# Characterization of rainbow trout (*Oncorhynchus mykiss*) and fathead minnow (*Pimephales promelas*) cell lines as models to study pulp and paper effluent effects in fish

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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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## List of Abbreviations

ANOVA: analysis of variance
AOX: absorbable oxygen halides
AR: androgen receptor
BLAST: basic local alignment search tool
BOD: biological oxygen demand
bp: base pairs
cDNA: complementary DNA
CEPA: Canadian Environmental Protection Agency
COD: chemical oxygen demand
Ct: cycle threshold
CYP1a: cytochrome p4501a
DMSO: dimethyl sulfoxide
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
ECF: elemental chlorine free
EDC: endocrine-disrupting compound
EEM: environmental effects monitoring
EF1α: elongation factor 1α
ER: estrogen receptor
EROD: ethyoxyresorufin-O-deethyase
FBS: fetal bovine serum
FHM: fathead minnow
IC: inhibition concentration
LC: lethal concentration
MMLV: Moloney murine leukemia virus
mRNA: messenger ribonucleic acid
PCDD: polychlorinated dibenzo-p-dioxins
PCDF: polychlorinated dibenzo-furans
PPER: pulp and paper effluent regulations
PPME: pulp and paper mill effluent
real-time PCR : quantitative real-time polymerase chain reaction
RNA: ribonucleic acid
TCF: totally chlorine free
TSS: total suspended solids
VTG: vitellogenin
v/v: volume per volume
WET: whole effluent testing
ZR: zona radiata

## ACKNOWLEDGEMENTS

I would like to take the time to thank the following people:

Dr. David Law for his support and his guidance throughout my honours and Masters research. Thank you for your time, patience and for contributing to my growth as a scientist and as a person.

Dr. Lucy Lee for graciously providing the cell lines used in my research.

Drs. Greg Pyle and Zacharias Suntres for their advice and participation on my committee.

Dr. Glen van der Kraak for his helpful suggestions as my external examiner.

Faculty, staff and students of Northern Ontario Medical School lab, Stephen Fratpietro and staff at the Paleo DNA lab and Dr. Wensheng Qin and students of his lab for the use of their facilities.

Past and present members of the Law lab (Lauren Sinnemaki, Sheena Makan, Jenna Piechota, Chris Edmunds, Jake Ouellet, Marlena Tassone, Paula Reguly, Antje Stahl, Nick Tkaczyk, Shannon Costigan, John Coccimiglio, Mike Broere) for sharing their thoughts and knowledge with me.

Julieta Werner. Thank you for your mentorship in the lab and for providing me with advice and assistance whenever it was needed. My experiences at the Law lab wouldn't have been the same without you.

Jessica Sheldon. Thank you for your kindness and your advice in the past year. "And another thing," it would not have been the same without coffee runs, vent fests, commiseration over the length of the hockey season and generally, your friendship.

My friends. Ashley, Stefan and Simon, biostats would have been torturous without you guys keeping spirits up with Dum Dums, skunk investigations and JibJab videos. Trish, weekly coffee dates and 'Rama runs made me look forward to Mondays. Marie-Eve, thanks for cheering me on, cheering me up, and always being there. To those I didn't mention, thank you for laughs, hugs and good times. I couldn't ask for better friends.

My parents and my sister. Simply saying thank you is not enough.

Andrew Parr. Admittedly through this arduous process, I haven't been the most pleasant person to live with. Thank you for enduring not one, but two theses with me. You help me de-stress, you make me laugh, and you provide me with comfort, encouragement, and occasionally, a motivational kick in the pants. Without your understanding and continued emotional support, the road to completing this Master's would have been a lot rougher.

## DEDICATION

I would like to dedicate this work to my parents and my sister. Thank you for being a positive influence in my life. To my father, you have taught me invaluable lessons in hard work, determination and character. To my mother, you have taught me that any task can be accomplished and any obstacle is surmountable if it is taken one step at a time. To my sister Stephanie, you have shown me that anything worth pursuing is worth pursuing with your whole heart. Your continued support in my education is appreciated beyond words. I could not have completed this thesis without your love and belief in me.

## ABSTRACT

The effects of aquatic toxicants on fish growth and development have been well-documented, in contrast to the changes in gene expression that necessarily precede them. Many of these toxicants are exogenous compounds that possess the ability to mimic steroid hormones or inhibit normal endocrine functions, which are known as endocrine disrupting compounds (EDCs). The Law laboratory previously obtained mRNA expression data indicating that exposure to aquatic effluents from a combined news/kraft pulp and paper mill changed the expression of genes associated with EDC exposure in the livers of fathead minnows (*Pimephales promelas*). Societal desire to reduce the use of animals in toxicity testing has encouraged the development and use of *in vitro* systems, including vertebrate cell models. In this study, the gene expression of the rainbow trout (*Oncorhynchus mykiss)* liver cell line RTL-W1 and the fathead minnow liver cell line FHM-L was examined following effluent exposure. In the RTL-W1 and FHM-L cell lines, 24 h exposure to effluents was sufficient for assessing changes in gene expression, with maximum changes observed between 4 and 6 h after exposure. Both cell lines showed upregulation of the estrogenic and androgenic-related genes, but this upregulation did not follow any consistent pattern in any of the effluent treatments. However, both lines were responsive to toxic compounds in the effluents in a time-dependent fashion, as indicated by the induction of cytochrome P450 1A (CYP1A). The expression of CYP1A was gradually up-regulated over time, peaking between 4 and 6 h after exposure and declining thereafter. Secondary treatment effectively removed toxic compounds from the effluent, as demonstrated by the decrease in fold induction of CYP1A mRNA levels after 6 h of exposure to combined mill outfall effluent compared to that of cells exposed to untreated and secondary treated kraft effluents This work, along with subsequent correlation analysis of gene expression in cell lines to that in the parent tissue, begins to validate fish cell lines as predictors of

changes in aquatic vertebrate health upon exposure to pulp and paper mill effluents and represents a critical step towards the Law lab goal of developing their use as a complement to whole fish life cycle testing that is currently employed in ecotoxicology studies.

# 1. LITERATURE REVIEW

## 1.1. PULP AND PAPER MILLS

The pulp and paper industry is a significant contributor to Canada's economy. However, the processes used in the pulping of wood and the production of paper products generate large amounts of wastewater and pollutants. Peck and Daley (1994) best described pulp and paper mill effluent (PPME) as "a Pandora's Box of waste chemicals," as it contains both natural extracts from wood and xenobiotic compounds formed during the pulping and paper making process, both of which may be considered pollutants. While each of the multiple steps in the pulping and papermaking process may generate toxicants, these vary based on the species of trees used and specific methods employed to produce specific paper products. If untreated effluents are discharged into water systems, they may cause considerable harm to the aquatic ecosystem. To reduce the toxicity of the effluents, they must first be subjected to multiple treatment processes.

### 1.1.1. TREATMENT OF EFFLUENT

The processes used to produce paper from wood require enzymes, chemicals and large volumes of water. In particular, the pulp and paper industry is the greatest user of freshwater among Canadian manufacturing industries, consuming more than 2.4 billion cubic meters per year or 32 % of the total volume of industrial freshwater used in 2004 (Figure 1) (Statistics Canada 2005). Before effluents can be safely discharged onto water systems, they must undergo multiple treatment processes (Figure 2).

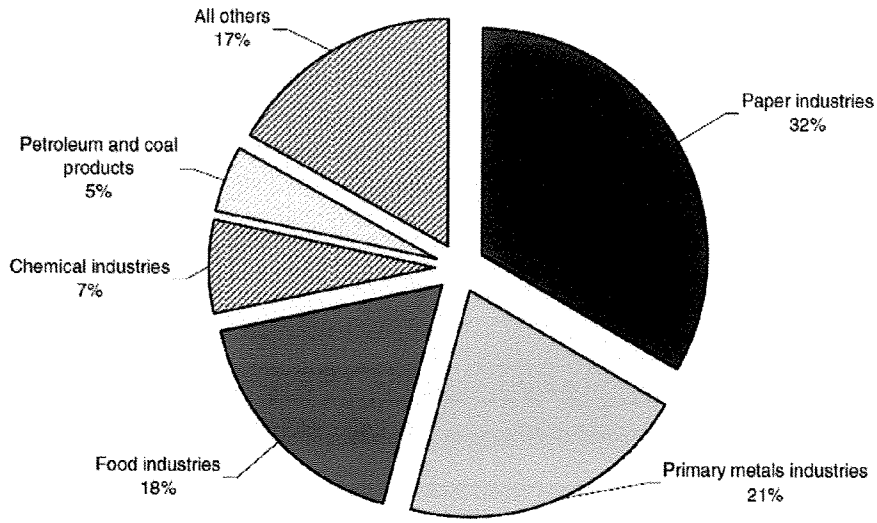


**Figure 1: Water usage by Canadian manufacturing sector** (Statistics Canada 2005).

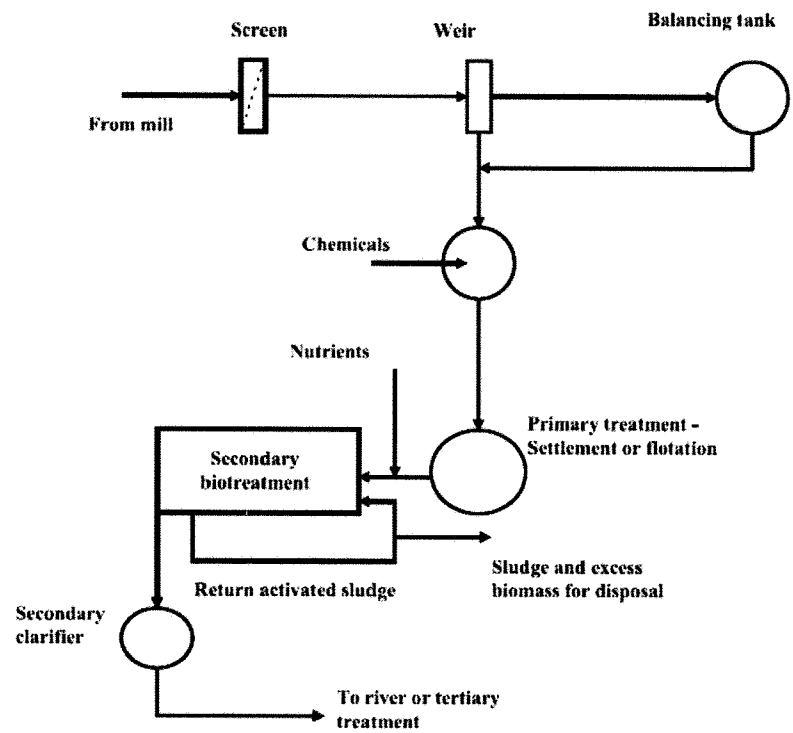


**Figure 2: A simplified schematic of a pulp and paper mill effluent treatment plant** (Thompson et al. 2001).

The first step in the treatment process is primary clarification that serves to remove suspended solids from the effluent. These largely consist of bark particles, fibre, filler, and coating material (Thompson et al. 2001). The removal of these solids is achieved by either sedimentation or flotation. Sedimentation, the preferred option, has been noted to achieve on average 80% removal of suspended solids. The primary clarification process in paper mills, however, does not address the considerable amount of organic material in the effluent (Saunamaki 1997). Organic matter is removed during secondary treatment.

The amount of pollutants is characterized by the rate at which biological organisms use up oxygen in water and the amount of organic compounds present in the water, termed biochemical oxygen demand (BOD) and chemical oxygen demand (COD) (Ali and Sreekirshnan 2001). Absorbable oxygen halides (AOX) may also be present in PPME and have been found to be toxic to aquatic organisms. Absorbable oxygen halides are products from the reaction between chlorine compounds and lignin or other organic matter (Kringstad and Lingstrom 1984). Secondary treatments take advantage of both aerobic and anaerobic environments to reduce the amount of organic pollutants present in the effluent. For example, passing clarified effluent through an activated sludge plant subjects it to an aerobic biological treatment that oxidizes complex organic molecules using aerobic organisms such as *Pseudomonas putida, Citrobacter sp.* and *Enterobacter sp.* (Chandra 2001). Pure or atmospheric oxygen bubbled through the effluent encourages the growth of these aerobic organisms and the formation of clumps of fine particulates and bacteria, termed a biological floc, in the tanks. While the precise mechanisms leading to biological floc formation are currently unknown, it is speculated that the development of a matrix by filamentous bacteria allows the attachment of the floc-forming bacteria. When the filamentous bacteria overgrow the floc, sludge settlement problems can occur (Sezgin et al. 1978) that result in

operational problems and a reduction in bioreactor efficiency (Morgan and Beck 1928). Other aerobic treatments include the use of aerated lagoons and aerobic biological reactors. The effectiveness of AOX removal by aerated lagoons is fairly inconsistent, ranging from 15-70%, even in a controlled batch study of kraft effluent (Pokhrel and Viraraghvan 2004). Chemical (COD) and biological (BOD) oxygen demand, chlorinated compounds, resin acids and lignin were substantially removed using aerobic biological reactors (Magnus et al. 2000). A two-stage activated sludge process in which the effluent is aerobically treated twice was particularly effective in reducing BOD and COD in PPME (Knudsen et al., 1994).

While anaerobic treatments can also be employed as a secondary clarification process, they are not as widely used as activated sludge processes (Pearson 1990). Anaerobic treatments are potentially advantageous when compared to their aerobic counterparts, resulting in reduced sludge production, lower chemical consumption, and/or smaller land requirements. Removal of COD is typically 80% efficient and constant over time. Although the anaerobic treatments are efficient in removing COD, they are slower acting than aerobic treatment equivalents (Paasschens et al. 1991). The ultimate desired effect of biological treatment is thus to reduce BOD and COD levels in PPME, thus producing effluent with a reduced capacity to promote the growth of aerobic organisms in the receiving waters that compete for dissolved oxygen with extant organisms in the aquatic ecosystem.

While tertiary treatment is currently rarely employed in PPME management, it may be adopted by more pulp and paper mills as more stringent regulations governing effluent quality come into effect. The main tertiary treatment methods in use are membrane processes, ozonation, and adsorption. Membrane processes, such as ultrafiltration, use a pressure-driven separation mechanism that may also serve as a secondary clarifier (Chen and Horan 1998). Kraft paper effluents yield a significant decrease in AOX, COD and colour when treated with ozone. Ozonation

is an expensive process and because high dosages of ozone are required to effectively decrease COD, it is rarely employed by pulp and paper mills (Hostachy et al. 1997). Adsorption requires the use of activated charcoal, fuller's earth and coal ash and has been reported to remove 90% of colour, COD, and AOX in bleached wastewater (Murthy et al. 1991; Shawwa et al. 2001). Adsorption was also found to remove a significant amount of lignin from effluent (Das and Patnaik 2000). Although small amounts of chemicals were still present in the effluent after the treatment process, levels were significantly reduced and the concentrations of potentially toxic compounds were generally sublethal to the wildlife in the aquatic ecosystem.

### 1.1.2. Environmental Effects of Effluent

Exposure to pulp and paper mill effluents has been found to have toxic effects on various species of fish. In studies conducted in the U.S. and Canada, fish populations demonstrated a variety of responses when living downstream from pulp and paper mills, including delayed sexual maturity, reduction in gonad size, changes in fish reproduction and a depression or emergence of secondary sexual characteristics (Munkittrick et al. 1997; Hewitt et al. 2008). Reproductive effects linked to PPME exposure were first observed in wild fish studies. Several studies examining wild fish downstream of North American pulp and paper mills reported decreased levels of endogenous steroid hormones (McMaster et al. 1991; Van Der Kraak et al. 1992; McMaster et al. 2006). Decreased gonad size and the appearance of male secondary sex characteristics in female fish were also observed (Parrott et al. 2002). Similarly, male-biased sex ratios were found in eelpout (*Zoarces viviparus*) located downstream from a Swedish pulp and paper mill (Larsson et al. 2000).

Laboratory studies have shown similar findings to observations in wild fish. Fish injected with pulp and paper mill effluents showed a decrease in steroid hormone levels (Orrego et al. 2010). Reductions in gonad size have been observed in life-cycle studies with fathead minnow as well as

artificial mesocosm studies with mummichog (*Fundulus heteroclitus heteroclitus)* (Dube et al. 2002; Parrott et al. 2004). Laboratory-based life-cycle studies have also found that the number of eggs produced by fathead minnows was consistently reduced when exposed to Canadian and U.S. kraft mill effluents (Kovacs et al. 1995; Borton et al. 2000).

Conversely, other studies have indicated that there are no significant adverse effects in fish and biota exposed to treated mill effluent (D'Surney et al. 2002). Stepanova et al. (2000) did not find any morphological or physiological changes in aquatic animals attributable to pulp and paper mill effluent discharged into the water system. Additionally, no changes in sex ratios or secondary sex characteristics were observed in fathead minnows exposed to diluted pulp mill effluent (Kovacs et al. 1995).

These opposite effects are likely because many components of PPME vary in concentration on a mill-to-mill basis as a result of differences in effluent treatment processes. Unsurprisingly, therefore, a variety of responses have been observed in fish populations living downstream from PPME sources as well as in PPME-exposed fish in the laboratory. Before the implementation of federal regulations in the 1970s (discussed in 1.1.5), habitat degradation, reduced oxygen concentrations in the water and acute lethality to fish were observed due to the high fibre and BOD content in the receiving environment (McLeay et al. 1987). Through the development of Pulp and Paper Effluent Regulations (PPER) and the Environmental Effluent Monitoring (EEM) program implemented by Environment Canada, many of the negative environmental effects of pulp and paper wastewaters have been alleviated. Though effluents are generally no longer acutely toxic, environmentally persistent compounds in wastewaters may be physiologically relevant and affect morphology and reproductive success. Effects of these wastewaters on aquatic populations include delayed sexual maturity, smaller gonads, decreases in egg production and depression of secondary

sexual characteristics (Johnsen et al. 1998; Erisction and Larsson 2000; Hewitt et al. 2008; Baer et al. 2009; Barrett et al. 2010).

### 1.1.3. Regulation of Mill Effluents

As the body of knowledge regarding the composition and potential toxicity of effluents has grown, provincial and federal regulations have evolved as well. In 1971, PPER were passed under the Fisheries Act, resulting in the federal control of PPME composition originating from Canadian mills. The PPER regulations established daily and monthly limits for BOD and total suspended solids (TSS) and required that the effluents were not acutely lethal to adult rainbow trout. These limits were only legally binding to mills constructed after the establishment of PPER, which only represented 10% of Canadian mills in the 1970s. By the late 1980s, concern regarding the presence of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzo-furans (PCDDs/PCDFs) became an increased area of concern by the public (Sodergren 1992). New regulations under the Canadian Environmental Protection Agency (CEPA) were passed to control the release of PCDDs and PCDFs in 1992 (CEPA 1999). The PPER were also updated to include stricter limits for BOD and TSS. Also, a review of the PPER acknowledged that uniform limits for the environmental parameters in effluent would not necessarily protect the health of all aquatic receiving environments (Walker et al. 2002). New regulations implemented through the PPER thus required environmental effects monitoring (EEM) at all mill sites in Canada. The objective of the EEM program is to evaluate the effects of effluent on fish, fish habitat and the use of fisheries resources. These data are then used to assess the adequacy of the regulations on a site-by-site basis.

In addition to complying with EEM regulations, pulp and paper mills are also subject to provincial environmental guidelines. In Ontario, pulp and paper mills are currently required to conduct monthly acute toxicity testing with water fleas (*Daphnia magna*) and rainbow trout fry.

Final undiluted effluent must be non-toxic to more than 50% of the testing organisms. In addition to the monthly assessments, mills are required to conduct chronic toxicity testing to assess reproduction inhibition and survivability of water flea species (*Ceriodaphnia dubia*) exposed to effluent for 7 days. Growth of adult fathead minnows is also assessed during a 7-day exposure period (Government of Ontario 2007). Mills are further required to conduct surveys on the effects of their PPME on microinvertebrates, adult fish, and sublethal toxicity every four years (Hardy 2002).

### 1.1.4. PPME Testing Methods

EEM uses short-term assays to measure survival and growth in native fish species exposed to effluent for one week. For pulp and paper mills that discharge into freshwater environments, these species include fathead minnow and rainbow trout. To represent marine environments, inland silverside (*Menidia berllin*) and topsmelt (*Atherinops affinis*) are examined. Short-term assays using invertebrates, namely *C. dubia*, sea urchin (*Strongylocentrotus purpuratus*) or sand dollar (*Dendraster excentricus*), expose these species to PPME for 7 to 21 days. For both invertebrates and fish, an acute IC25 test is conducted, which determines the concentration of the effluent that causes a 25% decrease in a health parameter such as liver-somatic index, gonadosomatic index or condition factor, compared to control fish (Parrott et al. 2006). While found effective for tracking changes in effluent quality when mills alter or install secondary treatment facilities, short-term assays lack environmental relevance (Lowell et al. 2003). An assessment of fish health indicators in both wild and laboratory fish for over 100 Canadian PPMEs suggested that there was little or no correlation between laboratory attained IC25 values and health indicators from wild fish captured downstream (Walker et al. 2002).

In addition to the toxicity tests employed by pulp and paper mills in fulfillment of federal regulations, other bioassays are performed to determine markers of toxicity that sublethal toxicity tests may overlook. Life-cycle studies are long-term exposures of fish to PPME that examine the growth, maturation and reproduction of selected fish species. Although laborious, time-consuming and costly, these provide the most convincing evidence linking exposure to PPME to aberrations in fish reproduction (Parrott et al. 2006). Various fish species have been used in life-cycle studies. The requirements for appropriate life-cycle test species are that they are small in size, are able to grow and breed in captivity, possess a short generation time and are sexually dimorphic. While the fathead minnow is often used in life-cycle tests because of its relevance to the Canadian environment (Kovacs et al. 1995; Parrott et al, 2004; Ankley and Villeneuve 2006), the marine mummichog has also been used in recent tests (Eisler 1979; Munkittrick et al. 1997; Courtenay et al. 2002). Life cycle exposures begin with fertilized eggs and fish are grown to maturity, where growth, secondary sex characteristics and breeding success are closely monitored. At the completion of the exposure, typically after 4 to 6 months, growth, liver weight and gonad weight of adults are assessed. These exposures are able to track improvements in final effluent quality and are considered the definitive test of assessing compounds for endocrine disruption and reproductive toxicity (Parrott et al. 2006) (Figure 3).

In studies that examine the effects of complex toxicant mixtures on fish reproduction and physiology, many researchers use a "whole mixture" approach where a combination of many chemicals, such as an effluent, is investigated as if it were a single agent (U.S. EPA 1986). In this approach, individual effects of the components that comprise the mixture are not assessed. Although this type of experiment is useful for examining complex mixtures on a case-by-case basis, difficulties arise when extrapolating to other mixtures because small differences between the

mixtures may significantly change their toxic effects (Kortenkamp 2007). Although this difficulty of reproducibility limits the utility of whole mixture testing, PPMEs are complex and cannot be successfully divided into individual components. The evaluation of the individual natural extracts from the wood and chemical additives used to process pulp may not account for new compounds formed by the heating of the pulp nor for additive or antagonistic interactions between compounds. Therefore, it can be argued that it is more appropriate to utilize a whole mixture approach to assess the toxicity of PPME on fish and fish cells (Sarakinos et al. 2000; Silva et al. 2002; Kortenkamp 2007).

Although current toxicity tests are ecologically relevant, producing endpoint data using them is expensive and requires considerable time and effort. Additionally, they do not provide insight into the mechanisms of action of the contaminants. The inconvenience of these tests is a primary motivation to examine the advantages and limitations of using cultured cells to develop a rapid toxicity assay.

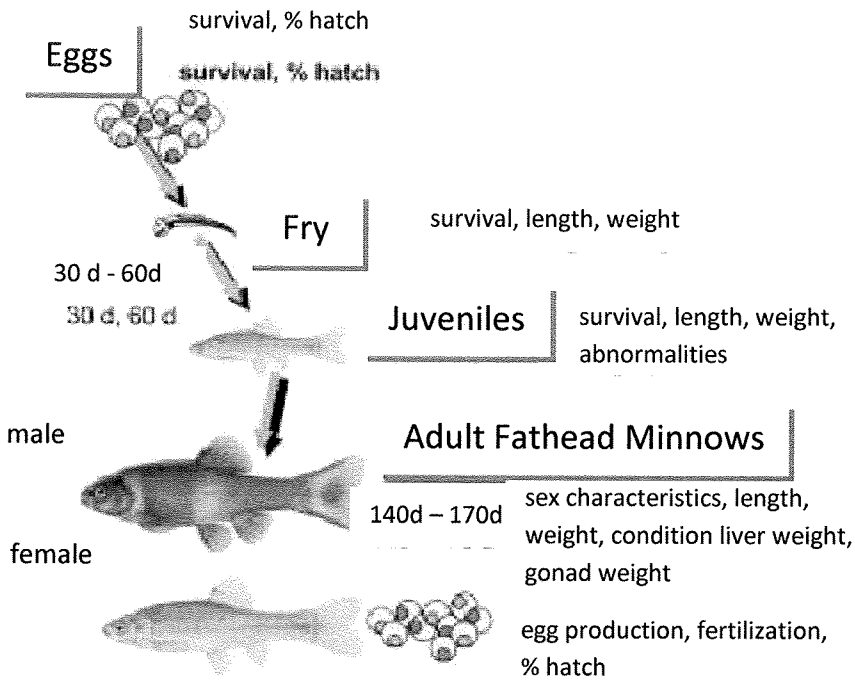


**Figure 3: Fathead minnow life cycle bioassay.** Each stage of exposure (eggs, larvae, juveniles and adults) is listed with the endpoints measured (Parrott et al. 2006).

### 1.2. Endocrine Disrupting Compounds

Endocrine disrupting compounds (EDCs) are exogenous natural or xenobiotic compounds or mixtures that can inhibit or alter normal endocrine functions. Consequentially, adverse effects of EDCs are observed in the organism, its progeny or subpopulations (Vos et al. 2000). Endocrine disrupting compounds can cause endocrine and reproductive effects in organisms by (1) mimicking endogenous hormones, (2) antagonizing normal, endogenous hormones, (3) altering the pattern of synthesis and metabolism of natural hormones, and/or (4) modifying hormone receptor levels (Sonneschein and Soto 1998). The aquatic environment is highly susceptible to pollution because of the release of effluents by various industries, including municipal sewage treatment plants, mines, textile industries and pulp and paper mills. Water pollution can also be attributed to the accidental release of chemicals through spills and run-off, and these have increased in frequency due to population growth and anthropogenic water use (Sumpter 2005). The effects of EDCs in the aquatic

environment at the subpopulation or population level threaten the sustainability of fish populations over time, inevitably impacting commercial and sport fisheries (Mills and Chichester 2005).

A major group of EDCs includes estrogenic chemicals, which is the most researched class of EDCs. The first key observations of apparent feminization of aquatic organisms were observed in alligators and fish in Floridian and English rivers, respectively (Guillette et al. 2000; Purdom et al. 1994; Jobling et al. 1998). The discovery of skewed sex ratios and feminized male organisms led to a search for the causative chemicals. In the aforementioned cases, there appeared to be natural and synthetic estrogens that were excreted by humans, incompletely degraded during sewage treatment and thus released in domestic sewage treatment effluents (Johnson and Sumpter 2001). Human-made estrogen-mimicking chemicals including pharmaceuticals including nonylphenol and bisphenol A, termed xenoestrogens, were observed to have effects in fish and while these are generally less potent than steroidal estrogens, they may occur at higher concentrations.

Phytoestrogens, such as β-sitosterol and stigmastanol, occur naturally within wood, and were detected in PPME after pulping of wood using the kraft process. These plant sterols have similarities in chemical structure to cholesterol and were found to compromise reproductive capability and elicit toxic effects in fish (Zacharewski et al. 1995; Denslow et al. 2004). For example, the phylogenetically widely distributed phytosterol β-sitosterol may be a crucial contributor to changes in gondal weight, gonadal hormone levels and other endocrine effects in fish (Cooper and Kavlock 1997; Sharpe et al. 2007). While long-term, multi-generational life-cycle studies performed on zebrafish exposed to 10 μg/L of β-sitosterol did not affect spawning success, induction of the EDC-responsive glycolipoprotein vitellogenin (VTG) and female-biased sex ratios in F2 generations were observed (Nakari and Erkomaa 2003).

The presence of chemicals with androgenic and anti-androgenic effects in the aquatic environment has also been reported. This was first recognized in mosquitofish (*Gambusia affinis*) downstream of PPME discharge into Elevenmile Creek in Florida (Howell et al. 1980). Both field and laboratory studies performed on some species of fish have observed induction of male secondary sex characteristics in females (Sumpter 1997; Ellis et al. 2003; Parrott et al. 2006).

### 1.3. Cell Cultures

Economic and ethical constraints and societal desires associated with live animal testing have encouraged the development of alternative toxicology techniques that reduce the use of animals in scientific research. One such technique is the use of cell cultures as an alternative to whole organism testing. The use of *in vitro* assays has therefore grown in importance in many fields, including aquatic toxicology (Shúilleabháin et al. 2004). Cell cultures offer several advantages as experimental tools, including (1) the ability to study cellular phenomena in a controlled environment without the complexities that are experienced with *in vivo* studies, (2) more rapid and cost effective results, (3) increased ease of exposure cells to toxicants, (4) lowered amounts of toxic waste generated due to reduced volumes used, and (5) the ability to compare the sensitivity of widely different species to contaminants and toxicants under equivalent conditions (Bols et al. 2005).

There are two general types of cell cultures: cell lines and primary cultures. The main difference between the two cultures is life span. Primary cultures are derived from organs taken directly from the organism of interest. The cells are either held in suspension or are spread and subsequently attach to a physical support such as the surface of plastic or glass culture vessels. The life span of a primary culture can vary based on the type of tissue used and the species it was extracted from. Primary cultures are occasionally favored because they are easily prepared from tissue obtained from a large organism and require less maintenance than cell lines. However, the life

span of primary cultures is limited to several months, with a maximum life span of up to one year, as observed in some kidney and spleen cultures (Ganassin and Bols 1998). A primary culture is considered to be a cell line when the cells are subcultured, or split into new culture vessels and successfully propagated. The cell line can be continually propagated through repeated cycles of cell proliferation followed by splitting into new culture vessels (Schirmer 2006). The use of cell lines is often favoured over the use of primary cultures because their reproducible growth over time makes them convenient for long-term studies. Furthermore, while some organs may be too small to supply sufficient material for primary cultures, cell lines derived from them can provide an unlimited amount of cells.

### 1.3.1. Use of Cell Cultures in Toxicology Research

Vertebrate cell cultures have been used in toxicological research since the late 1960s. At that time, mammalian cell lines were used to examine the potential impact of environmental toxicants on humans when it was recognized that bodies of freshwater are capable of receiving, accumulating and distributing hazardous substances. Mammalian cell cultures were also used by health laboratories and water boards to assess water quality (Richardson et al. 1977). Because of the abundance and diversity of fish species, piscine cell lines were developed and used as indicators of the toxicity of individual compounds. The use of cell cultures in bioassays as an alternative or a supplement to whole fish lethality tests was first proposed by Ahne (1985). The underlying philosophy for the use of cell lines is that interactions of a substance with the cells of an organism precede changes in tissue or organ function, ultimately affecting the function of the whole organism.

Cell cultures can be used to evaluate different aspects of cellular activities, such as cytotoxicity, cell viability, genotoxicity and effects on cell-specific functions and parameters (Castano et al. 2003; Bols et al. 2005). While primary cultures may be used for toxicity testing if no

suitable cell line is available, continuous cell lines are valuable for this purpose, especially if they can be stimulated to express the differentiated organ functions of their parent tissue. In the last decade, cell lines derived from desert topminnow (*Poeciliopsis lucida)*, hepatoma cells (Fent and Hunn 1996), and rainbow trout hepatocyte and gill epithelial cells (Sandbacka et al. 2000; Castano et al. 2003) have been shown to be suitable models for determining cytotoxicity endpoints. To definitively demonstrate that *in vitro* testing is a viable and relevant alternative to whole fish testing, a diversity of target sites and the degree of sensitivity of the cells lines to chemicals must be considered. However, toxicity studies indicate that many cell lines lack chemical sensitivity compared to tests involving whole organisms (Castano et al. 2003; Bols et al. 2005). This means that caution must be used prior to adopting the use of cell lines as alternatives to commonly used acute fish lethality test protocols.

Other research indicates the effectiveness of cell lines as environmental monitors. For example, Walker et al. (2008) found that the gill cell cultures exposed to single metals elicited the expression of marker genes that reflected the effects of metal exposure in whole animals. Lee et al. (2008) proposed the use of a rainbow trout gill tissue cell line as a novel method for whole effluent testing (WET). This test method does not analyse physiological changes to or lethality of test organisms but instead examines the viability of cells related to detoxification. Three different viability tests provided information on several cellular parameters, including cellular metabolism impairment, plasma membrane integrity and lysosome integrity (Dayeh et al. 2002; Lee et al. 2008). While these results indicate that the use of cell lines as a supplement to traditional whole organism testing is promising, adoption of WET must be preceded by the confirmation that the induction of biomarkers is of similar relative magnitude both *in vitro* and *in vivo.* It has been observed that VTG, a biomarker for endocrine disruption (discussed in 1.4.1.1), can be induced in both adult and

juvenile medaka (*Oryzias latipes*), and in primary cultures of medaka liver cells (Scholz et al. 2004). However, the use of cell lines for *in vitro* toxicity assays can be limited because some genes, including estrogen receptors in fish cell lines, cannot be induced (L.E.J. Lee, unpublished results). The inability of some cell lines to express certain genes or proteins can be attributed to the dedifferentiation of continuous cell lines over time in culture (Roberts 2005).

Although the use of cell lines has been proposed for toxicity testing because of their low cost and the short exposure times necessary to elicit a response, these bioassays do not account for tissue interactions, biotransformation, and chemical metabolism, and differ in sensitivity and activity compared to tissues within a whole organism. While there are limitations associated with *in vitro* systems, they provide important data on the activity of the compounds or mixtures at the cellular level.

### 1.3.2. Cell Lines in Focus

#### 1.3.2.1. Rainbow Trout Liver Cell Line (RTL-W1)

Rainbow trout is a salmonid native to North America and Asia. The rainbow trout's genome is distinctive, as it possesses a high occurrence of duplicated genes due to a tetraploidy event that occurred between 450 and 476 million years ago (Kumar and Hedges 1998). The rainbow trout is aquaculturally important and is one of the most intensively researched fish species in a variety of areas, including carcinogenesis (Dashwood et al. 1994; Stoner et al. 2002), immunology (Warr 1995), disease ecology (Okamoto et al. 1993; Hill 2007), and toxicology (Kleinow et al. 1987; Otto et al. 1994). Rainbow trout is also used as a model for acute, and occasionally chronic, toxicity testing performed by different industries, including pulp and paper mills (Couillard et al. 1988; Tremblay and van der Kraak 1999; Orrego et al. 2009). Its large size and ease of culture is favoured when large quantities of specific tissue and cell types are required, is the easiest to experimentally

control among the salmonids, and serves as a representative species for research needed on other related economically important fish species, including Atlantic salmon (*Salmo salar*) and Arctic char (*Salvelinus alpines*). Because of its wide usage and aquacultural importance, the genome of the rainbow trout has been sequenced (Young et al. 1998).

Primary cell cultures derived from rainbow trout have been used in toxicity testing of pulp and paper mills (Pesonen and Andersson 1992), municipal wastewater plants (Gagne and Blaise 1998) and other industries. Several continuous rainbow trout cell lines have been developed from a wide range of tissues, including liver (Lee et al. 1993), kidney (Andral et al. 1990), spleen (Morimoto et al. 1990), and gill (Bols et al. 1994). The RTL-W1 rainbow trout liver cell line was established from an adult male fish liver in 1984 and has undergone more than 200 passages (Lee et al. 1993) (Figure 4). These cells are polygonal shaped and grow in a monolayer in culture flasks and plates. RTL-W1 cells are also able to induce 7-ethoxyresorufin O-deethylase (EROD) activity, which is an enzymatic measurement of cytochrome P450 1A (CYP1A) induction (Lee et al. 1993). EROD activity has been regarded as a biomarker for exposure to chemicals, including polycylic aromatic hydrocarbons, polychlorinated biphenyls and dioxins, in investigations of PPME, contaminated sediments and chemical spills (Whyte et al. 2000). The ability of RTL-W1 cells to induce this important biomarker could be exploited for use in toxicity testing. Studies using this cell line range from testing single compounds, including polycyclic aromatic hydrocarbons and pesticides (Bols et al. 1999; Babin and Tarazona 2005), to mixtures such as river sediment (Kosmehl et al. 2004) and doxycycline-spiked aged manure (Fernandez et al. 2004).

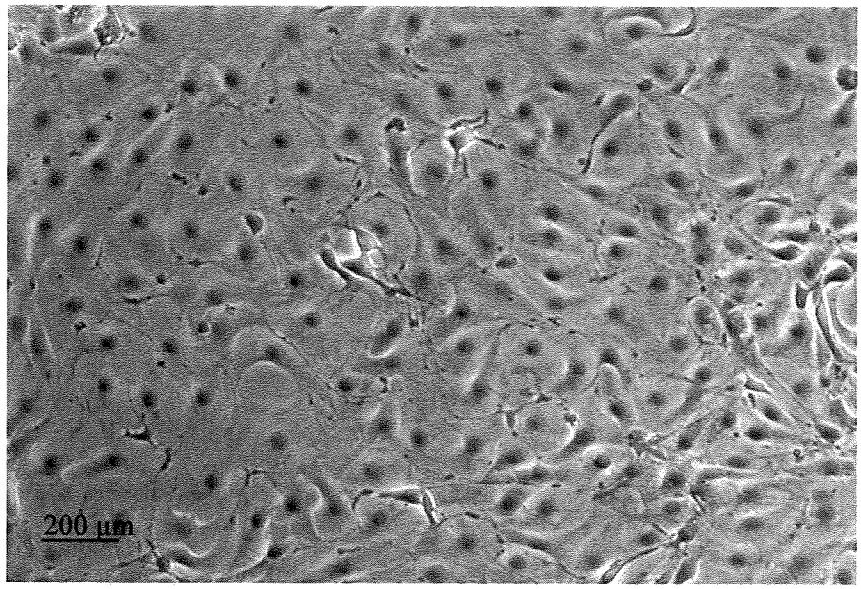


**Figure 4: Morphology of RTL-W1 cells.** Cells were visualised using an Olympus IX51 inverted light microscope at 100x (Lee et al. 1993).

### 1.3.2.2. Fathead Minnow Liver Cell Line (FHM-L)

The fathead minnow is a cyprinid that is ubiquitous across North America (Ankley and Johnson 2004). The species is sexually dimorphic: males are typically larger with blunt heads and display secondary sex characteristics, such as a vertical banding pattern, nuptial tubercles and a dorsal fatpad, whereas the females are lighter and silvery in colour and have a longer tapered head lacking tubercles (Miles-Richardson et al 1999; Ankley and Villeneuve 2006) (Figure 5). Sexual dimorphism in a model organism is important because it facilitates not only initiation of reproduction between morphologically known males and females but also monitoring of secondary sex characteristics, as these are controlled by sex steroids and can thus be used as endpoints for identifying the presence of EDCs in the aquatic environment. The fathead minnow is an important species used in aquatic toxicology, particularly in North America and Europe, where it has been used extensively in studies involving both acute and chronic testing of contaminants and effluents (Miracle et al 2003). This species is widely used in ecotoxicology, specifically in assessing pulp and

paper mill effluent quality (McLeod and Smith 1966; Kovacs et al. 1995; Parrott 2005; Rickwood and Dube 2007). Because the use of the fathead minnow in studying PPMEs is widespread, a cell line derived from an adult fathead minnow provides the opportunity to study the interactions of effluents with minimal usage of whole organisms and without the expense and complications of using primary cultures. However, there has been no continuous cell line initiated from this fish species to date. The fathead minnow liver cell line FHM-L was established by Dr. Lucy Lee in collaboration with Dr. David Law's lab from an adult male fish liver (Figure 6). This appears to be the first immortal fathead minnow liver cell line.

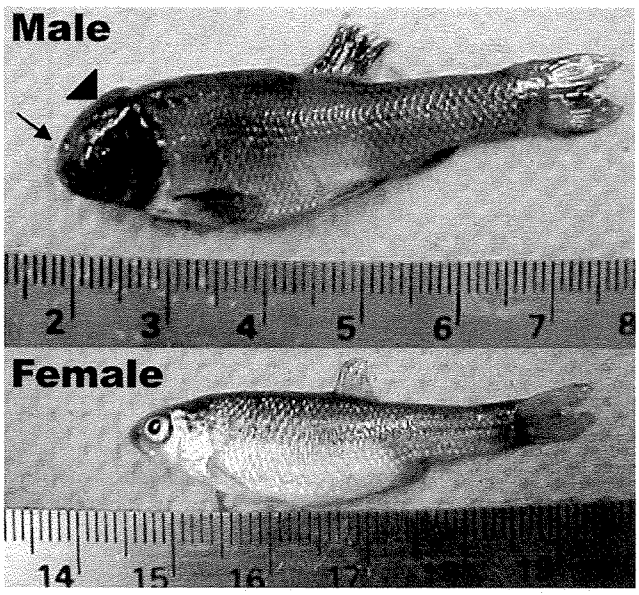


**Figure 5: Sexually dimorphic characteristics of adult male and female fathead minnows.** Male fish of this species exhibit a dorsally-located fatpad (arrowhead), nuptial tubercles (arrow), and dark symmetrical banding. Female fathead minnows do not possess these characteristics: females are smaller and silver in colour. Ruler measurements are in centimetres (adapted from Parrott 2005).

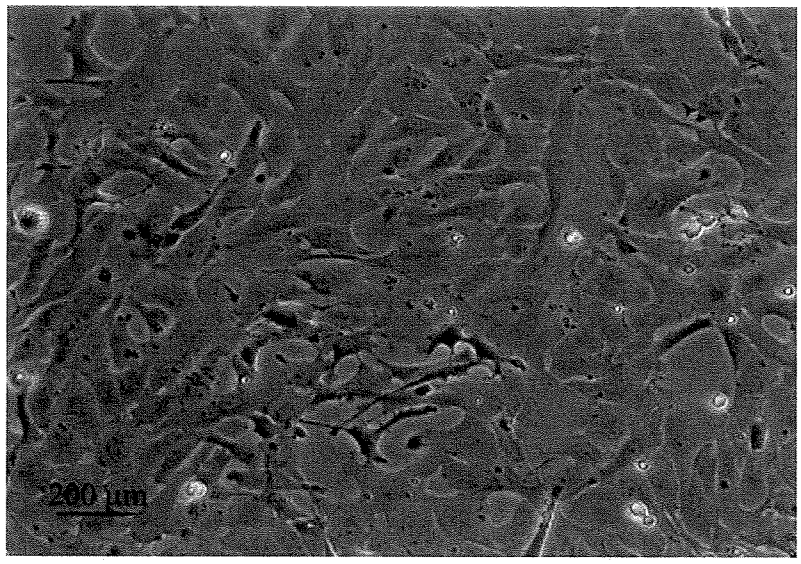


**Figure 6: Morphology of the FHM-L cell line.** Cells were visualised using an Olympus IX51 inverted light microscope at 100x.

## 1.4. Genes in Focus

### 1.4.1. Endocrine disruption indicators

The endocrine system is an organization of glands that each secrete specific hormones to regulate the functions of an organism. The endocrine system is central to the proper reproduction and development of vertebrates. External or internal cues are received by the brain and moderated by the hypothalamic-pituitary system in response to changes in hormone secretion. Gonadotrophin releasing hormone, corticotrophin releasing hormone, and thyrotrophin released by the hypothalamus trigger the release of hormones, namely gonadotrophins, from the pituitary gland. While several hormones are released by the pituitary gland, including adrenocorticotrophin and thyrotrophin, in the context of reproduction, gonadotrophins are the most important. Gonadotrophin stimulates the secretion of steroids by the gonads. These secreted steroids initiate changes in behaviour, secondary sex characteristics and the development of gametes (Kime 1998). Genes that are linked to endocrine function may be connected to estrogenicity, such as estrogen receptor, VTG and zona radiata, or to androgenicity, such as androgen receptor. As discussed in section 1.2, EDC

exposure can elicit physiological and reproductive aberrations in aquatic organisms. Long-term exposure to these compounds may affect the population dynamics of aquatic organisms within the receiving waters. Laboratory testing using biomarkers linked to endocrine disruption can be employed to assess the presence of active concentrations of pollutants (Bucheli and Fent 1996).

#### 1.4.1.1. VITELLOGENIN

Vitellogenin (VTG) is an essential egg yolk protein synthesized in the liver and transported into the ovary, where it is absorbed by growing oocytes as a food source (Sumpter and Jobling 1995). The expression of the VTG gene is controlled by multiple hormones, including 17β-estradiol, growth hormone, prolactin, triiodothyronine and thyroxine (Figure 7). Vitellogenin expression is dominantly regulated by 17β-estradiol as the concentration of plasma vitellogenin has been observed to increase concurrently with the increase of 17β-estradiol during the sexual maturation of female fish (Sumpter and Jobling 1995). Male fish possess the VTG gene but it is normally silent. Little if any VTG mRNA is detected in male fish because estrogen levels circulating in male fish are generally too low to trigger the expression of the VTG gene (Copeland et al. 1986). Vitellogenin is thus frequently used as a biomarker for endocrine disruption because of its ability to be induced in males by estrogen or estrogen-like compounds. If treated with natural or synthetic estrogens, male fish have been found to have significantly elevated plasma VTG protein levels. Exposure to high concentrations of environmental estrogens is capable of eliciting a feminizing response in male fish.

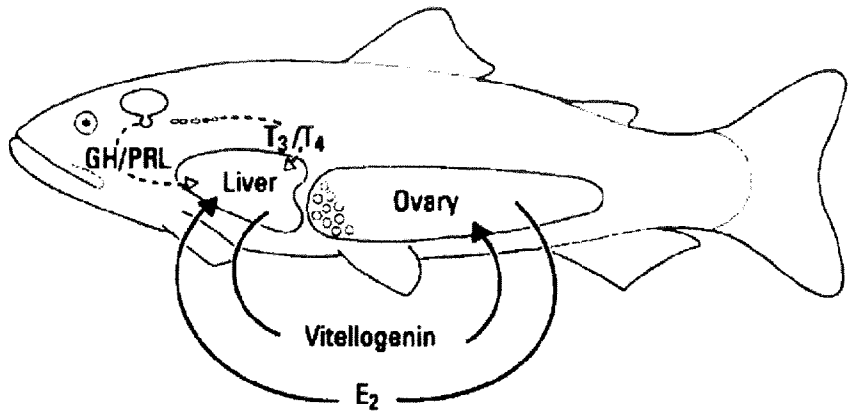


**Figure 7: Hormonal control and synthesis of vitellogenin.** Growth hormone (GH) and prolactin (PRL) from in the pituitary gland and triiodothyronine ($T_3$) and thyroxine ($T_4$) from the thyroid gland enhance the effect of estradiol ($E_2$). The presence of estradiol in the liver induces the synthesis of vitellogenin, which is subsequently transported to the ovary (Sumpter and Jobling 1995).

#### 1.4.1.2. ESTROGEN RECEPTOR

Natural estrogens are steroid hormones that are essential for the development and function of organs of the female genital tract, neuroendocrine tissues and mammary glands. At a physiological level, they are responsible for the development of female secondary sex characteristics and gonadal sex differentiation (Guiguen et al. 1999). At the cellular level, they induce the synthesis and secretion of cell-specific proteins. For these functions to be performed in a natural target cell, receptors are necessary. The liver of fish, particularly that of female fish, is rich in estrogen receptors (Campbell et al. 1994). Since estrogen receptors are similar in structure and function in fish and mammals, the estrogenic behaviour of natural and anthropogenic chemicals is similar throughout the vertebrates. The first estrogen receptor to be identified was estrogen receptor α subunit (ERα), which is present in classical estrogen target organs (Pakdel et al. 2000). Another isoform,estrogen receptor β subunit (ERβ), is present in the ovaries but has also been found in the prostate, testes, and some areas of the brain. The two subtypes of estrogen receptor are each present in two isoforms (α1/α2, β1/β2) in fleshy-finned fishes, such as rainbow trout as opposed to the

presence of only ERα and ERβ in fathead minnow. The presence of four isoforms is predicted by the hypothesis that the entire genome was duplicated when the fleshy-finned fishes Sarcopterygii that include rainbow trout, diverged from the bony-finned fishes Actinopterygii that include the fathead minnow (Amores et al. 1998).

#### 1.4.1.3. ZONA RADIATA

Zonagenesis is process that is vital to the growth of oocytes. During this process, the egg envelope surrounds the developing oocyte as a thick, highly-differentiated acellular zone (Arukwe and Goksoyr 2003). This acellular zone forms the eggshell and provides mechanical support for the embryo (Arukwe et al. 2001). Like vitellogenesis, zona radiata is estrogen-regulated, which drives synthesis of zona radiata mRNA. Similar to vitellogenin, zona radiata is synthesized exclusively in fish liver and is subsequently secreted and transported in the blood to the ovaries where it is taken up into maturing oocytes (Oppen-Berntsen et al. 1992). The synthesis of zona radiata has also been observed by the ovary itself in some fish species including carp (*Cyprinus caprio*) and pipefish (*Syngnathus scovelli*) (Arukwe and Goksoyr 2003). Environmental contaminants can disrupt the expression and formation of zona radiata in fish species in both laboratory exposures and field studies (Rotchell and Ostrander 2003).

#### 1.4.1.4. ANDROGEN RECEPTOR

Androgen receptor (AR) is an essential mediator of sexual differentiation, maturation and spermatogenesis in male vertebrates (Takeo and Yamashita 1999). Sertoli, Leydig and peritubular cells are found in the testes, express the AR protein and are considered to mediate androgen effects on germ cells, which do not express the AR protein (Zirkin 1998). Expression of the AR gene was detected in a broad spectrum of organs in Japanese eel (*Anguilla japonica*), including gill, heart, spleen, liver, kidney, muscle, testis and brain of the male and ovary in female fish (Ikeuchi et al.

1999). Androgen receptor regulates genes targeted by androgens by binding to an androgen responsive element, which is functionally similar to a glucocorticoid responsive element (Ham et al. 1988). The up- and down- regulation of this gene can be elicited by interactions with specific activators and co-activators (Yeh and Chang 1996). Exposure to known androgen mimics, such as the synthetic steroids methyltestosterone and trenbolone, is capable of inducing male secondary sex characteristics and decreasing fecundity in female fish (Jensen et al. 2006).

### 1.4.2. STRESS INDICATORS

All organisms possess the capability to respond to both chemical and physical stressors. This is controlled at the cellular level by increasing the synthesis of stress-responsive proteins. These proteins prevent stress-induced damage and limit or prevent further damage (Ryan and Hightown 1996). Stress-inducible proteins could be used as potential biomarkers for assessing environmental impacts industries may have on aquatic wildlife as well as aid in evaluating the progress of the remediation of polluted areas.

#### 1.4.2.1. CYP1A

One widely-studied stress-responsive gene family is that of the CYP genes, which play a critical role in oxidative metabolism of endogenous and exogenous chemicals. Cytochrome P450 proteins are monooxygenases that biotransform planar aromatic hydrocarbons such as polycyclic aromatic hydrocarbons through the addition of one atom of oxygen (Klaassen 2001). CYP1A is of particular interest to ecotoxicologists, as this enzyme plays a fundamental role in detoxification and metabolism of xenobiotics, as well as in the activation of hydrocarbon carcinogens (Parke et al. 1990). CYP1A is expressed in almost all tissues in fish (Urban et al. 2001). The induction of the CYP1A gene is mediated through aryl hydrocarbon receptors present in the cytosol of the cell. Planar aromatic hydrocarbons bind the aryl hydrocarbon receptor which complexes with the aryl

hydrocarbon nuclear translocator. This complex then moves into the nucleus of the cell where it activates xenobiotic response elements, causing the transcription of the CYP1A gene. The transcribed proteins increase the solubility of planar aromatic compounds in water, thus allowing for their easier elimination (Roberts 2005). Gene expression of CYP1A is a sensitive and adaptive response of organisms when exposed to environmental pollutants and is thus considered as a biomarker for exposure to pollutants in fish and fish cell systems (Fent 2001).

### 1.5. Gene Expression Quantification

Until the late 1990s, northern blotting was considered the gold standard of methods used for RNA expression quantification. This technique, introduced in 1977, was given this name after the DNA Southern blot invented by Sir Edwin Southern (Alwine et al. 1977) and uses denaturing gel electrophoresis and blotting with hybridization probe-dependent detection of target RNAs. While quantities of RNA can be compared between multiple samples on a single membrane, northern blotting lacks the accuracy of more modern methods of mRNA quantification, such as real-time polymerase chain reaction (PCR) (VanGuilder 2008). Northern blotting is still used in the study of RNA degradation and transcript size (Lee et al. 2005), but this method has several drawbacks as a method for quantifying relative gene expression. This method is not only slow, unreliable and inaccurate, but also requires a large quantity of total RNA, which may be difficult to obtain from experiments involving small biological samples (VanGuilder 2008).

The polymerase chain reaction was developed by Kary Mullis in the mid 1980s and is defined as "primer-mediated, enzymatic amplification of specific cDNA" (Saiki et al, 1985). Though innovative, traditional PCR, also known as endpoint PCR, has serious limitations for the quantification of gene expression. Reagent limitation and/or accumulation of pyrophosphate molecules or inhibitors of the polymerase reaction in the reaction prevent the PCR reaction from

generating template at an exponential rate. Because some reactions will generate more product compared to others, the use of endpoint PCR for quantification of amplicons is unreliable (Ginzinger, 2002). Because endpoint PCR analyzes the reaction after the completion of the predetermined number of cycles, the amount of product is independent of the initial amount of DNA template molecules present in the sample. Furthermore, an additional procedure, such as gel electrophoresis, is required after the reaction to visualize and quantify the PCR products. The ability to quantify PCR products through electrophoresis and subsequent densitometry of fluorescently-stained DNA is limited because of the potential oversaturation of amplicons that may yield inaccurate results.

In 1991, it was shown that the amount of PCR products could be evaluated in real-time within the reaction vessel using fluorescent dyes that bind to double-stranded DNA. This process was known as kinetic PCR (Ozawa et al. 1990). Around the same time, it was discovered that Taq DNA polymerase could be used to indirectly assess the amount of amplified DNA using fluorescent probes. This method eliminated the necessity of post-PCR amplicon visualization (Holland et al. 1991). One year later, Higuchi et al. (1992) coupled these two processes, which led to today's technology of fluorescence detection-based real-time PCR. While this relatively new technique uses the same principles as endpoint PCR, the amplification and the detection of specific DNA sequences are performed simultaneously and the amount of PCR products is measured as they accumulate (Gibson et al. 1996; Ginzinger 2002).

### 1.5.1. Fluorescent Dyes

Intercalating dyes bind to the minor groove of double-stranded DNA. Asymmetric cyanines such as SYBR Green I and BOXTO contain two aromatic systems, one of which is positively charged, that are connected with a methine bridge. These molecules vibrate when free in solution,

which converts electronic excitation energy into heat that dissipates in the solvent and therefore has virtually no fluorescence (Figure 8). However, when these dyes bind to the minor groove of the DNA, the rotation around the methine bond is restricted. This causes the dye to fluoresce brightly (Nygren et al. 1998). Intercalating dyes are relatively inexpensive and simple to use. Because they lack sequence specificity, they can be used for any reaction but will thus also recognize primer dimers and non-specific products. Melt curve analysis must therefore be performed to ensure the amplification of the desired product. Furthermore, they cannot be used for detecting multiple targets at once using multiplexing (Zipper et al., 2004).

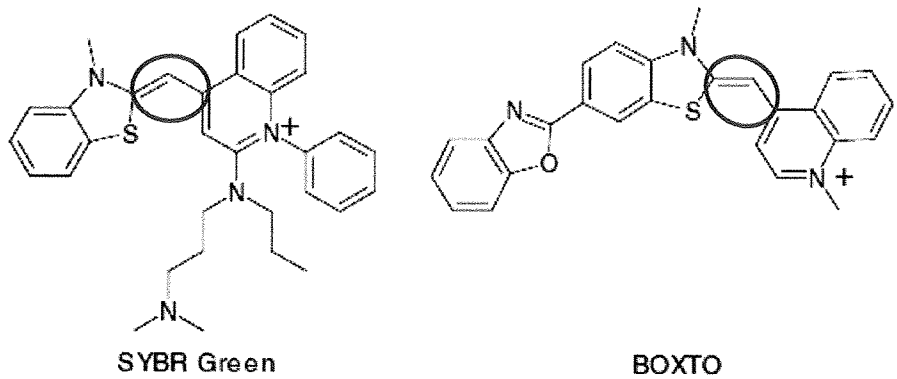


**Figure 8: The asymmetric cyanine dyes SYBR Green and BOXTO.** Circles indicate the location of the methine bond. When unbound, these molecules rotate around the methine bond (circled). When they bind to the major groove of double stranded DNA, rotation around this bond is restricted. The kinetic energy is converted to fluorescence (Kubista et al, 2006)

### 1.5.2. Cycle Threshold Value

Amplification curves from real-time PCR reactions of fluorescently-labelled product versus cycle number are graphed by software associated with the thermal cycler to allow for the determination of the cycle threshold (Ct) value in any sample of DNA. At the beginning of the reaction, a minute fluorescent signal, the background fluorescence, is detected. While the fluorescence threshold is selected differently by instruments and software manufacturer, most

software packages let the user set this value manually as well. The value of the threshold level is considered somewhat arbitrary. Although the change in the value of the threshold affects the individual Ct values, it does not significantly affect the differences between the Ct values. The threshold level is set above the background and the number of cycles required to reach the threshold, the Ct value, is registered and recorded. Initially, the fluorescence of the product is weak and cannot be distinguished from the background. As the amount of amplicons increases, the signal initially strengthens exponentially. As critical components of the reaction, such as dNTPs, primers or reporters, run out, the signal saturates and levels off (Figure 9). Because amplification on non-specific PCR products often occurs in small amounts throughout a reaction, a Ct value greater than 35 for any template can often be considered as the formation of these undesired amplicons rather than true target amplification (Kubista et al. 2001). The difference in the initial amounts of template molecules between samples is reflected by the separation of response curves in the growth phase of the reaction. Less abundant mRNAs thus possess higher relative Ct values.

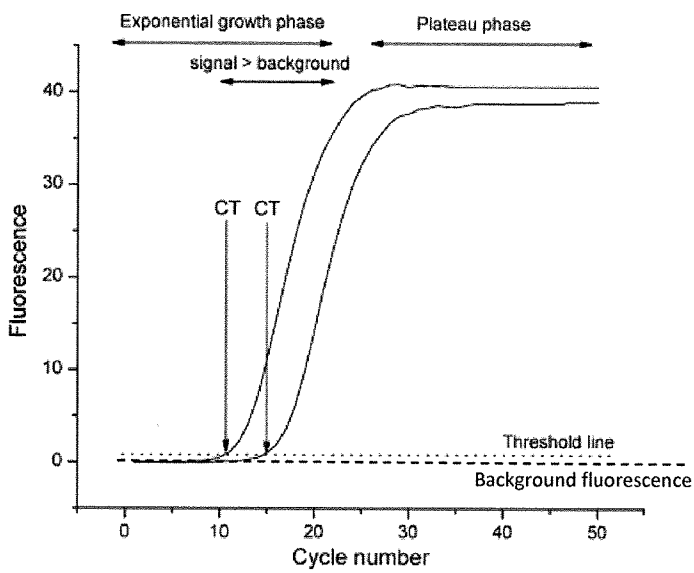


**Figure 9: Real-time PCR amplification curve.** The threshold is set sufficiently above the background fluorescence. The crossing point where the fluorescent signal produced by the intercalating dye exceeds the fixed threshold is termed the cycle threshold (Ct) value (modified from Kubista et al. 2006).

### 1.5.3. Melt Curve Analysis

Depending on the primers and PCR cycle parameters used, reaction artifacts, including primer-dimers, can be formed. Primer-dimers are small amplicons resulting from the extension of self-annealed primers. These artifacts and other amplification errors contribute to the fluorescence of the sample and during the exponential phase, are indistinguishable from the signal of specific amplicon accumulation (Zipper et al. 2004). Using real-time PCR, these undesirable products are identified by the completion of a melt curve analysis. Following PCR, fluorescence is measured as a function of temperature. Fluorescence decreases with increasing temperature because the increased thermal motion allows more internal rotation of the methine bond of the bound dye. When the temperature at which the double stranded DNA separates is reached, the dye detaches from the DNA, causing an abrupt drop in fluorescence (Ririe et al. 1997). The maximum of the first derivative of the fluorescence determines the melting temperature of the product. Because primer-dimers are generally shorter than the desired product, they melt at a lower temperature, which is easily recognized by melt curve analysis (Kubista et al. 2006).

### 1.5.4. Data Analysis

Ct values cannot be evaluated individually because of the subjectivity of the threshold and Ct values. Therefore, post-PCR analysis must be performed to evaluate the data. Two general methods have been employed to determine gene expression: absolute and relative quantification.

#### 1.5.4.1. Absolute Quantification

Absolute quantification measures the copy number of a specific gene in a given sample. In order for this ambitious task to be achieved, a sample of known quantity of the gene of interest must be diluted to generate a standard curve. This provides an external absolute standard (Kuhne and Oschmann 2002). The signal of the target gene in the sample is then compared to this standard curve

and the starting concentration is extrapolated from a curve fit using regression analysis (Wilkening and Bader 2004). The foremost limitations to the widespread use of absolute quantification are the acquisition of a reliable standard for each gene to be analysed and the necessity of performing concurrent standard curves during each assay (Valasek 2005). A computational method that does not require the calculation of a standard curve has been proposed but not developed (Wilkening and Bader 2004).

#### 1.5.4.2. Relative Quantification

In most experimental situations, the determination of the absolute quantity of a DNA template may be unnecessary and the calculation of relative expression of a gene is sufficient. Relative quantification measures the change in expression of the target gene relative to an untreated control, a sample at time zero in a time-course study, or a reference gene (Livak and Schmittgen 2001).

In order for relative expression of a gene to be determined, an appropriate reference gene must first be selected. A reference gene is a gene whose expression level remains constant between the cells of tissue being studied and under different experimental conditions (Thellin et al. 1999). Candidates for reference genes are often those involved in fundamental processes that are essential for cell survival and are thus expressed constitutively. These genes can be assessed separately or together in a multiplex assay with the unknown target (Ferre et al. 1994). Reference genes most frequently used include glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, ribosomal protein subunits and $\beta_2$-microglobulin (de Kant et al. 1994; Coutle et al. 1995). If reference genes are not consistently expressed between experimental conditions, small differences in the expression of genes of interest may be missed and could lead to erroneous real-time PCR results (Bustin 2000; Glare et al. 2002). Appropriate validation of selected reference genes is crucial to ensure the study

findings are correctly interpreted (Dheda et al. 2004). The best suited reference genes for normalization of real-time PCR data is determined by expressing each gene as $2^{-\Delta Ct}$. Delta Ct is calculated by subtracting the Ct value from a specific time point or treatment from the Ct value from control treatment or time zero in a time-course (Schmittgen and Zakrajsek 2000).

The most common method employed for the determination of relative quantification is the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), which calculates the fold induction of a target gene in relation to the reference gene of the same treatment as well as an untreated control. This calculation is based on the assumption that the amplification efficiencies of the target gene and the reference gene are approximately equal.

### 1.6. Project Overview

Fish cell lines have been established as valuable, cost-effective tools to preliminarily evaluate the effects of xenobiotics (Fent 2001). New techniques in molecular biology, namely real-time PCR, allow for the measurement of expression of specific genes and present a rapid, efficient means of assessing biomarkers and the effects of fish exposed to toxicants in the lab and in the field (McClain et al. 2003). Changes in gene expression are a relatively rapid biological response in comparison to other endpoints like protein expression, and often occur within hours of exposure (Haasch et al. 1993). Gene expression changes are also sensitive to exposure at low ecologically-relevant concentrations of contaminants (Levine and Oris 1999; Chung-Davidson et al. 2004). However, before cell lines can be used for rapid toxicity testing of PPMEs, the expression of target genes that are considered sentinels for EDC and stress, such as VTG, ER and CYP1A (Werner et al. 2010), must be determined. The focus of the research project discussed in this thesis is the elucidation of gene expression changes in the RTL-W1 and FHM-L cell lines after exposure to PPME, a complex mixture of natural extracts and xenobiotic compounds that is known to induce the

expression of EDC-linked genes *in vivo*. The evaluation of *in vitro* gene expression of PPMEs in cell lines may allow for a more rapid assessment of the potential effects that the compounds present in the effluent may have at the cellular level. If the acute or chronic effects of effluent could be determined by Canada's primary industries prior to the release of effluents into the receiving ecosystem, this might facilitate their ability to maintain aquatic ecosystem health.

Additionally, we will focus on the correlation of the gene expression profiles between the cell lines. Because *in vitro* assays using cell lines may be more reproducible, fast and economical, they have the potential to be used for routine toxicity testing by various industries. Cell cultures provide an alternative experimental system for studying toxic mechanisms of effluent at the molecular or cellular level, as the exposure environment can be closely controlled. These proposed testing methods have been met with scrutiny by regulatory authorities as they are not explicitly in the context of ecotoxicology (Fent 2001) because the physiological effects observed in fish may not be represented by the endpoints measured in a cell line. An important factor in the use of *in vitro* assays as models for *in vivo* studies is a high correlation of the cell line's expression profiles with those derived from the tissues of the species of origin to allow effects observed *in vitro* to be extrapolated to those *in vivo* (Bols et al. 2005). These systems must provide information on biological responses both at an organ system level and an ecological level. Cytotoxicity *in vitro* and lethality *in vivo* have been well correlated in several cell lines derived from goldfish (*Carassius auratus auratus*) (Saito et al. 1991), rainbow trout (Castano et al. 1996; Fent 2001) and desert topminnow (Brüschweiler et al. 1995; Fent and Hunn 1996).

Because gene expression in cell lines has not been well assessed, it is important to correlate the expression between cell lines of different model species used in ecotoxicology and from the cell lines to the parent tissue. Established cell lines may lose specialized functions when removed from

the originating tissue or organ, including mRNA and protein expression, or may become entirely undifferentiated (Ribas 2006). If any of the pre-existing functions are retained, it must be determined if they are similar in magnitude to those from the parent tissue. Before an *in vitro* test can be developed, it is important to ensure there is correlation to the *in vivo* test that it is attempting to accompany. The correlation analyses performed are statistically valid as the two cell line experiments were performed using identical experimental conditions. The comparison of endpoints between the two fish cell lines is important for the development of a novel toxicity test to ensure there is a universality of responses among teleosts. As previously mentioned, research examining protein expression levels has suggested that hepatocytes behave similarly *in vivo* and *in vitro* (Billiard et al. 2004; Christianson-Heiska and Isomaa 2008). The use of cell lines as a tool for environmental monitoring would reduce the need for whole animals for toxicity testing, a goal shared by the Canadian Council of Animal Care and animal care agencies worldwide.

Previous research in our laboratory examined the effects of PPME on gene expression in the livers of whole fathead minnows. EDC-responsive gene expression in adult male FHM livers was induced when exposed to effluents from an AbitibiBowater pulp and paper mill. When exposed to untreated kraft effluent and treated kraft effluent, the expression of genes linked with androgenicity, ERβ and AR, were upregulated (Naderi and Hughes-Davies 2008). VTG and ERα, genes linked with estrogenicity, were observed to be upregulated in the livers of fish exposed to combined mill outfall. These findings suggest that once the kraft and news mill effluents are combined before discharge into the Kaministiquia river, the compounds present in the kraft mill effluent were diluted and therefore their lower concentration effectively eliminated the significant effects observed in the untreated and treated kraft effluents (Werner et al. 2010). This thesis contains laboratory

experiments on cell lines that parallel our previous research that will assess the sensitivity of the cell lines and their ability to alter their gene expression subsequent to effluent exposure.

This project will contribute to the goal of supporting organizations to be stewards of the water supply they use. Although the quality of AbitibiBowater's effluent has improved since the installation of a secondary waste treatment system in the 1990s and is now considered a relatively clean mill as determined though multiple cycles of Environment Canada's EEM program, our current and ongoing data will aid in determining whether these modifications in the effluent treatment process have improved effluent quality and thus protected the health of Lake Superior and its associated ecosystems. The applications of this research could extend further than pulp and paper mill effluents, and be applied to mines, municipal wastewater treatment plants, textile industries and pharmaceutical companies to ensure that the output of effluents from industrial plants comply with federal and provincial environmental regulations. These methods can also be applied to mills that modify their effluent treatment configurations to ensure the process maintains its efficacy.

### 1.6.1. PROJECT OBJECTIVES

The first objectives of this thesis are to examine gene expression changes in both a rainbow trout hepatocyte cell line (RTL-W1) and a fathead minnow hepatocyte cell line (FHM-L) during effluent exposure. Three effluents will be used to characterize the cells' gene expression profile of these cells: untreated kraft effluent, treated kraft effluent and combined mill outfall (described in section 2.2). The objectives of this research project are:

1) To examine gene expression changes in the RTL-W1 and FHM-L cell lines exposed to three different PPMEs:
    a. Determine the appropriate effluent concentration to allow for cell growth in a 72 h viability assay
    b. Examine expression changes of genes involved in endocrine responses (ERα, ERβ, VTG, ZR, AR), genes involved in stress response (CYP1A), and reference genes (GAPDH and actin for FHM-L; GAPDH, actin, EF1α, L8 and ubiquitin for RTL-W1)
2) To compare gene expression profiles between the two cell lines studied:
    a. Determine which ERα and ERβ isoforms in RTL-W1 are more homologous to the FHM-L ERα and ERβ genes using the Basic Local Alignment Search Tool.
    b. Determine the correlation between the gene expression of both cell lines studied using Pearson correlation analysis.

# 2. MATERIALS AND METHODS

## 2.1. CELL CULTURE MAINTENANCE

Cells from the RTL-W1 cell line were grown in 25 $cm^2$ flasks (Falcon) containing Leibovitz's L15 medium (Gibco) with 1% (w/v) 100x penicillin/streptomycin (Gibco) and 5% (v/v) fetal bovine serum (FBS) (HyClone). Flasks of cells were cultured for four days in a Fisher Scientific Isotemp Incubator at 20°C and 26°C for the RTL-W1 and FHM-L cell lines, respectively. The ideal pre-exposure period was determined by visual analysis of cells using an inverted microscope (Fisher Scientific Micromaster).

Subcultivation occurred when cells reached 80-90% confluence. Cells were detached from growth surfaces with TrypLE (Gibco) for 5 minutes, and then L15 supplemented with FBS was subsequently added to deactivate the TrypLE. The aspirated cell solution was centrifuged at 1,000 x g for 5 minutes. The supernatant was removed and the pellet was either resuspended in 10 mL of fresh media to a homogeneous suspension and passaged into 2 or 3 sterile flasks, or alternatively resuspended in fresh media with a final concentration of 10% (v/v) dimethyl sulfoxide (DMSO) and stored at -80°C for cryopreservation. All cell culture reagents were tissue culture grade, autoclaved or filter sterilized prior to use to ensure sterility.

Prior to the exposure period, cells were dislodged from growth surfaces using TrypLE, centrifuged and resuspended in L15 media without phenol red (Gibco) as detailed above. The suspended cells were passaged into 12-well cell culture plates that were tissue culture treated (Falcon) and incubated for 2-3 days to 90% confluence at 20°C.

## 2.2. EFFLUENT COLLECTION

Three different effluents were obtained from holding tanks at AbitibiBowater: untreated kraft effluent (UTK) from the chemical kraft-making process, treated kraft effluent (TK) from the

secondary clarifier ponds after biological treatment, and combined mill outfall (CMO) from the mixing tank for treated kraft and newspaper effluent prior to their discharge into the Kaministiquia River (Figure 10). All effluents used in these experiments were collected in January 2009. Water quality measurements for river water and effluents were reported by Werner et al. (2010). Once collected, effluent was sterilized using a 0.22 μm filter (Millipore) and stored at -80°C in an effort to reduce any further chemical changes. Exposure medium (L15/ex) (Appendix 1) was combined with volumes of effluent and river water to obtain the desired concentration. For example, the 10% (v/v) UTK in the short-term exposure contained a final concentration of 10% (v/v) UTK, 10% (v/v) river water and 80% (v/v) L15/ex medium. L15/ex contained salts, pyruvate and galactose found in the cell maintenance medium but excludes amino acids, vitamins, growth factors and phenol red. The compounds present in L15/ex help maintain cellular survival and ionic concentration while avoiding or minimizing interactions between test chemicals and amino acids and proteins (Tanneberger et al. 2010).

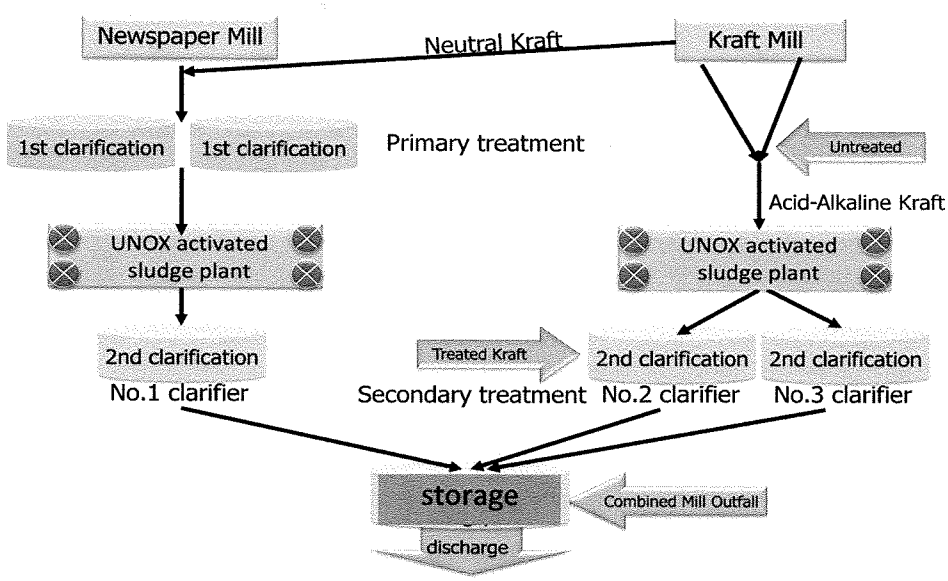


**Figure 10: Pulp and paper effluent treatment process at AbitibiBowater in Thunder Bay, Ontario, Canada.** Note that this process does not have an anaerobic secondary treatment or a tertiary clarification step. Large arrows indicate points at which effluent was removed for exposure testing. UNOX = aerated with pure oxygen (adapted from Werner et al. 2010).

## 2.3. Dose Response Assay

Dose response was performed prior to gene expression analysis to determine the optimal concentrations of effluents to use in experiments that resulted in maintenance of cell growth and integrity of mRNA extraction from the cell line. Cells were treated with 1 mL of 0%, 10%, 25%, 50%, 75% or 100% (v/v) of the effluent for 72 hours. In the RTL-W1 viability assay, a final effluent concentration of 20% (v/v) was also used. After the exposure period, cells were detached from the flask surface using 200 µL TrypLE for 5 minutes. Two hundred microliters L-15 media/well was added to deactivate trypsin. The viability of these cells was analysed using the ViCell XR Cell Viability Analyser (Beckman Coulter). The analyzer performs a trypan blue dye-exclusion assay and outputs the number of viable cells.

## 2.4. Time-course Assays

Cells were exposed to the concentrations of effluents determined from the dose-response assays. The optimal exposure period was determined through real-time PCR analysis of effluent responsive genes (Werner et al. 2010). Two time-course experiments were performed using the RTL-W1 cell line. First, a long time-course that included five time points (1, 6, 24, 48 and 72 h) was used to survey changes in the genes of interest in this cell line. As described in section 3.1, exposure periods of 72 h were inappropriate for the evaluation of gene expression as the majority of expression occurred before 24 h. A subsequent short time-course that included six time points from early in the exposure period (0.5, 1, 4, 6, 12 and 24 h) was then performed to refine our observations. Based on the results obtained in the RTL-W1 long time-course, only the short-term time-course was used in examining gene expression changes in the FHM-L cell line.

### 2.4.1. Effluent Exposure

Cells were subcultured from 25 $cm^2$ flasks to 12-well plates. The medium was changed to exposure media (L15/ex), which only contained salts. Cells were grown for 4 days in plates prior to the exposure period. Sets of three wells of cells were exposed to one of four treatments: 10% (v/v) UTK, 10% (v/v) TK, 10% (v/v) CMO, or an untreated control of only L15/ex media. In the short time-course, control cells were maintained on standard exposure media containing 10% (v/v) river water to account for the background gene expression responses that the river water may have provided.

### 2.4.2. RNA Extraction, RNA Quantification and Reverse Transcription

After the exposure period, wells were rinsed with 0.5 mL phosphate buffered saline (Farrell 2005). Then 380 µL TriZOL reagent (Invitrogen) was added to each well and the cells were removed and fractionated by repeated pipetting. The TriZOL-lysed cell mixtures from three wells of identical treatments were batched into a 1.5 mL tube and the RNA was purified as per the manufacturer's recommendation. Two microliters of RNA were quantified and its quality assessed using an Experion RNA StdSens Kit (Bio-Rad). Quality was assessed by a calculated ratio of the 28*S* and 18*S* ribosomal RNAs..If this ratio was less than 1.3, the sample was excluded from the study. One hundred nanograms total RNA was reverse-transcribed to generate cDNA using RevertAid Moloney murine leukemia virus enzyme (MMLV), following the manufacturer's recommendations. After synthesis, cDNA was stored at -20°C.

### 2.4.3. Gene Expression Analysis

Primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3) or taken from the literature to detect 13 genes associated with endocrine disruption, stress or reference (Table A2.1.1). A consistently expressed reference gene was identified using analyses of variance (ANOVA) and

used as a normalization reference. Real-time quantitative PCR reactions contained 1 μL of cDNA, the appropriate primer pair and Maxima SYBR Green/ROX real-time PCR Mastermix (Fermentas) at the manufacturer's recommended concentrations in a final volume of 25 μL. Samples were analysed using a Biorad CFX96 C1000 real-time PCR instrument. Samples were initially denatured at a 95°C for 150 s prior to the polymerase reaction. Each of 35 thermal cycles in the polymerase reaction consisted of a 95°C denaturation for 15 s, annealing at an appropriate temperature for 60 s and a 72°C extension for 60 s. Reaction conditions were optimized by analyzing melt curves of the final product (Kubista et al. 2006).

### 2.5. Data Analysis

Significant differences among treatments in the dose response assay were determined through analyses of variance and using post-hoc Tukey's Honestly Significant Difference test using R (http://r-project.org) and SigmaPlot. Fold-induction was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Log-transformation was applied to time-course assay data before analysis to ensure normal distribution of the gene expression data. Significance of the fold-induction of the target genes was determined using a pairwise fixed reallocation randomization test ($p<0.05$) (Pfaffl et al. 2002).

### 2.6. Sequence homology

Similarities between target mRNA sequences in rainbow trout and fathead minnow were determined using the nucleotide basic local alignment search tool (nBLAST; http:// blast.ncbi.nlm.nih.gov). The Megablast program was used to align the sequences, which is optimized for highly similar sequences. Accession numbers for each gene used in the sequence alignments are listed in Tables A2.1 and A2.2.

Due to the genome duplication that occurred during the divergence of the fleshy-finned fishes Sarcopterygii, from the bony-finned fishes Actinopterygii, rainbow trout has two isoforms of each estrogen receptor subunit, whereas fathead minnow only has one isoform of each gene (Nagler et al. 2007). Thus, target genes in fathead minnow were compared against both rainbow trout isoforms of the corresponding gene sequence where applicable. For example, the mRNA sequence of ERα in fathead minnow was compared with that of both the ERα1 and ERα2 isoforms that exist in rainbow trout.

### 2.7. Gene Expression Correlation

Pearson correlation analysis was performed in R. The means of the fold induction of target genes in each treatment were taken and log-transformed prior to analysis. Fold induction of ERα and ERß genes in fathead minnow were separately correlated to both isoforms of each target gene in rainbow trout.

# 3. RESULTS

## 3.1. VIABILITY ASSAY

A significant increase in the quantity of viable cells was observed in 10% (v/v) TK and 20% (v/v) UTK and TK treated RTL-W1 cells and FHM-L cells. A statistically significant reduction in cell viability was observed at 75% and 100% (v/v) with all effluents. All effluents promoted growth when present in the media at up to 20% (v/v). While cells were viable in 20% (v/v) effluent, RNA quantification suggested that RNA obtained from these cells was of poor quality (Figure 11 and Figure 12). For this reason, a final effluent concentration of 10% (v/v) was selected for determination of the effects of PPMEs on gene expression, as at this concentration cells remained viable and RNA obtained from them was intact and of good quality. Although a significant reduction in cell viability was observed in cells exposed to 10% (v/v) CMO, this concentration was chosen as it has been reported by AbitibiBowater that the final concentration of CMO in the receiving water is approximately 10% (v/v).

## 3.2. RTL-W1 LONG TIME-COURSE EXPERIMENT

### 3.2.1. LONG TIME-COURSE GENE SELECTION

GAPDH and β-actin were both tested for their appropriateness as reference genes (Filby and Tyler 2007). GAPDH showed variability among biological samples and treatments (Figure A2.1A), thus excluding this gene from being considered an appropriate reference gene ($p<0.001$). β-actin was subsequently selected as the reference gene for this study, as there was little variability of its gene expression among treatments ($p=0.223$) (Figure A2.1B).

### 3.2.2. LONG TIME-COURSE GENE EXPRESSION DATA

The majority of statistically significant up- or down-regulation occurred after 24 h of exposure for all eight target genes. Exposure to 10% (v/v) river water induced up-regulation of all

estrogen receptor isoforms 24 h after treatment (Figure 13C, D, E, and F). Cells exposed to river water showed an upregulation of ERα1 (8.6-fold) but this increase was not statistically significant. This statistical insignificance can be attributed to variability in expression levels among biological replicates (Figure 13C). Significantly increased expression of AR was observed at 24 h in cells exposed to river water and TK (4.4-fold and 4.9-fold, respectively), and at 72 h in all three effluents (UTK, 6.6-fold; TK, 8.0-fold; and CMO, 2.5-fold) (Figure 12G). Statistically significant upregulation of CYP1A was observed in liver cells exposed to UTK and TK after 6 h, with maximum fold induction at 72 h (78.6-fold and 50.3-fold, respectively). CYP1A expression was also induced in cells exposed to CMO for 72 h (9.2-fold) (Figure 12H). The expression of other genes did not vary significantly during the 72-h time-course. Although significant changes in gene expression were observed, no coherent trends were apparent among timepoints or exposures.

### 3.3. Short Time-course Experiments

#### 3.3.1. Short Time-course Reference Gene Selection

GAPDH, β-actin, elongation factor 1α (EF1α) and ubiquitin (Ubi) were tested as possible reference genes for real-time PCR result normalization and were all appropriate candidates to be used as a normalization reference as they did not vary among exposure treatments ($p>0.892$) (Figure 14; Figures A2.2 A, B, D, E). Ribosomal protein L8 (RPL8) was selected as the appropriate reference gene, as it was most consistently expressed among exposure treatments and was the least varied among the reference genes ($p=0.998$) (Figure A2.2C). In the FHM-L cells, it was observed that there was greater variability ($p<0.001$) in β-actin expression among treatments than in GAPDH in fathead minnow cells ($p=0.223$) (Figure 3.2.2A; Figure A2.3). GAPDH was therefore selected as the reference gene for this cell line, as it was consistently expressed among exposure treatments (Figure A2.3B).

### 3.3.2. Short Time-course Gene Expression Data

Expression of VTG was observed in RTL-W1 cells after exposure to UTK and TK effluents at 1 h (8.2-fold) and 24 h (9.3-fold), and 1 h (9.0-fold), respectively (Figure 15A). Upregulation of ERα1 was observed after 0.5 h of exposure to all three effluents. Statistically significant increases in ERα1 expression were also observed at 4 h, 6 h and 12 h (2.8-fold, 2.2-fold, 2.4-fold, respectively) after exposure to CMO (Figure 15C). ERα2 was significantly upregulated after 0.5 h in TK (2.8-fold) and at 4 h and 6 h after exposure to CMO (4.0-fold and 2.1 fold, respectively) (Figure 15D). No statistically significant induction of ERβ1, ERβ2, ZR, or AR mRNA levels was observed at any of the time points and exposures (Figure 15B, E, and F). After RTL-W1 exposure to UTK, TK and CMO, CYP1A expression rose over time to maxima at 4 h (88.0-fold), 6 h (20.0-fold), and 4 h (7.3-fold), respectively, and declined thereafter (Figure 2.2.4H).

No significant changes in β-actin were observed in any of the effluents and at any of the time points (Figure 16A) in FHM-L cell line. Upregulation of VTG was observed in cells exposed to UTK, TK and CMO (6.4-fold, 2.7-fold, and 2.4-fold, respectively) (Figure 16B) at 0.5 h. Cells exposed to TK for 0.5 h significantly upregulated ERα expression (2.5-fold) (Figure 16C). ERβ and AR mRNA were significantly upregulated in cells exposed to UTK for 1 h by 4.1-fold and 5.0-fold, respectively (Figure 16D and E). ERβ was upregulated 2.1-fold after 24 h of UTK exposure. Similar to RTL-W1 cells, CYP1A expression rose over time to maxima at 6 h (52.2-fold), 4 h (7.3-fold) and 6 h (3.9-fold), in UTK-, TK- and CMO-exposed cells, respectively (Figure 16F).

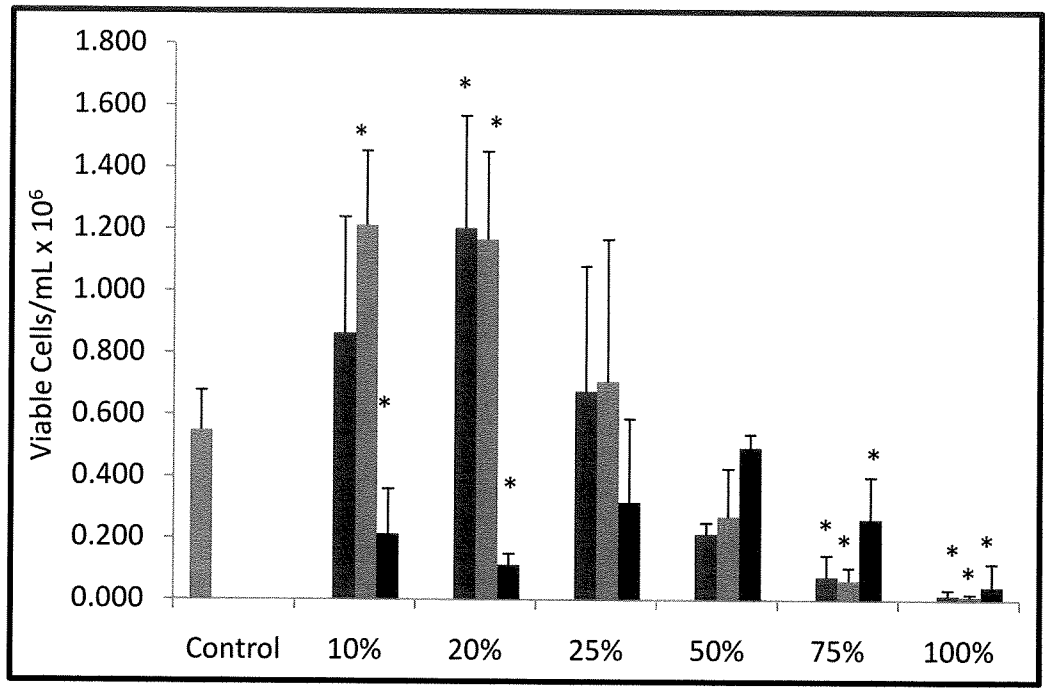


**Figure 11: Viability of RTL-W1 cells upon exposure to PPMEs.** The cells were exposed to an untreated control (▒) or varying concentrations of untreated kraft effluent (■), treated kraft effluent (▒), or combined mill outfall (■) for 72 hours ($n$=3). Concentrations of effluents in experimental media are represented as (v/v). Data is presented as mean number of viable cells ± standard error. Asterisks (*) indicate significant differences versus controls ($p$<0.05).

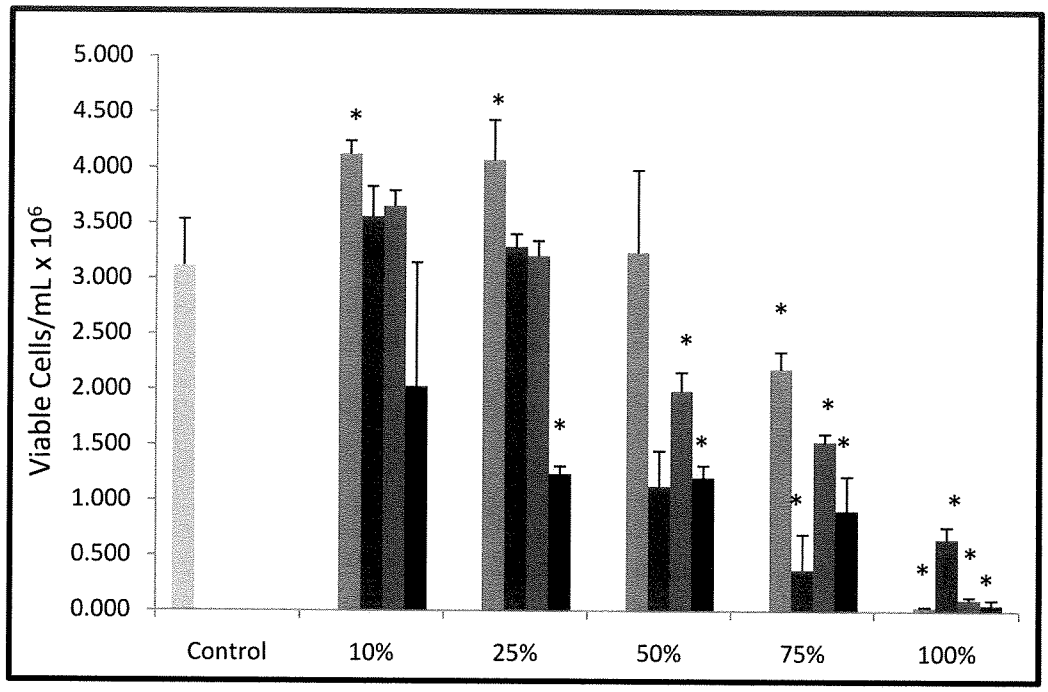


**Figure 12: Viability assay of FHM-L cells exposed to PPMEs.** Exposures included an untreated control (▒), river water (▒), untreated kraft effluent (■), treated kraft effluent (▒), or combined mill outfall (■) for 72 hours ($n$ = 3). Concentrations of effluent to experimental media are represented as (v/v). Data are presented as mean numbers of viable cells ± standard error. Asterisks (*) indicate significant differences versus controls ($p$<0.05).

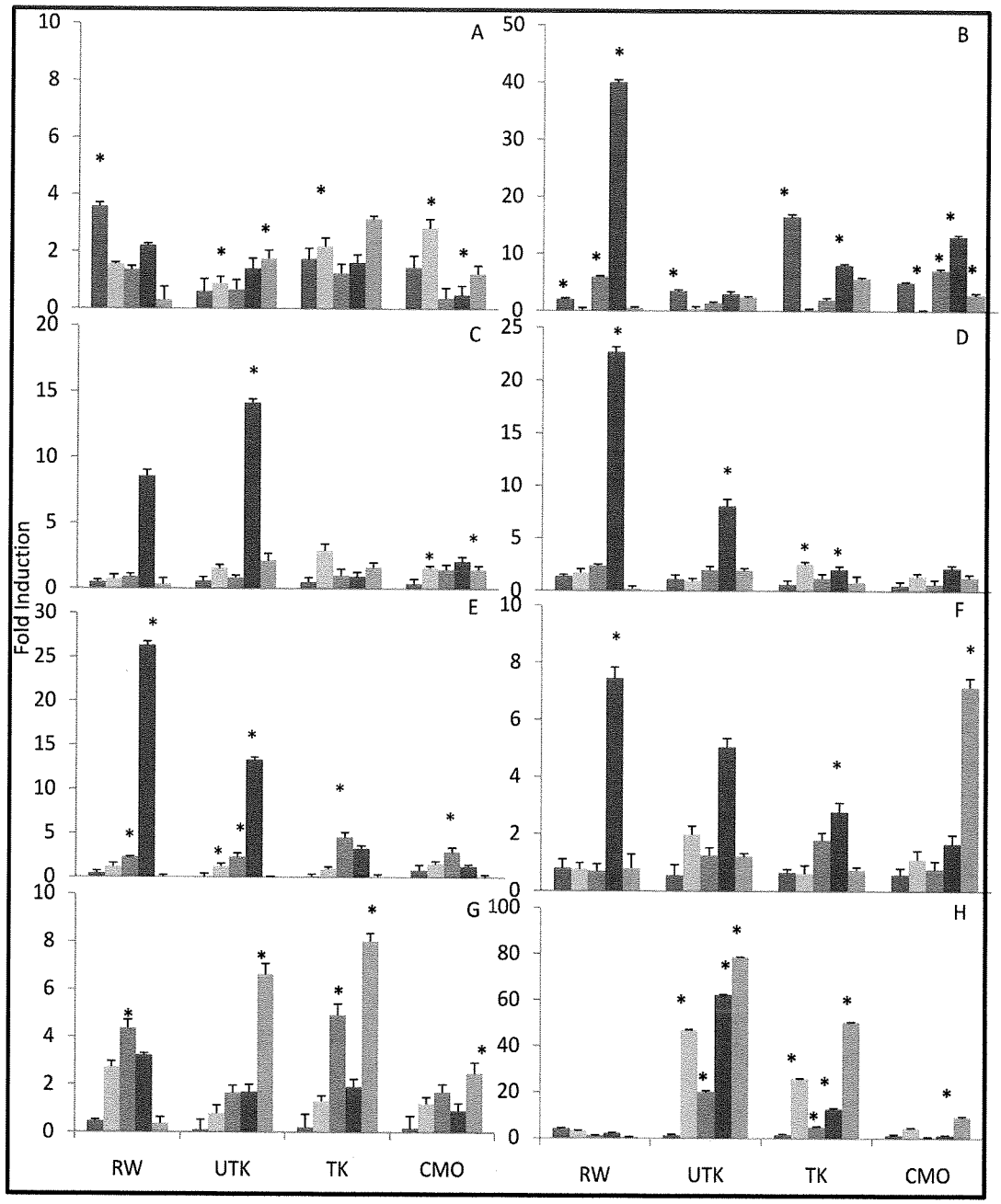


**Figure 13: Expression of target genes in RTL-W1 cells upon exposure to PPMEs in the long time-course.** The cells were exposed to river water (RW), untreated kraft effluent (UTK), treated kraft effluent (TK), or combined mill outfall (CMO) for 1 (■), 6 (■), 24 (■), 48 (■), or 72 h (■) ($n$=3). Effluents and river water were diluted to 10% (v/v) in exposure media. Genes surveyed were glyceraldehyde 3-phosphate dehydrogenase (**A**), vitellogenin (**B**), estrogen receptor alpha subunit isoforms 1 (**C**) and 2 (**D**), estrogen receptor beta subunit isoforms 1 (**E**) and 2 (**F**), androgen receptor (**G**) and cytochrome P4501A (**H**). Data are presented as mean fold induction ± standard error versus controls (L-15/ex media). Gene expression values were corrected using β-actin. Asterisks (*) indicate a significant difference versus controls ($p$<0.05).

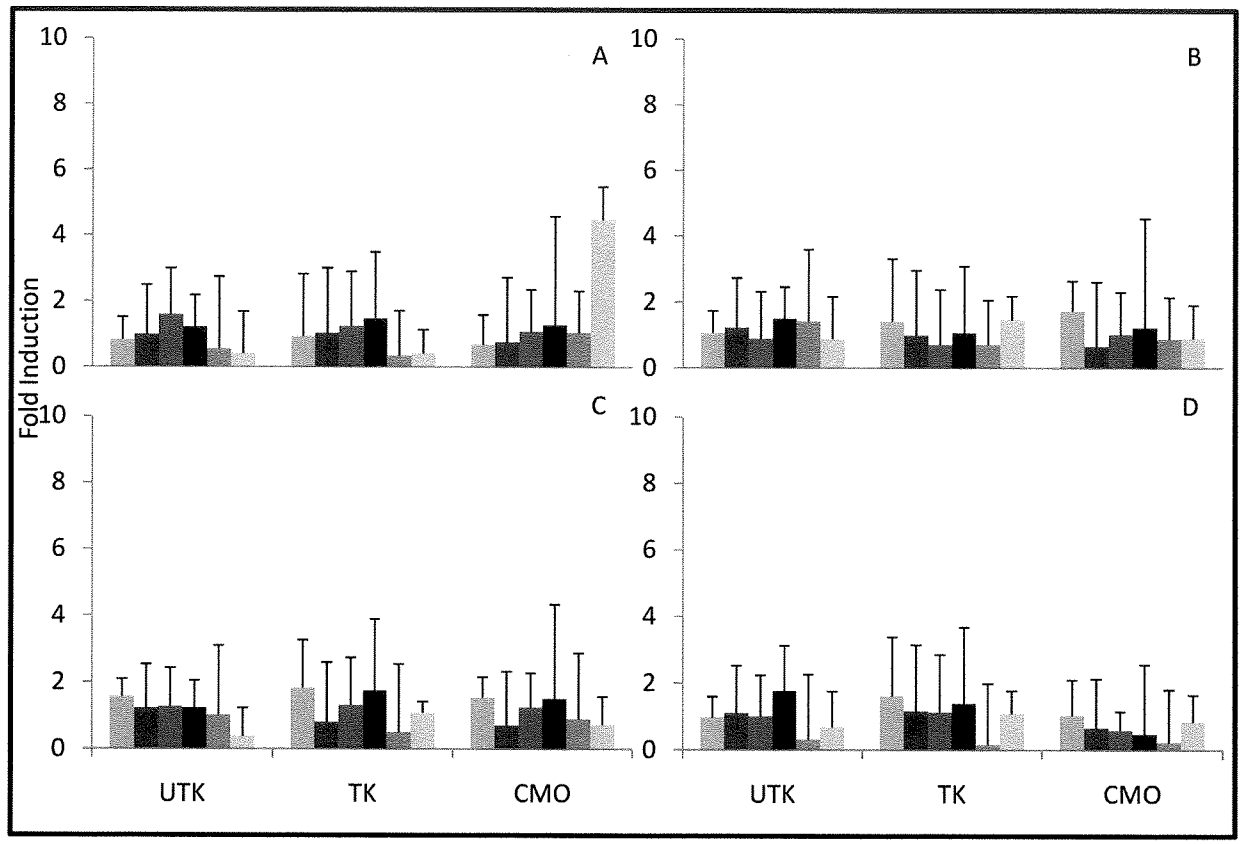


**Figure 14: Expression of housekeeping genes upon PPME exposure in RTL-W1 cells during the short time-course**. The genes examined were glyceraldehyde 3-phosphate dehydrogenase (**A**), β-actin (**B**), elongation factor 1a (**C**), and ubiquitin (**D**), exposed to 10% (v/v) untreated kraft effluent (UTK), treated kraft effluent (TK), or combined mill outfall (CMO) for 0.5 ( ), 1 ( ), 4 ( ), 6 ( ), 12 ( ), or 24 h ( ) ($n$=4). Effluents were diluted to 10% (v/v) in exposure media made with 10% (v/v) river water. Data are presented as mean fold induction ± standard error versus controls, which had a relative expression level of 1. Gene expression values were normalized using ribosomal protein L8 (RPL8). No statistical differences among treatments as compared to negative controls were observed ($p < 0.05$).

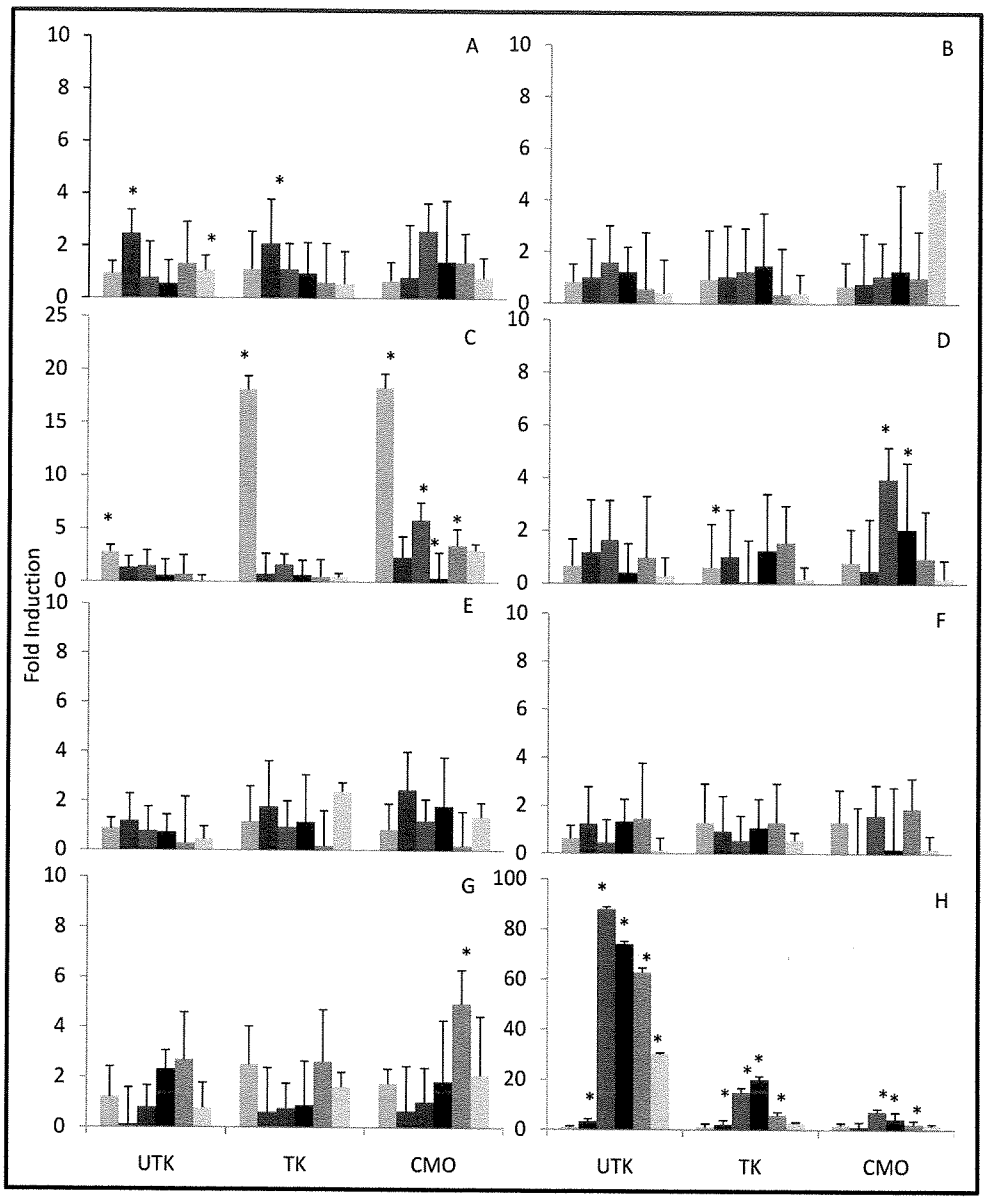


**Figure 15: Expression of EDC- and stress-indicator genes in PPME-exposed RTL-W1 cells during the short time-course.** The genes surveyed were vitellogenin (**A**), zona radiata (**B**), estrogen receptor subunit A isoforms 1 (**C**) and 2 (**D**), estrogen receptor beta subunit isoform 1 (**E**) and 2 (**F**), androgen receptor (**G**) and cytochrome P450 1A (**H**). The expression of target genes was assessed in RTL-W1 cells exposed to river water (RW), untreated kraft effluent (UTK), treated kraft effluent (TK), or combined mill outfall (CMO) at 0.5 (▒), 1 (▒), 4 (▒), 6 (▒), 12 (▒) or 24 h (▒) ($n$=4). Effluents were diluted to 10% (v/v) in exposure medium with 10% (v/v) river water. Data are presented as mean fold induction ± standard error versus controls (L-15/ex media with 10% (v/v) river water. Gene expression values were corrected using RPL8. Asterisks (*) indicate significant differences versus controls ($p$<0.05).

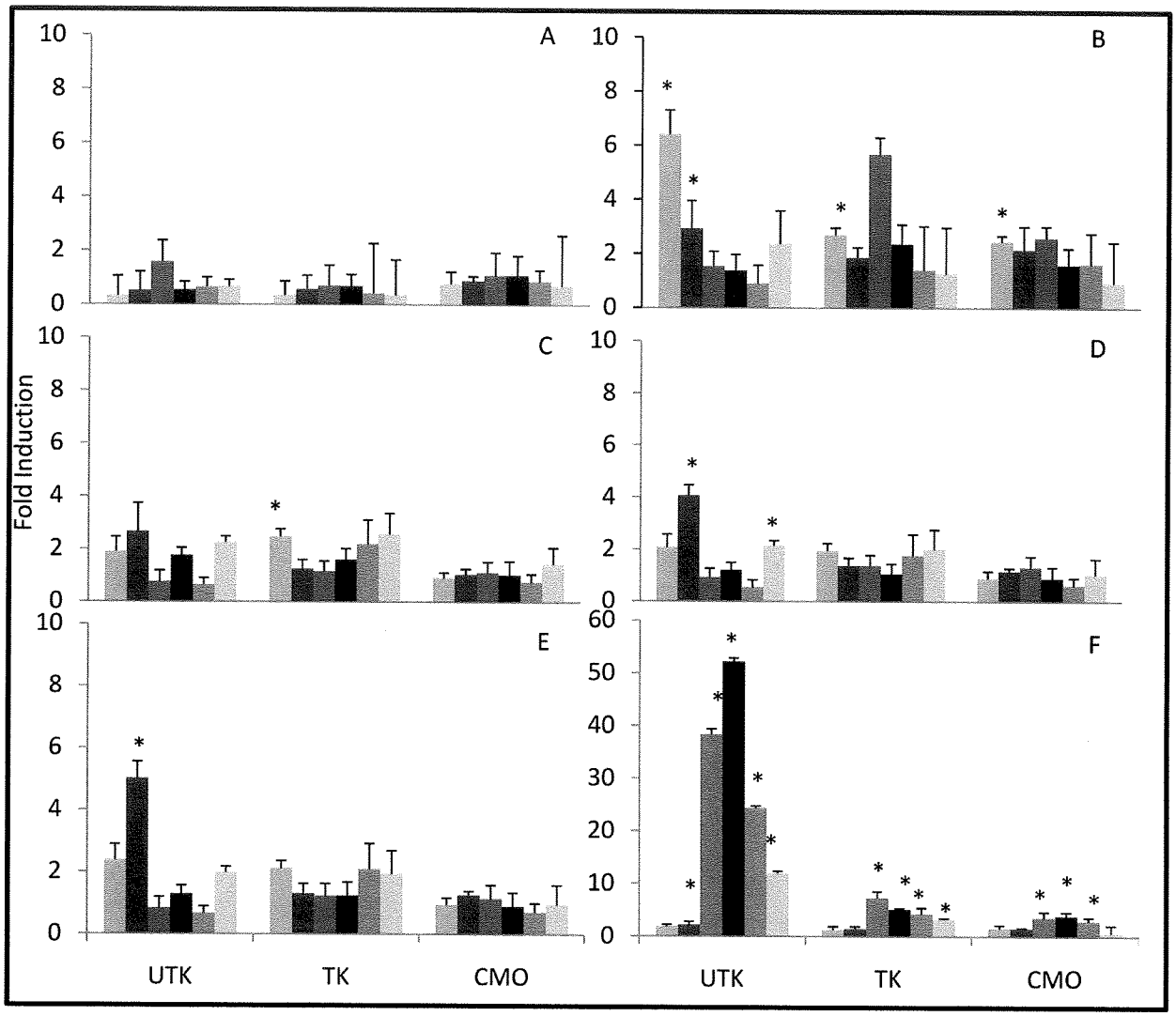


**Figure 16: Expression of housekeeping, EDC-responsive and stress-indicator genes in PPME-exposed FHM-L cells.** Real time PCR was used to examine the expression of β-actin (**A**), vitellogenin (**B**), estrogen receptor alpha subunit (**C**), estrogen receptor beta subunit (**D**), androgen receptor (**E**) and cytochrome P450 1A (**F**) mRNA levels in cells exposed to untreated kraft effluent (UTK), treated kraft effluent (TK), or combined mill outfall (CMO) for 0.5 (■), 1 (■), 4 (■), 6 (■), 12 (■) or 24 h (■) ($n = 4$). Effluents were diluted to 10% (v/v) in exposure media with 10% (v/v) river water. Data are presented as mean fold induction ± standard error versus controls (L-15/ex media with 10% (v/v) river water). Gene expression values were corrected using GAPDH. Asterisks (*) indicate significant differences in expression versus controls ($p<0.05$).

### 3.4. SEQUENCE COMPARISON

The gene sequences of the fathead minnow estrogen receptor subunits α and β and CYP1A were 70 to 80% homologous with those in rainbow trout. Both the ERα1 and ERα2 sequences in rainbow trout were 74% similar to that of the fathead minnow ERα sequence. The sequence of ERβ in fathead minnow was 81% and 78% homologous to that of the rainbow trout ERβ1 and ERβ2 genes, respectively. The sequences of CYP1A in both fish species were 78% similar. The sequences of the AR and VTG genes were found to be 77% and 79% similar in both fish species, respectively (Table 1).

### 3.5. GENE EXPRESSION CORRELATION

Pearson correlation analysis suggested that there was little correlation in the expression of AR (-0.316), VTG (-0.121), ERα isoforms 1 and 2 (-0.283 and 0.206, respectively) and ERβ isoforms 1 and 2 (0.014 and -0.131, respectively) in rainbow trout to that of the corresponding fathead minnow mRNAs (Table 1, Figure 17A-F). The expression of CYP1A had a correlation coefficient of 0.782 between the rainbow trout and fathead minnow (Table 4.2.1, Figure 17G).

**Table 1: Sequence homology and gene expression correlation between fathead minnow and rainbow trout liver cells.** The homology of sequences was determined using BLAST. Correlation of determination was calculated from log-transformed fold induction data using Pearson product-moment correlation. Refer to Tables A2.1 and A2.2 for accession numbers of genes.

| Fathead minnow gene | Rainbow trout isoform | Sequence homology | Expression correlation coefficient (R) |
|---|---|---|---|
| ERα | ERα1 | 74% | -0.283 |
| ERα | ERα2 | 74% | 0.206 |
| ERβ | ERβ1 | 81% | 0.014 |
| ERβ | ERβ2 | 78% | -0.131 |
| VTG | | 77% | -0.121 |
| AR | | 79% | -0.316 |
| CYP1A | | 78% | 0.782 |

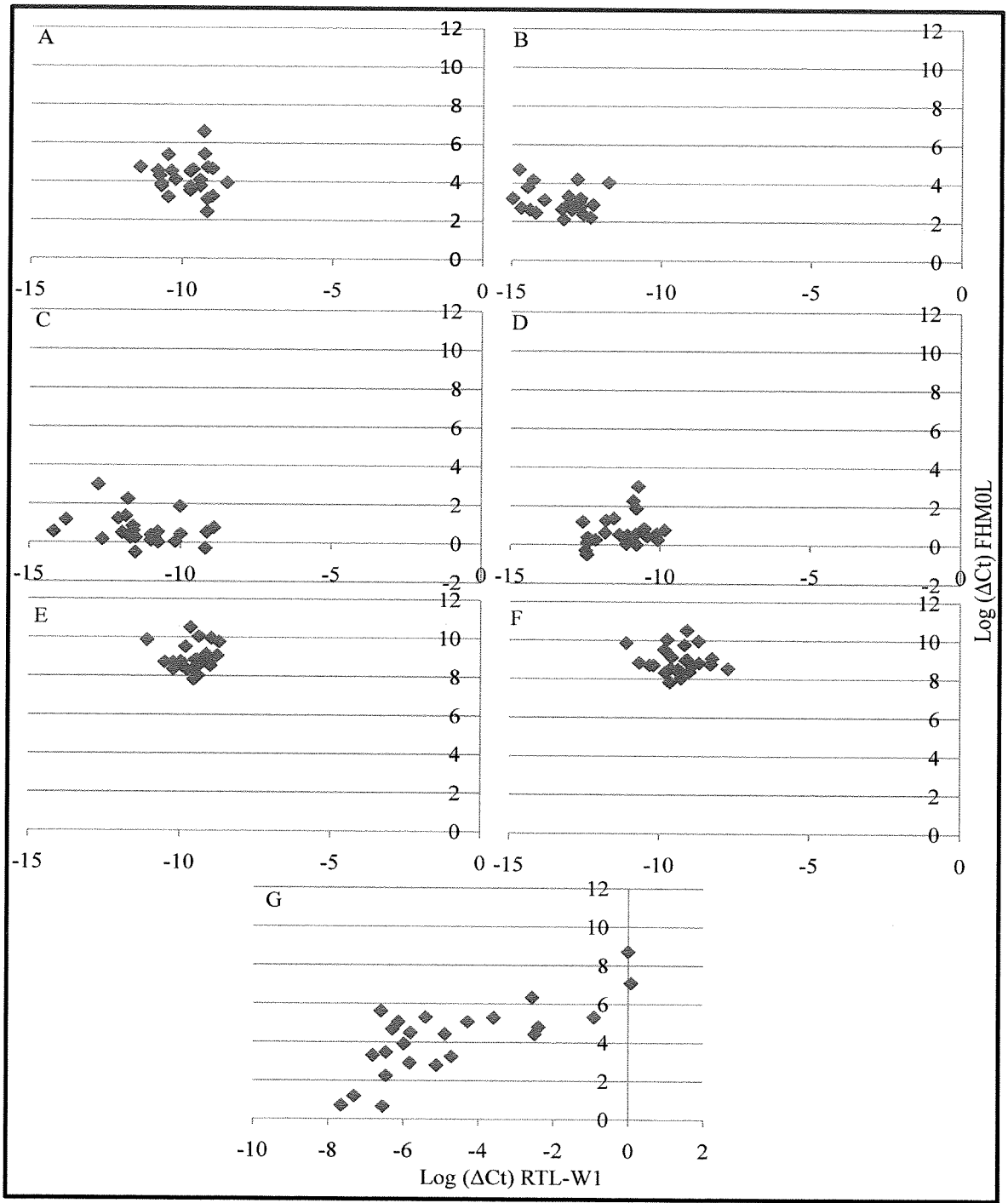


**Figure 17: EDC- and stress indicator gene expression in FHM-L and RTL-W1 cells.** Scatter plots show the expression patterns of VTG (A), AR (B), ERα1/ERα (C), ERα2/ERα (D), ERβ1/ERβ (E), ERβ2/ERβ (F), and CYP1A (G). Data represented is gene expression data from short-term time-courses and was normalized using untreated control. Pearson correlation coefficients (R) from these graphs are listed in Table 1.

# 4. DISCUSSION

### LOW CONCENTRATIONS OF EFFLUENTS PROMOTED HEPATOCYTE GROWTH

In both the rainbow trout cell line and the fathead minnow cell line, exposure to 10% (v/v) UTK, TK or CMO effluents increased cell viability compared to the untreated control. Fewer cells were viable when exposed to 10% (v/v) CMO than to the same concentration of UTK or TK. Organic compounds present in the UTK and TK may nutritionally enrich the effluent and encourage cell proliferation and cell viability (Owens 1991; Chambers et al. 2000). Organic matter in the effluents in the form of nitrogen, phosphorus and organic carbon enhances cell growth at low concentrations, but the precise identity of the compounds responsible and where in the pulp and paper making process they enter into the effluent are unknown (Amblard et al. 1990). Alternatively, this increase in cell viability can be attributed to the phenomenon of hormesis. Hormesis is an adaptive response that is characterized by a stimulatory response to low levels of toxins (Adams et al. 1996; Calabrese and Baldwin 2002). The toxicants present in effluent may trigger a hormetic response in the fish cell lines at low concentrations. At higher concentrations, the toxic effects of the effluents appear to overwhelm their ability to promote cell growth and thus cause a decline in the number of viable cells. The compounds that exert a toxic effect on the fish cells may be any combination of the pollutants previously mentioned, including resin acids, tannins and/or lignin. The less pronounced effects of CMO on cell growth suggests that this effluent lacks the high levels of nutrients and/or toxicants present in UTK and TK. This is reinforced by the CYP1A expression data (Figures 15H and 16F).

## LONG-TERM EFFLUENT EXPOSURE AFFECTED RTL-W1 GENE EXPRESSION

RTL-W1 cells that were exposed to any of the three effluents during the long time-course upregulated some target genes, but no coherent trend was observed. After 72 h of exposure to effluents or exposure media, cells were likely malnourished and declining in viability (Vo 2010). The expression of CYP1A, AR, VTG, ERα1 and ERβ2 at 72 h exposure could be attributed to a second wave of up-regulation in response to the sustained exposure to the chemicals in the effluent. Significant induction of GAPDH, VTG, ERα2, ERβ isoforms and AR were observed in cells exposed to river water. The induction of these genes can be attributed to the presence of hormones and pesticides from the agricultural and cattle farms upstream to the mill. River water had profound effects on gene expression in RTL-W1 cells. This effect could have masked the effects of the different effluents on the cells. Moreover, since fish exposed to the effluents in aquatic environments receive a mixture of effluents and runoff compounds from upstream sites, we decided to mimic this situation by adding 10% (v/v) river water to all the effluents and use 10% (v/v) river water as a control. The gene expression changes quantified from the long time course provided initial insight into the effects of effluents over time on the cell lines and led to changes in the effluent exposure protocols for the short time-courses used for in-depth examination of expression changes in the RTL-W1 and FHM-L cell lines upon PPME exposure. Comparisons between the long and short-time courses are inappropriate, as river water was not added to the exposure media prior to the dilution of the effluents during the long time course. Although longer time periods were used for the cell viability assay in comparison to the time periods used to assess gene expression, it was assumed that the cells would still be viable and the extracted RNA of high quality at time points less than 72 h. Because expression of all EDC and stress linked genes also occurred prior to 72 h, the expression of genes in response to compounds present in the PPMEs likely occurred between 6

and 48 h after effluent exposure. Previous research found that the maximal response of gene expression in various cell lines to occur generally between 24 h (Aluru and Vijayan 2007) and 8 days (Scholz et al. 2004) after exposure. Direct comparisons of the induction time for gene expression are difficult because the length of time in which target genes are optimally expressed varies depending on the toxicant or additive being studied, the nature of the gene itself, the cell line, and the experimental conditions of the study.

### FHM-L AND RTL-W1 CELL LINES LACK SENSITIVITY FOR EDC EXPOSURE

While VTG is often considered the gold standard for exposure to estrogenic compounds, VTG was not upregulated to the same extent in the liver cell lines as it normally is in the presence of estrogen or estrogen mimics. Other endocrine disruption- linked genes also were upregulated but did not follow any consistent pattern in any of the effluent treatments. While some statistically significant expression of EDC-linked genes was observed in both cell lines, the expression of these genes was not consistently correlated with exposure period after exposure to any of the effluents. This corroborates other research that found that RTL-W1 cells were not capable of estrogen-mediated induction of VTG mRNA, ER mRNA or VTG protein in a time-related fashion (Ackermann 2000). Also, exposure to estrogens for up to 72 h was unable to induce ER and VTG mRNA expression in FHM-L cells (Lee et al. 2009). By contrast, UTK and TK effluents collected from the same mill as in this study induced the expression of ERβ and AR in the livers of male fathead minnows, and induced the expression of VTG and ERα after exposure to CMO (Werner et al. 2010). Induction of VTG and ER mRNA in response to the exposure to a known or suspected estrogenic compound has been observed in primary cultures of hepatocytes from several fish species, including common carp (Bickley et al. 2009), Atlantic salmon (Braathen et al. 2009),

goldfish (Nelson and Habibi 2010), and rainbow trout (Flouriot 1993; Islinger 1999; Pawlowski et al. 2000). The lack of a consistent response in gene expression between livers extracted from whole fish, primary cultures of hepatocytes and a continuous hepatocyte cell line may be attributable to the loss of function of immortal cells when removed from their parent organ and grown *in vitro*. As cells are continually passaged and cultured in the absence of estrogens and other cells and tissues that participate in estrogenic VTG or ER expression induction, they may lose their sensitivity to estrogens and eventually their ability to induce the expression of estrogen-related genes. To examine the cells' responsiveness to estrogenic compounds, preliminary studies were performed using estradiol and ethnylestradiol, however DMSO, the solvent used to dissolve estradiol and ethynylestradiol, affected gene expression in both cell lines. Thus, any effect elicited in the cells could not wholly be attributed to the effect of the estrogenic compounds.

To circumvent this issue, other researchers have developed stable fish cell-based reporter systems that are sensitive to estrogenic compounds. The previously-established stable reporter gene systems HELN-rtER (Molina-Molina 2008) and MELN (Balaguer et al. 1999; Pillon et al. 2005) were developed from the human cell lines HeLa and MCF-7, and stably expressed rainbow trout ERα and human ERα, respectively. However, these cell lines are presently not used to assess environmental estrogens and were not derived from aquatic species with well-characterized morphological responses to estrogenic compounds. Ackermann et al. (2002) and Cosnefroy et al. (2009) developed estrogen-responsive fish cell lines by transfecting the rainbow trout gonad cell line RTG-1 and a desert topminnow hepatoma cell line with rainbow trout ERα cDNA and the luciferase reporter gene, respectively. Both reporter cell lines were able to induce ER in response to environmental xenoestrogen exposure. If a stable reporter system sensitive to steroid hormones in

the environment was developed using the RTL-W1 cell line, this could have potential as an appropriate assay to assess the estrogenic activity of PPMEs.

### EXPRESSION OF CYP1A WAS HIGHLY CORRELATED BETWEEN CELL LINES WHILE MAGNITUDES OF EXPRESSION OF EDC-RESPONSIVE GENES VARIED

The expression of EDC-responsive genes in RTL-W1 cells, namely those of the ERα isoforms and VTG, was greater in magnitude than in FHM-L cells. Correlation analysis revealed that the expression patterns of VTG, the ER isoforms and AR were dissimilar between the two fish cell lines. The poor correlation of expression is likely due to expression of genes at different time points and the differences in sensitivity of each cell line to EDCs (cf. Figures 2.2.4A and 3.2.2B). Alternatively, the relative lack of responsiveness to EDCs versus those from intact FHM liver (Werner et al. 2010) could also be a contributing factor to the lack of correlation.

### SHORT-TERM EXPOSURE TO PPMES INDUCED CYP1A EXPRESSION

CYP1A is considered a key enzyme in the metabolism of xenobiotics. Its induction is sensitive to several environmental pollutants, including PCDDs, PCDFs and polycyclic aromatic hydrocarbons (Bucheli and Fent 1996). While both RTL-W1 and FHM-L cells were responsive to single chemicals and compounds known to induce stress via CYP1A expression (Lee et al. 1993, 2009), little research has attempted to determine differential expression of the cell lines exposed to complex mixtures. Analysis of CYP1A expression in cells during long-term exposure to PPME did not show the same gene expression profile observed during short-term exposures of up to 24 h (cf. Figures 2.2.2H and 2.2.4H). Assessment of gene expression changes within 24 h of PPME exposure, most notably in CYP1A, in these cell lines may be sufficient for identifying acute levels of toxicants, including EDCs, in aquatic effluents.

The expression of CYP1A varied in FHM-L and RTL-W1 cells among treatments over time but levels were highest in UTK-exposed cells. Unsurprisingly, this suggests the presence of more compounds capable of binding the aryl-hydrocarbon receptor in cells in UTK than in the other two effluents, as this gene is often used as a biomarker indicative of exposure to pollutants in fish and fish cell systems (Fent 2001). The decrease in CYP1A expression after 4 to 6 h of exposure and the cells' continued viability (Figures 2.2.1 and 3.2.1) suggests that they acclimated to stress as time elapsed. The stepwise reduction of CYP1A expression between UTK and TK and subsequently between TK and CMO suggests that effluent clarification improved its short-term effects, reducing the quantity of stress-inducing compounds present, and also suggests the appropriateness of CYP1A as a putative biomarker indicative of relative effluent toxicity. However, the continued statistically significant induction of CYP1A in TK and CMO-exposed cells suggests that secondary treatment did not fully remove stress-inducing compounds from PPMEs. This result was similar to what was observed in fathead minnows exposed to PPMEs, where after a 6-day CMO exposure a significant increase in CYP1A (6.5-fold) was observed (Werner et al. 2010).

The RTL-W1 line induced CYP1A to a greater magnitude than the expression observed in the FHM-L cell line. Despite this difference, the expression of CYP1A in RTL-W1 and FHM-L was positively correlated (Table 1). The variation of expression of CYP1A in RTL-W1 is highly similar to that in FHM-L (cf. Figures 15H and 16F). This suggests that both cell lines were similarly responsive to stress-inducing compounds in the effluent. As discussed in section 1.3.1, the proposed use of cell lines in toxicity testing examines cells based on cytotoxicity, not gene expression based endpoints. However, the determination of cytotoxic endpoints requires exposure periods of up to 72 h and the use of protein biomarkers, such as CYP1A, requires longer exposure periods of up to 11 days (Schirmer et al. 2000). CYP1A mRNA induction appears to be a more sensitive endpoint, as

maximum expression changes were seen after as little as 4 h of PPME exposure at ecologically-relevant concentrations.

### FHM-L CELL LINE EXPRESSION DATA CANNOT BE CORRELATED TO PRE-EXISTING FATHEAD MINNOW LIVER EXPRESSION DATA

The next step in developing a toxicity test based on using gene expression changes in a fish cell line requires correlation of the expression in cell lines to that of the parent organ in the whole fish. Because cell lines do not represent the interactions between different cell types, the synergy of different organs within an animal, the biotransformation or metabolism of compounds *in vivo* and/or bioaccumulative effects PPME may have, it is essential to ensure there is concordance between observations in cell lines and those in the parent tissue. Exposure of whole fathead minnows to PPMEs has extensively demonstrated upregulation in EDC-responsive genes (Larkin et al. 2003; Wang et al. 2004; Denslow and Larkin 2006; Werner et al. 2010). However, this upregulation was not observed in the two cell lines examined in this study. Although CYP1A expression induction was observed in both cell lines, similar to livers from whole fish, the correlation of the FHM-L expression data and that of livers from whole organisms (Werner et al. 2010) is not appropriate in this case, because the exposure parameters differed between the two experiments and correlation using datasets with differing experimental parameters produces inaccurate results. However, gene expression data from similar short-term PPME exposure using whole organisms could be correlated to the cell line exposure data in the future.

# 5. Concluding Remarks and Future Directions

The overall objectives of this thesis were to determine the optimal PPME exposure conditions for the FHM-L and RTL-W1 cell lines and to use real-time PCR to examine the expression patterns of the EDC-linked and stress-induced genes previously examined in fish livers following exposure. At low concentrations, effluents appeared to contain organic compounds that promoted cell growth. Higher concentrations of PPMEs were cytotoxic and caused a decline in cell viability. Long-term (72 h) exposures of cell lines to effluents resulted in sampling times that were too late to capture the maximal increases in expression of these genes. Short-term exposures of 24 h were subsequently shown to be sufficient for examining gene expression of liver cell lines exposed to PPME, with maximum expression of EDC- and stress-induced genes observed between 4 and 6 h after exposure. These studies also demonstrated that while cells from both the RTL-W1 and FHM-L cell lines maintained some of the differentiated characteristics of hepatocytes from their parent tissues, such as induction of CYP1A in response to stress-inducing compounds, they were also unable to respond to effluents that are suspected to contain estrogenic and androgenic compounds via induction of EDC-sensitive genes, as in fish livers. The difference in relative sensitivity to PPMEs demonstrated by the two cell lines could be exploited as screening tools to evaluate environmental samples for pollutants.

Future studies that would further the potential of cell lines in toxicity testing likely require the transfection of cell lines with estrogen-responsive elements. The PPME-inducible expression of VTG, ER isoforms, and AR mRNAs in the cell lines would allow them to be used in environmental testing of effluents that contain EDCs. Protein analysis using western blotting is currently being performed to examine the expression of VTG and CYP1A proteins in the RTL-W1 and FHM-L cell lines subsequent to effluent exposure to ensure that changes in mRNA levels correlate with those in

protein expression. Microarray analysis of the cells would provide a more in-depth profile of the genes that are induced by PPME exposure and would further characterize the cell lines after exposure to PPMEs. This genomic analysis would identify consistently-expressed biomarkers, and allow them to be screened for environmental relevance prior to their further analysis. Correlation of whole organism exposures to similar exposures performed in cell lines would strengthen the use of cell lines as a supplementary tool for toxicity testing of PPMEs. As the literature shows, there are extensive challenges in identifying biomarkers among different species. Additionally, the evaluation of a single endpoint for effluent-exposed cells, such as gene expression, does not provide a complete assessment of contaminant exposure effects on the whole organism. The integration of data from various endpoints, including cytotoxicity, gene expression and cell viability assays, would supply a more complete assessment of effluent exposure and considerably enhance the ability of researchers to predict the effects of toxicant exposure on aquatic organisms and ecosystem health.

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# APPENDIX 1:

# COMPOSITION OF L15/EX MEDIA

Appendix 1: Composition of L-15/Ex Media

| Component | Amount (g) |
|---|---|
| NaCl | 8.00 |
| KCl | 0.40 |
| $MgSO_4 \cdot 7H_2O$ | 0.20 |
| $MgCl_2 \cdot 6H_2O$ | 0.20 |
| $CaCl_2 \cdot 2H_2O$ | 0.14 |
| $Na_2HPO_4$ (anhydrous) | 0.19 |
| $KH_2PO_4$ (anhydrous) | 0.06 |
| D-Galactose | 0.90 |
| Pyruvic Acid | 0.55 |

Note: Components are dissolved in 1 L e-pure water and filter sterilized. Exposure media can be stored at 4°C for up to 1 year.

# APPENDIX 2:

# SUPPLEMENTARY FIGURES AND TABLES

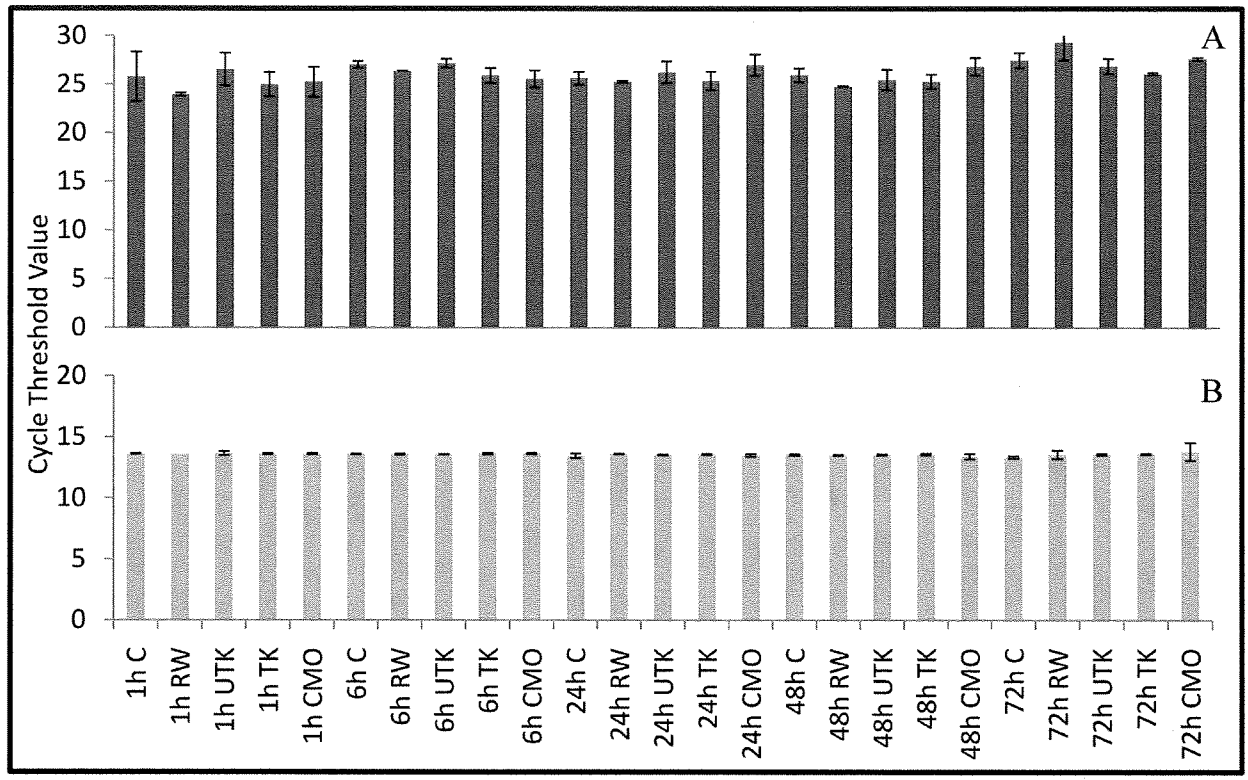


**Figure A2.1**: **Raw gene expression data of potential reference genes in the RTL-W1 cell line.** Expression of GAPDH (**A**) and β-Actin (**B**) was examined during a long time-course PPME exposure. Cells were exposed to an untreated control (C), river water (RW) or one of three effluent treatments: untreated kraft effluent, treated kraft (TK), or combined mill outfall (CMO) for 1, 6, 24, 48, or 72 h. Effluents were diluted in exposure media with 10% (v/v) river water. Data are presented as the mean cycle threshold value ± standard error.

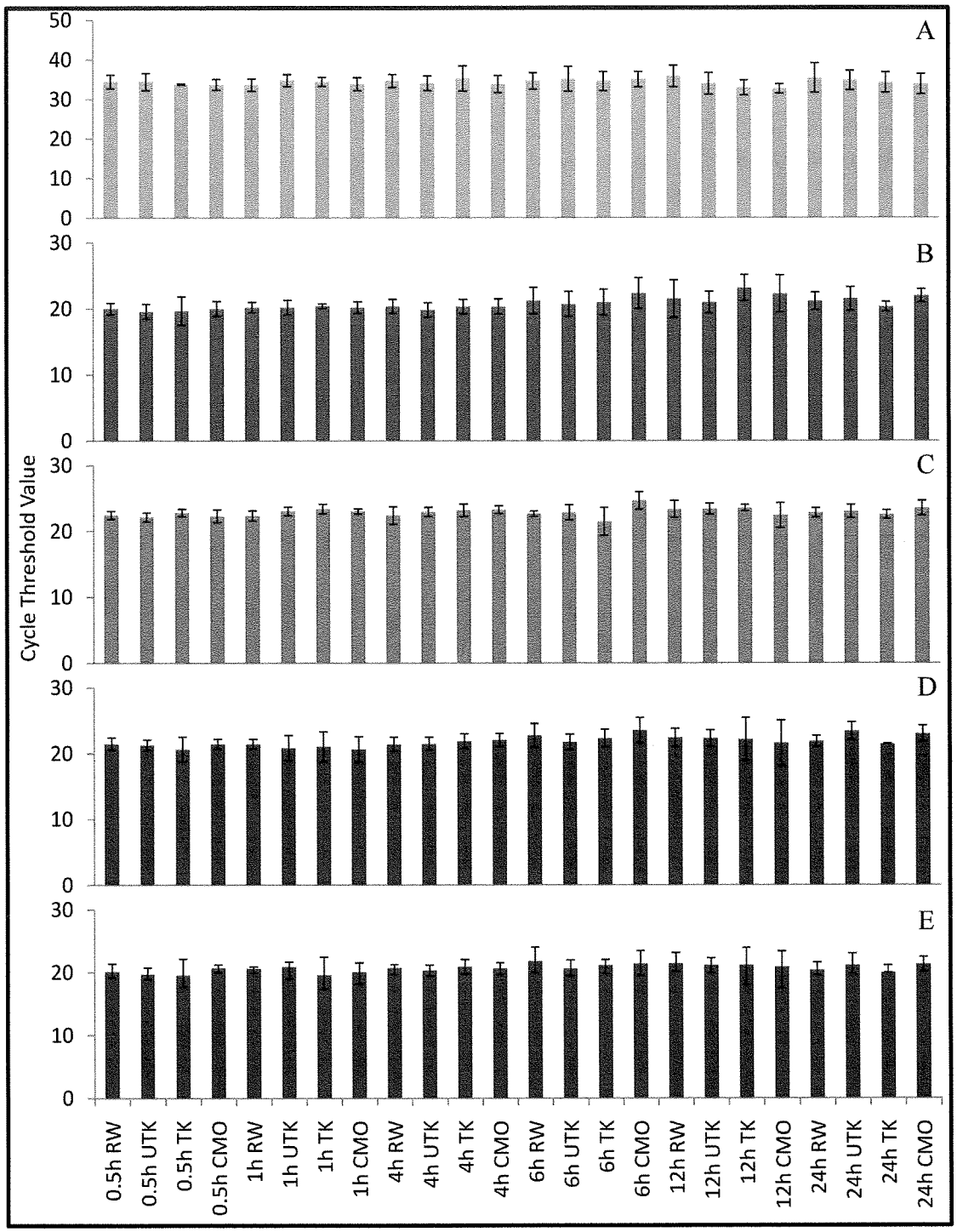


**Figure A2.2. Raw gene expression data of potential reference genes in the RTL-W1 rainbow cell line during a short time-course PPME exposure.** Potential reference genes examined were GAPDH (**A**), β-Actin (**B**), RPL8 (**C**), EF1α (**D**) and ubiquitin (**E**). Cells were exposed river water (RW) or one of three effluent treatments: untreated kraft effluent, treated kraft (TK), or combined mill outfall (CMO) for 0.5, 1, 4, 6, 12, or 24 h. Dilution conditions and data preparation are as in Figure A2.1.

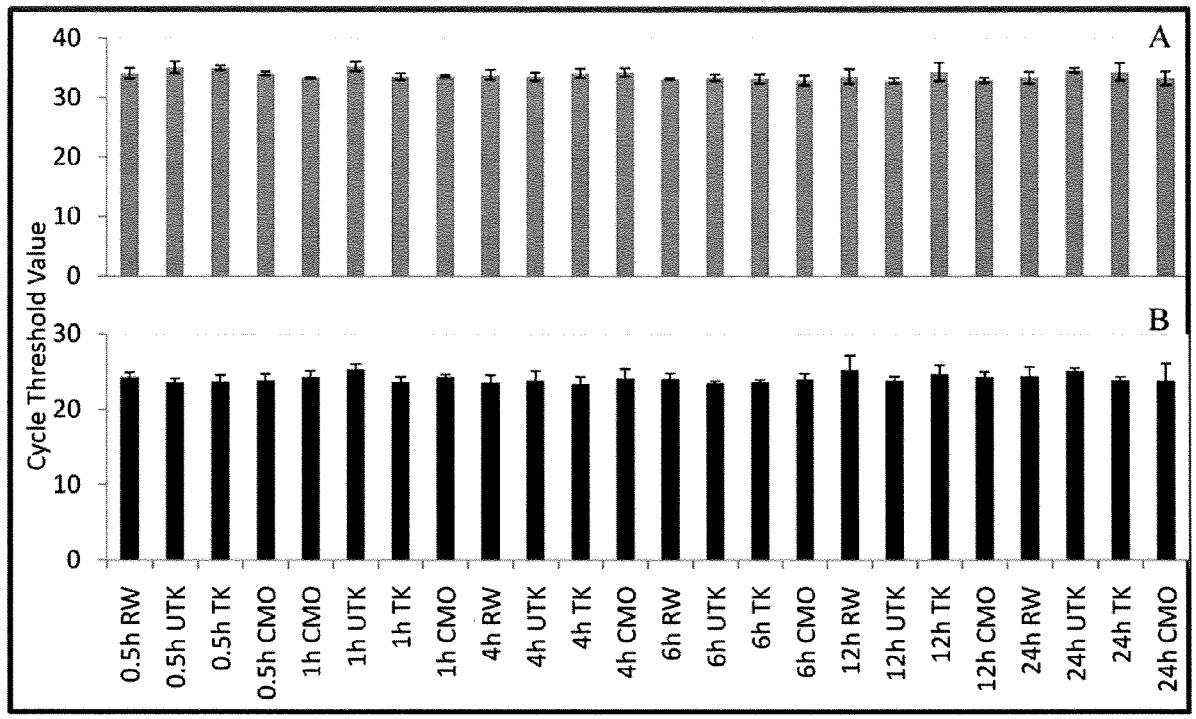


**Figure A2.3. Raw gene expression data of potential reference genes in FHM-L cells after PPME exposure in a short time-course**. The expression of GAPDH (**A**) and β-Actin (**B**) were examined as potential reference genes. Cells were exposed 10% (v/v) river water (RW) or one of three effluent treatments: untreated kraft effluent (UTK), treated kraft (TK), or combined mill outfall (CMO) for 0.5, 1, 4, 6, 12, or 24 h. Dilution conditions and data preparation are as in Figure A2.1.

**Table A2.1: Primer pairs used in real-time PCR analysis for rainbow trout cell line exposure.** Primer sequences are shown from 5' to 3'. Primers were taken from the literature (as indicated).

| Gene (GenBank No.) | Forward Primer Sequence | Reverse Primer Sequence |
|---|---|---|
| GAPDH [a] (AB066373) | GGGTAAAGCCGGTGCCGATTA | GCCTTCTTGACAGCCCCTTTG |
| β – actin [b] (AF157514) | AGAGCTACGAGCTGCCTGAC | CAAGACTCCATACCGAGGA |
| Ubiquitin [c] (AB036060) | ATGTCAAGGCCAAGATCCAG | TAATGCCTCCACGAAGACG |
| EF1a [d] (AF498320) | AGCGCAATCAGCCTGAGAGGTA | GCTGGACAAGCTGAAGGCTGAG |
| L8 [e] (AY957563) | GGTGTGGCTATGAATCCTGT | ACGACGAGCAGCAATAAGAC |
| ERα1 [f] (AJ242740) | CCCTGCTGGTGACAGAGAGAA | ATCCTCCACCACCATTGAGACT |
| ERα2 [f] (DQ177438) | GTGGCACTGCTGGTGACAAC | ACCACCGAAGCTGCTGTTCT |
| ERβ-1 [f] (DQ177439) | CCCAAGCGGGTCCTAGCT | TCCTCATGTCCTTCTGGAGGAA |
| ERβ-2 [f] (DQ248229) | CTGACCCCAGAACAGCTGATC | TCGGCCAGGTTGGTAGTG |
| VTG [g] (NM27651) | AACCAAGTCAGCCAGGTAATATG | AGAACGACAACTGGAAACTGTGT |
| ZR [h] (AF185274) | CAGTACCATTGTGGCTGTGGTT | GGCCCAGGAGCTATATCAGGAT |
| AR [i] (NM001124184) | AGCGCCAACTGGTCGAA | CACATGCAGATTCCGAAAACC |
| CYP1A [j] (AF015660) | GCTGATGGCACAGAACTCAA | CTTCATGGTGAGGCCTGTATT |

[a] Walker et al. 2008
[b] Aluru and Vijayan 2007
[c] von Schalburg et al. 2005
[d] Bobe et al. 2006
[e] Veldhoen et al. 2006
[f] Boyce-Derricott et al. 2009
[g] Schreer et al. 2005
[h] Celius et al. 2000
[i] Hook et al. 2006
[j] Roberts 2005

**Table A2.2: Primer pairs used in real-time PCR analysis for PPME exposures from FHM-L cells**

| Gene (GenBank No.) | Forward Primer Sequence | Reverse Primer Sequence |
|---|---|---|
| GAPDH (DT353565) | CAGACGCTTCCCACAAAC | TCACCAGACGCCCAATG |
| β – actin (EU195887) | CCTGAATCCCAAAGCCAACAG | GCCAATGGTGATGACCTGC |
| ERα (AY775183) | CATTGACAAGAGCCGACGC | TGAAAGCGTCAGCTCCACG |
| ERβ (AY566178) | GCGGTGTGCAGTGACTATGC | TGACGTGAACAGACAGCCG |
| VTG [a] (AF130354) | TGACAAGCCAACAGCAAGAG | TTAGCCGCCATAGGAATGAG |
| AR (AY727529) | TGATCGGATGCACCAAACG | GGGACAGCTTGCTTAAACGC |
| CYP1A (AF232749) | ATCACCGACTCGCTCATCG | TCTCAGTTCTCCCTGCAGGC |

Primer sequences are shown from 5' to 3'. Primers were designed using Primer3 or taken from literature (as indicated).

[a] Lattier et al. 2002