IMPROVING ENVIRONMENTAL RELEVANCE OF A STANDARD FISH BIOASSAY

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By

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ABSTRACT

The overall objective of the research conducted and described in this thesis was to develop an environmentally relevant bioassay to assess the effects of complex effluents on a sentinel fish species. A short-term fathead minnow (FHM) reproductive bioassay was utilized to assess the effects of industrial effluents on multiple levels of biological organization (sub-organismal to population endpoints). The FHM bioassay was tested in both lab and on-site investigations using an artificial stream system. The incorporation of trophic-transfer into the bioassay was also developed to quantify the importance of contaminated food as a source of exposure. This work was conducted in two key phases. Phase I focused on testing and developing the FHM bioassay, in the lab and on-site with pulp mill effluent (PME), to firstly document response patterns and, secondly, to conduct an investigation of cause study. Phase II focused on developing the trophic-transfer system to document responses to metal mine effluent (MME) in the lab and on-site in an artificial stream system. Development of the trophic-transfer system was also conducted during this phase to compare responses to standard water-only exposures.

In Phase I, exposure to PME in both the lab and field studies resulted in disruptions in egg production and spawning events. By focusing on identifying response patterns I was able to determine that the effects observed were indicative of an estrogenic response. I was also able to identify a process stream that was the potential cause of responses observed after exposure to final effluent. Isolation of this process stream will assist the mill in developing approaches for future mitigation. The results from this research will also provide additional data for the

environmental effects monitoring (EEM) program for pulp and paper and investigation of cause studies on a national basis.

In Phase II, in both the field and laboratory investigations, significant decreases in reproductive output (egg production and spawning events) were observed in the water-only system exposures. Significant decreases in hatching success and increases in deformities were observed in the trophic-transfer system only, suggesting that the combination of both food and water was important in assessing the effects on the F1 generation. Overall, the responses in the trophic-transfer system were not comparable between the lab and field studies. In the lab study, significant decreases in reproductive output occurred, compared to the field study where significant increases in egg production and spawning events occurred. In addition, the effects on the F1 generation in the field study were not as severe as those observed in the lab investigation. It was concluded that the presence of reference water and the environment within the trophic-transfer system were responsible for this reduction in toxicity.

Phases I and II of this research have made significant contributions to artificial stream development within Canada for the assessment of industrial effluents and their effects on aquatic biota. The results from these studies have also demonstrated that environmentally relevant testing is essential if we are to accurately assess effects on aquatic biota. Future development and application of this bioassay should be towards developing a standardized approach for not only assessing the effects of industrial effluents in a comparative manner, but also in investigation of cause studies.

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Finally, to my family (Mum, Dad, Mike, Kaye and Emily): I love you all without question – thank you for all your support.

"Goodbye, and thanks for all the fish."

D. Adams - Hitchhikers guide to the galaxy

To my loving parents:

You have shown me nothing but unconditional love and support.
I am very proud to be your daughter.

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LIST OF ABBREVIATIONS

Abbreviation

µg/g = micrograms per gram

µg/L = micrograms per litre

µL = microlitre

µm = micrometre

µM = micromolar

11-KT = 11- ketotestosterone

Admt = air dried meteric tones

Ag = silver

Ah = aryl-hydrocarbon

Al = aluminum

ANCOVA = analysis of covariance

ANOVA = analysis of variance

AR = androgen receptor

As = arsenic

ASB = aerated stabilization basin

B = boron

Ba = barium

Be = Beryllium

Bi = bismuth

BKME = bleached kraft pulp mill effluent

BOD = biochemical oxygen demand

CAC = combined acid stream

CaCO3 = calcium carbonate

CALK = combined alkaline stream

CCME = copper cliff mine effluent

CCMWW = copper cliff mine effluent and municipal waste water combined

CCWTP = copper cliff waste water treatment plant

Cd = cadmium

Cf = condition factor

ClO2 = chlorine dioxide

cm = centimetre

cm^2 = centimetres squared

CME = combined mill effluent

Co = cobalt

Cr = chromium

CSC = combined stripped condensates

Cu = copper

d = day

DDT = dichloro-diphenyl-trichloroethane

DHT = dihydrotestosterone

DO = dissolved oxygen

DOC = dissolved organic carbon

DOM = dissolved organic matter

EC50 = median effect concentration

EE_2 = ethynyl estradiol

EEM = environmental effects monitoring

ELISA = enzyme linked immunosorbent assay

ER = estrogen receptor

F = fluoride

Fe = Iron

FHM = fathead minnow

ft = foot

g = gram

GSI = gonadosomatic index

h = hour

H_2O_2 = hydrogen peroxide

H_2SO_4 = sulphuric acid

IC25 = median inhibition concentration

IOC = investigation of cause

kg = kilogram

km = kilometers

KS = Kolmogorov-Smirnov

KWALLIS = Kruskal Wallis

L = litre

LC50 = median lethal concentration

LCC = low contaminated condensates

LD = lack of data

Li = Lithium

LOEC = lowest observed effect concentration

LSI = liversomatic index

m = metre

m3 = cubic metre

m3/y = cubic meters per year

MeHg = Methyl mercury

MFO = mixed function oxygenase

mg = milligram

mg/kg = milligrams per kilogram

mg/L = milligrams per litre

mL = millilitre

mm = millimetre

MME = metal mine effluent

Mn = manganese

Mo = molybdenum

MT = methyltestosterone

MWTP = municipal waste water treatment plant

MWW = municipal waste water

n = number of samples

NaOH = sodium hydroxide

ND = non-detectable

ng =nanogram

ng/L = nangrom per litre

NH3 = un-ionised ammonia

NH4+ = ionized ammonia

Ni = nickel

nm = nanometre

NM = not measured

NOEC = no observed effect concentration

O2 = oxygen

ºC = centigrade

OECD = organisation for economic co-operation and development

P = phosphorus

PAH = Polycyclic aromatic hydrocarbon

Pb = lead

PME = pulp mill effluent

PWQG = Provincial Water Quality Guideline

Rb = rubidium

RIA = radioimmunoassay

RM = repeated measures ANOVA

RO = reverse osmosis

Ru = ruthenium

Se = selenium

SE = standard error

Sn = tin

TIE = toxicity identification evaluation
Tl = thallium

TOC = total organic carbon

TSS = total suspended solids

USEPA = United States Environmental Protection Agency

UV = ultra violet

V = vanadium

v/v = volume to volume ratio

WBL = weak black liquor

Zn = zinc

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Comparative toxicology is founded on the basis of using consistent bioassays to assess the relative toxicity of different contaminants. Standard, acute, short-term (<96h) toxicity tests with fish have been used for many years to determine the median lethal concentration (LC50) of contaminant exposure (Environment Canada, 2002). These bioassays are typically conducted as waterborne exposures with single species, single contaminants and under tightly controlled laboratory conditions (e.g., temperature, dissolved oxygen, water quality and photoperiod). The rainbow trout (*Oncorhynchus mykiss*) test, for example, exposes adults to contaminants for 48 to 96 hours to determine the lethal concentration at which 50% of the test population dies (Rand, 1995). These standardized tests have been invaluable in providing toxicologists with an understanding of acute chemical toxicity and for establishing conservative guidelines for the protection of freshwater aquatic life (Rand, 1995).

Aquatic organisms are exposed to a multitude of complex contaminants in both dissolved and particulate phases. Industrial effluents, in particular, are a challenge to toxicologists as they are a complex chemical mixture and predicting their fate and effects in the receiving environment has proven difficult (Kovacs et al, 1997; Munkittrick et al, 1997a). In Canada, acute toxicity tests are used to assess changes in effluent quality at the "end-of-pipe", however, these tests have limited usefulness for assessing toxicity of chemical mixtures on aquatic biota living in waters receiving complex effluents. Often the species used in the tests are not relevant to the habitat being studied and, with improvements in environmental legislation and stewardship, effluents are no longer discharged to the environment at lethal levels (Dubé, 2000). Therefore, using mortality as an assessment endpoint is not relevant in these instances.

Sub-lethal toxicity tests have been developed to detect more subtle changes in biota exposed to lower concentrations of contaminants (USEPA, 1994). Many studies now implement sub-lethal tests to determine a median effect concentration (EC50) so changes at the biochemical, physiological, behavioural or life cycle level can be measured (Mason, 1996). For example, fathead minnow (*Pimephales promelas*) are used to determine no observed and lowest observed effect concentrations (NOEC and LOEC respectively) by measuring growth, reproduction and survival (Rand, 1995). However, as with acute tests, sub-lethal toxicity tests are typically conducted with single species and contaminants, with endpoints focused on a particular life stage, and in tightly controlled laboratory conditions. Although these tests also serve a purpose for assessing effluent quality "end-of-pipe" or for contaminant screening, they have not proven to be effective in many cases for predicting how effluents may affect biota in the receiving environment (Robinson, 1994; Kovacs and Megraw 1995; Tucker and Burton, 1999).

The overall objective of the research conducted and described in this thesis was to develop an environmentally relevant bioassay to assess the effects of complex effluents on a sentinel fish species. Development of the bioassay focused on four key areas: 1) incorporation of population-level endpoints into the toxicological assessment of effluents by utilizing a standard short-term fish reproductive bioassay; 2) identification of consistent endpoints for use with the bioassay that would allow comparison of effects across different effluents; 3) transferability of the bioassay between the lab and the field using an artificial stream system; and 4) incorporation of trophic-transfer into the bioassay to quantify the importance of food as a source of exposure. If a more relevant bioassay could be developed for assessing fish responses to complex industrial effluents, this would improve our ability to assess effects of

effluent discharges on fish and provide a basis to compare the relative toxicity of different effluent mixtures.

1.2 Relevant endpoints

Industrial effluents from the pulp and paper and metal mining industry are of particular concern as they represent a significant part of total industrial discharges into Canadian waters (Environment Canada, 2005). In 2000, there were approximately 125 pulp and paper mills in operation across Canada generating approximately 90 to 130 million L of effluent per day per mill (Environment Canada, 2005). There are also currently 100 to 110 metal mines operating in Canada, typically discharging mine effluents into small, headwater streams where the effluent dominates the flow of the system (Environment Canada, 2005). Many studies within Canada have documented numerous effects in wild fish downstream of both pulp and paper mill (Munkittrick et al, 1991, 1998; Haley and Hall, 2000; Kovacs et al, 1997) and metal mine (Brady and Morris, 1996; Jaagumagi and Bedard, 2002) effluent discharges.

Effluents from pulp and paper mills (Munkittrick et al, 1997a) and metal mines (Dubé et al, 2006) often affect reproductive indicators and are known endocrine disruptors. Munkittrick et al (1997b) have consistently observed depressions in sex steroids and reduced gonad sizes in wild fish (white sucker [*Catostomus commersoni*]) and lake whitefish [*Coregonus clupeaformis*]) downstream of pulp mill effluent discharges. Dubé et al (2006) observed depressions in testosterone (five-fold reduction) and survival in an investigation into the effects of metal mine effluent on creek chub (*Semotilus atromaculatus*). In an executive summary from Environment Canada (2005) it was also stated that the present day concerns from metal mine effluent include the longer-term effects of chronic exposure to low levels of metals, bioaccumulation, sediment contamination and endocrine disruption

(Environment Canada, 2005). These investigations have shown that both pulp and paper and metal mine effluents have the potential to disrupt reproductive endpoints in fish. Thus, a bioassay that intends to assess these effluents needs to consider and incorporate these endpoints.

Currently, in Canada, the discharge of effluents from the pulp and paper and metal mining industries are regulated under the Environmental Effects Monitoring (EEM) program of the *Fisheries Act*. The EEM program aims to measure the effects of effluents on fish, fish habitat and the use of fisheries resources by humans. It goes beyond end-of-pipe assessments and directly examines effects on the receiving aquatic environment (Environment Canada, 2004a). Fish surveys conducted through the EEM program aim to assess changes in indicators of fish growth, reproduction, condition and survival. Individual endpoints (e.g., liver size, condition, gonad size, size-at-age) are used to assess these indicators in fish and the information gathered from the fish survey assists in interpretation of the health of the receiving environment.

Measuring these numerous physiological endpoints has provided a wealth of information regarding the health of sentinel fish downstream of effluent discharges. However, extrapolating these responses to determine ecosystem health has proved problematic and has, to date, not been accurately demonstrated (Calow, 2003; Forbes and Calow, 1999; Power and McCarty, 1997). Therefore, there is an immediate need to develop monitoring tools that can assess the effects of these industrial effluents on different levels of biological organization, so we can better understand the significance of these individual endpoints and how they relate to population level responses.

From an ecological perspective, populations and communities are of most concern, not individual organisms (Adams et al, 2002). It is, therefore, important that we are able to not only monitor individual responses, but also simultaneously quantify responses at a population and/or community level. We can link changes at the population level to the community level by measuring two main parameters:

1. Size of the population within the community at any given time (e.g. abundance, biomass); and
2. Persistence of the population within the community over a longer period of time (e.g. successful reproduction, recruitment). Persistence of a species within a community can be measured using parameters associated with reproductive output including survival of offspring, number of offspring produced, and successful production of gametes (Atrill and Depledge, 1997).

Toxicologists have long been aware of the importance of reproductive fitness as an indicator of population health (Kramer et al, 1998; Panter et al, 2002; Datson et al, 2003). Anthropogenic chemicals which disrupt reproductive ability through, for example disruption of the endocrine system, are a major cause for concern (Fox, 2001; Hewitt and Servos, 2001). Several studies have demonstrated the importance of reproductive endpoints for measuring population level effects in response to environmental pollution (Gray et al, 1999; Forbes and Depledge, 1992; Parrott and Wood, 2001). Measuring reproductive endpoints, therefore, allows a quantitative estimation of the persistence of the population and helps to understand the structure of the resulting community (Attrill and Depledge, 1997).

1.3 Fish life-cycle tests

Full and partial life-cycle tests offer an alternative bioassay approach to measure the effects of complex mixtures on reproductive endpoints. Life-cycle tests using small-bodied fish, such as the fathead minnow (FHM), are useful because they monitor multigenerational reproductive effects in a short amount of time (120 days) due to the rapid life cycle of this species. Life-cycle tests are currently being implemented in many monitoring programs (Haley and Hall, 2000; USEPA, 2002), but full life-cycle tests are resource intensive and logistically problematic. In response to the need for a more abbreviated assay, Ankley et al (2001) developed a short-term (21-day) reproductive bioassay using FHM that assesses reproduction, as well as aspects of early development in a time frame shorter than traditional life-cycle assays.

The Ankley protocol was designed to include endpoints that measure various levels of biological organization including biochemical (i.e., sex steroid levels, vitellogenin, histopathology), individual (i.e., weight, length, condition, secondary sex characteristics) and population (i.e., egg production, hatching success, larval deformities) level. However, to date, the endpoints measured with the short-term bioassay have not been consistently applied. For example, Ankley et al (2001) assessed the reproductive effects of estrogenic (methoxyclor) and androgenic (methyltestosterone) compounds. However, pathological assessment of the gonadal tissue and larval assessment was not included in the experimental design. Jensen et al (2004), in their investigation into the effects of the anti-androgen flutamide on FHM, did not include assessment of deformities or pathological assessment of the gonadal tissue. Depending on the hypothesis, the number of endpoints included within the experimental design was altered which affects the ability to compare the toxicity of contaminants.

A number of investigations have used the FHM short-term bioassay to monitor effects of estrogenic (Harries et al, 2000, Ankley et al 2001; Sohoni et al, 2001), androgenic (Ankley et al, 2003) and anti-androgenic (Jensen et al, 2004) compounds. These studies have predominantly focused on single contaminants that do not represent the complexity of effluents. A limited number of investigations into the effects of industrial effluents using the short-term FHM bioassay have been conducted (Martel et al, 2003; Parrott et al, 2005). In a study with pulp mill effluent, Martel et al (2003) showed that egg production had ceased in one of six effluents tested after 28 d exposure at 20% (v/v) effluent, and that vitellogenin induction was the most frequently observed response. However, all of these studies have used differences in methodology (e.g., number of independent replicates, number and type of endpoints measured) and were conducted under laboratory conditions. To date, no evaluation of the reproductive effects of metal mine effluent has been conducted using the FHM short-term bioassay. A national assessment to develop strategies to improve the quality of pulp and paper mill effluent in Canada has been initiated (Kovacs et al, 2006). One objective of this research is to standardize the endpoints and methods used to assess if effluents are causing reproductive effects in fish because, to date, assessments have been undertaken by individual groups using widely different approaches, making comparisons of effluent toxicity difficult. This national assessment is using both full and partial life-cycle FHM bioassays, but all investigations will be laboratory based.

After review of the literature, the short-term FHM bioassay appeared to be the most suitable starting point for development of a more environmentally-relevant bioassay to assess and compare complex mixture effects (i.e., pulp and paper and metal mining) on fish. A disadvantage of the bioassay however, is that high variability in egg production has been

documented for replicates within a treatment. Fathead minnow are repeat batch spawners and undergo rapid cyclical changes over short periods of time (3-5 days). Therefore, the size and stage of gonads can vary considerably between individuals (Harries et al, 2000). This variability has made interpretation of results on the effects of effluents on reproductive endpoints difficult (Martel et al, 2003). The short-term bioassay uses naïve reproductively-active adult fish in a 4:2 (4 females: 2 males) ratio. In an investigation to assess effects of endocrine disrupting chemicals, Harries et al (2000) noted that by using breeding pairs (1 male: 1 female) a more accurate comparison of reproductive performance before and during contaminant exposure could be conducted. Therefore, by using breeding pairs in my investigations, interpretation of results and identification of response patterns after exposure to complex effluents may be improved.

1.4 Fathead minnow

The aim of toxicity tests should be to assess the impact of contaminants on species that naturally inhabit the system under investigation, and so, are subject to that environment's ambient conditions (Chapman, 2002; Attrill and Depledge, 1997). FHM have been chosen as a biomarker species in previous laboratory investigations as they represent an ecologically significant part of the Cyprinidae family, they have been extensively tested, and a large database of knowledge exists regarding their culture and life-cycles (Panter et al, 2002; Ankley et al, 2001; Jensen et al, 2001). They are also used in risk assessment and government/industry monitoring studies on an international scale (USEPA, 1982, 1996, 1999, 2002; OECD, 1992; Shaw et al, 1995a,b). The use of small-bodied fish such as FHM has also been recommended in the EEM program in Canada to measure responses of aquatic organisms to significant point source discharges (i.e., pulp and paper and metal mining

effluents) (Munkittrick et al, 2002). This recommendation is founded on the basis that small-bodied fish species are typically more abundant, easier to catch and have reduced mobility improving their potential for effluent exposure compared to larger bodied fish (Gray et al, 2002; Munkittrick et al, 2002). In addition, FHM are small (average length of 6 cm and width of 1 cm), fractional, substrate spawners that, under specific conditions, can easily be manipulated in captivity to produce clutches of 50-150 eggs every 3-5 days. They are also an environmentally relevant species as they are abundant in freshwater systems across Canada. Thus, a standardized short-term reproductive bioassay that assesses the effects of effluent mixtures on FHM would be highly relevant.

1.5 Obtaining realism

By developing a consistent method using the short-term FHM bioassay it will be possible to routinely monitor the effects of industrial effluents. However, at the present time, this assay has only been used under controlled laboratory conditions and therefore has limited environmental relevance. Previous investigations have shown that species responses in the lab are not necessarily predictive of responses observed in the field (Robinson et al, 1994; Hall and Giddings, 2000). It has been problematic extrapolating results to those observed in the field due to the complex interaction between abiotic and biotic factors that are not replicated in laboratory studies (Kovacs and Megraw 1996; Sibley et al, 1999; Tucker and Burton, 1999). Therefore, there is a need to develop a method that improves the environmental relevance of the lab-based bioassay that will allow assessment of the effects of complex effluents in an environmentally realistic way.

Current laboratory toxicity tests are an integral part of investigating effects of contaminants on a species, however more complex studies are required if we are to predict

toxicity to organisms in the receiving environment (Chapman, 2002). Depending on the type of effect and contaminant being investigated, the endpoints measured in laboratory toxicity tests can be extensive. Laboratory studies allow accurate determination of an effect at a specific concentration whilst controlling variables (photoperiod, temperature, food, test species, water quality) that normally fluctuate in the field. Environmental relevance is therefore restricted and this lack of 'real world' interpretation may limit the potential of these assays to determine effects in situ (Munkittrick et al, 1994; Robinson et al, 1994).

By comparison, field studies are the ultimate indicator of realism and give an indication of the state of health of the ecosystem under investigation (Graney et al, 1995). When multiple monitoring techniques are used, it is possible to assess both community (e.g. changes in diversity indexes, biomass, or age) and individual (e.g., changes in biochemical, physiological and histopathological indicators) level responses. The weakness of field studies, however, lies in their inability to determine cause and effect relationships due to the diverse nature of an ecosystem (Langlois et al, 1997; Larsson et al, 1997). In addition, lack of replication and limited knowledge of exposure history (concentration and duration) can reduce the accuracy of assigning causality in field monitoring studies (Culp et al, 1996). There are many confounding factors that can make field studies difficult to interpret (Kovacs et al, 1997):

- Natural and anthropogenic sources of stress can elicit similar physiological responses in fish;
- Natural variability (season, time and habitat) is hard to separate from anthropogenic influences;

- Site accessibility can be a problem as well as obtaining adequate sample sizes; and
- Identifying an appropriate reference/control site with identical properties, except for the anthropogenic source, is problematic.

A few aquatic field studies have been highly successful in manipulating causal factors to demonstrate ecological consequences. For example, manipulation of the field environment has taken place in the Experimental Lakes Area of northeastern Ontario, Canada, where phosphorus enrichment (Schindler et al, 1971; Schindler 1980) and exposure to organochlorines (Kidd et al, 1999) were studied. These studies have provided extensive knowledge on the responses of ecosystems to contaminant exposures. However, the ability to replicate these experiments using industrial effluents is limited due to the scale and logistical requirements of the investigations.

Thus, laboratory and field studies have their benefits and disadvantages (Table 1.1) when investigating cause and effect relationships. The establishment of causality is an important final stage in any investigative framework (Clarke and Warwick, 1994). Confounding factors, such as multiple stressors and habitat and natural variability, make determination of cause in field studies difficult (Kovacs et al, 1997; Hall, 1996; Kloepper-Sams and Owens, 1993). In addition, assessing the impact of a complex mixture of contaminants when influenced by a suite of environmental variables is difficult to predict from laboratory experiments (Attrill and Depledge, 1997).

Many authors agree that by building a weight of evidence approach, by integrating results observed in the field and from controlled laboratory exposures, an accurate prediction of cause and effect may be made (Culp et al, 2000; Lowell et al, 2000). However, studies have

shown that the responses observed in lab tests are often less sensitive than those observed in the field. Laboratory studies assessing steroid levels in goldfish (McMaster et al, 1996) and FHM (Robinson, 1994) after exposure to pulp mill effluent (PME) showed that in general, results from the lab were less sensitive than the field observations in which circulating steroid levels in wild fish were significantly reduced (McMaster et al, 1995; Munkittrick et al, 1991, 1992, 1994). As such, the laboratory tests would have reduced ability to predict potential contaminant effects in the field (Munkittrick et al, 1998). There may be a number of factors contributing to this discrepancy in lab studies, such as:

1. Reduced volumes of effluent used in the lab studies;
2. Increased storage of contaminants in the lab studies and resultant changes in contaminant chemical characteristics;
3. Static renewal in the lab versus flow through conditions in the field;
4. Reduced influence of natural fluctuations in the lab; and
5. Increased challenge to field organisms due to complexity and natural stressors of the field environment.
6. Differences in species analysed and exposure periods

It is problematic when assessing risk if we are firstly unable to accurately quantify in the lab an effect that is indicative of a real world response. It is also problematic if we are unable to accurately assess whether the observations from field studies are a response to a contaminant or due to natural fluctuations. Inaccurate assessment of either lab or field studies could lead to an over- or under-estimation of a problem, potential or otherwise. It is therefore, quite clear that the gap between the lab and field needs to be filled by development

Table 1.1. The advantages and disadvantages of laboratory and field studies in risk assessment.

	LABORATORY	FIELD
DISADVANTAGES	• Difficult to extrapolate to other species • Difficult to extrapolate to the field • Lack of environmental realism • Short exposure duration reduces ability of test species to adapt • Effects often more severe than those in the field (contaminant bioavailability can differ in the field)	• Difficult to extrapolate to other ecosystems • Cause and effect determinations difficult • Less or no control of parameters (difficult to experimentally manipulate) • Difficult to determine dose-response • Difficulty in selecting appropriate reference sites or "controls" • Unavoidable pseudo-replication
ADVANTAGES	• Often fast and easy to perform • Statistically more precise • Low test variability • Increased replication • Provides directly quantifiable, causal data • Provides knowledge of dangerous chemicals • Eliminates indirect effects • Good controls	• Acclimation of species (Longer exposure may result in species adaptation and resilience) • Assesses realistic impacts on ecosystem • Specific climatic and physical environmental conditions taken into account • Historical contamination taken into account • Relevant species sampled

of a bioassay that can accurately assess effects of a particular contaminant source whilst maintaining environmental relevance.

1.6 Artificial streams

There has been substantial research over the last few decades in developing artificial streams to assess effects of contaminants/toxins on algae, invertebrate and fish populations (Lamberti & Steinmen, 1993; Dubé et al, 2002a,b). The field-based artificial stream systems that have been developed at the National Water Research Institute of Environment Canada are able to assess effects of point-source effluents on aquatic organisms in situ (Figure 1.1). Artificial streams bridge the gap between lab and field research by controlling many of the variables encountered in field studies, and at the same time increasing environmental relevance compared to laboratory studies. By using artificial streams it is possible to control the amount, concentration and duration of exposure in conditions that reflect the ambient environment, i.e., photoperiod, temperature and water quality. Another important benefit to using artificial streams is increased statistical power compared to field studies. By increasing the number of replicates used in a study, the power to accurately determine an effect is also increased (Dubé et al, 2002a; Culp et al, 1996).

For industries regulated in Canada conducting EEM (i.e., pulp and paper, metal mining), there is a critical need to develop improved methods due to a number of problems encountered in the field monitoring programs, e.g., hazardous sampling conditions, presence of confounding influences, or an inability to catch adequate numbers of fish species leading to an inability to measure the effects of effluents on fish (Munkittrick et al, 2002). In the latest EEM field cycle, Environment Canada recommended that alternative methods such as artificial streams be considered for fish and benthic sampling at confounded sites

(Environment Canada, 2002). Artificial streams, therefore, represent a vital area of research that can be utilized in the development of an environmentally realistic bioassay to determine population/community level effects in situ.

1.7 Trophic-transfer

Testing reproductive effects in FHM in artificial streams in situ will result in a bioassay with increased environmental relevance. However, as it is a single-species test and fish are currently fed during experiments, the test only evaluates contaminant transfer through the water. Taking the bioassay a step further, to a multi-species level, would allow assessment of not only water-borne, but also food-borne exposure effects, which would be more indicative of a real world situation. There have been numerous studies investigating the relative importance of trophic-transfer of metals (Chen et al, 2000; Mason et al, 2000; Ni et al, 2000; Xu and Wang, 2002) and organic contaminants (Clements et al, 1994; Nendzaa et al, 1997; Muir et al, 1999; Egeler et al, 2001) in aquatic environments highlighting dietary uptake as a significant route of exposure in addition to water-borne exposure in both fish (Liu et al, 2002; Besser et al, 2001) and invertebrates (Ingersoll et al, 1995). However, the majority of these studies were conducted under laboratory conditions and focused primarily on fate rather than effect. They were also concerned mainly with single contaminants, such as dichloro-diphenyl-trichloroethane (DDT), polychlorinated biphenyls (PCB's) and methyl mercury (MeHg) that have chemical properties (lipophilicity and chemical stability) amenable to biomagnification. There are a wealth of complex contaminants that continually enter into an ecosystem, and their occurrence and potential toxicity to aquatic life may present an environmental hazard (Reinfelder et al, 1998). There has been, to date, no research assessing

Figure 1.1. Small (a) and large (b) field-based mobile artificial stream systems developed by National Water Research Institute of Environment Canada (modified from Dubé et al, 2002a).

population level effects in fish through trophic-transfer of complex effluent mixtures. There is a need, therefore, to develop a multi-species bioassay that can assess the relative contribution of both trophic-transfer and water-borne exposures to effects on population end-points.

Multi-trophic bioassays have been developed in artificial streams to assess effects at a community level, but have predominantly been used to assess benthic foodwebs (e.g. algae and benthic invertebrates) (Dubé and Culp, 1996; Podemski and Culp, 1996; Culp et al, 2000). No comparisons have been made between multi-species and fish-only bioassays to assess how fish responses are affected by different routes of exposure (water versus food) (Dubé et al, 2002a; 2002b). Therefore, no quantitative method exists to assess the contribution of trophic-transfer of contaminants to toxicity. Furthermore, studies in artificial streams with fish have focused on endpoints such as growth and organ size changes. The reproductive endpoints that were measured included gonadosomatic index (GSI) and hormone levels, with no reproductive endpoints assessed at a population level (e.g., fecundity, hatching success) (Dubé et al, 2002a; 2002b). Therefore, there is a basis to develop a bioassay that can quantitatively assess whether food and/or water-borne exposure to complex effluents leads to population level effects by assessing reproductive endpoints such as fecundity and hatching success. This would be a significant development in identifying cause and effects in situ that has not been achieved with lab or field studies alone.

1.8 *Chironomus tentans*

A reasonable prey species to use for a trophic-transfer system are midge larvae *Chironomus tentans*. *Chironomus tentans*, a freshwater macroinvertebrate, is commonly used as an indicator of water quality because of its abundance in aquatic ecosystems (Mousavi et

al, 2003). They are present in freshwater systems across Canada within British Columbia, Alberta, Saskatchewan, Manitoba, Ontario and Quebec and, therefore, are an environmentally relevant species to use (Environment Canada, 1997).

Chironomus tentans are easy to culture and handle in the laboratory and they have been successfully reared on-site in previous artificial stream investigations (Hruska and Dubé 2004, 2005). They have several life stages consisting of an egg mass, larva, pupa and adult (mature fly). Many studies have shown impacts on growth, emergence and reproduction during numerous sediment toxicity studies (Ingersoll et al, 1995; Leppanen et al, 1998; Squires, 2006). Most importantly, previous investigations have shown that FHM reproduce well in both short- and long-term life-cycle tests when fed larval *C. tentans* (B. Blunt pers.comm, Environment Canada, Burlington, ON). We can also assess the reproductive responses of *C .tentans* (emergence and densities) after exposure to effluents and in the presence of predators. Therefore, they are an ideal species for use to develop a trophic-transfer bioassay.

1.9 Research objectives

My overall research objective was to develop an environmentally realistic bioassay for use in mobile artificial stream systems that could assess population level effects from complex effluent exposures on FHM and *C. tentans*. Research objectives by chapter have been provided in Table 1.2. Bioassay development was carried out in two phases.

Phase I: From lab to field - development of environmentally relevant FHM bioassay

- Objective 1: Laboratory testing of the FHM bioassay with a model contaminant mixture (PME) under controlled conditions.

- Objective 2: In situ testing of the bioassay with a model contaminant mixture (PME) using a known artificial stream system

Phase II: Stepping closer to 'real world' responses - development of an environmentally-relevant trophic-transfer bioassay

- Objective I: Laboratory development and testing of a trophic-transfer bioassay in an artificial stream system using a model contaminant mixture (MME).
- Objective II: *In situ* testing of the trophic-transfer bioassay with a model contaminant mixture (MME) in an artificial stream system.

Table 1.2 Research objectives by chapter

Chapter	Phase	Objectives	Description of Chapter
1		• Introduction	• Background information including development of fish bioassays, artificial stream systems, critical gaps in knowledge and description of FHM and *C. tentans* biology
2	PHASE I	• To conduct laboratory testing of the FHM bioassay to assess effects of treated PME	• Laboratory investigation conducted in 2003 assessing effects on FHM after exposure to control water and two concentrations of PME
3		• To conduct on-site testing of the FHM bioassay to assess effects of treated PME	• Field investigation conducted in 2003 in a temperature controlled bioassay trailer using reference water and two concentrations of PME. Published: Environmental Toxicology and Chemistry, *25, 191-201*
4		• To conduct on-site testing of the FHM bioassay to identify the source/cause of reproductive effects observed in Chapter 3	• Field investigation conducted alongside the Chapter 3 study using selected waste streams from within the mill. Published: Environmental Toxicology and Chemistry, *25, 202-211*
5	PHASE II	• To develop and conduct laboratory testing of the trophic-transfer bioassay to assess effects of MME	• Laboratory investigation conducted in 2004 in an artificial stream system under controlled temperature conditions with a single MME. Submitted: *Environmental Science and Technology*
6		• To conduct on-site testing of the trophic-transfer bioassay to assess effects of MME	• Field investigation conducted in 2005 in an artificial stream system on-site in ambient conditions using reference water, MME and municipal waste water
7		• General synthesis, discussion and conclusions	• An overview of results observed in each chapter and summary of key findings. Description of ecological and industrial significance of the results and suggested future directions

CHAPTER 2[a]

Development of a modified fathead minnow (*Pimephales promelas*) bioassay and testing in the laboratory with pulp and paper effluent

[a] This chapter has been submitted to the Water Quality Research Journal of Canada under joint authorship with Monique G. Dubé (University of Saskatchewan)

2.1 Introduction

The importance of reproductive fitness to the health of fish populations has been documented extensively (Kramer et al, 1998; Panter et al, 2002; Datson et al, 2003). Anthropogenic compounds that reduce the ability of organisms to reproduce by disrupting the endocrine system are a major cause for concern (Fox, 2001; McMaster, 2001). Several studies have demonstrated the importance of reproductive endpoints for measuring population level effects in response to environmental pollution (Forbes and Depledge, 1992; Attrill and Depledge, 1997; Gray et al, 1999; Parrott et al, 2001).

A number of studies over the last few decades have documented the potential of pulp mill effluents (PME) to affect reproductive health of fish (Andersson et al, 1988; Sandstrom, 1988; Van der kraak, 1992). Some of the first Canadian studies to assess the responses of wild fish downstream of a pulp mill were conducted in Jackfish Bay on the north shore of Lake Superior (Munkittrick et al, 1991, 1998; McMaster et al, 1995). A number of effects were observed in these fish including decreased gonad weights (gonadosomatic index [GSI]) and serum steroid levels in lake whitefish (*Coregonus clupeaformis*) and white sucker (*Catostomus commersoni*). Since these early studies, investigations into the health of sentinel fish populations downstream of pulp mill effluent discharges in Canada have been conducted under the direction of the EEM regulated under the *Fisheries Act.*

The EEM program was initiated to assess the environmental effects of PME entering Canadian waters by assessing fish health and fish habitat (Environment Canada, 2004b). The health of sentinel fish is assessed by measuring indicators of energy storage (e.g., liver size, condition) and energy use (gonad size, weight and length). However,

considerable debate exists over the ecological relevance of these endpoints as extrapolation from these individual level effects to assessing impacts at the population and community level has still not been accurately demonstrated (Robinson, 1994; Kovacs and Megraw, 1995; Kovacs et al, 1997; Kovacs et al, 2006). There is a need to assess the effects of PME at multiple levels of biological organization, e.g., reproductive output (egg production and spawning events), effects on F1 generation (hatching success and deformities) as well as endpoints at the individual level (gonad and liver size), so we may better understand the significance of these individual endpoints and how they relate to population level responses.

In Jackfish Bay, effects of PME continue to be documented in wild fish despite significant improvements in mill processing (Environment Canada, 2004a). To date, there are still a number of issues regarding causality, including difficulty quantifying exposure, ecological relevance of the reproductive indicators used in field studies (gonad size, steroid levels) and inconsistent responses between species and over time (Kovacs et al, 1997). Therefore, there is a need to investigate the potential of PME, discharged into Jackfish Bay, to affect the reproductive fitness of fish by assessing endpoints at the biochemical, individual and population level. By using controlled exposures and assessing different levels of biological organisation we may be able to establish a response pattern to better understand the effects observed.

A short-term (21 d) FHM bioassay, developed by Ankley et al (2001) assesses various levels of biological organisation i.e. biochemical (steroid levels, vitellogenin, histopathology), individual (weight, length, condition, secondary sex characteristics) and population (egg production, hatching success, larval deformities). This FHM bioassay has

been recently used to assess effects of treated PME on FHM reproduction (Martel et al, 2003; Borton et al, 2003). Martel et al (2003) showed that egg production ceased after 28 d exposure to 20% PME from a multi-process mill. In addition, vitellogenin induction was shown to be the most frequently observed response. The most sensitive and consistent endpoints affected by PME in this study was reproductive output i.e. egg production and spawning events. By using the short-term FHM bioassay, a better understanding of the effects of PME on various levels of biological organisation was achieved. Therefore, the FHM bioassay would be a useful tool to investigate the effects, and ultimately identify a response pattern for PME from Jackfish Bay.

The Ankley et al (2001) protocol combines four females and two males in one replicate to monitor reproductive performance. High variability in egg production has been documented in studies using this ratio (Martel et al 2003) as FHM are repeat, batch spawners and undergo rapid cyclical changes over short periods of time (3-5d). Therefore, the size and stage of gonads can vary considerably between individuals. Harries et al (2000) in their investigation into endocrine disruption on FHM used breeding pairs (1 male:1 female). By using pairs rather than a group scenario (4:2) a more accurate comparison of reproductive performance before and during contaminant exposure can be conducted (Harries et al, 2000; Ankley and Johnson, 2004). Therefore, using breeding pairs in this investigation may aid in identifying accurate response patterns after exposure to PME.

The objectives of this study were two-fold. Firstly, to modify the current short-term FHM reproductive bioassay to use breeding pairs to assess various levels of biological organisation. Secondly, to test the modified bioassay under laboratory conditions to

characterize a bioassay response pattern for a model compound (PME) and for a mill that has documented reproductive effects on wild fish

2.2 Methods

Experiments were conducted for 60 d during April and May, 2003 in a temperature controlled laboratory located at the National Water Research Institute, Saskatoon, SK. Six month old, naïve FHM were obtained from cultures at NWRI, Saskatoon, SK. Breeding pairs were placed into 16 L aquarium (1 pair per aquarium) that contained one spawning tile which was examined daily for eggs. The study was conducted under control conditions (16:8 light:dark photoperiod, temperature $20^{o}C \pm 1^{o}C$) and each pair was fed frozen brine shrimp twice daily ad libitum. A full description of the FHM bioassay is explained by Ankley et al (2001). A brief description of the protocol follows.

2.2.1 Pre-exposure

A breeding trial, consisting of 24 breeding pairs, was conducted over 21 d to determine activity of each pair and to acquire baseline data for the following endpoints: survival, egg production, spawning events and secondary sex characteristics of breeding adults, as well as hatching success and larval deformities in the F1 generation. Egg production was determined daily; tiles were checked for eggs at 10:00 am every morning, allowing time for spawning and fertilization to be completed and for eggs to water-harden (Ankley et al, 2001). The eggs were rolled off the tile into hatching cups (250 mL PVC jars with Nitex screened bottoms) which were then placed in aquaria with aerated laboratory water that was renewed daily. The total and viable numbers of eggs were counted daily allowing estimation of cumulative egg production (over 21 d) and mean

and total egg production (no. eggs/female). Eggs were determined to be viable if they had a defined yolk within the egg sac and their structural integrity was maintained. The eggs were checked daily for fungal infections and any dead eggs were removed and numbers recorded. Once larvae hatched (usually around 5 to 7 days after spawn) they were assessed for deformities under a dissecting microscope (6.4 x magnification); deformities included scoliosis, lordosis and yolk sac edema. The total number of viable larvae (hatching success expressed as % of viable eggs) and deformed larvae (% of total larvae hatched) were recorded. Secondary sex characteristics of FHM consist of banding, nuptial tubercles, dorsal pad and fin dot (male index) normally observed in males and ovipositor presence and size (ovipositor index) normally observed in females. Each secondary sex characteristic was evaluated based on a points system (Table 2.1) and were summed with equal weight to give an overall male and/or ovipositor index as outlined by Parrott and Wood (2001).

After 21 d, selection of breeding pairs for the effluent exposure was undertaken based on the following criteria: 100% (v/v) survival of all adults, presence of eggs in each replicate once a week, and >80% fertilization of eggs (OECD, 2003). Based on these criterion, three breeding pairs per treatment were randomly assigned to each of three treatments [control, 100% (v/v) and 50% (v/v) treated PME].

2.2.1.1 Pre-exposure data-analysis

Data were analyzed using SPSS 11.0 (SPSS, Chicago, Il, USA). Levene's Test was used to confirm parameteric assumptions for homogeneity of variance. One-way analysis of variance (ANOVA) was then conducted to determine if there were any differences

between tanks for the following endpoints: egg production (mean number per pair after 21d), hatching success (% of viable eggs produced) and deformities (% of total hatch). No significant differences were found ($\alpha = 0.05$), confirming that there were no significant differences in reproductive output between aquaria before exposures began. Thus, the unit of replication for egg production, spawning events, hatching success and deformities was breeding pairs (*n* = three per treatment). Unit of replication for weight, length, condition factor, gonadosomatic (GSI) and liversomatic index (LSI), testosterone and histopathology was three (*n* = three females and n = three males per treatment).

2.2.2 Exposure

2.2.2.1 Pulp mill effluent

The PME used in this experiment was collected from the Terrace Bay pulp mill which discharges into Jackfish Bay, Lake Superior, ON, Canada. The Terrace Bay mill is a bleached kraft pulp mill and features both hardwood and softwood pulp production in separate lines. The hardwood mill (mill 1) went on-line in 1948 and uses a wood furnish of poplar and aspen for pulp production. The softwood mill (mill 2) commenced operation in 1978 and uses furnish of spruce (predominantly black spruce), jack pine, and balsam fir. Each mill has its own bleach plant, with bleaching sequences DEDED (mill one) and DEopDEpD (mill two) (D = chlorine dioxide (ClO2) E = caustic extraction, Eop = caustic, peroxide and oxygen extraction, Ep = caustic and peroxide extraction).

Effluent for our study was collected from the final discharge point after secondary effluent treatment in a three-celled aerated stabilization basin with a total volume of 1.1 million m^3 and a residence time of approximately 10 d (Environment Canada, 2004b).

Exposure concentrations selected for this study were 100% (v/v) and 50% (v/v) PME. In Blackbird Creek, the natural receiving environment, documented concentrations of approx. 80% PME have been measured (Environment Canada, 2004b). Therefore 100% (v/v) PME was chosen to represent a worse-case exposure scenario and 50% (v/v) PME was chosen to document a dose-response.

Treated final PME was collected from the outflow point of the secondary treatment lagoon once per week and shipped to our laboratory (5 d) in Saskatoon where it was stored in a 1000 L holding tank, at room temperature, for a maximum of 7 d. Effluent (100 L for 100% (v/v) and 50 L for 50% (v/v)) was supplied daily to 116 L holding tanks. Effluent was delivered from each holding tank to three aquaria per treatment at a total flow rate of 16 L per d per aquarium. This maintained the turnover time to one full exchange per aquarium per 24 h period. Eggs and larvae were held in corresponding treatment tanks where water was changed daily. Daily observations and measurements, i.e. egg production, hatching success, deformities etc. were the same as those taken during pre-exposure. Conductivity and dissolved oxygen were monitored daily in each aquarium using a YSI meter (Yellow Springs Instruments, Yellow Springs, OH, USA). Ammonia (Rolf C. Hagen, Edmonton, Canada) and pH (Oakton pHTestr 3, Oakton Instruments, Vernon, IL, USA) were also recorded daily.

The fish were exposed for 21 d and biological endpoints were re-measured at this time. At the end of the exposure period the following endpoints were also measured: muscle testosterone, weight, length, gonad and liver weight and gonadal histopathology. Fish were anaesthetized (30µl per l clove oil), assessed for secondary sex characteristics, and fork length (mm) and total body weight (g) recorded. Fish were euthanised by spinal

Table 2.1. Point system for grading secondary sex characteristics in fathead minnow (*P. promelas*). Developed from Parrott and Wood (2002) and Martel et al (2003).

	MALE INDEX				OVIPOSITOR INDEX
CHARACTERISTIC	Nuptial Tubercles	Dorsal Pad	Dorsal fin dot	Banding	
SCORE	Absent: 0 Present: 1	Absent: 0 Small: 1 Medium: 2 Large: 3 Very Large: 4	Absent: 0 Present: 1	Absent: 0 Present 1	Absent: 0 Small: 0.5 Medium: 1 Large: 2 Very Large: 3

severance. Gonads in the adults were weighed (to 0.001 g) and placed in formalin (10%) for 24 h and then stored in ethanol (75%) until histological analysis was undertaken at the University of Saskatchewan using the quantitative method developed by Weber et al (2002). Gonadal tissue was paraffin-embedded, sectioned once and mounted on slides. The stage of ovarian development was assessed using an Olympus BH-2 light microscope at 200 x magnification and the number of ovarian follicles at oogonial, previtellogenic, vitellogenic, preovulatory and atretic stages were counted. The percent of follicles at each stage of development was expressed as a percent of the total number of follicles per view, and the mean calculated from four fields of view per slide. The stage of testes development was assessed at 1000 x magnification on an Olympus BH-2 light microscope, the number of spermatogonia, primary or secondary spermatocytes and spermatids or mature sperm stages were counted. The percent of each stage of development was expressed as a percent of the total number of stages per view and the mean calculated from four fields of view per slide. Histopathology in both males and females included the appearance of fibrosis and eosinophilia (excessive eosin staining), both scored according to severity (score 1 = minor to 5 = severe). In males, cell death was also assessed by counting the number of dead cells (from all stages) per view and the mean calculated from four fields of view per slide. In females, atretic follicles were also assessed by counting the number per field of view and calculating a mean from four fields of view per slide. Condition factor was calculated as Cf=100*[total weight (g)/standard length^3 (cm)]. Gonadosomatic index (GSI) and liversomatic index (LSI) were calculated as 100*[tissue weight (g)/total weight (g)]. Fish carcasses (excluding head and caudal fin) were then stored in a -80^{o}C freezer until muscle steroid testosterone

analysis was undertaken. Muscle sex steroids (i.e. testosterone) were measured using a radioimmunoassay (RIA) technique (McMaster et al, 1992b) optimized for muscle homogenate for FHM.

2.2.2.2 Exposure analysis

To test parametric assumptions for homogeneity of variance, Levene's test was used. If data did not require transformation, an ANOVA, followed by a post-hoc Tukey test, was performed on the following endpoints: hatching success (% of viable eggs produced), deformities (% of total hatch), stages of gonadal development (histological analysis), pathology (scored data were calculated as a % of total score possible) and muscle testosterone levels. If Levene's test for homogeneity was significant, the percentage-based data were arcsin (%) transformed and tested again for homogeneity. Data that were not percentage or ratio-based were log10 transformed. A one-way ANOVA, followed by a post-hoc Tukey test, was then performed on the transformed data. If assumptions could not be met for homogeneity of variance, a non-parametric Kruskal-Wallis test was performed on the non-transformed data. To assess responses over time Kolomogorov-Smirnov tests were conducted to assess the cumulative frequency of spawning events and eggs produced in each treatment over 21d compared to controls. Kolmogorov-Smirnov two-sample tests were also conducted on ovipositor development in both males and females. The appearance of male secondary sex characteristics in females was assessed using contingency tables. Water quality data (temperature, pH, ammonia, and dissolved oxygen) were analyzed using an ANOVA to determine if treatments differed significantly from control water. Samples taken over time within each

treatment were the unit of replication. Assumptions and post hoc tests were applied as discussed above.

2.3 Results

2.3.1 Water quality

No significant differences amongst treatments were observed for temperature, ammonia, or dissolved oxygen (ANOVA, p=942, p=0.147, p=0.963, respectively). A significant decrease (4%) in pH was observed in the 50% (v/v) PME treatment compared to control (ANOVA p=0.001). Specific conductivity was significantly increased (430%) in the 100% (v/v) PME treatment compared to control (ANOVA, p=0.047) (Table 2.2).

2.3.2 Individual endpoints

In males, a significant increase (109%) in GSI was observed after exposure to 100% (v/v) PME compared to control treatments (ANOVA, p=0.047). LSI increased by 53% in the 100% (v/v) PME treatment compared to control, however this was not significant (ANOVA, p=0.087). No other significant differences were observed in male FHM (Table 2.3). In females, a 40% increase in GSI was seen after exposure to 100% (v/v) PME although this was not statistically significant. No other endpoints were significantly different in either the 50% (v/v) or 100% (v/v) PME treatment compared to control (Table 2.3).

2.3.3 Biochemical endpoints and gonadal histology

In males, testosterone was not significantly altered after exposure to either 50% (v/v) or 100% (v/v) PME treatments (ANOVA, p>0.05) (Table 2.3). Gonadal staging in males

and females was also not significantly altered. However, the appearance of eosinophilia in male gonads was significantly increased in both the 50% (v/v) and 100% (v/v) PME treatments (ANOVA, $p=0.014$, $p=0.035$) (Figure 2.1).

2.3.4. Secondary sex characteristics

In females, no significant development of male characteristics was observed after exposure to either 50% (v/v) or 100% (v/v) PME (chi-square, $p>0.05$). In males, no significant ovipositor development was observed in either treatment (chi-square, $p >0.05$) (data not shown).

2.3.5 Reproductive output

A significant decrease in egg production (86%) and spawning events (82%) was observed throughout the 21 d exposure to 100% (v/v) PME (Kolmogorov-Smirnov, $p<0.001$, $p<0.001$ respectively) compared to control (Figures 2.2 and 2.3). A significant decrease in egg production (53%) was also observed after exposure to 50% (v/v) PME, however, spawning events were unaffected (Kolmogorov-Smirnov, $p=0.005$, $p>0.05$ respectively) (Figure 2.2 and 2.3).

2.3.6 F1 offspring

Hatching success was significantly reduced and occurrence of deformities significantly increased in the 50% (v/v) PME treatment compared to control (ANOVA, $p=0.026$, KWALLIS, $p=0.008$, respectively). No statistical analysis could be conducted on the 100% (v/v) PME treatment due to lack of egg production in this treatment ($n=2$ spawns). However, for the few eggs that did survive and hatch for this treatment, a severe

Table 2.2. Water quality parameters measured throughout the 21 d exposure to treated pulp mill effluent (50% (v/v) and 100% (v/v) PME) and control water. Values represent means ± standard error. Asterisk denotes significant difference from control where ** = $p<0.01$

PARAMETER	CONTROL	50%	100%
Temperature (^{o}C)	19.7 ± 0.23	19.9 ±0.23	19.6 ±0.22
Specific Conductivity (µS/cm)	245 ±43.5	933 ±78.7	1319 ±178*
pH	7.91 ±0.05	7.56 ±0.01***	7.79 ±0.01
Ammonia (mg/L)	0.07 ±0.01	0.08 ±0.01	0.08 ±0.01
Dissolved Oxygen (%)	93.6 ±0.18	93.2 ±0.58	93.9 ±0.96
Sample size	21	21	21

Table 2.3. Individual and biochemical endpoints measured in fathead minnow after 21 d exposure to treated pulp mill effluent (50% (v/v) and 100% (v/v) PME) and control water. GSI = gonadosomatic index, LSI = liversomatic index, CF = condition factor. Values represent means ± standard error. Asterisk denotes significant difference from control where * = $p<0.05$

PARAMETER	MALES			FEMALES		
	CONTROL	50%	100%	CONTROL	50%	100%
Wt (g)	4.42 ± 0.10	5.06 ± 0.52	4.29 ± 0.25	2.32 ± 0.14	2.51 ± 0.53	2.01 ± 0.02
GSI	1.10 ± 0.04	1.73 ± 0.25	2.30 ± 0.22*	10.5 ± 2.37	12.1 ± 2.43	14.8 ± 1.35
LSI	1.98 ± 0.23	1.62 ± 0.33	3.05 ± 0.52	3.29 ± 0.59	3.53 ± 0.62	4.28 ± 0.49
CF	1.74 ± 0.03	1.78 ± 0.12	1.83 ± 0.12	1.43 ± 0.15	1.62 ± 0.15	1.43 ± 0.12
Testosterone (ng/g)	1.41 ± 0.15	2.20 ± 0.29	1.85 ± 0.13	0.82 ± 0.01	0.78 ± 0.18	0.81 ± 0.08
Sample size	3	3	3	3	3	3

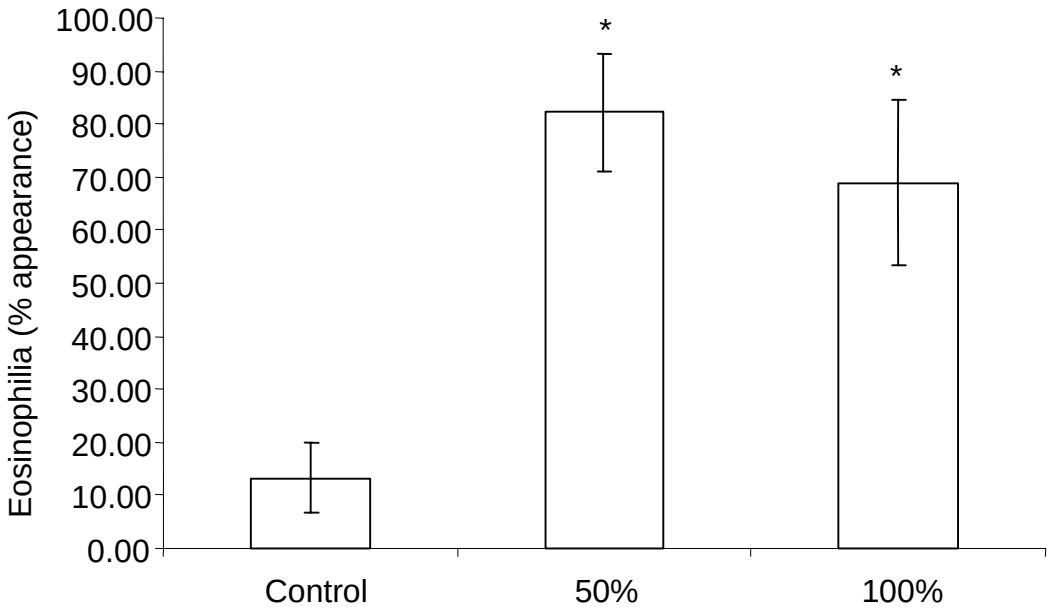

Figure 2.1. Appearance of eosinophilia (%) in male fathead minnow gonadal tissue after 21d exposure to treated pulp mill effluent (50% (v/v) and 100% (v/v) PME) and control water. Values represent means ± standard error (n=3). Asterisk denotes significant difference from control where * = $p<0.05$.

reduction in hatching success and an increase in deformities were observed compared to control (Figures 2.4 and 2.5). The predominant deformity observed in larvae was yolk sac edema (99%) in both the 50% (v/v) and 100% (v/v) treatments.

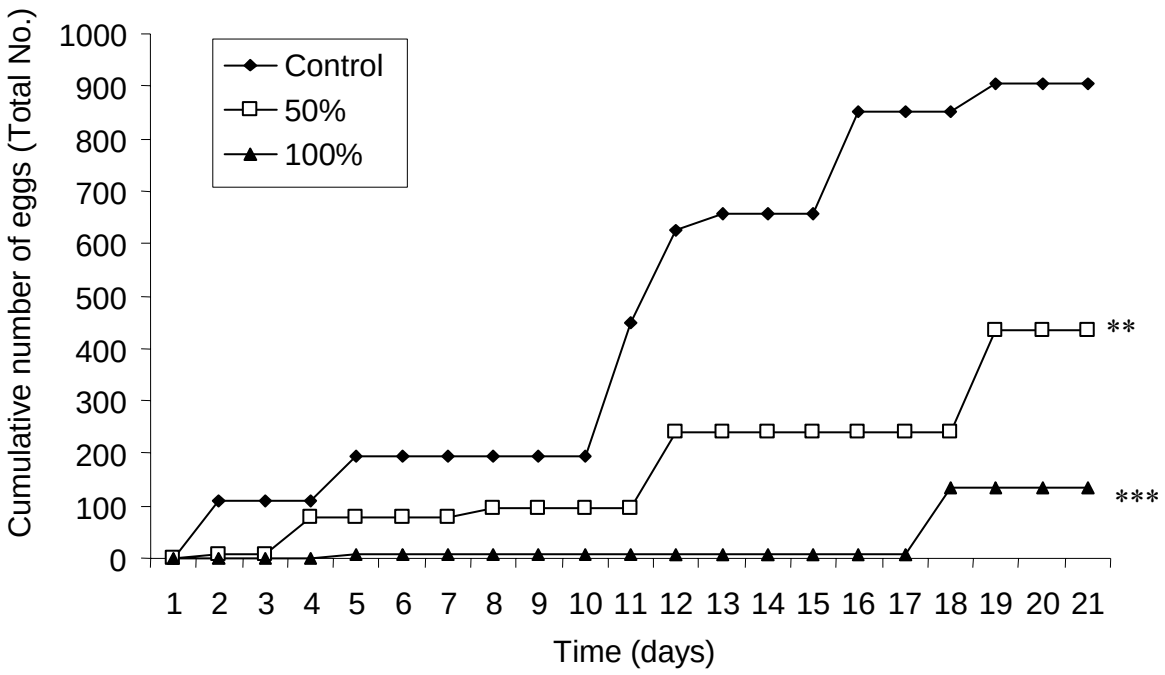

Figure 2.2. Cumulative number of eggs produced by fathead minnow during 21 d exposure to treated pulp mill effluent (50% (v/v) and 100% (v/v) PME) and control water. Asterisk represents significant difference from control where ** = $p<0.01$ and *** = $p<0.001$.

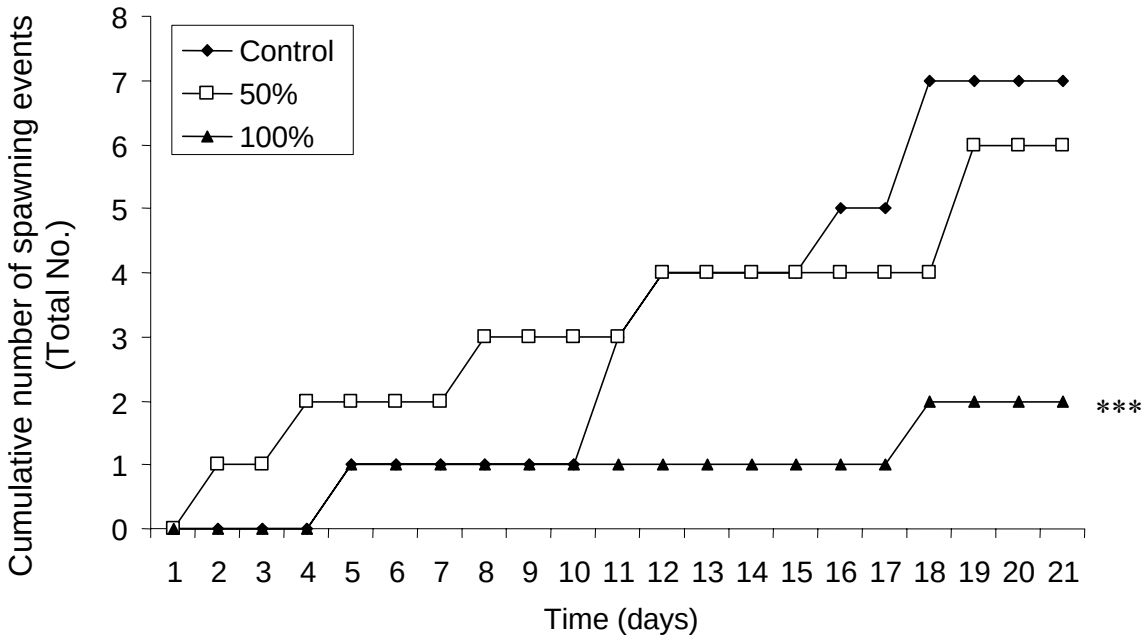

Figure 2.3. Cumulative number of spawning events for fathead minnow during 21 days exposure to treated pulp mill effluent (50% (v/v) and 100% (v/v) PME) and control water. Asterisk represents significant difference from control where *** = $p<0.001$.

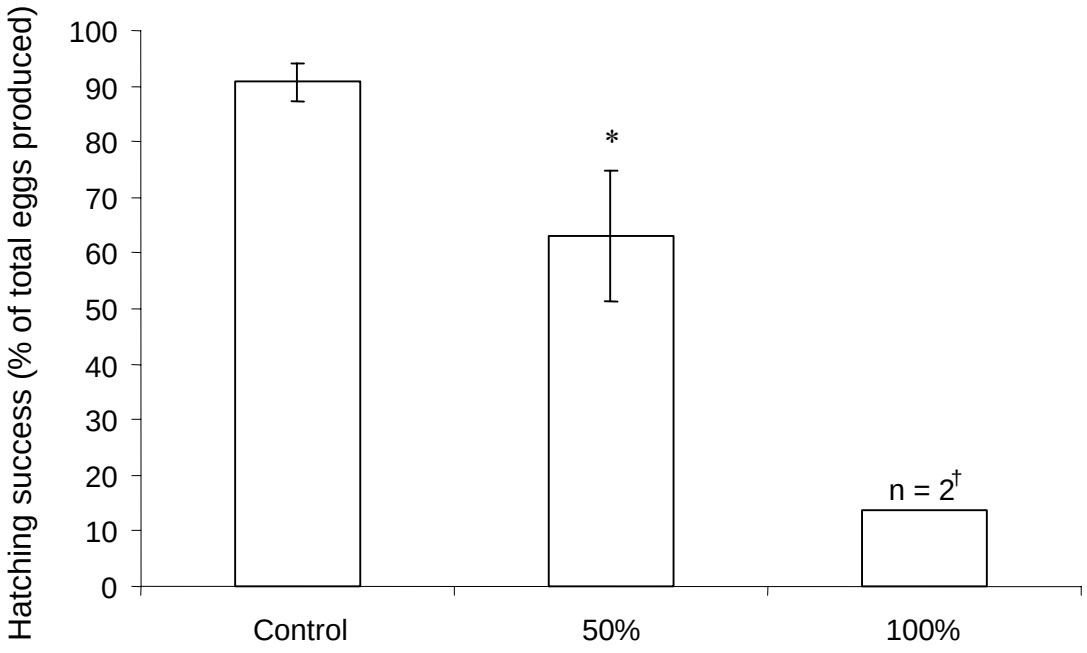

Figure 2.4. Hatching success (% of total eggs produced) of fathead minnow after 21 d exposure to treated pulp mill effluent (50% (v/v) and 100% (v/v) PME) and control water. Values represent means ± standard error. Asterisk denotes significant difference from control where * = $p<0.05$. † statistical analysis was not conducted on the 100% (v/v) PME treatment due to lack of data (n=2 spawning events), median value is shown.

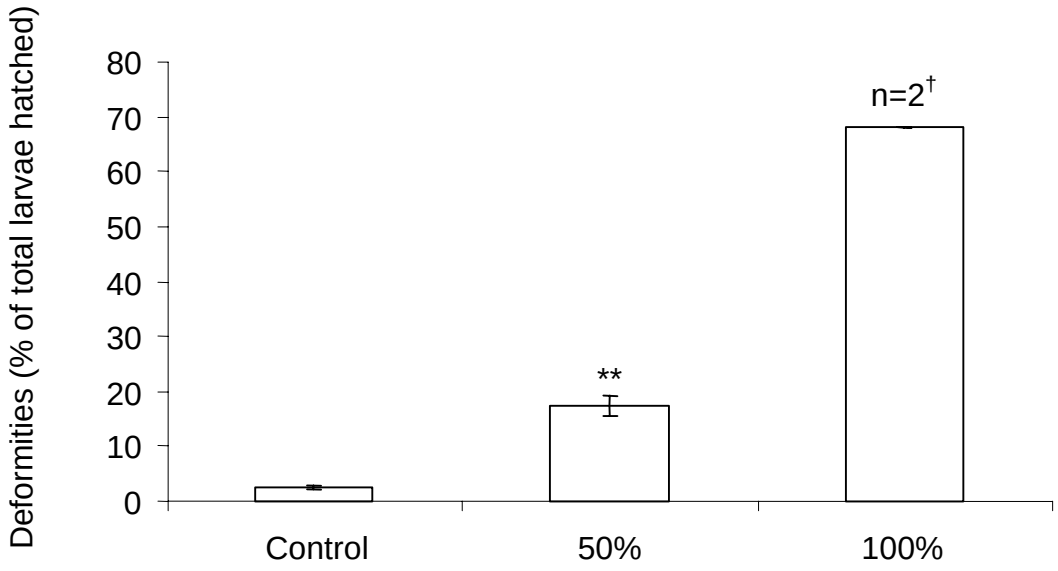

Figure 2.5. Deformities (% of total larvae hatched) observed in fathead minnow after 21 d exposure to treated pulp mill effluent (50% (v/v) and 100% (v/v) PME) and control water. Values represent means ± standard error. Asterisk denotes significant difference from control where ** = $p<0.001$. † statistical analysis was not conducted on the 100% (v/v) PME treatment due to lack of data (n=2 spawning events), median value is shown.

2.4 Discussion

In this study, we used a modified short-term FHM bioassay in the laboratory to assess effects of increasing concentrations of a PME on adults and offspring at multiple levels of biological organisation. Our primary objective was to identify a response pattern to PME using biochemical, individual and population endpoints. We observed effects on adult reproductive output and F1 survivorship as the most sensitive and consistent indicators of effect across PME concentrations.

2.4.1 Identification of a response pattern

The pattern of responses identified for adult FHM in this study (decreased egg production and spawning events, appearance of eosinophilic material in male gonads, increased gonad and liver sizes) suggests an estrogenic response was occurring after exposure to 50% and 100% PME in a dose-dependent manner.

The increase in GSI and the corresponding increase in eosinophilia in male gonads may be an indication of an estrogenic response. Eosinophilia is measured by assessing the degree of excessive eosin staining (eosinophillic material) in gonad tissue observed in histological examination. Previous investigations have observed accumulation of acellular and amorphous eosinophilic material around major organs in fish exposed to estrogenic chemicals (Wester and Canton, 1986; Metcalfe et al 2001; Zillioux et al 2001). In other studies, the eosinophilic material was identified as vitellogenin, the yolk precursor protein, and chorion glycoprotein, both produced in response to exposure to estrogens (Van Den Belt et al, 2002 cited in Weber et al, 2003). Palace et al (2002) suggested that eosinophilic deposits observed in the kidneys of FHM exposed to ethynyl-estradiol (EE2) were due to the accumulation of large quantities of vitellogenin. Palace et al (2002) also observed increased liver size which was attributed to the

production and accumulation of vitellogenin within hepatocytes. In our study, we observed significantly increased GSI, eosinophilic deposits (eosinophilia) and a 53% increase in LSI (non significant), in males exposed to 100% (v/v) PME. This pattern of response suggests that induction of vitellogenin in males possibly occurred due to exposure to estrogenic chemicals. This hypothesis of an estrogenic response pattern after exposure to PME corresponds with previous findings exposing FHM to PME from a multi-process mill where inductions in vitellogenin were a consistent and sensitive response (Martel et al, 2003). Unfortunately, we did not measure vitellogenin levels in either males or females, but it is an endpoint that is strongly recommended for future studies with this and other PMEs.

Exposure to estrogenic compounds within the PME would also explain the reduction in reproductive output observed in both 50% (v/v) and 100% (v/v) treatments. Exposure to EE2 (3 ng/L) and the weak estrogen methoxychlor (0.5 µg/L) has been shown to cause inhibition of spawning in FHM after 3 weeks of exposure (Ankley et al, 2001; Pawlowski et al, 2004). Disruption of egg production has been observed in previous PME exposure studies with FHM in full life cycle tests (Borton et al, 2000, 2003; Haley and Hall, 2000; Parrott and Wood, 2003). A complete halt, or severe reduction, of egg production was the most common response in these investigations immediately after exposure (without recovery). A possible hypothesis for the disruption in egg production is that endocrine disrupting chemicals in the effluent are acting as estrogen receptor agonists/antagonists, inhibiting the mechanisms required for spawning. Support for estrogenic activity in the PME can also be found in the appearance of eosinophilic material in the male gonads and increased GSI and LSI in the 100% (v/v) PME treatment.

One of the most interesting results observed in this study was the decrease in hatching success of larvae exposed to final PME (Figure 2.4). In both PME treatments, the number of normal

larvae hatched (hatching success %) was substantially reduced compared to control. Of those larvae that did survive an increased occurrence of deformities (67% occurrence) was observed in the 100% (v/v) effluent exposure compared to control (2% occurrence) (Figure 2.5). Due to the lack of egg production in the 100% (v/v) treatment (and hence a lack of offspring) (*n*=2 spawning events), statistical analysis could not be conducted, however, the increase in deformities is still worthy of discussion. The most common deformity observed in our study was yolk sac edema. The occurrence of deformities in fish larvae, including edema, can occur because of both nutritional, e.g., deficiency of certain vitamins and minerals (Halver, 1989; Lall, 1989) and environmental factors, e.g., specific contaminants or water quality (Hamilton, 2002). In previous investigations, exposure to PME has resulted in blue sac disease, the symptoms of which include pericardial/yolk sac edema, craniofacial malformations and mortality (Walker et al, 1991; Billiard et al, 1997). The specific compounds within the effluent causing these effects were hypothesized to be dioxins. However, there are no detectable dioxin compounds measured in the effluent from the mill we studied as pulp bleaching with elemental chlorine was phased out many years ago (Environment Canada, 2004b). Therefore, it is unclear from this study if the deformities observed were because of the presence of contaminants within the PME, or, because of nutritional deficiency, i.e., the maternal investment of nutrients into developing embryos was impacted.

2.4.2 Comparisons with field data

Studies of wild fish collected in the Jackfish Bay area exposed to PME have consistently observed reduced gonad weight and depressed sex steroids in adult fish (Munkittrick et al, 1997b). The depressions in reproductive hormones were associated with multiple disruptions in the endocrine pathway controlling reproduction (McMaster et al, 1995: Van Der Kraak et al,

1992). These observations correspond with our study where we observed effects on reproductive endpoints. Even though our conclusions were based on different endpoints to those measured in the Jackfish Bay studies, the mechanisms behind the changes observed in both investigations may be the same i.e. endocrine disruption controlling reproduction.

The response pattern observed in our study also corresponds with other studies investigating the effects of PME on FHM (Borton et al, 2003; Martel et al, 2003; Parrott and Wood, 2002). In a study with the short-term lifecycle bioassay, Martel et al (2003) observed impacts (i.e. decreased egg production and vitellogenin induction) after exposing FHM to a multi-process mill effluent. Similarly, Borton et al (2003), using a full life-cycle bioassay, observed significant reductions in egg production, spawning events and fecundity in FHM exposed to 100% (v/v) PME from a bleached kraft pulp mill. Impacts on egg production, in both short- and long-term FHM lifecycle studies, has been documented as one of the most sensitive parameters to be affected by PME (McMaster et al, 2004). The effects on reproductive output documented in our study, is consistent with these conclusions.

The differences in the response of specific endpoints (gonad size, testosterone levels) measured in the Jackfish Bay studies compared to our investigation could be due to a number of factors including length of exposure (21 d), differences in life history characteristics between the species studied and effluent quality.

Much of the research conducted in Jackfish Bay has been with synchronous annual spawners e.g. lake whitefish, white sucker (Munkittrick et al, 1997b). Comparatively, less work has been conducted with multiple or fractional spawners, such as FHM. The significance of decreased GSI to the life history of an annual spawner might be more significant than for a fractional spawner with multiple opportunities to spawn within a season.

The importance of exposure duration is worthy of discussion. The lack of effects on gonad sizes in the 50% (v/v) PME treatment, and testosterone levels in both 100% (v/v) and 50% (v/v) treatments suggests that the exposure was either not long enough or the concentrations were not potent enough to cause any significant changes in these endpoints. Additionally, effluent quality may also have been a factor that could explain differences observed between our study and other Jackfish Bay studies. Firstly, effluent was collected at the outfall from the secondary treatment basins whereas wild fish in Jackfish Bay are exposed to effluent that has traversed Blackbird Creek. It is unknown if effluent composition is altered in Blackbird creek which may change its toxicity. Secondly, storage of the effluent may also have changed its composition. The effluent was collected and shipped to Saskatoon where it was stored for a maximum of 7 d. The total storage time for the effluent was approximately 12 d including shipping. Unfortunately, we were not able to sample the effluent during this holding time to assess changes in its composition, but this is certainly a factor that needs to be accounted for in future studies.

The field studies conducted at Jackfish Bay have ultimately demonstrated that wild fish have reduced gonad sizes and altered reproductive hormone levels compared to fish from reference sites. Our controlled laboratory study, using the FHM short-term reproductive bioassay, also show that high concentrations of PME (50% and 100%) have the potential to affect FHM reproductive output in the adults as well as having the ability to affect hatching success and deformities in the F1 generation. These studies have demonstrated that the PME under investigation has the potential to affect fish reproduction, as identified in both field and lab studies. However, the consequences of these effects to the stability of the wild fish community in Jackfish Bay remain unknown. Further studies are required to investigate this critical gap in knowledge.

2.5 Conclusions

Overall, final PME (50% (v/v) and 100% (v/v)) significantly reduced the number of eggs produced and the ability of breeding pairs to spawn compared to control fish. The number of normal larvae hatched (hatching success %) was also reduced in both treatments, corresponding with an increased appearance in deformities compared to control. GSI and the appearance of eosinophilic material in male gonads may indicate that the reduced reproductive output was due to exposure to estrogenic compounds within the effluent. The use of breeding pairs in this investigation allowed assessment of endpoints at different levels of biological organization (e.g. biochemical, whole organism) for each individual fish to be compared. By using the short-term FHM bioassay we were able to identify a response pattern to PME that helped us to identify the type of potential compounds i.e. estrogenic, causing the responses observed.

CHAPTER 3[A]

Use of paired fathead minnow (*Pimephales promelas*) reproductive test: Part I: Assessing biological effects of final bleached kraft pulp mill effluent using a mobile bioassay trailer system

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3.1 Introduction

In the late 1980's, Scandinavian studies revealed impacts on fish communities including delayed maturation and low recruitment in areas receiving bleached kraft pulp mill effluent (BKME) (Andersson et al, 1988; Sandstron et al, 1988). Studies were conducted in Canada since the mid to late 1980's to investigate potential BKME-associated impacts (Munkittrick et al, 1991). Wild fish collected from Jackfish Bay, an area receiving BKME on the north shore of Lake Superior, ON, Canada, have shown changes in reproductive indicators (Munkittrick et al, 1991; McMaster et al, 1995). Lake whitefish (*Coregonus clupeaformis*), white sucker (*Catostomus commersoni*) and longnose sucker (*Catostomus catostomus*) were evaluated in field studies using indicators of fecundity, egg size, reproductive steroid levels, gonad weights, age to maturity, and secondary sex characteristics. These studies have most commonly and consistently shown decreased gonad weights (gonadosomatic index [GSI]) and serum steroid levels with lake whitefish being more sensitive to the effects of BKME than white or longnose sucker (Munkittrick et al, 1998). There is a need to establish whether BKME impacts occur at biochemical, individual and population levels using controlled exposures and to link changes in indicators at different levels of biological organization to better understand their ecological relevance.

Full and partial life cycle bioassays are approaches often used to examine contaminant effects on different life stages of an organism and over multiple generations. Use of these bioassays provides a mechanism to evaluate linkages between individual responses to contaminant exposure and population-level consequences. Long-term (120 d) and short-term (21 d) FHM lifecycle bioassays have been conducted in laboratories with various PME in an effort to determine consistent changes in reproductive indicators (Martel

et al, 2003; Robinson, 1994; Borton et al, 2003; Haley and Hall, 2000). Martel et al (2003) showed that egg production had ceased in one of six effluents tested, after 28 d exposure at 20% (v/v) effluent from a thermomechanical mill, but no effect was seen at 2% (v/v). In addition, vitellogenin induction was shown to be the most frequently observed response. Similarly Borton et al (2003) showed significant reductions in egg production, spawning events and fecundity in FHM exposed to 100% (v/v) BKME during spawning. Robinson (1994) also observed that lab studies with the full life cycle FHM test demonstrated decreased egg production, spawning events and gonadal steroid production, with basal levels of testosterone produced by fish testes 1.9-fold lower when held in 50% (v/v) PME compared to controls. Robinson concluded that, in the case of pulp and paper mill effluent, the most sensitive endpoint in fish may be the reproductive output of adult fish. While these investigations produced valuable results on direct effluent-induced changes in reproductive indicators, these laboratory studies generally showed effects at concentrations much higher than those found in the field studies.

Inconsistencies in effect threshold concentrations between lab and field studies make it difficult to extrapolate lab results to field responses and to confirm cause (Robinson, 1994; Kovacs and Megraw, 1995; Kovacs et al, 1995). Variability in effluent chemistry from mill to mill further confounds comparisons. Alterations in effluent quality with transport and storage and lack of environmental relevance, e.g. ambient water quality and dietary exposure, may result in the lack of sensitivity often observed in laboratory studies when compared to field observations (Munkittrick et al, 1998; Borton et al, 2003). There is a need to standardize assessments of PME on fish using standard biological assays linked to consistent physical/chemical effluent variables (e.g pH, conductivity). Although full and partial life

cycle tests with FHM provide an opportunity to link individual changes in reproductive indicators to higher level population and multi generational effects, their predominant use in laboratory settings has restricted their application for interpreting field responses in fish exposed to pulp mill effluents (PME's). One way to address these concerns is to apply life-cycle tests in situ.

Our laboratory has been developing in situ artificial stream techniques for the past decade to provide a mechanism to evaluate contaminant effects on fish under more realistic and natural exposure conditions (Dubé et al, 2002a,b). These systems are located outdoors and typically on-site at a pulp mill. This approach allows control of the amount, concentration and duration of effluent exposure in conditions reflecting the ambient environment i.e., water quality while also eliminating the need for long-term storage of effluent. Parrott and Wood (2001) conducted one of the first studies in Canada to assess FHM reproduction in situ. Their work was conducted at a bleached sulphite mill in New Brunswick, Canada and FHM were exposed to final treated effluent (1, 3.2, 10, 32, 48 and 100% (v/v)) using upstream water from the Saint John River in a closed and temperature controlled (25^{o}C) trailer system for a full lifecycle (120 d). However, the experiment was long-term (120 d) and labor intensive. In an effort to establish a shorter-term FHM test, Ankley et al (2001) have developed a 21 d protocol. This assay is used to measure the reproductive output of breeding adult FHM following a 21 d exposure and allows comparison of reproductive output to several biochemical, physiological, and morphological endpoints. The test also includes a pre-exposure breeding trial (21 d) that establishes baseline reproductive output for breeding fish so changes in reproductive indicators can be evaluated before and after effluent exposure and hence better capture variability amongst breeding

groups. This bioassay has been used with a number of model compounds including methyltestosterone and ethynylestradiol as well as various PME's (Martel et al, 2003; Parrott and Wood, 2001; Ankley et al, 2001). The time frame of a partial life-cycle test and its quantification of breeding group variability make it a suitable candidate for in situ development and application to evaluate reproductive effects associated with PME exposure. The Ankley et al (2001) protocol combines four females and two males in one replicate. Reproductive output is pooled which could reduce variability within a treatment. Using a 4:2 ratio reduces the sensitivity of tracking reproductive performance on a pair-by-pair basis making extrapolation from biochemical to population-level endpoints difficult (Harries et al, 2000). By using pair-breeding fish (1:1, male:female), it is possible to monitor individual performance throughout the experiment and accurately extrapolate the various biochemical and individual endpoint effects (GSI, sex steroid levels) to population-level effects (egg production).

The purpose of this study was to transfer a short-term, pair-breeding FHM bioassay into a mobile laboratory system for in situ testing at Jackfish Bay, ON, Canada with the objective of determining firstly the effect of BKME being discharged into Jackfish Bay on FHM at the biochemical, individual and population levels under more environmentally realistic conditions (i.e., ambient water and effluent quality); and secondly the suitability of using pair-breeding FHM to link BKME-induced changes in indicators at different levels of biological organization.

This paper is the first of two reports. The overall objectives of our study were to firstly determine if final effluent affected reproductive endpoints in FHM (this Chapter; Part I), secondly use effluent chemistry and toxicity tests to characterize and identify potential in-

mill sources of the reproductive effects, (Part II, Chapter 4) and thirdly test the effects of each candidate in-mill process sewer to determine effects on FHM (Part II, Chapter 4).

3.2 Methods

This study was conducted in July and August of 2003 at a bleached kraft pulp mill in Terrace Bay, ON, Canada. The effluent discharge point for the Terrace Bay pulp mill is Jackfish Bay, Lake Superior. This mill features both hardwood and softwood pulp production in separate lines, producing ~ 360 air dried metric tones per d and 805 air dried metric tones per d of market pulp respectively. The hardwood mill (mill 1) went on-line in 1948 and uses a furnish of poplar and aspen. The softwood mill (mill 2) commenced operation in 1978 and uses a furnish of spruce (predominantly black spruce), jack pine, and balsam fir.

Each mill has its own bleach plant, with bleaching sequences DEDED (mill one) and DEopDEpD (mill two) (D = chlorine dioxide (ClO2) E = caustic extraction, Eop = caustic, peroxide and oxygen extraction, Ep = caustic and peroxide extraction). A number of improvements in the mill have taken place including the installation of a spills elimination basin in 1995, a 5-stage counter current brownstock washing system for improved efficiency and a condensate stripper and turpentine recovery system in mill one in 1984 (Environment Canada, 2004).

Process water for both mills is taken from Lake Superior at an average rate of approximately 109,388 m^3 per day. Final effluent is treated as a combined discharge for both the hardwood and softwood mills. Treatment consists of clarification and biological treatment in a three-celled aerated stabilization basin with a total volume of 1.1m m3 and a residence time of approximately 10 d (Environment Canada, 2004b). After secondary treatment the effluent enters Blackbird Creek, which extends for 15km and includes two

lakes (Lake A and C). The estimated residence time of effluent within Blackbird Creek is 48 h (Environment Canada, 2004b). This system acts as a polishing and settling area and reduces loadings of biological oxygen demand (38%), total suspended solids (74%), total phenols (32%) and adsorbable organic halides or AOX (23%) (Environment Canada, 2004b).

3.2.1 Experimental design

Experiments were conducted for 60 d from July to August 2003 using a flow-through, enclosed bioassay trailer located on-site at the bleached kraft pulp mill. The bioassay trailer was 12 m by 3 m (40 x 10 ft) and housed thirty 35 L aquaria in a temperature controlled room containing an ultra violet (UV) light sterilization system and two 1000 L holding tanks. Each aquarium was subdivided into four sections with plastic mesh; each section housed one breeding pair and a spawning tile.

3.2.2 Pre-exposure design

The short-term FHM bioassay requires a pre-exposure trial and an exposure trial of approximately similar duration (21 d) (Ankley et al, 2001). The pre-breeding trial is conducted in the absence of effluent to establish baseline reproductive performance of breeding pairs. This approach ensures that the individual variability in the test is quantified and included in the statistical analyses when effluent treatment effects are examined. The initial breeding trial consisting of 120 breeding pairs (four pairs per aquarium) and was conducted over 21 d to determine reproductive capacity of the test animals and tank-specific baseline data for the following endpoints: survival, egg production, fertilization success, hatching success, larval deformities, and secondary sex characteristics of breeding adults. Six month old, naïve FHM were obtained from cultures at the National Water Research Institute,

Burlington, ON, Canada and acclimated to Lake Superior water for 24 h. After acclimation, fish total body weight (g), fork length (mm) and secondary sex characteristics were recorded. Secondary sex characteristics of FHM consisted of banding, nuptial tubercles, dorsal pad and fin dot in males and ovipositor size in females. Each secondary sex characteristic was evaluated based on a points system (Table 3.1) and were summed with equal weight to give an overall male and/or ovipositor index as outlined by Parrott and Wood (2001). The 120 breeding pairs were then randomly selected and placed into aquaria (four pairs per aquarium, 30 aquaria).

Lake Superior water was collected twice weekly from Terrace Bay and stored outside in a 6250 L polyethylene tank. This water was treated with UV light disinfection and pumped to two heated holding tanks (1000 L) inside the trailer for acclimation overnight to 25^{o}C. Smaller polyethylene holding tanks (200 L) were then filled daily and the heated water was pumped (Pulsatron Series E, Viking Pump of Canada, Edmonton, Canada) from each holding tank via a manifold system to the aquaria.

Each holding tank and manifold supplied three aquaria at a turnover rate of two volumes per day. Water temperature was maintained at 25oC (+/-1oC) and logged using Optic Stowaway temperature loggers (Optic stowaways©; Onset Computer, Bourne, MA, USA). Conductivity and dissolved oxygen were monitored daily in each aquarium using a YSI meter (Yellow Springs Instruments, Yellow Springs, OH, USA). Ammonia (Rolf C. Hagen, Edmonton, Canada) and pH (Oakton pHTestr 3, Oakton Instruments, Vernon, IL, USA) were also recorded daily. Fish were fed frozen brine shrimp (San Francisco Bay Brand, Newark, CA, USA) daily ad libitum. Photoperiod was maintained at 16 h:8 h light:dark.

Table 3.1. Point system for grading secondary sex characteristics in fathead minnow (*P. promelas*). Developed from Parrott and Wood (2001) and Martel et al (2003).

	MALE INDEX				
CHARACTERISTIC	Nuptial Tubercles	Dorsal Pad	Dorsal fin dot	Banding	OVIPOSITOR INDEX
SCORE	Absent: 0 Present: 1	Absent: 0 Small: 1 Medium: 2 Large: 3 Very Large: 4	Absent: 0 Present: 1	Absent: 0 Present 1	Absent: 0 Small: 0.5 Medium: 1 Large: 2 Very Large: 3

During the pre-exposure period survival, total number of spawning events, egg production, fertilization success, hatching success, larval deformities, and secondary sex characteristics of breeding adults were measured. Egg production was determined daily; tiles were checked for eggs at 10:00 am every morning, allowing time for spawning and fertilization to be completed and for eggs to water-harden (Ankley et al, 2001). The eggs were rolled off the tile into hatching cups (250 ml glass jars with screened bottoms) which were then placed in aquaria with aerated Lake Superior water that was renewed daily. The total and viable number of eggs were counted daily allowing estimation of cumulative egg production (over 21 d) and mean and total egg production (no. eggs per female). Eggs were determined to be viable if they had a defined yolk within the egg sac and their structural integrity was maintained. The eggs were checked daily for fungal infections and any dead eggs were removed and numbers recorded. Once larvae hatched (usually around five to seven days after spawn) they were assessed for deformities under a dissecting microscope (6.4 x magnification); deformities included scoliosis, lordosis and yolk sac edema. The total number of viable larvae (hatching success expressed as % of viable eggs) and deformed larvae (% of total larvae hatched) were recorded. Development of male-type secondary sex characteristics (Table 3.1) in both males and females were recorded at the start (day 0), mid (day 10) and end of the pre-exposure (day 21). Development of female-type characteristics (i.e., ovipositor presence and size) were not assessed at the mid or end of the pre-exposure to minimize disturbance of the breeding fish. The mean male and ovipositor index for each fish was then calculated to obtain index values for the pre-exposure period.

After 21 d, selection of breeding pairs for the effluent exposure was undertaken based on 100% survival of all adults, presence of eggs in each replicate once a week, and >80%

fertilization of eggs (OECD, 2003). Based on these criteria, 53% of the breeding pairs used in the pre-exposure period were selected for use in the exposure period resulting in three pairs per aquaria and three aquaria per treatment (n = nine). Statistical analyses were undertaken using an analysis of variance (ANOVA) on egg production (mean), spawning events (mean) and hatching success (mean) to determine there were no tank effects within a treatment. This provided justification for pooling fish across aquaria in a treatment for an n=9 and also confirmed that there were no significant differences in reproductive output between treatments before exposures began.

3.2.2.1 Pre-exposure analysis

Analysis between tanks within a treatment was conducted to determine if any tank effects could be observed. Levine's Test was used to confirm parameteric assumptions for homogeneity of variance. One-way analysis of variance (ANOVA) was then conducted to determine if there were any differences between tanks for the following endpoints: egg production and spawning events (mean number per pair after 21d), hatching success (% of viable eggs produced) and deformities (% of total hatch). No significant differences were found ($\alpha = 0.05$), therefore, breeding pairs were used as the unit of replication and pairs were pooled across aquaria within a treatment. Thus, the unit of replication for egg production, spawning events, hatching success and deformities were breeding pairs (n = nine per treatment). Unit of replication for weight, length, condition factor, GSI, LSI and testosterone was nine (n = nine females and n = nine males per treatment).

3.2.3 Exposure design

Three treatments were established for on-site testing: reference (Lake Superior mill intake water), 100% (v/v) BKME and 1% (v/v) BKME. 100% (v/v) BKME was chosen as a positive control based on a previous laboratory investigation using the same mill effluent (Chapter 2). 1% (v/v) BKME was chosen as a best-case scenario and an environmentally relevant concentration based on plume delineation studies conducted in Jackfish Bay (Environment Canada, 2004b). Nine breeding pairs were exposed to each of the three treatments for a further 21 d. Treated final BKME was collected from the outflow point of the secondary treatment lagoon twice weekly and stored in a 1000 L holding tank in the bioassay trailer. Effluent (200 L of 100% (v/v) and two L of 1% (v/v)) were supplied daily to 200 L holding tanks. Effluent was delivered to three aquaria per treatment at a total flow rate of 64 L per day per aquaria. This maintained the turnover time to two exchanges per aquaria per 24 h period. Eggs and larvae were held in corresponding treatment tanks that were changed daily. Daily observations and measurements, i.e. egg production, hatching success, deformities etc. were the same as those taken during pre-exposure. Water samples were collected from the reference water and treatment aquaria as well as the head tanks weekly during the three week exposure period and analyzed for general chemistry and nutrients. Samples were collected, preserved and analyzed as per standard methods and quality assurance and control procedures (National Laboratory of Environmental Testing, Burlington, ON, L7R 4A6).

At the end of the exposure period, fish were anaesthetized (30µl per L clove oil), assessed for secondary sex characteristics, and fork length (mm) and total body weight (g) recorded. Fish were euthanised by spinal severance. Gonads in the adults were weighed (to 0.001 g) and placed in formalin (10%) for 24h and then stored in ethanol (75%) until

histological analysis was undertaken at the University of Saskatchewan using the quantitative method developed by Weber et al (2002). Gonadal tissue was paraffin-embedded, sectioned once and mounted on slides. Condition factor was calculated as Cf=100*[total weight (g)/standard length3(cm)]. Gonadosomatic index (GSI) and liversomatic index (LSI) were calculated as 100*[tissue weight(g)/total weight(g)]. Fish carcasses (excluding head and caudal fin) were then frozen on dry ice for 24h whilst being shipped to the National Water Research Institute, Saskatoon after which time they were stored in a -80oC freezer until muscle steroid testosterone analysis was undertaken. Muscle sex steroids were measured using a radioimmunoassay (RIA) technique (McMaster et al, 1992a) optimized for muscle homogenate for FHM.

3.2.3.1 Exposure analysis

Data were analyzed using SPSS 11.0 (SPSS, Chicago, Il, USA). The % difference (increase or decrease) in egg production, spawning events, ovipositor development and secondary sex characteristics were calculated based on pre-exposure data ([(Exposure – Pre-exposure)/Pre-exposure]*100) and compared to control using an ANOVA. Paired t-tests were performed to compare the before and after data for egg production and spawning events within each treatment. To test parametric assumptions for homogeneity of variance, Levine's Test was used. If data did not require transformation, an ANOVA, followed by a post-hoc Tukey test, was performed on the following endpoints: egg production and spawning events (% differences compared to pre-exposure), hatching success (% of viable eggs produced), deformities (% of total hatch), stages of ovarian development (histological analysis) and muscle testosterone. If Levine's Test for homogeneity was significant, the percentage-based data were arcsin (%) transformed and tested again for homogeneity. Data that were not

percentage or ratio-based were log10 transformed. A one-way ANOVA, followed by a post-hoc Tukey test, was then performed on the transformed data. If assumptions could not be met for homogeneity of variance, a non-parametric Kruskal-Wallis test was performed on the non-transformed data. To assess responses over time Kolomogorov-Smirnov tests were conducted to assess the cumulative frequency of spawning events and eggs produced occurring in each treatment over 21d compared to controls. Kolmogorov-Smirnov two sample tests were also conducted on ovipositor development in both males and females. The appearance of male secondary sex characteristics in females were assessed using contingency tables. Water and effluent chemistry data were analyzed using an ANOVA to determine if treatments differed significantly from reference water. Samples taken over time within each treatment were the unit of replication. Assumptions and post hoc tests were applied as discussed above.

3.3 Results

3.3.1 Water and effluent chemistry

Highly significant differences were observed in 100% (v/v) treatment aquaria compared to control for the following endpoints: salinity, specific conductivity, nitrate, dissolved organic carbon, total nitrogen, calcium, chloride, hardness, potassium and sodium (ANOVA, $p<0.001$) (Table 3.2). In addition, colour, sulfate and total suspended solids also differed significantly in the 100% (v/v) PME treatment from control (KWALLIS, $p=0.027$). In the 1% (v/v) PME treatment aquaria only colour and sulfate (KWALLIS, $p=0.027$), as well as sodium (ANOVA, $p=0.004$), differed significantly from control (Table 3.2). Significant spatial changes from head tank to aquaria were observed with nitrate ($N0_3$) concentrations in 100% (v/v) treatment (t test, $p= 0.002$) (data not shown). $N0_3$ levels were

low in the head tank (0.38mg per L) and increased in aquaria (mean 3.97mg per L). No other spatial changes in water quality parameters were observed in any of the treatments.

3.3.2 Individual endpoints

No significant treatment differences were observed for GSI, condition or weight in either males or females. LSI significantly increased in 1% (v/v) treatment (ANOVA, p=0.049) compared to control in females and in 100% (v/v) treatment (ANOVA, p=0.002) compared to control in males (Table 3.3).

3.3.3 Spawning events

Spawning events were assessed throughout the 21 d exposure period (Figure 3.1). Total spawning events per treatment were also assessed as a percentage difference of the number of spawning events in the pre-exposure on a pair-specific basis (Figure 3.2). The number of spawning events over the 21 d period in the 100% and 1% PME (v/v) treatments were not significantly different from control. When data were assessed compared to pre-exposure a decrease in spawning events was observed in all treatments, including control (Figure 3.2). However this reduction was only significant in the 100% (v/v) treatment (paired t-test, p=0.004). When the percentage differences in both treatments (1% and 100% v/v PME) were compared with control no significant differences were observed (Figure 3.2).

3.3.4 Egg production

Egg production was assessed throughout the 21 d exposure period (Figure 3.3). The total number of eggs produced per treatment was also calculated as a percentage difference of the number of eggs produced in the pre-exposure period (Figure 3.4). When data were

Table 3.2. Water quality parameters and chemistry of pulp mill effluent (PME) (100% and 1% (v/v)) and Lake Superior (Canada) water (control) from 21-day exposure aquaria. Samples taken from one aquaria per treatment per week for 3 weeks plus head tanks. Values are mean (n=3) ± standard error of the mean. Temperature data continuously recorded by onset loggers throughout the 21-day pre- and post-exposure. Asterix denotes significant difference from control, where * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. [a] DO = dissolved oxygen; [b] DOC = dissolved organic carbon

Variable (mg/L unless indicated)		Control	1% PME	100% PME
General	pH (pH units)	7.85 ± 0.06	7.89 ± 0.06	7.87 ± 0.08
	Colour (pt-co)	4.20 ± 1.00	11.4 ± 0.45*	908 ± 30.2***
	Temp (^{o}C)	24.9 ± 0.24	23.8 ± 0.39	24.5 ± 0.26
	Salinity (ppt)	0.10 ± 0.00	0.10 ± 0.00	0.50 ± 0.03***
	Specific conductivity (us/cm)	113 ± 1.31	118.0 ± 2.94	1079 ± 68.7***
	DO (%)[a]	82.1± 2.43	92.0 ± 2.77	73.2 ± 3.71
Chemistry	Nitrate	0.60 ± 0.04	0.64 ± 0.05	3.97 ± 0.20***
	Ammonia	0.10 ± 0.08	0.02 ± 0.01	0.15 ± 0.04
	Nitrite	0.08 ± 0.05	0.05 ± 0.04	0.07 ± 0.01
	DOC[b]	1.93 ± 0.15	3.67 ± 0.30	139 ± 1.15***
	Total Nitrogen	0.95 ± 0.16	0.82 ± 0.06	5.28 ± 0.24***
	Calcium	15.5 ± 0.64	16.1 ± 0.58	53.0 ± 3.50***
	Chloride	2.14 ± 0.35	4.07 ± 0.33	187 ± 6.96***
	Hardness	51.0 ± 1.80	52.6 ± 1.91	155 ± 8.74***
	Sulfate	4.79 ± 0.47	6.17 ± 0.19*	153 ± 2.96***
	Potassium	0.67 ± 0.06	0.74 ± 0.04	7.52 ± 0.59***
	Magnesium	3.00 ± 0.07	3.02 ± 0.11	5.50 ± 0.00***
	Sodium	2.50 ± 0.31	5.04 ± 0.28**	216 ± 19.9***
	Total suspended solids	0.00	0.00	7.23 ± 1.29***
	Total Phosphorus	0.03 ± 0.01	0.03 ± 0.00	0.38 ± 0.19

assessed over time a significant difference in distribution was observed in the 1% (v/v) PME compared to control (Kolmogorov-Smirnov, $p=0.035$). A 40% and 178% increase was observed in egg production in the 1% (v/v) treatment in week 1 and 2 respectively compared to control; however, this was followed by a 20% reduction in the final week compared to control. In the 100% (v/v) PME treatment a slight reduction (14%) was observed in the first two weeks compared to control but was followed by a substantial increase (64%) in the final week of exposure. When total egg production after 21 d was compared to pre-exposure data (Figure 3.4) no significant changes were observed in either of the treatments or control (paired t-test, $\alpha=0.05$). When the percentage differences in both treatments (1% and 100% v/v PME) were compared with control no significant differences were observed (ANOVA, $\alpha=0.05$) (Figure 3.4). This would suggest that the differences observed in egg production in this treatment occurred throughout the 21 d exposure i.e. during the first two weeks.

3.3.5 Development of male and female sexual characteristics

Male FHM, showed a significant increase in ovipositor development in both 1% (v/v) and 100% (v/v) effluent treatments compared to controls (Kolmogorov-Smirnov, $p=0.037$ and 0.009 respectively) (Figure 3.5). No significant differences were observed in females (data not shown).

The appearance of male secondary sex characteristics (banding and fin dot; Table 3.1) in females in the 100% (v/v) treatment was significantly different from controls ($p<0.05$) (Figure 3.6) indicating that fin dot and banding development in female FHM were not independent of treatment. No significant differences were observed in males (data not shown).

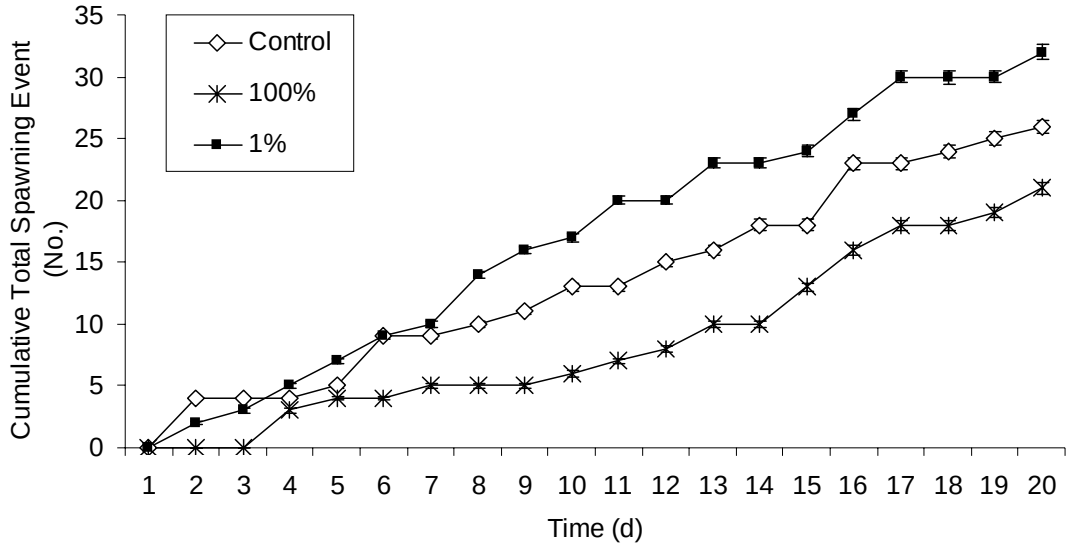

Figure 3.1. Cumulative number of spawning events of fathead minnow (*P. promelas*) breeding pairs during 21 d exposure to final bleached kraft mill effluent (1% and 100% (v/v)) and Lake Superior (Canada) water (control).

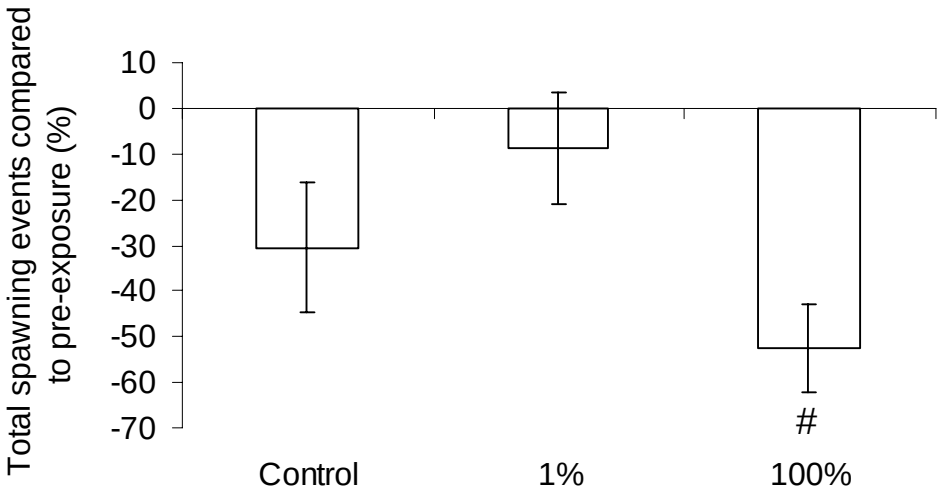

Figure 3.2. Spawning events of fathead minnow (*P. promelas*) breeding pairs. Total number compared to pair-specific pre-exposure data (%) after 21 d exposure to bleached kraft mill effluent (1% and 100% (v/v)) and Lake Superior (Canada) water (control). Pound denotes significant difference from pre-exposure data, where # = $p < 0.05$. Error bars represent +/- 1 SE

Table 3.3. Total body weight (g), condition factor (CF; %), gonadosomatic (GSI; %) and liversomatic (LSI; %) indices for adult male and female fathead minnow (*P. promelas*) after exposure to pulp mill effluent (PME) (100% and 1% (v/v)) and Lake Superior (Canada) water (control). Each value represents the mean ± standard error of the mean. Asterisk denotes significant difference from control, where * = $p<0.05$, ** = $p<0.01$.

Sex	Variable	Treatment		
		Control	1% Final PME	100% Final PME
Female	GSI (%)	10.2 ± 1.85	11.7 ± 1.53	12.7 ± 1.20
	LSI (%)	2.02 ± 0.32	3.21 ± 0.38*	2.93 ± 0.28
	CF (%)	1.55 ± 0.05	1.45 ± 0.07	1.53 ± 0.06
	Weight (g)	1.81 ± 0.13	1.72 ± 0.11	1.64 ± 0.10
Male	GSI (%)	1.29 ± 0.12	1.56 ± 0.28	1.16 ± 0.19
	LSI (%)	2.23 ± 0.14	2.31 ± 0.24	3.41 ± 0.24**
	CF(%)	1.74 ± 0.06	1.54 ± 0.12	1.64 ± 0.07
	Weight (g)	3.47 ± 0.31	3.37 ± 0.28	3.68 ± 0.15

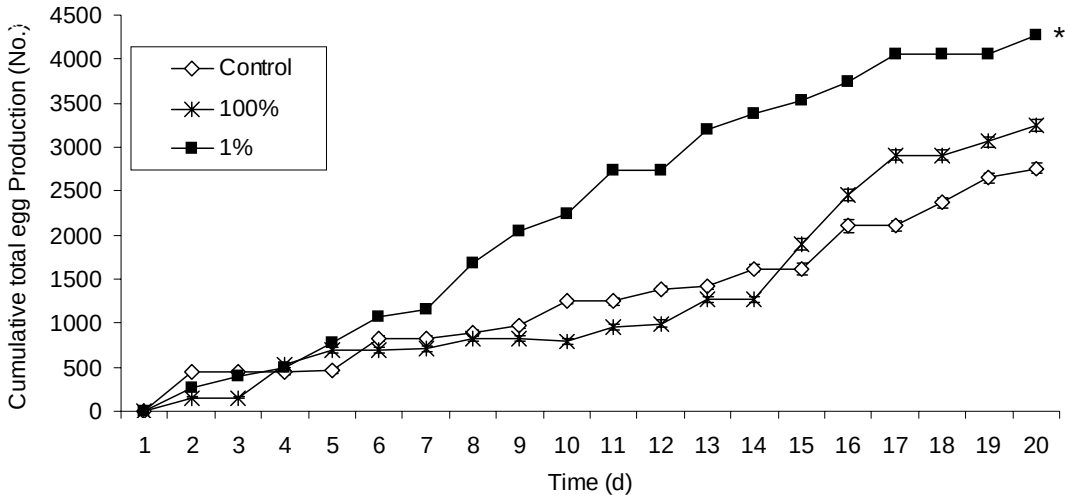

Figure 3.3. Cumulative number of total eggs produced by fathead minnow (*P. promelas*) breeding pairs during 21 d exposure to final bleached kraft mill effluent (1% and 100% (v/v)) and Lake Superior (Canada) water (control). Asterisk indicates significant difference from control, where * = $p < 0.05$.

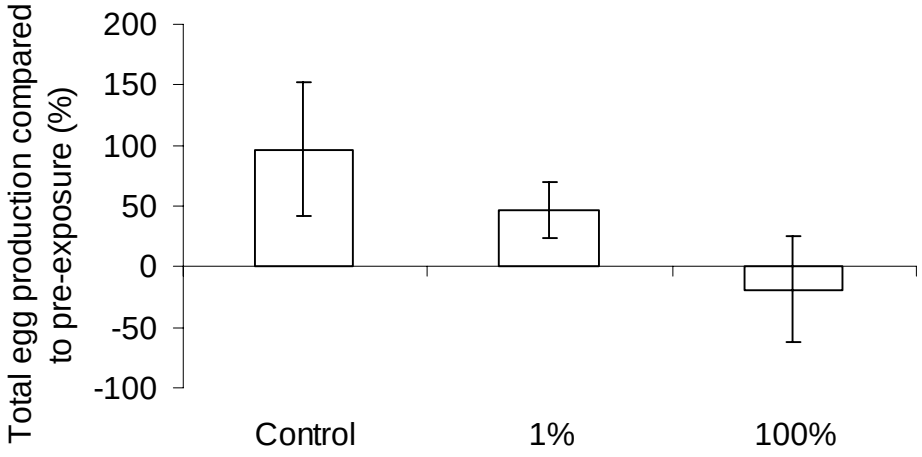

Figure 3.4. Egg production by fathead minnow (*P. promelas*) breeding pairs. Total number compared to pre-exposure data (%) after 21 d exposure to bleached kraft mill effluent (1% and 100% (v/v)) and Lake Superior (Canada) water (control). Error bars represent +/- 1 SE

3.3.6 Biochemical and histopathological analyses

In females, a significant increase (66%) in testosterone (ng per g body wt) was observed in the 100% (v/v) effluent treatment compared to control (ANOVA, p=0.003) (Figure 3.7A). There was a 24% decrease in female testosterone in the 1% (v/v) effluent treatment compared to controls although this was not statistically significant.

The changes in testosterone in both these treatments correspond with the 64% increase and 20% decrease in egg production in the last week of exposure in the 100% (v/v) and 1% (v/v) treatments respectively. In males, muscle testosterone significantly increased in the 1% (v/v) effluent treatment compared to controls (ANOVA, p=0.001) (Figure 3.7B).

Histological analysis of female gonads revealed a significant reduction in oogonia in the 100% (v/v) effluent treatment compared to controls (ANOVA, p=0.041) (data not shown). No significant differences were observed among stages of spermatocyte development in male gonads.

3.3.7 F1 offspring endpoints

There were no effects on hatching success in either of the treatments. Deformities in larvae did increase in the 1% (v/v) treatment, however, this was not significant and highly variable.

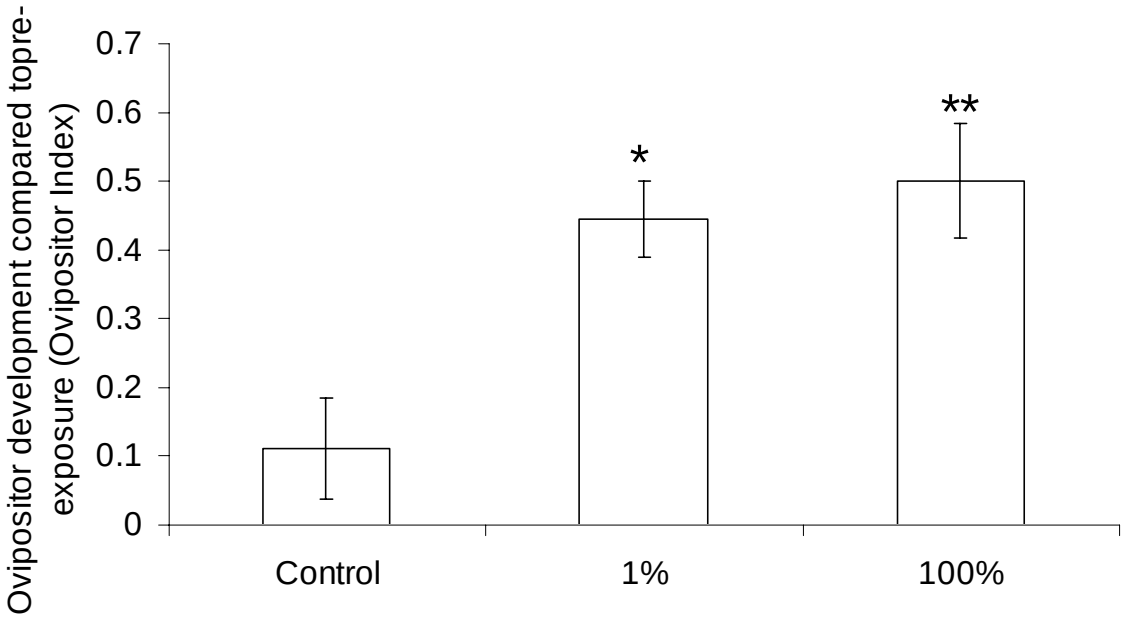

Figure 3.5. Ovipositor development (expressed as an index, see Table 3.1) in male fathead minnow (*P. promelas*) during 21 d exposure to final bleached kraft mill effluent (1% and 100%) and Lake Superior (Canada) water (control). Calculated as difference in ovipositor index from pre-exposure measurements. Asterisk indicates significant difference from control, where * = $p < 0.05$, ** = $p < 0.01$. Error bars represent +/- 1 SE

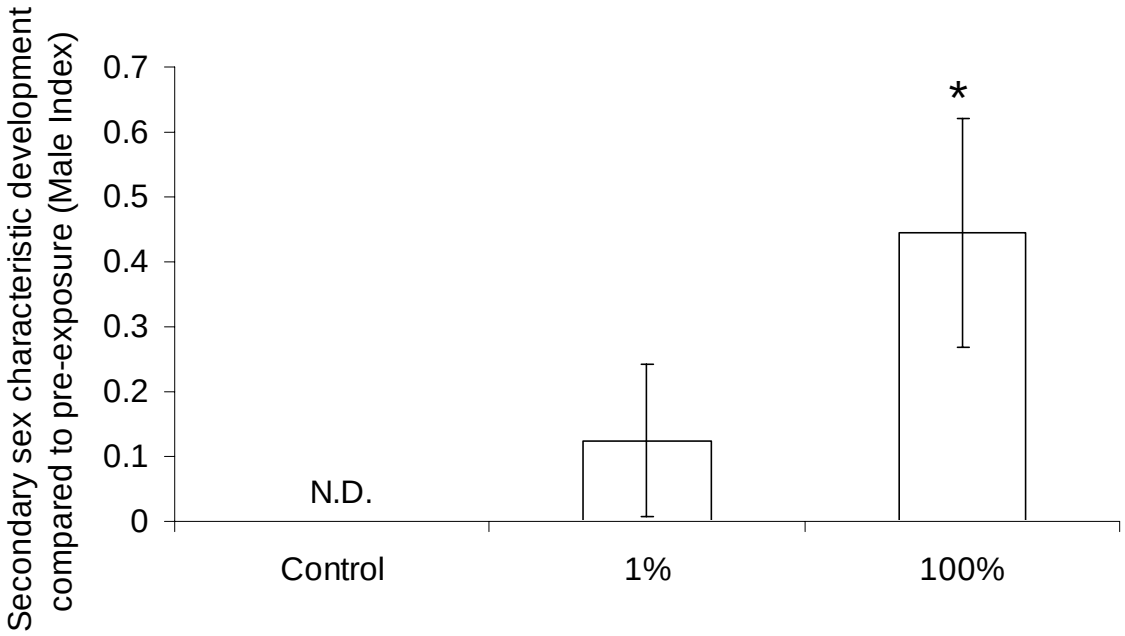

Figure 3.6. Secondary sex characteristic development (expressed as an index, see Table 3.1) in female fathead minnow (*P. promelas*) after 21 d exposure to final bleached kraft mill effluent (1% and 100%) and Lake Superior (Canada) water (control). Calculated as difference in male index from pre-exposure measurements. N.D. = non-detectable. Asterisk indicates significant difference from control, where * = $p < 0.05$. Error bars represent +/- 1 SE

3.4 Discussion

This study confirmed that effluent from the mill affected reproductive indicators in FHM although responses differed depending upon the effluent dose (1% (v/v), 100% (v/v)), the duration of exposure, and the method of data analysis. Two exposure concentrations of final PME were chosen for our study (1% and 100% v/v). Although drawing any firm conclusions as to mechanisms of action is difficult as a dose-response was not established, there are a range of possible hypothesis that could account for our results.

At low, best-case scenario, concentrations (1% (v/v)), an enhancement in reproductive output (i.e. cumulative egg production) was observed despite the development of ovipositors in males. The significant difference in cumulative eggs produced in the 1% (v/v) treatment throughout the 21 d could have resulted from spawning at a greater frequency or from an increase in the number of eggs available for release per spawn. The lack of any significant difference in spawning events however would suggest that is was the latter i.e. an increase in number of mature eggs.

It is not possible to speculate on the mechanism because egg production increased primarily in weeks 1 and 2 of the study whereas GSI and gonadal histology were measured at the end of the experiment when egg production had decreased 20% relative to controls. Mean GSIs of females (11.69 ± 1.53%) did not differ from controls and were within the mean range of 9.71 ± 0.51% reported by Jensen et al, (2001) investigating the basic reproductive physiology of FHM. Histological analysis revealed almost identical stages of ovarian development between the 1% (v/v) and control treatments.

High reproductive activity in the 1% (v/v) treatment was associated with increased LSI in females, and increased testosterone and ovipositor development in males. This response pattern i.e. increased egg production and hormones, suggests fish were predominantly investing energy

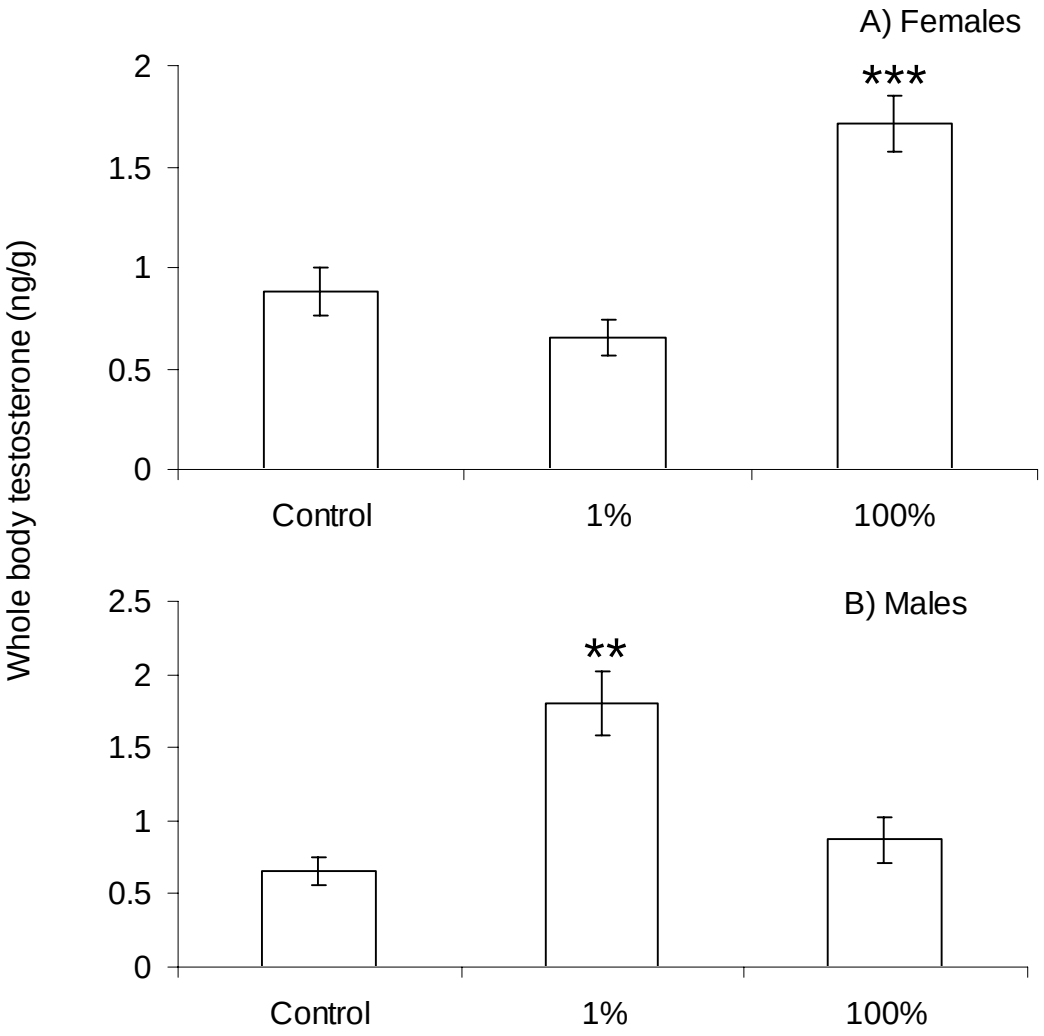

Figure 3.7. Muscle testosterone (ng per g) in female (A) and male (B) fathead minnow (*P. promelas*) after 21 d exposure to final bleached kraft mill effluent (1% and 100%) and Lake Superior (Canada) water (control). Asterisk indicates significant difference from control, where ** = $p < 0.01$, *** = $p < 0.001$. Error bars represent +/- 1 SE

into reproduction in this treatment over the 21 d. Increased LSI has been documented in fish exposed to PMEs (Andersson et al, 1988; Haley and Hall, 2000; Kloepper-sams et al, 1994; Munkittrick et al, 2000). These increases have been attributed to enhanced activity of xenobiotic biotransformation enzymes and/or nutrient enrichment effects, e.g. increased hepatic fat storage. It is not possible to conclude what led to increased LSI in this study as neither xenobiotic biotransformation enzymes nor histological analysis were conducted on liver tissue. However, it is possible that increased egg production may have resulted in increased hepatic vitellogenin and consequently larger relative liver size compared to control females. Previous studies have shown that increased vitellogenin production has resulted in increased hepatocyte size (Palace et al, 2002; Delahunty et al, 1980; Orlando et al, 1999). For example, Palace et al (2002) demonstrated that after exposure to 17α-ethinylestradiol (EE2) male FHM contained 9000-fold higher concentrations of vitellogenin and increased liver cell size. The enlargement of hepatocytes was concluded to be a direct result of the production and accumulation of vitellogenin within these cells.

Effluent exposure at low concentrations appeared to increase the reproductive output of FHM. In field studies, this type of response pattern is typically due to nutrient enrichment effects of PME where the low levels of the aquatic food web are enriched (i.e., algae, benthic invertebrates) resulting in an increased food availability or quality for exposed fish (Kloepper-sams et al, 1994). In our study, FHM were fed to satiation in a waterborne exposure. Thus, the possibility for trophic enrichment did not exist despite the high nutrient content of the effluent. This suggests that the stimulatory-type response was due to a direct effluent effect and not an indirect effect from food availability. The development of ovipositors in males suggests the increased reproductive activity in the 1% (v/v) treatment might be due to the presence of

estrogenic compounds in the PME. Preliminary work conducted by Hewitt et al (Hewitt L.M. Environment Canada, Burlington, ON, Canada) shows the presence of compounds with estrogenic activity in screenings of the final treated effluent from this mill. Development of ovipositors and feminization of mature male fish has been shown to be a sensitive indicator of estrogenic effects in FHM full lifecycle tests (Parrott and Wood, 2003). Development of ovipositors in mature male fish after exposure to bleached sulfite effluent has also been reported by Parrot and Wood (2001) where 18% of males exposed to 3.2% (v/v) effluent, 25% exposed to 10% (v/v) effluent and 47% exposed to 32% (v/v) effluent developed ovipositors. The potential for estrogenic compounds to increase egg production has been documented for a potent estrogen, EE2. Exposure of FHM significantly increased the mean number of eggs spawned per pair at low doses (0.1 and 1 ng per L) (Pawlowski et al, 2004). However, at higher EE2 concentrations, a dose-dependent decrease in the mean number of eggs per pair was apparent. Thus EE2 had a stimulatory effect on reproductive output in females at low doses and inhibited spawning when EE2 concentrations reached 3 ng per L.

In the 1% (v/v) PME treatment, no significant difference was observed in egg production compared to pre-exposure data. There was also no significant difference when this data was compared to control. Yet when the distributions were compared, a significant difference in the number of eggs produced over time occurred compared to control. A possible explanation for this is that analysis compared to pre-exposure data only uses total number of eggs produced at the end of the 21 d compared to total number of eggs produced at the end of the pre-exposure period. The apparent absence of any increase in the 1% compared to pre-exposure may be due to the reduction (20% compared to control) in the last week of exposure. This reduction would have reduced the total number of eggs produced overall, bringing the number in line with pre-

exposure levels. This would suggest then that the treatment effect occurred over time e.g. in the first two weeks of exposure.

In contrast to 1% (v/v) PME, the responses in the 100% (v/v) PME were very different. Histological analysis conducted on gonadal tissue at the end of the study revealed a significant reduction in oogonia, the first stage of oocyte development, in the 100% (v/v) treatment compared to controls. The ovary of a reproductively-active female contains oocytes in various stages of maturation. Jensen et al (2001) have shown that significant changes in oocyte maturational stage occur throughout the cycle of the FHM, a fractional spawner with primary growth stages present at a higher percentage on days 0 to one post-spawn which decrease by days one to two post-spawn with increased numbers of vitellogenic oocytes. When we look at the frequency of egg production in the 100% (v/v) PME throughout the 21 d, a reduction in the first two weeks followed by an increase in the last week of the experiment occurs. This may suggest that the reduction in oogonia was simply due to an acceleration of oocyte maturational state in the last week. A trend towards increased mature ovaries (pre-ovulatory, vitellogenic and pre-vitellogenic) and decreased oogonia in this treatment (100% (v/v)) might suggest that the ability to release mature ovaries was being affected. The significant decrease in spawning events compared to pre-exposure corresponds to this conclusion, suggesting that the ability to release the eggs was being affected rather than the ability to produce eggs for release.

Disruption in reproduction has been observed in previous PME exposure studies with FHM in full life cycle tests (Robinson, 1994; Borton et al, 2003; Kovacs and Megraw, 1995; Parrott and Wood, 2001; Borton et al, 2000). A complete halt or severe reduction in eggs was the most common response in these investigations immediately after exposure without recovery. A possible hypothesis for the disruption is that endocrine disrupting chemicals in the effluent are

acting as estrogen and/or androgen receptor agonists/antagonists inhibiting the mechanisms required to spawn. PME is dynamic and complex and could contain both anti/androgenic and anti/estrogenic chemicals with the potential for interaction (synergistic, antagonistic or additive). In addition, the compounds causing a reduction in spawning events would have been more prevalent in the 100% (v/v) effluent treatment which might explain the different responses observed between the 1% (v/v) and 100% (v/v) treatments. This pattern of response is indicative of a "hormesis" type response, with different disruptions at different effluent concentrations occurring. As previously mentioned, the estrogen EE2 has been shown to cause a stimulatory effect on reproductive output in female FHM at low doses and inhibit spawning at higher concentrations (3 ng per L) after 3 weeks of exposure [28]. Similar observations have been reported for FHM after exposure to a weak estrogen methoxychlor at a concentration of 0.5ug per L (Ankley et al, 2001). At day one, a reduction in fecundity was observed but at day 14 a rapid increase occurred, with no significant difference in testosterone at day 21, similar to our observations in egg production over time. Support for estrogenic activity in the PME can also be found in the development of ovipositors, the female reproductive organ, in male fish exposed to both 1% (v/v) and 100% (v/v) PME (Parrott and Wood, 2001).

Again, method of data analysis with this treatment revealed different responses. Although a significant difference was observed in spawning events compared to pre-exposure data no differences were observed over time. It could be that the frequency of spawning events over time was similar to controls throughout the 21 d exposure. However, this analysis was not sensitive enough to highlight the overall reduction in spawning events that occurred compared to pre-exposure data. This demonstrates the importance of using not only comparisons with control but also with pre-exposure data as this significance would not have been detected had we only used

the exposure data. Using multiple comparisons of data may also allow us to assess the severity of the response. It could be that significant differences in all of the analyses would occur if the effect was severe enough and as the severity decreased the number of significant differences would also be reduced.

A complete cessation in spawning in FHM has also been reported after exposure to the androgen methyltestosterone (Ankley et al, 2001). Ankley et al (2001) suggested that the mechanism behind this disruption was that androgens play some role in final maturation and/or release of eggs by the female FHM (i.e., that exposure to androgenic chemicals disrupts the ability to spawn). From this work, there was evidence of the presence of effluent androgens, based on the masculinization of females with development of secondary sex characteristics (Table 3.1). Banding and fin dots were the dominant male sex characteristics developed in females, which were absent in reference females. It is also possible that androgenic compounds in the PME resulted in ovipositor development in males. Some androgens such as methyltestosterone (1000 and 3200 ng per L) have caused development of ovipositors in male fish that also exhibited male characteristics (dorsal fin dot and tubercles) (Parrott and Wood, 2001). This contradictory response was caused by methyltestosterone possibly acting as a substrate for aromatase enzymes that would normally convert testosterone to estradiol. In previous studies with BKME, female FHM have shown increases in male characteristics, predominantly nuptial tubercles and fin dots; effects attributed to androgen-receptor agonist activity (Kovacs and Megraw, 1995; Parrott and Wood, 2001, 2003). After exposure to a bleached sulphite mill effluent, FHM males with sex characteristics e.g. nuptial tubercles, dorsal fin dots and some banding, were feminized with ovipositors (Parrott and Wood, 2001, 2003). Thus, it is possible that androgenic activity in the effluent was primarily responsible for the

reproductive/endocrine responses observed in our study. If the effluent was primarily estrogenic we would have expected a decrease in the presence of secondary sex characteristics in males (Parrott and Wood, 2001, 2003). It is important to highlight here that our evaluations are based on characteristic responses to androgen and estrogens, however, this is likely simplifying the issue as other responses may also be affected based on delayed maturity/spawning that has been previously observed in fish in Jackfish Bay (Munkittrick et al, 1998).

One main response that cannot be explained by androgenic activity is the increase in egg production in 100% (v/v) PME and decrease in 1% (v/v) PME in the final week of exposure. This difference could suggest a change in effluent composition and therefore effect. There were no reports of mill shutdowns or disruptions in processing and the final effluent composition was fairly static over the exposure period (Table 3.2). However, it is important to note the substantial colour change in the PME treatments (three-fold and 216-fold in the 1% (v/v) and 100% (v/v) PME respectively compared to control). Although changes in color per se cannot account for feminization and masculinization effects in the FHM, they may affect behavioral responses in FHM and subsequently spawning activity. It is also possible that the initial reduction in spawning events and egg production in 100% (v/v) PME was due to the hardness and conductivity, which were significantly higher (three-fold and five-fold respectively) compared to control (Table 3.2). The recovery of egg production towards the end of the exposure (around day 14) could have been due to acclimation.

The decrease in spawning events in the control compared to pre-exposure data is also worthy of discussion. This response would suggest that something other than treatment was reducing spawning. However, when this data was assessed using a paired t-test to compare before and after data there was no significant difference. The reduction in spawning events in the

control may suggest that the fish were "spawned out" but the lack of any significance in the spawning event and egg production data contradicts this observation. We could conclude then that the changes observed in the control were simply due to random sampling variability.

One main objective of our study was to conduct controlled exposure studies with this BKME and compare our results to the literature from this site collected over the past 15 years. Jackfish Bay has received BKME from a mill in Terrace Bay since 1949. In 1989, secondary effluent treatment was installed at the mill resulting in significant improvements in effluent quality including 93% and 19% reductions in biochemical oxygen demand and total suspended solids, respectively (Environment Canada, 2004). This improvement in effluent quality led to improvements in wild fish health in Jackfish Bay including some recovery in gonad size in both sexes of white sucker and in hormone levels in females from 1988 to 1993 (Munkittrick et al, 1997a,b). Despite these improvements reproductive responses in wild fish exposed to the BKME discharge are still being observed, and other issues are also emerging; effects on fecundity in fish sampled from Jackfish Bay were not observed until most recently (Environment Canada, 2004). In addition, effects on secondary sex characteristics have changed from estrogenic to androgenic (Munkittrick et al, 1997b). These examples highlight that the effluent and its effects have changed over time.

Partial recovery in gonad size and secondary sex characteristics in male white sucker and, most recently, increased fecundity, in female white and longnose sucker from Jackfish Bay have been observed in the near field exposure sites with effluent concentrations approximating 20% (v/v) (Environment Canada, 2004b). These results correspond with our findings in the 1% (v/v) effluent treatment (increased egg production). However, despite these improvements observed at Jackfish Bay, anomalies such as the increased appearance of male secondary sex characteristics

in female fish and reduced testosterone levels suggest exposure to androgenic compounds (Munkittrick et al, 1997b). Analysis of wild fish collected in the Jackfish Bay discharge area demonstrated that depressions in reproductive hormones were associated with multiple disruptions in the endocrine pathway which controls reproduction McMaster et al, 1995; Van Der Kraak et al, 1992). Van Der Kraak et al (1992) reported reduced plasma levels and secretion of gonadotropin II along with depressed ovarian steroid synthesis in white suckers in the receiving environment of this effluent. It was also observed that ovarian apoptosis was increased in BKME exposed white sucker, possibly due to alterations in hormone and gonadotropin levels, the latter of which is a known follicle survival factor (Janz et al, 1997). Work by Hewitt et al (2000) also reports the presence of compounds in hepatic tissues from male white sucker exposed to PME from Jackfish Bay, that act as ligands for the androgen receptor (AR), estrogen receptor (ER) and sex steroid binding protein (SSBP). The presence of ligands for the AR, ER and SSBP could indicate a possible mechanism for the effects observed in both wild fish in Jackfish Bay and our study, especially since the profile of accumulated ligands for the AR was the highest. Our results also indicate the presence of androgenic and estrogenic compounds in the effluent. The lack of effects on hatching success in both 1% (v/v) and 100% (v/v) treatments concur with observations of fertilization and survival of individual white sucker eggs and larvae sampled from the Jackfish Bay discharge area (McMaster et al, 1992b). White sucker from exposed sites were capable of spawning viable eggs and their ability to produce viable offspring were not reduced.

Our results are inconsistent, however, with the ongoing Jackfish Bay research of Munkittrick et al (Munkittrick et al, 1991, 1998, 1997b) and McMaster et al (1995) in that a reduction in GSI or sex steroids was not detected in either of our PME treatments with FHM.

Munkittrick et al (1998, 1997b) consistently report decreased relative gonad sizes in female white sucker. Differences in the response of these endpoints among the studies may be due to differences in life history characteristics between the species. Much of the physiology/endocrinology work in fish has been conducted with synchronous annual spawners, principally salmonids (Munkittrick et al, 1997a). The significance of delayed spawning and decreased GSI to the life history for an annual spawner might be more significant than for a fractional spawner that has multiple opportunities to spawn within a season. One other factor that might explain differences observed between our study and other Jackfish Bay studies is the effluent quality to which fish were exposed. Our effluent was taken at the outfall from the secondary treatment basins. Wild fish in Jackfish Bay would be exposed to effluent that has traversed Blackbird Creek which may have altered its composition.

The FHM assay provided an opportunity to measure the effects of effluent on a range of indicators using ambient water and effluent quality. The use of breeding pairs allowed for not only quantification of reproductive output throughout the exposure but also after exposure by using pre-exposure data. The ability to compare activity before and after exposure and then between treatments would not have been possible if multiple females were used. Obtaining pre-exposure data gave a sensitive indication of effluent effects and allowed accurate endpoint comparisons to be made. The use of breeding pairs also allowed endpoints at different levels of organization (e.g. biochemical, whole organism) for each individual fish to be compared.

3.4.1 Future Work

The pattern of effects observed in the final effluents (both 1% (v/v) and 100% (v/v)) were used to direct an investigation to determine the cause of reproductive effects observed in both this artificial stream study as well as in previous lab and field studies (Munkittrick et al, 1991,

1998, 1997b). Various in-mill process streams were tested using both acute toxicity tests and the partial FHM lifecycle bioassay. The results of this investigation are presented in our second paper (Chapter 4).

CHAPTER 4[a]

Use of paired fathead minnow (*Pimephales promelas*) reproductive test: Part II Source identification of biological effects at a bleached kraft pulp mill

[a] This chapter has been published in the journal of Environmental Toxicology and Chemistry 25, 202-211 under joint authorship with Monique G. Dubé (University of Saskatchewan), Mark Hewitt (Environment Canada), Tibor Kovacs (PAPRICAN) and Deborah MacLatchy (University of New Brunswick)

4.1 Introduction

Pulp mill effluents (PMEs) have been shown to alter some reproductive indicators in wild fish from Canadian (Munkittrick et al, 1997a; Kovacs et al, 1997; McMaster, 1995), Scandinavian (Andersson et al, 1988; Sandstron et al, 1988), New Zealand (Van Den Heuvel et al, 2004), and United States (Thomas and Hall, 2004; Parks et al, 2001) waters. Effects observed include low fish biomass, reduced recruitment, decreased gonad size and increased liver size. Extensive efforts have been invested to document these responses and to understand physiological and reproductive processes affected by effluent exposure (Kovacs et al, 1997). Whether these changes to reproductive indicators would affect the long-term viability of wild fish stocks has also been speculated (Munkittrick et al, 1997).

In our previous paper (Chapter 3), we described an approach where the short-term fathead minnow (FHM: *Pimephales promelas*) reproduction test of Ankley et al (2001) was modified for in situ testing at a pulp mill that discharges treated effluent into Jackfish Bay, Lake Superior, Ontario, Canada. The objective of that work was to determine if the discharge of final treated effluent (100% v/v and 1% v/v treatments) altered a suite of physiological, individual and population level indicators. Results showed that spawning was significantly reduced in FHM breeding pairs exposed to 100% (v/v) PME compared to pre-exposure data. In comparison, a stimulatory response was observed after exposure to 1% (v/v) PME resulting in increased cumulative egg production compared to controls. This enrichment-type response in the 1% (v/v) treatment corresponded with the increased fecundity in female white sucker (*Catostomus commersoni*) and longnose sucker (*Catostomus catostomus*) recently observed in the Jackfish Bay exposure sites (Environment Canada, 2004b). Exposure to PME in our study also resulted in ovipositor development in males in both the 100% and 1% (v/v) treatments and development of male secondary sex characteristics in females in the 100%

(v/v) treatment. This increased appearance of male secondary sex characteristics in female fish also correlated well with previous field observations (Munkittrick et al, 1997b).

In investigations of pulp and paper effluents on fish, Toxicity Identification Evaluation (TIE) approaches have been applied where the responses of organisms or appropriate bioassays are used to detect the presence of active agents Hewitt et al, 2003, 2005; Martel et al, 1997; Jenkins et al, 2001). In 1997, Martel et al (1997) surveyed process waste evaluations to identify candidate streams for chemical investigations at a thermomechanical pulp (TMP) mill. A flow-proportion approach was taken to evaluate waste streams and TMP condensates were ultimately identified as the major source of compounds causing elevated levels of mixed function oxygenase (MFO) activity in rainbow trout (*Oncorhynchus mykiss*). This investigation identified two potential causative agents; namely juvabione and dehydrojuvabione as ligands for the Ah receptor (Martel et al, 1997)

In 2001, Jenkins et al (2001) investigated water collected from a river known to contain paper mill effluent. Previous investigations at this site have shown androgenization of eastern mosquitofish (*Gambusia holbrooki*) and so fractionation of the river water was undertaken to identify androgenic components. Using cell culture assays, they identified two fractions that induced androgen receptor-dependent transcriptional activity, with androstenedione being identified as one of the fractions.

Parrott et al (2000) used a systematic waste stream approach to investigate sources of chemicals affecting circulating sex steroid levels in goldfish (*Carassius auratus*) at two different pulp mills. A number of waste streams and final effluent were evaluated at a bleached kraft mill (18 streams) and a bleached sulfite mill (14 streams). The individual waste streams within both mills did not affect steroid levels or steroid production. However,

final effluent from both mills after secondary treatment did cause significant steroid depressions e.g. 10-fold drop in testosterone. At the bleached sulfite mill, implementation of a number of process changes including increased ClO_2 substitution from 60-65%, a reduction in solids losses from the bleach plant, reduced liquor losses through spill management and increased aeration within secondary treatment may have resulted in elimination of effects on steroid levels (Parrott et al, 2000).

A number of studies were conducted by Dubé and MacLatchy [2000a, 2000b, 2001] to identify in-mill treatment options for minimizing final mill effluent toxicity at a bleached kraft mill in Saint John NB, Canada, that did not have secondary treatment. Systematic characterization of process stream quality and toxicity was conducted along with exposures of mummichog (*Fundulus heteroclitus*) to in-mill waste streams to identify the source(s) contributing to depressed sex steroids associated with exposure to final effluent. Chemical recovery condensates were identified as a primary source of substances depressing circulating testosterone in mummichog (Dubé et al, 2001). Reverse osmosis (RO) treatment of condensates before their re-use in brownstock washing was found to reduce the amount of active substances (Dubé and MacLatchy, 2000a,b) resulting in a recovery of testosterone levels in fish exposed to environmentally relevant (1% v/v) concentrations of final PME. Reverse osmosis treatment also resulted in a non-acutely lethal final effluent and reduced sublethal toxicity of final effluent in three different marine test species (Dubé and MacLatchy, 2000a)

The results from these Canadian studies have culminated in the development of an investigation of cause (IOC) framework for the pulp and paper industry legislated under the Fisheries Act to conduct Environmental Effects Monitoring (EEM) (Hewitt et al, 2003,

2005). The purpose of an IOC is to generate sufficient knowledge about the source and identity of the effects to identify potential sites within the mill process for mitigation (Hewitt et al, 2003, 2005).

The objective of this work was to employ the FHM test in an IOC approach using selected process streams at the BKPM in Terrace Bay, ON to isolate sources of contaminants causing reproductive effects that were observed after exposure to final BKME (outlined in Chapter 3) and in previous lab and field studies (Munkittrick et al, 1997b; McMaster, 2001). The conclusions from this study will assist in future investigations to characterize potential chemical classes or compound/s causing the reproductive responses.

4.2 Materials and Methods

4.2.1 Mill Description

This study was conducted in July and August of 2003 at a bleached kraft pulp mill in the town of Terrace Bay, Ontario, Canada. The process flow diagram for this mill is illustrated in Figure 4.1 with associated process stream information in Table 4.1. The Terrace Bay facility consists of separate hardwood (Mill 1; 450 air dried metric tones (ADMT)/d) and softwood (Mill 2; 1100 ADMT/d) mills (Figure 4.1). Mill 1 produces hardwood pulp from deciduous poplar and aspen furnish using six batch digesters. The pulp is then deknotted, washed in the brownstock washers and bleached in a 5-stage process of DEDED (D = chlorine dioxide (ClO_2), E = caustic extraction). Mill 2 produces softwood pulp from conifers including spruce, jack pine, and balsam fir in 12 batch digesters and uses 6 stages to bleach the pulp (DE1DE2D) (E1 = caustic, peroxide and oxygen extraction, E2 = caustic and peroxide extraction). Process water is taken from Lake Superior at an average rate of approximately 75,000 L/min. Final effluent is treated as a combined discharge for both the hardwood and

softwood mills and treatment consists of biological treatment in an aerated stabilization basin (ASB) before discharge (83,000 L/min) to Blackbird Creek, a tributary to Jackfish Bay.

Various process streams originating from different areas of Mills 1 and 2 contribute to the composition of the final treated effluent discharged to Blackbird Creek. Batch digestion of pulp requires chips to be cooked at high temperature and pressure in a white liquor mixture of sodium hydroxide and sodium sulphide to dissolve soluble resins, lignins, hemicelluloses, and extractives and yield the cellulose fibers or pulp (Smook, 1994). After digestion in either the hardwood or softwood mills, the pulp is screened and then washed in a series of brownstock washers (countercurrent washing in Mill 2) where the pulp is separated from the spent cooking liquor (weak black liquor or WBL). The WBL is concentrated to strong black liquor in a series of six countercurrent evaporators in the chemical recovery process for each respective mill. The strong black liquor is then concentrated further, heated, mixed with salt cake and ultimately burned in the recovery furnace to generate energy and recover the inorganic cooking chemicals. The latter are converted back to sodium hydroxide and sodium sulphide in the recausticizing plant. Some WBL from the brownstock washers is also mixed with the white liquor in the digesters for dilution and to ensure the wood chips are covered in the digesters during pulping.

Evaporation of WBL produces a series of vapors at six different stages of temperature and pressure. Condensates from WBL entering the 1st effect evaporator of each mill are termed high contaminated condensates and are combined, steam stripped, and then sent (8,000 L/min) to the mix tank of the ASB (Figure 4.1; Table 4.1). Condensates from the remaining 5 effect evaporators of each mill are termed low contaminated condensates (LCC) and are combined in a LCC mix tank (Figure 4.1). The softwood mill (Mill 2) contributes the largest

volume of LCC to the mix tank (8700 L/min) compared to the hardwood mill (2300 L/min) (Table 4.1). The combined LCC are used in both Mills 1 and 2 to wash the pulp in the brownstock washers. In the softwood mill (Mill 2) approximately 6060 L/min of LCC are used at the brownstock washers with an additional 1500 L/min of fresh water. After washing, the pulp is directed to the respective bleach plants.

The bleach plant for Mill 1 involves a 5-stage process of alternating acid (chlorine dioxide; D) and alkaline (caustic sodium hydroxide; E) stages. The bleach plant for Mill 2 involves ClO_2, first caustic extraction [NaOH + O_2 + hydrogen peroxide (H_2O_2)] stage (E), first ClO_2 and NaOH stage, second NaOH and H_2O_2 stage, and finally the last ClO_2 stage. Acid filtrates from the D stages contain chlorides and chlorinated lignin, fatty and resin acids (resin acids originating from softwoods only). The acid filtrates are collected into acid sewers for the hardwood and softwood mills and then combined into a CACID stream (Figure 4.1). The acid sewer also collects losses from the turpentine collection system and domestic sewage from the mill and is discharged directly into the mix tank of the ASB at a rate of approximately 19,000 L/min (Table 4.1). Each mill also contains an alkaline sewer (Mill 1: E; Mill 2: Eop, E2p) which is combined to form the CALK stream. Waste collected from Mill 1 (evaporators/recaustisizing sewer) and Mill 2 (boiler plant/evaporators/power boiler sewer, recausticizing plant, digester plant and boiler plant sewers as well as miscellaneous process spills and upsets (fibre/black liquor) and some nutrients) also contribute to the CALK stream. Mill 2 contributes approximately 50% greater volume (38,000 L/min) to the CALK stream compared to Mill 1 (19,000 L/min) (Table 4.1).

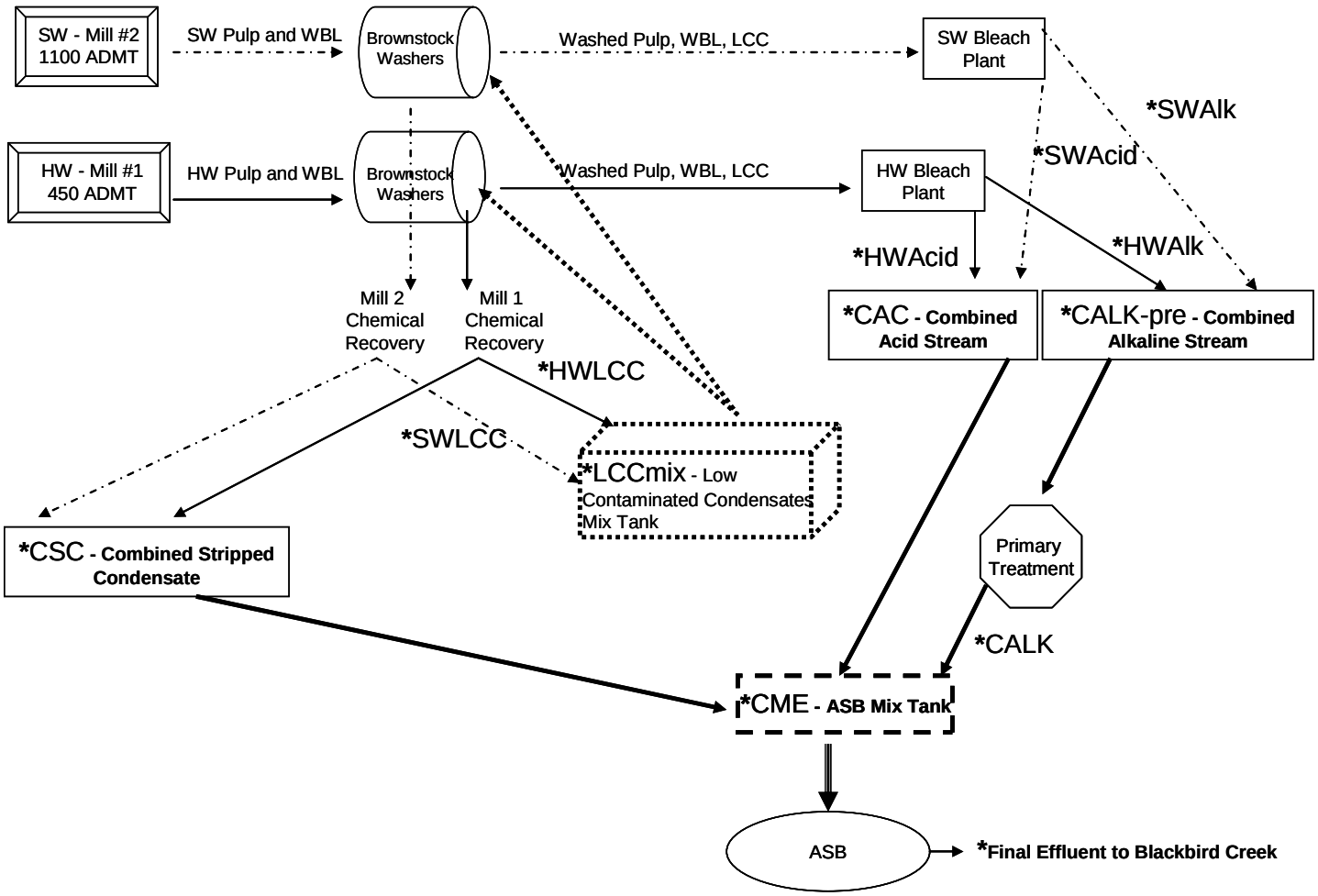

Figure 4.1. Schematic of process waste sewers (combine alkaline, combined acid and combined stripped condensate), the mix tank of the aerated stabilization basin (ASB) and ASB of a bleached kraft pulp mill (BKPM) in Terrace Bay, ON. * SWAlk (softwood alkaline), SWAcid (softwood acid), HWAlk (hardwood alkaline), HWAcid (hardwood acid), HWLCC (hardwood low contaminated condensates), SWLCC (softwood low contaminated condensates), CAC (combined acid), CALK-pre (combined alkaline preprimary treatment), CALK (combined alkaline post primarytreatment) CSC (combined stripped condensate), CME (combined mixed effluent).

Table 4.1. Flow (L/min), chemistry, acute toxicity and exposure concentrations of thirteen bleached kraft process streams including final secondary treated effluent (final treated), combined mill effluent (CME), combined alkaline stream post-primary treatment (CALK-Post), combined alkaline stream pre-primary treatment (CALK-Pre), combined acid (CACID), combined stripped condensate (CSC), softwood (SW) alkaline (SW Alk), SW acid, hardwood (HW) alkaline (HW Alk), HW acid, low contaminated condensate (LCC) mix tank (LCC Mix), HW LCC, and SW LCC. Streams are illustrated in Figure 1. All streams at 100% v/v concentration.

Variable:		Mill Process Streams												
		Final Treated	CME	CALK - Post	CALK - Pre	CACID	CSC	SW Alk	SW Acid	HW Alk	HW Acid	LCC Mix	HW LCC	SW LCC
Flow x 10^3 (L/min)		83.0	83.0	57.0	57.0	19.0	8.00	38.0	14.0	19.0	5.00	11.0	2.00	9.00
Proportion of flow to CME				68.0	NM	23.0	9.00	NM	NM	NM	NM	NM	NM	NM
Toxicity	Rainbow Trout LC50 (%)	>100	43.0	43.0	40.0	18.0	8.00	18.0	18.0	18.0	47.0	18.0	47.0	18.0
	Daphnia magna LC50 (%)	>100	89.0	>100	>100	>68.0	>32.0	82.5	79.0	>100	>100	68.0	>68.0	26.0
Chemistry	pH	7.40	6.30	100	11.5	2.90	9.60	6.20	2.80	9.40	3.10	8.90	9.00	8.80
	Conductivity (umhos)	1300	1200	800	1150	2000	210	1850	2700	900	1250	80.0	80.0	20.0
	DO (mg/L)	1.20	0.80	3.20	0.90	6.60	0.80	4.60	5.70	7.40	6.20	7.50	5.50	7.10
	Ammonia (mg/L)	1.90	2.90	0.03	0.30	NM	NM	NM	NM	NM	NM	NM	NM	NM
Exposure	Calculation	NM	20% of RBT LC50	68% of [CME]	NM	23% of [CME]	9% [CME]	NM	NM	NM	NM	NM	NM	NM
	Concentration tested %	100	8.50	5.75	NM	2.00	0.75	NM	NM	NM	NM	NM	NM	NM

The CALK sewer is treated in two primary treatment clarifiers operating in parallel and a 375,000m^3 settling basin and the clarified effluent is pumped (57,000 L/min) to the mix tank at the ASB. Combination of the acid, alkaline, and stripped condensate sewers at the ASB mix tank results in a relatively neutral pH of the effluent before treatment in the ASB. Secondary treatment is a three-celled ASB with a total volume of 1.1million m^3 and a residence time of approximately 8-10 d (Environment Canada, 2004).

4.2.2 Selection of process streams and test concentrations

Primary process streams were identified and numbered, beginning with the final effluent (Stream #1) and working upstream within the mill (Figure 4.1). Combined mill effluent (CME; Stream #2) consisting of combined alkaline stream after primary treatment (CALK; Streams # 3 and 4), the combined acid stream (CACID; Stream #5), and the combined stripped condensate stream (CSC; Stream #6). The contributions of the softwood and hardwood mills to the CALK, acid, and stripped condensate sewers are illustrated in Figure 4.1. Thirteen process streams were characterized with respect to flow, pH, conductivity, dissolved oxygen, ammonia and acute toxicity to rainbow trout (*Oncorhynchus mykiss*) and Daphnia magna (Table 4.1). Toxicity tests were conducted at the Pulp and Paper Research Institute of Canada and consisted of a single test on each process stream conducted over a 20 d period in 2003. Solutions used in these tests were aerated and adjusted to neutral pH and optimum dissolved oxygen. The capacity did not exist to test all 13 streams using FHM in the partial lifecycle bioassay. Therefore, Lake Superior control water and 5 primary process streams were selected for testing. The description of each treatment is as follows. Firstly, final treated effluent (Stream #1), which consists of combined mill effluent after secondary treatment in an aerated stabilization basin (ASB). Secondly, combined mill effluent (CME)

(Stream #2) which is a mixture of combined alkaline stream (CALK) (primary treated), combined acid (CACID) and combined stripped condensate (CSC) stream and was sampled from the mix tank of the ASB before secondary treatment. Thirdly, CALK stream post-primary treatment (Stream #3). This stream consists of alkaline sewers from both hardwood (Mill 1) and softwood (Mill 2) bleach plants, combined with waste collected from Mill 1 (evaporators/recaustisizing sewer) and Mill 2 (boiler plant/evaporators/power boiler sewer, recausticizing plant, digester plant and boiler plant sewers as well as miscellaneous process spills and upsets (fibre/black liquor) and some nutrients. Fourthly, CACID (Stream #5) contains acid filtrates that are collected into acid sewers for the hardwood and softwood mills and then combined into a CACID stream (Figure 4.1). The acid sewer also collects losses from the turpentine collection system and domestic sewage from the mill. Finally, CSC (Stream #6) consists of high contaminated condensates formed from the 1st effect evaporators in the chemical recovery areas of both Mill 1 and 2 that are combined and steam stripped. The CALK stream was tested after primary treatment as it is the clarified effluent which affects final effluent quality.

Single test concentrations were selected based upon acute toxicity results and flow proportions. Final effluent was tested at 100% (v/v) concentration. The CME stream was tested at 8.5% (v/v) which represented 20% of the lethal concentration (LC50) (43% v/v) for rainbow trout for this stream (Table 4.1). The CSC, CALK and CACID streams all contribute known flow proportions to the CME and their concentrations were set at 8.5% of their flow to correspond with the CME treatment (Table 4.1).

4.2.4 Methods for FHM exposures

Experiments were conducted for 60 d from July to August 2003 using a flow-through, enclosed bioassay trailer located on-site at the BKPM. Details of the site and enclosed bioassay trailer are outlined in Chapter 3.

4.2.4.1 Pre-exposure design

A pre-exposure and an exposure trial of approximately similar duration (21 d) was conducted. A full description of the short-term FHM bioassay, pre-exposure design and corresponding analyses is outlined in Chapter 3.

4.2.4.2 Exposure design

Nine breeding pairs were exposed to each of the six treatments for a further 21 d. Effluents were collected twice weekly. Treated final BKME was collected from the outflow point of the secondary treatment lagoon and stored in a 1000 L holding tank in the bioassay trailer at 25^{o}C. Waste water from the four process streams were sampled and stored in 70L polyethylene containers outside of the trailer. 200L (100% v/v), 58L (CME), 11.5L (CALK), 4L (CACID) and 1.5L (CSC) of effluent were supplied daily to 200 L holding tanks for supply to three treatment aquaria per treatment at a total treatment flow rate of 64 L/day/aquarium. This maintained the turnover time to two exchanges per aquarium per 24 h period. Daily observations and analyses conducted during the exposure period are outlined in Chapter 3.

4.2.4.2.1 Exposure analysis

Pre-exposure analysis between tanks was conducted to determine if any tank effects could be observed. A full description of the analyses undertaken is outlined in Chapter 3. A full description of the analyses undertaken after exposure is outlined in Chapter 3.

4.3 Results

4.3.1 Water and effluent chemistry

The most significant differences in chemistry were observed in the 100% (v/v) PME treatment compared to control (Table 4.2). Significant increases in specific conductivity and sodium were observed in 100% PME (v/v) (ANOVA, $p < 0.001$), CME (ANOVA, $p < 0.001$), CALK (ANOVA, $p<0.001$) and CACID (ANOVA, $p<0.001$) treatments compared to control. Dissolved organic carbon was only significantly increased in 100% PME (v/v) (ANOVA, $p=0.001$), CME (ANOVA, $p<0.001$) and CALK treatments (ANOVA, $p<0.001$). Sulfate and potassium increased in 100% PME (v/v) (KWALLIS, $p=0.027$; ANOVA, $p<0.001$ respectively), CME (ANOVA, $p<0.001$) and CALK treatments (ANOVA, $p<0.001$; ANOVA, $p = 0.002$ respectively). Significant increases in hardness, colour and chloride were only observed in 100% (v/v) PME treatments. No significant differences were observed in any of the parameters measured in the CSC compared to control.

4.3.2 Individual endpoints

There were no significant differences observed in any of the treatments for GSI, CF (%) or weight gain (g) compared to control (Table 4.3). An increase in LSI was observed for males in all treatments with statistically significant increases (ANOVA, $p = 0.029$) observed in the 100% PME treatment.

Table 4.2. Water quality parameters and chemistry of pulp mill effluent (PME) effluent: final secondary treated effluent (100% PME), combined mill effluent (CME), combined alkaline stream post-primary treatment (CALK), combined acid (CACID), combined stripped condensate (CSC) and Lake Superior, Canada, water (control) from 21-day exposure aquaria. Streams are illustrated in Figure 4.1. Samples taken from one aquaria/treatment/week for 3 weeks plus head tanks, values are mean (n=3) ± standard error. Temperature data continuously recorded by onset loggers throughout the 21-day pre and post-exposure. Asterisk denotes significant difference from control, where * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Variable (mg/L unless indicated)		Control	100% PME	CME	CALK	CACID	CSC
General	pH (pH units)	7.90 ± 0.06	7.90 ± 0.08	7.70 ± 0.05	7.70 ± 0.20	7.80 ± 0.05	7.60 ± 0.10
	Colour (pt-co)	4.20 ± 1.00	908 ± 30.2*	61.2 ± 4.60	46.4 ± 3.40	12.4 ± 0.70	4.70 ± 0.20
	Temp (oC)	24.9 ± 0.20	24.5 ± 0.30	25.0 ± 0.20	24.9 ± 0.20	24.7 ± 0.20	23.9 ± 0.40
	Salinity (ppt)	0.10 ± 0.00	0.50 ± 0.03***	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
	Specific conductivity	113 ± 1.30	1306 ± 68.7***	227 ± 1.80***	182 ± 4.80***	144 ± 2.90***	120 ± 3.50
	DO (%)	82.1± 2.40	73.2 ± 3.70	78.8 ± 3.90	80.7 ± 3.70	83.5 ± 2.50	82.3 ± 2.50
Chemistry	Nitrate	0.60 ± 0.04	4.00 ± 0.20	0.70 ± 0.70	0.30 ± 0.10	0.30 ± 0.10	0.80 ± 0.40
	Ammonia	0.10 ± 0.08	0.20 ± 0.04	0.05 ± 0.03	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.02
	Nitrite	0.10 ± 0.05	0.10 ± 0.01	0.10 ± 0.06	0.10 ± 0.01	0.10 ± 0.03	0.10 ± 0.03
	DOC	1.90 ± 0.20	139 ± 1.20***	14.0 ± 1.70***	10.3 ± 1.00***	5.20 ± 0.40	2.70 ± 0.20
	Total Nitrogen	1.00 ± 0.20	5.30 ± 0.20	1.20 ± 0.70	0.50 ± 0.03	0.60 ± 0.06	1.10 ± 0.40
	Calcium	15.5 ± 0.60	53.0 ± 3.50***	18.7 ± 1.20	16.5 ± 0.80	17.2 ± 0.80	15.7 ± 0.50
	Chloride	2.10 ± 0.40	187 ± 7.00*	14.4 ± 3.00	5.60 ± 1.00	9.00 ± 1.00	2.60 ± 0.20
	Hardness	51.0 ± 1.80	155 ± 8.70***	59.9 ± 3.30	53.5 ± 2.40	55.8 ± 2.40	51.6 ± 1.70
	Sulfate	4.80 ± 0.50	153 ± 3.00***	22.3 ± 2.20***	16.7 ± 1.50***	6.50 ± 0.30	6.00 ± 0.30
	Potassium	0.70 ± 0.10	7.50 ± 0.60***	1.5 ± 0.2***	1.2 ± 0.1**	0.70 ± 0.10	0.80 ± 0.10
	Magnesium	3.00 ± 0.10	5.50 ± 0.00***	3.2 ± 0.1	3.0 ± 0.1	3.10 ± 0.10	3.00 ± 0.10
	Sodium	2.50 ± 0.30	216 ± 19.9***	24.2 ± 1.0***	18.0 ± 0.5***	6.20 ± 0.40***	2.90 ± 0.20
	TSS	0.00	7.20 ± 1.30***	1.8 ± 0.9	2.1 ± 0.7	0.00	0.00
	Total Phosphorus	0.00 ± 0.01	0.40 ± 0.20	0.2 ± 0.1	0.0 ± 0.0	0.10 ± 0.01	0.10 ± 0.04

4.3.3 Spawning events

Spawning events were assessed throughout the 21 d exposure period (Figure 4.2). Total spawning events per treatment were also assessed as a percentage difference of the number of spawning events in the pre-exposure in each treatment (Figure 4.3). The number of spawning events during the 21d exposure showed significant reductions in both the CME (Kolmogorov-smirnov, $p=0.035$) and the CALK treatments (Kolmogorov-smirnov, $p<0.001$) compared to control (Figure 4.2). A decrease in spawning events was observed across all treatments compared to pre-exposure data (Figure 4.3) where the reduction in CALK stream > CME > 100% (v/v) > control with a significant reduction observed in the 100% (t test, $p=0.004$), CME (t test, $p <0.001$), CALK stream (t test, $p <0.001$) and CSC (t test, $p=0.008$). When the percentage differences in all treatments were compared with control a significant difference was observed in the CALK stream (ANOVA, $p=0.005$) (Figure 4.3).

4.3.4. Egg production

Egg production was assessed throughout the 21 d exposure period (Figure 4.4). Total number of eggs produced per treatment was also assessed as a percentage difference of the number of eggs produced in the pre-exposure period in each treatment (Figure 4.5). When data were assessed over time a significant difference in distribution was observed in the CALK stream compared to control (Kolmogorov-Smirnov, $p<0.001$). Overall, a 75% decline in egg production was observed in the CALK stream compared to control. When this stream was combined with the CSC and CACID stream in the CME treatment, egg production was decreased by 13% compared to control. After 21 d exposure an 18% reduction was observed in the 100% (v/v) treatment compared to control.

Table 4.3. Total body weight (g), condition factor (CF; %), gonadosomatic (GSI; %) and liversomatic (LSI; %) indices for adult male and female fathead minnow (*P. promelas*) after exposure to pulp mill effluent (PME) (final secondary treated effluent; 100% PME), combined mill effluent (CME), combined alkaline stream post-primary treatment (CALK), combined acid (CACID), combined stripped condensate (CSC) and Lake Superior, Canada, water (control). Streams are illustrated in Figure 4.1. Each value represents the mean ± standard error of the mean. Asterisk denotes significant difference from control, where * = $p<0.05$

Sex	Variable	TREATMENTS					
		Control	Final treated (1)	CME (2)	Comb. Alk (3)	Comb. Acid (5)	Comb. Stripped (6)
Female	GSI (%)	10.2 ± 1.85	12.7 ± 1.20	12.1 ± 1.28	13.3 ± 1.31	10.2 ± 1.27	10.8 ± 1.00
	LSI (%)	2.02 ± 0.32	2.93 ± 0.28	2.52 ± 0.32	2.29 ± 0.38	3.04 ± 0.37	2.07 ± 0.39
	Cf (%)	1.55 ± 0.05	1.53 ± 0.06	1.42 ± 0.08	1.62 ± 0.10	1.44 ± 0.05	1.50 ± 0.08
	Weight (g)	3.46 ± 0.31	3.68 ± 0.15	3.36 ± 0.15	3.71 ± 0.26	3.13 ± 0.28	3.63 ± 0.28
Male	GSI (%)	1.29 ± 0.12	1.16 ± 0.19	1.55 ± 0.21	0.99 ± 0.18	1.32 ±0.20	1.46 ± 0.27
	LSI (%)	2.23 ± 0.14	3.41 ± 0.24*	3.10 ± 0.37	2.61 ± 0.22	2.56 ± 0.27	2.65 ± 0.23
	Cf (%)	1.74 ± 0.06	1.64 ± 0.07	1.58 ± 0.09	1.54 ± 0.06	1.57 ± 0.05	1.52 ± 0.06
	Weight (g)	1.81 ± 0.13	1.64 ± 0.10	1.84 ± 0.14	1.65 ± 0.12	1.67 ± 0.09	1.43 ± 0.11

When compared to pre-exposure data egg production in the CALK stream was significantly reduced (t-test, p=0.002). When the percentage differences in all treatments were compared with control a significant difference was observed in the CALK stream (ANOVA, p=0.009) (Figure 4.5).

4.3.5 Development of female sexual characteristics

Ovipositor development was not observed in males during the pre-exposure period. However, ovipositors developed during the exposure period in all treatments including control (Figure 4.6). The greatest increase in male ovipositor development was observed in the CALK stream > 100% > CME > CACID stream. The CALK stream treatment was significantly greater than control (Kolmogorov-smirnov, p=0.009).

4.3.6 Development of male sexual characteristics

Development of male secondary sex characteristics in females was not observed in the pre-exposure period. However significant development of fin dots on the dorsal fin and banding was observed after exposure to the CME and 100% (v/v) PME treatments compared to controls ($p < 0.05$) (Figure 4.7).

4.3.7 Biochemical and histopathological analyses

Significant increases in whole-body testosterone levels in females was observed in 100% (v/v) PME (ANOVA, p = 0.001), CALK (ANOVA, p= 0.025) and CACID streams (ANOVA, p=0.033) with respective increases of 66%, 55% and 57% observed compared to control (Figure 4.8A). In males, significant differences in testosterone were also observed in the CME (ANOVA, p=0.043), CACID (ANOVA, p=0.003) and CSC stream (ANOVA,

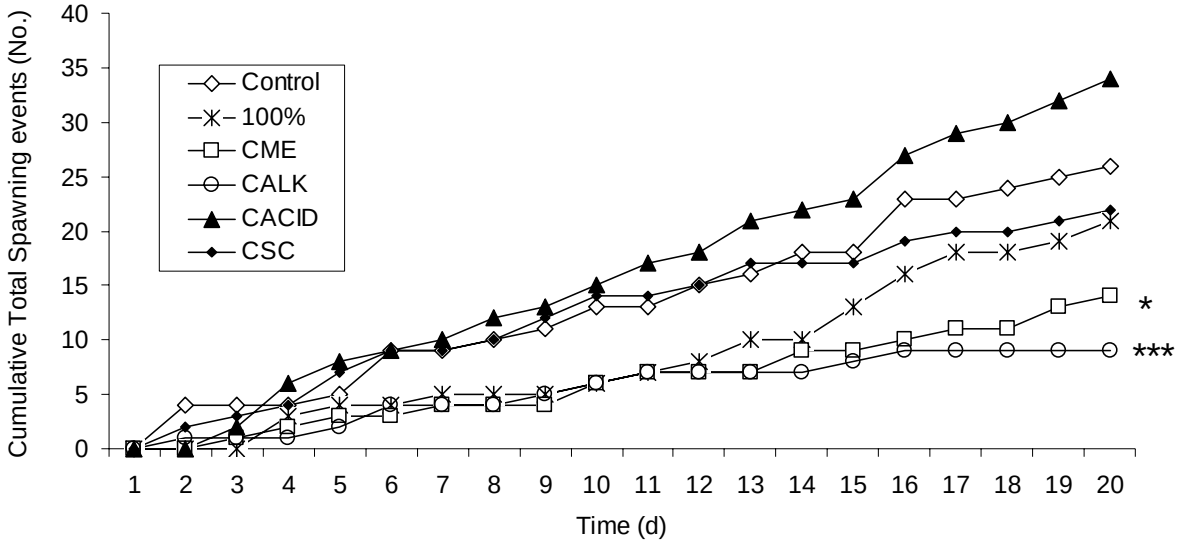

Figure 4.2. Cumulative number of spawning events of fathead minnow (*P. promelas*) breeding pairs during 21 d exposure to final bleached kraft mill effluent (100% v/v), selected process streams (CME = combined mill effluent, CALK = combine alkaline stream, CACID = combined acid stream, CSC = combined stripped condensate) and Lake Superior water (control). Asterisk denotes significant difference from control, where * = $p<0.05$ and *** = $p<0.001$.

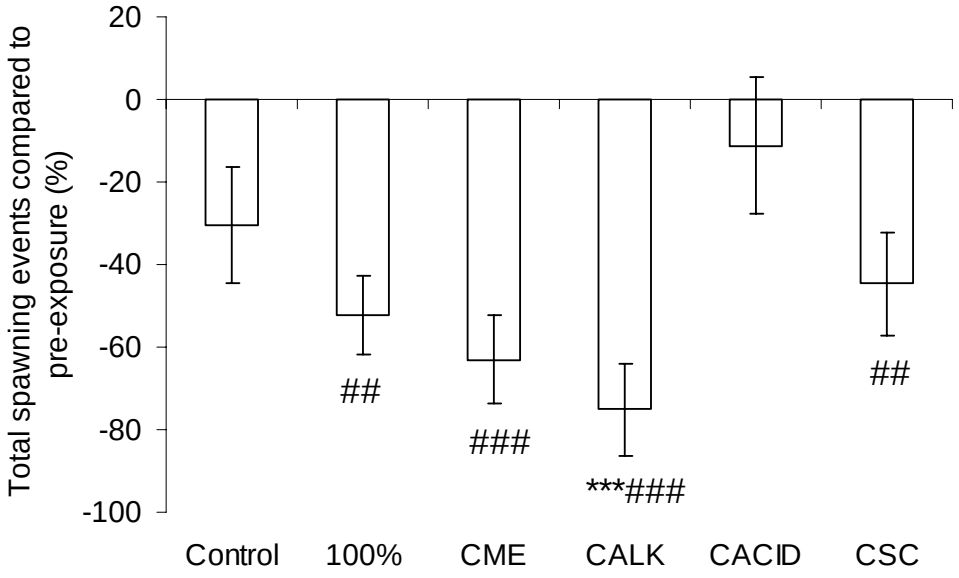

Figure 4.3. Spawning events of fathead minnow (*P. promelas*) breeding pairs. Total number compared to pair-specific pre-exposure data (%) after 21 d exposure to bleached kraft mill effluent (100% v/v), selected process streams (CME = combined mill effluent, CALK = combine alkaline stream, CACID = combined acid stream, CSC = combined stripped condensate) and Lake Superior water (control). Asterisk denotes significant difference from control, where *** = $p < 0.001$. Pound denotes significant difference from pre-exposure data, where ## = $p < 0.01$ and ### = $p < 0.001$. Error bars represent +/- 1 SE

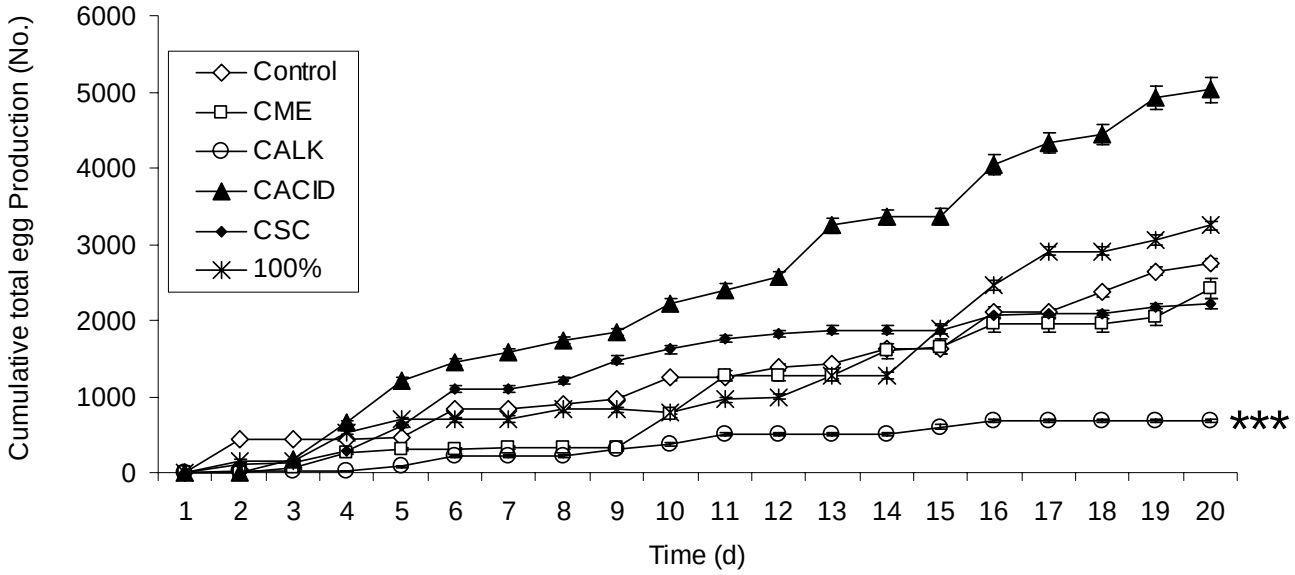

Figure 4.4. Cumulative number of total eggs produced by fathead minnow (*P. promelas*) breeding pairs during 21 d exposure to final bleached kraft mill effluent (100% v/v), selected process streams (CME = combined mill effluent, CALK = combine alkaline stream, CACID = combined acid stream, CSC = combined stripped condensate) and Lake Superior water (control). Asterisk denotes significant difference from control, where *** = $p < 0.001$.

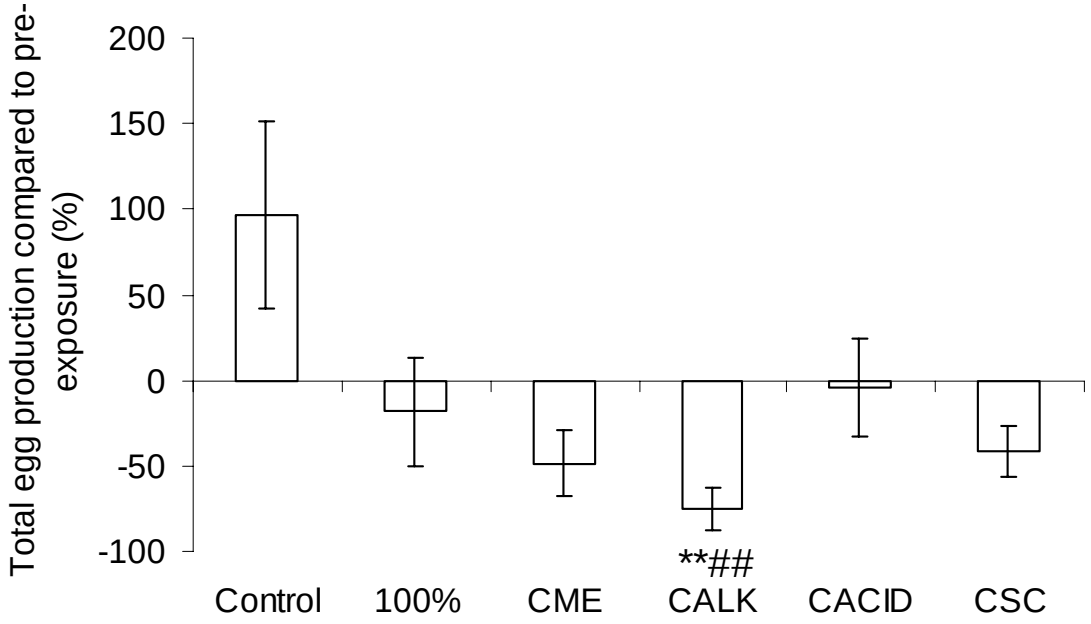

Figure 4.5. Egg production by fathead minnow (*P. promelas*) breeding pairs. Total number compared to pre-exposure data (%) after 21 d exposure to bleached kraft mill effluent (100% v/v), selected process streams (CME = combined mill effluent, CALK = combine alkaline stream, CACID = combined acid stream, CSC = combined stripped condensate) and Lake Superior water (control). Asterisk denotes significant difference from control, where ** = $p < 0.01$. Pound denotes significant difference from pre-exposure data, where ## = $p < 0.01$. Error bars represent +/- 1 SE.

p=0.002) with respective increases of 74%, 120% and 145% observed (Figure 4.8B).

In females, the percentage of oogonial follicles significantly decreased in the CALK (ANOVA, p=0.014), CME (ANOVA, p=0.008), and 100% (ANOVA, p=0.041) treatments relative to controls (data not shown). No significant differences were observed between stages of spermatocyte development in male gonads.

4.3.8. F1 offspring endpoints

There were no significant differences in hatching success in any of the treatments (data not shown). There were also no significant differences observed in occurrence of deformities in larvae.

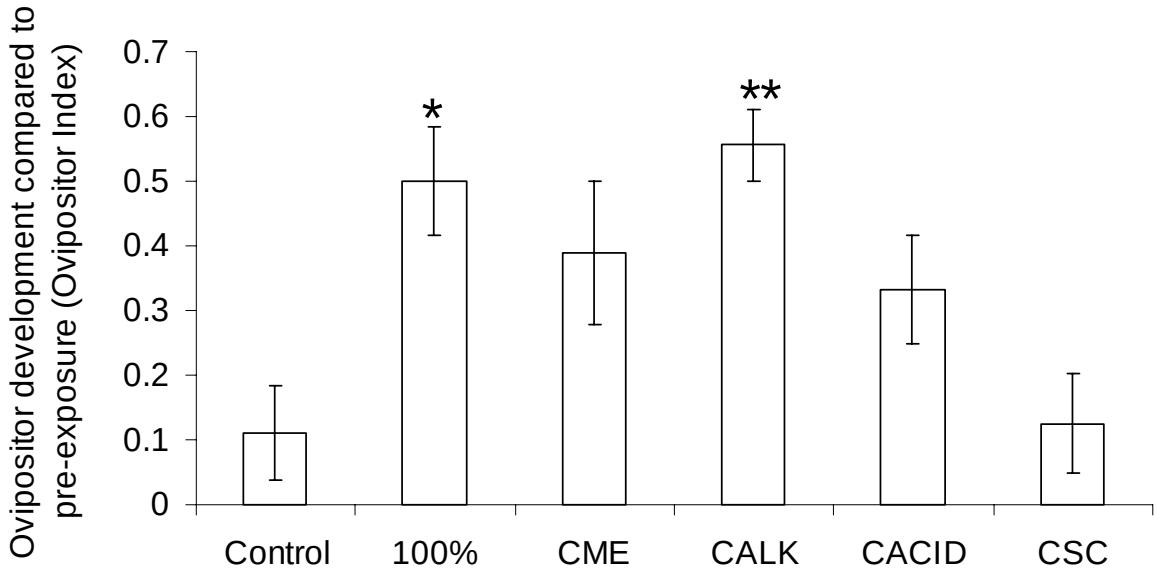

Figure 4.6. Ovipositor development in male fathead minnow (*P. promelas*) during 21 d exposure to final bleached kraft mill effluent (100% v/v), selected process streams (CME = combined mill effluent, alkaline = CALK stream, acid = CACID stream, stripped condensate = CSC) and Lake Superior water (control). Calculated as difference in ovipositor index from pre-exposure measurements. Asterisk indicates significant difference from control, where * = $p < 0.05$, ** = $p < 0.01$. Error bars represent +/- 1 SE

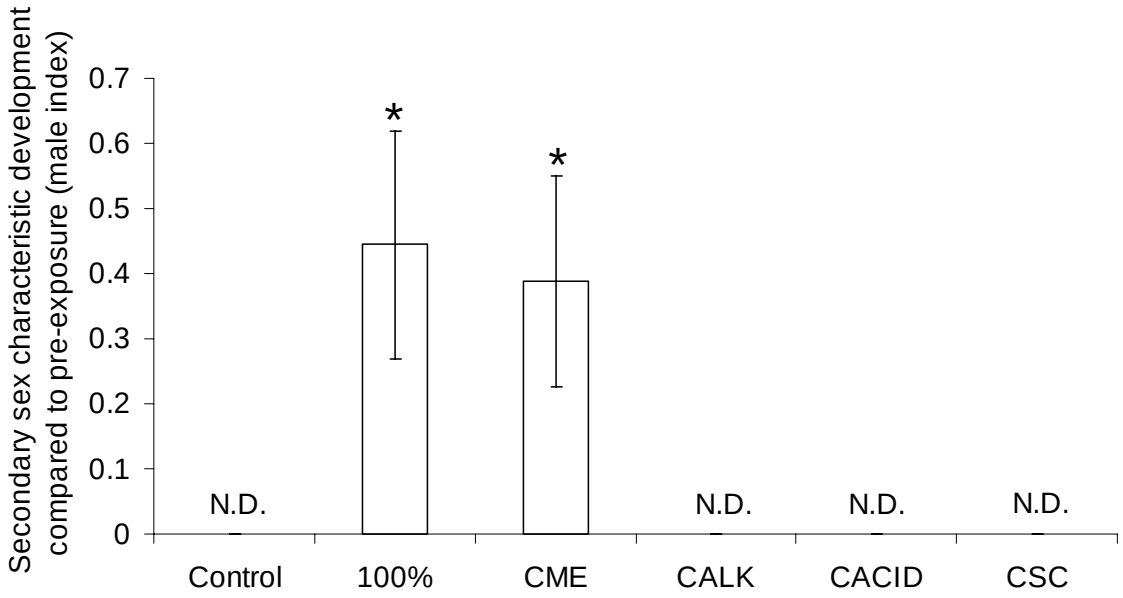

Figure 4.7. Secondary sex characteristic development (expressed as an index, see Table 4.1) in A) female fathead minnow (*P. promelas*) after 21 d exposure to final bleached kraft mill effluent (100%), selected process streams (CME = combined mill effluent, alkaline = CALK stream, acid = CACID stream, stripped condensate = CSC) and Lake Superior water (control). Calculated as difference in male index from pre-exposure measurements. Asterisk indicates significant difference from control, where * = $p < 0.05$. N.D. = non-detectable. Error bars represent +/- 1 SE

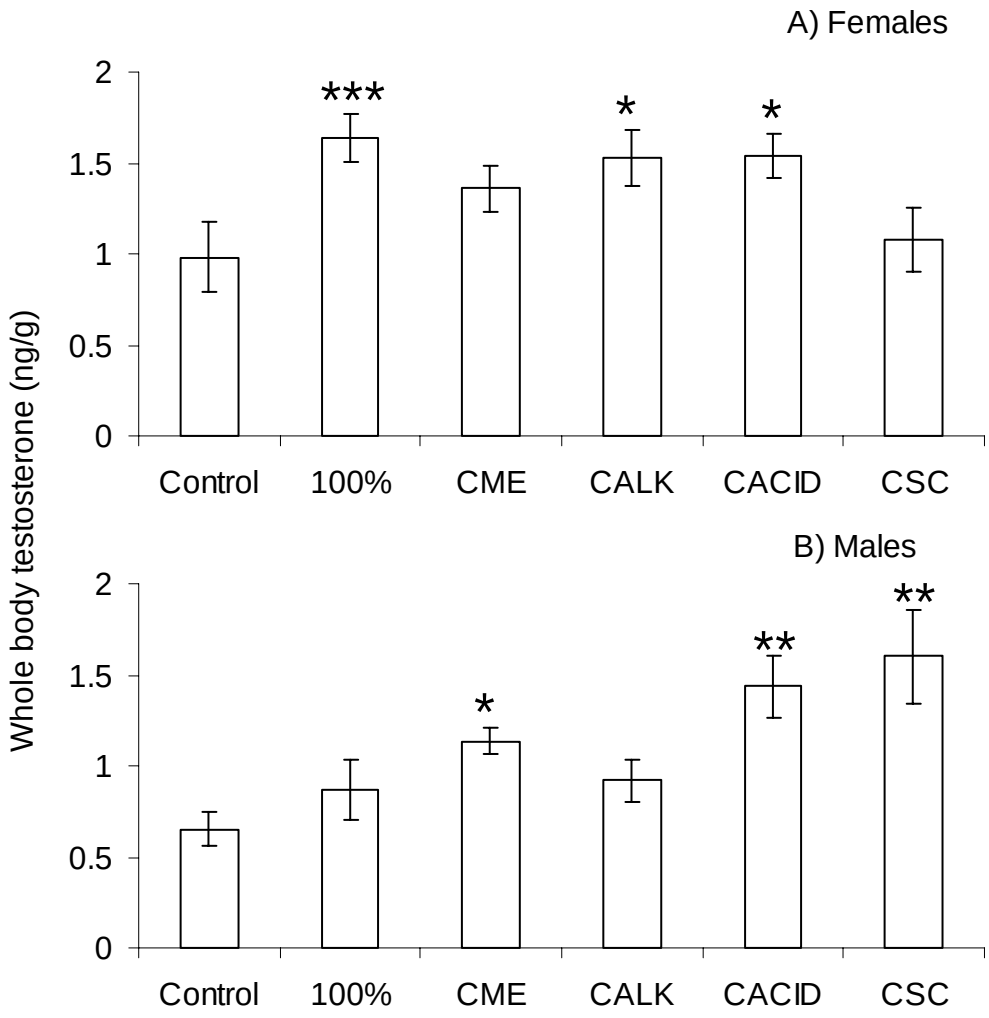

Figure 4.8. Whole body testosterone (ng/g) in female (A) and male (B) fathead minnow (*P. promelas*) after 21 d exposure to final bleached kraft mill effluent (100%), selected process streams (CME = combined mill effluent, alkaline = CALK stream, acid = CACID stream, stripped condensate = CSC) and Lake Superior water (control). Asterisk indicates significant difference from control, where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Error bars represent +/- 1 SE

4.4 Discussion

The objective of this work was to conduct a study to isolate potential contaminant sources causing reproductive effects in FHM that were observed after exposure to secondary treated effluent from the BKPM at Terrace Bay, ON (outlined in Chapter 3) and also observed in wild fish at this site.The confirmation of activity with the FHM bioassay after exposure to final PME (100% v/v and 1% v/v) led to the application of an investigation of cause (IOC) approach in the present study. The goal of this IOC was to isolate the source of compound/s to better understand the responses being observed in FHM exposed to final treated PME identified in Chapter 3 of this series. The information gathered in this IOC will help guide any further detailed investigation into identification of causative agents at this mill.

4.4.1 Combined stripped condensate stream

The combined stripped condensate is the smallest contributor to final PME but had the highest toxicity for rainbow trout (*O. mykiss*) and *D. magna* (Table 4.1). This process sewer consists of high contaminated condensates formed from the 1st effect evaporators in the chemical recovery areas of both Mill 1 and 2 that are combined and stripped. In our investigation, the CSC stream affected FHM the least compared to the other treatments. Responses, overall, were similar to those in the CALK but to a lesser extent e.g. reduced spawning events. Significantly increased testosterone levels in males was observed after exposure to CSC, this response was not seen in the CALK stream but increased testosterone was seen in the CACID stream. In addition, the CSC treatment did not induce ovipositor development in males signifying that at the concentration tested, the compound/s causing this development in the final effluent likely did not originate from this source. It is important to note that in previous studies, condensates were identified as a primary source affecting reproductive indicators in mummichog (Dubé and

MacLatchy, 2001). However, these condensates originated from the 5th effect evaporator of a BKPM in New Brunswick, Canada and are not the same as the stripped condensate stream reported here for the Terrace Bay mill. The 5th effect condensates are more similar to the LCC condensates at the Terrace Bay mill that are discharged to the CALK stream.

4.4.2 Combined acid stream

The CACID sewer, originating from the bleach plant, is the second largest contributor to final effluent at this mill. This stream had one of the lowest LC50's for rainbow trout and the highest conductivity. These results are reflected in our 21 d exposure where the only significant difference from control was an increase in testosterone. No other differences were observed.

4.4.3 Combined alkaline stream

The CALK stream is the dominant process sewer with the largest contribution of approximately 57 L/min into the ASB mix tank (Figure 4.1). Even though its flow contribution is greater than that of the CACID and stripped condensate streams, it has the lowest toxicity to rainbow trout and D. magna (Table 4.1). The alkaline stream collects process waste from the caustic extraction stages in the bleach plants as well as wastewater from various areas in both mills (Figure 4.1) and is treated in two primary clarifiers before entering the ASB mix tank. The CALK stream also receives LCC from the chemical recovery process that is used as wash water in the brownstocks. The responses of FHM exposed to the CALK treatment were the most dramatic resulting in a significant reduction in egg production and spawning events and a significant increase in ovipositor development in males. Spawning events and egg production in the CALK treatments were significantly reduced (65% and 74% respectively) compared to pre-

exposure data and when this data was compared with control, with no recovery during the 21-day exposure (Figures 4.2-4.5).

4.4.4 Combined mixed effluent and final treated pulp mill effluent

Combination of the CACID, CALK and CSC sewers occurs in the aerated stabilization basin (ASB) mix tank prior to secondary treatment (Figure 4.1). The toxicity of the CME stream to rainbow trout and *D. magna* is similar to that of the CALK stream (Table 4.1). Final PME is treated in a 300 million gallon clay-lined earthen ASB that provides biological treatment. After secondary treatment in the ASB, effluent toxicity diminished substantially with rainbow trout and *D. magna* (Table 4.1). This highlights the role of biotransformation in the ASB in reducing the overall toxicity of effluent.

The responses observed in FHM exposed to the CALK stream appeared to carry through to the CME and 100% (v/v) PME treatment. A significant reduction in spawning events in both treatments compared to pre-exposure data, and development of ovipositors in males in 100% (v/v) PME treatment, compared to controls, occurred. This suggests that the compound/s causing the reduction may have been diluted or transformed from the CALK to CME to 100% (v/v) PME. The CALK stream contributes 68% of wastewater into the ASB mix tank, this would mean that any compounds within the CALK stream would be diluted by ~ 45% when entering the mix tank of the ASB (CME treatment). These results suggest that the CALK stream is an important process stream affecting final effluent quality and its effects on FHM. The improvement in reproductive output in the 100% PME (v/v) treatment could have been due to the addition of compounds from the CACID and CSC stream that diluted the effects from the CALK stream, resulting in reduced responses of FHM indicators to the CME and 100% (v/v) PME streams. The CME in the ASB mix tank is not diluted any further but does undergo secondary treatment where

biotransformation could also account for the reduction in effects observed in 100% (v/v) PME. It is also possible that the initial reduction in spawning events and egg production in 100% (v/v) PME was due to the hardness and conductivity, which was significantly higher (3-fold and 5-fold respectively) than in any other treatment (Table 4.2). The recovery of egg production and spawning events towards the end of the exposure (around day 14) could have been due to acclimation. This is an area worthy of further investigation.

Results from seven-day FHM sub-lethal effluent toxicity testing (growth inhibition) showed that effluent from this mill was generally non-toxic to FHM with an inhibition concentration (IC25) >100% in six of the eight samples tested (Environment Canada, 2004). However, two tests conducted in April 2000 and 2001 resulted in growth IC25 values of 75.9% and 56.2% respectively. No explanation for the variability in the two samples from the other six samples was given, but, it would indicate that effluent from this mill has the potential to cause some sub-lethal effects in this species which is consistent with our results from final PME exposures (100% v/v). This study also demonstrates the dynamic nature of the final PME and how quality can change from one test to another or from week to week. This may therefore account for the variability in responses we observed in the last week of exposure in the 100% (v/v) treatment. This also raises the need for consistent characterization of final effluent quality if comparisons are to be made across mills or even for a single mill effluent over time.

4.4.5 Identification of cause

The goal of our study was to identify potential in-mill waste streams that affect final effluent quality and result in responses of FHM reproductive indicators. We achieved this goal by identifying the CALK stream as a source causing responses in FHM reproductive indicators. Although, identification of causative agents in these streams was beyond the scope of this work,

some interesting observations relative to the literature can be made. It is possible that the compounds affecting egg production and other reproductive endpoints are acting as estrogens. The responses in reproductive output (egg production and spawning events) in FHM from our study have also been observed in FHM after 21 d exposure to increasing concentrations of 17α-ethynylestradiol (EE2). A number of investigations (McMaster et al, 1995; Pawlowski et al, 2004b) have shown that at very low doses (<1ng/L) of EE2 a stimulation in egg production is observed, indicative of a hormesis effect. However, with increasing concentrations (>3ng/L), inhibition of reproductive output occurs. Assuming additivity toward an overall estrogenic response, the compounds causing the changes in reproductive output (egg production and spawning events) in our study could be estrogens and found in high concentrations (or high potency) in the CALK stream. This also corresponds well with the observations in the final effluent (Chapter 3) where we observed an increase in reproductive output in 1% (v/v) PME and a decrease in reproductive output at high concentrations (100% v/v). If the compounds causing changes in reproductive output in the final effluent (increased at low concentrations and decreased output at high concentrations) are estrogenic in nature, based on the response patterns documented in this and other studies, it would appear that the dominant source would be the CALK stream. Preliminary TIE work conducted by Belknap et al [Belknap. AM, University of Guelph, Guelph, ON, Canada] at the Terrace Bay mill show that the LCC condensates from the softwood mill contain similar compounds as those identified as endocrine disrupting chemicals in the 5th effect condensates at a New Brunswick mill. Interestingly, the LCC condensates are a component of the CALK stream. Quantification of the LCC contribution to the CALK stream in future studies at this mill should be considered.

The cessation in spawning with no recovery in FHM exposed to the CALK stream and CME has also been observed after exposure to the androgen methyltestosterone (MT) (Ankley et al, 2001; Pawlowski et al, 2004a). The mechanism behind the cessation in spawning is thought to be due to androgens playing an inhibitory role in final maturation and/or release of eggs. The decrease in oogonia and increase in pre-ovulatory ovarian follicles in female FHM from all treatments is consistent with this hypothesis. The development of male secondary sex characteristics in females in the CME would also correspond with the presence of androgens, a response that has been documented in previous investigations with FHM exposed to the androgens dihydrotestosterone (DHT) (Parrott and Wood, 2001) and methyltestosterone (Ankley et al, 2001; Pawlowski et al, 2004a). However, the lack of any development of male secondary sex characteristics in females exposed to the process streams contributing to the CME suggests otherwise. From this study it appears that the development of male secondary sex characteristics occurs only when the process streams are combined in the mix tank of the ASB (CME treatment) and undergo secondary treatment (100% (v/v) PME treatment).

A final postulation in examining the overall responses from this study is the possibility that the combination of individual streams is necessary to produce the effect associated with combined final effluent. Final treated effluent has been shown to affect sex steroids in previous investigations (Parrott et al, 2000). Significant reductions in goldfish testosterone were observed in final treated effluent at a bleached sulphite mill but no corresponding reductions were seen in isolated process streams (Parrott et al, 2000). It remains to be seen whether the effects of final combined mill effluent are related to the combination of individual streams (in the mix tank of the ASB) or whether hormonally active compounds are produced during the course of effluent treatment in the ASB itself. In either case, our observations of male secondary sex characteristic

development in females are consistent with that of previous studies. Further investigation is required to ascertain the role of secondary effluent treatment in effects on fish reproduction at this mill and other mills across Canada.

4.4.6 Summary and future work

The short-term FHM bioassay was a useful tool in measuring reproductive responses to PME in this investigation. By transferring the bioassay into an artificial stream trailer for on-site exposure we avoided transport and storage-related complications that have been linked to reduced effluent potencies in previous studies (Hewitt et al, 1996). We identified that responses to final effluent were concentration and time dependent and we were able to isolate one stream causing responses, the CALK stream. In summary, the major responses observed in 100% (v/v) PME were reduced spawning events, compared to pre-exposure data, with a corresponding increase in ovipositors in males. A reduction in spawning events, compared to pre-exposure data, was also identified in the CME treatment. The most severe responses were observed in the CALK stream identifying the latter as a major source of compounds causing effects at high final PME concentrations (100% v/v). In addition, a number of endpoints measured in our study corresponded with observations from previous studies on wild fish from the effluent receiver. Building on the results of this study, future investigations should include TIE's, to identify causative agents, and characterization for estrogenic versus androgenic activity for the CALK stream. One possible direction in future studies would be to breakdown the CALK stream into the two alkaline streams from mill one and two. Using the FHM short-term bioassay, as well as physical-chemical analysis, it may be possible to identify the contributions of each stream to the overall combined effect observed in the CALK stream. If the primary source is identified, a treatment process may help to alleviate any effects originating from this source. Previous

investigations (Dubé and MacLatchy, 2000a) have shown that by implementing a treatment process (reverse osmosis) at the identified source, reproductive effects on fish were significantly reduced. In addition, investigations into the effects of biotransformation during secondary treatment on effluent quality should also be conducted.

CHAPTER 5 [a]

Assessing effects of metal mining effluent on fathead minnow (*Pimephales promelas*) reproduction in a laboratory trophic-transfer system

[a] This chapter has been accepted to the journal of Environmental Science and Technology under joint authorship with Monique G. Dubé, Lynn Weber, Kim Driedger and David Janz (University of Saskatchewan)

5.1 Introduction

The effects of metal mining operations in the Sudbury, ON region have been well documented over the last few decades, the most severe of which were acidification and metal contamination of both aquatic and terrestrial environments (Brady and Morris, 1986; Griffiths, 1992). Remediation efforts over the last 30 years have led to extensive improvements in the surrounding environment (Gunn et al, 1995), however, some issues remain.

Junction Creek flows through the city of Sudbury and receives direct effluent inputs from three metal mine operations (Garson, Nolin and Copper Cliff) in a cumulative manner. The creek also receives municipal waste-water from the city, storm run-off, atmospheric deposition and is subject to historical contamination (Jaagumangi and Bedard, 2002). Previous studies conducted in Junction Creek have shown decreased diversities and densities in both fish and benthos downstream of metal mine effluent (MME) discharges (Jaagumangi and Bedard, 2002; Sein, 1993). However, due to the confounded nature of the creek, identifying the potential cause of these effects has been an issue. In response to this problem, artificial stream studies were conducted with isolated MME to determine effects on both benthos (Hruska and Dubé, 2004, 2005) and fish (Dubé et al, 2006). The results of these investigations revealed reduced survival in creek chub and reduced body weights in male and female dace along with increases in Ni, Ru, Sn, Fe, Li, Tl and Se body burdens after exposure to Copper Cliff and Garson MME. Hruska and Dubé (2004, 2005) also reported reduced emergence, hatching success and survival of the benthic invertebrate, *Chironomus tentans* after exposure to Copper Cliff MME.

Linking the effects measured in the artificial stream studies to field effects has been difficult firstly because only individual endpoints were measured (gonadosomatic index, liversomatic index, condition, length and weight) in the artificial stream studies; reproductive endpoints at the population level (e.g., fecundity, hatching success) have not been assessed (Dubé et al, 2002a,b). Secondly, trophic-transfer of contaminants via a food source was not considered in the exposure designs. There have been numerous studies investigating the relative importance of trophic-transfer of metals (Ni et al, 2000; Chen et al, 2000; Mason et al, 2000; Xu and Wang, 2002) and organic (Egeler et al, 2001; Muir et al, 1999; Nendzaa et al, 1997; Clements et al, 1994) contaminants in aquatic environments highlighting that dietary uptake can be a significant route of exposure in addition to water-borne exposure.

There is a need to develop a bioassay that can quantitatively assess whether food and/or water-borne exposure to complex metal mine effluents leads to population level effects by assessing reproductive endpoints such as fecundity and hatching success. This need is urgent because in Canada under the federally legislated Environmental Effects Monitoring Program for base metal mines, the use of artificial stream systems are approved alternative monitoring methods to assess biological effects. However, all applications of this approach to date have used water-borne exposures (Dubé et al, 2002a). Furthermore, many bioassays employed to assess the effects of complex sewage, mining and pulp mill effluents (fathead minnow life-cycle and partial life-cycle bioassays) are conducted using waterborne exposures (Parrott and Wood, 2001) ignoring the potential contribution of trophic-transfer as a significant route of exposure.

Our objectives were to: 1) develop a trophic-transfer system using a short-term fathead minnow (FHM; *Pimephales promelas*) reproductive bioassay developed by Ankley et al

(2001) and 2) assess the effects of 45% Copper Cliff Mine Effluent (CCME) via both food and water (trophic-transfer system) compared to a water-only (water-only system) exposure in a laboratory environment.

5.2 Methods

5.2.1. Trophic-transfer system development

Prior to the start of the exposure experiments, a trophic-transfer system was developed. A feeding trial with FHM was conducted to firstly assess whether reproductive output (egg production and spawning events) significantly differed between the two food sources, brine shrimp or *C. tentans*. Brine shrimp are the standard food source used in the FHM reproductive bioassay (Ankley et al. 2001) whereas *C. tentans* was the desired food source to develop the trophic-transfer system because maintenance of self-sustaining cultures over multiple generations is fairly straight forward. There was no statistical difference in reproduction ($p>0.05$) so *C. tentans* was used as the food-source in the system.

The artificial stream system used for this study has been described in detail by Hruska and Dubé (2004). Briefly, each system consists of a table (one/treatment) with five to eight replicate, 10.3-L, circular, high-density polyethylene streams on each table. Each stream has a diameter of 0.3 m and a depth of 0.2 m. The replicate streams sit on a common table that drains into an 85-L dilution reservoir. The streams, table, and dilution reservoir are self-contained on a shipping pallet.

The trophic-transfer system consisted of a culture of *C. tentans* on the bottom of each replicate stream with a spawning tile and one breeding pair of FHM. Chironomid cultures were firstly set-up in each artificial stream (eight streams per system, two systems). One inch of pre-cleaned (rinsed 5 times with control water) silica sand was placed in the bottom of

each stream. The number of *C. tentans* eggs sacs that needed to be placed into the substrate to obtain a larval density that was comparable to the amount of *C. tentans* being fed to the water-only fish was calculated. Based on previous investigations conducted by Hruska and Dubé (2004, 2005) hatching success is estimated at ~250 larvae per egg sac. Approximately 100, 3rd instar *C. tentans* weighed approximately 1g, which was the amount of food the water-only FHM received on a daily basis. One gram of invertebrate food is equivalent to the ad libitum rate fed to FHM in previous studies with this bioassay (Rickwood et al, 2006a,b). Therefore, based on this data, 120 *C. tentans* were required per day for three weeks or a minimum of 1 egg sac every two days (250/100 = 2.5) to maintain densities of *C. tentans* that were comparable to 21 days of food.

C. tentans egg sacs were added to each stream at a rate of five per week for three weeks to culture adequate densities of larvae to sustain a breeding pair of FHM for 21 days. Covers (250 μm mesh) were placed over the streams to limit escape of eggs, larvae, and any emerged adults. *C. tentans* were fed 5mL of tetramin slurry (100g of tetramin blended in 1L dechlorinated water) once per day. Cores were taken every 7 days within the trophic-transfer system to firstly calculate survival and secondly to compare the densities between the streams. Densities were calculated by taking three replicate cores (core sampler area = $9cm^2$) and calculating densities per cm^2 (mean core density/9) and total stream densities (densities per cm^2*total stream area). Finally, densities were checked at the end of the three weeks to determine that both adequate and similar amounts of food were present in each stream. Once the total number of invertebrates per stream reached the target of 2100 (3 per cm^2) egg sacs were no longer added.

A breeding trial was then conducted to assess the reproductive output of FHM in the trophic-transfer system in the absence of effluent in order to ground-truth the bioassay. Breeding pairs and one spawning tile were placed into each stream under optimal breeding conditions (water temperature of 25^{o}C +/- 1^{o}C, 16:8 light:dark photoperiod). This reproductive activity was compared to FHM held in the water-only system that were being fed *C. tentans* (1g/day) obtained from our lab cultures. Density checks of *C. tentans* larvae were conducted every 3 days in the trophic-transfer system to monitor the amount of food available. The FHM endpoints measured in both systems were egg production (no of eggs produced per spawn), fertilization success [(no of eggs/no of viable eggs)*100], weight and length of fish before and after trial. After 2 days, the densities of *C. tentans* in the trophic-transfer streams had dropped extensively due to excessive feeding by FHM. The trial was aborted and a feeding barrier was designed to limit the amount of food to which the FHM had access too. The feeding barrier was a circular, course plastic wire mesh "platform" (mesh size of 1cm^2) with a central PVC standpipe that fitted over the central standpipe of the artificial stream. This allowed the barrier to rest approximately 1.5cm over the sand substrate. A section (1/10th) was cut out of the mesh (piece of the pie) that allowed access of FHM to a controlled streambed area that had an estimated *C. tentans* density equal to 1g/*C. tentans*/day. The barrier was designed to turn once per day to allow FHM to feed on uncovered *C. tentans*. Once the trophic-transfer system had been developed with the barrier, the CCME (45%) exposure experiment was conducted.

5.2.2 CCME exposure

This study was conducted during August and September 2004 in our laboratories in Saskatoon, SK. Experiments were conducted for 60 d using the artificial stream system

(water only and trophic-transfer system) and consisted of a pre-exposure period (no effluent) and an exposure period of similar duration (21 d) (Ankley et al, 2001).

5.2.2.1 Pre-exposure design

The pre-breeding trial is conducted in the absence of effluent to establish baseline reproductive performance of breeding pairs. Artificial stream tables were set-up with eight replicate streams for each table, giving a total of 48 breeding pairs. Six month old, naïve FHM were obtained from cultures at our laboratories. Fish total body weight (g), fork length (mm) and secondary sex characteristics were recorded. Secondary sex characteristics of FHM consisted of banding, nuptial tubercles, dorsal pad and fin dot in males and ovipositor size in females. Each secondary sex characteristic was evaluated based on a points system and were summed with equal weight to give an overall male and/or ovipositor index as outlined by Parrott and Wood (2001). The 48 breeding pairs were then randomly selected and placed into the streams.

Pre-heated de-chlorinated water was collected once a day and stored in a 1200 L polyethylene tank. This water was pumped (Pulsatron Series E, Viking Pump of Canada, Edmonton, Canada) from the holding tank to the reservoir tanks for each artificial stream system at a turnover rate of two reservoir tank volumes per day. Water from the reservoir was then supplied via a March pump (Bancroft Western, Vancouver, BC, Canada) and a manifold continuously redistributed the water to the eight replicate streams per table at a turnover rate of 2 L/min resulting in a residence time of around 5 minutes for each stream. Water temperature was maintained at 25^{o}C (±1^{o}C) and logged using Optic Stowaway temperature loggers (Optic stowaways©; Onset Computer, Bourne, MA, USA). Conductivity

and dissolved oxygen were monitored daily in each stream using a YSI meter (Yellow Springs Instruments, Yellow Springs, OH, USA). Ammonia (Rolf C. Hagen, Edmonton, Canada) and pH (Oakton pHTestr 3, Oakton Instruments, Vernon, IL, USA) were also recorded daily. All fish were fed frozen brine shrimp during the pre-exposure period (San Francisco Bay Brand, Newark, CA, USA) at a rate of 1g per breeding pair per day. *C. tentans* were fed 5mL of tetramin slurry (100g of tetramin blended in 1L dechlorinated water) once per day. Photoperiod was maintained at 16 h:8 h light:dark.

During the pre-exposure period survival, total number of spawning events, egg production, fertilization success, hatching success, larval deformities, and secondary sex characteristics of breeding FHM were measured. Egg production was determined daily, by rolling the eggs off each spawning tile into hatching cups (250 ml PVC cups with screened bottoms), which were then placed into flow-through tubs (maintaining 24 hr turnover time) with aerated water. The total and viable number of eggs was counted daily allowing estimation of cumulative egg production (over 21 d) and mean and total egg production (no. eggs per female). Eggs were determined to be viable if they had a defined yolk within the egg sac and their structural integrity was maintained. Once larvae hatched (usually around five to seven days after spawn) they were assessed for deformities under a dissecting microscope (6.4 x magnification). Deformities included scoliosis, lordosis and yolk sac edema. The total number of viable larvae (hatching success expressed as % of viable eggs) and deformed larvae (% of total larvae hatched) were recorded. Development of male-type secondary sex characteristics in both males and females were recorded at the start (day 0), mid (day 10) and end of the pre-exposure (day 21). Development of female-type characteristics (i.e., ovipositor presence and size) were not assessed at the mid or end of the pre-exposure to minimize

disturbance of the breeding fish. The ovipositor index for each fish (both male and females) was then calculated to obtain index values for the pre-exposure period. For a full description of the bioassay see Ankley et al (2001).

After 21 d, selection of breeding pairs for the effluent exposure was undertaken based on 100% survival of all adults, presence of eggs in each replicate once a week, and >80% fertilization of eggs (OECD, 2003). Based on these criteria, 42% of the breeding pairs used in the pre-exposure period were selected for use in the exposure period resulting in five pairs per treatment (n = five).

5.2.2.2 Pre-exposure analysis

Analysis between tables was conducted to determine if any effects could be observed in reproductive endpoints prior to effluent exposure. Levene's Test was used to confirm parametric assumptions for homogeneity of variance. One-way analysis of variance (ANOVA) was then conducted for the following endpoints: egg production and spawning events (mean number per pair after 21d), hatching success (% of viable eggs produced) and deformities (% of total hatch). No significant differences were found ($\alpha = 0.05$) confirming that there were no differences amongst replicate breeding pairs on the four tables during the pre-exposure. The unit of replication for egg production, spawning events, hatching success and deformities were breeding pairs (n = five per treatment). Unit of replication for weight, length and condition factor was five (n = five females and n = five males per treatment).

5.2.2.3 Trophic-transfer system

During the pre-exposure period of the FHM bioassay the trophic-transfer system was established. Once *C. tentans* cultures had reached adequate densities the addition of egg sacs

ceased and the feeding barrier was added along with a spawning tile. Densities were checked at the end of the three weeks to determine that both adequate and similar amounts of food were present in each stream.

5.2.3 Exposure design

The exposure study was a two-factor ANOVA design with water treatment (control or effluent) and system (water-only or trophic-transfer) as factors. This experimental design allowed for assessment of the effects of effluent on FHM exposed only through the water (water-only) or when exposed through both food and water (trophic-transfer). The experimental design was not fully factorial as we did not assess the effects of food cultured under effluent exposure conditions on FHM contained in clean water. This type of experimental manipulation was not possible in a self-sustaining trophic-transfer system. By conducting a two-way ANOVA we were able to assess effects of effluent, effects of the system used (water-only and trophic-transfer), and assess if the effects of the effluent depended upon the system (i.e., interaction effect). With this in mind four artificial stream tables were established (two reference [trophic-transfer and water-only] and two CCME [trophic-transfer and water-only]) with five replicate streams per table.

5.2.3.1 Copper Cliff mining effluent (CCME)

In Sudbury (400 km north of Toronto, ON, Canada), INCO Limited operates the largest fully integrated mining, milling, smelting and refining complex in Canada, producing nickel, copper, precious metals, platinum-group metals, sulphuric acid and liquid sulphur dioxide. Three waste-water treatment plants discharge treated MME into Junction Creek including Garson (approx. 982,030 m^3/y in 2004), Nolin (6,050,023 m^3/y in 2004) and Copper Cliff

(43,870,000 m^3/y in 2004). The effluents are all treated by conventional hydroxide precipitation (lime addition and settling) and subsequent pH adjustment prior to discharge.

Mine effluent from the Copper Cliff Waste-water Treatment Plant (CCME) was chosen for this study based on previous studies that have shown effects on fish and benthos (6, 8). The concentration of 45% was selected as an environmentally relevant concentration based on discharge rates into Junction Creek and volume calculations. The Copper Cliff Waste-water Treatment Plant receives inputs from active and inactive tailings, several mines, a nickel refinery, a mill, collected surface drainage, a copper refinery, a smelter complex, surface runoff from the Town of Copper Cliff, ON and sewage from the mine-related housing/administration offices. Thus the CCME is a highly complex effluent mixture.

5.2.3.2 Exposure

After the pre-exposure, selected FHM breeding pairs were moved to either the water-only system (control or CCME 45%) or trophic-transfer system (control or CCME 45%). Treated final CCME was collected at the waste-water treatment plant in Sudbury once a week in two 1000L totes that were shipped to our lab in Saskatoon. The average holding time for the effluent was ~10-12 days (5 days shipping, 5-7 days use). The CCME (405 l, 100% (v/v)) was supplied every day to a 900 L holding tank where it was mixed with de-chlorinated City of Saskatoon municipal water (495 L). Diluted CCME (45%) was delivered to each artificial stream reservoir at a total flow of 0.24 L/hour to maintain a turnover time of two exchanges per system per 24 h period. Eggs and larvae were held in corresponding flow-through treatment tanks that were fed with pre-mixed CCME (45%) and reference water via a manifold and pump to maintain an overall turnover rate of 2 volumes/day. Daily observations and measurements, i.e., egg production, hatching success, deformities, etc. were the same as

those taken during pre-exposure. Fish in the water-only system were fed *C. tentans* during the exposure period, at a rate of 1g/day to be consistent with fish in the trophic-transfer system.

Water samples were collected from the reference water and treatment streams as well as the head tanks on a weekly basis during the three week exposure period and analyzed for general chemistry, nutrients and metals. Samples were collected, preserved and analyzed as per standard methods and quality assurance and control procedures (National Laboratory of Environmental Testing, Burlington, ON, L7R 4A6). Total stream densities in the trophic-transfer system were checked once every week and one section/stream was checked before exposure, after exposure (24h) and 6 days later to determine *C. tentans* re-colonization rates.

At the end of the exposure period, fish were anaesthetized (30µl per l clove oil), assessed for secondary sex characteristics, and fork length (mm) and total body weight (g) were recorded. Fish were euthanized by spinal severance. Gonads and livers in the adults were weighed (to within 0.001 g). Gonads were placed in formalin (10%) for 24h and then stored in ethanol (75%) until histological analysis was undertaken at the University of Saskatchewan using the quantitative method developed by Weber et al (2003). Gonadal tissue was paraffin-embedded, three 5µm sections were taken at mid-gonad and mounted on slides. Ovaries and testes were assessed for both stages of development and histopathology. In brief, the percentage of oogonia, pre-vitellogenic, vitellogenic and mature follicles were calculated from four fields of view for each female. Atretic follicles (No. per field of view), the presence of fibrosis and eosinophilic (excessive eosin stain) material were also assessed for each female. The percentage of spermatogonia, primary and secondary spermatids and mature sperm were calculated from four fields of view for each male. Cell death (No. per

field of view), the presence of fibrosis and eosinophilic material were also assessed for each male. Gonad and liver sizes were assessed relative to both body weight and length. Condition was assessed with body weight relative to length. Fish carcasses (excluding head and caudal fin) were then stored in a -80^{o}C freezer until muscle sex-steroids, vitellogenin and metal content were measured. Sex steroids (11-ketotestosterone [11-KT; males only], 17β-estradiol [females only] and testosterone [males and females]) and vitellogenin were measured using enzyme-linked immunosorbent assays (ELISA). EIA kits for testosterone and 17β-estradiol were supplied from Assay Designs, MI, USA. EIA kits for 11-KT and vitellogenin (Carp, pre-coated) were supplied from Cayman Chemical Company, MI, USA. *Chironomus tentans* were sampled from the trophic-transfer streams for each treatment at the end of the 21 day exposure. A whole-body scan of 27 metals was conducted on both *C. tentans* (whole body) and FHM (muscle tissue) using standard methods (Inductively Coupled Plasma-Sector Field Mass Spectrometry [ICP-SFMS]) and quality assurance and control procedures (National Laboratory of Environmental Testing, Burlington, ON, L7R 4A6).

5.2.3.3 Exposure Analysis

Data were analyzed using SPSS 11.0 (SPSS, Chicago, Il, USA). To test parametric assumptions for homogeneity of variance, Levene's Test was used. If data did not require transformation, a two-way ANOVA was performed on the following endpoints: hatching success (% of viable eggs produced), deformities (% of total hatch), stages of ovarian development (histological analysis), muscle sex steroids, vitellogenin and metal body burdens. A two-way ANOVA was performed with water treatment (control or 45% CCME) and route of exposure (water-only or trophic-transfer) as factors. If an interaction was

observed a t-test was conducted on the water-only treatments and trophic-transfer treatments separately to determine differences. If Levene's Test for homogeneity was significant, the percentage-based data were arcsin (%) transformed and tested again for homogeneity. Data that were not percentage or ratio-based were log10 transformed. A two-way ANOVA was then performed on the transformed data. If assumptions could not be met for homogeneity of variance, a non-parametric two-way ANOVA test (Scheirer-Ray-Hare extension of the Kruskal-Wallis test using ranked data) was performed (Sokal and Rohlf, 2003). Two-way ANCOVAs were used to assess gonad and liver weights (with body weight as covariate) and body weight (with length as a covariate). If an interaction was observed between treatment and source an ANCOVA was conducted on the water-only and trophic-transfer treatments separately to determine differences. To assess responses over time, Kolmogorov-Smirnov (KS) tests were conducted to assess the cumulative frequency of spawning events and eggs produced in each treatment over 21d compared to controls. Kolmogorov-Smirnov two-sample tests were also conducted for ovipositor development in both males and females. The appearance of male secondary sex characteristics in females was assessed using contingency tables. The severity score data for gonadal histopathology in both males and females (eosinophilia and fibrosis) were analyzed with the non-parametric Kruskal-Wallis test. Water and effluent chemistry data were analyzed using a two-way ANOVA to determine if treatments differed significantly from reference water. Samples taken over time within each treatment were the unit of replication. Assumptions were applied as discussed above.

5.3 Results

5.3.1 Water quality and metal analysis

Alkalinity and pH were significantly reduced after exposure to CCME (45%) (two-way ANOVA, $p<0.001$ and $p=0.001$ respectively) (Table 5.1). Significant increases were observed in this treatment in chloride (two-way ANOVA, $p=0.046$), conductivity (two-way ANOVA, $p<0.001$), nitrate (two-way ANOVA, $p=0.021$), hardness (two-way ANOVA, $p<0.001$), calcium (two-way ANOVA, $p<0.001$), magnesium (two-way ANOVA, $p<0.001$), sodium (two-way ANOVA, $p<0.001$) and sulfate (two-way ANOVA, $p<0.001$) (Table 5.1).

Significant increases in boron (two-way ANOVA, $p=0.026$), barium (two-way ANOVA, $p=0.024$), copper (two-way ANOVA, $p<0.001$) and nickel (two-way ANOVA, $p=0.049$), occurred in both water-only and trophic-transfer 45% CCME systems compared to controls (Table 5.2). Significant decreases were observed with molybdenum (two-way ANOVA, $p=0.025$) and uranium (two-way ANOVA, $p=0.034$) in both water-only and trophic-transfer systems (Table 5.2).

5.3.2 Individual endpoints

In both male and female FHM, no significant changes in gonad size with body weight as a covariate (ANCOVA, $p=0.831$, $p=0.174$ respectively) were observed after exposure to CCME. In males and females, body weights were reduced by 8% and 6.8% respectively, but these differences were not significant (ANOVA $p=0.464$, $p=0.452$). Length was also unaffected by exposure to CCME in both males (ANOVA, $p=0.255$) and females (ANOVA, $p=0.616$) (data not shown). Survival was determined by number of adults (male and female)

Table 5.1. Summary of water quality variables conducted throughout the October 2004 laboratory study with Copper Cliff mine effluent (CCME) (45%) and control water in water-only and trophic-transfer exposure systems. Asterisk represents significant difference from water-only control (* = $p<0.05$, *** = $p<0.001$). Pound denotes significant difference from trophic-transfer control (# = $p<0.05$, ###=$p<0.001$).

Parameter	Detection limit	Water-only		Trophic-transfer	
		Ref	CCME	Ref	CCME
Ammonia (mg/L)	0.05	0.05 ± 0.00	0.15 ± 0.10	0.05 ± 0.00	0.15 ± 0.10
DOC (mg/L)	1	1.67 ± 0.33	3.00 ± 1.15	2.33 ± 0.67	3.33 ± 1.45
TOC (mg/L)	1	2.33 ± 0.67	3.00 ± 1.15	2.33 ± 0.67	3.67 ± 1.76
Alkalinity (total) (mg/L)	5	82.3 ± 0.88	40.0 ± 3.00***	81.0 ± 0.58	36.3 ± 2.33###
Chloride (mg/L)	1	30.0 ± 18.0	66.7 ± 18.0*	19.3 ± 6.44	41.7 ± 0.33#
pH	0.1	8.00 ± 0.1	7.63 ± 0.10***	7.97 ± 0.07	7.57 ± 0.07###
Conductivity (µS/cm)	10	456.7 ± 61.7	1787 ± 61.7***	450 ± 30.6	1730 ± 43.6###
Nitrate (mg/L)	0.1	0.60 ± 0.06	3.00 ± 0.06*	0.70 ± 0.10	1.80 ± 0.78#
Hardness (mg/L)	5	111 ± 9.07	874 ± 9.07***	124 ± 15.9	882 ± 29.5###
Calcium (mg/L)	2	21.3 ± 1.86	304 ± 1.86***	26.3 ± 5.04	307 ± 9.29###
Potassium (mg/L)	1	24.7 ± 22.2	56.0 ± 22.2	10.7 ± 7.69	29.7 ± 7.69
Magnesium (mg/L)	1	14.0 ± 1.15	27.7 ± 1.15***	14.0 ± 1.00	28.0 ± 2.00###
Sodium (mg/L)	1	40.0 ± 2.65	78.0 ± 2.65***	41.0 ± 4.36	77.7 ± 1.79###
Sulfate (mg/L)	6	91.3 ± 1.33	924 ± 1.33***	105 ± 14.7	932 ± 11.1###
F (mg/L)	0.01	0.50 ± 0.01	0.51 ± 0.01	0.50 ± 0.00	0.50 ± 0.01
Total P (mg/L)	0.0005	0.06 ± 0.04	0.03 ± 0.04	0.28 ± 0.02	0.40 ± 0.11

Table 5.2. Summary of water metal analysis measured in the October 2004 laboratory study with Copper Cliff mine effluent (CCME) (45%) and control water in water-only and trophic-transfer exposure systems. Asterisk represents significant difference from water-only control (* = $p<0.05$, *** = $p<0.001$). Pound denotes significant difference from trophic-transfer control (# = $p<0.05$, ### = $p<0.001$). Values represent mean (µg/L) ± 1 standard error. nd (non-detect).

Parameter (µg/L)	Detection limit (µg/L)	Water Only		Trophic-transfer	
		Control	CCME	Control	CCME
Ag	0.001	nd	nd	nd	nd
Al	0.2	89.5 ± 10.3	83.2 ± 19.6	155 ± 13.5	101 ± 39.3
As	0.01	0.42 ± 0.16	0.53 ± 0.16	0.31 ± 0.03	1.07 ± 0.36
B	0.5	36.2 ± 10.6	46.1 ± 11.7*	26.2 ± 1.27	76.6 ± 16.7#
Ba	0.05	35.1 ± 5.74	41.0 ± 7.30*	28.2 ± 0.98	58.8 ± 10.4#
Be	0.001	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Bi	0.001	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Cd	0.001	0.04 ± 0.02	0.06 ± 0.03	0.04 ± 0.01	0.15 ± 0.05
Co	0.002	1.28 ± 1.24	3.75 ± 2.59	0.15 ± 0.07	3.12 ± 0.61
Cr	0.005	0.71 ± 0.05	0.77 ± 0.06	0.76 ± 0.07	0.63 ± 0.12
Cu	0.02	9.31 ± 1.46	81.7 ± 21.7***	8.14 ± 4.81	93.8 ± 16.1###
Fe	0.5	12.6 ± 5.45	17.0 ± 5.68	15.3 ± 4.02	55.9 ± 34.1
Li	0.2	25.3 ± 10.9	35.4 ± 11.5	14.7 ± 0.95	66.5 ± 17.3
Mn	0.05	0.87 ± 0.47	1.05 ± 0.20	2.18 ± 0.48	3.73 ± 1.27
Mo	0.005	2.15 ± 0.16	2.04 ± 0.09*	2.26 ± 0.05	1.53 ± 0.31#
Ni	0.02	24.0 ± 21.8	44.6 ± 21.7*	3.00 ± 0.80	114 ± 51.2#
Pb	0.005	0.13 ± 0.04	0.20 ± 0.03	0.21 ± 0.05	0.24 ± 0.05
Rb	0.01	12.8 ± 11.6	22.7 ± 10.8	1.78 ± 0.56	51.4 ± 16.4
Se	0.05	0.28 ± 0.04	6.25 ± 3.11	0.39 ± 0.08	7.37 ± 0.66
Sr	0.05	301 ± 140	431 ± 143	164 ± 8.54	809 ± 220
Tl	0.001	0.00 ± 0.00	0.74 ± 0.21	0.03 ± 0.02	1.02 ± 0.53
U	0.0005	0.71 ± 0.17	0.59 ± 0.13*	0.87 ± 0.16	0.31 ± 0.06#
Zn	0.05	10.2 ± 3.00	14.3 ± 0.91	9.16 ± 1.62	12.5 ± 1.99

surviving the 21d exposure in each treatment and the unit of replication was breeding pair. Differences in survival were not significant (chi-square, $p>0.05$) (data not shown). In males, liver weight (with body weight as a covariate) was not significantly different from control (ANCOVA, $p = 0.835$, $p=0.514$). Condition (body weight with length as a covariate) was reduced by 8%, but this was not significant (ANCOVA, $p = 0.068$) (data not shown). In females, liver weight was reduced by 21%, but this was not significantly different from control with body weight as a covariate (ANCOVA, $p=0.284$). Condition was also reduced by 7%, but, again, this was not significantly different (ANCOVA, $p=0.629$) (data not shown). No interactions or source effects were observed in any of the endpoints conducted with a two-way ANOVA or ANCOVA.

5.3.3 Reproductive investment

Reproductive output was quantified by calculating cumulative number of eggs produced and spawning events over the 21 d exposure period. A KS test revealed a significant difference in egg production in both the water-only (KS, $p<0.001$) and trophic-transfer (74%) (KS, $p<0.001$) after exposure to CCME (Fig 5.1A, B). Total egg production was reduced by 76% and 74% in the water-only and trophic-transfer CCME treatments respectively. Spawning events were also significantly altered after exposure to CCME in the water-only (63% reduction) (KS, $p<0.001$) and trophic-transfer systems (50% reduction) (KS, $p<0.001$) (Fig 5.2A, B).

Viability of offspring was quantified by assessing hatching success (% hatched from viable eggs produced) and deformities (% deformed from total hatched). A significant interaction was observed in hatching success (ANOVA, $p=0.036$) so the analysis was split and ANOVA's were conducted on both water-only and trophic-transfer data. In the water-

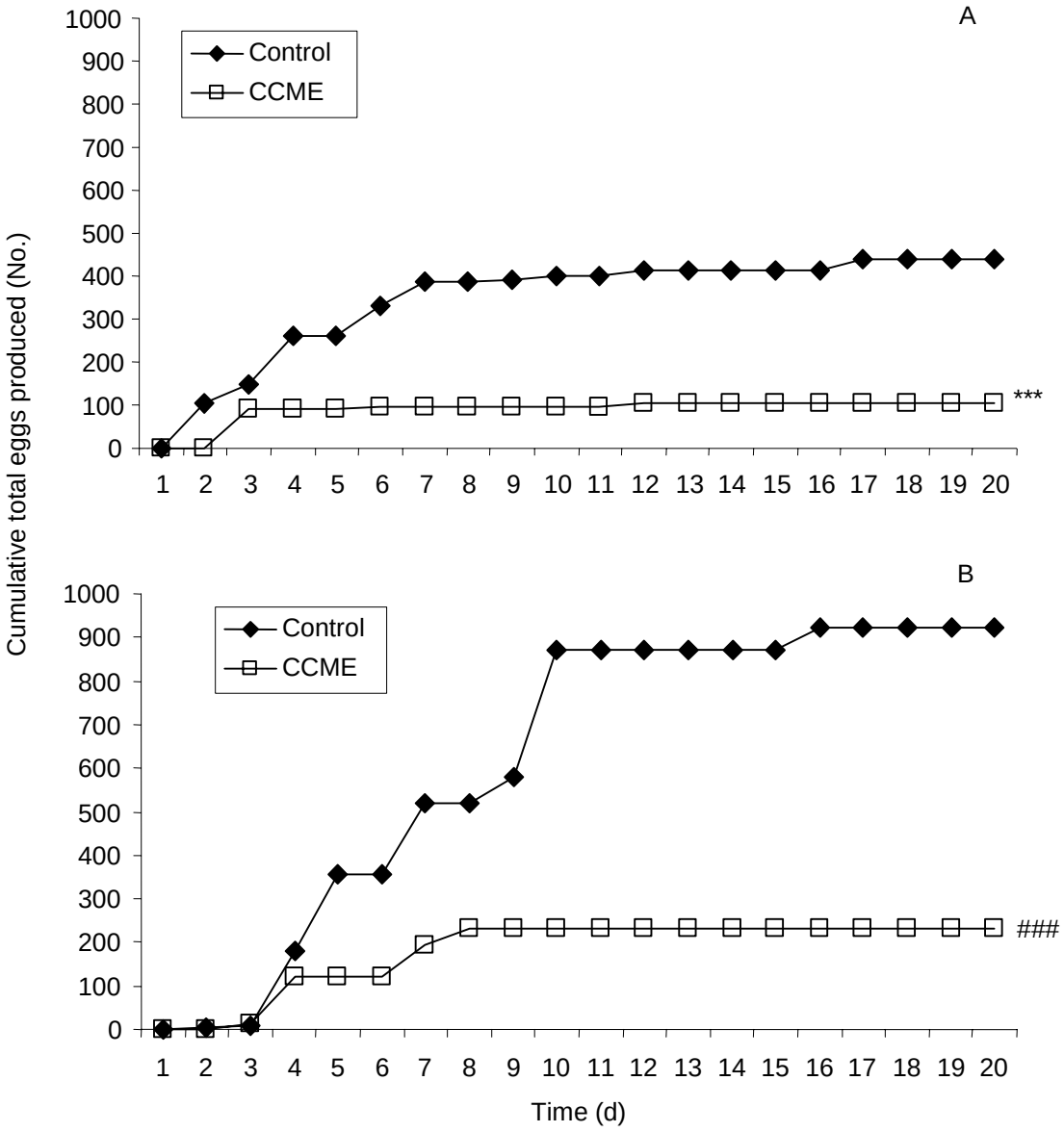

Figure 5.1 . Cumulative number of eggs produced by fathead minnow (*P. promelas*) breeding pairs during the 21 d exposure to Copper Cliff mine effluent (CCME, 45%) and control water in A) water-only and B) trophic-transfer system. Asterisk denotes significant difference from water-only control, where *** = $p<0.001$. Pound denotes significant difference from trophic-transfer control, where ### = $p<0.001$.

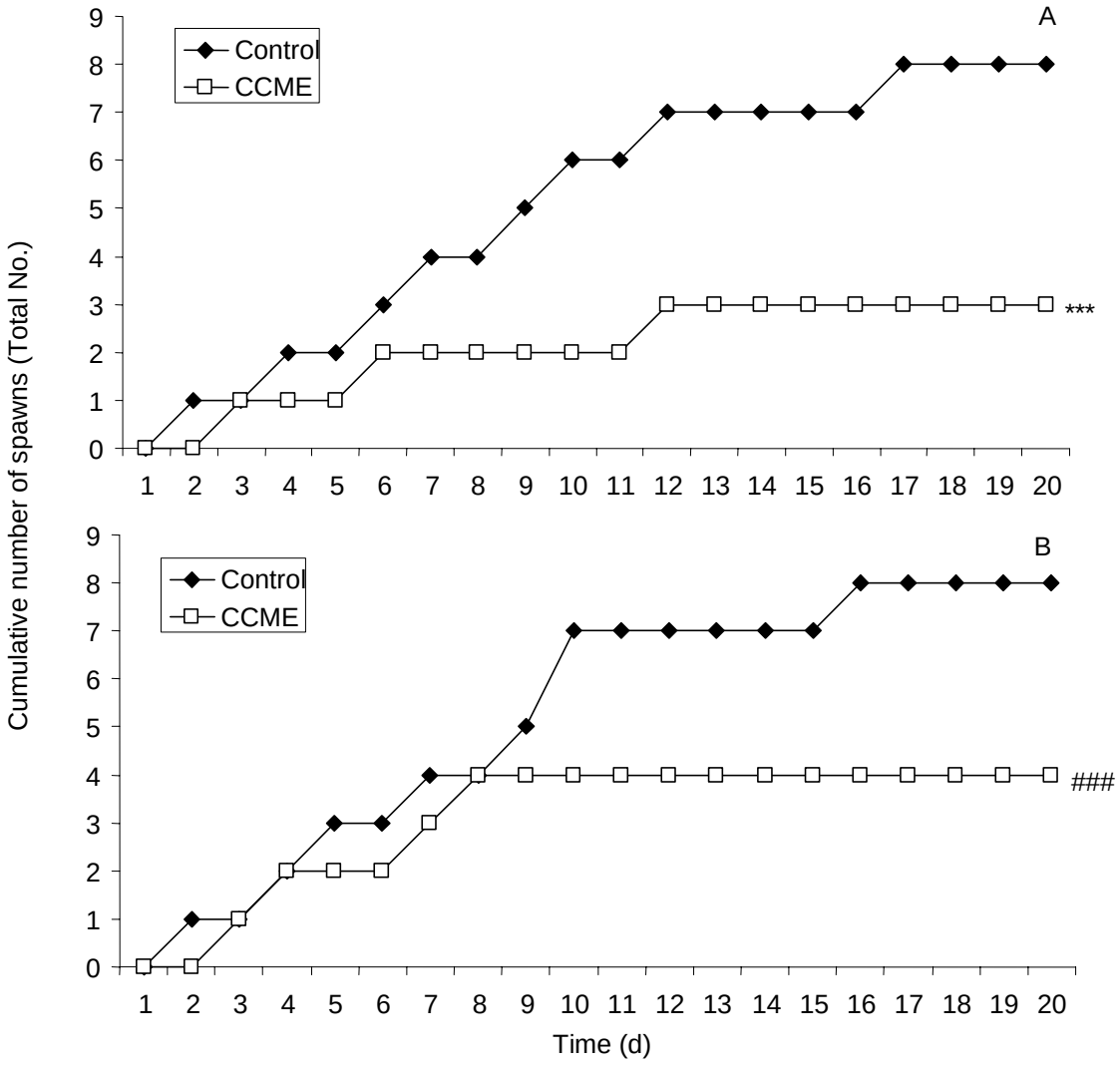

Figure 5.2. Cumulative number of spawning events of fathead minnow (*P. promelas*) breeding pairs during the 21 d exposure to Copper Cliff mine effluent (CCME, 45%) and control water in A) water-only and B) trophic-transfer system. Asterisk denotes significant difference from water-only control, where *** = $p<0.001$. Pound denotes significant difference from trophic-transfer control, where ### = $p<0.001$.

only system no significant effects were observed (ANOVA, p=0.400), however, a significant decrease in hatching success occurred in the trophic-transfer system (KWALLIS, p=0.049) (Fig 5.3).A similar response was observed with deformities. A significant interaction occurred with the two-way ANOVA (p=0.031) suggesting that the effect of treatment was dependent upon the route of exposure. The analysis was split and an ANOVA run for each system. No significant treatment effects were observed in the water-only system (ANOVA, p=0.562), however, a significant increase (101%) in deformities (i.e., scoliosis, lordosis, edema) was observed in the trophic-transfer system after exposure to CCME (45%) (ANOVA, p=0.022) (Fig 5.4).

5.3.4 Biochemical endpoints

In male FHM, no significant differences were observed in levels of testosterone (two-way ANOVA, p=0.574), 11-KT (two-way ANOVA, p=0.116) or vitellogenin (two-way ANOVA, p=0.612) after exposure to CCME (45%) compared to control. In females, however, a significant interaction was observed with estradiol (two-way ANOVA, p=0.013). The analysis was split and an ANOVA run for each system. A significant increase (399%) was observed after exposure to CCME (45%) in the trophic-transfer system (ANOVA, p=0.006) but not in the water-only system (ANOVA, p=0.73) (Fig 5.5A). Testosterone in females also significantly increased in both the water-only (250% compared to control) and trophic-transfer (546% compared to control) system (ANOVA, p=0.035) (Fig 5.5B). No source (p=0.97) or interaction (p=0.303) effect occurred. A significant treatment effect was observed with vitellogenin in female FHM with an approximate 50% induction in exposed fish compared to control (two-way ANOVA, p=0.001) (Fig 5C). There were no source (p=0.203) or interaction (p=0.497) effects observed.

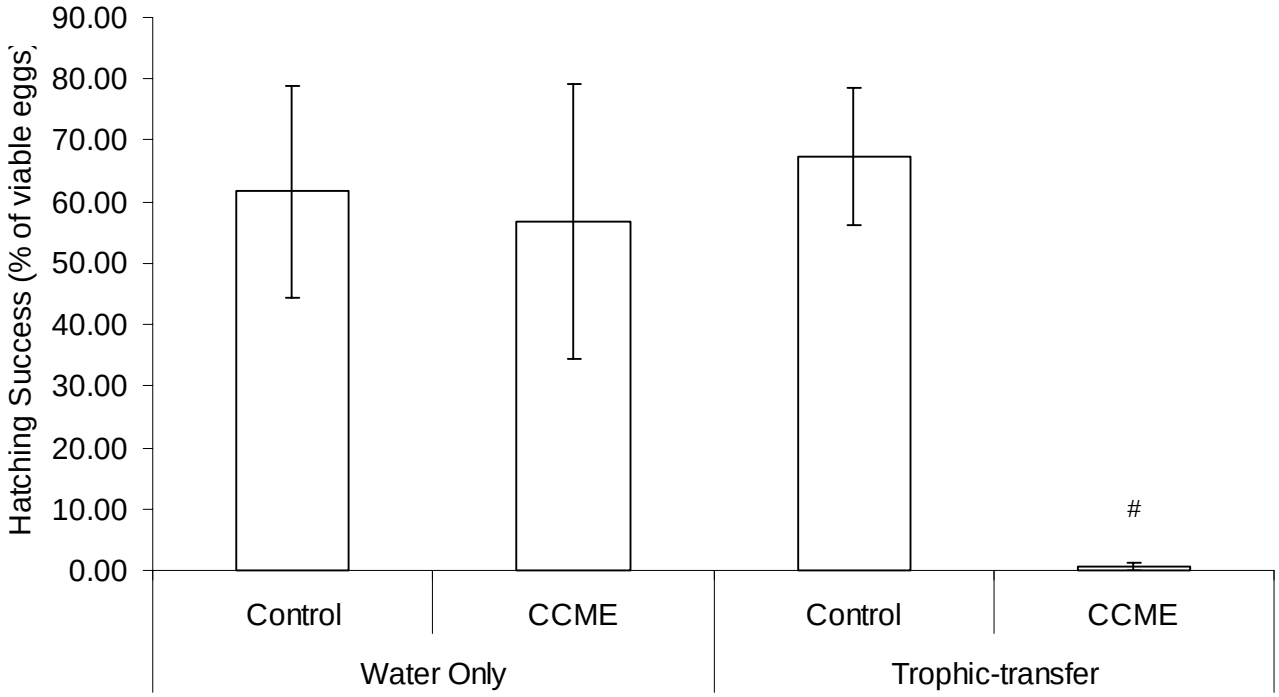

Figure 5.3. Hatching success (% of viable eggs) of fathead minnow (*P. promelas*) after 21 d exposure to Copper Cliff mine effluent (CCME, 45%) and control water in water-only and trophic-transfer systems. Pound denotes significant difference from trophic-transfer control, where # = $p<0.05$. Error bars represent standard error of the mean.

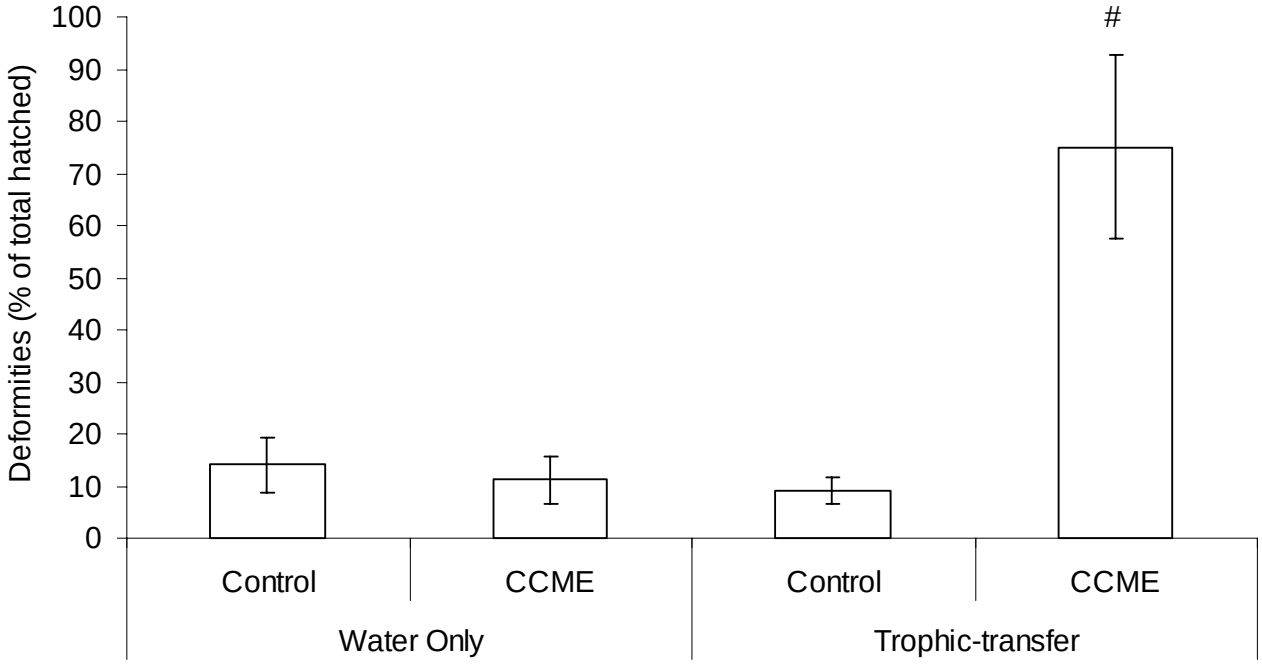

Figure 5.4. Deformities (% of total hatched) of fathead minnow (*P. promelas*) after 21 d exposure to Copper Cliff mine effluent (CCME, 45%) and control water in water-only and trophic-transfer systems. Pound denotes significant difference from trophic-transfer control, where # = $p<0.05$. Error bars represent standard error of the mean.

5.3.5 Secondary sex characteristics

No significant changes in secondary sex characteristics in either males or females were observed after exposure to CCME (45%) (chi-square, $p>0.05$) (data not shown).

5.3.6 Gonadal histopathology

A significant increase in cell death was observed in male gonads (t-test, $p<0.001$) which was accompanied by a significant increase in fibrosis in both systems exposed to 45% CCME. When female gonadal staging was analysed a significant interaction was observed (two-way ANOVA, $p=0.001$). When the analysis was split, significant differences were observed in oogonia in both systems, however a significant increase occurred in the water-only system (t-test, $p=0.022$) whereas a significant decrease in the trophic-transfer system was observed (t-test, $p=0.001$). No significant differences in the occurrence of either eosinophilia (two-way ANOVA, $p=0.826$) or fibrosis (two-way ANOVA, $p=0.834$) in females were observed.

5.3.7 Metal body burdens

In males, a significant treatment effect was observed in rubidium (two-way ANOVA, $p<0.001$) and thallium (two-way ANOVA, $p<0.001$) after exposure to CCME (45%) where 31-46% and 400-1500% increases, respectively, were observed in muscle tissues in both the water-only and trophic-transfer systems (Table 5.3). A significant treatment effect and interaction was observed with selenium (two-way ANOVA, $p=0.001$, $p=0.024$ respectively). The analysis was split and revealed a significant increase in selenium in the trophic-transfer

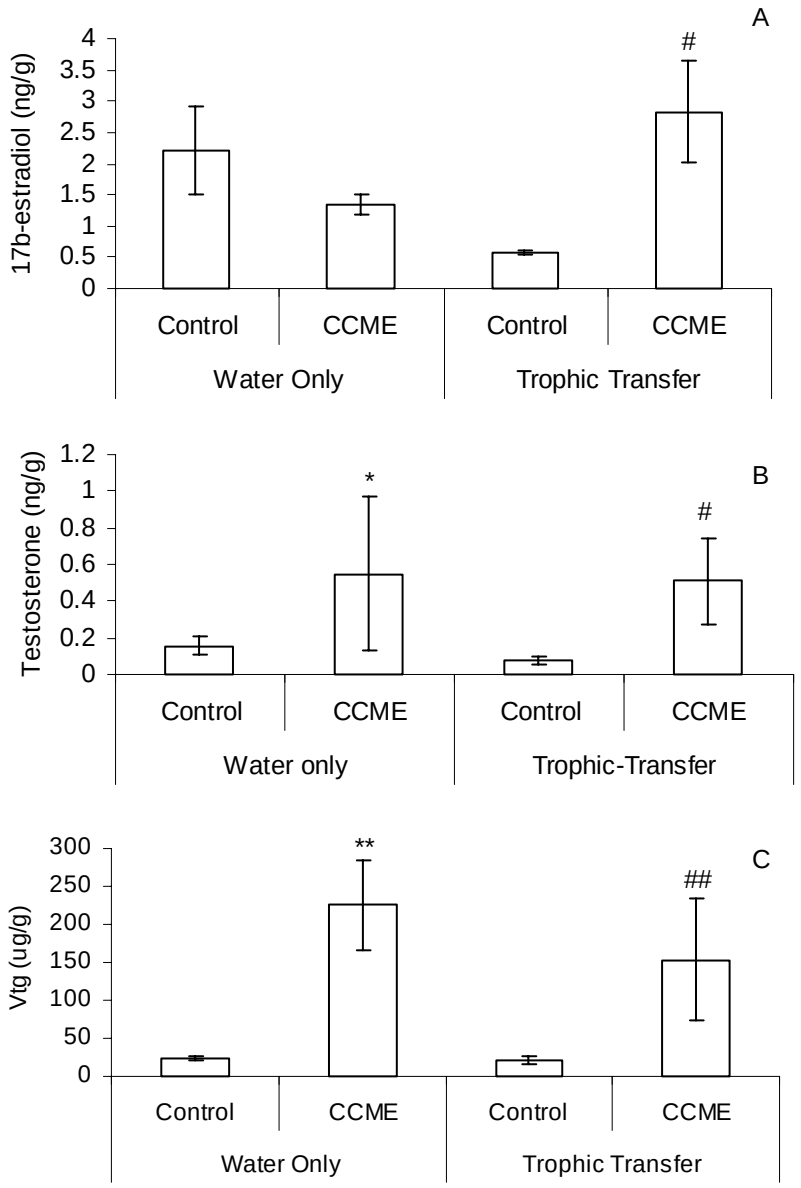

Figure 5.5. Levels of A) 17β-estradiol (ng/g) B) testosterone (ng/g) and C) vitellogenin (µg/g) in female fathead minnow (*P. promelas*) muscle tissue after 21 d exposure to Copper Cliff mine effluent (CCME, 45%) and control water in water-only and trophic-transfer systems. Asterisk denotes significant difference from water-only control, where * = $p<0.05$, ** = $p<0.001$. Pound denotes significant difference from trophic-transfer control, where # = $p<0.05$, ## = $p<0.01$. Error bars represent standard error of the mean. Sample size for water-only = 5 (control), 4 (CCME). Sample size for trophic-transfer = 4 (control), 5 (CCME).

exposure (ANOVA, p=0.004) but no significant difference in the water-only exposure (ANOVA, p=0.158).

In females, in both the water-only and trophic-transfer systems, significant treatment effects were observed for rubidium (two-way ANOVA, p=0.005), selenium (two-way ANOVA, p<0.001) and thallium (two-way ANOVA, p<0.001) where 23-75%, 62-116% and 500-1700% increases occurred, respectively, (Table 5.3). A significant decrease (-59%) in cobalt (two-way ANOVA, p=0.021) also occurred.

5.3.8 *C. tentans* densities and metal body burdens

The repeated measures ANOVA revealed a significant time effect in *C. tentans* densities (p<0.001) indicating that over the 21d exposure period the densities changed. However, the lack of any significant treatment or interaction effect between control and CCME (RM ANOVA, p=0.943, p=0.243, respectively) suggests that this change was uniform in both treatments (Figure 5.6).

Chironomus tentans metal body burdens are shown in Table 5.4. Increases in aluminum, copper, iron, nickel, lead and selenium were observed. No statistical analysis was conducted on the data as the measurements were conducted on a pooled sample from each treatment, therefore n=1.

Table 5.3. Metal body burdens in male and female fathead minnow assessed in the October 2004 laboratory study with Copper Cliff mine effluent (CCME) (45%) and control water in water-only and trophic-transfer exposure systems. Asterisk represents significant difference from water-only control (* = p<0.05, ** = p<0.01, *** = p<0.001). Pound denotes significant difference from trophic-transfer control (# = p<0.05, ## = p<0.01, ### =p<0.001). Values represent mean (µg/g dry weight) ± 1 standard error.

Parameter (µg/g)	Detection Limits (µg/g)	Males				Females			
		Water-only		Trophic-transfer		Water-only		Trophic-transfer	
		Control	CCME	Control	CCME	Control	CCME	Control	CCME
Ag	0.0001	0.31 ± 0.06	0.22 ± 0.07	0.20 ± 0.05	0.24 ± 0.07	0.49 ± 0.10	0.65 ± 0.21	1.11 ± 0.25	0.51 ± 0.16
Al	0.02	3.83 ± 0.58	2.83 ± 0.35	3.93 ± 1.98	5.40 ± 2.21	3.23 ± 0.38	5.97 ± 1.59	4.50 ± 0.91	2.57 ± 0.18
As	0.002	1.28 ± 0.02	1.54 ± 0.33	0.69 ± 0.16	1.04 ± 0.38	0.43 ± 0.11	0.88 ± 0.08	0.69 ± 0.13	0.82 ± 0.17
Ba	0.005	17.7 ± 3.38	15.9 ± 1.31	12.4 ± 1.68	17.2 ± 4.14	14.5 ± 1.89	15.9 ± 1.25	19.0 ± 0.42	16.8 ± 3.25
Be	0.0001	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Cd	0.0001	0.12 ± 0.05	0.09 ± 0.01	0.15 ± 0.05	0.16 ± 0.04	0.24 ± 0.02	0.27 ± 0.01	0.35 ± 0.05	0.22 ± 0.05
Co	0.0002	1.39 ± 0.66	0.32 ± 0.14	0.44 ± 0.16	0.43 ± 0.19	0.79 ± 0.12	0.34 ± 0.02*	0.75 ± 0.14	0.30 ± 0.08$^{\#}$
Cr	0.001	2.84 ± 1.31	1.17 ± 0.13	1.66 ± 0.66	2.03 ± 0.81	1.51 ± 0.14	1.54 ± 0.40	0.94 ± 0.18	0.44 ± 0.09
Cu	0.01	854 ± 153	597 ± 201	531 ± 121	636 ± 193	1298 ± 290	1629 ± 566	2310 ± 228	1360 ± 348
Fe	0.05	51.5 ± 9.07	43.8 ± 4.29	46.3 ± 5.56	71.6 ± 11.7	38.6 ± 1.73	59.9 ± 5.24	95.5 ± 39.0	45.2 ± 1.68
Li	0.01	0.10 ± 0.00	0.13 ± 0.03	0.10 ± 0.00	0.13 ± 0.03	0.10 ± 0.00	0.13 ± 0.03	0.10 ± 0.00	0.13 ± 0.03
Mn	0.005	2.49 ± 0.56	2.66 ± 0.41	2.54 ± 0.16	3.48 ± 0.62	1.85 ± 0.23	2.01 ± 0.31	2.28 ± 0.32	1.50 ± 0.23
Mo	0.001	0.28 ± 0.13	0.14 ± 0.01	0.21 ± 0.09	0.26 ± 0.11	0.21 ± 0.03	0.23 ± 0.06	0.14 ± 0.02	0.06 ± 0.03
Ni	0.005	5.48 ± 0.68	3.13 ± 0.79	3.50 ± 0.93	4.52 ± 0.69	6.94 ± 1.38	9.14 ± 2.41	14.1 ± 3.18	6.88 ± 1.82
Pb	0.001	73.1 ± 12.2	55.9 ± 19.6	50.5 ± 9.02	64.2 ± 19.1	126 ± 28.2	161 ± 55.7	280 ± 59.7	136 ± 42.8
Rb	0.001	5.67 ± 0.22	7.41 ± 0.25***	5.15 ± 0.37	7.55 ± 0.19$^{\#\#\#}$	5.55 ± 0.45	9.73 ± 0.86**	9.42 ± 0.40	11.6 ± 1.28$^{\#\#}$
Se	0.01	1.60 ± 0.12	1.80 ± 0.00	1.37 ± 0.09	2.00 ± 0.06$^{\#\#}$	1.17 ± 0.03	2.53 ± 0.18***	1.40 ± 0.30	2.27 ± 0.15$^{\#\#\#}$
Sr	0.005	291 ± 114	468 ± 63.8	296 ± 99.9	482 ± 57.1	379 ± 81.8	322 ± 45.5	436 ± 47.8	313 ± 54.4
Tl	0.0001	0.00 ± 0.00	0.02 ± 0.00***	0.00 ± 0.00	0.02 ± 0.00$^{\#\#\#}$	0.00 ± 0.00	0.02 ± 0.00***	0.01 ± 0.00	0.03 ± 0.01$^{\#\#\#}$
Zn	0.01	95.2 ± 1.50	142 ± 44.7	116 ± 17.1	101 ± 13.9	192 ± 15.9	193 ± 36.6	218 ± 55.4	151 ± 13.6

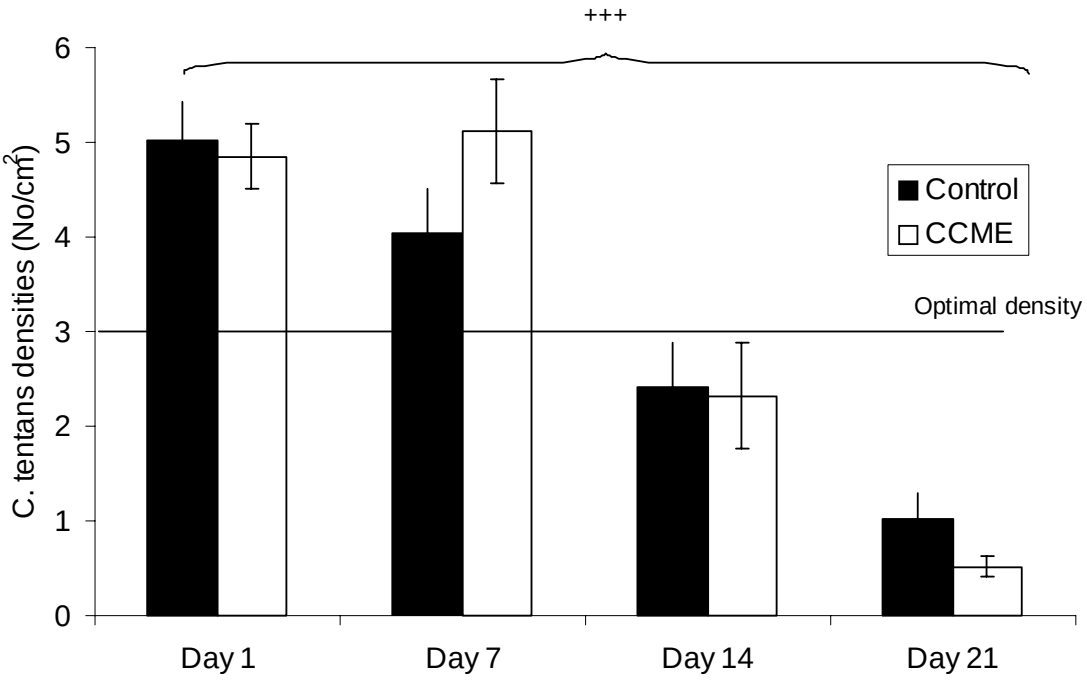

Figure 5.6. *C. tentans* densities (no/cm^2) measured throughout a 21 d exposure to Copper Cliff mine effluent (45%) and control water in a trophic-transfer system. Plus denotes significant time effect, where +++ = $p<0.001$. Error bars represent standard error of the mean.

Table 5.4. Metal body burdens in *C. tentans* assessed in the October 2004 laboratory study with Copper Cliff mine effluent (CCME) (45%) and control water in water-only and trophic-transfer exposure systems. Values represent pooled value (µg/g dry weight)

Parameter (µg/g)	**Control**	**CCME**
Ag	1.71	0.58
Al	1440	2480
As	1.03	1.53
Ba	29.2	33.4
Cd	2.07	2.47
Co	7.84	16.0
Cr	178	1170
Cu	2000	4320
Fe	1820	5630
Li	0.70	1.20
Mn	65.9	182
Mo	22.6	145
Ni	196	893
Pb	152	582
Rb	2.26	9.45
Se	1.40	11.0
Sn	74.4	138
Sr	55.6	75.8
Tl	0.01	0.05
U	4.20	7.63
Zn	346	453

5.4 Discussion

Development of a trophic-transfer system improved the environmental relevance of a standard FHM lab bioassay. The combined contribution of food (*C. tentans*) and water as sources of exposure to CCME were assessed compared to exposure to CCME through the water alone. Overall, the effects on the adult FHM were comparable in both systems, but it was only with the trophic-transfer system that the effects on the F1 generation were observed. Hypotheses as to the mechanisms behind these effects are discussed here.

Significant reductions in egg production, spawning events and increased cell death in male gonads and vitellogenin induction in females observed in both CCME treatments (water-only and trophic-transfer) would suggest that water was the primary route of exposure affecting these endpoints. Spawning events and egg production have been shown in previous investigations with pulp and paper effluents to be a sensitive indicator of effects in FHM (Rickwood et al, 2006a,b). The significant increase in cell death in male gonads would suggest that the mechanism behind reduced reproductive output was disruption of viable gametes. This observation is supported by the increase in fibrosis that was also documented. Similar observations were documented in male FHM sampled downstream of the copper cliff and municipal waste-water treatment discharge in Junction Creek (Weber et al, 2006a,b) indicating that this is a potentially important mechanism of action for CCME and should be investigated further.

A decrease in hatching success and increase in deformities in the F1 offspring along with increased estradiol levels in females were observed only in the trophic-transfer system. This could suggest that exposure through the food was a contributing factor. However, we were not able to examine the effects of contaminated food in isolation as this was not possible in the self-sustaining system. Our primary objective was to assess the combination of the two as this is more indicative of "real world" responses. As such, the effects observed only in the trophic-

transfer system suggests that the combination of both food and water exposure may be required to exert the effects observed.

The significant increases in estradiol, testosterone and vitellogenin in females in the trophic-transfer system would indicate that an up-regulation of the testosterone-estradiol-vitellogenin pathway occurred. However, levels of estradiol in the trophic-transfer exposure were similar to those observed in the water-only controls. The slight vitellogenin induction, in females in the trophic-transfer system, may have been caused by the presence of estrogenic compounds within the CCME. CCME does contain domestic sewage from the town of Copper Cliff and from within the mine itself. In 2005, domestic sewage accounted for approximately 1-3% of total effluent discharged into Junction Creek. Studies at other sites exposed to domestic sewage have documented a direct link between exposure and the induction of vitellogenin, predominately due to the presence of estrogenic compounds within the effluent (Purdom et al, 1994; Harries et al, 1996, 1997, 1999). It could be that the small amount of domestic sewage present in the CCME accounted for the slight induction in vitellogenin observed in our study. However, as these levels do not exceed normal baseline levels observed in breeding females they are not thought to represent any significant physiological disruption.

Water quality e.g. pH, conductivity, alkalinity, and metal content showed significant differences in the effluent treatment systems. The pH was reduced in both CCME exposure systems, however, the range of pH in the exposures were 7.5 to 7.7 compared to controls that ranged from 7.8 to 8.1. It is unlikely that pH would have caused the responses observed in reproductive output in this exposure as FHM spawning has been documented to successfully occur between pH 6.6 to 9.5 (Mount, 1973 cited in USEPA, 2002). The levels of pH were also within the limits of the water quality guidelines for the protection of freshwater aquatic life (6.5

to 9.0) (CCME, 1999). The reduction in alkalinity (50%) was greater than the Provincial Water Quality Objectives (PWQO) in ON, Canada (>25% reduction) which would mean that the buffering capacity was reduced in these 45% CCME treatments. However, the level of pH and the significant increases in hardness, i.e., calcium and magnesium would suggest that the buffering capacity was not of concern and any acidification effect resulting in increased bioavailability of metals was highly unlikely.

Increased ion levels (magnesium, calcium and sodium) may be of interest as causal factors in the effluent affecting FHM due to their potential to cause stress associated with osmoregulation (Evans, 2000). Reproduction may be impaired under periods of stress and the reduction in reproductive output could simply be due to the change in water quality, i.e., high conductivity and hardness. 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). Thus, this is an area worthy of further investigation.

Increased metal content in the effluent may have played a significant role in the reduction of reproductive output. Significant increases in boron (96%), barium (58%), copper (900%) and nickel (486%) were all observed in both CCME systems. Copper and nickel both exceeded PWQO (>5 and 25ug/L respectively) measuring 81 and 44 µg/L, respectively, in the water-only system and 93 and 113 µg/L, respectively, in the trophic-transfer system (Table 5.2). These increases in Cu and Ni are consistent with a number of studies conducted on Junction Creek downstream of the CCME discharge (Jaagumagi and Bedard, 2002; Weber et al, 2006a,b; Gauthier et al, 2005). However, none of these metals in the water were significant in the body burdens of either male or female FHM suggesting that even though they were elevated in the

water column, their bioavailability and uptake appear limited. This is consistent with our previous mesocosm studies with pearl dace (*Semotilus margarita*) in 2002 (Dubé et al, 2006). In this study a number of metals, including copper and nickel, were significantly increased in the water but only selenium, thallium and lithium were significantly increased in whole bodies of both males and females (Dubé et al, 2006). The only metals that were consistently elevated, in our study, in males and/or females in both systems were thallium (400-1700%), rubidium (23-75%) and selenium (46-116%). The significant increase in rubidium is comparable to our previous field investigation where we also observed significant increases in FHM body burdens downstream of the Copper Cliff and sewage treatment discharge (Weber et al, 2006a,b). The levels of rubidium in our study were higher but comparable (approximately 7 µg/g in males and 9-11 µg/g in females) to those observed in the field investigation (5 and 4 µg/g in males and females, respectively). Rubidium has been found to consistently biomagnify in diverse aquatic food webs (Campbell et al, 2005). However, no reports on its sub-lethal effects have been documented and, therefore, it is not possible for us to speculate as to its significance in this study.

Limited scientific data exists regarding the aquatic toxicity of thallium. A study was conducted by Pickard et al (2001) to document its sub-lethal and lethal toxicity to various aquatic organisms. In a 7-d *Ceriodaphnia dubia* reproduction bioassay they documented 0.10 mg/L as the IC25 (inhibition concentration at which 25% of the test population are effected) and 4.27 mg/L as the average 96hr LC50 (lethal concentration at which 50% of the test population are effected) value for rainbow trout. The International Programme on Chemical Safety (IPCS, 1996) reported the lowest thallium concentration to affect aquatic species is 0.008 mg/L, which caused a reduction in growth of aquatic plants. They also reported 96-h LC50 values for

daphnids (2.2 mg/L) and for a freshwater fish (120 mg/L). Lethal concentration for rainbow trout has also been documented at 10-15 mg/L and 0.03 mg/L for Atlantic salmon (*Salmo salar*) (Zitko and Carson cited in Pickard et al, 2001). LeBlanc and Dean (1984), in an exposure with fathead minnow eggs and larvae, documented a lowest observed effect concentration (LOEC) of 0.04 mg/L where larval survival was significantly reduced. Abnormal FHM larvae were also observed after 28 days exposure to thallium at 0.292 mg/L (Kimball, undated report cited in Stephenson and Spry, 1995). Stephenson and Spry (1995) in a review of thallium toxicity report that thallium is chronically toxic to FHM at 0.04 – 1.2mg/L. Levels of thallium in the CCME treatment from our study were 0.0007-0.001 mg/L (water concentrations) and 0.016-0.03 µg/g in muscle tissue. Levels in the water in both CCME treatments are an order of magnitude lower than the concentrations reported to cause chronic toxicity to FHM (0.04 mg/L). Unfortunately, no data have been found that document body burden levels and corresponding effects in fish. There is not enough evidence to either rule out or confirm that thallium could be playing a part in the responses observed in our study. We can say that the documented effects of reduced hatching success and abnormal larvae after exposure to thallium in FHM (LeBlanc and Dean, 1984) correspond to observations made in our study. Further investigation into the sub-lethal effects of thallium on FHM is warranted before any further conclusions can be made.

Selenium was significantly elevated in female tissues in both systems (62% in trophic-transfer and 116% in water only), but only in the trophic-transfer system in males (46%). Again, this increase is comparable to our previous field investigation where we also observed significant increases in selenium body burdens in FHM (Weber et al, 2006a,b). The levels in the field investigation were higher (approximately 4 µg/g in males and females) than those observed in our study (approximately 2 µg/g). This difference may simply be due to the duration of exposure

(21 days versus life-time) or due to the contribution from other sources in the Junction Creek watershed. From the muscle levels and endpoints affected, it appears that selenium may play a role in the responses observed on reproduction. The endpoints affected in the trophic-transfer system only (hatching success and deformities) correspond with this hypothesis. The effects of selenium on fish reproduction have been documented and reviewed extensively (Hamilton, 2004; Lemly, 2002; Hamilton et al, 2002). The basic chemistry and physical properties of selenium are very similar to sulfur. When present in excessive amounts, selenium is substituted for sulfur which prevents the formation of essential disulfide chemical bonds (S-S) resulting in impaired protein biosynthesis (Lemly, 2002). Effects of selenium in fish include necrosis and rupturing of mature egg follicles in reproducing adults, (Sorensen et al, 1984) and, most notably larval deformities including spinal curvature (kyphosis, lordosis, scoliosis) and edema (Lemly, 2002). Uptake via food by adults is thought to be the primary route of exposure for larval effects to manifest (Hamilton, 2004). It is thought that selenium is incorporated into vitellogenin (egg yolk protein) which is synthesized in the liver. Vitellogenin is transported from the liver to the ovary where it is deposited within the embryo and eventually absorbed, along with selenium, by the developing larvae (Kroll et al, 1991).

The increase in selenium in males and females in the trophic-transfer system corresponds to the responses observed in egg production, hatching success and deformities. Selenium concentrations of >4.6 µg/g in food organisms have been shown to adversely affect survival of razorback sucker larvae (*Xyrauchen texanus*) and the effects of survival were more prominent from dietary exposure compared to waterborne exposure (Hamilton et al, 2002). The majority of literature supports whole-body thresholds of 3 µg/g in the diet of fish (Hamilton, 2002). We observed an 8-fold increase in selenium in *C. tentans* in the trophic-transfer exposure with a

pooled value of 11 µg/g compared to 1.4 µg/g in controls, this is nearly four times higher than the threshold concentration documented by Hamilton (2002). The concentration in the food (*C. tentans*) in the trophic-transfer system was at levels reported in the literature and could be a causal source affecting the F1 offspring.

Exposure via food can, therefore, be an important route in causing F1 generation effects, i.e., hatching success and deformities. This route of exposure was not present in the water-only system, which would account for why we did not see any effects on these endpoints. A study with FHM larval survival after exposure to Kelly Lake water from Sudbury, ON also found no significant differences in embryonic mortality (Gauthier et al, 2005). This study was a water-only exposure of eggs from lab-reared FHM, similar to our exposure. These results would suggest that the important route of exposure for assessing the effects of CCME on the F1 offspring is via trophic-transfer.

Even though we did not see reduced hatching success or appearance of larval deformities in the water-only CCME system we did observe impacts on adult egg production. The CCME is a complex mixture and thus there are a variety of potential causative factors that could be involved in these biological responses including selenium, thallium, rubidium, increased ions and alkalinity. All of these factors require closer scrutiny before causal linkages can be made between the effluent and the biological changes observed. That being said, significant research has been conducted on selenium and offers a potential explanation as to why adults were affected in both systems and F1 endpoints were only affected in the trophic-transfer system.

Hamilton (2002), in his review on selenium thresholds in freshwater fish, states that once selenium tissue concentrations reach a critical threshold, regardless of the route of exposure, adverse effects will occur. This is based on a number of separate studies that looked at water

only exposures with Chinook salmon (*Onchorhynchus tshawytscha*) (Hamilton et al, 1986; Hamilton and Wiedmeyer, 1990 cited in Hamilton, 2004) and dietary exposures (Hamilton and Buhl, 1990). Both exposure routes resulting in similar whole-body selenium residues of 4-5 µg/g in adults were associated with the same adverse effects (reduced growth and survival). In our study, females from both CCME systems (water-only and trophic-transfer) had very similar muscle tissue levels of selenium which could correspond to the significant decrease in reproductive output in both systems (egg production and spawning events). Pyle et al (2005) suggest that selenium could be an issue in the contaminated systems around Sudbury, ON. In their study on wild fish populations selenium was related to all condition metrics studied. A previous field investigation conducted by our lab also observed elevated selenium in FHM downstream of Copper Cliff effluent and municipal sewage discharge in Junction Creek, a finding not found downstream of either Nolin or Garson discharges (Weber et al, 2006a,b). FHM could not be sampled between the Copper Cliff discharge and the municipal sewage outfall and thus, although our study suggests that CCME may be a potential source of selenium in Junction Creek causing reproductive impairment in FHM, we cannot make a direct causal link for the field studies.

Although both selenium and rubidium levels measured in adult FHM muscle tissue in our CCME treatments were significantly higher than controls, those in the trophic-transfer system were not higher than those in the water-borne only exposure. This is surprising, as both selenium and rubidium have been documented to bioaccumulate and/or biomagnify (Campbell et al, 2005; Hamilton, 2004). We would have expected much higher levels in adult tissue in the trophic-transfer system where we documented concentrations of 9.45 and 11 µg/g of rubidium and selenium respectively in *C. tentans*. However, we only measured body burdens in muscle tissue.

Investigations in our laboratory with selenium (A. Belknap, pers. comm. University of Saskatchewan, Saskatoon, SK, Canada) have shown that bone, whole-body and muscle tissue have the lowest total selenium levels in northern pike (*Escox lucius*), whereas the kidneys, liver and ovaries have the highest. A recommendation for future studies with CCME is to sample other tissue as well as muscle in adult females to determine and compare body burden levels.

The lack of effect in any of the individual endpoints (condition, gonad or liver size, etc.) in either males or females suggests that the exposure was either not long enough or not potent enough to cause any significant changes in these endpoints. These individual endpoints, compared to the reproductive endpoints, seem to be less sensitive to CCME over the 21 d exposure. A longer period of exposure may be required to see any significant difference in these individual endpoints, or exposure at a more sensitive life stage i.e. with a full life-cycle bioassay over 120 days. However, a previous field investigation (Weber et al, 2006a,b) in Junction Creek also found that the only individual endpoint that was significantly different from reference in FHM was increased liver weights in males downstream of the CCME and municipal sewage discharge. No difference was observed in condition or gonad weights in either males or females. Therefore it would seem that the lack of effect in these endpoints is consistent with field observations and therefore these endpoints may not be sensitive or relevant for assessing changes after exposure to CCME.

5.5 Conclusion

The most sensitive endpoints were reproductive; a conclusion that agrees with our previous field investigation with FHM. Trophic-transfer was an important route of exposure for assessing effects of CCME on the F1 generation. If only waterborne exposures had been conducted, these responses would not have been observed. We recommend that dietary exposure pathways be

conducted in future studies with this effluent and other complex mining effluents. The only significant elements of concern based on consistent detection in water and uptake into muscle tissues were rubidium, thallium and selenium. The lack of data for rubidium and thallium make it difficult to draw specific causal links to the responses observed. Thallium, rubidium and selenium are recommended as elements for more focused investigations in the future. Alterations in experimental design for future studies with this effluent have been made based on the results of this paper, they include collection of ovaries for selenium analysis and deformed larvae collection for more detailed microscopic analysis.

CHAPTER 6[a]

Assessing effects of metal mining effluent on fathead minnow (*Pimephales promelas*) reproduction in a field-based trophic-transfer artificial stream system

[a] This chapter has been submitted to the journal of Aquatic Toxicology under joint authorship of Monique G. Dubé, Lynn P. Weber, Kimberlea L. Driedger and David M. Janz (University of Saskatchewan)

6.1 Introduction

The Junction Creek watershed, located in Sudbury, ON, Canada, has been the subject of numerous investigations into the impacts of mining operations that have been conducted in Sudbury for over 100 years (Keller et al, 1992). Junction Creek flows through the city of Sudbury, is approximately 52 kilometres in length, and has five main tributaries: Nolin Creek, Copper Cliff Creek, Frood Creek, Maley Creek and Garson Creek (Figure 6.1). Nolin, Copper Cliff and Garson Creeks receive mine effluent from three operations located around the city of Sudbury (Garson [GME], Nolin [NME] and Copper Cliff [CCME]). The creek also receives municipal waste-water (MWW) from the city, storm run-off, and atmospheric deposition has created significant historical contamination including acidification and metals loading (Jaagumagi and Bedard, 2002). A recent survey in 2004 of fish populations within the creek showed that 12 main species are resident, including creek chub (*Semotilus atromaculatus*), finescale dace (*Phoxinus neogaeus*), common shiner (*Notropis cornutus)*, brown bullhead (*Ameiurus nebulosus*) and fathead minnow (*Pimephales promelas*) (Lemieux et al, 2004).

A field investigation conducted by our laboratory in 2004 in Junction Creek observed increases in metal muscle burdens (Cd, Cu, La, Rb, Se), decreased egg size and increased fecundity in fathead minnow (FHM) downstream of the CCME and MWW discharge (Weber et al, 2006a;b). Total fecundity and egg size were also significantly reduced in female creek chub downstream of GME. This investigation supports other studies (Sein, 1993; Jaagumagi and Bedard, 2002) that suggest fish and benthos within Junction Creek are being affected by anthropogenic discharges. Identifying the potential cause of these effects has been an issue due to the confounded nature of the creek. Previously, artificial stream studies were

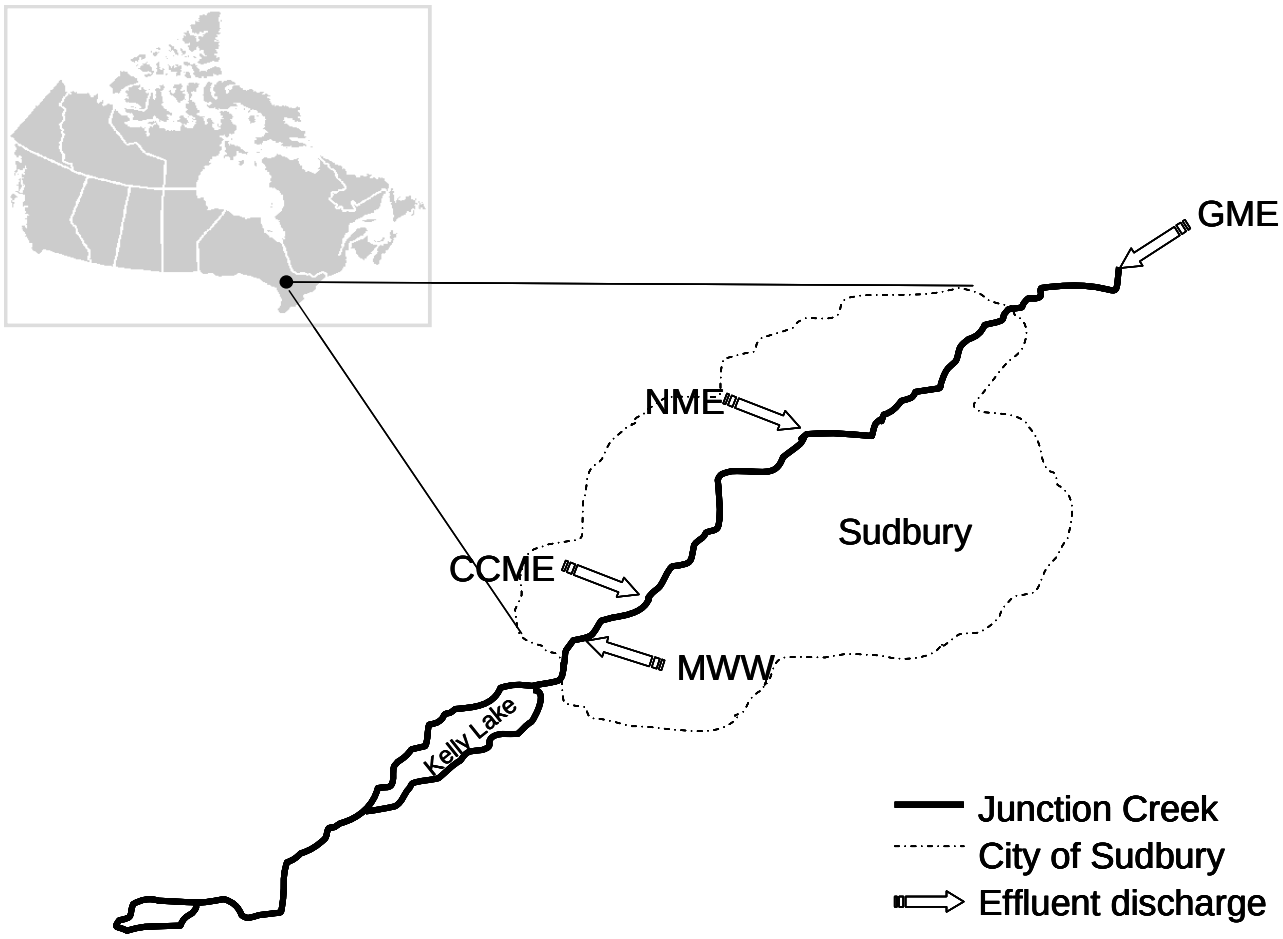

Figure 6.1. Map of Junction Creek, Sudbury, ON, Canada and the four major effluents discharging into the creek: Garson Mine Effluent (GME), Nolin Mine Effluent (NME), Copper Cliff Mine Effluent (CCME) and Municipal waste-water (MWW). Dotted line represents the city of Sudbury (not to scale).

conducted in 2001 and 2002 to determine effects on both benthos (Hruska and Dubé, 2004; 2005) and fish (Dubé et al, 2006) in an attempt to tease apart the effects of the individual MMEs. The results of these investigations revealed reduced survival in creek chub and reduced body weights in male and female dace along with increases in metal muscle burdens (Ni, Ru, Sn, Fe, Li, Tl and Se) after exposure to CCME and GME. Hruska and Dubé (2004; 2005) also reported reduced emergence, hatching success and survival of the benthic invertebrate, *Chironomus tentans* after exposure to CCME.

Even though these studies allowed identification of specific changes in biological endpoints from individual mine effluents, there were a number of limitations. Firstly, the artificial stream studies conducted with fish on-site in Sudbury (Dubé et al, 2006) only measured individual endpoints, which limited extrapolation of the results to infer effects on the populations within the creek. Secondly, trophic-transfer of contaminants via a food source was not considered in the exposure designs. Dubé et al (2005) conducted a multi-trophic exposure in an artificial stream study at a New Brunswick mine. They observed reduced weight, condition and survival of slimy sculpin (*Cottus cognatus*) after exposure to 20% and 80% MME. There have also been numerous studies investigating the relative importance of trophic-transfer of metals (Ni et al, 2000; Chen et al, 2000; Mason et al, 2000; Xu and Wang, 2002) in aquatic environments. Finally, the studies conducted have focused only on MME entering Junction Creek with no investigation into the additional municipal waste-water discharge entering the creek around 200m below the Copper Cliff Creek confluence. In our previous field studies, FHM could only be caught downstream of both the CCME and MWW discharge locations. Therefore there is a need to test not only CCME in

isolation but also to combine this with the MWW, thereby allowing a more accurate interpretation and comparison of responses observed in the field study.

In response to these issues we developed a trophic-transfer system using a short-term FHM bioassay (Chapter 5). This system allows us to assess the contribution of food and/or water-borne exposure of the effluents on both population and individual endpoints. This trophic-transfer system was tested in the laboratory in 2004 to assess the affects of CCME in both a water-only and trophic-transfer exposure. Results showed that CCME significantly reduced egg production, regardless of the route of exposure, however, hatching success and the appearance of larval deformities only appeared after exposure in the trophic-transfer system. This suggests that trophic-transfer was an important route of exposure with CCME in determining effects in the offspring. As the trophic-transfer bioassay was developed within an artificial stream system we are not only able to test the effects of CCME in a controlled laboratory environment (Chapter 5) but also transfer the system to the field, thereby improving its environmental relevance (temperature, photoperiod and ambient reference water).

The objectives of this study were to: 1) assess the effects of Copper Cliff mining effluent (CCME), and a combined mixture of CCME and municipal waste-water (CCMWW) on FHM reproduction in an artificial stream system on-site at Sudbury; and 2) use the trophic-transfer system to assess the importance of food as a source of exposure of CCME and CCMWW to FHM.

6.2 Methods

6.2.1 Study site.

The Junction Creek watershed encompasses an area of 329 km^2. The mainstem is 52 km in length, with a width of 2 m (headwaters) to 30 m (mouth), and a mean annual discharge of 1.36 m^3/s at the City of Sudbury with peak freshet flows in April (4.94 m^3/s). Five tributaries drain into the mainstem including (from headwaters to mouth of Kelly Lake), Garson Creek, Maley Creek, Frood Creek, Nolin Creek and Copper Cliff Creek. The mainstem flows southwest from Garson Mine, through the City of Sudbury into Kelly Lake. A 762 m section of Junction Creek and a section of Nolin Creek at its confluence are directed underground at Sudbury by concrete box culverts constructed in 1966. Junction Creek is largely surrounded by urban development and is exposed to aerial deposition and liquid MME discharges from mining operations, as well as storm water and treated municipal sewage. Based on average annual MME discharges, flows in Junction Creek at Sudbury, and proportions of the respective watershed drainage areas, the concentrations of the Garson, Nolin, and Copper Cliff waste-water treatment plant discharges at their respective Junction Creek confluences were estimated at 30%, 20% and 45%.

In Sudbury (400 km north of Toronto, ON, Canada; Figure 1), INCO operates the largest fully integrated mining, milling, smelting and refining complex in Canada, producing nickel, copper, precious metals, platinum-group metals, sulphuric acid and liquid sulphur dioxide. Three waste-water treatment plants discharge treated MME into Junction Creek including Garson (approx. 982,030 m^3/y in 2004), Nolin (6,050,023 m^3/y in 2004) and Copper Cliff (43,870,000 m^3/y in 2004). The effluents are all treated by conventional hydroxide precipitation (lime addition and settling) and subsequent pH adjustment prior to discharge. The Garson treatment plant treats water from ore processing at the active Garson mine. The

Nolin treatment plant treats surface water draining from decommissioned mining pits. Due to low water levels in Sudbury during the course of this experiment, Nolin waste-water treatment plant was not discharging effluent into Junction Creek. Therefore investigations into the effects of this effluent were not assessed. The Copper Cliff waste-water treatment plant receives inputs from active and inactive tailings, several mines, a nickel refinery, a mill, collected surface drainage, a copper refinery, a smelter complex, surface runoff and sewage from the mine-related housing/administration offices. By volume, the Copper Cliff treatment plant represents the largest mining-related input into Junction Creek (~ 45% of Junction Creek flow at the point of input).

The Sudbury waste-water treatment plant treats municipal sewage prior to discharge to Junction Creek. The outlet for the discharge is upstream of Kelly Lake and located approximately 200 m downstream from where the CCME enters Junction Creek. The MWW treatment plant has a current capacity of 79,625 m^3. The current treatment process is secondary with bar screens, detritors, aeration tank, clarifiers, chlorine contact chamber and sludge thickening tanks (for dewatering the sludge using a polymer). Phosphorus is chemically removed utilizing ferric sulphate. Average daily flows of the MWW were 60,000m^3 with a maximum daily flow of 90,000 m^3 in 2005.

6.2.2 Experimental design

This study was conducted during July and August 2005 at the Vermillion water treatment plant, Lively, ON, Canada. The Vermillion River was the source of the reference water and the effluents tested were copper cliff mine effluent (CCME) from Copper Cliff waste-water treatment plant (CCWTP) and municipal waste-water (MWW) from the Municipal Treatment Plant (MWTP) in Sudbury, ON.

The artificial stream system used for this study has been described in detail by Hruska and Dubé (2004). Briefly, each system consists of a table (one/treatment) with five to eight replicate, 10.3-L, circular, high-density polyethylene streams on each table. Each stream has a diameter of 0.3 m and a depth of 0.2 m. The replicate streams sit on a common table that drains into an 85-L dilution reservoir. The streams, table, and dilution reservoir are self-contained on a shipping pallet. The trophic-transfer system design has been described fully in Chapter 5.

6.2.2.1 Pre-exposure design

The short-term FHM bioassay requires a pre-exposure and exposure trial of approximately similar duration (21 d) (Ankley et al, 2001). The pre-breeding trial is conducted in the absence of effluent to establish baseline reproductive performance of breeding pairs. The initial breeding trial consisting of 58 breeding pairs (eight pairs per table) was conducted over 21 d and is explained in detail in Chapter 4.

After 21 d, selection of breeding pairs for the effluent exposure was undertaken based on 100% survival of all adults, presence of eggs in each replicate once a week, and >80% fertilization of eggs (OECD, 2003). Based on these criteria, 66% of the breeding pairs used in the pre-exposure period were selected for use in the exposure period resulting in five pairs per treatment (n = five).

Analysis between artificial stream tables using streams as replicates (one breeding pair per stream) was conducted to determine if any differences could be observed in reproductive endpoints prior to effluent exposure. Levine's Test was used to confirm parametric assumptions for homogeneity of variance. One-way analysis of variance (ANOVA) was then

conducted to determine if there were any differences between tables for the following endpoints: egg production and spawning events (mean number per pair after 21d), hatching success (% of viable eggs produced) and deformities (% of total hatch). No significant differences were found ($\alpha = 0.05$). The unit of replication for egg production, spawning events, hatching success and deformities were breeding pairs (n = five per treatment).

6.2.2.2 Trophic-transfer system

During the pre-exposure period of the FHM bioassay the trophic-transfer system was set-up. Cultures of *C. tentans* were established on-site. Due to the reduced survival of *C. tentans* eggs in the first week, 7-day old larvae and egg sacs were used to establish cultures. The 7-day old larvae and egg sacs were supplied from cultures at the University of Saskatchewan, Saskatoon, SK, Canada, where they were shipped overnight in sampling tubs (larvae) and scintillation vials (one egg sac per vial). The egg sacs were evenly distributed into small hatching tubs that contained approximately one inch of silica sand and aerated reference water. Partial water exchanges were conducted each day within the tubs and 1mL of tetramin slurry was added every day. After 7 days, three cores (core sampler area = $9cm^2$) from each tub were taken to estimate densities within the tubs. Based on this estimation, cores were distributed evenly throughout the trophic-transfer streams and densities were calculated based on the number of cores supplied to each stream and the original densities calculated in the cores. Cores were taken every 7 days within the trophic-transfer system to firstly calculate survival and secondly to compare the densities between the streams. Densities were calculated by taking three replicate cores (core sampler area = $9cm^2$) and calculating densities per cm^2 (mean core densities/9) and total stream densities (densities per cm^2 * total stream area). Finally, densities were checked at the end of the three weeks to determine that

both adequate and similar amounts of food were present in each stream. Based on these criteria, five streams/table (three tables) were selected for the exposure period. One gram of fourth instar *C.tentans* were weighed to calculate the optimal daily amount (per cm^2) required by each breeding pair in the trophic-transfer treatment. It was estimated that 1080 fourth instar *C.tentans* would be required for the duration of the exposure (21 days). Densities per cm^2 were then calculated based on the total surface area of the stream. Once densities were established, the FHM feeding barrier was added along with a spawning tile. The barrier was designed to turn once every two days to allow FHM to feed on uncovered *C. tentans* at a target feeding amount of 1g/pair/day (see Chapter 5).

Statistical analysis of the trophic-transfer streams was conducted to determine if any differences in densities of *C. tentans* existed between artificial stream tables before effluent exposure commenced using streams as replicates. An ANOVA was conducted using total densities in each stream. No significant differences existed ($p>0.05$).

6.2.2.3 Exposure design

Six treatments were established: Reference water, Copper Cliff Mine Effluent (CCME) (45%) and a mixture of CCME and MWW combined (CCMWW) (30% and 30% respectively = 60%) in both the water-only and trophic-transfer systems. The concentrations of 45% and 60% v/v in the CCME and CCMWW respectively were selected as environmentally relevant concentrations based on discharge rates into Junction Creek and volume calculations. The combined CCMWW was chosen based on a previous field investigation conducted downstream of both of these discharges (Weber et al, 2006). Breeding pairs were then transferred to either the water-only system (Ref, CCME or

CCMWW) or trophic-transfer system (Ref, CCME or CCMWW) with a total of five breeding pairs per system.

Treated final CCME was collected at the Copper Cliff water treatment plant in Sudbury once a week in four 1000L totes that were shipped to the Vermillion Water Treatment plant. The average holding time for the effluent was between 1-7 days. The MWW was collected at the Sudbury municipal treatment plant twice a week in 1000L totes that were shipped to the Vermillion Water Treatment plant. The average holding time for the MWW was between 1-4 days. Prior to exposures, both CCME and MWW was collected and stored for 7 days. Daily sampling of the effluents was conducted each day to assess general chemistry, nutrients and metals. Samples were collected, preserved and analyzed as per standard methods (Inductively Coupled Plasma-Sector Field Mass Spectrometry [ICP-SFMS]) and quality assurance and control procedures (National Laboratory of Environmental Testing, Burlington, ON, L7R 4A6). This analysis was conducted to determine if any changes in effluent chemistry occurred during the 7-day holding period. Based on these results mine effluent was collected once a week and MWW was collected twice a week. The CCME (574 L, 100% (v/v))and CCMWW mix (383 L each, 100% (v/v)) was supplied every day to 1200 L holding tanks where the effluent was mixed with Vermillion River water to make up to 1200 L. For the reference treatments, Vermillion River water was collected once a day and supplied to a 1200 L polyethylene tank. Reference and treatment waters were pumped (Pulsatron Series E, Viking Pump of Canada, Edmonton, Canada) from the holding tanks to the reservoir tanks for each artificial stream system at a total flow of 0.24L/hour which maintained a turnover time to two exchanges per system per 24 h period. Water from the reservoir was then supplied via a March pump (Bancroft Western, Vancouver, BC, Canada)

and manifold that continuously redistributed the water to five streams at a turnover rate of 2L/min resulting in a residence time of around 5 minutes for each stream. Water temperature underwent diurnal fluctuations and was logged using Optic Stowaway temperature loggers (Optic Stowaways©; Onset Computer, Bourne, MA, USA). Conductivity and dissolved oxygen were monitored daily in each stream using a YSI meter (Yellow Springs Instruments, Yellow Springs, OH, USA). Ammonia (Rolf C. Hagen, Edmonton, Canada) and pH (Oakton pHTestr 3, Oakton Instruments, Vernon, IL, USA) were also recorded daily.

Eggs and larvae were held in corresponding flow-through treatment tanks that were fed pre-mixed CCME or CCMWW and reference water via a manifold and pump that maintained an overall turnover rate of 2 volumes/day. Daily observations and measurements, i.e. egg production, hatching success, deformities etc. were the same as those taken during pre-exposure. Fish in the water-only system were fed live *C. tentans* (1g/breeding pair/day) obtained from cultures on-site at the Vermillion water treatment plant. Fish in the trophic-transfer system were self-sustaining and did not require additional feeding. Densities in the trophic-transfer system were checked once every week using core samplers (surface area = 9 cm^2) to obtain densities/stream and densities/cm^2. One section/stream was also checked before being uncovered by the feeding barrier, after 48h and 6 days later to determine re-colonization rates.

Water samples were collected from the reference water and treatment streams as well as the head tanks weekly during the three week exposure period and analyzed for general chemistry, nutrients and metals. Samples were collected, preserved and analyzed as per standard methods (Inductively Coupled Plasma-Sector Field Mass Spectrometry [ICP-

SFMS]) and quality assurance and control procedures (National Laboratory of Environmental Testing, Burlington, ON, L7R 4A6)..

At the end of the exposure period, fish were anaesthetized (MS222), and fork length (mm) and total body weight (g) recorded. Fish were euthanized by spinal severance. Gonads and livers in the adults were weighed (to 0.001g) and placed in formalin (10%) for 24h and then stored in ethanol (75%) until histological analysis was undertaken at the University of Saskatchewan using the quantitative method developed by Weber et al (2003). Gonadal tissue was paraffin-embedded, and three 5µm sections were taken at mid-gonad and mounted on slides. Ovaries and testes were assessed for both stages of development and histopathology. In brief, the percentage of oogonia, pre-vitellogenic, vitellogenic and mature follicles were calculated from four fields of view for each female. Atretic follicles (No. per field of view), the presence of fibrosis and eosinophilic (excessive eosin stain) material were also assessed for each female. The percentage of spermatogonia, primary and secondary spermatids and mature sperm were calculated from four fields of view for each male. Cell death (No. per field of view), the presence of fibrosis and eosinophilic material were also assessed for each male. A section of ovarian tissue was also sampled and stored in 5% buffered formalin for fecundity and egg size analysis. Both fecundity and egg size were determined using an Olympus SZ61 stereomicroscope and Image-Pro Discovery 4.5 software (Media Cybernetics Inc., Silver Springs, MD). Each ovarian lobe was taken from the midsection, briefly blotted dry and weighed. After dissection and separation of all yolked ovarian follicles, follicles were counted (20-200 eggs in each sub sample) and egg diameters of at least 15 yolked eggs measured using digital images at 8x magnification. Only the most mature cohort of eggs was counted to give an estimate of batch fecundity (Weber et al,

2006). Fish carcasses (excluding head and caudal fin) were then stored on dry ice whilst being shipped (24hr) to our laboratory at the University of Saskatchewan, Saskatoon, Canada. Upon arrival all samples were stored in a -80^{o}C freezer until sex-steroids, vitellogenin and metal content analyses were undertaken in muscle tissue. Muscle tissue was homogenized and ether-extracted for sex steroid analysis. Extraction efficiency for testosterone in male muscle tissue was calculated at 87%. Sex steroids (11-ketotestosterone [11-KT; males only], 17β-estradiol [females only] and testosterone [males and females]) and vitellogenin were measured using enzyme-linked immunosorbent assays (ELISA). EIA kits for testosterone and 17β-estradiol were supplied from Assay Designs, MI, USA. EIA kits for 11-KT and vitellogenin (Carp, pre-coated) were supplied from Cayman Chemical Company, MI, USA. Metals in muscle tissue were analysed, using ICP and ICP-MS, and calculated as dry wt as per standard methods and quality assurance and control procedures (Testmark Laboratories, Sudbury, ON, Canada). Ovarian selenium analysis was conducted using Hydride Generation Atomic Absorption Spectrometry (HG-AAS) at the University of Saskatchewan, SK, Canada.

6.2.2.4 Exposure analysis

Data were analyzed using SPSS 11.0 (SPSS, Chicago, Il, USA). To test parametric assumptions for homogeneity of variance, Levine's Test was used. If data did not require transformation, a two-way ANOVA was performed on the following endpoints: hatching success (% of viable eggs produced), deformities (% of total hatch), stages of ovarian development (histological analysis), muscle sex steroids, muscle vitellogenin, metal muscle-burdens and water and effluent quality data. A two-way ANOVA was performed to take into consideration the effect of the trophic-transfer system i.e. the contribution of food and water

exposure. The experimental design was not fully factorial as we did not assess the effects of contaminated food in isolation. If an interaction was observed, a one-way ANOVA was conducted on the water-only treatments and trophic-transfer treatments separately to determine where the difference lied. Post-hoc analysis (Tukey test) was also conducted to determine treatment differences. If Levine's Test for homogeneity was significant, the percentage-based data were arcsin (%) transformed and tested again for homogeneity. Data that were not percentage or ratio-based were log10 transformed. A two-way ANOVA was then performed on the transformed data. If assumptions could not be met for homogeneity of variance, a non-parametric two-way ANOVA test (Scheirer-Ray-Hare extension of the Kruskal-Wallis test) was performed (Sokal and Rohlf, 2003).

Two-way ANCOVAs were used to assess gonad and liver weights (with body weight as a covariate) and body weight (with length as a covariate). If an interaction was observed between treatment and source of exposure an ANCOVA was conducted on the water-only treatments and trophic-transfer treatments separately to determine where the difference lied.

To assess responses over time Kolmogorov-Smirnov (KS) tests were conducted to assess the cumulative frequency of spawning events and eggs produced occurring in each treatment over 21 d compared to controls. KS two sample tests were also conducted on ovipositor development in both males and females. The appearance of male secondary sex characteristics in females was assessed using contingency tables.

Densities (no. of *C. tentans*/cm^2) and emergence (no. of animals emerged) were assessed with a repeated measures ANOVA to determine responses over 21 d exposure period. Temperature was also assessed with a repeated measures ANOVA for all treatments. The temperature at four time points during the day (6am, 12noon, 6pm and midnight) were the

repeated measurements and replication was day, i.e., n=20 for each treatment. Diurnal fluctuations (time effect) would be assessed as well as the differences in these diurnal fluctuations between treatments (treatment effect) and system (source effect). Assumptions were applied as discussed above.

6.3 Results

Statistical analysis of exposure data was altered due to a system failure in the CCME trophic-transfer treatment. A power outage during the exposure period resulted in the CCME trophic-transfer treatment being discontinued after two weeks of effluent exposure. To maintain analysis of source and interaction effects between the two systems a two-way ANOVA was conducted with the CCMWW treatments. One-way ANOVAs were then conducted on the water-only treatments to include the CCME in this system. Endpoints that would not be affected by the system failure (e.g., hatching success, deformities) were analysed as described in section 6.2.2.4.

6.3.1 Water quality

6.3.1.1 Water quality parameters

In the water-only system, significant increases were observed for ammonia, chloride, conductivity, hardness, calcium, potassium, magnesium, sodium, sulfate, fluoride and total phosphorus in CCME treatment compared to reference (Table 6.1). Similar significant increases were observed in the CCMWW treatment with the exception of ammonia which was not significantly increased. Overall, the levels were lower in the CCMWW treatment, compared to CCME, for conductivity (17% lower than CCME), hardness (29%), calcium

(30%), potassium (24%), magnesium (14%), sulfate (29%) and total phosphorus (13%). Only chloride (17%) and fluoride (13%) were higher in the CCMWW compared to the CCME.

In the trophic-transfer system, the same significant increases as those observed in the water-only system occurred in the CCME and CCMWW treatments. The exception was ammonia where no significant difference was identified (Table 6.1). Similar to the water-only system the levels in the CCMWW treatment were lower than those measured in the CCME for conductivity (16% lower than CCME), hardness (28%), calcium (29%), potassium (24%), magnesium (19%), sulfate (28%) and total phosphorus (26%). Again the only parameter that was increased in the CCMWW treatment compared to CCME was chloride (14%).

Water quality in the trophic-transfer system was generally comparable to the water-only system where similar levels were observed. However, a number of parameters were elevated in all three treatments (including reference) in the trophic-transfer system compared to water-only. These were TSS, DOC, TOC and total phosphorus. On average DOC, TOC and TSS increased by 73%, 91% and 175% respectively, compared to water-only system. Total phosphorus increased by over 1000% in the reference treatment, 100% in CCME and 25% in the CCMWW compared to the water-only system treatments. Significant system effects were observed for TSS (two-way ANOVA, $p=0.008$) and total phosphorus (two-way ANOVA, $p=0.001$) suggesting that these parameters increased in the trophic-transfer system independent of treatment.

6.3.1.2 Trace metal analysis

In the water-only system, significant increases in copper, arsenic, boron, barium, cadmium, lithium, molybdenum, thallium and selenium occurred in the CCME treatment. In

the CCMWW treatment similar elevations were observed except for copper, arsenic and selenium where significant increases did not occur. Similar to the general water quality parameters, metal levels in the CCMWW treatment were generally lower. Barium, lithium and thallium were 27%, 36% and 31% lower than the levels measured in the CCME treatment. Molybdenum and boron, in contrast, were both elevated in the CCMWW treatment by 116% and 64% respectively compared to CCME (Table 6.2). Iron, manganese and nickel were also elevated in the CCMWW treatment compared to controls; these increases were not observed in the CCME. A significant decrease in aluminum in the CCMWW treatment was also not observed in the CCME treatment (Table 6.2).

In the trophic-transfer system, elevations in metal content were similar to the water-only system for both CCME and CCMWW. Again, levels in the CCMWW were generally lower than those observed in the CCME for barium (27% lower than CCMW), lithium (35%), and thallium (31%). Elevated levels of molybdenum and boron in the CCMWW compared to CCME also occurred in the trophic-transfer system. Metal content in the trophic-transfer system was comparable to the water-only system except for copper and iron where concentrations in the trophic-transfer system were 23- and 8-fold higher, respectively, in the trophic-transfer reference compared to the water-only reference treatment.

6.3.2 Individual endpoints

In the water-only system, no significant differences were observed in female body weight (ANCOVA, $p=0.675$), gonad weight (ANCOVA, $p=0.729$) or liver weight (ANCOVA, $p=0.609$) in either the CCME or CCMWW treatment compared to reference. In males, no significant difference occurred in body weight (ANCOVA, $p=0.653$), gonad size (ANCOVA, $p=0.692$) or liver size (ANCOVA, $p=0.442$) in either treatment (data not

Table 6.1. Summary of water quality parameters conducted throughout the August 2005 artificial stream study with Copper Cliff mine effluent (CCME) (45%) and a CCME and municipal waste-water (CCMWW) (30%, 30%) mixture in water-only and trophic-transfer exposure systems. Data for the CCME trophic-transfer system is based on 14 days of exposure. Values represent means ± standard error. Asterisk represents significant difference from reference when analysed with a two-way ANOVA where * = $p<0.05$, ** =$p<0.01$, *** = $p<0.001$. ‡ indicates significant difference from water-only reference, where ‡ = $p<0.05$. DOC = dissolved organic carbon, TOC = total organic carbon, F = fluoride, P = phosphorus, TSS = total suspended solids, Ra = radium.

Parameter	Unit	Detection Limit (mg/L)	Water-only			Trophic-transfer		
			Ref	CCME	CCMWW	Ref	CCME	CCMWW
Ammonia	mg/L	0.05	0.00 ± 0.00	2.25 ± 0.08‡	2.02 ± 2.00	0.41 ± 0.22	2.57 ± 0.45	2.78 ± 1.86
DOC	mg/L		2.96 ± 1.57	2.60 ± 1.31	3.63 ± 1.84	5.67 ± 0.65	5.54 ± 0.40	6.11 ± 0.32
TOC	mg/L		3.47 ± 1.85	3.35 ± 1.69	4.04 ± 2.03	6.17 ± 0.98	6.00 ± 0.47	6.66 ± 0.18
Alkalinity	mg/L	1	28.8 ± 2.35	22.0 ± 0.13	23.6 ± 12.3	32.5 ± 1.81	23.4 ± 0.98	26.1 ± 11.1
Chloride	mg/L	0.1	12.4 ± 0.60	62.8 ± 2.92***	73.0 ± 4.72***	13.3 ± 0.30	63.3 ± 3.43***	72.4 ± 5.06***
pH	pH	0.05	8.01 ± 0.09	7.89 ± 0.12	7.69 ± 0.50	8.13 ± 0.16	8.03 ± 0.30	7.59 ± 0.48
Conductivity	uS/cm	10	192 ± 3.09	1859 ± 67.9***	1547 ± 103**	241 ± 41.3	1848 ± 88.0***	1559 ± 108**
Nitrate	mg/L	0.1	1.28 ± 1.13	4.36 ± 1.16	2.88 ± 0.59	23.0 ± 22.8	2.38 ± 0.15	2.93 ± 0.84
Hardness	mg/L	0.05	51.5 ± 2.85	750 ± 9.25***	533 ± 48.5**	56.0 ± 1.06	744 ± 24.7***	533 ± 43.7**
Calcium	mg/L	0.008	15.5 ± 0.88	285 ± 2.95***	200 ± 19.5***	17.0 ± 0.30	281 ± 9.43***	200 ± 17.7***
Potassium	mg/L	0.019	0.82 ± 0.06	20.6 ± 0.76***	15.9 ± 1.71***	0.96 ± 0.18	20.8 ± 1.32***	16.1 ± 1.66***
Magnesium	mg/L		3.11 ± 0.16	9.26 ± 0.78***	7.95 ± 0.09**	3.31 ± 0.08	9.92 ± 0.29***	8.03 ± 0.17**
Sodium	mg/L		6.40 ± 0.19	61.9 ± 2.03***	61.4 ± 3.40***	6.85 ± 0.15	62.9 ± 0.94***	61.8 ± 2.98***
Sulfate	mg/L	0.1	35.6 ± 1.95	953 ± 37.2***	681 ± 75.3***	34.6 ± 1.26	944 ± 18.6***	681 ± 78.0***
F	mg/L	0.1	0.05 ± 0.00	0.23 ± 0.04**	0.26 ± 0.05**	0.05 ± 0.00	0.20 ± 0.01**	0.21 ± 0.05**
Total P	mg/L		0.01± 0.00	0.40 ± 0.01***	0.35 ± 0.03**	0.21 ± 0.07	0.78 ± 0.31***	0.44 ± 0.03**
TSS	mg/L	2	3.40 ± 0.60	9.87 ± 3.25	5.67 ± 0.24	15.0 ± 0.07	20.7 ± 11.6	9.87 ± 2.07
Ra226	Bq/L	0.0025	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00

Table 6.2. Summary of water metal analysis conducted throughout the August 2005 artificial stream study with reference water, Copper Cliff mine effluent (CCME) (45%) and CCME and municipal waste-water (CCMWW) (30%, 30%)) in water-only and trophic-transfer exposure systems. Data for the CCME trophic-transfer system is based on 14 days of exposure. Values represent means (µg/L) ± standard error, ND (non-detectable). Asterisk represents significant difference from reference when analysed with a two-way ANOVA where * = $p<0.05$, ** =$p<0.01$, *** = $p<0.001$.

Parameter	Detection limit (µg/L)	Water-only			Trophic-transfer		
		Ref	CCME	CCMWW	Ref	CCME	CCMWW
Ag	0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01		ND	
Al	0.02	36.8± 7.92	10.9 ± 0.56	5.05 ± 1.67**	40.4 ± 13.3	29.5 ± 15.7	10.8 ± 2.10**
As	0.1	0.91 ± 0.10	1.57 ± 0.11**	0.97 ± 0.08	1.01 ± 0.09	1.61 ± 0.24**	1.29 ± 0.04
B	6	3.00 ± 0.00	16.9 ± 1.73**	27.6 ± 4.48***	3.00 ± 0.00	15.0 ± 0.97**	27.5 ± 3.42***
Ba	1	2.88 ± 0.57	25.6 ± 2.31***	18.7 ± 4.56**	2.37 ± 0.37	27.1 ± 4.56***	17.6 ± 3.76**
Be	0.2	0.10± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
Bi	30				ND		
Cd	0.01	0.01 ± 0.00	0.08 ± 0.01***	0.10 ± 0.03***	0.00 ± 0.00	0.06 ± 0.02***	0.10 ± 0.03***
Co	5				ND		
Cr	0.5	0.53 ± 0.15	0.80 ± 0.26	0.50 ± 0.14	0.60 ± 0.18	0.71 ± 0.31	0.86 ± 0.02
Cu	1	0.50 ± 0.00	54.8 ± 11.2*	36.0 ± 16.5	11.9 ± 9.41	58.9 ± 29.0*	33.6 ± 16.8
Fe	7	3.50 ± 0.00	51.2 ± 17.3	94.7 ± 10.8**	28.0 ± 14.7	136 ± 105	97.3 ± 40.3**
Li	1	0.50 ± 0.00	22.1 ± 0.79***	14.2 ± 2.78***	0.50 ± 0.00	22.6 ± 1.08***	13.7 ± 2.55***
Mn	0.01	0.67 ± 0.24	1.61 ± 0.61	10.1 ± 6.39**	1.25 ± 0.17	3.52 ± 1.43	20.1 ± 9.82**
Mo	0.01	0.24 ± 0.01	0.73 ± 0.11***	1.58 ± 0.16***	0.26 ± 0.02	0.66 ± 0.08***	1.78 ± 0.04***
Ni	9	4.50 ± 0.00	38.0 ± 8.87	35.6 ± 15.6*	4.50 ± 0.00	33.2 ± 14.4	32.5 ± 14.1*
Pb	15				ND		
Se	0.5	0.76 ± 0.51	9.22 ± 0.71**	4.14 ± 2.24	0.97 ± 0.72	6.79 ± 3.33**	7.20 ± 0.90
Tl	0.01	0.01 ± 0.00	0.82 ± 0.13***	0.56 ± 0.05***	0.01 ± 0.00	0.82 ± 0.11***	0.52 ± 0.05***
Zn	5	5.50 ± 3.00	2.50 ± 0.00	16.4 ± 7.43	2.50 ± 0.00	5.83 ± 3.33	10.0 ± 7.50

shown). All of the measurements in both males and females in the CCMWW treatment were comparable to the values observed in the CCME treatment.

For the trophic-transfer system, analysis of data from the CCME treatment could not be conducted for these endpoints because of the system failure. Therefore, only analysis of the CCMWW treatment was conducted and compared to the trophic-transfer controls. No significant differences in females were observed in body weight, gonad or liver weight in the CCMWW treatment (two-way ANCOVA, p=0.619, p=0.808, p=0.919 respectively) (data not shown). In males, no significant differences were observed in body weight, gonad size or liver size after exposure to CCMWW treatment (two-way ANCOVA, p=0.133, 0.289 and 0.219 respectively).

When the two systems are compared, values in the reference treatments were similar with the exception of male liver weight. Liver weight in males, in the trophic-transfer system was 67% larger than liver weight in the water-only system. A similar increase was seen in the CCMWW treatments where a 138% increase in male liver weight was observed in the trophic-transfer system compared to the water-only system. All other measurements were comparable between the two systems.

6.3.3 Reproductive investment

6.3.3.1 Egg size and fecundity

In the water-only system, no significant differences were observed in either egg size (ANOVA, p=0.873) or fecundity (ANOVA, p=0.596) in CCME and CCMWW treatments compared to reference (Table 6.3).

For the trophic-transfer system, analysis of data from the CCME treatment could not be conducted for these endpoints because of the system failure. Therefore only analysis of the

CCMWW treatment was conducted. In the trophic-transfer system no significant treatment effects were observed in either egg size (two-way ANOVA, $p=0.495$) or fecundity (two-way ANOVA, $p=0.613$) in the CCMWW treatment compared to reference (Table 6.3).

When the two systems are compared egg sizes in the reference treatments were smaller in the trophic-transfer system. Egg size was 66% smaller in the trophic-transfer compared to the water-only reference, however fecundity did not change. By comparison egg size and fecundity in the CCMWW treatment increased slightly by 15% and 33% respectively in the trophic-transfer compared to the water-only system.

6.3.3.2 Egg production and spawning events

In the water-only system, cumulative spawning events and egg production were significantly reduced by 65% (KS, $p<0.001$) and 77% (KS, $p<0.001$) respectively in the CCME treatment (Figure 6.2A, 6.3B). A similar significant reduction was observed in both spawning events (KS, $p<0.001$) and egg production (KS, $p=0.017$) in the CCMWW treatment. The reduction in egg production for CCMWW was not as severe as observed in the CCME alone. Compared to controls, egg production was reduced by 17% in the CCMWW compared to 77% in the CCME treatment.

In the trophic-transfer system, significant increases of 116% in spawning events (KS, $p=0.002$) and 65% in egg production (KS, $p=0.008$) occurred in the CCME treatment after 14 days of exposure compared to reference (Figures 6.2B, 6.3B). Significant increases in spawning events (KS, $p<0.001$) and egg production (KS, $p=0.002$) were also observed in the CCMWW treatment compared to reference after 21 days of exposure. The increase in reproductive output in the CCMWW treatment was less than that observed in the CCME. Compared to controls, spawning events were increased by 125% in the CCMWW mixture, compared to

225% in CCME treatment after 14 days exposure. Egg production was increased by 20% in the CCMWW treatment compared to 65% in the CCME after 14 days of exposure (Figures 6.2B, 6.3B).

When the two systems are compared quite different responses were observed. In reference, CCME and CCMWW treatments, significant increases in reproductive output occurred in the trophic-transfer system, whereas significant reductions were observed in the water-only system.

6.3.3.3 Hatching success and deformities

In the water-only system, hatching success and deformities could not be assessed due to the lack of offspring and hence a lack of data for both the CCME and CCMWW treatments (n=2 for both treatments). Despite the lack of data, an apparent reduction in hatching success (40%) was observed in the CCME treatment (Figure 6.4A). In the CCMWW treatment the reduction in hatching success was not observed.

In the trophic-transfer system, no significant difference was observed in hatching success in either CCME (ANOVA, p=0.997) or CCMWW (ANOVA, p=0.429) treatments compared to reference (Figure 6.4A). However, a significant increase in deformities (did occur in both CCME (ANOVA, p=0.006) and CCMWW (ANOVA, p=0.026) compared to reference (Figure 6.4B). The predominant deformity to occur in the CCME treatment was scoliosis in the abdominal and caudal region (40% of the time), followed by lordosis and yolk sac edema (25% and 16% of the time, respectively). In comparison, the predominant deformity to occur in the CCMWW treatment was yolk sac edema (46% of the time) followed by scoliosis and lordosis (24% and 16% of the time, respectively).

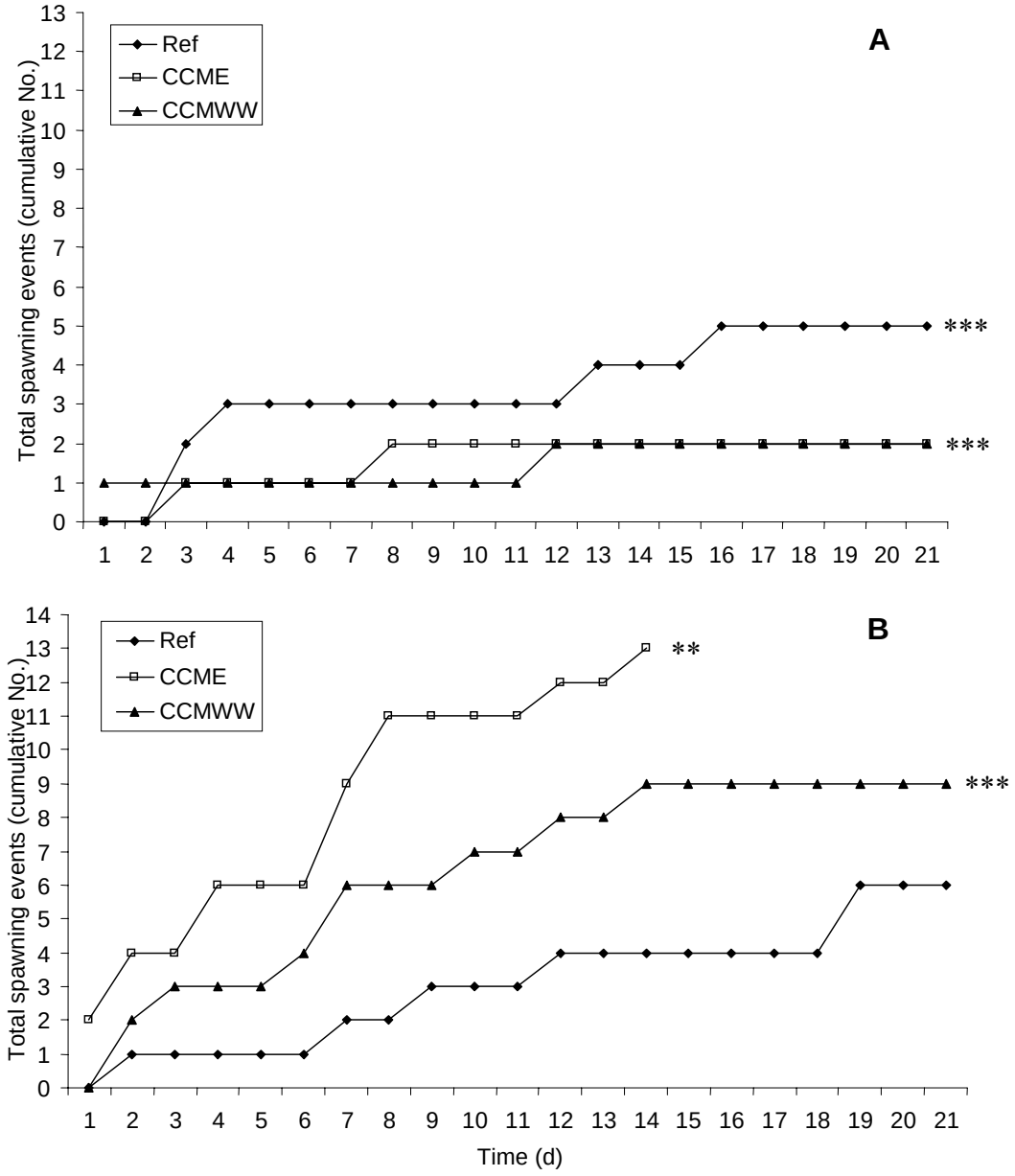

Figure 6.2. Cumulative number of spawning events of fathead minnow (*P. promelas*) breeding pairs during 21 d exposure to Copper Cliff Mine Effluent (CCME 45%), CCME and municipal waste-water combined (CCMWW) and reference water in a water-only (A) and trophic-transfer (B) system. Asterisk represents significant difference from reference treatment, where ** = $p<0.01$, *** = $p<0.001$

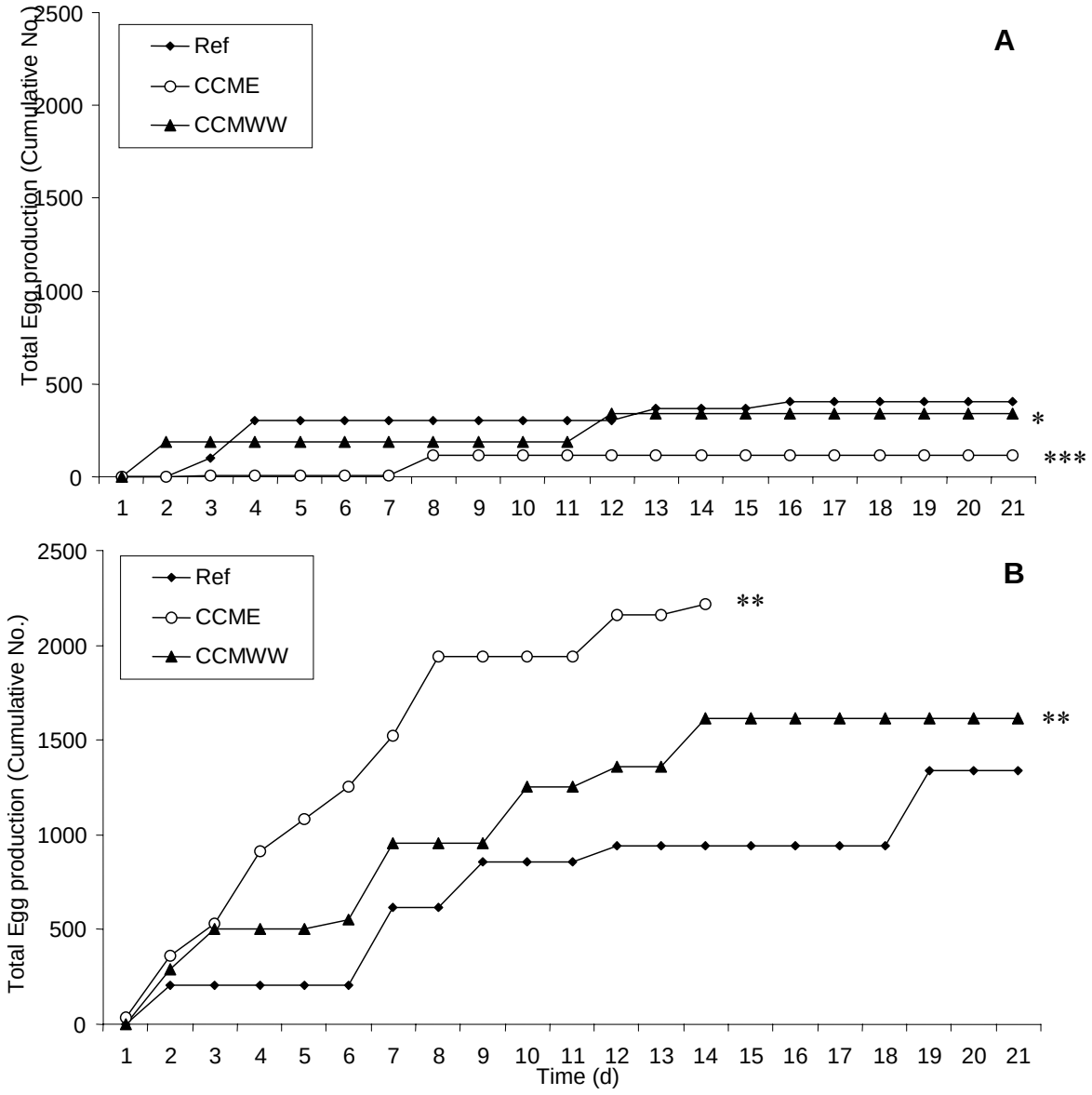

Figure 6.3. Cumulative egg production by fathead minnow (*P. promelas*) breeding pairs during 21 d exposure to Copper Cliff Mine Effluent (CCME 45%), CCME and municipal waste-water combined (CCMWW) and reference water in a water-only (A) and trophic-transfer (B) system. Asterisk represents significant difference from reference treatment, where * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Similar to the water-only treatments hatching success was higher in the CCMWW treatment compared to CCME (Figure 6.4A). It is difficult to compare the two systems (water-only and trophic-transfer) for the F1 endpoints as statistical analysis could not be conducted due to the lack of offspring in the water-only system. There were no differences in hatching success and deformities between the reference treatments in each system. However, the appearance of deformities did occur in both the CCME and CCMWW treatments in the trophic-transfer system.

6.3.4. Biochemical endpoints

In the water-only system, no significant differences in muscle testosterone (ANOVA, p=0.306), 11-KT (ANOVA, p=0.217) or vitellogenin (ANOVA, p=0.313) levels were observed in males after exposure to CCME or CCMWW compared to reference (Table 6.4). In females in the CCME treatment, no significant changes were observed in testosterone or estradiol (ANOVA, p=0.695, p=0.463), however vitellogenin levels were significantly increased (ANOVA, p=0.036). In the CCMWW treatment, estradiol was significantly increased compared to reference (two-way ANOVA, p=0.001) however no changes in vitellogenin (two-way ANOVA, p=0.290) or testosterone (ANOVA, p=0.722) were observed (Table 6.4).

For the trophic-transfer system, analysis of data from the CCME treatment could not be conducted for these endpoints because of the system failure. Therefore only analysis of the CCMWW treatment was conducted and compared to control trophic-transfer. In the trophic-transfer system no significant differences in muscle testosterone (two-way ANOVA, p=0.462), 11-KT (two-way ANOVA, p=0.769) or vitellogenin (two-way ANOVA, p=0.229) were observed in males, after exposure to CCMWW compared to reference treatments. In

females, estradiol was significantly increased compared to reference in the CCMWW treatment (two-way ANOVA, p=0.001). No significant changes in either vitellogenin (two-way ANOVA, p=0.290) or testosterone (ANOVA, p=0.722) were observed (Table 6.4).

When both systems are compared, testosterone, 11-KT and vitellogenin levels in reference males were 96%, 141% and 118% higher in the trophic-transfer system compared to the water-only system. A similar increase in testosterone and 11-KT in the CCMWW treatment in the trophic-transfer system was also observed where levels in males were 395 and 134% higher respectively than those in the water-only treatment. Female testosterone levels in contrast, were 69% and 31% lower in the reference and CCMWW treatments in the trophic-transfer system compared to the water-only system. This response is reflected in the significant system effect observed in the two-way ANOVA (two-way ANOVA, p=0.01) when assessing testosterone levels in females. Therefore, changes in testosterone were occurring between the two systems regardless of treatment.

6.3.5 Secondary sex characteristics

In the water-only system, development of secondary sex characteristics (male characteristics in females; ovipositor development in males) was not observed in males or females in either treatment (chi-square, p>0.05).

In the trophic-transfer system, development of ovipositors in males was observed in the CCMWW treatment (20% developed ovipositors) but not in the CCME, however, this was not significant (chi-square, p>0.05). Development of male characteristics was not observed in any of the females in either treatment (results not shown).

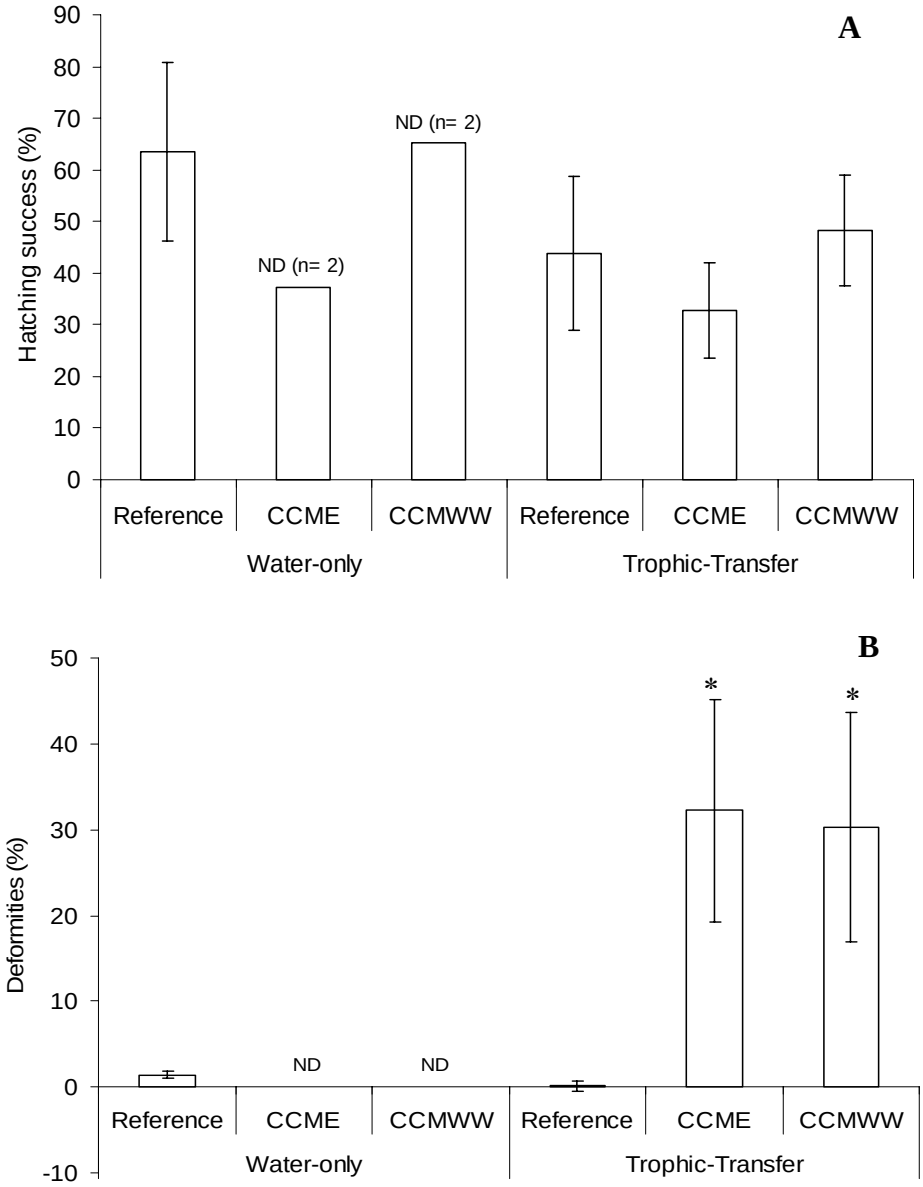

Figure 6.4. a) Hatching success (% of total viable eggs produced) and b) deformities (% of total hatched) of fathead minnow (*P. promelas*) after exposure to Copper Cliff Mine Effluent (CCME 45%), CCME and municipal waste-water combined (CCMWW), and water in a water-only and trophic-transfer system. ND = inadequate data for conducting statistical analysis.

Table 6.3 Summary of male and female biochemical endpoints measured in the August 2005 artificial stream study with Copper Cliff mine effluent (CCME) (45%) and CCME and municipal waste-water (CCMWW) (30%, 30%)) in a water-only and trophic-transfer exposure system. Values represent means ± standard error. Asterisk represents significant difference from reference when analysed with a two-way ANOVA where ** = $p<0.01$. ‡ denotes significant difference from water-only reference where ‡ = $p<0.05$.

Sex	Parameter	Unit	Water-only			Trophic-transfer		
			Ref	CCME	CCMWW	Ref	CCME	CCMWW
Males	Testosterone	pg/g	14.7 ± 7.15	25.6 ± 8.80	8.10 ± 2.72	28.7 ± 13.5	N/A	40.1 ± 10.9
	11-KT	ng/g	0.77 ± 0.30	1.98 ± 0.64	0.82 ± 0.18	1.86 ± 0.82		1.92 ± 0.68
	Vitellogenin	µg/g	0.49 ± 0.26	0.75 ± 0.35	0.45 ± 0.13	1.07 ± 0.97		0.09 ± 0.05
Females	Testosterone	pg/g	128 ± 45.8	94.1 ± 31.3	89.9 ± 19.4	38.6 ± 8.33		62.1 ± 11.9
	Estradiol	pg/g	191 ± 27.8	303 ± 81.3	345 ± 103**	154 ± 14.9		421 ± 49.8**
	Vitellogenin	µg/g	37.5 ± 18.6	194 ± 42.7‡	88.8 ± 30.6	108 ± 44.6		207 ± 67.3
	Egg size	mm	0.63 ± 0.12	0.60 ±0.04	0.64 ± 0.17	0.62 ± 0.02		0.85 ± 0.09
	Fecundity	No.	196 ± 65.6	91.8 ± 31.1	135 ± 24.4	65.8 ± 6.56		157 ± 80.6

reference When the two systems are compared it is only in the trophic-transfer system in the CCMWW treatment that we see an appearance of ovipositors in males. This is not observed in the water-only CCMWW treatment.

6.3.6 Gonadal histopathology

In the water-only system, no significant differences in percentage of spermatogonia (ANOVA, p=0.096), spermatocytes (ANOVA, p=0.941) or spermatids (ANOVA, p=0.079) in males occurred after exposure to either CCME or CCMWW. No significant differences in the occurrence of eosinophilia or fibrosis occurred in males in either of the treatments compared to reference males (chi-square, p>0.05). In females, no significant difference in percentage of oogonia, pre-vitellogenic, vitellogenic or mature follicles were observed in either treatment compared to reference females (ANOVA, p>0.05, chi-square, p>0.05). However, significant increases in eosinophilia in ovaries from both CCME and CCMWW treatments were observed (KWALLIS, p=0.046; two-way ANOVA, p=0.001 respectively). The appearance of eosinophilia was reduced in the CCMWW treatment (25% appearance) compared to CCME (46% appearance).

For the trophic-transfer system, analysis of data from the CCME treatment could not be conducted for these endpoints because of the system failure. Therefore, only analysis of the CCMWW treatment was conducted. No significant differences were observed in either spermatocytes (two-way ANOVA, p=0.857) or spermatids (two-way ANOVA, p=0.065) in males in the CCMWW treatment. However, a significant decrease was observed in spermatogonia after exposure to CCMWW compared to reference (two-way ANOVA, p=0.049). In females, no significant difference in percentage of oogonia, pre-vitellogenic,

vitellogenic or mature follicles were observed after exposure to CCMWW treatment (two-way ANOVA, $p>0.05$).

When the two systems are compared, gonadal staging and histopathology were comparable between the water-only and trophic-transfer systems in both reference and CCMWW treatments.

6.3.7 Metal muscle burdens

6.3.7.1 Females

In the water-only system, a significant increase in muscle selenium concentration occurred in females in both the CCME (223%) and CCMWW (64%) treatments. Muscle selenium levels in the CCMWW treatment were slightly less than those observed in the CCME compared to reference.

In the trophic-transfer system, increases in muscle selenium occurred in both the CCME (25%) and CCMWW (133%) treatments compared to reference (Table 6.4). Levels in the CCMWW treatment were higher than the CCME compared to reference, which was probably due to the reduced exposure time in the CCME (14 days) compared to the CCMWW (21 days). Muscle chromium concentration was also significantly increased in females from the CCME treatment (180%) compared to reference females.

When the two systems are compared, levels of selenium were slightly elevated in the CCMWW treatment in the trophic-transfer system, which had the highest muscle burden value of 2.80 mg/kg (Table 6.4). Selenium values were also slightly higher in the reference treatment in the trophic-transfer (1.2mg/kg) compared to water-only (0.8mg/kg) system. Chromium was also elevated in the CCME treatment in the trophic-transfer system where

concentrations were 136% higher compared to the CCME treatment in the water-only system.

6.3.7.2 Males

In the water-only system no significant increases in male muscle burdens of metals occurred in either CCME or CCMWW treatments compared to controls. Aluminum (96%), barium (42%) and strontium (47%) were all significantly decreased in CCME compared to reference males (two-way ANOVA, $p< 0.001$, $p=0.008$ and $p=0.029$, respectively). In the CCMWW treatment, barium, chromium, cobalt, manganese and molybdenum all significantly decreased compared to reference (two-way ANOVA, $p=0.008$, $p=0.005$, $p=0.01$, $p=0.006$ and $p=0.008$, respectively) (Table 6.5).

In the trophic-transfer system, muscle concentrations of boron, rubidium, thallium and selenium significantly increased (two-way ANOVA, $p<0.001$, $p=0.029$, $p<0.001$, $p<0.001$ respectively) and chromium, cobalt, manganese and molybdenum were all significantly decreased (two-way ANOVA, $p=0.005$, $p=0.01$, $p=0.006$, $p=0.008$, respectively) in the CCMWW treatment compared to reference males. Overall, the number of elevated metals was greater in the CCMWW than the CCME treatment, which was probably due to the reduced exposure time in the CCME (14 days) compared to the CCMWW (21 days) exposures (Table 6.5).

When we compare the two systems, it was only in the trophic-transfer system that we see significantly elevated muscle burdens in both the CCME and CCMWW. However, chromium, cobalt, manganese and molybdenum were all consistently decreased in both systems in the CCMWW treatments.

Table 6.4. Summary of female metal muscle burdens measured in the August 2005 artificial stream study with Copper Cliff mine effluent (CCME) (45%) and CCME and municipal waste-water (CCMWW) (30%, 30% respectively) in a water-only and trophic-transfer exposure system. Data for the CCME trophic-transfer system is based on 14 days of exposure. Values represent means (µg/g dry wt) ± standard error. Asterisk represents significant difference from reference when analysed with a two-way ANOVA where * = $p<0.05$. # denotes significant difference from trophic-transfer reference where # = $p<0.05$.

		Detection Limit	FEMALES					
			Water-only			Trophic-transfer		
Parameter	Unit	(µg/g)	Ref	CCME	CCMWW	Ref	CCME	CCMWW
Ag	µg/g	0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.00
Al	µg/g	1	17.3 ± 3.90	0.27 ± 0.04	0.30 ± 0.06	14.1 ± 8.95	0.38 ± 0.05	8.13 ± 2.80
As	µg/g	0.01	0.13 ± 0.03	0.05 ± 0.02	0.17 ± 0.03	0.37 ± 0.09	0.28 ± 0.11	0.88 ± 0.21
Ba	µg/g	0.01	7.66 ± 1.58	5.85 ± 1.00	7.00 ± 0.63	5.22 ± 1.77	7.50 ± 1.52	5.74 ± 0.97
Be	µg/g	0.1	0.09 ± 0.01	0.12 ± 0.02	0.13 ± 0.03	0.13 ± 0.03	0.18 ± 0.03	0.16 ± 0.04
B	µg/g	1	0.48 ± 0.03	0.47 ± 0.03	0.65 ± 0.18	0.67 ± 0.21	0.88 ± 0.24	4.20 ± 1.16
Cd	µg/g	0.01	0.09 ± 0.02	0.05 ± 0.01	0.04 ± 0.01	0.01 ± 0.03	0.08 ± 0.04	0.08 ± 0.03
Co	µg/g	0.01	3.91 ± 0.16	2.62 ± 0.52	2.69 ± 0.55	4.36 ± 1.22	5.56 ± 1.21	3.41 ± 0.74
Cr	µg/g	0.1	300 ± 28.2	221 ± 45.4	221 ± 53.0	186 ± 60.1	522 ± 129#	179 ± 38.4
Cu	µg/g	0.1	8.73 ± 0.42	7.97 ± 1.55	11.8 ± 1.72	9.02 ± 2.25	49.1± 23.0	12.2 ± 2.54
FE	µg/g	1	912 ± 101	766 ± 188	790 ± 207	1206 ± 236	2025 ± 595	1086 ± 284
Li	µg/g	0.1	0.25 ± 0.04	0.27 ± 0.04	0.30 ± 0.06	0.27 ± 0.06	0.38 ± 0.05	0.32 ± 0.05
Mn	µg/g	0.1	36.4 ± 1.60	26.1 ± 3.84	26.5 ± 5.04	35.2 ± 9.96	67.3 ± 16.8	30.3 ± 6.77
Mo	µg/g	0.01	25.1 ± 1.12	15.0 ± 2.08	18.3 ± 4.29	24.1 ± 6.65	60.4 ± 16.4	22.4 ± 5.19
Ni	µg/g	0.1	151 ± 10.6	140 ± 33.2	133 ± 27.7	173 ± 32.4	384 ± 113	170 ± 41.8
Pb	µg/g	0.1	0.08 ± 0.01	0.08 ± 0.02	0.08 ± 0.02	0.07 ± 0.02	0.98 ± 0.88	0.18 ± 0.08
Rb	µg/g	0.01	19.3 ± 1.55	17.4 ± 0.62	18.4 ± 2.22	26.0 ± 4.41	29.3 ± 3.88	27.0 ± 3.40
Se	µg/g	0.1	0.83 ± 0.18	2.67 ± 0.33*	2.33 ± 0.33*	1.20 ± 0.20	1.50 ± 0.62*	2.80 ± 0.86*
Sn	µg/g	0.1	44.8 ± 6.83	52.2 ± 9.85	62.5 ± 7.24	42.5 ± 13.6	72.0 ± 19.0	55.1 ± 8.62
Tl	µg/g	0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.07 ± 0.00
Zn	µg/g	0.1	113 ± 30.6	154 ± 44.1	157 ± 36.0	151 ± 50.3	157 ± 37.7	156 ± 29.3

Table 6.5. Summary of male metal muscle burdens measured in the August 2005 artificial stream study with Copper Cliff mine effluent (CCME) (45%) and CCME and municipal waste-water (CCMWW) (30%, 30%)) in a water-only and trophic-transfer exposure system. Data for the CCME trophic-transfer system is based on 14 days of exposure. Values represent means (µg/g dry wt) ± standard error. Asterisk represents significant difference from reference when analysed with a two-way ANOVA where *= p<0.05 and **=p<0.01. If the analysis was split due to a significant interaction then ‡ indicates significant difference from water-only reference, where ‡=p<0.05, ‡‡=p<0.01, ‡‡‡=p<0.001. Pound denotes significant difference from trophic-transfer reference where $^{\#}$=p<0.05, $^{\#\#}$=p<0.01, $^{\#\#\#}$=p<0.001.

		Detection Limit	MALES					
			Water-only			Trophic-transfer		
Parameter	Unit	(µg/g)	Ref	CCME	CCMWW	Ref	CCME	CCMWW
Ag	µg/g	0.01	0.04 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Al	µg/g	1	183 ± 118	5.99 ± 1.71‡‡‡	0.13 ± 0.01‡‡‡	18.6 ± 9.48	9.32 ± 7.46	5.50 ± 1.02$^{\#\#\#}$
As	µg/g	0.01	0.95 ± 0.40	0.71 ± 0.13	0.82 ± 0.21	1.05 ± 0.24	0.70 ± 0.17	1.33 ± 0.18
Ba	µg/g	0.01	6.39 ± 0.45	3.60 ± 0.66‡‡	3.62 ± 0.32‡‡	3.04 ± 0.55	4.24 ± 0.79$^{\#\#}$	3.68 ± 0.53$^{\#\#}$
Be	µg/g	0.1	0.11 ± 0.05	0.05 ± 0.00	0.05 ± 0.00	0.06 ± 10	0.05 ± 0	0.06 ± 10
B	µg/g	1	0.63 ± 0.29	0.34 ± 0.02	0.31 ± 0.02	0.43 ± 0.15	0.28 ± 0.02	3.40 ± 0.40$^{\#\#\#}$
Cd	µg/g	0.01	0.06 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.03 ± 0.01
Co	µg/g	0.01	2.98 ± 0.67	1.89 ± 0.14	1.59 ± 0.28*	2.79 ± 0.51	1.81 ± 0.33	1.72 ± 0.29*
Cr	µg/g	0.1	175 ± 29.2	134 ± 13.7	95.1 ± 4.24**	126 ± 16.9	145 ± 39.4	64.9 ± 11.5**
Cu	µg/g	0.1	12.1 ± 3.55	7.30 ± 0.78	5.84 ± 0.31	11.5 ± 4.69	8.28 ± 1.54	8.52 ± 0.91
Fe	µg/g	1	594 ± 69.7	498 ± 55.3	364 ± 13.6	728 ± 203	522 ± 120	450 ± 78.6
Li	µg/g	0.1	0.24 ± 0.09	0.15 ± 0.00	0.12 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.15 ± 0.03
Mn	µg/g	0.1	22.8 ± 2.61	16.7 ± 1.14	13.2 ± 0.28**	20.7 ± 2.41	18.9 ± 4.69	14.4 ± 2.62**
Mo	µg/g	0.01	14.8 ± 2.21	10.6 ± 0.83	8.36 ± 0.63**	13.3 ± 1.93	14.2 ± 5.30	8.86 ± 1.51**
Ni	µg/g	0.1	93.5 ± 12.2	83.4 ± 6.30	59.4 ± 3.33	91.7 ± 14.4	93.7 ± 23.3	70.7 ± 11.2
Pb	µg/g	0.1	0.19 ± 0.11	0.05 ± 0.01	0.09 ± 0.04	0.21 ± 0.10	0.09 ± 0.03	0.10 ± 0.03
Rb	µg/g	0.01	17.6 ± 2.90	16.7 ± 1.23	15.4 ± 0.56	22.3 ± 1.08	17.6 ± 1.54	27.6 ± 1.07$^{\#}$
Se	µg/g	0.1	0.88 ± 0.13	1.80 ± 0.20‡	1.60 ± 0.24	1.10 ± 0.23	1.40 ± 0.24	3.20 ± 0.28$^{\#\#\#}$
Sn	µg/g	0.1	82.4 ± 18.2	38.7 ± 8.30‡	52.1 ± 5.50	30.3 ± 3.48	31.8 ± 2.67	44.9 ± 7.26
Tl	µg/g	0.01	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.01$^{\#\#\#}$
Zn	µg/g	0.1	191 ± 17.3	131 ± 24.2	152.0 ± 18.6	105 ± 16.9	106 ± 17.4	174 ± 25.9

6.3.7.3 Ovarian selenium analysis

In the water-only system, ovarian selenium concentrations were significantly increased in the CCME and CCMWW treatments (ANOVA, p=0.046, two-way ANOVA, p=0.006); increases of 195% and 115% were observed, respectively, compared to reference values (Figure 6.5). Levels of selenium in ovary in the CCMWW treatment were lower than those observed in the CCME.

For the trophic-transfer system, analysis of data from the CCME treatment could not be conducted for these endpoints because of the system failure. Therefore, only analysis of the CCMWW treatment was conducted. Selenium was also significantly increased in female ovaries in the CCMWW treatments (two-way ANOVA, p=0.006) where an approximately 100% increase was observed compared to reference values (Figure 6.5).

When the two systems are compared values of selenium in the ovarian tissue were similar in both the reference and CCMWW treatments. CCME treatments could not be compared due to the system failure in the CCME trophic-transfer treatment.

6.3.8 *C. tentans* metal body burdens

In the trophic-transfer system, significant increases in copper (350%), lead (55%) and selenium (236%) were observed in *C. tentans* body burdens in the CCME treatment compared to reference (ANOVA, p=0.003, p=0.027, p=0.013, respectively) (Table 6.6). In the CCMWW treatment significant increases in lead (73%) and nickel (75%) were observed compared to reference (ANOVA, p=0.007, 0.039, respectively). Lead levels in *C. tentans* were substantially higher in the CCMWW (74%) compared to the CCME (56%).

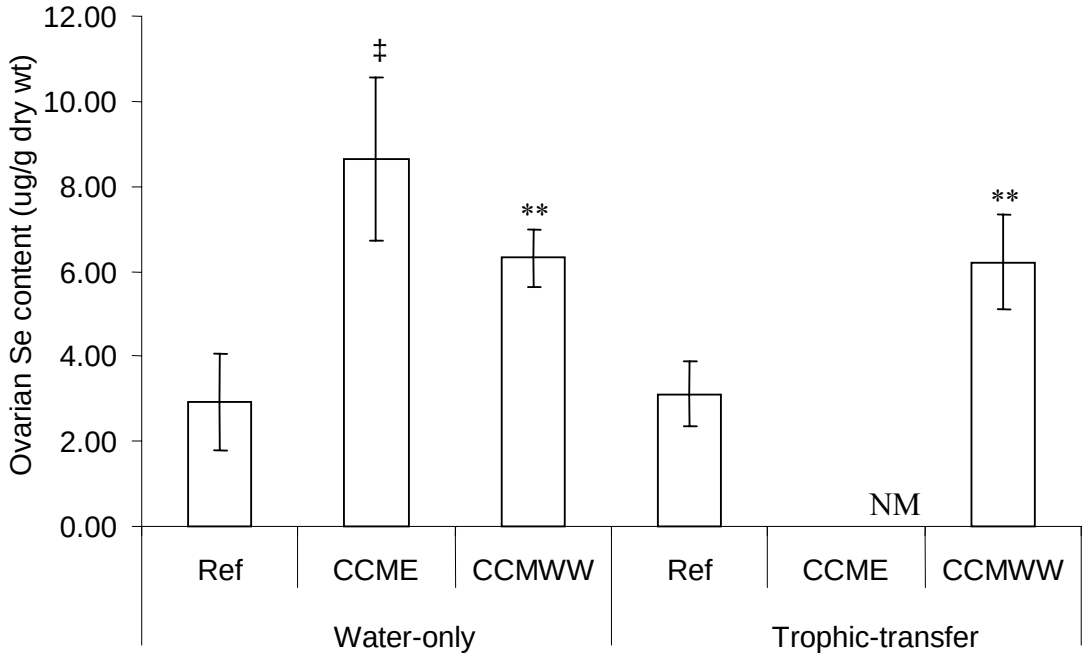

Figure 6.5. Selenium concentrations in female FHM ovaries after 21 days exposure to Copper Cliff mine effluent (CCME) (45%) and CCME and municipal waste-water (CCMWW) (30%, 30%)) in a water-only and trophic-transfer exposure system. Values represent means ± standard error. Asterisk represents significant difference from reference when analysed with a two-way ANOVA where ** =$p<0.01$. ‡ indicates significant difference from water-only reference when analysed with a one-way ANOVA, where ‡ = $p<0.05$. NM = not measured.

6.3.9 *C.tentans* densities and emergence

In the trophic-transfer system, no significant differences were observed in emergence (RM ANOVA, p=0.482) or densities (RM ANOVA, p=0.358) after 14 days exposure to CCME compared to reference (Figure 6.6 and 6.7, respectively). A significant treatment and time effect was observed with emergence (RM ANOVA, p=0.044, p<0.001) and densities (RM ANOVA, p=0.004, p=0.031) in the CCMWW treatment throughout the 21 days of exposure compared to reference (Figure 6.6 and 6.7 respectively). The ability to detect differences in the CCME treatment may have been affected by the reduced exposure time (14 days) compared to the CCMWW treatment (21 days).

6.3.10 Temperature fluctuations

In both the water-only and trophic-transfer systems temperature fluctuations were not significantly different between treatments (RM ANOVA, p=0.593) and no source effect (RM ANOVA, p=0.143) or interaction (RM ANOVA, p=0.448) was observed. A significant time effect (RM ANOVA, p=0.004) illustrated that diurnal fluctuations were occurring but these were uniform in all treatments.

Table 6.6 Summary of *C. tentans* metal body burdens measured in the August 2005 artificial stream study with Copper Cliff mine effluent (CCME) (45%) and CCME and municipal waste-water (CCMWW) (30%, 30%)) in a trophic-transfer exposure system. Data for the CCME trophic-transfer system is based on 14 days of exposure. Values represent means (µg/g dry wt) ± standard error. Pound denotes significant difference from trophic-transfer reference where # = $p<0.05$, ## = $p<0.01$.

Parameter	Unit	Detection Limit (µg/g)	Trophic-transfer		
			Ref	CCME	CCMWW
Al	µg/g	0.01	37600 ± 4590	34600 ± 6188	43566 ± 2857
As	µg/g	1	1.29 ± 0.10	1.30 ± 0.06	1.81 ± 0.29
Ba	µg/g	0.01	7.50 ± 0.75	6.52 ± 0.50	8.23 ± 2.59
Be	µg/g	0.01	0.23 ± 0.07	0.18 ± 0.07	0.37 ± 0.03
B	µg/g	0.1	7.30 ± 1.12	8.73 ± 0.72	13.3 ± 2.03
Cd	µg/g	1	0.52 ± 0.06	0.71 ± 0.12	0.67 ± 0.09
Ca	µg/g	10	3566 ± 121	5173 ± 301##	2630 ± 283
Cr	µg/g	0.01	1053 ± 128	790 ± 46.4	1876.67 ± 468
Co	µg/g	0.01	19.3 ± 1.88	14.9 ± 1.01	25.5 ± 4.67
Cu	µg/g	0.1	43.0 ± 5.50	197 ± 29.1##	107 ± 12.2
Fe	µg/g	0.1	6686 ± 716	4633 ± 202	8980 ± 1326
Hg	µg/g	0.01	0.29 ± 0.10	0.01 ± 0.05	0.07 ± 0.06
Li	µg/g	1	15.0 ± 2.71	15.2 ± 0.79	19.7 ± 2.43
Mg	µg/g	1	787 ± 7.31	1134 ± 182	579 ± 98.1
Mn	µg/g	0.1	221 ± 15.8	171 ± 6.24	294 ± 55.6
Ni	µg/g	0.01	999 ± 178	668 ± 11.6	1446 ± 274#
Pb	µg/g	0.1	1.73 ± 0.12	2.70 ± 0.15#	3.01 ± 0.27##
Rb	µg/g	0.1	24.4 ± 2.63	19.9 ± 1.56	26.3 ± 0.49
Se	µg/g	0.01	0.37 ± 0.03	1.23 ± 0.39#	0.67 ± 0.03
Sr	µg/g	0.1	19.5 ± 1.72	21.2 ± 3.61	21.3 ± 5.10
U	µg/g	0.001	0.24 ± 0.04	0.39 ± 0.11	0.18 ± 0.02

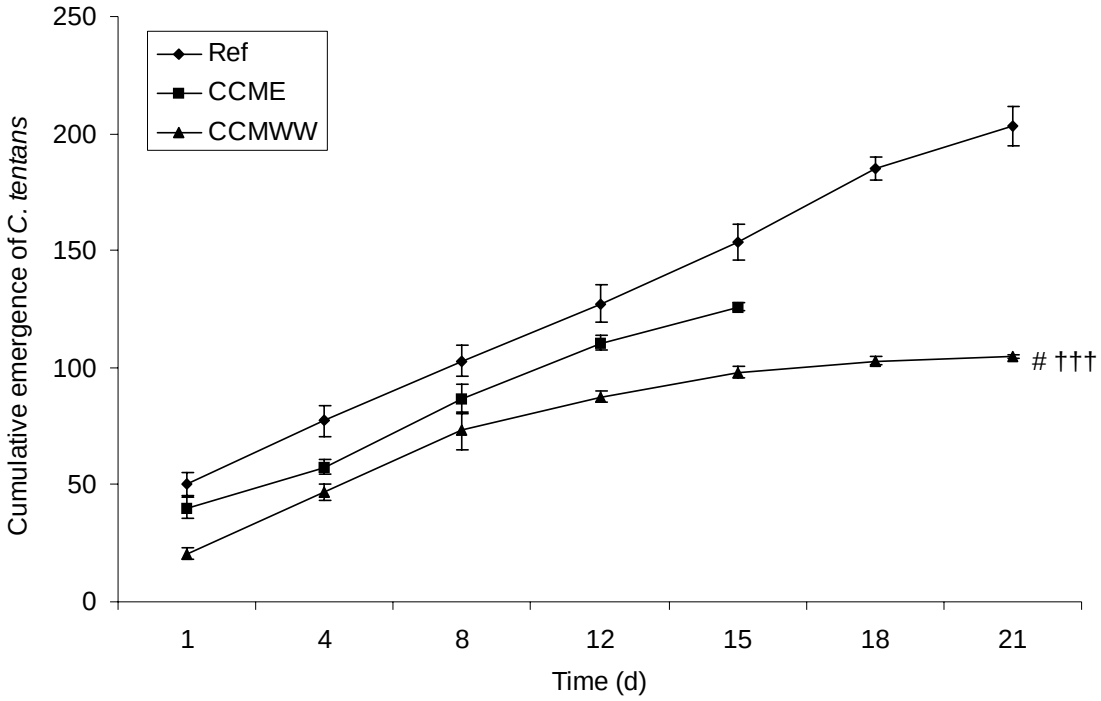

Figure 6.6 *C. tentans* emergence measured throughout a 21 d exposure to reference water, Copper Cliff mine effluent (CCME) (45%) and CCME and municipal waste-water mixture (CCMWW) (30%, 30%)) in a trophic-transfer exposure system. Pound denotes significant treatment effect, where # = $p<0.05$. Cross denotes significant time effect, where ††† = $p<0.001$.

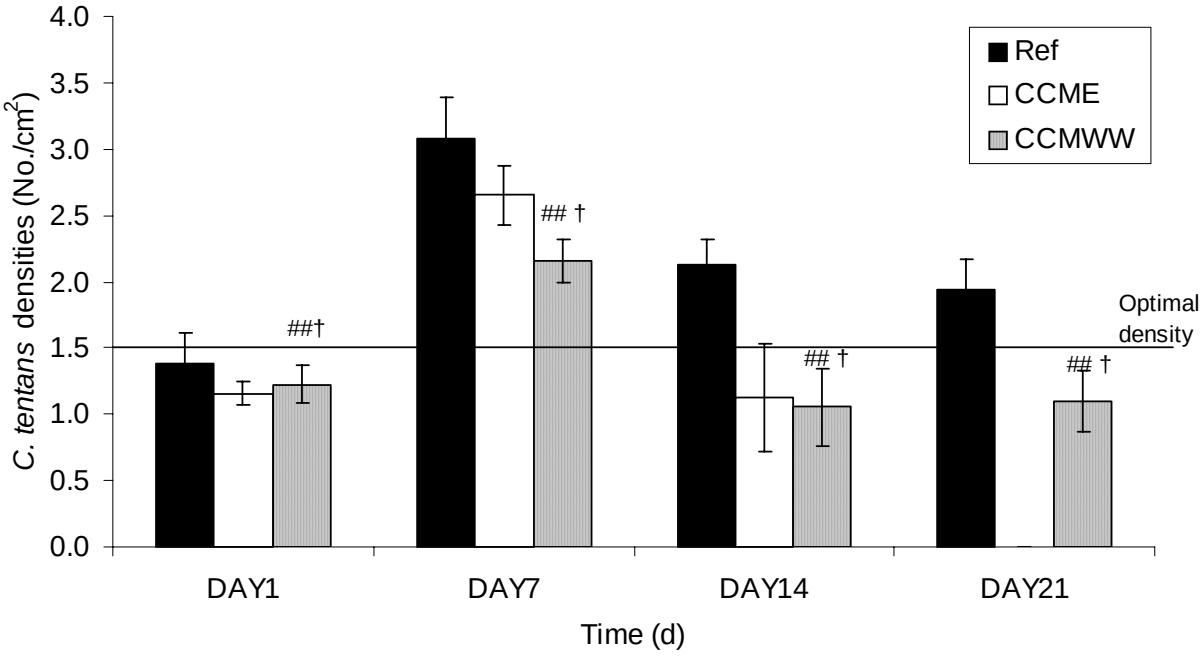

Figure 6.7 *C. tentans* densities (no/cm^2) measured throughout a 21 d exposure to reference water, Copper Cliff mine effluent (CCME) (45%) and CCME and municipal waste-water mixture (CCMWW) (30%, 30%)) in a trophic-transfer exposure system. Pound denotes significant treatment effect, where ## = $p<0.001$. Cross denotes significant time effect, where † = $p<0.05$

6.4 Discussion

The objectives of this study were to firstly assess the effects of CCME and CCMWW on FHM and secondly, to evaluate the contribution of trophic-transfer as a source of exposure to both treatments. Using the trophic-transfer system on-site allowed us to increase environmental relevance of a standard lab bioassay and compare responses to water-borne exposures. Overall, exposure to both CCME and CCMWW in the water-only system reduced reproductive output (i.e., egg production and spawning events) of adult FHM, induced vitellogenin in females, increased eosinophilia in female ovarian tissue and resulted in increased selenium in female muscle and ovarian tissues. Hatching success and deformities in the F1 generation could not be assessed in the water-only treatments due to very low egg production by breeding pairs. In general, when CCME was mixed with MWW the effects on a number of endpoints were lessened. This may correspond to the reduced concentration of CCME in the mixed treatment (45% in the CCME versus 30% in the CCMWW).

In the trophic-transfer system, exposure to CCME and the CCMWW mixture resulted in a differing response in reproductive output compared to the water-only system. Our ability to compare the CCME treatment was affected by the loss of this treatment one week short of completion of the experiments. Thus comparisons for the CCME trophic-transfer treatment are based on two weeks of exposure and were only conducted for egg production, spawning events, muscle metal burdens, hatching success and deformities. Significant increases in egg production and the number of spawning events were observed in both effluent treatments with greater increases observed in the CCME treatment compared to the CCMWW treatment. Selenium increased in muscle tissues for both males and females exposed to the CCMWW mixture and for females exposed to the CCME. Barium, boron, rubidium and thallium increased in male muscle after exposure to effluent (particularly CCMWW). F1 hatching success in the trophic-transfer

system was not affected by exposure to either effluent although there were greater deformities observed.

Reproductive hormone analyses, gonadal histology and selenium analyses in female ovaries were not conducted on fish from the CCME trophic-transfer treatment because we believed that these endpoints may have been affected by the premature death of fish in this system with the system failure. Thus, comparisons for these endpoints in the trophic-transfer system were conducted only for the CCMWW relative to reference. After exposure to the CCMWW treatment, muscle estradiol concentrations and selenium concentrations in ovaries increased in females and the number of spermatogonia decreased in males.

6.4.1 Effects of CCME and CCMWW

The most consistent response from exposure to both CCME and CCMWW in the water-only system was reduced reproductive output. The effects on reproductive output in the water-only treatments (CCME and CCMWW) were consistent with our laboratory investigation (Chapter 5; Rickwood et al, 2006d). No such disruptions were observed in either treatment in the trophic-transfer system. A number of possible reasons for the reproductive disruption in the water-only system are discussed here.

Total ammonia was elevated in both treatments which may have affected reproductive performance. The chronic toxicity of ammonia on fathead minnow has been assessed by Thurston et al (1986). They observed effects on growth, survival and egg production at concentrations of 0.91 mg/L (un-ionized ammonia) and estimated a chronic-effects threshold concentration of 0.27 mg/L. Total ammonia (NH_3 + $NH4^+$) concentrations in the water-only system were 0.0025 mg/L in reference treatment and 2.24 mg/L in the CCME treatment. When unionized ammonia (NH_3) is calculated with regards to pH and temperature, the concentrations

range from 0.3mg/L to 0.1mg/L in CCME and CCMWW. Clearly, the concentrations in all treatments were lower than the chronic-effect threshold documented by Thurston et al (1986) indicating that unionized ammonia (NH_3) may not have been the cause of reduced reproductive output for FHMs exposed to effluents in the water-only system.

Selenium was identified as one of the potential contaminants of concern in the laboratory investigation (Chapter 5) due to the increase in female muscle burdens, reduced egg production and increased appearance of deformities in the offspring. The effects of selenium on fish reproduction have been documented and reviewed extensively (Lemly, 2002; Hamilton et al, 2002; Hamilton, 2004). Effects of selenium in fish include ovarian cell necrosis and rupturing of mature egg follicles in reproducing adults, (Sorensen et al, 1984) and, most notably larval deformities including spinal curvature (kyphosis, lordosis, scoliosis) and edema (Lemly, 2002). Selenium was the only compound to be significantly increased in female muscle burdens in the CCME and CCMWW treatments in the water-only system. Selenium values in the ovaries of females were also significantly increased in the CCME and CCMWW treatments compared to reference. The levels of selenium in female ovaries ranged between 6µg/g for the CCMWW treatments and 8µg/g in the CCME water-only treatment. These concentrations are below the effect-threshold level of 10µg/g in the ovaries of fathead minnow that has been shown to result in elevated frequencies of larval deformities (Lemly, 1996 cited in Hamilton, 2004). Selenium concentrations in female ovaries exposed to the CCMWW treatment ranged from 5-8µg/g in the water-only system which reflects the reduced concentration of CCME in this mixture and possibly the reduced toxicity, i.e., greater egg production and spawning events compared to the CCME treatment. The significant increase of selenium in the CCME treatment water suggests

that this effluent could be a significant source of selenium which could account for the decrease in reproductive output observed.

When we compare the responses between the CCME and CCMWW treatments in the water-only system, we observe a consistent amelioration of responses measured in the latter treatment. Although egg production, spawning events, hatching success and selenium concentrations in both muscle burdens (male and female) and ovarian tissue were all significantly different from reference fish, effects were lessened after exposure to CCMWW compared to CCME in the water-only treatments. This lessened response can also be observed in the lack of induction of vitellogenin, an estrogen-responsive protein, in females in the CCMWW treatment. The significant induction of vitellogenin in females in the CCME is consistent with our previous lab study where we observed a 780% induction in female vitellogenin (Chapter 5). It was thought that this induction could have been due to the presence of estrogenic compounds within the CCME, as the effluent contains approximately 1-3% of domestic sewage from the town of Copper Cliff. MWW has been documented in the past to induce vitellogenin in fish and is commonly used as a marker for exposure to MWW (Purdom et al, 1994; Harries et al, 1997, 1999). Interestingly, we also observed vitellogenin induction in male FHM collected downstream of the CCME and MWW discharges in our Junction Creek study (Weber et al, 2006a,b). It was unknown at that time whether the compounds causing vitellogenin induction were from the CCME or MWW due to confounded nature of the creek. However, the lack of induction in the CCMWW treatments in our study suggests that either the MWW does not contain estrogenic compounds or, when mixed with CCME, the potency of the compounds causing the induction of vitellogenin is reduced. The latter suggestion corresponds with the improvement of multiple endpoints observed after exposure to CCMWW in this study. However, further investigations

into the toxicity of MWW, such as studies examining the MWW in isolation, are required before any firm conclusions can be made.

In contrast, a significant increase in production of estradiol was only observed in females from the CCMWW treatments. An increase in estradiol was observed in the CCME treatment but this was not significant. This would suggest that the compounds causing the induction of estradiol were possibly present in both CCME and MWW and that when combined the effect was additive. Again, further investigations into the MWW in isolation need to be investigated before any conclusions can be made. Overall, the patterns of improved responses in the CCMWW treatment compared to the CCME suggest that CCME is the driving force behind the responses observed and that the improved responses in the CCMWW could merely be a reflection of the reduced concentration of CCME in this treatment.

The pattern of effects observed in the water-only system correspond with observations from our previous laboratory investigation (Chapter 5) and with a Junction Creek field study conducted by our lab in 2004 (Weber et al, 2006a,b). In our laboratory study, we observed significant decreases in reproductive output, increased appearance of cell death in male gonadal tissue and elevated levels of selenium and increased vitellogenin in female tissues (Chapter 5). In the Junction Creek study conducted in 2004, FHM were sampled downstream of the municipal waste-water and CCME discharge. Similar to the lab study, males were found to have significantly increased occurrence of eosinophilia and cell death in gonadal tissue. Females were observed to have decreased egg sizes and increased fecundity and elevated selenium muscle burdens were observed in both male and female FHM (Weber et al, 2006a,b). The appearance of pathology in gonadal tissue (cell death, eosinophilia), disruptions in reproductive endpoints (decreased egg production and size) and elevated selenium values in males and females have

been consistently observed in this study (water-only exposures), our previous lab study (Chapter 5) and the field investigation conducted at Junction Creek in 2004 (Weber et al, 2006a,b).

The lack of effects on individual endpoints (i.e., condition, liver size, gonad size) also corresponds with our previous investigations (Weber et al, 2006a,b, Chapter 5). The only individual endpoint that was significantly different from reference in FHM sampled from Junction Creek was increased liver weights in males downstream of the CCME and municipal sewage discharge (Weber et al, 2006a,b). No difference was observed in condition or gonad weights in either males or females which is consistent with our investigation. Therefore, these endpoints may not be the most relevant or sensitive for assessing CCME, CCMWW, or effluents similar to these.

6.4.2 Contribution of trophic-transfer

The decrease in reproductive output observed for FHM exposed to effluents in the water-only system was not observed in the trophic-transfer system. This result is not consistent with our laboratory study (Chapter 5) where we saw significant decreases in reproductive output and severe effects on hatching success and deformities in the trophic-transfer system exposed to the effluents. A few possible reasons for these differences exist and are briefly discussed here.

Increased food availability may have accounted for the greater egg production in the trophic-transfer system compared to the water-only system. Egg production in the self-sustaining reference trophic-transfer treatment was greater than that in the reference water-only treatment where fish were fed a controlled amount of 1g of *C.tentans* per day. A similar response was observed in our laboratory study, which we concluded might have been due to increased food availability, i.e., access to higher densities of *C. tentans* in the multi-trophic streams compared to the optimal daily amount fed to the fish in the water-only streams (Chapter 5). To account for

this in the current study, we attempted to better control densities of *C. tentans* by reducing the number of egg sacs and 7 day old larvae added. We also attempted to better control FHM access to food by turning the feeding barrier from once per day to once every two days. On the first day, *C. tentans* densities were consistent with the optimal daily feeding amount, however, after 7 days densities doubled. This could be due to the emergence of adult *C. tentans* and in-stream reproduction resulting in deposition of new egg sacs into the system. Therefore, the increase in FHM egg production in the trophic-transfer systems could be due to the increased amount of food available to them in the second week compared to fish in the water-only treatment systems. A suggestion for future studies would be to remove emerged flies in the streams to limit the input of additional egg sacs by breeding invertebrates in the contained system, thereby controlling densities. This being said, in our laboratory studies despite a difference in food availability between system types, we measured significant effects of CCME on adults and F1 in the trophic-transfer streams, results not observed in this on-site study.

A change in water quality may also have been responsible for the reduced responses to effluent for FHM in the trophic-transfer treatments. The only differences in water quality parameters between the water-only and trophic-transfer treatments were DOC, TOC, TSS and total phosphorus where >2-fold increases were observed in both CCME and CCMWW trophic-transfer treatments. Bioavailability of metals, predominantly cationic metals (Cu^{2+}, Ni^{2+} and Ag^{2+}) has been shown to decrease in the presence of increasing amounts of organic material, e.g., dissolved and/or total organic carbon (DOC/TOC) which binds waterborne metals and decreases their availability for uptake by fish (Playle et al, 1993; Pyle et al, 2005). It is possible that the increase in organic material in the trophic-transfer system may have reduced the toxicity of the

effluent in these treatments which could have led to the increased reproductive output and in general, fewer changes measured in assessed endpoints.

When metals in the water, *C. tentans* tissue, and female and male FHM muscle tissue are compared, the only metal analyzed that consistently increased in these media after exposure to the effluents was selenium. In the water-only system, selenium levels were elevated in the water by 1113% and 444% in the CCME and CCMWW treatments, respectively, compared to reference. Selenium was also increased by 63% and 190% in males and 122% and 95% in females in the CCME and CCMWW treatments, respectively. Levels of selenium in ovarian tissue also increased significantly by 195% and 115% in the CCME and CCMWW treatments, respectively. Similar increases in the trophic-transfer system were observed. Selenium values were elevated in the water by 675% and 644% in the CCME and CCMWW treatments respectively. Increases in selenium muscle burdens by 27% and 45% in males and 25% and 133 % in females in the CCME and CCMWW treatments also occurred compared to reference. Levels of selenium in ovarian tissue also increased significantly by 100% in the CCWWW treatment (CCME was not assessed for this endpoint due to system failure). Additionally in the trophic-transfer system, selenium increased in *C. tentans* body burdens by 232% and 81% in the CCME and CCMWW treatments respectively compared to reference. The increases in selenium in the water, *C. tentans,* male and female muscle burdens and ovarian tissue are consistent between the water-only and trophic-transfer systems where we observed increases and accumulation among responses. Selenium was highlighted as a possible contaminant of concern causing the effects on reproduction in the water-only system. However, no effects on reproduction were observed in the trophic-transfer system, despite having similar increases in selenium in the water, muscle-burdens and ovaries. The decreased toxicity observed in the

trophic-transfer system could possibly be due to reduced bioavailability. Wang and Dei (2001) found that selenite (Se (IV)) accumulation in diatoms and green alga was inversely dependent on the ambient phosphate and silicate concentrations. When 7.2 μM of phosphate was added to treatment water, concentration factors for selenite decreased by 2.4-8.1 times after 5 h of exposure. Similar decreases in bioconcentration factors were also observed with the addition of 105 μM silicate, which reduced the concentration of selenite by 1.5-4.6 times. Total phosphorus measured in our study ranged from 0.4mg/L in the water-only treatments to 0.8mg/L in the trophic-transfer treatments. Comparisons with the Wang and Dei (2001) study are difficult as it is unknown what proportion of phosphate was in the total phosphorus measured and, in addition, silicate was not one of the parameters measured in our study. However, it is possible that increases in total phosphorus, or organic carbon in the trophic-transfer exposures reduced bioavailability of selenium.

The reduced toxicity in the trophic-transfer system could be due to complexation of contaminants with other compounds within the effluent. For example, Berntssen et al (1999, 2000) have shown that elevated dietary copper reduced the concentrations of selenium in the liver of Atlantic Salmon (*Salmo salar*). This is possibly due to the formation of insoluble copper-selenium complexes in the intestinal lumen, which ultimately reduces selenium bioavailability. Therefore, if the FHM in the trophic-transfer system were exposed to higher dietary copper concentrations than those in the water-only treatments, this may have reduced the bioavailability of selenium in this system. Significantly increased copper concentrations were observed in *C. tentans* in the CCME treatment and were elevated in the CCMWW treatment compared to reference in the trophic-transfer system. This would indicate that FHM in the exposure treatments in the trophic-transfer system were being exposed to higher dietary concentrations of

copper compared to FHM in the reference treatment, and, in all three treatments in the water-only system which were being fed "clean" food. Therefore, the formation of copper-selenium complexes could have reduced the toxicity of the effluent in the trophic-transfer system. However, the lack of any significant increases in copper in either male or female muscle tissue, compared to reference fish, suggests that any additional exposure via the food was not resulting in higher muscle tissue concentrations of this element. It is important to note here, that since we only measured metals in muscle tissue, it is possible that the concentration of metals, e.g. copper, would have been higher had we measured metals in the gill, plasma or whole-body. A suggestion for future studies would be to analyse the distribution of metals throughout the body for a complete analysis of metal uptake and distribution.

Bacterial degradation may also be a contributing factor in reducing the toxicity of effluents, specifically organic compounds, in the trophic-transfer system. Previous investigations have noted that enzymes produced by native bacterial populations can biodegrade or biotransform contaminants in ecosystems (Alexander, 1999; Rittmann and McCarty, 2001). It is possible that the primary source of bacteria was from the Vermillion river (i.e. reference water). In addition, the presence of sediment in the trophic-transfer system may have been required to support a population of microorganisms that could potentially produce these degrading enzymes. Therefore, the combination of reference water and the trophic-transfer system was required to reduce the toxicity of the effluents. No measurements of bacterial communities were taken during this investigation and, as such, we cannot conclude that biodegradation was occurring. However, this is certainly an area worthy of further investigation.

It is beyond the scope of this investigation to make any further conclusions regarding the speciation, complexation or biological degradation of compounds within the effluent. We are

only able to conclude that in the trophic-transfer system the toxicity of the effluent was reduced compared to both the water-only and laboratory investigations (Chapter 5). The obvious explanation is that different dilution water quality between the lab studies (City of Saskatoon treated municipal water) versus these field studies (Vermillion River water) resulted in the differing responses. However, this does not explain the difference fully as the water-only treatments in the present study were diluted with the same reference water as those in the trophic-transfer system. If the reference water that was higher in organic content and nutrients was the sole basis for reduced effluent toxicity, then the CCME water-borne exposures should have had similar results as the CCME trophic-transfer; namely limited effects on FHM reproduction and F1 hatching success. Thus, it appears that the combination of reference water with the productivity of the trophic-transfer foodweb resulted in fewer effects of the effluents on FHM. This also suggests that effects observed after exposure to CCME through the water-only and/or under controlled laboratory conditions may be more severe than those occurring in natural systems such Junction Creek. This highlights the importance of environmental relevance when assessing the toxicity of CCME and CCMWW effluents and the difficulties of using toxicity tests in the lab or with water-borne exposure routes in an effort to extrapolate potential effects on organisms in the field.

The significant decrease in *C. tentans* densities in the CCMWW treatment in the last two weeks and the significant decrease in emergence suggests that CCMWW affected the development of *C.tentans* larvae. The lack of any significant response in emergence or densities in the CCME treatment is likely due to the reduced exposure time (14 days). When we compare the toxicity of CCME to *C. tentans* in our study with previous exposures conducted in an identical system with *C.tentans* (Hruska and Dubé, 2004) we observe similar responses but to a

lesser extent. Hruska and Dubé, (2004) observed an approximately 80% decrease in emergence in *C.tentans* after 21 days exposure to CCME. We observed a 60% decrease in emergence after exposure to CCMWW after 21 days and only 20% decrease in emergence after exposure to CCME at day 14. When we compare the water quality between the two studies, there were higher concentrations of copper (83 µg/L) and selenium (64 µg/L) in the study conducted by Hruska and Dubé (2004). In the present study we report 58µg/L and 6.7µg/L in copper and selenium concentrations, respectively. The concentrations of selenium, in particular, in the 2002 study were an order of magnitude higher than that observed in our field investigation and previous lab investigations with this effluent (Hruska and Dubé, 2005; Chapter 5) where a consistent concentration of approximately 7µg/L was measured. In addition, the concentrations of TOC, DOC and total phosphorus were 28%, 31% and 38%, higher respectively, in our CCME trophic-transfer treatment compared to concentrations reported in the 2002 field exposure with this effluent (Hruska and Dubé, 2004). We were not able to compare TSS between the studies as it was only measured in our 2005 field investigation. The greater extent of effects observed with *C. tentans* in the 2002 study compared to our investigation could be due to the combination of elevated selenium in the effluent in that year and reduced concentrations of TOC, DOC and total phosphorus in the dilution water.

Hatching success in the trophic-transfer treatments was not significantly affected by exposure to either CCME (~30% success) or CCMWW (~48% success) compared to reference (~45% success). The lack of effects on hatching success is not consistent with our previous lab study where we observed only 2% hatching success in the CCME trophic-transfer treatment. This suggests that the impacts on the F1 generation in the trophic-transfer system were lessened in the field study. Hatching success in the water-only exposures could not be assessed because of the

lack of eggs produced in these treatments (n=2), therefore only trends can be discussed. We observed a 46% reduction in hatching success in the CCME treatment, and no reduction in the CCMWW treatment. The lack of offspring and reduced hatching success in the CCME treatment suggests that the impacts on the F1 generation were more severe in the water-only system compared to trophic-transfer.

Because of the lack of eggs produced in the water-only system we were also unable to assess deformities. However, of the few larvae that did hatch in these treatments no deformities were recorded. In the trophic-transfer exposures, the appearance of deformities in the offspring was increased. Similar to hatching success, the percentage of deformed larvae observed in the CCME trophic-transfer system (~30%) was less than those measured in our previous lab investigation where approximately 70% of the larvae hatched were deformed in the CCME trophic transfer treatment (Chapter 5). This suggests again that the toxicity of effluent in the trophic-transfer CCME treatment in the field investigation was lessened compared to the lab study (Chapter 5).

The increased appearance of deformities in the trophic-transfer system was thought to be due to selenium in the laboratory study and could also be of concern here, despite the observation that the magnitude of effects were reduced in the field. Uptake of selenium via food is thought to be the primary route of exposure for larval effects to manifest (Hamilton, 2002). It is thought that selenium is incorporated into vitellogenin (egg yolk protein) which is synthesized in the liver. Vitellogenin is then transported to the ovary where it is deposited within the embryo and eventually absorbed, along with selenium, by the developing larvae (Kroll and Doroshov, 1991). If deformities in the offspring only occur after dietary exposure then this accounts for why we observed increases in the trophic-transfer system and not the water-only treatments regardless of muscle burden and water concentrations. It also corresponds to the significantly higher selenium

body burdens in *C. tentans* in the CCME treatment. To investigate this relationship further selenium muscle burdens in the females were plotted against the appearance of deformities (% of total hatched) for each breeding pair. The R^2 value was 0.64 indicating that there was a significant relationship between the two parameters (Pearson, p=0.014), this plot is shown in Figure 6.8. Therefore, it is quite possible that selenium, the only compound significantly elevated in females, was contributing to the appearance of deformities in these exposures. The decreased appearance of deformities observed in this study compared to our previous laboratory investigation also corresponds to the potential reduced bioavailability of compounds causing these responses. This highlights, again, the importance of environmental relevance when assessing the toxicity of CCME on the F1 generation.

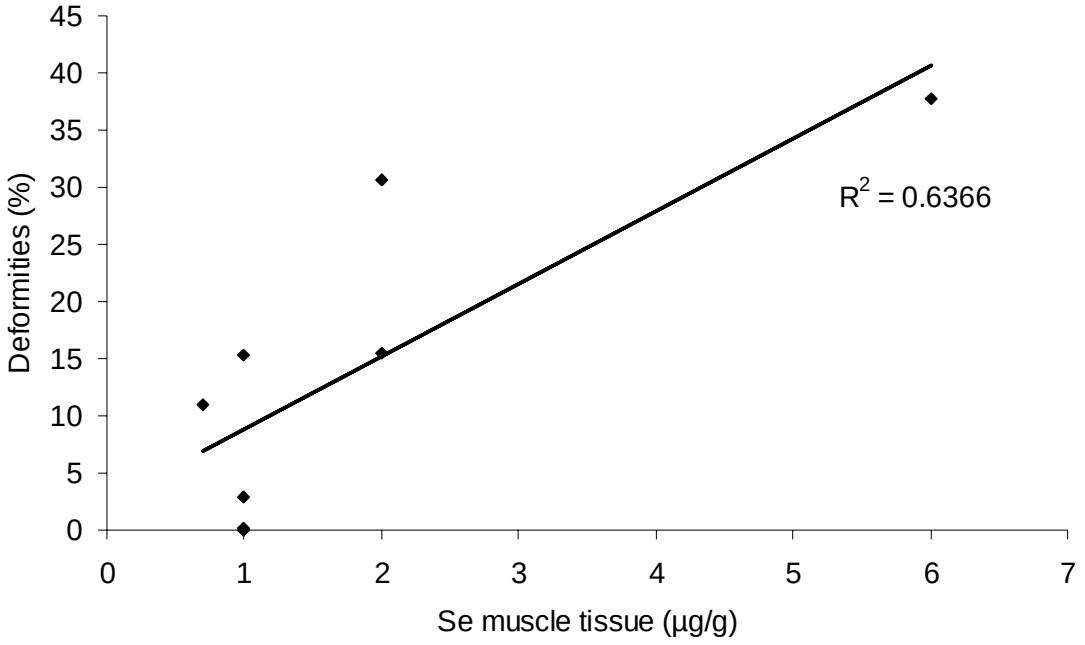

Figure 6.8. Appearance of deformities and concentrations of selenium in female fathead minnow (*P. promelas*) muscle tissue (dry wt), after exposure to reference water, Copper Cliff Mine Effluent (CCME 45%) and CCME and municipal waste-water combined (CCMWW) in a trophic-transfer system.

6.5 Conclusion

Overall, effects of CCME and CCMWW on adult FHM were more apparent in the water-only exposures. The severity of responses in this system corresponds with increased selenium concentrations in the water, female muscle burdens and gonadal tissue compared to reference fish. The reduced toxicity in CCMWW treatments (increased egg production and hatching success compared to CCME) corresponds to the reduced CCME concentrations in this treatment (CCME was only 30% in the mixture treatment compared to the CCME treatment alone which was at 45%). This reduced toxicity may also have been due to the increased organic matter (TOC, DOC) from the MWW that could have reduced bioavailability of elements causing toxicity in the CCME treatment. The lack of effects in the trophic-transfer system by comparison may also result from reduced toxicity of the effluent, possibly due to increased nutrients and organic matter which may have reduced metal bioavailability. The increase in appearance of deformities in the trophic-transfer system indicates that this endpoint is sensitive to CCME exposure as it is a consistent response pattern seen both in the lab and in the field. More detailed examinations of metals in the sediments, water column and muscle burdens of FHM and *C.tentans* is recommended to get an understanding of the speciation of potential factors such as selenium within the different compartments. It is also recommended for future investigations, that an assessment of bacterial community composition be conducted and modifiers such as carbon be measured in all aquatic components (including sediments).

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

The overall objective of the research conducted and described in this thesis was to develop an environmentally relevant bioassay to assess the effects of complex effluents on a sentinel fish species. Development of the bioassay focused on four key areas: 1) Incorporation of population-level endpoints into the toxicological assessment of effluents by utilizing a standard short-term fish reproductive bioassay; 2) Identification of consistent endpoints for use with the bioassay that would allow comparison of effects across different effluents; 3) Transferability of the bioassay between the lab and the field using an artificial stream system; and 4) Incorporation of trophic-transfer into the bioassay to quantify the importance of food as a source of exposure.

7.2 Identification of response patterns

One of the key objectives of this project was to identify consistent endpoints using the FHM short-term life-cycle bioassay that would allow comparison of effects of industrial effluents.

7.2.1 Pulp mill effluent

In the laboratory investigation (Chapter 2) we observed a complete halt in reproduction after exposure to 100% PME and a significant reduction in egg production after exposure to 50% PME. This was accompanied by increased appearance of pathological lesions (eosinophilic material) in male gonads as well as increased GSI. The F1 offspring were also affected after exposure to both 50% and 100% PME (where reduced hatching success and appearance of deformities was observed). In both Chapter 3 and 4, when the bioassay was applied on-site, we observed differing responses in reproductive output (decreased egg production and spawning events in 100% PME, increased egg production and spawning events in 1% PME), increased LSI

and testosterone, and development of ovipositors in males, a female secondary sex characteristic, in both 1% and 100% PME treatments. The endpoints that responded to PME in both the lab and field are summarized in Table 7.1. Consistent responses at high concentrations of PME (50% and 100%) were decreased egg production and spawning events. At low concentrations (1%) we observed increases in LSI but this was accompanied by increased egg production and spawning events. The endpoints that did not respond consistently to exposure to PME were testosterone (observed in the field but not the lab) and hatching success and deformities (observed in the lab but not the field). Induction of vitellogenin (measured in the field but not the lab) and gonadal pathology, i.e., the appearance of eosinophilic material (measured in the lab but not the field) were significantly altered in the experiments they were measured in.

It was concluded that the response patterns observed in both the lab and field studies were indicative of an estrogenic response based on comparisons with the literature. Previous investigations assessing the effects of estrogenic compounds (E_2) on FHM in the short-term bioassay have observed decreases in egg production at high concentrations and increased egg production at low concentrations (Ankley et al, 2001). In addition, Parrott and Wood (2003) observed appearance of ovipositors in male FHM in a full lifecycle bioassay with a PME. It was thought that this development could well be in response to estrogenic compounds within the effluent. A supporting investigation conducted in our laboratory assessed vitellogenin induction and the estrogen/androgen ratio in male FHM to try to determine an estrogenic response. It was observed in both the 1% and 100% final PME exposures from the field study that vitellogenin was significantly induced in male FHM (Rickwood et al, 2006c).

One main objective of this study was to conduct controlled exposures with this PME and compare our results to the literature from this site collected over the past 15 years. PME from the

Table 7.1 Summary of responses identified after exposure to pulp mill effluent (PME) in a controlled lab study (Chapter 2) and on-site in a bioassay trailer (Chapters 3&4). NM (not measured). LD (lack of data to perform statistical analysis). Arrows represent direction of change compared to control (lab study) and reference (field study) treatments where ↓ or ↑ = $p<0.05$, ↓↓or ↑↑ = $p<0.01$, ↓↓↓ or ↑↑↑ = $p<0.001$. m = males only, f = females only

LEVEL	ENDPOINT	LAB STUDY		FIELD STUDY	
		50%	100%	1%	100%
POPULATION	Egg production	↓↓	↓↓↓	↑	–
	Spawning events	–	↓↓↓	–	↓
	Hatch Success	↓	LD	–	–
	Deformities	↑↑	LD	–	–
INDIVIDUAL	LSI	–	–	↑f	↑m
	GSI	–	↑m	–	–
	Histopathology	↑m	↑m	NM	NM
	Ovipositor (Males)	–	–	↑	↑↑
BIOCHEMICAL	Testosterone	–	–	↑↑m	↑↑↑f
	Vitellogenin	NM	NM	↑↑m[a]	–

[a] Rickwood et al, 2006c

mill in Terrace Bay has been discharging into Jackfish Bay, Lake Superior since 1949. Despite significant improvements in processing and treatment of effluent, reproductive responses in wild fish exposed to the PME discharge are still being observed. In addition, other issues are also emerging, for example, effects on fecundity in fish sampled from Jackfish Bay were not observed until most recently (Environment Canada, 2004).

The increased egg production observed in our 1% treatment corresponds with the partial recovery in gonad size and, most recently, increased fecundity, in female white and longnose sucker from Jackfish Bay observed in the near field exposure sites (Environment Canada, 2004). Detailed analysis of wild fish collected in the Jackfish Bay discharge area demonstrated that depressions in reproductive hormones were associated with multiple disruptions in the endocrine pathway which controls reproduction (McMaster et al, 1995; Van Der Kraak et al, 1992).

The lack of responses in gonad size or sex steroids in our investigations is not consistent with previous research at Jackfish Bay conducted by Munkittrick et al (Munkittrick et al, 1991, 1998, 1997b) and McMaster et al (1995). Munkittrick et al (1998, 1997b) consistently report decreased relative gonad sizes in female white sucker. It was identified that the differences in the response of these endpoints may be due to differences in life history characteristics between the species, i.e., annual versus fractional spawner. It was also suggested that effluent quality to which fish were exposed may have differed. The effluent for our studies was taken at the outfall from the secondary treatment basins, whereas, wild fish in Jackfish Bay would be exposed to effluent that has traversed Blackbird Creek and may have been altered in composition.

In a review conducted by Kovacs et al (1997) a number of issues regarding the current assessment practices of PME were highlighted. Firstly, the ecological relevance of current reproductive indicators, e.g., gonad size, serum steroids is poorly understood. Secondly, natural

variability in fish populations can affect interpretation of results as habitat, lack of steady-state conditions and genetic-related issues can all affect variability in endpoints being measured. Thirdly, current laboratory investigations assessing the effects of FHM detect responses at concentrations much higher than that found in the field and are, therefore, not predictive of PME effects in receiving environments. Kovacs et al (1997) made a number of recommendations for future studies, most specifically to conduct more integrative studies using lab, artificial stream and field investigations, as well as assess multiple levels of biological organization to better understand the relevance of current reproductive indicators, i.e., gonad size and serum steroids.

In our investigations we were able to utilise the FHM short-term bioassay in both a lab and artificial stream setting, something that to date has not been achieved elsewhere to the best of our knowledge and review of literature. By conducting the investigation in both settings we were able to record and compare responses to PME at multiple levels of biological organization. This enabled us to identify that responses on-site were lessened compared to those observed in the laboratory investigation and this was possibly due to the inclusion of reference water and improved effluent quality. In addition, identification of response patterns from both the lab and on-site studies allowed us to use the FHM bioassay effectively in an investigation of cause study. We were able to successfully identify a potential source of compounds (e.g., combined alkaline process stream) within the mill that was causing effects in the final PME treatments. By using the short-term FHM bioassay and modifying it for use in an artificial stream setting we have made a significant contribution to the current database of knowledge regarding PME effects.

7.2.2 Metal mine effluent

The studies outlined in Phase II were conducted from 2004 to 2005 and included an integrated approach of laboratory (Chapter 5) and artificial stream (Chapter 6) studies at the INCO mine in

Sudbury, ON. The studies were conducted to determine response patterns in FHM and a dominant benthic invertebrate, *C. tentans,* exposed to treated MME, and to assess potential causative agents and to explore mechanisms of toxicity (e.g., life stages and endpoints most affected and pathways of exposure).

The laboratory study, conducted in August and September of 2004 (Chapter 5), examined the effects of CCME on fathead minnow (FHM) under controlled laboratory conditions. In both males and females no significant changes in survival, condition, relative gonad or liver size were measured after exposure to 45% CCME. However, significant decreases in reproductive output (egg production and spawning events) were observed in exposed fish along with significant increases in male gonadal cell death and fibrosis. The significant increase in vitellogenin in exposed female FHM compared to controls indicates that CCME has estrogenic properties. Alongside the water-only exposure to CCME (45%) we also ran a trophic-transfer exposure to assess the effects of dietary and water exposure compared to the water-only exposure. Significant decreases in hatching success and increases in deformities were observed in the trophic-transfer system suggesting that the combination of both food and water was important in assessing the effect on the F1 generation.

The field-based artificial stream study was conducted in 2005 to evaluate the effects of the CCME and the CCMWW (Chapter 6). In the water-only exposures, in both sexes of FHM, no significant changes in survival, relative gonad or liver size were observed after exposure to CCME or CCMWW. Overall egg production decreased by 77% and 17% in the CCME and CCMWW treatments, respectively. A significant reduction (60%) was also observed in spawning events in both treatments compared to reference. Significant decreases in spawning events and egg production were consistent with results from the lab study (Chapter 5). Due to low egg

production and few spawns in the treatment streams, hatching success and deformities could not be statistically tested.

In the trophic-transfer system, no significant differences were observed in any of the individual endpoints measured in females. In comparison to the water-only treatments, a significant increase in egg production and spawning events occurred in the trophic-transfer system. A 65% and 20% increase in egg production and a 116% and 50% increase in spawning events in the CCME and CCMWW treatments occurred, respectively. No significant difference was observed in hatching success in either CCME or CCMWW treatments although a significant increase in deformities did occur in both treatments.

In the 2005 field investigation, *C. tentans* endpoints were also assessed as part of the trophic transfer study. A significant decrease in emergence and densities were observed in the CCMWW treatment compared to reference. Effects on *C. tentans* densities and adult emergence in the CCMWW treatment suggests that CCMWW affected the development of *C.tentans* larvae. This is consistent with previous exposures of CCME to *C. tentans* (Hruska and Dubé, 2004).

In both the lab and field studies, endpoints such as condition, growth, body weight, length, and organ size (relative liver size and gonad size) did not respond as consistently as other endpoints. Our conclusions and assessments of responses to mine effluents are largely based upon changes in reproductive output (most frequently egg production and spawning events) and F1 survival and occurrence of deformities as primary endpoints. This is supported by detailed histological analyses of ovarian and testicular tissues. A summary of endpoints that responded to MME is outlined in Table 7.2.

Compared to the extensive research that has been conducted on the effects of PME on fish and benthos limited studies exist for MME. The majority of research has been conducted on

individual metals using single species tests (Sorensen, 1991; Woodward et al, 1994; Gensemer and Playle, 1999). This lack of data makes comparisons of response patterns, observed in our studies, with previous investigations difficult, especially with regards to population level endpoints, i.e., egg production and larval survival. However, of the limited data that exist, consistently elevated metal body burdens have been documented in fish sampled downstream of MME discharges (Farag et al, 1998; Olsvik et al, 2000; Dubé et al, 2005) which correspond to our study. The most extensive research examining biological responses to MME in field surveys, partial and full life-cycle bioassays and mesocosm studies in both lab and field settings has been conducted in Sudbury, ON.

Sudbury has been the site of extensive mining activity over the last 100 years and investigations to assess the health of fish populations in the lakes/rivers within and surrounding the Sudbury area have identified a number of changes, particularly metal accumulation in fish and benthos (Jaagumagi and Bedard, 2002). A field investigation of Junction Creek conducted by our lab in 2004 identified a number of biological responses in wild fish (fathead minnow and creek chub) including increases in metal body burdens (Cd, Cu, La, Rb, Se), decreased egg size and increased gonadal pathology in FHM (Weber et al, 2006a,b). However, due to the number of point and non-point source stressors affecting Junction Creek, attributing cause to a particular source or sources is difficult. A number of recommendations for further studies were made after this initial field investigation was completed. Specifically, it was stated that focusing solely on adult field surveys in Junction creek was not advisable due to the great difficulty in obtaining fish from Junction Creek, the confounded nature of the system and the unknown biological consequences of the changes in the sub-organismal end-points that were measured in the wild fish field survey (e.g increased cell death in male gonads and decreased egg size in females).

Table 7.2 Summary of responses identified in FHM after exposure to metal mine effluent (MME) in a controlled lab study (Chapter 5) and in an on-site study (Chapter 6). Arrows represent direction of change compared to control (lab study) and reference (field study) treatments. Dash represents no change. NM = not measured.

LEVEL:	ENDPOINT:	LAB STUDY		FIELD STUDY			
		Water-only	Trophic-transfer	Water-only		Trophic-transfer	
		CCME	CCME	CCME	CC-MWW	CCME	CC-MWW
POPULATION	Egg production	↓	↓	↓	↓	↑	↑
	Spawning events	↓	↓	↓	↓	↑	↑
	Hatch Success	–	↓	–	–	–	–
	Deformities	–	↑	–	–	↑	↑
INDIVIDUAL	Liver size	–	–	–	–	NM	–
	Gonad size	–	–	–	–	NM	–
	Histopathology	↑	↑	↑	↑	NM	↑
	Ovipositor (Males)	–	–	–	–	–	↑
BIOCHEMICAL	Sex steroids	↑	↑	–	↑	NM	↑
	Vitellogenin	↑	↑	↑	–	NM	–

It was recommended that an integrative approach be used to investigate the effects of MME entering the creek. In our lab and on-site studies we were able to address these points.

Firstly, we were able to identify and compare response patterns in a water-only and trophic-transfer system after exposure to individual MME's in both a lab and on-site setting. Secondly, we were able to document changes at multiple levels of biological organization gaining further insight into the linkages between sub-organismal and population-level endpoints measured in the field studies. These linkages are critical to begin to understand and document biological response patterns to MME, potential causative sources, and potential mechanisms of action. In addition, we were also able to assess the effects of MME and the contribution of trophic-transfer, in both a lab and on-site setting. These investigations have made a significant and unique contribution to understanding the potential effects of a MME on fish and benthos, how responses differ in different settings and how responses are altered under different conditions. They will provide valuable information to the Canadian Environmental Effects Monitoring (EEM) Program.

7.2.3 Comparison of effluent response patterns

Consistent endpoints that responded to both effluents tested (MME and PME) were identified. For example, egg production, spawning events, histopathology and alterations in sex steroids were observed after exposure to both PME and MME in the water-only exposures. However, there were differences in responses. Most noteably, larval survival and deformities were more severe in the MME exposures where deformities appeared in both lab and field studies. In comparison, hatching success and deformities were only affected in the lab PME study (Chapter 2), with no appearance of deformities in the field investigation (Chapter 3 and 4). In addition, it was only in the PME studies that significant increases in LSI appeared, none of the individual level responses were observed in the MME exposures. It is likely that the response patterns for

both PME and MME, identified in Tables 7.1 and 7.2, differed slightly because of the different mechanisms of action for each effluent. For example, the pattern of response identified in the MME studies was indicative of toxicity to certain metals/elements. By comparing our response patterns to the literature we were able to isolate certain elements that could have been responsible for the responses observed (Chapter 5 and 6). The responses to these specific metals, based on the literature, were reduced hatching success, increased deformities in the F1 generation, disruption in reproductive output and appearance of gonadal pathology. Again, in the PME studies, by comparing the response patterns to the literature we were able to identify that the responses observed were indicative of endocrine disruption, specifically the presence of estrogenic compounds. This estrogenic response, based on the literature, includes induction of vitellogenin in males, altered reproductive output (increased at low concentrations, decreased at high concentrations) and the appearance of female sex characteristics (ovipositor development) in males. Most importantly, even though similar responses were observed after exposure to both PME and MME (e.g. decreased reproductive output) the mechanisms behind these responses were different. By assessing the response patterns using a suite of endpoints it was possible to characterize specific types of compounds that were most likely causing the responses observed, i.e., estrogenic (PME) or specific metals/elements (MME). The ability to use these response patterns to identify the types of compounds causing the responses observed, suggests that the FHM bioassay could be developed as a standardised test to assess the toxicity of complex effluents.

Comparison of response patterns between PME and MME is limited to water-borne only exposures as trophic-transfer was not conducted with PME investigations. However, the observations made in the trophic-transfer system are, perhaps, the most relevant for this

discussion. The consistent responses observed after exposure to MME (reduced egg production, spawning events, hatching success) in the water-only exposures were not observed in the trophic-transfer system in the field investigation (Chapter 6). It was concluded that the presence of reference water and the environment within the trophic-transfer were responsible for this change in response. If this is the case, then this raises the important question of whether a similar change in response would be observed if the trophic-transfer system were applied to PME? This is certainly an area of further investigation that is required to understand the complexity of these effluents and how bioavailability/toxicity is altered in this system, and ultimately whether this system is indicative of the receiving environment.

The use of the short-term FHM bioassay throughout this project has enabled us to identify consistent and sensitive endpoints and characterize response patterns to both PME and MME. The concentrations used in our exposures were based on environmentally relevant levels. To establish this bioassay as a standardized tool for assessment of complex effluents, future studies should focus on generating comparative dose-response curves. The concentrations used in future studies must be consistent, and the inclusion of relevant endpoints must be used. By conducting dose response studies with this bioassay, it may be possible to calculate a sublethal dose (EC50s) for complex mixtures. The endpoints identified throughout this research have been compiled for each effluent and recommendations for future studies are given in Table 7.3. These endpoints are suggested as a minimum for each effluent. It is recommended that future studies include a suite of endpoints reflective of different levels of biological organization to generate response patterns that are relevant to the effluent under investigation. It is also recommended that the importance of trophic-transfer be considered in future investigations with both PME and MME.

Table 7.3 Recommendation of endpoints to be included in the assessment of both pulp mill and metal mine effluents.

LEVEL:	PULP MILL EFFLUENT	METAL MINE EFFLUENT
POPULATION	Egg production Spawning events Hatching Success Deformities	Egg production Spawning events Hatching Success Deformities
INDIVIDUAL	Liver size Gonad size Gonadal pathology Secondary sex characteristics	Gonadal pathology
BIO-CHEMICAL	Vitellogenin Sex steroids	Vitellogenin Sex steroids
OTHER	Trophic-Transfer	Metal body burdens Trophic-transfer

7.3 Bioassay Development

7.3.1 Fathead minnow life-cycle bioassay

The inclusion of the FHM bioassay into our investigation was a key decision based on the lack of understanding, or significance, of individual endpoints and how they might relate to the health and sustainability of fish populations. Extrapolation of sub-organismal effects to determine ecosystem health has, to date, not been accurately demonstrated (Power and McCarty, 1997; Forbes and Calow, 1999; Calow, 2003). The FHM bioassay was designed to include endpoints that measure various levels of biological organization including biochemical, individual and population (Ankley et al, 2001). Therefore, by using this bioassay in our studies, we were able to develop a monitoring tool that could assess the effects of industrial effluents on these different levels of biological organization. This ultimately allowed us to better understand the significance of the individual endpoints measured and how they relate to both biochemical, physiological and population level responses. Most importantly it was our goal to use the FHM bioassay to develop a standardized method for assessing effluent effects in fish. Incorporation of this bioassay allowed assessment of multiple endpoints and identification of response patterns for both PME and MME.

7.3.2 Artificial streams

The incorporation of the FHM bioassay into an artificial stream system was based on previous investigations that have shown species responses in the lab are not predictive of responses observed in the field (Robinson et al, 1994; Kovacs et al, 1997). It is generally thought the issues in extrapolating results to those observed in the field are due to the complex interaction between abiotic and biotic factors that are not replicated in laboratory studies (Kovacs and Megraw, 1995; Sibley et al, 1999; Tucker and Burton, 1999). Transferring the bioassay on-site increased

environmental relevance of both PME and MME studies as we were able to use reference water, reduce effluent holding time and conduct exposures under ambient conditions.

In Phase I, the biological response of FHM to PME in both the lab and field studies were indicative of reproductive effects, however, different endpoints responded and those which consistently responded, did so at a lesser magnitude in the field. Certainly the pattern of FHM response to PME from this mill was reproductive but factors such as effluent storage times, sources of dilution water, turnover times or exposure exchange rates, and different levels of replication, likely affected which endpoints responded and the magnitude of the response. This information is critical as it demonstrates the importance of standardizing assessment approaches to examine effects for the same effluent let alone across different effluents. These results should be carefully considered when laboratory investigations are used in an effort to better understand responses observed in the field.

In Phase II, effects in the on-site MME exposures, similar to Phase I, were lessened compared to the lab study demonstrating, again, the importance of environmental relevance in toxicity testing. Specifically, reproductive output was not reduced in either of the CCME or CCMWW trophic-transfer treatments on-site. This was hypothesized to be due to the cumulative contribution of reference water and the productive foodweb environment within the trophic-transfer system. The combination of these factors may have increased organic matter (significantly higher BOD and TSS) in these treatments to a level which would reduce the bioavailability of metals/elements that were the cause of reproductive impairment in the laboratory study. The fate and bioavailability of many compounds, including metals, can be affected by numerous parameters in receiving waters and should not be overlooked in future investigations.

In conclusion, the effects observed in the on-site investigations were lessened compared to the lab studies in both the pulp mill and metal mine investigations. These studies highlight the importance of environmental relevance when conducting standard toxicity tests. By using an integrative approach throughout our investigations, we were able to not only compare the toxicity of effluents under differing exposure scenarios but also develop our knowledge with regards to the capabilities of artificial streams in effluent toxicity testing. The information gathered in these studies has made a significant contribution to the development of artificial stream systems.

7.3.3 Trophic-transfer

The incorporation of trophic-transfer into the FHM bioassay in Phase II was conducted to improve environmental relevance of standard water-only toxicity testing. Prior to this study, research assessing population level effects in fish through trophic-transfer of effluents had not been conducted. The results from our studies have demonstrated that environmentally relevant testing is essential if we are to accurately assess effects on aquatic biota and move towards realistic solutions for mitigation. In both the lab and field studies, exposure in the trophic-transfer system was important in determining F1 generation effects, i.e. hatching success and deformities. Had we conducted water-only exposures these responses would not have been observed. It is not possible for us to conclude that food was solely responsible for the effects observed in this system as we did not measure the effects of contaminated food in isolation. However, we can speculate that the cumulative contribution of exposure through both food and water was the cause of these responses.

In summary, the combination of reference water with the productivity of the trophic-transfer food web resulted in significantly fewer effects of the effluents on FHM. This suggests that

effects observed after exposure to MME through the water-only and/or under controlled laboratory conditions may be more severe than those occurring in natural systems such as Junction Creek. This highlights the importance of environmental relevance when assessing the toxicity of effluents and the difficulties of using toxicity tests in the lab or with water-borne exposure routes in an effort to extrapolate potential effects of MMEs on organisms in the field. The research conducted in this investigation has begun to address a critical gap in knowledge regarding relevance of standard water-only exposures in metal mine effluent testing.

7.4 Recommendations

During the development of the bioassay and trophic-transfer system a number of suggestions/improvements were highlighted. These are briefly discussed here.

7.4.1 Sediments

During the lab investigation of the trophic-transfer system (Chapter 5) we were interested in developing cultures within the artificial stream systems that would first and foremost be the primary food source for FHM. As such, the primary substrate used was silica sand as per the standard protocol for the *C.tentans* assay as this was the primary substrate for optimal growth. However, if the focus of investigations is to assess the importance of contaminated sediments and their effects on both benthos and fish, it is suggested that using sediments from the site of interest would improve environmental realism. Experimental investigations can then be applied using the trophic-transfer system; firstly, with contaminated sediments (with and without contaminated water) and, secondly, with non-contaminated sediments (with and without contaminated water). In systems that have historical contamination this would be a significant contribution for investigating cause in confounded environments.

7.4.2 Pre-exposure of *C.tentans* cultures

Our initial experimental design for our laboratory investigation was to start exposures of both *C. tentans* and FHM at the same time. However, previous investigations have observed that at least 3-5 days of exposure to metals is required before *C. tentans* reach a steady state concentration (Muscatello, 2003). This means that FHM in our system were consuming relatively clean food for approximately 3 days into the exposure period. It was suggested after the lab investigation that *C. tentans* should be exposed 5 days prior to FHM entering the system. This was initially incorporated into the field study, however, problems with obtaining adequate cultures on site resulted in exposures of both *C. tentans* and FHM at the same time. It should be noted here that if the trophic-transfer system is to be improved, a pre-exposure period of 5 days for the *C. tentans* cultures should be included in the experimental design.

7.4.3 Incorporating bacterial and algal measurements

During the field investigation we observed substantial algal growth in both the water-only and trophic-transfer systems. This was especially prevalent in the CCMWW treatment probably due to the high organic load in these exposures. It is unknown whether the presence of algae or bacterial communities in these systems had any effect on bioavailability of contaminants. In addition, the reduced toxicity observed in the trophic-transfer system in the field investigation may have been due to the presence of algae or bacterial communities. Investigations have shown that the presence of microorganisms can degrade hazardous organic compounds from water, and the treatment of industrial waste streams before discharge is utilized in many bioremediation processes (Godlewska-Zylkiewickz, 2006). In addition, microorganisms have also been used in the reclamation of heavy metals and radionuclides from waste streams, industrial effluents and

mine water (Valls and de Lorenzo, 2002; Malik, 2004). These investigations highlight how the presence of bacterial communities can alter or reduce the toxicity of both organic and inorganic compounds. As such, if the trophic-transfer system is to be improved, incorporation of algal measurements, i.e., calculation of biomass and contaminant loads, and measurement of bacterial communities, i.e., types of bacteria present, is strongly suggested.

7.4.4 Reduction of the breeding trial

The standard laboratory protocol of the FHM short-term bioassay requires a 1 week period for acclimation followed by a 3 week period in reference/control water to establish breeding pairs. Although this time period has obvious benefits, i.e. increases the number of breeding pairs available to use during the exposure period, 7 weeks is a substantial period of time for breeding pairs to maintain a constant level of reproductive output under optimal conditions. This is especially important in the short-term bioassay as exposure only occurs in the last 21 days after certain breeding pairs may have been reproducing for over three weeks. In addition, consistent reproductive output is an important measurement in the reference treatments. If the length of the assay resulted in a naturally decreased temporal trend in egg production in reference streams, could this lead to incorrect interpretation of results and increased variability in these reproductive endpoints? If this trend was occurring, all treatments including controls would experience a similar temporal trend and likely treatment–related differences would not change or affect interpretation of our results. However, to improve upon this issue we would suggest increasing the number of breeding pairs in the pre-exposure to approximately three times the amount required in the exposure and limiting the pre-exposure period to 3 weeks including acclimation. Throughout all of our investigation we consistently had ~ 50% success rate, i.e., 50% of breeding pairs in the pre-exposure period met exposure criteria. By increasing the number of breeding

pairs available in the pre-exposure period it may be possible to obtain adequate numbers in a shorter time period, thereby limiting the effects of time on breeding activity. Obviously, logistical constraints may confound this improvement, especially in field situations where space is limited.

7.5 Environmental Effects Monitoring program

The primary focus of these investigations has been to develop a standardized bioassay that has the capacity to measure effects of PME and MME on aquatic organisms. Currently, in Canada, both PME and MME are monitored under the EEM program. Results from our studies will provide fundamental information on biological response patterns to these complex mixtures under controlled exposure conditions and the endpoints that define them. Our research has also developed an alternative monitoring tool as defined under the EEM program to assess if biological effects due to these effluents are occurring and potential sources of the effects. By developing a bioassay that can measure endpoints at multiple levels of biological organization in both fish (FHM) and benthos (*C. tentans)* in an environmentally relevant way, this study has allowed us to step closer in establishing a method that can monitor population and possibly community level effects. It is certainly not our goal to develop a method that will be used instead of field investigations. Rather, we believe that an integrative, weight of evidence approach is required. Utilizing field assessments and conducting exposures in an environmentally relevant way will improve our understanding of effluent effects.

7.6 Conclusion

In Phase I we used the FHM bioassay to go beyond standard laboratory assessment of pulp mill effluent and conducted an investigation of cause study on-site. By focusing on identifying

response patterns in fish using both physiological and population level endpoints we were able to identify a process stream that was the potential cause of responses observed after exposure to final effluent. Isolation of this process stream will assist the mill in developing approaches for future mitigation. The results from this research will also provide additional data for the EEM program for pulp and paper and investigation of cause studies on a national basis.

In Phase II, development of the trophic-transfer bioassay in an artificial stream, resulted in a quantitative method to assess the effects of exposure to a mine effluent for both fish and benthos. We measured population-level effects in both fish and benthos as defined by changes in reproductive endpoints such as egg production and hatching success in fish and emergence and densities in benthos. Phases I and II of this research have made significant contributions to artificial stream development within Canada for the assessment of industrial effluents and their effects on aquatic biota.

The results from this study have also demonstrated that environmentally relevant testing is essential if we are to accurately assess effects on aquatic biota. Future development and application of this bioassay should be towards developing a standardized approach for not only assessing the effects of industrial effluents in a comparative manner but also in investigation of cause studies.

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