EFFECTS OF METAL MINE AND MUNICIPAL WASTEWATER ON GROWTH AND ENERGY STORES IN JUVENILE FISHES

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ABSTRACT

The Sudbury, ON, Canada region has been the site of metal mining and processing operations for more than 100 years. The study site for my thesis, Junction Creek, flows southwest through the City of Greater Sudbury and receives cumulative inputs including from the Garson Mine wastewater treatment plant (WWTP), Nolin Creek WWTP (stormwater) and CVRD Inco Limited WWTP (process water) as well as effluent from municipal WWTPs and untreated urban runoff, aerial deposition and historical contamination from multiple sources. Elevated levels of ammonia, Ni, Cu, Co, Pb and As, as well as reduced benthic invertebrate community diversity and density have been observed in the Junction Creek system below certain mine inputs. In addition, the Sudbury region has cold winters, with average daily air temperatures below 0°C from November to March.

The winter stress syndrome hypothesis proposes that the combination of winter conditions and contaminants (acting as physiological stressors) in the aquatic environment could reduce fish condition and deplete energy (lipid) reserves to the point of decreased survival, thus negatively impacting wild fish populations. However the winter stress syndrome hypothesis has rarely been tested in the field. I hypothesized that juvenile fish challenged with a physiological stressor (treated wastewater) in combination with winter conditions would have decreased growth and energy stores as a result of increased metabolism.

The approach I used to examine the potential effects of treated metal mine and municipal wastewaters on bioenergetics and growth, as they related to overwinter survival potential and the winter stress syndrome, of juvenile fish was a combination of a field study and a laboratory experiment. The first objective was to test the winter stress syndrome hypothesis under field conditions. Juvenile fathead minnows (*Pimephales promelas*), creek chub (*Semotilus atromaculatus*) and white sucker (*Catostomus commersoni*) were collected in fall and the following spring from sites along Junction Creek, Sudbury, ON downstream of two metal mining wastewater treatment plants as well as a municipal wastewater treatment plant. The second objective was to test the winter stress syndrome hypothesis in the laboratory by determining the effect of diluted (45 percent) treated CVRD Inco Limited wastewater effluent (CCWWTP) on juvenile fathead minnow growth and energy storage under simulated summer and winter conditions of reduced temperature, photoperiod and food ration. The effect on growth and energy storage of exposure to environmentally relevant ammonia concentrations was also assessed. In both the field and laboratory portions of this study, overwinter survival potential was assessed indirectly through measurements of growth (length, weight, muscle RNA/DNA ratio, muscle proteins) and energy stores (whole body triglycerides). There were inconsistent effects between the field study and the laboratory experiment. In contrast to my hypothesis, fathead minnows in the field study were larger with greater triglyceride stores at exposure sites compared to the reference site. White suckers were smaller at exposure sites but did not differ in triglycerides among sites and creek chub had no clear trend. For the laboratory portion of this study, only fathead minnows were used. After a 90 day exposure to reference or diluted CCWWTP water under simulated winter or summer conditions, juvenile fathead minnows raised in winter CCWWTP water (4°C) had lower whole body triglyceride concentration than those raised in winter reference water. There was no difference in triglycerides in fathead minnows raised in diluted CCWWTP or reference water under summer conditions. This lends support to the winter stress syndrome hypothesis, but the traditional measures of growth showed no significant differences in any of the treatments. In a separate experiment, fathead minnows were exposed from 10-100 days post hatch to graded concentrations of ammonia (0.02 to 0.40 mg unionized NH_3/L) under summer conditions only. There was no effect of ammonia exposure on growth parameters, but a significant increase in total body triglycerides at the highest exposure concentration (0.40 mg/L) was observed.

The results of this study emphasize that laboratory-based hypotheses must be tested in the field to determine their environmental significance. The winter stress syndrome may not apply to northern fish adapted to living and feeding in colder climates and was not strongly supported by my study.

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LIST OF ABBREVIATIONS

CCME Canadian Council of Ministers of the Environment

DPH days post hatch

DNA deoxyribonucleic acid

RNA ribonucleic acid

YOY young-of-the-year

WWTP waste water treatment plant

CCWWTP treated CVRD Inco Limited Copper Cliff wastewater effluent

MWW municipal waste water

PREFACE

This thesis has been organized as two manuscripts for publication in scientific journals. Thus, there is some repetition of introductions, materials and methods and figures throughout. As well, abstracts for each data chapter are included.

Chapter 2 was published in *Environmental Toxicology and Chemistry* 28:296-304 (2009). Chapter 3 will be submitted to the *Archives of Environmental Contamination and Toxicology*.

1. GENERAL INTRODUCTION

1.1. Study Area: Sudbury, Ontario, Canada

Metal mining is an important industry in Canada. As with other types of mining operations, metal extraction produces wastes that may have a negative impact on the surrounding environment. Effects on the aquatic and terrestrial environment from metal mines must be monitored and minimized whenever possible. The study area (Sudbury, Ontario) has been a focal point for Canada's metal mining industry for over 100 years. The first smelter in the area began operation at Copper Cliff in 1888 (Jaagumagi and Bedard 2002). Nickel, copper, zinc, and cobalt are currently mined and processed in the area. Although metal pollution has been well documented in the past, the current major metal mining company in the Sudbury area, CVRD Inco Limited, has attempted to control metal discharge into the aquatic ecosystem (Nriagu et al. 1998; Jaagumagi and Bedard 2002).

Junction Creek flows Southwest through Sudbury, Ontario and receives many anthropogenic inputs such as multiple metal mine discharges, aerial deposition, urban development, treated municipal waste water and more than 100 years of mining and smelting in the area. Conducting ecotoxicological field research in the Junction Creek, Sudbury, ON system is challenging because of the multiple potential sources of contamination to the receiving water. The study site for my field research, Junction Creek, receives cumulative inputs including those from the Garson Mine wastewater treatment plant (WWTP), Nolin Creek WWTP and CVRD Inco Limited's Copper Cliff WWTP as well as effluent from municipal WWTPs and untreated urban runoff, aerial deposition and historical contamination from multiple sources (Figure 1.1).

Elevated levels of ammonia, nickel, copper, cobalt, lead and arsenic as well as reduced benthic community diversity and density have been observed in the Junction Creek system below certain mine inputs (Jaagumagi and Bedard 2002; Weber et al. 2008). It is unclear if potential effects on fish result from metal or ammonia contamination or some other unidentified factor in the environment. Ammonia levels as high as 5.15 mg/L total ammonia (approximately 0.12 mg/L un-ionized ammonia at pH 7.9 and 15^{o}C) in Junction Creek, compared to reference sites with no direct mine input where maximum total ammonia was 0.02 mg/L (approximately 0.21 x 10^{-3} mg/L un-ionized ammonia), have been reported (Weber et al. 2008). The Canadian Water Quality Guidelines for the Protection of Aquatic Life criterion for ammonia is 0.019 mg/L un-ionized ammonia. A 96-hour median lethal concentration (96-hour LC50) of un-ionized ammonia for adult fathead minnows (*Pimephales promelas*) ranges from 0.75 mg/L to 3.4 mg/L where increased temperature decreased ammonia toxicity (Thurston et al. 1983). Increased total water hardness has been reported to decrease the toxicity of ammonia and metals to fish (Tomasso et al. 1980; Tabata 1969). Junction Creek has total hardness ranging from 25 mg/L upstream of direct mine input to 463 mg/L downstream of Copper Cliff after it enters Junction Creek (Weber et al 2008). At the confluence of Junction Creek and the CCWWTP discharge, treated wastewater from the plant makes up approximately 45 percent of the creek volume. Previous laboratory and field studies using Copper Cliff waste water have shown effects on adult fish as well as benthic invertebrates (Hruska and Dubé 2004; Dubé et al. 2006; Rickwood et al. 2006; Weber et al. 2008).

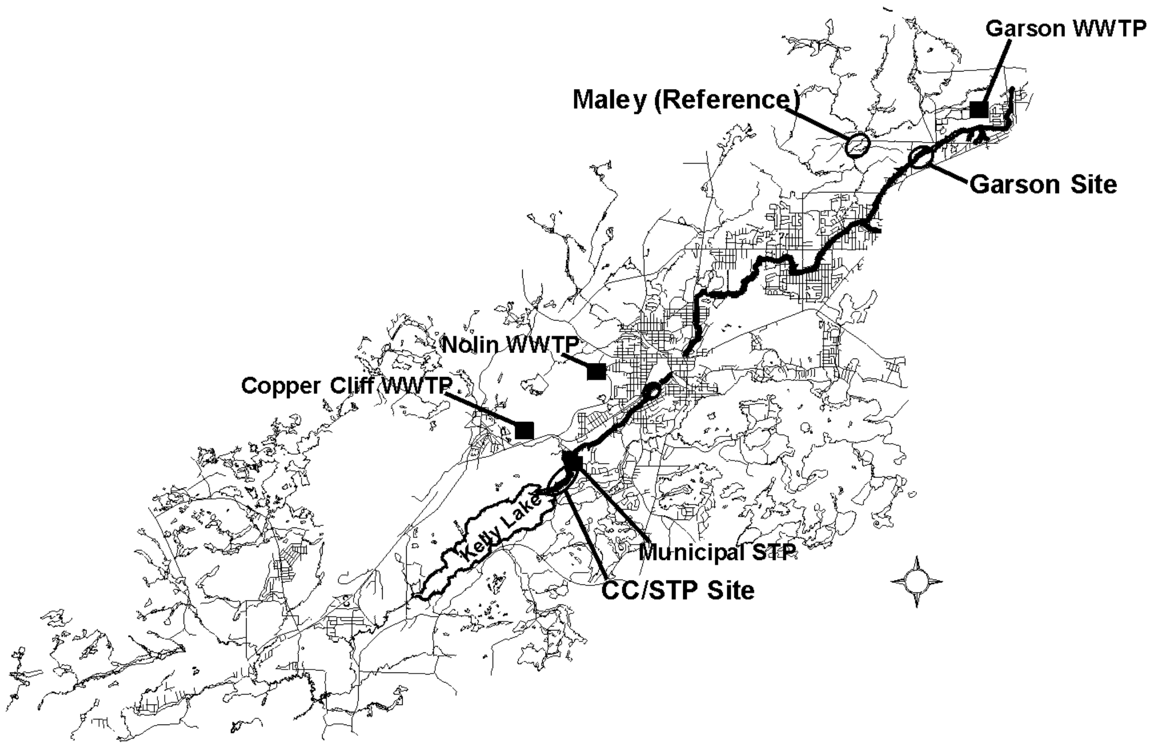

Figure 1.1: Map of the Junction Creek, Ontario area showing two treated effluent sources (Garson WWTP and Copper Cliff WWTP) as well as the municipal WWTP (STP) in relation to fish collection sites.

1.2. Juvenile Fish Recruitment in Relation to Overwinter Stress

Recruitment of young fish into the population is critical for its success. Factors that decrease survival of young fish are likely to have effects on year-class strength and potentially the population (Hurst and Conover 1998; Mills et al. 2000). Northern and temperate winters pose a survival challenge to fish due to conditions such as reduced oxygen, temperature, food, and light (Johnson and Evans 1991; Biro et al. 2004; Byström et al. 2006). Because of their small body size and relatively higher basal metabolic rate, juvenile fish are more vulnerable to predation and starvation than larger adult fish (Eckman 2004; Post and Evans 1989; Biro et al. 2004). Surviving the first winter of life is a critical step in the recruitment of young-of-the-year (YOY) fish into the population.

In order to survive winter in north temperate aquatic systems, fish must achieve sufficient body size and stored lipids (Lemly 1996; Post and Parkinson 2001; Eckmann 2004). Appropriate energy allocation to growth, energy storage, basic metabolism, and other processes such as reproduction is critical for survival. Stressors including temperature change, disease, and pollutants can increase the amount of energy individual fish need to maintain basic metabolic processes and repair damaged biological systems (Calow 1991; Adams 1999; McGeer et al. 2000). For example, rainbow trout (*Oncorhynchus mykiss*) chronically exposed to Cu had increased metabolic requirements, indicated by increased oxygen consumption during exercise (McGeer et al. 2000). These same fish were found to have increased appetite compared to reference fish, but showed no difference in growth rates. Thus, increased energy allocation to basic maintenance can result in decreased growth and/or energy storage (Calow 1991; McGeer et al. 2000). In energy-limited situations, increased metabolic demand for biological maintenance and stress-resisting systems may result in decreased growth rates, decrease energy storage and decreased survival (Calow 1991; Lemly 1993; Congdon et al. 2001). Small increases in energy allocation to any one function, away from growth and energy storage, can result in decreased fitness and perhaps survival (Adams 1999; Congdon et al. 2001).

The winter stress syndrome hypothesis, put forward by Lemly (Lemly 1993; Lemly 1996), proposes that the combination of winter conditions and contaminants (acting as physiological stressors) in the aquatic environment could reduce fish condition and deplete lipid reserves to the point of decreased survival, thus negatively impacting wild fish populations. The combination of normal winter conditions including reduced temperature, food, and light availability along with a physiological or metabolic stressor such as the presence of a contaminant, a parasite or less than ideal water chemistry may increase metabolic demands of individuals to the extent that overwinter survival may be decreased (Lemly 1993; Levesque et al. 2002). For example, juvenile bluegill sunfish (*Lepomis macrochirus*) exposed to selenium under simulated winter conditions (low temperature and reduced photoperiod) in the laboratory exhibited higher mortality, decreased condition factor and reduced lipid stores than fish exposed under summer conditions (Lemly 1993). Winter stress syndrome is the term used to describe lipid depletion, decreased growth, and increased mortality due to a physiological stressor acting in combination with the typical decrease in feeding that most fish experience during winter (Lemly 1996).

The winter stress syndrome hypothesis has rarely been tested in the field. Recent studies (Bennett and Janz 2007a,b) found no evidence of winter stress syndrome occurring in YOY northern pike (*Esox lucius*) or burbot (*Lota lota*) exposed to metal mining effluents in lentic habitats (lakes) in northern Saskatchewan, Canada. In contrast, slimy sculpin (*Cottus cognatus*) inhabiting lotic habitats (creeks) did exhibit evidence of winter stress syndrome (Bennett and Janz 2007b). Further research is needed to investigate the combined effects of natural and anthropogenic stressors in wild fish species in laboratory and field conditions.

1.3. Growth and Bioenergetics – Measures of Overwinter Survival Potential

Gross morphometric endpoints such as weight and length have traditionally been used to estimate the overall condition of fish. In addition to length and weight, condition factor is used as an indicator of relative general health or growth of fish. It is calculated as [weight/length^3] * 100 (Bervoets 2003). Fish with higher condition factor are presumed to be in better general health and condition than those with lower condition factor. Because biochemical changes may become apparent before gross morphological endpoints are affected, these may be more sensitive and earlier indicators of changes in fish condition at the time of sampling (De Boeck et al. 1997; Weber et al. 2003; Bennett and Janz 2007a, b). Biochemical measures, such as whole body lipids, muscle RNA/DNA ratio and muscle protein concentration can be combined with conventional parameters in order to more sensitively detect differences in fish growth and condition (Clemmesen 1988; Adams 1999; Congdon et al. 2001; Weber et al. 2003).

Triglycerides, a family of lipids, are the main form of energy storage in fish (Lochmann et al. 1995; Adams 1999). Whole body triglyceride concentration provides an estimate of the energy stored in individual fish (Bligh and Dyer 1959; Weber et al. 2003). Stored triglycerides can be utilized by individual fish at times when energy requirements are greater than what is available from diet (Sheridan 1988; Benton et al. 1994; Tocher 2003). Sullivan (1986) found that lipid content at the onset of winter, as well as the rate at which it is utilized for energy, is critical to overwinter survival of freshwater fish. Metal contamination from mining activity has been reported to decrease condition factor and increase energetic costs in fish sampled in the field (Levesque et al. 2002). Whole body triglyceride levels represent the main source of stored potential energy in freshwater fish (Adams 1999).

The RNA/DNA ratio is considered an instantaneous measure of growth rate (Buckley 1984) and represents changes in growth over the last 1-3 days. It is based on the assumption that quantity of RNA varies according to protein synthesis and thus reflects recent growth (McLaughlin et al. 1995; Weber et al. 2003). Fish with a higher RNA/DNA ratio are presumed to have higher growth rates at the time of sampling.

Muscle protein concentration is used as an indicator of growth in recent weeks; it is also a potential source of energy in times of increased stress (McLaughlin et al. 1995; Weber et al. 2003). Since only a small amount of muscle tissue (10-20 mg) is required for RNA/DNA isolation and protein measurement, all three biochemical methods as well as traditional measures of growth and condition can be completed in the same individual fish (Weber et al. 2003).

1.4. Study Species

The fathead minnow is a small-bodied forage fish that is part of the Cyprinid family including carps and minnows. It is widely distributed across Canada and the central United States and is commonly used in toxicity testing (Environment Canada 1992; Scott and Crossman 1998; United States Environmental Protection Agency 2002). Because the fathead minnow is such a common test species, its spawning behaviour is well documented and researchers can take advantage of predictable spawning patterns by collecting and rearing embryos for experimentation (Environment Canada 1992; Jensen et al. 2001). Due to its small body size, prolific nature, wide natural distribution and environmental relevance, the fathead minnow represents an excellent fish species for both laboratory and field research in aquatic toxicology.

Similar to the fathead minnow, creek chub (*Semotilus atromaculatus*) are commonly found in eastern and central Canada (Scott and Crossman 1998). Another small-bodied fish that are commonly used for bait, creek chub spawn mainly in May and June. Within the first year, juvenile creek chub can attain a length of 5-9 centimetres and reach sexual maturity in their third or fourth year (Scott and Crossman 1998). Creek chub are found in the study area of Junction Creek (Jaagumagi and Bedard 2002; Lemieux et al. 2004; Dubé et al. 2006; Weber et al. 2008).

Juvenile white sucker (*Catostomus commersoni*) were also plentiful at the study sites in both fall and spring field collections. The YOY white suckers captured were approximately the same size as the intended study species, making it an ideal candidate for comparison of growth and bioenergetic endpoints using the methods in this study. It also is considered a habitat generalist (Schultz 2003) but is a large-bodied fish (up to 40 centimetres) that does not reach sexual maturity until it is between three and five years of age (Schultz 2003).

1.5. Research Objectives and Hypotheses

Due to the complexity of natural systems, and the particularly high number of confounding variables in Junction Creek, the present study was initiated to compare the potential effects of CCWWTP discharge on bioenergetics and growth of juvenile fish in the field and in isolation in the laboratory under more controlled conditions. The first objective was to test the winter stress syndrome hypothesis under field conditions. Juvenile fathead minnows, creek chub and white sucker were collected in fall and the following spring from Junction Creek directly downstream of Garson Mine and Copper Cliff WWTPs and directly downstream of the City of Sudbury municipal wastewater treatment facility. Overwinter survival potential was assessed indirectly through growth measurements (length, weight, muscle RNA/DNA ratio, muscle proteins) and energy stores (whole body triglycerides). We hypothesized that fish collected from exposure sites would exhibit decreased size, body condition and energy stores compared to fish from a reference site in both spring and fall due to the potential increased metabolism caused by physiological stress of effluent exposure. In addition, we hypothesized that fish collected in the spring from all sites would have decreased energy stores compared to those caught the previous fall due to energy mobilization and use during the overwinter period.

The second objective of this study was to test the winter stress syndrome hypothesis in the laboratory by determining the effect of diluted (45 percent) CCWWTP effluent on juvenile fathead minnow growth and energy storage under simulated summer and winter conditions. Since ammonia is known to be toxic fish and was one of the major constituents of the treated waste water, the effects of ammonia in isolation on growth and energy storage of juvenile fathead minnows was also assessed. I hypothesized that juvenile fathead minnows challenged with both simulated winter and exposure to 45 percent CCWWTP water would have decreased growth and energy stores as a result of potentially increased metabolism compared to those not exposed to CCWWTP water and living under simulated summer conditions. In addition, I hypothesized that exposure to one component of the CCWWTP effluent, ammonia, at environmentally relevant concentrations would produce similar deficits in growth and energy storage in juvenile fathead minnows.

2. OVERWINTER ALTERATIONS IN ENERGY STORES AND GROWTH IN JUVENILE FISHES INHABITING AREAS RECEIVING METAL MINING AND MUNICIPAL WASTEWATER EFFLUENTS

2.1. Abstract

The winter stress syndrome hypothesis proposes that the combination of winter conditions and contaminant exposure reduces overwinter survival in juvenile fishes, mainly due to increased depletion of stored energy (lipids). To test this hypothesis in the field, juvenile fathead minnows, (*Pimephales promelas*) creek chub (*Semotilus atromaculatus*) and white sucker (*Catostomus commersoni*) were collected from three exposure sites along Junction Creek, Sudbury, ON Canada, representing cumulative inputs from metal mining and municipal waste water. Overwinter survival potential was determined through measurements of growth (length, weight, muscle RNA/DNA ratio, muscle proteins) and energy stores (whole body triglycerides) in fish collected just prior to and following the overwinter period. We hypothesized that fish collected from exposure sites would exhibit reduced growth and energy storage compared to reference fish in both fall and spring, and that fish from all sites would exhibit reduced energy storage in spring compared to the previous fall. Whole body selenium concentrations were elevated (11-42 µg/g dry weight) in juvenile fathead minnows and white sucker collected at two exposure sites in comparison to fish collected from the reference site (3-6 µg/g dry weight). In contrast to our hypothesis, fathead minnows were larger with greater triglyceride stores at exposure sites compared to the reference site. White suckers were smaller at exposure sites but did not differ in triglycerides among sites. Overall, the results in these fish species exposed to metal mining and municipal wastewaters do not support the winter stress syndrome hypothesis. It is recommended that future studies focus on relating growth and energy storage with other environmental factors such as habitat and food availability in addition to anthropogenic contamination.

2.2. Introduction

Recruitment of juvenile fish into a population is important for its sustainability, and recruitment failure caused by environmental stress can cause local population extinction (Mills et al. 2000). In north temperate regions, fish face prolonged periods of reduced temperature, light, and food availability. Due to their smaller size and relatively higher basal metabolic rate, juvenile fish are more vulnerable to predation and starvation than larger, adult fish (Eckmann 2004; Post and Evans 1989; Biro et al. 2004). During the summer, juvenile fish must optimize allocation of energy to both growth and storage in order to maximize winter survival (Eckmann 2004; Post and Evans 1989; Lemly 1996). Surviving the first winter is critical to the recruitment of young-of-the-year (YOY) fish into the population, and the threat of overwinter mortality in subsequent years decreases afterwards (Post and Parkinson 2001; Calow 1991).

Increased environmental stress including temperature change, disease and pollutants can increase the amount of energy individual fish need to maintain basic metabolic processes and repair damaged biological systems (Adams 1999; McGeer et al. 2000; Gongdon et al. 2001). For example, rainbow trout (*Oncorhynchus mykiss*) exposed to copper showed increased oxygen consumption compared to unexposed fish when forced to swim against high water velocities, indicating increased metabolism (McGeer et al. 2000). These same fish were found to have increased appetite compared to reference fish, but showed no difference in growth rates. Thus, increased energy allocation to basic maintenance can result in decreased growth and/or energy storage (Calow 1991; Adams 1999; McGeer et al. 2000; Congdon et al 2001). Stressors that are normally tolerable in summer due to abundant food and warmer water temperatures may become lethal under winter conditions. For example, juvenile bluegill sunfish (*Lepomis macrochirus*) exposed to selenium under simulated winter conditions (low temperature and reduced photoperiod) in the laboratory exhibited higher mortality, decreased condition factor and reduced lipid stores than fish exposed under summer conditions (Lemly 1993). The winter stress syndrome hypothesis, put forward by Lemly (Lemly 1996; Lemly 1993), proposes that the combination of winter conditions and contaminants (acting as physiological stressors) in the aquatic environment could reduce fish condition and deplete lipid reserves to the point of decreased survival, thus negatively impacting wild fish populations. However the winter stress syndrome hypothesis has rarely been tested in the field. Recent studies (Bennett and Janz 2007a,b) found no evidence of winter stress syndrome occurring in YOY northern pike (*Esox lucius*) or burbot (*Lota lota*) exposed to metal mining effluents in lentic habitats (lakes) in northern Saskatchewan, Canada. In contrast, slimy sculpin (*Cottus cognatus*) inhabiting lotic habitats (creeks) did exhibit evidence of winter stress syndrome (Bennett and Janz 2007b). Further research is needed to investigate the combined effects of natural and anthropogenic stressors in wild fish species.

The Sudbury, ON, Canada region has been the site of metal mining and processing operations for more than 100 years. Junction Creek flows southwest through the City of Greater Sudbury receiving cumulative inputs including those from the Garson Mine wastewater treatment plant (WWTP), Nolin Creek WWTP and Copper Cliff WWTP as well as effluent from municipal WWTPs and untreated urban runoff, aerial deposition and historical contamination from multiple sources. Elevated levels of ammonia, Ni, Cu, Co, Pb and As, as well as reduced benthic community diversity and density have been observed in the Junction Creek system below certain mine inputs (Jaagumagi and Bedard 2002). In addition, the Sudbury region has cold winters, with average daily air temperatures below 0°C from November to March. The objective of this study was to test the winter stress syndrome hypothesis in the field. Juvenile fathead minnows (*Pimephales promelas*), creek chub (*Semotilus atromaculatus*) and white sucker (*Catostomus commersoni*) were collected in fall and the following spring from Junction Creek directly downstream of Garson Mine and Copper Cliff WWTP and directly downstream of the City of Sudbury municipal wastewater treatment facility. Overwinter survival potential was assessed indirectly through growth measurements (length, weight, muscle RNA/DNA ratio, muscle proteins) and energy stores (whole body triglycerides). We hypothesized that fish collected from exposure sites would exhibit decreased size, body condition and energy stores compared to fish from a reference site in both spring and fall due to the potential increased metabolism caused by physiological stress of effluent exposure. In addition, we hypothesized that fish collected in the spring from all sites would have decreased energy stores compared to those caught the previous fall due to energy mobilization and use during the overwinter period.

2.3. Materials and Methods

2.3.1. Study sites

The CVRD Inco operation located in Sudbury, ON, Canada is the largest fully integrated mining, milling, smelting, and refining complex in Canada. Products of the Sudbury operation include Ni, Cu, precious metals, platinum-group metals, sulphuric acid and liquid sulphur dioxide. Junction Creek flows southwest for approximately 25 km from its headwaters near Garson Mine, through the City of Greater Sudbury and into Kelly Lake.

In October 2004 and May 2005, juvenile (refers to fish that were not yet sexually mature) fathead minnows, creek chub and white sucker were collected downstream of Garson Mine WWTP (46^{o} 31' 44" N, 80^{o} 55' 29" W; referred to as Garson from this point forward), Copper Cliff WWTP (46^{o} 27'56"N, 81^{o} 02'11" W; referred to as Copper Cliff from this point forward), and the City of Sudbury municipal WWTP (46^{o} 27'32" N, 81^{o} 02'20" W; referred to as MWW from this point forward). The exposure site downstream of Copper Cliff is about 200 m upstream of the effluent discharge from MWW. These two sites were chosen in an attempt to distinguish potential differences in exposure and effects between metal mine vs. municipal discharges (Weber et al. 2008). The reference site, Maley Branch of Junction Creek (46^{o}31'44"N, 80^{o}55'29"W), is upstream of direct mining wastewater inputs within the same catchment as the exposure sites and has received historical atmospheric deposition inputs of trace metals from a century of human activity (Jaagumagi and Bedard 2002).

2.3.2. Fish collection

Juvenile fathead minnows, creek chub and white sucker were collected using a combination of backpack electrofishing (Model LR-24, Smith-Root, Vancouver, WA, USA) and minnow traps baited with beef liver wrapped in cheesecloth (to prevent ingestion by trapped fish). Where a sufficient number of fish were caught (> 15), juveniles were selected by excluding any fish exhibiting secondary sex characteristics (such as breeding colors and/or tubercles in the case of fathead minnows and creek chub). Certain sites yielded catches of only 5 to 15 fish, so all were collected and later excluded (when necessary) on basis of age. Fish were over-anaesthetized with MS-222 and fork lengths (to the nearest 0.01 cm) were recorded in the field. Each fish was placed individually in a Ziploc® bag, frozen on dry ice and transported to a -85 ^{o}C freezer at the University of Saskatchewan. Fish weights (to the nearest mg) were recorded in the laboratory at the time of first thaw for biochemical endpoints. Separate fish were used for whole body trace metal analyses and biochemical analyses, as described below.

2.3.3. Water chemistry and metal body burdens

At each fish collection site, one water sample was collected in an acid-washed polyethylene bottle. These water samples were kept on ice and transported within 6 hr to the CVRD Inco Central Laboratory (Copper Cliff, ON, Canada) for analysis of basic water chemistry and trace metal concentrations by ICP-AES. Dissolved oxygen, temperature, pH, conductivity and total dissolved solids were all measured once at each study site in both fall and spring fish collections using a handheld YSI meter (YSI Environmental, Yellow Springs, OH, USA) approximately 10 cm above the sediment.

Metal body burdens in juvenile fathead minnows and white sucker were determined by ICP-MS at Testmark Laboratories (Sudbury, ON, Canada). Frozen fish were finely minced with scissors and homogenized in reagent-grade water for 2 x 10 sec bursts with a Tissue Tearor (BioSpec Products, Bartlesville, OK, USA). Homogenates were transferred to acid-washed polyethylene bottles and freeze-dried before shipping to Testmark Laboratories. Blank metal values were determined by using the same amount of reagent-grade water and homogenization time as for actual fish samples.

2.3.4. Fish age

Fish age in calendar years was used to exclude non-juveniles from the growth and bioenergetic analyses. Our goal was to use only YOY fish (age 1+ in spring collections) but insufficient numbers of fish were collected from all sites to allow statistical comparisons. Thus, sexually immature fish less than 2 years old were considered juvenile for the purpose of this study. White sucker age was determined using otoliths. Creek chub and fathead minnows were aged using scales taken from just below the anterior end of the dorsal fin. All fish aging was performed by the Ontario Federation of Anglers and Hunters (Peterborough, ON, Canada).

2.3.5. Whole body triglyceride concentration

For biochemical assays, all chemicals and reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless specified otherwise. Fish carcasses (except 10 to 20 mg caudal muscle removed for muscle RNA/DNA ratio and protein concentration) were used to determine whole body triglycerides (Weber et al. 2003). Fish were finely minced with scissors and homogenized in 2x volume reagent-grade water for 3 x 10 sec with a Tissue Tearor on ice. Equal volumes of the homogenized sample and 0.4 M sodium citrate were mixed and heated at 100^{o}C for 5 min before placed to cool on ice. Samples were then diluted 1/5 (v/v) with 100 percent isopropanol, vortexed vigorously, then centrifuged at 2500 rpm for 5 min. Whole body triglyceride concentrations were determined in the supernatant using a method developed for serum triglycerides (McGowan et al. 1983) and modified for use in fish (Weber et al. 2003). Glycerol was used as a standard. Whole body triglyceride concentration was expressed as mg triglycerides/g fish.

2.3.6. Muscle RNA/DNA ratio

A 10 to 20 mg portion of frozen fish muscle was removed at the caudal peduncle, weighed, and placed on ice in a microcentrifuge tube. Nucleic acids were isolated as described previously (Weber et al. 2003). A modification of the dual fluorescent dye method described by Clemmesen (1988) was used to determine RNA/DNA ratio (Weber et al. 2003). Calf thymus DNA and calf liver RNA were used as standards.

2.3.7. Muscle protein determinations

Muscle proteins were measured using a modification of the Lowry (1951) protein assay (BioRad DC, Hercules, CA, USA). An aliquot of the initial muscle sample homogenate prepared for RNA/DNA ratio determination was used to determine protein. Bovine serum albumin was used to create the standard curve.

2.3.8. Statistical analyses

Two-way analysis of covariance (ANCOVA) with site and season as factors and age as covariate was used for comparisons of fish lengths and weights. Fish weights were also compared using two-way ANCOVA with length as covariate. Differences in whole body triglyceride concentration, muscle RNA/DNA ratio and muscle proteins were detected using two-way analysis of variance (ANOVA) with site and season as factors. Both ANCOVAs and ANOVAs were followed by Tukey's post-hoc test if the overall *P*-value was significant ($p < 0.05$). Bartlett's test and the Kolmogorov-Smirnov test were used to examine data for homogeneity of variance and normality, respectively. Length and weight were $\log_{10}$ transformed before statistical analysis if necessary. When data were missing (i.e. spring creek chub downstream of MWW) or when there were significant interactions between factors (site and season), one-way ANOVA was used to detect differences among sites and seasons separately, followed by Tukey's test as appropriate. Data are presented as mean ± standard error of the mean (SEM). Metal body burden data were analyzed using two-way ANCOVA with site and season as factors. Age was not used as a covariate because it was not found to be a significant factor influencing metal body burdens for either species.

2.4. Results

2.4.1. Abiotic environment

Exposure sites were characterized by elevated conductivity and total dissolved solids, with no differences in dissolved oxygen or pH (Table 2.1). Temperatures ranged from 12.2 to 15.6°C in fall and 9.5 to 11.0°C in spring (Table 2.1). Detailed water chemistry of samples collected in spring 2005 from all study sites, including trace metal concentrations that exceeded the Canadian Council of Ministers of the Environment

(CCME) water quality guidelines (1999) or were increased at least ten-fold over the value measured at the reference site are shown in Table 2.2. Conductivity, hardness, ammonia, nitrate, and sulphate generally increased in a cumulative manner at each sample collection point from the reference site downstream to MWW along Junction Creek. Four metals exceeded the CCME guideline for the protection of aquatic life (Table 2.2). Aluminum exceeded the CCME guideline at all three exposure sites and increased in a cumulative manner from 37.3 µg/L at the reference site to 246 µg/L downstream of MWW. Copper exceeded the CCME guideline at all fish collection sites and ranged from 5 µg/L downstream of Garson to 230 µg/L downstream of Copper Cliff. Iron exceeded the CCME guideline at the reference site and MWW. Nickel was exceeded at all study sites and increased in a cumulative manner downstream from the reference site (103 µg/L) to downstream of MWW (267 µg/L). In general, the remaining trace metal concentrations increased in a cumulative manner along Junction Creek with the reference site being the lowest, downstream of Garson having slightly higher concentrations, and downstream of Copper Cliff and MWW similar to each other but higher than the reference site (Table 2.1).

Table 2.1: Basic water chemistry recorded from the reference site and the three exposure sites (downstream Garson, Copper Cliff and Municipal Waste Water) in fall 2004 and spring 2005 field collections.

		Reference		Garson		Copper Cliff		Municipal Waste Water	
Analyte	Units	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005
Conductivity	µS/cm	107	125	2051	1370	2161	2256	2164	2402
Dissolved Oxygen	mg/L	7.9	n/a	7.5	n/a	9.5	5.7	9.1	7.0
pH		7.3	7.1	7.1	7.8	7.0	7.4	7.3	7.7
Total Dissolved Solids	mg/L	0.09	0.05	1.69	0.70	1.86	1.20	1.72	1.20
Temperature	^{o}C	12.2	11.0	13.9	9.5	12.3	10.9	15.6	10.5

n/a = data not available

Table 2.2: Summary of water chemistry and trace metal concentrations for metals of interest (metals that exceeded the Canadian Council for Ministers of the Environment Guidelines for the Protection of Aquatic Life (1999) are in bold; metals that were increased at least tenfold in water at exposure sites compared to the reference site are in italics; and metals that were significantly different in either fathead minnow or white sucker (whole body) from exposure sites compared to the reference site are in plain text). Samples were collected in spring 2005 from the reference site (Maley) and the three exposure sites (downstream Garson, Copper Cliff and Municipal Waste Water). Values preceded by the < symbol represent the detection limit for that analyte.

Analyte	Units	CCME Guideline	Reference	Garson	Copper Cliff	Municipal Waste Water
Conductivity	µS/cm		114.9	*1391*	*2538*	*2396*
Total Hardness	mg/L		23	*518.1*	*1113*	*1024*
Ammonia (as NH_3)	µg/L	19	< 100	< 100	*4270*	*5500*
NO_3	mg/L	13.0	< 0.10	10	7.2	6.1
SO_4	mg/L		18.2	*412.8*	*1281*	*1194*
Ca	mg/L		8.42	*172*	*363*	*334*
Cl	mg/L		1.63	*155*	*114*	*118*
F	µg/L		< 0.1	< 0.1	*0.2*	0.2
K	mg/L		0.56	*9.2*	*29*	*27*
Li	µg/L		< 1.2	8.0	*44*	*39*
Mg	mg/L		3.0	22	*50*	*46*
Na	mg/L		1.7	*74*	*112*	*111*
Al	**µg/L**	**100**[(a)]	37.3	**104**	**271**	**246**
As	µg/L	5	< 65.6	< 65.6	< 65.6	< 65.6
B	µg/L		< 12.0	36.7	53.4	54.3
Ba	µg/L		13.4	51.2	48.1	44.4
Be	µg/L		< 0.3	< 0.3	< 0.3	< 0.3
Bi	µg/L		< 59.2	< 59.2	< 59.2	< 59.2
Cd	µg/L	0.017	< 12.7	< 12.7	< 12.7	< 12.7
Co	µg/L		< 9.6	< 9.6	36.4	30.9
Cr	µg/L	1	< 6.00	< 10.3	15.9	9.35
Cu	**µg/L**	**2-4**[(b)]	**24**	**5**	**230**	**207**
Fe	**mg/L**	**0.30**	**0.57**	0.12	**0.30**	**0.41**

Mn	µg/L		69.6	63.1	45.4	56.6
Mo	µg/L	73	< 16	< 16	< 16	< 16
Ni	**µg/L**	**25 to 150[b)]**	**103**	**161**	**285**	**267**
P	mg/L		< 0.06	< 0.25	*0.69*	*0.68*
Pb	µg/L	1 to 7[(b)]	< 31	< 31	< 31	< 31
S	mg/L		5.51	132	430	391
Se	µg/L	1.0	< 196	< 196	< 196	< 196
Si	mg/L		0.21	1.0	1.5	1.8
Tl	µg/L		< 4.80	10.4	24.8	24.3

[(a)] Guideline is pH dependent, value reported is for range observed in water bodies during this water sample period (May 2005)
[(b)] Guideline is hardness dependent, increasing with hardness

2.4.2. Sample size

The October 2004 juvenile fish collection yielded a total of 50 white sucker, 75 creek chub and 70 fathead minnows from the four study sites combined. The May 2005 juvenile fish collection yielded a total of 50 white sucker, 94 creek chub, and 57 fathead minnows. These represent only the numbers of the study species collected, not total catch because of large numbers of other species (e.g. dace) at certain sites that were not counted for logistical reasons.

2.4.3. Morphometric endpoints – length-at-age, weight-at-age, weight-at-length

Fathead minnows

Fathead minnows had significantly increased length-at-age and weight-at-age at all three exposure sites when compared to the reference site in both fall and spring collections (Table 2.3). Fathead minnows collected from both the reference site and downstream of MWW showed increased length-at-age in the spring collection compared to the previous fall (Table 2.3). There were significant increases in weight-at-age at the reference site, downstream of Copper Cliff, and downstream of MWW in the spring collection compared to the previous fall. For weight-at-length, there was a significant interaction between the site and season using two-way ANCOVA. Comparing site and season separately did not detect any significant differences in weight-at-length among fathead minnows collected from the exposure sites and reference site or between fall and spring within each site (Table 2.3).

Creek chub

Creek chub length-at-age was significantly different among sites in each season (Table 2.4). Chub collected downstream of Garson were significantly shorter while those collected downstream of Copper Cliff were significantly longer than the reference fish in the fall (Table 2.4). Chub collected downstream of Garson were also shorter than reference creek chub in the spring. Creek chub collected from both the reference site and downstream of Garson had increased length-at-age in the spring compared to the previous fall. There was a significant difference in creek chub weight-at-age among sites in fall and spring. In fall, weight-at-age was significantly decreased downstream of Garson and increased downstream of Copper Cliff compared to the reference site. In spring, creek chub downstream of Garson and Copper Cliff had decreased weight-at-age compared to the reference site. Seasonally, creek chub collected from the reference site and downstream of Garson had increased weight-at-age in spring compared to fall. Creek chub collected downstream of Garson had decreased weight-at-length compared to the reference site in both fall and spring. In spring, creek chub collected downstream of Garson had increased weight-at-length compared to the fall, while those downstream of Copper Cliff had decreased weight-at-length in spring compared to fall (Table 2.4).

Table 2.3: Length and weight of juvenile fathead minnows (*Pimephales promelas*) collected at the reference site (Maley) and three exposure sites (Garson, Copper Cliff and Municipal Wastewater) in fall 2004 and spring 2005. Data are mean ± standard error of the mean.

	Sample Size	Length (cm)	Weight (g)
Reference			
Fall 2004	15	4.57 ± 0.19	1.08 ± 0.17
Spring 2005	16	5.38 ± 0.11†††	1.64 ± 0.11†
Garson			
Fall 2004	14	6.20 ± 0.14***	2.91 ± 0.18***
Spring 2005	10	6.26 ± 0.23**	3.14 ± 0.43***
Copper Cliff			
Fall 2004	18	5.95 ± 0.19***	2.53 ± 0.27***
Spring 2005	5	6.36 ± 0.49***	3.22 ± 0.49***††
Municipal Waste Water			
Fall 2004	15	6.11 ± 0.22**	3.03 ± 0.36**
Spring 2004	10	6.78 ± 0.17***†††	4.03 ± 0.35***†††

*Significant difference between exposure site and reference site in that season (**$p<0.01$, ***$p<0.001$).

†Significant difference between seasons within a study site (†$p<0.05$, ††$p<0.01$, †††$p<0.001$).

Significant differences were detected using two-way ANCOVAs (length-at-age, weight-at-age and weight-at-length) with site and season as factors, followed by Tukey's post hoc test. There were no significant differences in weight-at-length.

Table 2.4: Length and weight of juvenile creek chub (*Semotilus atromaculatus*) collected at the reference site (Maley) and three exposure sites (Garson, Copper Cliff and Municipal Wastewater) in fall 2004 and spring 2005. Data are mean ± standard error of the mean.

	Sample Size	Fork Length (cm)	Weight (g)
Reference			
Fall 2004	19	4.92 ± 0.12	1.18 ± 0.09
Spring 2005	28	6.23 ± 0.23†††	2.66 ± 0.35††
Garson			
Fall 2004	15	3.95 ± 0.06***	0.65 ± 0.04***$^{\#\#\#}$
Spring 2005	35	4.71 ± 0.21***††	1.19 ± 0.19***$^{\dagger\#\#@@@}$
Copper Cliff			
Fall 2004	7	6.01 ± 0.25*	2.28 ± 0.32**
Spring 2005	16	5.84 ± 0.15	1.95 ± 0.15*$^{@}$
Municipal Waste Water			
Fall 2004	18	5.06 ± 0.13	1.32 ± 0.11
Spring 2005	0	n/a	n/a

*Significant difference between exposure site and reference site in that season (*p<0.05, **p<0.01, ***p<0.001).
†Significant difference between seasons within a study site (†p<0.05, ††p<0.01, †††p<0.001).
$^{\#}$Significant difference in weight-at-length between exposure site and reference site ($^{\#\#}$p<0.01,$^{\#\#\#}$p<0.001).
$^{@}$Significant difference in weight-at-length between seasons within a study site ($^{@}$p<0.05, $^{@@@}$p<0.001).
Significant differences were detected using two-way ANCOVAs (length-at-age, weight-at-age and weight-at-length) with site and season as factors, followed by Tukey's post hoc test.
n/a = no creek chub were collected.

White sucker

In fall, white sucker collected downstream of Copper Cliff had significantly decreased length-at-age compared to the reference site (Table 2.5). In spring, white sucker collected at all three exposure sites had decreased length-at-age compared to the reference site. White sucker collected from the reference site and Copper Cliff had increased length-at-age in spring compared to fall. White sucker collected in fall downstream of Copper Cliff had significantly lower weight-at-age than those from the reference site. In spring, white sucker collected at all three exposure sites had decreased weight-at-age compared to the reference site. There were no significant differences detected in weight-at-length when comparing exposure and reference sites, or within sites between seasons (Table 2.5).

Table 2.5: Length and weight of juvenile white sucker (*Catostomus commersoni*) collected at the reference site (Maley) and three exposure sites (Garson, Copper Cliff and Municipal Wastewater) in fall 2004 and spring 2005. Data are mean ± standard error of the mean.

	Sample Size	Fork Length (cm)	Weight (g)
Reference			
Fall 2004	17	7.80 ± 0.41	5.84 ± 0.94
Spring 2005	5	9.09 ± 0.30††	7.69 ± 0.98
Garson			
Fall 2004	8	6.09 ± 0.13	2.45 ± 0.21
Spring 2005	15	5.97 ± 0.25***	2.31 ± 0.26***
Copper Cliff			
Fall 2004	16	6.08 ± 0.15***	2.30 ± 0.19***
Spring 2005	15	7.77 ± 0.21**†††	4.58 ± 0.39**†††
Municipal Waste Water			
Fall 2004	8	6.56 ± 0.17	3.01 ± 0.21
Spring 2005	14	7.70 ± 0.24**	4.57 ± 0.39**

*Significant difference between exposure site and reference site in that season (**p<0.01, ***p<0.001).
†Significant difference between seasons within study site (††p<0.01, †††p<0.001).
Significant differences were detected using one-way ANCOVAs (length-at-age, weight-at-age and weight-at-length) with site and season as factors, followed by Tukey's post hoc test. There were no significant differences in weight-at-length.

2.4.4. Biochemical endpoints – whole body triglyceride concentration, muscle RNA/DNA ratio, muscle proteins

Fathead minnows

In the fall, fathead minnows collected downstream of Garson and MWW had increased whole body triglyceride concentrations compared to the reference site (Figure 2.1). In spring, fathead minnows collected from all three exposure sites had increased triglyceride concentrations compared to the reference site. Fathead minnows collected from the reference site and downstream of Garson had decreased whole body triglyceride concentrations in the spring compared to the fall (Figure 2.1). In the fall, fathead minnows collected downstream of Garson had decreased muscle RNA/DNA ratio compared to the reference site (Figure 2.1). There were no differences in muscle RNA/DNA ratio among fathead minnows collected from the reference and exposure sites in the spring. Within sites, muscle RNA/DNA ratio was greater in fathead minnows collected downstream of Garson and Copper Cliff in the spring compared to the fall (Figure 2.1). Compared to fathead minnows collected from the reference site in the same season, muscle protein concentration was significantly decreased downstream of Garson and Copper Cliff in the fall as well as Copper Cliff in the spring.

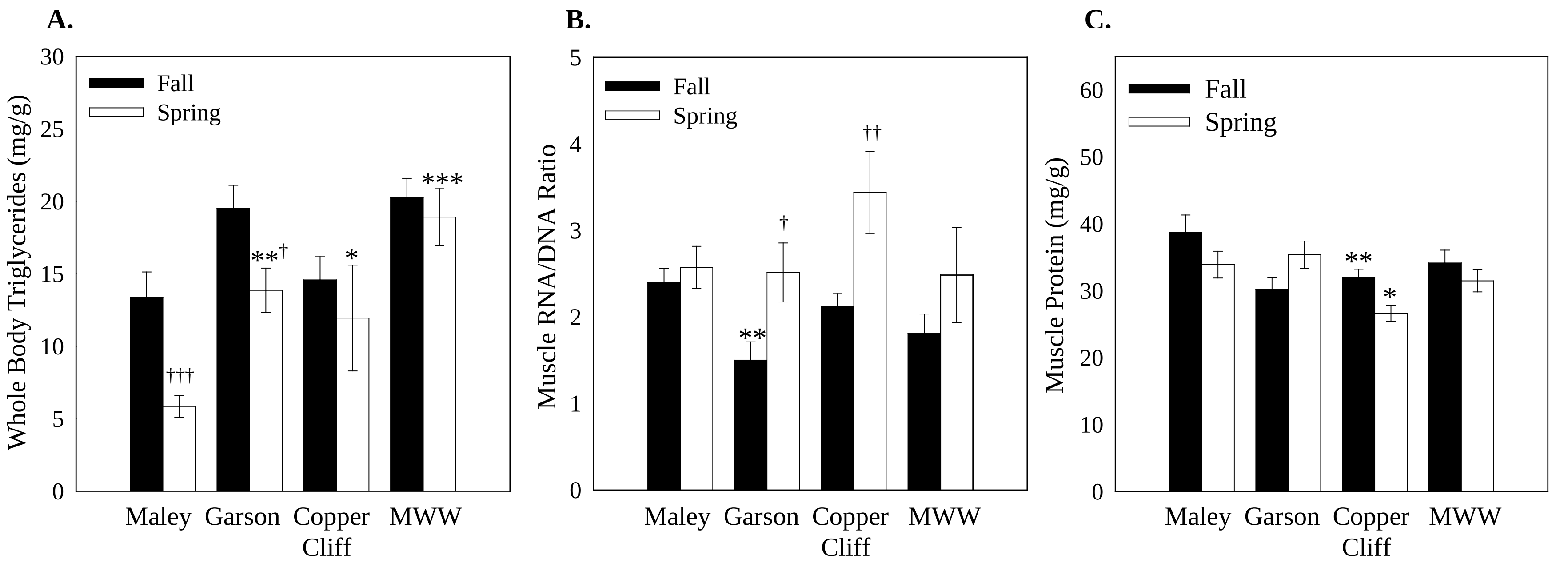

Figure 2.1: Whole body triglycerides (A), muscle RNA/DNA ratio (B) and muscle protein concentration (C) determined in juvenile fathead minnows (*Pimephales promelas*) collected in fall 2004 (n = 14 - 15) and spring 2005 (n = 5 – 16) from three effluent exposure sites (Garson, Copper Cliff and Municipal Waste Water) and one upstream reference site (Maley) on Junction Creek, Ontario, Canada. Data are mean ± standard error of the mean. Significant differences were detected using two-way ANOVA with site and season as factors, followed by Tukey's post hoc test. *Significant difference between exposure site and reference site within a season (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$); † Significant difference between seasons within a study site († $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$).

Creek chub

In the fall, triglyceride concentration was lower in fathead minnows from Garson compared to the reference site but higher downstream of MWW compared to the reference site (Figure 2.2). Creek chub collected downstream of Copper Cliff had higher whole body triglycerides than those from the reference site in the spring. Seasonally, the only difference in triglyceride concentrations was observed at the reference site where triglycerides were lower in creek chub in spring compared to fall (Figure 2.2). Creek chub muscle RNA/DNA ratio was not different in the fall among sites, but a site difference was detected in the spring, where Garson creek chub had decreased RNA/DNA ratio compared to reference (Figure 2.2). Muscle RNA/DNA ratio in creek chub was lower in the spring than the fall at the reference site and downstream of Garson. There were no statistically significant differences detected in muscle protein concentrations among sites in the fall collection, or within sites between seasons (Figure 2.2). In spring, muscle protein concentration was significantly higher in creek chub collected downstream of Garson compared to the reference site.

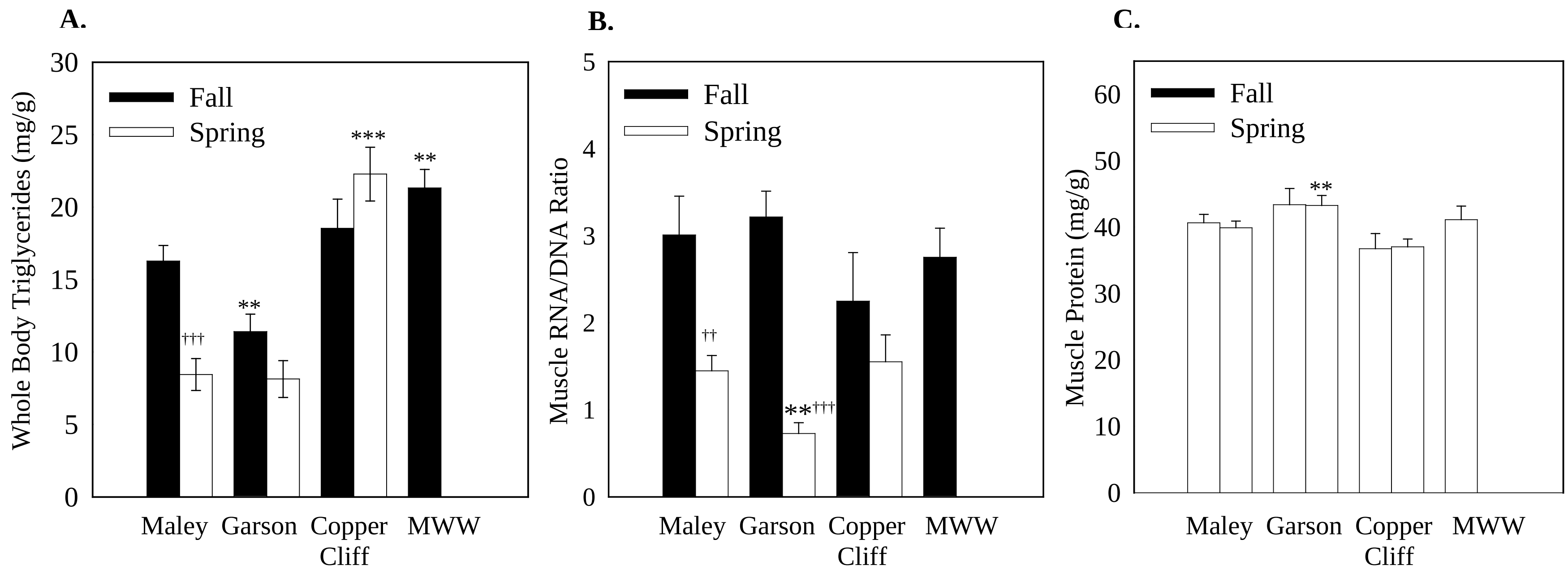

Figure 2.2: Whole body triglycerides (A), muscle RNA/DNA ratio (B) and muscle protein concentration (C) determined in juvenile creek chub (*Semotilus atromaculatus*) collected in fall 2004 (n = 7 - 18) and spring 2005 (n = 7 - 16) from three effluent exposure sites (Garson, Copper Cliff and Municipal Waste Water) and one upstream reference site (Maley) on Junction Creek, Ontario, Canada. Data are mean ± standard error of the mean. Significant differences were detected using two-way ANOVA with site and season as factors, followed by Tukey's post hoc test. *Significant difference between exposure site and reference site within a season (**p < 0.01); † Significant difference between seasons within a study site (††p < 0.01, †††p < 0.001).

In fall there was no difference in white sucker triglyceride concentrations among exposure and reference sites. In spring, white sucker collected downstream of Garson had higher triglyceride concentrations than those collected from the reference site (Figure 2.3). Seasonally, white sucker collected from the reference site had significantly decreased whole body triglycerides in the spring compared to the previous fall. There were no significant differences in muscle RNA/DNA ratio among white sucker collected from reference and exposure sites. White sucker collected downstream of Copper Cliff and MWW in the spring had decreased muscle RNA/DNA ratio compared to the previous fall (Figure 2.3). There were no significant differences in white sucker muscle protein concentrations among sites or between seasons (Figure 2.3).

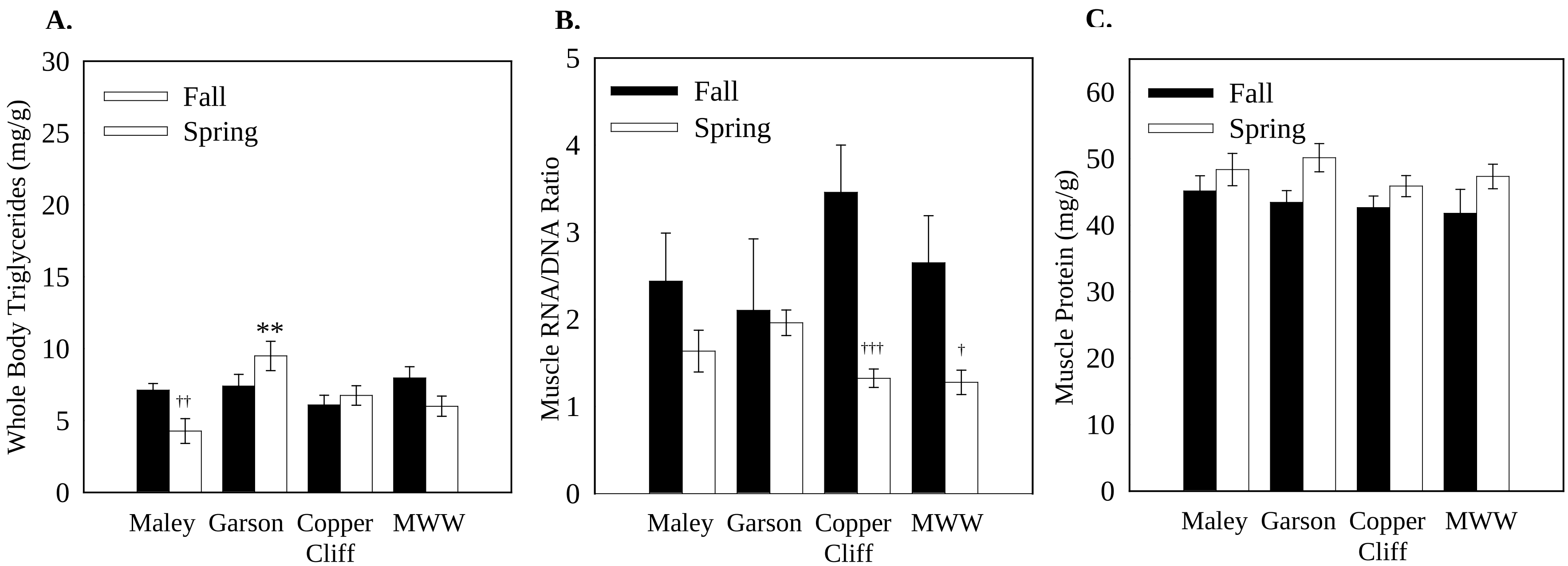

Figure 2.3: Whole body triglycerides (A), muscle RNA/DNA ratio (B) and muscle protein concentration (C) determined in juvenile white sucker (*Catostomus commersoni*) collected in fall 2004 (n = 8 - 17) and spring 2005 (n = 5 - 15) from three effluent exposure sites (Garson, Copper Cliff and Municipal Waste Water) and one upstream reference site (Maley) on Junction Creek, Ontario, Canada. Data are mean ± standard error of the mean. Significant differences were detected using two-way ANOVA with site and season as factors, followed by Tukey's post hoc test. *Significant difference between exposure site and reference site within a season (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$); † Significant difference between seasons within a study site (†$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$).

2.4.5. Metal body burdens

Fathead minnows

Trace metal body burdens that were elevated significantly in fathead minnows collected downstream of both Garson and Copper Cliff compared to reference were Pb (spring only) and Cr (fall only) (Table 2.6). Fathead minnows collected downstream of Garson also had elevated Cs and Zr (in both seasons), Sr (fall only), and As and U (spring only). Fathead minnows collected from downstream of Copper Cliff had significantly higher body burdens of Cr, Se and Tl (fall only) and Bi and Y (different in spring only) than those collected from the reference site. In contrast, fathead minnows collected downstream of Garson and Copper Cliff had significantly lower body burdens of Cd and Ga in spring only, and Fe, Mn, Mo, Ni and Rb in fall only. Fathead minnows collected downstream of Garson also had lower body burdens of Ba in spring, while As and Be were lower in fish collected downstream of Copper Cliff in fall (Table 2.6). Fathead minnows collected downstream of MWW displayed a somewhat different pattern of trace metal body burdens than those collected downstream of either metal mine exposure site (Table 2.6). Only Se (both fall and spring), Cr and Tl (fall only), and Bi (spring only) were significantly greater in fathead minnows downstream of MWW compared to the reference site. Arsenic, Ga, Mn, Rb and Sr were lower at MWW compared to reference in both fall and spring. Beryllium, Co, Fe, Mo, and Ni were significantly lower at MWW compared to reference in fall only. Barium and Cd were significantly lower at MWW compared to reference in spring only. There were also significant seasonal differences in body burdens of certain trace metals in fathead minnows among sites (Table 2.6).

Table 2.6: Summary of fathead minnow (*Pimephales promelas*) body burdens for trace metals that showed a significant difference from fathead minnows collected at the reference site (Maley) in at least one of the three exposure sites (Garson, Copper Cliff and Municipal Wastewater) in fall 2004 or spring 2005. Data are mean (µg/g dry weight) ± standard error of the mean.

Metal (µg/g)	Reference		Garson		Copper Cliff		Municipal Waste Water	
	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005
N	*13*	*16*	*13*	*10*	*13*	*5*	*14*	*10*
As	1.57 ± 0.25	1.24 ± 0.26	1.41 ± 0.20	2.33 ± 0.29***††	0.58 ± 0.08**	0.97 ± 0.30	0.52 ± 0.07**	0.60 ± 0.07*
Ba	9.87 ± 1.50	27.8 ± 4.47†††	3.41 ± 0.38	16.7 ± 4.27*††	8.70 ± 1.71	22.0 ± 11.2†	2.61 ± 1.07	3.99 ± 0.99***
Be	0.04 ± 0.02	0.01 ± 0.01†	BD	BD	0.00 ± 0.00**	0.03 ± 0.03	0.00 ± 0.00**	BD
Bi	0.00 ±0.00	BD	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.02***††	0.00 ± 0.00	0.01 ± 0.01*
Cd	0.29 ± 0.04	0.51 ± 0.06†††	0.15 ± 0.06	0.14 ± 0.04***	0.18 ± 0.03	0.24 ± 0.12**	0.13 ± 0.02	0.15 ± 0.03***
Cs	0.02 ± 0.01	0.01 ± 0.00	0.05 ± 0.01***	0.06 ± 0.01*†††	0.06 ± 0.01	0.03 ± 0.02	0.04 ± 0.01	0.04 ± 0.01
Cr	5.23 ± 12.9	25.7 ± 12.41†	9.72 ± 1.64**	18.0 ± 5.59	17.5 ± 3.36**	16.4 ± 4.18	13.4 ± 3.77**	8.03 ± 1.56
Co	2.53 ± 0.57	2.10 ± 0.85	1.12 ± 0.26	2.95 ± 0.74†	0.84 ± 0.28*	1.80 ± 0.81	0.62 ± 0.15*	0.66 ± 0.21
Ga	0.75 ± 0.11	1.56 ± 0.33††	0.17 ± 0.02*	0.69 ± 0.18**†	0.42 ± 0.08	0.77 ± 0.36*	0.14 ± 0.03*	0.16 ± 0.03***
Fe	836 ± 129	344 ± 80.1†††	316 ± 43.4***	502 ± 83.4	303 ± 57.2***	562 ± 178	297 ± 34.2***	288 ± 49.2

Metal (µg/g)	Reference		Garson		Copper Cliff		Municipal Waste Water	
	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005
Mn	193 ± 40.6	92.8 ± 22.2†††	17.2 ± 1.76***	92.7 ± 14.71††	16.1 ± 2.31***	38.7 ± 17.1	10.7 ± 1.32***	11.4 ± 1.38**
Mo	7.06 ± 1.65	2.17 ± 0.14†††	0.84 ± 0.24***	1.55 ± 0.60	1.94 ± 0.58***	1.54 ± 0.62	0.86 ± 0.30***	0.16 ± 0.09
Ni	65.1 ± 12.2	26.4 ± 6.98†††	33.1 ± 5.79**	51.4 ± 9.68*	25.9 ± 5.39***	41.4 ± 16.9	16.1 ± 2.48***	12.7 ± 2.69
Rb	39.7 ± 4.82	33.9 ± 3.17	29.8 ± 2.01**	29.3 ± 1.88	19.3 ± 1.37***	18.1 ± 2.41**	15.5 ± 1.53***	18.3 ± 1.25***
Pb	0.14 ± 0.05	0.13 ±0.07	0.87 ± 0.17***	0.33 ± 0.12**††	0.24 ± 0.11	0.77 ± 0.45***	0.15 ± 0.06	0.33 ± 0.10
Se	3.09 ± 0.55	3.49 ± 0.37	4.36 ± 0.36	3.71 ± 0.38	29.7 ± 5.68***	11.0 ± 3.64†††	23.6 ± 2.41***	18.7 ± 1.83***
Sr	29.7 ± 4.55	38.9 ± 3.91	75.5 ± 3.5***	46.9 ± 2.89††	24.1 ± 2.17	35.4 ± 11.2	15.8 ± 1.73*	19.3 ± 2.21**
Tl	0.01 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.21 ± 0.04***	0.06 ± 0.01†††	0.10 ± 0.02**	0.08 ± 0.02
U	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.03 ± 0.01**††	0.00 ± 0.00	0.02 ± 0.01†	0.00 ± 0.00	0.01 ± 0.00
Y	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.02***††	0.00 ± 0.00	0.01 ± 0.00
Zr	0. 30 ± 0.13	0.27 ± 0.09	0.89 ± 0.15**	0.71 ± 0.22*	0.35 ± 0.10	0.57 ± 0.34	0.32 ± 0.09	0.64 ± 0.13

BD means below detection limit.
*Significant difference between exposure site and reference site in that season (*p<0.05, **p<0.01, ***p<0.001).
†Significant difference between seasons within study site (†p<0.05, ††p<0.01, †††p<0.001).
Significant differences were detected using two-way ANOVA with site and season as factors, followed by Tukey's post hoc test.

White sucker

Juvenile white sucker collected downstream of both treated metal mine effluent sites Garson and Copper Cliff had significantly increased body burdens of Cs, Pb and Ni (fall only) and decreased body burdens of Ba and Ga (fall only) and Cd (spring only) compared to white sucker collected at the reference site (Table 2.7). In addition, Sr (both seasons) and As, B, Cr, Hg, Mo and Ti (spring only) were elevated in white sucker downstream of Garson compared to those collected at the reference site. White sucker collected from downstream of Copper Cliff had significantly higher body burdens of Mn and Se (both seasons), Bi, Ce, Cu, La, Li, Tl and Y (fall only) but lower As and Rb (fall only) compared to white sucker collected from the reference site. White sucker collected downstream of MWW had significantly higher Se (both seasons) and Al (fall only) compared to reference, while Cd and Mn (both seasons), As, Ba, Co, Ga, Fe and Rb (fall only) were significantly lower compared to reference. Similar to fathead minnows, there were significant seasonal differences in white sucker trace metal body burdens among sites (Table 2.7).

Table 2.7: Summary of white sucker (*Catostomus commersoni*) body burdens for trace metals that showed a significant difference from white sucker collected at the reference site (Maley) in at least one of the three exposure sites (Garson, Copper Cliff and Municipal Wastewater) in fall 2004 or spring 2005. Data are shown as mean (µg/g dry weight) ± standard error of the mean.

	Reference		Garson		Copper Cliff		Municipal Waste Water	
Metal (µg/g)	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005
N	*15*	*5*	*8*	*14*	*16*	*15*	*7*	*15*
Al	18.7 ± 35.5	102 ± 35.4	183 ± 30.6	88.0 ± 21.8	219 ± 54.7	49.6 ± 11.1††	86.2 ± 23.6*	50.7 ± 10.3
As	1.04 ± 0.12	0.43 ± 0.12††	1.08 ± 0.10	0.95 ± 0.12*	0.67 ± 0.12**	0.47 ± 0.05	0.48 ± 0.09***	0.43 ± 0.06
Ba	12.9 ± 1.68	6.36 ± 2.00†	5.17 ± 0.90***	8.15 ± 1.22	7.29 ± 2.40**	1.77 ± 0.36†	3.52 ± 0.87***	2.18 ± 0.41
Bi	BD	BD	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.02***	BD	0.00 ± 0.00	0.00 ± 0.00
B	0.23 ± 0.16	0.19 ± 0.19	0.32 ± 0.24	1.68 ± 0.62*††	0.14 ± 0.07	0.58 ± 0.17	0.42 ± 0.28	0.60 ± 0.27
Cd	0.20 ± 0.02	0.22 ± 0.07	0.06 ± 0.01***	0.06 ± 0.01***	0.15 ± 0.03	0.07 ± 0.01***††	0.06 ± 0.01***	0.06 ± 0.01***
Ce	0.06 ± 0.01	0.03 ± 0.01	0.08 ± 0.02	0.03 ± 0.01†††	0.11 ± 0.03*	0.02 ± 0.01†††	0.03 ± 0.01	0.02 ± 0.01
Cs	0.01 ± 0.00	0.01 ± 0.01	0.18 ± 0.03***	0.08 ± 0.02**†††	0.06 ± 0.01**	0.02 ± 0.01	0.02 ±0.01	0.03 ± 0.01
Cr	7.21 ± 1.17	7.87 ± 3.45	7.62 ± 1.05	23.78 ± 5.30*††	17.1 ± 6.53	4.86 ± 0.84†	4.55 ± 0.78	4.62 ± 0.84
Co	2.70 ± 0.96	1.02 ± 0.39	1.94 ± 0.23	1.31 ± 0.36	2.41 ± 0.56	0.261 ± 0.10††	1.07 ± 0.25*	0.56 ± 0.21
Cu	8.05 ± 0.97	6.93 ± 1.68	17.5 ± 1.98	11.6 ± 2.22	31.4 ± 5.83***	13.1 ± 1.52†††	13.5 ± 2.08	15.4 ± 2.30
Ga	0.52 ± 0.09	0.31 ± 0.15	0.26 ± 0.04**	0.42 ± 0.07	0.30 ± 0.08**	0.12 ± 0.03	0.18 ± 0.06***	0.10 ± 0.01

	Reference		Garson		Copper Cliff		Municipal Waste Water	
Metal (µg/g)	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005	Fall 2004	Spring 2005
Fe	564 ± 83.6	273 ± 68.9†	362 ± 1.65	305 ± 59.4	538 ± 115	145 ± 37.2††	336 ± 7.61*	175 ± 35.5
La	0.03 ± 0.01	0.01 ± 0.01	0.04 ± 0.01	0.01 ± 0.01†††	0.05 ± 0.01*	0.01 ± 0.00†††	0.02 ± 0.01	0.01 ± 0.00
Pb	0.13 ± 0.04	0.06 ± 0.02	1.46 ± 0.25***	0.37 ± 0.17†††	0.93 ± 0.26***	0.14 ± 0.04†††	0.20 ± 0.08	0.15 ± 0.05
Li	0.05 ± 0.02	0.00 ± 0.00	0.05 ± 0.03	0.03 ± 0.02	0.19 ± 0.07**	0.00 ± 0.00††	0.00 ± 0.00	0.01 ± 0.01
Mn	261 ± 25.5	79.1 ± 29.5†††	16.7 ± 1.65***	31.8 ± 5.50	12.6 ± 2.73***	6.63 ± 1.67**	24.5 ± 16.5***	5.55 ± 1.51**
Hg	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.07 ± 0.03*††	BD	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
Mo	0.19 ± 0.13	0.44 ± 0.44	0.17 ± 0.12	2.29 ± 0.65*††	1.21 ± 0.61	0.12 ± 0.00	0.00 ± 0.00	0.17 ± 0.11
Ni	15.5 ± 2.26	11.4 ± 4.25	45.8 ± 6.26**	32.9 ± 6.05	39.3 ± 8.65**	8.51 ± 1.83†††	10.1 ± 2.15	8.93 ± 1.78
Rb	31.7 ± 2.63	15.9 ± 3.56†††	26.9 ± 2.85	20.2 ± 1.59†	13.1 ± 1.26***	14.2 ± 1.09	14.0 ± 1.82***	14.60 ± 1.18
Se	3.56 ± 0.53	6.42 ± 4.12	3.06 ± 0.65	3.66 ± 0.86	42.6 ± 5.03***	21.5 ± 3.35*†††	37.3 ± 2.76***	21.1 ± 2.42*††
Sr	24.4 ± 1.83	21.6 ± 6.44	81.2 ± 10.7***	49.7 ± 4.89***†††	17.3 ± 1.80	16.4 ± 1.51	13.9 ± 1.27	15.7 ± 2.14
Tl	0.01 ± 0.00	0.01 ± 0.01	BD	0.00 ± 0.00	0.09 ± 0.02***	0.04 ± 0.00†††	0.03 ± 0.01	0.03 ± 0.01
Ti	75.8 ± 4.70	59.6 ± 15.9	59.4 ± 4.23	84.6 ± 5.84*†	61.3 ± 6.13	82.4 ± 4.91†	66.7 ± 6.97	72.7 ± 6.77
Y	0.001 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.02 ± 0.01**	0.00 ± 0.00†††	0.01 ± 0.00	0.00 ± 0.00

BD means below detection limit.
*Significant difference between exposure site and reference site in a season (*p<0.05, **p<0.01, ***p<0.001).
†Significant difference between seasons within study site (†p<0.05, ††p<0.01, †††p<0.001).
Significant differences were detected using two-way ANOVA with site and season as factors, followed by Tukey's post hoc test.

2.5. Discussion

Since the exposure sites occur in sequence along Junction Creek, it is not surprising that certain water chemistry variables associated with current metal mine effluent discharges (e.g. conductivity, hardness, ammonia, nitrate, sulphate and other ions [Ca^{2+}, Cl^-, F^-, K^+, Li^+, Mg^{2+} and Na^+]) all increased in a cumulative manner at each successive sampling site, with levels of each variable 10-100 times greater than the reference site in water sampled downstream of Copper Cliff and MWW in spring. The increases in these water chemistry variables further downstream are likely due to the volume of effluent leaving Copper Cliff relative to Garson. From 2002 to 2004, Copper Cliff released approximately 50 times the effluent volume compared to Garson, and concentrations of certain trace metals, particularly Cu and Ni, were also higher in Copper Cliff wastewater than at Garson (Rickwood et al. 2006; Rickwood et al. 2008; Dubé et al. 2006; C. Brereton, CVRD Inco, personal communication). Similar to results from May 2004 (Weber et al. 2008), wastewater discharge data from 2001 and 2002 indicated that Cu, Fe, Li, Ni, and Se were higher in Copper Cliff effluent than Garson, while Sr was highest from Garson (Dubé et al. 2006). In contrast, a gradient of increasing trace metal concentrations in water was not apparent for all metals at successive sites along Junction Creek. Of 22 trace metals determined in water in the present study, Al, Cu, Fe, and Ni were observed to exceed Canadian water quality (CCME) guidelines (1999) at all sites except Al at the reference site and Fe downstream of Garson. Although Se analysis in the present study was limited by a high detection limit (196 μg/L), our field survey of Junction Creek conducted in May 2004 (Weber et al. 2008) reported that waterborne Se exceeded the CCME guideline (1 μg/L) only downstream of Copper Cliff (7.8 μg/L) and MWW (7.2 μg/L).

Of the ten trace metals that exhibited consistent differences in body burdens among reference and exposure sites, seven (As, Ba, Cd, Co, Mn, Mo and Rb) were greater in fish from the reference site compared to exposure sites. A previous study of creek chub and pearl dace (*Semotilus margarita*) exposed to diluted Garson or Copper Cliff waste water in artificial streams (mesocosms) reported increased muscle concentrations of Al, Fe and Ni in creek chub exposed to Garson effluent and increased Fe, Ni and Se in creek chub exposed to Copper Cliff effluent (Dubé et al. 2006). Selenium was also increased in pearl dace exposed to Copper Cliff wastewater (Dubé et al. 2006). In a recent study, fathead minnows exposed to diluted Copper Cliff wastewater in mesocosms displayed increased Rb, Tl, and Se (Rickwood et al. 2006). In contrast, the current study showed body burdens of Cs, Pb, and Se to be consistently elevated at exposure sites compared to reference in both fish species. From these recent studies (Weber et al. 2008; Dubé et al. 2006; Rickwood et al. 2006) and the current study, it is apparent that the most consistently elevated trace metal in fish exposed to Copper Cliff effluent was Se. In addition, these results clearly show the importance of historical (i.e. sediment) contamination and aerial deposition in this catchment, and further illustrate the difficulty in selecting appropriate reference site(s) for ecotoxicological investigations in confounded aquatic systems. There were also significant seasonal differences in body burdens within each sampling site in the present study, with no consistent trends for increasing or decreasing trace metals in spring compared to the previous fall. We do not know the underlying reasons for these seasonal differences, but future field work could focus more specifically on temporal changes in water chemistry between seasons, particularly during the overwinter period.

An objective of the present study was to use resident small-bodied fish species to attempt to distinguish patterns of exposure and effect among the different point source inputs into Junction Creek, such as Copper Cliff and MWW. However, trace metal concentrations in water, fathead minnows and white sucker collected from these two exposure sites were similar, with no trace metal appearing in greater concentration downstream of MWW compared to Copper Cliff. Similar to recent mesocosm studies using diluted Copper Cliff effluent (Rickwood et al. 2006) or a mixture of diluted effluents from both Copper Cliff and MWW (Rickwood et al. 2008), the overall trend was for trace metals to decrease in water and fish collected downstream of MWW compared to Copper Cliff, except for Fe, Mn and Si, indicating a possible dilution effect of MWW discharge on the trace metals determined in the present study. However the influence of fish migration may confound interpretations of the field data presented in the current study, especially between Copper Cliff and MWW. Due to the larger distances between Garson, the reference site, and these downstream sites (several kilometres), it was assumed that juvenile fish migration would be minimal. Nevertheless, Junction Creek is an open system with no natural or artificial barriers, and it is possible that juvenile fish could migrate among all study sites.

Of all trace metals analyzed in fathead minnows and white sucker, Se exhibited the greatest increase in fish collected downstream of Copper Cliff and MWW compared to the reference site. Whole body Se concentrations ranged from 11-30 μg/g in juvenile fathead minnows and 21-43 μg/g in juvenile white sucker collected downstream of Copper Cliff and MWW compared to 3-6 μg/g and 3-4 μg/g in both species collected from the reference site and Garson, respectively. Due to the bioaccumulative nature of selenium through aquatic food webs and potential adverse effects on fish populations due to impaired reproduction, there is currently much focus and debate regarding the ecotoxicology of this element (Maier and Knight 1994; Chapman 1999; Deforest et al. 1999). There is currently general agreement among scientists that a tissue-based chronic selenium guideline for protection of fish populations be developed instead of a guideline based on waterborne or sediment selenium concentrations (Chapman 1999; Deforest et al. 1999; U.S. Environmental Protection Agency 2004; Hamilton 2002). Recently the USEPA proposed a guideline for selenium at a whole body fish concentration of 7.91 μg/g dry weight (U.S. Environmental Protection Agency 2004). Of particular relevance to the present study, the USEPA recommended that this criterion be reduced to 5.85 μg/g dry weight if fish are sampled in the late summer or fall (U.S. Environmental Protection Agency 2004), due to the potential for winter stress syndrome to exacerbate Se toxicity (Lemly 1993, 1996). The present study was designed to test aspects of Lemly's winter stress syndrome hypothesis in the field by determining whole body triglycerides, weight-at-length, and other measures of growth and condition in juvenile fishes exposed to metal mining effluent containing Se. Importantly, relationships between juvenile fish energetics/condition and the whole body Se levels determined in juvenile fathead minnows and white sucker in the present study (11-43 μg/g) may be of particular relevance from a regulatory standpoint since they exceed the Se tissue criteria recently proposed by the USEPA (5.85-7.91 μg/g) (U.S. Environmental Protection Agency 2004), suggesting that the winter stress syndrome may not apply to all fish species, especially those inhabiting cold water aquatic systems.

There were considerable species differences in whole body triglycerides among study sites in the present study. In contrast to our hypothesis, fathead minnows had consistently greater triglycerides at exposure sites compared to the reference site. However the whole body triglyceride levels observed in creek chub and white sucker were not consistently greater at exposure sites. Among all three species, there was only one incidence of lower triglycerides in fish collected at exposure sites, indicating an overall trend for elevated triglycerides at exposure sites compared to the reference site. The higher whole body triglycerides observed in effluent-exposed fish in the present study is similar to recent work in juvenile (YOY) northern pike and burbot collected downstream of uranium mining and milling operations in northern Saskatchewan (Bennett and Janz 2007a,b), sites that are also characterized by elevated Se levels in water and fish tissues (Muscatello et al. 2006; 2008). This common response of elevated energetic lipids may be due to a combination of direct and indirect effects, as discussed below.

A possible explanation for the greater energetic lipids observed in juvenile fish collected from exposure sites in Junction Creek is a potential indirect effect of effluents on food (invertebrate) abundance and/or quality. This could result from the observed elevations in nitrogen (as ammonia and nitrate) and phosphorus downstream of treated mine and municipal sewage effluents. Nutrient enrichment to the system could increase productivity and available food energy for the juvenile fish which would allow them to attain larger size and store more energy (Eckmann 2004, Post and Evans 1989). In another field study in the Sudbury area, Iles and Rasmussen (2005) speculated that decreased growth observed in yellow perch in metal-contaminated Sudbury area lakes was more closely tied to prey availability and quality than metal contamination. We did not investigate benthic invertebrate abundance or diversity in the present study, so cannot verify this possibility. However, previous work has reported reduced benthic invertebrate density and diversity downstream of certain mine discharges in Junction Creek (Jaagumagi and Bedard 2002) and toxicity to benthic invertebrates (*Chironomus tentans*) exposed to Copper Cliff effluent in artificial stream mesocosms (Hruska and Dubé 2004).

Whole body triglycerides were markedly lower (40 to 60 percent) in spring compared to the previous fall in all three fish species collected from the reference site, which supports the majority of literature reporting that energy stores (lipids) decrease during the overwinter period in fish inhabiting temperate and Arctic aquatic ecosystems (Eckmann 2004; Post and Evans 1989; Biro et al. 2004; Lemly 1996; Post and Parkinson 2001). However, there was only one observation of an overwinter decrease in triglycerides (fathead minnows collected at Garson) from exposure sites. This was contrary to our prediction of reliance on stored energy over the winter at exposure sites. This could indicate that feeding during the winter months at exposure sites, generally thought to decrease significantly in fish (Johnson and Evans 1991), may continue in the species studied here. Another possibility for this difference between reference and exposure sites is size- or energy-dependant overwinter mortality. Size-dependant mortality where larger individuals survive reduced food and temperature more often than smaller individuals has been reported in situations where individuals are unable to feed (Thompson et al. 1991; Byström et al. 2006). Stored energy (triglycerides) is thought to play the major role in this selective overwinter mortality (Biro et al. 2004; Adams 1999; Muscatello et al. 2006). Since there were no consistent seasonal differences in triglyceride concentrations in fish collected from exposure sites, it is possible that the fish collected in spring represented survivors that had more stored energy. Since fish were needed for whole body triglyceride and trace metal analyses, individual fish were not released for recapture in spring making it impossible to directly measure overwinter mortality and energy storage in the same fish. Future work should attempt to directly measure temporal changes in triglyceride stores in the same juvenile fish during the overwinter period.

Weight-at-length, similar to condition factor, is used to assess the general health of individual fish. Decreased condition factor has been reported in fish collected from metal contaminated lakes in Canada (Muscatello et al. 2008; Johnson and Evans 1991; Levesque et al. 2003; Munkittrick and Dixon 1988). In the present study, fathead minnow and white sucker displayed no significant differences in weight-at-length between exposure sites and the reference site. Similarly, exposure of juvenile northern pike to metal mining effluent in the field had no effect on condition factor, while the same fish had increased whole body triglycerides compared to reference fish (Bennett and Janz 2007 a,b). Smaller (length-at-age and weight-at-age) white sucker collected downstream of all three exposure sites could indicate compromised growth related to metal exposure. However, the lack of differences in weight-at-length among sites in these fish, coupled with the fact that triglyceride stores were not depleted, indicates that although the white sucker individuals were smaller, their overall condition was not impaired.

Muscle RNA/DNA ratio is used to estimate short-term growth and nutritional status of an individual in the days to weeks prior to fish sampling (Weber et al. 2003; Clemmesen 1988), while protein concentration can provide a longer term measure of growth (weeks to months), and in extreme cases may also indicate starvation (Weber et al. 2003; McLaughlin et al. 1995). RNA/DNA ratio is an indicator of growth through protein synthesis because cellular RNA increases with increased protein synthesis while cellular DNA remains fairly constant. Decreased growth and RNA/DNA ratio have been reported in fish exposed to Cd and other contaminants (Barron and Adelman 1984; Miliou et al. 1998). Since RNA/DNA ratio showed no consistent differences among sites in the present study, this measure of growth indicated that fish were growing at similar rates at all study sites. We predicted that RNA/DNA ratio would be lower in fish in spring if feeding and growth were reduced over the winter months. Decreased RNA/DNA ratio in creek chub and white sucker in spring compared to fall supported this prediction. There was not a consistent increase in either length or weight, which also indicates little (if any) overwinter body growth in these two species.

Overall, this study suggests that winter stress syndrome does not apply to the three fish species we assessed at three exposure sites along Junction Creek. There were no consistent exposure-related reductions in growth or energy storage in any of the species collected, despite significant exposure to certain trace elements including Se, Cu and Ni. Elevated water hardness at exposure sites may have mitigated the potential toxic responses to certain trace metals (e.g., Cu and Ni) that may have exacerbated overwinter stress. Although not addressed in the present study, it is also possible that native fish species are able to acclimate to effluent exposure in the face of winter conditions. It is important to note that the experimental approach used in the present study was not able to determine overwinter mortality directly, so that the fish collected in spring may represent survivors that displayed more favourable growth or energy storage from the previous autumn. Species-specific and site-specific factors should be important considerations if applying overwinter stress to regulations aimed at protecting fish populations.

3. GROWTH AND ENERGY STORAGE IN JUVENILE FATHEAD MINNOWS EXPOSED TO METAL MINE WASTE WATER IN SIMULATED WINTER AND SUMMER CONDITIONS: TESTING THE WINTER STRESS SYNDROME HYPOTHESIS

3.1. Abstract

In aquatic ecotoxicology, the term winter stress syndrome has been proposed to describe the potential for contaminants to exacerbate lipid depletion, decreased growth, and increased mortality during the overwinter period in fishes, particularly juveniles. The present study was designed to test the winter stress syndrome hypothesis in juvenile fathead minnow (*Pimephales promelas*) exposed from 10 to 100 days post-hatch (dph) to diluted metal mining effluent or municipal water (reference) under simulated summer (20°C and 16:8 light: dark) or winter (4°C and 8:16 light: dark) conditions in the laboratory. There was no effect of effluent exposure or temperature on survival of juvenile fish, which ranged from 85 to 98 percent among treatments. Body mass and body length were greater under summer conditions compared to winter; however there was no effect of effluent exposure on these growth parameters. The condition factor of fish at 100 dph was similar between seasons, and was significantly greater in the summer effluent exposure group compared to the summer reference. In support of the winter stress syndrome hypothesis, whole body triglyceride concentration was significantly lower in the effluent exposure group compared to reference under winter conditions, but not summer. Whole body burdens of several trace metals were greater in the effluent exposed fish at 100 dph; interestingly there were seasonal differences in bioaccumulation. In a separate experiment, fathead minnows were exposed from 10-100 dph to graded concentrations of ammonia (0.02 to 0.40 mg unionized NH_3/L) under summer conditions only. There was no effect of ammonia exposure on growth parameters, but a significant increase in total body triglycerides at the highest exposure concentration (0.40 mg/L). Overall, the results of the present study provide limited support of the winter stress syndrome hypothesis in this fish species.

3.2. Introduction

Recruitment of young fish is critical for the success of a population and factors that decrease recruitment are likely to have effects on year-class strength and potentially at the population level (Mills et al. 2000). Due to limited food resources and cold temperatures, winter is considered a recruitment challenge for young-of-the-year (YOY) fish in temperate climates (Johnson and Evans 1991; Biro et al. 2004; Byström et al. 2006). Young (small) fish with less stored energy can be particularly vulnerable to starvation and predation over winter and populations may experience size-dependant mortality in winter (Post and Evans 1989; Byström et al. 1998; Post et al. 1998; Biro et al. 2004). The addition of a physiological stressor may increase the basic metabolic energy demands of an individual (Calow 1991; Adams 1999; McGeer et al. 2000). For example, rainbow trout (*Oncorhynchus mykiss*) chronically exposed to Cu had increased metabolic requirements, indicated by increased oxygen consumption during exercise and increased appetite. Especially when energy resources are limited, increased metabolic demand for biological maintenance and stress-resisting systems may result in decreased growth rates (Calow 1991; Lemly 1993; Congdon et al. 2001). In winter, normal environmental conditions of cold temperatures and reduced food intake can combine with a chemical stressor to further decrease the ability of an individual to allocate sufficient energy to storage to survive (Lemly 1993; Levesque et al. 2002). Winter stress syndrome is the term used to describe lipid depletion, decreased growth and increased mortality due to a physiological stressor acting in combination with the typical decrease in feeding that most fish experience during winter (Lemly 1996). The three conditions of Lemly’s (1996) winter stress syndrome include presence of a significant metabolic stressor, cold water temperatures, and fish respond to cold temperatures by reduced feeding (Lemly 1996).

The Copper Cliff Waste Water Treatment Plant (CCWWTP) operated by CVRD Inco is located in the Greater Sudbury area and releases treated waste water into Junction Creek. At the confluence of Junction Creek and the CCWWTP discharge, treated waste water from the plant makes up approximately 45 percent of the creek volume. Treated CCWWTP effluent is composed of a large number of potentially harmful components. Elevated levels of ammonia, Ni, Cu, Co, Pb, Se and As, as well as reduced benthic invertebrate community diversity and density, have been observed in Junction Creek below certain mine release points (Jaagumagi and Bedard 2002; Weber et al. 2008). Previous laboratory and field studies using Copper Cliff waste water have shown effects on adult fish as well as benthos (Hruska and Dubé 2004; Dubé et al. 2006; Rickwood et al. 2006; Weber et al. 2008).

Contrary to the winter stress syndrome hypothesis, a recent field study reported immature fathead minnows (*Pimephales promelas*) collected downstream of treated metal mine and municipal waste water discharges had increased triglyceride stores compared to fish collected from an ecologically similar reference site (Driedger et al. 2009). Similarly, field studies of YOY northern pike (*Esox lucius*) and burbot (*Lota lota*) also reported increased triglyceride and glycogen stores in fish exposed to metal mine discharges during the overwinter period (Bennett and Janz 2007a,b).

Ontario's Sudbury region has been continuously mined for valuable metals such as copper and nickel since the 1880s. Conducting ecotoxicological field research in the Junction Creek, Sudbury, ON system is challenging because of many anthropogenic influences such as multiple metal mine discharges, aerial deposition, urban development, treated municipal waste water and more than 100 years of mining and smelting in the area. Due to the complexity of natural systems, and the particularly high number of confounding variables in Junction Creek, the present study was initiated to examine the potential effects of CCWWTP discharge on bioenergetics and growth of juvenile fish in isolation.

Along with trace metal contamination, elevated ammonia concentrations have been reported in Junction Creek downstream of WWTP inputs in past field assessments when compared to reference (Jaagumagi and Bedard 2002; Dubé et al. 2006; Weber et al. 2008; Driedger et al. 2009). While the Canadian Council of Ministers of the Environment (CCME; 1999) ammonia guideline for the protection of aquatic life is 0.019

mg/L unionized ammonia in fresh water, field studies on Junction Creek, ON have reported total ammonia values ranging from 0.9 mg/L to 4.27 mg/L in water samples collected from 2002 to 2005 (Dubé et al. 2006; Weber et al. 2008; Driedger et al. 2009). Laboratory studies using diluted CCWWTP discharge have also reported total ammonia concentrations ranging from 0.15 mg/L to 3.2 mg/L in 45 percent CCWWTP discharge diluted with dechlorinated municipal water (Hruska and Dubé 2004; Hruska and Dubé 2005; Rickwood et al. 2006). Ammonia is known to be toxic fish and was one of the major constituents of the treated waste water.

The main objective of this study was to test the winter stress syndrome hypothesis in the laboratory by determining the effect of diluted (45 percent) CCWWTP effluent on juvenile fathead minnow growth and energy storage under simulated summer and winter conditions. A secondary objective was to evaluate the effects of environmentally relevant ammonia exposures on juvenile fathead minnow growth and energy storage in order to investigate the potential effects of ammonia in isolation.

3.3. Materials and Methods

This study was conducted from January to April, 2005 in temperature and photoperiod controlled chambers at Environment Canada, National Water Research Institute (NWRI) in Saskatoon, SK. Fertilized eggs were collected from the breeding fathead minnow colony at NWRI and fry were raised until 10 days post hatch (dph). All exposures lasted 90 days (i.e. 10 - 100 dph). The goal of *Part One* of this study was to determine the effect of 45 percent Copper Cliff waste water (from now on referred to as 45 percent CCWWTP) on various morphometric and bioenergetic endpoints in juvenile fathead minnows under simulated winter and summer conditions. The objective of *Part Two* was to determine the effect of pulsed exposure to environmentally relevant concentrations of ammonia on the same morphometric and bioenergetic endpoints for larval fathead minnows under simulated summer conditions.

3.3.1. Part one: Copper Cliff WWTP effluent experiment

The CVRD Inco Limited operation located in Sudbury, Ontario, Canada is the largest fully-integrated mining, milling, smelting and refining complex in Canada. Products of the Sudbury operation include Ni, Cu, precious metals, platinum-group metals, sulphuric acid and liquid sulphur dioxide. Copper Cliff WWTP is only one of many inputs to the receiving waters of Junction Creek including treated effluents from Garson Mine WWTP, Nolin Creek WWTP, City of Sudbury municipal WWTP, aerial deposition and untreated urban runoff. Copper Cliff WWTP plant released 43,870,000 m^3 of liquid effluent into Junction Creek in 2004 (Rickwood et al. 2006). The 45 percent dilution of Copper Cliff WWTP effluent chosen for the laboratory exposure was based on the rates of discharge into Junction Creek in 2004 and 2005 and to be consistent with previous exposures using diluted CCWWTP discharge (Hruska and Dubé 2004; Hruska and Dubé 2005; Dubé et al. 2006; Rickwood et al. 2006).

3.3.2. Part two: pulsed ammonia exposure

Because ammonia is a potentially harmful component of the CCWWTP discharge, a secondary laboratory exposure of larval fathead minnows to varying concentrations of this contaminant was conducted. Ammonia concentrations chosen for this experiment were low compared to other chronic ammonia toxicity tests on juvenile fish (Person-Le Ruyet et al. 1997; Fairchild et al. 2005), ranging from just above the CCME guideline (0.02 mg/L) to 0.16 mg/L unionized ammonia. The purpose of the secondary experiment was to evaluate potential effects of chronic low level ammonia exposure on larval fathead minnow growth and energy stores, in an attempt to evaluate potential effects of ammonia in isolation from other components of CCWWTP effluent.

3.4. Experimental Setup

For both *Part One* and *Part Two*, twenty 10dph fathead minnows were randomly assigned to each treatment tank. Replicate tanks were randomly assigned to each treatment. Each treatment in *Part One* had four replicate tanks while each treatment in *Part Two* had three replicate tanks. A continuous flow-through system eliminated the need for complete water changes and flow rates were such that half the volume in each tank was replaced every 24 hours. Due to the extremely small size of 10dph fathead minnows (approximately 4 – 5 mm long) and low density (20 individuals per replicate tank), 500 mL polycarbonate plastic containers were used initially and volume was eventually increased to 2.5 L polycarbonate containers in order to keep fish density less than 0.5 g/L.

Treatment water for *Part One* was CCWWTP effluent shipped weekly from Sudbury, ON to Saskatoon SK. After the waste water was acclimated to the appropriate temperature, it was diluted to 45 percent with dechlorinated City of Saskatoon municipal water. *Part Two* attempted to duplicate the ammonia concentration, hardness and pH found in 45 percent CCWWTP water but remove the effect of dissolved trace metals. Ammonium bicarbonate and calcium carbonate (Sigma-Aldrich; Oakville, ON, Canada) were mixed with dechlorinated municipal water that was pH-adjusted with HCl to 7.7 ± 0.2. Unionized ammonia concentrations were nominally 0, 0.02, 0.04, 0.08 and 0.16 mg/L. Reference water for both experiments was temperature-acclimated dechlorinated municipal water with hardness and pH adjusted to match treatment water for *Part Two*.

3.4.1. Simulated winter and summer environments

During the study period, environmental chambers were initially kept at 20^{o}C (±1^{o}C) with a light/dark cycle of 16/8 hours. Larvae were fed freshly hatched brine shrimp once per day until satiation. After 30 days, the simulated winter temperature chamber underwent gradually (over a period of 30 days) decreased light, temperature and food ration to simulate fall, while environmental conditions in the simulated summer temperature chamber and the entire *Part Two* ammonia experiment remained unchanged. The final simulated winter conditions lasted 30 days: light/dark was 8/16 hours, temperature was 4^{o}C and food was 3 percent of estimated body weight while the simulated summer environment remained unchanged throughout the study period.

3.4.2. Water chemistry and metal body burdens

For both parts of the experiment, temperature, dissolved oxygen and chlorine levels were monitored daily while conductivity, total dissolved solids, pH, hardness, nitrate and ammonia were recorded weekly. For *Part One* only, four water samples were randomly chosen for trace metal analysis using inductively coupled plasma mass spectrometry (ICP-MS) at Testmark Laboratories Ltd (Sudbury, ON). *Part One* also included determination of trace metal body burden analysis of fish by ICP-MS at Testmark Laboratories Limited. Because individual fish were too small to perform metal analysis, a subsample of fish from each replicate tank was pooled for one whole body trace metal determination. Frozen fish were weighed then finely minced with scissors and homogenized in reagent-grade water for 2 x 10 second bursts with a Tissue Tearor (BioSpec Products Inc., Bartlesville, OK, USA). The homogenate was transferred to nitric acid washed polyethylene bottles and freeze-dried before shipping to Testmark Laboratories Limited Blank metal values were determined by using the same amount of reagent-grade water and homogenization time as for actual fish samples.

3.4.3. Endpoints

3.4.3.1. Length, weight, condition factor

Fork length and weight were recorded after fish were over-anesthetized with MS-222 (3-aminobenzoic acid) and used to calculate Fulton's condition factor ($weight/length^3*100$).

3.4.3.2. Whole body triglyceride concentration

For biochemical assays, all chemicals and reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless specified otherwise. Fish carcasses (except approximately 1-5 mg caudal muscle removed for muscle RNA/DNA ratio and protein concentration) were used to determine whole body triglycerides (Weber et al. 2003). Fish were finely minced with scissors and homogenized in 2x volume reagent-grade water for 3 x 10 sec with a Tissue Tearor on ice. Aliquots of the homogenate were then frozen at $-85^{o}C$. On the day of the assay, homogenate samples were thawed on ice then vortexed. Equal parts of the homogenized sample and 0.4 M sodium citrate were homogenized with a Tissue Tearor for 2 x 10 sec on ice; the resulting homogenate was heated at 100^{o}C for five minutes before it was placed to cool on ice. Samples were then diluted 1/5 (v/v) with 100 percent isopropanol in glass tubes and capped to prevent isopropanol evaporation. Tubes were vortexed vigorously, then centrifuged at 2500 rpm for 5 minutes. Whole body triglyceride levels were determined in the supernatant using a method developed for serum triglycerides (McGowan et al. 1983) and modified for use in fish (Weber et al. 2003). Glycerol was used as a standard (0.04-2.5 mg/mL) and was diluted with isopropanol as needed. To a 96-well microplate, 180 μL of GPO-Trinder Reagent A (glycerol kinase reagent and chromogen) was added before a 10 μL volume of each sample or standard was added in duplicate. Whole body triglyceride concentration was then calculated and expressed as mg/g fish.

3.4.3.3. Muscle RNA/DNA ratio

For nucleic acid isolation and determination a 1-5 mg portion of frozen fish muscle was removed at the caudal peduncle, weighed, and placed on ice in a microcentrifuge tube containing 30 μL of TE homogenization buffer (10 mM Tris, 1 mM EDTA, 0.2 M NaCl, 0.01 percent RNase Zap®, pH = 8.0). Samples were finely minced with scissors and homogenized for 2 x 10 sec with a Tissue Tearor. After 2 μl of 1 percent sodium dodecyl sulphate was added, samples were shaken by hand and incubated at 65°C for two hours. Samples were centrifuged at 5200xg for 10 min at 4°C, and the supernatant was removed to a sterile tube. Nucleic acids were semi-purified by adding 0.15x volume 3 M sodium acetate and 2.5x volume ice-cold isopropanol, mixed by repeated inversion, and precipitated overnight at -20°C. Tubes were centrifuged for 30 min at 4°C and 16,000xg and the supernatant was discarded. The resulting pellet was re-suspended in 1x volume TE (buffer) and incubated at 65°C in a water bath for 1.5 hours, vortexing tubes every 15 minutes and storing tubes overnight at 4°C. The next day, tubes were stored at -85°C until assay.

A modification of the method described by Clemmeson (1988) was used to determine RNA/DNA ratio (Weber et al. 2003). Calf thymus DNA standard, calf liver RNA standard and samples were diluted as appropriate with TE buffer plus 2 M NaCl for RNA/DNA determinations. Two identical microplates (white 96-well fluorescence assay microplates, Nunc, Naperville, IL, USA) were used and 100 μl of samples or standards were added to duplicate wells on each plate. The first plate was used for DNA determination and had a DNA standard curve with all wells receiving 50 μl of 3 μg/mL Hoechst 33258. The second plate was for combined DNA and RNA determination and had both a DNA and an RNA standard curve with all wells receiving 50 μL of 15 μg/mL ethidium bromide. Both fluorescent dye stock solutions were made in TE buffer plus 2.7 U/mL heparin sulphate. The plates were mixed by gently swirling then covered with aluminum foil. The ethidium bromide plate was read with an excitation wavelength of 544 nm and emission wavelength of 590 nm while the Hoechst 33258 plate was read with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. DNA concentrations in fish samples were calculated directly from the DNA standard curve using the Hoechst 33258 fluorescence. The expected ethidium bromide fluorescence of each sample's DNA was then interpolated from the ethidium bromide DNA standard curve and subtracted from the sample's actual ethidium bromide fluorescence. The remaining sample ethidium bromide fluorescence was attributable to RNA and was calculated from the ethidium bromide RNA standard curve (Weber et al. 2003).

3.4.3.4. Muscle protein determinations

Muscle proteins were measured using a modification of the Lowry et al. (1951) protein assay method (BioRad DC protein assay, Hercules, CA, USA). An aliquot of the initial muscle sample homogenate prepared for RNA/DNA ratio determination was used to determine protein. Bovine serum albumin was used to create the standard curve.

3.4.4. Statistical analyses

Percent survival was evaluated by the number of individuals surviving from each replicate tank at the end of the study period (n = the number of replicate tanks per treatment). Analysis of variance (ANOVA) was used to compare individual fish endpoints (morphometric and bioenergetic) between replicate tanks to ensure they could be pooled (no significant differences were detected between replicate tanks). Thus, for all other comparisons, individual fish were the experimental unit.

In *Part One*, two-way ANOVA with exposure (reference or 45 percent CCWWTP effluent) and simulated season as factors was used for determining significant differences of all endpoints. If an interaction occurred between the two factors, a one-way ANOVA was performed for each factor individually to determine significant differences between exposure and simulated season. In *Part Two*, a one-way ANOVA for treatment (ammonia concentration) was used to determine significant differences. In both *Part One* and *Part Two*, one or two-way ANOVAs were followed by Tukey's post-hoc test if the overall P-value was significant ($p < 0.05$). Bartlett's test and the Kolmogorov-Smirnov test were used to examine data for homogeneity of variance and normality, respectively. Data are presented as mean ± standard error of the mean (SEM). Water chemistry data are presented as the mean of four randomly chosen sample dates and when value was below detection, half the detection limit was used in calculations. Metal body burden data were analyzed using two-way ANOVA with exposure and simulated season as factors.

3.5. Results

3.5.1. Effluent/water chemistry

3.5.1.1. Copper Cliff WWTP experiment

Although the basic water chemistry between reference and 45 percent CCWWTP treatments was not compared statistically, 45 percent CCWWTP effluent was characterized by elevated ammonia, conductivity, hardness, total dissolved solids and certain trace metals (Table 3.1). Analysis of 45 percent CCWWTP effluent revealed that Ca, Co, Ni, Rb and Se were elevated at least an order of magnitude compared to reference water; and Cd, Cu and Se concentrations exceeded CCME water quality guidelines (CCME 1999). The remaining trace metals in Table 3.1 (Al, Ba, Ce, Cr, Ga, Hg, Mo, Nb, Pb, Sc, Sr, Sn and U) had comparable concentrations in both reference and 45 percent CCWWTP effluent treatments, but were included because they had significant differences in fathead minnow metal body burdens (Table 3.2).

Table 3.1: Summary of water chemistry and trace metal concentrations for metals of interest (metals that exceeded the Canadian Council of Ministers of the Environment Guidelines for the Protection of Aquatic Life (1999) are in bold; trace metals that were increased at least tenfold in 45 percent Copper Cliff waste water compared to reference (dechlorinated municipal) water are in italics; and trace metals that had significantly different body burdens due to either treatment or simulated season from reference water fish are in plain text). Samples were taken from four different days; data are presented as means. Values preceded by the < symbol represent the detection limit for that analyte.

Analyte	Units	CCME Guideline	Reference	45% Copper Cliff
Conductivity	µS/cm		370	1750
pH		6.5 to 9	8.32	7.83
Total Hardness	mg/L		120	793
Total Dissolved Solids (mg/L)	mg/L		0.25	1.14
Ammonia (total)	mg/L		0.10	2.75
Ammonia (as NH_3)	mg/L	0.019	0.009	0.083
NO_3	mg/L	13.0	< 1	5
Ca	mg/L		*26.1*	*336*
Al	µg/L	100[(a)]	65.5	49.0
Ba	µg/L		27.0	38.3
Cd	**µg/L**	**0.017**	**< 0.1**	**0.16**
Ce	µg/L		< 1	< 1
Co	µg/L		*< 0.1*	*11.0*
Cr	µg/L	1	< 1	1.75
Cu	**µg/L**	**2 to 4[(b)]**	***7.65***	***136***
Ga	µg/L		1.15	1.50
Hg	µg/L		< 1	< 1
Mo	µg/L	73	1.80	4.63
Ni	µg/L	25 to 150[(b)]	*2.50*	*149*
Nb	µg/L		< 1	< 1
Rb	µg/L		*<1*	*31.0*
Pb	µg/L	1 to 7[(b)]	< 1	0.70
Sc	µg/L		< 1	< 1
Se	**µg/L**	**1**	*0.63*	***14.8***
Sr	µg/L		146	575
Sn	µg/L		<1	< 1
U	µg/L		0.65	< 1

[(a)] Guideline is pH dependent, value reported is for range observed in water bodies during this water sample period (May 2005).
(b) Guideline is hardness dependent, increasing with hardness

Table 3.2: Summary of trace metal body burdens for those metals with significant differences detected in juvenile fathead minnows (*Pimephales promelas*) exposed to either dechlorinated municipal water (reference) or 45 percent Copper Cliff waste water (45 percent CCWWTP) under simulated winter or summer conditions from 10-100 dph (N = 4 pooled samples). Data are shown as mean (µg/g dry weight) ± standard error of the mean.

Metal (µg/g)	Winter		Summer	
	Reference	45% CCWWTP	Reference	45% CCWWTP
Al	73.3 ± 64.7	19.8 ± 9.09	135 ± 31.4	252 ± 24.9††
Ba	12.6 ± 0.47	3.18 ± 1.32***	12.1 ± 0.49	4.34 ± 0.40**
Ce	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.00**†††
Cr	413 ± 127	388 ± 130	105 ± 4.34†	61.9 ± 6.66†
Cu	30.8 ± 15.8	76.8 ± 24.5*	26.6 ± 2.41	56.3 ± 4.29
Ga	0.66 ± 0.11	0.21 ± 0.03***	0.58 ± 0.03	0.29 ± 0.03**
Pb	0.37 ± 0.14	0.35 ± 0.21	2.26 ± 0.16†††	2.96 ± 0.46†††
Hg	3.20 ± 0.49	5.45 ± 1.54	1.52 ± 0.17	2.49 ± 0.14†
Mo	31.5 ± 8.72	28.4 ± 10.1	9.61 ± 0.75†	5.71 ± 0.85†
Ni	233 ± 71.2	219 ± 75.0	72.5 ± 3.76†	61.7 ± 7.34
Nb	0.02 ± 0.01	0.03 ± 0.02	BD	BD†
Rb	10.4 ± 2.60	27.6 ± 10.5*	5.53 ± 0.27	13.7 ± 0.51
Sc	0.03 ± 0.02	0.03 ± 0.02	BD	0.11 ± 0.02***
Se	2.24 ± 1.19	36.2 ± 17.5*	2.19 ± 0.36	12.0 ± 1.89
Sr	71.2 ± 20.3	43.3 ± 13.7	60.6 ± 2.4	21.9 ± 0.60*
Sn	BD	0.05 ± 0.05	BD	0.60 ± 0.19**††
U	0.00 ± 0.00	0.02 ± 0.02	0.09 ± 0.01	0.89 ± 0.22***†††

BD means below detection limit.
*Significant difference between treatments within a season ($*p<0.05$, $**p<0.01$, $***p<0.001$).
†Significant difference between simulated seasons within a treatment ($^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$).
Significant differences were detected with a two-way ANOVA with treatment and simulated season as factors, followed by Tukey's post hoc test.

3.5.1.2. Ammonia experiment

Basic water chemistry data for the ammonia experiment are shown in Table 3.3. As expected, the unionized ammonia concentration increased with each treatment compared to reference but the measured mean ammonia concentrations did not match the nominal concentrations, with actual concentrations of 0.00, 0.02, 0.08, 0.19 and 0.40 mg/L instead of 0.00, 0.02, 0.04, 0.08, and 0.16 mg/L as NH_3.

Table 3.3: Summary of general water chemistry measurements taken during a 90 day experiment where juvenile fathead minnows (*Pimephales promelas*) were raised from 10 to 100 dph in water with varying concentrations of ammonia. Treatments consisted of reference (dechlorinated municipal water; *n* = 3 replicate tanks), 0.02 mg/L (*n* = 2 replicate tanks), 0.04 mg/L (*n* = 3 replicate tanks), 0.08 mg/L (*n* = 3 replicate tanks), or 0.16 mg/L ammonia (*n* = 3 replicate tanks). Mean values are presented.

Analyte	**Units**	**CCME Guideline**	**Reference**	**0.02 mg/L**	**0.04 mg/L**	**0.08 mg/L**	**0.16 mg/L**
Conductivity	µS/cm		0.413	0.412	0.427	0.427	0.462
pH		6.5 to 9	7.71	7.38	7.74	7.78	7.67
Total Hardness	mg/L		169	144	140	144	144
Total Dissolved Solids (mg/L)	mg/L		251	251	278	286	300
Ammonia (total)	mgL		0.00	0.703	1.81	3.85	7.56
Ammonia (as NH_3)	mg/L	0.019	0.00	0.02	0.08	0.19	0.40
NO_3	mg/L	13.0	3	2	1	2	1

3.5.2. Percent survival

A two-way ANOVA (n = four replicate tanks) revealed no difference in percent survival between treatments or simulated seasons in the CCWWTP exposure experiment. Percent survival varied from 85 percent to 98 percent.

In the ammonia experiment, percent survival in the highest ammonia exposure group (0.16mg/L nominal) was lower than the rest of the treatments (0.02mg/L – 0.08mg/L ammonia), although this difference was not statistically significant and there was no difference in survival rates between any treatment compared to reference. The statistical analysis did not include the 0.02 mg/L ammonia treatment because one replicate tank was removed after the air bubbler failed partway through the exposure (n = 2). Percent survival varied from 47 percent to 87 percent among treatments.

3.5.3. Morphometric endpoints

Two-way ANOVA revealed an overall effect of simulated season (summer vs. winter) on fathead minnow length and weight ($p < 0.001$) but no difference between exposure treatments (reference vs. 45 percent CCWWTP). Fathead minnows raised in simulated summer had significantly greater length and weight than those raised in simulated winter ($p < 0.001$ for both; Figure 3.1). The two-way ANOVA for condition factor revealed an exposure effect ($p = 0.02$) but no seasonal effect. Under summer conditions, fathead minnows raised in 45 percent CCWWTP effluent had significantly increased condition factor compared to those raised in reference water ($p = 0.001$; Figure 3.1).

For the ammonia experiment, there were no significant differences in fathead minnow length, weight or condition factor of any ammonia treatment compared to reference (Figure 3.2).

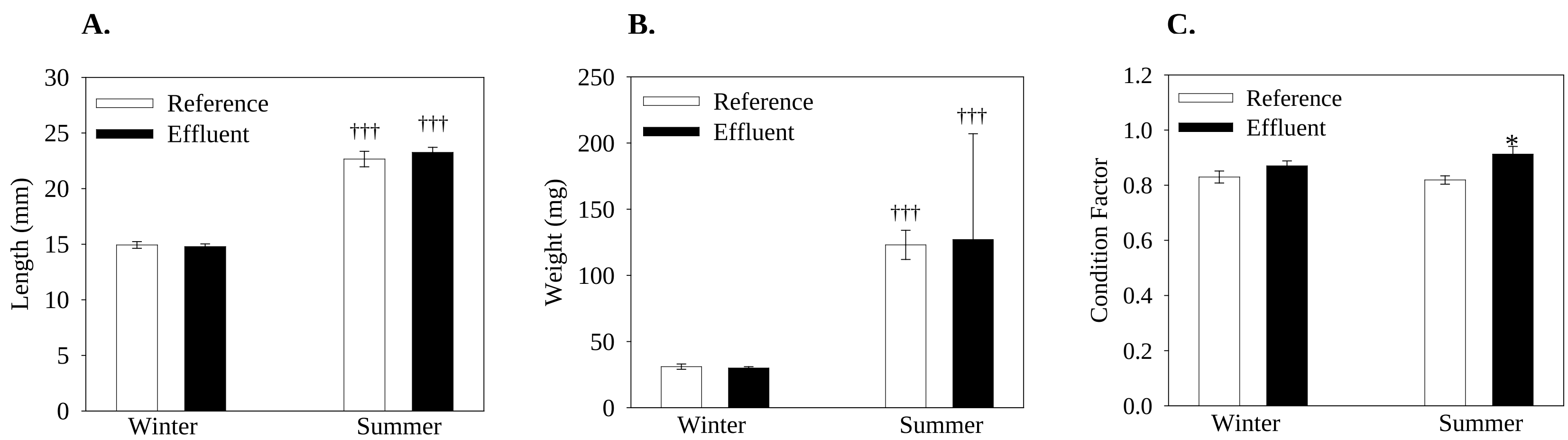

Figure 3.1: Length (A), weight (B) and condition factor (C) for juvenile fathead minnows (*Pimephales promelas*) after 90 days (from 10 to 100 days post hatch) exposure to either reference (dechlorinated municipal water; n = 73-77 fish) or 45 percent Copper Cliff effluent treatments (n = 86-78 fish) under simulated winter or summer conditions. Data are mean ± standard error of the mean. Significant differences were detected using two-way ANOVA with treatment and simulated season as factors, followed by Tukey's post hoc test as appropriate. *Significant difference between exposures ($p < 0.05$); †††Significant difference between simulated seasons within an exposure ($p < 0.001$).

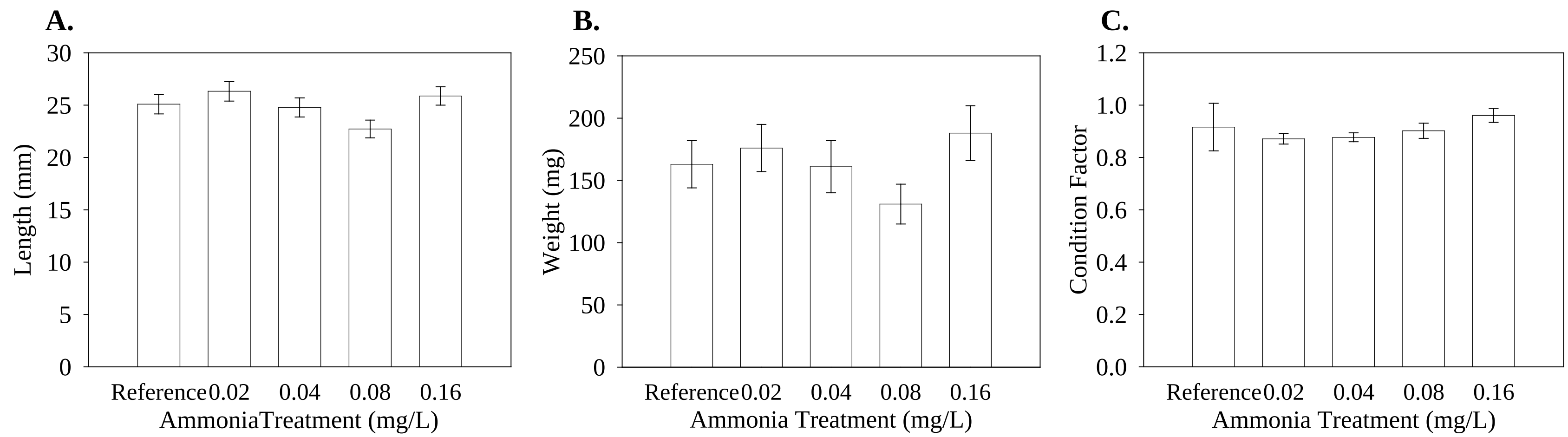

Figure 3.2: Length (A), weight (B) and condition factor (C) for juvenile fathead minnows (*Pimephales promelas*) after 90 days (from 10 to 100 days post hatch) exposure to varying concentrations of ammonia. Treatments consisted of reference (dechlorinated municipal water; n = 44 fish), 0.02 mg/L (n = 29 fish), 0.04 mg/L (n = 45 fish), 0.08 mg/L (n = 51 fish), or 0.16 mg/L ammonia (n = 28 fish). Data are mean ± standard error of the mean. Significant differences were detected using two-way ANOVA with treatment and simulated season as factors, followed by Tukey's post hoc test as appropriate. *Significant difference between exposures ($p < 0.05$); †††Significant difference between simulated seasons within an exposure ($p < 0.001$).

3.5.4. Bioenergetic endpoints

Two-way ANOVA detected an overall significant difference in whole body triglyceride concentration between seasons (p = 0.016) but not treatments (Figure 3.3). Under winter conditions only, fathead minnows exposed to 45 percent CCWWTP effluent had significantly lower whole body triglycerides compared to reference fish (p = 0.001). No overall effect of season on whole body triglyceride concentrations was detected. Two-way ANOVA revealed an effect of season on RNA/DNA ratio (p = 0.004). For fish raised in 45 percent CCWWTP, those in the simulated summer environment had a significantly decreased RNA/DNA ratio compared to those raised under simulated winter conditions (p = 0.017). There was no seasonal effect on RNA/DNA ratio between fish raised under simulated summer compared to winter conditions. Two-way ANOVA revealed that neither treatment nor simulated season had a significant effect on muscle protein concentration of fathead minnows in this experiment.

There was an overall significant effect of ammonia concentration on whole body triglycerides of juvenile fathead minnows (p = 0.005). There was significantly greater triglycerides in fathead minnows raised in the highest ammonia concentration (0.16 mg/L) compared to those raised in reference water (p = 0.007; Figure 3.4). There were no significant differences in muscle RNA/DNA ratio or muscle protein concentrations of juvenile fathead minnows raised in varying concentrations of ammonia compared to reference fish.

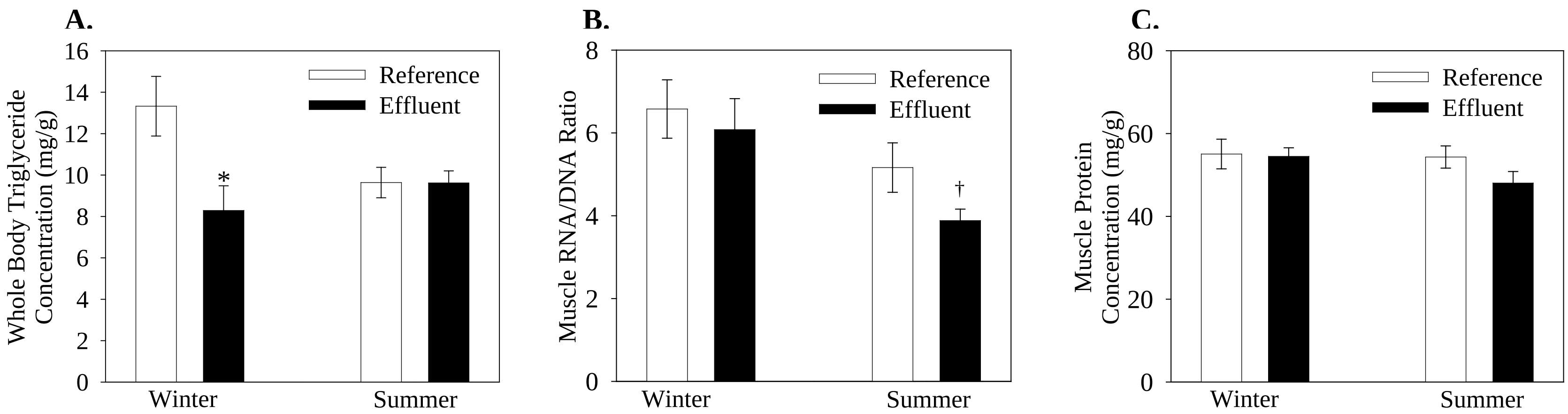

Figure 3.3: Whole body triglycerides (A), muscle RNA/DNA ratio (B) and muscle protein concentration (C) for juvenile fathead minnows (*Pimephales promelas*) after 90 days (from 10 to 100 days post hatch) exposure to either reference (dechlorinated municipal water; n = 15-20 fish) or 45% Copper Cliff effluent treatments (n = 12-20 fish) under simulated winter or summer conditions. Data are mean ± standard error of the mean. Significant differences were detected using a two-way ANOVA with exposure and simulated season as factors except for whole body triglycerides because there was a significant interaction between the two factors. Significant differences in whole body triglycerides were detected by repeating ANOVAS between exposures then between simulated seasons and followed by Tukey's post hoc test. *Significant difference between exposures ($p < 0.05$); †Significant difference between simulated seasons within an exposure (†$p < 0.05$).

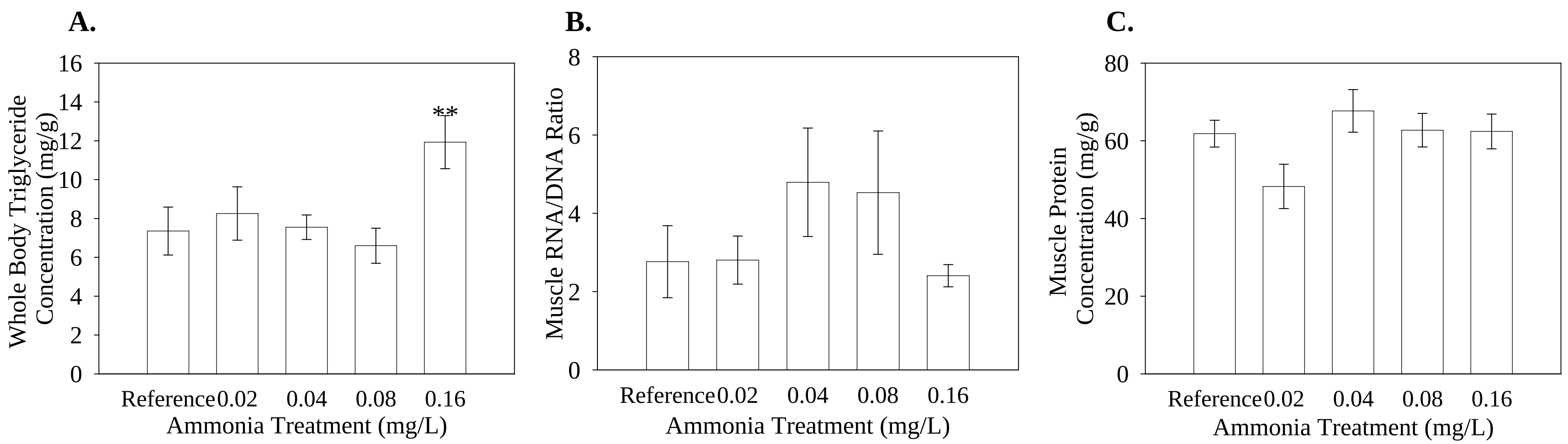

Figure 3.4: Whole body triglycerides (A), muscle RNA/DNA ratio (B) and muscle protein concentration (C) for juvenile fathead minnows (*Pimephales promelas*) after 90 days (from 10 to 100 days post hatch) exposure to varying concentrations of ammonia. Treatments consisted of reference (dechlorinated municipal water; n = 11-15 fish), 0.02 mg/L (n = 8-10 fish), 0.04 mg/L (n = 15-17 fish), 0.08 mg/L (n = 14-18 fish), or 0.16 mg/L ammonia (n = 18-19 fish). Data are mean ± standard error of the mean. Significant differences between ammonia treatments were detected with one-way ANOVA followed by Tukey's post hoc test as appropriate. **Significant difference between treatments ($p < 0.01$).

3.5.5. Metal body burdens

3.5.5.1. Copper Cliff experiment

Trace metals that did not exceed the CCME (1999) water quality guidelines and were not elevated in treatment water, but were found to have significantly different concentrations in body burden analysis (due to exposure treatment or simulated season) were Al, Ba, Ce, Cr, Ga, Hg, Mo, Nb, Pb, Sc, Sr, Sn and U (Table 3.1, p. 55).

Overall, both treatment (reference versus 45 percent CCWWTP) and simulated season had significant effects on fathead minnow trace metal body burdens. Table 3.2 shows body burdens of trace metals selected due to their concentration (exceeded the CCME Guidelines for the Protection of Aquatic Life (1999) or were increased at least tenfold in 45 percent CCWWTP compared to reference water); or trace metals where significant differences in body burdens were detected due to treatment or simulated season.

Two trace metals (Ba and Ga) had body burdens that were different between both simulated seasons; Ba and Ga were both decreased in fish raised in 45 percent CCWWTP effluent compared to reference fish. Strontium was also decreased in fish raised in 45 percent CCWWTP effluent, but only under summer conditions. For fathead minnows raised in summer conditions and exposed to 45 percent CCWWTP effluent, Ce, Sc, Sn and U body burdens were significantly increased, while those raised in simulated winter conditions and exposed to 45 percent CCWWTP effluent had increased Cu, Rb and Se body burdens (Table 3.2, p. 56).

Within seasons, body burdens of fish raised in 45 percent CCWWTP effluent were increased for Al, Ce, Pb, Sn and U under summer conditions compared to winter, while Cr, Hg, Mo and Nb were decreased. Among reference fish, the body burden of Pb was greater under summer conditions, while Cr, Mo and Ni were lower in fish raised under summer conditions compared to those under winter conditions.

3.6. Discussion

Energy storage appears critical for overwinter survival of fish in temperate climates (Calow 1991; Post and Evans 1989; Post and Parkinson 2001; Lemly 1996). Results supporting the winter stress syndrome hypothesis have been reported in laboratory exposures to trace metals in the past but less so in the field (Lemly 1993; McGeer et al. 2000; Bennett and Janz 2007b; Driedger et al. 2009). In the current study, reduced whole body triglyceride concentrations in winter 45 percent CCWWTP effluent exposed fish supports Lemly's (1996) winter stress syndrome hypothesis. The 45 percent CCWWTP effluent may have acted as a physiological stressor, leaving the fathead minnows less able to store energy than the reference fish under simulated winter conditions. Copper, Rb and Se were elevated in body burden analysis in the same group that had decreased lipid concentrations (winter 45 percent CCWWTP). Copper and Se may be associated with the decreased energy (lipid) concentration in the winter 45 percent CCWWTP exposed fish (Lemly 1996; McGeer et al. 2000). Aqueous Cu in the present study was 136 µg/L in 45 percent CCWWTP effluent. Copper was previously identified as a physiological stressor causing increased metabolic demand to rainbow trout at 75 µg Cu/L (McGeer et al. 2000).

Selenium concentration in the 45 percent CCWWTP (14.8 µg/L) exceeded the CCME (1999) guideline of 1 µg/L. Lemly (1993) reported that Se accumulation was increased in juvenile bluegill under winter conditions, and that condition factor and survival were decreased in fish exposed to 5 µg Se/L and 5 µg Se/g dw (aqueous and diet, respectively). Selenium is known to accumulate in aquatic food webs (DeForest et al. 1999; Hamilton 2002; Muscatello et al. 2008, 2009). In the current study, the mean whole body Se concentration for 45 percent CCWWTP exposed fish was 36.2 µg Se/g dw under simulated winter conditions and 12.0 µg Se/g dw under simulated summer conditions, compared to 2.19 to 2.24 µg Se/g dw in reference fish. The U.S. Environmental Protection Agency proposed Se guideline for whole fish is 7.91 µg/g (U.S. EPA 2004). Thus, fish exposed to 45 percent CCWWTP effluent accumulated Se at levels much greater than this proposed guideline.

The winter stress syndrome hypothesis assumes that feeding activity of fish in the winter is reduced. Despite the fact that the food ration was reduced under winter conditions, both reference and 45 percent CCWWTP exposed fish still grew (although at a lower rate). One condition for the winter stress syndrome, that feeding and activity are reduced (Lemly 1996), may not have been completely met in the present study. Sullivan (1981) reported that the overwinter survival of YOY largemouth bass (*Micropterus salmoides*) was dependant on the amount of stored lipids at the onset of winter, while the overwinter survival of yellow perch (*Perca flavescens*) was dependant on food availability throughout winter, demonstrating differences in winter survival strategies among fish species. Sullivan (1981) identified two main strategies as (1) fish continue actively feeding and growing during winter, and (2) fish reduce metabolism and conserve energy stores. Food is likely still available to fathead minnows during winter as detritus and invertebrates make up the majority of their diet (Price et al. 1991; Herwig and Zimmer 2007). Fathead minnows may be less dependent on energy stores and may continue to feed in reduced temperatures, enabling growth and energy storage to continue when challenged with winter conditions.

Condition factor is a metric that compares the ratio of weight to length and is used as a standard assessment of overall fish health. Decreased condition factor has been reported in fish exposed to trace metals (Johnson and Evans 1991; Levesque et al. 2002; Dubé et al. 2006). The current study found increased condition factor in 45 percent CCWWTP exposed fish under summer conditions. Bervoets and Blust (2003) reported that there was a threshold concentration for trace metals in certain body tissues (liver, kidney and gill) above which condition factor was reduced. It is possible that the differences in accumulation of certain trace metals between simulated seasons and between exposure treatments in the current study contributed to the difference observed in condition factor. In addition, livers were not removed and weighed separately due to the small body size of individual fish. Liver enlargement or increased liver triglycerides have been reported in trace metal-exposed fish (De Boeck et al. 1997; Dubé et al. 2005; Weber et al. 2008; Kelly and Janz 2008). Enlarged livers could increase the condition factor and account for the apparent improvement in condition of summer 45 percent CCWWTP effluent exposed fish.

Muscle RNA/DNA ratio can be used to assess short-term growth rate (hours to days prior to capture) of fish (Buckley and Lough 1987; Clemmesen 1994; McLaughlin et al. 1995; Weber et al. 2003). Muscle protein concentration can indicate long term growth (days to weeks) as well as conditions of extreme stress, when proteins may be used as an energy source (Weber et al. 2003; McLaughlin et al. 1995). Decreases in RNA and protein have been reported in fish exposed to toxicants (Barron and Adelman 1984). McLaughlin et al. (1995) reported that fed fish had higher concentrations of body proteins compared to starved fish. In the current study, fish may not have been subjected to the level of extreme stress required to affect muscle protein concentrations. Decreased RNA/DNA ratio in 45 percent CCWWTP effluent exposed fish during summer conditions may indicate that they were growing at a lower rate than winter 45 percent CCWWTP effluent exposed fish (who were about half as large) or that they were producing fewer enzymes or proteins.

Environmentally relevant exposures to ammonia did not appear to act as a physiological stressor to fish under summer conditions in the present study. Increased whole body triglycerides in the highest ammonia concentration group (0.16 mg/L unionized ammonia) without accompanying increased length, weight or condition factor may indicate that these fish were accumulating lipids in their organs. Lipids may accumulate in the liver of fish exposed to effluents containing elevated ammonia (Bennett and Janz 2007a,b; Greenfield et al. 2008; Kelly and Janz 2008) and increased liver size has been reported in fish exposed to metal mine and municipal waste water (Weber et al. 2008; Dubé et al. 2006). Similar to the current study, an earlier study reported that growth was not affected in larval fathead minnows exposed to 0.3 mg /L unionized ammonia for 28 days, although there was reduced survival at concentrations of 0.60 and 0.93 mg NH_3 /L (Fairchild et al. 2005). The USEPA (1999) found fathead minnow growth to be affected at unionized NH_3 concentrations of 0.16 and 0.28 mg/L (EC20s) and Brinkman et al. (2009) reported that survival, growth and biomass of rainbow trout swim-up fry were unaffected by ammonia concentrations below 7.44 mg/L. Further research is required at low temperatures and a larger range of exposure concentrations to determine if the ammonia ranges seen in 45 percent CCWWTP effluent could act as physiological stressors and contribute to decreased energy storage, growth or survival under winter conditions.

3.7. Conclusion

The major finding of the current study was that the winter stress syndrome hypothesis was supported by decreased whole body triglyceride concentrations in juvenile fish exposed to complex metal mine effluent under winter conditions. The functional effect on the population, however, is unknown as other growth and energy storage indicators were not negatively affected. Environmentally relevant ammonia exposures did not affect the growth and bioenergetic endpoints assessed here. Further research is needed to assess the mechanisms and potential ecological effects of increased condition factor (summer) and decreased triglyceride concentration (winter) in effluent exposed fish.

4. DISCUSSION

4.1. Evaluation of Winter Stress Syndrome Hypothesis

By and large, it is believed that many fish species enter a period of relative dormancy during the winter months and exhibit reduced activity and feeding (Cunjak 1988; Pratt and Fox 2002; Biro et al. 2004; Finstad et al. 2004). Winter stress syndrome is the term used to describe lipid depletion, decreased growth and increased mortality in fish when challenged with a physiological stressor acting in combination with the typical decrease in feeding that most fish experience during winter (Lemly 1996). Lemly's (1996) winter stress syndrome hypothesis relies on the assumption that cold temperatures are present and that fish reduce activity and feeding in response. Essentially, the winter stress syndrome hypothesis suggests that a contaminant, acting as the physiological stressor, could reduce fish condition (through depletion of energy reserves) to the point of decreased survival (Lemly 1996).

The physiological stressor(s) for the field study (Chapter 2) was exposure to treated mine and mill process waste water as well as treated municipal waste water. Aqueous and dietary exposure to metals such as Cu and Se have been shown in the laboratory to increase metabolic demands of fish (De Boeck et al. 1997; Lemly 1993; McGeer et al. 2000). The laboratory exposure experiment (Chapter 3) attempted to evaluate the effect of simulated winter conditions on juvenile fathead minnows exposed to diluted effluent (45 percent CCWWTP) from a CVRD Inco Limited site that discharges treated waste water into Junction Creek, Sudbury, ON. Decreased energy storage (triglyceride concentration) observed in the 45 percent CCWWTP winter-raised laboratory portion of this study supported the winter stress syndrome hypothesis, but that conditions were not extreme enough to affect juvenile fathead minnow growth and survival. This may not be completely environmentally relevant, as the juvenile fish in the laboratory study were fed a reduced ration, in order to test their response to decreased resources. A similar response (decreased triglyceride concentration) was not observed in the field study. Only white suckers had decreased growth downstream of exposure sites and none of the species had lower triglyceride stores.

It was especially surprising that given the increased Se body burden in fish downstream of exposure sites on Junction Creek, there were no signs of winter stress syndrome in the present study. Lemly's (1993) study used Se as the physiological stressor to juvenile bluegill. Increased metabolic demands were measured as increased respiratory demand and oxygen consumption. Bluegill kept in cold water showed reduced feeding and depletion of body lipids while those kept in warm water continued to feed actively and did not exhibit lipid depletion. However, Lemly's (1993) study examined winter stress syndrome effects of Se as a single element, while I studied the effect of a complex mixture (whole effluent) that included metals, organics and nutrients. It is unknown how complex mixtures may contribute to the winter stress syndrome.

The conditions of presence of a metabolic stressor cold water temperatures appear to have been met in both the field study and the laboratory experiment. The third condition, that fish undergo a period of reduced feeding activity and reliance on stored energy, however, may not have been met by the species examined in the field. Bennett and Janz (2007b) observed that at least one northern Saskatchewan species, slimy sculpin, exhibited signs of winter stress syndrome while others, northern pike and burbot, did not. Perhaps the fish species studied here are able to feed in winter, like the cold-adapted char (Byström et al. 2006). Fish feeding in winter would not need to rely on stored energy to the same extent that dormant fish would. They could also even grow over the winter period.

It should also be noted that the experimental approach to the field study did not allow the same individuals to be recaptured in the spring to determine overwinter mortality, rather an estimate of overwinter survival potential was made. Smaller individuals with fewer energy reserves may not have survived the winter to be collected in the spring.

4.2. Comparison Between Field Collection and Laboratory CCWWTP Exposure

As with any field research, it is important to note that there are many other influences to consider when attempting to assess the potential impact of a single constituent of the complex system that is Junction Creek. The laboratory portion of this study attempted to isolate two factors (treated waste water from CVRD Inco Limited operation and elevated ammonia) to determine if there were similarities to effects observed in the natural environment.

Water chemistry was somewhat similar between the field collection and laboratory exposure portions of this study. Water samples collected from Junction Creek, Sudbury, ON downstream of the CCWWTP discharge point showed elevated ammonia, conductivity, hardness and a variety of minerals such as Ca, Cl, F, K, Li, Mg, Na and P compared to the reference site (Maley branch of Junction Creek). Water sample analysis from the laboratory exposure portion of this study showed that diluted 45% CC was also characterized by elevated ammonia, conductivity, hardness, total dissolved solids. Unlike the water samples collected from Junction Creek, minerals such as Ca, Co, Cu, Ni, Rb, and Se were elevated in 45 percent CCWWTP when compared to reference (dechlorinated municipal) water. Years of historical mining and smelting in the Sudbury, ON area may partially account for the differences in water chemistry, as well as the natural or background mineral composition of the receiving water compared to dechlorinated municipal water from Saskatoon, SK.

Tissue metal concentrations in juvenile fathead minnows collected from Junction Creek were quite different from those exposed to 45 percent CCWWTP in the laboratory. This may be due to individual factors such as the age of the fish (10 – 110 dph in the laboratory versus up to two years in the field) or to altered absorption and excretion of minerals and metals found in combination with one another in the environment (Sandström 2001; Niyogi and Wood 2006; Paulino et al. 2007). Hardness has also been shown to affect the absorption of harmful metals such as Zn and Cu (Bradley and Sprague 1985; Erickson et al. 1996). The measured pH of both the reference site and downstream of 45 percent CCWWTP ranged from 7.0-7.4 while laboratory pH ranged from 7.8-8.3, possibly affecting the solubility and bioavailability of the metals present.

4.2.1. Growth and condition

Fish growth and condition factor downstream of the exposure sites in the field was likely influenced by habitat and productivity of the creek. Juvenile fathead minnows collected downstream of CCWWTP were larger (length and weight) overall than those from the reference site, while a similar increase in length and weight of exposure fish was not observed in the laboratory exposure where treatment and reference fish were the same size. It was noted at the time of field collection that minnows were abundant at exposure sites, especially downstream of Garson. The reference site (Maley) required the most collection effort (both electrofishing and minnow trapping) to collect enough fish for the study, while downstream of CCWWTP and the municipal WWTP had an intermediate abundance. Superior habitat including food and shelter availability may have played a role in the increased size and apparent condition of minnows collected in the field compared with those in the laboratory. Thus, the potential effects of contaminants on fish size may have been mitigated by abundant resources.

4.2.2. Biochemical endpoints

A striking difference between the field study and the laboratory exposure is that triglyceride concentration was increased in juvenile fathead minnows collected downstream of Junction Creek exposure sites in spring, while fish exposed to 45 percent CCWWTP in the laboratory displayed the opposite effect (in winter conditions). One reason for this may be selective winter mortality of smaller individuals and those with lower energy stores. This study required analyzing whole body constituents, therefore did not allow for recapturing the same individuals in the spring 2005 as fall 2004 to assess individual overwinter survival, growth and biochemical endpoints. In future, it would be important to evaluate how much size-selective mortality accounts for the perceived increase in size and lipid concentration in fish downstream of exposure sites.

There was no difference between the RNA/DNA ratio of fathead minnows collected downstream of the CCWWTP discharge point (field) or the 45 percent

CCWWTP (laboratory) compared to the reference water. This indicates that muscle growth rates were approximately the same in the treatment fish as in the reference fish at the time of collection. There was, however decreased muscle protein concentration in fathead minnows exposed to CCWWTP in the field that was not seen in the laboratory. Muscle protein concentration can be an indicator of either longer-term growth or extreme stress/starvation (Weber et al. 2003). Since the fish collected were large and in good condition, relative to reference fish, perhaps they were growing at a slower rate as they approached adult size.

4.2.3. Species differences

In general, fathead minnow and creek chub experienced some similar trends at exposure sites compared to the reference site (increased length and weight). Juvenile white suckers, on the other hand, had decreased length and weight at exposure sites compared to the reference site. White sucker growth could be more sensitive to the metabolic effects of treated metal mining and milling water as well as municipal waste water. The difference in growth pattern may also be due to differences in life history, mobility and home range, or habitat requirements. The white sucker is a larger-bodied fish with a longer lifespan, and different food sources than both minnow species. Jaagumani and Bedard (2002) reported decreased benthic density and diversity in Junction Creek below certain mine discharges and indirect effects of exposure on food source may also have affected the growth rate of the juvenile white suckers.

The biochemical endpoints, whole body triglycerides, muscle RNA/DNA ratio and muscle protein concentration, were less consistent across species. Juvenile fathead minnows collected downstream of exposure sites generally had increased triglyceride concentrations while creek chub and white sucker did not. Muscle RNA/DNA ratio and protein concentration did not indicate any consistent effect of site for any of the species. It is interesting to note, however, that they may be employing different growth strategies, as juvenile fathead minnows had higher RNA/DNA ratios (indicates increased protein synthesis/growth rate) in the spring than fall, while creek chub and white sucker were the opposite. This may have to do with life history, as fathead minnows may spawn at one year old (or less), while creek chub generally spawn in their second year and white suckers their fourth or fifth (Scott and Crossman 1973). Perhaps an examination of relevant species to determine the most sensitive would be advantageous when assessing the potential effects of CCWWTP waste water, or other complex mixtures of contaminants, in the field.

4.2.4. Pulsed ammonia experiment

The pulsed ammonia exposure was added to this study because of the elevated ammonia levels found in Junction Creek, ON as well as the water chemistry from the CVRD Inco Ltd operation (CCWWTP). Further breaking down the waste water constituents may assist in identifying one or more parameters that could be responsible for the observed effects. The laboratory exposure to 45 percent CCWWTP did not produce similar effects to those seen in the field, and the concurrent low level pulsed ammonia exposure did not have much effect on the endpoints chosen for this study. Therefore, chronic pulsed ammonia exposures (at the low levels used here) do not seem to have an effect on growth or selected bioenergetic endpoints in juvenile fathead minnows.

4.3. Comparison of traditional fish growth endpoints (morphometric) to bioenergetic endpoints

Growth and condition have traditionally been assessed using length, weight and condition factor. Recently, there has been interest in biochemical endpoints that are perhaps more sensitive to more subtle changes in individual fish (Beamish et al. 1996; Buckley et al. 1999; Levesque et al. 2002; Rajotte and Couture 2002; Couture and Rajotte 2003; Weber et al. 2003). Part of the purpose of this study was to consider the appropriateness of three biochemical endpoints (whole body triglyceride concentration, muscle RNA/DNA ratio and muscle protein concentration) in assessing Lemly's (1996) winter stress syndrome hypothesis in the field and the laboratory.

From the laboratory portion of the current study, it seems that triglyceride concentration could be a more sensitive endpoint than length and weight under certain circumstances, as it was affected by 45 percent CCWWTP under winter conditions in laboratory when no other endpoints were. Triglyceride concentration is a relevant endpoint when assessing the overwinter survival potential of fish, as it represents stored energy. The current study was 90 days, perhaps other indicators of growth (or survival) would have been affected had the experiment been longer. Effects of triglyceride concentration in the field were less clear. As expected, the group of fathead minnows with increased length and weight was found to have increased triglyceride concentration, but for creek chub and white sucker, this was not true.

The environmental significance of higher triglyceride concentration in fathead minnows from exposure sites is unclear. One would normally assume more triglycerides indicated a healthier fish population; but in the case of this field study, they could be an indication of the increased productivity of the exposure sites. Since the mechanism of increased triglyceride accumulation is not known in this case, it could also be due to increased fat deposits in the liver or muscle of exposure fish. Further research is needed to better understand the relationship between whole body triglycerides and overwinter survival potential in response to environmental stressors.

The current study did not find analysis of muscle RNA/DNA ratio or protein concentration in the field or the laboratory a sensitive measure of growth. In instances where changes in length and weight were clearly represented (field fathead minnows and laboratory summer versus winter conditions), RNA/DNA ratio and protein concentration were inconsistent. RNA/DNA ratio has been successfully used to detect differences in growth of larval fish (Raae *et al*. 1988, Steinhart and Eckmann 1992). Perhaps the fathead minnows in the laboratory and the species collected in the field were already past this period of very rapid growth where RNA/DNA ratio may have been more appropriate. Since muscle protein can be an indicator of extreme stress, it may not have been a sensitive endpoint in a study such as this one where fish were not near the point of starvation (McLaughlin et al. 1995; Weber et al. 2003).

Future research could focus on evaluation of both the traditional growth and biochemical endpoints used here to determine the sequence of responses and perhaps more causal associations between biochemical and traditional growth measurements. Future work could also involve further refining the timeline and environmental conditions where muscle RNA/DNA ratio and protein concentration would be best utilized as sensitive endpoints.

4.4. Conclusions

As with any field study, it is important to consider that there are many other influences to consider when attempting to assess the potential impact of a single constituent of the complex system that is Junction Creek. The laboratory portion of this study attempted to isolate two factors (treated waste water from CVRD Inco Limited operation and elevated ammonia) to determine if there were similarities to effects observed in the natural environment. There were inconsistent effects between the field study and the laboratory component. This emphasizes that hypothesis must be tested in the field to determine the environmental relevance.

The winter stress syndrome may not apply to northern fish adapted to living and feeding in colder climates. Different life histories and habitat requirements may account for some of the variability in response seen in the field study. Overall, the winter stress syndrome was not strongly supported by either the field or the laboratory studies, but the condition of greatly decreased feeding activity may not have been met.

In this case, the traditional measures of length and weight appeared to be the most useful estimates of growth, but triglyceride concentration also appears relevant to this study. Low-level ammonia exposure did not affect the growth and bioenergetic endpoints assessed here.

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