EXAMINATION OF THE EXPOSURE PATHWAYS AND EFFECTS OF METAL MINING MIXTURES IN FATHEAD MINNOW (*Pimephales promelas*)

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ABSTRACT

The overall objective of the work described in this thesis was to examine the effects of both waterborne and dietary routes of exposure to fathead minnow (*Pimephales promelas*) when exposed to complex metal mining mixtures. This was conducted using a 21-day, multi-trophic, short-term fathead minnow (FHM) reproductive bioassay. The endpoints that were measured were used to assess the effects on multiple levels of biological organization (sub-organismal to population endpoints).

The first phase of this research was conducted *in situ* using environmentally realistic concentrations of 3 separate metal mining effluents [20% surface water effluent (SWE), 30% mine water effluent (MWE), 45% process water effluent (PWE)] from Sudbury, Ontario, Canada. Metals were analyzed in several media (water, sediments) and tissues (biofilm, *Chironomus dilutus*, female fathead minnow carcass, ovaries, liver and gills). The incorporation of the biofilm (primary producers) into the bioassay also added another level of organization that was novel to this study. Significant increases in metal concentrations were observed in the water and biofilm tissues in all treatments [SWE, MWE, PWE], compared to reference. Cobalt and nickel increased significantly in *C. dilutus* tissues in SWE (1.4-fold and 1.5-fold respectively), and copper and selenium in PWE (5.2-fold and 3.3-fold respectively), however no significant increases occurred in MWE compared to reference. There were no significant increases in metal concentrations in female FHM tissues (carcass, liver, gonads, gills) in any of the treatments, suggesting that metal bioavailability was reduced. Cumulative number of eggs per female per day increased significantly (+127%) after exposure to SWE and decreased significantly (-33%) after exposure to PWE when compared to the reference fish. Mean total number of days to hatch was also reduced in PWE compared to reference.

In order to gain a better understanding of the routes of exposure causing toxicity in FHM, the second phase of this research examined the effects of exposure through diet, through water or through both using a fully factorial food exposure design in a laboratory setting. In this experiment we pre-exposed *C. dilutus* to both 45% PWE and laboratory control water until they reached the 3^{rd}-4^{th} instar stage of development (approximately 21 days) where they were collected and frozen until the start of the FHM reproductive bioassay. We further examined the role of food quality on fish toxicity by assessing differences between multi trophic (where fish

were fed both a live and frozen diet of *C. dilutus*) in the laboratory. This research was conducted at the Toxicology Centre in Saskatoon, Saskatchewan, Canada. The results showed that significant effects were observed when fish were fed a live diet versus a frozen diet. Condition factor and body weight increased, although inconsistent effects were observed for liver somatic index (LSI) in fathead minnows in both experiments when exposed to one or both routes of exposure. Cumulative total egg production and cumulative spawning events were both significantly affected by both waterborne and dietborne exposures with the greatest effects seen in the multi-trophic streams and particularly when fish were fed a live diet.

This significance of this research has demonstrated the importance of including both routes of exposure when assessing effects of mine effluent. This research also shows that the artificial stream technology is a useful tool in isolating the effects of a particular point source input (metal mining mixtures) when a system is highly confounded. The results suggest that under environmentally relevant exposure conditions, trophic transfer and live diet may lead to greater reproductive effects and increased fish toxicity. This also suggests that trophic transfer is an important route of exposure that is virtually impossible to attain using typical laboratory bioassay techniques (food-borne study using artificial diets or waterborne exposures only).

PREFACE

This thesis has been organized in a manuscript format for publication in scientific journals. Therefore there may be some repetition of introduction, materials and methods and figures throughout. Abstracts have not been included in each data chapter to reduce the redundancy.

Chapter 2 was submitted to *Ecotoxicology and Environmental Safety*.

Chapter 3 was submitted to *Aquatic Toxicology*.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APHA	American Public Health Association
AURIVAS	Australian River Assessment System Model
B-IBI	Bioassessment Index of Biotic Integrity
CCME	Canadian Council of Ministers of the Environment
CCWWTP	Copper Cliff Waste Water Treatment Plant
CEFD	Cumulative Eggs/Female/Day
CEH	Centre for Ecology and Hydrology
CES	Critical Effects Size
CS	Cumulative Spawning Events
CTE	Cumulative Total Egg Production
CWCB	Control Water Control Benthic organisms
CWEB	Control Water Effluent Benthic organisms
CWQG	Canadian Water Quality Guidelines
DOC	Dissolved Organic Carbon
DQO	Data Quality Objectives
EEM	Environmental Effects Monitoring
EPT	Ephemeroptera, Plecoptera, Trichoptera
ES	Egg Size
EWCB	Effluent Water Control Benthic organisms
EWEB	Effluent Water Effluent Benthic organisms
FHM	Fathead Minnow
GSI	Gonadal Somatic Index
HDPE	High Density Polyethylene
ICP-MS	Inductively Coupled Plasma - Mass Spectrometry
INC	Incorporated
KS	Kolmogorov Smirnov
LSI	Liver Somatic Index
MME(s)	Metal Mining Effluent (s)
MMER	Metal Mining Effluent Regulations
MT	Multi-Trophic
MWE	Mine Water Effluent
n	Sample Size
NAQWA	National Water Quality Assessment Program
NLFTS	National Lake Fish Tissue Study
NOEL	No Observed Effect Level
NRHP	National River Health Program
ON	Ontario
PRD	Relative Percent Difference
PSA	Particle Size Analysis
PTC	Population Threshold Concentration
PVC	Poly Vinyl Chloride
PWE	Process Water Effluent
PWQO	Provincial Water Quality Objectives
QC	Quality Control
RIVPACS	River Invertebrate Prediction and Classification System
RO	Reverse Osmosis
SEM	Standard Error of the Mean
SK	Saskatchewan

SW	Solid Waste
SWE	Surface Water Effluent
TIE	Toxicity Identification Evaluation
TOC	Total Organic Carbon
TRE	Toxicity Reduction Evaluation
TSS	Total Suspended Solids
US EPA	United States Environmental Protection Agency
WRF	Water Reform Framework
WTP	Water Treatment Plant
WWTP	Waste Water Treatment Plant

Chapter 1

GENERAL INTRODUCTION

1.0 INTRODUCTION

The overall objective of my thesis research was to assess the effects of waterborne and dietborne exposures to metal mining effluents (MMEs) in fathead minnow (*Pimephales promelas*). Emphasis on reproductive response endpoints in fathead minnow (FHM) was also a primary focus because of the link between these endpoints and population stability. This study also provided an indication of the population level effects of effluent exposure in Junction Creek, Ontario, Canada. Comparisons between the multi-trophic bioassay (fish fed a live diet of *Chironomus dilutus*) and traditional feeding experiments (fish fed pre-frozen *C. dilutus*) were also conducted to assess the role of food quality. Causative metals in the effluent were examined by assessing the accumulation in tissues [biofilm, *C. dilutus*, FHM (carcass, gonads, liver, gills)]. Understanding the causes of reproductive effects by examining routes of exposure and identifying causative metals is the first step towards mitigating the effects of complex metal mining mixtures.

1.1 Study site

Sudbury, Ontario (ON), Canada is home to some of the richest ore bodies in North America and was the second largest nickel producer in the world in 2007 (DOIR, 2008). Mining activities have taken place in the Sudbury region (~46.5° N, 81° W) for centuries and the continuous mining and smelting of ores in open roast beds conducted from about 1883 until 1929, have led to significant environmental degradation of the landscape and watersheds in the area (Jaagumagi and Bedard, 2001). The Junction Creek watershed in particular has been the receiving environment of a variety of point and non-point source discharges (Jaagumagi and Bedard, 2001; Stantec and C. Portt & Associates, 2006). Junction Creek is located in Sudbury, ON , approximately 400 km north of Toronto, ON, Canada (Figure 1.1). Junction Creek spans a length of approximately 25 km and can vary in width between 3-16 m. The main branch of the creek flows in a south-westerly direction from the town of Garson, ON, through the city of Greater Sudbury, ON, terminating at Kelly Lake (Figure 1.1) and contains five main tributaries. Point sources include three Vale Inc. wastewater treatment plant (WWTP) discharges; Garson,

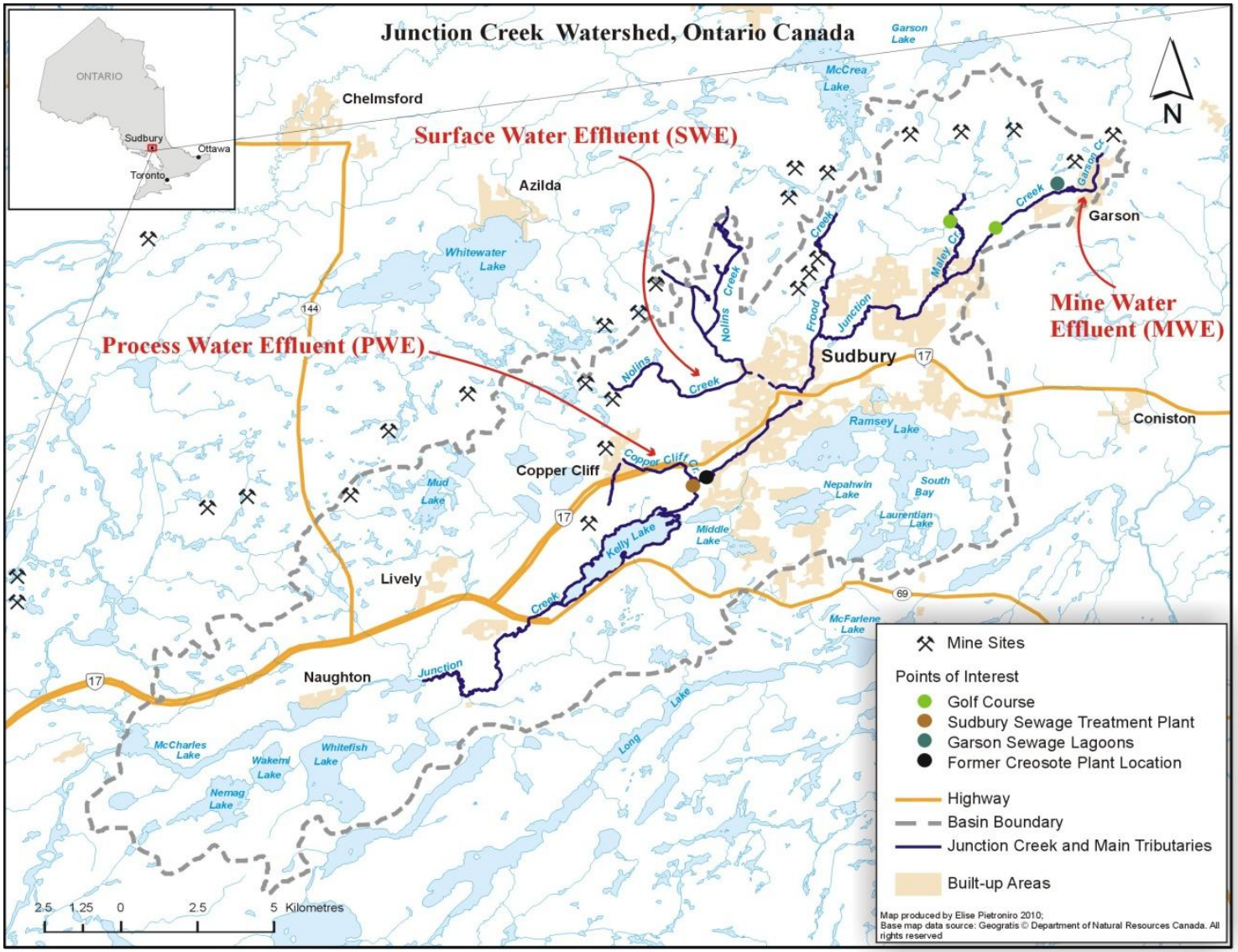


Figure 1.1: Location of Junction Creek watershed near Sudbury, Ontario, Canada, and its tributaries.

referred from this point forward as mine water effluent (MWE) (2,987 m^3/d in 2009), Nolin referred from this point forward as surface water effluent (SWE) (19,161 m^3/d in 2009) and Copper Cliff referred from this point forward as process water effluent (PWE) (141,345 m^3/d in 2009), as well as municipal sewage wastewater from the Sudbury WWTP (102,375 m^3/d in 2009). Non-point discharges have included atmospheric deposition from smelting and refining, seepage from acid generating waste rock and slag, runoff from a historical creosote plant and urban runoff (Jaagumagi and Bedard, 2001; Stantec and C. Portt & Associates, 2006). Historical studies of Junction Creek have shown a decrease in the fish population in all reaches of the creek, suggesting the possibility of reproductive failure and population-level effects (Jaagumagi and Bedard, 2001).

1.2 Examination of metal exposure to fish

The effects of waterborne metal toxicity in freshwater species have been well documented in the Sudbury region (Bradley and Morris, 1986; Eastwood and Couture, 2002; Couture and Rajotte, 2003; Pyle et al., 2005; Gauthier et al., 2006). Elevated Cu and Ni levels have been reported in the livers of several fish species (yellow perch, *Perca flavescens*; walleye, *Stizostedion vitreum*; northern pike, *Esox lucius*; white sucker, *Catostomus commersoni* and lake whitefish, *Coregonus clupeaformis*) (Bradley and Morris, 1986). Elevated levels of (Cd, Cu, Ni, Pb, Zn) have also been observed in the kidneys, livers, intestines and muscles of resident lake trout and yellow perch (Eastwood and Couture, 2002; Couture and Rajotte, 2003; Pyle et al., 2005). Furthermore, increased metal body burdens of Cd, Cu, Rb, Se and Sr have been observed in resident fathead minnow (*Pimephales promelas*) and creek chub (*Semotilus atromaculatus*) from Junction Creek (Weber et al., 2008).

The gills and the gut are the main routes of exposure to toxic agents in teleost fish (DiGiulio and Hinton, 2008). The liver is a main target organ of metal toxicity because it receives a large supply of blood (transporter of metals), and is directly involved in the biotransformation, metabolism and excretion of metals from the body (DiGiulio and Tillitt, 1997). The substitution of nutrient metals (a metal which is required for normal health at low concentrations but can become toxic at higher concentrations (e.g., Se)), by exogenous metals and/or an overload of nutrient metals in the body could lead to alterations in cellular function,

DNA and proteins resulting in a reduction in viable offspring and reduced egg production (DiGiulio and Tillitt, 1997). Furthermore, increased metal accumulation in the liver could affect circulating hormone levels in the blood resulting in increased embryo toxicity and altered vitellogenin levels (DiGiulio and Tillitt, 1997). Vitellogenin is the egg yolk pre-cursor protein produced in the livers of reproductively mature female fish.

The gills function in the exchange of ions and respiratory gases as well as for acid-base regulation in freshwater fish, and thus can be greatly affected by the exposure to environmental chemicals (DiGiulio and Hinton, 2008). One of the many functions of fish gills is to regulate the ion homeostasis in fish (Niyogi and Wood, 2004). The acute exposure to metals such as Cu, Ni and Zn could severely affect the ability of the fish gill to regulate ions, and could also lead to other adverse health effects such as reduced gas exchange, excess mucus secretion, gill swelling, gill damage (epithelial lifting, lamellar fusion, necrosis and apoptosis), cellular damage, oxidation of proteins, membrane lipid peroxidation, cleavage of DNA and RNA molecules and ultimately death (Niyogi and Wood, 2004). Due to the importance of gills as a vital organ and the primary site accumulation of waterborne metals, one of the key aspects of this study was to examine metal concentrations in gill tissues. This aspect has not yet been examined previously in Junction Creek and will help in understanding the importance of water as route of exposure leading to fish toxicity.

Evidence in the literature suggests that metals taken up from the water and the diet have different dispositions within the body of fish and invertebrates (DiGiulio and Tillitt, 1997; Meyer, 2002; Niyogi and Wood, 2003). Gills are mainly affected by waterborne exposure to chemicals, however blood borne chemicals accumulated from diet via the gut could also enter the gills, in addition to other vital organs such as liver, gut and kidney, albeit in small proportions, and affect its structure and function (DiGiulio and Hinton, 2008). Significantly elevated levels of metal accumulation in the gills compared to other internal organs may indicate that the route of exposure is primarily waterborne, whereas significantly higher accumulation in other organs (e.g., intestines, liver) compared to the gill may suggest a primarily dietborne exposure (Meyer, 2005). In order to gain a better understanding of the various routes of exposure, metal assimilation through the diet was also examined using the three MME's in Junction Creek. Evidence of toxic effects of dietborne metals have already been reported in the literature, where increased accumulation of dietborne metals such as As, Cd, Cu, Ni, Zn, Pb, Se

in the liver, gut, kidneys and gonads have been linked to reduced survival, growth and reproduction in fish (Lemly, 2002; Ng and Wood, 2008; Boyle et al., 2008; Muscatello et al., 2008).

Our research team of Dubé et al., has been examining exposure pathways (waterborne and dietborne) at the study site for over ten years. In total we have conducted more than seven different studies using a variety of invertebrate and fish species. The early experiments (prior to 2006) were strictly waterborne exposures and did not include a dietary component. Consequently, we felt that these studies lacked environmental realism and did not fully assess both exposure pathways. Therefore, in 2006, development of a modified fish bioassay was implemented at the study site using a modular mesocosm (artificial stream) system (Rickwood et al., 2006b). We further tested the application of the mesocosm system using different treatment waters (e.g., PWE and municipal sewage wastewater) (Rickwood et al., 2008). Overall we have observed increased metal tissue burdens in all of the studies to date in one or more tissue type (Hruska and Dubé, 2004; Dubé et al., 2006a; Rickwood et al., 2006a, 2008). We have also seen reduced survival in both fish and invertebrates in half of the experiments when exposed to one or more routes of exposure (Hruska and Dubé, 2004; Dubé et al., 2006a; Rickwood et al., 2006a). Reproduction (e.g., gonad size, testosterone levels, spawning, egg size, egg production, hatching and emergence) in both fish and invertebrates were also affected in some way in all of the studies and remain the main focus of the research (Hruska and Dubé, 2004; Dubé et al., 2006a; Rickwood et al., 2006a, 2008). However conflicting reproductive results among the field and lab studies (e.g., spawning and egg production) have been observed (Rickwood et al., 2006a, 2008). In Rickwood's lab study (2006a), reproductive output in both the waterborne-only and multi-trophic streams was reduced. Rickwood's study (2006a) also showed a significant reduction in larval responses (hatching & deformities) in the multi-trophic streams suggesting that diet was an important route of exposure. In contrast, Rickwood's field study (2008), showed that exposure through both routes of exposure had a stimulatory effect on fish reproduction (increased egg production and spawning). These discrepancies were mainly attributed to differences in treatment water used (PWE vs. municipal sewage/PWE combination). It was postulated that the mixture of sewage treatment effluent along with the process water effluent may have affected the bioavailability of the metals due to increased organic content in the water. Consequently, one of the key results to come out of Rickwood's studies was that greater effects were observed in the

multi-trophic streams in comparison to the waterborne streams alone, which suggested that dietary exposure was far more important than was originally anticipated. In light of the fact that diet may play a dominant role in the fish responses that we saw in the multi-trophic studies, we recognized the need to investigate the exposure pathways of water, diet and the combination of the two in greater detail and their responses to FHM using a factorial design.

1.3 Assessing food quality

Another important aspect of our study was to examine the differences in metal accumulation in FHM when food quality differed. It has been identified that many mesocosm studies are not multi-trophic and most use a pre-frozen or artificial diet to feed the fish (Wren and Stephenson, 1991; Alves et al., 2006). Furthermore, most studies in the laboratory that determine critical thresholds or toxicity guidelines for metals are based on waterborne exposure studies (Borgmann et al., 2005; Roussel et al., 2007; Kolts et al., 2009). This does not allow for an environmentally relevant assessment of the biological pathways. Our previous work seems to suggest that multi-trophic bioassays in mini food webs make the most sense ecologically. However, we have observed conflicting effects in FHM responses when fish were exposed multi-trophic compared to waterborne exposures, which indicates that the quality of the prey (*C. dilutus*) may play a dominant role. Therefore, we wanted to assess whether fish responses differed when FHM were fed live or frozen diets of *C. dilutus* held in both lab and effluent water (PWE). It has been suggested in the literature that feeding with live prey may result in greater metal toxicity due to biological incorporation into living tissues (DeSchamphelaere and Janssen, 2004).

1.4 The addition of primary producers (biofilm) to the bioassay

As a result of historical and current industrial activities in the region, all waterbodies within 17,000 km^2 of the Sudbury area have shown elevated metal concentrations in surface water, sediment and biota (Bradley and Morris, 1986; Rajotte and Couture 2002; Pyle et al., 2005; Gauthier et al., 2006). In particular, Cu, Ni, Zn (Bradley and Morris, 1986; Nriagu et al., 1998; Couture and Rajotte, 2003; Dubé et al., 2006a) and Pb (Couture and Rajotte, 2003; Dubé et al., 2006a) have been found above background levels in water and sediments. Regardless of reduced smelter emissions and efforts to clean-up the Sudbury region, Se, Cu and Ni

concentrations in sediment and surface waters remain high throughout the area (Couture and Rajotte, 2003; Pyle et al., 2005). Elevated total metal concentrations in the water and sediment however do not necessarily correlate to high tissue accumulations in aquatic organisms. Water hardness, metal speciation, pH, organic matter and cationic competition among metals for binding sites on membrane surfaces can all influence the bioavailability of certain metals (Meyer, 2002; Peakall and Burger, 2003). For instance, waterborne Se can be removed from the water column through adsorption to sediments and organic matter, complexation with other metals or ions in the water, and taken up by plants and algae, which form the basis of the food chain (Muscatello et al., 2008). Selenium in particular is highly assimilated by primary producers (e.g., phytoplankton) and is transformed into more bioavailable forms (e.g., seleno-amino acids) contributing to increased Se accumulation in higher trophic level organisms (Muscatello et al., 2009). Therefore, the presence of primary producers plays a critical role in the accumulation of Se and possibly other metals in the food chain following exposure to effluent mixtures. In order to gain a better understanding of the role of the periphyton/biofilm growth in the artificial streams, we allowed the biofilm to naturally develop in the streams enabling us to examine a natural primary producer (algae). Metal accumulation in the biofilm has not been assessed previously in any artificial stream studies with MME – an aspect that may provide a better understanding of metal partitioning and bioavailability in biota.

1.5 Relevance to the Environmental Effects Monitoring program

In the late 1990's, Environment Canada initiated a multi-stakeholder consultation to develop the Environmental Effects Monitoring (EEM) program for all metal mining operations in Canada (ENV Canada, 2002). The EEM program was designed to achieve national uniformity in monitoring the effects of mining to the aquatic environment, while taking into consideration site-specific factors (ENV Canada, 2002). Since 2002, all metal mining operations in Canada, to which the Metal Mining Effluent Regulations (MMER) apply, have been required to conduct an EEM study. EEM is a requirement of the MMER under the authority of the *Fisheries Act,* and is used to evaluate the effects of metal mining effluents on the environment (Lowell et al., 2007). The Metal Mining EEM Program is structured into "phases", whereby a mine conducts an EEM study every two to six years with both monitoring and interpretation components (Lowell et al., 2007). These phases include: i) initial monitoring (the first biological study), ii) periodic

monitoring (to confirm results of previous studies and to ensure no changes), iii) focused monitoring (to assess magnitude and geographical extent of the effects), iv) investigation of cause (to identify the cause of the effect).

The EEM program primarily focuses on the biological monitoring of three main areas: 1) a fish population survey to assess fish health, 2) a benthic invertebrate community survey to assess effects on fish habitat, and 3) a study of mercury levels in fish tissue to assess effects on the usability of the fisheries resources (Lowell et al., 2007). Sentinel fish populations (both sexes of two species) and benthic invertebrate communities are collected and assessed at reference sites and sites exposed to the treated effluent discharge. The response endpoints to be measured are specified for fish (condition [body weight against length], relative liver size [liver weight against body weight] , relative gonad size [gonad weight against body weight], size at age [body weight against age], age) and benthos (total density [total No. of individuals of all taxonomic groups collected at the sampling station], taxon richness [No. of different taxonomic groups collected at the sampling station], Bray-Curtis [index that measures dissimilarity in community structure among sites], Simpson's evenness [measures the proportion of individuals that contribute to the total sample for each taxonomic group]) with "an effect" defined as a statistically significant different between reference and exposed populations for any of the effect endpoints measured. One of the ways that this is measured is by comparing the critical effect sizes (a measured percentage difference or magnitude of change) between exposure and reference fish for 5 main fish endpoints [i) condition, ii) gonad weight, iii) liver weight, iv) weight at age and v) age]. The critical effect sizes (CES) have been set at ±10% for condition and CES = [(exposure mean - reference mean / reference mean) * 100)], ±25% for the other 4 endpoints (ENV Canada, 2002; Lowell 2010 pers. communications). The monitoring program is one of a kind in the world, of the highest level of scientific review and adaptive management, and key management actions and decisions are tied to the presence of effects and their magnitude. Due to the consistency and scientific rigor of the EEM approach at all mine sites, national assessments of mine effects across the country are possible. In 2010 (Phase 2 of the EEM program), the national assessment reported decreased condition, liver size and growth rate in fish across Canada indicating an inhibitory response pattern (Lowell et al., unpublished). For benthos, the national assessment for mines across Canada also indicated an inhibitory response pattern after exposure to MME for taxon richness but indicated a stimulatory response pattern for

density. Since the development of the mesocosm technology was primarily for use as an alternative method to the standard fish field survey, we were able to assess all of the EEM-based endpoints in order to compare our data with the national EEM program. We did not, analyze mercury in the fish tissues in any of our studies, nor did we fully assess the benthic invertebrate community of Junction Creek. The main focus of this research was solely to address the fisheries component of the EEM program and to further assess the use of the modified mesocosm bioassay in conducting these types of studies.

1.6 Mesocosm technology and historical research at the study site

In Junction Creek, there are three regulated final discharge locations (SWE, MWE and PWE) that fall under the EEM monitoring program. Concentrations of the three mine effluents have been estimated to be 20% SWE, 30% MWE and 45% PWE respectively at the point of convergence of the mine's discharge with Junction Creek at low creek flow (Figure 1.1) (Dubé et al., 2006a). Due to the site-specific historical contamination and the confounded nature of the study site, Vale, with the approval of Environment Canada, have been studying the fish population of Junction Creek using an alternative mesocosm technology. Field studies have shown that complex mixtures such as mining effluents are extremely difficult to mimic in the laboratory and that although laboratory studies allow for the isolation of various environmental components, they cannot replace the value of field experimentation. Furthermore, assessment of the effects of contaminants and development of guidelines and criteria for the protection of freshwater aquatic life have almost exclusively been developed through the use of waterborne dose-response studies conducted under highly controlled laboratory conditions (ASTM, 1980; EPS, 1980; Mayer et al., 1986; Mayer and Ellersieck 1988; Pascoe et al., 1989; Canada Gazette, 1989; Wren and Stephenson, 1991; CCME, 1991; USEPA, 1972, 1985abc, 1994ab; CCME, 1991, 1999). Furthermore, in those studies, the test organisms were either: not fed; were fed uncontaminated food; or fed an artificial diet (e.g., fish pellets). The relevance of these criteria to the field has been questioned (Fisher and Hook, 2002; Lemly, 2002; Hamilton, 2004; DeSchamphelaere and Janssen, 2004; Reash et al., 2006; Farag et al., 2007; Boyle et al., 2008; Muscatello et al., 2008). Alternative methods such as field-based mesocosm studies have shown increasing ability to maintain the environmental realism of a field study while still allowing for

variable control (e.g., sample size, sex ratios, ambient temperature, natural reference water quality as dilution water, etc).

Mesocosm technology or artificial stream systems have been used at the study site for a number of years. The early use of the technology in 2001 and 2002 identified that 20% SWE, 30% MWE and 45% PWE, affected fish endpoints in a waterborne exposure (Dubé et al., 2006a). Creek chub (*Semotilus atromaculatus*) survival was decreased in all exposures and pearl dace (*Semotilus margarita*) survival was decreased by as much as 40% in MWE and 50% in PWE (Dubé et al., 2006a). In addition, total body weight for both male and female pearl dace was reduced in MWE and PWE respectively (Dubé et al., 2006a). Furthermore, metal analysis in water and muscle tissue (body burdens) showed increased Ni, Rb, Sr, Fe, Li, Tl and Se for both species of fish exposed to each of the MWE and PWE (Dubé et al., 2006a). In 2002, field-based, life-cycle bioassays were conducted in artificial streams using the freshwater midge (*Chironomus dilutus*, formerly *Chironomus tentans*). Midges were exposed to PWE for 37 days in field mesocosms and showed reduced survival, reduced total emergence, increased time to emergence and reduced hatching success (Hruska and Dubé, 2004). Subsequent *C. dilutus* lab studies (Hruska and Dubé, 2004; 2005) showed that the freshwater midge was a good test species for use in field-based mesocosms and could be used as a self-sustaining diet for fish in developing a multi-trophic bioassay.

A lab-based multi-trophic mesocosm was developed and implemented in the field at the Vale site by Rickwood et al., (2006a,b). Two environmentally relevant species, the fathead minnow (*Pimephales promelas*) and the freshwater midge (*C. dilutus*) were used by Rickwood et al., (2006a,b). A self-sustaining multi-trophic bioassay was developed to comparatively assess the effects of an effluent (45% PWE) and effluent/sewage waste water blended effluent (PWE-WWT) through water alone compared to water plus food (trophic transfer or multi-trophic) pathways on FHM reproduction (Rickwood et al., 2006b). Results of the field study showed that exposure to PWE-WWT significantly increased egg production and larval deformities but significantly decreased larval hatching success which were evident only in the trophic transfer treatments (Rickwood et al., 2006a). This work suggested that the dietary pathway was an important route of exposure for determining the effects of mine effluents on the offspring of fathead minnow (Rickwood et al., 2006a).

Subsequent lab studies showed significant reductions in cumulative spawning events and egg production as well as decreased hatching success after waterborne exposure to PWE (Rickwood et al., 2006a). In contrast, the trophic transfer system showed significant increases in spawning events, egg production and larval deformities after exposure to the PWE (Rickwood et al., 2006a; 2008). Rickwood et al., (2008) suggested the possibility of a nutrient enhancement effect due to the use of municipal wastewater and subsequent increase in phosphorus concentrations (Rickwood et al., 2008). Differences in food availability between the multi-trophic and water-only exposures were also hypothesized but could only account for a small portion of the discrepancies observed among the studies conducted by Rickwood (Rickwood et al., 2006b;). In the multi-trophic system, fish may have been able to access higher densities of *C. dilutus* compared to the water-only system in which the fish were fed an optimum daily amount of 1 gram *C. dilutus*/day in each stream (Rickwood et al., 2006a; 2008). Consequently, increased food availability may have resulted in increased egg production. It was also suggested that increases in water quality parameters (DOC, TOC, TSS and total phosphorus) in the multi-trophic streams may have contributed to increased egg production in FHM since the bioavailability of metals has been shown to decrease with exposure to increased organic matter (Rickwood et al., 2008). Furthermore, it was hypothesized that perhaps increased bacterial load in the municipal wastewater may have contributed to decreased toxicity of the effluent in the multi-trophic streams (Rickwood et al., 2008). Bacteria are capable of producing enzymes, which can biodegrade or biotransform contaminants in the environment (Rickwood et al., 2008).

1.7 Treatment water

Two reference treatments and 3 effluent treatments were used in the current studies. The first reference treatment (Vermilion River) considered the resident field conditions at the study site and contained similar background levels of contamination as Junction Creek in order to eliminate differences in physical parameters (climate, geography) and allow for a better assessment of the current effluent effects. The second reference treatment was formulated using a combination of reverse osmosis and de-chlorinated Saskatoon tap water. This was conducted so that effluent effects could be examined in isolation of historical contamination, while keeping water quality parameters that are known to modify toxicity such as hardness as close as possible to Sudbury levels.

Three mine effluent treatments (SWE, MWE, PWE) were used in the field experiment, whereas only PWE was used in the laboratory experiment. Since PWE was shown to elicit the greatest number of effects in the field experiment as well as in previous experiments conducted by our lab, it was the only effluent treatment used in the laboratory experiments. All effluents were treated by conventional hydroxide precipitation (lime addition and settling) and subsequent pH adjustment prior to discharge (Stantec, 2009). It should be noted that under the MMER and the EEM program, effluents are regulated in the same manner despite their origin. However, the nature of each effluent is quite unique and it is critical to understand how different each is to truly be able to understand mine discharge effects on waters.

1.7.1 Control/reference treatment water

The Vermilion Water Treatment Plant (WTP) is located approximately 18 km west of Greater Sudbury, north of the town of Lively, ON, Canada and was the location of the field experiment conducted in 2009. It is owned and operated by Vale and provides potable water to the communities of Copper Cliff, Lively, Whitefish, Naughton, Whitefish Lake First Nations as well as all Vale mine properties (Creighton Mine, Nickel Refinery, Copper Refinery, Smelter South Mine, North Mine, Clarabelle, Tailings area) west of Sudbury (Vale, 2008). Raw water was transported to the plant through underground piping system from the Vermilion River, located approximately 5 km west of the WTP, and was used as the reference/control and dilution water for the field experiment. Despite the fact that the Vermilion River is situated in what is considered the "zone of impact" [an area within 17,000 Km^2 of the City of Greater Sudbury that has been significantly impacted by atmospheric deposition from the smelting of ore (Keller,1992)], it was chosen as our reference site for two main reasons. First, the Vermilion River was located in the adjacent watershed and within the same geographic region, which helps to eliminate differences unrelated to contamination (e.g., climate and geography) (Weber et al., 2008). Second, we thought that by using a reference site that was similarly contaminated historically, it may allow for a better assessment of the current effluent effects, since the EEM program is mainly concerned with current effects and not historical effects (Weber et al., 2008). Furthermore, logistically the Vermilion River provided a reference waterbody that had not received direct mine or municipal sewage water effluent; it was easily accessible and had been used as reference water in previous mesocosm studies at the site.

Reference water for the lab experiment was conducted using a combination of 60% reverse osmosis (RO) water and 40% de-chlorinated Saskatoon city water. The use of RO water was a key component of this study design since our goal was to closely approximate the reference water conditions (pH, hardness, alkalinity) in the Sudbury region. This had enabled us to truly analyze the effluent effects on the aquatic organisms without the confounding effects of the environmental legacies and/or the confounding effects of geographic reference/control water quality differences (Sudbury vs. Saskatoon).

All reference water was used as a control or reference treatment in the experiments and was also used to dilute the 100% effluent to the appropriate environmentally relevant concentrations.

1.7.2 20% Surface water effluent (SWE)

The Nolin Creek Waste Water Treatment Plant is the final discharge point for the 20% Surface Water Effluent (SWE). The treatment plant was designed to treat the surface water drainage from the Nolin West Branch watershed including runoff from the Murray Mine pit and Clarabelle Mill. It also treats water from two storage reservoirs (Clarabelle and Nolin), but does not treat any direct process (mining, milling, smelting, refining) water discharges (Stantec, 2009). In 2009 it treated and discharged a total of 19,161 m^3/d effluent to Junction Creek. The water from these sources is pH adjusted with lime and settled to remove heavy metals and suspended solids with the aid of flocculent addition in the clarifier prior to discharge into Nolin Creek, which joins Junction Creek from the northwest (see Figure 1.1) (Jaagumagi and Bedard, 2001; Stantec, 2009). The confluence of these two waterbodies is located inside a large culvert, beneath the downtown area of the city and is not readily accessible (Jaagumagi and Bedard, 2001). Based on flow data and conductivity readings, the effluent concentration has been estimated to be ~20%, at the confluence of Nolin Creek and Junction Creek (Dubé et al, 2006). Nolin Creek also receives non-point source inputs in the form of urban runoff from the City of Greater Sudbury as well as runoff from the main city snow dump (Jaagumagi and Bedard, 2001). Elevated levels of Ba, Bo, Ca, Co, Cu, Li, Mg, Ni, Rb, Se and Sr were observed in the 100% treated effluent in 2009 compared to reference water. Hardness and calcium levels were also increased compared to reference.

1.7.3 30% Mine water effluent (MWE)

The Mine Water Effluent (MWE) discharge was located just downstream of the Garson Mine in the Town of Garson. The Garson Mine is an underground Copper and Nickel Mine, which produces about 2,150 tons of ore per day. Surface runoff and dewatering of the underground workings are directed to a reservoir on the surface where they are subsequently treated (pH adjusted with lime) in a relatively small treatment system, then pumped to a polishing pond prior to being discharged to the receiving environment (Stantec, 2009). The final discharge point was located at the headwaters of Junction Creek and the effluent concentration located 250 m downstream of it has been estimated at ~30%. In 2009 the Garson Mine treated and discharged 2,987 m^3/d of effluent to Junction Creek. Effluent concentrations in 2009 showed all of the same elevated metals as in the SWE (Ba, B, Ca, Co, Cu, Li, Mg, Mo, Rb, Se, Sr), along with As, hardness and Total Organic Carbon (TOC) compared to reference values. The MWE showed a slightly increased nutrient enrichment compared to the SWE, much greater hardness levels and a greater concentration in metals overall.

1.7.4 45% Process water effluent (PWE)

The final mining effluent discharge into Junction Creek is the Process Water Effluent (PWE) discharge from the Copper Cliff Waste Water Treatment Plant (CCWWTP). The CCWWTP receives a number of process water effluents including mine water and tailings from the Creighton Mine, Frood Stobie, North Mine, South Mine, Nickel Refinery, Copper Refinery, Smelter Complex and Clarabelle Mill (Stantec, 2009). It also receives inputs from active and inactive tailings, collected surface runoff from the Town of Copper Cliff, ON and sewage from the mine-related housing/administration offices and the Copper Cliff municipal sewage treatment plant (Stantec, 2009). Effluent is discharged directly to Copper Cliff Creek, one of the major tributaries of Junction Creek, where it flows northwest approximately 3 km and enters Junction Creek. A total of 141,345 m^3/d of treated effluent was discharged to Junction Creek in 2009. The estimated effluent concentration at the confluence of Copper Cliff Creek and Junction Creek has been estimated to be ~45%. The PWE represents the greatest volume of effluent that is discharged into Junction Creek and is the largest facility among the three processing facilities described above. Consequently it has been the effluent of most concern in the studies that have

been conducted by our lab to date. Effluent concentrations in 2009 and 2010 showed elevated levels of all of the same metals as in the SWE and MWE (Ba, B, Ca, Co, Cu, Li, Mg, Mo, Rb, Se, Sr), though metal concentrations were much higher in the PWE than in either SWE or MWE for most metals. In addition to these, elevated levels of Al, As, Tl, TOC and Hardness levels were observed. The PWE appears to be characteristically more similar to the MWE with respect to nutrient enhancement, with the exception of it having a slightly greater increase in overall metals when observed at the 100% effluent concentration. Furthermore, there are other constituents in the effluents from metal beneficiation (mineral extraction) processes that may or may not be toxic to fish (e.g., xanthates, alcohols, flocculants, polymers, organic reagents, hydrocarbons, estrogenic compounds from sewage waste etc.), though it was beyond the scope of the current study to assess their effects on fish.

1.8 Study species

1.8.1 Fathead minnow (*Pimephales promelas*)

Fathead minnow (FHM) was used for both field-based and laboratory experiments of my study. FHM is one of the most-commonly used fish species for acute and chronic toxicity testing because it is an environmentally relevant species and ubiquitous throughout North America (Rand, 1995). FHM is an ideal test species for this study in particular because it is naturally found in Junction Creek and can be readily cultured in the laboratory (Rand, 1995). In particular, FHM species are small, fractional substrate spawners that produce clutches of 50-150 eggs every 3-5 days both in the field and the laboratory (Rickwood, 2006a). Additionally, the natural history, reproduction and spawning behaviour of the FHM are well known, since they have been extensively tested and used for regulatory purpose by the government as well as the industry (Benoit et al., 1982; Rand, 1995; Ankley et al., 2001; OECD, 2004; Rickwood, 2006a; Ankley and Villeneuve, 2006; USEPA, 2007).

1.8.2 Freshwater midge (*Chironomus dilutus*)

Chironomus dilutus (C. dilutus), formerly *Chironomus tentans,* was used in both field-based and laboratory mesocosm experiments in our study. Similar to FHM, *C. dilutus* are commonly used for freshwater toxicity testing, because their life cycle is well characterized and they can be easily cultured and handled in the laboratory and field (Benoit et al., 1997; EPS,

1997). They also represent an environmentally relevant species for my study since they are ubiquitous and abundant in freshwater environments in Sudbury and throughout North America (Benoit et al., 1997; Rickwood, 2006a). *C. dilutus* undergoes complete holometabolous metamorphosis (egg, larvae, pupae, adult), in approximately 23-30 days at 23ºC (Benoit et al., 1997). Eggs begin to hatch within two days of oviposition and may take up to six days to complete the hatch (Benoit et al., 1997). The larvae pass through four instars and after 23 days enter the pupae stage where they terminate feeding (Benoit et al., 1997). The pupal stage generally lasts one day before emergence into adulthood (Benoit et al., 1997). Adults mate within days of emergence and females produce a single egg mass within 24 hrs of mating (Benoit et al., 1997). Both males and females die within seven days of emergence, just long enough to reproduce and complete the life cycle (Benoit et al., 1997). Due to their relatively short life cycle, and numerous life stages, *C. dilutus* are an appropriate species for use in the multi-trophic bioassay because they will naturally replenish the FHM food supply in each self-contained artificial stream.

1.9 Research objectives and hypotheses

The mesocosm technology has been developed for the EEM program because it has allowed us to measure all of the EEM-based endpoints under environmentally relevant exposure conditions in isolation of other confounding factors in the watershed. This technology has allowed us to measure direct reproductive outputs in invertebrates and fish under environmentally relevant exposure conditions using a live diet, which to our knowledge, has not been developed in any other country. We have also been able to show how we can manipulate the study design to assess various exposure scenarios (e.g., sediment vs water exposures; dietary vs waterborne routes of exposure; whole effluent vs single metal exposures). The ability to be able to conduct hypothesis-driven manipulative studies using a fish bioassay will be especially useful as we move towards investigation of cause in the EEM program.

Based on previous work conducted at the Vale study site over the last 10 years, our studies involving the multi-trophic streams have suggested that the diet may play a dominant role in the fish responses that we have seen. Therefore more research was required to assess exposure pathways using all three of Vale's effluents (SWE, MWE, PWE) in a multi-trophic bioassay study design. Since metals assimilate differently in the body based on exposure route, tissue type

and species, there was also a need to examine metal accumulation in several different tissue types (gills, gonads, liver, carcass) and in several species including the biofilm. The assessment of metals in the FHM gills and the biofilm was novel to this study and had not been conducted previously. In addition we modified the methodology so that the prey base (*C. dilutus*) was exposed to the effluent prior to the addition of the fish so that once the exposure began the FHM would be exposed through both routes of exposure simultaneously from the onset of the experiment. We have attempted to address these data gaps in the Phase I field-based mesocosm study of this thesis. Our research objectives and hypotheses follow.

Having verified once again that the PWE elicited the greatest toxic responses to FHM compared to the other 2 effluents in Phase I, there was still a need to investigate in greater detail the exposure pathways of water, diet and the combination of the two and their responses to FHM. This second study was novel in that it followed a complete factorial food design (see chapter 3 for greater details). Another unique feature of this experiment was that the prey was exposed to the PWE and control water for about 3 weeks prior to freezing them into 1 gram aliquots. This was a key component to this study in order to allow sufficient time for metal tissue concentrations to reach equilibrium with the water concentrations to ensure dietary exposure. Another novel component to the study was the need to control for differences in water quality parameters such as hardness since they are known modifiers of toxicity. Previous studies have not been able to address the differences in water quality between Sudbury and Saskatoon. We were able to do this using a mixture of 60% reverse osmosis water and 40% de-chlorinated lab water. Furthermore, since many mesocosm studies are not multi-trophic and most use a pre-frozen or artificial diet, (as was used in the exposure pathway experiment above), we wanted to assess the role of food quality with respect to fish responses. This was addressed in our food quality experiment when FHM were fed both a live and frozen diet and held in differing treatment waters (lab vs. effluent water). We have attempted to address these data gaps in Phase II of this thesis, the laboratory-based mesocosm study. Our research objectives and hypotheses follow.

1.9.1 Phase I – Field-based mesocosm study

Objective 1: To determine responses of FHM to three different MME's (20%SWE, 30%MWE, 45%PWE) discharging to the same receiving environment *in situ* using a multi-trophic, self-sustaining bioassay.

H_o 1: There are no significant differences among FHM egg production, larval deformities, and larval survival following exposure to MME and responses do not differ according to effluent type (surface water effluent, mine water effluent, process water effluent).

Objective 2: To examine the combined effects of both waterborne and dietary routes of exposure, using cultured *C. dilutus*, under environmentally realistic multi-trophic exposure conditions.

H_o 2: There are no significant differences among FHM responses when exposed to effluent exposed food and water.

Objective 3: To identify key water quality parameters and target metals in the effluent and in environmental components of the mesocosm system (e.g., biofilm, sediments, *C. dilutus* bodies, and FHM gills, ovaries, liver and muscle).

H_o 3: There are no significant differences in metal accumulation among tissue types, species or effluent treatments.

1.9.2 Phase II - Laboratory-based mesocosm study

Objective 1: To understand the relative importance of water versus diet as pathways of exposure to a PWE causing effects in FHM using a fully factorial cross-over experimental design.

H_o1: There is no significant difference in fish response when exposed through the water, diet or both routes of exposure.

Objective 2: To examine the role of food quality (exposed or unexposed to MME) on FHM through a comparison of a combined multi-trophic bioassay (fish were fed a live diet of *C. dilutus*) versus fish fed a frozen diet (laboratory prepared and frozen *C. dilutus*).

H_o2: There is no significant difference in fish response when fish are fed a live or frozen diet or when fish are held in reference or effluent treatment water.

Chapter 2[a]

EXAMINING THE EFFECTS OF METAL MINING MIXTURES ON FATHEAD MINNOW (*PIMEPHALES PROMELAS*) USING FIELD-BASED MULTI-TROPHIC ARTIFICIAL STREAMS

[a] This chapter has been submitted to the Journal of Ecotoxicology and Environmental Safety under joint authorship with Monique Dubé, Carrie Rickwood and Som Niyogi.

2.0 INTRODUCTION

MME's can vary significantly in quality even for the same discharge site based on several factors, including: (i) the type/quality of the ore being milled, (ii) the geological formation of the rock, (iii) the effluent treatment process, (iv) the type of mining facility, and (v) the size of the operation. As with most rivers in Canada, multiple discharges (point sources) from the same mine or from different mining operations can occur within the same waterbody or watershed. In some cases, several other confounding factors, such as urban run-off, historical contamination, leaching of metals from soils and sediment and inputs from other users of the same waterbody, add to the complexity of assessing effects of individual mine effluent discharges into natural waters. However, it is critical for mitigation, regulation and management, when studying the receiving environment, to know which discharge (point source) is having the greatest effect on the environment in isolation of the other inputs. While this seems to be an obvious consideration for watershed management it is often overlooked when the same regulations are applied across very different discharge types for the same industry, in this case metal mining. To date, much of the research on the effects of metal contamination in fish populations has focused primarily on single metal exposures, either through water or diet (McGeer et al., 2000; Kolts et al., 2009). However, there is evidence that metal mixtures (e.g., mining effluent) and their interactions with surface water can act in both a synergistic and antagonistic manner in the natural environment (Weber et al., 2008). Due to the complexity of mixtures, the tendency in many studies is to focus hypothesis testing on several elements of interest despite the receiving environment being exposed to the effluent as a whole. While this may focus the experimental design, the overall conclusions can be misleading and of lower ecological relevance since no mine or milling operation discharges a single metal, micronutrient or element. Therefore our current study allowed us to examine three major point sources simultaneously in order to determine which point source was of greatest ecological importance with respect to FHM responses.

Junction Creek is the final receiving waterbody of three treated MME discharges. In addition to these inputs, it receives municipal sewage treatment plant effluent, urban and industrial discharges and seepage from historical contamination (Jaagumagi and Bedard, 2001). Moreover, inputs into Junction Creek from tributaries carry drainage from soils and sediments that are historically contaminated with metals (Nriagu et al, 1998). The effects of mining

effluents/metal mixtures on freshwater species have been well documented downstream of mining discharges throughout North America, with elevated metals observed in fish (whole body, gonads, blood, liver, kidney, carcass) and invertebrate (whole body) tissues (Dubé et al., 2006a; Schmitt et al., 2009). Furthermore, increased body burdens of Cd, Cu, Rb, Se and Sr, have been recorded in resident FHM and creek chub (*Semotilus atromaculatus*) downstream of the discharge locations within Junction Creek in Sudbury, Ontario, the site of the current research study (Weber et al., 2008).

Furthermore, there are other constituents in the effluents from metal beneficiation (mineral extraction) processes that may or may not be toxic to fish (e.g., xanthates, alcohols, flocculants, polymers, organic reagents, hydrocarbons, estrogenic compounds from sewage waste etc.), and there is little to no understanding of how these constituents interact in the effluent. When mining effluents act in a synergistic and interactive manner with the other confounding factors described above, it is nearly impossible to decipher between contributing influences using standard field fisheries surveys. Conventional field studies were unable to distinguish current mining effluent effects from all other inputs (Jaagumagi and Bedard, 2001; Eastwood and Couture, 2002).

Very few studies have been able to isolate the major point sources in a river system and even fewer have been able to assess all relevant exposure pathways (water & diet) in a controlled hypothesis-driven experimental design. This study allowed us to examine mine effluent and metal/micronutrient effects on fish and fish reproduction that incorporated a full trophic structure in a manipulative experimental design. Since it is known that many elements are transferred through the diet, the trophic transfer was of key importance to the study design. The significance of this research is important to the mining industry, Environment Canada and Canadians who utilize fisheries resources. The assessment of potential effluent effects from mine sites is Federally regulated under the Environmental Effects Monitoring program (EEM). The EEM program was implemented in 2002 by Environment Canada under the Metal Mining Effluent Regulations (MMER) to monitor and assess the effects of MME on fish, fish habitat and fisheries resources across Canada (MMER, 2002). Artificial streams (mesocosms) have been used at the current study site for approximately 10 years to study these effects and to develop a modified multi-trophic bioassay that could be used to test point source discharges in isolation of all other inputs, in consideration of the guidelines and regulatory standards of the EEM program. Early

mesocosm studies were conducted using waterborne exposures and were determined to lack environmental realism in that the dietary exposure pathway was not considered as a confounding factor (Dubé et al., 2006a). Although this approach remained consistent with many sublethal toxicity bioassays and fish mesocosm studies, it was recognized that there was a need to build a food web into the biological exposure system. The first multi-trophic reproductive bioassay studies using the Junction Creek effluents were conducted in 2004-05 using *Chironomus dilutus* as the diet and FHM breeding pairs (Rickwood et al., 2006a, 2008). In the current study, another trophic level was included to allow the natural biofilm in the water to colonize the artificial streams.

The main objective of the current study was to assess the combined effects of both waterborne and dietborne routes of exposure to FHM adults and larvae exposed to three separately treated MME's [surface water effluent (SWE), mine water effluent (MWE), and process water effluent (PWE)], under environmentally relevant concentrations using a self-sustaining multi-trophic mesocosm bioassay. The three mining effluents were examined concurrently and comparatively to determine if and how exposure to the effluents affected FHM responses. In addition to the biological and reproductive endpoints measured in previous multi-trophic experiments, metal and micronutrient accumulation in specific target organs (e.g., gill, liver and gonad) were also measured. Using this power of integrated results (weight of evidence approach) allowed for the identification of the most prominent elements in the effluent, and allowed for the characterization of their exposure pathways and potential toxicity to fish.

2.1 MATERIALS AND METHODS

2.1.1 Study site

Sudbury, Ontario, Canada is home to the second largest nickel deposit in the world and has been continuously mined for copper and nickel for over a century (Weber et al., 2008). The mine complex itself produces nickel, copper, precious metals, platinum-group metals, sulphuric acid and liquid sulphur dioxide (Rickwood et al., 2008).

Junction Creek is the receiving environment of three discharges (MWE, SWE, PWE) of treated mine effluent (Figure 1.1). The main branch of Junction Creek spans a length of approximately 25 km and flows southwest through the City of Greater Sudbury. The MME's are

all treated by conventional hydroxide precipitation and subsequent pH adjustment (using Lime). In addition, flocculants and filtering agents (e.g., Percol 338 polymer) are added to aid in settling of particles (Stantec, 2009). Final effluents are then pH adjusted with either CO_2 or SO_2 prior to being discharged into the environment (Stantec, 2009). Although the nature of the MME's varies among the three mine discharges, they are subjected to the same environmental regulations regardless of their composition. MWE discharge is located at the headwaters of Junction Creek and is comprised of mine water and surface water from an underground mining operation. MWE is discharged directly into Garson Creek, a tributary before it enters the main branch of Junction Creek. The second effluent discharge, SWE, is mainly comprised of surface water from decommissioned mining pits and reservoirs and flows directly into the Nolin Creek tributary, prior to entering Junction Creek, downstream of MWE. The third mine discharge, PWE, constitutes the greatest volume of effluent entering Junction Creek. The composition of PWE is very different from the other effluents in that it is the only one that receives process water from the beneficiation of the ores as well as sewage waste from the surface mine buildings. PWE is discharged first to the Copper Cliff Creek tributary prior to entering Junction Creek, just downstream of the Sudbury Municipal sewage wastewater facility. Environmental concentrations of the three effluent discharges have been estimated at 30% MWE, 20% SWE and 45% PWE at their respective Junction Creek confluences (Dubé et al., 2006a).

2.1.2 Experimental design

This study was conducted from July to September, 2008 using *in situ* modular mesocosm systems in Sudbury, Ontario. It consisted of a 10-day pre-exposure period and 21-day exposure period. Each modular mesocosm unit consisted of a table (one table per treatment) with eight 10.3-L, circular, high-density polyethylene streams. The mesocosm system consisted of polyethylene holding tanks and a series of pumps (centrifugal and metering pumps) for the delivery of the treatment concentrations to the four modular mesocosm units (see Hruska and Dubé, 2004 for detailed description of mesocosm systems and design). There were three effluent treatments (SWE, MWE, PWE) as well as a reference treatment. Flows within the system were partially re-circulated at rate of 0.26 L/min to achieve a reservoir exchange rate of twice daily. Reference water was obtained from the Vermilion River located west of Junction Creek within the adjacent watershed. Despite the fact that the Vermilion River is situated in what is considered

the "zone of impact" [an area within 17,000 km^2 of the City of Greater Sudbury that has been significantly impacted by atmospheric deposition from the smelting of ore (Keller, 1992)], it was chosen as our reference site for several reasons. The Vermilion River was located in the adjacent watershed and within the same geographic region, which helped to eliminate differences unrelated to contamination (e.g., climate and geography) (Weber et al., 2008). It was decided that by using a reference site that was similarly contaminated historically, it might allow for a better assessment of the current effluent effects, since we were mainly concerned with current effects and not historical effects (Weber et al., 2008). Furthermore, logistically the Vermilion River provided a reference waterbody that had not received direct mine or municipal sewage effluent; it was also easily accessible and had been used as reference water in previous mesocosm studies at the site. Effluent water (100%) was collected in 1,000 L cubic totes and transported to the site on a weekly basis. Treatment solutions were mixed in 300-gallon (1,136L) polyethylene tanks at their pre-determined concentrations (45% PWE, 30% MWE, 20% SWE) using Vermilion River water as the dilution water.

2.1.3 Pre-exposure phase

A pre-exposure phase was conducted over a 10-day period (from August 4 to 14, 2008) using Vermillion reference water in order to establish baseline reproductive performance of the FHMs. Fish were fed frozen bloodworms (*C. riparius*) twice daily at a feeding rate of 1 gram/day. Breeding tiles (~15 cm section of PVC pipe cut in half) were checked daily for egg production. Eggs were removed from the tiles, placed in a Petri dish and photographed using a Cannon Powershot digital camera (Model A620, Mississauga, ON) and examined using a Vista vision™ (Model 48402-00, VWR International, Mississauga, ON) tri-nocular microscope to determine fertilization success. Eggs were then placed into PVC cups with 250 µm screen mesh and placed into rearing chambers and continuously aerated using air stones. Treatment water was heated in the mesocosm reservoirs and in the mixing tanks using submersible aquaria heaters. *In situ* water quality monitoring was conducted each morning at 8:00 am. Sampling equipment was cleaned using de-ionized water between measurements. Samples were collected starting from the reference treatment and in order of increasing effluent concentration in a sequential and orderly fashion to prevent cross-contamination. Temperature was monitored on an hourly basis in each treatment using data loggers (Optic Stowaways, Onset Computer, Bourne, MA, USA) and varied

between 18-25°C in all streams over the course of the day based on the diurnal fluctuations and ambient environmental conditions. The length of daylight also varied according to natural diurnal fluctuations but averaged a daily photoperiod of 13 hrs of day light : 11 hours of darkness. Other *in situ* water quality parameters (pH, dissolved oxygen, temperature, conductivity) were monitored using a YSI meter (Yellow Springs Instruments, Yellow Springs, OH, USA). Ammonia was monitored using an ammonia test kit (Rolf C. Hagen, Edmonton, AB, Canada). All meters and pumps were calibrated daily and maintained in good working order at all times throughout the experiment. Hatching success was determined once larvae hatched (3-5 days post spawn). Ten month old, naive (fish that had not previously spawned prior to the experiment) FHM were used in the study from stock cultures supplied by Osage Catfisheries Inc. (Osage Beach, MO, USA). A total of 64 breeding pairs were randomly selected for the pre-exposure phase of the study. At the end of the breeding trial, 32 pairs were selected for use in the exposure phase of the experiment. Breeding pairs were selected on the basis that there was 100% survival of all adults, eggs were produced in each replicate at least once in the immediately preceding 7 days, and >80% fertilization of eggs had occurred (Ankley et al., 2001; OECD, 2006; USEPA, 2007). Statistical analyses were conducted on the breeding pairs selected for the exposure phase to ensure that there were no significant differences among treatments.

2.1.4 Trophic transfer set-up

Each stream of the trophic-transfer system consisted of a sediment layer (2.54 cm of pre-cleaned silica sand), a breeding tile and a feeding barrier. The feeding barrier was a circular, plastic wire mesh "platform" (mesh size of $1cm^2$) with a pie section removed ($1/10^{th}$ of the circle) that allowed access of FHM to a controlled streambed area that had an estimated *C. dilutus* density equal to 1g/*C. dilutus*/day (Rickwood et al., 2006a). Each stream also was covered with a mesh screen (500 microns) so any emerging insects were retained, counted, and left in the streams to reproduce. Seven day-old midge larvae (*C. dilutus*) were added on three occasions (July 25, August 1 and August 8) to establish a mixed age chironomid larvae population (egg, larvae, pupae, adult) in the artificial streams. Egg sacs were isolated from brood stocks and grown to 7 days at the Western College of Veterinary Medicine at the University of Saskatchewan and shipped to the site in snap lid containers. Chironomids were acclimated to both temperature and water over a 16 hour period by static renewal of 25% of the water every 4

hours. The total number of egg sacs required for the 21-day exposure period was calculated based on optimal food supply (1g/day) for the FHM breeding pair in each of the streams (see Rickwood et al., 2006a, 2008 for greater details). The chironomids were cultured under respective treatment conditions approximately three weeks prior to the introduction of the FHM to ensure dietary exposure. The *C. dilutus* were fed 10 ml in the first week, 20 ml in the second week and 30 ml in the third and subsequent weeks with a Tetramin™ slurry blend (100g of Tetramin™ flakes to 1000 mL of control water), 3 times per week. Core samples were obtained on a weekly basis in order to estimate survival and compare stream densities (number chironomids per cm^2 x total stream area ($9cm^2$)) among treatments.

2.1.5 Exposure phase

The exposure phase was conducted immediately following the pre-exposure phase for a duration of 21 days (August 15- September 4). Breeding pairs (32 in total) selected from the pre-exposure phase were randomly placed into the exposure and reference streams which were heated to 18-25°C using submersible aquaria heaters. Lighting averaged a 13h light: 11h dark photoperiod based on natural field conditions and flow rates remained the same as during the pre-exposure phase at 0.26 L/min to achieve a reservoir exchange rate of twice daily. During the exposure phase of the experiment, breeding tiles were checked on a daily basis. Eggs were checked each morning by 9:00 am in the same manner as described above in section 2.1.3. Eggs were aerated continuously and after a 2-day period, (once eyes had formed), were re-photographed, and re-counted. Once the larvae hatched (5 days post-hatch), larvae were examined for deformities using the Vista Vision microscope. Frequency of deformities was observed in three main categories: craniofacial, edema and skeletal based on criteria outlined in Holm et al., (2003). At the end of the 21-day exposure period, fish were anaesthetized using clove oil (30µl reference water per 1µl clove oil) and were then euthanized by spinal severance prior to dissection according to the University of Saskatchewan approved animal care protocols. Liver, gonads, gills and carcass tissues were removed and weighed (to 0.001 g) and placed into the appropriately pre-labelled sample vial and frozen for metals analysis. Visual observations for abnormalities or presence of parasites were also noted during dissections. Secondary sex characteristics including: banding, nuptial tubercles, dorsal pad and fin dot in males and ovipositor size in females were also assessed. Each secondary sex characteristic was evaluated

using a point system outlined by Parrot and Wood (2002). Fish were analyzed for the standard EEM fish survey endpoints including: relative liver weight, relative gonad weight, condition, and survival. One biofilm and chironomid sample was also collected from each stream at the end of the exposure period. Chironomids were physically picked from the bottom of the streams using forceps. They were dried and weighed to 0.001 g and then placed into pre-labeled sample collection vials and placed in the freezer. Final *C. dilutus* densities (number of *C. dilutus*/cm^2) were determined at the end of the exposure period by obtaining a pooled sample from three sediment cores (area per core 9 cm^2). Biofilm was scraped from the sides of each stream using a plastic spatula and placed into pre-labelled sample vials and into the freezer. All tissue samples and biofilm were immediately shipped on ice to the lab (Testmark Laboratories, Garson, ON, Canada) after experimental take-down and analyzed in accordance with the American Public Health Association (APHA) and the US EPA solid waste (SW) analytical methods.

Water samples from each treatment were collected on a weekly basis for the analysis of general water chemistry parameters (e.g., pH, alkalinity, dissolved oxygen), major anions (e.g., Cl, NO3, PO4, SO4), Total Organic Carbon (TOC), Dissolved Organic Carbon (DOC), total metals (e.g., Al, Cr, Ni, Rb, Sr), metalloids (e.g., As, B, Ce, Si, Te) and micronutrients (e.g., Se, Co, Zn, Cu). Grab samples were collected, in pre-labelled high density polyethylene (HDPE) sample bottles from each mesocosm reservoir. Sample bottles were rinsed three times with the respective treatment water prior to sample collection. Each suite of samples were placed in a plastic bag and immediately placed into the fridge or cooler and shipped directly to the lab on ice to maintain samples at 4°C. A suite of 41 elements were analyzed. Water samples were acidified, and analyzed for total metals using inductively coupled plasma-mass spectrometry (ICP-MS). Water hardness was also analyzed using ICP-MS without acidification of the water. The total water hardness was calculated from ICP-MS using both Ca^{2+} and Mg^{2+} cations and was expressed as the equivalent of calcium carbonate ($CaCO_3$) in the water. Conductivity and alkalinity in water were measured using a Metrohm analyzer, pH was analyzed using electrometric methodology, and total suspended solids were evaluated using a gravimetric method. Anions in the water were analyzed by ion chromatography. Ammonia, total Kjeldahl nitrogen, and total phosphorus were filtered in acid (persulfate or sulfuric acid) and analyzed by auto-colorimetry. TOC and DOC were both analyzed using a Dohrman TOC analyzer, and for DOC estimation water samples were filtered through a 0.45 µM nylon filter. One sediment

sample was collected from each stream at the end of the experiment. Grab samples were collected in 250 mL glass jars and analyzed for total metals, particle size (PSA) and total organic carbon (TOC). Sediment samples were digested in aqua regia and total metals were analyzed by ICP-MS. All of these above water quality measurements were conducted by the Testmark Laboratories, in accordance with the analytical methodology of APHA and US EPA.

Four types of quality control (QC) practices were followed by the laboratory (method blanks, in-house control standards, laboratory duplicates and matrix spike recovery samples). All four were used for water and sediments and three (method blanks, in-house control standards and spike recovery samples) were used for the tissue analysis. Data quality objectives (DQO) of 10% for water and sediments and 20% for biota were established to serve as criteria for data quality assessments. Three laboratory duplicates were run for water and one for sediment in order to assess the variability which may occur during laboratory analysis. The relative percent difference (RPD) among original samples and duplicates were analyzed, and showed that the laboratory [RPD = 100 * ABS (Sample A - Duplicate B) / mean (Sample A : Duplicate B)] precision was achieved for 91% of the comparisons made for water and 81% of the sediment comparisons. Spike recovery samples for water, ammonia, total phosphorus, anions, DOC and TOC were assessed using aqueous certified reference material purchased from various manufacturers by the lab. Percentage recovery for aqueous samples was within the range of 85.8%-113%. DOLT-3 dogfish (*Squalus acanthias*) liver reference materials were used in the calibration procedures to analyze all biological tissues; the percentage recovery was within the range of 94.1% - 112.5% for select elements (As, Cd, Cu, Fe, Pb, Hg, Ni, Se, Ag, Zn). Field blanks or field duplicates were not collected during the study.

2.1.6 Data analysis

All statistical analyses were performed using SPSS® 17 (SPSS Inc., Chicago, Il, USA) and graphed using Sigmaplot® Version 10 (San Jose, CA, USA). One-way analysis of variance (ANOVA) or the non-parametric equivalent (Kruskal Wallis) tests were used to assess most of the data (mean egg production, hatching success, percent deformities, metal tissue burdens, water quality, condition, LSI, GSI). All data analyzed using an ANOVA were first tested for parametric assumptions (normal distribution and homogeneity of variance using Shapiro-Wilk's and Levene's tests). Kolmogorov Smirnov (KS) tests were used to assess cumulative frequency

data (e.g., egg production, spawning events), and Chi-square analysis was used to assess the attribute data (e.g., secondary sex characteristics). Transformation was conducted (log transformation of continuous or derived data and angular transformation of percentage-based or ratio scaled data) when required. Differences from reference and among treatments were assessed using a Tukey's or Dunnett's *post hoc* or non-parametric Mann-Whitney-U test applying the appropriate *Bonferonni* correction (α (0.05)/number of comparisons made) to reduce the Type I error rate. Chironomid densities (number of *C. dilutus*/cm^2) and emergence (number of adults emerged) data were analyzed using a two-way ANOVA with the duration of the exposure (in days) and treatment type as the two factors. All results were significant when $p \leq 0.05$.

2.2 RESULTS

2.2.1 Water quality and metal analysis

Significant increases in conductivity, calcium, and total hardness were observed in all three treatments (SWE, MWE, PWE) compared to the reference (one-way ANOVA and multiple comparisons for all variables, $p<0.001$). Other general water quality parameters including temperature did not differ significantly among treatments at any point in time during the experiment. Likewise, there were significant increases in concentrations of Ba, B, Li, Rb, and Sr in all treatments compared to reference (Table 2.1). Nitrate, total nitrogen and Mg significantly increased in MWE and PWE treatments compared to reference (Table 2.1). The concentrations of Cu, Ni, Se and Tl increased significantly in PWE compared to the reference, SWE or MWE (Table 2.1).

2.2.2 Metal burdens in all matrices

Copper in PWE was the only metal that increased significantly (~830%) in the sediment among all treatments (2.32 ± 0.56 mean µg/g ± SE), compared to the reference (0.25 ± 0.00 mean µg/g ± SE) (sediment data not shown). Several elements (e.g., Cu, Se, Ni, Co, Sr) were significantly elevated in the biofilm relative to reference in all three treatments (Figure 2.1). Co (~141% data not shown) and Ni (~150%) were the only two elements found to increase significantly in chironomids in the SWE treatment (Figure 2.2). Cu (~515%) and Se (~327%) increased significantly however in chironomids in the PWE treatment (Figure 2.2). However,

these increases did not directly transfer into the invertebrate (*C. dilutus*) or female fish tissues, and, in many cases, elements that increased in the biofilm decreased in chironomid and female fish tissues in the PWE treatment relative to the reference (Figure 2.2 selected metals). There were also no significant increases in metal or micronutrient burdens in chironomids exposed to MWE compared to the reference (Figure 2.2 selected metals). Metal burdens in FHM were analyzed solely in the females in the current study based on the assumption that females have a greater ability to affect the F1 generation and pass any potential contaminants to the offspring via maternal transfer. In addition, previous work conducted on resident FHM population in Junction Creek (Weber et al., 2008), suggested that female fish accumulated greater metal burdens relative to males. There were no significant increases in metal accumulation in any FHM tissues analyzed (carcass, ovaries, gills, liver) in any of our treatments of the present study. An increasing trend of selenium accumulation was observed in the carcass (~120%) of fish exposed to PWE (Figure 2.2). Similarly, an increasing trend, though not statistically significant, was observed in FHM liver with copper accumulation in the MWE treatment (~72%) and the PWE treatment (~180%) compared to reference (Figure 2.2).

2.2.3 Biological endpoints

Significant differences among fish body weight (p=0.588) or fork length (p=0.615) among the four treatments were not observed. Variability of fish condition [100(body weight (g)/ length(cm)^3], gonadosomatic index (GSI) [100(gonad weight/ body weight)] and liver somatic index (LSI) [100(liver weight/body weight)] were assessed in FHM. A significant increase in female FHM condition was observed in the SWE treatment compared to the reference (p=0.002) (Table 2.2). Since body weight or length did not differ among treatments, these results would suggest that the observed differences were the result of a synergistic relationship between body weight and length. GSI and LSI were not significantly affected in females when exposed to MME's. However, a significant increase in LSI (p=0.041) was observed in male FHM in the MWE treatment compared to reference (Table 2.2), with no appreciable differences in condition

Table 2.1: Summary of water chemistry and mean total metals (mean ± standard error, n=3) showing statistically significant differences analyzed in water samples collected from Reference, SWE (surface water effluent), MWE (mine water effluent) and PWE (process water effluent) treatments in artificial streams during the 2008 field study. Asterisks represent significant system effects where *p<0.05, **p<0.01, ***p<0.001 (data analyzed using a One-Way ANOVA or Kruskal Wallis).

	Parameter	Units	Canadian Water Quality Guidelines (Aquatic Life)	Reference	20% SWE	30% MWE	45% PWE
	Temperature	°C	-	21.7±0.44	22.4±0.63	21.7±1.44	23.17±0.40
General Chemistry	**pH**	pH	6.5-9	7.52 ± 0.15	7.29 ± 0.12	7.33 ± 0.16	6.69 ± 0.43
	TOC [a]	mg/L	-	10.33 ± 0.67	9.67 ± 0.33	9.00 ± 0.58	7.33 ± 0.88*
	DOC [b]	mg/L	-	8.67 ± 0.88	9.33 ± 0.33	8.00 ± 1.15	7.00 ± 0.58
	TSS [c]	mg/L	-	3 ± 0	3± 0	3 ± 0	5± 2
	Alkalinity	mg/L	-	43.7 ± 0.1	43.5 ± 1.4	43.2 ± 4.0	25.1 ± 6.6
	Ammonia	mg/L	-	0.17 ± 0.05	0.32 ± 0.11	0.74 ± 0.35	1.89 ± 0.77
	Conductivity	µS/cm	-	148 ± 6	465 ± 14***	769 ± 33***	1616 ± 53***
	Nitrate	mg/L	-	0.10 ± 0.05	0.12 ± 0.04	1.97 ± 0.43**	1.26 ± 0.76*
	Total Calcium	mg/L	-	15.5 ± 0.8	60.7± 5.8***	108.9 ± 7.6***	259.3 ± 20.7***
	Total Magnesium	mg/L	-	3.3 ± 0.3	3.9 ± 0.2	1.0 ± 0.4***	8.2 ± 1.4**
	Total Hardness (as $CaCO_3$)	mg/L	-	54.5 ± 3.8	163.0 ± 20.4***	308.3 ± 5.5***	651.3 ± 23.9***
	Total Phosphorus (as P)	mg/L	-	0.25 ± 0.20	0.29 ± 0.23	0.05 ± 0.01	0.10 ± 0.02
	Total Nitrogen	mg/L	-	1.31 ± 0.01	1.61 ± 0.22	4.06 ± 0.75**	4.16 ± 0.51**
Metals	**Total Barium**	µg/L	-	13.57 ± 0.20	16.83 ± 0.78*	80.50 ± 55.25*	28.43 ± 1.47*
	Total Boron	µg/L	-	12.33 ± 1.20	18.00 ± 0.58*	30.87 ± 1.92***	42.30 ± 5.31***
	Total Cobalt	µg/L	-	0.30 ± 0.16	0.39 ± 0.05	0.36 ± 0.14	1.45 ± 0.12*
	Total Copper	µg/L	2-4	9.00 ± 0.38	9.23 ± 1.07	7.93 ± 1.20	50.80 ± 5.45***
	Total Lithium	µg/L	-	2.50 ± 0.00	8.10 ± 0.65***	8.47 ± 1.33***	26.33 ± 3.38***
	Total Nickel	µg/L	25-150	11.47 ± 1.54	32.67 ± 4.96	27.10 ± 10.60	53.27 ± 15.49*
	Total Rubidium	µg/L	-	2.33 ± 0.03	6.00 ± 0.50***	11.77 ± 1.02***	35.47 ± 2.74***
	Total Selenium	µg/L	1.0	0.80 ± 0.30	0.50 ± 0.00	1.37 ± 0.44	11.10 ± 0.92*
	Total Strontium	µg/L	-	57.93 ± 1.01	137.7 ± 10.17***	482.3 ± 36.06***	485.0 ± 32.51***
	Total Thallium	µg/L	0.8	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.30 ± 0.04*

[a]TOC = Total Organic Carbon
[b]DOC = Dissolved Organic Carbon
[c]TSS = Total Suspended Soli

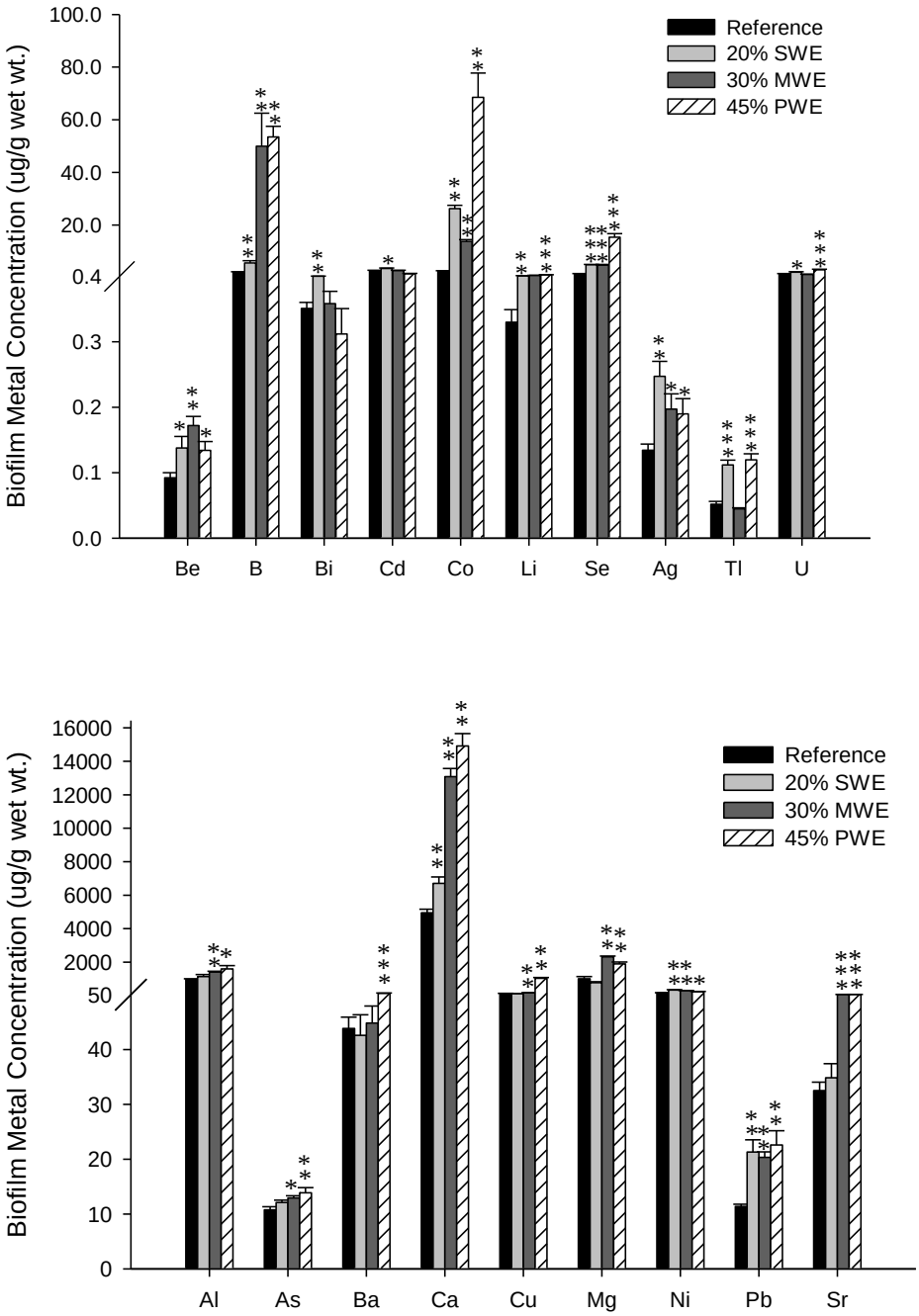


Figure 2.1 Selected metals (mean ± standard error, n=5) that were significantly increased in one or more treatment, SWE (surface water effluent), MWE (mine water effluent), and PWE (process water effluent) in the biofilm (µg/g) over a 21-day exposure period. Asterisks represent significant difference from reference where *p<0.05, **p<0.01, ***p<0.001 (data analyzed using a One-Way ANOVA or Kruskal Wallis).

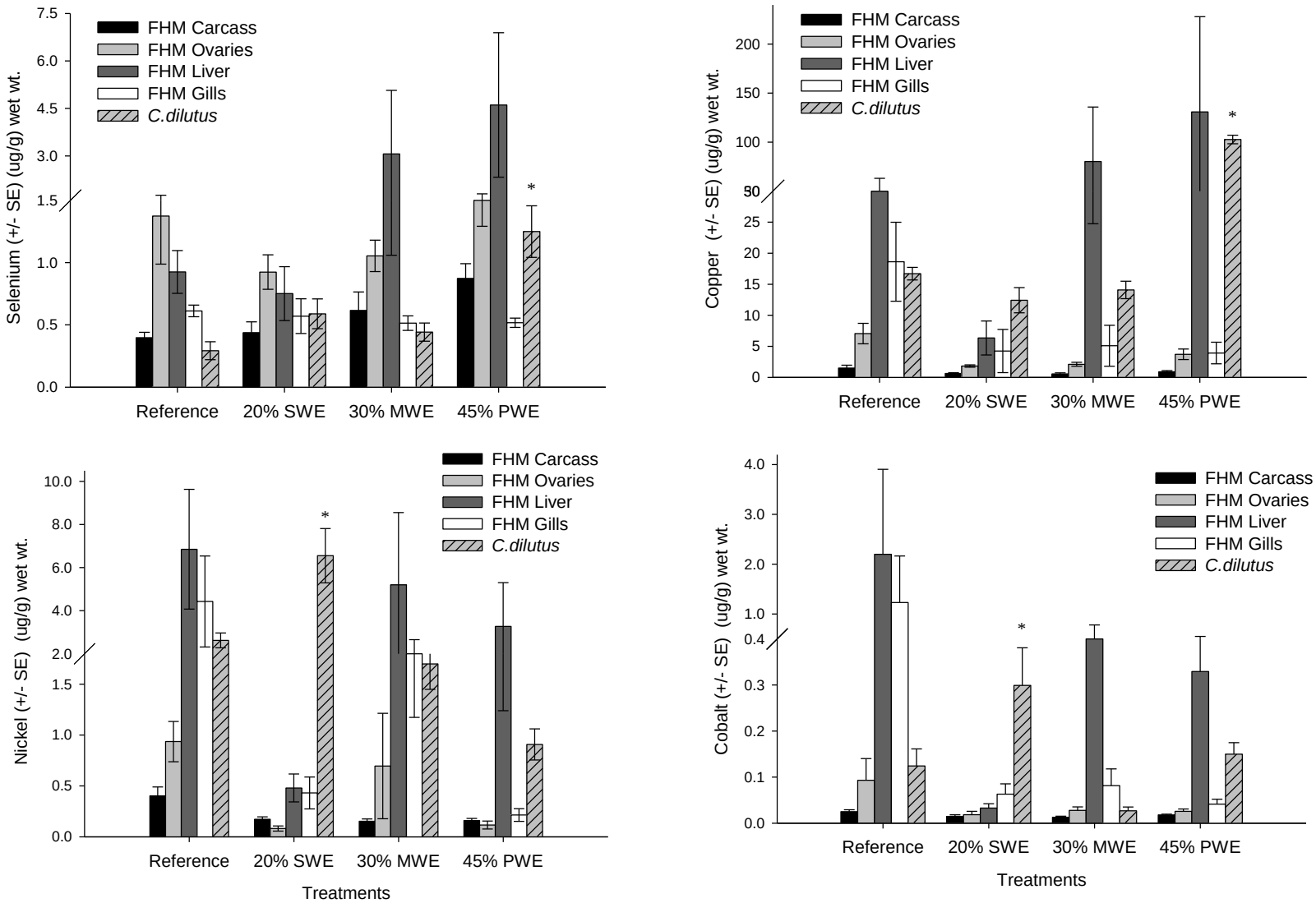


Figure 2.2 Selected metal concentrations [Cu, Se, Ni, Co] in tissues analyzed in fathead minnow (*P.promelas*) [Carcass, Ovaries, Liver, Gills], and midge larvae (*C. dilutus*) (mean ± standard error, n=5) after exposure to SWE (surface water effluent), MWE (mine water effluent), and PWE (process water effluent) over 21-days. Asterisks denote a significant increase in metal concentrations compared to reference, where *p<0.05, **p<0.01, ***p<0.001 (data analyzed using a One-Way ANOVA or Kruskal Wallis)

or GSI in any of the treatments when compared to reference. Again, since body weight did not significantly differ among fish we assume that liver weight was mainly responsible for the observed differences in LSI.

2.2.4 Reproductive endpoints

There was a significant difference in fecundity when compared to both body weight and fork length (p= 0.046 and 0.049, respectively) in the SWE treatment only (Table 2.2). This is supported by results showing a significant increase in egg production [Cumulative total number of eggs produced (p=0.006), cumulative eggs per female per day (p=0.017) and cumulative spawning events (p=0.042) see Table 2.2 and Figure 2.3 for selected parameters] after exposure to SWE compared to that in the reference. In comparison, a significant decrease was observed in cumulative eggs per female per day p=0.001), cumulative total number of eggs produced (p=0.001) (Table 2.2; Figure 2.3) with no significant differences in spawning events (p=0.095) (Table 2; Figure 2.3) in the PWE treatment compared to that in the reference (Table 2.2; Figure 2.3). No significant changes in any of these endpoints were observed in MWE treatment relative to the reference (p>0.05). Egg size (as a function of body weight) was significantly decreased following PWE exposure (p<0.05) (Table 2.2).

A significant reduction in mean total number of days to hatch was also observed after exposure to both SWE and PWE compared to the reference (p<0.05) (Table 2.2). A significant increase in fertilization success was observed after exposure to PWE compared to the reference (p=0.018) (Table 2.2). No significant changes in gonad weight, mean total egg production (the total number of eggs produced by each female in a given stream/replicate, over the 21-day exposure period) and mean egg production (average number of eggs produced by each female in a given stream over the 21 day exposure period), hatching success, adult survival, larval survival or larval deformities were observed in any of the effluent treatments when compared to the reference (Table 2.2). Similarly, there were no significant differences in either male or female secondary sex characteristics in any of the effluent treatments compared to the reference (p>0.05). When development of characteristics was assessed over time, no significant changes occurred in any of the treatments compared to the reference (p>0.05).

2.2.5 *Chironomus dilutus* density and emergence

Chironomid densities in each treatment at the end of the exposure period are shown in Figure 2.4. After 21 days of the study, the food resource averaged 3.5 larvae/cm^2 in the reference treatment and decreased to 2.5 larvae/cm^2 in the SWE, 1.75 larvae/cm^2 in PWE and 1.0 larvae/cm^2 in MWE. A statistically significant difference in chironomid densities among effluent and reference exposed invertebrates was observed ($p<0.001$). Post-hoc analysis revealed a significant difference from the reference for all three treatments (SWE $p=0.001$, MWE $p<0.001$, PWE $p<0.001$). Satiation density of 1.48 *C. dilutus*/cm^2 was, however, achieved in most treatments by the end of exposure. The optimum density was based on a daily feeding amount of 1g/breeding pair/day established during the development of the modified bioassay (Rickwood et al., 2006a), which was based on the amount of food that was required by the FHM to achieve satiation (Ankley, 2001). Densities were then based on the number of *C. dilutus* that were equivalent to one gram (50 larvae in our case) over 21 days of exposure (1050 total chironomids) per total stream area (706 cm^2), resulting in the satiation density of 1.48 *C. dilutus*/cm^2. Densities did not appear to affect FHM egg production since densities in the SWE treatment were significantly lower than the reference, yet this treatment had the highest egg production. Similarly, MWE had the lowest densities but similar egg production to the reference.

Results showed that the duration of exposure (time) and the type of effluent (treatment), as well as the interaction between time and treatment had a significant effect on the emergence of adult chironomids ($p<0.001$ for all). In the first two weeks of exposure, emergence appeared consistent across the three effluent treatments (Figure 2.5). A decrease of adult emergence in the reference and SWE occurred during the intermediate phase of the exposure, which could be due to the increased consumption by fish and/or the stage of larval development at the time of sample collection. Emerging adults were counted but not removed from the streams, thus any chironomid breeding resulted in the deposition of egg sacs and corresponding generational cycling in the streams. Chironomid egg sacs were observed being laid in the streams throughout the study. The final week of adult emergence corresponded to the high larval densities observed in the sediment cores in all of the treatments including the reference.

Table 2.2 Percent magnitude of change (+, -, 0, n=8) relative to reference for selected biological and reproductive endpoints measured in both male and female fathead minnow (*P.promelas*) after exposure to SWE (surface water effluent), MWE (mine water effluent), and PWE (process water effluent) over 21-days**.** Asterisks represent significant treatment effect where *p<0.05, **p<0.01, ***p<0.001 (data analyzed using a One-Way ANOVA or Kruskal-Wallis for mean data, Kolmorov Smirnov for cumulative data and Chi-square for attribute data).

Biological Endpoints	**Females**			**Males**		
	SWE	**MWE**	**PWE**	**SWE**	**MWE**	**PWE**
Fork length	-4%	0%	-1%	+8%	+8%	+2%
Body weight	+14%	+11%	+12%	+32%	+33%	+9%
Liver weight	+26%	-5%	-9%	+118%	+161%	+47%
Condition [(body wt/length(cm)^3) *100]	+26%**	+11%	+13%	+4%	+6%	+1%
Liver weight vs body weight (LSI)	+6%	-14%	-20%	+59%	+97%*	+34%
Reproductive Endpoints						
Gonad weight	+76%	+58%	+65%	+51%	+40%	+33%
Gonad weight vs body weight (GSI)	+49%	+45%	+46%	+13%	+20%	+31%
Mortality	0%	0%	0%	0%	0%	0%
Fecundity	+97%*	+2%	-52%	N/A	N/A	N/A
Egg size vs length	0%	-4%	-8%**	N/A	N/A	N/A
Egg size vs body weight	-11%	-13%	-18%*	N/A	N/A	N/A
Cumulative eggs/female/day	+75%*	-5%	-60%***			
Cumulative total egg production	+122%**	+7%	-39%***			
Cumulative spawning events	+29%*	+9%	-17%			
Mean eggs produced	+98%	+2%	-41%			
Mean eggs/female/day	+68%	+9%	0%			
Secondary sex characteristics	+13%	+20%	+7%	+26%	+11%	+12%
Mean days to hatch	-11%*	-3%	-15%**			
Hatching success	-23%	-47%	+9%			
Mean fertilization success	+2%	0%	+6%*			
Mean larval survival (Day 5)	-6%	-28%	-21%			
Mean larval deformities	+13.9%	-25.8%	-40%			

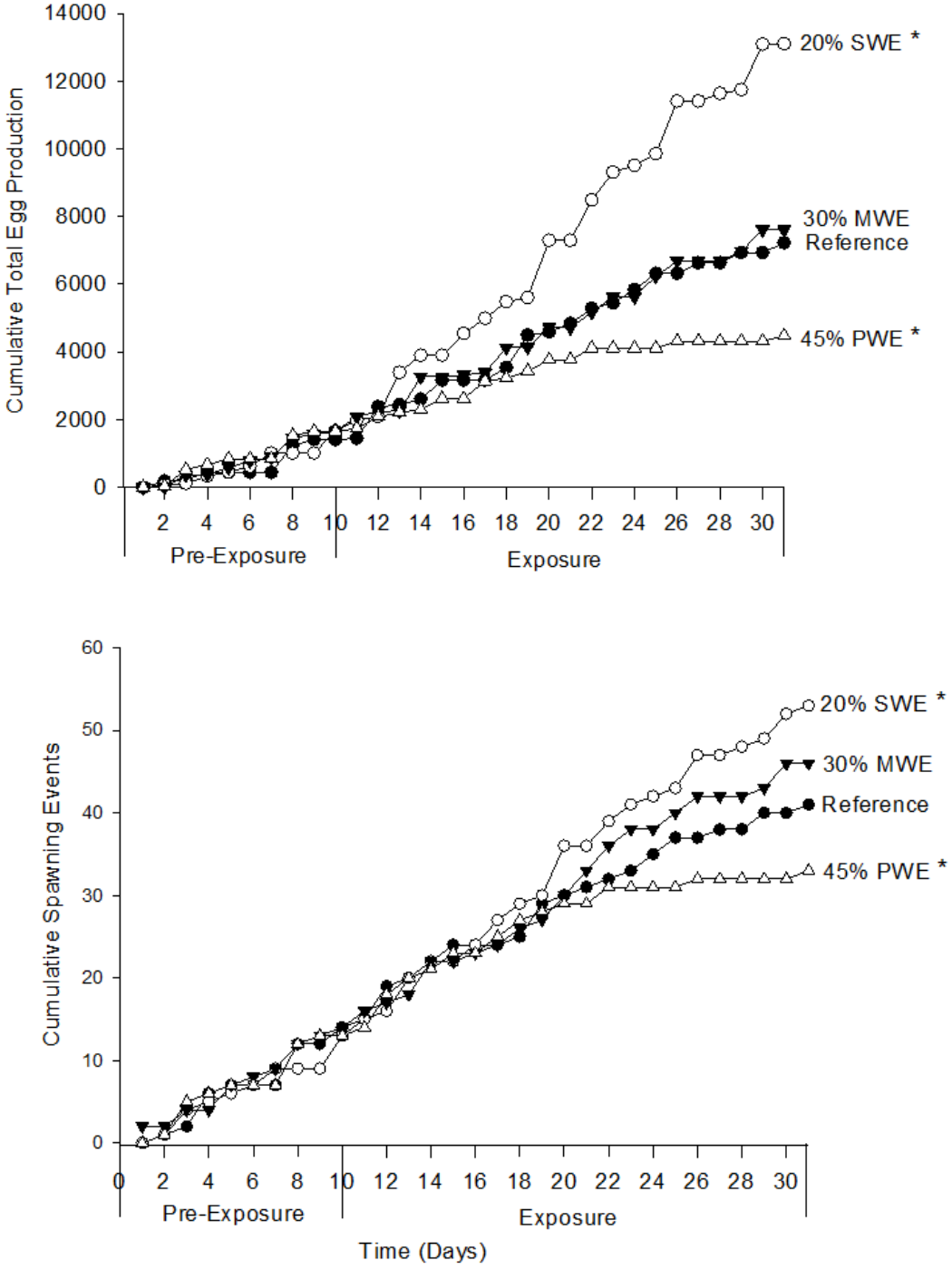


Figure 2.3. Cumulative total egg production and cumulative number of spawning events by fathead minnow (*P.promelas*) breeding pairs during a 10-day pre-exposure and 21-day exposure period to 20%SWE (surface water effluent), 30% MWE (mine water effluent), and 45% PWE (process water effluent) compared to reference water. Asterisks denote a significant difference from reference, n=8 during the exposure phase (data analyzed using Kolmorov Smirnov).

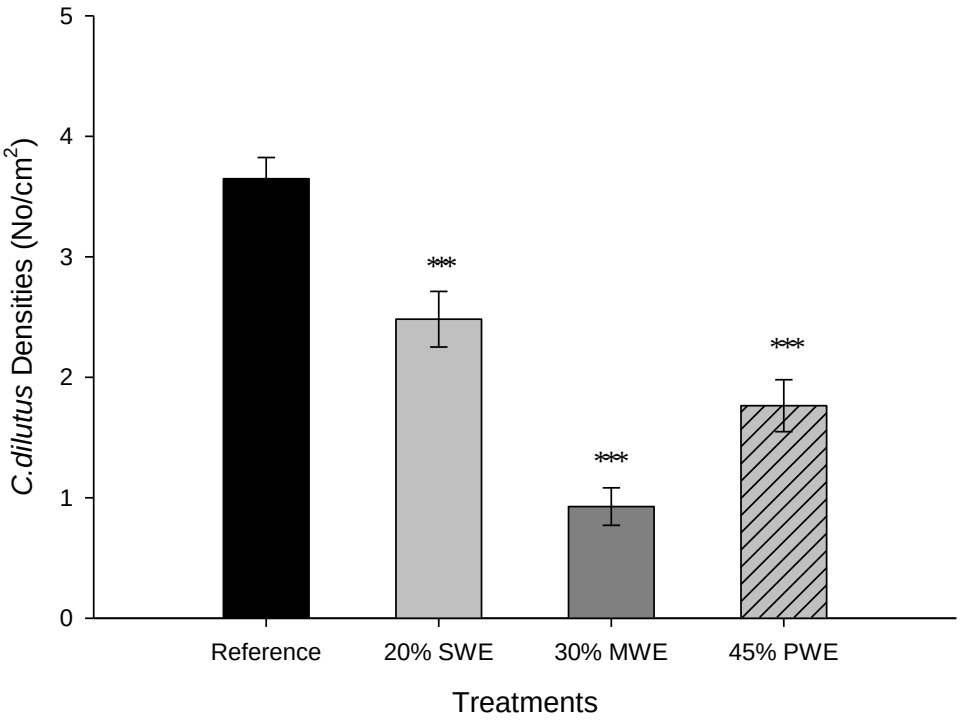


Figure 2.4 Comparison of *C. dilutus* densities (mean ± SE, n=8) in replicate streams after exposure to 20% SWE (surface water effluent), 30% MWE (mine water effluent), 45% PWE (process water effluent) after the 21-day exposure period. Asterisks represent significant difference from reference where *p<0.05, **p<0.01, ***p<0.001 (data analyzed using a One-Way ANOVA).

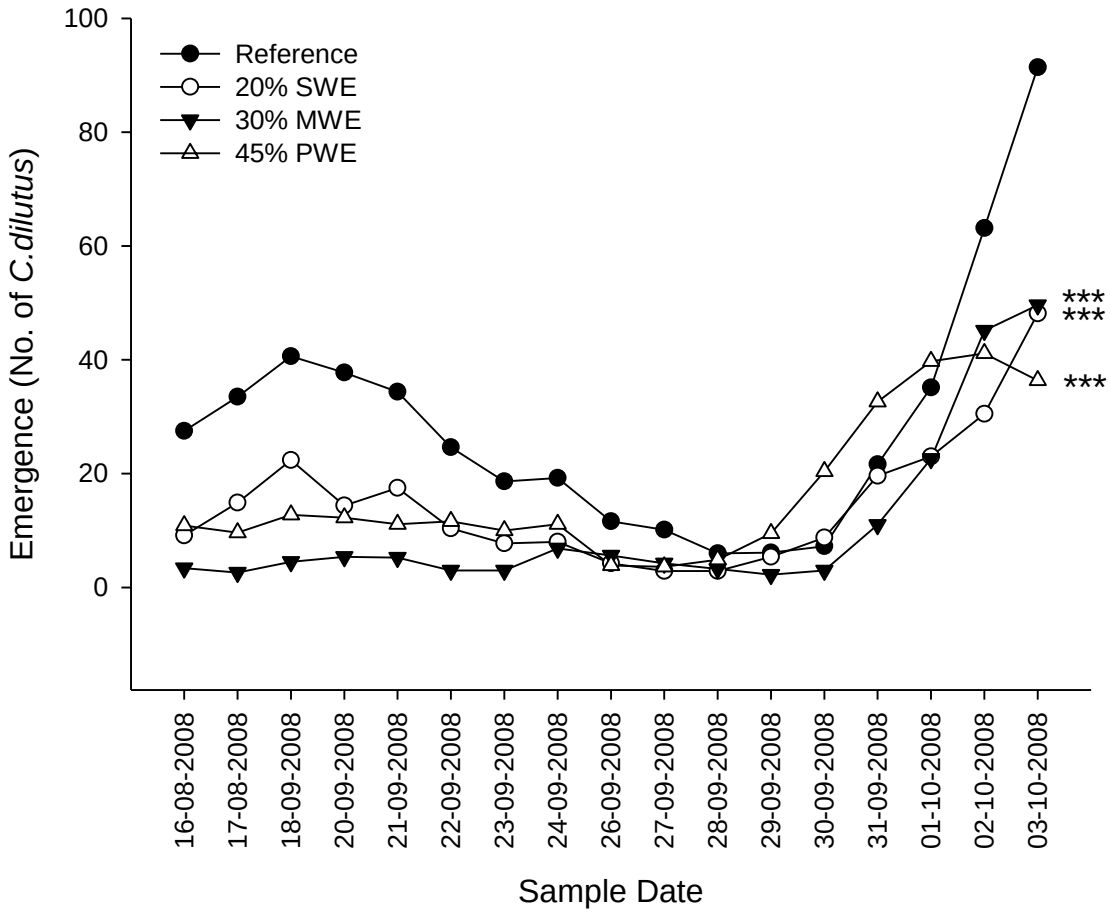


Figure 2.5 Comparison of *C. dilutus* adult emergence in replicate streams after exposure to 20% SWE (surface water effluent), 30% MWE (mine-water effluent), 45% PWE (process-water effluent) after the 21-day exposure period. Asterisks represent significant difference from reference where *p<0.05, **p<0.01, ***p<0.001 (data analyzed using a Two-Way ANOVA, n=8).

2.3 DISCUSSION

Most mining operations in Canada are now moving towards investigating the cause of effluent effects as years of monitoring have determined that effects exist and subsequent mitigation requires an understanding of cause. The ability to assess the effects of each individual effluent can be a difficult task to undertake using traditional field studies, particularly when the study site is impacted by multiple confounding stressors. Multi-trophic mesocosms combining both food and water as a source of exposure have proven to be a good alternative to traditional field studies.

2.3.1 Water quality

Total metal concentrations in the effluents increased in a concentration-dependent fashion with the lowest metal concentrations in the 20%SWE, followed by 30%MWE, and the highest metal concentration in the 45%PWE. These findings were consistent with previous field and lab studies of Junction Creek (Hruska and Dubé, 2004; Dubé et al., 2006a; Weber et al., 2008; Rickwood et al., 2006a, 2008). Although many of the causative elements found in all three mining effluents (SWE, MWE, PWE) (e.g., Co, Cu, Li, Mg, Ni, Rb, Se, Sr, Tl) were significantly higher than in the reference water and were above Canadian Water Quality Guidelines for the Protection of Aquatic Life (CCME, 1999),(Cu, Ni, Cr, Fe, Cd, Co, Pb, Mo, Cr, Tl), there were no significant increases of metal accumulation in any of the female FHM tissues (carcass, gills, gonads and liver) analyzed. Adult survival was similar among all treatments relative to the reference (88% survival in reference).

Water chemistry parameters such as hardness, dissolved and suspended organic carbon, pH and alkalinity are important modifiers of metal bioavailability and toxicity to aquatic organisms (Paquin et al., 2002; Niyogi and Wood, 2004). In the current study, 3-12 fold elevated water hardness concentrations as well as 4-16 fold increase in Ca (the primary hardness cation) concentrations in the MMEs (SWE, MWE, PWE) were observed compared to the reference water. Since it has been found that metal bioavailability decreases with increasing water hardness, it was likely the most important factor among all the water chemistry variables in reducing waterborne metal accumulation by MME exposed fish in the study.

2.3.2 Biofilm and chironomids

Biofilm is composed of algae, bacteria, and fine detrital matter that adhere to substrates in waterbodies. This study showed that several elements (e.g., boron, cobalt, lead, strontium and selenium) were elevated in the biofilm of MME treatments relative to the reference (Figure 2.2). Similarly, cobalt and nickel in the SWE treatment, and copper and selenium in the PWE treatment were elevated in the chironomid tissues (Figure 2.2, select elements). However, increased metal and micronutrient accumulation both in the biofilm and chironomids did not result in significant increased burdens in FHM, although increasing trends were visible in FHM livers, ovaries and carcass tissues (Table 2). Farag et al. (2007) have also reported significantly higher metal levels in the biofilm relative to benthic macro-invertebrates and fish tissues in a watershed impacted by MMEs, suggesting that biofilm plays a key role in influencing the transfer of metals into the food chain. Considering the highest concentration of elements were detected in biofilm across all effluent treatments, it is possible that, in addition to water hardness, the growth of biofilm in the streams played an important role in reducing waterborne metal bioavailability in our treatments. Dissolved elements in the water column can easily be transferred to the abiotic (colloids in the water) and biotic components through biological processes (diffusion, binding, uptake) within the biofilm (Hamilton, 2004; Orr et al., 2006; Buffle et al., 2009). It is also possible that only a limited transfer of elements occurred through the food chain because the chironomids and possibly the fish were not feeding entirely on the biofilm during our study. Chironomids were supplied a diet of Nutrafin^{TM} slurry 3 times per week throughout the entire experiment, and therefore had an alternate food source. The consumption of biofilm by FHM or the chironomids as a food source has not been addressed under the current study design, and should be examined in future investigations. Furthermore, chironomid larvae in the natural environment are primarily collector-gatherers but can occupy a variety of niches including: collector-filterers, scrapers and predators feeding on fine sediment, detritus, algae as well as other midge larvae. (Voshell, 2002). Due to the omnivorous nature of the chironomids, future studies may also consider the addition of a scraper (at the nymph stage) to the streams (e.g., mayflies, caddisflies), since they would feed entirely on the natural biofilm, which may provide a better indication of metal transfer through the food chain.

2.3.3 Fathead Minnow tissue metal burdens

One of the objectives of the present study was to assess the accumulation of elements by fish both through the water and the food chain. In the natural environment, FHM's feed from the bottom on aquatic insect larvae, zooplankton and algae (Holm et al., 2009). This natural feeding behaviour was facilitated within each of the streams by allowing the biofilm (algae) to establish along with the prey base (chironomids) in the bottom sediments prior to the introduction of the FHM. This also ensured that both the biofilm and the chironomids were exposed to the effluent prior to the fish consuming them. It was hypothesized that increased metal content in the effluents would lead to increased metal accumulation in fish tissues through trophic transfer. Although we observed statistically elevated levels of Cu and Se, in the water, biofilm and chironomid tissues in the PWE treatment, statistically significant accumulations of these micronutrients in the fish tissues were not observed. Often in the natural sciences, important biological differences can fail statistical detection due to a variety of reasons (e.g., small sample size, high variance in the data, low power to detect a significant difference) (Johnson, 1999; Bosker et al., 2009). Conversely, unimportant differences can turn out to be significant due to abnormally large sample sizes (Johnson, 1999). In some cases a judgment call between statistical significance (non-significance) and biological significance (non-significance) must be made. Consequently, upon examination of the data biologically (though not statistically), both selenium (in the carcass and liver) and copper (in the liver) showed an increasing trend of metal accumulation in FHM exposed to PWE (Figure 2). Previous studies conducted on Junction Creek (Eastwood and Couture, 2002; Jaagumagi and Bedard, 2001, Weber et al., 2008) have consistently reported elevated levels of Cu in fish tissues. Studies conducted by our lab using PWE have also consistently reported elevated levels of both Cu and Se in fish tissues (Dubé et al., 2006a; Rickwood et al., 2006a, 2008). It is possible that the sample size was too small and/or the elevated micronutrient concentrations in the reference water limited the ability to detect a significant difference among treatments, however we could not discount the biological significance of the increasing trend in both Cu and Se in fish tissues.

The dominant pathway of selenium uptake in fish is thought to be the diet, where it is passed on to the egg mainly through maternal transfer (Ogle and Knight, 1989; Lemly, 2002). The bioaccumulation of Se through trophic transfer has been well documented in the literature, and it has been implicated in fish reproductive impairment, reduced fish biomass, population

declines and drastic alterations in fish community structure (Ogle and Knight, 1989; Heinz et al., 1996; Lemly, 2002). Therefore, it is a metal of great concern for all mining operations in Canada and throughout the world and has been gaining substantial interest in the literature over the last few years. Although waterborne Se concentrations exceeded the toxicity thresholds established by the USEPA (2004) of 5 µg/l in PWE by a factor of 2, Se accumulation in the eggs were well below the level reported in the literature (Lemly, 1993; Hamilton, 2004) to elicit teratogenic effects (10 µg/g dry weight), with concentrations ranging from 0.924 to 1.548 µg/g wet weight (approximately 3.926 to 6.579 µg/g dry weight based on 76.47% moisture [Muscatello et al., 2006]). There were also no significant differences in larval deformities in any of the effluent treatments (SWE, MWE, PWE) compared to the reference. Previous studies conducted by our lab have recorded increased deformities in larvae exposed to PWE, despite lowered total Se concentrations in the water (Rickwood et al., 2008). Although we found significantly higher Se concentration in chironomids, and increasing trends in the FHM livers and carcasses, the Se levels in the ovaries and eggs were similar across all of the treatments. These results suggest that although trophic transfer of Se might have occurred in the PWE treatment to an extent, it did not result in a substantial maternal transfer of Se. Speciation analysis conducted in our lab have shown that the inorganic Se present in the PWE in our study was largely in selenate form, which is known to be less bioavailable and less toxic to aquatic animals than selenite (Lemly, 2002; Orr et al., 2006). However, it is known that primary producers can transform inorganic forms of Se into organic species (e.g., selenomethionine) which are readily transferred through food webs (Muscatello et al., 2009). It is possible that this could have occurred in the current study though we have no way of confirming this theory. Nevertheless, based on the re-occurrence of significant Se accumulations in biotic tissues in all of our studies to date, inconsistent larval deformity results and minimal speciation data, it can be suggested that Se is a metal of concern in the effluent water with regards to the reproductive effects observed in fish exposed to the PWE.

Cu also transferred through the food chain (water, biofilm, chironomids, FHM [liver]) and exhibited an increasing trend, though not statistically significant in the PWE. Cu toxicity can occur through both waterborne and dietborne routes of exposure. Cu is a micronutrient that is known to be tightly regulated in fish through the homeostatic mechanisms to ensure toxicity and/or deficiencies do not occur (Schlenk and Benson, 2001). Due to this homeostatic regulation of Cu, levels in the water or diet would have to remain at a relatively high level in order for

measurable bioaccumulation into the tissues and subsequent toxicity to occur (Schlenk and Benson, 2001). Population threshold concentration (PTC) in the literature has been estimated at 27 µg/l for waterborne Cu exposure of FHM, at hardness levels of 218 mg/L (Iwasaki et al, 2010). Since Cu levels were found at concentrations well above the estimated PTC in the PWE treatment (50 µg/l at hardness of 651 mg/L in 45% PWE), it is plausible that chronic waterborne Cu exposure could lead to population-level effects in FHM. Consequently, chronic exposure to elevated levels of Cu has been shown to impair the homeostatic mechanisms resulting in cellular damage, oxidation of proteins, membrane lipid peroxidation and cleavage of DNA and RNA molecules (Schlenk and Benson, 2001). Significant Cu accumulation in the gills was not observed in any of the treatments, which suggests that the accumulation in the tissues was predominantly dietary related. This was also reflected in the tissue analysis which showed concentrations (wet.wt.) between 1.8-7.1 µg/g in the ovaries, 0.5-0.9 µg/g in the carcass and 3.9-80.3 µg/g in the livers among the three treatments. Due to the elevated levels of Cu in the PWE, the observed metal accumulations in FHM (ovaries, carcass and liver) tissues and the ability of elevated Cu in fish to affect critical life stages, fish growth and development (Schlenk and Benson, 2001), Cu remains a metal of concern in the PWE.

Reproductive effects have been consistently observed in both fish and benthic invertebrates in all of the studies that we have conducted with Junction Creek MMEs to date including our current study (Dubé et al., 2006a; Weber et al., 2008; Rickwood et al., 2006a, 2008). We believe that the nature of the effluent and its composition are the main contributing factors affecting toxicity. As previously mentioned, PWE is mainly comprised of mine process water from mining, and milling activities whereas the other effluents are mixtures of surface runoff and underground mine operation water. The milling process for most mines requires the use of a variety of chemical reagents in the flotation process which may or may not include such things as: modifiers (e.g., Lime, sodium carbonate) used to increase the pH and activators (e.g., sodium sulfide, sodium hydrosulfide) used to float the metal of interest. After the flotation process the concentrated product goes through a de-watering and thickening process where flocculants and filtering agents are used such as: sodium isopropyl xanthate, sodium isobutyl xanthate, polymers, nonionic surfactants, polyacrylate and anionic and nonionic polyacrylamides (EPA, 1994b). At all steps in the process, the waste products generated are sent to the tailings basin. In addition, there are other wastes produced by the mine which could end up in the tailings

basin such as surface runoff from fuel storage areas and maintenance shops and effluent from waste water treatment processes. Though it was beyond the scope of the current study design to examine the organic constituents in the MME's, they too have the potential to affect FHM responses and could be partially responsible for the reproductive effects observed in our current study. Also PWE constitutes the greatest volume of effluent at the highest concentration of metals entering Junction Creek. Therefore, it is not surprising that we observed greater effects associated with PWE exposure. However, we cannot discount the importance of effluent characteristics and composition in assessing effluent-mediated effects. Elevated concentrations of elements in the tissues of biota exposed to PWE suggest that the reproductive effects documented are associated with micronutrient exposure. Though not statistically significant, we did find clear trends for increasing Cu and Se accumulation in livers of fish exposed to PWE. Our fish metal accumulation data had a high degree of variability surrounding the mean and the sample size was fairly low (n=8,) therefore the power (P=0.115 to 0.618) to detect a significant difference between PWE and the reference fish was low. Furthermore, the lack of statistical power to detect a significant difference could be attributed to the relatively shorter duration of exposure (21 days) since Cu accumulation occurs rather slowly in fish due to homeostatic regulation (Kamunde and Wood, 2004). Cu and Se levels increased by 6-fold and 4-fold, respectively, in *C. dilutus* tissues exposed to PWE when compared to the reference. Although we did not observe significant metal accumulations in FHM ovaries, reproductive effects are still possible via other important organs (e.g., liver). The liver is a target organ of metal toxicity in fish because it receives a large supply of blood (transporter of metals), is directly involved in the biotransformation, metabolism and excretion of metals, and plays a critical role in maintaining internal homeostasis in fish (DiGiulio and Tillitt, 1997). The biotransformation process could lead to alterations in cellular function, DNA and proteins resulting in a reduction of viable offspring and reduced egg production (DiGiulio and Tillitt, 1997; Schlenk and Benson, 2001). This does not contradict with our findings since we did observe a significant decrease in cumulative total egg production (Figure 2.3) in PWE exposed fish compared to the reference. Furthermore, increased metal and micronutrient accumulation in the liver could affect circulating hormone levels in the blood resulting in increased embryo toxicity and altered vitellogenin levels (DiGiulio and Tillitt, 1997). Se is incorporated into vitellogenin (yolk proteins) in the liver and gets transported to the ovary where it can cause necrosis and rupturing of the egg follicles (Kroll

et al., 1991). Furthermore, if it is incorporated into the yolk sac, larval absorption could lead to deformities (e.g., kyphosis, lordosis, scoliosis) (Kroll et al., 1991). Reduced mRNA transcript levels of vitellogenin have been observed in the livers of female zebrafish (*Danio rerio*) fed with metal-laden polychaetes (*Nereis diversicolor*) (Boyle et al., 2008). Since vitellogenin levels were not measured in our current study, we can only speculate that it might have been a factor for the decreases in cumulative egg production and cumulative spawning events that we have observed with PWE in the study (Figure 2.3).

2.3.4 Metal effects on Fathead Minnow reproductive/biological endpoints

On a national level, the EEM program has shown that fish exposed to mining effluents have reduced condition factor, liver size and growth rates compared to the reference fish (Lowell et al., unpublished). Similarly, benthic invertebrates have shown consistent decreases in taxon richness but inconsistent results with respect to densities (Lowell et al., unpublished). In the current study, increased condition (+26%) was observed in fish exposed to SWE. Increased liver size (+133% in females and +27% in males) and increased gonad size (+31% in females to +49% in males) were observed for all MME's (Table 2). In each instance the magnitude of change exceeded the Critical Effect Size (CES) for condition factor (±10%), liver size (± 25%) and gonad size (± 25%) as specified in the EEM program (MMER, 2002). Increased liver size is not all that surprising since the liver is a target organ for metal accumulation however, the increase in condition and gonad size in the MME's compared to reference was of special interest to the study. Differences in fecundity were observed in the SWE and PWE treatments when compared to reference treatments. This would suggest that fish may be larger in size in SWE and smaller in PWE and that larger fish in any given treatment would be expected to produce more eggs. However, changes in fecundity were not a factor of the size (body weight or length were not statistically different) of the fish, they just produced more eggs in the SWE and less eggs in PWE. Furthermore, it has been postulated that perhaps the differences in FHM reproduction (cumulative total egg production, cumulative spawning events) could be linked to differences in food abundance in the streams. Previous studies (Hruska and Dubé, 2004; Rickwood et al., 2006a) have shown time dependent effluent-related effects on chironomid emergence patterns. When adult emergence was analyzed using a two-factor ANOVA, a significant interaction between time and treatment was observed in all treatments. This suggested that the effect of

time was dependent on the presence of the effluent and exposure duration. Biologically, successful chironomid adult emergence was significantly reduced by both increasing effluent concentration and exposure time, which has been observed in previous studies (Hruska et al., 2004). At the end of the exposure period for this study, chironomid densities were significantly reduced in all treatments compared to reference, although all treatments exceeded our optimal feeding amount (1.0 -1.5/cm^2). This suggests that the greater than 100% increase in egg production in SWE and the 39% reduction in egg production in PWE may be due to the greater food availability or lack thereof in MME treatments compared to the reference.

It was also hypothesized that differences in reproductive output between the two treatments (PWE/SWE) may be associated to differences in effluent type or temperature differences among treatments. Although temperature fluctuated between 18-25°C in all streams throughout the experiment, the mean temperature did not differ significantly among treatments. Therefore temperature was not elevated in this particular treatment compared to the others. The nature of the three effluents however, differed considerably in both composition and concentration. Elevated levels of Ba, B, Ca, Co, Cu, Li, Mg, Mo, Rb, Se, Sr and Total Phosphorus (TP) were observed in the SWE compared to reference water. TP was the only nutrient that was elevated, though not statistically, in SWE relative to all other treatments (Table 2.1). In nutrient-limited systems, TP enrichment can increase periphyton (biofilm) biomass and the production and abundance of benthic invertebrates such as chironomids, which use periphytic algae as a primary food source (Chambers et al., 2000; Culp et al., 2000). Consequently, increased food supply has been linked to increased condition, gonad size and egg masses (Gibbons et al, 1998). Though TP levels in SWE were not statistically different among treatments there may have been a slight nutrient enhancement effect since elevated chironomid densities were observed relative to PWE. Therefore, increased condition factor and egg production in SWE was most likely due to an elevated food supply resulting in increased energy storage in the fish. There is also the possibility that a hormetic effect (e.g., increased egg production at low-dose) may have occurred in SWE, though further toxicity testing using a dilution gradient with doses near to the no observed effect level (NOEL) would be required to confirm this theory (Giesy, 2001). Mine effluent mixtures are very complex and it is unknown how the various compounds interact. Since speciation data was not conducted on this effluent we can only speculate that the species present in SWE may have been less toxic forms than those

found in the PWE. Furthermore, a reduced concentration of overall elements in SWE may have resulted in overall reduced metal exposure, reduced oxidative stress and reduced bioavailability in SWE compared to PWE.

Effluent concentrations in MWE showed all of the same elevated elements as in the SWE (Ba, B, Ca, Co, Cu, Li, Mg, Mo, Rb, Se, Sr), along with As, hardness and total organic carbon (TOC) compared to reference values. The MWE showed much greater hardness levels and a greater concentration of elements overall.

Effluent concentrations in PWE showed elevated levels of all of the same elements as in the SWE and MWE (Ba, B, Ca, Co, Cu, Li, Mg, Mo, Rb, Se, Sr), though metal concentrations were much higher in the PWE than in either SWE or MWE for most elements. In addition to these variables, elevated levels of Al, As, Tl, TOC and hardness were observed. The PWE appears to be characteristically more similar to the MWE with respect to nutrient concentration, with the exception of it having a slightly greater increase in overall metal concentrations in the 100% effluent. In addition, PWE contains a number of beneficiation products which may or may not affect reproduction in fish. Furthermore, PWE contains sewage wastewater from the associated mining operations, whereas SWE and MWE do not. It is possible that PWE could contain sufficient levels of estrogenic compounds which could greatly hinder reproductive output in FHM. Hardness and calcium levels were elevated in all effluents compared to reference, which is likely an artifact of the treatment process since all three MME's are pH adjusted with lime and settled prior to discharge into Junction Creek. This may also explain why some elements were elevated in the reference matrices (water, sediment, biofilm, chironomids, fish tissues) compared to exposure. In addition to the physical uptake of elements, stressful exposure conditions, especially in the PWE, could have resulted in a shift in energy production with more energy concentrated on survival and less on reproduction (Franssen, 2009).

Increased TP and subsequent increased growth of biofilm and chironomid densities in SWE streams compared to PWE also has led us to consider that increased bacterial growth in the streams may have also contributed to reduced toxicity in SWE due to the potential for bacteriological reactions, particularly by sulphate reducing bacteria to reduce metal content in effluent water by precipitating them as metal sulphides (Choudhury et al., 2006).

2.4 CONCLUSION

Through the use of an environmentally relevant bioassay, the point source discharge into Junction Creek of greatest priority has been identified as PWE. It is difficult to determine whether the composition of the effluent, the volume discharged, the treatment processes or a combination of factors affected metal accumulation in PWE. However, a weight of evidence approach was used to identify the prominent elements in the metal mixture which may have contributed to reproductive effects in FHM. The high metal content in the MME waters did not transfer appreciably through the food chain for most of the elements except Cu and Se in the PWE. Effluent quality (increased water hardness, organic matter, presence of sewage in the effluent and differences in metal concentrations), fish energetics, and differences in bacterial (biofilm) growth in the streams appear to have played a role in reducing waterborne metal bioavailability to FHM. We also observed significantly higher accumulation of elements by the biofilm than chironomids and FHM, which also likely decreased waterborne metal bioavailability and toxicity in FHM exposed to MME's. Cu and Se remain micronutrients of concern in the PWE since trophic transfer of both were observed in our current study as well as many other studies that have been conducted in the Sudbury area. Reduced cumulative egg production and cumulative number of spawning events were recorded in FHM exposed to PWE, possibly induced by dietary Cu and Se exposure and accumulation. Though Se certainly appears in the effluents and is sufficiently high in PWE to raise cause for concern, there is insufficient speciation data to directly link it as a cause to the reproductive effects seen in the current study.

Future studies should consider assessing dissolved metals, the presence of organic constituents (flotation agents, frothing and de-foaming compounds, polymers, flocculants etc.), and estrogenic compounds in MME's especially in PWE. Vitellogenin levels in gonad tissues would help to assess cause of reproductive impairment and may give an indication of organic or estrogenic exposure. Dissolved metals analysis and speciation of Se, and the analysis of organic forms of selenium in the food chain (e.g., selenomehionine) would also help to better predict metal bioavailability in the presence of these organic compounds. It would also be beneficial to examine the bacterial component of the biofilm for the presence of sulphur reducing bacteria in conjunction with metal content, in order to better understand the role of biofilm in metal

bioaccumulation, and to determine its significance in metal uptake from complex metal-mining mixtures.

Chapter 3[a]

EXAMINING WATERBORNE AND DIETBORNE ROUTES OF EXPOSURE AND THEIR CONTRIBUTION TO BIOLOGICAL RESPONSE PATTERNS IN FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

[a]This chapter has been submitted to the journal of Aquatic Toxicology under joint authorship with Monique G. Dubé, Allison J. Squires and Som Niyogi.

3.0 INTRODUCTION

Drainage from historical mining activities, mine waste, process mill tailings, mine effluent and refining activities can enter surrounding watersheds and become sources of metals in the sediments, water and biota (fish, benthic invertebrates, biofilm). However, due to the complexities of aquatic ecosystems, our understanding of how metals and metal mixtures affect the food chain is limited. Previous studies conducted by our research group have shown waterborne effects in both fish (decreased egg size, increased tissue metal burdens) and invertebrates (reduced hatching success, reduced emergence) when exposed to the treated metal mining effluents (MME) (Hruska & Dubé, 2004; Dubé et al., 2005). Similar effects of metal-mining effluent exposure on fish have also been demonstrated in both lab-based as well as field-based studies (Bradley and Morris, 1986; Eastwood and Couture, 2002; Couture and Rajotte, 2003; Pyle et al., 2005).

There is no dispute that water-borne exposure is an important route of metal uptake in aquatic organisms and has been the basis for setting environmental water quality criteria and standards for metals in Canada and the USA (Meyer, 2005). However, metals are assimilated from the water and the diet very differently in the tissues of invertebrates and fish (Meyer et al., 2005). Diet-borne exposure can result in whole body burdens, (often several orders of magnitude higher), or specific tissue burdens far greater than caused by water-borne exposure alone (Meyer et al., 2005). Even low levels of exposure in the water could lead to acutely toxic levels of dietary exposure in higher-level predators due to biomagnification and trophic transfer up the food chain (Meyer et al., 2005). Diet-borne metals such as Cu, Ni, Zn, Se can also reduce the survival, growth and reproduction of aquatic invertebrates and fish (Meyer et al., 2005). More recent studies have demonstrated that diet can be an important pathway of metal exposure in fish leading to reproductive impairment and toxicity (Rickwood et al., 2006a,2008; Rasmussen et al., 2008; Ng and Wood, 2008; Boyle et al., 2008; Muscatello et al., 2008).

It is often assumed that the waterborne exposure pathway is primarily mediated through the gills and the dietary pathway is mediated through other internal target organs, such as the gut and liver. However, metals accumulated from diet can be transported to the gill, and can subsequently affect the gill structure and function, suggesting that the dietary pathway is more important than previously identified (DeSchamphelaere and Janssen, 2004; DiGiulio and Hinton, 2008). Nevertheless, it is well documented that the gill is usually the primary organ of metal

accumulation if the exposure is predominantly waterborne, whereas gill accumulation of metals is much less relative to that in other vital organs (e.g., liver and gut) during dietary exposure.

The objectives of our current study are two-fold; i) to examine the effects of exposure through diet, through water or through both routes of exposure using a fully factorial exposure design in the laboratory (Experiment I), and ii) to examine the role of food quality on fish toxicity by assessing differences in FHM responses when fish were fed a live diet of *C. dilutus* (multi-trophic) versus when fish were fed a frozen, laboratory-prepared diet of *C. dilutus* (Experiment II). This information is critical to bettering our understanding of the significance of diet for chronic toxicity testing of MMEs to reproducing FHM and their offspring and to consider how this information may affect our interpretation and application of different bioassays.

3.1 METHODS AND MATERIALS

3.1.1 Source area of mining effluents

The study site was located in Junction Creek, Sudbury, ON, Canada approximately 400 km north of Toronto, Ontario, Canada from January to May, 2009 (Figure 1.1). Junction Creek spans a length of about 25 km and can vary in width between 3-16 m. The main branch of the creek flows in a south westerly direction from the community of Garson, ON, through the city of Greater Sudbury, ON, terminating at Kelly Lake (Figure 1.1). It contains 5 main tributaries and receives several point and non-point source inputs, which may affect the system in a cumulative manner, including urban runoff, historical and current atmospheric deposition from mining activities, municipal wastewater treatment and landfill seepage (Jaagumagi and Bedard, 2001). It is also the final receiving environment of three treated MME discharges. The MME's are all treated by conventional hydroxide precipitation and subsequent pH adjustment prior to discharging into Junction Creek (Rickwood et al., 2008). However, the nature of the MME varies among the three mine discharges. This study was conducted using only one of the MME discharges, a process water effluent (PWE) diluted to its natural environmental concentration in

Junction Creek of 45% PWE comprised of a mixture of several mining inputs (e.g., mining, milling, smelting and refining from the Sudbury area. The 45% PWE was chosen for this study since previous research undertaken by our lab has shown it to elicit the greatest effects to fish among the three MME's (Hruska & Dubé, 2004; Dubé et al., 2005; Rickwood et al., 2006a, 2008).

3.1.2 Experimental design

The current study was conducted from January to May, 2009 at the Aquatic Toxicology Research Facility, in the Toxicology Centre of the University of Saskatchewan in Saskatoon, SK, Canada, using artificial streams (mesocosms). Process water effluent (PWE) was shipped to the University on a weekly basis from Sudbury, ON, Canada. Laboratory control water was comprised of a mixture of 40% de-chlorinated tap water and 60% reverse osmosis (RO) water. The use of RO water in the current study was a key component of this study design since our goal was to closely approximate the reference water conditions (pH, hardness, alkalinity) in the Sudbury region. This has enabled us to truly analyze the effluent effects on the aquatic organisms without the confounding effects of the environmental legacies and/or the confounding effects of geographic reference/control water quality differences (Sudbury vs Saskatoon). The laboratory control water was also used to dilute the 100% effluent to the appropriate environmentally relevant concentration of 45% PWE. Both water-only and multi-trophic artificial streams were used in the current study. The water-only streams expose FHM to effluent only through the water phase. The multi-trophic streams refer to a food chain of *C. dilutus*, and FHM in a self-sustaining system (no external food supply) so that exposure of FHM is through both water and dietary pathways.

Each mesocosm table consisted of eight replicate, 10.3 L, circular, high-density polyethlylene artificial streams and each stream contained a 1" (2.54 cm) layer of pre-washed silica sand. The table then drained into an 85-L reservoir from which water was re-circulated through an 8-port manifold via a Marsh pump (Model LC-3CP-MD, March Manufacturing, Glenview, IL, USA), and evenly distributed to each of the streams (for more details regarding the mesocosm design see Hruska and Dubé, 2004). Treatment water was aerated with air stones and heated to 25 ± 2 °C using submersible aquaria heaters. Lighting was adjusted to a 16h light:8h dark photoperiod. In addition, there were two corresponding flow through treatment tanks that

housed the egg and larval cups for each treatment. Two peristaltic pumps (Masterflex® L/S, Model 7524-50, USA) were used to supply the appropriate fresh reference and effluent water to the larval and egg tanks to achieve the same turnover rates as the artificial streams (0.014 L/min). This ensured that at least 1 turnover per day was maintained in each of the streams and egg/larval hatching tanks throughout the experiment.

3.1.3 Exposure pathway - full factorial set-up

A total of 4 mesocosm tables were used in the factorial study for a total of 32 streams. Two of the tables were supplied with reference water and the other two tables were supplied with 45% PWE. Each stream contained a PVC breeding tile and a 250 µm mesh screen to prevent the escape of FHM. The factorial portion of the study required that the culturing of the fish diet (*C. dilutus*) be prepared in advance and frozen. Diet varied between *C. dilutus* raised in effluent water (45% PWE) and, *C. dilutus* raised in laboratory control water (60% RO water and 40% dechlorinated laboratory water) as shown in Figure 3.1. The treatments used in our exposure pathway study design were as follows:

- Table 1 contained control water and FHM were fed *C. dilutus* raised in control water (CWCB treatment).
- Table 2 contained control water and FHM were fed *C. dilutus* raised in 45% PWE water (CWEB treatment).
- Table 3 contained 45% PWE water and FHM were fed *C. dilutus* raised in control water (EWCB treatment).
- Table 4 contained 45% PWE water and FHM were fed *C. dilutus* raised in 45% PWE water (EWEB treatment).

3.1.4 Food quality – multi-trophic set-up

The laboratory-based multi-trophic system involved the use of 2 mesocosm tables as described above for a total of 16 streams (Figure 3.2). Each stream contained a breeding tile, feeding barrier and 250µm mesh screen to prevent the escape of biota and to ensure any emerging insects were retained, counted, and left in the streams to reproduce. Treatments used in the food quality study design included the following:

- Table 1 contained control water and FHM were fed artificial diet of *C. dilutus* raised in control water (CWCB treatment as above).
- Table 2 contained 45% PWE water and FHM were fed artificial diet of *C. dilutus* raised in 45% PWE water (EWEB treatment as above)
- Table 3 contained the multi-trophic streams with FHM held in control water, which grazed on live *C. dilutus* raised in the streams (MT-control treatment).
- Table 4 contained the multi-trophic streams with FHM held in 45% PWE water, which grazed on live *C. dilutus* raised in the streams (MT-effluent treatment).

	Control Water	**Effluent Water**
Control Benthic Organisms	CWCB (Complete Control)	EWCB (Waterborne-Only)
Effluent Benthic Organisms	CWEB (Dietary-Only)	EWEB (Complete Exposure)

Figure 3.1. Exposure pathway (full factorial) experimental design used to investigate the importance of water-only, dietary-only and complete exposures when fathead minnow are exposed to both control water and 45% PWE (Process Water Effluent). Where C=control, E=effluent, W=water and B=benthic organisms.

	Control Water	**Effluent Water**
Frozen/artificial diet	CWCB (Complete Control)	EWEB (Complete Exposure)
Multi-trophic/live diet	MT-control (MT-Control)	MT-effluent (MT-Effluent)

Figure 3.2 Food quality experimental design used to investigate differences in fathead minnow responses when fed a live diet (multi-trophic) or a laboratory prepared artificial diet (frozen) of *Chironomus dilutus* when exposed to both control water and 45% PWE (Process Water Effluent). Where C=control, E=effluent, W=water, B=benthic organisms and MT=multi-trophic.

3.1.5 Water requirements

Effluent from the mine was shipped to the University of Saskatchewan on a weekly basis for approximately 12 weeks in order to culture chironomids and for the fish exposures. Chironomids were cultured as larvae exposed to either laboratory control water (40% de-chlorinated lab water and 60% RO water), or 45% PWE. The culturing of the chironomids in effluent water required that the mine send 126 L of effluent on a weekly basis for the first 4 weeks of the experiment. Effluent was collected weekly by mine personnel in 60 L pails and shipped immediately upon collection to the Toxicology Centre at the University of Saskatchewan. The 100% PWE water was mixed in a 90 gallon (341 L) polyethylene tank to a concentration of 45% PWE using laboratory control water. The water was heated with 400 W submersible aquaria heaters to approximately 23 ± 2°C, aerated, and added to the 40 L chironomid aquaria via static renewal (e.g., 25-50% treatment water replacement twice a week) for 4 weeks or until the larvae reached the third or fourth instar stage.

The fish pre-exposure period spanned 21 days and required the use of approximately 15,000 L of laboratory control water per week to achieve 1 turnover per day in each of 96 streams. During the fish exposure period of the study, effluent was shipped from the mine to the University of Saskatchewan in 1,000 L totes for approximately 7 consecutive weeks. The effluent water was mixed in 300 gallon (1,136 L) polyethylene tanks and heated to 25 ±2 °C using 400 W submersible aquatic heaters to maintain optimum breeding temperatures based on OECD (2004, 2006) and US EPA (2007) fish bioassay protocols. Normal summer temperatures of 15-23 °C have been reported in Junction Creek but have been shown to vary as much as 10 degrees in a single day in the same sampling area (JCSC, 2008). Therefore, we are certain that we were able to achieve environmentally relevant temperatures in the streams throughout the experiment. Over the entire exposure period (21 days), a total of 3 mesocosm tables were supplied with laboratory control water, and 3 were supplied with 45% PWE (Figures 3.2 and 3.3).

3.1.6 Fathead minnows (*Pimephales promelas*)

A pre-exposure breeding trial was conducted for approximately similar duration as the exposure period of 21 days. Six-month-old naïve FHM were obtained from Osage Catfisheries Inc. (Osage Beach, MO, USA), and 192 fish were randomly selected from this culture for the

pre-exposure period. Breeding pairs (1male and 1 female) were placed into each stream containing a PVC breeding tile and a 250 µm mesh screen to prevent the escape of FHM. The pre-breeding trial consisted of 96 breeding pairs (8 pairs per table) and was conducted in the absence of effluent to establish baseline reproductive performance. Breeding tiles were checked daily for egg production. Eggs were removed from the tiles, placed in a Petri dish and photographed using a Cannon Powershot digital camera (Model A620, Mississauga, ON) and examined using a Vista vision^{TM} (Model 48402-00, VWR International, Missisauga, ON) trinocular microscope to determine fertilization success. Photographs were obtained in order to count and measure egg size using Image Pro Plus 6.1 software (Media Cybenectics Inc., Maryland, USA). Eggs were then placed into PVC cups with 250 µm screen mesh and placed into rearing chambers and continuously aerated using air stones. Hatching success was determined once larvae hatched (3-5 days post spawn). After 21 days, selection of breeding pairs for the exposure period of the study was undertaken. The selection was based on 100% survival of all adults, that the pair had bred at least once and there was a >80% fertilization of the eggs (Rickwood et al., 2008). Based on these criterion only 31% of the pre-exposed breeding pairs met the requirements and were selected for exposure testing, therefore our sample size during the exposure phase was reduced to 5 (n=5 pairs per treatment).

Statistical analysis was conducted in order to determine if there were differences among reproductive endpoints prior to effluent exposure. Fertilization success was analyzed using a one-way ANOVA providing assumptions of normality (Shapiro-Wilks) and homogeneity of variance (Levene's) were met. Alternatively, the data was log transformed or the non-parametric equivalent (Kruskal-Wallis test) was applied to any data that did not meet the assumptions (e.g., mean egg production, mean total egg production). A chi-squared test was used to analyze differences in the number of spawning events among the groups. No significant differences were found among the 6 groups ($\propto$>0.05).

Selected breeding pairs were placed into the appropriate treatment groups in individual streams. Fish that were placed into the factorial streams were fed 1 gram of pre-frozen *C. dilutus* daily, which were raised in either laboratory control water or in 45% PWE depending on the treatment group (Figure 3.1). The fish in the trophic transfer mesocosms grazed on live *C. dilutus* that were cultured in the same streams before the fish were added. Feeding barriers were placed in the trophic transfer streams to ensure that the FHM were getting the same dietary exposure of

1g/day based on satiation density following the same methodology as previous studies conducted by our research team (Rickwood et al., 2006a, 2008) and others (Ankley, 2001).

We collected a subsample of larvae to assess larval deformities and whole body metal uptake at two critical time periods where metal exposure to the larvae was anticipated to be the greatest (day 5 and day 10 post-hatch). Breeding tiles were checked on a daily basis for egg production. Eggs laid were gently rolled off the breeding tile and placed into a Petri dish to obtain a photo for enumeration, sizing and determination of fertilization rates using a Cannon Powershot digital camera (Model A620, Mississauga, ON). Eggs were immediately placed into a PVC egg cup with 250 µm nitex screen on the bottom and placed in the appropriate egg-hatching tank in the flow through wet table. Eggs were aerated continuously and after a 2-day period, (once eyes had formed), were re-photographed and re-counted. Once the larvae hatched (3-5 days), 5-20 larvae were collected, stored in 10% buffered formalin and examined for deformities at a later date using a Vista vision^{TM} (Model 48402-00, VWR International, Missisauga, ON) trinocular microscope. Frequency of deformities was recorded based on four main categories (craniofacial, edema, spinal and hemorrhage) using criteria outlined by Holm et al., (2003). A sub-sample of 5 to 10 larvae were placed back into the larval tanks where they were allowed to further develop for a 5-day period (10 days total) and then re-assessed using the same protocol as the day-5 larvae. Remaining larvae were dried, collected and frozen for metals tissue analysis. At the end of the exposure period, fish were anaesthetized using methane tricainesulfonate (MS222, ~1,000 mg/L) following the University of Saskatchewan's animal care protocols. Fork length (mm), whole body weight (g) and secondary sexual characteristics were recorded based on protocols developed by Parrot and Wood (2002). Fish were euthanized by spinal severance and, liver, gills, gonads and carcass weights were recorded, collected and frozen for analysis of metals.

3.1.7 Exposure pathway study - *Chironomus dilutus*

Chironomid culturing was conducted for the factorial feeding component of the current study. Four to five egg sacs were isolated from the University of Saskatchewan culture and placed into a 40 L culturing aquaria (culture chambers) containing either laboratory control water or 45% PWE. Given that the PWE has been found to reduce hatching success in *C. dilutus* (Nebeker et al., 1988; Pascoe et al., 1989; Hruska and Dubé, 2004), the 45% PWE was

introduced to the culture chambers once hatching had occurred. Each culture chamber contained a 1" (2.54 cm) layer of pre-washed silica sand. Treatment water was mixed in two separate, 90 gallon (341 L) polyethylene mixing tanks that were aerated and heated to approximately 23°C ± 2 prior to transferring into the appropriate aquaria. Water in the culture chambers was renewed manually every 48 hours to prevent excessive spikes in ammonia and to maintain a healthy culture. During culturing period (21 days) *C. dilutus* were fed 30 ml of Tetramin™ slurry (100g of Tetramin™ flakes to 1000 mL of control water), 3 times per week. Once larvae reached the 3rd or 4th instar stage, they were aspirated from the top layers of sand, dried and separated into 1 g samples and frozen for use in the full factorial experiment as the laboratory-based artificial (non-living) food source for the FHM.

3.1.8 Food quality study - *Chironomus dilutus*

Chironomid culturing was also conducted for the set-up of the multi-trophic systems. A total of two mesocosm tables (16 streams) were used in the establishment of the multi-trophic cultures. In order to achieve a similar feeding rate (1g/day) as the factorial system, egg sacs were isolated and placed into 16 individual, 11.4 L culturing chambers. Once hatched, the larvae were grown under both treatment conditions (laboratory control water and 45% effluent water) for 7-10 days and then placed into each mesocosm stream. This process was repeated each week for three weeks until the food base was established in each stream. A feeding barrier and breeding tile were then placed in each of the multi-trophic streams prior to the introduction of the FHM to ensure that all emerging insects were retained, counted, and left in the streams to reproduce. The *C. dilutus* were fed 10 ml in the first week, 20 ml in the second week and 30 ml in the third and subsequent weeks with a Tetramin™ slurry blend. One core sample (9cm^2) was obtained from each of the streams on a weekly basis after each new batch of chironomids had been added to the stream in order to calculate densities and to ensure that all streams contained a similar density of larvae 1.48 chironomids/cm^2 based on satiation. At the end of the experiment, 3 core samples were also taken from each stream in order to calculate the final densities in the streams. Samples were preserved with 10% buffered formalin to be counted under a proper fumehood at a later date. At the end of the exposure period, a 1 g sample of *C. dilutus* larvae was collected from each of the streams for metals tissue analysis.

3.2 ANALYSES

3.2.1 Laboratory analysis

All samples were analyzed using standard methods and quality assurance at Testmark Laboratories in Sudbury, ON, Canada. Matrices tested included: water, fish tissues (carcass, gonad, gills, liver), fish larvae and *C. dilutus* tissues. Water samples were collected once per week during the exposure phase from each of the mesocosm reservoirs and from the 100% effluent tank in week two. Samples were collected according to standard sampling protocol and kept at 4 ºC in a cooler on ice and shipped immediately to Testmark Laboratories in Sudbury, ON, Canada where all the analyses were carried out. The water samples were analyzed for various water chemistry parameters (see Tables 3.1 and 3.2) using the American Public Health Association (APHA) and the US EPA solid waste (SW) analytical methods and procedures. Total metals were analyzed in water and tissue samples using Inductively Coupled Plasma - Mass Spectrometry (ICP MS). The tissue samples were block digested prior to analysis, and the concentration of metals were derived on the basis of wet mass. Quality assurance/quality control for the metals analysis were maintained using calibration standards, control standards, aqueous and tissue certified reference standards (85.8% to 116% recovery rate), calibration blanks, method blanks and the analysis of duplicate samples.

3.2.2 In-situ water quality analysis

Daily in-situ water quality measurements were obtained for general chemistry in all of the treatments during both the pre-exposure and exposure phases of the experiments. Conductivity, dissolved oxygen and temperature were measured using a handheld YSI portable meter (Yellow Springs Instrument, Yellow Springs, OH). In addition, ammonia levels (Hannah Instruments, Hungary, Europe), pH (Oakton phTestr30, San Francisco, CA), and hardness (Hatch Test Kit Model 5-EP MG-L, Loveland, CO) were also measured.

3.2.3 Data analysis

At the end of the exposure period, fish metrics, reproductive endpoints and larval endpoints were analyzed. All statistical analyses were performed using SPSS® 17 (SPSS Inc., Chicago, Il, USA) and graphed using Sigmaplot® Version 11 (San Jose, CA, USA). Most of the

data were analyzed using a two-way Analysis of Variance (2-way ANOVA) providing that the data met the assumptions (normal distribution and homogeneity of variance) when analyzed using Shapiro Wilk's and Levene's tests. 2-way ANOVAs were performed for experiment I data in order to try and decipher if there was a significant difference among treatments when fish were exposed through water only, diet only and through both routes of exposure. Whereas, the data from Experiment II was examined to determine whether there was a significant difference among water treatments (control vs effluent), food quality (live vs frozen diet) or both (interaction). Transformation was conducted (Log transformation of continuous or derived data and angular transformation of percentage-based or ratio scaled data) when normality failed and the non-parametric equivalent of the 2-way ANOVA (Shreirer-Ray-Hare extension of the Kruskal-Wallis test) was conducted when assumptions were not met. A two-way ANOVA or its equivalent was conducted on the following endpoints: hatching success, percent deformities, LSI (liver weight(g)/body weight(g)*100), GSI (gonad weight(g)/body weight(g) * 100), condition [(body weight(g)/total length(cm)^3) * 100] metal tissue burdens and water quality. Kolmogorov Smirnov (KS) tests were used to assess cumulative frequency data including: cumulative eggs/female [Cum. # eggs produced per treatment/# of living females/# of days] which factors in the effects of mortality on egg production and represents population effects over time; cumulative total egg production [Cum. total # eggs produced/treatment/day] which measures the distribution of total egg production (when and how much) for each treatment over time; and cumulative spawning events [Cum. total # spawning events/treatment/day]). Two-way ANOVA's or non-parametric equivalent (Kruskal Wallis) tests were conducted on mean egg data. Mean total egg production [total # of eggs produced per breeding group/ # of females in group/ # of exposure days] assessed the total number of all eggs produced in each replicate over a 21 day period; and, mean egg production [mean # eggs produced per stream/ # of females in group/# of exposure days]) assessed the size of the brood produced for each replicate. Differences among treatment groups were further assessed using a Tukey's *post hoc* or non-parametric Mann-Whitney-U test applying the appropriate *Bonferonni* correction (α (0.05)/number of comparisons made) to reduce the Type I error rate. Chi-square tests were used to analyze discrete data (e.g., number of spawning events). Two-way ANOVAs were used to assess gonad, liver and body weight of FHM. When an interaction was observed, graphical interpretation of the data was conducted. Any interactions that could not be deciphered readily

were split and a t-test was performed on the water only exposures or dietary only/diet quality exposures separately to determine where the difference lay. Finally, chironomid densities (number of *C. dilutus*/cm^2) and emergence (number of adults emerged) were assessed using 2-way ANOVAs to determine responses over 21 days of exposure. All results were significant when $p<0.05$.

3.3 RESULTS

3.3.1 Water quality

3.3.1.1 Exposure pathway study (Experiment I)

Treatment Effects: Significant treatment effects were observed in all general water quality parameters and in most metals (Al, Ca, Co, Cu, Li, Mn, Ni, Rb, Se) exposed to EWCB and EWEB treatments in the exposure pathway study compared to control treatments (CWCB, CWEB) (Table 3.1).

Food-Type Effects: Food-type effects were not as prominent in the water quality parameters with significant effects only seen in the CWEB and EWEB treatments for alkalinity, and no significant food-type metal effects (Table 3.1).

Interaction Effects: Waterborne Pb was the only water quality parameter to elicit a significant interaction (Table 3.1). However, closer examination of the raw data showed high variability among treatment groups. Furthermore, half of the data analyzed were below detection limits, which is likely a contributing factor of the spurious results observed. Pb levels in all treatments were well below the Canadian water quality guidelines (CWQG) for the protection of aquatic life and it is not anticipated to contribute appreciably to fish toxicity in the current study (CCME, 2007).

3.3.1.2 Food quality (Experiment II)

Treatment Effects: A significant treatment effect was observed for most of the general water quality parameters with the exception of K, and for most metals with the exception of Al, Pb and Tl in the effluent treatments (EWEB, MT-effluent) when compared to controls (CWCB, MT-control) (Table 3.2).

Food-Type Effects: Significant food-type effects were observed in the general water quality parameters (DOC, TOC, pH) and for several metals (Cd, Co, Pb, Ni) in the multi-trophic

streams compared to the artificially fed streams regardless of treatment water (control/effluent water) indicating an increase in DOC, TOC, pH and metals when insects were alive (Table 3.2).

Interaction Effects: Significant interactions occurred for Nitrate (NO_3), Al, and Ni (Table 3.2). However, Ni was the only interaction of concern in these three parameters since it increased approximately 36-fold (3,478%) in the effluent treated streams regardless of food quality (live or frozen benthic organisms) when compared to the controls. Although there was no effect on the presence of the control benthic organisms (live vs frozen), there was a small effect in the effluent treated streams, which resulted in a 14% increase in Ni when live benthic organisms were present compared to the frozen *C. dilutus*.

3.3.2 Metal tissue burdens

3.3.2.1 Exposure pathway study (Experiment I)

Treatment Effects: There were no significant effects among treatment types (effluent or reference water) observed in FHM tissues in the exposure pathway study ($p>0.05$).

Food-Type Effects: Food-type effects were observed for Rb in carcass ($p=0.029$) and gonad ($p<0.001$) tissues in fish exposed to effluent-raised benthic organisms (effluent benthic organisms). An increase of about 2-fold was observed in both carcass and gonads compared to fish exposed to control-raised benthic organisms (Figure 3.3).

Interaction Effects: A significant interaction occurred for Rb ($p=0.005$) in liver tissues of the FHM. Closer examination of the interaction graphs suggested that regardless of treatment type (control water or effluent water), Rb appeared to accumulate in FHM liver in a similar fashion when exposed to effluent benthic organisms (CWEB, EWEB). Conversely, when FHM were exposed to control benthic organisms, there was substantially more accumulation (~2-fold increase) of Rb in the effluent exposed fish (EWCB) compared to control fish (CWCB). These results also suggest that greater accumulation of Rb occurred when FHM were exposed only through waterborne routes of exposure (EWCB) and less accumulation occurred when exposed through diet (CWEB) or both routes of exposure (EWEB).

Table 3.1 Summary of mean (±SEM, n=3) water quality parameters measured in the exposure pathway study (factorial design).Significant treatment effect (**a)**, dietary effect (**b)** and, interaction (**c)** observed when p<0.05. Where C=control, E=effluent, W=water, and B=benthic organisms (data analyzed using a Two-Way ANOVA or Scheirer Ray Hare extension of the Kruskal Wallis).

Parameter	Units	Canadian Water Quality Guidelines (Aquatic Life)	Full Factorial CWCB	CWEB	EWCB	EWEB
General WQ						
Ammonia	mg/l	-	0.06 ± 0.01	0.11 ± 0.01	0.27 ± 0.11 **a**	0.18 ± 0.04 **a**
DOC	mg/l	-	4.65 ± 0.43	4.06 ± 0.58	3.15 ± 0.55 **a**	3.0 ± 0.42 **a**
TOC	mg/l	-	5.06 ± 0.28	5.44 ± 0.26	4.09 ± 0.26 **a**	3.90 ± 0.12 **a**
pH	-	6.5-9	7.11 ± 0.05	7.09 ± 0.01	6.82 ± 0.11 **a**	6.81 ± 0.13 **a**
Alkalinity	mg/l	-	44.7 ± 0.35	45.3 ± 0.55 **b**	23.4 ± 1.27 **a**	22.2 ± 1.79 **ab**
Chloride	mg/l	-	2.75 ± 0.06	2.72 ± 0.08	47.13 ± 1.84 **a**	45.03 ± 2.33 **a**
Conductivity	mg/l	-	156 ± 4.1	157 ± 4.4	1307 ± 140 **a**	1257 ± 95 **a**
Nitrate	mg/l	-	0.19 ± 0.02	0.21 ± 0.01	1.44 ± 0.19 **a**	1.47 ± 0.24 **a**
Hardness	mg/l	-	48.5 ± 1.81	46.9 ± 0.57	458.7 ± 61.82 **a**	437 ± 44.98 **a**
Calcium	mg/l	-	10.1 ± 0.95	9.4 ± 0.64	149.3 ± 23.51 **a**	142.7 ± 16.83 **a**
Potassium	mg/l	-	1.23 ± 0.04	1.23 ± 0.04	16.73 ± 2.25 **a**	16.5 ± 1.80 **a**
Magnesium	mg/l	-	5.66 ± 0.34	5.71 ± 0.30	20.73 ± 2.27 **a**	19.7 ± 2.12 **a**
Sodium	mg/l	-	8.99 ± 0.49	9.07 ± 0.55	79.20 ± 7.79 **a**	46.50 ± 20.04 **a**
Total Phosphorus (as P)	mg/l	-	0.018±0.01	0.016±0.00	0.012±0.00	0.013±0.00
Sulfate	mg/l	-	23 ± 0.38	23 ± 0.46	608 ± 115.40 **a**	584 ± 66.81 **a**
Metals						
Aluminum	µg/l	5-100	20.63 ± 4.75	18.0 ± 1.61	4.63 ± 2.07 **a**	5.2 ± 1.96 **a**
Cadmium	µg/l	-	0.05 ± 0.00	0.05 ± 0.00	0.08 ± 0.03	0.11 ± 0.03
Cobalt	µg/l	-	0.05 ± 0.00	0.05 ± 0.00	2.85 ± 0.32 **a**	2.03 ± 0.91 **a**
Copper	µg/l	2-4	4.13 ± 0.12	3.9 ± 0.40	53.53 ± 24.27 **a**	70.1 ± 11.07 **a**
Lead	µg/l	1-7	1.47 ± 0.19 **c**	0.5 ± 0.00 **c**	0.5 ± 0.00 **c**	2.53 ± 1.28 **c**
Lithium	µg/l	-	2.5 ± 0.00	2.5 ± 0.00	12.8 ± 5.20 **a**	18.0 ± 0.58 **a**
Magnesium	µg/l	-	5.7x10^3± 120	5.8x10^3± 279	1.4x10^5 ± 6.2 x10^3	2.04x10^5 ± 1.9 x10^3
Manganese	µg/l	-	0.5 ± 0.00	0.5 ± 0.00	6.77 ± 3.31 **a**	9.63 ± 1.92 **a**
Nickel	µg/l	25-150	1.87 ± 0.33	1.57 ± 0.29	54.9 ± 23.45 **a**	74.2 ± 1.81 **a**
Rubidium	µg/l	-	0.5 ± 0.00	0.5 ± 0.00	17.3 ± 7.93 **a**	22.63 ± 3.76 **a**
Selenium	µg/l	1.0	0.5 ± 0.00	0.5 ± 0.00	6.07 ± 2.87 **a**	8.0 ± 1.59 **a**
Strontium	µg/l	-	68 ± 2.60	69 ± 3.89	254 ± 113.59	341 ± 41.68
Thallium	µg/l	0.8	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.09 ± 0.02

Table 3.2 Summary of mean (± SEM, n=3) water quality parameters measured in the food quality study. Significant treatment effect (**a)**, dietary effect (**b)** and, interaction (**c)** observed when p<0.05. Where C=control, E=effluent, W=water, and B=benthic organisms (data analyzed using a Two-Way ANOVA or Scheirer Ray Hare extension of the Kruskal Wallis).

Parameter	Units	Canadian Water Quality Guidelines (Aquatic Life)	Full Factorial		Multi- Trophic	
			CWCB	EWEB	Reference	Effluent
General WQ						
Ammonia	mg/l	-	0.06 ± 0.01	0.18 ± 0.04 **a**	0.17 ± 0.04	0.36 ± 0.07 **a**
DOC	mg/l	-	4.65 ± 0.43	3.0 ± 0.42 **a**	5.85 ± 0.35 **b**	4.95 ± 0.54 **ab**
TOC	mg/l	-	5.06 ± 0.28	3.90 ± 0.12 **a**	7.47 ± 0.81 **b**	6.21 ± 0.43 **ab**
pH	-	6.5-9	7.11 ± 0.05	6.81 ± 0.13 **a**	6.99 ± 0.01 **b**	6.65 ± 0.12 **ab**
Alkalinity	mg/l	-	44.7 ± 0.35	22.2 ± 1.79 **a**	42.7 ± 2.35	22.4 ± 3.27 **a**
Chloride	mg/l	-	2.8 ± 0.06	45.0 ± 2.33 **a**	3.5 ± 0.25	48.7 ± 2.26 **a**
Conductivity	mg/l	-	156 ± 4	1.258x10^3 ± 95 **a**	168 ± 4	1.32x10^3 ± 142 **a**
Nitrate	mg/l	-	0.19 ± 0.02 **c**	1.47 ± 0.24 **ac**	0.66 ± 0.11 **c**	0.70 ± 0.29 **ac**
Hardness	mg/l	-	48 ± 1.81	437 ± 44.98 **a**	48 ± 0.55	467 ± 54.11 **a**
Calcium	mg/l	-	10.1 ± 0.95	142.7 ± 16.83 **a**	9.8 ± 0.71	153.3 ± 20.85 **a**
Potassium	mg/l	-	1.2 ± 0.04	16.5 ± 1.80	1.5 ± 0.07	12.3 ± 6.34
Magnesium	mg/l	-	5.66 ± 0.34	19.70 ± 2.12 **a**	5.63 ± 0.30	20.50 ± 1.95 **a**
Sodium	mg/l	-	8.99 ± 0.49	46.50 ± 20.04 **a**	10.12 ± 0.09	47.18 ± 23.76 **a**
Total Phosphorus (as P)	mg/l	-	0.018±0.01	0.012±0.00	0.121±0.07	0.092±0.02
Sulfate	mg/l	-	22.8 ± 0.38	583 ± 66.81 **a**	23.8 ± 0.55	602 ± 51.94 **a**
Metals						
Aluminum	µg/l	5-100	20.63 ± 4.75 **c**	5.20 ± 1.96 **c**	6.67 ± 3.11 **c**	30.33 ± 18.80 **c**
Cadmium	µg/l	-	0.05 ± 0.00	0.11 ± 0.03 **a**	0.07 ± 0.03 **b**	0.30 ± 0.04 **ab**
Cobalt	µg/l	-	0.05 ± 0.00	2.03 ± 0.91 **a**	0.12 ± 0.07 **b**	3.24 ± 0.53 **ab**
Copper	µg/l	2-4	4.1 ± 0.12	70.1 ± 11.07 **a**	2.1 ± 0.07	53.5 ± 5.19 **a**
Lead	µg/l	1-7	1.47 ± 0.19	2.53 ± 1.28	0.5 ± 0.00 **b**	0.5 ± 0.00 **b**
Lithium	µg/l	-	2.5 ± 0.00	18.0 ± 0.58 **a**	2.5 ± 0.00	17.3 ± 1.20 **a**
Magnesium	µg/l	-	5.7x10^3 ± 120	2.0x10^5 ± 1.9 x10^3 **a**	5.6x10^5 ± 55	2.0x10^5 ± 1.4x10^3 **a**
Manganese	µg/l	-	0.5 ± 0.00	9.63 ± 1.92 **a**	1.93 ± 0.46	14.1 ± 4.52 **a**
Nickel	µg/l	25-150	1.87 ± 0.33 **c**	74.20 ± 1.81 **ac**	2.57 ± 1.78 **bc**	84.67 ± 3.07 **abc**
Rubidium	µg/l	-	0.5 ± 0.00	22.6 ± 3.76 **a**	0.5 ± 0.00	23.7 ± 3.74 **a**
Selenium	µg/l	1.0	0.5 ± 0.00	8.0 ± 1.59 **a**	0.5 ± 0.00	8.13 ± 1.05 **a**
Strontium	µg/l	-	67.7 ± 2.60	341 ± 41.68 **a**	75.3 ± 3.71	352 ± 49.41 **a**
Thallium	µg/l	0.8	0.05 ± 0.00	0.09 ± 0.02	0.05 ± .00	0.08 ± 0.03

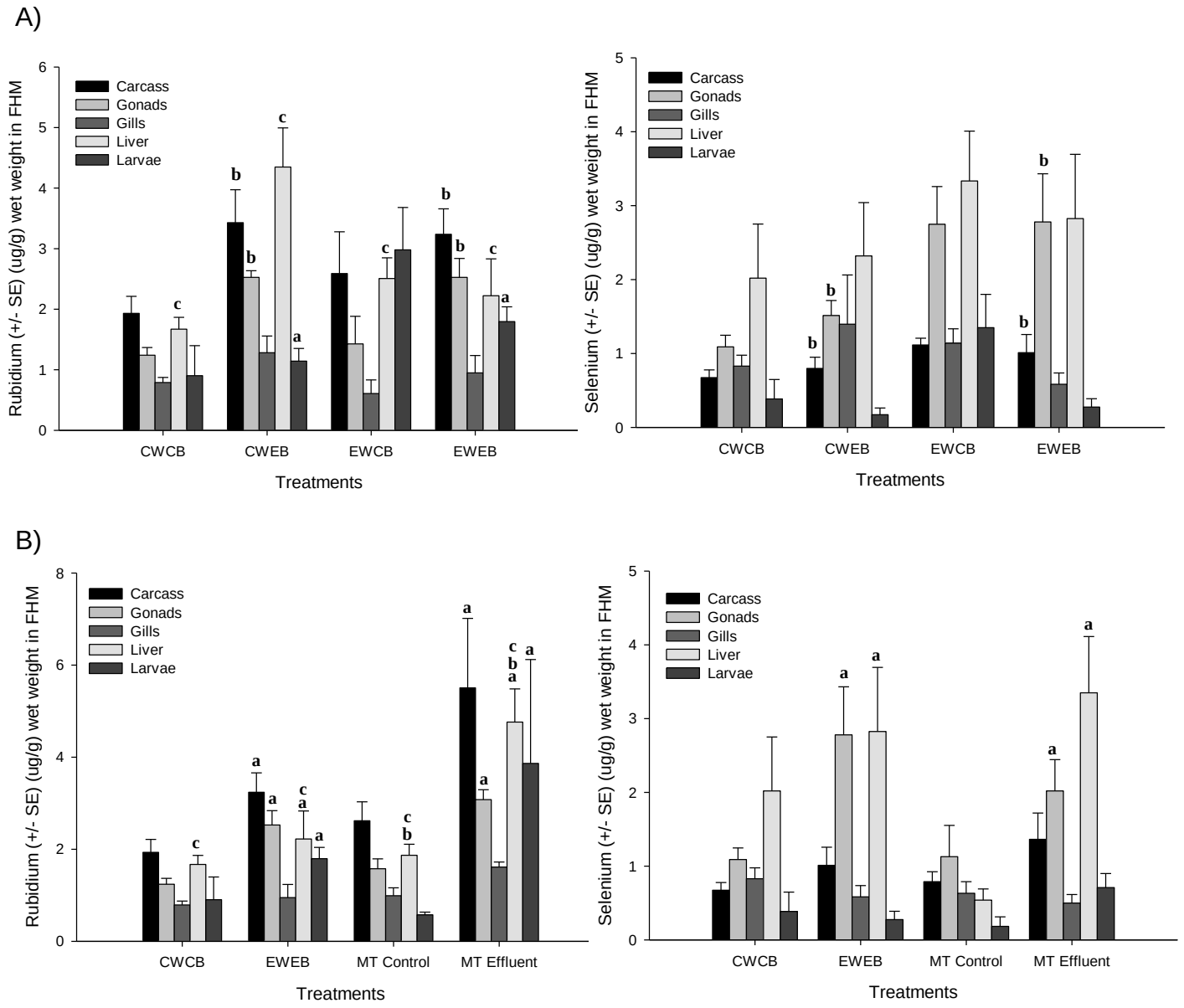


Figure 3.3 Selected metal concentrations [Rb, Se] in tissues analyzed in fathead minnow (*P.promelas*) [carcass, ovaries, liver, gills, larvae] (mean ± standard error, n=5) after exposure to 45% PWE in the A) exposure pathway and B) food quality experiments over 21-days of exposure. Letters denote a significant increase in metal concentrations compared to reference when $p<0.05$. Where, a=significant treatment effect, b=significant dietary effect and c=significant interaction and where C=control, E=effluent, W=water, B=benthic organisms and MT=multi-trophic (data analyzed using a Two-Way ANOVA or Scheirer Ray Hare extension of the Kruskal Wallis).

3.3.2.2 Food quality study (Experiment II)

Treatment Effects: Significant treatment effects were observed for Tl (p=0.010) and Rb (p=0.002) in FHM carcass tissues when exposed to effluent water regardless of food quality (artificial or live diet of *C. dilutus*) (Se and Rb results shown in Figure 3.3). Thallium levels increased 3-fold and Rb levels increased 2-fold. Similarly, Se (p=0.002) and Rb (p<0.001) both significantly increased (2-fold) in the gonads (ovaries) of female FHM exposed to effluent water compared to fish exposed to control water (Se and Rb results shown in Figure 3.3). Rb (p=0.004), Sr (p=0.022), Se (p=0.002), and Cu (p=0.029) all significantly increased in FHM livers (2 to 4-fold) in the fish exposed to the effluent water compared to control regardless of food quality (Se and Rb results shown in Figure 3.3, all other data shown in Appendix B).

Food-Type Effects: Rb increased significantly (p=0.015) in the livers (2-fold) when fish were fed a live diet of *C. dilutus* compared to when fish were fed a frozen diet (Rb results shown in Figure 3.3).

Interaction Effects: A significant interaction was observed in the liver of FHM for Rb (p=0.032) (Se and Rb results shown in Figure 3.3). Interaction graphs showed that Rb increased in liver tissues by 3-fold (~155%) in fish fed a live diet and by about 33% in fish fed an effluent treated frozen diet relative to the controls. A significant interaction for Cd (p=0.049) was also observed in carcass tissues of FHM. Closer examination of the interaction graphs showed that there were no changes in Cd burdens in the fish fed an artificial diet regardless of the treatment type (effluent/control), however there was a 3-fold reduction of Cd in the multi-trophic system in the effluent treatment compared to control which resulted in the interaction observed.

3.3.3 *Chironomus dilutus* tissue burdens

The data presented in Figure 3.4 shows the concentration of a select number of metals in the chironomid tissues. Although statistical analysis could not be conducted since samples were pooled for each treatment, it still provides an indication of the tissue concentrations in the chironomids. The data appears to coincide with the FHM tissue metal uptake in that we see increased metal concentrations (Co, Ni, Rb, Se) in the effluent exposed chironomids compared to

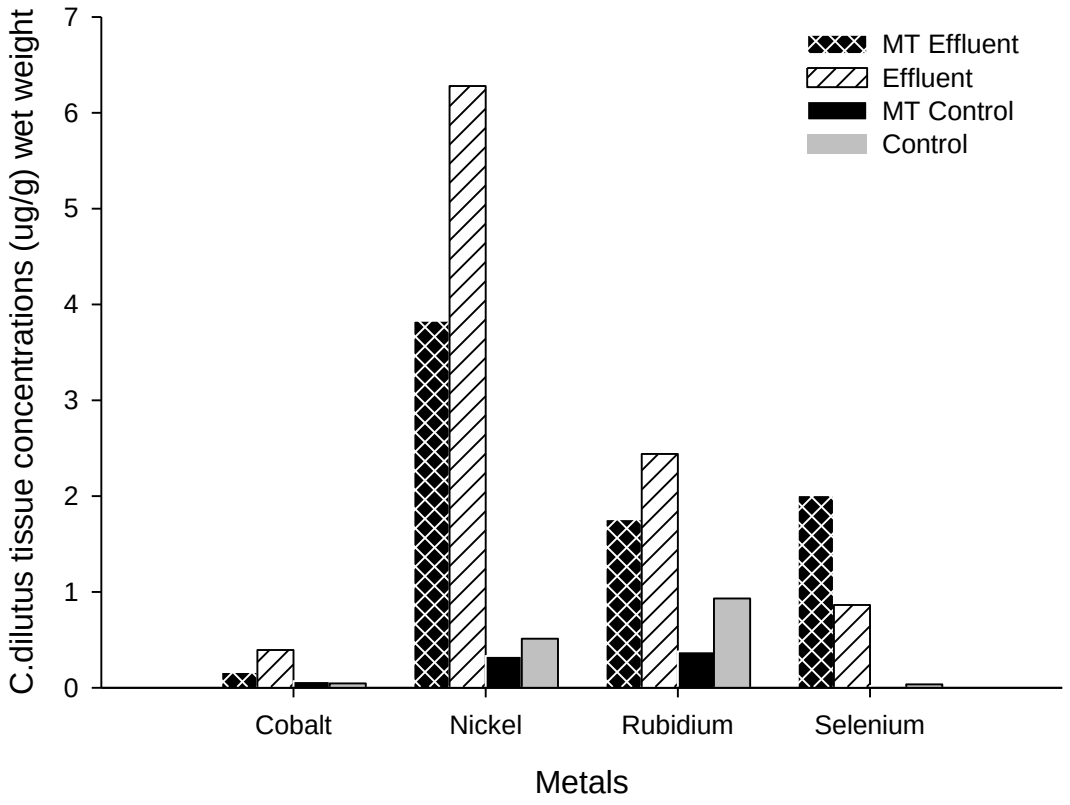


Figure 3.4 Selected metal concentrations [Co, Ni, Rb, Se] in tissues analyzed in freshwater midge (*C. dilutus*) exposed to control water [MT Control, CWCB] and 45% PWE (Process Water Effluent) [MT Effluent, EWEB] over a 21-day period. Where C=control, E=effluent, W=water, B=benthic organisms and MT=multi-trophic.

the control exposed chrionomids in both systems. This indicates the importance of trophic transfer and dietary exposure of metals to fish.

3.3.4 Morphological endpoints

Treatment Effects: There was a significant treatment effect for female condition factor, liver somatic index (LSI), egg size and body weight in both studies (exposure pathway (Exp 1) and food quality (Exp 2)) ($p<0.05$). Interestingly, in both systems, condition factor and body weight showed about a 30% increase when exposed to effluent water whereas LSI and egg size showed about a 35% and 6% decrease respectively when exposed to effluent water. No significant differences were observed in male FHM in any of the same morphological endpoints measured (see Appendix A).

Food-Type Effects: In the exposure pathway system, significant food-type effects were observed for LSI ($p<0.001$), which showed a decrease of about 41% in FHM fed effluent benthic organisms compared to those fed control benthic organisms.

A significant food-type effect was also observed for female condition factor and body weight in the food quality system (Experiment 2) ($p<0.001$ for both). Condition factor increased about 39% and body weight increased 49% in the multi-trophic streams compared to the artificially fed streams. No significant differences were observed in similar endpoints measured for male FHM (see Appendix A).

Interaction Effects: A significant interaction in females was seen for condition factor ($p=0.005$) and body weight ($p=0.004$) in the exposure pathway system (Experiment 1). Graphical representation of the data showed condition was similar when FHM were fed effluent benthic organisms regardless of the treatment type (effluent vs control). However, when FHM were fed control benthic organisms, condition was much greater in the effluent treatments than in the controls. These findings were also observed with body weight (Appendix A).

A significant interaction was also seen for female LSI in the food quality system (Experiment 2) ($p=0.020$). Interaction graphs depicted LSI's to be similar in the multi-trophic streams regardless of treatment type (control vs effluent). In contrast LSI's decreased when FHM were fed the frozen diet of effluent insects. No significant interactions were observed for male FHM in either system for any of the morphological endpoints measured (see Appendix A).

3.3.5 Reproductive endpoints

3.3.5.1 Cumulative egg production and spawning events

In the exposure pathway system (Experiment 1), there was a significant increase in cumulative total egg production ($p<0.001$) and cumulative egg production ($p=0.006$) in CWEB compared to CWCB (Figure 3.5A). Conversely, there was a significant decrease in cumulative spawning events in both EWCB and EWEB ($p<0.001$ respectively) (see Appendix A).

In the food quality system (Experiment 2), there was a significant increase in cumulative total egg production ($p=0.017$), cumulative spawning events ($p<0.001$) and cumulative egg production ($p=0.017$) in the MT control treatment when FHM were fed a live diet compared to the artificially fed CWCB treatment (Figure 3.5B). However, there was a significant decrease in cumulative total egg production, spawning events and egg production ($p<0.001$ for all) in MT effluent compared to CWCB (Figure 3.5B). There was also a significant decrease in cumulative egg production ($p=0.017$) and cumulative spawning ($p<0.001$) in EWEB when compared to CWCB but no significant difference in cumulative total egg production ($p=0.194$) (see Appendix A).

3.3.5.2 Mean egg production

Treatment Effects: Mean total egg production (total eggs produced/treatment/exposure period) was the only endpoint that showed a significant treatment effect in the food quality system (Experiment 2) ($p=0.020$). There was a 2.6-fold decrease in mean total egg production in the effluent treatments compared to the control treatments. However, there were no significant treatment effects in the exposure pathway system and, there were no significant food-type effects in either system.

Interaction Effects: A significant interaction occurred for mean egg production [(total eggs produced per replicate/number of broods)/number of females in a breeding group/ number of exposure days)] ($p=0.042$), and mean total egg production ($p=0.015$) in the full factorial system (Experiment 1) indicating that diet and water were not acting independently in this system. Further examination of the interaction graphs showed that overall there was greater mean egg production in FHM held in control water compared to effluent water. However we also saw elevated mean egg production when FHM were fed effluent benthic organisms regardless of the treatment type (control vs effluent). Mean total egg production was similar in

FHM when fed control benthic organisms regardless of treatment water (control vs effluent). However, there was much greater mean total egg production when FHM were fed effluent benthic organisms and held in control water (CWEB) than when held in effluent water (EBEW) resulting in the interaction observed.

3.3.6 Larval endpoints

Treatment Effects: In the exposure pathway experiment (Experiment 1) there was a significant hastening in the number of days to hatch ($p=0.001$). However, an increase in the number of larvae to successfully hatch ($p=0.005$) was observed when larvae were exposed to the effluent water. Results showed a 2-fold delay in days to hatch and about a 25% increase in hatching success when compared to larvae in the control treatments. Metal tissue burdens 5 days post-hatch also increased for Rb (2-fold), B (7-fold), Cu (3-fold) and Ni (2-fold) in the larvae exposed to effluent compared to control (see Figure 3.3 for Se and Rb results).

There was a significant treatment effect on days to hatch in the food quality experiment (Experiment 2) ($p=0.009$), which showed that larvae hatched 22.7% quicker in the control water than the effluent water. Cu and Rb both increased by about 2-fold and were the only metals that were elevated in larvae tissues exposed to effluent treatment water in the food quality (see Figure 3.3 for Rb results). There were no significant food-type effects or interactions seen in the larvae endpoints for either system.

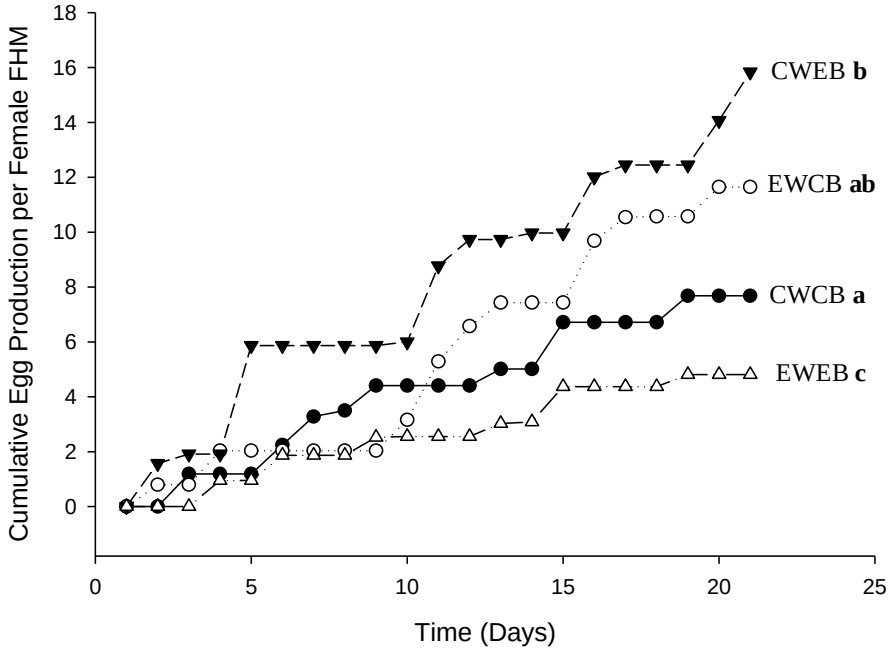
3.3.6.1 Larval deformities

The frequency of deformities was normalized based on the brood size and analysis revealed that there were no significant effects observed for either system 5 and 10 days post hatch (Figure 3.6). Although there was a slight increase in the percentage of edema and spinal malformations in the food quality system at day 5, and a slight increase in the percentage of edema, hemorrhage and spinal deformities in the exposure pathway system at day 10, statistical analysis failed to show significant treatment, food or interactive effects (Figure 3.6).

3.3.6.2 *Chironomus dilutus* densities and emergence

Statistically significant differences among *C. dilutus* densities were not observed among treatment water nor were any food-related effects observed in either of the systems (Experiment 1 or 2) (Figure 3.7). However, a significant treatment effect ($p=0.007$), food-type effect ($p=0.014$) and significant interaction ($p=0.001$) were observed in the emergence data (Figure 3.7). Emergence in the MT-effluent was significantly reduced due to treatment, food and interactive effects compared to the MT-control streams (Figure 3.7). Conflicting density and emergence results may be partially attributed to the high degree of variance around the mean for the density data and the inability to statistically show a difference. However, it was apparent that densities were inhibited in the effluent exposed streams.

A)



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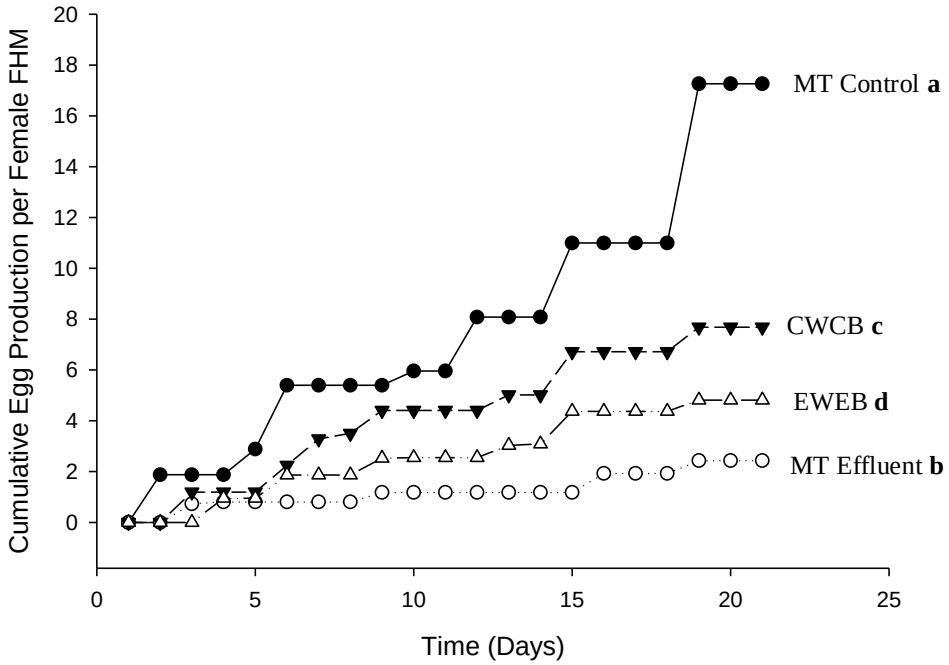


Figure 3.5 Cumulative egg production per female fathead minnow (*P.promelas*) per day in the (A) exposure pathway study and, (B) food quality study during a 21-day exposure period. Letters denote a significant difference from CWCB (control water control benthic organisms) when α=0.05, n=5. Where C=control, E=effluent, W=water, B=benthic organisms and MT=multi-trophic (data analyzed using Kolmorov Smirnov).

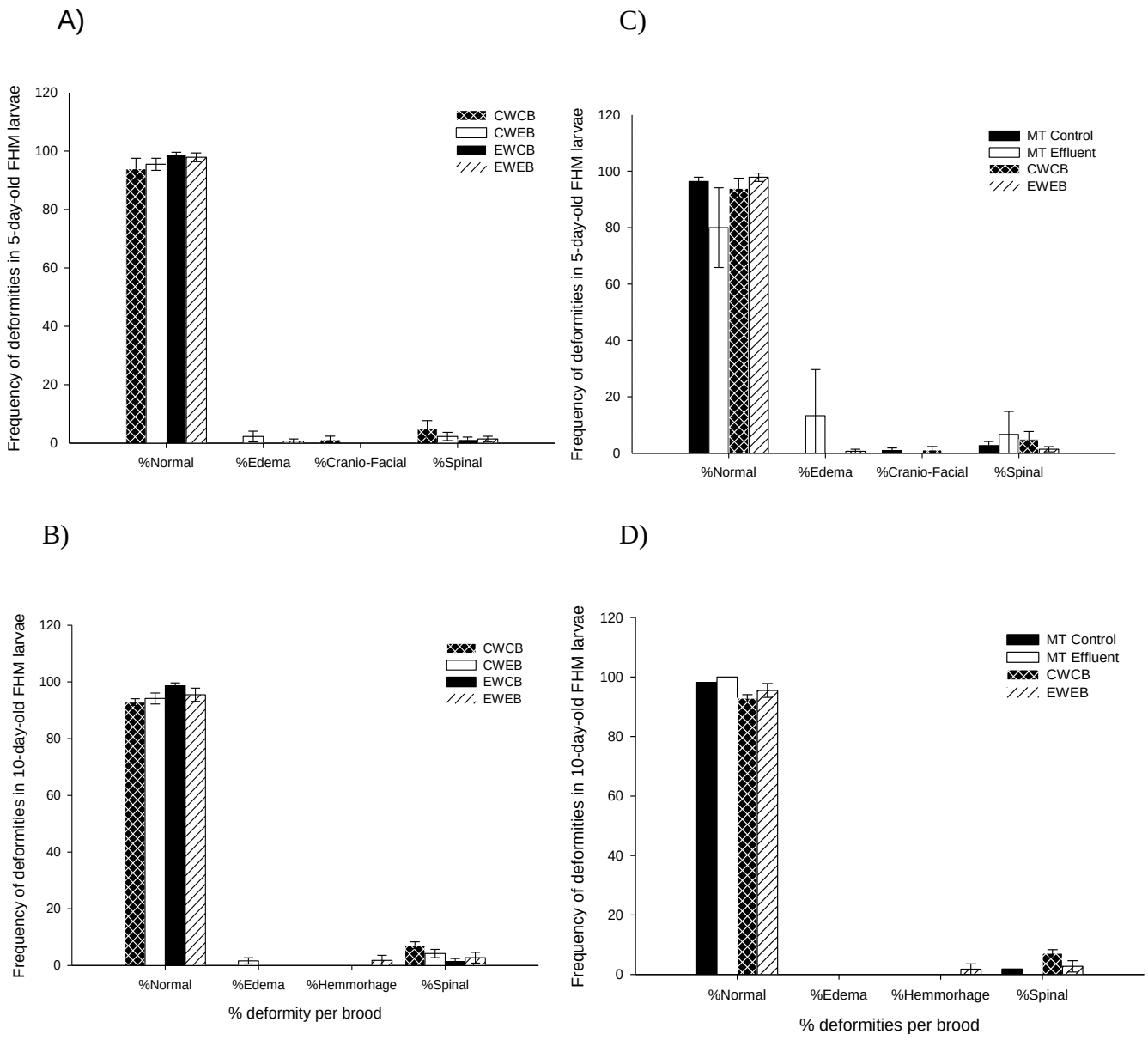
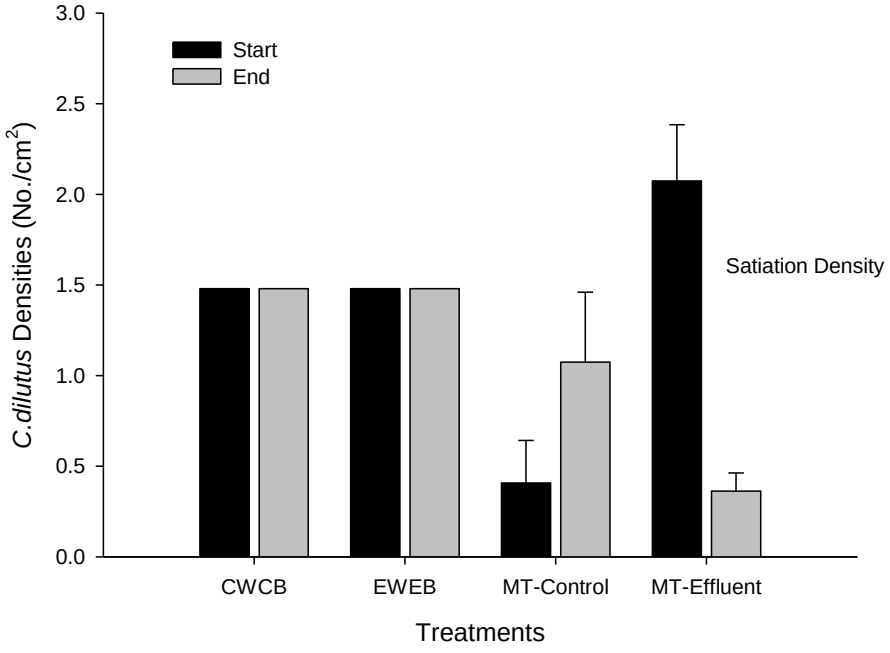


Figure 3.6 Frequency of fathead minnow larval deformities from birth to 5 and 10 days post-hatch in the exposure pathway study (A, B) and food quality study (C, D). Where C=control, E=effluent, W=water, B=benthic organisms and MT=multi-trophic (data analyzed using a Two-Way ANOVA or Scheirer Ray Hare extension of the Kruskal Wallis).

A)



B)

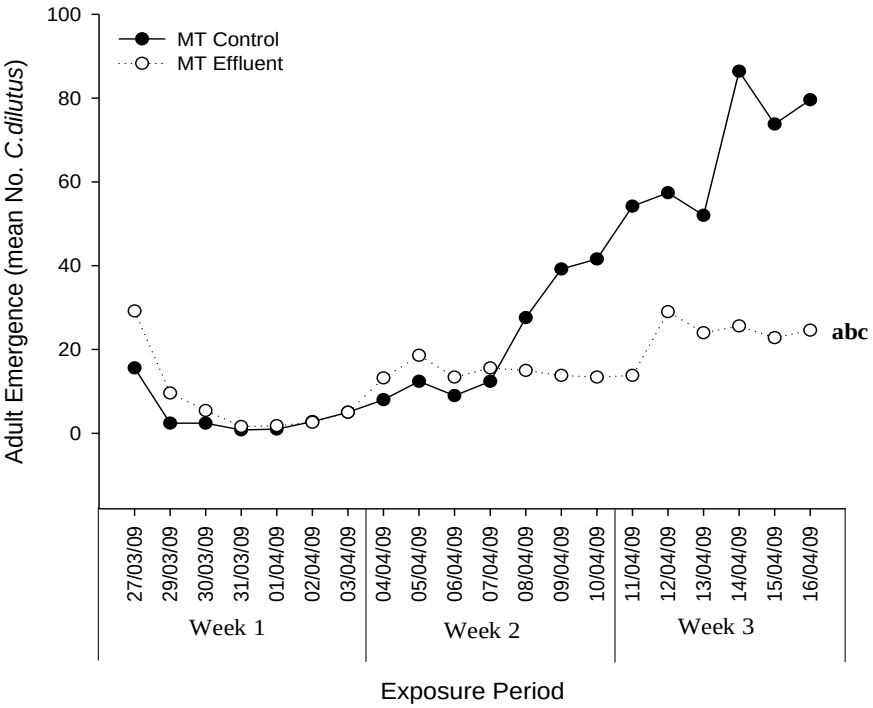


Figure 3.7 *C. dilutus* densities (A) and adult emergence (B) measured throughout the 21-day exposure period in the multi-trophic streams. Letters denote a significant treatment effect (a), significant food effect (b), and significant interaction (c), where C=control, E=effluent, W=water, B=benthic organisms and MT=multi-trophic (data analyzed using a Two-Way ANOVA).

3.4 DISCUSSION

3.4.1 Water chemistry

Dissolved organic carbon (DOC), total organic carbon (TOC), Cd, Co, and Ni all were significantly elevated whereas Pb and pH were significantly decreased in the multi-trophic effluent streams in the food quality system compared to control streams. Alkalinity however, was the only water quality parameter in the effluent factorial streams (exposure pathway system) to exhibit significantly reduced values compared to control streams. Water chemistry parameters such as dissolved and suspended organic carbon, pH and alkalinity are important modifiers of metal bioavailability and toxicity to aquatic organisms (Paquin et al., 2002; Niyogi and Wood, 2004). Furthermore, elevated TOC and DOC levels in the environment have been associated with reduced uptake of various divalent metals (e.g., Cu, Pb, Cd) in aquatic organisms due to complexation (Sprague, 1985; Winter et al., 2005; Chakraborty et al., 2006), and has probably played a prominent role in influencing metal bioavailability in our multi-trophic streams. Multi-trophic streams were administered a blend of Tetramin™ slurry as sustenance for the chironomids. Tetramin™ slurry was formulated from a blend of Tetramin™ flakes containing a mixture of both plant and fish-based materials and water. It is possible that excess Tetramin™ may have contributed to an increase in the dissolved and organic carbon content in the multi-trophic streams. Furthermore, the Tetramin™ may have provided a source of algae or bacteria, which may have enhanced carbon levels in the multi-trophic streams.

Alkalinity levels were lower (2-fold) in the factorial 45% PWE streams compared to the control streams likely due to acidic nature of the raw effluent, though it is unlikely that it contributed to any physiological and/or reproductive effects observed in fish.

3.4.2 Metal tissue burdens

Metal burdens in FHM were conducted solely on the females in the current study so that a greater diversity of tissue samples could be analyzed. It was also assumed that the females would have a greater ability to affect the F1 generation and pass any potential contaminants to the offspring via maternal transfer. In addition, previous work conducted on resident FHM at the studied site (Rickwood et al., 2006a, 2008; Weber et al., 2008) suggested that female fish accumulated greater metal burdens and suffered significantly greater biochemical effects than males.

Rb was the only element analyzed in the chironomid and fish tissues that consistently assimilated in tissues when exposed to the effluent both through waterborne and dietborne exposure. Rb is an abundant alkali earth metal found in the earth's crust (Anke & Angelow, 1995). Despite its abundance in the environment, it remains an element that is rarely studied. However, there is increasing evidence to suggest that it biomagnifies in the food chain and that it could cause reproductive impairment in fish (Campbell et al., 2005; Yamaguchi et al., 2007). Biomagnification studies using stable nitrogen isotopes (^{15}N) conducted using invertebrates, freshwater fish, seabirds and ringed seals showed that Rb biomagnified through the food web in diverse ecosystems in a similar fashion to methylmercury (Campbell et al., 2005). Although it was not possible to assess the biomagnification potential of Rb in our study, we did observe significant increases of Rb in water, chironomid tissue, and FHM carcass, liver and gonad tissues ($p<0.05$) in the effluent treatments compared to controls. Similar findings have also been recorded in our previous field and lab studies (Rickwood et al., 2006a, 2008; Weber et al., 2008). Furthermore, Rb was also one of the only metals of significance found in the F1-generation (larvae) tissues in our current study. More recent studies have also shown Rb has the potential to inhibit spermatogenesis in fish (Yamaguchi et al., 2007). Due to the fact that there is very little aquatic toxicological data available for Rb in the literature, it is difficult to hypothesize safe levels of exposure of Rb to FHM. Consequently, we cannot discount Rb as a potential metal of concern in mining effluents. In addition, histopathological analyses of male testes in our previous lab and field studies have shown significant increased cell death (necrosis and apoptosis) in gonads, increased fibrosis, reduced sperm production, reduced spermatogonia and spermatocytes in males when exposed to PWE effluent water (Rickwood et al., 2006a; Weber et al., 2008). At present, we cannot speculate whether Rb is linked to the cause of the reproductive effects seen in the current study. However, due to the biomagnification potential, its recent link to male reproductive toxicity and its prevalence in all of our previous studies at the study site and other mine sites, it remains a metal of concern, warranting further investigation.

Cd accumulated significantly in the liver (1.18 ± 0.79 µg/g wet wt. or ~4.54 µg/g dry wt. based on 74.01% moisture [Muscatello et al., 2006]) of FHM exposed to waterborne effluent in the full factorial system. Previous field studies conducted by our lab have also confirmed that Cd increases significantly in the bodies of resident fish at the study site (Weber et al., 2008). In a comparable study, increased concentrations were observed in the liver of rainbow trout exposed

to 2.2 µg/l waterborne Cd (Farag et al., 1994). No significant increases in FHM tissue burdens occurred when exposed to Cd through the diet in either system. Despite these findings, Cd exposure through the diet has been identified as a route of exposure, which could result in reproductive impairment to aquatic organisms (Farag et al., 1994). Studies using marine copepods have shown that Cd exposure through the diet significantly affected reproduction through the alteration of vitellogenesis and decreased hatching due to lowered protein levels in the egg at concentrations of 0.562 µg/g dry wt. (2.16 µg/g wet wt. based on 74.01% moisture) Cd with no appreciable effects seen with waterborne exposure (Fisher and Hook, 2002). Though we did not observe any significant increases in dietary Cd levels in FHM, the levels of exposure are well within levels known to elicit reproductive toxicity. Moreover, levels of Cd observed in the PWE were approximately 91% greater than the safe level established by the CWQG for the protection of aquatic life (CCME, 2007). Though we cannot speculate as to the significance of Cd on the reproductive effects we have seen in FHM, its known persistence in the environment (1/2 life >30years) and its relative toxicity to fish, identify it as a constituent in the PWE worthy of further investigation.

Similar to our previous findings (Rickwood et al, 2006b, 2008), selenium (Se) was increased in the gonads in effluent exposed FHM. Selenium effects (ovarian cell necrosis, reproductive failure, ruptured egg follicles) on fish reproduction and developmental abnormalities in larvae have been well documented in the literature (Lemly, 2002; Hamilton, 2004; Muscatello et al., 2008). The currently proposed threshold for ovarian Se has been identified at >10µg/g dry wt. by the US EPA (Lemly, 2002). Ovarian Se concentrations in our study averaged approximately 2.51 µg/g wet wt. [9.66 µg/g dry wt. based on 74.01% moisture content (Muscatello et al., 2008)], in both exposure systems. Based on the current threshold levels, the concentrations recorded in our current study were quite close to the levels expected to elicit toxic response in FHM. However, this was not reflected in our larval investigation, which showed no significant differences in deformities among treatments over two critical time periods (day 5 &10) post-hatch. The majority of the larvae in all of the treatments were normal (>90%), followed by the appearance of spinal deformities at 7% with edema and hemorrhage at 2% respectively. In order to enable the investigation of all endpoints of interest, only a sub-set of larvae were analyzed for deformities since they were collected at two time periods (day 5 & 10), and the majority of larvae were required for metals analysis. Furthermore, the deformities that

were observed did not appear to be associated with selenium exposure. Skeletal deformities are more indicative of Se exposure through maternal transfer rather than other types of deformities (edema, hemorrhage, spinal), which were observed in our study (Lemly, 2002; Hamiliton, 2004). From Se speciation studies conducted by our research team, it is known that the selenium present in PWE is predominantly in the form of selenate, a less toxic and less bioavailable form of Se. Nevertheless, based on our findings it can be suggested that selenium is also a metalloid of concern with regards to the reproductive effects observed in fish exposed to the PWE.

The current study was the first in our lab to analyze metal tissue burdens in the FHM larvae (F1-generation). Early life stages (egg, yolk-sac larvae, swim-up) are the most sensitive to developmental effects of metal exposure (DiGiulio and Tillitt, 1997). Metal uptake, accumulation and deposition during early life stages has been linked to the direct exposure of the eggs and larvae to toxins through maternal transfer as well as waterborne exposure after the eggs have been laid and/or after the larvae have hatched (DiGiulio and Tillitt, 1997). Additionally, as the yolk-sac is consumed and the larvae begin to start feeding on their own (swim-up stage), metals can be further mobilized into the tissues from the yolk-sac, increasing the metal exposure to the larvae (DiGiulio and Tillitt, 1997). Studies have identified that the yolk-sac may be fully consumed in FHM anywhere from 2-9 days post hatch (USEPA, 1996; Crane et al., 2004; DiGiulio and Hinton, 2008). Analysis showed that both Cu and Rb significantly increased in the larvae tissues through one or more exposure pathway (food/water) in both of the systems (exposure pathway & food quality). Copper increased 29-78% and Rb increased 26-43% in larvae tissues when exposed through waterborne routes of exposure. This suggests that metal uptake and accumulation in the larvae was predominantly dermally and bronchially mediated. We also observed a 16% increase in Cu and a 61% increase in Rb in the multi-trophic streams when the adults were exposed through dietborne routes of exposure. This suggests the possibility of metal exposure through maternal transfer and the mobilization of both Cu and Rb from the yolk-sac since the larvae had not yet reached the swim-up stage of development, and were not feeding on their own.

3.4.3 Morphological endpoints

Results of our study showed significantly increased condition factor (+53%) and body weight (+51%) and significantly decreased LSI (-30%) and egg size (-3%) in effluent exposed

FHM in both systems. There was also a significant dietary effect on LSI in the exposure pathway system, which showed that liver size decreased (- 41%) when fish ate an artificial diet of effluent-exposed chironomids. There was also a significant dietary effect for condition factor and body weight in the food quality system. Both condition factor (+65%) and body weight (+96%) significantly increased in FHM in the multi-trophic streams compared to the FHM in the streams fed an artificial diet. The increase in condition and decrease in egg size coincide with our previous field studies conducted using resident fish species collected downstream of the effluent discharge (Weber et al., 2008). However, where the two studies differ is in the effects on liver size. The field studies (Weber et al., 2008), showed significantly increased liver size, whereas our current study showed significantly decreased liver size. These discrepancies however could be related to differences in treatment water among the lab and field studies. In Weber's study (2008) fish were caught downstream of the Sudbury sewage treatment plant outfall. Increased organic nutrient enrichment has been linked to increased liver size in fish exposed to municipal and industrial wastewater (Yeom et al., 2007), and maybe partially responsible for the increased liver sizes in the resident FHM of Junction Creek. The relatively low sample size used in our current lab study may also have reduced the power to detect a statistically significant difference.

Our current study results do coincide with the national environmental effects monitoring (EEM) findings, showing that most mines across Canada exhibited similar inhibitory responses in fish liver when exposed to mine effluent (Lowell et al., pers. communication). Condition factor did not exhibit a similar inhibitory response pattern as observed in the national EEM findings, however when we examined reproductive output in our effluent exposed streams, we had significantly less egg production especially in our EWEB and MT-effluent treatments where fish were exposed to effluent through both routes of exposure. It would appear that reproductive investment in FHM was severely reduced and females seemed to be sequestering their eggs. Although it may apparently indicate that they are healthier, fatter and in better condition, when in fact it is possible the fish are allocating more energy towards metal detoxification and thereby survival, and less on reproduction. Perhaps a longer exposure period is necessary to determine whether or not the eggs would be reabsorbed or eventually released. The similarity of findings from our current study with both the field and national EEM program findings further validate

the mesocosm technology as a useful alternative for investigating fisheries effects due to effluent exposure when field studies cannot be conducted.

3.4.4 Exposure Pathway

The first objective of our study was to examine exposure pathways (dietary, waterborne and both) independently and simultaneously within the same experiment. We are not aware of any studies in the literature that have assessed environmentally relevant effluent mixtures and three separate routes of exposure simultaneously using a modified fish bioassay. In this study, fish were fed a pre-frozen diet of chironomids that had been grown in either laboratory control water or 45% PWE. This was important to the study design since metal enrichment of the diet (chironomids) needed to reflect environmental exposure conditions found in Junction Creek without the confounded effects of historical sediment contamination. Results showed that when fish were exposed through the water it did not result in significant metal tissue burdens in the FHM, however female condition, LSI, egg size and body weight increased. Previous waterborne-only studies conducted by our research team have shown increased metal tissue accumulations in Creek Chub and FHM exposed to PWE however, no changes in body weight, condition or liver size were observed (Dubé et al., 2006a; Rickwood et al., 2006b). Discrepancies among studies may be attributed to species differences (Creek Chub vs FHM) and/or differences in reference-control water used in each study (Garson flux pit vs, dechlorinated laboratory control water vs RO-dechlorinated lab water mix). Since the reference water was used as dilution water to mix the 100% effluent to the appropriate 45% concentration, variations in the reference water used could account for the contrasting fish responses since water quality parameters (e.g., pH, hardness, alkalinity, DOM) are known modifiers of toxicity to aquatic organisms.

When fish were exposed through the diet, elevated levels of Rb were observed in the carcass and gonads. Elevated metal concentrations have also been observed in rainbow trout carcass when exposed to dietary metals (Cd, Cu, Zn, Ag) relative to fish exposed waterborne metals or control fish suggesting that metals behave differently depending on their routes of exposure (Galvez et al., 2001; Szebedinszky et al., 2001).

Significant interactions were observed for Rb in liver tissues and for condition and body weight in females suggesting that both diet and water exposure were required for effects to be observed. Differences in physiological parameters and accumulation rate based on route of metal

exposure (diet or water) has also been observed in rainbow trout (Galvez et al., 2001). Increased condition has also been observed in previous mesocosm studies using (Rickwood et al., 2006b) as well as wild fish in the Sudbury area exposed via both routes of exposure (Pyle et al., 2005; Weber et al., 2008).

A significant increase in egg production (cumulative total egg production and cumulative egg production) was also observed when fish were exposed to effluent through the diet only. This suggested that the effluent had a stimulatory effect on egg production when exposed through one exposure pathway. It is possible that the control water used in these particular streams contained high levels of RO water and did not provide sufficient micronutrients/metals for optimum reproduction. We suspect that this may have occurred because of improved food quality due to the leaching of metal from the frozen chironomids, resulting in a decrease in dietary metal exposure. Studies have shown that frozen organisms can depurate approximately 30% of the metal burdens when placed in water (Mount et al., 1994; Ng and Wood, 2008). Although it was rare for un-eaten food to remain in the streams for any length of time, it is possible that mortality of one of the pairs could have led to increased depuration and reduced metal uptake. However, when fish were exposed through both routes of exposure (e.g., EWEB), there was significantly decreased egg production and spawning events observed. This suggested that effluent exposure through both exposure pathways acted in an additive manner. These conflicting results showed how complex these natural systems are and that omitting an exposure pathway (dietary or waterborne) can lead to very different results. Similar findings have been observed in a factorial food study using *Daphnia magna*, which showed that combined waterborne and dietary exposures significantly affected reproduction and growth at higher exposure concentrations and results differed from when *D. magna* were exposed through only one route of exposure (DeSchamphelaere and Janssen, 2004).

3.4.5 Food quality and quantity

The second objective of our study was to assess whether relative toxicity to FHM was affected by the quality of food (live versus frozen) that they were eating. Studies have reported absorption efficiencies up to 5-fold higher in rainbow trout fed with live prey compared to those fed an artificial diet (Harrison and Curtis, 2002). It has been suggested that feeding with live prey may result in greater metal toxicity due to biological incorporation into living tissues

(DeSchamphelaere and Janssen, 2004). Metals bound to cytosolic proteins of prey may increase metal bioavailability to the predator far greater than metals bound to insoluble fractions (Ng and Wood, 2008). This suggests that multi-trophic pathways may play a greater role in metal toxicity than a frozen diet (Ng and Wood, 2008). However, in our current study we did expose the *C. dilutus* to PWE for three weeks prior to freezing which should have enabled sufficient incorporation of metals into the tissues of the prey. Furthermore, analysis of the *C. dilutus* tissues confirmed that metals were in fact similar in both multi-trophic and frozen insects, yet we observed very different response patterns in the predators (FHM) with respect to food quality. We can only assume that this may be attributed to the subcellular distribution of metals in the prey, metabolic availability of metals in the prey, assimilation efficiency of the predator, weight specific ingestion rate of the predator and/or metal depuration from the artificial/frozen prey (DeSchamphelaere and Janssen, 2004; Ng and Wood, 2008). Similar studies have shown that 20-30% of metals (Cd, Cu, Zn) depurated from frozen prey to the treatment water when thawed and fed to fish (Mount et al., 1994; Ng and Wood, 2008). Since, depuration studies were not conducted on the frozen insects in our study, it is possible that FHM fed the artificial diet were not exposed to the same concentration of metals as the multi-trophic FHM due to depuration of metals from the frozen insects.

Nevertheless, our study showed that the quality of the food significantly affected the FHM condition factor and body weight. Condition is a commonly used morphometric endpoint to assess the well being of fish in a specific population and is generally thought to reflect the recent feeding activities in fish (Couture and Pyle, 2008). Elevated condition corresponds to increased energy storage (e.g., fat deposition, egg production) relative to physiological energy requirements, whereas decreased condition may indicate reduced food availability and/or increased physiological demand for energetic resources (Couture and Pyle, 2008). Therefore it is possible that fish may allocate significant energetic resources to metal detoxification and less to energy storage in metal contaminated systems (Smith et al., 2001). When fish were fed a live diet, we saw a significant increased condition and body weight compared to fish fed frozen chironomids. At first glance it would appear that the fish are of superior health in the PWE multi-trophic streams. However their highly elevated condition factor, well above the EEM-based critical effect level of ± 25%, suggests that the significant increase in condition and body weight, may actually be related to a reduction in egg production and spawning events in the

effluent exposed FHM. Increased condition has also been observed in studies of fish inhabiting metal-contaminated lakes (Farkas et al.2003; Pyle et al. 2005) as well studies conducted in Junction Creek (Jaagumaji and Bedard, 2001; Weber et al., 2008) relative to fish inhabiting reference waterbodies.

Fish in the multi-trophic control streams (MT-control) had significantly greater egg production (+55% cumulative eggs per female and +37% total egg production) compared to fish fed an artificial diet held in control water (CWCB). Whereas, fish in the multi-trophic effluent streams (MT-effluent) had significantly decreased egg production (-49% cumulative eggs per female and -68% total egg production) when compared to effluent exposed fish fed a frozen diet (EWEB). The fish fed a frozen diet maintained statistically similar cumulative egg production (eggs/female/day and total egg production) regardless of treatment water. We suspect that this might have occurred because of improved food quality due to the leaching of metals from the frozen insect tissue to the exposure water. We observed significantly increased condition factor and body weight and significantly decreased egg production (both cumulative and mean) and spawning events, which suggests that the females were sequestering their eggs. One hypothesis that explains this effect could be that energy allocations in fish were focused primarily on metal detoxification and maintaining homeostasis rather than reproduction due to the increased accumulation of metals from PWE exposure water.

Another hypothesis is that food abundance differed in the multi-trophic treatments compared to the artificially fed treatments. When we established our prey base in the multi-trophic streams, our goal was to attain a density of 1.48 *C. dilutus*/cm^2 to achieve a feeding rate of 1gram *C. dilutus*/day, similar to that of the non multi-trophic streams (exposure pathway - factorial design). Prior to the insertion of the fish, at week 1, chironomid densities in the multi-trophic control streams reached levels of 0.41/cm^2 with levels of 2.07/cm^2 (5-fold increase) achieved by the end of the 21-day exposure. This showed that food was not a limiting factor in the multi-trophic control streams. Conversely, the densities in our multi-trophic effluent streams began with densities of 1.07/cm^2 then decreased to levels of 0.36/cm^2 (3-fold decrease) by the end of the 21-day exposure period, which suggests that food may have been limiting in the effluent streams. Statistical analyses also detected a significant treatment effect on chironomid emergence (P=0.007). Previous studies are consistent with these findings, which suggest that PWE exposure to the chironomids resulted in reduced hatching and emergence, indirectly

affecting fish reproduction in these streams (Hruska and Dubé, 2004). The lowered densities may have contributed to the decrease in reproduction that we recorded in this study, however statistical analysis of the densities in the streams compared to the optimum levels required to achieve FHM satiation of 1gram chironomids/day (48% wet body wt./ 1.48/cm^2) failed to detect a statistically significant difference among treatments ($P>0.05$). It should also be noted that many studies in the literature and other bioassay protocols suggest a feeding rate anywhere from 3.5-6% wet body weight in order to maintain healthy fish reproduction (Mount et al., 1994; Farag et al., 1994; USEPA, 1996; Dubé et al., 2006a; Ng and Wood, 2008). The nature of the multi-trophic mesocosms allows us to establish a multi-generational prey base while controlling the FHM feeding rate using a feeding barrier which we turn every second day. In theory this should provide the appropriate density of chironomids for ~34 days of exposure without having to add further chironomids. Excess chironomids in PWE streams at the start of the experiment, prior to when the fish were introduced and densities at the end of the experiment suggest that levels between 12-35% body weight were maintained in the streams at all times throughout the study (Figure 3.7). Furthermore, despite the indication that relatively high environmental metal concentrations resulted in a decrease in food availability (e.g., reduced chironomid densities) in the PWE streams, the elevated condition in the FHM suggests that there was sufficient food resources for maintaining fat deposition though not sufficient for maintaining egg production.

Based on the fact that adult emergence was significantly affected by the PWE, we can only assume that the FHM had sufficient food for the duration of the study however, chironomid reproduction was significantly altered with effluent exposure resulting in a reduced density by the end of the experiment. Since we were unable to obtain core samples throughout the entire exposure phase, we cannot discount the fact that lowered reproduction could have been at least partially due to reduced food availability in the MT-effluent streams. Similar studies have also shown that fish fed a lower ration of food (~4.62% of their initial mean body mass) exhibited greater effects (increased mortality, reduced growth and increased fin erosion) than fish fed higher rations (18.48% and 44.03% of their initial mean body mass) (Hopkins et al., 2002). Most toxicity bioassays control for confounding food limitations in their designs, but under environmentally realistic exposure conditions, prey abundance is often limited (Hopkins et al., 2002). Future studies should focus on food quantity and its role in affecting FHM reproduction.

3.5 CONCLUSION

Assessing the relative importance of dietary and waterborne routes of exposure showed that metals assimilated differently in FHM tissues depending on the exposure route. Elevated metals were observed in one or more tissue type (carcass, liver and gonads) when FHM were exposed through water or diet or both routes of exposure. Condition, body weight in female adult FHM and tissue metal burdens in 5-day FHM larvae increased significantly and cumulative spawning decreased when exposed to effluent water. Conversely, LSI, cumulative total egg production and cumulative eggs/female/day increased in female FHM exposed through the diet. The examination of food quality effects on FHM toxicity showed that for some metals (e.g., Tl, Rb, Se, Sr, Cu) effluent water, regardless of food type (live or frozen), was predominantly responsible for increased FHM tissue burdens (carcass, liver, gonads). However, Rb and Cd appeared to increase significantly in FHM livers when exposed to through live diet (MT-effluent). Interestingly though, a majority of the reproductive and biological responses to FHM were found in the multi-trophic effluent streams where FHM fed on live chironomids. Condition and body weight increased and cumulative total egg production, cumulative spawning, cumulative eggs/female/day significantly decreased. This suggests that multi-trophic and dietary exposure are the predominant pathways responsible for reproductive impairment in FHM exposed to complex metal mining effluent. These effects appear to be most notable when FHM were fed a live diet vs an artificial diet of frozen *C. dilutus* which holds importance for methodological testing approaches with metals and mine effluents.

Chapter 4

GENERAL DISCUSSION

4.0 PROJECT RATIONALE

The mining industry in Canada is of significant economic importance. It also holds potential for contributing to environmental change in waters receiving significant volumes of treated effluents. In the most recent review of aquatic effects in Canada (Lowell et al., 2007) it was revealed that there are over 70 mines currently operating and discharging effluent. It is difficult to quantify the total effluent loading into Canadian aquatic systems however if we consider an average discharge rate of a mine to be about 41,500 m^3/day (based on the three mine discharges at the study site), we can estimate the daily effluent loading in Canadian waters to be about 2.9 million m^3/day. While all effluents discharged are treated under Canadian regulations, effects are documented in the aquatic environments receiving the effluent discharges. After 2 phases of monitoring (phase 1 [2004-2005]; phase 2 [2007-2008]), the national assessment team (Lowell et al., 2007; 2010 pers. communication) reported some key changes in aquatic environments receiving effluents. There were significant decreases in condition, liver sizes, and growth rates in fish as well as significant decreases in benthic invertebrate density and taxon richness, demonstrating an inhibitory response pattern on a national level.

Understanding the significance of the industry to our country, the potential for environmental change associated with effluent discharges, and the fact that significant changes have been detected as evidenced in biological response patterns of Lowell et al. (2007), the broad objectives of this thesis were to address significant research gaps limiting our understanding of mine effluent effects on aquatic systems. Firstly, I wished to develop an approach to assess individual mine effluent contributions to a watershed receiving multiple point source discharges including three different types of mine effluents. Secondly, I wished to assess effects in a manner that allowed experimental manipulation of critical response endpoints (e.g., fish survival and reproduction) in a highly realistic and more environmentally relevant manner than approaches currently used in the literature. Environmental realism was achieved by inclusion of multiple routes of exposure (waterborne and dietborne) in an in-field food web bioassay. Thirdly, I wished to better understand the role of diet in assessing the effects of metal mine effluents on fish reproduction through direct manipulative studies. Finally, based on tissue accumulation of metals, my intention was to determine if particular metals appeared to potentially cause any of the effects observed.

4.1 INTRODUCTION

The field study was conducted from July to September, 2008 and the laboratory studies were conducted from January to May, 2009 using *in situ* modular mesocosm systems. The mesocosm system was based on a modified reproductive bioassay developed by Rickwood et al., 2006a which allowed the assessment of up to three separate point source discharges (effluents) simultaneously in a field experiment. This also enabled the determination of which effluent was having the greatest effects on the aquatic environment in isolation of any other confounding inputs. Further application of the mesocosms was administered in the laboratory studies to assess the various routes of exposure as well as food quality to evaluate their role in affecting fish responses. Very few studies have been able to isolate the major point sources in a river system and even fewer have been able to assess exposure pathways (water and diet) in a controlled hypothesis-driven experimental design using effluent mixtures in a multi-trophic system. To our knowledge this study was also the first to investigate effects of metal mining effluent on fish responses based on food quality when fish were fed a live diet versus a laboratory prepared diet. This research provides a more environmentally realistic bioassay by incorporating a trophic transfer component. The technology has proven to be effective as a surrogate to a resident fish survey for mines in Canada that may need to assess their current effluent effects using an alternative approach to the standard fish survey. A total of 38 endpoints were analyzed over three studies however, this discussion will focus only on selected biological (condition factor, gonad size (GSI), liver size (LSI), body weight) and reproductive (cumulative eggs/female/day, cumulative total egg production, cumulative spawning events and egg size) endpoints in FHM as well as two main endpoints that were measured in the chironomids (densities, adult emergence).

4.2 ENVIRONMENTAL EFFECTS MONITORING ENDPOINTS

The Canadian EEM program requires all mines to evaluate the effects of mine effluent by assessing several response endpoints [fish population surveys, benthic invertebrate community surveys and fish usability (Hg in tissues)] (MMER, 2002). Similar endpoints are assessed around the world. In the USA this is conducted through nation-wide monitoring programs such as: the National Water Quality Assessment Program (NAQWA), Biomonitoring of Environmental Status and Trends Program, the National Lake Fish Tissue Study (NLFTS) and the Bioassessment Index of Biotic Integrity (B-IBI) program (CCME, 2006). Benthic

macroinvertebrates and fish are most commonly monitored. However, some States also monitor perhiphyton (CCME, 2006). Similar programs have also been implemented in Australia using the Water Reform Framework (WRF), the National River Health Program (NRHP) and the Australian River Assessment System model (AURIVAS) (CCME, 2006). The AURIVAS model incorporates a number of community assemblages such as benthic macroinvertebrates, fish, diatoms, macrophytes and riparian vegetation (CCME, 2006). Similarly in Europe, the Centre for Ecology and Hydrology (CEH) manages biomonitoring programs in Scotland, Northern Ireland, Wales and England (CCME, 2006). The CEH uses the River Invertebrate Prediction and Classification System (RIVPACS) model to analyze routine benthic macroinvertebrate monitoring data that are collected in spring and fall (CCME, 2006). However, there has been limited use of fish and aquatic vegetation in the RIVPACS model thus far (CCME, 2006).

The most common biological indicator used in monitoring programs to assess environmental degradation throughout the world appears to be the benthic macroinvertebrate community, followed by fish and to a lesser degree periphyton and aquatic vegetation. Common endpoints measured for benthic invertebrates include: taxanomic richness, abundance, EPT (Ephemeroptera Plecoptera, Trichoptera) richness, diversity, evenness, Bray-Curtis Index, Shannon-Wiener Index, Hilsenhoff Biotic Index among others. Fish endpoints measured include the index of biological integrity or the index of well being, as well as assessing relative weight, abundance and condition. Assessment of the periphyton community includes an algal community assessment, chlorophyll "a" content and algal biomass. The EEM program in Canada however, uses both fish and benthic response endpoints to assess mine impacts to the environment. The response endpoints considered in the EEM program include condition (total body weight vs total length), relative liver weight, relative gonad weight, weight at age and age for fish; and density, taxa richness, Simpson's evenness index, and Bray-Curtis dissimilarity index for benthic macroinvertebrate communities. An assessment of fish usability is also conducted where fish tissues are analyzed for mercury. The EEM program has attempted to encompass many of the valuable assessment programs available internationally into one comprehensive program that can be applied to all mines across Canada. The program is currently on its third Phase of studies for most mines in Canada. On a national level, Phase 1 of the EEM program found a significant decrease in condition and relative liver weight in the fish population survey as well as decreased taxon richness and total density in the benthic invertebrate community survey exposed to treated

effluent for many mines across Canada. In Phase 2, a decrease in condition, relative liver weight and weight at age was observed in fish populations as well as decreased taxon richness in the benthic invertebrate communities. Total invertebrate densities at exposure sites for mines significantly increased across Canada.

In the current field study, magnitude of changes in exposed fish relative to the reference fish was significantly increased for condition in female FHM exposed to SWE. Significant increases in relative liver size (both sexes) in all MME's were also observed as was a significant increase in relative gonad size (both sexes) in all MME's. In each instance changes exceeded the Critical Effect Size (CES) for condition factor (±10%), liver size (± 25%) and gonad size (± 25%) as specified in the EEM program (MMER, 2002). Overall, the field study showed that the MME's have a stimulatory effect on fish in Junction Creek, which was not consistent with the observations made by the national findings in both phases. Similar to the national findings of Phase 1, decreased chironomid densities were observed in all MME's relative to reference streams.

Results of the lab studies however showed significantly increased condition and body weight in female FHM and significantly decreased egg size in fish as well as decreased chironomid densities when exposed to PWE (Table 4.1). Conflicting results for LSI was observed in our lab studies (Table 4.1). However, our food quality experiment (MT-Lab) in which fish were fed live chironomids, did coincide with the national findings, showing inhibitory responses in fish liver when exposed to mine effluent in both phase 1 and 2 (Lowell et al., 2007 and 2010 pers. communication). Condition factor and body weight however, exhibited a stimulatory response pattern in all current experiments, contrary to those observed by the national EEM findings in both Phases. We have observed significant increases in condition in both fish and invertebrates in our studies to date (Table 4.1). Despite discrepancies with the national findings, the results do coincide with other studies conducted in the Sudbury area, which have shown that condition in yellow perch was either unchanged or increased when compared to

Table 4.1. Summary of studies (field and mesocosm) conducted by our research team (Dubé et al.) over the last ten years using the 45% PWE (Process Water Effluent). Arrows depict a significant increase (↑) or decrease (↓) in the effects observed in female FHM when compared to the control/reference treatment. Effects for *C. dilutus* rather than FHM.

	FIELD STUDY	**MESOCOSM STUDIES**						
Effect Observed	Weber et al. 2008 (Resident Fish)	Dubé et al., 2006a (Fish-Field)	Hruska & Dubé, 2004 (*C. dilutus* - Lab & Field)	Rickwood et al., 2006b (MT-Lab)	Rickwood et al., 2008 (MT-Field)	Ramilo et al., Field (Exp 1)	Ramilo et al., Exposure Pathway (Exp 2)	Ramilo et al., Food Quality (Exp 2)
[a]Density	-	-	↓	↓	↓	↓	↓	NS
[b]GSI	NS	↓	NS	-	NS	NS	NS	NS
[c]LSI	↑	↓	-	-	NS	↑	↓	↑
Testosterone	-	↓		↑	NS	-	-	-
Survival	-	↓	↓	NS	↓	NS	NS	-
Condition	NS	NS	↑			↑	↑	↑
Metal Accumulation in Tissues	↑	↑	↑	↑	↑	↑	↑	↑
Spawning Events	-	-	-	↓	↑	↓	↓	↓
Egg Size	↓	NS	-	-	NS	↓	↓	↓
Egg Production		NS	-	↓	↑	↓	↓	↓
Hatching Success			↓	↓	NS	NS	↑	↑
Larval Deformities	-	-	-	-	↑	NS	NS	NS

[a]Densities refer to *C. dilutus* stream densities
[b]GSI = Gonadal Somatic Index
[c]LSI = Liver Somatic Index
NS= not significantly different from reference values
MT= multi-trophic
Exp = Experiment

reference fish (Pyle et al., 2005). Furthermore, 2 out of 5 of our studies have shown inhibitory effects in LSI (Table 4.1). Inconsistent effects on liver size (LSI) have also been observed in fish exposed to other mine effluents (Woodward et al., 1995; Farag et al., 1999; Eastwood and Couture, 2002). All of our studies conducted to date have shown an inhibitory effect of PWE on chironomid density (Table 4.1), which is consistent with the national findings in Phase 2 as well as with the field studies conducted in Junction Creek in 2009 (Stantec, 2009). In addition, several reproductive endpoints were measured in the MME's and have been shown to inhibit egg production (cumulative eggs/female/day and cumulative total egg production), egg size and cumulative spawning events when exposed to PWE (Table 4.1), have no effect when exposed to MWE (Chapter 2) and a stimulatory effect on reproduction when exposed to SWE (Chapter 2). Similar reproductive impairment (e.g., reduced egg production, reduced hatching success) has also been observed in fish exposed to metal contaminated sites (Munkittrick and Dixon, 1988; Fisher and Hook, 2002; Boyle et al., 2008; Franssen, 2009). Decreased egg size has also been observed in yellow perch in the Sudbury area (Pyle et al., 2005).

4.3 GENERATION OF HYPOTHESES

Several hypotheses have been presented to explain why FHM condition has been increased in SWE (Chapter 2) and PWE in our current field study despite reduced spawning events and eggs produced in the PWE treatment. One theory is that the fish may have grown larger in the SWE treatments and therefore ate more, grew larger and produced more eggs than the fish in PWE. However, there were no significant differences among total body weight or fork length among treatments. Increased egg production was observed for SWE compared to reference, however this was not a factor of fish size. The fish in the SWE did not grow larger than the fish in the other treatments; they simply produced more eggs even when compared to the reference treatments. Some investigators compute condition based on the "gonad-free" body weight to remove the effect of temporally varying gonad weight from the denominator. Condition was calculated using both methods however, the end results were not appreciably different therefore condition indices were calculated based on total body weight. Furthermore, it is not clear in the EEM guidance document whether condition is computed using total body weight or gonad-free body weight. However, since the fish used in the study were all laboratory reared, were naive fish, at a similar stage of reproduction and of the same age, it is unlikely that

temporal variability played a substantial role in affecting condition. We also investigated the possibility that a greater abundance of food (*C. dilutus*) was available in the SWE compared to the other treatments however, chironomid density analysis showed that there was less food available in the SWE compared to the control streams (Chapter 2), which suggested that food abundance was not a factor in the increased egg production observed. However, in our food quality experiment (Table 4.1, Experiment 2), it was possible that lowered densities of chironomids in the effluent exposed multi-trophic treatments could have partially attributed to a decrease in reproduction (Table 4.1). The findings from previous studies conducted in our lab are consistent with the current findings, which suggest that PWE exposure severely alters reproduction in chironomids (Hruska and Dubé, 2004, Rickwood et al., 2006a, 2008). Both the current field and lab experiments showed that the duration of exposure (time) and type of treatment (effluent vs control), regardless of the effluent, as well as the interaction between time and treatment had a significant effect on adult chrionomid emergence.

Interestingly when food abundance was controlled for in the exposure pathway experiment (Table 4.1, Experiment 1) a significant reduction in egg production was still observed when fish were exposed to PWE through both routes of exposure, which seems to suggest that food abundance may not be a limiting factor in reproductive effects in our current studies.

One proposed hypothesis for the increased condition factor, decreased egg size, spawning events and overall egg production in fish exposed to PWE may be that energy allocations were focussed primarily on maintaining metabolic homeostasis and metal detoxification rather than reproduction, due to increased metal exposure, and that significantly reduced egg size may indicate the possibility of resorption of their eggs under these stressful conditions (Helfman et al., 1997). Egg resorption is a common process in oviparous fish which allows the female to re-use proteins, fats and minerals in the eggs for maintenance and growth under stressful conditions (Helfman et al., 1997). Furthermore, fish have been shown to adjust their egg size in response to environmental stress (Kamaran et al., 2007; Driessnack et al., 2011). Although we can only speculate as to what may be occurring, gonadal development and histopathology analysis in previous studies appear to agree with this theory and have shown significant increased rates of zona radiata breakdown, enlarged granulose cells and yolk resorption in ovaries of FHM (Weber et al., 2008). This suggests that perhaps 21 days of

exposure was not sufficient for resorption of the eggs to occur and could have resulted in the elevated condition factor that we have observed in our latest studies. In addition, fish mortality increased in some streams likely as a result of increased stress due to effluent exposure or disruption of normal reproductive functions. Mortality alters the sample size, increases variability and reduces the power to detect a significant effect, which could be partially attributed to the variability in some of the biological endpoints measured in this study. Discrepancies among our latest studies and the national findings could also be attributed to fact that most mines conduct EEM studies on resident fish downstream of their final discharge point. There is no way of controlling the migration of wild fish and they may actually spend more time closer to the effluent discharge, where there is less dilution, and therefore be subjected to greater exposure than in the mesocosm studies. This may explain why a stimulatory effect was prominent in our studies and an inhibitory effect was more prominent in the national findings.

4.4 ROUTES OF EXPOSURE

Our exposure pathway study was conducted specifically to examine the relative importance of dietary and waterborne routes of exposure to PWE in a laboratory setting (Chapter 3). This study enabled us to alter the experimental design of the bioassay to allow for the application of a factorial study design allowing us to assess both routes of exposure simultaneously in a controlled hypothesis-driven study. Most studies do not assess routes of exposure independently from each other, and concurrent exposures to both waterborne and dietborne exposures can complicate the interpretation of the experimental data (Meyer et al., 2005). Examining the exposure pathways independently and simultaneously within the same experiment can provide valuable insight about mechanisms of metal uptake, bioavailability, bioaccumulation and toxicity (Meyer et al., 2005). We are not aware of any studies in the literature that have assessed environmentally relevant effluent mixtures and three separate routes of exposure (dietary, waterborne and both) simultaneously using a modified fish bioassay. In this study, fish were fed a pre-frozen diet of chironomids that had been grown in either laboratory control water or 45% PWE. This was important to the study design since metal enrichment of the diet (chironomids) needed to reflect environmental exposure conditions found in Junction Creek. There were 4 treatments: a complete control where fish were held in control water and fed benthic organisms raised in control water (CWCB), a waterborne-only exposure

where fish were held in effluent water and fed control benthic organisms (EWCB) a dietborne-only exposure where fish were held in control water and fed effluent raised benthic organisms (CWEB) and finally a complete exposure treatment (EWEB) where fish were held in effluent water and fed benthic organisms raised in effluent water. The results showed that metals accumulated differently in FHM tissues depending on the exposure route. Elevated metals were observed in one or more tissue type (carcass, liver and gonads) when FHM were exposed through water (Al, Ce, Sr, Cd), diet (Rb, Co, Al, Ce) or both routes (Mg, Pb, Cr, Va) of exposure. Condition factor and body weight significantly increased, whereas LSI and egg size significantly decreased when FHM were exposed through waterborne routes of exposure. LSI was also significantly decreased when exposed through dietary exposure pathways (CWEB). Results showed that both waterborne and dietborne routes of exposure can significantly affect liver size (LSI) and can do so independently of each other. A significant interaction was observed for condition and body weight in female FHM which suggested that both effluent water and effluent exposed chironomids were dependent on each other and acted in an additive manner for effects to occur. This was an important discovery since it was apparent that both routes of exposure were required to elicit significant effects.

A significant increase in egg production (cumulative total egg production and cumulative egg production) was observed when fish were exposed through the diet only. This suggested that the effluent had a stimulatory effect on egg production when exposed through one exposure pathway. However, when fish were exposed through both routes of exposure (EWEB), there was significantly decreased egg production and spawning events observed. This suggested that effluent exposure through both exposure pathways acted in an additive manner. These conflicting results showed how complex these natural systems are and that omitting an exposure pathway (dietary or waterborne) can lead to very different results. Similar findings have been observed in a factorial food study using *Daphnia magna*, which showed that combined waterborne and dietary exposures significantly affected reproduction and growth at higher exposure concentrations and results differed from when *D. magna* were exposed through only one route of exposure (DeSchamphelaere and Janssen, 2004).

4.5 FOOD QUALITY

The food quality study was conducted concurrently with the exposure pathway study in the lab in order to assess whether fish responses differed when prey (*C. dilutus*) were live or frozen. We are only aware of one other study design which has attempted to assess biological effects through various routes of exposure using a live diet (DeSchamphelaere and Janssen, 2004), however the current study to our knowledge, is the first to examine a live and laboratory prepared frozen diet simultaneously using fish held in an effluent mixture. Results showed that several FHM responses (biological and reproductive) were affected by food quality.

A significant treatment effect (PWE treatment vs reference treatment) was observed for LSI and egg size. Both were significantly decreased when exposed to the PWE treatment compared to reference treatment, regardless of whether fish were fed live or frozen food. A significant interaction was also observed for LSI suggesting that both effluent exposure and live benthic organisms interacted in an additive manner to cause a decrease in LSI. However based on the fact that a similar response was observed when fish were held in PWE treatments (waterborne only exposure), it would suggest that the predominant route of toxicity is through waterborne exposure and food quality plays only a small role in eliciting toxic response to FHM livers when exposed to metal mixtures. Egg size and egg volume have also been shown to significantly decrease when exposed to waterborne concentrations of Se (selenate form) as low as 3 ug/l (Driessnack et al., 2011). Waterborne Se concentrations in all MME's discharged to Junction creek have exceeded this threshold value (Table 4.2). Furthermore in the same study, a 21 day depuration phase showed that when fish were removed from the contaminant source, egg size/ volume rapidly increased to reference conditions (Driessnack et al., 2011). This study showed a direct cause and effect of selenate on FHM egg reproduction and would be of value to reproduce using Vale's MME's in future studies.

A significant treatment effect and food-type effect was observed for condition and body weight in female FHM. Both condition and body weight increased when exposed to the PWE, and when fish were fed live benthic organisms. However, significantly decreased egg production (cumulative total egg production, cumulative eggs/female/day) and spawning was observed when fish were held in the PWE and fed live benthic organisms. Increased condition and body weight in conjunction with decreased egg production would suggest that the females were sequestering their eggs or that one or both sexes may have altered energy allocation (e.g., from

reproduction to ionoregulation) in order to deal with the stress of increased metal exposure. Conversely, a significant increase in cumulative total egg production, cumulative spawning events and cumulative egg production was observed when fish were fed live benthic organisms in the control treatment compared to when they were held in effluent water and fed live benthic organisms.

Overall, similar reproductive effects were observed when fish were fed a frozen laboratory prepared diet regardless of the treatment water they were exposed to. Whereas we observed significantly contrasting reproductive effects when fish were fed live benthic organisms and exposed to different treatment water.

Furthermore, when metal accumulation in the fish tissues was examined, Rb and Se both significantly increased in liver and carcass when fed live prey. Several possible reasons for this were discussed previously in Chapter 3 but these findings suggests that multi-trophic live dietary pathways may play a greater role in metal toxicity than a frozen laboratory prepared diet of chironomids.

4.6 EFFLUENT QUALITY

Both field and lab studies showed a high degree of variability for most of the biological endpoints measured (egg production, egg size, larval deformities), even when the same effluent treatment was used (PWE) (Table 4.1). This variability may be attributed to a number of factors including: 1) the chemical composition and nature (e.g., surface water, mine water, process water) of the mining effluent; 2) other organic constituents in the effluent; and 3) the sample size.

Upon closer examination of the reproductive output in our effluent exposed streams, we have observed significantly less egg production and spawning events in most studies to date, especially in the PWE treatments (Table 4.1). It would appear that reproductive investment in FHM was severely reduced and females seemed to be sequestering their eggs which directly affected condition. We have hypothesized that effluent quality may have been partially responsible for this and could have resulted in a shift in energy allocation, whereby more energy was concentrated on ionoregulation and less on reproduction (Franssen, 2009). MME's are continually changing based on the geological properties of the rock and the milling process, which makes them extremely difficult to work with and to obtain repeatable results. Over the last

Table 4.2 Key Effluent Parameters Measured in Eight Mesocosm and One Resident Fish Field Study Using One or More Treated MME's [Surface Water Effluent (SWE), Mine Water Effluent (MWE), Process Water Effluent (PWE)] Discharged to Junction Creek Over the Last Ten Years.

MESOCOSM STUDIES									
	Dubé et al., 2001 (Field Study)			Dubé et al., 2002 (Field Study)			Hruska & Dubé, 2002 (Field Study)	Hruska & Dubé , 2003 (Lab Study)	Rickwood et al., 2004 (Lab Study)
Key Effluent Parameters	SWE	MWE	PWE	SWE	MWE	PWE	PWE	PWE	PWE-MT
Conductivity (µS/cm)	711.5 ± 22.5	1128.0 ± 1.0	1628.0 ± 190.0	197.8 ± 6.3	525.9 ± 18.1	1065.6 ± 37.0		1864 ± 48	1730 ± 43.6
pH	8.1 ± 0.01	8.0 ± 0.01	7.8 ± 0.04	7.7 ± 0.7	7.5 ± 0.06	7.5 ± 0.06	7.7 ± 1.4	7.8 ± 0.1	7.57 ± 0.07
Total Ammonia (mg/l)	0.20 ± 0.00	0.55 ± 0.25	0.9 ± 0.10	0.32 ± 0.18	0.87 ± 0.16	1.35 ± 0.07	1.39 ± 0.07	2.3 ± 0.9	0.15 ± 0.10
Dissolved Organic Carbon (mg/l)	-	-	-	-	-	-	4.2 ± 0.1	5.6 ± 1.7	3.33 ± 1.45
Total Organic Carbon (mg/l)	-	-	-	8.88 ± 2.58	5.32 ± 1.08	4.22 ± 0.12	4.7 ± 0.5	-	3.67 ± 1.76
Total Hardness (mg/l as CaCO3)	-	-	-	103.1 ± 9.8	287.1 ± 23.1	729.2 ± 52.6	793 ± 21	1035 ± 116	882 ± 29.5
Total Suspended Solids (mg/l)	-	-	-	-	-	-	-	-	-
Cadmium (µg/l)	-	-	-	-	-	-	-	0.99 ± 0.23	0.15 ± 0.05
Cobalt (µg/l)	-	-	-	-	-	-	2.3 ± 0.4	15.03 ± 0.47	3.12 ± 0.61
Copper (µg/l)	-	-	-	-	-	-	83 ± 10	63.7 ± 8.6	93.8 ± 16.1
Nickel (µg/l)	-	-	-	-	-	-	78 ± 12	199 ± 25	114 ± 51.2
Rubidium (µg/l)	-	-	-	-	-	-	29 ± 0.8	47.7 ± 5.6	51.4 ± 16.4
Selenium (µg/l)	-	-	-	-	-	-	64 ± 28	6.84 ± 0.36	7.37 ± 0.66
Strontium (µg/l)	-	-	-	-	-	-	576 ± 96	722 ± 82	809 ± 220
Thallium (µg/l)	-	-	-	-	-	-	-	0.664 ± 0.052	1.02 ± 0.53
Vanadium (µg/l)	-	-	-	-	-	-	-	0.17 ± 0.02	-
Zinc (µg/l)	-	-	-	-	-	-	9 ± 1	4.5 ± 0.7	12.5 ± 1.99

MESOCOSM STUDIES						FIELD STUDY		
	Rickwood et al., 2005 (Field Study)	Ramilo et al., 2008 (Field study)			Ramilo et al., 2009 (Lab Study)	Weber et al., 2004 (Field Study)		
Key Effluent Parameters	PWE MWW-MT	SWE-MT	MWE-MT	PWE-MT	PWE	PWE-MT	SWE	MWE	PWE
Conductivity (µS/cm)	1559 ± 108	465 ± 14	769 ± 33	1616 ± 53	1257 ± 95	1316 ± 142	316	933	1322
pH	7.59 ± 0.48	7.29 ± 0.12	7.33 ± 0.16	6.69 ± 0.43	6.81 ± 0.13	6.65 ± 0.12	7.32	7.92	7.05
Total Ammonia (mg/l)	2.78 ± 1.86	0.32 ± 0.11	0.74 ± 0.35	1.89 ± 0.77	0.18 ± 0.04	0.36 ± 0.07	0.05	0.01	1.19
Dissolved Organic Carbon (mg/l)	6.11 ± 0.32	9.33 ± 0.33	8.00 ± 1.15	7.00 ± 0.58	3.0 ± 0.42	4.95 ± 0.54	11	12	8.1
Total Organic Carbon (mg/l)	6.66 ± 0.18	9.67 ± 0.33	9.00 ± 0.58	7.33 ± 0.88	3.90 ± 0.12	6.21 ± 0.43	-	-	-
Total Hardness (mg/l as CaCO3)	533 ± 43.7	163.0 ± 20.4	308.3 ± 5.5	651.3 ± 23.9	437 ± 44.98	467 ± 54.11	74	276	463
Total Suspended Solids (mg/l)	9.87 ± 2.07	3 ± 0	3 ± 0	5 ± 2	-	-	5	<3	15
Cadmium (µg/l)	0.1 ± 0.03	0.08 ± 0.03	0.07 ± 0.02	0.09 ± 0.04	0.11 ± 0.03	0.30 ± 0.04	0.28	<0.1	0.26
Cobalt (µg/l)	-	0.39 ± 0.05	0.36 ± 0.14	1.45 ± 0.12	2.03 ± 0.91	3.24 ± 0.53	10.6	1.9	10.9
Copper (µg/l)	33.6 ± 16.8	9.23 ± 1.07	7.93 ± 1.20	50.80 ± 5.45	70.1 ± 11.07	53.5 ± 5.19	61	6	106
Nickel (µg/l)	32.5 ± 14.1	32.67 ± 4.96	27.10 ± 10.60	53.27 ± 15.49	74.2 ± 1.81	84.67 ± 3.07	299	153	280
Rubidium (µg/l)	-	6.00 ± 0.50	11.77 ± 1.02	35.47 ± 2.74	22.63 ± 3.76	23.7 ± 3.74	2.3	8.2	19
Selenium (µg/l)	7.2 ± 0.90	0.50 ± 0	1.37 ± 0.44	11.10 ± 0.92	8.0 ± 1.59	8.13 ± 1.05	<1	2.2	7.8
Strontium (µg/l)	-	137.67 ± 10.17	482.33 ± 36.06	485.00 ± 32.51	341 ± 41.68	352 ± 49.41	61	380	270
Thallium (µg/l)	0.52 ± 0.05	0.05 ± 0	0.05 ± 0.00	0.30 ± 0.04	0.09 ± 0.02	0.08 ± 0.03	<0.1	<0.1	0.6
Vanadium (µg/l)	-	1.03 ± 0.53	1.07 ± 0.35	0.50 ± 0.0	-	-	1.3	2.1	1.3
Zinc (µg/l)	10 ± 7.50	9.17 ± 8.17	4.77 ± 2.78	5.4 ± 2.06	7.7 ± 4.53	8 ± 2.02	23	7.9	19

MT=multi-trophic

In particular, conductivity has varied by as much as of 4-fold in SWE, 2-fold in MWE and 2-fold in PWE (Table 4.2).Ammonia has also varied by about 2-fold in SWE and MWE, and by as much as 19-fold in PWE in various studies, with the highest concentrations of ammonia observed in Rickwood's field study at 2.27 mg/l total ammonia (~0.052 mg/l un-ionized) in PWE (Table 4.2). Un-ionized ammonia has been associated with decreased liver somatic index (LSI) at concentrations between 0.834 mg/l and 1.112 mg/l in slimy sculpin (*Cottus cognatus*) (Spencer et al., 2008). In the same study, gonadosomatic index (GSI) was elevated in fish exposed to 1.668 mg/l un-ionized ammonia (Spencer et al., 2008). Ammonia levels in all three of Vale's effluents appear at concentrations below this toxic threshold value, but may be partially associated with the increase in GSI and decrease in LSI observed in some of our studies to date.

Increased ion levels (magnesium, calcium and sodium) have also been implicated as causative factors in MME affecting FHM due to their potential to cause stress associated with osmoregulation (Rickwood et al., 2006b; Evans, 2000). Reproduction may be impaired under periods of stress and the reproductive effects observed could simply be due to the change in water quality, e.g., high conductivity and hardness (Rickwood et al., 2006b). However, it is not possible to make any comparisons to previous literature as studies could not be found that have investigated these parameters and their effects on reproductive output in FHM (either directly or indirectly) (Rickwood et al., 2006b). Furthermore, the nature of the effluent varies considerably in composition from each other as described in Chapter 1. SWE is mainly comprised of surface runoff and water from collection ponds, MWE is mainly comprised of underground mine water and surface runoff from the Garson Mine whereas the PWE is comprised of process water from the mining, milling, refining and smelting of ores from the Vale operations. Despite the variability in effluent quality temporally, PWE has been identified in all of our studies to date as the effluent of greatest concern, not only because it contains the highest concentration of metals and constitutes the greatest volume of effluent that enters into Junction Creek but also because it is the only effluent that receives waste from the beneficiation of ores and sewage waste water from surface mine operations simultaneously.

In this study and other studies conducted in Junction Creek (Jaagumagi and Bedard, 2001; Weber et al., 2008) a concentration gradient of metal contamination occurs in the water with SWE at the lower end and PWE at the higher end and MWE in between. We have seen this gradient in a number of water quality parameters such as hardness, conductivity and ammonia as

well as a number of metals and mettaloids (Cu, Ni, Rb, Se, Sr) (Table 2). This gradient has also been observed in the biological and reproductive endpoints that have been measured using the three effluents in our 2001-2002 studies (Dubé et al., 2006a) as well as in our field study conducted in 2008 (Chapter 2). Overall a stimulatory effect has been observed in the SWE and an inhibitory effect has been observed in the PWE with a mixture of both effects observed in the MWE when compared to reference values, which could be partially due to effluent quality (Chapter 2). We have also observed a reverse gradient with TOC and DOC appearing at higher concentrations in SWE (Table 4.2). The effluent quality and general water quality parameters play a major role in the bioaccumulation of metals and metal uptake in the aquatic environment (see Chapter 1 section 1.4 for greater details). One of the most notable differences among the three MME's however is the fact that the PWE contains a number of organic constituents in the effluent that are not present in the other MME's that have not yet been identified. The milling process in particular requires the use of chemical reagents in the beneficiation process (e.g., lime, sodium cyanide, xanthates, polymers, surfactants). Furthermore, PWE also contains sewage waste water as well as runoff from fuel storage areas and maintenance shops as was noted in Chapter 2. Though it was beyond the scope of the current study as well as beyond the regulatory requirements of the EEM program to examine the organic constituents in the effluent they have the potential to affect biological and reproductive endpoints in fish and invertebrates and require further attention in future studies.

4.7 METALS OF CONCERN

Specific metals in the effluent have the ability to directly affect fish reproduction. At the end of the 21 day exposure period, both fish and invertebrates were collected to assess metal accumulation in determining any potential causative metals of concern which may have led to reproductive effects in FHM. Overall among all of the studies that have been conducted by our lab to date, including the current studies; Cu, Ni, Rb, Se, Sr and Tl appear to be the metals most consistently observed in the tissues of biota and are the metals of most concern at this time (Table 4.3).

In particular Rb has been found to accumulate in fish 5 of the 8 studies and in the greatest variety of tissue types. Studies have shown that Rb has the potential to inhibit spermatogenesis in fish at concentrations of 18 µg/g in the testes (Yamaguchi et al., 2007). Though Rb

accumulation was not analyzed in the FHM testes in any of our previous experiments our current study results have shown mean concentrations in the ovaries of females varied from 2.34 to 4.37 µg/g in the effluent treatments, much lower than Yamaguchi's study (2007). Despite differences in gonadal tissue type and species differences, the results suggest that Rb in Vale's MME's were below levels that would elicit similar reproductive impairment. However, previous histological testing of male testes has shown evidence of male reproductive impairment (testicular cell death, delayed testicular development, fibrosis) when exposed to PWE (Rickwood et al., 2006a; Weber et al., 2008) and remains a metal of potential concern.

Selenium has been implicated in fish reproductive impairment, reduced fish biomass, and population declines (Ogle and Knight, 1989; Heinz et al., 1996; Lemly, 2002), and is a known teratogen causing severe larval deformities and impaired biological functions at egg concentrations of ≥10 µg Se/g dry wt. (DeForest et al., 1999; Muscatello et al., 2006). Se has been found consistently elevated in a number of tissue types in a number of studies conducted to date (Table 4.3). It has mostly been found in the whole body, carcass and carcass tissues but has also been found in the gonad tissues in some studies including the most recent studies. Selenium egg concentrations in FHM exposed to PWE treatments in the lab reached levels of 2.78 µg/g wet wt. [10.7 0 µg Se/g dry wt. based on 74% moisture (Muscatello et al., 2006)], which was sufficiently elevated to elicit teratogenic effects in FHM.

Both Cu and Ni have shown increased tissue accumulations in three different studies. Both Cu and Ni are highly regulated in fish and though may not be directly linked to reproductive impairment they do have the potential to elicit cellular and sub-cellular impairment and the development and growth of the F1 generation (Johnson et al., 2007; LaPointe and Couture, 2009). Population threshold values for Cu have been estimated at 27 µg/l for waterborne Cu exposure in FHM (Iwasaki et al., 2010). Copper levels in the MME's have been known to vary anywhere from 6-106 µg/l with concentrations in the PWE that have never existed at a lower concentration than 33 µg/l in all of the studies that have been conducted to date (Table

Table 4.3 Summary of metal tissue accumulations in fish exposed to Vale's three MME's [Surface Water Effluent (SWE), Mine Water Effluent (MWE), Process Water Effluent (PWE)] over the last ten years that have been consistently elevated across studies.

Study	Treatment	Tissue Type	Metals of Potential Concern					
			Cu	Ni	Rb	Se	Sr	Tl
Dubé et al., 2006a (creek chub)	SWE	whole body		X				X
	MWE	whole body		X				X
	PWE	whole body						X
Dubé et al., 2006a (pearl dace)	SWE	whole body		X				
	MWE	whole body						
	PWE	whole body				X		X
Rickwood et al., 2006a (FHM)	PWE	muscle				X		
	PWE	ovaries				X		
Rickwood et al., 2008 (FHM)	PWE	whole body			X	X		X
Weber et al., 2008 (Creek chub & FHM)	SWE	whole body	X		X	X	X	
	MWE	whole body	X		X	X	X	
	PWE	whole body	X		X	X	X	
Ramilo et al., 2008 Field (FHM)	SWE	gonads	X	X			X	X
		carcass		X	X			
	MWE	gills			X			
		gonads	X					X
		carcass	X	X	X			
	PWE	gills		X	X			
		gonads	X	X				
Ramilo et al., 2009 Exposure Pathway (FHM)	PWE	gonads			X			
		carcass			X			
		liver			X		X	
Ramilo et al., 2009 Food Quality (FHM)	PWE MT	gonads			X	X		
		carcass			X			X
		liver	X		X	X	X	

4.2) and therefore it is highly probable that population effects could occur in the PWE due to Cu exposure. Cellular and sub-cellular effects have been observed in FHM exposed to waterborne Ni concentrations of 16 µg/l and dietary exposures of 10 µg/l (LaPointe and Couture, 2009). Waterborne concentrations of Ni in all MME's and in all studies have exceeded 16 µg/l (Table 4.2), which suggests cellular-level effects were possible and could affect reproduction in FHM. Strontium and Tl have also been consistently elevated in MME's. Strontium is not believed to directly affect reproduction but has been known to affect ionic regulation in fish and could affect survival and therefore has implications for population-level effects. Thallium is known to be acutely toxic to Atlantic Salmon at a concentration of 30 µg /L in effluent (Zitko et al. 1975). Though thallium levels in the MME's were well below these levels in all of our current studies (between 0.05-0.50 µg g/L), it does consistently appear elevated in tissues and the toxic effects in FHM are not well known.

4.8 RECOMMENDATIONS

4.8.1 Recommendations for regulators

Under the current EEM guidelines "effluent" is defined as: mine water effluent, milling facility effluent, tailings impoundment area effluent, treatment pond effluent, treatment facility effluent (other than sewage treatment effluent) and seepage and surface drainage that contains deleterious substances (MMER, 2002). Despite the obvious differences among effluent types, they are all currently regulated in the same manner under the *Fisheries Act*. National response patterns have been evaluated using meta-analyses and bivariate and multivariate plotting tools to evaluate mines on the basis of habitat, ore type, fish gender, fish species, effluent concentration and continuous vs intermittent effluent discharge (Lowell et al., 2007). Meta-analyses are valuable tools for assessing individual studies that research the same questions using established effect sizes, and then combining these effects to get a more accurate idea of the true effect to the population (Field, 2009). However, our research has shown that differences in effluent type elicited significantly different effects on both fish and benthic invertebrates yet there is no distinction among them under the current regulation, nor any indication of their importance in the meta-analysis. In light of our current findings our suggestion to the regulators would be that greater attention be given to meta-analysis based on effluent type.

Much of the research and all of the guidelines that have been developed to date are based on toxicity testing of waterborne exposure pathways (Environment Canada EPS1/RM/13; EPS1/RM/14; EPS1/RM/21; EPS1/RM/22; EPS1/RM/37) or sediment contamination (Environment Canada EPS1/RM/32; EPS1/RM/33; EPS1/RM/41; EPS1/RM/42). However, if our goal is to assess the effects of mine effluents on resident fish and invertebrate species under environmentally realistic conditions, changes should be made to the Environment Canada biological test methods to reflect this. Our exposure pathway study has shown that in some cases both waterborne and dietborne routes of exposure were required to elicit significant effects in female FHM for condition, body weight, egg production (cumulative total egg production and cumulative egg production) when exposed to PWE and by omitting an exposure pathway (dietary or waterborne) can lead to very different results. Similar findings have been observed in a factorial food study using *Daphnia magna*, which showed that combined waterborne and dietary exposures significantly affected reproduction and growth at higher exposure concentrations and results differed from when *D. magna* were exposed through only one route of exposure (DeSchamphelaere and Janssen, 2004). Our recommendation is that these test methods also include a live dietary component since our food quality study has shown that similar reproductive effects were observed when fish were fed a frozen laboratory prepared diet regardless of the treatment water they were exposed to. However, significantly contrasting reproductive effects were observed when fish were fed live prey and exposed to different treatment water.

Under the current EEM guidelines reproduction is expressed as relative gonad size, reproductive effort/success (e.g., number of young-of-the-year, age class composition, larval density), fecundity (number of eggs produced by a female), and relative egg size as a function of body weight (MMER, 2002). Both fecundity and egg size estimates are based on sub samples from female ovaries at the time of sacrificial sampling of the fish. Relative gonad size is the "effect endpoint" where as fecundity and egg size are "supporting endpoints". This means that relative gonad size is used as a surrogate to actual reproductive output. The most direct measurement of reproductive output is to obtain actual measurements of egg numbers and egg size. This is not possible in the EEM program because in wild fish surveys the adults are sampled (or young-of-the-year) rather than nests of spawned eggs. Furthermore, a number of studies (~72%) have not properly considered the reproductive biology of the sentinel species

prior to the implementation of the field studies and have collected reproductive data at the wrong time of year (Barrett and Munkittrick, 2010). Gonad size is commonly used as a surrogate for reproductive output because it is much easier to obtain in the field. Our research has consistently shown that egg size was significantly affected by effluent exposure (Weber et al., 2008; Driessnack et al., 2011, Chapter 2 and 3). Furthermore, when fish were removed from the effluent source, their egg sizes increased significantly over a relatively short time period back to reference conditions (Driessnack et al., 2011). Female gonads often contain non-viable, partially resorbed eggs amongst the viable eggs, especially in fractional spawners such as FHM's. Consequently, assessing the gonad size as a whole, may not provide a clear indication of reproductive effects. Our recommendations would be that regulators consider a concurrent wild fish field study with a live diet multi-trophic FHM bioassay for a mine where significant fish reproductive effects (i.e., significant reductions in gonad size, and perhaps ovarian fecundity and egg size) have been observed in the EEM program. This would provide an opportunity for surrogate measures of changes in relative gonad size for a wild fish species to be compared to actual egg counts and sizes as well as relative gonad size in FHM.

4.8.2 Recommendations for the mining industry

Often in mining there are several mining operations within a relatively small geographic area due to the nature of the ore bodies, and often they may discharge effluent into the same receiving environment. However, the definition of effluent under the current regulations is all encompassing even though it could vary in type from surface runoff, to mine dewatering, to mill process water, and tailings water. It is critical for mitigation, regulation and management, when studying the receiving environment, to know which discharge (point source) is having the greatest effect on the environment in isolation of the other inputs. While this seems to be an obvious consideration for watershed management it is often overlooked when the same regulations are applied across very different discharge types for the same industry, in this case metal mining. To date, much of the research on the effects of metal contamination in fish populations has focused primarily on single metal exposures, either through water or diet (Carreau and Pyle, 2004; Kolts et al., 2009). However, there is evidence that metal mixtures (e.g., mining effluent) and their interactions with surface water can act in both a synergistic and antagonistic manner in the natural environment (Weber et al., 2008). Due to the complexity of

mixtures, the tendency in many studies is to focus hypothesis testing on several elements of interest despite the receiving environment being exposed to the effluent as a whole. While this may focus the experimental design, the overall conclusions can be misleading and of lower ecological relevance since no mine or milling operation discharges a single metal, micronutrient or element. It has often occurred where inferences of effects of single elements have resulted in significant regulatory focus and industry cost to undertake fate and distribution studies and the relevance to the mixture is minimized. The recent international focus on Se is an example of this especially as it relates to the discharge of treated mine effluents. Furthermore, treatment of whole effluent mixtures is rarely single element focused. Any modification to the effluent treatment process to reduce effects typically alters/reduces many elements concurrently. Thus, modifications of effluent quality should focus on reducing effects with specific identification of the causative element a secondary focus. Therefore our field study allowed us to examine three MME's simultaneously in order to determine which effluent was of greatest ecological importance with respect to FHM responses. We have been able to confirm that the PWE has consistently elicited the greatest effects to the aquatic biota of the three MME's tested and similar studies could help other mines isolate their research efforts as they move towards Investigation of Cause.

Furthermore there is a growing body of evidence to support the understanding of watershed-scale cumulative effects assessments in highly confounded watersheds in order to better understand the context and significance of individual effluent dischargers to effects in a broader watershed context (Munkittrick et al., 2000; Dubé and Munkittrick, 2001; Dubé, 2003; Dubé et al., 2006b; Squires et al., 2009). While regulation of individual mine discharges is currently required under the *Fisheries Act* if unregulated non-point source discharges upstream are causing much greater significant effects, for example, perhaps emphasis should be placed on management and mitigation of the non-point discharges while the mine discharge is expected to ensure its effluent quality, and associated and lesser effects, do not change or become worse. A cumulative effects assessment could be offered as a potential alternative study design to the control/impact, gradient or reference condition approach currently offered in the guidelines in cases where multiple discharges or industries are using the same receiving environment.

4.8.3 Recommendations for Vale

Focus should be placed on the PWE effluent stream with far lesser emphasis on the SWE and MWE. As mentioned previously, all of our research to date has shown that the PWE has elicited the greatest effects to the aquatic biota. Our field study has shown that this is primarily due to the nature of the effluent. The PWE differs considerably from the other two MME's because it receives a number of process water effluents including mine water and tailings from the Creighton Mine, Frood Stobie, North Mine, South Mine, Nickel Refinery, Copper Refinery, Smelter Complex and Clarabelle Mill (Stantec, 2009). It also receives inputs from active and inactive tailings, collected surface runoff from the Town of Copper Cliff, ON and sewage from the mine-related housing/administration offices and the Copper Cliff municipal sewage treatment plant (Stantec, 2009). As part of the metal beneficiation (metal extraction) process, especially during the milling process, many organic constituents are added to the process in order to separate the ore from the waste. There is little to no understanding of how these constituents (xanthates, alcohols, flocculants, polymers, organic reagents) interact in the effluent and furthermore, we have little understanding of their toxicity to aquatic organisms. During the course of our lab experiment we also discovered that the mine alters its treatment process in the winter due to extremely cold temperatures in order to maintain copper levels to below acceptable levels in the effluent. This is conducted through the addition of Nalmet to the effluent stream. Little information regarding the toxicity of Nalmet is available however, it has been shown to be toxic to daphnia magna (EC_{50} = 10 ppm) and rainbow trout (LC_{50} = 20ppm) (Vigneault et al., 2010 unpublished). Because very little is known about Nalmet it may be of benefit to conduct a toxicity identification evaluation (TIE) and/or toxicity reduction evaluation (TRE) to determine whether it is responsible for causing toxic effects in PWE. (http://maxxam.ca/services/ ecotoxicology/tre-tie-tte-2). Xanthates, thiosalts and lime ($CaCO_3$) have also been identified as constituents in effluent water that can drastically alter toxicity. TIE studies conducted at other mine sites have shown that thiosalts in particular mask the presence of other toxic substances in the effluent, xanthates are more toxic in effluent water than lab water (Novack 2010, unpublished), and an over abundance of lime could be toxic (Vigneault et al., 2010, unpublished). It may be of benefit to conduct a TRE of the PWE in conjunction with a TIE to identify other potential causative toxicant(s), isolate their source, evaluate toxicity control options and confirm a reduction of PWE toxicity (http://maxxam.ca/services/ecotoxicology/tre-

tie-tte-2). The TIE procedures within TREs are designed to identify specific substances responsible for effluent toxicity (http://maxxam.ca/services/ecotoxicology/tre-tie-tte-2). TIEs often consist of three phases:

- Characterization of the toxicant group
- Identification of specific causative toxicants
- Confirmation of causative toxicants

This data would help the mine to confirm the causative toxicants within the effluent and may indicate what adjustments to their ore processing could be made to reduce chemical inputs to the environment. Making adjustments to their milling processes may also indirectly remove some of the current metals of concern or improve the effluent effects as a whole.

4.8.4 Recommended improvements to mesocosm methodologies

4.8.4.1 Increased sample size

Increasing the sample size would help to reduce the variance of the mean and increase the power to detect a significant difference among treatments. A power analysis was conducted using an online program available from the Vanderbilt Biostatistics Department, University of Vanderbilt, Nashville, TN, using the means of four of the commonly tested endpoints in the EEM program [liver somatic index (LSI), gonadal somatic index (GSI), condition and egg size (ES)] (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize). Although cumulative egg production is one of the most sensitive endpoints for measuring reproductive effects in FHM it could not be conducted using a power analysis because the power analysis calculation is based on mean data and there is currently no method that we are aware of which can be used to calculate power on cumulative frequency data. Overall the power to detect a significant difference among the four endpoints was sufficiently high for assessing GSI, condition and egg size in the field experiment, however was not sufficient for determining effects in LSI (Table 4.4). In the lab experiments, the power to detect a statistical difference for LSI and GSI was low, however was sufficient for condition and egg size (Table 4.4). When this situation occurs it increases the probability of making a type II error (you conclude based on your stats that there is no effect when in fact there may be). Since the variance could also affect the power to detect a statistical difference, all attempts have been made to create a more homogeneous test group through the inclusion of one specific age group (<1 year old fish), analysis of one sex (females)

and by using only one breed of fish (FHM). Since we have already made all possible attempts to reduce the variance in the sample population our only solution for future studies would be to increase our power by increasing the sample size. Based on our field study, we have shown that a sample size of 8 was sufficiently powerful to detect a significant difference for most of the EEM-based endpoints (Table 4.4). However, data may be compromised when sample size dips down to 5. Sample size analysis of our current studies indicated that we would require anywhere from a sample size of 14-207 to have sufficient power to detect effects 95% of the time at a critical effect size of 25% (Table 4.4). A critical effect size of 25% has been proposed for the EEM program and has been associated with the ability to detect ecologically relevant differences among most species and endpoints (Munkittrick et al., 2009). However, it is unrealistic and unmanageable to propose a sample size of 207 for future studies in order to enable sufficient power to detect a statistical difference. Studies by Munkittrick (1992) have found that variance estimates in white sucker do not improve substantially beyond a sample size greater than 16, therefore our recommendation to increase the sample size to between 8 and 16 seems both manageable and appropriate for future mesocosm studies.

4.8.4.2 Pumping system

Small changes to the mesocosm system design could be made that would allow for greater replication and subsequently greater statistical power by replacing the centrifugal and diaphram pumps that are currently being used with a peristaltic pump for low turnover rates and low effluent dilution concentrations. The metering and centrifugal pumps were originally designed for use in larger mesocosm systems where flow rates of 80 L/h (1.33 L/min) were required to achieve one turnover per day and when used in this manner are highly accurate. The Pulsatron metered diaphram pumps have the ability to pump 500 gallons per day (1.31 L/min) and can handle large volumes of water more precisely. However, Masterflex peristaltic pumps are designed to be more accurate at lower flow rates of 0.001 to 34 ml/min and would be better suited for the smaller stream designs where flow rates of 0.14 L/min are required to achieve 1 turnover per day. Based on personal experience with the various pumps and the current design of the mesocosms, the peristaltics are much easier to use, they are easily calibrated and hold their flow rates at these low levels much more consistently and evenly compared to the metered pumps when the proper tubing is used. This new system configuration would also make it easier

Table 4.4 Summary of power analysis and sample size estimate of four key EEM endpoints [liver somatic index (LSI), gonadal somatic index (GSI), condition and egg size (ES)] measured in the three current mesocosm experiments comparing PWE (Process Water Effluent) to reference/control data. Critical effect sizes (CES) of 25% were used to estimate appropriate sample size at a power level of 95%.

							Reference			Exposure		
Treatment	Endpoint	CES	COV	α	β	Power	Mean	SD	Sample Size @ 95% power	Mean	SD	Sample Size @ 95% power
Field Experiment (n=8)	LSI	25	33.95	0.05	0.05	0.258	2.916	0.990	48	2.326	0.335	48
	GSI	25	7.59	0.05	0.05	1.000	8.682	0.659	2	12.703	4.624	2
	Condition	25	14.40	0.05	0.05	0.897	1.340	0.193	9	1.513	0.086	9
	ES	25	4.93	0.05	0.05	1.000	1.358	0.067	1	1.224	0.076	1
Exposure Pathway Experiment (n=5)	LSI	25	39.38	0.05	0.05	0.120	8.330	3.280	64	3.680	0.990	65
	GSI	25	51.88	0.05	0.05	0.088	22.300	11.570	112	10.690	2.670	112
	Condition	25	4.76	0.05	0.05	1.000	0.420	0.020	1	0.840	0.050	1
	ES	25	2.29	0.05	0.05	1.000	1.310	0.030	0	1.280	0.060	0
Food Quality Experiment (n=5)	LSI	25	24.72	0.05	0.05	0.252	4.410	1.090	26	4.770	2.030	26
	GSI	25	67.64	0.05	0.05	0.072	12.730	8.610	200	15.500	6.900	207
	Condition	25	18.18	0.05	0.05	0.450	1.100	0.200	14	1.150	0.160	15
	ES	25	1.53	0.05	0.05	1.000	1.310	0.020	0	1.290	0.030	0

COV= SD/mean*100
CES = Critical effect sizes at 95% power to detect statistically significant differenc

to maintain an even population of chironomids among the streams throughout the experiment by reducing turbulent flows within the streams. This would also enable us to increase our sample size up to 16 replicates per treatment with the use of 1 pump. By re-configuring the system design, we would be able to increase our sample size, reduce variability and increase our power to detect small differences in EEM endpoint fish responses. Overall, this may strengthen our studies while still maintaining some manageability in the field and lab. If we were to double our sample size but decrease the turnover rate to 1 turnover/day, we would still use the same volume of effluent and logistics would remain manageable.

4.8.4.3 Develop statistical tool to calculate power analysis on cumulative data

Our research (Dubé et al., 2006a, Rickwood et al., 2006a,b; 2008, Driessnack et al., 2011, Chapters 2 and 3) and others (Parrott and Wood, 2002; Parrott et al., 2004; Kovacs et al., 2005; Parrott, 2005) have shown that the reproduction endpoints are the most sensitive indicators of reproductive effects in FHM. Our studies in particular have identified: cumulative total egg production (total number of eggs produced/breeding pair/21 days of exposure); cumulative eggs/female/day (cumulative number of eggs produced/female/ each day - factoring mortality into the equation); cumulative spawning events (cumulative number of spawning events/breeding pair/21 days of exposure); mean egg production (mean number of eggs produced/female/21 days of exposure - factoring in mortality); mean total egg production (total mean number of eggs produced/breeding pair/21 days of exposure), and egg size (mean egg size/treatment), to be the most sensitive endpoints in determining effluent effects in fish. We have consistently shown over the last ten years that these reproductive endpoints are extremely valuable and our methodology could be a viable tool for the investigation of cause of effluent effects in the EEM program.

This endpoint however remains a topic of much debate because the method of quantification statistical evaluation cannot be agreed upon and is highly inconsistent in the literature. Furthermore, differences in methodologies have made it difficult for true comparisons to be made across studies, despite what has been reported in the literature. Some of the main differences in methodology are related to the sample size, number of replicates, experimental units of replication and statistical methods. The short-term reproduction bioassay developed elsewhere in the literature (Ankley et al., 2001; OECD, 2006; US EPA, 2007), and studies conducted by others (Parrott and Wood, 2002; Kovacs et al., 2005; Parrott et al., 2006; Bosker et

al., 2010) use multiple fish in a single replicate (e.g., 3-5 females and 2-4 males).This method of pooling the reproductive output would increase both the sample size and replication and would reduce the within treatment variability among streams. However, it also reduces the ability to track reproductive performance on an individual breeding pair basis, making extrapolation from biochemical-level responses to population-level effects impossible to determine. In addition it does not allow the assessment of individual reproductive endpoints or track metal accumulation or effluent effects from the parent to the offspring. Without the ability to determine which fish is affected, individual reproductive endpoints cannot be truly assessed.

We have been able to address all of the endpoints of interest (metal accumulation, LSI, GSI, reproduction, larval effects) while taking into account different levels of biological organization (biochemical, individual and population) and survivorship (mean, total and cumulative). Our research has shown that the most significant endpoints are associated with the replication changes which factor mortality into the equation (e.g., cumulative eggs/female/day, mean eggs/female/day, cumulative spawning). The experimental unit of replication for the Ankley/US EPA bioassays is the tank rather than the individual fish whereas the unit of replication of our modified bioassay is either the number of males/females (condition, LSI, GSI), breeding pair (mean egg production, spawning events, hatching success, deformities) or time (all cumulative endpoints). Consequently the manner in which these endpoints have been statistically tested has also varied considerably among studies. Despite the fact that cumulative number of eggs spawned has been portrayed in the literature with the number of days (time) used as the unit of replication, the actual statistical analyses have been conducted using ANOVA's (e.g., based on means) where the unit of replication is the tank, in many of these bioassay tests (Ankley et al., 2001; Bosker et al., 2010). This of course can be very misleading since it is more statistically robust to analyse cumulative data using a non-parametric Kolmorov Smirnov test because differences in egg distribution over time is what is actually being assessed in these cases.

We believe that the cumulative data in particular has been misrepresented in the current literature and our recommendation is that a proper protocol be developed for calculating the cumulative endpoints. Furthermore, there is currently no method to determine statistical power or sample size of the cumulative data in this way, which may be why this calculation remains a controversial topic of debate. When the unit of replication is time (day) and there is a minimum of 21 days of exposure, we can only assume that this unit of replication is sufficiently high to

provide enough power to detect a statistical difference. However currently there is no way of proving that this method of analyzing cumulative data is more powerful. We propose that a methodology be developed for calculating power and sample size on cumulative data so that there is a viable and defendable alternative method for researchers to use so that comparisons among studies can truly be made. We also recommend that this method consider the value of the pre-exposure data. Our lab statistically tests for any "pre-treatment" differences among breeding fish after randomly assigned to treatment groups and before effluent exposure begin. This ensures that fish with equal breeding output are allocated to treatment groups without bias. Statistical tests using pre-exposure data is not commonly reported in the literature and is recommended. In addition, our lab has observed very high pre-exposure breeding output that is then completely and instantly terminated with the start of effluent exposure and for the same fish. It is recommended that this "transitional" change between pre-exposure and exposure for each unit of replication be considered in the quantification of power. The "transition" is a highly powerful and obvious response not available in other chronic bioassays and should be considered.

4.8.4.4 Chironomus dilutus cultures

Although effluent effects on chironomids have already been established (Hruska and Dubé, 2004), with respect to the trophic transfer of metals through the food chain, it may be more beneficial to allow the chironomids to feed entirely on natural algae or biofilm from Junction Creek. Currently we have been supplementing their diet with Tetramin^{TM} slurry (Chapter 2 & 3), however our studies have shown that the Tetramin^{TM} appears to increase the organic matter in the streams (TOC and DOC) which may affect the bioavailability of the metals to the biota in the streams. Future studies should allow the chironomids to feed solely on the biofilm in order to experience a true dietary trophic transfer to accurately assess the role of the biofilm in the multi-trophic bioassay.

4.8.4.5 Biofilm measurements

In our field study we noted that the biofilm accumulated a significant level of metals compared to any other matrix analyzed and noted that it played an important role in the biavailability of metals in the streams. However, very little is known about the composition of

the biofilm or the species of algae and bacteria present. Investigations of the algae and bacterial communities in Junction Creek and the Vermillion River should be conducted to determine whether similar species are present in both the reference and exposure areas, since different species have different metal assimilation capacities. Furthermore, sulphate-reducing bacteria can play a key role in reducing the metal content in effluent water by precipitating them as metal sulphides. It would be highly beneficial to determine the species present to assess whether the biofilm is truly affecting bioavailability of metals in the effluent. Additionally, algal biomass as a measurement of the chlorophyll a content in the streams was not analyzed in this study and could also help quantify both metal bioavailability and the biomass of the algae portion of the biofilm in the water.

4.8.4.6 Cellular-level responses

Results of the tissue metals analysis identified a number of key metals of concern in the MME's. These studies give us a good indication of the ecosystem and population-level effects that may be occurring at the study site but lack the ability to link the metal of concern to a direct cause. By including cellular-level responses to the study design would allow a direct link to a potential cause and effect and provides a more meaningful result for a particular metal of concern. Future studies should consider both male and female histopathology and vitellogenesis analysis using single metal and effluent exposures to establish cause in order to validate or contest it as a metal requiring mitigation in the effluent treatment process. The current research indicates that gonad histopathological analyses would be most beneficial for cadmium, rubidium and selenium since they have the ability to alter vitellogenin, spermatogenesis/ oogenesis and reproduction in fish. Future studies may also wish to assess levels of estrogen mimics especially in the water and fish exposed to PWE since it is known to contain sewage waste, in order to verify or eliminate it as a potential cause for altered reproductive effects (vitellogenin, spermatogenesis etc.).

Liver and gonad histopathology would also be most beneficial for copper and nickel exposure since cellular damage and oxidation of proteins have been associated with both. Both are also highly regulated and cellular damage is most likely to be observed in the livers. Again, the studies should be conducted as single metal in conjunction with effluent mixtures in order to validate or contest each one as a metal requiring mitigation in the effluent treatment process.

4.8.4.7 Future Applications of the Mesocosm Design

Regardless of whether or not the power to detect a significant difference was sufficient in our current studies, biologically there was a distinct reduction in reproductive output and spawning events and egg size that cannot be ignored since we have consistently observed similar findings in most of our studies to date (Table 4.1). These findings have implications for the natural population in Junction Creek and for how studies may be conducted in the future. The most noteworthy finding of this research was associated with the food quality study. This study maintained statistically significant power (P=0.824) to detect differences among cumulative total egg production in FHM. This study was able to determine that when fish were held in the PWE and fed live benthic organisms, cumulative total egg production was reduced and we had significant power and confidence in our data to confirm this. This was a key finding which showed that multi-trophic live dietary pathways play a greater role in reproductive effects than a frozen laboratory prepared diet of chironomids. Furthermore, our initial exposure route lab study showed that omitting an exposure pathway (dietary or waterborne) can lead to very different results and thus the inclusion of the dietary component to these industrial studies is important and our recommendation would be to always include a multi-trophic or dietary component to all effluent studies.

We have also hypothesized that reduced egg production in PWE has been partially attributed to food quantity in the multi-trophic streams since the fish naturally graze on live chironomids in the streams and it is difficult to fully quantify how much they are eating compared to the waterborne only streams. Food quantity experiments where chironomid densities should be manipulated to gain better control over feeding rates would be the next logical progression of this research in order to determine if food quantity is directly associated with reproductive impairment. This information would be valuable to the EEM program since most mines across Canada have shown inhibitory effects with respect to the benthic invertebrate community [decreased taxon richness and density (phase 1)] (Lowell et al., 2007), but it is currently unknown how this relates to fish health and reproduction.

Also in light of the association of Se and egg size identified by Driessnack et al., (2011), similar egg size studies could be conducted using Vale's three MME's to try and assess possible linkages between Se exposure and egg size to better assess cause and effect. Furthermore,

metals of potential concern that have been identified in our research to date (e.g., Cu, Ni, Rb, Se, Sr and Tl) could be tested using single metal versus whole effluent testing to try and isolate cause and effect based on reproductive or biological endpoints (including cellular-level responses) to attempt at deriving similar cause and effect type relationships. The importance of testing single metal vs whole effluent is of importance to the development of guidelines for mines since metals are never found in the effluent in isolation of other metals and there is the potential for them to act in an additive and synergistic manner. There has been little to no work conducted on this issue and there are huge implications for mining operations since it is known how complex these natural systems are.

Other important applications of this technology could include the addition of natural sediment from the exposure and reference areas to assess current effluent effects from historical contamination and loading. Mesocosms could be used to assess current sediment toxicity in the natural reference and exposure areas to determine whether the mine has an effluent or sediment quality issue as a starting point in assessing environmental effects. The application of this technology offers endless possibilities for the mining industry as the EEM program moves towards investigation of cause. Mesocosms offers an environmentally relevant option that can assess current effluent effects in isolation of historical contamination in a multi-trophic system, using all components of the environment (water, sediment, fish, benthic organisms, algae), which is currently not possible with any of the alternative methods.

4.9 CONCLUSION

The field study allowed us to identify the point source discharge into Junction Creek of greatest priority. A weight of evidence approach was used to identify the prominent elements in the effluent mixtures which may have contributed to reproductive effects in FHM. The high metal content in the MME waters did not transfer appreciably through the food chain for most of the elements except Cu, and Se in the PWE. Effluent quality (increased water hardness, organic matter, presence of sewage in the effluent and differences in metal concentrations), fish energetics, and differences in bacterial (biofilm) growth in the streams appears to have played a role in reducing waterborne metal bioavailability to FHM. We also observed significantly higher accumulation of elements by the biofilm than chironomids and FHM, which also likely decreased waterborne metal bioavailability and toxicity in FHM exposed to MME's. Cu and Se

remain micronutrients of concern in the PWE since trophic transfer of both were observed in our current study as well as many other studies that have been conducted in the Sudbury area. Reduced cumulative egg production and cumulative number of spawning events were recorded in FHM exposed to PWE, possibly induced by dietary Cu and Se exposure and accumulation.

In the lab study, we assessed the relative importance of dietary and waterborne routes of exposure, and showed that metals assimilated differently in FHM tissues depending on the exposure route. Elevated metals were observed in one or more tissue type (carcass, liver and gonads) when FHM were exposed through water (Al, Ce, Sr, Cd), diet (Rb, Co, Al, Ce) or both routes (Mg, Pb, Cr, Va) of exposure. Condition factor and body weight was significantly increased, whereas LSI and egg size was significantly decreased when FHM were exposed through waterborne routes of exposure. Both routes of exposure were required to elicit significant effects for LSI. PWE had a stimulatory effect on egg production when exposed through one exposure pathway. However, when fish were exposed through both routes of exposure (EWEB), there was significantly decreased egg production and spawning events observed. These conflicting results showed that omitting an exposure pathway (dietary or waterborne) can lead to very different results.

In the food quality experiment (lab experiment 2), LSI and egg size were both significantly decreased when exposed to the PWE treatment compared to reference treatment, regardless of whether fish were fed live or frozen prey. Condition and body weight increased when exposed to the PWE, and when fish were fed live benthic organisms. However, significantly decreased egg production (cumulative total egg production, cumulative eggs/female/day) and spawning was observed when fish were held in the PWE and fed live benthic organisms. Conversely, a significant increase in cumulative total egg production, cumulative spawning events and cumulative egg production was observed when fish were fed live benthic organisms in the control treatment. Most of the reproductive and biological responses to FHM were elevated in the multi-trophic effluent streams where FHM fed on live chironomids confirming that the trophic transfer of metals is at least partially dependent upon food quality.

Overall, we identified several metals of concern (Cu, Ni, Rb, Se, Sr and Tl) in the MME's among all of the field and lab studies conducted by our lab. Future studies should consider assessing histopathology of the liver and gonads and the presence of estrogenic compounds in

MME's especially in PWE in both a single metal and effluent exposure. In addition, the algal and bacterial components of the biofilm should be identified and quantified since it has been identified as a significant sink of metals in the effluent and can greatly affect bioavailability. Other constituents in the effluent (Nalmet, xanthates, thiosalts and lime ($CaCO_3$)) should be examined through a TIE or TRE investigation to assess their role in toxic responses to FHM. Vitellogenin levels in gonad tissues would also help to assess cause of reproductive impairment and may give an indication of organic or estrogenic exposure. Dissolved metals analysis and speciation of the key metals would also help to better predict metal bioavailability in the presence of these organic compounds. Future studies should also be conducted to specifically address the development of a sound method for estimating power and determining appropriate sample sizes so that cumulative data may be included in the alternative methods section of the EEM program as a viable endpoint.

These studies have made a significant contribution to our understanding of the effects of MMEs on fish and benthic invertebrates by assessing differences in response patterns under different conditions (field vs lab). They also further validate the mesocosm approach as a viable alternative to field studies and provide valuable information to the Canadian Environmental Effects Monitoring (EEM) Program.

Chapter 5

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APPENDIX A

APPENDIX A: Table 1 Summary of mean (± SEM, n=5) biological and reproductive endpoints measured in FHM females in the exposure pathway (Experiment I) and food quality (Experiment II) studies. Significant treatment effects (a), dietary effects (b) and interactions (c) were observed when $p < 0.05$ when data was analyzed using a Two-Way ANOVA or Scheirer Ray Hare test. Cumulative data was analyzed using, Kolmorov-Smirnov, asterisks represent significant effects when *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Biological Endpoints	**Exposure Pathway (Exp 1)**				**Food Quality (Exp 2)**			
	CWCB	**CWEB**	**EWCB**	**EWEB**	**CWCB**	**EWEB**	**MT-Control**	**MT-Effluent**
Total length (cm)	4.82 ± 0.06	4.8 ± 0.07	4.87 ± 0.29	4.7± 0.21	4.82 ± 0.06	4.7± 0.21	5.02 ± 1.31	5.08 ± 0.13
Body weight (g)	0.48 ± 0.06	1.00 ± 0.08**c**	1.24 ± 0.16**ac**	1.00 ± 0.13**ac**	0.48 ± 0.06	1.00 ± 0.13**a**	1.41± 0.16**b**	1.51± 0.14**ab**
Liver weight (g)	0.043 ± 0.01	0.042 ± 0.0	0.063 ± 0.01	0.038 ± 0.0	0.043 ± 0.02	0.038 ± 0.0	0.065 ± 0.01	0.08 ± 0.02
Adult Survival	0.90 ± 0.10	1.00 ± 0.0	0.70 ± 0.12	0.90 ± 0.10	0.90 ± 0.10	0.70 ± 0.12	0.80 ± 0.12	0.60 ± 0.19
Condition [(body wt/length(cm)^3) *100]	0.424 ± 0.02	0.89 ± 0.04**c**	1.07 ±0.04**ac**	0.94 ± 0.02**ac**	0.424 ± 0.02	0.94 ± 0.02**a**	1.10 ± 0.10**b**	1.15 ± 0.08**ab**
LSI [Liver weight vs body weight]	8.33 ± 1.47	4.16 ± 0.32**b**	5.06 ± 0.23**a**	3.68 ± 0.0**ab**	8.33 ± 1.47	3.68 ± 0.0**c**	4.41 ± 0.54**bc**	4.77 ± 1.01**bc**
Reproductive Endpoints								
Gonad weight	0.13 ± 0.04	0.10 ± 0.01	0.14 ± 0.01	0.11 ± 0.02	0.13 ± 0.04	0.11 ± 0.02	0.19 ± 0.07	0.24 ± 0.06
Gonad weight vs body weight (GSI)	25.3 ± 5.2	10.0 ± 0.89	11.1 ± 0.33	10.7 ± 1.34	25.3 ± 5.2	10.7 ± 1.34	12.7 ± 4.3	15.5 ± 3.5
Fecundity	52.7 ± 20.6	97.3 ± 34.2	35.9 ± 21.6	68.1 ± 18.8	52.7 ± 20.6	68.1 ± 18.8	74.0 ± 31.0	22.3 ± 11.6
Egg size vs length	0.027 ± 0.0	0.028 ± 0.0	0.027 ± 0.0	0.027 ± 0.0	0.027 ± 0.0	0.027 ± 0.0	0.026 ± 0.0	0.027 ± 0.0
Egg size vs body weight	2.92 ± 0.43	1.42 ± 0.18	1.14 ± 0.15	1.34 ± 0.19	2.92 ± 0.43	1.34 ± 0.19	0.87 ± 0.0	0.86 ± 0.01
Cumulative eggs/female/day	4.30 ± 1.14	8.01 ± 1.98**	5.52 ± 1.83	2.65 ± 0.75	4.30 ± 1.14**	2.65 ± 0.75	7.71 ± 2.30**	1.24 ± 0.32***
Cumulative total egg production	471± 110	1006 ± 208***	506 ± 119	411 ± 78	471± 110	411 ± 78	882 ± 164**	118 ± 26***
Cumulative spawning events	10.95 ± 2.9	9.14 ± 2.38	5.62 ± 1.45***	5.09 ± 1.30***	10.95 ± 2.9	5.09 ± 1.30***	6.52 ± 1.36***	3.71 ± 0.74***
Mean eggs produced	37.0 ± 22.8	79.2 ± 52.6**c**	37.9± 22.6**c**	30.3 ± 21.2**c**	37.0 ± 22.8	30.3 ± 21.2	59 ± 42.8	9.67 ± 9.37
Mean eggs/female/day	0.38 ± 0.43	0.75 ± 0.50**c**	0.55 ± 0.33**c**	0.29 ± 0.20**c**	0.38 ± 0.43	0.29 ± 0.20	0.82 ± 0.70	0.11 ± 0.11
Mean days to hatch	2.27 ± 0.14	2.24 ± 0.11	3.80 ± 0.36**b**	2.89 ± 0.26**b**	2.27 ± 0.14	2.89 ± 0.26**b**	2.40 ± 0.16	3.67 ± 0.33
Hatching success	0.66 ± 0.17	0.83 ± 0.01	0.95 ± 0.03**b**	0.96 ± 0.01**b**	0.66 ± 0.17	0.96 ± 0.01**b**	0.69 ± 0.15	0.84 ± 0.12
Mean fertilization success	0.97 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.98 ± 0.00	0.97 ± 0.00	0.98 ± 0.00	0.99 ± 0.00	0.98 ± 0.01
Mean larval survival (Day 5)	0.63 ± 0.15	0.79 ± 0.04	0.93 ± 0.04	0.76 ± 0.09	0.63 ± 0.15	0.76 ± 0.09	0.78 ± 0.09	0.88 ± 0.06
Mean larval deformities	1.78 ± 1.5	1.46 ± 0.50	0.34 ± 0.27	1.13 ± 0.66	1.78 ± 1.5	1.13 ± 0.66	0.07 ± 0.09	0 ± 0

APPENDIX A: Table 2 Summary of mean (± SEM, n=5) biological endpoints measured in FHM males in the exposure pathway (Experiment I) and food quality (Experiment II) studies. Significant treatment effects (a), dietary effects (b) and interactions (c) were observed when p <0.05 when data was analyzed using a Two-Way ANOVA or Scheirer Ray Hare test. Cumulative data analyzed using, Kolmorov-Smirnov, asterisks represent significant effects when *p<0.05, **p<0.01 and ***p<0.001.

Biological Endpoints	**Exposure Pathway (Exp 1)**				**Food Quality (Exp 2)**			
	CWCB	**CWEB**	**EWCB**	**EWEB**	**CWCB**	**EWEB**	**MT-Control**	**MT-Effluent**
Total length (cm)	6.00 ± 0.15	6.22 ± 0.09	6.1 ± 0.09	6.12 ± 0.09	6.00 ± 0.15	6.12 ± 0.09	6.8 ± 0.30	6.28 ± 0.29
Body weight (g)	2.10 ± 0.25	2.30 ± 0.12	2.31 ± 0.08	2.08 ± 0.07	2.10 ± 0.25	2.08 ± 0.07	3.73 ± 0.51	2.53 ± 0.40
Liver weight (g)	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.01	0.15 ± 0.00	0.109 ± 0.01
Adult Survival	0.80 ± 22.4	1.00 ± 0.00	0.80 ± 22.4	1.00 ± 0.00	0.80 ± 22.4	1.00 ± 0.00	0.40 ± 24.5	0.80 ± 22.4
GSI [gonad weight vs body weight]	1.38 ± 0.12	1.17 ± 0.25	1.54 ± 0.21	2.00 ± 0.38	1.38 ± 0.12	2.00 ± 0.38	1.16 ± 0.29	1.46 ± 0.21
Condition [(body wt/length(cm)^3) *100]	0.96 ± 0.07	0.97 ± 0.08	1.02 ± 0.03	0.91 ± 0.06	0.96 ± 0.07	0.91 ± 0.06	1.18 ± 0.00	1.00 ± 0.09
LSI [Liver weight vs body weight]	3.01 ± 0.61	2.66 ± 0.31	3.10 ± 0.45	3.07 ± 0.80	3.01 ± 0.61	3.07 ± 0.80	4.06 ± 0.51	4.59 ± 0.91