Changes in physiology and reproductive success in fathead minnows (*Pimephales promelas*) exposed to pulp and paper mill effluent

by

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A thesis submitted to the Faculty of Graduate Studies at Lakehead University in partial fulfillment of requirements for the degree of Master of Science in Biology.

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Your file Votre référence
ISBN: 978-0-494-43430-7
Our file Notre référence
ISBN: 978-0-494-43430-7

Acknowledgements

I would like to begin by thanking my supervisor, Dr. David Law, for providing me with the opportunity to pursue a Master's degree and providing me with guidance, encouragement, and advice along the way. I would also like to thank Dr. Peter Lee (committee), and Dr. Vince Palace (external examiner – Research Scientist, Department of Fisheries and Oceans Canada), for their helpful advice and suggestions.

Many other people at Lakehead University cannot be forgotten. First, Dr. Julieta Werner who provided much needed support, advice, and was willing to help whenever help was needed. Young-Jun Ju, Sheri Skerget, Caroline Cheng, and Susanne Walford were always helpful with setting up experiments and taking care of my fish.

Several people at AbitibiBowater must also be acknowledged. Chris Walton for his interest in the project and providing me lab space at the mill and Brent Lindberg for helping me find my way around the mill and answering an endless number of questions.

Next, I would like to thank my family, friends, and everyone around the lab and at Lakehead University who made the 2 years in Thunder Bay enjoyable and memorable.

Finally, I would like to thank AbitibiBowater, FedNor, the Northern Ontario Heritage Fund, the Canada Foundation for Innovation, and Lakehead University for the financial support they provided.

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Abbreviations

ANOVA – analysis of variance
AOX - adsorbable organically bound halogens
BOD - biochemical oxygen demand or biological oxygen demand
CACID - combined acid
CALK - combined alkaline stream
CME - combined mixed effluent
COD – chemical oxygen demand
CSC - combined stripped condensate
CTMP - chemi-thermomechanical pulping
CYP1A - Cytochrome P450 1A
DO – dissolved oxygen
EDC - endocrine disrupting compounds
EEM - environmental effects monitoring
EPA – Environmental Protection Agency
GSI - gonadosomatic index
ISO - International Standards Organization
K – condition factor
LSD – least significant difference
LSI – liver somatic index
LUEL - Lakehead University Environmental Laboratory
MDL – method detection limit
MEDAL - Molecular, Environmental and Developmental Biology Lab
MFO - mixed-function oxygenase
ND – not detected
PAPEER – pulp and paper effluent ecotoxicology research
PPER - pulp and paper effluent regulations
RMP – refiner mechanical pulp
RW – river water
SCMP - semichemical-mechanical pulping
SGW – stone groundwood pulp
SK – secondary treated kraft
TMP – thermomechanical pulp
TSS – total suspended solids
UK – untreated acid-alkaline kraft

Abstract

As part of their manufacturing process, pulp and paper mills release effluent into waterways that may affect the fecundity, morphology, and physiology of invertebrates and vertebrates in the receiving ecosystem (Kovacs *et al.* 2006, McMaster *et al.* 2003, Munkittrick *et al.* 1998). Treatment systems within the pulp and paper mills are effective at removing many toxicants and improving effluent quality; however, pulp and paper mill effluents may still negatively impact the aquatic environment. Fathead minnows (*Pimephales promelas*) are a useful vertebrate model species for environmental monitoring because they spawn frequently, reproduction can easily be monitored, and a significant quantity of data has been published regarding their responses to chemicals and effluents (Rickwood and Dubé 2007, Kovacs *et al.* 2005, Parrott and Wood 2004, among numerous others).

Our research involved using a short-term fathead minnow reproductive bioassay (which includes a 15-day pre-exposure period and a 6-day exposure period) in order to assess consistency and predictability of spawning, determine reproductive and physiological changes resulting from exposure to 10% (v/v) untreated kraft mill effluent (UK), 25% (v/v) secondary treated kraft mill effluent (SK), and 100% (v/v) combined mill outfall (CMO), and analyze the applicability and relevance of our 6-day reproduction test. Two set of experiments were run: river water vs. kraft mill effluent, and river water vs. combined mill outfall (CMO) effluent.

Pre-exposure and control fish showed predictable spawning, although a number of breeding pairs were required in order to ensure a sufficient sample size. Ten percent (v/v) UK decreased egg production dramatically, and 10% (v/v) UK and 25% (v/v) SK

each caused masculinization in a female fathead minnow. While clarification and secondary effluent treatment appeared to improve the short-term reproductive impacts on fathead minnows observed in kraft mill effluent, these processes did not entirely remove the source of endocrine disruption causing masculinization. In contrast, the 100% (v/v) CMO effluent did not cause any observable reproductive or physiological changes. A short-term (6-day) exposure period appears to be sufficient for analysis of the effect of EDCs on vertebrate morphology and fecundity, although it is unclear whether responses were not observed in SK and CMO effluents because of the short observation period.

1.0 Literature Review and research rationale

1.1 Pulp and paper

1.1.1 Introduction to pulp

Smook (2002) defines pulp as "the fibrous raw material for papermaking". Pulp is primarily obtained from wood, although other plant material may be used, such as straws and grasses, canes and reeds, and bamboo (Smook 2002). Recycled fibres may be reused and can make up a substantial percentage of the material needed for pulp production (Bowyer *et al.* 2007). Several methods exist in order to extract the fibres useable in pulp production from wood. These processes include mechanical, chemical, and thermal pulping, as well as combinations of the three (Bowyer *et al.* 2007, Smook 2002) (see section 1.1.4 for details).

1.1.2 Main components of wood

The main components of wood are carbohydrates (Bowyer *et al.* 2007, Smook 2002, LaFleur 1996) (Table 1). Cellulose is present in the highest quantity and is also the plant fibre used for producing paper (Bowyer *et al.* 2007, Smook 2002, LaFleur 1996). It is a straight chain polymer of repeating units of glucose, which provides its strong structure. Hemicellulose is the next most common component of wood and is a polymer of five different sugars (glucose, mannose, galactose, xylose, and arabinose) (Bowyer *et al.* 2007, Smook 2002). As opposed to cellulose, it is a branched-chain polymer of repeating sugar units.

Lignin is also present in relatively high quantities in wood (Bowyer *et al.* 2007, Smook 2002). It is a complex polymer added onto phenylpropane units and, although it

is made of carbon, hydrogen, and oxygen, it is not a carbohydrate. It provides a major component of the rigidity of wood (Bowyer *et al.* 2007, Smook 2002).

Extractives are also present in wood and include terpenes, resin acids, fatty acids, and alcohols (Bowyer *et al.* 2007, Smook 2002, LaFleur 1996). They are soluble in water or neutral organic solvents and make up a small percentage of the components of wood.

Table 1 – The major components of wood [a] (Smook 2002)

Wood type	Cellulose	Hemicellulose	Lignin	Extractives
Hardwood	45±2%	30±5%	20±4%	5±3%
Softwood	42±2%	27±2%	28±3%	3±2%

[a] normal range by percentage of dry weight

1.1.3 Types of wood

Wood may be classified into one of two groups, softwoods or hardwoods (Smook 2002). Softwoods are gymnosperms, which consist of coniferous trees, such as jack pine (*Pinus banksiana*), white spruce (*Picea glauca*), and black spruce (*P. mariana*), among numerous others (Ritchie 2007). Hardwoods are deciduous angiosperms; among those common in northwestern Ontario are trembling aspen (*Populus tremuloides*) and white birch (*Betula papyrifera*) (Ritchie 2007). Both types of wood are used for pulp and paper making due to their high cellulose content, although hardwoods may contain a higher percentage of cellulose and hemicellulose (Table 1). Hardwoods may also have shorter fibre lengths than softwoods and therefore the relative amount of each used in the pulping

process has a significant impact on the type of paper produced (Bowyer *et al.* 2007, Smook 2002).

1.1.4 Methods of fibre extraction

Mechanical pulping involves the use of either an abrasive stone or wheel (stone groundwood pulping), or steel disks (refiner mechanical pulping) in order to grind and separate fibres from the wood (Bowyer *et al.* 2007, Smook 2002). Stone groundwood pulping (SGW) is considered to be an outdated technology and is not generally used, while refiner mechanical pulping (RMP) is used in most mechanical pulp mills currently operating worldwide (Bowyer *et al.* 2007).

Semimechanical pulping includes thermomechanical pulping (TMP), chemi-thermomechanical pulping (CTMP), and semichemical-mechanical pulping (SCMP). These methods limit damage to fibres and allow for extraction of stronger fibres than by mechanical pulping alone (Bowyer *et al.* 2007, Smook 2002). TMP uses a refiner, steam, and high pressure to soften and separate fibres (Bowyer *et al.* 2007). CTMP is similar; however, it also involves chemicals added to the wood chips that further soften and separate fibres from dense woods (Bowyer *et al.* 2007). SCMP also uses chemicals and a refiner, however it does not use high pressure or high temperature Bowyer *et al.* 2007).

While semichemical pulping requires some mechanical treatment, it relies mainly on chemicals to soften and extract the fibres (Bowyer *et al.* 2007). Because less mechanical force is used, the extracted fibres remain strong and are less damaged than in mechanical pulping, allowing for high pulp yields (Bowyer *et al.* 2007).

Chemical pulping is reported to be the most frequently used method of fibre extraction and consists of placing wood chips in a chemical solution prior to heating in a

digester (Bowyer *et al.* 2007). Chemical pulping may be accomplished by one of two methods that require the use of different chemicals: the sulfite process or the kraft process. The sulfite process uses sulfurous acid and ammonium, in addition to magnesium, calcium, or sodium bisulfites, while the kraft process employs sodium hydroxide and sodium sulphide (Bowyer *et al.* 2007, Saka and Matsumura 2004). These chemical pulping methods result in the highest quality of paper because of their ability to extract large quantities of undamaged fibres and the removal of lignin, a major aspect in the yellowing of old paper. However, pulp yields are also significantly lower than with mechanical extraction methods (Bowyer *et al.* 2007).

Wood fibres may also be extracted from recycled paper products. Contaminants and inks are first removed from the fibres, and from 11 and 33% of recycled fibres are typically damaged or lost and thus cannot be reused (Bowyer *et al.* 2007).

While these modern pulping processes extract wood fibres with high efficiency, they also result in the production of aquatic effluents that may harm the environment if discharged without treatment.

1.2 Water Pollution

1.2.1 Introduction to water pollution

Water pollution can be considered as "any change in the condition of water which is detrimental to some beneficial use" (Smook 2002). Pulp and paper mills release waste in the form of effluent into receiving waterways that can potentially pollute it. The chemical components of these effluents are difficult to determine and have been seen to change as a result of the method of pulp extraction, paper processing, and effluent treatments (Servos *et al.* 1996).

1.2.2 Effluent components

Pulp and paper mill effluents contain wood components, chemicals used in the pulp or paper making processes, additives, and degraded wood and chemical products. This chemicals mixture makes identification of toxic or biologically harmful compounds within the effluent difficult (Kovacs *et al.* 2006, LaFleur 1996). During the 1980s and early 1990s, the components of pulp and paper mill effluents were heavily characterized, although little has been done since (Hewitt *et al.* 2008, Kovacs *et al.* 2006).

General effluent parameters such as chemical oxygen demand (COD), biological (or biochemical) oxygen demand (BOD), adsorbable organic halogens (AOX), and colour, as well as specific organic compounds, including chlorinated phenolic compounds, chlorinated acetic acids, chloroform, polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), fatty and resin acids, and sterols have all been recognized as compounds of potential environmental concern (Strömberg *et al.* 1996, Owens 1991). The concentrations of many of these chemicals in pulp and paper effluents have been reduced as a result of changes in the bleaching process to lower or eliminate chlorine use, a shift to increased TMP and CTMP pulping, and upgrades to secondary treatment processes (Kovacs *et al.* 2006). Kraft mills still using chlorine bleaching to remove colour from the lignin are considered likely to release chlorinated phenolics and chlorinated organic acids in addition to chemicals present in wood which may include β-sitosterol, isoharpontigenin, juvabione, dehydrojuvabione, and pinosylvin (Kime 1998). Kraft mill discharges have been shown to contain degraded wood components in ratios of 60% fatty acids: 4% resin acids: 9% sterols: 27% triterphenyl alcohols (based on the percentage of weight of degraded wood components) (Vidal *et al.* 2007). Fatty and resin

acids are toxic to aquatic organisms (Werker and Hall 1999) while sterols have been linked to hormonal changes in aquatic vertebrates (Kostamo & Kukkonen 2003). Between 50,000 and 150,000 cubic metres of effluent can be discharged into waterways by a pulp mill on any particular day (Robinson *et al.* 2004). The high volume of effluent released into the environment highlights the need for effective treatment systems.

1.2.3 Effluent treatment

Treatment of pulp and paper mill effluent includes primary treatment, secondary treatment, tertiary treatment, and dilution. Each mill has its own combination of treatment processes and not all treatments are performed in the same manner at all mills.

Primary treatment is the removal of suspended materials through the use of a primary clarification system. The purpose is primarily to remove debris and suspended materials before secondary treatment (Smook 2002, Gibbons *et al.* 1992). High concentrations of fibre and suspended material in pulp and paper mill effluents have been shown to decrease the number of some benthic organisms and increase the loss of suitable feeding and reproductive habitats for others (Owens 1991). However, there is evidence that primary treatment also removes some resin and fatty acids, as well as sterols. Kostamo and Kukkonen (2003) found that primary treatment removed up to 60% of resin acids and up to 64% of sterols from kraft mill effluent. Kostamo *et al.* (2004) found that 33 to 82% of fatty acids and 13 to 78% of resin acids were degraded or transformed during primary treatment while 2.1 to 17% and 3.9 to 34% of fatty acids and resin acids, respectively, were adsorbed in the primary sludge. Kostamo *et al.* (2004) also found that 21 to 76% of sterols were degraded or transformed during primary treatment and 2.4 to 15% were adsorbed to the primary sludge.

Secondary treatment includes the use of biotreatment and a sludge system within an aerobic environment. The secondary treatment process is responsible for reducing organic and nutrient loads in effluent, controlling adsorbable organically bound halogens (AOX), chemical oxygen demand (COD), and biochemical oxygen demand (BOD), removing resin acids, fatty acids, and sterols, as well as removing acute lethal toxicity and reducing sublethal toxicity and mutagenic activity (Kostamo *et al.* 2004, Kostamo and Kukkonen 2003, Strömberg *et al.* 1996, Gibbons *et al.* 1992, Owens 1991). Kostamo *et al.* (2004) found that overall removal of wood extractives ranged from 35 to 99% during secondary treatment, although between 74 and 99% were discharged in particles. Kostamo and Kukkonen (2003) found that an activated sludge system degraded or transformed over 94% of resin acids and 41% of sterols from kraft mill effluents. Strömberg *et al.* (1996) analyzed both activated sludge systems and aerated lagoons as secondary treatment options and saw effluent improvements in both. Secondary treatment reduced AOX between 34 and 56%, COD between 39 and 71%, BOD between 81 and 99%, resin acids between 68 and 100%, fatty acids between 42 and 100%, and sterols between 53 and 99%. Gibbons *et al.* (1992) found that biological treatment reduced wood extractive concentrations by more than 99%, BOD by more than 80%, and COD by more than 60%. By contrast, Cook *et al.* (1997) demonstrated accumulation of stigmasterols in aerobic systems, although overall sterol concentrations (campesterol, β-sitosterol, and stigmastanol, with stigmasterol excluded) decreased between 56 and 95%. Recently, the benefits of adding anaerobic pre-treatment prior to secondary aerobic treatment have been observed and over 200 mills worldwide have installed anaerobic pre-treatment systems, with 75% of these in Europe, 13% in Asia, and 9% in North America

(Habets and Driessen 2007). Vidal *et al.* (2007) found that anaerobic biodegradation has the ability to remove 77 to 100% of β-sitosterols and 87 to 95% of stigmasterols.

Tertiary treatment is the removal of colour and may not be done to all effluents. The need for colour removal depends on the natural river conditions at the discharge site as light penetration in dark water with high sediment loads is less likely to change with the input of darkened effluents. Increasing the water colour through effluent discharges has been shown to absorb light, thus reducing photosynthesis and primary production. It may also lead to interference in fish sending and/or receiving visual cues necessary for normal fish reproduction or feeding (Owens 1991).

Dilution is a part of the final treatment process whereby effluent is released into the environment and the concentration is lowered by mixing with the receiving waterway. Because environmental changes are generally concentration dependant, dilution may mitigate some environmental impacts, although Kovacs *et al.* (2006) recommend further studies with receiving water because different biological endpoints have different concentration thresholds. The use of dilution is becoming less acceptable as an effluent treatment process, especially from a regulatory point of view.

1.2.4 Traditional effluent monitoring

During treatment, the quality of effluent must be monitored to ensure that toxic compounds are removed prior to discharge. Pulp and paper mill effluent monitoring began in the 1950s as a result of observations of fish habitat loss due to low oxygen content and high sedimentation, in addition to acute toxicity of fish in receiving waters (McMaster *et al.* 2003, Owens 1996). At this time, dilution was the primary method for effluent treatment (Folke 1996). These observations led to regulation of total suspended

solids (TSS) content and BOD in receiving waters and improvements to effluent quality in order to reduce acute toxicity to less than 50% in a test population (McMaster *et al.* 2003, Owens 1996). Consequently, pulp and paper mills installed treatment systems in order to consistently follow these regulations (McMaster *et al.* 2003).

The Pulp and Paper Effluent Regulations (PPER) program came into effect in Canada through adoption of the 1971 Fisheries Act, which set limits on effluents released by pulp and paper mills (McMaster *et al.* 2003). The goals were to eliminate acute toxicity caused by pulp and paper mill effluent, in addition to setting limits for TSS and BOD (McMaster *et al.* 2003). Throughout the 1970s and 1980s, the chemical identification and analysis of resin acids, fatty acids, chlorinated phenolics, dioxins, and furans in effluents was of primary concern in order to reduce their toxicity (McMaster *et al.* 2003, Owens 1996, Owens 1991). AOX became a primary measurement for regulating several Scandinavian mills (McMaster *et al.* 2003, Folke 1996), and chronic bioassays using species such as the fathead minnow (*Pimephales promelas*) and *Ceriodaphnia* were designed in order to assess chemical toxicity (Owens 1996).

In 1992, PPER was updated in order to set regulatory binding limits to TSS and BOD levels (McMaster *et al.* 2003). It also requires mills to perform environmental effects monitoring (EEM) in order to evaluate whether fish, fish habitat, and the use of fisheries resources are affected by the release of effluents into waterways. Periodic studies on fish populations, benthic invertebrate communities, and fish tissues are required in order to establish a consistent nationwide program for effluent monitoring. The purpose of EEM is also to evaluate whether PPER is able to achieve the desired goals. It has been reported that PPER reduced dioxin and furan release into the aquatic

environment around mills by over 99%, BOD by 94%, and TSS by 70%. Canadian pulp mills have been able to meet BOD and TSS limits at a rate of 99.8% and rainbow trout acute lethality limits at a rate of 94.9% (McMaster *et al.* 2003).

PPER also includes short-term acute bioassays involving either fathead minnows or rainbow trout (*Oncorhynchus mykiss*) for mills releasing effluent into freshwater, and inland silverside (*Menidia beryllina*) or topsmelt (*Atherinops affinis*) for mills releasing effluent into marine waterways (McMaster *et al.* 2003). These bioassays have been useful for assessing effluent quality over time and improvements to mill treatment systems, as well as examining sublethal effects caused by exposures to pulp and paper mill effluents (McMaster *et al.* 2003).

1.2.5 Biomonitoring

Using live organisms to assess aquatic conditions, also known as biomonitoring, allows development of an overall picture of the effects of pollutants in that environment (Kime 1998). This approach is especially useful when considering the unknown effects of chemicals. Biomonitoring can successfully detect the accumulation of pollutants, damaged immune systems, changes in lifespan over generations, or the reproductive fitness of individuals or a population (Kime 1998). It is a useful tool for assessing the effects of sublethal stress, predicting future trends, and providing evidence into cause and effect relationships at both the community and ecosystem levels (Adams *et al.* 1989). Measuring a number of responses of live organisms also allows for an increased chance of observing a significant effect of the pollutant. For example, monitoring reproductive rates, behaviour, and morphology provides a more complete picture of impacts from pollutants than monitoring morphology alone.

Biomonitoring may be extended to the cellular level by examining the concentration and activity of toxicant-responsive biomacromolecules, which are known as biomarkers. This technology has been introduced into the effluent monitoring process (Owens 1996, Kloepper-Sams and Owens 1993). Stressors have been shown to cause biochemical or cellular changes that lead to a response in a particular organism (van der Oost *et al.* 2003, Kloepper-Sams and Owens 1993). These stressors can be physical or chemical factors found in pulp and paper mill effluents (Kloepper-Sams and Owens 1993). As a result, molecular changes may cause physiological and morphological changes within an organism, and thus ultimately affect the organism, the population, the community, and eventually the ecosystem. These measurable changes in hormone or protein level and/or activity are known as biomarkers. Fish reproduction, steroids, and enzyme induction are thought to be important sublethal effects (Owens 1996). For example, it is hypothesized that some environmental stressors may alter metabolism and detoxification in fish by affecting the P450 enzyme system and cytochrome P450 1A (CYP1A) genes and thus harm the organism (Rees *et al.* 2005). A combination of biomarkers and bioassays using fish have been suggested as a valid method for assessing and linking endocrine disrupting compounds (EDCs) to sublethal effects (Leino *et al.* 2005, van der Oost *et al.* 2003, Kloepper-Sams and Owens 1993).

1.2.6 Changes in wild and caged fish exposed to mill effluents

Early observations of reproductive changes in fish exposed to pulp and paper mill effluents began in the 1970s in Florida (Kovacs *et al.* 2006). Female mosquitofish (*Gambusia affinis holbrooki*) found downstream from a bleached kraft mill were observed with elongated anal fins, a male secondary sexual characteristic (Kovacs *et al.*

2006). During the 1980s, perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) were observed with smaller than normal gonads in Sweden, while white suckers (*Catostomus commersoni*) living downstream from a bleached kraft pulp mill in Terrace Bay, Ontario, were found to have changes in secondary sexual characteristics, delayed sexual maturity, reduced gonad size, circulating sex hormone levels, and fecundity (Kovacs *et al.* 2006, McMaster *et al.* 2003, Munkittrick *et al.* 1998). During the late 1980s, the Terrace Bay mill installed and began secondary treatment, and although effluent quality and acute toxicity improved, biomarker (e.g., hormone and hepatic mixed-function oxygenase [MFO]) changes were still observed (McMaster *et al.* 2003, Parrott *et al.* 2000, Munkittrick *et al.* 1998). MFOs are detoxifying enzymes found in the liver of fish and their activity can be used as one measure of the toxicity of mill effluents (Coakley *et al.* 2001). Sampling performed following a maintenance shutdown in 1990, however, showed an improvement in both fish hormonal system function and lower MFO activity, indicating that impacts from effluents might be short-term and that secondary treatment did not resolve the problem (McMaster *et al.* 2003, Munkittrick *et al.* 1998). Further observations upon effluent exposure at the Terrace Bay site included increases in MFO enzyme and steroid concentrations in lake whitefish (*Coregonus clupeaformis*) and longnose sucker (*Catostomus catostomus*), in addition to physiological changes, such as significant decreases in gonad size and later maturation in lake whitefish (McMaster *et al.* 2003, Munkittrick *et al.* 1997, Munkittrick *et al.* 1995). Janz *et al.* (1997) observed decreases in both ovary size and plasma testosterone levels in white sucker at the same site.

Additional studies at Canadian and American mills showed similar results. White suckers exposed to bleached kraft mill effluent in the St. Maurice River, Quebec, had changes in MFO activity (McMaster *et al.* 2003, Hodson *et al.* 1992, Gagnon *et al.* 1994), and reproductive steroids, including lower levels of 11-ketotestosterone in males, and higher testosterone levels in females (McMaster *et al.* 2003, Gagnon *et al.* 1994). White suckers in the Spanish River, Ontario, also were found to have changes in MFO activity, although reproductive effects were minimal (McMaster *et al.* 2003, Servos *et al.* 1992). In Florida, reduced gonad size, altered sex hormone levels, and decreased vitellogenin in females were observed in largemouth bass (*Micropterus salmoides floridanus*) exposed to bleached kraft mill effluent in the St. Johns River (Kovacs *et al.* 2006, McMaster *et al.* 2003, Sepúlveda *et al.* 2002).

A review by Sandström (1996) on the possible effects of pulp mill effluents on wild fish populations from 1983 to 1993 showed that in 8 of 10 populations that were studied, sexual maturation was delayed. In 4 of 6 species (14 of 24 studies), gonad size was reduced compared to normal populations, and in 3 of 5 studies, a change in fecundity was observed. Munkittrick *et al.* (1998) and McMaster *et al.* (1995) suggested that changes in endogenous steroid levels were most correlated with these types of reproductive changes.

1.2.7 Improvements in treatment processes leading to recovery

Despite the consistent changes in fish fecundity found in waterways in the vicinity of several pulp and paper mills, not all mills have been shown to cause reproductive changes in wild fish populations. In fact, several studies have shown improvements and recovery of fish populations living in the vicinity of the mill over time. For example,

studies performed at the Wapiti River in Alberta downstream from a bleached kraft mill showed little change in cytochrome P4501A induction (a biomarker for MFO activity) and no significant physiological changes in longnose sucker or mountain whitefish compared to control populations (McMaster *et al.* 2003, Kloepper-Sams and Swanson 1992). This mill used chlorine substitution bleaching and its effluent passed through secondary treatment (Munkittrick *et al.* 1997). Changes to chlorine dioxide substitution bleaching at the Terrace Bay mill in 1993 coincided with a decreased effect on steroid and hormonal changes in goldfish and wild fish, as well as recovery of female gonad size and secondary sexual characteristics in male fish, although not in MFO activity (Munkittrick *et al.* 1997). It is unknown whether the change to chlorine substitution bleaching was the primary factory that led to these improvements (Munkittrick *et al.* 1997).

Effluents from mills in Kapuskasing and Smooth Rock Falls in Ontario in 1991 increased liver detoxification enzyme activity and liver sizes, decreased plasma sex steroid hormone levels, decreased gonad sizes, and induced MFO (Munkittrick *et al.* 1997). By 1995, gonad sizes in white sucker had began to increase near the Kapuskasing mill and liver sizes at both Kapuskasing and Smooth Rock Falls sites had began to return to near normal levels (Munkittrick *et al.* 1997). Changes to the mill treatment processes at these sites after the 1991 studies included the installation of an activated sludge treatment system at Kapuskasing and the installation of an aeration lagoon in Smooth Rock Falls. Kapuskasing switched to TMP pulping over that time and Smooth Rock Falls began using 100% chlorine dioxide substitution bleaching.

Several studies in the United States also reported recovery in fish populations after improvements to pulp production systems and effluent treatment during the 1990s. Researchers found that fish community structure, sex ratios, and MFO induction measured after changes in the pulping process improved near a bleached kraft pulp mill on the Pigeon River, Tennessee (Munkittrick *et al.* 1997). The masculinization of females also was reported to be decreasing in Eleven Mile Creek and the Fenholloway River in Florida (Munkittrick *et al.* 1997), although Toft *et al.* (2004) reported changes in sexual characteristics of mosquitofish in the Fenholloway River during a 2001 study. Worldwide, despite modifications to mill effluent treatment systems, changes in fish reproductive systems continue to be observed (Hewitt *et al.* 2008). Many of these changes are ascribed to pulp effluent component effects on the endocrine system of aquatic vertebrates.

1.3 Endocrine system

1.3.1 Endocrine disruption

The endocrine system is a hormone signal transduction chain within an organism that is used to regulate a large number and variety of biological processes, such as body fluid homeostasis, stress, reproduction, and fertility (Kime 1998). Nearly all organisms, whether vertebrate or invertebrate, have an endocrine system. The testes, ovaries, liver, pituitary gland, hypothalamus, and thyroid gland are all part of the endocrine system (Kime 1998). Endocrine disruption occurs as a result of either a natural or synthetic substance that interferes with the normal communication taking place between cells using chemical messengers (Larkin *et al.* 2003, EC 1999, Kime 1998). Disruption may occur if a compound mimics a natural hormone by binding to a receptor and causing a similar

response (agonist response), or if a compound binds to a receptor and prevents a normal response (antagonist response) (EC 1999, Kime 1998) (figure 1).

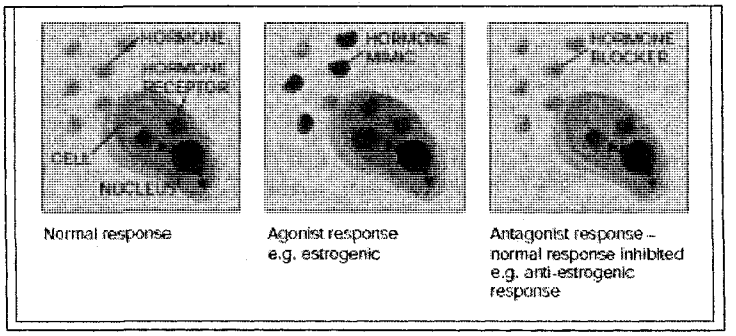

Figure 1 - Endocrine disruption responses (from EC 1999)

1.3.2 Endocrine disrupting compounds (EDCs)

Endocrine disrupting compounds (EDCs) are released from a variety of sources, including municipal and industrial effluents, agricultural runoff, and incinerators and landfills (EC 1999). Over 87,000 chemicals have been identified that could potentially act as EDCs, but how to appropriately test all of these chemicals for toxicity is a significant challenge (EDSTAC 1998).

Compounds present in pulp and paper mill effluents that have been investigated as potential EDCs include genistein, abietic acid, β-sitosterol, stigmastanol, campesterol, and stigmasterol (Hewitt *et al.* 2008, Parrott *et al.* 2006, van den Heuvel 2004, Cook *et al.* 1997). These compounds are grouped into three categories: polyphenolics, plant sterols, and flavones (van den Heuvel 2004). Polyphenolics, including lignins, have been linked to a reduction of steroid hormone production (van den Heuvel 2004). Plant sterols include β-sitosterol, stigmastanol, campesterol, and stigmasterol, and are suspected to cause androgenic or estrogenic effects (Hewitt *et al.* 2008, Parrott *et al.* 2006, van den

Heuvel 2004). Flavones include genistein, which has been linked to estrogenic effects (van den Heuvel 2004).

Although the previously mentioned plant-derived compounds are suspected of causing endocrine disruption, their modes of action are still in question. It is believed that the structural similarity of these compounds to cholesterol or related steroid hormones plays a significant role. For example, plant sterols are suspected of disrupting the endocrine system because of their structural similarity to cholesterol (figure 2) (Gilman *et al.* 2003). Cholesterol is the chemical precursor of all of the steroid hormones and is also the main sterol in vertebrates (Gilman *et al.* 2003). β-sitosterol has been shown to reduce plasma cholesterol levels and gonadal steroid production in fish, although the mechanism of action is still largely not understood (Gilman *et al.* 2003).

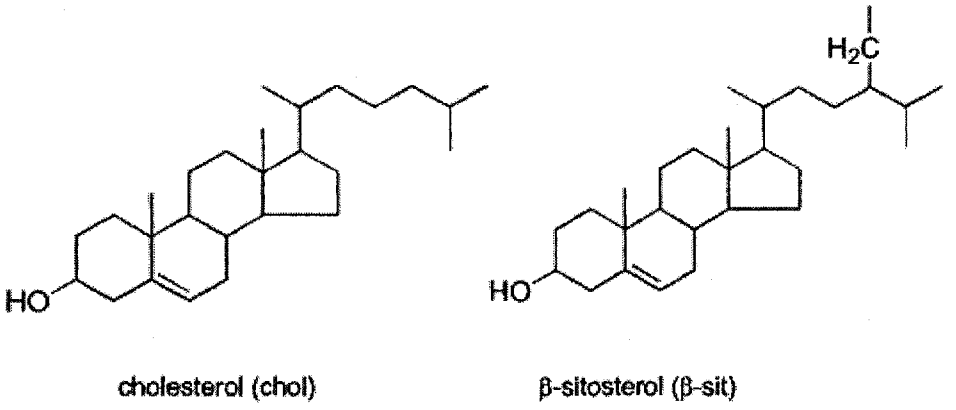

Figure 2 – The chemical structures of cholesterol and a suspected EDC, β-sitosterol (from Gilman *et al.* 2003). Other plant sterols, such as campesterol, stigmasterol, and stigmastanol, are also structurally similar to cholesterol.

Further compounding difficulties in our understanding of EDCs is that studies with pulp and paper mill effluents have shown that different fish species may respond uniquely to exposure and that some documented hormonal changes may not necessarily

lead to reproductive changes (Munkittrick *et al.* 1998). Still, effects have been observed to persist up to 95 km downstream from effluent release sites (McMaster *et al.* 2003, Hodson *et al.* 1992), and as a result effective standardized bioassays are required for assessing effluents and the effects of EDCs that may be present. The major component of these bioassays is a fish species to use as a predictive model for gauging the response of the aquatic environment to challenge with toxicants.

1.4 Choosing a model species for ecotoxicological research

1.4.1 Fathead minnow (*Pimephales promelas*)

The fathead minnow (*Pimephales promelas*) belongs to the family Cyprinidae and is a ray-finned, bony fish with a distribution that includes most of North America, extending from New Brunswick to Alberta, Canada, and south to Mexico (Watanabe *et al.* 2007). It is a relatively short-lived species; adults live for approximately 2 years (Hartviksen & Momot 1989) and become sexually mature in 4 to 5 months (Ankley *et al.* 2001). Mature males weigh 4 to 5 grams and mature females weigh 2 to 3 grams (Ankley *et al.* 2001). Immature males and females are similar in appearance. They are silver in colour on the sides and dark olive green or brown on the back (Hartviksen & Momot 1989). Mature, sexually active males develop a black, fleshy fat pad that extends from the nape to the dorsal fin, cranial nuptial tubercles, and distinct black vertical bands along their sides. Females do not undergo noticeable morphological changes upon maturity; instead, they develop only a distinct ovipositor near the urogenital opening (Ankley *et al.* 2001) (figure 3).

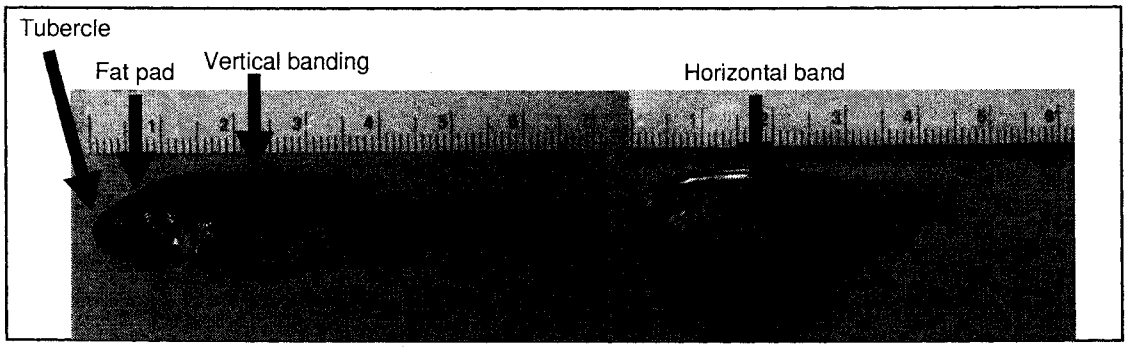

Figure 3 – Typical sexually mature male and female fathead minnows. Distinct characteristics of the male fathead minnow (left) include prominent nuptial tubercles, a fleshy fat pad, and vertical banding. Distinct characteristics of the female fathead minnow (right) include a horizontal band and the ovipositor. Ruler measurements are in cm.

Breeding behaviour begins with the males, who search out a suitable nest site, clean it, and defend it from other males. Suitable nests can consist of overhanging logs, rocks, plants or any other similar overhead structure (Watanabe *et al.* 2007). A single female enters the nest and is stimulated by the male pressing the female upwards against the nest. The female releases the eggs against the nest while the male releases milt. Generally, 50 to 150 fertilized eggs subsequently become attached to the nest (Ankley *et al.* 2001). The male continues to protect and clean the nest until the eggs are hatched. Females normally spawn in 3- to 4-day intervals and embryos typically hatch between 4 and 5 days later. Feeding begins within 2 days of hatching (Ankley *et al.* 2001).

1.4.2 Fathead minnow as an EDC test species

The fathead minnow has been used in environmental toxicity assessment since the 1950s and is one of the most commonly used test species for regulatory ecotoxicology work (Ankley and Villeneuve 2006). Several standardized test procedures exist for using fathead minnows in toxicity and EDC testing.

Short-term lethality tests are frequently used to assess the toxicity of both new and existing chemicals and pollutants, or to determine a concentration range for longer, more extensive testing (Ankley and Villeneuve 2006). These can be either 48 or 96 hours in duration and normally use juvenile fathead minnows.

Full life-cycle tests are extensive and begin with less than 24 hour old embryos. Fathead minnows are tested through maturity and into reproduction of the F1 generation, generally being completed 30 days into the F1 generation (Ankley and Villeneuve 2006). Full life-cycle tests are normally used for assessing chemicals or pollutants considered to be a potential ecological threat, but as they may last up to 6 months, they are time-intensive and infrequently used (Ankley and Villeneuve 2006).

Partial life-cycle tests are more commonly used and can be a 30-day early life-stage test, a 7-day larval survival and growth test, or a reproduction test (Ankley and Villeneuve 2006). These tests are typical for assessing the lethal and sublethal effects of either single chemicals or mixtures (Ankley and Villeneuve 2006).

The 30-day early life-stage test includes the use of embryos less than 24 hours old until 30 days post-hatch in order to assess effects on survival, growth, and morphology, or to assess chronic toxicity (Ankley and Villeneuve 2006).

The 7-day larval survival and growth test is similar to the 30-day test except that it is shorter in duration, concluding 7 days post-hatch. It is normally done with mixtures of complex chemicals (e.g., pulp and paper mill effluents) and used to determine when mandatory toxicity identification or treatment alterations must be done (Ankley and Villeneuve 2006). The 7-day reproduction and growth test has been one of the short-term

test methods most frequently used by the U.S. Environmental Protection Agency (EPA) (Kovacs *et al.* 1995b).

The reproduction test is recommended for EDC testing as it includes the use of biomarkers and may help predict impacts at the population level (Ankley and Villeneuve 2006). It is performed with mature spawning fathead minnows where reproductive success is measured daily along with assessment of physical appearance, behaviour, and fecundity. Hatching success, developmental rate, and mutations may also be assessed if desired. The gonadosomatic index (GSI) (100 x gonad weight/body weight – gonad weight) of each fish is also measured at the conclusion of the 21- day exposure (Ankley and Villeneuve 2006, Ankley *et al.* 2001). The test utilizes a 14- to 21-day pre-exposure phase, along with a 21-day exposure period in order to assess baseline reproductive output (Ankley *et al.* 2001). Reproductive output is generally determined using breeding groups of 4 females and 2 males with 4 replicates (Ankley *et al.* 2001), although Rickwood and Dubé (2007) recommend pair-breeding (using 1 female and 1 male) in order to accurately determine and relate reproductive output to the individual.

1.4.3 Changes in fathead minnows exposed to mill effluents – Full life-cycle

Full life-cycle laboratory exposures to pulp and paper mill effluents can cause changes in egg production, secondary sexual characteristics, and sex hormone levels, as well as delayed sexual maturation (Hewitt *et al.* 2008, Kovacs *et al.* 2006). These studies have included exposures to effluents from bleached kraft mills, bleached sulfite mills, and TMP mills (Hewitt *et al.* 2008, Kovacs *et al.* 2006).

Changes in egg production may result from exposure to several types of pulp and paper mill effluents. Kovacs *et al.* (1995b) reported a decrease in egg production due to exposure to treated bleached kraft mill effluents at greater than 2.5% (v/v) concentrations, although these changes were not seen in a later 1994-1995 study (Kovacs *et al.* 2002). Similarly, Borton (1997) reported a decrease in egg production at several concentrations of treated bleached kraft mill effluent, although significant changes took place at concentrations of at least 18% (v/v). Parrott *et al.* (2004) found a significant decrease in egg production in fathead minnows when exposed to 10% (v/v) bleached sulfite mill effluent and a complete absence of egg production at concentrations of 30% (v/v) or greater. Changes in secondary sexual characteristics and sex ratios have been reported in bleached kraft mill effluents at greater than 2.5% (v/v) concentrations (Kovacs *et al.* 1995b) and bleached sulfite mill effluents at concentrations of 3.2% (v/v) and higher (Parrott *et al.* 2004, Parrott *et al.* 2003, Parrott and Wood 2002).

However, not all full-life cycle tests using fathead minnows have found changes in reproductive endpoints. Kovacs *et al.* (1995a) found no changes in any reproductive endpoints with fish exposed to secondary treated TMP effluent, while Kovacs *et al.* (1996) found no changes in any reproductive endpoints with fish exposed to bleached kraft mill effluent. Both experiments assessed the endpoints at concentrations of 1.25%, 2.5%, 5%, 10%, and 20% (v/v).

Despite the numerous full-life cycle studies assessing the impacts of different mills using different pulp extraction methods and treatment processes, the reasons for changes in reproductive endpoints are still largely unresolved and poorly understood.

1.4.4 Changes in fathead minnows exposed to mill effluents – reproduction test

Although full life-cycle testing has been performed with pulp and paper mill effluents, it is considered time-consuming and labour intensive (Rickwood *et al.* 2006a). As a result, application of the short-term reproduction test using fathead minnows has been suggested as a useful tool for assessing the impacts of pulp and paper mill effluents on fish reproduction, as well as determining whether or not a response pattern exists in order to assess the effectiveness of effluent treatment and improve it, if necessary (Rickwood and Dubé 2007).

Kovacs *et al.* (2005) exposed breeding groups (4 females and 2 males) for 21 days to 2% (v/v) and 20% (v/v) concentrations of treated outfall effluents from two softwood TMP mills, two kraft mills (one hardwood mill and one softwood and hardwood mill), and one softwood and hardwood multiprocess mill that used chemical and mechanical pulping. In addition, they also exposed breeding groups to 40% (v/v) concentration from the softwood and hardwood kraft mill for 21 days. It was found that effluent did not affect weight, length, or condition factor (K) of males; however, females exposed to 2% (v/v) effluent from one of the kraft mills had reduced caudal tail fork lengths, despite no physiological effects observed at 20% (v/v) concentration. Females exposed to the 40% (v/v) kraft mill effluent had increased weight and condition factor. Egg production decreased only in 20% (v/v) effluent from the multiprocess mill, and percent fertilization and hatch success was affected only in 2% (v/v) effluent from the same mill. Secondary sexual characteristics were not seen to change as a result of exposure to the effluents. Kovacs *et al.* (2005) concluded that the 21-day test can be used to assess the potential of

effluents to affect some reproductive endpoints (including egg production) and that the type of mill did not appear to be related to changes in reproductive endpoints.

Rickwood *et al.* (2006a) exposed breeding pairs of fathead minnows to bleached kraft mill outfall effluent at 100% (v/v) and 1% (v/v) concentrations. Results indicated that 100% (v/v) caused a decrease in egg production and number of spawning events, whereas 1% (v/v) stimulated total egg production over the 21 day exposure. Both 100% (v/v) and 1% (v/v) effluent was also seen to cause ovipositor development in males and 100% (v/v) were seen to cause banding and fin dots (male secondary sexual characteristics) in females.

Rickwood *et al.* (2006b) exposed breeding pairs of fathead minnows to different effluent treatment stages within a bleached kraft mill. This kraft mill included both a softwood and a hardwood mill. GSI, liver somatic index (LSI) (100 x liver weight/body weight – liver weight), K (100 x [body weight/length^3]), weight, cumulative number of spawning events, cumulative number of eggs produced, change in egg production and spawning events from the pre-exposure to exposure periods, and the development of secondary sexual characteristics in both males and females were assessed with breeding pairs exposed to effluent from 5 treatment streams. These streams included 100% (v/v) final treated, 8.5% (v/v) combined mixed effluent (CME), 8.5% (v/v) combined alkaline stream postprimary treatment (CALK), 8.5% (v/v) combined acid (CACID), and 8.5% (v/v) combined stripped condensate (CSC). Final treated effluent in this mill was collected after secondary treatment in an aerated stabilization basin; CME was combined primary treated alkaline stream, combined acid, and combined condensate before secondary treatment; CALK was primary treated alkaline effluent from both mills and

several waste streams; CACID was combined acid filtrates from both mills and several waste streams; and CSC was high contaminated condensates from the first evaporators in the chemical recovery areas from both mills. No significant differences in GSI, K, or weight were seen between any of the treatment groups and the controls. LSI increases were noted in males from all treatments compared to the controls, although only the final treated males were statistically different. The cumulative number of spawning events decreased in the CME and CALK pairs, while the CALK pairs also had a significant decrease in cumulative egg production. Final treated, CME, CALK, and CSC had a significant decrease in total spawning events, and CALK showed a significant decrease in total egg production from the pre-exposure to exposure period compared to controls. Male ovipositor development was seen in the CALK stream and females in the CME were seen to develop male secondary sexual characteristics. No changes in hatch success or larval deformities were seen in any of the treatments.

Rickwood and Dubé (2007) also used the 21-day reproduction test to determine the effects of 100% (v/v) and 50% (v/v) secondary treated bleached kraft mill effluent on fathead reproduction. They found that both GSI and LSI were higher in male fish exposed to 100% (v/v) effluent when compared to the controls; however, no significant changes were seen with GSI or LSI in the females. No changes in secondary sexual characteristics were observed at either concentration, although 100% (v/v) and 50% (v/v) effluent was seen to decrease egg production compared to the controls. Effluent at 100% (v/v) concentration was also seen to decrease the number of spawning events compared to the controls. Rickwood and Dubé (2007) also observed a decrease in hatching success

and increase in larval deformities as a result of exposure to both concentrations of secondary treated effluent.

Although the above studies have addressed several issues concerning the effect of pulp and paper mill effluent on fish development, there is still significant concern regarding the impacts of these effluents on the environment. As a result, additional studies are needed that include different pulp and paper mills employing a variety of pulp extraction and treatment methods in order to improve our understanding of the mode of action of EDCs. This thesis project addresses some of these data gaps.

1.5 PAPEER, Research objectives, and research questions

1.5.1 Pulp and paper effluent ecotoxicology research (PAPEER)

The Pulp and Paper Effluent Ecotoxicology Research (PAPEER) project collaboratively links several interrelated projects with the broad goal of improving our understanding and monitoring of pulp and paper mill effluents and their environmental and toxicological effects. The research of this thesis is a primary component of PAPEER. Research within PAPEER conducted in the Molecular, Environmental and Developmental Biology Lab (MEDAL) group at Lakehead University includes: assessing the impacts of pulp and paper mill effluents on fathead minnow reproduction and physiology; assessing immediate gene expression changes resulting from exposure to pulp and paper mill effluents; assessing gene expression changes using fathead minnow and rainbow trout liver cell cultures exposed to pulp and paper mill and sewage effluents; and developing effluent monitoring tools using DNA microarrays. The data obtained from the fathead minnow reproduction test developed during this thesis work provided tissue samples for the gene expression studies. Together, this work will provide a more

complete understanding of the effects of pulp and paper mill effluent on vertebrate development and reproduction. The PAPEER group's ultimate goal is to determine genome-wide changes in gene expression in effluent-exposed fish, and to use this data to develop an environmental test suitable for industrial effluent monitoring.

1.5.2 Research objectives and rationale

The primary objective of the work in this thesis is to complete the first step of PAPEER. Specifically, this involves assessing the impacts of pulp and paper mill effluent on fathead minnow reproduction and physiology using a short-term reproduction test that is similar to that of Ankley *et al.* (2001). A shorter 6-day exposure period was chosen instead of the standard 21-day test in order to allow for step 2 of PAPEER to proceed, as toxicant-responsive organs (liver, gonads, gills, brain, and kidneys) obtained from the 6-day exposure period were designated for gene expression analysis.

1.5.3 Hypotheses and research questions

The primary endpoint of the MEDAL reproduction test developed as part of the work in this thesis is fathead minnow egg production. This test assumes that egg production is relatively constant and predictable over the 15-day pre-exposure period and should be relatively constant during the 6-day exposure period if treatments have no effect on reproduction. As such, it is necessary to test this hypothesis (research question #1). The second assumption made in the MEDAL reproduction test is that egg production will be consistent during the pre-exposure and exposure periods. Testing this hypothesis will allow us to address several effluent treatment and effluent quality issues

(research questions #2 and #3). Lastly, assessment of the 6-day exposure period will be provided in order to determine its overall validity (research question #4).

Overall, the following research questions will be addressed:

1) Are fathead minnows predictable spawners that can be used in a short-term reproduction test, as reported by Ankley *et al.* (2001)?

2) Does treatment improve effluent quality, as measured by reproductive and physiological changes in fathead minnows?

3) Does short-term exposure to pulp and paper mill combined mill outfall (CMO) cause reproductive and physiological changes in fathead minnows?

4) Is a 6-day, short-term test method useful for determining reproductive changes as a result of exposure to pulp and paper mill effluents?

2.0 Study site, materials and methods

2.1 Pulp and paper mill

2.1.1 AbitibiBowater

AbitibiBowater (formerly Bowater Canadian Forest Products) is situated on the Kaministiquia River in Thunder Bay, ON, Canada. It began operating in 1926 and is one of the largest pulp and paper mills in Canada. The AbitibiBowater Thunder Bay site includes both a kraft pulp mill and a newsmill. The kraft mill produces softwood and hardwood pulps, while the newsmill produces TMP pulp and operates two paper machines. In addition to TMP, recycled pulp and some kraft pulps are used for paper making in the newsmill (B. Lindberg and C. Walton, pers. commun.).

At AbitibiBowater – Thunder Bay, newsmill TMP is made primarily from white spruce and black spruce. Newsmill products include newsprint, telephone directories, basestock for flyers, and bowbook for novels. Hardwood kraft pulp is made primarily from trembling aspen, and softwood kraft pulp is made mostly from jack pine (*Pinus banksiana*), white spruce, and black spruce, although balsam fir (*Abies balsamea*) and tamarack (*Larix laricina*) are also sometimes used. Kraft products include paper towels, toilet paper, and office papers.

AbitibiBowater Thunder Bay uses substituted chlorine dioxide bleaching in both the kraft mill and newsmill to obtain brightness of 90% ISO (International Standards Organization) in the pulp. The kraft mill and newsmill are able to produce 1,100 tonnes of pulp and 1,100 tonnes of paper per day, respectively.

2.1.2 Effluent treatment process

The effluent treatment process at AbitibiBowater was updated in November of 2006. Effluents are treated in one of two streams (Figure 4). Acid kraft and alkaline kraft effluent are combined and treated, whereas neutral kraft is first combined with newsmill effluent and then treated together. The combined newsmill-neutral kraft effluent undergoes primary treatment in two primary clarifiers and secondary treatment in an activated sludge plant using on-site produced oxygen (UNOX), before undergoing further clarification in the secondary clarifiers. Acid-alkaline kraft effluent does not undergo primary treatment and is directed into the activated sludge plant before undergoing secondary treatment in one of two clarifiers.

The AbitibiBowater-Thunder Bay UNOX activated sludge plant used for secondary (biological) effluent treatment employs species of Ciliates, Flagellates, Amoeboids, Rotifers, and Nematodes (C. Walton, pers. commun.). The holding capacity of each of the two kraft secondary clarifiers is approximately 18,200 cubic metres of effluent. The single news secondary clarifier can hold nearly 27,000 cubic metres of effluent. Effluent is held in the oxygen reactor (UNOX sludge plant) for approximately 5 to 7 hours and in the secondary clarifiers for approximately 2 to 3 hours (C. Walton, pers. commun.).

Some lignin and waste chemicals from kraft processing are not released in the effluent but are instead captured in the recovery boiler. These black liquor wastes are burned to generate steam and additional power at the AbitibiBowater site.

2.1.3 Effluent release

Effluent from the newsmill and kraft secondary clarifiers are combined and released into the Kaministiquia River. Approximately 60% of the outfall effluent is newsmill effluent and approximately 40% is kraft mill effluent. Effluent flow into the river averages approximately 110,000 cubic metres per day and effluent concentrations at the outfall range from approximately 2 - 6% depending on flow conditions in the Kaministiquia River.

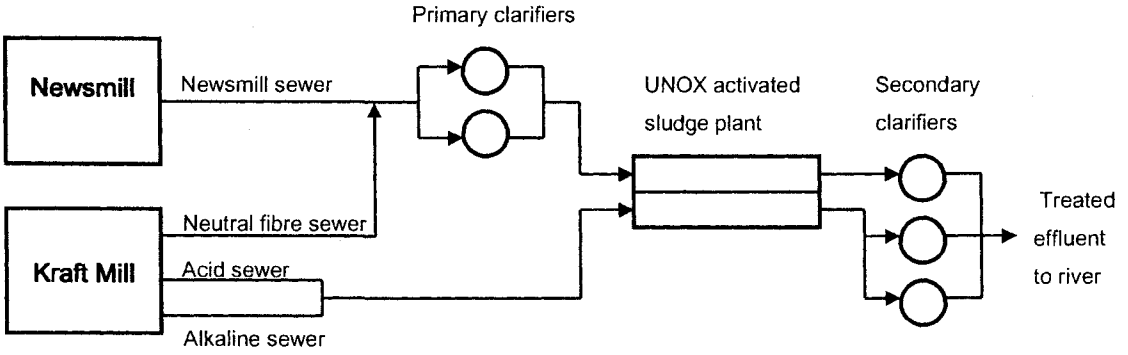

Figure 4 – Effluent treatment process of AbitibiBowater - Thunder Bay (modified from Hardy 2002).

2.2 Bioassay design

2.2.1 Introduction

All laboratory work was performed on-site at the AbitibiBowater mill in Thunder Bay, Ontario. In order to expose breeding fathead minnows to pulp and paper mill effluent, an on-site bioassay was designed. The original storage and flow design was developed by Hardy (2002) and modified for Ingram (2006). The system was further modified for its current use with the fathead minnow reproduction test (figure 5). This

bioassay system consisted of 3 parts: effluent collection, a cold room and holding tanks, and a laboratory flow-through system. It was distributed over 3 floors in the newsmill.

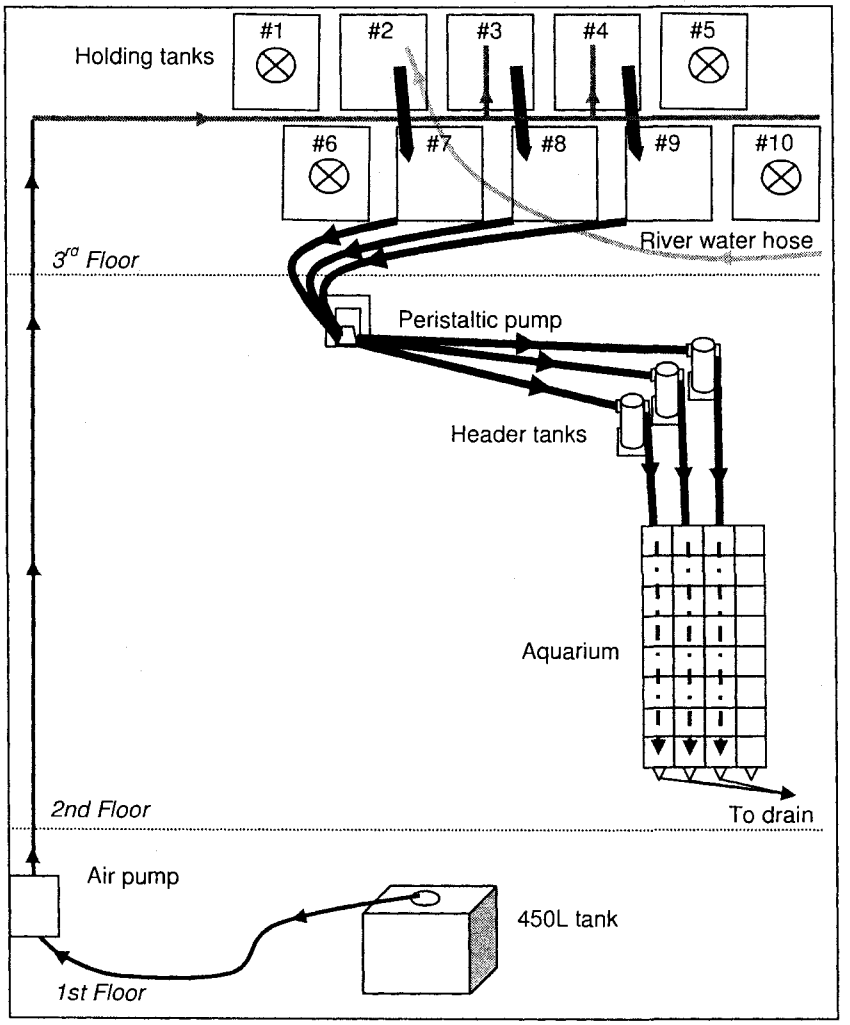

Figure 5 – Diagram of the bioassay setup at AbitibiBowater - Thunder Bay.

2.2.2 Effluent Collection

Effluent was collected using a 450-L Equinox polypropylene tank and a truck (figure 6a). Effluent was pumped into the tank using a Honda WX10 4-stroke gas powered pump (model #WATJ 1017391). The effluent was then brought to the 1st floor

of the paper mill (figure 6b) where a wall mounted Selfilco air-operated diaphragm pump (model #C-55-7120A, connected to ¾" HDPE tubing) (figure 6c) was used to pump the effluent into the cold room for holding and chilling.

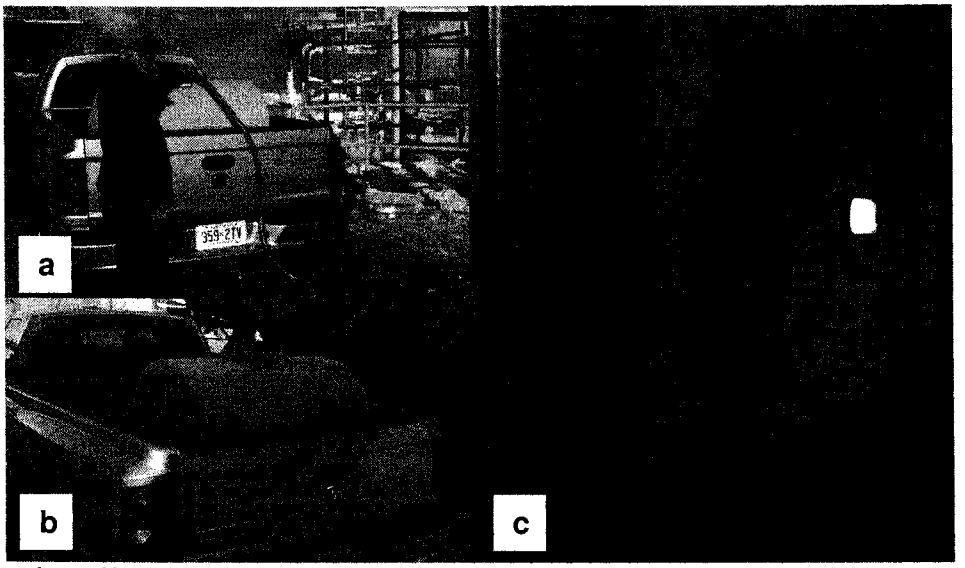

Figure 6 – Effluent collection steps at the AbitibiBowater - Thunder Bay pulp and paper mill: a – effluent collection. b – effluent transportation. c – pump used to transport effluent into the cold room

2.2.3 Cold room and holding tanks

A 7.62 x 2.44 x 2.44 metre cold room on the 3rd floor of the paper mill was used to hold river water and effluent at 4±2 degrees Celsius during the duration of the test period (figure 7a). Effluent was stored for no longer than 5 days after collection prior to use in the bioassays. A water-cooled Keeprite condenser (model #KW300M) and Keeprite evaporator coils (model #KUC153A) were used in order to maintain temperature and thus chemical composition of the effluents. Ten 1050-L polypropylene tanks (numbered 1 through 10) were arranged in two rows of five tanks each in the cold room (figure 7b). Chlorinated river water for controls or effluent dilution was pumped

into the tanks through a hose located outside of the cold room, and effluent was pumped in from the 1st floor through the HDPE tubing. The HDPE tubing was connected to a ½" 316SS header pipe located against the top row of tanks. 316SS valves attached to ¾" HDPE tubing were used to control flow from the header pipe into the upper row of tanks.

Tanks #2 and #7 were used only for holding river water. Tanks #3, #4, #8, and #9 were used for holding river water during the acclimation and pre-exposure phases of the bioassay and effluent during the exposure periods. Tanks #1, #5, #6, and #10 were not used during the fathead minnow reproduction test. River water or effluent was filled in tanks #2, #3, and #4 prior to use in the laboratory flow-through system. When the contents were ready to be used, they were emptied into the lower tanks through a ¾" water heater hose attached to a ¾" valve. An agitator motor (1/3 HP Farm duty MixPro Marathon F101 with a 41" x 0.625" 316SS shaft and 3.5" 316SS diameter impeller) was used to stir the contents of the lower tanks, as well as to dechlorinate the river water (figure 7c). Tanks #7, #8, and #9 were connected to the 2nd floor laboratory by ½" HDPE pipes. Effluent dilution, when necessary, was performed using river water. Dilution took place in the holding tanks to provide time to dechlorinate the river water and to provide accurate dilution concentrations.

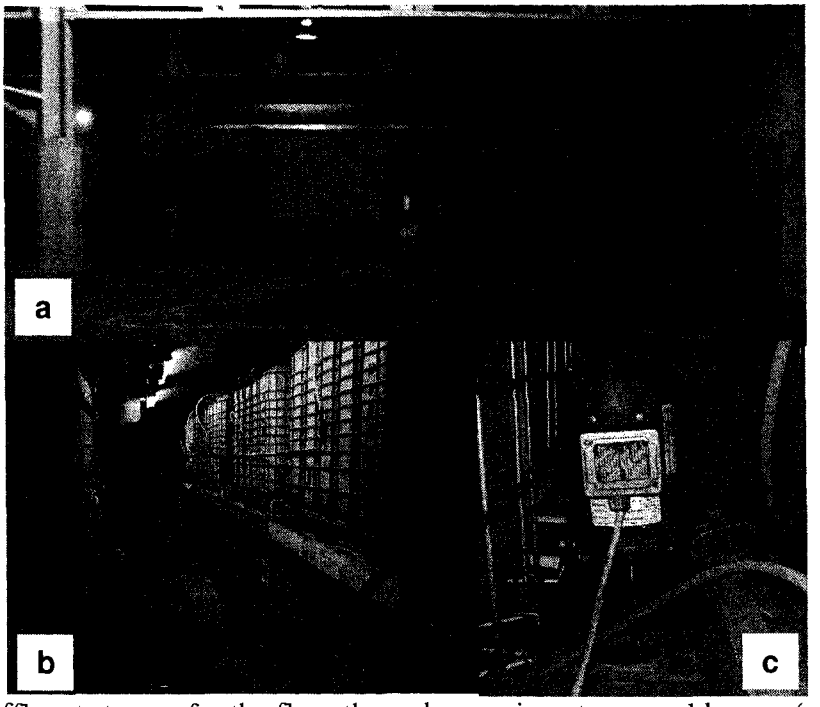

Figure 7 - Effluent storage for the flow-through experiments: a – cold room (outside) on 3rd floor of AbitibiBowater newsmill. b - cold room (inside) and 1050L storage tanks. c - agitator motor used for stirring and dechlorination

2.2.4 Laboratory flow-through system

River water and effluents were pumped into the 2nd floor laboratory through HDPE pipes connected to Masterflex C-flex L/S 17 tubing and run through a Masterflex L/S 7-200 rpm economy drive peristaltic pump (model #07519-05), Masterflex cartridge pump (model #7519-05), and large L/S cartridges (model #07519-70) into three separate header tanks (figure 8a). The contents of the header tanks were heated by two aquarium heaters (one 150W and one 200W) and stirred to maintain effluent homogeneity using magnetic stir plates located under a spill tray (2" magnetic stir bars and VWR magnetic stirring plates – model #361) (figure 8b). River water and effluent flowed out of the header tanks through Fisherbrand clear PVC tubing attached to the opposite end of the incoming flow. From there, the contents entered the flow-through aquarium (figure 8c).

The aquarium was made from clear acrylic plastic and was divided into 4 independent flow-through columns. Each column measured 160 cm in length and was divided into 8 individual compartments measuring 15 cm in width, 20 cm in length, and 17.55 cm in height (figure 9). Each compartment held approximately 5.2 L. Divisions between columns were 20 cm in height in order to prevent spilling and overflow between columns. A 4.5 mm gap was present at the bottom of each compartment within a column in order to allow river water and effluent to flow through the compartments.

Air stones were placed in every second compartment, beginning with the first compartment of the aquarium, in order to maintain dissolved oxygen levels above 60% and effluent mixing within a compartment. Air flow through the airstones was highest in the first compartment and was lower in the remaining compartments in order to avoid unnecessary disturbance to the fathead minnows.

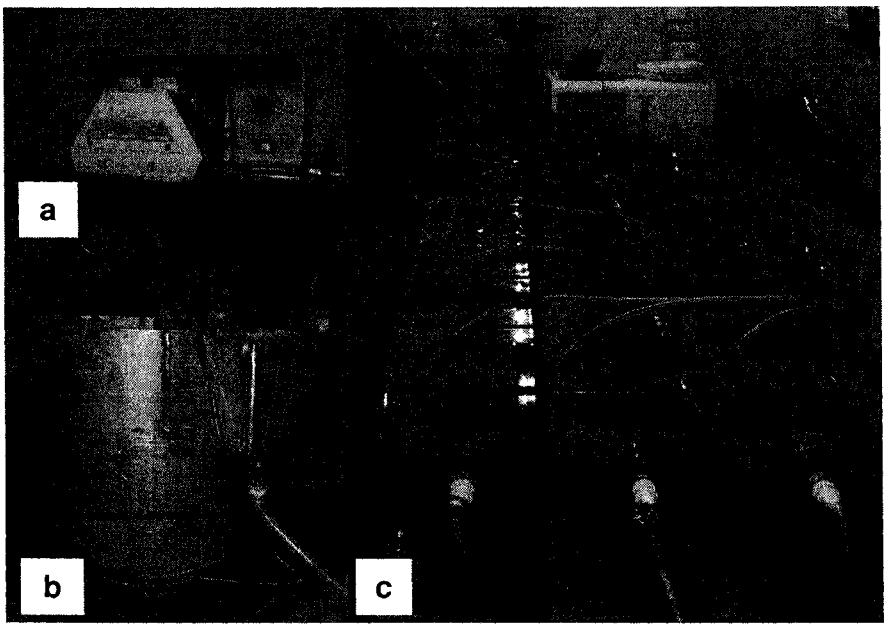

Figure 8 - Flow-through aquarium setup: a – peristaltic pump used to control flow from the cold room to the header tanks. b – header tank with 2 aquarium heaters. c – flow through aquarium with spawning substrates and aeration.

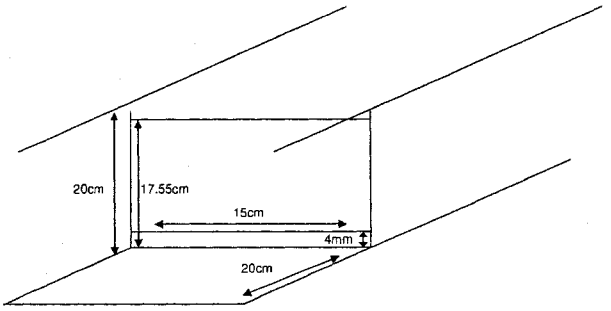

Figure 9 - Dimensions of a compartment within the flow through aquarium. Each compartment housed a breeding pair of fathead minnows and a spawning substrate (modified from Hardy 2002).

2.2.5 River Water

River water was chosen as a reference to ensure that the results were as environmentally relevant as possible. In order to ensure that river water was an appropriate control, samples were taken from the holding tank and aquarium during the pre-exposure period of the first preliminary flow-through experiment and brought to the Lakehead University Environmental Laboratory (LUEL) for analysis prior to beginning the exposure period. Samples from the holding tank were again taken and brought to LUEL during the final experiment to verify consistency within the control. River water used within the bioassay was pumped upstream from the AbitibiBowater site through an intake pumphouse that provided water from a tap next to the coldroom and holding tanks.

2.3 Fathead minnow reproductive bioassays

2.3.1 Introduction

The fathead minnow reproductive bioassay that was used was based on that of Ankley *et al.* (2001). Overall, 7 experiments were performed. Experiments 1 through 3

were performed to assess the flow-through system, methodology, and allow for a basis for effluent concentrations chosen for experiments 4 through 7. Several changes in experimental methods occurred as a result of preliminary testing.

Fathead minnows used for preliminary testing were obtained from Paprican, Montreal, Canada. The remaining fathead minnows were obtained from Aquatic Bio Systems, Colorado, USA. Prior to their use at the AbitibiBowater laboratory, male and female fish were held at Lakehead University (CB 0026J) in separate 830-L holding tanks. These tanks possessed a flow-through system fed with dechlorinated municipal water, and a controlled photoperiod (16 h light: 8 h dark). During holding at CB 0026J, fish were fed twice daily (Nutrafin basix Goldfish Food by Hagen in the morning and frozen Brine Shrimp, *Artemia* with nutrients from Aquamarin in the afternoon). AbitibiBowater experiments were performed between January 2007 and February 2008.

2.3.2 Requirements of the fathead minnow reproduction test

In order to assess the reproductive rate of fathead minnows, a single spawning substrate (1/2-cut PVC piping – 10 cm diameter, length 7 to 10 cm) (figure 10) was placed in each compartment within the flow-through aquarium. Females released the eggs directly onto the underside of the spawning substrate in a single layer. Eggs were counted daily after 1 pm to avoid disruption of spawning which has been reported as normally taking place prior to 10 am (Ankley *et al.* 2001) and were photographed for accurate data assessment. Fathead minnows used during the tests were between 5 and 7 months of age and were sexually naive. Temperature requirements were 25 (±1) degrees Celsius with extreme fluctuations of ±2 degrees Celsius. Water temperature was measured daily. Lighting requirements included a photoperiod of 16 hours of light and 8

hours of darkness (7 am to 11 pm light) using wide-spectrum fluorescent lighting with light intensity of 10-20 $\mu E/m^2/s$ at the surface of the aquarium. pH was maintained between 6.5 and 8.5 and adjusted with concentrated HCl or NaOH if necessary. pH measurements were taken at least every 2nd day. Dissolved oxygen (DO) was required to be greater than 60% for minnow survival and preferably between 80 and 100% for reduced stress. DO measurements were taken at least every 3rd day (every 2nd day was recommended). Conductivity was also measured at least every 3rd day. Fish were fed twice each day (as during the holding period), once upon arrival to the laboratory and once after all measurements were taken. Flow rate into the aquarium was maintained at a minimum of 6 exchanges daily (approximately 175 mL/min) and was measured every 3rd day.

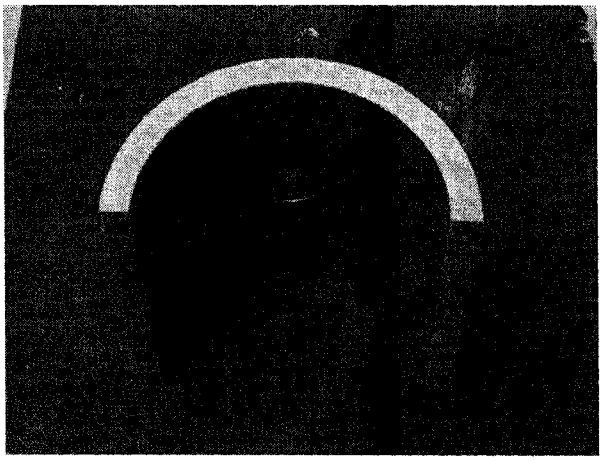

Figure 10 – Spawning substrate for fathead minnow breeding pairs. This consisted of ½ of a PVC pipe. Eggs were released directly onto the underside of the substrate by the females.

2.3.3 Dissolved Oxygen (DO), pH, and conductivity measurements

DO was measured using a portable DO reader (Fisher accumet portable AP64) (figure 11a). The DO probe was calibrated once every third use (approximately every 6^{th} day). pH was measured using a benchtop pH meter and probe (Orion 410A pH meter with Orion Ag/AgCL electrode (model #917007) (figure 11b). The pH meter and probe underwent a 3-point calibration at pH 4, 7, and 10 at least every second use (every 4th day). Conductivity was measured using a benchtop conductance meter (YSI model 35) (figure 11c). Accuracy of the conductance meter was verified using quality control solutions (conductivity 100 μs and 1000 μs) prior to each experiment. Measurements were taken twice in each column, once upon flow entering the aquarium and once prior to flow exiting the aquarium, in order to ensure similar DO, pH, and conductivity within the flow through system.

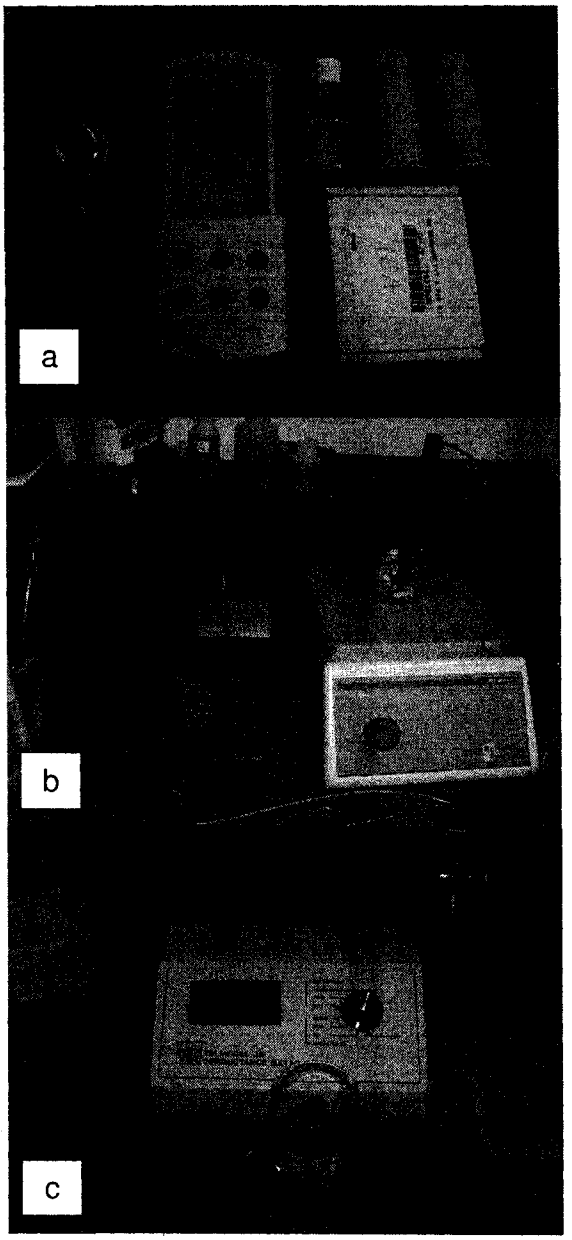

Figure 11 - Apparatus used for water quality monitoring at AbitibiBowater – Thunder Bay: a – portable DO probe and reader. b – benchtop pH meter. c – conductance meter.

2.3.4 Acclimation Period

Fathead minnows underwent a strict acclimation period to minimize their stress. Fish were not fed for 24 hours before transportation to AbitibiBowater. Acclimation took place over a period of 1 day. This began with removal of the fathead minnows from the holding tanks at Lakehead University. For this process, water from the fish tanks was used to fill 3 clear plastic bags that were placed into a styrofoam cooler. Fish were taken from the aquaria and placed in the plastic bags as quickly as possible to minimize stress. Males and females were placed in separate bags. Three extra fish (of any sex) were placed in the 3rd plastic bag. The Styrofoam coolers were sealed using duct tape to maintain the temperature during transportation. The coolers were then brought directly to AbitibiBowater (approximate transportation time of 30 minutes).

To acclimate the fish at AbitibiBowater, the coolers were opened immediately upon arrival to the laboratory. The laboratory flow-through aquarium was filled with river water prior to arrival and the aquarium heaters in the head tanks were set to 28°C in order to maintain a temperature of 25°C. Flow was also maintained at approximately 200 mL/min. The 3 extra fish were used to ensure that the flow-through aquarium and river water were clean and non-toxic to the fish. This process included the following steps:

1) The bag of 3 extra fish slowly received river water until it was full.
2) If the 3 fish appeared stress free (no rapid breathing, difficulty swimming, loss of balance, etc.) then they were placed into the flow-through aquarium for 30 minutes in order to continue acclimation.
3) At this point the rest of the water was siphoned from the bags into the flow through aquarium.

4) Fish were monitored for 30 minutes.

Steps 1 through 4 were repeated for the male and female fish. If <5% of the fish died or appeared highly stressed then they were replaced with the extra fish undergoing acclimation. If >5% of the fish died or appeared highly stressed, then fish were to be removed from the flow-through aquarium and brought back to Lakehead University. Behaviour and physical characteristics were monitored throughout the acclimation period. Fish were not fed again until 24 hours after acclimation began.

2.3.5 Preliminary experimentation (experiments 1 through 3)

In the first experiment, pairs of fathead minnows (1 male and 1 female) were exposed only to river water during a 5 day pre-exposure period (n=16 pairs). A clog in an HPDE pipe caused a stop in flow into the aquarium and low DO levels (DO<60%), resulting in the death of 15 females and 4 males. As a result, the test was terminated. Light aeration with airstones was added to the header tanks at this point and used during the second experiment.

The second experiment involved a pre-exposure period of 5 days (river water) and an exposure period of 5 days. Breeding pairs of fathead minnows were exposed to either river water (control), 5% (v/v) untreated acid-alkaline kraft (UK), or 25% (v/v) untreated acid-alkaline kraft (UK) (n=5 pairs for each treatment) during the exposure period. Egg production during the pre-exposure period was low in all 3 columns. Two of 5 pairs spawned in two of the columns and 3 of 5 spawned in the remaining column. During the exposure period DO dropped significantly (40%<DO<60%) in the 25% (v/v) UK and all 10 fish in this treatment group died. No deaths occurred in the 5% (v/v) UK treatment group. Airstones were moved from the header tanks directly into the aquarium in order

to improve DO levels throughout the aquarium and were used in this manner for the remainder of the experiments.

Breeding groups of 2 females and 1 male per compartment were used during the third experiment in order to improve egg production. In addition, the pre-exposure and exposure periods were increased to 6 days each. Breeding groups were exposed either to river water (control) (n=4) or 10% (v/v) UK (n=4) during the exposure period. Egg production was adequate during the pre-exposure period as all 8 breeding groups spawned; however, egg numbers were not high enough for accurate comparisons to be made with egg production during the pre-exposure period. For this reason, the pre-exposure period was increased to 15 days. Lastly, using breeding groups of 2 females did not allow for determination of which females were spawning; thus, paired-breeding with 1 male and 1 female was chosen for future testing.

2.3.6 Final experimentation methods

Final experimentation methods involved a 15-day pre-exposure period in which breeding pairs of fathead minnows were placed in river water only and monitored daily for egg production, colouration, and behaviour. Following the pre-exposure period, breeding pairs were exposed to either river water (control) or effluent (treatment) during the 6-day exposure period. The type of treatment was assigned randomly to a particular flow-through column. During the exposure period, fathead minnows were also monitored daily for egg production, colouration, and behaviour. Immediately following the exposure period, the fathead minnows were sacrificed with MS-222 (see appendix 1), measured (total length), weighed, photographed, and dissected in order to obtain liver and gonad weights. Fish heads were removed, brought back to Lakehead University, and

frozen prior to counting tubercles in males and checking for tubercle development in females.

Spawning substrates containing eggs were removed from the aquarium daily and replaced with a clean substrate. Used substrates were cleaned using a mild chlorine bleach solution (approximately 5 to 10 % [v/v] commercial bleach) and rinsed extensively with river water. Substrates were replaced with clean substrates every 3rd day, regardless of whether spawning had taken place or not.

2.3.7 Fathead minnow bioassay using kraft mill effluents

Experiments 4 and 5 involved assessing the impacts of 10% (v/v) UK and 25% (v/v) SK, compared to the river water (RW) control. Following the 15-day pre-exposure period, breeding pairs were exposed to either river water (RW) (control), 10% (v/v) UK, or 25% (v/v) SK (n=14 pairs each for the control and two treatments). 10% (v/v) UK was collected from the acid-alkaline sewer (figure 12a). 25% (v/v) SK was collected from the #1 kraft secondary clarifier (figure 12b).

2.3.8 Fathead minnow bioassay using final outfall effluent

Experiments 6 and 7 involved assessing the impacts of 100% (v/v) combined mill outfall (CMO) effluent compared to the RW control. Following the 15-day pre-exposure period breeding pairs were exposed to either RW (n=19) or 100% (v/v) CMO (n=21). CMO effluent was collected from the CMO outfall sewer (figure 13).

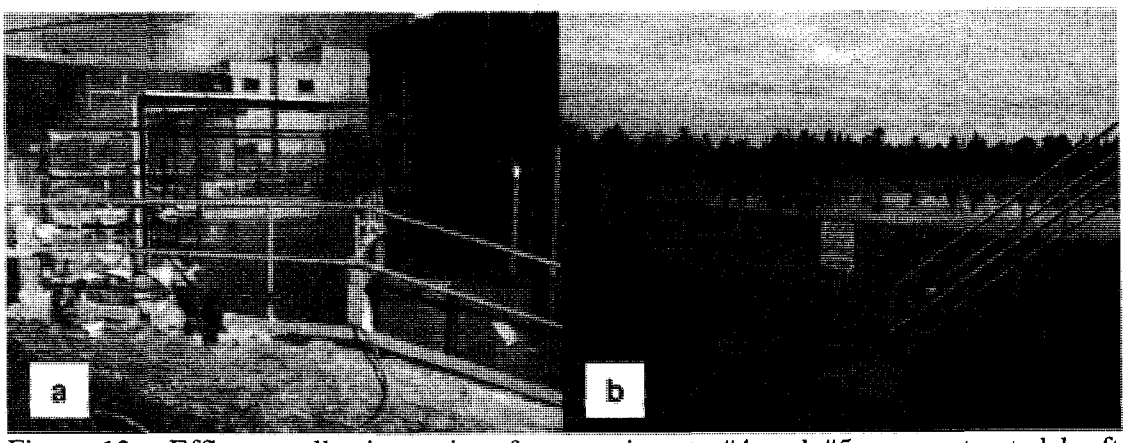

Figure 12 - Effluent collection points for experiments #4 and #5: a – untreated kraft effluent collected from the acid-alkaline sewer. b – secondary treated kraft effluent collected from the #1 kraft secondary clarifier

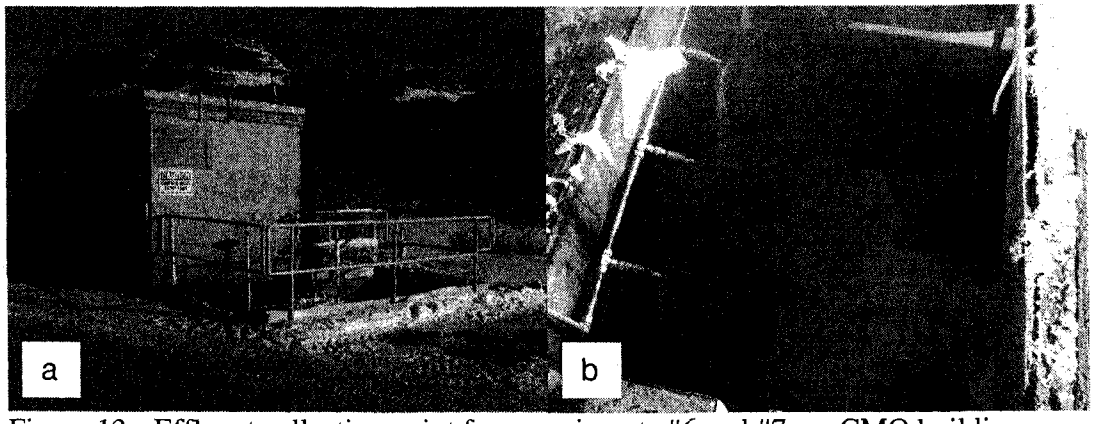

Figure 13 - Effluent collection point for experiments #6 and #7: a –CMO building. b – final treated effluent collected from CMO outfall sewer

2.3.9 Analysis and statistics

Gonadosomatic (GSI) and liver somatic (LSI) indices were calculated by the following formula: 100 x organ weight/body weight – organ weight (Rickwood and Dubé 2007). Condition factor (K) was calculated using: K = 100 x (body weight/length^3) (Rickwood and Dubé 2007).

Statistical analysis was performed using Minitab 15.1.0.0 (2006). Breeding pairs spawning less than 150 eggs during the 15-day pre-exposure period were considered not to be reproductively capable and were excluded from data analysis. This also prevented the problem of determining whether effluent or timing caused spawning to begin in pairs that did not spawn during the pre-exposure period. The Anderson-Darling test for normality was used in order to ensure that egg production during the pre-exposure period was normally distributed. Analysis of Variance (ANOVA) and Fisher's least significant difference (LSD) method were used to determine whether the 5 experimental groups (2 controls and 3 treatments) differed in egg production during the pre-exposure periods and thus whether the controls could be grouped for analysis. Consistency and predictability of pre-exposure egg production was analyzed using one-way ANOVA to ensure that reproduction test assumptions (as discussed in section 1.5.3) were true. Following this, pre-exposure period egg production was compared to exposure period egg production using one-way ANOVA. When significant differences were found Dunnett's method (with 5% significance level) was used to determine whether or not the treatment differed from the control.

3.0 Results

3.1 Water Quality

3.1.1 River water anion concentrations

Water was sampled from holding tank #9 and from the flow-through aquarium during experiment #1, and again from holding tank #9 during experiment #7. Anion concentrations were determined and are shown in Table 2. Total ammonia (NH4+NH3) in the aquarium was at a toxic level due to a clogged line and fish deaths in the aquarium.

3.1.2 River water metal concentrations

River water metal analysis taken during experiment #1 can be seen in Table 3. Canadian freshwater guidelines (CCME 2007) (when provided) are given for reference. Metals tested for but not detected (ND) through analysis are also included. Copper levels were found to be above Canadian limits for protection of aquatic life, however this value was below what EPA (2007) considers normal.

Table 2 – Anion concentrations in river water used for effluent exposure experiments.

Parameter	MDL	Holding exp. #1	Aquarium exp. #1	Holding exp. #7
Bromide	0.05	0.38	0.32	---
Chloride	0.05	11.66	12.22	8.3
Total ammonia NH4+NH3	0.010	0.046	0.776*	0.032
Nitrite NO2-N	0.005	<DL	0.025	<DL
Nitrate NO3-N	0.009	0.176	0.176	0.177
Phosphate PO4-P	0.010	<DL	0.026	<DL
Sulphate SO4	0.05	3.84	4.27	3.91

*indicates a potentially toxic value for fathead minnows (EPA 2002)
<DL indicates a value below the method detection limit (MDL) used by LUEL
All values are given in mg/L.

Table 3 – River water metal analysis from holding tank #9 taken during experiment #1 [a]

	Al	B	Ba	Ca	Cu	Fe	K	Mg	Mg-hi	Mn	Na	S	Si	Sr	Zn
Holding tank water	0.039	0.007	0.012	12.690	0.022	0.205	0.925	4.559	4.013	0.065	2.301	1.455	3.975	0.026	0.002
Canadian Guidelines	0.100	--	--	--	0.004[b]	0.300	--	--	--	--	--	--	--	--	0.030

	Cd	Be	Co	Cr	Li	Mo	Ni	P	Pb	Sn	Ti	V
	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] All results are expressed as µg/mL
[b] EPA 2007 reports values ≤0.03 as normal in freshwater
ND represent values not detected

3.2 Pulp and paper mill effluents

3.2.1 Analysis

Egg production (total number of eggs/pair) was significantly lower during the pre-exposure periods of experiments #6 and #7 (RW2 and 100% [v/v] CMO) compared to the pre-exposure periods of experiments #4 and #5 (RW1, 10% UK [v/v], 25% [v/v] SK) ($p \leq 0.001$) (figure 14). As a result, the control groups from all 4 experiments were not combined and analysis was performed based on experiments #4 and #5 (kraft mill effluents vs. river water 1) (see section 3.2.2) and experiments #6 and #7 (CMO vs. river water 2) (see section 3.2.3).

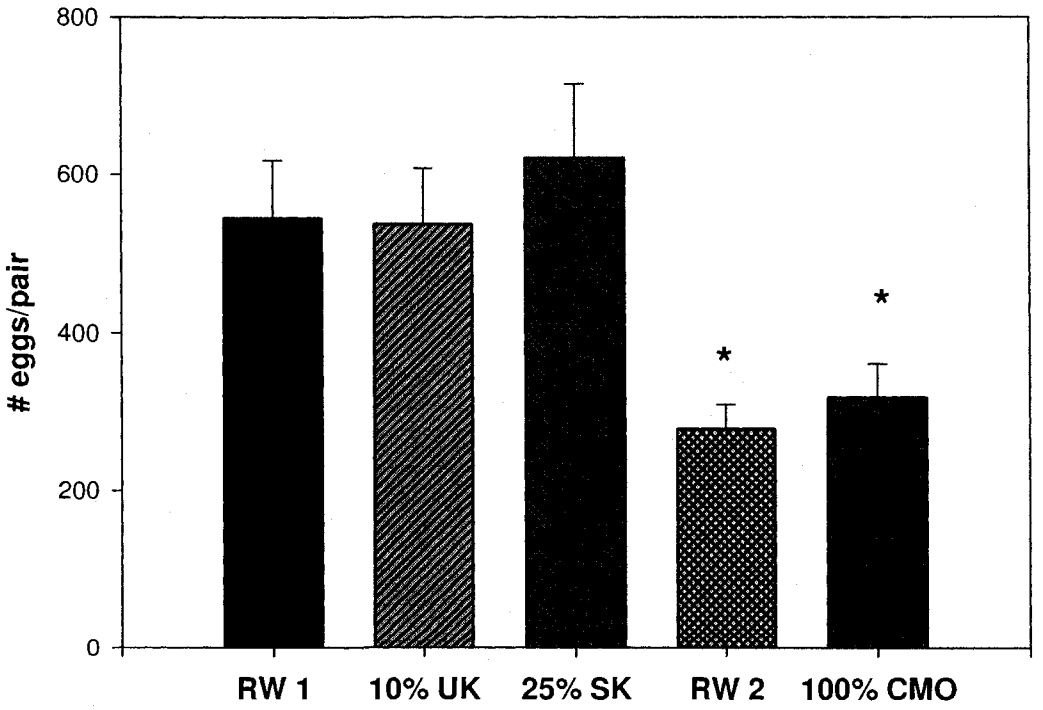

Figure 14 - Pre-exposure period egg production (# of eggs/pair) compared between the 5 experimental groups (2 controls: RW1 and RW2; and 3 treatments; 10% [v/v] UK, 25% [v/v] SK, and 100% [v/v] CMO). An * indicates a significant difference (ANOVA; $p \leq 0.001$) from RW 1, 10% (v/v) UK, and 25% (v/v) SK. Error bars represent the standard error.

3.2.2.0 Kraft mill egg production: pre-exposure period (experiments #4 and #5)

One female fish died during the pre-exposure period. Five pairs of fish were removed from analysis because they did not pass the quality control standard of 150 eggs spawned during the pre-exposure period. This included one pair from the control group and 2 pairs from each of the two groups bound for the effluent treatments. As a result, 12 breeding pairs per control and each treatment were used for data analysis.

There were a total of 116 spawning events between the 36 pairs of fathead minnows during the pre-exposure periods of experiments #4 and #5. Breeding pairs in the 3 groups spawned 34, 44, and 38 times, respectively. Spawning was most frequent every 3rd day (24.4%) and every 4th day (25.6%) across all 3 groups. A total of 20,426 eggs were counted. Fathead minnows in the 3 groups spawned 36.3 ± 4.9, 35.8 ± 4.7, and 41.4 ± 6.2 eggs/pair/day (mean ± standard error) during the pre-exposure period. There was no significant difference in the number of eggs/pair/day between the 3 groups ($p=0.71$) (figure 15).

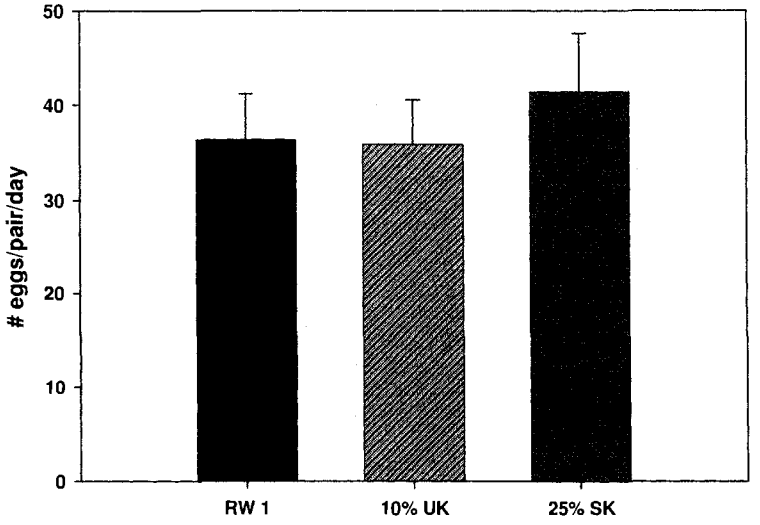

Figure 15 - Mean number of eggs/pair/day during the pre-exposure period of experiment #4 and #5. All 3 groups were exposed only to river water during this period. Error bars represent the standard error.

3.2.2.1 Kraft mill and river water quality (experiment #4 and #5)

Temperature, pH, DO, and conductivity values measured during the exposure period of experiments #4 and #5 can be seen in Table 4. Temperature, pH, and DO measurements were taken to ensure test guidelines were met. Conductivity was measured to ensure accurate dilution and homogeneity throughout the flow-through system. Temperature was consistently measured at 25°C in the control and both treatment. pH was within the required 6.5 to 8.5 in the control and both treatments. DO was within acceptable limits (60 to 100%) although 10% (v/v) UK was often lower than the control and 25% (v/v) SK. Low standard errors indicate relative homogeneity achieved within the flow-through system over the entire exposure period.

Table 4 - Kraft mill effluent and river water quality parameters (experiments #4 and #5) [a]

	Temperature (°C)			Dissolved Oxygen (%)			pH	conductivity (µmhos)
	max.	min.	mean	max.	min.	mean	mean	mean
RW 1	26.1	24.3	25.2 (0.1)	103.8	81.8	94.0 (1.6)	7.11 (0.03)	99 (2)
10% UK	25.9	23.8	25.0 (0.1)	95.6	64.0	85.2 (1.9)	7.64 (0.04)	466 (23)
25% SK	26.3	24.3	25.2 (0.1)	102.3	80.6	93.7 (1.3)	7.91 (0.02)	621 (33)

[a] values given in () represent the standard error

3.2.2.2 Kraft mill effluent egg production: exposure period (experiment #4 and #5)

Control, 10% (v/v) UK, and 25% (v/v) SK pairs spawned 14, 7, and 13 times, respectively, during the exposure period. A total of 5,403 eggs were counted. Of these, 40.7% were observed in the controls, 8.0% were observed in 10% (v/v) UK, and 51.3% were observed in 25% (v/v) SK. There were no changes in mean number of eggs/pair/day between the pre-exposure and exposure periods in both the control ($p=0.48$) and 25% (v/v) SK ($p=0.81$), however there was a significant decrease in egg production in 10% (v/v) UK ($F=31.0$; $p<0.01$) (figure 16).

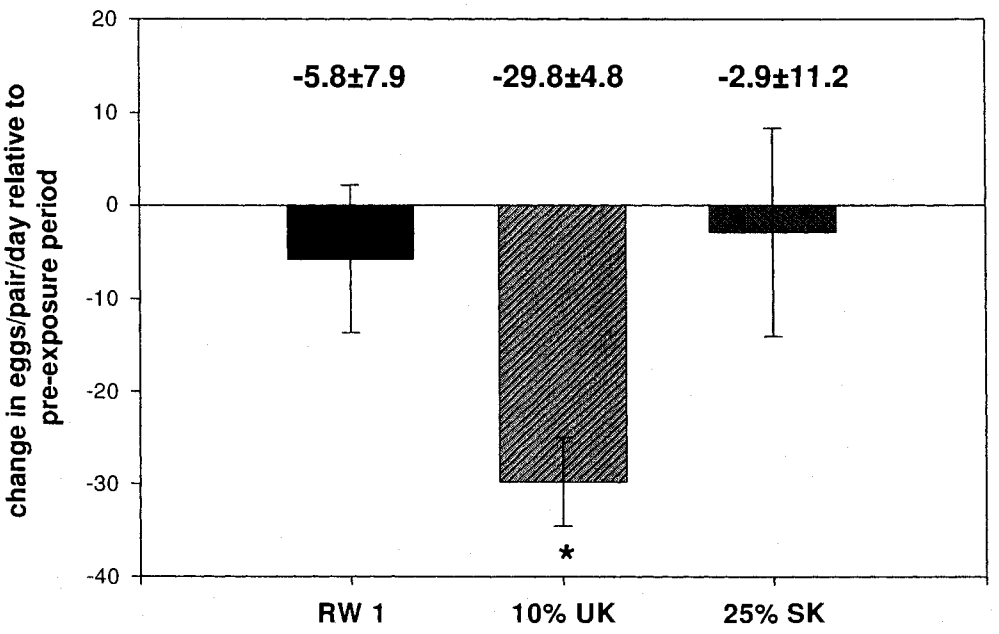

Figure 16 - Changes in mean number of eggs/pair/day between the pre-exposure and exposure periods during experiments #4 and #5. An * represents a significant change from the pre-exposure to exposure period ($p<0.01$). Values given are changes in mean ± standard error.

3.2.2.3 Kraft mill effluent: cumulative egg production

As seen in figure 17, cumulative egg production remained similar between groups until day 0 (the beginning of the exposure period). Cumulative egg production was significantly lower over the exposure period in 10% (v/v) UK ($p<0.01$). Egg production (# of eggs per day) remained relatively constant in the control and 25% (v/v) SK. Cumulative egg production in 25% (v/v) SK appeared slightly higher during the exposure period, however this was not statistically significant (figure 17). Egg production peaked on day 3 of the exposure period in the control, 10% (v/v) UK, and 25% (v/v) SK groups, with 611, 247, and 897 eggs, respectively.

3.2.2.4 Male individual endpoints

Measurements taken for individual endpoints in males are shown in Table 5. Males were seen to have a decrease in LSI following exposure to 25% (v/v) SK compared to the controls ($p<0.05$). There were no other significant differences in endpoints seen in males between the control and treatment groups.

3.2.2.5 Female individual endpoints

Measurements taken for individual endpoints in females are reported in Table 6. There were no significant differences in endpoints in females between the control and treatment groups.

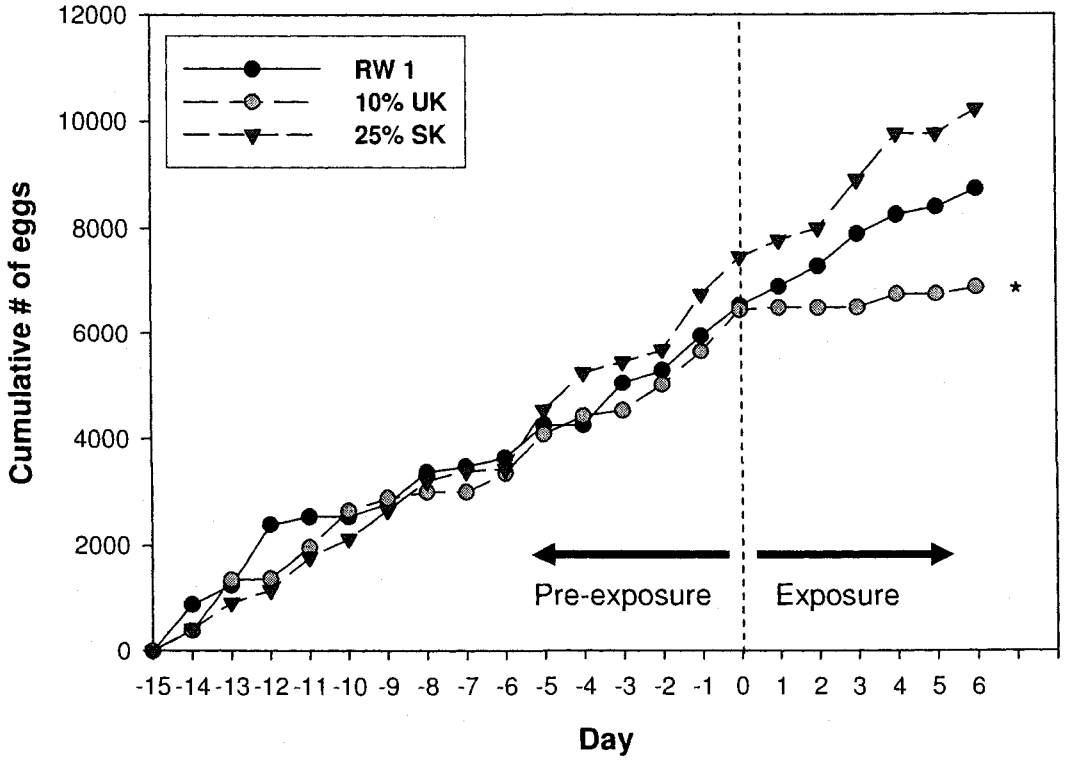

Figure 17 - Cumulative egg production by fathead minnow breeding pairs during experiments #4 and #5. Total egg numbers were noted during the 15 day pre-exposure period (day -15 to day 0) and the 6 day exposure period (day 0 to day 6) to 10% (v/v) UK, 25% (v/v) SK and RW (control) (n=12 breeding pairs for each treatment and the control). An * represents a significant difference in egg production per day from the control during the exposure period ($p<0.01$).

Table 5 - Male individual endpoints after the exposure period (experiments #4 and #5) [a]

Treatment	**Length** (cm)	**Weight** (g)	**K**	**LSI**	**GSI**
RW 1	6.11 (0.17)	3.31 (0.31)	1.41 (0.04)	3.41 (0.19)	1.65 (0.17)
10% (v/v) UK	6.18 (0.24)	3.43 (0.35)	1.40 (0.04)	2.85 (0.23)	1.85 (0.22)
25% (v/v) SK	6.01 (0.13)	2.95 (0.22)	1.33 (0.03)	2.65 (0.16)*	1.66 (0.16)

[a] Values given in () represent the standard error

* indicates a significant difference from the control ($p<0.05$)

K represents condition factor; LSI represents liver somatic index; GSI represents gonadosomatic index

Table 6 – Female individual endpoints after the exposure period (experiment #4 and #5) [a]

Treatment	Length (cm)	Log_{10} Weight (g)	K	Log_{10} LSI	GSI
RW 1	5.47 (0.14)	0.28 (0.04)	1.18 (0.04)	0.54 (0.04)	13.68 (1.08)
10% (v/v) UK	5.32 (0.24)	0.24 (0.01)	1.17 (0.14)	0.60 (0.04)	13.40 (2.39)
25% (v/v) SK	5.25 (0.30)	0.24 (0.02)	1.22 (0.14)	0.64 (0.03)	12.22 (3.50)

[a] Values given in () represent the standard error
K represents condition factor; LSI represents liver somatic index; GSI represents gonadosomatic index. Weight and LSI values were non-normal, therefore log_{10} data was transformed for ANOVA

3.2.2.6 Behaviour, colouration, and tubercles

There were no observed changes in behaviour or colouration between the control and either treatment group. Males continued to guard the substrates and both females and males continued to eat upon feeding. No loss of equilibrium or uncoordinated swimming was observed in any of the minnows. All males had typical vertical banding and nuptial fat pads. Females had a typical thin horizontal band. Control males had a mean of 18.6 ± 1.0 tubercles (mean ± standard error). Males exposed to 10% (v/v) UK and 25% (v/v) SK had on average 14.8 ± 1.6 tubercles and 14.2 ± 1.4 tubercles (mean ± standard error), respectively, which were both lower than the controls; however, statistically significance was also low ($p=0.06$). Two females were found that were developing a single small tubercle each, one in 10% (v/v) UK and one in 25% (v/v) SK. All females had normal ovipositors.

3.2.3.0 CMO egg production: pre-exposure period (experiments #6 and #7)

Two control group females died during the pre-exposure period and one female died during the exposure period in the 100% (v/v) CMO. An additional 5 control and 8 treatment breeding pairs did not pass the quality control standard of 150 eggs spawned during the pre-exposure period and were thus removed from data analysis. As a result, there were 12 pairs in each of the control and treatment groups included for data analysis.

There were a total of 64 spawning events between the 24 pairs of fathead minnows during the pre-exposure period. Breeding pairs spawned a total of 40 times. Spawning was most frequent every 3rd day (25%), every 4th day (17.5%), and in back to back days (17.5%). A total of 7,168 eggs were counted. Fathead minnows spawned an average of 18.6 ± 2.1 and 21.3 ± 2.9 eggs/pair/day in both columns (mean ± standard error). There was no statistical difference in the number of eggs/pair/day between the 2 groups (p=0.45) (figure 18).

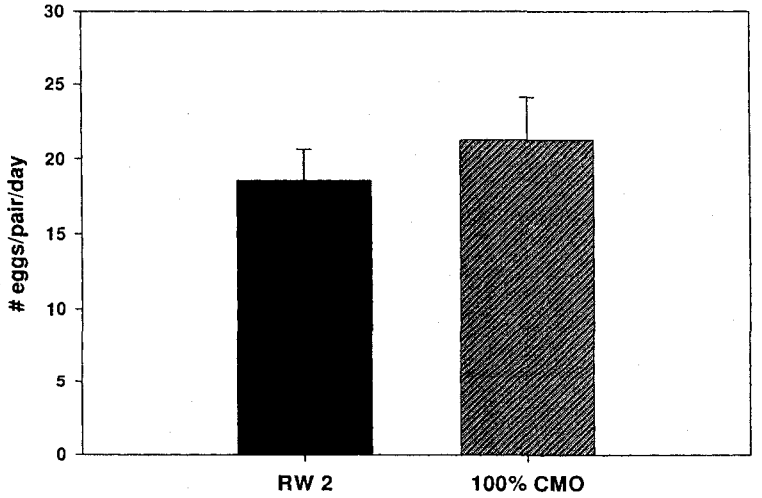

Figure 18 - Mean number of eggs/pair/day during the pre-exposure period (experiments #6 and #7). Both groups were exposed only to river water during this period. Error bars represent standard error.

3.2.3.1 CMO effluent and river water quality (experiment #6 and #7)

Temperature, pH, DO, and conductivity values measured throughout the exposure period of experiments #6 and #7 can be seen in Table 7. Temperature, pH, and DO measurements were taken to ensure test guidelines were met. Conductivity was measured to ensure accurate homogeneity throughout the flow-through system. Temperature was consistently measured near 25°C in the control and treatment. pH was within the required 6.5 to 8.5 in the control and treatment. DO was within acceptable limits (60 to 100%) although 100% (v/v) CMO was slightly lower than the control at times. Low standard errors indicate relative homogeneity achieved within the flow-through system over the entire exposure period.

3.2.3.2 CMO effluent egg production: exposure period (experiment #6 and #7)

Control and 100% (v/v) CMO exposed pairs bred 13 and 11 times, respectively, during the exposure period. A total of 2,384 eggs were counted, with 48.2% occurring in the control effluent exposed pairs and 51.8% occurring in the final treated outfall exposed pairs. There were no changes in the mean number of eggs/pair/day between the pre-exposure and exposure periods in both the control ($p=0.62$) and the 100% (v/v) CMO ($p=0.45$) (figure 19).

Table 7 – CMO and river water quality parameters (experiments #6 and #7) [a]

	Temperature (°C)			Dissolved Oxygen (%)			pH	conductivity (µmhos)
	max.	min.	mean	max.	min.	mean	mean	mean
RW 2	26.4	22.3	24.9 (0.2)	102.3	83.8	93.7 (1.6)	6.79 (0.03)	105 (2)
100% CMO	25.9	21.9	24.7 (0.2)	100.1	78.9	89.0 (1.9)	6.94 (0.09)	1583 (18)

[a] values given in () represent the standard error

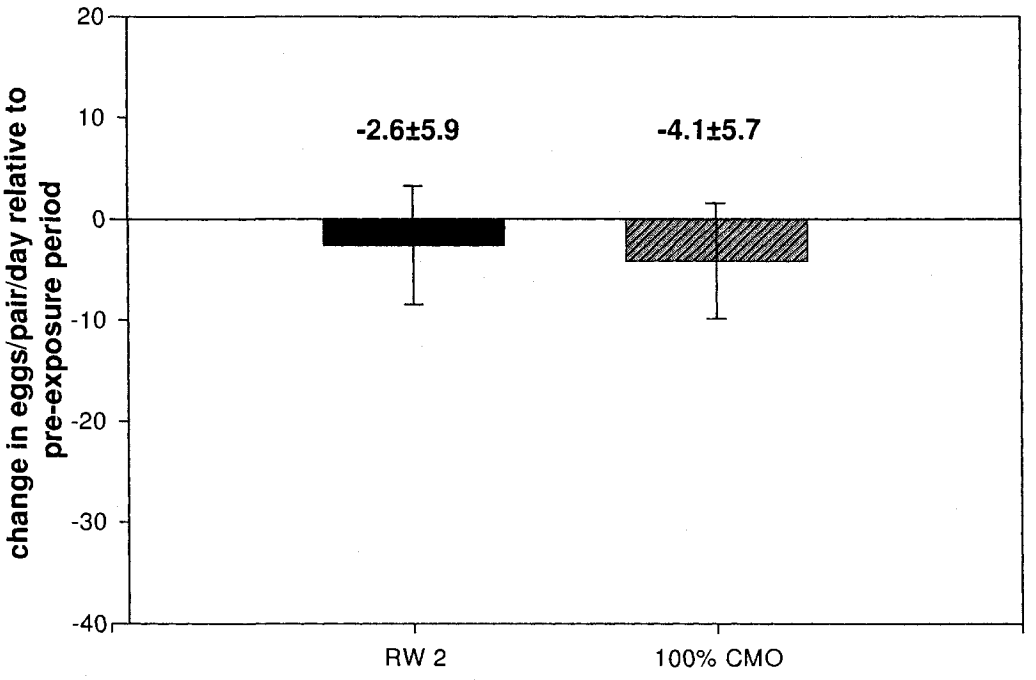

Figure 19 - Changes in mean number of eggs/pair/day between the pre-exposure and exposure periods during experiments #6 and #7. There were no significant differences in changes from the pre-exposure to exposure period in either the control (RW 2) (p=0.62) or the treatment (100% [v/v] CMO) (p=0.45). Values given are changes in mean ± standard error.

3.2.3.3 CMO effluent: cumulative egg production

As can be seen in figure 20, cumulative egg production was slightly higher in the treatment group than the control group until day 0 (the beginning of the exposure period). Cumulative egg production was not significantly different between the control and treatment group during the exposure period (p=0.86). In both controls and the 100% (v/v) CMO-exposed fish, daily egg production peaked on day 3 of the exposure period, with 344 and 369 eggs, respectively. A total of 138 eggs were spawned on days 5 and 6 in the control, and 2 were spawned in the treatment on the same final 2 days of exposure.

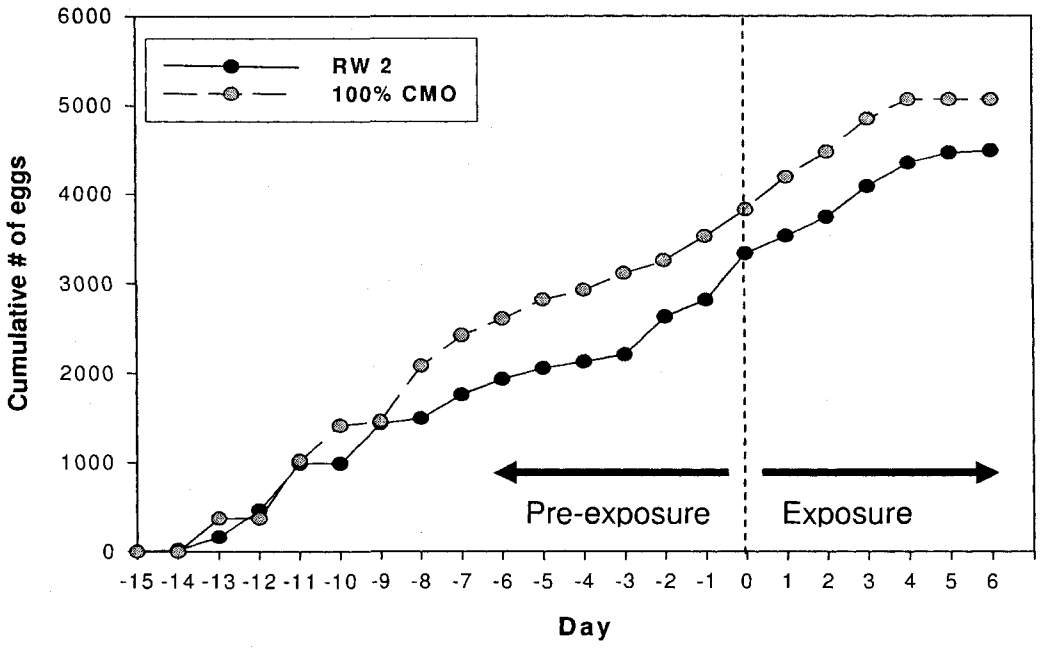

Figure 20 - Cumulative egg production by fathead minnow breeding pairs during experiment #6 and #7. Total egg numbers were noted during the 15 day pre-exposure period (day -15 to 0) and the 6 day exposure period (day 0 to 6) to 100% (v/v) CMO and Kaministiquia River water (control) (n=12 breeding pairs each for the treatment and the control). There was no significant difference in egg production per day during the 6 day exposure period between the control and the treatment (p=0.86).

3.2.3.4 Male individual endpoints

Measurements taken for individual endpoints in males are shown in Table 8. Males exposed to 100% (v/v) CMO were seen to have a decreased condition factor compared to the controls ($p<0.05$). There were no other significant differences in endpoints seen in males between the control and treatment groups.

Table 8 - Male individual endpoints after the exposure period (experiments #6 and #7) [a]

	Length (cm)	Weight (g)	K	LSI	GSI
RW 2	5.93 (0.13)	2.74 (0.22)	1.28 (0.03)	2.66 (0.24)	1.65 (0.10)
100% (v/v) CMO	5.88 (0.15)	2.35 (0.20)	1.14 (0.06)*	2.44 (0.21)	1.47 (0.16)

[a] Values given in () represent the standard error
* indicates a significant difference from the control ($p<0.05$)
K represents condition factor; LSI represents liver somatic index; GSI represents gonadosomatic index

3.2.3.5 Female individual endpoints

Measurements taken for individual endpoints in females are shown in Table 9. There were no significant differences in endpoints seen in females between the control and treatment groups.

Table 9 - Female individual endpoints after the exposure period (experiments #6 and #7) [a]

	Length (cm)	Weight (g)	K	LSI	GSI
RW 2	5.00 (0.09)	1.47 (0.08)	1.16 (0.03)	3.32 (0.25)	12.43 (1.46)
100% (v/v) CMO	5.11 (0.09)	1.52 (0.09)	1.13 (0.03)	3.59 (0.32)	13.41 (0.93)

[a] Values given in () represent the standard error
K represents condition factor; LSI represents liver somatic index; GSI represents gonadosomatic index

3.2.3.6 Behaviour, colouration, and tubercles

There were no obvious changes in behaviour or colouration between the control and treatment groups. Males continued to guard the substrates and both females and males continued to eat during the exposure period. No loss of equilibrium or

uncoordinated swimming was observed in any of the minnows. All males had typical vertical banding and nuptial fat pads. Females had a typical thin horizontal band. There were no significant differences in tubercle numbers in control males compared to 100% (v/v) CMO exposed males, with 17.1 ± 1.4 tubercles and 15.6 ± 1.8 tubercles, respectively (mean ± standard error) ($p=0.51$). No females in either the control or treatment were found with any tubercle development. All females had normal ovipositors. Two control (RW 2) females were observed with slight caudal fin rot (figure 21). One female and one male in the CMO effluent were observed with caudal fin rot (figure 22). An additional CMO male was abnormally thin (figure 23).

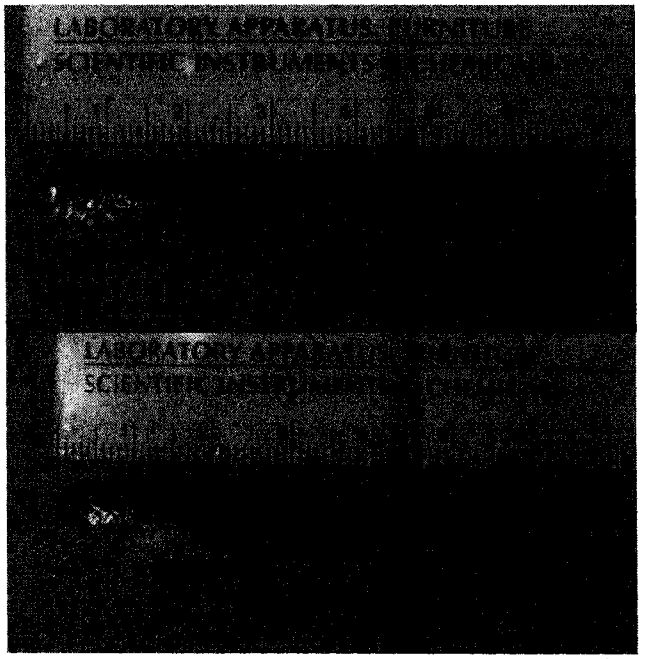

Figure 21 – Caudal fin rot observed in two control females during experiments #6 and #7. Location of fin rot is indicated by the black arrow. Ruler measurements are in cm.

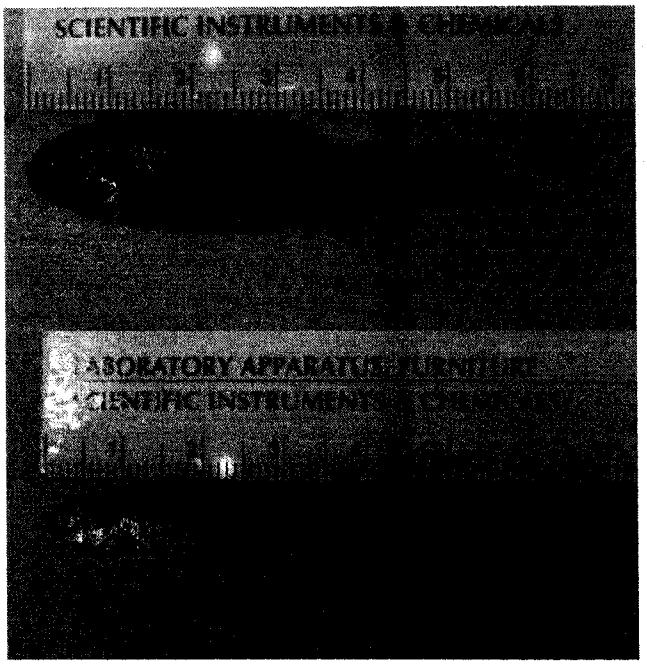

Figure 22 - Caudal fin rot in one male (top) and one female (bottom) after exposure to 100% CMO (v/v) effluent. Location of fin rot is indicated by the black arrow. The ruler measurements are in cm.

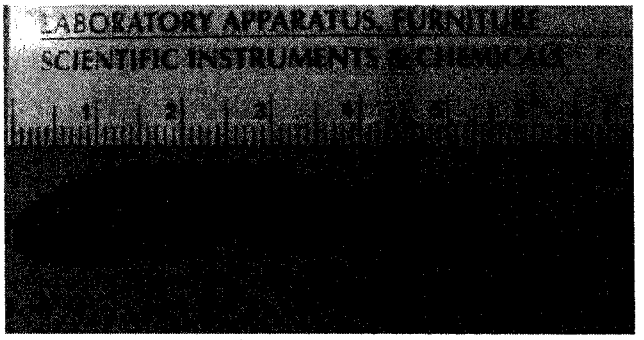

Figure 23 – Atypical male fathead minnow observed after 6-day exposure to 100% (v/v) CMO effluent. This was the only sickly male that appeared in this treatment group. The ruler measurements are in cm.

4.0 Discussion

4.1 Research questions

4.1.1 Are fathead minnows predictable spawners that can be used in a short-term reproduction test?

Pre-exposure period and control fathead minnows showed predictable spawning patterns throughout our study. In all four experiments (#4, #5, #6, and #7), pre-exposure period spawning normally took place at intervals of 3 or 4 days. This is similar to reported values of mean spawning intervals of 3.9 (Watanabe *et al.* 2007), 3.7 (Jenson *et al.* 2001), and 3.3 to 4.8 days (Thorpe *et al.* 2007).

Egg production in control pairs ranged from 35.8 to 41.4 eggs/pair/day during experiments #4 and #5, and from 18.6 to 21.3 eggs/pair/day during experiments #6 and #7. This is relatively consistent with reported values of 19 eggs/pair/day from Jenson *et al.* (2001) and 21 eggs/pair/day from Watanabe *et al.* (2007), although lower than the 64 eggs/pair/day reported in Thorpe *et al.* (2007). It is, however, still above the 15 eggs/female/day recommended for a test to be considered valid by the U.S. EPA. Variation in egg production between studies makes direct comparisons between different effluents and mills difficult, although patterns can still be assessed. For example, comparing patterns of change as a result of kraft mill acid effluent exposure against kraft mill alkaline effluent exposure within a mill is still possible in order to evaluate similarities and differences in their environmental effects thus providing improvements to treatment options. In addition, although variation in egg production is natural, large variation (such as that reported between experiments #4 and #5 compared to #6 and #7) provides evidence that fathead minnows used within a particular experiment should be

from the same cohort and obtained from the same source in order to attempt to minimize natural variation and improve analysis. Still, the pre-exposure period served as an additional line of quality control that allowed for recognition of egg production that varied significantly between batches of fish.

Although spawning is relatively predictable, successful spawning within a certain time-frame may not occur in all breeding pairs. As a result, a high number of breeding pairs is needed in order to ensure a suitable sample size for analysis (>12 pairs are recommended). Strict guidelines for removal of non-successful breeding pairs from analysis are necessary in order to allow for accurate comparisons between studies. Rickwood and Dubé (2007), and Rickwood *et al.* (2006a, 2006b) followed the Organisation for Economic Co-operation and Development (OECD) guidelines that involved selecting breeding pairs based on pre-exposure survival (100% survival in adult minnows), egg production (each pair spawned at least once each week), and fertilization rate (greater than 80% fertilization of eggs). Of 24 breeding pairs that were used in the pre-exposure period of Rickwood and Dubé (2007), only 9 were included in the exposure period (37.5% spawning success rate). Of the 120 breeding pairs used in the pre-exposure period of Rickwood *et al.* (2006a), 63 were used in the exposure period (52.5% spawning success rate). Breeding pairs from our study were slightly more successful. Thirty-six of 42 pre-exposure pairs passed our quality control guidelines and were included in the exposure period during experiments #4 and #5 (85.7% spawning success rate), while 24 of 40 pre-exposure pairs passed our quality control guidelines and were included in the exposure period of experiments #6 and #7 (60.0% spawning success rate). However, it should be noted that our study did not measure fertilization rate, which may

have contributed to the removal of additional minnow pairs. Nonetheless, we did observe predictable and consistent spawning within our groups during the pre-exposure periods making analysis with effluent exposure possible.

4.1.2 Does treatment improve effluent quality as measured by reproductive and physiological changes in fathead minnows?

Clarification and secondary effluent treatment appeared to improve short-term reproductive effects resulting from exposure to kraft mill effluent. However, secondary biological treatment did not appear to entirely remove the source of endocrine disruption. Exposure to 10% (v/v) UK resulted in a significant decrease in egg production from the pre-exposure to exposure period which was not seen in secondary treated kraft effluent or control breeding pairs (figure 16). This suggests that short-term egg production may be improved by secondary treatment, although additional evidence supporting this conclusion is lacking. Rickwood *et al.* (2006b) found that several untreated and primary treated effluent streams decreased the number of spawning events in fathead minnows, although they also found a significant decrease in spawning events after secondary treatment. They did not report a statistically significant decrease in egg production in the effluent stream just prior to secondary treatment, although egg production was higher in pairs exposed to secondary treated effluent than those in effluent just prior to the secondary treatment (as measured by changes in egg production from the pre-exposure to exposure periods).

Differences in concentrations are unlikely to explain the differences in results between untreated effluents, as our concentration of 10% (v/v) UK was lower than that used in Rickwood *et al.* Still, Rickwood *et al.* reported a decrease in egg production

(both cumulative spawning events and cumulative egg production) in untreated alkaline kraft effluent. This could be a primary source of the reproductive changes observed in fathead minnows, as our untreated effluent was a mixture of acid and alkaline effluent. In addition, Hardy (2002) reported that the alkaline kraft sewer was responsible for the majority of sterols (44%) found in AbitibiBowater effluent. β-sitosterol was the most common sterol in this effluent, followed by stigmastanol, and a smaller amount of campesterol. Hardy also reported a decrease in sterol concentration of only 2% following secondary treatment. Although β-sitosterol is thought to be an estrogenic compound (Kovacs *et al.* 2005, Van Der Kraak *et al.* 1998) it has been suggested that it could be modified into an androgenic compound during the effluent treatment process (Hewitt *et al.* 2008, Kovacs *et al.* 2005, Newman and Unger 2002, Giesy *et al.* 2000, Jones *et al.* 2000). Despite research on β-sitosterol, its mechanisms of action and effects on vertebrate fecundity are still in question (Kovacs *et al.* 2005, Gilman *et al.* 2003).

Although changes in egg production were observed in our study, we saw few impacts of either 10% (v/v) UK or 25% (v/v) SK effluent on individual endpoints. Condition factor and GSI in both males and females were similar to reported values, as was female LSI (Rickwood and Dubé 2007, Watanabe *et al.* 2007, Rickwood *et al.* 2006a, Rickwood *et al.* 2006b, Kovacs *et al.* 2002), although males and females from our experiments were generally smaller and weighed less. Still, males exposed to 25% (v/v) SK had a significantly lower LSI than control males. It should be noted that control males in our study were seen to have an LSI mean of 3.41 during experiments #4 and #5, and 2.66 during experiments #6 and #7, both of which are higher than the reported value of 2.02 for normal males (n=154 combined from research over 6 years) provided in

Watanabe *et al.* (2007). Other reported LSI values for control males include 2.23 (Rickwood *et al.* 2006a and 2006b) and 1.98 (Rickwood and Dube 2007). Generally, it is believed that effluent exposure results in increases to LSI. Rickwood *et al.* (2006a, 2006b) and Rickwood and Dubé (2007) reported an increase in LSI resulting from exposure to secondary treated kraft mill effluent. Similar increases have also been reported in juvenile fathead minnows exposed to bleached sulfite mill effluent (Parrott *et al.* 2003).

LSI increases, such as those previously reported, are thought to be caused by enhanced vitellogenin production resulting from increases in liver metabolism (Li and Wang 2005). Since males exposed to 25% (v/v) SK in our study had increased levels of vitellogenin mRNA (J. Werner, C. Cheng, R.D. Law, unpublished data) a decrease in liver size caused by exposure to the effluent seems unlikely. There are two other possibilities. The first, and most likely, is that the decreases in mean LSI values we observed in 25% (v/v) SK exposed males was simply a type 1 error (false positive), resulting from the large LSI values seen in our control males. The second possibility is that the river water that we used as a control contained compounds which induced vitellogenin production and thus increased LSI. This is unlikely as control females were not seen with increased LSI measurements. Furthermore, increased vitellogenin production suggests estrogenic effects (Werner *et al.* 2003) of the river water which is again unlikely as there is no evidence to support this theory (e.g., female traits seen in control males and decreased egg production). Lastly, river water was used for dilution in the two treatment effluents. Therefore, if river water increased vitellogenin induction then LSI values should show some increase in the treatment groups as well.

Lastly, tubercle numbers in males were slightly lower in both of the treatment groups versus the control groups (although with a p-value of 0.06, statistical significance was borderline). This could suggest the presence of anti-androgenic compounds in both acid-alkaline untreated kraft and secondary kraft mill effluent, as anti-androgenic compounds have been shown to lead to tubercle formation inhibition in male fathead minnows (Panter *et al.* 2004). Tubercle formation was observed in one female from each of the 10% (v/v) UK and 25% (v/v) SK groups, which as opposed to our observations in males, would suggest the presence of androgenic compounds in the effluent (Panter *et al.* 2004, Ankley *et al.* 2001). Two scenarios are possible as a result of these tubercle observations. It is possible that these kraft mill effluents contain both androgenic and anti-androgenic compounds. However, because the statistical significance was low (p=0.06) and fish were young and still developing, it is more likely that the appearance of higher tubercle numbers in males is simply artificial. Mean tubercle counts of 18.6 (control), 14.8 (10% [v/v] UK), and 14.2 (25% [v/v] SK) were within normal values of 18 to 38 reported in Jenson *et al.* (2001), although slightly higher than the 4.9 to 12.0 reported in Kovacs *et al.* (2005) and 10 to 13 reported in Panter *et al.* (2004). Again, it could be suggested that compounds in the river water were androgenic, however this is unlikely as male control LSI values were high and river water was used for dilution in both of the experimental kraft mill treatments.

Regardless, the occurrence of one masculinized female in each of the untreated kraft and secondary treated kraft groups suggest that EDCs are not completely removed during treatment and that changes in secondary sexual characteristics may occur rapidly. Male secondary sexual characteristics have been observed in females following life-cyle

tests with bleached sulfite mill effluent (dorsal fin dots and nuptial tubercles) (Parrott and Wood 2004, Parrott *et al.* 2004, Parrott and Wood 2002), and in females exposed to kraft mill effluent following secondary treatment (fin dots on the dorsal fin and banding) (Rickwood *et al.* 2006a, Rickwood *et al.* 2006b) although not in all cases (Rickwood and Dubé 2007, Kovacs *et al.* 2005). It should be noted that the sample size in Rickwood and Dubé (2007) was small and only 3 pairs of fish were analyzed in each of their two effluent treatments, although their test duration was 15 days longer than the 6-day exposure period used in our experiment.

4.1.3 Does short-term exposure to pulp and paper mill CMO cause reproductive and physiological changes in fathead minnows?

Although we found that secondary treatment and secondary clarification of kraft mill effluent improved effluent quality as measured by egg production, final CMO was also assessed as it is the effluent released into the environment and is thus the most relevant. CMO effluent at 100% (v/v) concentrations did not induce any statistically significant change in any of the reproductive or physiological parameters measured in our study. No decrease in egg production was noted, nor were any changes in individual endpoints, behaviour, or colouration observed. Still, egg production during the final 2 days of exposure period in the treatment group consisted of only 2 eggs, compared to 138 eggs in the control group. Unfortunately, it is difficult to conclude whether or not this is merely 2 days of decreased egg production or part of a trend that might have continued if the exposure period was extended past 6 days. Regardless, the biological significance of short-term exposure to CMO appears relatively low, as control- and CMO-exposed pairs produced similar cumulative egg counts over the 6-day period (figure 20). If egg

production was to slow permanently in CMO-exposed pairs after day 4, as was observed, a long term decrease in egg production might be seen. This change would likely be biologically significant, however our study exposed breeding pairs to CMO concentrations significantly higher than what would be found in the Kaministiquia River (estimated at 2-6%). Kovacs *et al.* (2005) reported a decrease in egg production in multiprocess mill effluent (combined TMP and kraft) at concentrations of 20% (v/v) but not 2% (v/v). Therefore, the biological significance of these short-term exposures with CMO outfall is again debatable. Kovacs *et al.* (2005) reported that egg production decreased at a time when the mill did not meet toxicological regulations, however a decrease in egg production was again observed during a second survey where toxicology limits were met. Unfortunately, these researchers did not provide day-by-day egg production data. Therefore, no comparison can be made between the pattern of egg production during the first 6 days of their exposure period and our cumulative egg production results.

Kovacs *et al.* (2005) also reported no changes in individual endpoints or secondary sexual characteristics resulting from exposure to the concentrations of 2% (v/v) or 20% (v/v) effluent from the multiprocess mill, although they did observe a decrease in hatch rate in 2% (v/v) effluent, as well as an increase in vitellogenin protein in males (although this increase was not statistically significant). In our study, a lack of physiological changes observed in 100% (v/v) CMO is surprising, considering that a masculinized female with a tubercle was found in 25% (v/v) SK. It would appear that mixture with newsmill effluent may have diluted any EDCs present in the kraft SK effluent, resulting in a concentration that is low enough to remove its physical impacts.

Rickwood *et al.* (2006b) came to a similar conclusion following their analysis of several effluent streams and final outfall effluent.

4.1.4 Is a 6-day, short-term test method useful for determining reproductive changes as a result of exposure to pulp and paper mill effluents?

A short-term (6-day exposure period) appears to be sufficient for analysis of egg production and tubercle formation, as seen in untreated kraft mill effluent, although it is unclear whether egg production responses were not observed in secondary treated and CMO as a result of the short observation period. Days 5 and 6 of the CMO exposure period did show a decrease in egg production, however we cannot conclude whether this was simply a temporary change or the beginning of a significant decrease in daily egg production.

Rickwood and Dubé (2007) showed decreases in egg production within 6 days of exposure to final treated bleached kraft mill effluent, although statistical analysis was performed only on the 21-day data. Still, they did show that exposure to 100% (v/v) final treated bleached kraft mill effluent reduced spawning to 1 event in the first 17 days of exposure, a process that normally occurs every 3 to 4 days. Rickwood *et al.* (2006b) also saw decreases in egg production in 8.5% (v/v) untreated alkaline kraft effluent that was observable within the first 6 days, but again statistical analysis was performed only on the 21-day data. Rickwood *et al.* (2006a) found that changes in egg production as a result of exposure to 1% and 100% (v/v) kraft mill effluent were seen primarily in the first 2 weeks. This suggests that reproduction might return to normal (or close to normal) after a short period of time. Thus, assessing reproductive changes within the first 2 weeks after exposure may be sufficient for pulp and paper mill effluent monitoring, even though

it may not accurately represent long term impacts seen downstream from pulp and paper mills. Regardless of its biological significance, short-term monitoring, coupled with adding additional assessment (i.e., biomarker measurement) is likely to improve our overall understanding of impacts of pulp and paper mill effluent on short-term fish reproduction and physiology, providing further tools for improving effluent treatment, environmental monitoring, and our biological knowledge of EDCs.

4.2 Conclusions

4.2.1 Overall conclusions

As previously reported, the fathead minnow spawning rate was predictable and useful for environmental monitoring (Dubé and Rickwood 2007, Rickwood *et al.* 2006a and 2006b, Ankley *et al.* 2001). It was necessary to use large sample sizes to allow for sufficient egg production for statistical analysis. However, our measurement of changes in the same fish as they pass from pre-exposure to exposure periods allows for some flexibility in data collection and analysis. We found that while secondary treatment and clarification of kraft mill effluent improved short-term egg production in fathead minnows, these processes did not remove all impacts of EDCs. We also found that 100% (v/v) CMO did not produce statistically significant changes in egg production, although it is unknown whether or not egg production would have decreased during a longer exposure period. Overall, a 6-day exposure period was long enough to assess several impacts of treatment on effluent and effects of final CMO effluent, although without concomitant assessment of biomarkers (e.g., mRNA levels of EDC-responsive genes), it was not sufficient to gauge long-term effects on the health of aquatic ecosystems receiving the treated effluent. Supplementation of the data in this study with final

PAPEER project biomarker levels and cell culture data should provide further evidence that will permit identification of morphological and biochemical parameters in vertebrate species used for routine effluent quality monitoring.

Overall, the evidence from this study indicates that the 100% (v/v) CMO effluent from AbitibiBowater – Thunder Bay caused no significant impacts on fathead minnow physiology or fecundity as measured by the 6-day reproduction test. As such, it is highly likely that environmentally relevant concentrations (of approximately 6%) would also show no impacts on fathead minnow reproduction during a 6-day exposure test.

4.2.2 Recommendations

Although measured water quality parameters (temperature, DO, pH, conductivity) were within acceptable values (controls, 10% UK [v/v], 25% [v/v] SK, and 100% [v/v] CMO), it would be interesting to assess what impacts differences in these variables might have on reproduction between groups. It would also be beneficial to perform a 6-day reproduction test using newsmill and neutral kraft mill effluent in order to give a complete picture of sources of EDCs and areas where effluent treatment processes may be improved. Further analyzing effluents with a 21-day reproduction test coupled with biomarker analysis would allow for optimization of a short-term reproduction test.

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Appendix 1

Standard operating procedure

MEDAL lab: Written by Sheri Skerget (2006)

MS-222 Protocol for Euthanization of Fathead Minnow

Based on guidelines set out by the American Veterinary Medical Association

Preparation of a 10g/L stock solution

(protocol prepares enough stock for 2 uses)

1. Weigh out 0.6g of MS-222 (Ethyl 3-aminobenzoate methanesulfonic acid salt 98%) and add to a 250ml flask.
2. Add 45mLs of ddH_2O to flask.
3. Using a pH meter, buffer the MS-222 solution with sodium bicarbonate until the pH is in the range of 7-7.5.
4. Accurately bring the volume up to 60mLs by adding ddH_2O.
5. Transfer solution to a dark brown bottle or a clear container wrapped in tin foil. Solution can be stored at -20°C for 1 month, or until solution turns brown.

Euthanization of Fathead Minnow using 10g/L stock solution of MS-222

1. Add 30mLs of 10g/L stock solution of MS-222 to H_2O up to 1L. (Lesser volumes can be prepared depending on the number of fish being euthanized.)
2. Monitor fish activity. Ensure that fish remain in MS-222 solution for at least 10 minutes after opercular (gill flap) movement has ceased.
3. Remove fish from MS-222 solution and rise with cold sterile water. Immediately place on ice or store in freezer in properly labeled tubes at -80°C.