figures 5 and 6). The R^2 values for the first (0.01) and second swim trials (0.19) did not indicate a correlation between exposure to ferric iron and changes in U_{crit} (cm s^{-1}). There were no significant differences between the mean U_{crit} (cm s^{-1}) of the first and second swim trials (Table 3, Figure 6). The correlation coefficient for the combined U_{crit} (cm s^{-1}) from the first and second swim trials was 0.05, and did not indicate a concentration-dependent effect on U_{crit} (cm s^{-1}) (Figure 7).

Table 3. Two-tailed T-test results comparing mean U_{crit} (cm s^{-1}) results from first and second swim trials (one and three weeks of exposure respectively).

[Fe^{3+}]mg/L	0.0	1.0	3.0	9.0
P value	0.904	0.952	0.103	0.798
Degrees freedom	4	5	4	7
Sample size	12	11	12	11
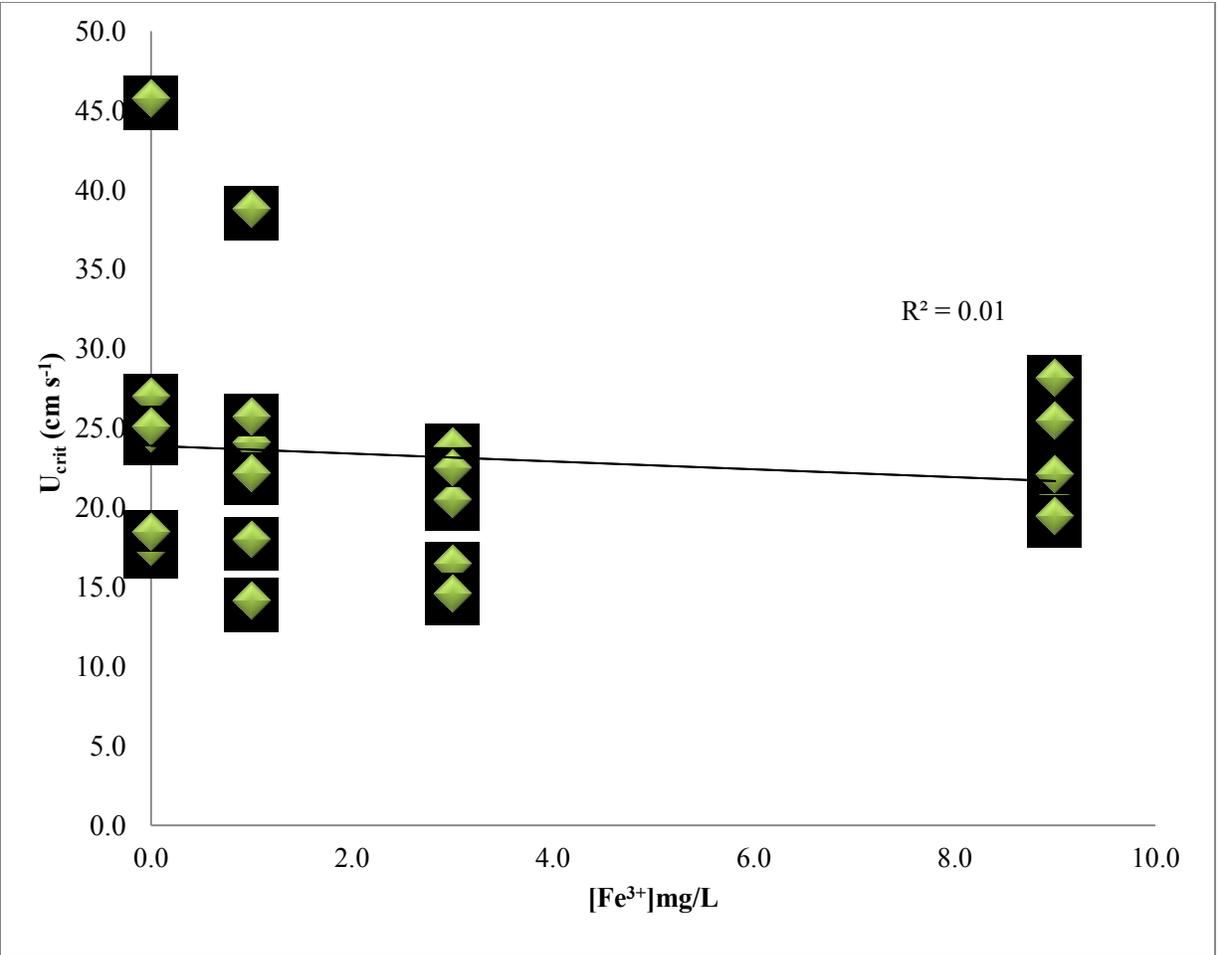
Figure 5. U_{crit} (cm s^{-1}) of juvenile rainbow trout exposed to one week of ferric iron with trendline.
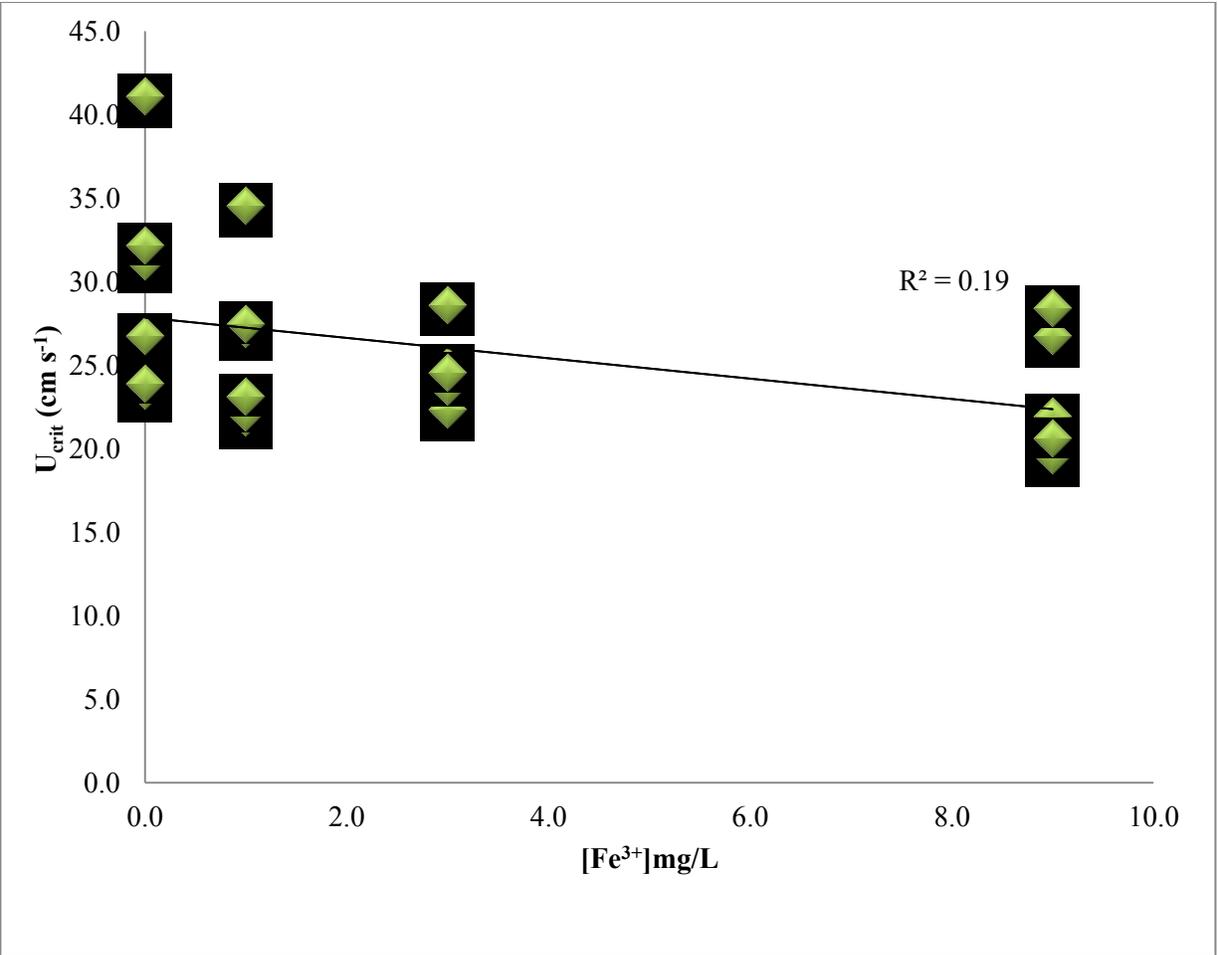
Figure 6. U_{crit} (cm s^{-1}) of juvenile rainbow trout exposed to three weeks of ferric iron with trendline.
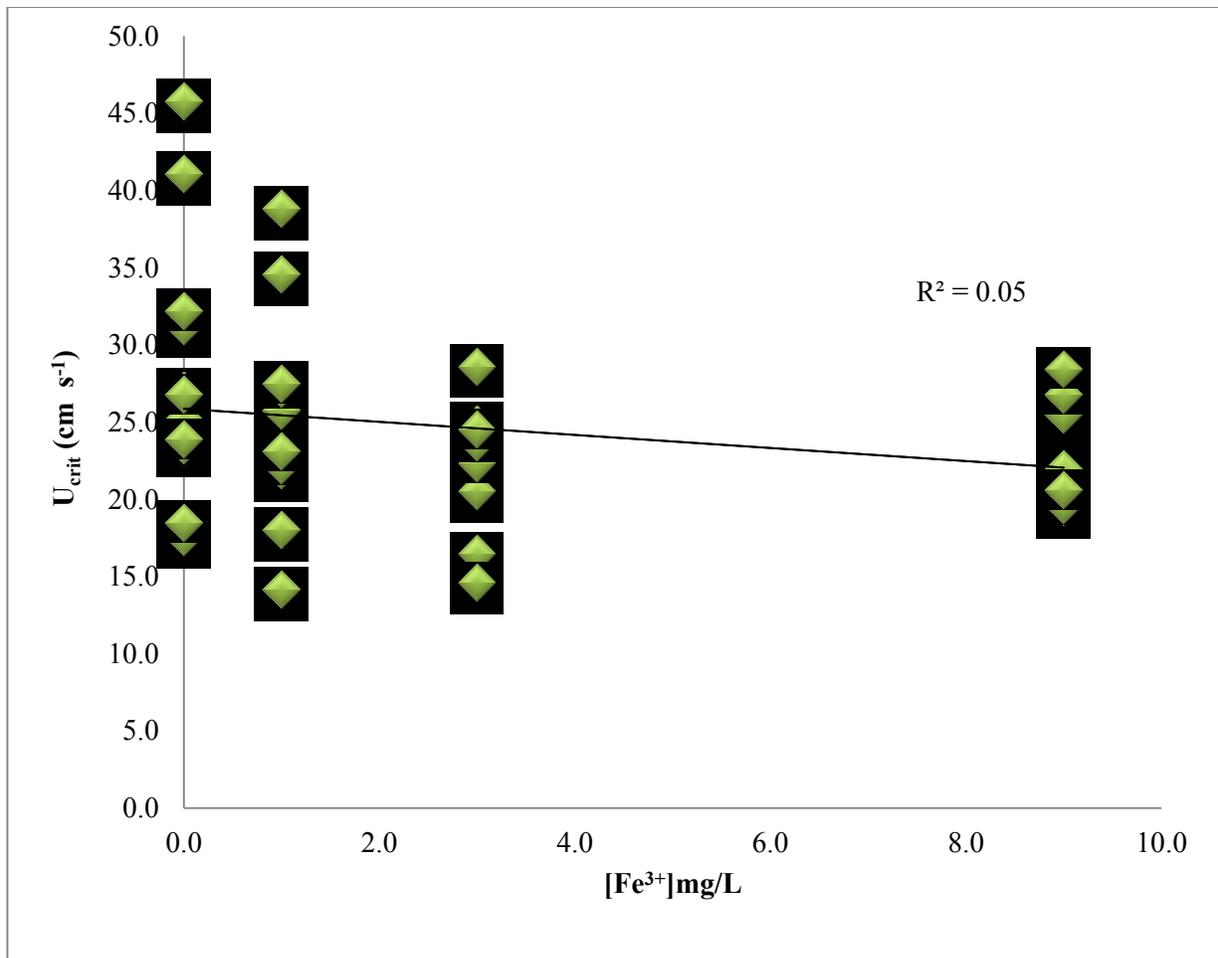
Figure 7. Combined U_{crit} (cm s^{-1}) of juvenile rainbow trout exposed to one and three weeks of ferric iron with trendline.

The mean weight of juvenile rainbow trout from the first swim trials did not significantly differ by treatment group (Figure 8). There were no significant differences in the mean weight of juvenile rainbow trout used in the second swim trials (Figure 9). The lack of significant effect is further confirmed by the first and second week's low correlation coefficient of 0.03 and 0.0 (Figures 10 and 11).
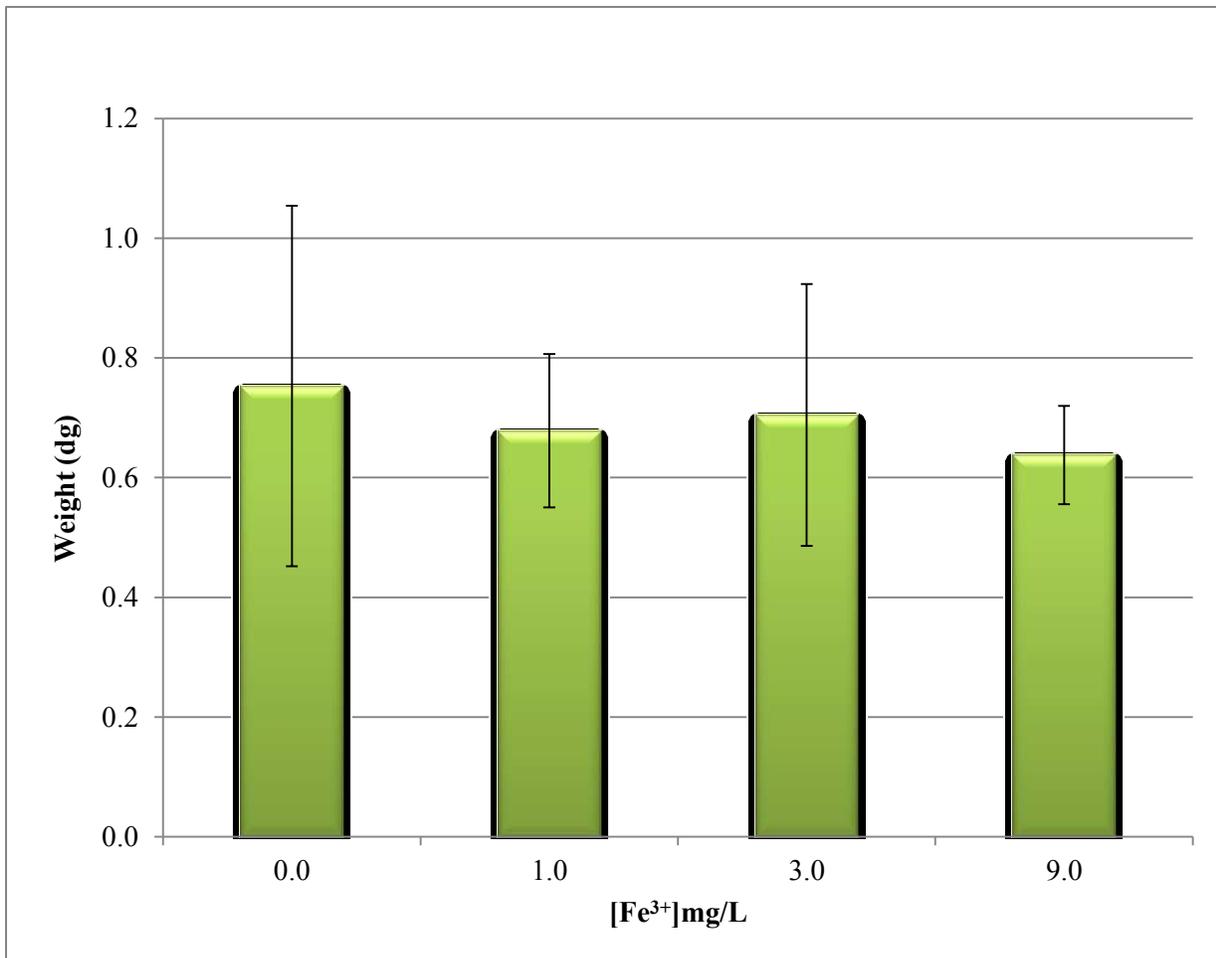
Figure 8. Mean weight of juvenile rainbow trout exposed to one week of ferric iron. Error bars indicate standard deviation.
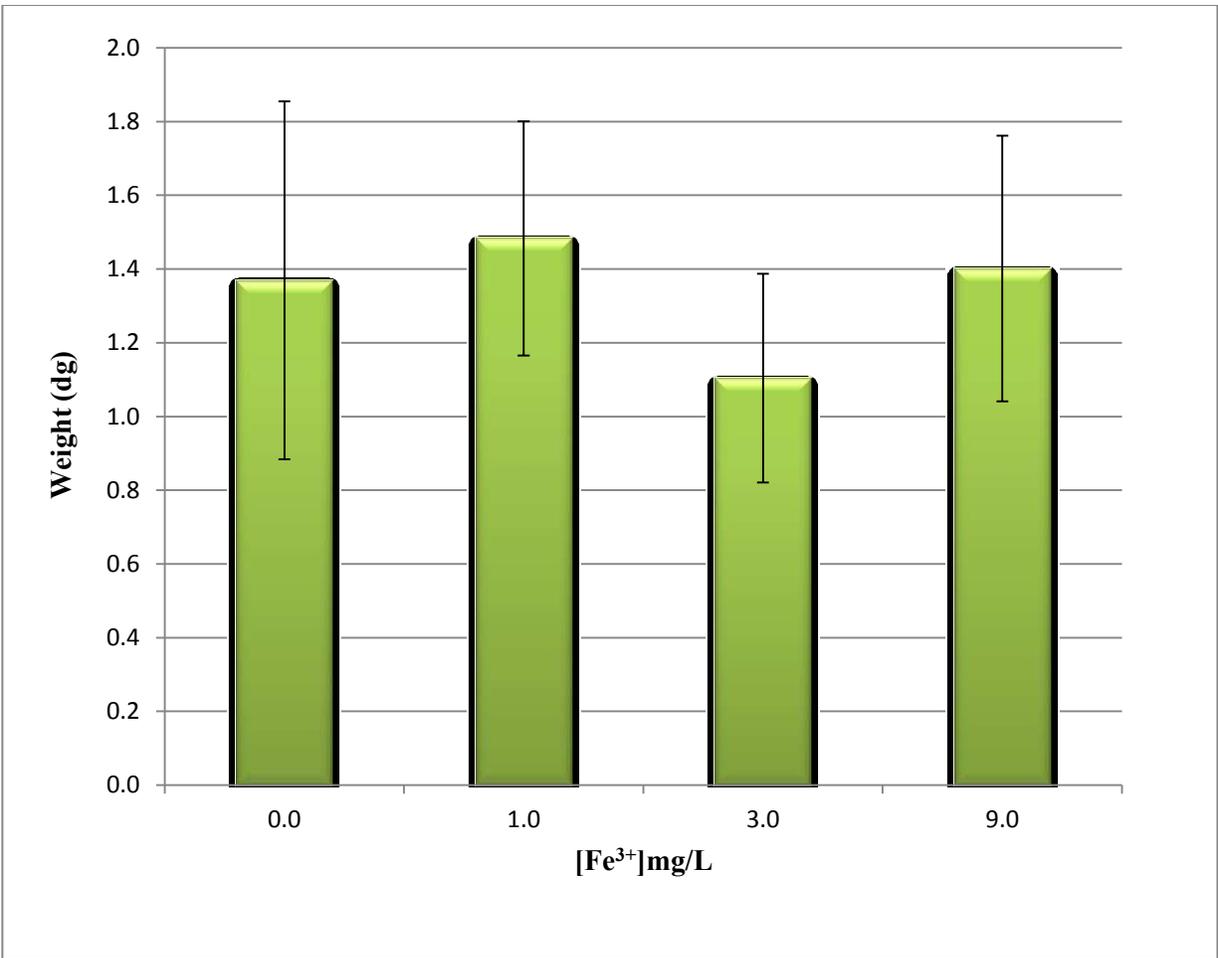
Figure 9. Mean weight of juvenile rainbow trout exposed to three weeks of ferric iron. Error bars indicate standard deviation.
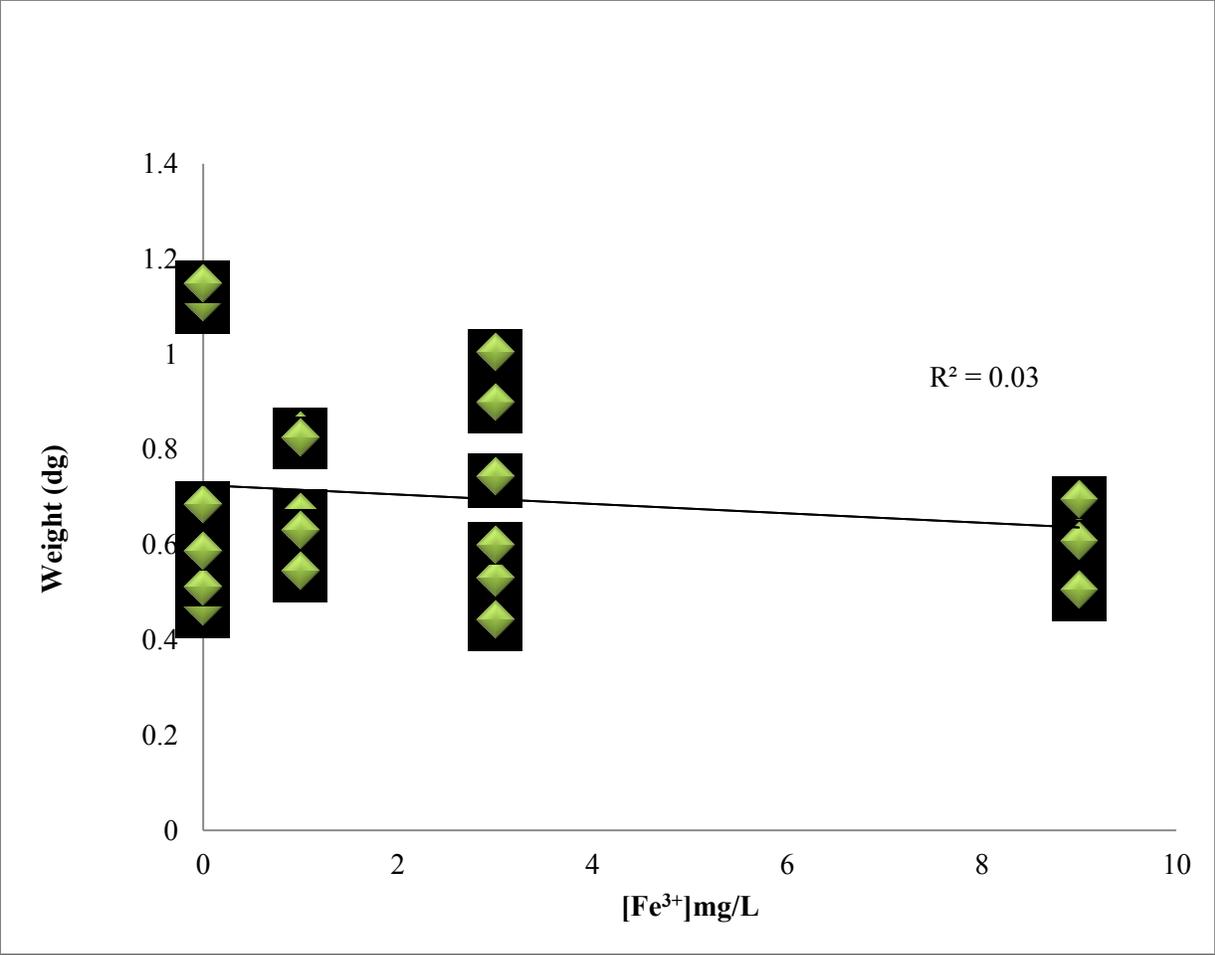
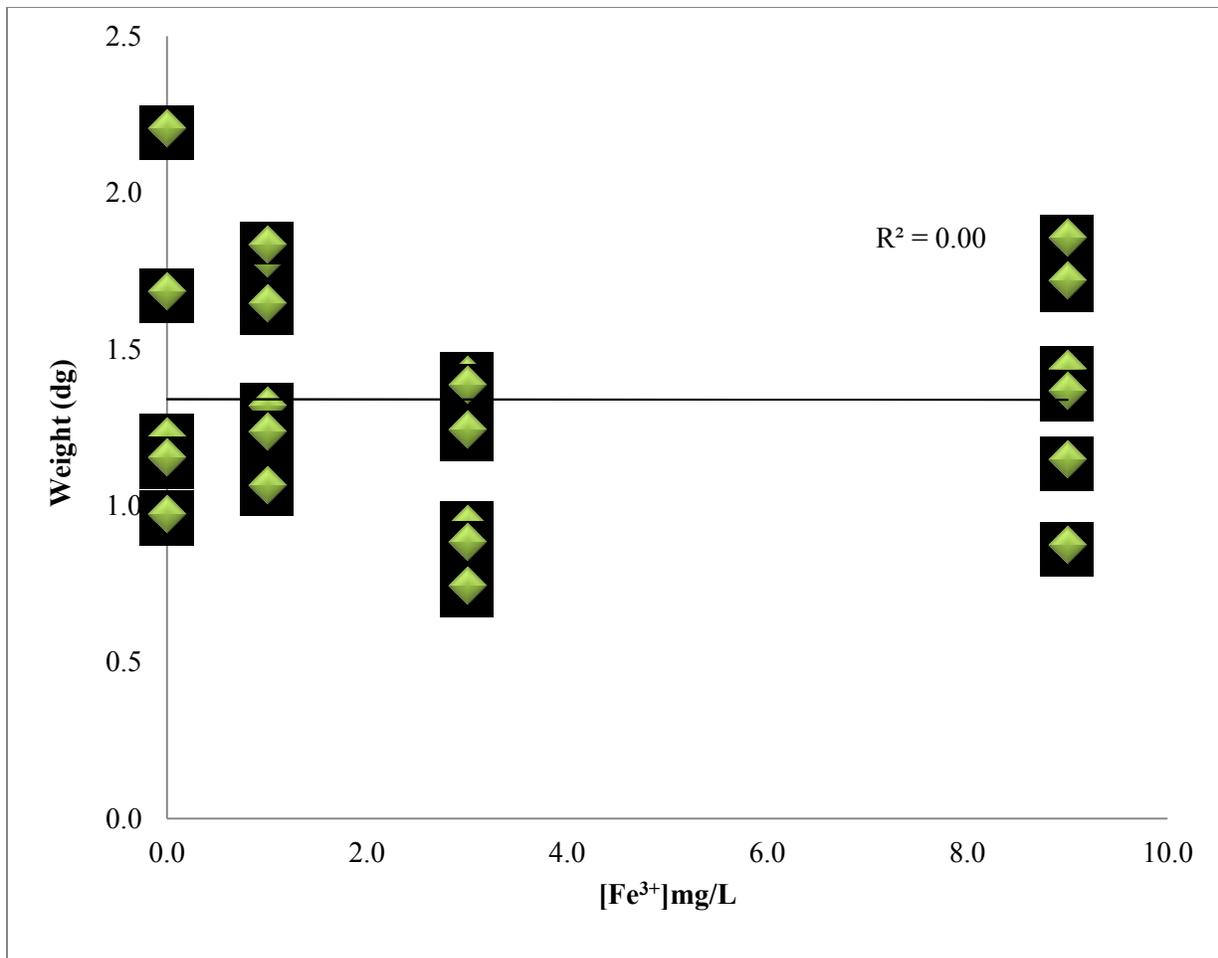Figure 10. Weight of juvenile rainbow trout exposed to one week of ferric iron with trendline.

Figure 11. Weight of juvenile rainbow trout exposed to three weeks of ferric iron with trendline.

There were no significant effects of ferric iron exposure on mean total length (TL) of the fish exposed to one or three weeks of ferric iron (Figures 12-15). For the first swim trials (one week of exposure), the mean total length of the fish ranged from 42.45 mm to 49.96 mm. For the second swim trials (three weeks of exposure), the mean total length of the fish tested ranged from 47.70 mm to 51.70 mm. The low correlation coefficients between length and iron concentration of 0.00 and 0.01 for the first and second swim trials further support the conclusion that there was no significant effect from ferric iron exposure on the total length of juvenile rainbow trout (Figures 14 and 15).
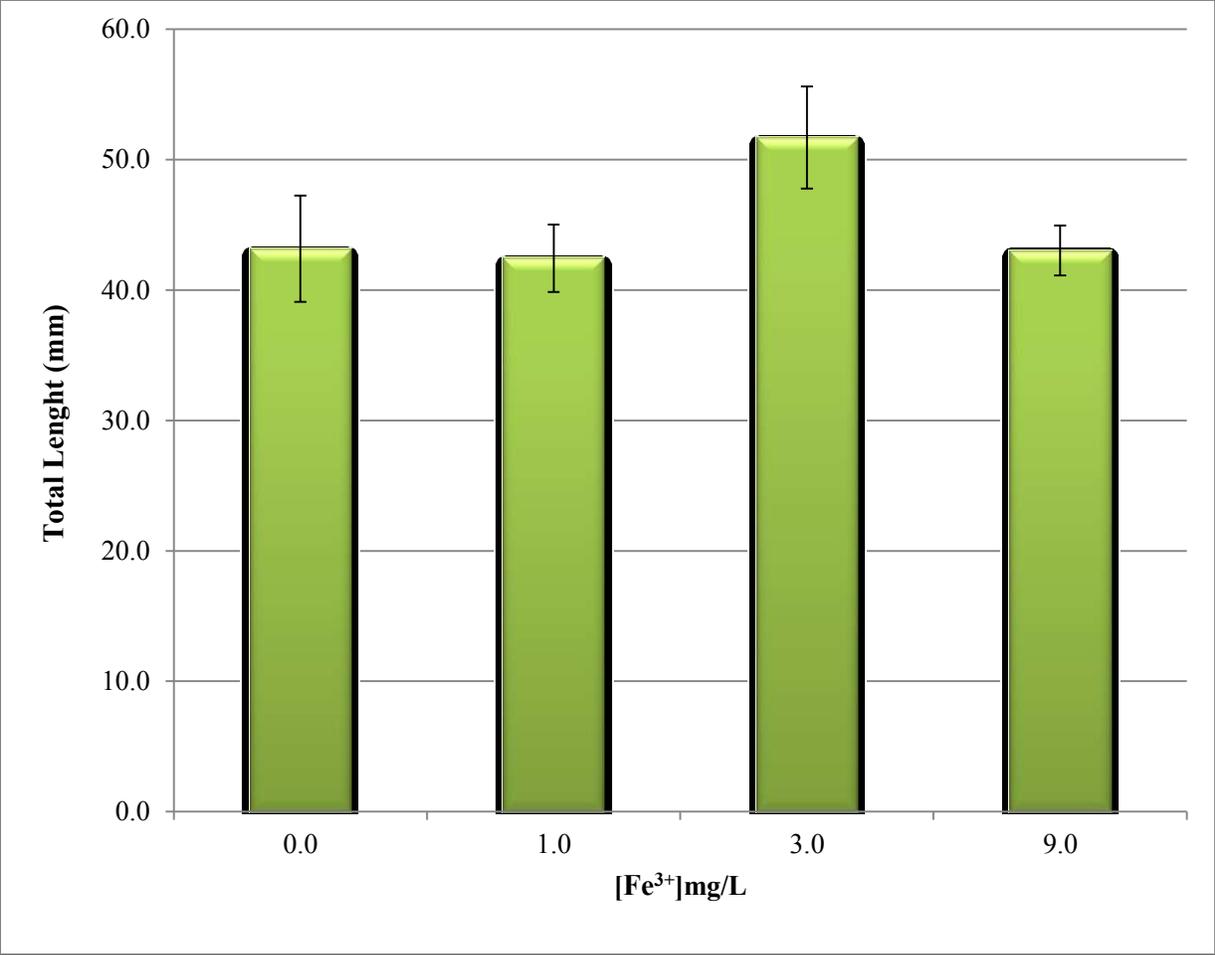
Figure 12. Mean total length of juvenile rainbow trout exposed to one week of ferric iron. Error bars indicate standard deviation.
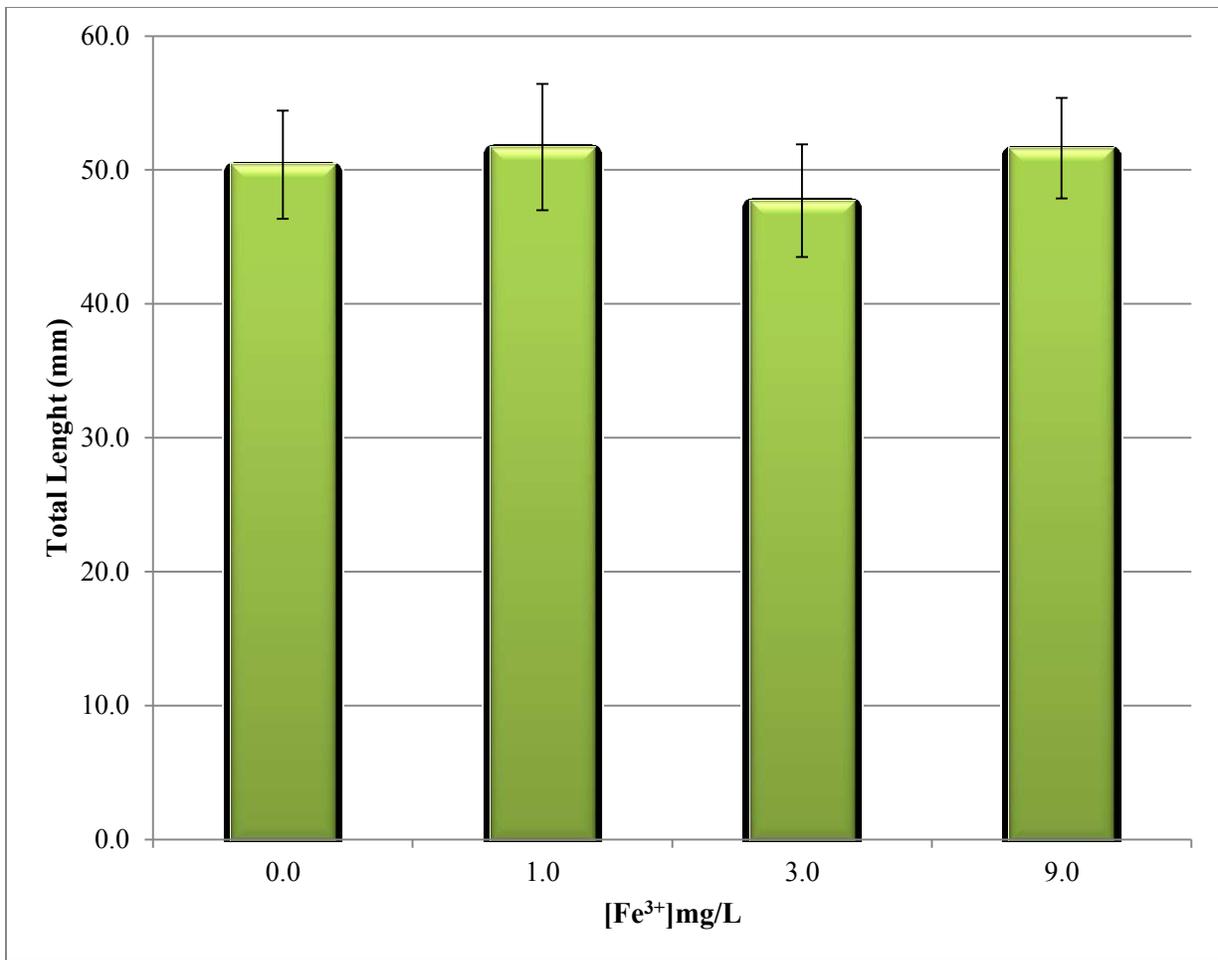
Figure 13. Mean total length of juvenile rainbow trout exposed to three weeks of ferric iron. Error bars indicate standard deviation.
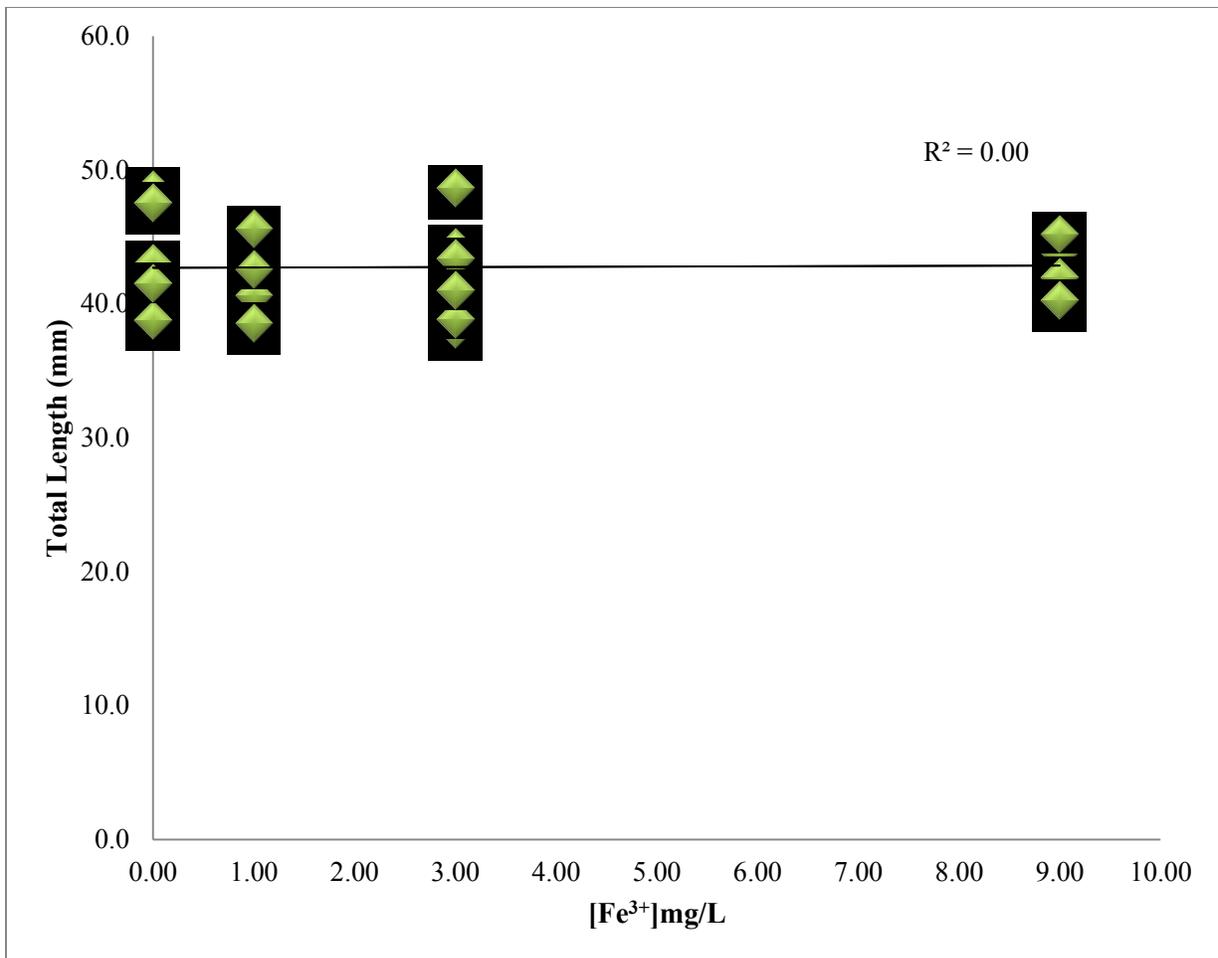
Figure 14. Total length of juvenile rainbow trout exposed to one week of ferric iron with trendline.
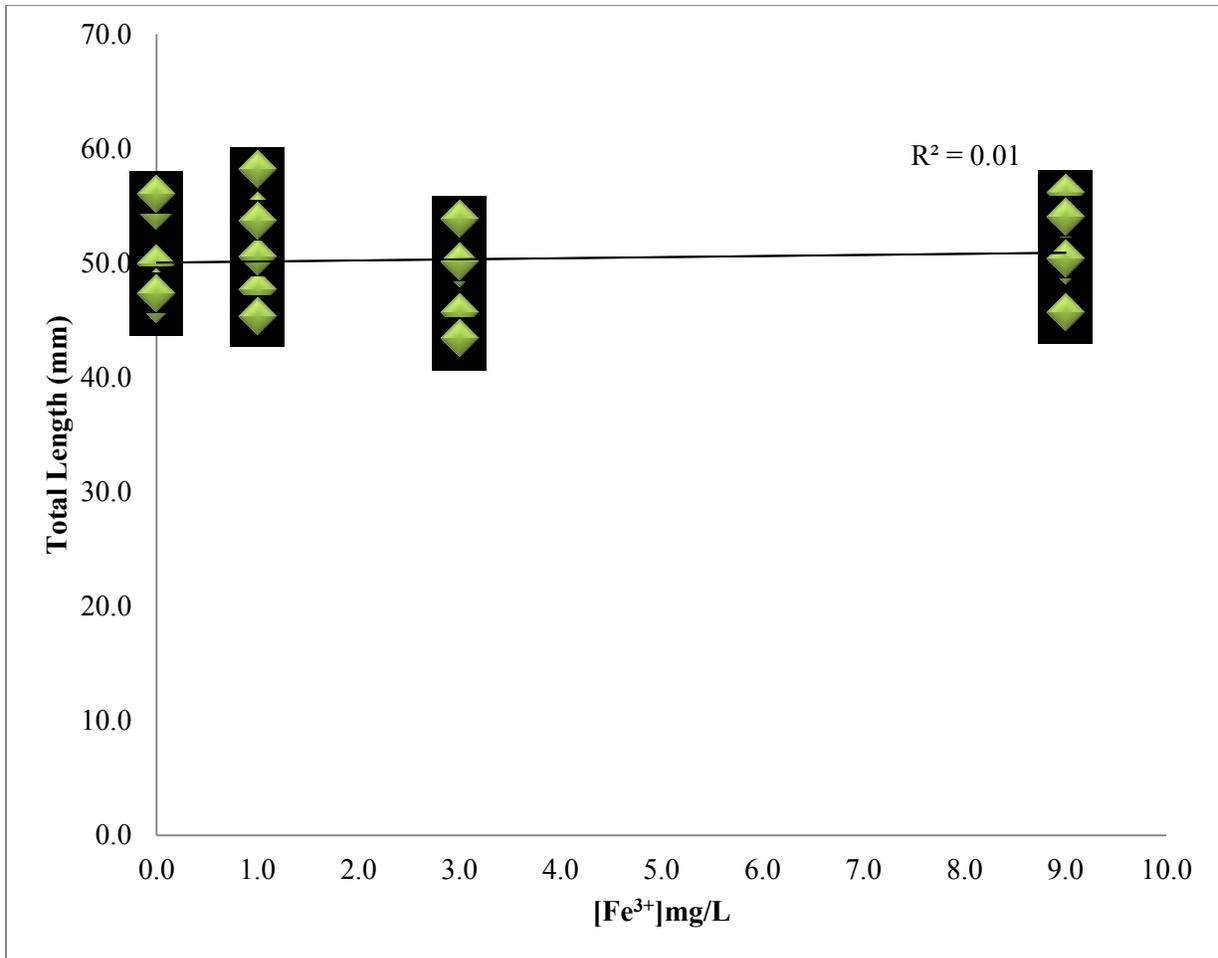
Figure 15. Total length data of juvenile rainbow trout exposed to three weeks of ferric iron with trendline.

Mean diffusion distance of juvenile rainbow trout lamellae was measured following the U_{crit} swim trials (Table 4). An ANOVA indicated that there was no significant effect measured in changes to diffusion distance by ferric iron exposure following either one or three weeks of exposure. Although a significant effect was not noted, the control fish have the shortest measured diffusion distances for primary and secondary lamellae for both sets of swim trials (Table 4). The fish exposed to 9.0 mg/L of ferric iron have the longest diffusion distance for all measurements, except for the primary lamellae diffusion distance after one week of exposure (Table 4). Tables 5 and 6 show the results from two-tailed T-test comparing the mean diffusion distances of primary and secondary lamellae from the data from the first and second swim trials. The diffusion distances for primary and secondary lamellae from one week of exposure were not significantly different from the fish exposed to three weeks of ferric iron. The results from the two-tailed T-test comparing mean diffusion distances of combined primary and secondary lamellae from fish exposed to one and three weeks of ferric iron are shown in Table 7. The correlation coefficients for primary lamellae from fish exposed to one and three weeks of ferric iron are 0.01 and 0.31 (Figures16 and 17). The results do not indicate a concentration-dependent effect on the diffusion distance of the primary lamellae for either exposure duration. These results are supported by the first and third week's secondary lamellae results (Figures 19-20). Although the results for the third week of exposure had a greater correlation coefficient (0.28) than the first week's secondary diffusion distance (0.18), both are far below a correlation coefficient that would indicate a significant effect from exposure to ferric iron. The combined secondary lamellae diffusion distance data from the results from one and three weeks of ferric iron exposure further supports the conclusion that there was not a significant effect from ferric iron exposure on diffusion distance (Figure 21). Table 8 shows the mean diffusion distance of primary and

secondary lamellae from fish exposed to one week of ferric iron. Table 9 shows the mean

diffusion distance of primary and secondary lamellae from fish exposed to three weeks of ferric

iron. Although in Table 9, the greatest diffusion distances are consistently seen in the 9.0 mg/L

exposed fish, the results were not found to be significant (23.39 µm, 5.45 µm, 14.42 µm).

Table 4. Mean lamellae diffusion distance of juvenile rainbow trout by swim trial and ferric iron exposure.

1 Week of Fe^{3+} Exposure	0.0	1.0	3.0	9.0
1° Lamellae diffusion distance (µm)	16.38[a.] (4.63)	16.45 (3.81)	18.83 (4.04)	17.44 (4.55)
2° Lamellae diffusion distance (µm)	3.91 (0.97)	4.50 (1.05)	4.69 (0.61)	5.83 (2.92)
3 Weeks of Fe^{3+} Exposure	0.0	1.0	3.0	9.0
1° Lamellae diffusion distance (µm)	15.97 (3.46)	16.43 (2.35)	20.10 (5.23)	23.39 (6.66)
2° Lamellae diffusion distance (µm)	4.20 (0.69)	4.25 (0.53)	4.75 (0.50)	5.45 (1.40)

[a.] Mean (standard deviation)

Table 5. Two-tailed T-test results comparing mean diffusion distances of primary lamellae from one and three weeks of ferric iron exposure.

[Fe^{3+}]mg/L	0.0	1.0	3.0	9.0
P value	0.859	0.813	0.173	0.156
Degrees freedom	5	4	6	6
Sample size	11	12	10	10

Table 6. Two-tailed T-test results comparing mean diffusion distances of secondary lamellae from one and three weeks of ferric iron exposure.

[Fe^{3+}]mg/L	0.0	1.0	3.0	9.0
P value	0.423	0.884	0.896	0.701
Degrees freedom	5	4	4	2
Sample size	11	12	10	10

Table 7. Two-tailed T-test results comparing mean diffusion distances of combined primary and secondary lamellae from one and three weeks of ferric iron exposure.

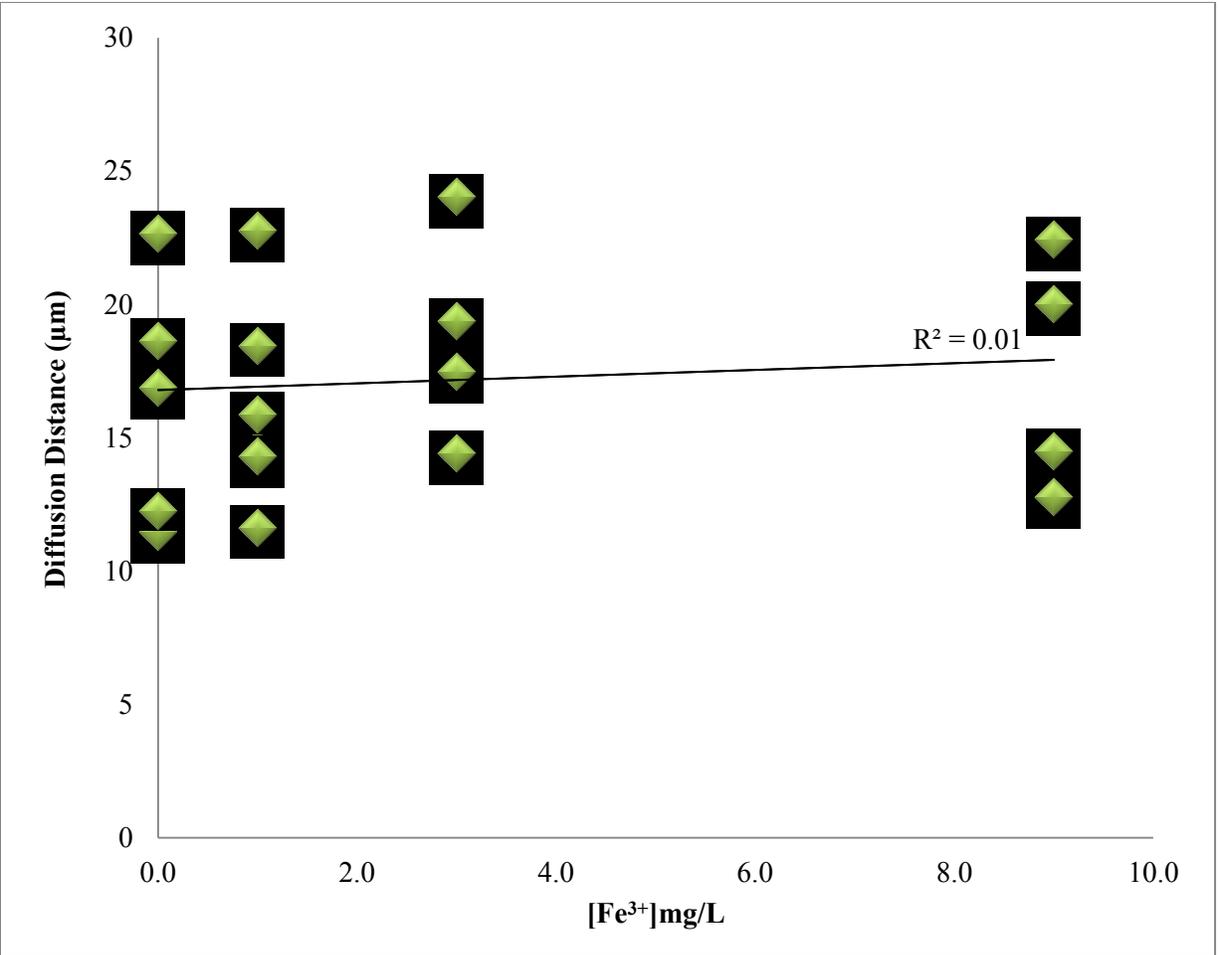| $[Fe^{3+}]$mg/L	0.0	1.0	3.0	9.0
P value	0.777	0.779	0.215	.0358
Degrees freedom	5	4	6	4
Sample size	22	24	20	20

Figure 16. Diffusion distance of primary lamellae of juvenile rainbow trout exposed to one week of ferric iron with trendline.
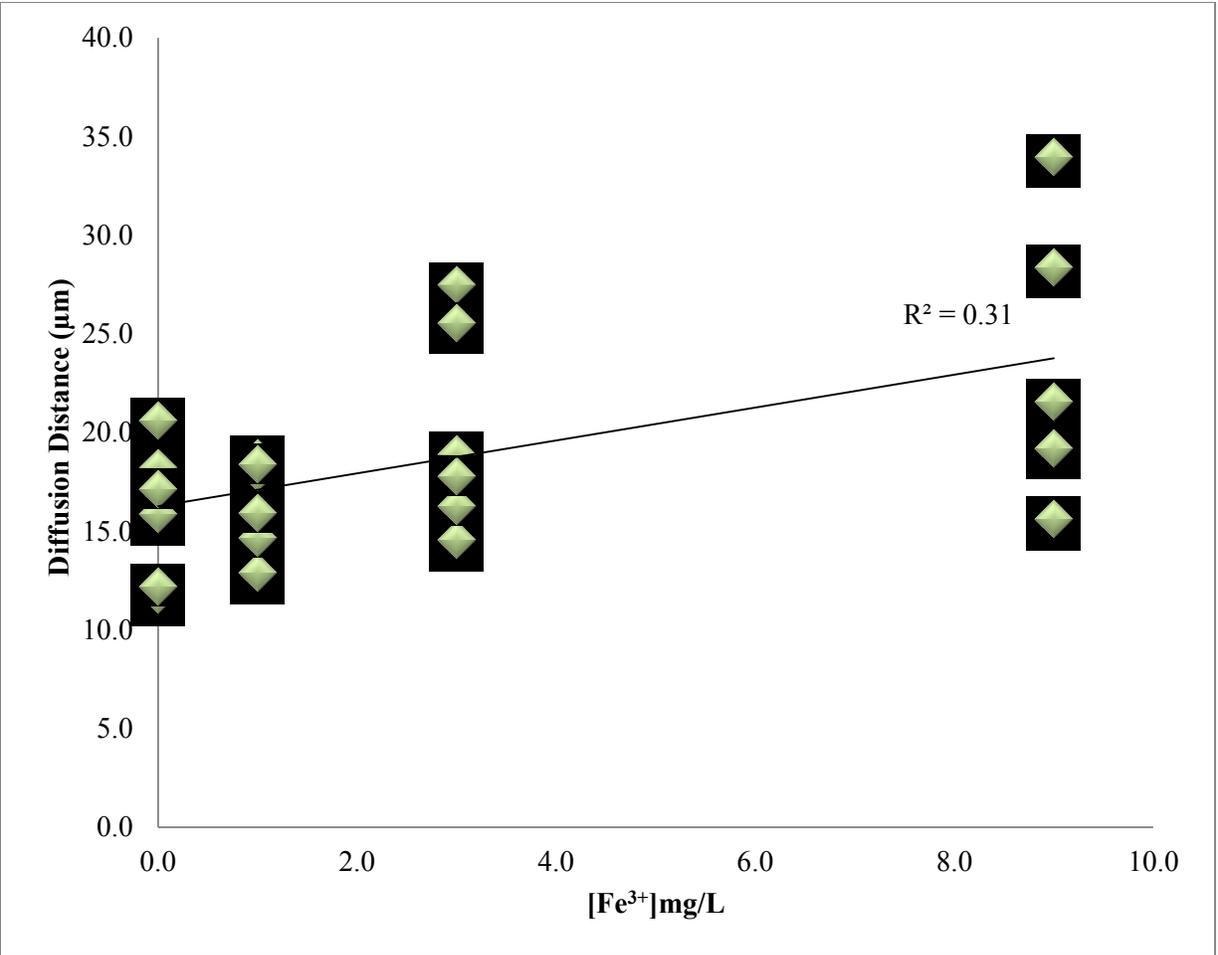
Figure 17. Diffusion distance of primary lamellae of juvenile rainbow trout exposed to three weeks of ferric iron with trendline.
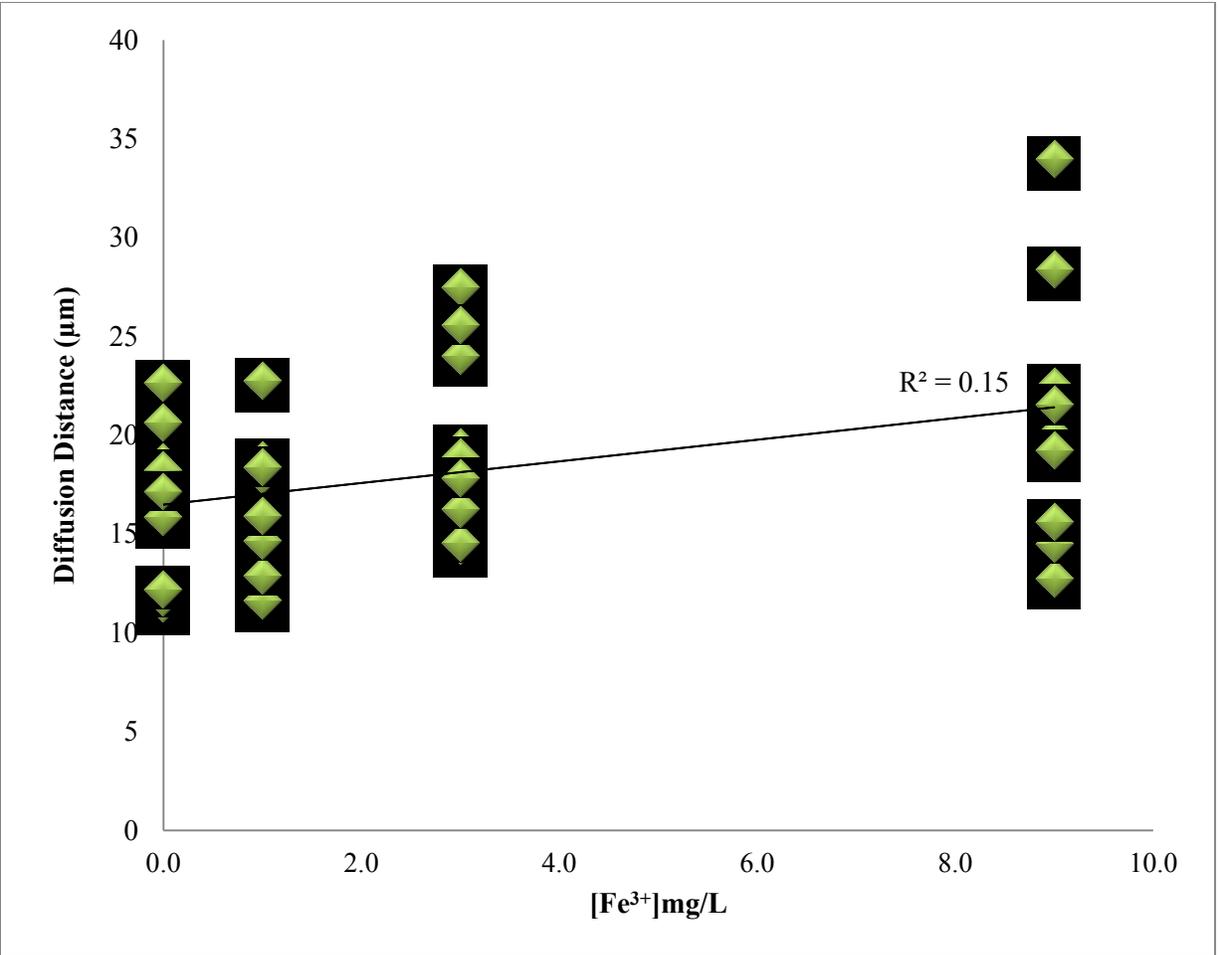
Figure 18. Combined diffusion distance of primary lamellae of juvenile rainbow trout exposed to one and three weeks of ferric iron with trendline.
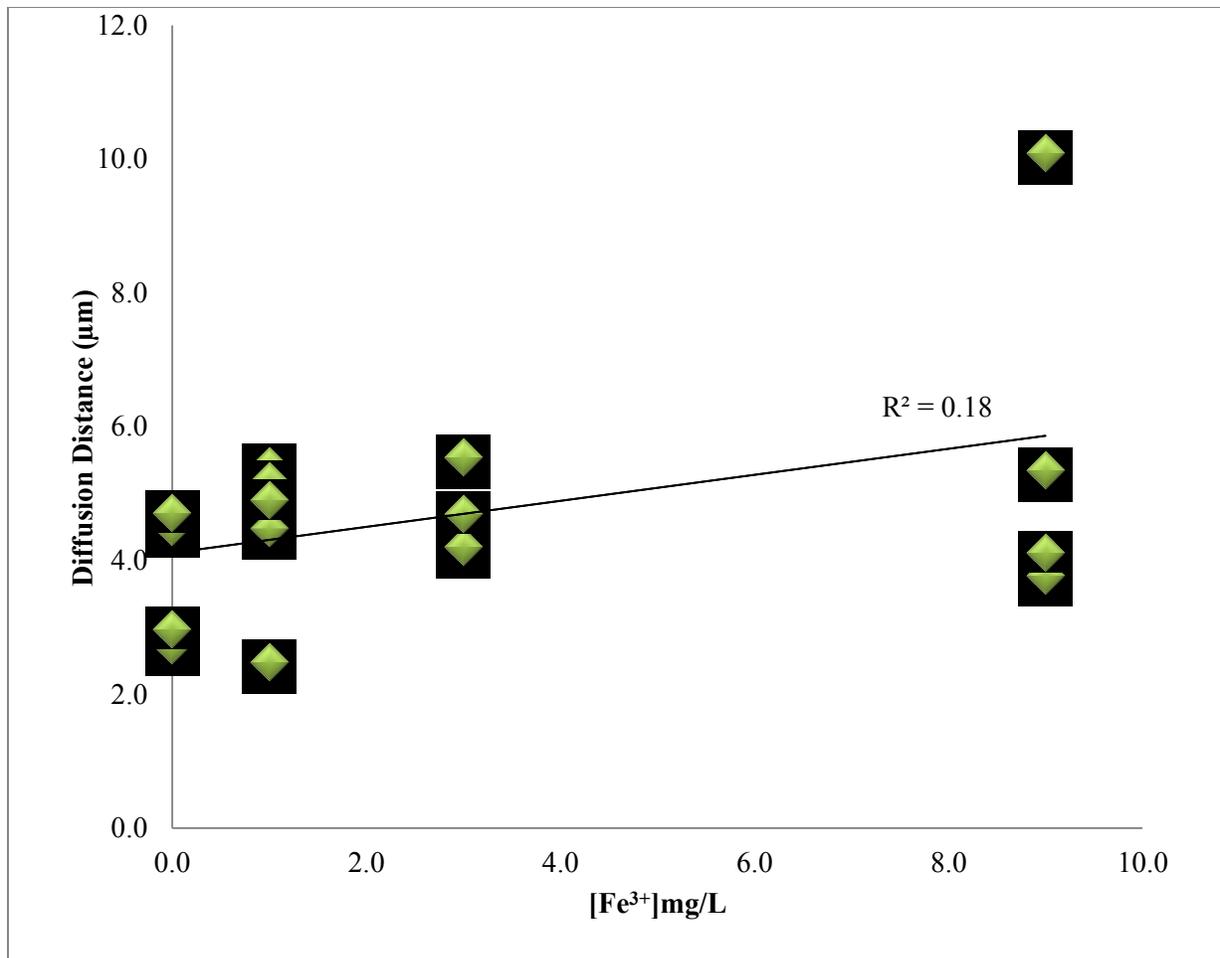
Figure 19. Diffusion distance of secondary lamellae of juvenile rainbow trout exposed to one week of ferric iron with trendline.
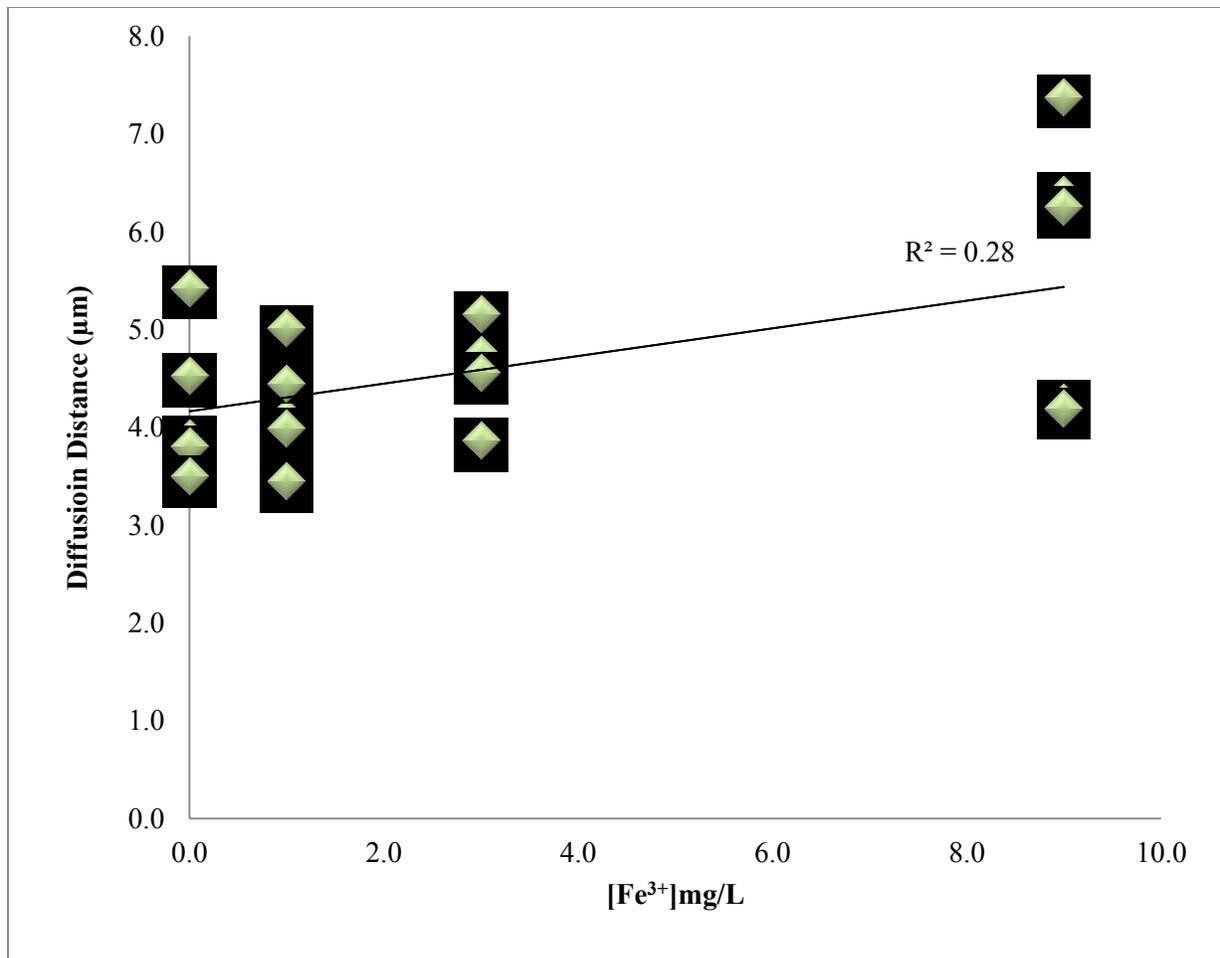
Figure 20. Diffusion distance of secondary lamellae of juvenile rainbow trout exposed to three week of ferric iron with trendline.
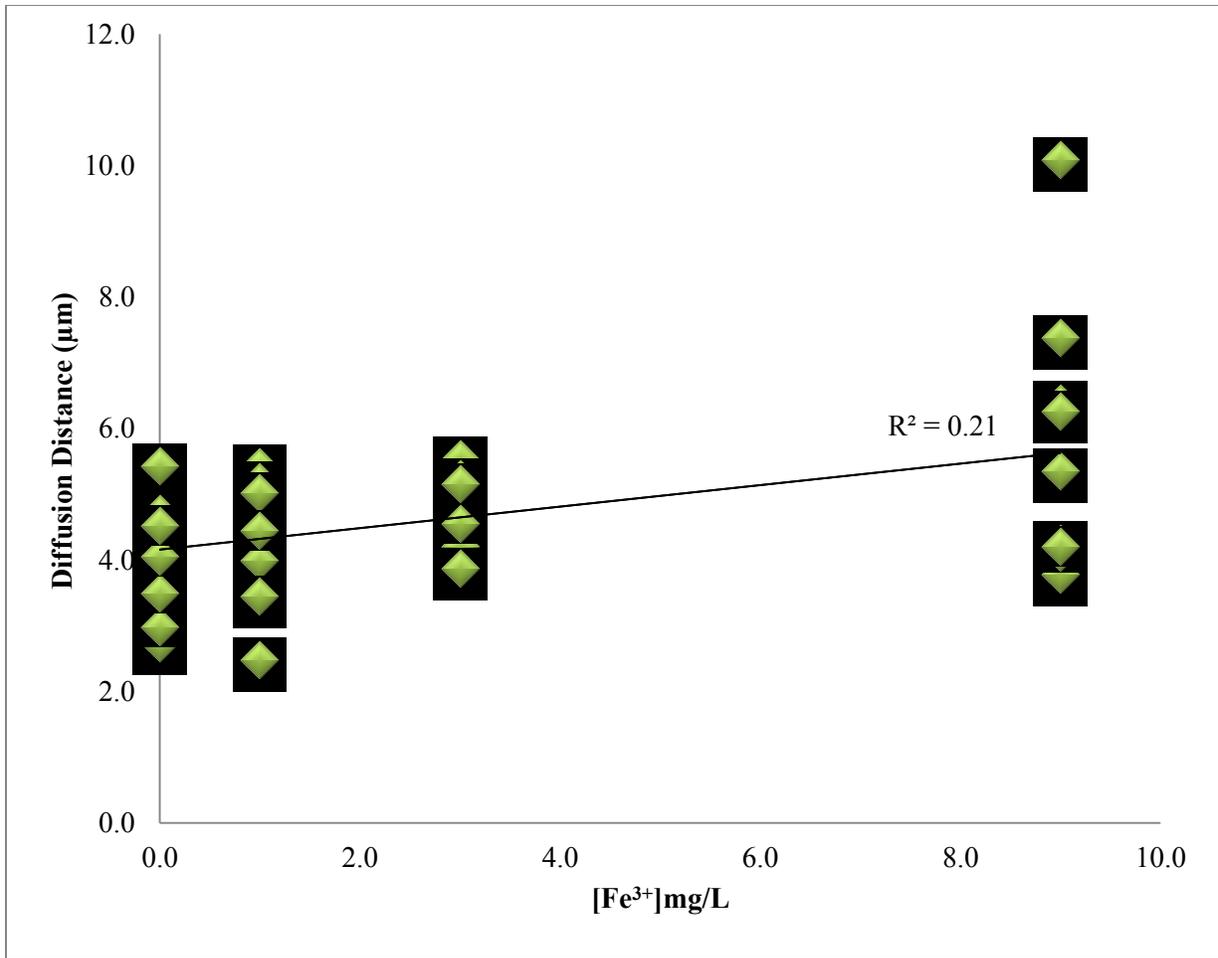
Figure 21. Combined diffusion distance of secondary lamellae of juvenile rainbow trout exposed to one and three weeks of ferric iron with trendline.

Table 8. Mean diffusion distance of primary & secondary lamellae of juvenile rainbow trout exposed to one week of ferric iron.

	1° Lamellae	2° Lamellae	1° & 2° Lamellae
Mean diffusion distance (µm) [Fe^{3+}] 0.0mg/L	16.16 [a] (4.63)	4.07 (0.97)	10.12 (7.29)
Sample Size	5	5	10
Mean diffusion distance (µm) [Fe^{3+}] 1.0 mg/L	16.45 (3.81)	4.50 (1.05)	10.48 (6.79)
Sample Size	6	6	12
Mean diffusion distance (µm) [Fe^{3+}] 3.0 mg/L	18.83 (4.04)	4.69 (0.61)	11.76 (8.02)
Sample Size	4	4	8
Mean diffusion distance (µm) [Fe^{3+}] 9.0 mg/L	17.44 (4.55)	5.83 (2.92)	11.63 (7.14)
Sample Size	4	4	8

[a.] Mean (Standard deviation)

Table 9. Mean diffusion distance of primary and secondary lamellae of juvenile rainbow trout exposed to three weeks of ferric iron.

	1° Lamellae	2° Lamellae	1° & 2° Lamellae
Mean diffusion distance (μm) [Fe^{3+}] 0.0 mg/L	15.98 [a] (3.46)	4.20 (0.69)	10.09 (6.59)
Sample Size	6	6	12
Mean diffusion distance (μm) [Fe^{3+}] 1.0 mg/L	16.42 (2.34)	4.25 (0.53)	10.34 (6.57)
Sample Size	6	6	12
Mean diffusion distance (μm) [Fe^{3+}] 3.0 mg/L	20.10 (5.23)	4.75 (0.50)	12.49 (8.76)
Sample Size	6	6	12
Mean diffusion distance (μm) [Fe^{3+}] 9.0 mg/L	23.39 (6.66)	5.45 (1.40)	14.42 (10.43)
Sample Size	6	6	12

[a.] Mean (Standard deviation)

Figure 22 shows normal gill morphology from a fish exposed to the control concentration of 0.0 mg/L of Fe^{3+}. In the control fish gills in Figure 22 there is a lack of hypertrophy or hyperplasia of the primary and secondary lamellae. Figure 23 shows altered gill histology from a fish exposed to three weeks of ferric iron at the concentration of 9.0 mg/L. Hyperplasia of primary epithelium, hypertrophy of secondary lamellae epithelium, clubbing of the secondary lamellae and epithelial separation can be seen in this gill slide. Figure 24 was taken from a fish exposed to the 3.0 mg/L Fe^{3+} for three weeks. Mild swelling of the secondary lamellar epithelium and hyperplasia of the primary lamellae can be seen. The arrow in Figure 25 points to a mitotic figure in a gill cell from a fish exposed to three weeks of the 1.0 mg/L Fe^{3+} treatment. The arrows in Figure 26 points to epithelial lifting/separation possibly caused by the extended time in MS-222.
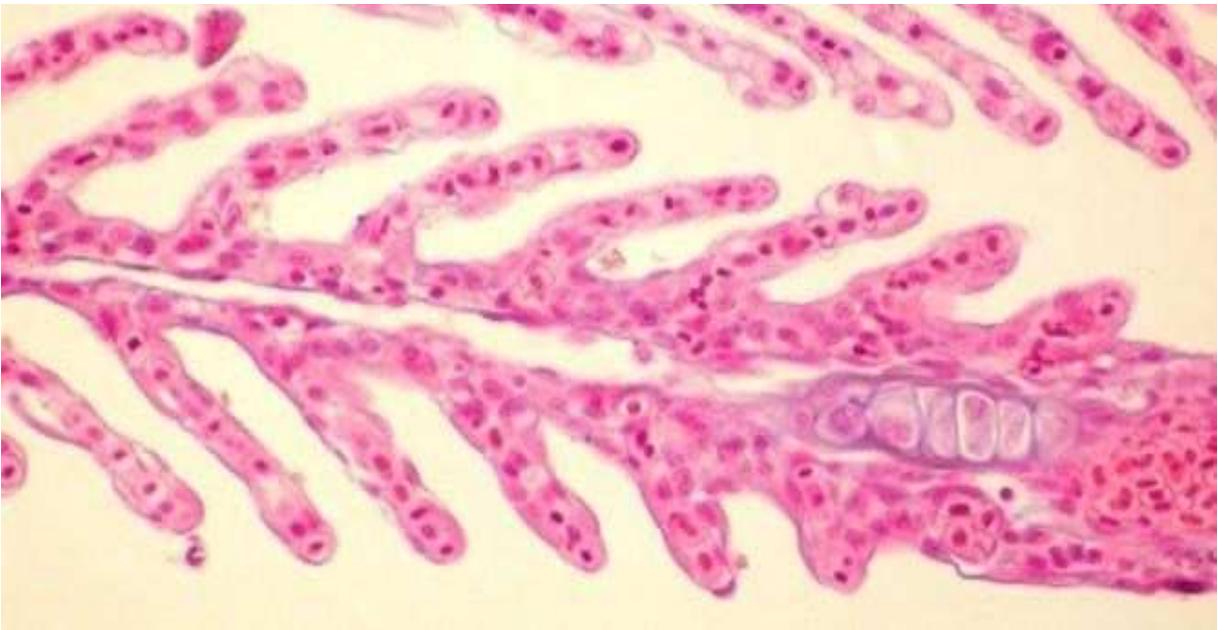

Figure 22. Normal gill lamellae with normal epithelial cells and normal distance between lamellae. 0.0 mg/L Fe^{3+} H&E x40 magnification.
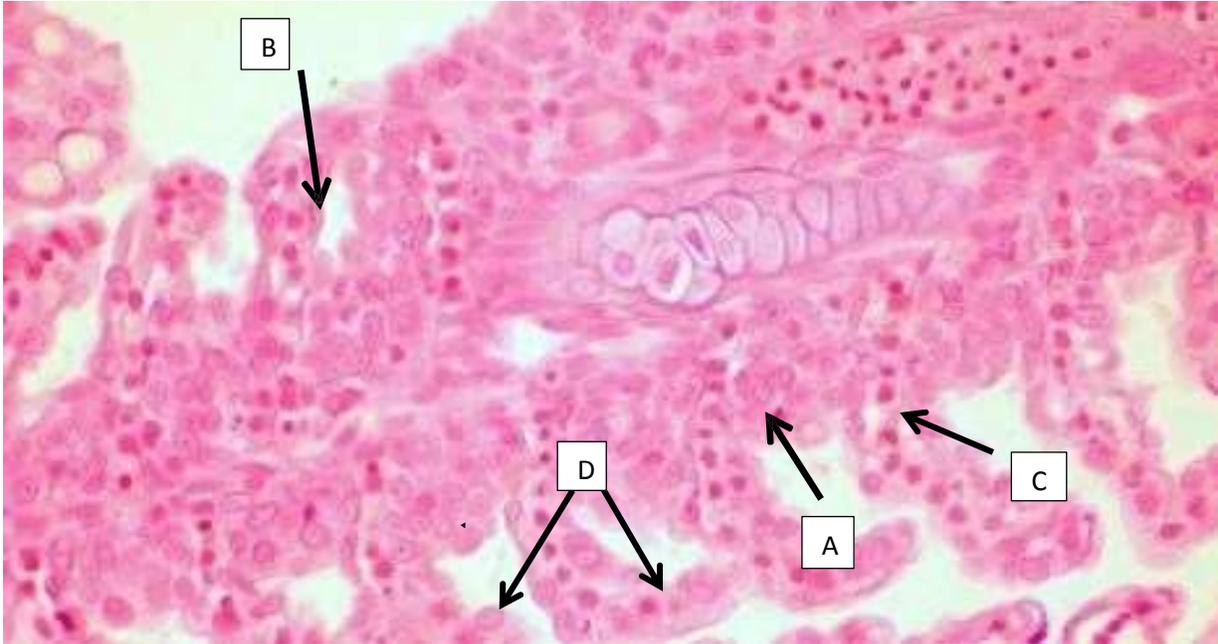
Figure 23. A. hyperplasia of primary epithelium, B. hypertrophy of secondary lamellar epithelium, C. epithelial separation, D. secondary showing clubbing. 9.0 mg/L Fe^{3+} H&E x40 magnification.

Figure 24. Mild swelling of secondary lamellar epithelium (arrow) and primary epithelial hyperplasia between lamellae on upper filament. 3.0 mg/L Fe^{3+} H&E x40 magnification.

Figure 25. Arrow points to mitotic figure 1.0 mg/L Fe^{3+} H&E x40 magnification.
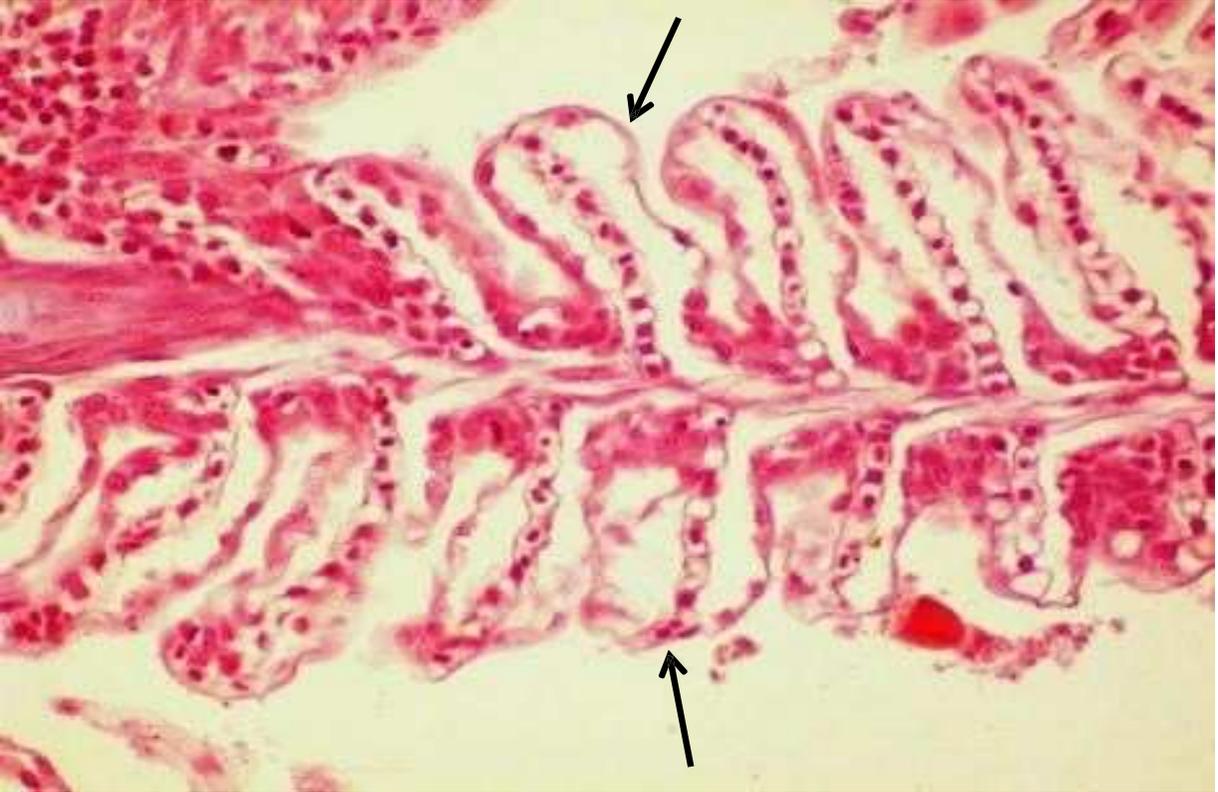
Figure 26. Arrows point to possible artifact from extended exposure to MS-222. 0.0 mg/L Fe^{3+} H&E x10 magnification.

In order to better quantify changes in mucous cells and iron accumulation on the gill tissue, additional slides were made from the remaining fish gill tissue preserved in paraffin wax. Due to cost concerns, the number of additional slides was limited to four fish. Two of these additional slides were stained with PAS, a dye used specifically for staining mucous cells and mucous. Two other slides were stained with Prussian blue stain, a dye used to detect precipitated iron. Two of the original slides were also restained with H&E in an effort to better identify and quantify chloride cells. The two PAS slides did not contain much gill tissue and were difficult to assess for mucous cell number. The Prussian blue stained slides did not show any accumulated iron on the gill tissue, although a few internal areas in the fish head were stained, indicating the presence of precipitated iron in those areas. The attempt to detect iron

via staining did not have expected results. The lack of iron detected on the fish gills stained

with Prussian blue may be due to the low pH of the Bouin solution that the fish were preserved

in. It is probable that any ferric iron precipitate located on the gill tissue dissolved off the gills

and went into solution when exposed to the low pH and was not detected by the Prussian blue

staining. The two slides restained with H&E did not assist in detecting and quantifying

chloride cells. Given these difficulties, it was decided to use measured diffusion distance as the

primary histological endpoint as opposed to grading the slides using gill damage or changes in

chloride and mucous cell number.

4.2. Experiment Two: Critical Thermal Maximum of Juvenile Cutthroat Trout

Thermal tolerance was tested in cutthroat trout exposed to ferric iron for one and two

weeks. The mean daily temperature during the exposure period was 13.06°C in the three

treatment tanks. The standard deviation of the mean temperature within each aquarium

averaged 0.53°C. The control (0.01 mg/L) was closest to the desired concentration of 0.0 mg/L

(Table 10). Survival rate was 100% and all fish tested recovered during the 20 minute post-

experiment recovery period.

Filtered and unfiltered water samples were used to assess ferrous and ferric iron

concentrations respectively, using flame atomic absorption spectrometry. There was no

detectable iron measured in the filtered water samples. The results for each exposure

concentration from the unfiltered water samples are listed in Table 10. The results from the

unfiltered water samples indicated that the concentration of the control was closest to the

target concentration, with a background measurement of 0.01 mg/L Fe^{3+}. The 1.0 mg/L Fe^{3+}

treatment had a measured Fe^{3+} concentration 35% lower than the target Fe^{3+} concentration. In the 10.0 mg/L target treatment tank, the mean measured Fe^{3+} was 0.08% lower than the target concentration. The control iron concentration had a relative standard deviation (RSD) of 0%. The 1.0 mg/L target ferric iron treatment had a RDS of 28% and the 10.0 mg/L target treatment had a RDS of 2% for the measured iron concentrations.

No significant differences in CT_{max} or weight were observed between the treatment and control fish or in fish tested week one versus week two (Tables 11 and 12). There are no meaningful trends noted in CT_{max} following exposure to different concentrations of ferric iron (Figures 27 and 28). The same is true of the combined data from week one and week two results (Figure 29). The correlation coefficients for graphs in Figures 30 and 31 are 0.01 for the first week and 0.10 for the second week of exposure, further supporting the lack of a significant correlation between changes in CT_{max} and ferric iron exposure.

Table 10. Mean measured Fe^{3+} mg/L by exposure concentration.

Treatment[Fe^{3+}]mg/L	0.0	1.0	10.0
Measured Mean [Fe^{3+}]mg/L	0.01[a] (0.01)	0.65 (0.18)	9.16 (0.22)
Sample Size	3	4	3

[a.] Mean measured concentration of Fe^{3+} mg/L (Standard deviation)

Table 11. Mean CT_{max} (°C) and weight (dg) of juvenile cutthroat trout exposed to ferric iron.

Fe^{3+} mg/L	CT_{max} (°C)	Weight (g)	Sample size
1st Week of Exposure			
0.0	29.67 (0.31)[a]	2.84 (0.35)	10
1.0	29.85 (0.50)	2.36 (0.65)	10
10.0	29.83 (0.43)	2.82 (1.23)	10
2nd Week of Exposure			
0.0	29.34 (0.50)	2.27 (1.19)	10
1.0	29.70 (0.38)	3.05 (1.13)	9
10.0	29.76 (0.25)	2.81 (1.09)	10

[a.] Standard deviation.

Table 12. T-test comparing mean CT_{max}, (°C) from juvenile cutthroat trout exposed to one and two weeks of ferric iron.

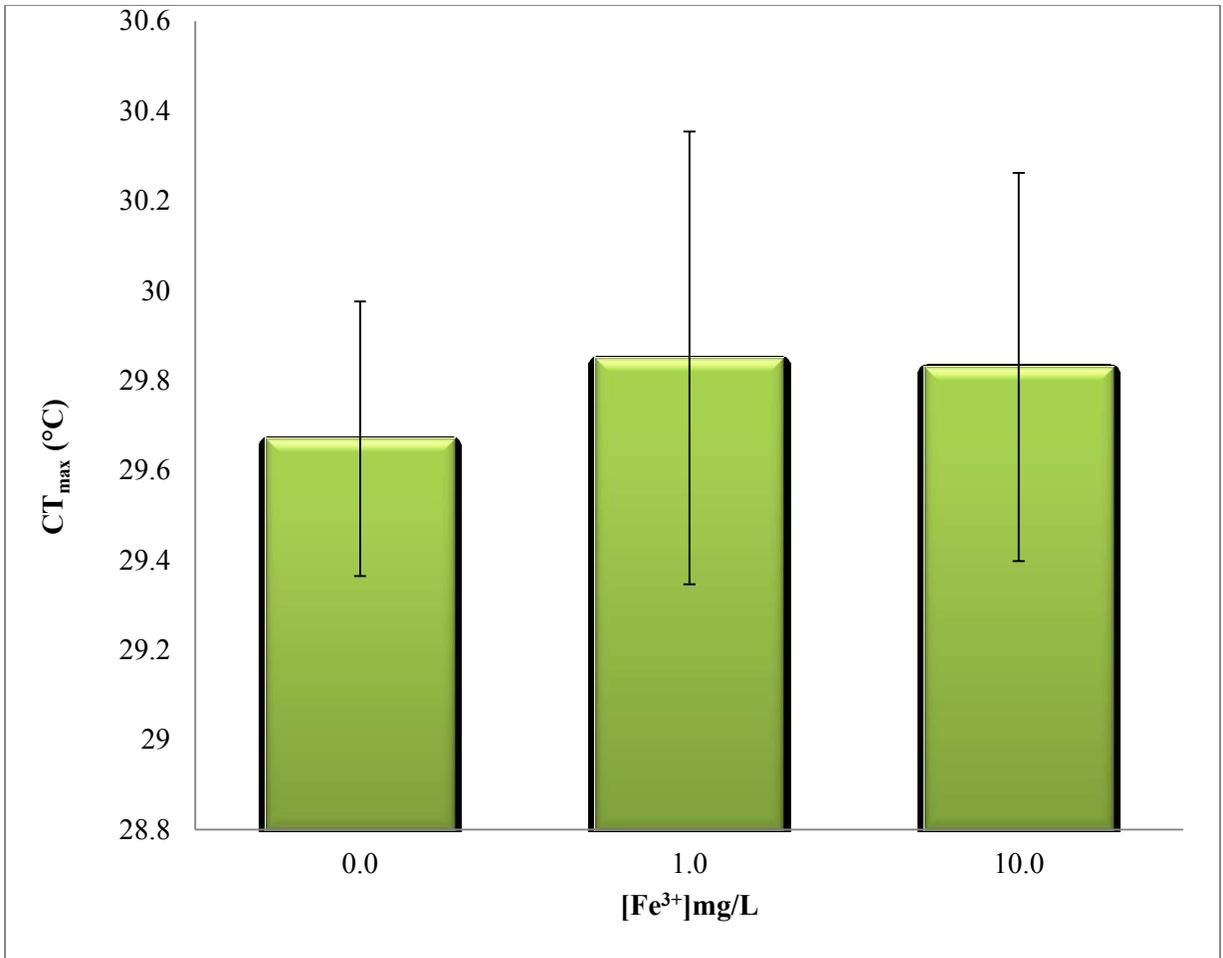| $[Fe^{3+}]mg/L$	0.0	1.0	10.0
P value	0.122	0.368	0.704
Degrees of Freedom	8	14	9
Sample Size	20	19	20

Figure 27. Mean CT_{max} of juvenile cutthroat trout exposed to one week of ferric iron. Error bars indicate standard deviation.

Figure 28. Mean CT_{max} of juvenile cutthroat trout exposed to two weeks of ferric iron. Error bars indicate standard deviation.

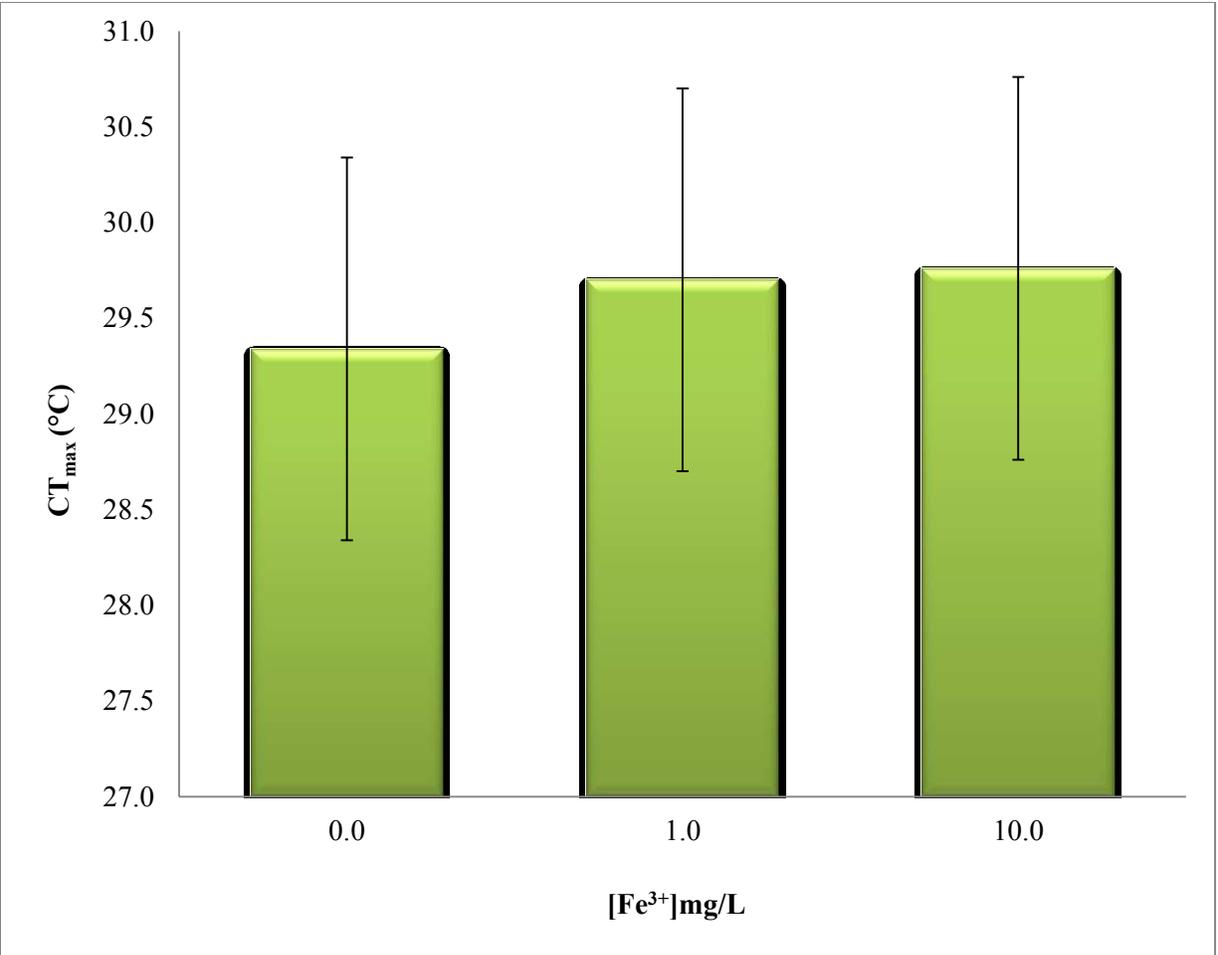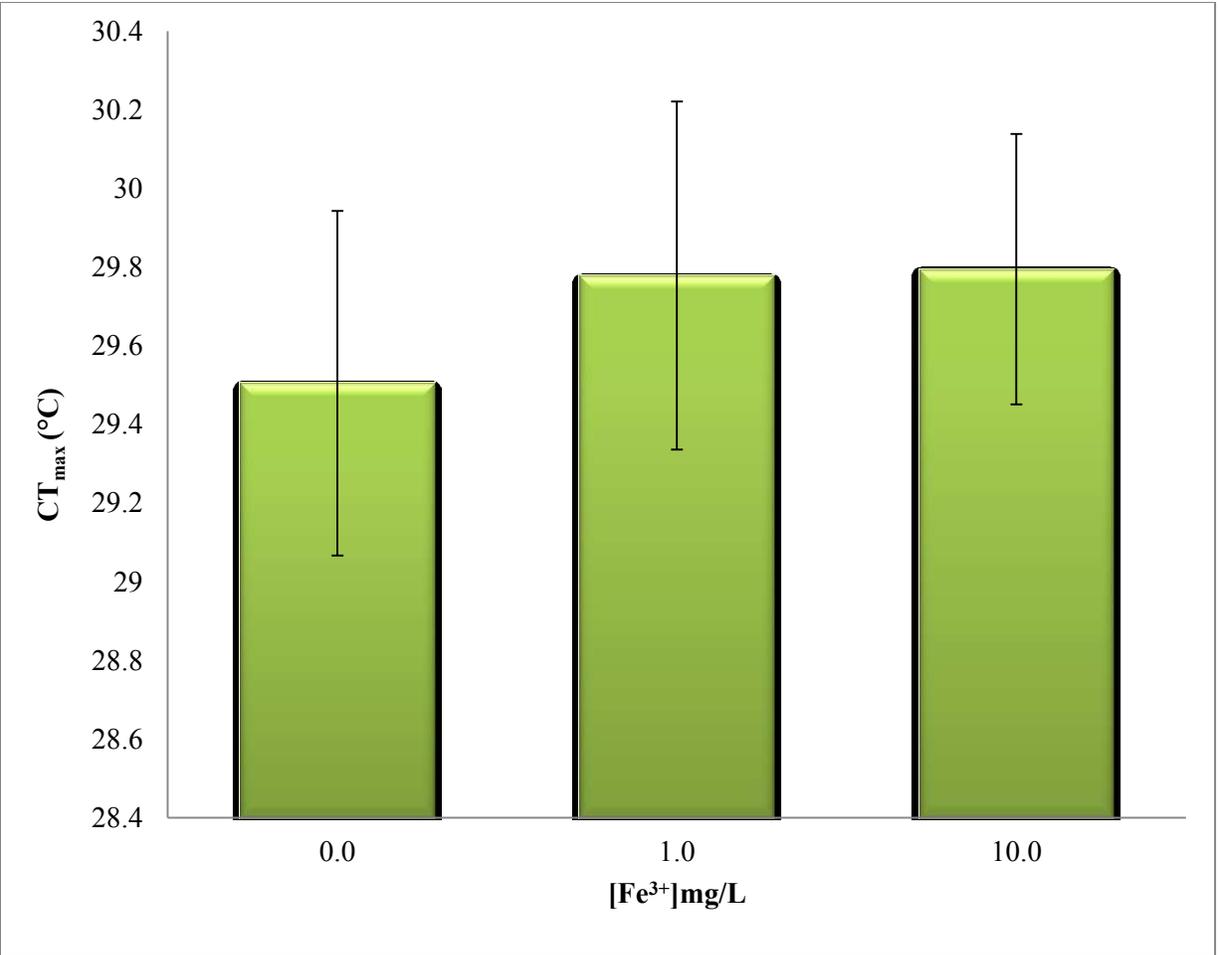Figure 29. Combined mean CT_{max} of juvenile cutthroat trout exposed to one and two weeks of ferric iron. Error bars indicate standard deviation.
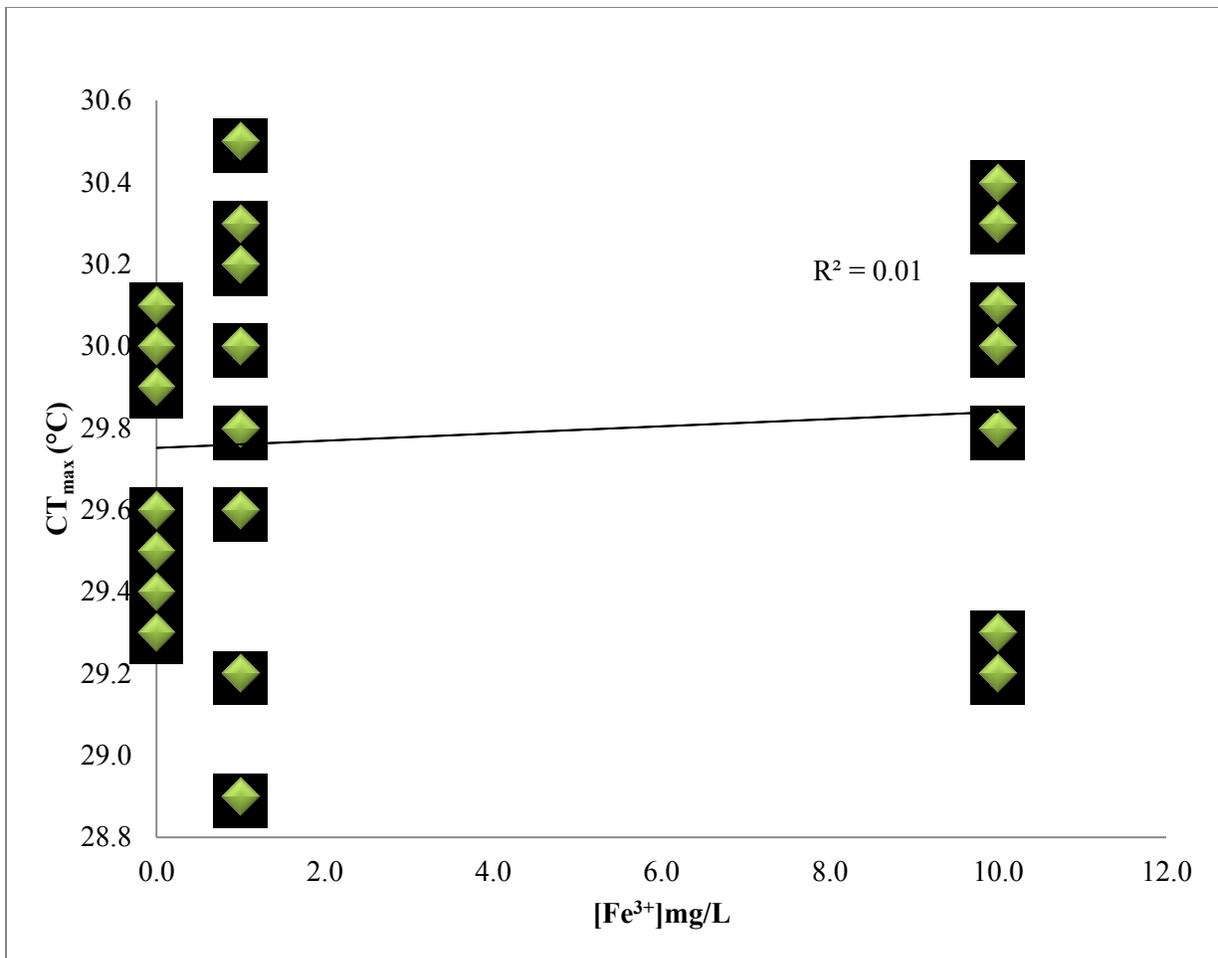
Figure 30. CT$_{max}$ of juvenile cutthroat trout exposed to one week of ferric iron with trendline.

Figure 31. CT_{max} of juvenile cutthroat trout exposed to two weeks of ferric iron with trendline.

There were no significant effects of ferric iron exposure on mean weight for either week of the CT_{max} experiments (Figures 32-35). The mean weight of the fish used in the first week of CT_{max} trials ranged from 2.36 g to 2.84 g (Figure 32). The mean weight of the fish used in the second week of the CT_{max} experiment, ranged from 2.27 g to 3.05 g (Figure 33). The correlation coefficient for both weeks was 0.01, further supporting a conclusion that there was no significant effect from ferric iron exposure on the weight of juvenile cutthroat trout (Figures 34 and 35).

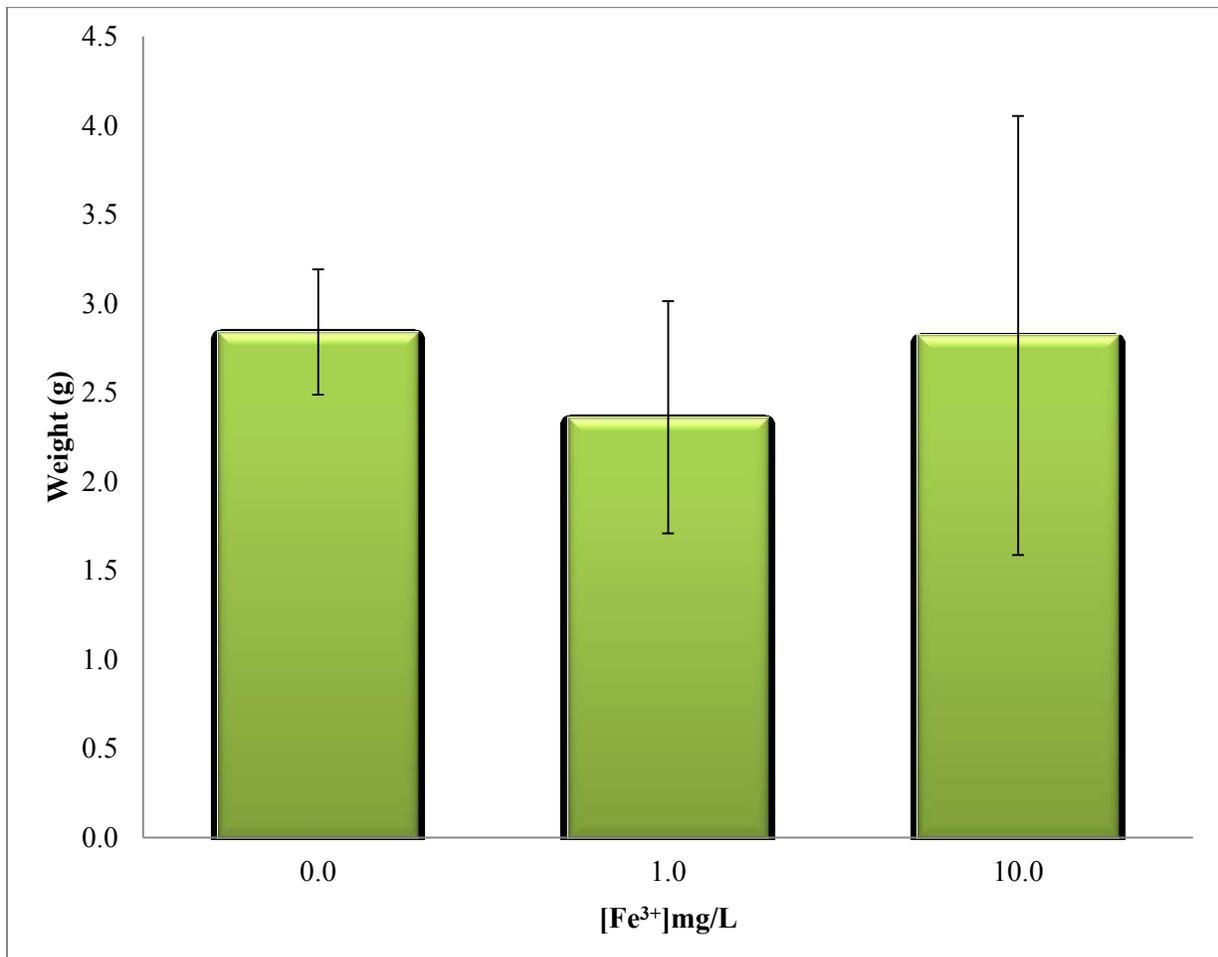Figure 32. Mean weight of cutthroat trout exposed to one week of ferric iron. Error bars indicate standard deviation.
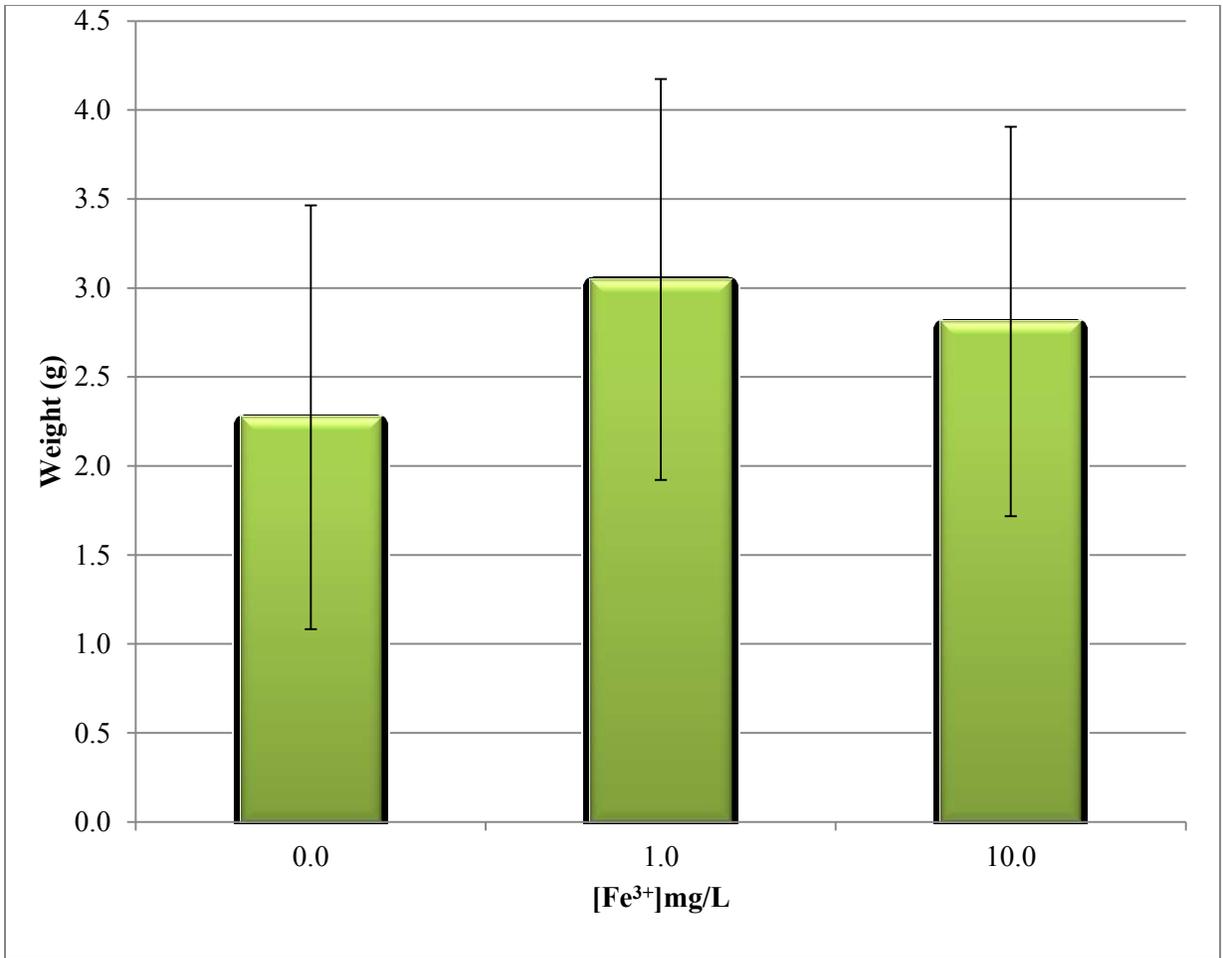
Figure 33. Mean weight of cutthroat trout exposed to two weeks of ferric iron. Error bars indicate standard deviation.
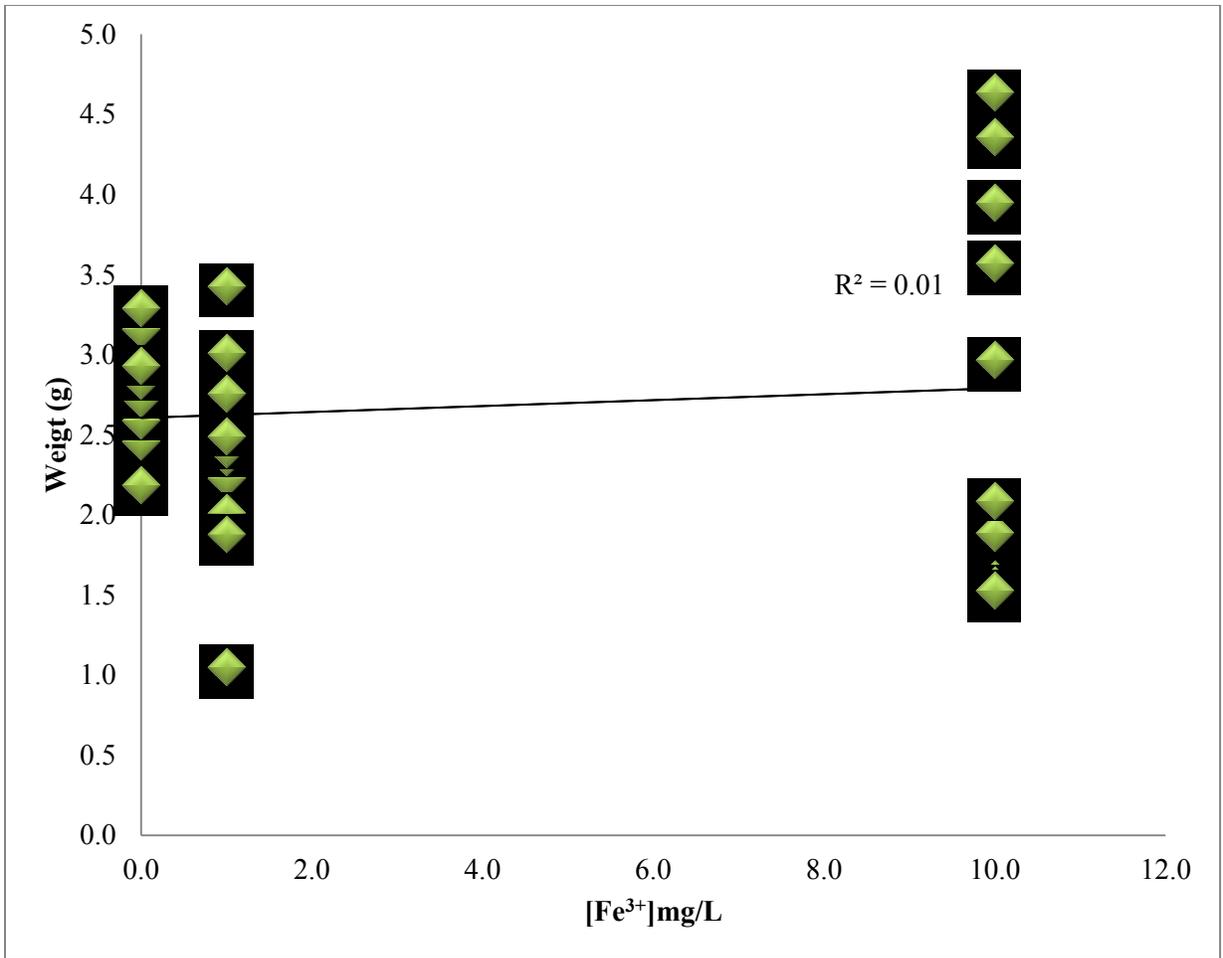
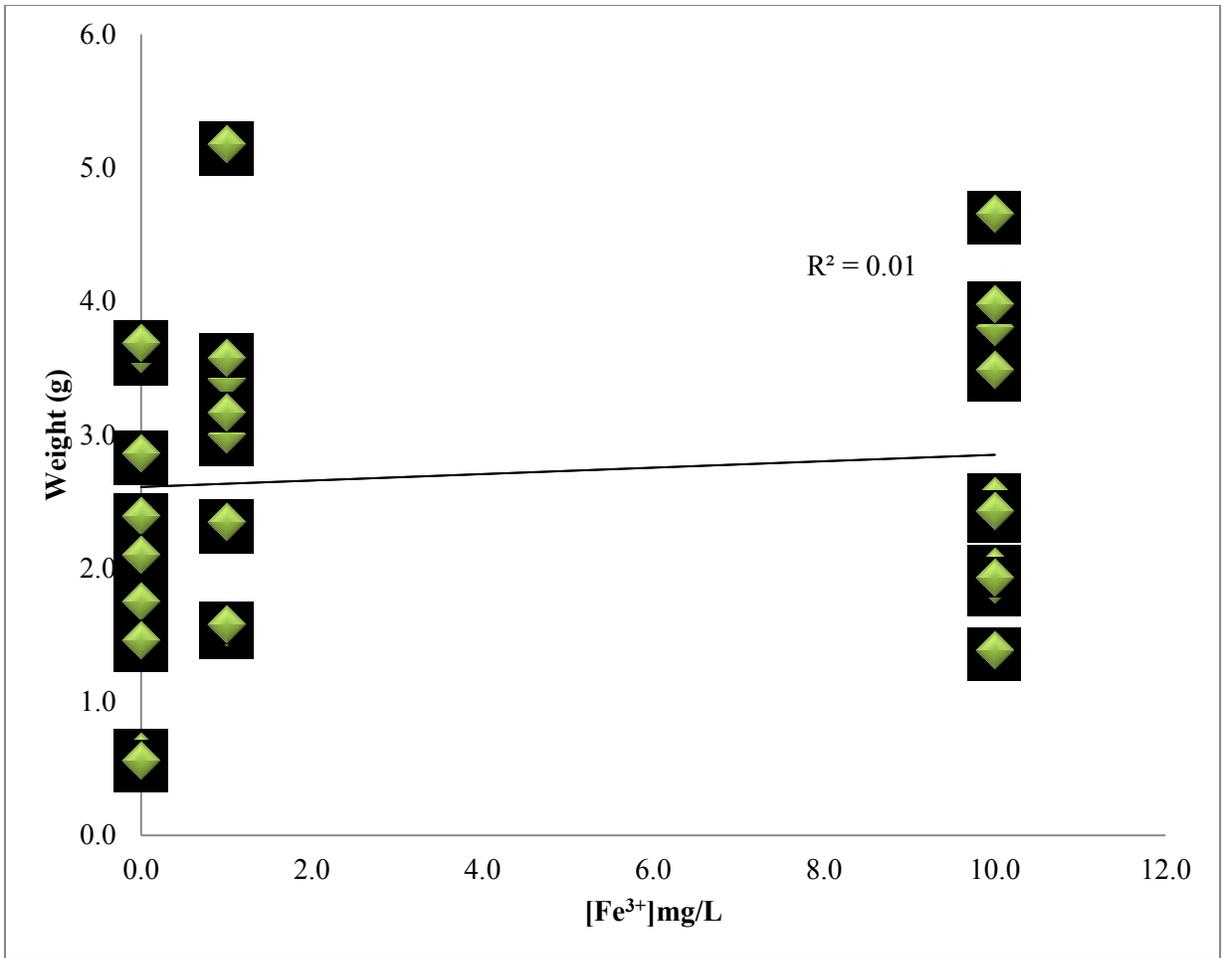Figure 34. Weight of juvenile cutthroat trout exposed to one week of ferric iron with trendline.

Figure 35. Weight of juvenile cutthroat trout exposed to two weeks of ferric iron with trendline.

V. DISCUSSION

The critical swim speed (U_{crit}) and critical thermal maximum (CT_{max}) studies were conducted to aid in establishing biologically relevant Colorado chronic iron water quality standards for ferric iron. The results of the U_{crit} and CT_{max} experiments did not identify statistically significant effects in the endpoints tested. There are some weak trends observable in the graphs and tables, although some of the trends were not consistent with the expected results. The inconclusive results from this experiment did not aid in establishing new, biologically relevant Colorado water quality criteria for ferric iron.

In the U_{crit} experiment, specifically, the size of the juvenile fish proved to be challenging and may have affected the study results. Due to the diminutive size of the juvenile fish, available adult-sized swim chambers were too large to be utilized for this study. A novel swim chamber was designed and built to accommodate the juvenile fish size. The design of the swim chamber itself resulted in challenges and unexpected problems that complicated successful data collection. Two other challenges encountered in this experiment were the learning curve for the experiment operator to successfully conduct the U_{crit} study and conditioning the fish to swim in the Brinkman swim chamber. The dynamic and multiple challenges in the U_{crit} experiment increased the difficulty of conducting the U_{crit} experiment and collecting accurate data.

5.1. Experiment One: Critical Maximum Swim Speed of Juvenile Rainbow Trout

The results of the U_{crit} experiment did not show statistically significant changes from Fe^{3+} exposure in juvenile rainbow trout for the endpoints of U_{crit}, weight, length, or gill

lamellae diffusion distance. Although we did not see statistically significant differences in the U_{crit} of fish exposed to ferric iron and the control fish, there was a slight trend towards decreased swim speeds in the fish exposed to 1.0 mg/L, 3.0 mg/L and 9.0 mg/L Fe^{3+} when compared to the control fish (Table 3,Figures 5-7). The correlation coefficient for the trendlines for Figures 5 to 7 are well below 0.5, but the general slope is negative in all graphs, with a trend towards decreased U_{crit} as ferric iron concentration increased. The U_{crit} results from the second set of swim trials following three weeks of ferric iron exposure had a larger correlation coefficient than the first set of swim trials (one week of ferric iron treatment), which is consistent with the expectation that prolonged exposure to ferric iron would have increased impact on the endpoint of U_{crit} than would shorter-duration exposures (Figures 5 and 6). This general trend is consistent with previous study findings of decreased overall U_{crit} in fish exposed to chronic, sublethal toxic metals (Wilson and Wood 1992).

Performing U_{crit} swim tests on juvenile rainbow trout proved problematic on multiple levels. The difficulties may have impacted the results from the U_{crit} experiment or inadvertently introduced new, unexpected variables into the study that confounded the results. One issue was that many of the fish in the U_{crit} study appeared to have difficulty adjusting to swimming in the current of the swim chamber. The fish that had difficulty acclimating to the swim chamber had an extended period of adjustment before they swam with more ease in the swim chambers. This period of adjustment was beyond the ten minute period provided for the fish to acclimate to the swim chamber. At the onset of the swim trials, most fish needed to be repeatedly encouraged to swim. Without the repeated encouragement of tapping on the swim chamber, fish frequently remained stationary, resting their tails on the rear screen of the swim chambers. Some fish never mastered swimming in the chambers and appeared to struggle at

lower than expected velocities or never began to swim, even when the pumps were turned off and there was not a detectable current. Other fish, usually after an initial period of hesitant and rough swimming, often at around thirty minutes or more into the swim trial, would begin to swim with minimal body movement at much higher velocities for extended periods of time. We may have been inadvertently measuring the fish's ability to learn how to swim in the swim chamber or in the current, rather than objectively determining the U_{crit} that the fish could achieve had it been better able to acclimate itself to the novel situation of swimming in the swim chamber in a higher velocity current. Preliminary testing indicated that our initial experimental design needed to be revised. The initial design utilized 24 replicate rectangular tanks that had little current for the fish in the tanks to swim in. The environment of the rectangular tanks resulted in fish that neither knew how to swim in a current nor were conditioned to swim against a current. The performance of these fish in the swim chamber was less than expected, with many fish refusing to swim even at lower velocities than the initial velocity of the swim trial. We modified our treatment tank design to a circular tank and generated a slight circular current with the aid of air bubbles (Figure 36). We utilized the circular experimental tanks in this experiment in an effort to better condition the fish to swimming. An extensive review by Hammer (1995) found that fish trained to swim in a current performed better than untrained fish and appeared less excited during the trial. The current generated by the air bubbles was slight. Prior to the beginning of the U_{crit} experiment, we tested a group of fish that were exposed to increased current velocities for two weeks. The fish exposed to the increased velocity in this holding tank had marked improvement in their swim chamber performance. These test fish performed better in the swim chamber than any of the.
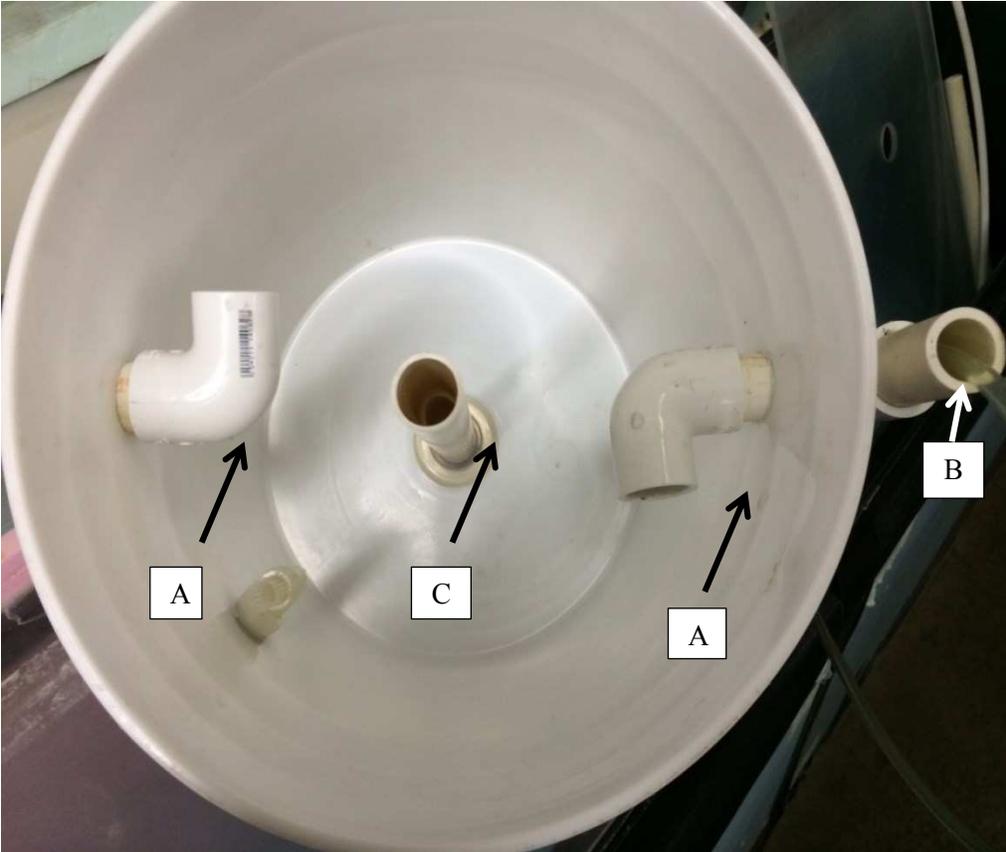
Figure 36. Circular treatment tank. A. bubble inlets - directed bubbles generated a circular current. B. outside portion of bubble inlet- airline tubing brought air-stream of bubbles into inlets. C. drain tube for exiting water.

other groups of fish we tested. Due to logistics and funding issues, we were unable to provide similar current velocities in our 24 experimental tanks during the exposure period of this experiment.

Another factor that may have affected the U_{crit} results is that not all fish used the same types of swim techniques or a variety of swimming techniques were utilized at different times by an individual fish. Burst swimming is primarily produced by fast twitch contraction of white muscle (Johnston 1977; Jayne and Lauder 1994). Subcarangiform swimming has been shown to employ primarily red muscle, although some studies have found that subcarangiform swimming employs both red and white muscle (Johnston 1977; Jayne and Lauder 1994). Fast

twitch muscle is considered to be primarily aerobic, red muscle. The goal of our study was to test aerobic swimming ability. If fish were instead employing burst swimming followed by periods of time resting on the screen, we may not have been testing aerobic capacity. If fish were utilizing anaerobic or a combination of anaerobic and aerobic swimming techniques, our results may have been affected and additional variables may inadvertently have been added to the study.

The swim chamber design itself was a factor that may have affected how well and at what velocities fish would swim (Figure 37). The Brinkman swim chamber was constructed to test swimming speed of juvenile fish. Previous studies have typically utilized older fish that are of a larger size and have used a variety of swim chamber design. Although we were careful when constructing the swim chambers, some fish seemed to find lull spots in the water velocity inside the swim chambers. Three fish preferred to swim in the same location of swim chamber one and maintained their position precisely at that location throughout the swim trial. These fish appeared to swim with less effort than expected. When tapping stimuli were used to frighten the fish out of this particular area of the swim chamber, the fish would usually return to the same precise spot as soon as the tapping stimuli stopped, resuming their smooth swimming once they were back in the preferred location of the swim chamber. Another issue encountered was small objects clogging the intake grates of the Brinkman swim chamber (Figure 38). When objects caught on the intake grate, it created an area that had decreased current in the swim chamber. Fish would line up with the clogged area and swim with what appeared to be less effort. Once the obstruction on the grate was detected and removed, the fish often had difficulties maintaining its position in the swim chamber and was often pushed to the back screen. The data for these fish were discarded. The fish were replaced and the U_{crit}

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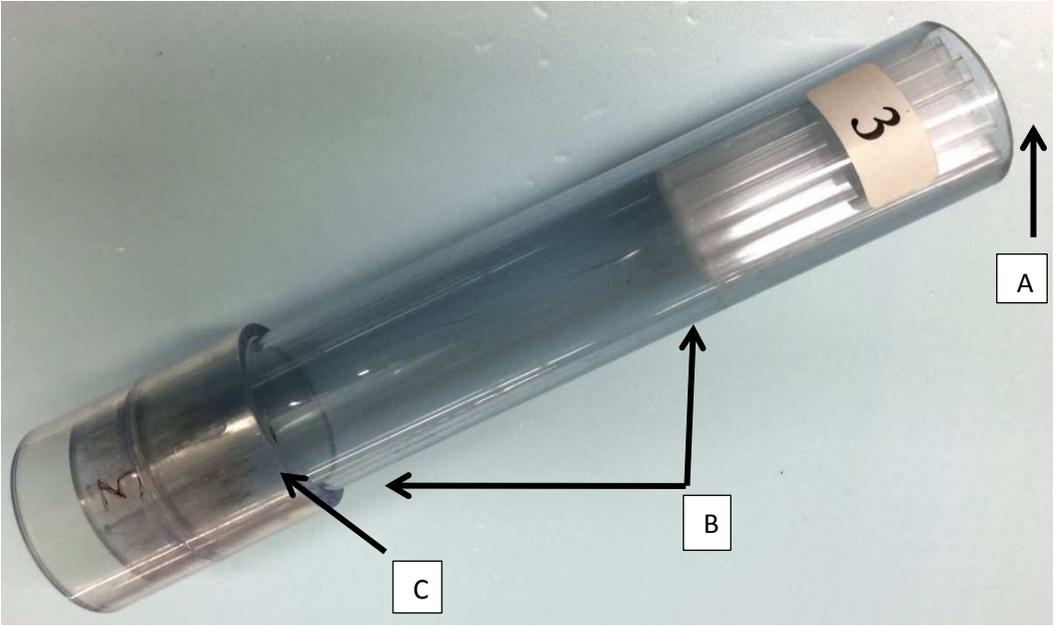
Figure 37. Brinkman swim chamber. A. front grates where water enters into swim chamber. B. swimming area. C. back screen.

swim teste was restarted using new fish. On two occasions, the replacement fish also did not

begin to swim, even at the lowest velocity. The data for these fish were also discarded.

The results of the U_{crit} experiment may have been further affected by the considerable

antagonistic behavior of stronger fish towards weaker individuals in the experimental tanks.

Social stress has been shown to impair the subordinate fishes' ability to respond to additional

stressors (LeBlanc et al. 2011). The fish that were targeted were smaller in length and weight,

swam weakly in the experimental tanks, hovered close to the surface and were easily caught in

the net. These fish often had to be given more coaxing at the onset of the test to swim and

often failed at lower U_{crit} than their more robust looking tank-mates.

Figure 38. Front grate on Brinkman swim chamber.

Contrary to the results from previous studies, we did not see a significant difference in the length or weight of the Fe^{3+} exposed fish and the control fish (Figure 8 to 15). Brinkman and Viera (2011) found that chronic exposure of ferric iron significantly affected the growth rate of mountain whitefish *(Prosopium williamsoni)*, although no significant effects in brown trout (*Salmo trutta*) growth rate were found. Two other studies indicated that length and weight of boreal toad tadpoles (*Bufo boreas*) were significantly reduced at the higher ferric iron concentration tested (8115 μg/L) and the biomass of to the oligochaete, *Lumbriculus variegate,* was significantly reduced when exposed to 4000 μg/L and 8000 μg/L (Brinkman 2012; Brinkman 2013).

The mean weight of the fish used in the first week of U_{crit} swim trials (one week of ferric iron exposure) had a slight trend of decreased weight as ferric iron exposure increased

(Figure10). There was not a detectable trend in the weight data in the fish exposed to ferric iron for three weeks (Figure 11). The length data collected from the U_{crit} experiment did not have a detectable trend for either one week or three weeks of ferric iron exposure (Figures 14 and 15).

Lamellar diffusion distances in the control versus the treatment fish were not found to be significantly different. A slight trend towards increased diffusion distance with increased ferric iron exposure can be seen in secondary lamellae of the fish exposed to one week of ferric iron (Figure 19). The results from the primary and secondary lamellae diffusion distances from the fish exposed to ferric iron for three weeks were the most consistent with what was expected and had the highest correlation coefficient of any of the data collected for the U_{crit} study (Figures 17 and 20).

When euthanizing the fish, some fish were left for longer periods than five minutes in the MS- 222, which may have resulted in artifacts in the gill-histological slides (Spear 1989). It was difficult to establish whether there was increased gill damage in the exposed fish compared with the controls due to the possibility of artifacts in both the control and iron exposed fish. Epithelial lifting, hypertrophy and necrosis of epithelial cells were observed in many of the gill histology slides. These types of gill damage are consistent with changes to fish gills from toxic exposure and other irritants but are nonspecific and can result from exposure to a variety of stressors in the aquatic environment (Mallatt 1985). Four of the gill slides could not be measured due to the amount of possible artifact and the data were not used.

Future chronic, sublethal ferric iron exposure experiments that test for measurable precipitated iron in the gill tissue are suggested. Combining a measurement for precipitated

iron with tests for increased internal, bioavailable iron would be helpful to determine if ferric

iron is reduced and taken up into the fish via divalent metal transporters. An experiment testing

serum malondialdehyd levels after ferric iron exposure would also be helpful in determining if

exposure to ferric iron results in increased reactive oxygen species formation and tissue

damage.

Future U_{crit} studies may benefit from having a substantially increased acclimation

period. Allowing the fish more time to adjust to the swim chamber and become calm may

reduce some of the issues that were encountered in this study with fish swimming

performance. Repeated exposure over a period of days to swimming in the chamber might also

be a helpful in reducing variables caused from the fish being unfamiliar with the swim

chamber and would condition the fish to swim against the current. It is also suggested that a

more substantial current in the exposure tanks be used to aid in conditioning the fish to

swimming.

Larger swim tanks or tanks with decreased or increased fish numbers may assist in

limiting some of the interspecies aggression that was observed in this study and may have

altered some of the results in fish weakened by antagonistic behavior.

Brauner et al. (2011) observed that prolonged swimming causes gill remodeling in

carp. Future studies that compared the gill histology of exposed fish used in an U_{crit} trial with

exposed fish that were not subject to swimming tests would help to determine if vigorous

swimming may have caused changes in gill histology.

Avoiding predation is critical to juvenile fish that, due to their size, are more likely to be prey for a variety of larger aquatic creatures. The ability of juvenile fish to rapidly flee via anaerobic burst swimming is a biologically relevant endpoint measurement. Future studies that examine if there is a correlation between burst swimming and U_{crit} would aid in creating a larger picture of fish fitness and help determine if toxicants such as ferric iron are having broader-ranged impacts on juvenile fish survival rates from predation.

5.2. Experiment Two: Critical Thermal Maximum of Juvenile Cutthroat Trout

The juvenile cutthroat trout in this experiment were not significantly affected by ferric iron exposure at either the lower range of this experiment (1.0 mg/L) or the highest exposure concentration (10.0 mg/L). There was a slight trend seen in both CT_{max} experiments from week one and two for fish exposure to the higher ferric iron treatments to have a higher CT_{max} (Figures 27 to 31). This positive trend is the opposite of what was expected and is not consistent with previous experiments' results that indicated that gill damage and clogging of the gills from exposure to precipitated iron reduces CT_{max} in exposed fish (Dalzell and Macfarlane 1999; Peuranen et al. 2003). Underwood et al. (2012) found the CT_{max} of cutthroat trout acclimated to a mean temperature of 12.5°C had a mean CT_{max} of 28.15°C. The mean CT_{max} for cutthroat trout in this experiment exposed to 1.0 mg/L was 29.78°C, an increase in 1.63°C over Underwood's study. The mean CT_{max} in this experiment for fish exposed to 10.0 mg/L was 29.80°C; a 1.65°C increase in CT_{max} over the previous Underwood study. The mean CT_{max} of all fish (control and exposed fish) in this experiment was 29.69°C, an increase of 1.54°C from the CT_{max} results of the Underwood study. The fish used in the Underwood study

were more than one year old; several studies have found that larger fish had a lower CT_{max} than smaller fish. This difference in fish size and age may account for the 1.64°C and 1.54°C differences in CT_{max} between the two experiments (Selong et al. 2001; Bear et al. 2007; Underwood et al. 2012). It should also be noted, however, that a recent CT_{max}, study by Roberts et al. (2012) found little to no difference between CT_{max} of smaller vs. larger sized cutthroat trout. Variation in thermal tolerance between genetically diverse populations of brook and rainbow trout species has been noted in previous studies and may partially account for the differences in CT_{max} results of the current study and the Underwood study (Carline and James 2011). The treatment tanks for the juvenile cutthroat trout used in this CT_{max} study had a mean daily temperature of 13.06°C, which is 0.56°C higher than the temperature of the treatment tanks in the Underwood study, and another factor that may have altered the mean CT_{max} for this study. The acclimation temperature for the CT_{max} test tanks of the current experiment was 13.0°C, 0.5°C above the acclimation temperature of the Underwood study and may have impacted the CT_{max} results for the current study.

The results of previous experiments have found that exposure to toxic metals results in significant decreased growth rate; our results (Figures 32 to 35) did not find a significant reduction in weight between the treated and control fish (Javed and Saeed 2010; Hussain et al. 2011). There was a very slight trend of increased weight in fish as ferric iron exposure increased (Figures 32 to 35). This result is the opposite of the expected results. Length data were not measured for the CT_{max} fish due to the length board being absent when the CT_{max} trials were conducted.

The heater in one CT_{max} test tank failed to heat at a constant rate of 0.3°C/min for five

of the fish tested in week two. The data for these fish were discarded. Five new fish were obtained randomly from the appropriate treatment tank and each trial was restarted with a beginning water temperature of 13°C. The malfunctioning heater was replaced with a new, functioning heater that performed adequately. One timer was unplugged; there were no replacement fish available at that time. The data for the fish associated with the unplugged timer were discarded.

Future CT_{max} experiments employing replicate treatment tanks are suggested in order to decrease the probability of a type 2 error. Increased sample size and an experimental design that included replicate treatment tanks would have improved the validity of the results from this experiment. Experimental design would further be improved upon by including additional exposure concentrations between 1.0 mg/L and 10.0 mg/L. Increasing the number of exposure concentrations would make detecting statistically significant results more likely and would assist in determining the No Observed Effects Level (NOEL) for ferric iron exposure in juvenile cutthroat trout.

An experimental setup that investigates multiple stressors such as the combined effects on CT_{max} of chronic ferric iron exposure with chronically elevated water temperature may provide valuable insight on possible ecologically relevant interactions between chronic precipitated iron exposure and temperature. Future experiments that look at environmentally relevant iron fluctuations on aquatic organism health would also be beneficial. Another possible improvement to our study would be to test the fish multiple times throughout their growth to adult size in order to measure if the CT_{max} changes in an individual fish as the body size increases and the fish ages.

Funding was not available to study the gill histology of the CT_{max} fish in this study. Future studies that combined the CT_{max} results with the results from gill histology may be beneficial to better understand the effects of changes in gill histology on maximum temperature tolerance.

5.3. Conclusion

The data collected in the U_{crit} and CT_{max} studies did not render the expected nor statistically significant results. A variety of challenges were involved with testing swim speed with juvenile rainbow trout in the U_{crit} experiment. The CT_{max} results had a low sample size with few concentrations, making it more challenging to find statistically significant results. Although the intent for the U_{crit} and CT_{max} studies was to help create a more scientifically based water quality standard for iron, the results from the experiments were inconclusive.

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