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THE EFFECTS OF SHORT-TERM EXPOSURE TO MUNICIPAL WASTEWATER EFFLUENT ON SIGNALLING PATHWAYS IN THE LIVER OF FISH.

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This thesis is dedicated to my father, Jonas Arstikaitis.

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List of Abbreviations

3β-hsd	3β-hydroxysteroid dehydrogenase
11-KT	11-Ketotestosterone
17β-HSD	17β-hydroxysteroid dehydrogenase isoform 8
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif 1
AES	amino-terminal enhancer of split
Ag	silver
AhR	aryl hydrocarbon receptor
Al	aluminum
ANGPT1	angiopoietin 1
AP	alkylphenols
APC	adenomatous polyposis coli
AR	androgen receptor
ARIH2	ariadne homolog 2
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
BCL2L13	BCL2 like 13
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein
BPA	bisphenol A
cAMP	cyclicAMP
Casp4	caspase 4
CBFA2T2	core-binding factor, runt domain, alpha subunit 2
Cd	cadmium
CDC42	cell division cycle 42
CEP152	centrosomal protein 152kDa
CK1	casein kinase 1
Co	cobalt
Cr	chromium
Cryab	alpha-crystallin B chain
CTNNB1	beta-catenin
Cu	copper
Cx	connexin
Cx26	connexin26
Cx30	connexin30
Cx32	connexin32
Cx43	connexin43
CYP1A1	cytochrome P450 family 1 subfamily A polypeptide 1
CYP19A1	cytochrome P450 family 19 subfamily A polypeptide 1
DACT1	dapper antagonist of CTNNB1, homolog 1
Ddit3	DNA-damage inducible transcript 3
Dieldren	1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethano-naphtalene
Dkk1	high-affinity dickkopf homolog 1
DNA	deoxyribonucleic acid
DNAJB1	DNAJ (Hsp40) homolog subfamily B member 1

DNAJC3	DNAJ homolog subfamily C member 3
DNAJC5	DNAJ (Hsp40) homolog subfamily C member 5
dsh	dishevelled
dvl2	dishevelled dsh homolog 2
E1	estrone
E2	estradiol
E3	estriol
EE2	ethinyl estradiol
EGF	epidermal growth factor
ENPEP	glutamyl aminopeptidase
ER	estrogen receptor
esr1	estrogen receptor 1
esr2B	estrogen receptor 2b
Fe	iron
FKHR	forkhead transcription factor
FoxB1	forkhead box b1
FoxD1	forkhead box d1
Fz	frizzled
FSH	follistatin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCLM	glutamate-cysteine ligase modifier subunit
GJ	gap junctions
GJA1	gap junction alpha 1
GJB1	gap junction beta 1
GJB2	gap junction beta 2
GJB6	gap junction beta 6
GJIC	gap junction intercellular communication
GnRH	gonadotropin-releasing hormone
GPR128	G protein-coupled receptor 128
GSI	gonadosomatic index
GSK3	glycogen synthase kinase 3
GST	glutathione-S-transferases
H3	histone protein 3
HAO1	hydroxyacid oxidase 1
HCB	hexachlorobenzene
HEPACAM	hepatocyte cell adhesion molecule
Hg	mercury
Hmox1	heme oxygenase 1
HSPA5	heat shock 70kDa protein 5
HSP1A	heat shock 70 kDa protein 1a
HSP1B	heat shock 70 kDa protein 1b
HSPB8	heat shock protein beta-8
IGF1	insulin-like growth factor 1
ILK	intergrin-linked kinase
JAK	janus kinase
KCNA1	potassium voltage-gated channel, shaker-related subfamily, member 1

LMNA	lamin A/C
LRP6	low density lipoprotein receptor-related protein 6
KREMEN1	kremen protein 1
MAGEA10	melanoma antigen family A 10
MAPK	mitogen-activated protein kinase
MCAM	melanoma cell adhesion molecule
MGAT5	mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase
Mn	manganese
MT	metallothionein
MUC	Montreal Urban Community
MWWE	Montreal wastewater effluent
MWWTP	Montreal wastewater treatment plant
Mx2	Interferon-induced GTP-binding protein Mx2
MXC	methoxychlor
MYB	myeoblastosis oncogene
Ni	nickel
Nrf2	nuclear factor erythroid 2-related factor 2
OH-MXC	mono-demethylated methoxychlor
OP	4-tert-octylphenol
OV	ovariectomized
NELL2	NEL-like 2
NME1	non-metastatic cells 1
NP	4-nonylphenol
$p34^{cdc2}$	cyclin B-dependent $p34^{cdc2}$ kinase
PAH	polyaromatic hydrocarbon
Pb	lead
PCB	polychlorinated biphenols
PLG	plasminogen
PP1	protein phosphatase 1
PPCP	pharmaceutical and personal care products
PHKA1	phosphorylase kinase alpha 1
PKA	protein kinase A
PKC	protein kinase C
p,p'-DDE	2,2-Bis(4-chlorophenyl1)-1,1,-dichloroethylene
PPP1R15A	protein phosphatase 1 regulatory subunit 15A
PPP2R5E	protein phosphatase 2 regulatory subunit B epsilon isoform
PTGS2	prostaglandin-endoperoxide synthase 2
PTK2	protein tyrosine kinase 2.2
PVC	polyvinyl chloride
RACGAP1	Rac GTPase activating protein 1
RNA	ribonucleic acid
SASH3	SAM and SH3 domain containing 3
SIPA1L2	signal-induced proliferation-associated 1 like 2
SKOR1	SKI family transcriptional corepressor 1
SMAD3	MAD homolog 3

Sox2	SRY-box 2
SPOCK2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan
sqstm1	sequestosome 1
StAR	steroid acute regulatory protein
STAT1	signal transducer and activator of transcription 1
T3	3,3`,5-Triiodo-L-thyronine
T4	3,3`,5,5`-Tetraiodo-L-thyronine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCF/LEF	T cell specific transcription factor/ lymphoid-enhancing transcription factor
TH	thyroid hormone
TNFS11	tumor necrosis factor (ligand) superfamily, member 11
TOPORS	topoisomerase I binding arginine serine-nich
TR	thyroid hormone receptor
UBQLN4	ubiquilin 4
VTG	vitellogenin
WNT	wingless-type MMTV integration site family
WNT4	wingless-type MMTV integration site family member 4
ZM	ZM-182780
Zn	zinc
ZO1	tight junction protein 1
ZO2	tight junction protein 2
ZP3	zone pellucida glycoprotein 3

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Summary

Characterization of Montreal municipal wastewater effluent (MWWE) has identified the presence of pharmaceuticals and personal care products (PPCP), alkyphenols (AP), polychlorinated biphenyls (PCB), polyaromatic hydrocarbons (PAH), and heavy metals. MWWE contains mixtures of xenobiotics that have been shown to disrupt normal metabolic and cellular functions.

Previous studies have shown that fish exposed to MWWE have resulted in altered cellular signaling in the testis. These processes are facilitated by transmembrane proteins called connexins (Cx) which form gap junctions (GJ). GJ allow the passage of small molecules between neighbouring cells, called gap junction intercellular signaling (GJIC). Brook trout exposed to MWWE for 4 and 12 weeks showed altered testicular Cx levels at both time points. At 4 weeks there was a significant increase in both Cx43 and Cx31. At 12 weeks a decrease in Cx31 and an increase in Cx43 and Cx43.4 was observed. The presence of xenobiotics has been shown to target and disrupt Cx mRNA levels resulting in altered cellular functions. Similar results were reported in Spottail shiners living within the effluent plume of MWWE. Shiners displayed altered testicular Cx levels resulting in delayed spermatogenesis, increased vitellogenin levels, intersex characteristics and decreased sperm motility. MWWE has been shown to target Cxs in the testis causing an alteration in key signaling pathways. Interestingly, few studies have examined the effects of MWWE on cellular signaling in the liver considering the important role it plays in xenobiotic metabolism.

Xenobiotics can accumulate in the liver if the complex signaling required to carry out the detoxification process is disrupted. GJIC plays an important role in the regulation of normal homeostatic processes in the liver including xenobiotic metabolism, bile secretion and glycogenolysis. Fish exposed to MWWE have demonstrated altered Cx expression levels and cellular signaling in the testis, it can be hypothesized that a similar disruption of GJIC could occur in the liver.

There are presently no studies that have examined the effects of MWWE on the liver and currently only one Cx has been identified in the liver of fish, Cx43 in the zebrafish. In the

present study two Cxs were identified in the liver of the brook trout, Cx43 and Cx30. When compared to mammalian Cxs, brook trout Cx43 was partially homologous to GJA1 in the mouse and rat. Brook trout Cx30 shared some homology to mouse GJB6 and human Cx26. This is the first report of Cx30 in the liver of fish.

After exposing brook trout for 12 weeks to MWWE, a large increase in Cx30 mRNA levels was observed at the two highest effluent concentrations. Similar results have been previously reported in the testis and were shown to disrupt normal GJIC. There were no changes to Cx43 levels suggesting either individual contaminants targeting Cx43 were not present or the exposure period was too short for accumulation to occur.

To further investigate the effects of MWWE on key signaling pathways in the liver, juvenile fathead minnow were exposed to 20 % MWWE for 21 days. This study aimed to identify key signaling pathways affected by toxicants contained within the effluent mixture. Using a 15 208 cDNA microarray and pathway analysis software, three groups of genes, organized by function, were identified as being altered suggesting effects on various signaling pathways. These groups include genes involved in cell adhesion, inflammation and kinases. As well, two key signaling pathways were identified as having a large number of genes affected, including the anti-estrogenic and Wnt signaling pathways.

The anti-estrogenic and Wnt signaling pathways are critical during fish development, alterations have been shown to cause developmental malformations, cancer and are implicated in a number of pathologies. The present study found that over ten genes were altered in these pathways suggesting a disruption in normal developmental processes. Thirteen genes involved in the anti-estrogenic pathway were altered and suggested an overall inhibition of the pathway further confirming previous studies that found a long-term (12 weeks) exposure was required to measure an induction of estrogenic effects. These results also suggest that a short-term exposure may elicit an initial inhibitory effect of estrogen.

The Wnt signaling pathway is involved in cell-to-cell signaling during key developmental periods including embryogenesis. Thirteen genes involved in the Wnt signaling pathway were

altered including the down-regulation of Wnt genes required for the activation of the pathway and the up-regulation of Wnt signaling inhibitors. These findings suggest that a short-term exposure to MWWE can result in the inhibition of the Wnt signaling pathway which plays a critical role during development.

Lastly, to assess whether the inhibition of the Wnt signaling pathway was detected at the protein level a western blot was carried out to measure and compare beta-catenin (CTNNB1) levels. A small decrease in nuclear CTNNB1 levels was observed in exposed fish but when statistically compared to cytosolic protein levels this difference was not signficiant.

Overall, this study identified two Cxs in the liver of the brook trout and showed that Cx levels can be altered by the xenobiotics readily found in MWWE. The use of microarray technology allowed for thousands of genes to be studied in parallel and to identify three groups of genes and two key signaling pathways that were altered after a short-term exposure to MWWE.

Student

Director of Research

INTRODUCTION

Studies have shown that xenobiotics found in Montreal wastewater effluent (MWWE) enter aquatic ecosystems and can alter organismal homeostasis (Osterman et al., 1990; Gagnon et al., 2006; Marcogliese et al., 2009; Lajeunesse et al., 2011). Direct communication between neighbouring cells is required to carry out a number of functions required for homeostasis including bile secretion, xenobiotic biotransformation, spermatogenesis, glycogenolysis and albumin secretion (Neveu et al., 1994; Nelles et al., 1996; Stumpel et al., 1998; Nathanson et al., 1999; Shoda et al., 1999; Shoda et al., 2000; Hamilton et al., 2001; Temme et al., 2001; Bode et al., 2002; Yang et al., 2003; Aravindakshan et al., 2004a,b; de Montgolfier et al. 2007). To facilitate communication between neighbouring cells, six transmembrane proteins called connexin (Cx) combine to form a gap junction (GJ) (Cruciani et al., 2006; Vinken et al., 2009, 2011). Two GJ, one from each neighbouring cell, form channels and allow the passage of small molecules, called gap junction intercellular signaling (GJIC) (Cruciani et al., 2006).

A number of studies have shown that xenobiotics can alter Cx mRNA expression levels in the testis causing a disruption of GJIC and altering homeostatic functions (Elaut et al., 2006; Papeleu et al., 2006; de Montgolfier et al., 2008). Presently, there are no publications that have examined the effects of xenobiotics in the liver of fish. The first part of this thesis aims to address this research gap and hypothesizes that a number of Cx are expressed in the liver of the brook trout and that their mRNA expressions levels are altered, similar to those observed in the testis, after a short-term exposure to MWWE. To address this hypothesis brook trout, an invasive species in the St. Lawrence River and a well known species for toxicology studies were exposed to MWWE for 12 weeks. Further analysis was carried out using degenerate primers, RT-PCR, and real-time PCR (qPCR) (Fisherins and Oceans Canada, 2012).

The second part of this thesis applies the knowledge of the first study at the gene expression level to determine if changes in Cx mRNA levels can be observed using a genomic approach. It is hypothesized that if Cx mRNA levels are altered in the first study a genomic approach would allow changes in key regulatory pathways to be observed. In order to carry out this hypothesis juvenile fathead minnow are exposed for 21 days to MWWE and using a microarray, key regulatory pathways in the liver are examined. In order to test this hypothesis and evaluate

thousands of genes in parallel a 15 208 oligonucleotide fathead minnow microarray was used and further analyses was carried out using pathway analysis software. Fathead minnow were used in the second experiment because they are found in the St. Lawrence River, they are easily reared in a laboratory setting, and a specie specific microarray was available (Ankley and Villeneuve, 2006).

This thesis is divided into five chapters; the first chapter is an extensive literature review of the characterization of MWWE, as well as information on gap junctions and the use of genomic technology. The second chapter contains original research titled: Exposure to Municipal Effluent Results in Increased Level of the Gap Junction Protein, Connexin30, in the brook trout. The third chapter contains original research titled: Short-term exposure of fathead minnows to Municipal Wastewater Effluent Inhibits the Canonical Wnt Signaling Pathway in the Liver. The implications of these studies and general conclusions are discussed in chapter four. Finally chapter five contains a French summary of the thesis.

CHAPTER 1: LITERATURE REVIEW

1.0 Characterization of Montreal Municipal Wastewater Effluent

The island of Montreal is inhabited by 1.8 million people and approximately 8000 commercial establishments and industrial plants (Sabik et al., 2003; Marcogliese et al., 2009). All of the sewage from the Montreal Urban Community (MUC), 187 x 10^6 m^3/year is received at the Montreal wastewater treatment plant (MWWTP) located on the eastern tip of the island (Pham et al., 1997; Sabik et al., 2003; Marcogliese et al., 2009). The MWWTP typically treats 1.3 million m^3 of raw wastewater daily, equivalent to 88 m^3/sec of effluent discharge (Sabik et al., 2003). During periods of heavy rain or thaw, the volume of water increases and can reach up to 7.6 million m^3 (Pham et al., 1997; Sabik et al., 2003; Marcogliese et al., 2009). Effluent from the treatment plant is released directly into the St. Lawrence River using an underwater pipe at a depth of approximately 7 m and at a distance of 4.5 km from the shore of the island of Montreal (Pham et al., 1997; Sabik et al., 2003; Marcogliese et al., 2009).

Presently, over 90 % of Montreal wastewater effluent (MWWE) undergoes some form of wastewater treatment before reaching the St. Lawrence River (Chambers et al., 1997). The MWWTP is the largest primary physico-chemical treatment plant in North America (Sabik et al., 2003). This process includes mechanical screening, grit removal, a coagulant, usually ferric chloride, and an anionic polymer that are added to act as an aid to increase the settling of suspended particles (Pham et al., 1997; Chambers et al., 1997; Gagne et al., 2002). Sludge settles to the bottom of the treatment tank and is then incinerated and ash is sent to landfill sites or is applied as fertilizer to agriculture lands. Large particles are sieved and removed (Gagne et al., 2002). The goal of treatment is to remove toxicants, decrease phosphorous levels and improve water clarity (Gagne et al., 2002). The MWWTP does not utilize a disinfection step thereby allowing large amounts of bacteria to be released into the St. Lawrence River (Gagne et al., 2002).

Treatment facilities that use a secondary treatment apply a biological process to remove anaerobic and/or aerobic microorganisms and are able to further reduce total suspended solid level. These processes target the removal, or degradation, of biological content from wastewater

and can include aerated activated sludge, slag filtration, plastic filtration, membrane biological reactor and secondary sedimentation (Kirk et al., 2002). The most common method for secondary treatment mixes the results of primary treatment with activated sludge and for a specified length of time the mixture is aerated, during this holding time organisms within the sludge degrade organic matter (Yang et al., 2011). The resulting suspended solids are then settled out in a separate tank allowing the wastewater to undergo further treatment (Yang et al., 2011). The alternative treatment systems including slag filtration, and plastic filtration are based on similar technologies where an additional coagulant is added and the mixture is then forced through a unique type of filter (Chiemchaisri et al., 2003; Pratt et al., 2010). The most effective treatments are at the tertiary level where chemical and biological treatments are used to remove specific compounds or materials that remain after secondary treatment (Chambers et al., 1997).

As MWWE is discharged into the St. Lawrence River the warmer effluent rises to the surface creating movement within the water and immediate dilution (Osterman et al., 1990). Natural currents in the hydrology of the river carry the wastewater downstream creating an effluent plume (Osterman et al., 1990). Wastewater is not uniformly distributed in the plume, pockets of effluent occur or areas of higher flow have greater dilution (Osterman et al., 1990). As the effluent moves downstream it is diluted; at 0.3 km from the pipe the concentration is approximately 20 %, at 1 km the concentration is reduced to 10 % and at 10 km the concentration is diluted to 1 % of the original effluent (Gagnon et al., 2003; de Montgolfier et al., 2008).

Characterization of MWWE identified a number of contaminants including pharmaceuticals and personal care products (PPCP), alkylphenols (AP), polychlorinated biphenyls (PCB), polyaromatic hydrocarbons (PAH), and heavy metals (Pham et al., 1997; Quemerais et al., 1998; Gagne et al., 2001; Sabik et al., 2003; Aravindakshan et al., 2004a; Aravindakshan et al., 2005; Matozzo et al., 2008; Martin-Diaz et al., 2009; Bhavsar et al., 2011; Lajeunesse et al., 2011). Characterization of water samples at each site has helped to identify the composition of individual contaminants and determined the most persistent and therefore most highly toxic compounds to the aquatic environment (Pham et al., 1997; Pham et al., 1999; de Lafontaine et al., 2000; Gagnon et al., 2003; Gagnon et al., 2006; Lajeunesse et al., 2011). One of the most

problematic groups is the PPCP because of their increasing use and ability to bioaccumulate in the environment (Howard and Muir, 2011).

1.1.0 Contaminants in Montreal Municipal Wastewater Effluent

1.1.1 Pharmaceuticals and Personal Care Products

As the average age of the population increases there is a correlation in the use of pharmaceuticals and as a result an increase in the amount of pharmaceuticals that enter wastewater (Quinn et al., 2008). Pharmaceuticals are primarily metabolized in the liver by phase I and phase II metabolic reactions and excreted through urine and/or faeces (Corcoran et al., 2010). In some instances metabolism renders these drugs inactive while others produce metabolites that are biologically active (Quinn et al., 2008). Pharmaceuticals that are transported into wastewater treatment plants have three possible outcomes: the substance is mineralized to carbon dioxide and water; the substance is retained in the sludge after wastewater treatment due to their lipophilic nature; or the substance is metabolized to a more hydrophilic form of the parent lipophilic substance (Halling-Sorensen et al., 1998; Trudeau et al., 2005; Corcoran et al., 2010).

The most common pharmaceuticals and personal care products (PPCP) found in MWWE include antidepressants (desmethylvenlafaxine, venlafaxine, paroxetine, citalopram) detected at concentrations between 10.5-55.3 ng/L (Lajeunesse et al., 2011); anti-inflammatory drugs (ibuprofen, naproxen; 217-1191 ng/L); lipid regulators (bezafibrate, gemfibrozil; 59-72 ng/L); anti-convulsion drug (carbamazepine; 33 ng/L); antibiotics (sulfapyridine, trimethoprim, oxytetracycline, sulfamethoxazole) between 46-440 ng/L; and caffeine with the highest concentration of 22 μg/L (Quinn et al., 2008). Depending on the drug, toxic effects to aquatic organisms have been observed at concentrations ranging from the high μg/L to low ng/L (Hernando et al., 2006). The levels of pharmaceuticals in the environment are below lethal concentrations and therefore have a low risk of acute toxicity, however, since fish have a lower capacity than mammals to metabolize xenobiotics there is a potential for accumulation of these drugs in fish species (Brun et al., 2006).

Gagne et al. (2006) found that the majority of pharmaceuticals present in the MWWE were capable of inducing toxic drug-drug interactions by inhibiting the P450 (CYP) enzymes that would otherwise metabolize the contaminants (Lam et al., 2002). Drug-drug interactions can occur when two drugs act in an inductive or inhibitory fashion, inhibiting enzymes required to carry out detoxification (DeVane et al., 2004). Oxidative metabolism is required to degrade many of these drugs however long-term exposure can result in oxidative stress (Gagne et al., 2006). Trout hepatocytes exposed for 48 h to a mixture of pharmaceuticals from the MWWE showed effects from oxidative stress (Gagne et al., 2006; Martin-Diaz et al., 2009). As well, many fish and bivalve species do not have the necessary metabolizing enzymes, specifically CYP P4502C, to break down and excrete many of these drugs (Gagne et al., 2006). In 2008, a study was carried out using MWWE and aimed to examine the effects of chronic exposure to PPCP on invertebrates such as cnidarians and bivalves (Quinn et al., 2008; Martin-Diaz et al., 2009). This study determined that the levels of gemfibrozil, ibuprofen and naproxen measured in the freshwater Hydra (Hydra attenuate) and the freshwater mussel (Elliptio complanata) were sufficient to cause toxic effects (Quinn et al., 2008; Martin-Diaz et al., 2009). Carbamazepine, bezafibrate, sulfapyridine, oxytetracycline and novobiocin were all classified as harmful to aquatic species due to the moderate concentrations required to induce toxic biological effects (Quinn et al., 2008). Finally, sulfamethoxazole, trimethoprim and caffeine were detected at concentrations below the levels required to produce biological effects (Quinn et al., 2008).

Synthetic and natural hormones including ethinylestradiol (EE2) found in birth control pills and hormone replacement therapy (Desbrow et al., 1998; Allen et al., 1999; Liu J. et al., 2011; Liu S. et al., 2011) as well as endogenous estrone (E1), 17β-estradiol (E2) and estriol (E3) have been detected in MWWE (Fig. 1) (Fishman et al., 1960; Quiros et al., 2005; Liu S. et al., 2011). EE2 concentrations detected in wastewater are usually lower than the levels found for naturally occurring steroids (Fishman et al., 1960; Quiros et al., 2005; Liu J. et al., 2011). Although EE2 is detected at lower concentrations than the naturally occurring steroids it is 10- to 50-fold more potent in vivo because of its longer half-life and ability to accumulate (Lange et al., 2001; Thorpe et al., 2003; Gorelick et al., 2011). Estrogens are involved in a number of physiological processes including growth and development, reproduction, lipid and glucose metabolism.

These are all mediated via the interaction between estrogens and the estrogen receptor (ER) (Kuiper et al., 1997; Bryzgalova et al., 2006).

In the zebrafish (Danio rerio), fathead minnow (Pimephales promelas) and Japanese medaka (Oryzias latipes) E2 concentrations between 0.1-15 ng/L were capable of altering normal sexual development and differentiation, as well as reproductive ability (Van Aerle et al., 2002; Van den Belt et al., 2003; Andersen et al., 2003; Nash et al., 2004;Kidd et al., 2007). Adult fathead minnow and zebrafish exposed to EE2 concentrations between 1-10 ng/L can cause a reduction in both fertilization and embryo viability (Lange et al., 2001; Hill et al., 2003; Segner et al., 2003).

Japanese medaka fed 5 mg/kg of E2 for 170 days resulted in an inhibition of egg production and a high rate of fish mortality (Oshima et al., 2003). Similar results were reported for female fathead minnow exposed to 2.7 µg/L of E2 for only 19 days (Kramer et al., 1998). A 21-day study in which male fathead minnow were exposed to 1 µg/L of E2 displayed decreased gonadosomatic indices (GSI) and reduced testicular growth (Panter et al., 1998). Similar findings were observed when fathead minnow were exposed to comparable concentrations of E1 (Panter et al., 1998). Metcalfe et al. (2001) observed the induction of intersex (testis-ova) in male medaka exposed to 10 µg/L of E3 for 100 days. Clearly these studies show that estrogenic compounds can have marked effects on sexual function in both male and female fish.

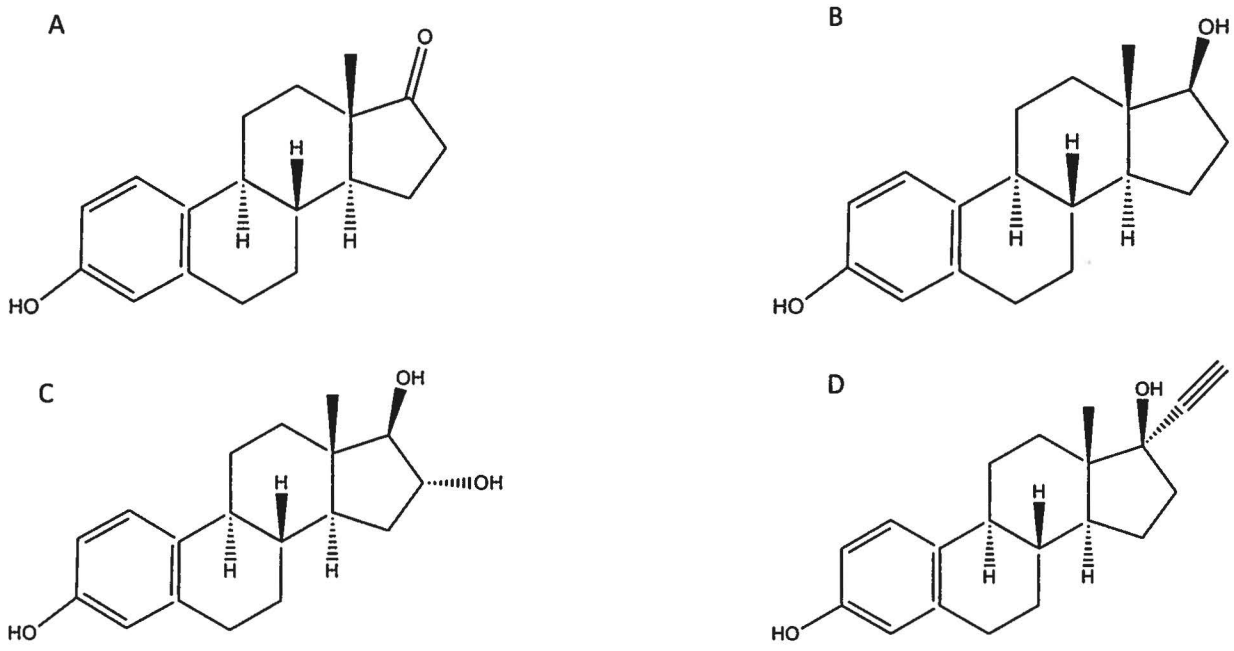

Figure 1: Chemical structure of natural and synthetic estrogens: estrone (E1) (A), estradiol (E2) (B), estriol (E3) (C) and ethinylestradiol (EE2) (D).

1.1.2 Alkylphenols

Concentrations of alkylphenols (AP) detected in MWWE ranged from 0.01 to 10 µg/L (Sabik et al., 2003). These concentrations are similar to those found in the treated effluent of other wastewater treatment plants in Canada and are within the doses reported to cause biological affects in aquatic organisms (Sabik et al., 2003).

AP are a large family of organic compounds characterized by a phenol ring and an alkyl chain on the para position (Fig. 2) (Jobling et al., 1996; Wu et al., 2011). AP have been used since the 1940s and are often found in household and industrial detergents, paints, herbicides, cosmetics, skin care products, emulsifiers, antistatic agents, and food packaging material (Soto et al., 1991; Ahel et al., 1994; Jobling et al., 1996; Sabik et al., 2003; Deng et al., 2007). AP are made up of ethoxylate chains that are gradually removed by ethoxylation (Ahel et al., 1994; La Guardia et al., 2001; Vetillard and Bailhache, 2006). This process results in a mixture of ethoxylated metabolites of different lengths (Ahel et al., 1994; La Guardia et al., 2001; Vetillard and Bailhache, 2006). Under anaerobic conditions, including those found in sludge and sediments,

biodegradation occurs very slowly resulting in the accumulation of short-chain AP (Shang et al., 1999).

Shorter chain AP, including 4-tert-octophenol (OP) and 4-nonylphenol (NP), are toxic due to their lipophilicity and ability to bind to suspended solids, sediments and sewage sludge (McLeese et al., 1981; Ahel et al., 1994). OP has been detected at low levels in the MWWE (1-3 ng/L) but can accumulate in sludge and sediments (18.9 μg/kg) (Sabik et al., 2003). Similar levels have been reported for NP in MWWE but levels in sludge and sediment were found to be almost ten times higher (192 μg/kg) (Ahel et al., 1994; Bennie et al., 1999; Sabik et al., 2003). Both NP and OP are slowly metabolized under anaerobic conditions and have a half-life of approximately 60 years (Ahel et al., 1993; Ahel et al., 1994; Shang et al., 1999; Soares et al., 2005; Baker et al., 2009).

AP have been shown to competitively bind to both the thyroid hormone receptor (TR) and the ER (White et al., 1994). In fish, thyroxine (T4) is converted to triiodothyronine (T3) in target tissues by the microsomal enzyme deiodinase (Brown et al., 2004). T3 can then bind to the nuclear TR (Bres and Eales, 1990) where they regulate processes implicated in development, growth, and reproduction (Cyr and Eales, 1992; Scholz et al., 2008). Schmutzler et al. (2007) examined the effects of NP on secretion of thyroid hormone (TH) levels using rat thyroid FRTL5 cells. NP inhibited iodide uptake by FRTL5 cells as well as thyroid peroxidase, an enzyme implicated in the synthesis of TH. NP could also stimulate TR agonist transcriptional activation (Schmutzler et al., 2007). These results suggest that NP and TH share structural similarities allowing NP to competitively bind to TR altering normal endocrine functions (Schmutzler et al., 2007).

AP can also bind with low affinity to ER (Meucci and Arukwe, 2006; Writer et al., 2010; Gorelick et al., 2011). Jobling et al. (1996) exposed male rainbow trout (Oncorhynchus mykiss) to four different AP and measured vitellogenin (VTG) induction. VTG is a hepatic egg yolk protein that is regulated by estradiol in females during oogenesis (Arukwe and Goksoyr, 2003). VTG is not sex-linked and can therefore be stimulated by estradiol in males who normally do not express the VTG gene (de Montgolfier, 2008). Since VTG induction is dependent on the

estrogenic potency of each of the chemicals, the authors could determine the estrogenicity of both OP and NP as compared to EE2 (Jobling et al., 1996). OP acts as a more potent estrogen than NP (Jobling et al., 1996). At a concentration of 30 μg/L OP induced VTG levels to the same extent as 2 ng/L of EE2 (Jobling et al., 1996). While NP shares certain similarities to E2, in comparison NP has a lower binding affinity for ER and is therefore considered a weak xenoestrogen (Baker et al., 2009).

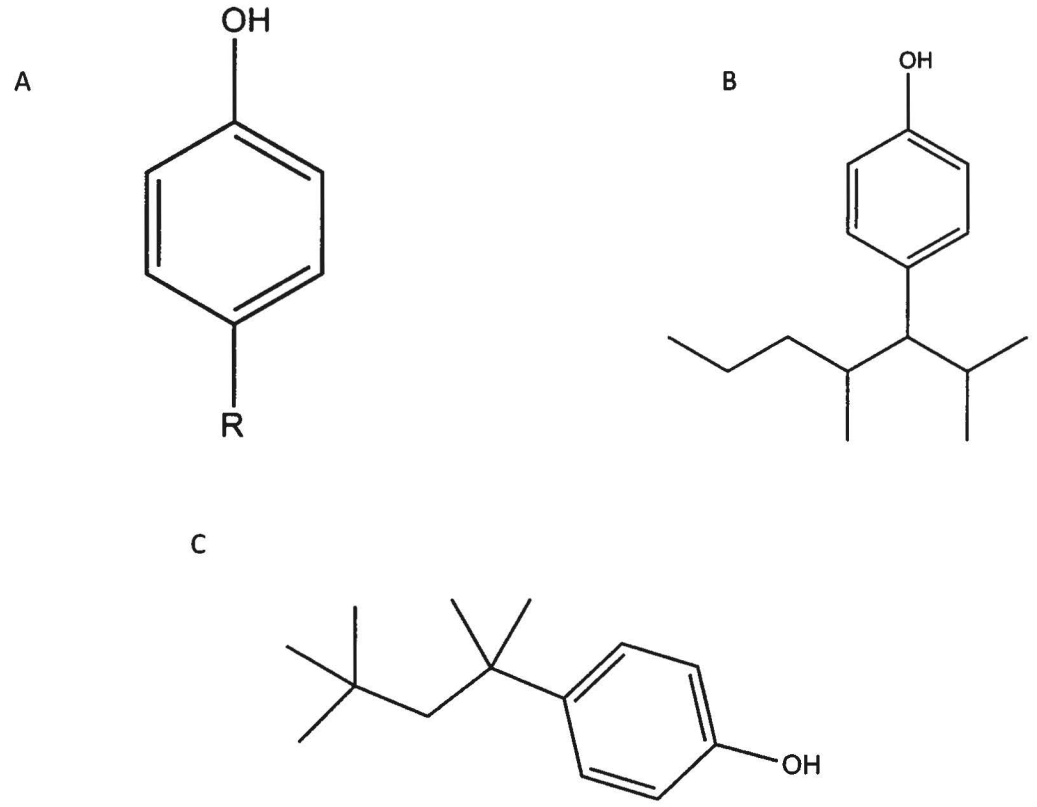

Figure 2: Chemical structure of alkyphenols. A generic alkylphenol structure (A) as well as the structures of 4-nonylphenol (B) and 4-tert-octylphenol (C) are presented.

1.1.3 Polychlorinated Biphenyls

The average concentration of 13 polychlorinated biphenyl (PCB) congeners in untreated MWWE was 18 ng/L, however, after treatment these levels were reduced to 6 ng/L (Pham et al., 1997; Pham et al., 1999). These concentrations are lower than those reported in the effluent of other Canadian cities (Harris et al., 2000).

PCB are synthetic compounds consisting of two phenyl rings and two to ten chlorine atoms attached to the biphenyl ring (Fig. 3) (Simon et al., 2007). There are two distinct groups of PCBs: co-planar PCBs in which the two phenyl rings are in the same plane and non-coplanar where the chlorine substitutes are in the ortho positions (Babut et al., 2009). PCBs are most often used as coolants and insulating fluids for transformers, as well as plasticizers in paints, polyvinyl chloride (PVC) and as flame retardants (Simon et al., 2007). The variation in degree of chlorination enables the production of over 209 different congeners each with a unique fate and toxicity (Ross et al., 2004; Simon et al., 2007). PCBs are capable of being passed up through the food chain and biomagnified in top predators (Cleverly, 2005). Studies have found that PCB mixtures containing larger amounts of moderately chlorinated homologues are more toxic because of their affinity to bind to the aryl hydrocarbon receptor (AhR) (Giera et al., 2011). The AhR is a ligand-activated transcription factor that regulates the activity of a number of genes encoding phase I and II biotransformation enzymes (Giera et al., 2011). AhR and aryl hydrocarbon receptor nuclear translocator (ARNT) proteins interact with AhR response elements in the promoter region of the cytochrome P4501A1 (CYP1A1) gene (Alexeyenko et al., 2010). Giera et al. (2011) showed that 12 different PCB congeners can activate the AhR causing an induction of CYP1A1, which hydroxylates PCB to form metabolites that can act as TR agonists (Van der ven et al., 2005). Van den Berg et al. (2006) found that of these 12 congeners 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic (Giera et al., 2011).

TCDD has been shown to block estrogen action by activating AhR and overlaps the estrogen response element of estrogen-dependent genes thereby preventing the binding of the ER (Safe et al., 1998). AhR activity has been shown to mediate sex steroid hormone-related actions through ER-AhR cross-talk activation (Mortensen and Arukwe, 2008a; Mortensen and Arukwe, 2008b; Beischlagl et al., 2008). PCB congeners bind to ER resulting in increased VTG mRNA levels, zona radiata protein, estrogen receptor-alpha (ERα) mRNA and ERα protein levels (Mortensen and Arukwe, 2008a; Mortensen and Arukwe, 2008b). Activation of AhR inhibits ER activity whereas activation of ER has a positive effect on the induction of AhR (Matthews et al., 2006). Mixture of planar and co-planar PCB congeners were used to examine the cross-talk between AhR and ER in juvenile sea breams (Spaurus aurata) (Calo et al., 2010). After 12 h there was an induction of VTG whereas after 24 h the estrogenic activity was inhibited by the activation of

AhR (Calo et al., 2010). Alexeyenko et al. (2010) exposed embryonic zebrafish to 1 ng/mL dioxin for 1 hour at 4-5 h post-fertilization and examined the effects after 1 day, 2, 3, 4, and 5 days. The results showed an induction of CYP1A1 genes that have previously been shown to be induced by dioxin through AhR (Gonzalez et al., 1998; Matthews et al., 2006; Alexeyenko et al., 2010).

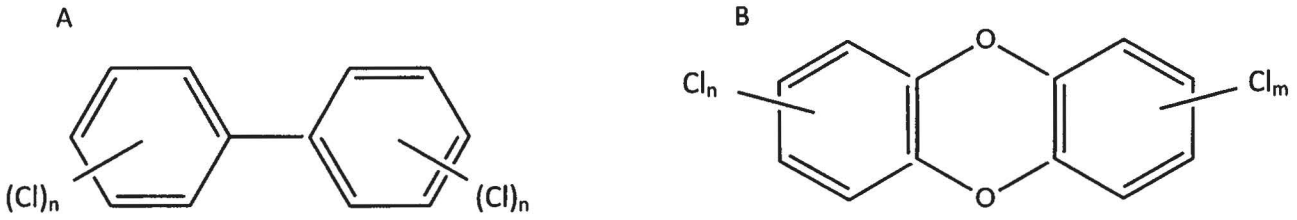

Figure 3. The general chemical structure of polychlorinated biphenols (A). Each of the carbon atoms of the benzene rings are possible positions of chlorine atoms. The chemical structure of polychlorinated dibenzodioxins (PCDD) also known as dioxins (B). The n and m can range from 0 to 4.

1.1.4 Polycyclic Aromatic Hydrocarbons

In 1999 polycyclic aromatic hydrocarbon (PAH) concentrations in MWWE were measured between 3.4 to 125.6 ng/L (Pham et al., 1999) which is similar to levels found in Sydney Harbour sediment (4.02 to 44.8 μg/g) (Smith et al., 2009).

PAHs are a heterogenous class of chemicals (Blaha et al., 2002). The most common PAHs are fluorine, phenanthrene, and pyrene. In general PAH contain between two and four fused benzene (aromatic) rings (Fig. 4) (Pham et al., 1997; Goncalves et al., 2008). The majority of low molecular weight PAH (2- and 3-benzene ring compounds) are from spilled diesel fuel, crude oil and wood products whereas higher molecular weight compounds (4-and 5-benzene ring compounds) have been linked to the incomplete burning of fossil fuels, forest fires and some are natural byproducts of decaying organic matter (Pham et al., 1997; Blaha et al., 2002; Dahle et al., 2003; Yanagida et al., 2011). PAH have strong hydrophobic properties that allow them to resist biodegradation and bind to sediments and sludge (Roger et al., 1996).

PAHs have been linked to nongenotoxic, genotoxic and estrogenic effects in exposed organisms (Babich and Borenfreund, 1991; Blaha et al., 2002). Genotoxic effects include activation of AhR mediated genes (Bols et al., 1999), activation of mitogen-activated protein kinase (MAPK) (Rummel et al., 1999), inhibition of intracellular signaling (Rummel et al., 1999) and alterations of Ca^{2+} levels (Tannheimer et al., 1997). Fish embryos exposed to environmentally relevant concentrations of PAH resulted in symptoms resembling blue sacs disease including reduced growth, yolk sac and pericardial edema, as well as subcutaneous hemorrhaging (Hawkins et al., 2002; Carls et al., 2008).

Chronic exposure to mixtures of PAHs can result in oxidative stress due to increased induction of CYP1A1 via the AhR pathway (Burczynski et al., 1999). DNA damage can occur when PAHs covalently bind to the DNA forming unstable adducts (Burczynski et al., 1999). Juvenile chinook salmon were exposed to a mixture of PAHs with a median concentration of 0.66 μg/g/day for 50 days (Bravo et al., 2011). This study showed that the metabolism of PAHs by CYP1A1 in the liver produced toxic PAH metabolites from the parent compounds through activation of AhR (Bravo et al., 2011). Furthermore, the salmon displayed high levels of reactive oxygen species and reactive nitrogen species indicative of oxidative stress (Bravo et al., 2011). Oxidative stress and chronic induction of CYP1A1 may have contributed to the decreased fitness observed as an increase in disease susceptibility in salmon exposed to PAHs (Bravo et al., 2011).

PAHs have been shown to exert anti-estrogenic effects by reducing circulating E2 levels in flounder resulting in lower VTG levels (Monteiro et al., 2000). This reduction in endogenous E2 is thought to result from increased biliary excretion of E2 in the liver of PAH exposed fish (Nicolas et al., 1999). The anti-estrogenic properties of PAHs are thought to be mediated through its activation of AhR which in turn inhibits ER (Nicolas et al., 1999). PAHs have also been shown to have a weak estrogenic effect under synergistic conditions where an increased estrogenic response is possible (Nicolas et al., 1999).

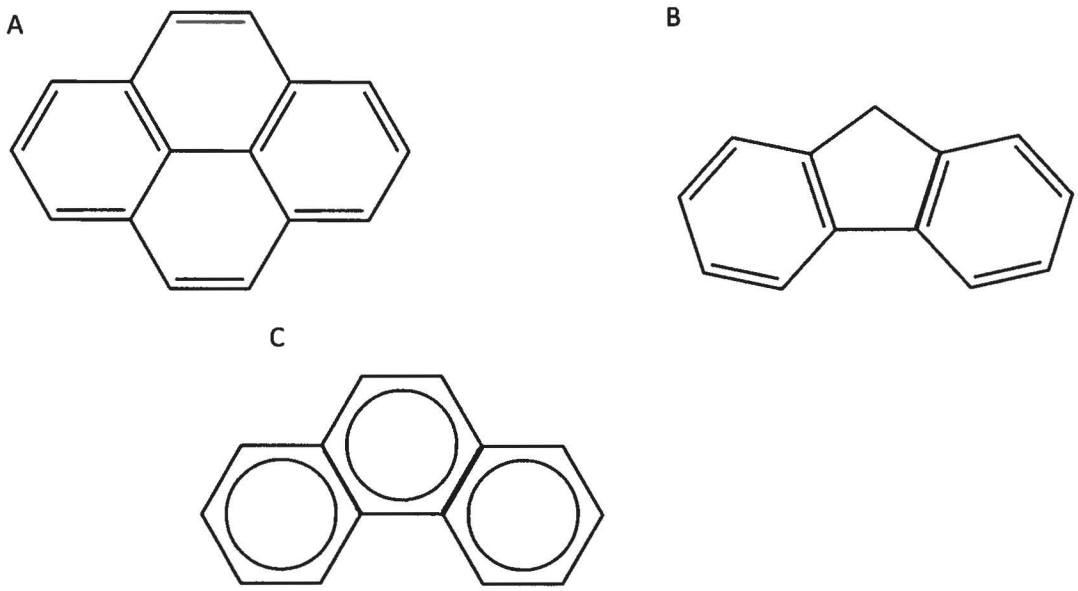

Figure 4. Chemical structures of three common polycyclic aromatic hydrocarbons. The structures of pyrene (A), fluorene (B) and phenanthrene (C).

1.1.5 Heavy Metals

Heavy metals are inorganic pollutants such as Iron (Fe), Copper (Cu), Chromium (Cr), and Zinc (Zn) which are essential for metabolic activities at moderate concentrations but can become toxic as levels increase (Qadir et al., 2011). Metal bioaccumulation in fish organs is dependent on the method of uptake, absorption, storage, regulation and excretion (Qadir et al., 2011). Uptake and accumulation can occur through multiple mechanisms including ingestion of food, suspended particulate matter, metal ion exchange through gills and skin, and water intake (Qadir et al., 2011).

In the MWWE, Aluminum (Al), Cr, Manganese (Mn), Fe, Cobalt (Co), Nickel (Ni), and Lead (Pb) made up less than 1 % of the effluent while Cu, Zn, and Cadmium (Cd) made up between 3-5 % (Gagnon et al., 2003; Gobeil et al., 2005). MWWE is a key source for high Silver (Ag) concentrations that reach the St. Lawrence River and bind to sediments (Gobeil et al., 2005). The effluent is responsible for contributing up to 60 % of current Ag concentrations in the river (Gobeil et al., 2005).

When metals are taken up by organisms they can bind to metallothionein (MT) proteins which are low-molecular weight proteins containing 25-30 % cysteine that sequester divalent heavy metals in cells (Cosson, 2000; Bonneris et al., 2005; Nordberg, 2009). Bound metals are less biologically active and therefore considered less toxic (Gagne et al., 1990; Dragon et al., 2004; Bonneris et al., 2005). The induction of MT levels in the liver and kidney represents an effective biological marker of metal exposure (Baudrimont et al., 2003; Gagnon et al., 2006).

An example of MT induction as an indicator of metal exposure was observed in both a time and dose dependent manner when goldfish were injected with cadmium chloride ($CdCl_2$) (Choi et al., 2007). MT mRNA levels were significantly increased in the brain, liver, kidney and intestine of goldfish tissue at 12, 24 and 36 h post-injection (Choi et al., 2007). Yellow perch (Perca flavescens) from Rouyn-Noranda, Quebec, were chronically exposed to Cd, Zn and Pb and showed altered hormonal, physiological and reproductive status (Levesque et al., 2003). These fish showed abnormal seasonal cycling of liver glycogen and triglyceride reserves suggesting that energy stores were redirected for biotransformation and detoxification processes; decreased sodium (Na^+)-potassium (K^+)-adenosine-5'-triphosphate (ATP), a key enzyme in osmoregulation and acid base balance was coupled with abnormalities in gill morphology including a thickening of the lamellae; impaired cortisol stress response levels and smaller nuclei in adrenocortical cells; atrophy of adrenocortical cells consistent with the decreased cortisol secretion; and decreased plasma thyroid hormone levels and thyroid epithelial cell height (Levesque et al., 2003). Fish had an overall reduction in GSI and gonads were at a less mature stage when compared to controls (Levesque et al., 2003). Female exposed fish had lower levels of E2 possibly due to the actions of MT on Zn availability. Zn is required for the synthesis of sex steroid hormones and if bound to MT is unavailable for conversion therefore causing a reduction in E2 levels (Levesque et al., 2003).

Within contaminant mixtures a number of synthetic chemicals have been shown to induce MT levels including non-metal substances. In a 2007 study, caged mussels were placed within the MWWE plume in the St. Lawrence River (Gagne et al., 2007a). After 90 days exposed mussels were found to have high levels of MT induction with no corresponding metal accumulation in tissues (Gagne et al., 2007a). After further investigation it was hypothesized that the MT

induction occurred from the inflammation and metal scavenging processes within the plume rather than from direct metal exposure (Gagne et al., 2007a). Further studies are needed to determine the spread of chemicals that are able to induce MT proteins.

1.2 Mixture Effects

Most environmental contaminants exist as a mixture of compounds that can act in an inhibitory, additive or synergistic manner (Gardner Jr et al., 1998; Monosson, 2005; Sexton and Hattis, 2007). The composition of complex mixtures is dependent on the industrial and household sources contributing to the make-up of the wastewater (Monosson, 2005; Sexton and Hattis, 2007; Filby et al., 2007). Among the most frequently studied chemical groups are PCBs, xenoestrogens, PPCPs, PAHs and other industrial pollutants (Kortenkamp et al., 2008). When chemicals act in combination to inhibit normal organismal activity they can negatively alter normal metabolic and cellular functions (Kortenkamp et al., 2008). Inhibitory actions can occur when a contaminant acts as an antagonist on a receptor, for example BPA inhibits TR (Goto et al., 2006). As well, the actions of a transporter can be altered when targeted by a xenobiotic, such as the antagonistic affect of antidepressants on the 5-HT (5-hydroxytryptamine) transporter (Bal et al., 1997; Owens et al., 1997). Finally, contaminants can also have an inhibitory affect on the biosynthesis of enzymes, for example heavy metals cadmium and zinc can inhibit the biosynthesis of α-Galactosidase and β-Galacsidase (Nweke and Okpokwasili, 2011). These inhibitory actions can occur in different combinations or they can occur at the same time leading to negative consequences for the organism (Bal et al., 1997; Owens et al., 1997; Nweke and Okpokwasili, 2011).

Toxicants targeting enzymatic processes within the liver can cause alterations of the detoxification processes allowing contaminants to accumulate within the organism (Luckenbach and Epel, 2005). Besselink et al. (1998) exposed European flounder (Platichthys flesus) to a mixture of either non-dioxin PCB congeners, a mixture of dioxin-like PCB congeners, or a mixture of the two groups (Besselink et al., 1998). Studies have shown that individual dioxin-like PCB congeners induce toxic effects by acting on the AhR, however, few studies had looked at the effects of a mixture of PCB congeners (Besselink et al., 1998). The results indicated that mixtures of PCB congeners can have inhibitory effects on CYP1A1 induction in the liver of

flounder (Besselink et al., 1998). These findings suggest that PCB congeners interfere with CYP1A1 catalytic activity by having an inhibitory effect (Besselink et al., 1998). Other studies with similar findings have shown a low functioning or non-functioning AhR pathway in response to PCB mixtures (Besselink et al. 1996). Overall, this study showed that exposing flounder to a mixture of PCB congeners can cause an inhibition of CYP1A1 activity in the liver which can lead to alterations in the expression of genes encoding xenobiotic metabolizing enzymes (Besselink et al., 1998; Bemanian et al., 2004). Thus the behavior of chemicals within a mixture can result in drastically different effects than individual chemicals.

Studies have shown that exposure to contaminant mixtures containing estrogenic chemicals can have inhibitory effects on the reproductive processes including egg production and the development of secondary sexual characteristics (Jobling et al., 1996; Brian et al., 2007; Thorpe et al., 2009). Exposure to individual estrogenic chemicals has been shown to inhibit reproduction in fish populations found in the plume of wastewater discharge pipes (Jobling et al., 1996; Thorpe et al., 2009). Pair-breeding fathead minnows were exposed to effluent containing environmental estrogens in order to determine their reproductive success (Thorpe et al., 2009). Fish were exposed for three weeks to one of three effluent groups with varying degrees of estrogenic potency (Thorpe et al., 2009). The study found that the measured estrogen potencies of the effluents were proportional to the observed effects on reproductive success (Thorpe et al., 2009). The effluent with the highest estrogenic activity had the greatest inhibitory effect on egg production and the effluent with the weakest estrogenic activity had no impact on egg production (Thorpe et al., 2009). Of the three different effluent mixtures only two had estrogen levels high enough to alter reproduction and induce VTG levels above 1 mg/ml (Thorpe et al., 2009). Xenoestrogens in effluent are capable of altering reproductive success and can modify the three ER subtypes found in fish which may heighten the effects and lead to a suppression of egg production in exposed fish (Thorpe et al., 2009).

A second study examined the effects of mixtures of natural and synthetic estrogenic contaminants on pair breeding adult fathead minnows. The fish were grouped in pairs to measure the reproductive performance and secondary sexual characteristics after one week exposure to a mixture of xenoestrogenic contaminants (Brian et al., 2007). The stock estrogenic

mixture was made up of 3 ng/L of EE2, 125 ng/L of E2, 35 µg/L of NP, 225 µg/L of OP and 750 µg/L of BPA (Brian et al., 2007). This stock mixture was then diluted to concentrations of 40, 20 and 10 % mixture dilutions (Brian et al., 2007). The results found that reproductive success decreased in a concentration dependent manner, however, there were no differences in the number of eggs produced during each spawning event (Brian et al., 2007). In male fish the prominence of the secondary sexual characteristic, nuptial tubercles, was reduced after exposure to treatments relative to controls (Brian et al., 2007). When the contaminant concentrations were reduced to 0.6 ng/L EE2, 25 ng/L E2, 7 µg/L NP, 45 µg/L OP, and 150 µg/L BPA applied as either a mixture or individually to measure the same reproductive endpoints, the number of spawning events and the number of eggs produced by fish exposed to the low-dose mixture decreased in a concentration dependent manner (Brian et al., 2007). The authors found that when pair-breeding fish were exposed to OP alone the negative impact on reproductive ability was similar to fish pairs exposed to the mixture, suggesting that OP is a more potent xenoestrogen (Brian et al., 2007). All of the other mixture components individually did not produce similar reductions in reproductive success despite inducing VTG in the male fathead minnows (Brian et al., 2007).

Mixtures containing contaminants with similar modes of action can react in an additive fashion and have consequences greater than the sum of their individual effects (Faust et al., 2003; Kortenkamp et al., 2008). Chemicals having an additive effect often interact with the same sub-system of an organism and contribute to the toxicity of a mixture in a dose dependent manner (Kortenkamp et al., 2008). Wastewater effluent is often made up of classes of contaminants which individually can alter toxicant metabolism, reproductive processes, altered cellular signaling and induced stress response and when in combination can have a negative effect on all of these targets (Faust et al., 2003; Kortenkamp et al., 2008).

Spottail shiners sampled within the MWWE effluent plume in the St. Lawrence River displayed delayed spermatogenesis, increased VTG levels, intersex characteristics and reduced sperm motility (Aravindakshan et al., 2004b). Furthermore, exposure of brook trout (Salvelinus fontinalis) to MWWE indicated that VTG induction occurred only following long-term exposure suggesting that the chemicals responsible for the estrogenic effects need to accumulate in the fish

(de Montgolfier et al., 2008). Since the MWWE contains a large number of chemicals, it is therefore likely that short-term accumulation of chemicals from the effluent may inhibit the estrogenic effects of xenoestrogens and natural hormones in the effluent. Following long-term exposure the bioaccumulation of certain chemicals may be sufficient to tilt the balance towards estrogenic effects and the induction of VTG.

1.3 Gap Junctions

Gap junctions (GJ) are clusters of transmembrane channels that allow neighbouring cells to communicate through the exchange of small molecules including: secondary messengers (Ca^{2+}), nucleotides, amino acids, ions, siRNA and metabolites (Fig. 5) (Goodenough et al., 1996; Sohl et al., 2004; Cruciani et al., 2006; Plante et al., 2007). GJ typically gather in groups of 10 to 10 000 channels, called plaques, on the surface of the plasma membrane (Vinken et al., 2006). GJ channels are made up of two hemichannels or connexons, one from each cell (Cruciani et al., 2006; de Montgolfier et al., 2007). Six connexins (Cx) are oligomerized to form each connexon. A connexon can be homologous, composed of the same Cxs, or heterologous, composed of two or more different Cxs (Cruciani et al., 2006). The structural difference among connexon composed of different Cxs will determine the channels permeability to molecules (Echevarria and Nathanson, 2004).

Cxs are a family of transmembrane proteins that can be further subdivided into α, β, ε, γ, and σ subgroups and a number in the order of their discovery, for example gap junction alpha 1 (GJA1) (Vinken et al., 2006; Li et al., 2010). A second, more common, nomenclature is based on the molecular weight (in kDa) of each Cx, for example Cx43 is 43 kDa (also known as GJA1) (Vinken et al., 2006; Li et al., 2010). Cxs have nine main domains: four transmembrane domains, two extracellular loops, an intracellular loop and cytoplasmic N- and C- terminal ends (Hertzberg et al., 1988; Falk et al., 1994) (Fig. 5). The four transmembrane domains, two extracellular loops and N-terminus are highly conserved among fish and mammals while the cytoplasmic domains vary between different isoforms which allows for different interaction and function (Fig. 6) (Hertzberg et al., 1988; Falk et al., 1994; Cruciani et al., 2006; Dbouk et al., 2009; Li et al., 2010).

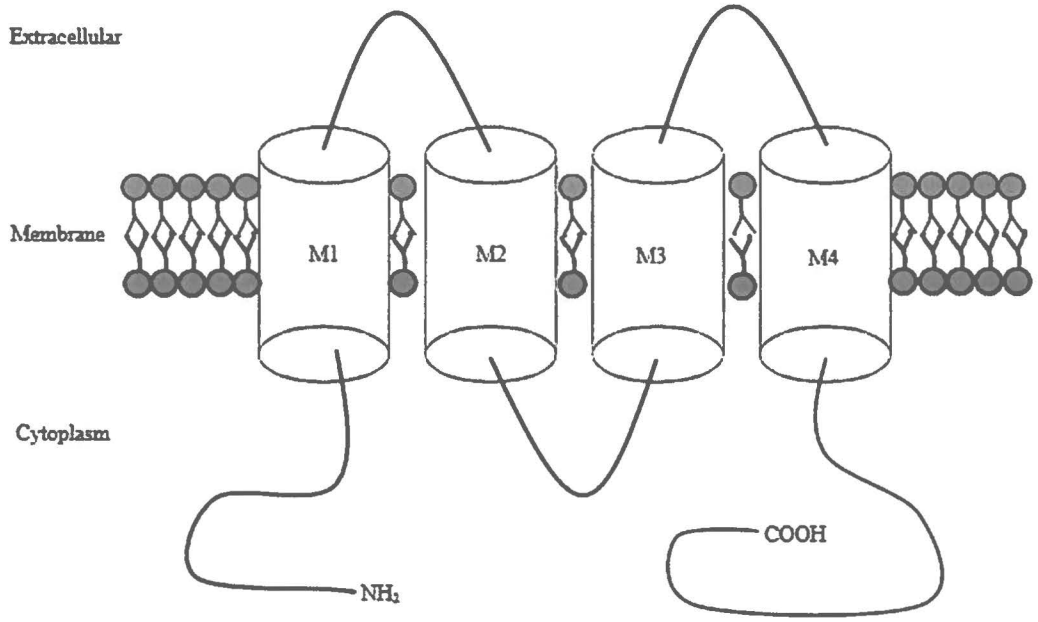

Figure 5. Detailed structure of the connexin. The nine domains of the connexin structure includes four transmembrane domains (M1, M2, M3 and M4), two extracellular loops, one intracellular loop, and cytoplasmic N- and C- terminal ends.

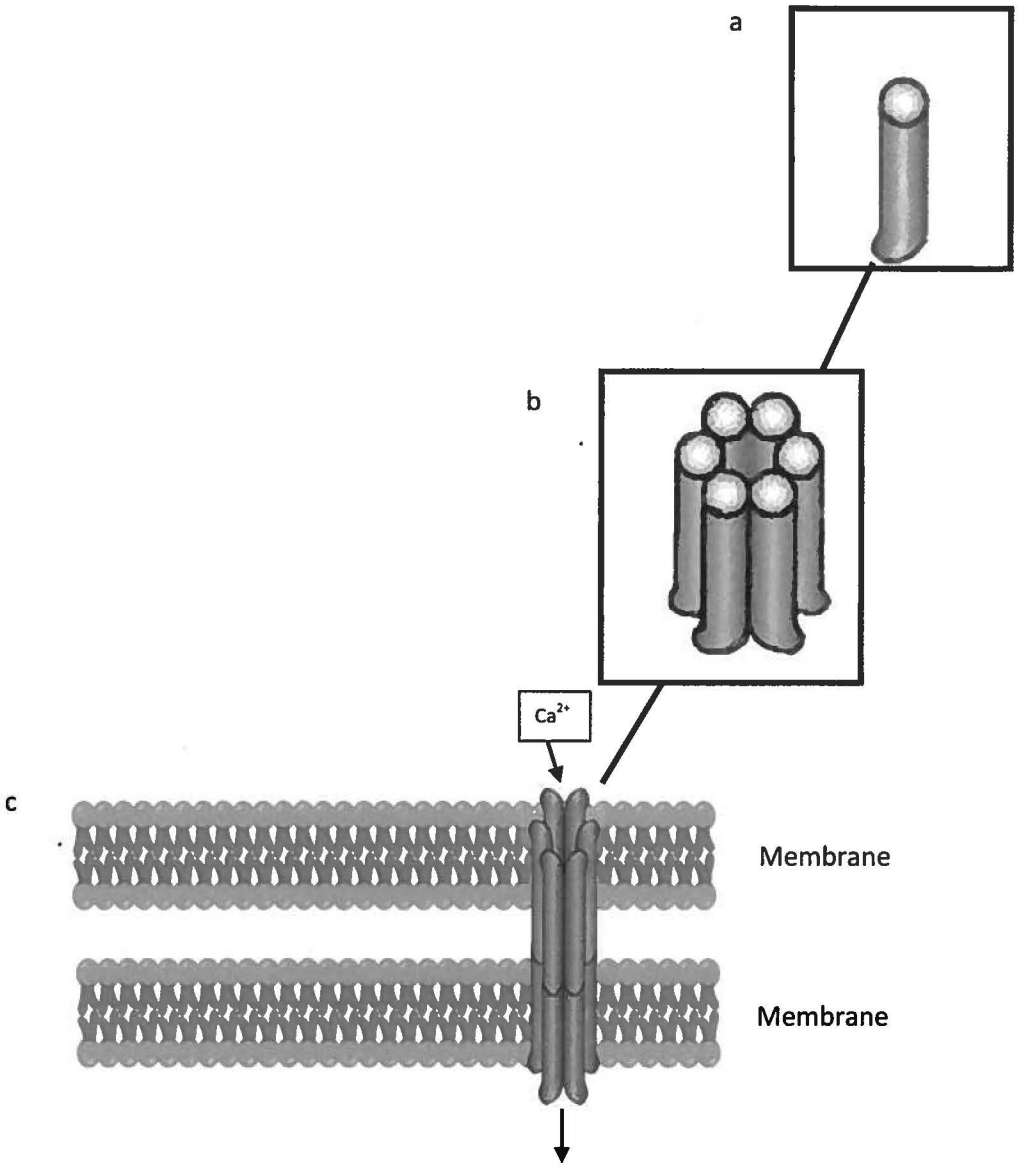

Figure 6. Basic structure of the gap junction. (A), a single connexin (B) six connxin oligomerize to form a connexon and (C) two neighbouring connexon form a gap junction channel across a membrane allowing secondary messengers, such as Ca^{2+} to pass through the channel.

Different cell types express different Cxs (Rackauskas et al., 2010). In mammals, three Cxs are expressed in the liver, Cx43 found in biliary and non-parenchymal epithelial cells, Cx32 (GJB1), and Cx26 (GJB2) both found in hepatocytes (Yang et al., 2003; Plante et al., 2007; Vinken et al., 2008; Fukumasu et al., 2010; Rackauskas et al., 2010; Vinken et al., 2011). Cx32 is located throughout the lobules and is thought to be the major component of liver GJs (Yang et al., 2003).

Cx26 is located in the periportal zone of the lobules (Yang et al., 2003). Each Cx, therefore, appears to be implicated in forming GJs that have separate physiological functions.

The flux of molecules through the GJ channel is referred to as gap junction intercellular communication (GJIC) and is important in the control of cellular homeostasis (Vinken et al., 2006). The regulation of GJIC ranges from Cx gene transcription to GJ degradation (Vinken et al., 2006). Deficient or improper GJ formation has been associated with a number of diseases including some forms of neuropathy, hereditary deafness, cataracts, skin disease, heart disease and cancer (Willecke et al. 2002). Phosphorylation sites located on the C-terminal, in addition to the make up of the connexon, will determine the permeability properties of the channels (Warn-Cramer and Lau, 2004).

GJIC can be regulated by two kinetic courses: fast control (millisecond range) and long-term control (hour range) (Vinken et al., 2006). Fast control is also called, gating, and is triggered by a number of factors including voltage sensitivity, intracellular Ca^{2+} levels, intracellular pH and phosphorylation (Rackauskas et al., 2010; Li et al., 2010). Voltage sensitivity is important for the intercellular coupling between excitable cells where each connexon contains voltage sensitive gates (Rackauskas et al., 2010; Li et al., 2010). For example, Cx30, Cx26 and Cx43 channels close under depolarization conditions (Van der ven et al., 2005). Changes in intracellular Ca^{2+} can cause channels to close and plays an important role in protecting healthy cells from membrane depolarization and leakage of metabolites through GJ by disconnecting them from surrounding damaged cells (Rackauskas et al., 2010; Li et al., 2010). This process is known as the by-stander effect (Rackauskas et al., 2010; Li et al., 2010). Finally, phosphorylation by different protein kinases may alter cell-to-cell communication resulting in the rapid turnover of GJ channels (Vinken et al., 2006; Rackauskas et al., 2010; Li et al., 2010). Phosphorylation acts by modifying electrical and metabolic communication by changing the channel structure altering its conductive capabilities (Rackauskas et al., 2010; Li et al., 2010). A number of kinases have been shown to phosphorylate Cxs including epidermal growth factor (EGF), MAPK, protein kinase C (PKC), protein kinase A (PKA), casein kinase 1 (CK1) and cyclin B-dependent $p34^{cdc2}$ kinase ($p34^{cdc2}$) (Vinken et al., 2006). Substances that increase intracellular cyclicAMP (cAMP) levels can increase GJIC (Lampe and Lau, 2000). This

increase in communication has been attributed to increased rates of Cx transcription, phosphorylation, increased levels of Cx mRNA or protein levels, and changes in Cx43 trafficking (Lampe and Lau, 2000). GJIC can play a role in tumor suppression through its control in cell cycling and proliferation (Vinken et al., 2006). Over-expression of Cxs in tumor cells has been shown to decrease cellular proliferation and increase cell death activities suggesting a role in tumor suppression (Vinken et al., 2006).

GJ are present between cells in a number of different tissues and a wide range of species (Bennet and Goodenough, 1978). These GJ share basic structural similarity but vary in their detailed morphologies (Friend and Gilula, 1972) and in their sensitivities to physiological manipulations (Spray et al., 1981). In mammals, Cx43, Cx32 and Cx26 have been identified by electron microscopy in the liver of a number of species including the rat, mouse, cow and human (Paul, 1986; Chen et al. 2012; Parthasarthi, 2012). Currently, Cx43 is the only Cx that has been identified in the fish liver and was identified in the zebrafish (Christie et al., 2004; Cruciani et al., 2006). GJ play a significant role in the regulation of liver function by regulating glucose release from the liver, by contributing to the regulation of bile secretion and through the regulation of hepatocellular growth and tumor formation suppression (Echevarria and Nathanson, 2004). Although only one Cx has been identified in the liver of fish it has been suggested that these same functions in fish are controlled by GJIC and possibly unidentified Cxs (Echevarria and Nathanson, 2004).

Cx43 is a ubiquitous Cx found in mammals and has been identified in four fish species: zebrafish, rainbow trout, brook trout and common carp (Cyprinus carpio) (Van der Heyden et al., 2004; de Montgolfier et al., 2007, 2008). It is homologous to Cx43 in carp, rainbow trout and GJA1 in the rat and human (Chatterjee et al., 2001; Cheng et al., 2003; Christie et al., 2004; Chatterjee et al., 2005; Cruciani et al., 2006; de Montgolfier et al., 2007).

Immunohistochemical analysis determined that in mammals Cx32 is the Cx predominately expressed in hepatocytes (Bruzzone et al.1996; Bone et al., 1997; Echevarria and Nathanson, 2004; Laird et al., 2005). During hepatocyte differentiation Cx32 mRNA levels significantly increase and have been shown to play a role in maintaining the normal structure and organization

of hepatocytes (Yang et al., 2003; Echevarria and Nathanson, 2004). In the liver Cx32 is about 10-times more abundant than Cx26 and has been suggested to play a role in stabilizing Cx26 levels (Plante et al., 2002; Echevarria and Nathanson, 2004). Cx32 plays a key role by forming metabolic coupling between neighboring hepatocytes for the proper distribution of secondary messengers involved in liver-specific functions (Yang et al., 2003). Channels formed between hepatocytes can be homotypic, containing only Cx32 or heterotypic which can be formed with Cx26 and allow for the selective passage of messages (Yang et al., 2003).

Plante et al. (2002) showed that Cx32 mRNA levels were significantly higher in ovariectomized (OV) rats 10 days after surgery. When OV rats were implanted with E2, Cx32 mRNA levels decreased below control levels suggesting that Cx32 is under partial regulation by E2 (Plante et al., 2002; Saito et al. 2004). This finding is important in environmental conditions contaminated by natural and synthetic estrogens which can mimic E2 and alter Cx32 expression levels.

Alterations in Cx32 expression levels and the induction of CYP P450 isoenzymes have been suggested to be important for the biotransformation of xenobiotics (Vinken et al., 2008). A second major function of the liver and a component of GJIC is the excretion of bile from the liver (Vinken et al., 2008). Bile excretion is a route of elimination for degradation products of xenobiotics (Di Giulio et al., 2008). Bile flow originates from both hepatocytes and the bile duct epithelial and requires GJIC-dependent Ca^{2+} signaling (Vinken et al., 2008). The secretory activity of bile in the liver is controlled by fluctuations in Ca^{2+} levels (Echevarria and Nathanson, 2004).

Cx26, is also expressed in hepatocytes and follows a unique path during GJ formation as it bypasses the Golgi apparatus during the translocation into the plasma membrane (Garcia-Rodriquez et al., 2011). Cx26 is the only reported Cx that is not phosphorylated, possibly due to its short C-terminal tail (Traub et al., 1989; Laird et al., 2005). There is little known about the role that Cx26 plays in liver function but studies have shown that the over-expression of Cx26 and Cx32 can serve an anti-carcinogenic function (Echevarria and Nathanson, 2004).

Modification of the expression of Cxs has been associated as a marker for cellular distress (Segner et al., 2003). The impairement of GJIC can be associated as a protection mechanism to prevent the proliferation of disease to healthy neigbouring cells (Segner et al., 2003). These studies have shown that exposure to environmentally relevant contaminants can alter Cx expression levels in the liver and cause an impairment to GJIC therefore having negative consequences to liver homeostasis and function.

The liver is a target for many xenobiotics because of its role in detoxification. Several liver specific functions require GJIC for regulating albumin secretion, ammonia detoxification, glycogenolysis, bile secretion and xenobiotic phase I biotransformation (Vinken et al., 2008). Xenobiotic-metabolizing enzymes are responsible for mediating the toxicity of chemicals and protecting the organisms by quickly metabolizing contaminants so they can be eliminated (Vinken et al., 2008). Alterations to the components of this system either in the expression or phosphorylation of Cx or by altering one of the protein kinases involved in phosphorylation of Cx can greatly affect GJIC and the functions in which it controls. Contaminants can therefore alter GJIC by modifying Cx phosphorylation and expression. Previous studies have shown that individual contaminants and mixtures can alter GJIC but in order to understand the underlying mechanisms to these complex pathways a more global genomic approach should be taken.

Contaminants targeting the liver can alter the levels of Cx expression which can impair GJIC and have negative consequences to homeostatic conditions (Habeebu et al. 1998; Vinken et al., 2008). Acute Cd exposure has been shown to induce cellular proliferation and death in the liver (Habeebu et al. 1998). Adult male mice were given an intraperitoneal injection of 30 μmol $CdCl_2$/kg and samples were collected at 1.5, 3, 6, 9, 14, 24 and 48 h to determine time-response effects (Jeong et al., 2000). A second dose-response experiment was carried out for comparison (Jeong et al., 2000). Mice were injected with 0.9 μg/L,1.83 μg/L,3.6 μg/L,5.5 μg/L, 7.2 μg/L and 11 μg/L, liver samples were collected 9 h later (Jeong et al., 2000). GJIC activity was measured using the fluorescent dye Lucifer Yellow CH and determined that Cd caused a time-dependent and dose-dependent inhibition of GJIC (Jeong et al., 2000). Both Cx32 and Cx26 mRNA levels were down-regulated in a dose- and time-dependent manner (Jeong et al., 2000). Findings suggest that Cd exposure results in a decrease of Cx26 and Cx32 proteins as well as the number

of GJ channels per cell (Jeong et al., 2000). This inhibition occurred before the Cd-induced apoptosis suggesting that GJIC may play a regulatory role in the induction of apoptosis by Cd (Jeong et al., 2000). The cellular toxicity of Cd has been attributed in part to the induction of oxidative stress which has been shown to inhibit GJIC (Upham et al., 1997; Shaikh et al., 1999). The highest level of inhibition was achieved at 6 h whereas the maximum level of cell death occurred at 9 h (Habeebu et al., 1998; Jeong et al., 2000). GJIC decreased and apoptosis increased as time and dose progressed (Jeong et al., 2000). These findings suggest that GJIC plays a role in the initiation of Cd-induced apoptosis (Jeong et al., 2000). A rapid decrease in GJIC occurs before cellular proliferation suggesting proliferation is partially controlled by GJIC (Jeong et al., 2000). This study found that Cd induces alterations in Cx levels simultaneously in the liver whereas other studies have shown that contaminant exposure can affect Cx26 and Cx32 (Jeong et al., 2000). One study found that exposure to TCDD decreases Cx32 mRNA in isolated hepatocytes but has no effect on Cx26 mRNA levels (Baker et al., 1995).

A rat liver epithelial cell line (WB-F344) was used to determine the effects of PAH on GJIC (Blaha et al., 2002). Cells were exposed for 15, 30 or 60 min to a mixture of 35 different PAHs (Blaha et al., 2002). At 15 and 30 min, exposed cells showed the strongest inhibitory effects with a transient recovery 60 min after the initial exposure (Blaha et al., 2002). The low molecular weight PAH: fluorine; phenanthrene; fluoranthene; and their methylated derivatives showed the strongest inhibition of GJIC most likely due to their structural characteristics (Blaha et al., 2002). Higher molecular weight PAHs elicited between a 25-50% inhibition of GJIC where a plateau was reached and higher concentrations showed no further effects (Blaha et al., 2002). PAHs potentially inhibited GJIC at the post-translational level by altering the phosphorylation of Cx or by altering one of the protein kinases, such as PKC or MAPK, involved in the process (Lampe and Lau, 2000).

Rats exposed to hexachlorobenzene (HCB), an epigenetic carcinogen, were shown to be more susceptible to chemically induced liver carcinogenesis (Larouche et al., 1993). Several studies have shown that the formation of tumors in the liver is associated with a loss of GJIC and exposure to HCB specifically results in the decrease of Cx32 and Cx26 mRNA and protein levels in the liver of female rats (Yamasaki et al., 1999; Plante et al., 2002; Plante et al., 2007). After

HCB exposure Cx levels decrease followed by the formation of tumors in the liver, suggesting that this reduction in Cx levels could be an early event in the process of liver carcinogenesis (Plante et al., 2002). The decrease in Cx32 is thought to occur through the over-expression of the integrin-linked kinase (ILK) pathway which leads to the activation of Akt and a decrease in Cx32 levels (Plante et al., 2006).

Exposure to MWWE has been shown to alter Cx levels in the brook trout testis. Cx43 mRNA levels were increased after exposure to MWWE dilutions of 1 and 10% but not at the highest concentration (de Montgolfier et al., 2008). Cx31 mRNA levels were reduced in the 1 and 10 % groups but levels were not altered at the highest concentration (de Montgolfier et al., 2008). There was no significant affect on Cx30 mRNA levels (de Montgolfier et al., 2008).

While contaminants may alter Cx levels by acting directly on their expression levels via hormone receptors, as is the case for endocrine disruptors, certain contaminants may also affect intracellular signaling pathways implicated in the regulation and phosphorylation of Cxs. NP for example has been shown to reduce GJIC in Sertoli cells by decreasing both Cx43 and phosphorylated Cx43 by targeting the p-38 MAPK pathway (Aravindakshan et al., 2005). Thus the effects of contaminants on intracellular signaling leading to altered GJIC may represent critical mechanisms leading to toxicity. Identifying how these pathways are altered however requires a broad analysis of gene expression and may best be assessed using genomic and proteomic approaches.

1.4 Genomics

Toxicogenomics provides the resources to examine a large number of changes in the expression of genes, proteins and physiological metabolites from an organism after exposure to an environmental stressor or toxicant (Ankley et al., 2006). As the technology and resources have evolved, the scope of the analysis has progressed from assessing hundreds of genes using macroarrays, to microarrays which can contain a hundred thousand genes (Ankley et al., 2006). Current microarray technology allows the partial or whole genome of an organism to fit onto a single substrate which allows the comparison of multiple treatments in one experiment (Hook et al., 2006; Ankley et al., 2006). Before genomic technology, genes were studied individually,

ignoring the complex interactions and cascade pathways which can be elucidated with microarrays (Ankley et al., 2006; Hook et al., 2006). Examining such pathways is necessary, particularly in assessing low-dose effects that can cause subtle but critical cellular and molecular effects.

Microarray technology requires samples of interest to hybridize to closely related cDNA sequences on a slide. The development of cDNA microarrays requires knowledge of the species genome in order to create the array (Hook et al., 2010). The development of a unique microarray for new species is both time consuming and costly and while there has been some progress for certain wildlife species it has been very limited (Hook et al., 2010). Fish are a highly diverse group with more species than any other vertebrate class, however, divergence in gene sequences has occurred throughout evolution and using microarrays across species poses experimental limitations (Miller and Maclean, 2008). Likewise, the differences in gene sequences between fish species limits the use of microarrays in field-based environmental toxicology.

Technological improvements to software programs used in bioinformatics has enabled the analysis of interacting gene products providing a theoretical picture of large affects occurring along cascading pathways (Garcia-Reyero et al., 2009). This is important since most biological processes occur through functional pathways rather than focusing on the effects of individual genes for each experiment.

Azzam et al. (2003) examined the effects of ionizing radiation and other environmental stressors on GJIC, specifically Cx43 expression levels, in human diploid fibroblast cells (AG1522) (Azzam et al., 2003). In order to gain insight into the mechanisms underlying the radiation-induced bystander effect the study utilized a global approach to identify alterations in gene expression using a cDNA microarray (Azzam et al., 2003). Cells were exposed to one centigray unit of α-particles for 3 h (Azzam et al., 2003). At this low exposure dose there was an initial up-regulation (5-fold) of Cx43 mRNA relative to controls suggesting an α-particle induced bystander effect (Azzam et al., 2003). This study showed that Cx43 responded to stress by undergoing post-translational modifications (Azzam et al., 2003). As indicated in the previous section, phosphorylation processes are implicated in the regulation of Cxs (Azzam et al., 2003).

Rapid changes in phosphorylation are thought to be a response to stress in order to restrict or aid in the transfer of particular signals between cells (Azzam et al., 2003). Microarray analysis showed an up-regulation of Cx43 and a down-regulation of cytoskeletal genes including y-actin and β-tropomyosin (Azzam et al., 2003). This is consistent with reports in literature where an inverse relationship between Cx43 and cytoskeletal protein expression in smooth muscle cells had been reported (Ko et al., 2001). Furthermore, a number of down-regulated genes that encode for cell-adhesion molecules (collagen and laminin) were noted. This was also consistent with reports that cell adhesion proteins and Cxs interact using signal transduction pathways such as the Wnt pathway (Azzam et al., 2003). Cell adhesion proteins are regarded as participants in the expression of the bystander effect through their role in the extracellular matrix (Barcellos-Hoff and Brooks, 2001).

Metals including, Ni, Cd and Cr in the environment can be found at toxic concentrations for a variety of organisms including the brook trout, yellow perch, mussel and the rat (Levesque et al., 2003; Choi et al., 2007; Gagne et al., 2007a; Permenter et al., 2011). H4-II-E-C3 rat liver cells were exposed to $NiCl_2$ (Ni; 51 mg/l), $CdCl_2$ (Cd; 2 mg/l) or $Na_2Cr_2O_7$ (Cr; 2.6 mg/l) for 24 h. Ni, Cd and Cr are heavy metals that have been shown to induce oxidative stress and DNA damage in a number of organs, however the underlying mechanisms of action are poorly understood. Permenter et al. (2011) exposed hepatic cells to three heavy metals and utilized a genomic approach to help identify the pathways involved in the toxicity of these metals. A common biological process for all three metals was the induction of genes associated with oxidative stress. All three metals induced levels of the nuclear factor erythroid 2-related factor 2 (Nrf2) as well as a number of genes downstream including heme oxygenase 1 (hmox1), sequestosome 1 (sqstm1) and glutathione-S-transferases (GST) (Permenter et al., 2011). Hmox1 is a ubiquitous stress response protein involved in reducing the effects of oxidative stress and apoptosis (Permenter et al., 2011). Sqstm1 has been previously shown to play a role in the sustained activation of Nrf2 in response to oxidative stress. GST has been shown to contribute to the phase II biotransformation of xenobiotics and altered expression of GST is associated with oxidative DNA damage (Permenter et al., 2011). A number of genes that respond in oxidative stress conditions were down-regulated including hydroxyacid oxidase 1 (HAO1) a liver specific enzyme that converts α-hydroxy acids to α-keto acids while reducing oxygen to hydrogen

peroxide (Permenter et al., 2011). A second gene down-regulated in response to conditions of oxidative stress is forkhead transcription factor (FKHR) which stimulates the expression of metal containing antioxidant proteins (Permenter et al., 2011). Cells exposed to Cd demonstrated a unique profile of oxidative stress leading to endoplasmic reticulum stress including the induction of the unfolded protein response and apoptosis (Permenter et al., 2011). Six genes encoding for the chaperone proteins (Hspa1a, Hspa1b, Hspb8, Dnajb1, Dnajc3, and Cryab) were all up-regulated and were unique to Cd exposure (Permenter et al., 2011). The role of chaperone proteins in apoptosis has been well documented as well as the folding and degradation of damaged proteins in the unfolded protein response (Permenter et al., 2011). Stress in the endoplasmic reticulum can lead to apoptosis and is observed in Cd exposure by the induction of caspase 4 (Casp4) which encodes an apoptosis-related cysteine peptidase (Permenter et al., 2011). Two other genes were up-regulated in response to Cd and have been previously shown to be associated with apoptosis: protein phosphatase 1 regulatory subunit 15A (Ppp1r15a) and DNA-damage inducible transcript 3 (Ddit3) (Permenter et al., 2011). This study determined common and unique pathways for induced oxidative stress in response to heavy metal exposure using a genomic approach (Permenter et al., 2011).

Fish are an excellent sentinel species of the aquatic environments and can therefore act as models for studies to examine the underlying mechanisms of action as a result of exposure to contaminants (Hook et al., 2010). Persistent organic pollutants, including insecticides and pesticides, are present in the environment at high concentrations because they are not easily broken down (Garcia-Reyero et al., 2006). Microarray technology can help to identify the pathways that are altered after exposure to mixtures of persistent organic pollutants (Garcia-Reyero et al., 2006).

Two chlorinated hydrocarbon insecticides, dichlorodiphenyldichloroethylene (DDE) 45.9 μg/g and 0.81 μg/g of 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanona-phthalene (dieldrin) were incorporated into pellets and fed to largemouth bass (Micropterus salmonides) for 120 days (Garcia-Reyero et al., 2006). Dieldrin and DDE are organochlorine insecticides and known endocrine disrupting chemicals (Garcia-Reyero et al., 2006). Endocrine disruptors can act through three main pathways: first through the interaction of

endocrine disrupting chemicals with hormone receptors, second alteration in the processes involved in steroid synthesis and sex steroid metabolism (Garcia-Reyero et al., 2006). The expression of steroid acute regulatory protein-related (StAR), the rate limiting step in steroid synthesis, was up-regulated in the gonads of fish exposed to DDE and dieldrin (Garcia-Reyero et al., 2006). Increased expression of StAR was consistent with the elevated 11-keto-testosterone (11-KT) levels observed after DDE exposure but not dieldrin. Increased expression of StAR occurs when androgen receptor (AR) activity is down-regulated and p,p'-DDE has been shown to act as an anti-androgen and may be acting directly as an AR antagonist to increase gonadal StAR expression. On the other hand, dieldrin exposure resulted in a decrease in the 11-KT levels which is unexpected with increased StAR expression. A decrease in the expression of CYP19, also known as aromatase, the determining step in the conversion of testosterone to E2 was down-regulated in the gonads following DDE treatment. The decrease in aromatase was 8-fold higher in females than in males which is consistent with the lowering of plasma E2 levels in females but not in males where plasma E2 levels remained constant. 11-KT levels were higher suggesting that overall steroidogenesis was increased in males. Dieldrin exposure resulted in an increase in aromatase mRNA in the gonads of females but decreased in males. E2 levels decreased in both sexes. Low plasma E2 levels could be a result of feedback mechanisms to increase the release of gonadotropin-releasing hormone (GnRH) in an effort by the brain to maintain sex steroid homeostasis. This feedback mechanism may also be responsible for the increased expression of StAR. This study determined that p,p'-DDE acted as a weak estrogen and inhibited the expression of AR and altered genes involved in the synthesis of endogenous hormones. In contrast dieldrin acted primarily to down-regulate the expression of mRNA for some hormone receptors but did not mimic an estrogen. Thus the use of microarrays enabled the analyses of complex cellular pathways leading to altered physiological responses caused by dieldrin.

In another study, methoxychlor (MXR), an insecticide that has been shown to negatively affect fish reproduction by interacting directly or its metabolites with the binding sites of the fish ER and inducing VTG production in males (Martyniuk et al., 2011). It is readily metabolized in the liver by both CYP1 and CYP3 family enzymes and is degraded into OH-MXC and HTPE (Martyniuk et al., 2011). Martyniuk et al. (2011) aimed to characterize the hepatic response of male largemouth bass (Micropterus salmoides) to MXR using microarray analysis in order to

identify the underlying molecular responses (Martyniuk et al., 2011). Male bass were injected intraperitoneally with 1 mg/kg E2, a reference estrogen, or 25 mg/kg MXC and were sampled after 48 h. The results of this study supported the hypothesis that MXC can act through ER-mediated pathways and suggests that MXC may also act through other steroid receptor pathways in the liver of the bass including androgen- and glucocorticoid-receptor mediated pathways (Martyniuk et al., 2011). Three genes involved in steroid hormone synthesis that were differentially expressed after MXC exposure were StAR (decreased), 3β-hydroxysteroid dehydrogenase (3β-HSD) (decreased) and 17β-hydroxysteroid dehydrogenase isoform 8 (17β-HSD) (increased). Vaithinathan et al. (2008) showed that in adult male rats exposed to MXC there was a significant decline in the activities of testicular 3β-HSD and 17β-HSD at 6 h as well as reductions in the protein levels of StAR at 6 h and 12 h (Vaithinathan et al., 2008). In bass testis, StAR mRNA expression is inhibited in a time-dependent manner by both E2 and MXC (Guerra et al., 2012). Collectively these results suggest that there is a common gene response in the steroidogenic pathway in the gonad and liver of both fish and mammals (Martyniuk et al., 2011). Results also found a 4-fold increase in insulin-like growth factor 1 (IGF1) mRNA levels. Previous studies have shown that estrogens and anti-androgens decrease IGF1 mRNA expression in the liver whereas androgens increase IGF1 mRNA in the testis. IGF1 is a stimulator of cell growth and proliferation and an inhibitor of apoptosis. The induction of IGF1 in the liver of bass after MXC exposure may be due to its anti-estrogenic effects (Martyniuk et al., 2011). MXC induced both estrogen receptor 1 (ESR1) and estrogen receptor 2b (ESR2b) expression in the liver. The activation profile of MXC on ER has been reported to be similar to expression profiles from E2 as well as a number of other xenoestrogens (Martyniuk et al., 2011). Twenty-four hours after the MXC injection AR mRNA levels were lower than in control fish but greater than in E2-treated fish suggesting that MXC does not act solely as an estrogen or anti-androgen. After 48 h of exposure no known AR activators were induced and activation of ESR1 by MXC would not directly lead to an increase in AR therefore it is possible that AR transcription is directly increasing as a result of MXC acting as an androgen or as a result of a complex time based signaling cascade from the activation of the ER by MXC. This study demonstrated that MXC induces genomic responses in the liver similar to, and different from, the gene expression profiles of E2 (Guerra et al., 2012).

Microarray analyses can help to identify the individual profiles of contaminants within a mixture acting individually or synergistically (Monosson, 2005; Sexton and Hattis, 2007). Garcia-Reyero et al. (2009) used a genomic approach to compare individual steroid hormone profiles with those of a mixture of estrogens and an ER antagonist ZM-182780 (ZM) (Garcia-Reyero et al., 2009). The mixture profile showed that ZM has the ability to block the effects for some estrogenic activated genes, however it does not do so for all genes indicating two different expression patterns under individual contaminant and mixture conditions (Garcia-Reyero et al., 2009). This study was also able to uncover genes that were affected by the mixture but not by the individual contaminants (Garcia-Reyero et al., 2009). Pathway analyses isolated the prostaglandin-endoperoxide synthase 2 (PTGS2) pathway affected by the mixture EE2 and ZM (Garcia-Reyero et al., 2009). PTGS2 was induced almost 2-fold by EE2 alone but the mixture had no effect (Garcia-Reyero et al., 2009). PTGS2 is involved in the synthesis of prostaglandins from arachidonic acid and is influenced by E2 in mammalian tissue (Shah, 2005), PTGS2 induces signal transducer and activator of transcription 1 (STAT1) which is used in the Janus kinase (JAK) JAK-STAT signaling pathway and is important for immunity and apoptosis. As well, myeoblastosis oncogene (MYB) is a transcription factor downstream of PTGS2 however it is down-regulated and is known to be involved in estrogen signaling in some breast cancer cells (Drabsch, 2007). Overall, these findings suggest a cellular reaction for survival by increasing immunity and apoptosis and the down-regulation of cellular proliferation to prevent cancer development.

These studies highlight the usefulness of using microarray technology to identify mechanisms of toxicity, and in understanding the effects of endocrine disruptors. Combining cellular markers, such as gap junction, and gene expression profiling should provide powerful tools to understand the mechanisms of toxicity and predict pathological consequences associated with exposure to complex mixtures such as municipal wastewater effluent.

2.0 Objectives

Little is known about how xenobiotics affect molecular communication within the liver. The first objective of this study is to identify which Cxs are expressed in the liver of the brook trout

and the second objective is to determine whether Cx expression levels in the liver are altered after exposure to graded concentrations of MWWE.

In order to assess potential changes in liver communication, the second study aims to identify alterations to key regulatory pathways in the liver of fathead minnow exposed to 20 % MWWE using genomic technology.

In contribution to this article, Jennifer Arstikaitis carried out the laboratory work and analyses, as well as the writing.

Sampling was carried out by Benjamin de Montgolfier.

Daniel Cyr oversaw the study procedures and contributed to the writing of the article.

CHAPTER 2: ARTICLE

FRENCH ABSTRACT

Résumé

Les jonctions intercellulaires jouent un rôle essentiel dans la coordination de la fonction épithéliale. Des études de notre laboratoire ont montré que l'exposition de l'omble de fontaine aux effluents municipaux modifie l'expression et la localisation de protéines des jonctions lacunaires, les connexines (Cx), dans les testicules. Ces changements sont considérés comme étant responsables du retard de spermatogenèse observée chez les poissons vivant dans le fleuve Saint-Laurent en aval de Montréal. L'objectif de cette étude était de déterminer si la communication hépatique par les jonctions lacunaires était modifiée chez les poissons exposés aux effluents municipaux et de déterminer le mécanisme par lequel ces changements peuvent se produire. Les ombles de fontaine ont été exposés à 0 (témoin), 1, 10 et 20 % d'effluents municipaux pendant 12 semaines. Chez les poisons exposés à 10 et à 20 % d'effluents municipaux, une induction de l'ARNm de la vitellogénine a été observée, indiquant la présence de produits chimiques oestrogéniques dans les effluents. Les Cx hépatiques de truite ont été amplifiées par RT-PCR en utilisant deux paires d'amorces dégénérées qui amplifient toutes les sous-familles de vertébrés Cx. Deux produits spécifiques ont été amplifiés, clonés et séquencés. Ils ont été identifiés comme Cx43 et Cx30. La technique de la courbe standard en PCR en temps réel a été utilisée pour déterminer les changements d'expression des Cx43 et Cx30. Les résultats obtenus indiquent que les niveaux d'ARNm de Cx43 n'ont pas été modifiés par l'exposition aux effluents alors que les niveaux d'ARNm de Cx30 ont augmenté de manière dose-dépendante. Ces résultats indiquent que l'exposition aux eaux usées municipales peut entraîner des modifications dans l'expression des Cx hépatiques. Les changements d'expression des Cx entraînent probablement une modification des messages échangés puisque les Cx forment les pores intercellulaires entre les cellules adjacentes.

EXPOSURE TO MUNICIPAL EFFLUENT RESULTS IN INCREASED LEVEL OF THE GAP JUNCTION PROTEIN, CONNEXIN 30, IN THE BROOK TROUT (Salvelinus fontinalis).

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ABSTRACT

Intercellular gap junctions are critical for coordinating epithelial function. Studies from our laboratory indicate that exposure of brook trout to municipal wastewater effluent results in alteration in the expression and cellular localization of gap junctional proteins, connexins (Cx), in the testis. These changes may be responsible for delayed spermatogenesis observed in fish living in the St. Lawrence River downstream from Montreal. The objective of this study was to determine whether or not hepatic Cx were altered in fish exposed to wastewater effluent. Brook trout were exposed to 0 (Ctrl), 1, 10 and 20 % municipal wastewater effluent for 12 weeks. Trout exposed to 10 and 20 % municipal effluent had induced levels of vitellogenin mRNA, indicating the presence of estrogenic chemicals in the effluent. Trout hepatic Cx were amplified by RT-PCR using two pairs of degenerate primers which amplify all vertebrate Cx subfamilies. Two specific products were amplified, cloned and sequenced. These were identified as Cx43 and Cx30. Real-time PCR using the standard curve protocols were used to determine changes in both Cx43 and Cx30. Results indicated that Cx43 mRNA levels were unaltered by effluent exposure while Cx30 mRNA levels increased in a dose-dependent manner. These results indicate that exposure to municipal wastewater effluent can result in alterations in the expression of hepatic Cx. Changes in the expression of Cxs likely results in the modification of messages exchanged since Cx gate the intercellular pores between adjacent cells.

INTRODUCTION

Direct intercellular communication in the liver is mediated by gap junctions (GJ) that form channels between neighboring cells (Cruciani et al., 2006; Vinken et al., 2009; Vinken et al., 2011). These channels are made up of two connexons, one from each neighboring cell, while each connexon is made up of six connexin (Cx) proteins (Cruciani et al., 2006). There are over 20 different connexins that have been identified in mammals (De Boer et al., 2005). The composition of each connexon will determine the selectivity of the connexon and resulting intercellular pore (Bevans et al., 1998). Gap junction intercellular communication (GJIC) is the passive exchange of small hydrophilic molecules of less than 1 kDa and include: nucleotides, ions, secondary messengers and metabolites (Cruciani et al., 2006).

In the adult mammalian liver, nonparenchymal cells and epithelial cells predominantly express Cx43 (Berthoud et al., 1992; Greenwel et al., 1993; Saez, 1997), whereas hepatocytes predominately express Cx32 and small amounts of Cx26 (Cascio et al., 1995; Neveu et al., 1995). de Montgolfier et al. (2009) showed that in the brook trout, testicular Cx43 mRNA levels were regulated in a dose-dependent manner by 3,5,3′-triiodo-L-thyronine, suggesting that exposure to hormones could alter Cx43 levels (de Montgolfier et al., 2009). Connexins are known to interact with a wide range of partners, including β-catenin and zona occludens proteins (ZO-1 and ZO-2). They can also interact with enzymes (e.g. serine/threonine kinase and protein kinase C (PKC)) and other cellular proteins (e.g. caveolin) (Herve et al., 2004; Herve et al., 2007; Dbouk et al., 2009).

GJ play a critical role in the maintenance of liver homeostasis. Several liver-specific processes depend on GJIC, including albumin secretion (Yang et al., 2003), ammonia detoxification (Yang et al., 2003), glycogenolysis (Nelles et al., 1996; Stumpel et al., 1998), bile secretion (Nathanson et al., 1999; Temme et al., 2001; Bode et al., 2002) and cytochrome P450 mediated xenobiotic biotransformation (Neveu et al., 1994; Shoda et al., 1999; Shoda et al., 2000; Hamilton et al., 2001). Wilson et al. (2000) demonstrated, in a serum-deprived rat epithelial cell line, that GJIC is temporarily induced in the early phases of apoptosis and declines as cell death progresses. This study suggested that the transient induction of GJIC may play a role in the initial wave of cell death, followed by a decline in communication acting as protection for healthy neighbouring

cells (Wilson et al., 2000; Krutovskikh et al., 2002; Contreras et al., 2004). Other studies in Xenopus embryos have shown that Wnt1 and Wnt8 expression induces GJIC through the activation of the Wnt-mediated signal transduction pathway (van der Heyden et al., 1998). Wnt genes are highly expressed during development and interference of Wnt1 or Cx43 during murine brain formation results in mid/hindbrain defects (van der Heyden et al., 1998). Therefore, alteration of Cx expression levels or Wnt protein levels during development can have detrimental effects (van der Heyden et al., 1998).

The liver is targeted by a number of xenobiotics because of its location in the body and its role in the biotransformation of foreign molecules (Elaut et al., 2006; Papeleu et al., 2006). Previous studies have shown that exposure of brook trout to Montreal wastewater effluent (MWWE) for 12 weeks resulted in the alteration of the expression and cellular localization of Cx in the testis and increased vitellogenin (VTG) mRNA levels in the liver (de Montgolfier et al., 2008). Alterations in both Cx expression levels and their phosphorylation have been shown to cause delays in spermatogenesis and a reduction in sperm motility and sperm concentration in spottail shiners living in the St. Lawrence River downstream from the discharge site (Aravindakshan et al., 2004a,b). Shiners collected from this site were fed to pregnant rat dams; when the rat pups reached adulthood they displayed reduced sperm motility and sperm counts (Aravindakshan et al., 2004a). In addition, histological sections of the testis revealed abnormal distributions of the stages of spermatogenesis, and mislocalization of Cx43 indicating important alterations in the coordination of testicular function and development (Aravindakshan et al., 2004a).

Given the evidence of MWWE induced GJIC alteration in the testis of brook trout, we attempted first, to identify which Cxs are expressed in the liver of the brook trout, and second, determine whether exposure to MWWE alters Cx expression levels in the liver.

MATERIALS & METHODS

Brook trout

One year old male juvenile brook trout were purchased from a fish farm (Pisciculture D'Arthabaska, Chesterville, QC) and transported to the Montreal wastewater treatment plant (Montreal, QC). Male fish were used in order to alleviate any gender-specific effects. Trout

(n=300) were housed in 1700 L fibreglass tanks with a flow-through water system. Fish were acclimated for two weeks at a constant temperature of 15°C under natural photoperiod before starting the experiment, and were fed trout pellets (St-Clet, QC) at a ration of 1 % body weight per day.

Experimental protocol

Identification of hepatic connexins

Immature brook trout were anesthetized in 3-aminobenzoic-ethyl-ester-acid (MS222; 0.05g/L) and subsequently euthanized by decapitation. Livers were then dissected, rapidly frozen in liquid nitrogen and stored at -80°C. All animal procedures were approved by the university Animal Care Committee.

Exposure to MWWE

A flow-through exposure system was used to examine the effects of MWWE exposure on hepatic Cx in brook trout. Dechlorinated MWWE flowed at a rate of 50 L/h through 60 L fibreglass tanks with male brook trout (n=30). Two replicate tanks per treatment were carried out. Trout were acclimated for 2 weeks at a constant temperature of 15°C under natural photoperiod before starting the exposure experiment and were fed trout pellets (St-Clet, QC) at a ration of 1 % body weight per day.

Trout were exposed to four different concentrations of treated wastewater effluent ratios (v/v): 0 (control group), 1, 10 and 20 %. Effluent concentrations corresponded to concentrations found at 10, 1, and 0.3 km, respectively, downstream from the discharge site in the St. Lawrence River (de Montgolfier et al., 2008). After 12 weeks, fish were placed in a 10 L tank containing 3-aminobenzoic-ethyl-ester-acid (MS222; 0.05 g/L) as an anaesthetic. The fish were then euthanized, the livers rapidly dissected, frozen liquid nitrogen and stored at -80°C.

RNA extraction

Total RNA was extracted from the livers of untreated brook trout using the Illustra RNAspin Mini kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Extracted RNA was aliquotted and stored at -80°C.

RT-PCR protocol

Two pairs of degenerate primers (F1R1 & F2R2, Table 1) were designed according to two highly conserved regions of the mammalian Cx multigene family (Itahana et al., 1996). These regions include the amino terminal cytoplasmic domain and the two extracellular loops (Itahana et al., 1996). This method was previously used to identify epididymal Cxs in the rat (Dufresne et al., 2003) and testicular Cxs in the rainbow trout and in brook trout (de Montgolfier et al., 2007). Degenerate primers were used because complete genome information is not available for the brook trout. These primers were designed based on known sequences of the Cx family but are not specific to the brook trout.

An aliquot of 2 µl (500 ng) total RNA from immature brook trout was subjected to reverse transcription (RT). RT was performed in a 20 µl reaction volume with a final concentration of 1.5 mM 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 15 mM $MgCl_2$), supplemented with 3.5 mM $MgCl_2$, 1 mM dNTP, 20 U RNase inhibitor, 20 U M-MLV reverse transcriptase, 0.75 mM of the 3'-Oligo dT primer forward primer, and 0.75 mM of the 3'- Oligo dT reverse primer. The 2 µl (500 ng) total RNA was heated at 65°C for 10 min, then cooled on ice for 5 min and subsequently added to the RT mixture and heated for 60 min at 42°C.

RT-PCR was carried out with a 50 µl reaction volume and a final concentration of 1.5 mM 10X PCR buffer, 0.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 U Taq DNA polymerase, 0.5 µM forward primer and 0.5 µM reverse primer. Amplifications were carried out for 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 60 sec and elongation at 72°C for 90 sec. A final extension at 72°C for 15 min was done to create 3' A-overhangs. The F1R1 and F2R2 PCR products were then separated on 2 % agarose gel and visualized by ethidium bromide staining.

Cloning protocol

The amplified RT-PCR products were then extracted from the gel (QIAEX II Gel Extraction Kit; Qiagen, Missiauga, ON, Canada) and cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). The cloning reaction was then transfected into E. Coli competent cells (Invitrogen, Carlsbad, CA, USA). Plasmids were amplified and purified from individual colonies using the Qiagen Plasmid Purification Kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions. The insert was digested from the plasmid using EcoR1 restriction enzyme (Amersham Biosciences, Baie d'Urge, Quebec) and sequenced by a commercial automated sequencer (Genome Quebec, QC).

To confirm the identity of the products, the resulting sequences were compared to other known Cxs using BLAST homology comparisons (GenBank, National Center for Biotechnology Information, Bethesda, MD). Real-time PCR (qPCR) specific primers were designed using these sequences and Oligo Primer Analysis Software (Molecular Biology Insights, Cascade, CO, USA).

Real-time PCR protocol

Template for the RT reaction consisted of a 2 µl (500 ng) aliquot of total RNA from immature brook trout. RT was performed in a 20 µl reaction volume with a final concentration of 1.5 mM 10X PCR buffer, 3.5 mM $MgCl_2$, 1 mM dNTP, 20 U RNase inhibitor, 20 U M-MLV reverse transcriptase, 0.75 mM of the 3'-Oligo dT primer forward primer, and 0.75 mM of the 3'- Oligo dT reverse primer. The 2 µl (500 ng) total RNA was heated at 65°C for 10 min, then cooled on ice for 5 min and subsequently added to the RT mixture and heated for 60 min at 42°C. The resulting cDNA templates were amplified using qPCR specific primers for Cx30 and Cx43 (Oligo Primer Analysis software, Molecular Biology Insights, Cascade, CO, USA).

qPCR was carried out with a 50 µl reaction volume with a final concentration of 1.5 mM 10X PCR buffer, 0.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 U Taq DNA polymerase, 0.5 µM forward primer and 0.5 µM reverse primer. A Rotor-Gene RG3000 was used to carry out qPCR with the following protocols: Cx43 was amplified for 40 cycles of denaturation at 95°C for 2 min, annealing at 95°C for 15 sec, annealing at 59.7°C for 30 sec and 72°C for 30 sec; Cx30

amplification was carried out for 40 cycles of denaturation at 95°C for 15 sec, annealing at 53°C for 30 sec and elongation at 72°C for 30 sec.

In order to standardize mRNA levels for Cx43 and Cx30 a standard curve was created using relative actin expression levels. Actin amplification by qPCR was carried out for 35 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 30 sec and elongation at 72°C for 30 sec. Relative actin levels were measured in the liver of both control and treated fish with no significant difference. Cx expression levels were then normalized using actin expression levels for each sample. Technical replicates were carried out in triplicate to control for technical variability and biological replicates were used to calibrate for biological diversity. Melting curve analysis was carried out to differentiate amplicons and identify any false positives.

Statistical analyses

Statistical differences in mRNA levels were determined by one-way ANOVA. A comparison between groups was made using the Holm-Sidak method. Statistical analyses were performed using SigmaStat software (SPSS Inc., Chicago, IL, USA), and results were considered significant when $p<0.05$. Data are expressed as the mean ± SEM.

RESULTS

Identification of hepatic Cx

Amplification of hepatic mRNA by RT-PCR with F1R1 primers resulted in two amplicons, 605 bp and 425 bp (Fig. 1a). The resulting amplified cDNA was sequenced and sequence homology determined by BLAST (GenBank, National Center for Biotechnology Information, Bethesda, MD). It was confirmed that both amplicons were Cx43. Although two amplicons were observed for Cx43, the amplicon at 425 bp was a segment of the larger amplicon found at 605 bp. Amplification with the F2R2 primers resulted in one amplicon of 450 bp (Fig. 1b). Sequencing and blasting results indicated that this cDNA amplicon was Cx30.

Using multiple sequence alignment tools Cx43 and Cx30 were aligned with published sequences of other mammals. Cx43 in the liver of the brook trout showed 100 % homology to zebrafish

Cx43, 75 % to rainbow trout (Onchorhynchus mykiss) Cx43 , and 30 % with mouse and rat gap junction alpha 1 (GJA1). Sequence alignment of Cx30 from the liver of the brook trout showed 38 % homology to gap junction beta 6 (GJB6) of the mouse and 33 % GJB6 of the rat.

qPCR protocol

In order to quantify Cx43 and Cx30 mRNA in the liver of brook trout, a qPCR protocol was developed. qPCR cycling protocols were developed and optimized for Cx30 and Cx43 mRNA expression in the liver of brook trout. Melt curve analysis was carried out for Cx30 (Fig. 2a) and indicated one qPCR product after 40 cycles of amplification. Despite the presence of two Cx43 amplicons present after visualization in 2 % agarose gel, the melt curve analysis after 40 cycles of amplification indicated only one major product (Fig. 2b).

Effect of Montreal municipal effluents on hepatic connexin levels

After 12 weeks of exposure to 0 (ctrl), 1 %, 10 % and 20 % MWWE brook trout Cx30 and Cx43 mRNA levels were shown to be altered when compared with controls. Control and 1 % Cx43 mRNA levels showed no significant difference. Cx43 mRNA levels from the livers of fish exposed to 10 and 20 % were slightly lower than the levels in controls, however this was not statistically significant (Fig. 3a). Cx30 hepatic mRNA levels were lower in the 1 % exposure group when compared to controls, and were significantly higher than controls in fish exposed to 10 and 20 % MWWE (Fig. 3b).

A previous study from our lab showed that a short-term (4 weeks) exposure at 0, 1, 10 and 20 % MWWE concentrations did not result in an increase in hepatic VTG mRNA levels. These results were compared to a second experiment from the same study, in which fish were exposed for 12 weeks; fish demonstrated induced VTG mRNA levels at the two highest concentrations (Fig. 3c) (de Montgolfier et al., 2008).

DISCUSSION

Characterization of MWWE has shown that the effluent contains a wide range of xenobiotics including pharmaceuticals and personal care products, alkylphenols, polychlorinated biphenyls,

polyaromatic hydrocarbons, and heavy metals (Pham et al., 1997; Quemerais et al., 1998; Gagne et al., 2001; Sabik et al., 2003; Aravindakshan et al., 2004a,b; Aravindakshan et al., 2005; Matozzo et al., 2008; Martin-Diaz et al., 2009; Bhavsar et al., 2011; Lajeunesse et al., 2011). Exposure to individual contaminants has been shown to alter Cx expression levels and subsequently GJIC. Modified Cx expression levels have been associated with cellular stress (Segner et al., 2003). When cells are damaged intercellular communication shuts down to prevent the spread of disease to nearby healthy cells (Segner et al., 2003).

In the present study, we showed two Cxs expressed in the liver of the brook trout: Cx43 and Cx30. A previous study has reported Cx43 expression in the liver of the zebrafish (Cheng et al., 2003) and Cx43 expression in the liver of mammals has been well established (Echevarria and Nathanson, 2004; Cruciani et al., 2006; Vinken et al., 2008). The region of Cx43 amplified in the brook trout liver was found to be 100 % homologous to Cx43 in the zebrafish and 75 % homologous to rainbow trout Cx43. This is interesting, since rainbow trout and brook trout belong to the same phylogenetic family, Salmonidae, and zebrafish belong to the Cyprinidae family (Cruciani et al., 2006). Through species divergence, fish have become the most diverse group of vertebrates, with more species than in any other class (Miller et al., 2008). It is possible that through species divergence and mutations during gene duplication, rainbow trout and brook trout diverged. When brook trout Cx43 was compared with mammalian GJA1 sequences it should a 30 % homology for the mouse and rat but <1 % homology for human GJA1. Of the nine Cx domains, only the four transmembrane domains, two extracellular loops and the N-terminus are highly conserved among fish and mammals, meaning that most differences occur in the C-terminal and intracellular loop (Hertzberg et al., 1988; Falk et al., 1994; Dbouk et al., 2009; Li et al., 2010). The C-terminal tail contains sites for the phosphorylation of Cx proteins, which have been shown in mammals to be somewhat conserved; however, these sites have not been well-characterized in fish and differences may be responsible for the low level of homology.

The resulting expression of Cx30 in the liver of brook trout is unique, since Cx30 has not been previously detected in the liver of humans and rodents. This is the first time Cx30 has been identified in the liver of any fish species. When the amplified region of the brook trout Cx30

sequence was compared against all mammalian Cx sequences, it showed a 30.3 % homology to GJB6 in the mouse and 31.8 % homology to Cx26 in the human. Human Cx30 is primarily expressed in the ear and when compared to brook trout Cx30 no similarity was found. This finding suggests that human Cx26 is more similar to brook trout Cx30.

Interestingly, GJB6 in the mouse is not expressed in the liver but is localized in the ear. In the liver of rodents, Cx26, Cx32 and Cx43 have been shown to be expressed (Vinken et al., 2008). Human Cx26 is expressed in the skin, ear and hepatocytes (Plante et al., 2002; Echevarria and Nathanson, 2004). In mammalian hepatocytes Cx32 is 10-times more abundant than Cx26, suggesting it plays a larger role when compared to Cx26 (Plante et al., 2002; Echevarria and Nathanson, 2004). The African clawed frog (Xenopus laevis) expresses Cx29 in the developing liver and is 65.5 % and 63.6 % homologous with mouse Cx26 (liver) and Cx30 (brain and skin), respectively (de Boer et al., 2006). The differences in homology and nomenclature are most likely attributed to the evolutionary distance between fish and mammals; however more work is needed to elucidate the role and localization of Cx43 and Cx30 in the liver of fish.

The results of this study found an increase in Cx30 in the liver of brook trout exposed to the two highest concentrations of effluent after 12 weeks. In rainbow trout, Cx30 has been localized between the interstitial Leydig cells within the testis (de Montgolfier et al., 2007). Cx30 expression levels were shown to be present in all stages of spermatogenesis, and communication between GJ allowed the elucidation of the complex processes of spermatogenesis to be carried out (de Montgolfier et al., 2007). Previous studies have shown that exposure to MWWE which contains a wide range of xenoestrogens, can alter Cx expression in the testis of fish, resulting in delayed spermatogenesis, decreased sperm motility and concentration (Aravindakshan et al., 2004a,b; de Montgolfier et al., 2007). To our knowledge there have been no publications regarding the expression of Cx in the liver of brook trout and no reports on the effects of effluent exposure in the liver of fish.

Murine Sertoli cells (TM4 cell line) were exposed to NP for 24 h and resulted in a reduction of GJIC in a time- and dose-dependent manner (Aravindakshan et al., 2005). At the highest dose of NP (11 mg/L) GJIC was almost completely inhibited (Aravindakshan et al., 2005). NP was

shown to inhibit the phosphorylation of Cx43, indicating its actions at the level of the Cx (Aravindakshan et al., 2005). This study showed that NP can exert its effects on either Cx turnover or their synthesis, or both (Aravindakshan et al., 2005). The results of the present study showed a decrease in Cx43 levels at the two highest MWWE concentrations in the liver of brook trout after 12 weeks of exposure to MWWE. This decrease in Cx43 expression could result in altered GJIC in the liver.

Similar alterations in Cx levels were observed in the testis of brook trout exposed to MWWE for 12 weeks. This study showed a decrease in Cx43 mRNA levels at the two highest concentrations and a small increase in Cx30 mRNA levels. These altered levels of Cx expression resulted in modifications to GJIC in the testis (de Montgolfier et al., 2008). This study also looked at the effects of MWWE exposure in the liver of brook trout by measuring VTG mRNA levels. The results indicated that in order to elicit an induction of VTG mRNA in the liver, a long-term exposure is required, suggesting that xenoestrogens must accumulate before they are present at detectable levels (de Montgolfier et al., 2008). In this study, we showed elevated levels of Cx30 mRNA in hepatocytes of brook trout after exposure to MWWE suggesting that long-term exposure can modulate hepatic-GJIC (Kojima et al., 1994).

VTG is an egg yolk-precursor protein normally expressed in the liver of female fish during ovarian follicular development (Aravindakshan et al., 2004). Male fish express the VTG gene but do not normally exhibit increased VTG levels unless exposed to estrogenic compounds (Aravindakshan et al., 2004). de Montgolfier et al. (2008) exposed brook trout for 4 and 12 weeks and measured the resulting VTG mRNA levels in the liver (Fig. 3). The results showed that male fish exposed to MWWE containing xenoestrogenic compounds exhibited induced VTG levels after only 12 weeks (de Montgolfier et al., 2008). These results indicate that certain hepatic effects may only be apparent after long-term exposures are carried out, and that accumulation of contaminants may be required before these effects can be detected.

In conclusion, results from this study showed, for the first time, the presence and identification of Cx43 and Cx30 in the brook trout liver. The results from this study also showed that brook trout exposed for 12 weeks to graded concentrations of MWWE exhibited altered Cx expression

levels. Similar studies have shown that altered Cx expression levels can result in the interruption of GJIC, which is critical for maintaining homeostasis in a number of organs.

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Table 1: DNA sequences of RT-PCR primers used to identify different Cx present in the brook trout liver.

Primer Name	Primer sequence (5'-3')
CxF1	ATGGGTGACTGGAGYKYCYTRG
CxR1	ACCACCARCATRAAGAYRATGAAG
CxF2	GGCTGYRASAAYGTCTGCTAYG
CxR2	GCCKGGARAYRAARCAGTCCAC

Table 2: Sequence of oligonucleotide primers used to amplify Cx30, Cx43 and actin in the brook trout liver for this study.

Primer name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Cx30	CGTGACTTACAGGAAGCGTG	TGTGATGGGCAGACGACT
Cx43	GCCCTTCCTCTTTGCGGTTAAT	CGCCGACTCTTCTCTACCTTGC
Actin	GGACCTGTACGCCAACAC	GCTTGCTGATCCACATCTG

Table 3: Similarity of brook trout Cx sequences with other vertebrate species.

Trout Cx	Homology (%)	Cx and species similarity
Cx43	100	Cx43 Zebrafish
	75	Cx43 Rainbow trout
	60	GJA1 Mouse
	58	GJA1 Rat
Cx30	38	GJB6 Mouse
	33	GJB6 Rat

FIGURE LEGENDS

Figure 1: RT-PCR amplification of mRNA from brook trout liver. Panel A shows two isoforms both identified as Cx43 amplified from F1R1 primers. The first amplicon was 605 bp and the second was 425 bp. Panel B shows a single product amplified using F2R2 primers. The amplified product was identified by sequencing and blasting to be Cx30 and 450 bp.

Figure 2: Panel A shows the RT-PCR melt curve for Cx30 indicating one product after 40 cycles of amplification. The RT-PCR melt curve for Cx43 indicating one product after 40 cycles of amplification is shown in panel B. The individual lines in each panel are representative of samples tested (n=27). Five points from an appropriate standard curve were used to ensure accuracy.

Figure 3: Panel A shows the resulting RT-PCR amplification of Cx43 mRNAs from brook trout liver using gene specific primers. Fish were exposed to 0, 1, 10 and 20 % MWWE. There was no statistically significant difference between the groups. The data was expressed relative to actin mRNA levels. ($p<0.05$; n=8 per effluent group; ±SEM). Panel B shows the RT-PCR amplification of Cx30 mRNA using gene specific primers. Different letters indicate a significant difference. The data was expressed relative to actin mRNA levels. ($p<0.05$; n=8 per effluent group; ±SEM). Panel C shows previously published hepatic VTG mRNA levels in male brook trout exposed to different concentrations of municipal effluent for 12 weeks. VTG mRNA levels were measured by semi-quantitative RT-PCR and GAPDH mRNA levels were used for standardization. Different letters indicate a significant difference ($p<0.05$ ANOVA; 0% n=6, 1% n=8, 10% n=9 and 20% n=10).

Figure 1

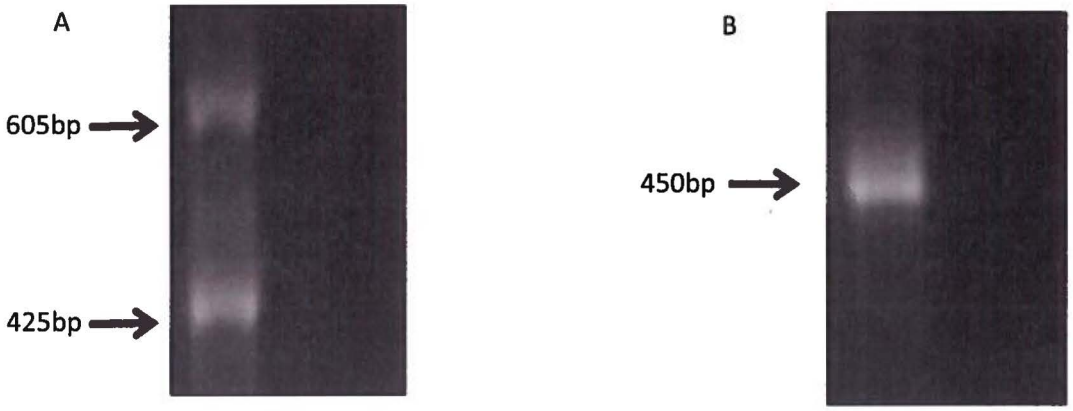


Figure 2

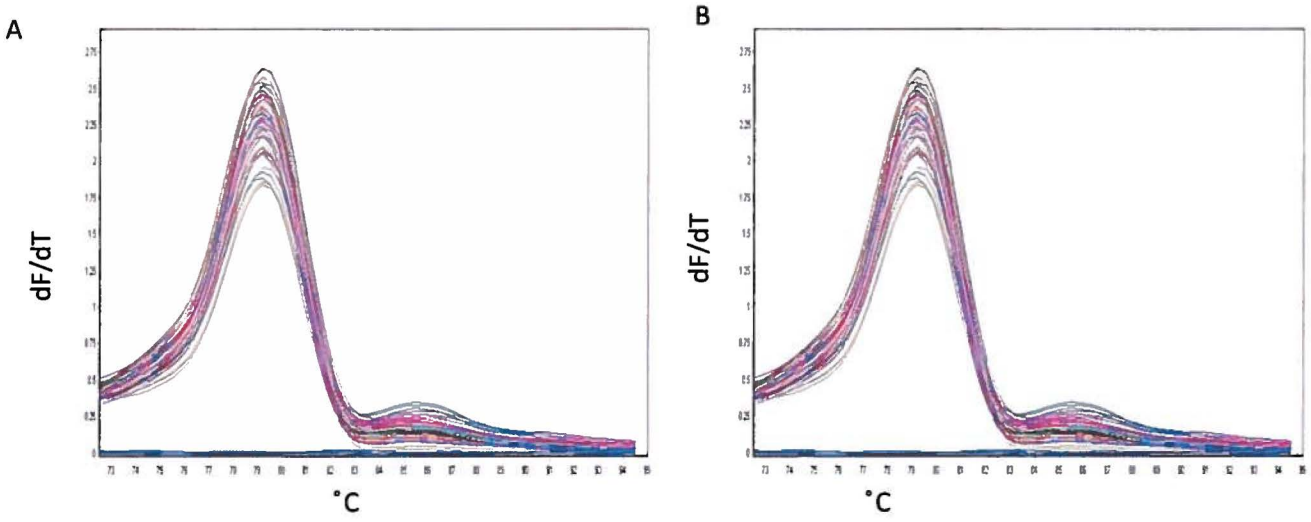
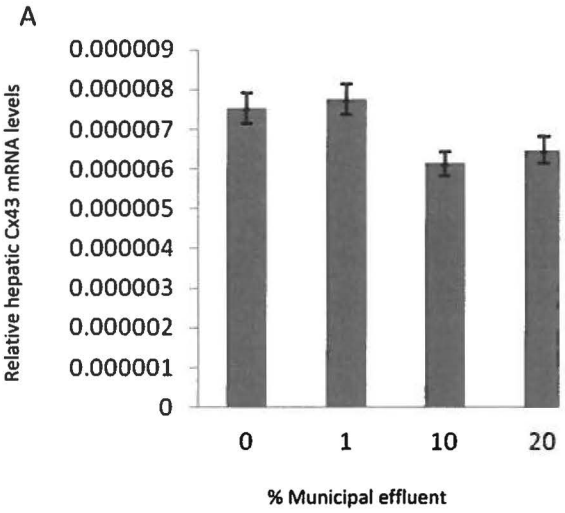
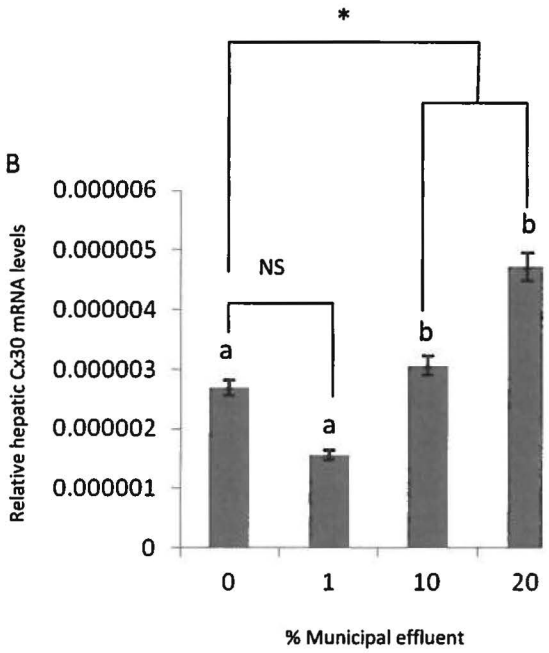


Figure 3


A
Relative hepatic Cx43 mRNA levels
0.000009
0.000008
0.000007
0.000006
0.000005
0.000004
0.000003
0.000002
0.000001
0
0
1
10
20
% Municipal effluent


B
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NS
a
a
b
b
Relative hepatic Cx30 mRNA levels
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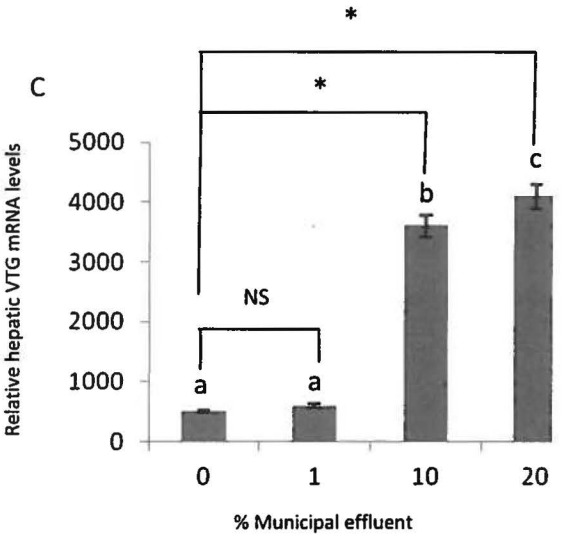
0.000005
0.000004
0.000003
0.000002
0.000001
0
0
1
10
20
% Municipal effluent


C
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NS
a
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Relative hepatic VTG mRNA levels
5000
4000
3000
2000
1000
0
0
1
10
20
% Municipal effluent

In contribution to this article, Jennifer Arstikaitis carried out the sampling, laboratory work and analyses, as well as the writing.

François Gagné provided the exposure setting as well as the help to carry out the sampling.

Daniel Cyr oversaw the study procedures and contributed to the writing of the article.

CHAPTER 3: ARTICLE

FRENCH ABSTRACT

Résumé

Les effluents municipaux peuvent affecter négativement l'environnement d'accueil. Dans le fleuve Saint-Laurent, les poissons mâles vivant en aval de Montréal repésentent une augmentation de vitellogénine hépatique, d'intersexualité, de retard de spermatogenèse et une diminution des fonctions des spermatozoïdes. Certains de ces effets peuvent être transmis à des humains par ingestion de poissons contaminés. Peu d'études ont examiné sur l'ensemble du génome les conséquences associés à une exposition des poissons aux effluents municipaux afin de déchiffrer les mécanismes par lesquels les processus physiologiques sont altérés. L'objectif de cette étude était d'identifier les voies de signalisation cellulaires hépatiques affectées chez le ménés à grosse tête après l'exposition aux eaux usées municipales. Des méné à grosse tête immatures ont été exposés pendant 21 jours à 0 % (contrôle) ou à 20 % d'effluents municipaux, la plus forte concentration présente dans le fleuve Saint-Laurent. Les ARN hépatiques ont été extraits et utilisés pour hybrider une puce à ADN 15K méné à grosse tête (Ecoarray). Un total de 309 gènes étaient exprimés différentiellement après une exposition aux effluents municipaux. Ces gènes comprennent 118 régulés à la hausse et 191 régulés à la baisse. Les gènes altérés classés selon leur function indiquent des effets sur diverses voies de signalisation incluant l'apoptose, les réponses immunitaires et le métabolisme cellulaire. Divers éléments de la voie canonique Wnt, incluant KREMEN1, Frizzled, DACT1, et DVL2, étaient considérablement régulés à la baisse. Plusieurs gènes de la voie non-canonique Wnt, tels que Wnt4, LRP6, et PPP2R5E (inhibiteurs de la voie Wnt étaient régulés à la hausse. Il s'agit de la première etude démontrant une modification de la voie Wnt suite à une exposition aux effluents municipaux. Les altérations de la voie Wnt canonique peuvent provoquer des malformations du développement, le cancer et sont impliquées dans diverses autres pathologies. Le fait que ces effets puissent être transmis ou non aux humains ou d'autres animaux qui mangent du poisson du Saint-Laurent demeure inconnu.

SHORT-TERM EXPOSURE OF FATHEAD MINNOWS TO MUNICIPAL WASTEWATER EFFLUENT INHIBITS THE CANONICAL WNT SIGNALING PATHWAY IN THE LIVER

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ABSTRACT

Municipal wastewater effluent can negatively impact its receiving environment. In the St. Lawrence River, male fish living downstream from Montreal exhibit increased hepatic vitellogenin, intersex, delayed spermatogenesis and decreased sperm functions. Few studies have examined the consequences of genome-wide effects associated with municipal effluent exposure in fish to decipher the mechanisms by which these alter physiological processes. The objective of this study was to identify hepatic cellular signaling pathways altered in fathead minnows following exposure to municipal wastewater effluent. Immature minnows were exposed for 21 days to either 0 % (Control) or 20 % municipal effluent, the highest concentration in the St. Lawrence River. Hepatic RNA was extracted and used to hybridize a fathead minnow oligonucleotide microarray containing approximately 15K gene sequences. A total of 309 genes were differentially expressed following exposure to wastewater effluent. Of those, 118 were up-regulated and 191 down-regulated. Altered genes grouped according to function indicated effects on various signaling pathways, apoptosis, immune responses, and cellular metabolism. Various components of the canonical Wnt pathway, including KREMEN1, Frizzled, DACT1, and dishevelled dsh homolog 2 (DVL2), were dramatically down-regulated while several other genes involved in the non-canonical Wnt pathway, such as Wnt4, LRP6, and PPP2R5E and which are know to inhibit the canonical Wnt pathway were increased. This is the first report of altered Wnt signaling following exposure to wastewater effluent. Alterations in the Wnt canonical pathway can cause developmental malformations, cancer, and other pathologies.

INTRODUCTION

There is well-established evidence of the deleterious impact of municipal wastewater effluent MWWE on fish living in the receiving environment (Cleuvers, 2003; Gregory et al., 2008; Vidal-Dorsch et al., 2012; Cyr et al., 2013). Sewage is collected from all municipalities on the island of Montreal, representing approximately 1.8 million people. Previous studies have shown that spottail shiners (Notropis hudsonius) caught in the St. Lawrence River downstream from the Montreal wastewater effluent (MWWE) discharge point had induced hepatic vitellogenin (VTG) mRNA levels, delayed spermatogenesis, produced fewer sperm that were less motile, and had a higher incidence of intersex (Aravindakshan et al., 2004). These fish also displayed suppressed immune function (Menard et al., 2010) and altered parasitic abundance (Marcogliese et al., 2006). Likewise, short-term exposure of shiners to this municipal effluent under laboratory conditions altered immune function and decreased thyroid function (Menard et al., 2008).

Long-term laboratory-based exposure studies, in which brook trout (Salvelinus fontinalis) were exposed to 1, 10 and 20 % MWWE concentrations for 4 and 12 weeks, indicated that hepatic VTG mRNA levels were unaltered after 4 weeks of treatment. However, VTG mRNA levels increased significantly at the two highest exposure concentrations at 12 weeks suggesting that estrogenic compounds in the wastewater effluent likely accumulated over time (deMontgolfier et al., 2008). The expression of gap junction proteins, connexins, in the testes of these fish was also altered at both 4 and 12 weeks which may, at least in part, explain the delayed spermatogenesis observed in wild-caught shiners in the St. Lawrence River downstream from Montreal (de Montgolfier et al., 2008). These data suggest that intracellular pathways may be targeted by exposure to these effluents, leading to the alterations in gap junctions, and likely other cellular targets.

Several studies have reported that components of the Wnt/β-catenin signaling pathway may be altered following exposure to environmental contaminants. Wnt signaling is implicated in liver development, cell proliferation, apoptosis and tissue homeostasis (Logan and Nusse, 2004; Lu et al., 2011). Ung et al. (2010) reported that in zebrafish (Danio rerio), $HgCl_2$ exposure induced glycogen synthase kinase 3 (GSK3) mRNA and decreased levels of cadherin-1 (also known as E-

cadherin) immunostaining. In the canonical Wnt signaling pathway, GSK3β phosphorylates beta-catenin (CTNNB1) resulting in its degradation via the ubiquination pathway (Faux et al., 2010; Su et al., 2008). When GSK3β is inactive, CTNNB1 is stabilized and in concert with TCF/LEF can act as a nuclear transcription factor regulating the expression of a variety of genes, including cadherin-1 whose cytoplasmic tail also binds to CTNNB1 at the level of the intercellular adherens junction (Cadigan and Nusse, 1997; Harris and Peifer, 2005).

In another study, Mirbahai et al. (2011) collected dab (Limanda limanda) from five sampling sites along the Irish Sea and Bristol Channel and compared healthy livers with livers with lesions and/or hepatocellular carcinoma. Liver of fish sampled from two of the sampling sites that have elevated levels of polychlorinated biphenyl, polybrominated diphenyl ethers, and heavy metals such as cadmium and lead (Mirbahai et al., 2011) displayed altered DNA methylation of genes implicated in the Wnt/β-catenin signaling pathway (Mirbahai et al., 2011). These included dishevelled, a mediator of Wnt signaling, frizzled, the Wnt receptors, the tumor suppressor gene APC (adenomatous polyposis coli) and c-myc, a TCF/LEF regulated gene. In each case there was an increase in the methylation pattern of the promoters of these genes in fish collected from the contaminated sites (Mirbahai et al., 2011). To date there have been no reports of alterations in Wnt/β-catenin signaling in fish exposed to MWWE. However, differences in the composition of wastewater effluents due to differences in the industrial base of municipalities or treatment of effluents may alter specific signaling pathways that are activated or inhibited by exposure to MWWE.

The objectives of this study were to examine the effects of MWWE on gene expression in the liver of fathead minnow (Pimephales promelas) in order to identify key regulatory pathways implicated in the toxicity of the effluent.

MATERIALS & METHODS

Experimental protocol

Immature fathead minnows were raised at the Quebec Aquarium (Québec, QC) and transferred to the Montreal wastewater treatment plant (Montreal, QC). Fish were maintained in 15 L glass

aquariums with flowing water at a constant temperature (25°C ± 2°C) under 16 hours of light and 8 hours of dark. Fish were fed 4 mL of brine shrimp (Ocean Nutrition, Dartmouth, NS) twice per day. Minnows (n=6 per group) were exposed to either 0 (control) or 20 % v/v wastewater effluent in a flow-through system. Each treatment condition was carried out in duplicate. Effluent concentrations corresponded to concentrations previously determined to be equivalent to those measured 0.3 km downstream from the discharge pipe in the St. Lawrence River (de Montgolfier et al., 2008). After 21 days, juvenile minnows were anesthetized in 3-aminobenzoic-ethyl-ester-acid (MS222; 0.05g/L). Fish were euthanized and liver tissue was removed, frozen and stored at -80°C. All animal procedures were approved by Environment Canada's Animal Care Committee according to the guidelines of the Canadian Council on Animal Care.

RNA Extraction

Frozen liver samples were ground under liquid nitrogen using a mortar and pestle and total RNA was extracted using the Illustra RNAspin Mini kit (GE Healthcare, Baie D'Urfe, QC) according to the manufacturer's protocol. The quality of the RNA was verified using an RNA 6000 Nano kit (Agilent) and Bioanalyzer model 2100 (Agilent Technologies, Palo Alto, CA, USA). The isolated RNA was treated with DNase (1U/μg of RNA, deoxyribonuclease I, amplification grade; GE Healthcare) to remove any contaminating genomic DNA. The RNA was then reverse transcribed using oligo d(T) 12-18 (R&D Systems, Minneapolis, MS, USA) and M-MLV reverse transcriptase (Sigma Aldrich, Mississauga, ON), according to the suppliers instructions.

Microarray processing

Gene expression profiling was carried out using a fathead minnow oligonucleotide array containing 15208 oligonucleotides designed by EcoArray (Fathead Minnow Microarray 016349, Alachua, FL, USA) and purchased from Agilent (Agilent Technologies, Palo Alto, CA, USA). An aliquot of 500 ng of total RNA was prepared for amplification and labeling with Cy3 using the Low RNA Input Linear Amplification kit and Quick Amp Labeling Kit, according to the manufacturer's instructions (Agilent Technologies). A 40 μl-aliquot of labeled cRNA was hybridized to each microarray according to the manufacturer's instructions using the In Situ Hybridization Kit Plus (Agilent Technologies). Following hybridization, the microarray slides

were scanned using a ScanArray Express laser scanner (Perkin Elmer, Woodbridge, ON) and imported into Gene Pix Pro 7 (Molecular Devices Inc., Sunnyvale, CA, USA). Two slides, each containing 8 one-color microarrays, were used to hybridize RNA from control (n=4) and MWWE-exposed (n=4) fish.

Microarray analysis

Fluorescent images were processed using the GenePix Pro 7 software (Molecular Devices Inc.). Data were imported into GeneSpring GX11 software (Agilent Technologies) for further analysis. The intensity of each spot was characterized by the median pixel intensity and between-array scale normalization on median intensities was carried out using GeneSpring GX11 (Zahurak et al., 2007). Genes with a greater than 2-fold change in at least seven of the eight samples were included for further analysis. Analyses were done according to MIAME standards. Network analysis and functional analysis (biological functions and biological/ toxicological processes) of the differentially expressed genes with a greater than 4-fold change was carried out using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA). For these analyses, mammalian signaling pathways were considered to be similar to those in fish. Statistical analyses was carried out using a one-way ANOVA (significance level set at $p<0.05$) using SigmaStat (SPSS Inc., Chicago, IL).

Real-time PCR (qPCR)

Total RNA (500 ng) was reverse transcribed using oligo d(T) 12-18 (R&D Systems, Minneapolis, MN, USA) and M-MLV reverse transcriptase (Sigma Aldrich), according to the suppliers' instructions. Three genes were selected for validation: BCL2 like 13 (apoptosis facilitator) (BCL2L13), VTG and Forkhead Box B1 (FOXB1). Forward and reverse real-time PCR (qPCR) primers were designed for each gene using the Oligo Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA) (Table 1) based on homologous sequences found in GenBank. Total RNA was extracted from the liver of immature fathead minnow using Illustra RNAspin Mini kit (GE Healthcare) and qPCR carried out using a Rotor-Gene RG3000 (Corbett Life Science, San Francisco, CA, USA) . A 2 µl-aliquot of the RT reaction was amplified in a 15 µl solution containing 1X Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Burlington, ON, Canada), and 0.3 µM of both reverse and forward primers. The

PCR cycling protocols were optimized to maximize the reaction efficiency and ensure that only the target product was contributing to the SYBR Green fluorescence signal. For each quantification, a standard curve was created using suitably appropriate cDNA. qPCR amplification of BCL2L13 was done using 45 cycles of denaturation at 95°C for 15 sec, annealing at 53°C for 30 sec and elongation of 72°C for 30 sec; FOXB1 was amplified using 45 cycles of denaturation at 95°C for 15 sec, annealing of 56°C for 30 sec and elongation at 72°C for 30 sec; VTG was amplified using 40 cycles of denaturation at 95°C for 15 sec, annealing at 51°C for 30 sec and elongation at 72°C for 30 sec. Gene expression levels were normalized using Actin mRNA levels for each sample. Each sample was measured in triplicate. Melting curve analysis was carried out to ensure the accuracy of quantification.

Western Blot analyses

Nuclear and cytosolic proteins were extracted from liver of control and MWWE-exposed (20 % w/w) fish using the Nuclear Extract kit (Active Motif, Carlsbad, CA, USA). Frozen tissue was homogenized with 3 ml of 1X Hypotonic Buffer (containing 3 µl of 1M DTT and 3 µl of detergent/g of tissue) and incubated on ice for 15 min. Samples were centrifuged for 10 min at 850 x g at 4°C and the supernatant transferred to a chilled microcentrifuge tube. The pellet was resuspended in 500 µl of 1X Hypotonic Buffer and incubated on ice for 15 min. After incubation, 25 µl of detergent was added to each sample and the tubes were vortexed for 10 sec. Samples were subsequently centrifuged for 45 sec at 14 000 x g in a pre-cooled microcentrifuge (4°C). The supernatant was used as the cytosolic fraction. The nuclear pellet was resuspended in 50 µl of complete lysis buffer (10mM DTT, lysis buffer AM1 and protease inhibitor cocktail) and vortexed for 10 sec. The suspension was then incubated for 30 min on a rocking platform at 4°C. Samples were then vortexed for 10 sec and centrifuged at 4°C for 10 min at 14 000 x g. The supernatant, containing the nuclear fraction, was then transferred to pre-cooled microcentrifuge tubes. Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON). The purity of the nuclear fraction was determined using alpha-tubulin (α-tubulin) as a marker for the cytoplasmic fraction and histone protein 3 (H3) as a marker for the nuclear fraction. Overexposed films showed <7 % cross-contamination between cytoplasmic and nuclear extracts. Aliquots of control and treated samples containing 25 µg of nuclear proteins and 12.5 µg of cytosolic protein were loaded onto a 4-20 % gradient gel

(Bio-Rad Laboratories) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were then transferred onto a nitrocellulose membrane at 100 V for 30 min and then at 80 V for 1 hour at 4°C. The resulting membranes were stained with Ponceau red S to evaluate the transfer efficiency.

Membranes containing transferred proteins were blocked with phosphate-buffered saline (PBS) containing 5 % powdered milk and 0.05 % Tween and then hybridized overnight at 4°C with the appropriate primary antibody (rabbit anti-H3, 6ng/ml, Cell Signaling Technology, Danvers, MA, USA; rabbit anti-β-catenin, 48 ng/ml, Cell Signaling Technology; rabbit anti-alpha-tubulin, 60-120 ng/ml, Abcam, Toronto, ON, Canada). Following the hybridization, the membranes were washed in PBS containing 0.05 % Tween and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (0.08 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein signal was revealed by chemiluminescence using a commercial kit (Lumilight, Roche Diagnostic, Laval, QC, Canada).

Protein loading was verified using Ponceau red (5 % Ponceau red in 5 % acetic acid) coloration and quantified using a Fluor-S MultiImager (Bio-Rad Laboratories). A comparison of cytosolic and nuclear protein levels of CTNNB1 for control and treated samples was carried out. Cytoplasmic protein loading was standardized by measuring α-tubulin levels and nuclear protein loading was standardized according to H3 levels. Since levels for these two proteins were not affected by treatment, they were considered appropriate loading controls.

RESULTS

The fathead minnow oligonucleotide microarray used in this study contained 15 208 genes. A total of 309 genes were differentially expressed in the hepatic tissue of exposed fish with a greater than 2-fold change in steady-state mRNA levels as compared with controls (Fig. 1). Of the 309 differentially expressed genes, 118 were up-regulated and 191 were down-regulated. Three genes were chosen at random for qPCR analysis to validate the results from the microarray data. FOXB1 was down-regulated, BCL2L13 was up-regulated, and VTG showed no change, these results were consistent between the microarray and qPCR analysis (Fig. 2).

Mammalian homologues were identified for the differentially expressed genes and found to be associated with metabolism, development, signaling, cellular organization and assembly, and cell death (Fig. 3; Table 2). Further analysis was conducted using differentially expressed genes by a greater than 4-fold change: 17 genes were up-regulated and 18 genes were down-regulated. A large number of the up-regulated genes were implicated in cellular signaling, organization and development (Table 2). MCAM, SIPA1L2, SASH3 and MAGEA10 were the most highly up-regulated genes (Table 2; Table 3). Of the differentially expressed genes three functional groups were identified with a large number of altered genes including cellular adhesion, inflammation and kinase activity (Fig. 3).

Altered genes involved in cellular adhesion include the down-regulation of PCDHB7, ARHGAP17, HEPACAM, RIKEN 2009E01, and the up-regulation of MCAM, PTK2, and L1CAM (Fig. 3; Table 4). Down-regulated genes associated with inflammatory activities include MAVS, CXCR2, SMAD3, TNSF11, AMBP, ADAMTS, and ATG16L1 (Fig 3.). Finally, the majority of genes involved in kinase activity were down-regulated including ADPGK, NME1, Raf-like, Aromatase, C9orf98, AKAP6, PAK6, GUC2C, EPHA2, PHKA1 and GAPDH with only one gene up-regulated, PFKL (Fig. 3).

At least ten genes were identified that are known to be associated with the estrogen receptor signaling pathway (Table 5; Fig. 4). The majority of these genes were found to be down-regulated after exposure to MWWE. These genes included: SPOCK2, heat shock 70kDa protein 5 (HSPA5), aromatase (CYP19A1), BNIP2, NME1, phosphorylase kinase alpha 1 (PHKA1), centrosomal protein 152kDa (CEP152), and CBFA2T2. Three of these genes were up-regulated including: DNAJ (Hsp40) homolog subfamily C member 5 (DNAJC5), NEL-like 2 (NELL2), glutamyl aminoepeptidase (ENPEP). One gene involved in this pathway, VTG, was shown to have no change in expression levels.

Several genes (13) implicated in the Wnt/CTNNB1 signaling pathway were up-regulated in exposed fish when compared to controls (Table 5; Fig 5). These included Wingless-type MMTV integration site family, member 4 (Wnt4), low density lipoprotein receptor-related protein 6

(LRP6), amino-terminal enhancer of split (AES), ariadne homolog 2 (ARIH2) and protein phosphatase 2, regulatory subunit B, epsilon isoform (PP2A/ PP2R5E). Several other genes involved in this pathway were down-regulated in treated fish when compared to controls. These included dapper antagonist of CTNNB1 homolog 1 (DACT1), kremen protein 1 (KREMEN1), G protein-coupled receptor 128 (GPR128), SKI family transcriptional corepressor 1 (SKOR1), SMAD family member 3 (SMAD3), FOXB1, BCL2/adenovirus E1B 19kDa interacting protein (BNIP2), and nucleoside diphosphate kinase A (NME1) . These changes are consistent with an overall inhibition of the Wnt/β-catenin signaling pathway.

To determine if the Wnt/CTNNB1 signaling pathway was in fact inhibited, we examined the levels of nuclear CTNNB1 in the liver of treated and control fish as this is the key mediator in the canonical Wnt signaling pathway. CTNNB1 protein levels in both the cytosolic and nuclear fractions were lower in MWWE-treated fish as compared to controls, although the effect was not statistically significant (Fig. 6).

DISCUSSION

Exposure of fish to MWWE has been associated with a variety of physiological alterations including reproductive and immune dysfunction. Pathway analysis software identified three altered pathways including the cell adhesion, inflammation, and intracellular signaling.

In vertebrate adherens junctions, tight junctions and desmosomes, are responsible for epithelial integrity and the formation of immune privileged sites within the body (Battle et al., 2006; Cyr and Dubé, 2012). In the present study, seven transcripts coding for proteins implicated in cell adhesion were altered. Three of these genes (MCAM, PTK2 and L1CAM) were up-regulated while four others were down-regulated (PCDHB7, ARHGAP17, HEPACAM and Riken 2009E01). Among these genes, PCDHB7 is a protocadherin which has been reported to be implicated in the establishment and function of specific cell-cell neural connections (Yagi and Takeichi, 2000). ARHGAP17 encodes for a protein involved in cell polarity and the maintenance of tight junctions by regulating the activity of CDC42. In mammals, ARHGAP17 is induced by GTPase-activating proteins (Kobayashi et al., 2013). HEPACAM has been shown to be down-

regulated in many cancer cell lines and therefore may act as a tumor suppressor gene due to its role in cell motility and cell-matrix interactions (Moh and Shen, 2009). In this study HEPACAM was down-regulated by almost 6-fold suggesting that normal developmental functions that require cell motility and cell interactions were being inhibited (Moh and Shen, 2009). Another cell adhesion protein, Riken 2900E01, responsible for hepatocyte cell adhesion was also down-regulated, further suggesting that cell adhesion and motility processes in the liver of MWWE-exposed fish were being disrupted. L1CAM, which was upregulated, is normally induced during development because of its role in axon guidance and cell migration (Dou et al., 2013). MCAM is a cell adhesion molecule that acts as a surface receptor that can trigger tyrosine phosphorylation of PTK2 and cause a transient increase in intracellular Ca^{2+} concentrations. Its induction has been shown to allow melanoma cells to interact with cellular elements of the vascular system potentially increasing the spread of tumors (Chen et al., 2013; Ward et al., 2012). While the function of these genes in fish liver function is largely unknown, alteration in cell adhesion suggests that epithelial function in the liver may be altered and that these may predispose fish to other pathologies.

Studies have shown that fish exposed to mixtures of toxicants, similar to those found in MWWE, present with altered immune function and an induction of the stress-related pathways (Hebert et al., 2008a, b; Muller et al., 2009; Ings et al., 2011). Ings et al. (2011) sampled juvenile rainbow trout (Oncorhynchus mykiss) placed downstream from a municipal wastewater discharge pipe for 14 days (Ings et al., 2011). Using a cDNA microarray, a number of genes involved in immune response including low molecular mass protein 2, major histocompatibility complex 1, Mx2 and Sox24 were down-regulated suggesting an inhibition of the immune system (Ings et al., 2011). A second study showed that juvenile rainbow trout exposed to 1 % wastewater showed an increase in phagocytic activity after 28 days but a decrease in phagocytic activity after 90 days suggesting accumulation of toxicants can inhibit the immune response (Hebert et al., 2008). A number of genes implicated in the inflammatory response were also altered by exposure to municipal effluent. These include: MAVS, CXCR2, SMAD3, TNSF11, TNSF13, AMBP, ADAMTS, and ATG16L1. An overall inhibition of the inflammatory response was observed in a number of studies where aquatic organisms were exposed to effluent mixtures (Hoole et al., 2003; Gagne et al., 2005; Gagne et al., 2007; Gagne et al., 2008; Bouchard et al., 2009; Ferraro

et al., 2012; Gagne et al., 2012; Tetreault et al., 2012). In the present study a number of cell derived mediators of the inflammation response were down-regulated including MAVS which is required for the activation of transcription factors which regulate the expression of beta interferon. During a stress response leukocytes are mobilized towards toxicants reducing the levels available within the organism. As well, CXCR2, a receptor for the cytokine interleukin 8 and chemokine liagand 1, mediates neutrophil migration to sites of inflammation suggesting a reduction of key mediators in the inflammatory pathway response. The inhibition of AMBP, a glycoprotein and ADAMTS, a peptidase, both play important roles in the regulation of inflammatory processes including tissue organization, coagulation, and cell migration. Finally, three genes involved in the T cell-dependent immune response, TNSF11, TNSF13 and ATG16L1 which play a role in cell-mediated immunity were down-regulated. Overall, the present study showed a reduction in inflammation response, shown by a reduction in proteins expressed by T cells as well as a decrease in cytokines and other key mediators.

Several genes transcripts for protein kinases were also altered by exposure to MWWE. Eleven genes in the present study were down-regulated including ADPGK, NME1, Raf-like,C9orf98, AKAP6, PAK6, GUCY2C, EPHA2, PHKA1, and GAPDH while PFKL was upregulated (Fig 3). ADPGK is responsible for the catalyzing ADP-dependent phosphorylation of glucose to glucose-6-phosphate in the glycolytic cycle. GAPDH catalyzes the breakdown of glucose for energy and carbon molecules (Tarze et al., 2007). It has also been recently been implicated in several non-metabolic processes, including transcription activation, and initiation of apoptosis (Tarze et al., 2007). C9orf98 is a phosphotransferase enzyme that catalyzes the interconversion of adenine nucleotides that play a role in cellular energy homeostasis. The PAK6 gene encodes for an enzyme implicated in the regulation of a number of cellular processes, including cytoskeleton rearrangement, apoptosis, and the MAP kinase signaling (Ye and Field, 2012; Furnari et al., 2013). As well, PAK6 interacts with the androgen receptor which is important for male sexual differentiation and development (Lee et al., 2002), although this unlikely implicated in liver function. An overall inhibition of kinase activity observed in this study suggests that various intracellular signaling pathways may be directly targeted by exposure to MWWE.

Analyses of gene expression data indicated that 13 estrogen (E2)-regulated genes were altered after exposure to MWWE (Table 5; Fig. 4). These genes are implicated in numerous cellular functions and have been shown in mammals to be regulated either directly or indirectly by estradiol. Genes that are estrogen-dependant and whose expression was decreased by exposure MWWE include aromatase, NELL2, HSPA5, SPOCK2 and BNIP2 (Choi et al., 2010; Guzel et al., 2011; Watanabe et al., 2003; Ivanga et al., 2007; Belcredito et al., 2001). Aromatase, a member of the cytochrome P450 superfamily, is plays an important role in the biosynthesis of estrogens. Inhibition of aromatase suggests a reduction in the synthesis of the hormone estrogen, a key hormone for both sexual differentiation and morphogenesis (Bonleon de Castro, 1998; Cooke and Naaz, 2001; McCarthy et al., 2006). In the present study, aromatase was down-regulated providing further support that E2 may have been inhibited in MWWE-exposed fish. Furthermore, there were eight genes that have been shown be regulated downstream of the E2 receptor including ENPEP, DNAJC5, PLXNA3, and UBQLN4.

While the data suggest an inhibition of the estrogenic pathway, it should be noted that estradiol levels in these immature fish is likely to be very low, and thus the significance of these is unknown, nor can we draw any conclusions at this time on the potential response of these fish to E2. Previous studies have suggested that in brook trout VTG mRNA induction in male and juvenile fish occurred after only 12 weeks of exposure (de Montgolfier et al. 2008) suggesting the need for contaminants to accumulate in these fish prior to the induction of an estrogenic response. The present data would support these conclusions.

There were a number of genes involved in the Wnt/CTNNB1 signaling pathway that were differentially expressed in MWWE-exposed fathead minnows (Table 5: Fig 5). Wnt signaling is implicated in numerous cellular functions including cell-cell signaling during embryogenesis, cellular differentiation, cell adhesion, cell cycle progression and differentiation (Hendriks and Reichmann, 2002; Monga and Michalopoulos, 2005).

Several genes implicated in the canonical Wnt pathway, including KREMEN1, Frizzled, DACT1, and dishevelled dsh homolog 2 (DVL2), were dramatically down-regulated. The Wnt pathway can be activated when the Fz-LRP complex is formed or with the autophospohorylation

of LRP6 both actions send a signal through intermediate proteins to activate dishevelled (dsh) (Lade and Monga, 2011; Monga and Michalopoulos, 2005; Veeman et al., 2003). Dsh then blocks CTNNB1 degradation by recruiting GSK3β, allowing the axin APC complex to form and inactivating CTNNB1 ubiquitination (Lade and Monga, 2011; Monga and Michalopoulos, 2005; Veeman et al., 2003). Dkk-1 interacts with LRP5/6 and the co-receptor KREMEN1 preventing the formation of the LRP5/6-Wnt-Frizzled complex (Osada et al., 2006). In this study KREMEN1 was down-regulated suggesting a reduction in the formation of LRP5/6 Wnt-Frizzled complex (Cadoret et al., 2002; Davidson et al., 2002; Mao et al., 2002; Osada et al., 2006) DACT1 binds to dsh and impedes the degradation of CTNNB1 enhancing the transcriptional activity of genes targeted by the Wnt pathway. In this study fathead minnow exposed to MWWE resulted in a down-regulation of DACT1 suggesting one mechanism of controlling CTNNB1 levels is inhibited. Dsh signaling through the Wnt pathway can be transduced by one of two branches depending on the target gene and end function.

Several other genes involved in the non-canonical Wnt pathway, such as Wnt4, LRP6, and PPP2R5E and which are known to inhibit the canonical Wnt pathway were increased. Wnt4 has been shown to inhibit Wnt signaling to the nucleus by redirecting CTNNB1 to the plasma membrane (Bernard et al., 2008). Thus the increase in Wnt4 may decrease overall Wnt signaling of the canonical Wnt signaling pathway. Exposed minnows showed higher levels of LRP6 mRNA. The role of LRP6 in Wnt signaling has been reported to be either stimulatory or inhibitory. Activation of Wnt signaling can occur when LRP6 inhibits GSK3β (Metcalfe and Bienz, 2011) resulting in increased CTNNB1 in the cytosol and allows it to translocated to the nucleus where it can act as a transcription factor (Fang et al., 2000). However, LRP can also interact with DKK1 which can then inhibit Wnt signaling.

In the absence of Wnt signaling, APC, axin and GSK3 phosphorylate CTNNB1 marking it for degradation by an E3 ubiquitination protein (Hendriks and Reichmann, 2002; Polakis et al., 2001). This study showed that fish exposed to MWWE resulted in an increase in three E3 ubiquitination proteins, ARIH2, topoisomerase I binding arginineserine-nich (TOPORS) and ubiquilin 4 (UBQLN4). A lack of Wnt signaling in the nucleus could act as a repressor of target gene transcription in conjunction with the co-repressor Groucho/AES (Monga and

Michalopoulos, 2005). Two Wnt pathway inhibitors, AES and phosphatase 2A (PPP2R5E) were up-regulated in fish further confirming an inhibition of the canonical Wnt signaling pathway. The decrease in Wnt signaling is supported by the results of the western blot in which there was a consistent decrease in both cytosolic and nuclear Wnt protein levels in the liver of fish exposed to MWWE.

Results from the present study suggest that a short-term exposure to MWWE results in an inhibition of estrogenic signaling and Wnt signaling pathway. Recent studies have shown that the presence of E2 suppresses hippocampal elevation of Dkk1 allowing the deregulation of the Wnt/CTNNB1 pathway (Scott et al., 2012). Further studies have examined whether long-term deprivation of E2 can lead to the dysregulation of the Wnt/CTNNB1 pathway (Scott et al., 2012). Using female Sprague Dawley rats, E2 was deprived for 10 weeks to determine whether a dysregulation of DKK1 and the Wnt pathway is observed (Scott et al., 2012). After 10 weeks it was evident that E2 was responsible for the modulation of the Wnt pathway through basal levels of Dkk1 causing the down-regulation of CTNNB1 (Scott et al., 2012). This regulation is thought to occur through the p53 signaling pathway, which was markedly decreased during E2 deprivation (Scott et al., 2012).

Signaling pathway analyses suggest that other pathways mediated by several kinases and inflammation are also targeted following exposure to MWWE and likely result in a variety of altered physiological functions. The effects on Wnt signaling is particularly interesting and suggests that in addition to the adult, the effluent may exert significant effects during embryonic development or in the development of pathological conditions when Wnt signaling play an important role in cellular differentiation and development.

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Table 1. qPCR primers designed to amplify BCL2-like 13 (BCL2L13), forkhead box B1(FOXB1) and vitellogenin (VTG).

Primer name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Pubmed Accession Number
BCL2L13	GTTCAGTCCAGCCAATCCA	CCTGCAGGGCTTGTAACAATA	NM_001044891
FOXB1	CAGCGATTATAGTGCCTACGG	GAGATTGCGGAGAGTTCGATA	NM_131285.1
VTG	AGACATTCTCAAGGGCAACTA	TTCTGACCACGGCATAAGT	NM_001044897.2

Table 2. Panel A is a list of differentially expressed genes associated with development and Panel B is a list of differentially expressed genes associated with cellular signaling and organization.

A

Gene ID	Gene Symbol	Fold Change	Pubmed Accession Number
EA_Pp_54861a	FOXB1	-11.63	NM_012182.2
EA_Pp_68848a	NME1	-5.717	NM_000269.2
EA_Pp_50367a	SPOCK2	-3.889	NM_001134434.1
EA_Pp_70277a	ADAMTS1	-3.132	NM_006988.3
EA_Pp_53515a	GATA5	-2.753	NM_080473.4
EA_Pp_64501a	TBOX20	-2.665	NM_001077653.2
EA_Pp_55993a	CBFA2T2	-2.563	NM_001032999.2
EA_Pp_55893a	CYP19A1	-3.138	NM_000103.3
EA_Pp_52477a	CXCR2	-2.578	NM_001168298.1
EA_Pp_53694a	TNFSF11	-5.915	NM_003701.3

B

Gene ID	Gene Symbol	Fold Change	Pubmed Accession Number
EA_Pp_53694a	TNFSF11	-5.915	NM_003701.3
EA_Pp_55893a	CYP19A1	-3.138	NM_000103.3
EA_Pp_61467a	CYP17A1	-2.664	NM_000102.3
EA_Pp_52477a	CXCR2	-2.578	NM_001168298.1
EA_Pp_69039a	PLA2G1B	-2.416	NM_000928.2
EA_Pp_52520a	PPP1R14B	-2.364	NM_138689.2
EA_Pp_63409a	SYNGAP1	-2.335	NM_006772.2
EA_Pp_69192a	Cdc42/Rac	-2.221	NM_006772.2
EA_Pp_56356a	GUCY2C	-2.205	NM_004963.3
EA_Pp_56645a	DNAJC5	4.964	NM_025219.2
EA_Pp_69307a	PPP2R5E	3.708	NM_006246.2
EA_Pp_58090a	PTK2	2.868	NM_001199649.1
EA_Pp_63591a	SMPD3	2.751	NM_018667.3
EA_Pp_66108a	VRK2	2.363	NM_001130480.2
EA_Pp_51246a	SPTA1	2.016	NM_003126.2

Table 3. List of up-regulated genes with a greater than 4-fold change.

Gene ID	Gene Symbol	Fold change	Gene Function	Pubmed Accession Number
EA_Pp_62041a	MCAM	10.153	Cell adhesion, anatomical structure morphogenesis	NC_018922.1
EA_Pp_56813a	SIPA1L2	8.282	Regulation of small GTPase mediated signal transduction	NM_020808.3
EA_Pp_52712a	SASH3	6.548	Proliferation	NM_018990.3
EA_Pp_69607a	CPEB4	6.523	Nucleotide binding	NM_030627.2
EA_Pp_58287a	MAGEA10	6.511	May play a role in embryonal development and tumor transformation	NM_001011543.2
EA_Pp_64305a	SLC40A1	5.815	Ion transport, anatomical structure morphogenesis	NM_014585.5
EA_Pp_54032a	ATP6V0A2	5.533	Ion transport, immune response, ATP synthesis coupled proton transport	NM_012463.3
EA_Pp_70960a	PDZRN3	5.126	Protein ubiquitination	NM_015009.1
EA_Pp_57661a	PNOC	5.092	Signal transduction, neuropeptide signaling pathway, synaptic transmission, sensory perception	NM_006228.3
EA_Pp_56645a	DNAJC5	4.964	Protein folding, negative regulation of neuron apoptosis	NM_025219.2
EA_Pp_69299a	KIAA0748	4.876	Protein coding	NM_001261844
EA_Pp_70787a	PHF13	4.631	Transcription regulator	NM_153812.2
EA_Pp_59744a	TLCD1	4.620	Integral to the membrane	NM_001160407.1
EA_Pp_52997a	UBQLN4	4.462	Cell death, negative regulation of transcription	NM_020131.3
EA_Pp_67710a	LCMT2	4.398	tRNA processing	NM_014793.4
EA_Pp_57375a	C10orf27	4.370	Multicellular organismal development, cell differentiation	NM_152710.2
EA_Pp_69299a	KIAA0319	4.307	Cell adhesion	NM_001168374.1

Table 4. List of down-regulated genes with a greater than 4-fold change.

Gene ID	Gene Symbol	Fold Change	Gene Function	Pubmed Accession Number
EA_Pp_54861a	FOXB1	11.630	DNA dependent transcription regulation	NM_012182.2
EA_Pp_58527a	ADPGK	11.472	Carbohydrate metabolic process, glycolysis	NM_031284.4
EA_Pp_70091a	RACGAP1	10.711	Transporter	NM_001126103.1
EA_Pp_52153a	DCLRE1A	8.567	Nucleotide-excision repair	NM_001271816.1
EA_Pp_50170a	TMEM63B	7.152	Protein binding	NM_018426.1
EA_Pp_53694a	TNFSF13	5.915	Immune response, signal transduction, positive regulation of cell proliferation	NM_001198622.1
EA_Pp_68848a	NME1	5.717	Nervous system development, negative regulation of cell proliferation, cell differentiation, regulation of apoptosis	NM_000269.2
EA_Pp_68213a	DACT1	5.390	Multicellular organismal development, Wnt receptor signaling pathway	NM_001079520.1
EA_Pp_54650a	KREMEN1	5.329	Cellular communication, Wnt receptor signaling pathway	NM_001039570.2
EA_Pp_68856a	TIMM50	5.104	Mitochondrial membrane organization, protein transport	NM_001001563.1
EA_Pp_63441a	SKOR1	4.878	Regulation of transcription, negative regulation of BMP signaling pathway	XM_236329.4
EA_Pp_55978a	UCHL3	4.798	Ubiquitin-dependent protein catabolic process	NM_001270952.1
EA_Pp_52942a	AMBP	4.637	Cell adhesion, negative regulation of immune response	NM_001633.3
EA_Pp_59061a	HEPACAM	4.604	Cell cycle, cell arrest, cell adhesion, regulation of growth	NM_152722.4
EA_Pp_55468a	TMEM151A	4.547	Integral to the membrane	NM_153266.3
EA_Pp_68927a	SGCD	4.397	Cytoskeleton organization, muscle organ development	NM_000337.5
EA_Pp_58058a	TTPAL	4.384	Transporter activity	NM_001145527.1
EA_Pp_51446a	PBX3	4.076	Transcription regulator	NM_001134778.1

Table 5. Panel A lists differentially expressed genes associated with the anti-estrogenic pathway and Panel B lists differentially expressed genes associated with the Wnt/beta-catenin signaling pathway.

A

Gene ID	Gene Symbol	Fold Change	Pubmed Accession Number
EA_Pp_54861a	FOXB1	-11.631	NM_012182.2
EA_Pp_68848a	NME1	-5.717	NM_000269.2
EA_Pp_68213a	DACT1	-5.391	NM_001079520.1
EA_Pp_54650a	KREMEN1	-5.329	NM_001039570.2
EA_Pp_53009a	SKOR1	-4.878	XM_236329.4
EA_Pp_70693a	SMAD3	-2.825	NM_001145102.1
EA_Pp_64316a	GPR128	-2.207	NM_032787.2
EA_Pp_55342a	BNIP2	-2.187	NM_004330.2
EA_Pp_69307a	PP2R5E	3.708	NM_006246.2
EA_Pp_50988a	ARIH2	3.323	NM_006321.2
EA_Pp_69288a	AES	3.028	NM_001130.5
EA_Pp_56793a	Wnt4	2.601	NM_030761.4
EA_Pp_55010a	LRP6	2.545	NM_002336.2

B

Gene ID	Gene Symbol	Fold Change	Pubmed Accession Number
EA_Pp_54861a	FOXB1	-11.631	NM_012182.2
EA_Pp_68848a	NME1	-5.717	NM_000269.2
EA_Pp_68213a	DACT1	-5.391	NM_001079520.1
EA_Pp_54650a	KREMEN1	-5.329	NM_001039570.2
EA_Pp_53009a	SKOR1	-4.878	XM_236329.4
EA_Pp_70693a	SMAD3	-2.825	NM_001145102.1
EA_Pp_64316a	GPR128	-2.207	NM_032787.2
EA_Pp_55342a	BNIP2	-2.187	NM_004330.2
EA_Pp_69307a	PP2R5E	3.708	NM_006246.2
EA_Pp_50988a	ARIH2	3.323	NM_006321.2
EA_Pp_69288a	AES	3.028	NM_001130.5
EA_Pp_56793a	Wnt4	2.601	NM_030761.4
EA_Pp_55010a	LRP6	2.545	NM_002336.2

FIGURE LEGENDS

Figure 1: Volcano plot comparing genes found to be statistically significant (unpaired t-test) compared with their p-value. A total of 309 genes were differentially expressed. Of these genes, 118 were up-regulated and 191 were down-regulated. The darker spots were found to have a greater than 2-fold change and a p-value of less than 0.05.

Figure 2: Microarray and qPCR analysis of mRNA levels for FOXB1, VTG and BCL2L13. Data are presented as percent fold change from control fish. qPCR data results show similar trends as results obtained by microarray.

Figure 3: Functional classification of differentially expressed genes by at least 2-fold in fathead minnow exposed to MWWE when compared to control fathead minnow. The genes were grouped based on their biological function. Panel A are genes associated with cell adhesion, Panel B inflammation and Panel C are genes associated with kinases.

Figure 4: Pathway analysis predicted beta-estradiol inhibition. Several beta-estradiol receptor dependent genes are down-regulated suggesting that an inhibition of beta-estradiol receptor is occurring from short-term MWWE exposure. Genes in green are down-regulated and genes in red are up-regulated.

Figure 5: Wnt/CTNNB1 Signaling pathway. A number of molecules involved in the Wnt signaling pathway are down-regulated. As well, several Wnt signaling inhibitors are up-regulated. This suggests an overall inhibition of the Wnt Signaling pathway. Genes in green are down-regulated and genes in red are up-regulated.

Figure 6: Western blot analysis of CTNNB1 protein expression in fathead minnow exposed to 20 % MWWE when compared with controls. Cytosolic protein loading was standardized using α-tubulin as a protein loading control (A and B) and nuclear protein loading was standardized using H3 as a protein loading control (C and D). Data are expressed as the relative CTNNB1 values. There was no statistical significant difference found between control and exposed fathead minnow both for the cytosol and nuclear protein fractions (E/F).

Figure 1

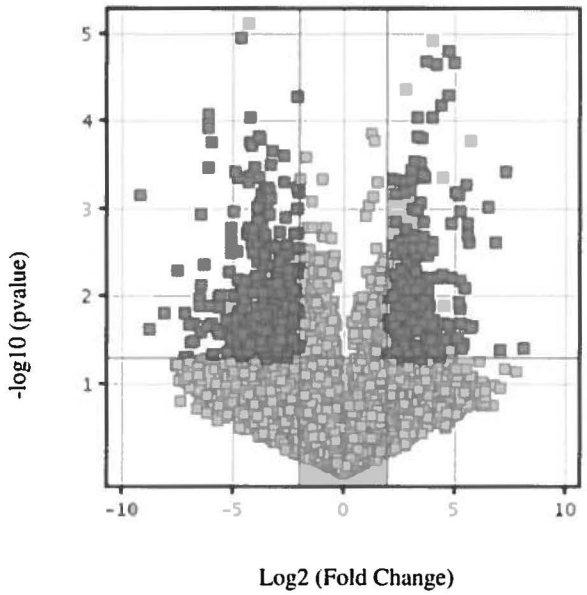


Figure 2

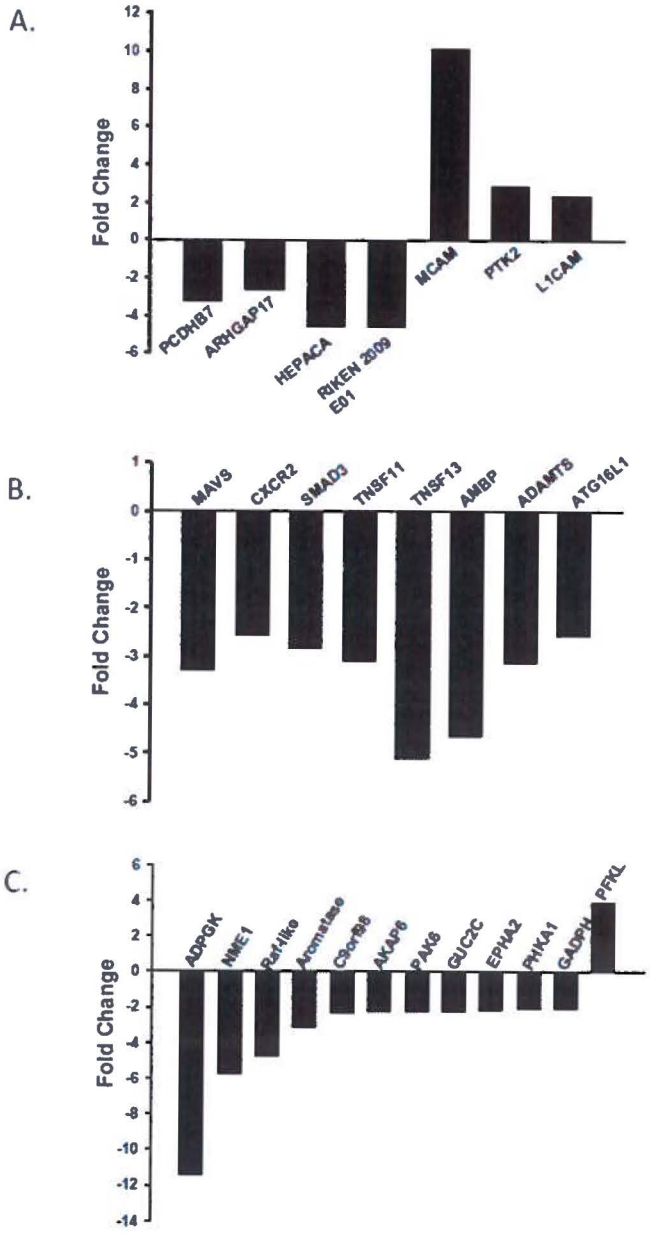

A.
Fold Change
12
10
8
6
4
2
0
-2
-4
-6
PCDHB7
ARHGAP17
HEPACA
RIKEN 2009 E01
MCAM
PTK2
L1CAM
B.
Fold Change
1
0
-1
-2
-3
-4
-5
-6
MAVS
CXCR2
SMAD3
TNSF11
TNSF13
AMBP
ADAMTS
ATG16L1
C.
Fold Change
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
ADPGK
NME1
Rel-like
Aromatase
C9orf58
AKAP6
PAK6
GUC2C
EPHA2
PHKA1
GADPH
PFKL

Figure 3

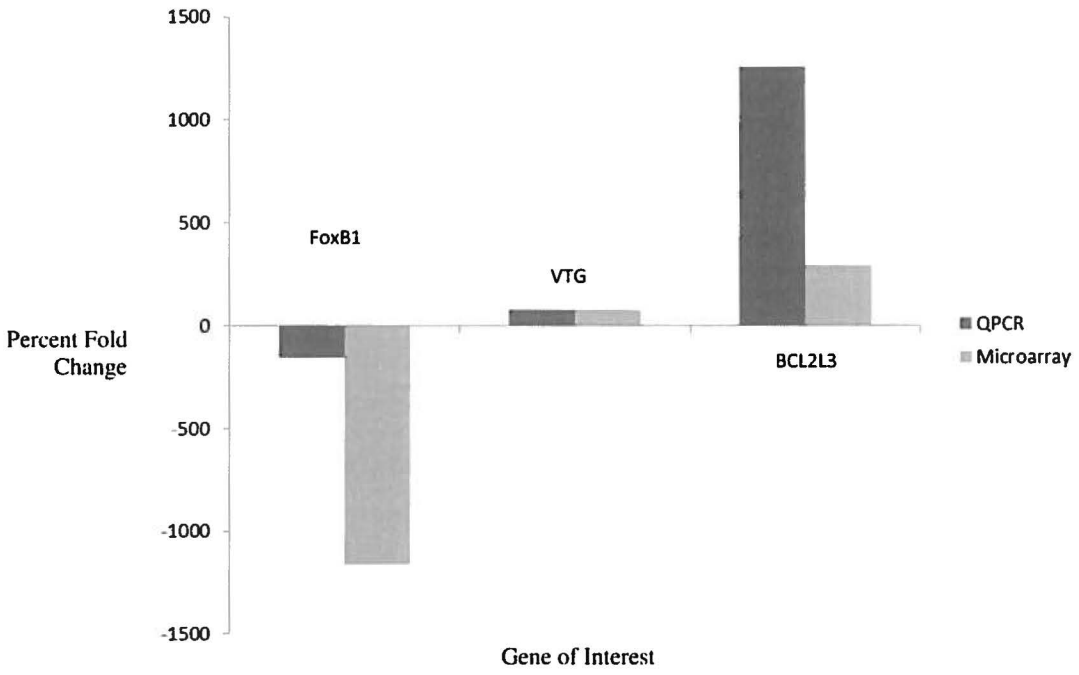
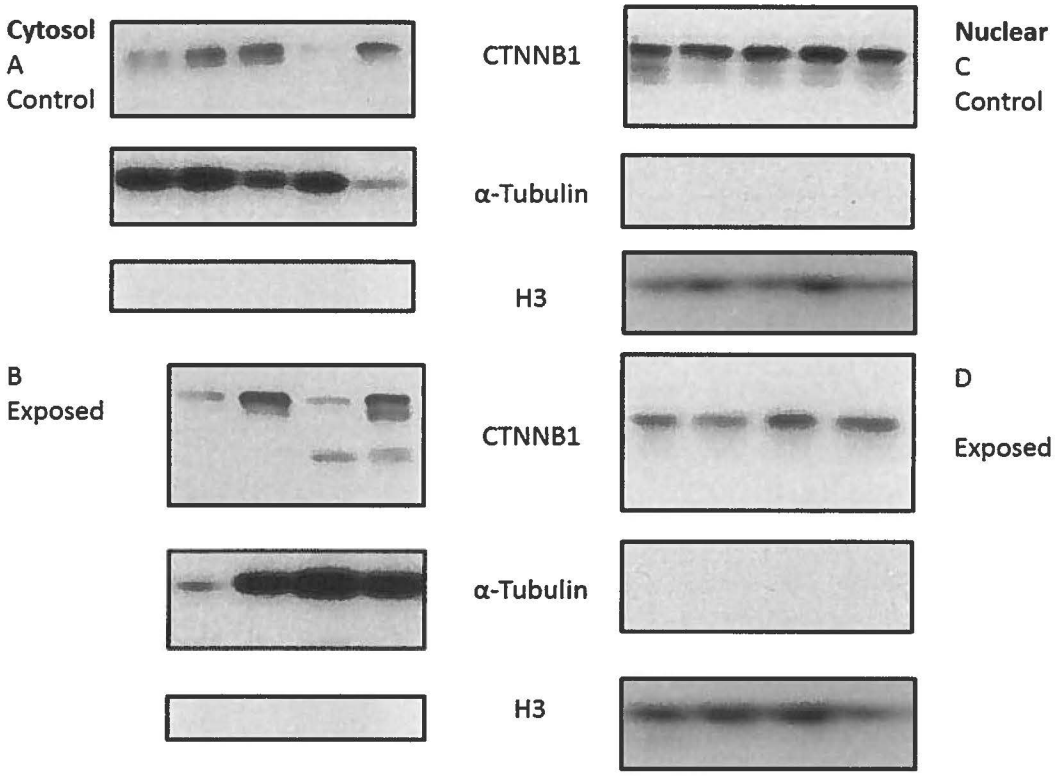
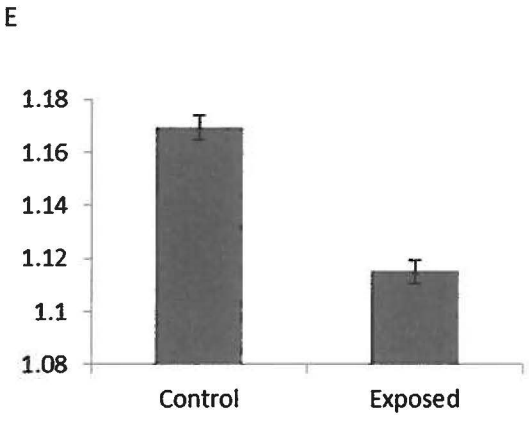

1500
1000
500
0
-500
-1000
-1500
Percent Fold Change
FoxB1
VTG
BCL2L3
QPCR
Microarray
Gene of Interest

Figure 4


Cytosol
A
Control
CTNNB1
Nuclear
C
Control
α-Tubulin
H3
B
Exposed
CTNNB1
D
Exposed
α-Tubulin
H3


E
1.18
1.16
1.14
1.12
1.1
1.08
Control
Exposed

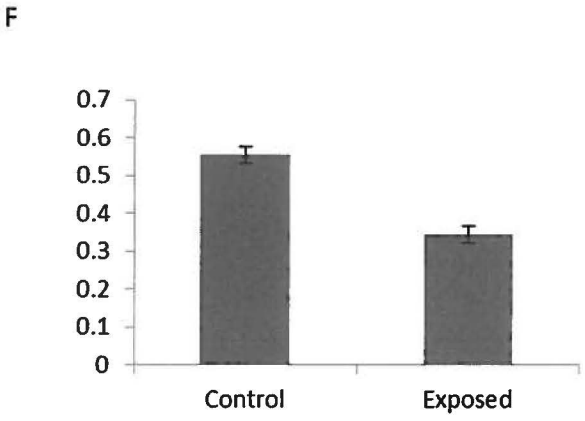

F
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Exposed

Figure 5

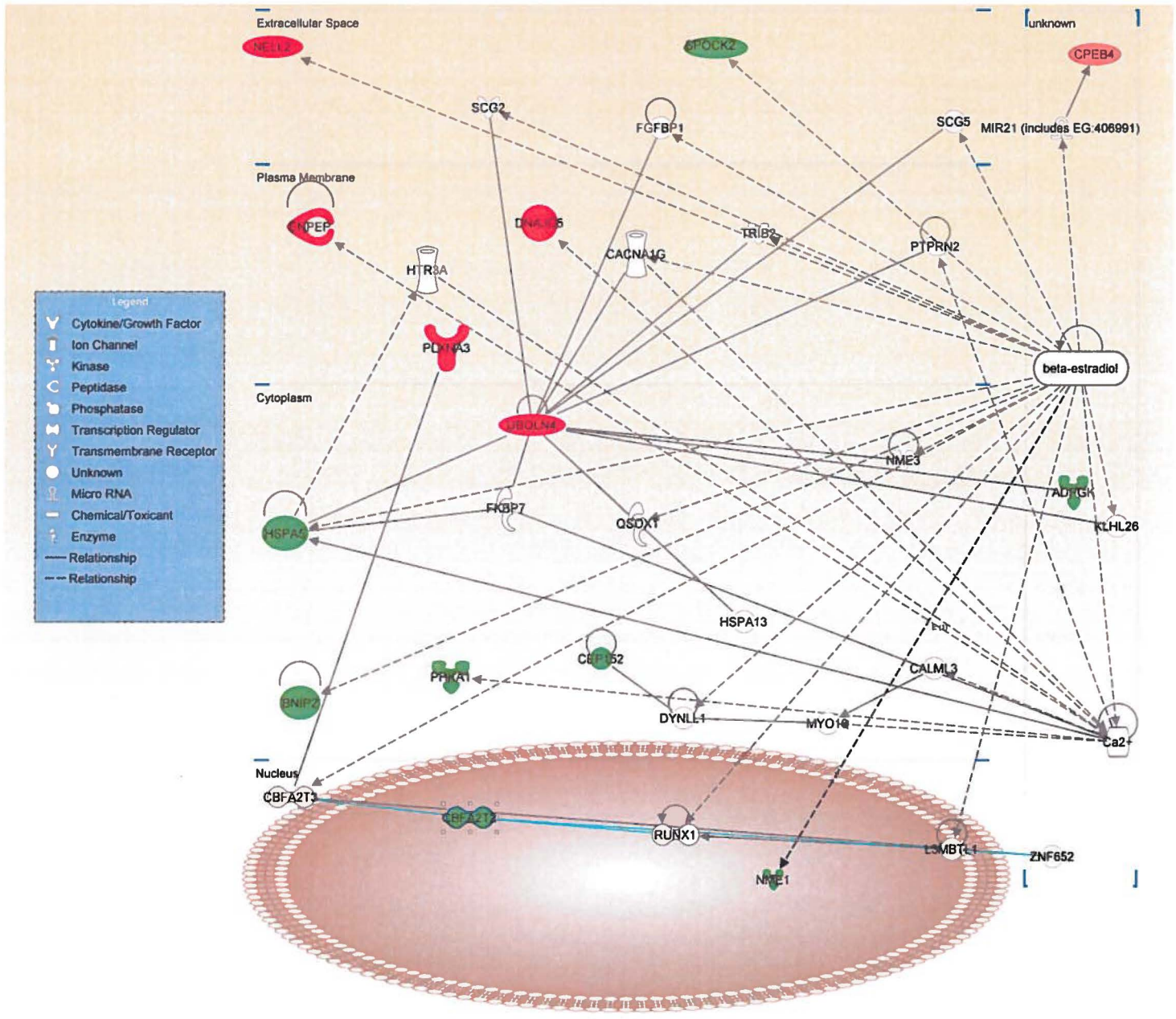
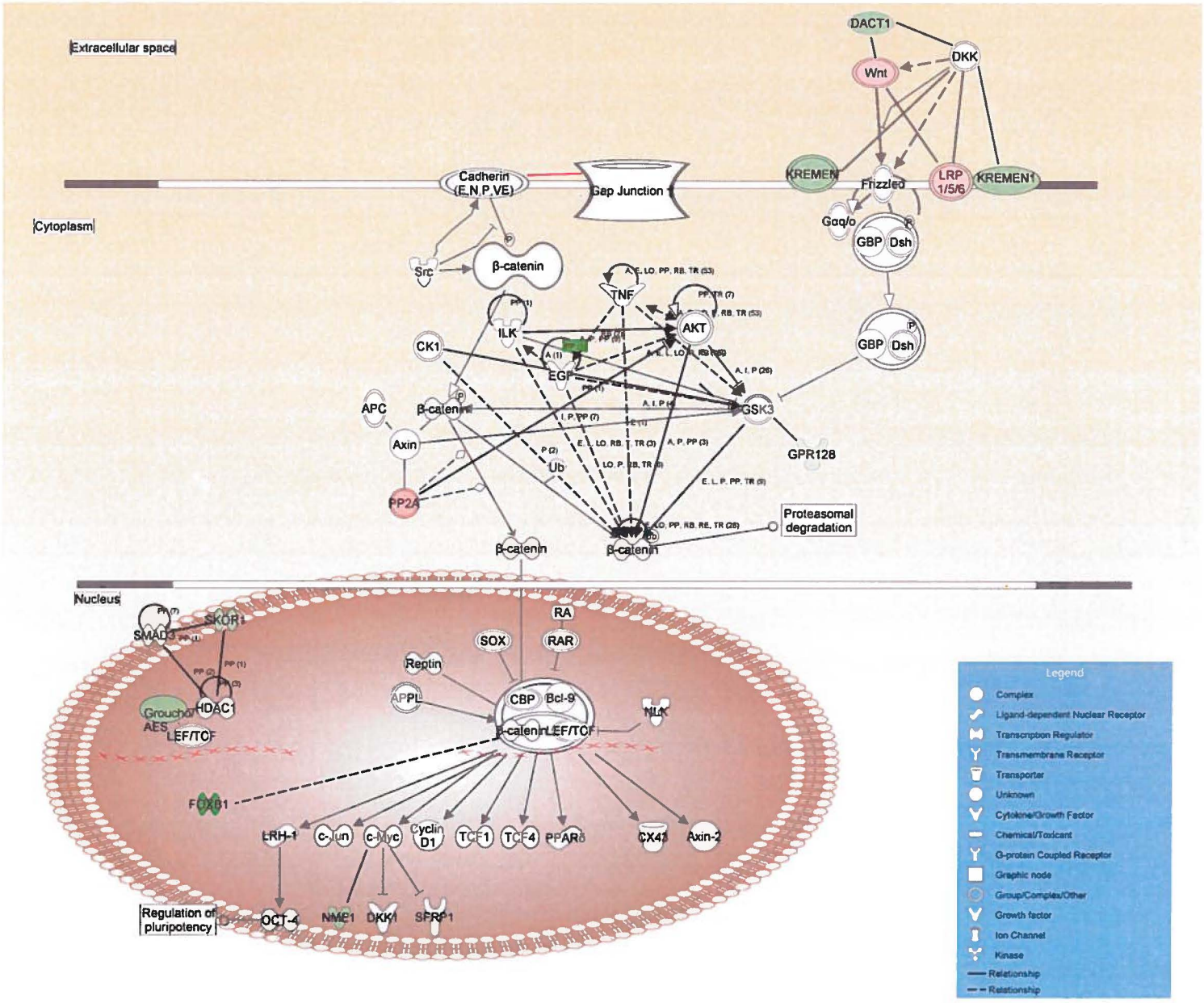

Extracellular Space
Unknown
SPOCK2
CPEB4
SCG2
FGFBP1
SCG5
MIR21 (includes EG:406991)
Plasma Membrane
HTR3A
CACNA1G
TRIB2
PTPRN2
PDYN
beta-estradiol
Cytoplasm
UBQLN4
NME3
FKBP7
QSOX1
KLHL26
HSPA13
CALML3
PPIA1
DYNLL1
MYO1B
Ca2+
Nucleus
CBFA2T3
RUNX1
L3MBTL1
ZNF652
Legend
Cytokine/Growth Factor
Ion Channel
Kinase
Peptidase
Phosphatase
Transcription Regulator
Transmembrane Receptor
Unknown
Micro RNA
Chemical/Toxicant
Enzyme
Relationship
Relationship

Figure 6


Extracellular space
Cytoplasm
Nucleus
DACT1
DKK
Wnt
KREMEN
KREMEN1
Frizzled
LRP 1/5/6
Cadherin (E,N,P,VE)
Gap Junction
Gαq/o
GBP
Dsh
Src
β-catenin
TNF
AKT
ILK
CK1
EGF
APC
Axin
PP2A
GSK3
GPR128
Ub
Proteasomal degradation
β-catenin
SKOR1
SMAD3
HDAC1
Groucho/ AES
LEF/TCF
RA
SOX
RAR
Reptin
APPL
CBP
Bcl-9
NLK
β-catenin/LEF/TCF
FOXB1
LRH-1
c-Jun
c-Myc
Cyclin D1
TCF1
TCF4
PPARδ
CX43
Axin-2
Regulation of pluripotency
OCT-4
NMP1
DKK1
SFRP1
Legend
Complex
Ligand-dependent Nuclear Receptor
Transcription Regulator
Transmembrane Receptor
Transporter
Unknown
Cytokine/Growth Factor
Chemical/Toxicant
G-protein Coupled Receptor
Graphic node
Group/Complex/Other
Growth factor
Ion Channel
Kinase
Relationship
Relationship

CHAPTER 4: GENERAL DISCUSSIONS AND CONCLUSIONS

The aim of these studies was to investigate the effects of MWWE exposure on signaling in the liver of fish. Overall, the results found that a number of key pathways in the liver were altered after a short-term exposure to MWWE.

The first part of this study identified two Cxs, Cx43 and Cx30, in the liver of the brook trout. This is the first identification of Cx in the liver of fish. These results were surprising since three Cxs have been identified in the liver of mammals, Cx43, Cx32 and Cx26 (Yang et al., 2003; Plante et al., 2007; Vinken et al., 2008; Fukumasu et al., 2010; Rackauskas et al., 2010; Vinken et al., 2011). Sequence homology showed that the brook trout Cx shared a greater degree of homology with the zebrafish, a member of the Cyprinidae family than the rainbow trout which is part of the same Salmonidae family as the brook trout (van der Hayden et al., 2004). This anomaly can be explained through species divergence with alterations occuring within the cytoplasmic domain, the less conserved section of the Cx structure (Hertzberg et al., 1988; Falk et al., 1994; Cruciani et al., 2006; Dbouk et al., 2009; Li et al., 2010).

After identifying Cx43 and Cx30 in the liver of brook trout it was important to determine whether these expression levels would be altered after exposure to MWWE for 21 days. Cx43 expression levels showed no change at the lowest concentration but at the two highest concentrations a small decrease was observed. Changes in Cx expression levels in the liver can act as a marker of cellular distress and suggests an impairment of GJIC as a protective mechanism to prevent the proliferation of disease and contaminants to healthy neighbouring cells (Segner et al., 2003). Other studies which have examined the effects of individual contaminants showed that murine Sertoli cells exposed to NP resulted in a decrease in GJIC and an inhibition of the phosphorylation of Cx43 (Aravindakshan et al., 2005).

A previous study that examined the effects of MWWE in the testis of the brook trout showed an increase in Cx43 levels after both 4 and 12 weeks (de Montgolfier et al., 2008). This study suggested that this small increase reflects cellular changes required to carry out testicular maturation and spermatogenesis (de Montgolfier et al., 2008). After 12 weeks Cx43 levels had

been significantly increased at all concentrations suggesting a greater susceptibility to disease and an increase in infertility (de Montgolfier et al., 2008). Variations in Cx43 expression levels between the testis and the liver can be explained by the different role that Cx43 plays in GJIC or the year-to-year variations in the characterization of MWWE.

The present study showed that after 4 weeks a significant increase in Cx30 expression levels at the two highest concentrations was observed. A number of liver specific functions require GJIC including albumin secretion, ammonia detoxification, glycogenolysis, bile secretion and xenobiotic phase I biotransformation (Vinken et al., 2008). In particular, induction of GJIC after exposure to xenobiotics could be an indicator of an increase in metabolism of these contaminants. Similar results were observed in the testis where Cx30 levels were significantly increased after 4 weeks of exposure to MWWE (de Montgolfier et al., 2008). This same study showed a decrease in Cx30 levels at all concentrations after 12 weeks of exposure to MWWE (de Montgolfier et al., 2008). In order to further investigate the effects of MWWE on key signaling pathways in the liver a microarray was used in order to study thousands of genes in parallel.

Immature fathead minnow were exposed to the highest concentration of MWWE for 21 days and using a fathead minnow 15 208 oligonucleotide microarray, alterations to key cellular signaling pathways were observed. Using pathway analysis software, three functional groups were identified as having a large number of genes affected; these groups included cell adhesion, inflammation and protein kinases. Further analysis identified two prominent pathways that included a minimum of 13 genes that were differentially expressed. These pathways were identified as the anti-estrogenic and Wnt/CTNNB1 signaling pathways.

There are known endocrine disrupting contaminants in the MWWE which cause disruptions in key pathways over time, however, there are often contaminants that would remain unidentified without the help of systems toxicology and genomics. Exposing fish to xenobiotics can alter normal homeostatic function and in order to survive fish must adapt or react using organismal resources altering homeostasis (Ings et al., 2012). Cellular adhesion proteins are required for the coupling of neighbouring channels in order to facilitate gap junction intercellular signaling (Barcellos-Hoff and Brooks, 2001; Battle et al., 2006). In the present study three cell adhesion

proteins were up-regulated, suggesting some normal developmental functions requiring cellular communication were still occurring. While under stressful conditions the normal cellular adhesion and junction formation processes can be disrupted by altering protein levels. Four cellular adhesion genes were down-regulated; this reduces the available number of proteins and disrupts normal junction formation processes. This interruption reduces cellular differentiation, migration and motility activities required during development (Ishicuhi et al., 2009; Dou et al., 2013).

The inflammatory reaction allows fish to mobilize a defense against invading contaminants in order to prevent their spread and to reestablish homeostasis (Rottmann et al., 1992). Leukocytes are sent to infected areas to target toxins and immbolize any possibility for further spread of infection (Ellis, 1986). A number of these leukocytes, namely cytokines and chemokines were observed as being down-regulated in the present study suggesting that leukocytes are being drawn away to fight toxicants present in the organisms liver. A decrease in leukocytes can be problematic as it may increase an organisms vulnerability to the damage caused by toxins.

The final group of genes altered by exposure to MWWE were protein kinases. A number of kinases that were down-regulated were involved in the glycolytic pathway and suggested a reduction in the conversion and breakdown of glucose. As well, two genes that encode proteins required during sexual differentiation were down-regulated suggesting a disruption of morphogenesis and development. Overall, a number of genes that encode proteins acting as transcription factors were reduced suggesting a decrease in the target molecules. Protein kinases are involved in a number of different signal transduction pathways and can have a localized affect on a single reaction or have down-stream affects for more complex processes.

Through the use of microarray technology two pathways were identified as having a large number of genes differentially expressed after a short-term exposure to MWWE. Previous studies from our lab have demonstrated that brook trout exposed for 4 weeks to MWWE showed no change in VTG levels but after 12 weeks VTG levels had significantly increased (de Montgolfier et al., 2008). These results suggest that estrogenic contaminants are present in small concentrations and require time to accumulate within the organism (de Montgolfier et al., 2008).

In the present study, similar results were found suggesting that a short-term exposure to MWWE can result in an inhibition of the estrogenic pathway. The difference between the VTG levels after 4 weeks between the two studies could be explained by the differences in threshold values for VTG induction between the two species (Hiramatsu et al., 2006). These results are important as it suggests that estrogenic contaminants are present at low concentrations within the MWWE and can provide insight into the mechanism by which estrogenic compounds disrupt homeostasis.

The Wnt/CTNNB1 signaling pathway is the second pathway identified by pathway analysis software. This pathway is a network of highly conserved proteins that regulate cell-to-cell signaling interactions during growth and development (Monga and Michalopoulos, 2005). In the present study, exposing fish to MWWE for 21 days resulted in an inhibition of the canonical Wnt signaling pathway which suggests a decrease in the amount of CTNNB1 available to translocate to the nucleus. Studies have shown in mice that CTNNB1 levels increase significantly during liver development suggesting they play an important role in organogenesis (Apte et al., 2007). This same study looked at liver development in beta-catenin KO mice and found that these mice had smaller livers and decreased hepatocyte proliferation (Apte et al., 2007). There are currently no studies that have looked at the specific role of the canonical Wnt signaling pathway in the liver of fish but mammalian studies suggest this key signaling pathway plays a critical role during liver growth and development (Apte et al., 2007).

Overall the findings of these studies suggest that a short-term exposure to MWWE is enough time to cause disregulation of several key signaling pathways in the liver of exposed fish. The use of microarray technology provides the platform to view a large number of genes at one time in order to examine how toxicants interact with one another and their possible mechanisms of action.

CONCLUSIONS

There are two Cxs expressed in the liver of the brook trout, Cx30 and Cx43. After a short-term exposure to MWWE there was no significant change in Cx43 expression levels, however, Cx30 increased significantly at the two highest exposure concentrations.

Using a fathead minnow microarray, gene expression levels were measured in exposed fish to determine whether alterations in key signaling pathways occurred after 21 days in 20 % MWWE. Three functional groups were identified, cell adhesion, inflammation and kinases, as having a large number of genes altered. Seven transcripts coding for proteins linked to cell adhesion were altered suggesting that epithelial function in the liver may be compromised. Results of the microarray analysis found that a number of genes associated with immune function were shown to be down-regulated potentially leading to an inhibition of the immune response in exposed fish. As well, an overall inhibition of kinase activity was observed with 11 gene transcripts coding for protein kinases were altered, however implications in the liver are largely unknown. These genes are all part of a larger network of signaling pathways which suggests the targeted affects to these genes can have a more global affect on the entire pathway. Interestingly, two signaling pathways were identified as having a large number of genes altered including the anti-estrogenic pathway and the Wnt/CTNNB1 signaling pathway. Both of these pathways play a key role during development and were found in this study to be down-regulated.

Our findings suggest that a short-term exposure to MWWE can have a significant impact on key signaling pathways in the liver causing a disruption to homeostasis. To the best of our knowledge this is the first time that Cx have been identified in the liver of the brook trout.

CHAPTER 4: SYNTHÈSE DE LA MÉMOIRE REDIGE EN FRANÇAIS

1.0 Caractérisation des eaux usées effluentes de la municipalité de Montréal

Il y a 1.8 millions d'habitants sur l'Île de Montréal et approximativement 8 000 établissements commerciaux et industriels (Pham et al., 1997; Sabik et al., 2003; Marcogliese et al., 2009). Les eaux usées de la Communauté Urbaine de Montréal (MUC), 187 x 106 m^3 par an, est traitée par l'usine de traitement de Montréal (MWWTP) qui se trouve sur la Rive-Est, au bord de l'île (Pham et al., 1997; Sabik et al., 2003; Marcogliese et al., 2009). Typiquement, le MWWTP traite 1.3 millions de m^3 d'eau usée quotidiennement, équivalent à 88 m^3 par seconde de décharge effluente (Sabik et al., 2003). Durant les périodes de précipitation et de dégel, le volume de l'eau augmente et peut atteindre jusqu'à 7.6 millions m^3 (Pham et al., 1997; Sabik et al., 2003; Marcogliese et al., 2009). L'effluent de l'usine de traitement est relâché directement dans le fleuve St-Laurent par l'intermédiaire d'un tuyau souterrain qui se trouve approximativement à 7 m de profondeur et 4.5 km de la côte de l'île de Montréal (Pham et al., 1997; Sabik et al., 2003; Marcogliese et al., 2009).

Alors que l'effluent municipal de Montréal (MWWE) est relâché dans le fleuve St-Laurent, l'effluent plus chaud remonte à la surface créant des remous (Osterman et al., 1990). L'eau usée n'est pas uniformément distribuée dans les remous—des pochettes effluentes se manifestent où il y a plus de dilution dans les zones fluides (Osterman et al., 1990). Alors que l'effluent se déplace en aval, sa concentration est de 20% à 0.3 km du tuyau et 10% à 1 km du tuyau. À 10 km, la concentration est diluée à 1% de l'effluent original. (Gagnon et al., 2003; de Montgolfier et al., 2008).

En caractérisant le MWWE, on a identifié un certain nombre de contaminants incluant des produits pharmaceutiques et d'hygiène (PCPP), des alkylphénols (AP), biphényls polychlorés (BPC), des hydrocarbures aromatiques polycycliques (PAH), et des métaux lourds (Pham et al., 1997; Quemerais et al., 1998; Gagne et al., 2001; Sabik et al., 2003; Aravindakshan et al., 2004a; Aravindakshan et al., 2005; Matozzo et al., 2008; Martin-Diaz et al., 2009; Bhavsar et al., 2011; Lajeunesse et al., 2011). En analysant des échantillons d'eau à chaque endroit, on a pu déterminer les contaminants les plus persistants et selon les résultats, les plus toxiques pour l'environnement aquatique.

1.1.0 Contaminants dans l'eau usée de la municipalité de Montréal

1.1.1 Produits pharmaceutiques et d'hygiène

Alors que l'âge moyen de la population augmente, on observe une corrélation entre l'utilisation des produits pharmaceutiques et leur présence dans l'effluent (Quinn et al., 2008). Les produits pharmaceutiques sont métabolisés dans le foie selon la phase I et la phase II et excrétées par l'urine et/ou les matières fécales (Corcoran et al., 2010). Dans certain cas, ces composés sont rendus inactifs par le métabolisme, et dans d'autres cas, ils produisent des métabolites biologiquement actifs (Quinn et al., 2008). Il y a trois résultats possibles pour les produits pharmaceutiques qui sont transportés aux usines de traitement: la substance est transformée en dioxyde de carbone et en eau; la substance reste dans la boue à cause de lipophile; ou la substance est métabolisée en une forme hydrophile d'une autre substance lipophile et peut facilement se déplacer à travers les usines de traitement et dans l'eau potable (Halling-Sorensen et al., 1998; Trudeau et al., 2005; Corcoran et al., 2010).

Les PCPP produits pharmaceutiques et de soins personnels les plus communs que l'on trouve dans le MWWE incluent les antidépresseurs (desmethylvenlafaxine, venlafaxine, paroxetine, citalopram) selon les concentrations entre 10.5 à 55.3 ng/L (Lajeunesse et al., 2011); les anti-inflammatoires (ibuprofène, naproxène; 217 à 1191 ng/L); les régulateurs de lipides (bezafibrate, gemfibrozil; 59 à 72 ng/L); les médicements anticonvulsifs (carbamazepine; 33 ng/L); les antibiotiques (sulfapyridine, trimethoprim, oxytetracycline, sulfamethoxazole) entre 46 et 440 ng/L; et la caféine avec la plus haute concentration de 22 µg/L (Quinn et al., 2008). Selon les médicaments, on a découvert des effets toxiques dans les organismes aquatiques avec différentes concentrations allant des µg/L pour la fourchette basse aux ng/L pour la fourchette haute.

Gagne et al. (2006) ont trouvé que dans la majorité des produits pharmaceutiques, présents dans le MWWE, étaient capables de créer des interactions toxiques entre médicaments empêchant les enzymes P450 (CYP) qui autrefois métabolisaient les contaminants (Lam et al., 2002). Le métabolisme oxydatif est requis pour dégrader la plupart de ces médicaments. Cependant, l'exposition à long terme peut engendrer un stress oxydatif (Gagne et al., 2006). Les truites hépatocytes exposées à un mélange de produits pharmaceutiques du MWWE pendant 48 heures

ont démontré des effets de stress oxydatif (Gagne et al., 2006; Martin-Diaz et al., 2009). Ainsi, plusieurs poissons et espèces bivalves ne possèdent pas les enzymes nécessaires, spécifiquement CYP P4502C, pour réduire et excréter la plupart de ces médicaments.

Les hormones synthétiques et naturelles, incluant l'ethinylestradiol (EE2), qui se trouvent dans les pilules de contraception et les hormones de traitement substitutif (Desbrow et al., 1998; Allen et al., 1999; Liu J. et al., 2011; Liu S. et al., 2011) ainsi que l'estrogène endogène (E1), 17β-estradiol (E2) et l'estriol (E3), ont été détectées dans le MWWE (Fishman et al., 1960; Quiros et al., 2005; Liu S. et al., 2011). Les concentrations de EE2 détectées dans les eaux usées sont habituellement moins élevées que celles des stéroïdes qui existent naturellement (Fishman et al., 1960; Quiros et al., 2005; Liu J. et al., 2011). Bien que l'EE2 soit détectée avec des concentrations moins élevées, elle est 10 à 50 fois plus forte in vivo grâce à sa demi-vie plus longue et sa capacité à s'accumuler dans les tissus de l'organisme (Lange et al., 2001; Thorpe et al., 2003; Gorelick et al., 2011). Les estrogènes sont impliqués dans plusieurs processus physiologiques, incluant la croissance et le développement, la reproduction, en plus du métabolisme des lipides et du glucose. Ceci est facilité via l'interaction entre les estrogènes et le récepteur d'estrogènes (ER) (Kuiper et al., 1997; Bryzgalova et al., 2006).

1.1.2 Alkylphénols

On a détecté des concentrations d'alkylphénols (AP) dans le MWWE de 0.01 à 10 µg/L (Sabik et al., 2003). Ces concentrations sont semblables à celles qui se trouvent dans l'effluent traité des autres usines de traitement au Canada et qui sont classées parmi les quantités causant des effets biologiques sur les organismes aquatiques.

L'AP est une famille de mélanges organiques moléculaires caractérisé par un cycle de phénol et une chaîne de neuf carbones sur la position para (Jobling et al., 1996; Wu et al., 2011). L'AP est utilisé depuis les années 1940 et se trouve souvent dans les détergents domestiques et industriels, les peintures, les herbicides, les cosmétiques, les produits de soin pour la peau, les émulsifiants, les produit antistatiques, et les emballages pour la nourriture (Soto et al., 1991; Ahel et al., 1994; Jobling et al., 1996; Sabik et al., 2003; Deng et al., 2007). L'AP est métabolisé par l'ethoxylation dont les chaines d'etholyxate sont progressivement enlevées, produisant des mélanges de métabolites de longueurs variées (Ahel et al., 1994; La Guardia et al., 2001;

Vetillard and Bailhache, 2006). La biodégradation se produit lentement dans les conditions anaérobiques, incluant celles dans la boue et les sédiments. Comme résultat, il y a une accumulation de la chaîne courte AP (Shang et al., 1999).

Il y a évidence que l'AP s'attache au récepteur de l'hormone de la thyroïde (TR) et l'ER (White et al., 1994). Chez les poissons, la thyroxine (T4) est transformée en triiodothyronine (T3) dans les tissus ciblés par l'enzyme micro-deiodinase (Brown et al., 2004). Le T3 peut alors s'attacher au TR nucléaire (Bres and Eales, 1990) où il règle le processus du développement, de croissance et de reproduction (Cyr and Eales, 1992; Scholz et al., 2008). Schmutzler et al. (2007) ont examiné les effets du 4-nonylphenol (NP) sur la sécrétion des hormones de la thyroïde (TH) en utilisant les cellules thyroïdes des rats FRTL5. NP a empêché la prise d'iodite par les cellules FRTL5, ainsi que de thyroïde peroxydase, un enzyme impliqué dans la synthèse de la TH. Le NP peut aussi stimuler l'activation transcriptionnelle du TR (Schmutzler et al., 2007). Ces résultats suggèrent que le NP et la TH possèdent des similarités structurelles, permettant au NP de s'attacher au TR, ce qui modifie les fonctions endocrines (Schmutzler et al., 2007).

L'AP peut aussi s'attacher à la l'ER avec une affinité peu élevée (Meucci and Arukwe, 2006; Writer et al., 2010; Gorelick et al., 2011). Jobling et al. (1996) ont exposé la truite arc-en-ciel mâle à quatre AP différents et ils ont mesuré l'induction de la vitellogénine (VTG). La VTG est une protéine du jaune d'œuf hépatique qui est réglée par l'œstradiol des femelles durant l'oogenèse (Arukwe and Goksoyr, 2003). La VTG n'est pas liée au sexe et peut par conséquent être stimulée par l'œstradiol des mâles qui ne manifestent pas la gêne VTG (de Montgolfier, 2008). Puisque l'induction de VTG dépend de la puissance de chaque produit chimique, les auteurs ont pu déterminer le taux d'estrogène de l'OP et du NP comparé à l'EE2 (Jobling et al., 1996). L'OP agit comme un estrogène plus fort que le NP (Jobling et al., 1996). Lorsque la concentration est de 30 µg/L, les taux de VTG induits par l'OP sont équilibrés à 2 ng/L de l'EE2 (Jobling et al., 1996). Alors que le NP partage des similarités avec l'E2, en comparaison, le NP a une affinité moindre pour l'ER et est considéré comme étant un xéno-estrogène faible (Baker et al., 2009).

1.1.3 Biphényls polychlorés

La concentration moyenne de 13 bisphénols congénères chlorés (BPC) dans le MWWE non-traité était de 18 ng/L. Cependant, suivant le traitement, les taux ont été réduits à 6 ng/L (Pham et al., 1997; Pham et al., 1999). Ces concentrations sont moins élevées que celles trouvées dans les effluents des autres villes canadiennes (Harris et al., 2000).

Les BPC sont des composés synthétiques qui sont formés de deux anneaux de phénol et de deux à dix atomes de chlore attachés à la chaîne bisphénol (Simon et al., 2007). Il y a deux groupes distincts de BPC: BPC coplanaire dont deux anneaux phénols sont sur le même plan et non-coplanaire dont les substituts de chlore sont en position ortho (Babut et al., 2009). Les BPC sont utilisés le plus souvent comme liquide de refroidissement et fluides isolants, ainsi qu'en tant que plastifiant dans les peintures, polychlorure de vinyle (PVC) et matériaux ignifuges (Simon et al., 2007). La variation du taux de chloration permet la production de plus de 209 différents congénères, chacun avec son propre taux de toxicité (Ross et al., 2004; Simon et al., 2007). Les BPC sont capables de passer à travers la chaîne alimentaire et peuvent se retrouver à différents degrés chez certains prédateurs (Cleverly, 2005). Grâce aux études, on a découvert que les mélanges de BPC contenant plus d'homologues modérément chlorés, sont plus toxiques à cause de leur affinité avec le récepteur hydrocarboné aryle (AhR) (Giera et al., 2011). L'AhR est un facteur de transcription activé par le ligand qui règle l'activité d'un nombre de gènes qui codent la phase I et II de la biotransformation des enzymes (Giera et al., 2011). Les protéines AhR et aryle de récepteur hydrocarboné nucléaire transporté (ARNT) interagissent avec les éléments de l'ADN sur le site qui règle la synthèse du gène cytochrome P450-1A1 (CYP1A1) (Alexeyenko et al., 2010). Giera et al. (2011) ont démontré que 12 différentes congénères peuvent activer l'AhR causant une induction du CYP1A1, qui introduit l'hydroxyle du BPC pour former des métabolites pouvant agir comme agoniste TR (Van der ven et al., 2005). Van den Berg et al. (2006) ont découvert que parmi ces 12 congénères, 2,3,7,8-tétrachlorodibenzo-p-dioxine (TCDD) est la plus toxique (Giera et al., 2011).

1.1.4 Hydrocarbures polycycliques aromatiques

En 1999, des concentrations d'hydrocarbures polycycliques aromatiques (PAH) dans le MWWE ont été mesurées entre 3.4 à 125.6 ng/L (Pham et al., 1999) soient semblables aux taux trouvés dans le sédiment du port de Sydney (4.02 to 44.8 µg/g) (Smith et al., 2009).

Les PAHs sont une classe hétérogène de produits chimiques (Blaha et al., 2008). Les PAHs les plus communs sont la fluorine, le phénanthrène, et le pyrène. En général, les PAHs contiennent entre deux à quatre cycles de benzène (aromatique) (Pham et al., 1997; Goncalves et al., 2008). La majorité des PAHs ayant un poids moléculaire moins élevé (composés à 2 et 3 cycles benzéniques) viennent du combustible diésel, du pétrole brut et des produits de bois. Tandis que les composés avec un poids moléculaire plus élevé (composés à 4 et 5 cycles benzéniques) sont liés à la combustion incomplète des fossiles, des feux de forêt, et certains sont des produits dérivés des organismes en décomposition (Pham et al., 1997; Blaha et al., 2002; Dahle et al., 2003; Yanagida et al., 2011). Les PAHs ont des caractéristiques hydrophobiques fortes qui leur permettent de résister à la biodégradation et de s'attacher aux sédiments et à la boue (Roger et al., 1996).

Les PAHs sont liés aux effets de gènes non toxiques, de gènes toxiques et estrogènes toxiques dans les organismes exposés (Babich and Borenfreund, 1991; Blaha et al., 2002). Les effets des gènes toxiques incluent l'activation des gènes au travers de la voie AhR (Bols et al., 1999), l'activation mitogène de la protéine kinase (MAPK) (Rummel et al., 1999), l'inhibition du signal intracellulaire (Rummel et al., 1999) et les modifications des taux de Ca^{2+} (Tannheimer et al., 1997). Les embryons de poissons exposés aux concentrations de PAHs dans l'environnement ont démontré des symptômes semblables à la maladie des sacs bleus, incluant une croissance réduite, en plus d'une réduction du vitellus et de l'œdème péricardique, ainsi que l'hémorragie sous-cutanée (Hawkins et al., 2002; Carls et al., 2008).

On a démontré que les PAHs emploient des effets anti-estrogènes en réduisant les taux de E2 circulants dans les flets (Monteiro et al., 2000). Par conséquence, les taux de VTG sont réduits (Monteiro et al., 2000). On pense que cette réduction en E2 endogène est le résultat de l'excrétion biliaire E2 plus élevée dans le foie d'un poisson exposé au PAH (Nicolas et al., 1999). Il est possible que les caractéristiques anti-estrogènes du PAH soient facilitées par

l'activation du AhR, qui, par conséquent inhibe la ER (Nicolas et al., 1999). Dans les conditions synergique où une réaction d'estrogènes est possible, les PAHs ont aussi démontré un faible taux d'estrogènes (Nicolas et al., 1999).

1.1.5 Métaux lourds

Les métaux lourds sont des polluants inorganiques comme le fer (Fe), le cuivre (Cu), le chrome (Cr), et le zinc (Zn), qui sont essentiels pour les activités métaboliques en concentrations modérées, mais peuvent devenir toxiques lorsque les taux augmentent (Qadir et al., 2011). La bioaccumulation du métal dans les organes des poissons dépend de la méthode d'absorption, de l'accumulation, de la régulation et de l'excrétion (Qadir et al., 2011). L'absorbation et l'accumulation peuvent se produire au travers de multiples mécanismes, incluant l'ingestion de la nourriture et des particules, l'échange des ions de métal à travers les bronches ou la peau, et l'absorption de l'eau (Qadir et al., 2011).

Dans le MWWE, l'aluminium (Al), Cr, le manganèse (Mn), Fe, le cobalt (Co), le nickel (Ni), et le plomb (Pb) représentent moins de 1 % du MWWE tandis que Cu, Zn, et Cadmium (Cd) représentent entre 3 à 5 % (Gagnon et al., 2003; Gobeil et al., 2005). Le MWWE est une source majeure de concentration d'argent (Ag) qui atteint le fleuve Saint-Laurent et s'attache au sédiments (Gobeil et al., 2005).

1.2 Effets des mélanges

La plupart des contaminants environnementaux existent sous forme de mélange de composés qui peut agir selon une manière inhibitrice, additive ou synergique (Gardner Jr et al., 1998; Monosson, 2005; Sexton and Hattis, 2007). Les sources industrielles et domestiques contribuant aux toxines dans les eaux usées, favorisent la création de mélanges complexes (Monosson, 2005; Sexton and Hattis, 2007; Filby et al., 2007). Les composés industriels BPC, xenoestrogènes, PCPP, PAHs et autres polluants industriels sont parmi les groupes chimiques les plus fréquemment étudiés (Kortenkamp et al., 2008). Lorsque les produits chimiques agissent ensemble pour arrêter l'activité normale des organismes, ils peuvent avoir un effet négatif sur les fonctions métaboliques et cellulaires (Kortenkamp et al., 2008). Des actions inhibitrices peuvent se produire lorsqu'un de ces produits chimiques fait augmenter le taux d'excrétion d'un autre ou

en bloquant directement ses actions toxiques (Kortenkamp et al., 2008). Les toxines qui ciblent les processus enzymatiques du foie peuvent modifier les processus de détoxification, permettant aux contaminants de s'accumuler dans l'organisme (Luckenbach and Epel, 2005). Grâce aux études, on a pu démontrer que les congénères du BPC de type dioxine induisent des effets toxiques en agissant avec l'AhR. Cependant, il y a peu d'études qui ont examiné les effets d'un mélange de congénères du BPC. (Besselink et al., 1998). Leurs résultats ont démontré que les mélanges de congénères du BPC peuvent avoir des effets inhibiteurs sur l'induction du CYP1A dans le foie du flet (Besselink et al., 1998). Ces résultats suggèrent que les congénères du BPC interviennent avec l'activité catalytique, ayant un effet inhibiteur (Besselink et al., 1998).

Des études ont démontré que l'exposition au mélanges de contaminants contenant des produits chimiques composés d'estrogènes peuvent avoir des effets inhibiteurs sur le processus de reproduction, incluant la production des œufs et le développement des caractères sexuels secondaires (Jobling et al., 1996; Brian et al., 2007; Thorpe et al., 2009). On a démontré que l'exposition aux produits chimiques composés d'estrogènes individuels inhibe la reproduction chez les poissons qui se trouvent dans les tuyaux d'évacuation des eaux usées (Jobling et al., 1996; Thorpe et al., 2009). Une paire reproductrice de vairons a été exposée aux effluents contenant des estrogènes environnementaux pour déterminer le succès de leur reproduction (Thorpe et al., 2009). Les vairons ont été exposés durant trois semaines à un des trois groupes d'effluents avec différents degrés d'estrogènes (Thorpe et al., 2009). L'étude a prouvé que le degré d'activité de l'estrogène des effluents était proportionnelle aux effets observés sur le succès de la reproduction (Thorpe et al., 2009). L'effluent avec le plus d'estrogènes avait l'effet le plus inhibiteur sur la production des œufs et l'effluent avec le plus faible taux d'estrogènes avait aucun effet sur la production des œufs (Thorpe et al., 2009). Seulement deux des trois mélanges avaient des taux d'estrogènes assez élevés pour altérer la reproduction et induire des taux de VTG supérieur à 1 mg/ml (Thorpe et al., 2009). Les xéno-œstrogènes de l'effluent sont capables d'altérer le succès de la reproduction et peuvent modifier les trois sous-types ER chez les poissons pouvant ainsi augmenter les effets et stopper la production d'œufs chez les poisson exposés (Thorpe et al., 2009).

Les mélanges contenant des contaminants avec des modes d'action similaires peuvent réagir de manière additive et avoir des conséquences supérieures à la somme de leurs effets individuels

(Faust et al., 2003; Kortenkamp et al., 2008). Les composés chimiques ayant un effet additif interagissent le plus souvent avec le même sous-système que celui de tout organisme vivant et contribuent à la toxicité d'un mélange, dépendamment de la dose (Kortenkamp et al., 2008). Les eaux usées sont souvent composées de classes de contaminants qui, individuellement peuvent altérer le métabolisme des toxines, le processus de reproduction, les signaux cellulaires et générer des réactions de stress. Ces classes de contaminants, lorsque combinées, peuvent avoir des effets négatifs sur tous les processus cités ci-dessus (Faust et al., 2003; Kortenkamp et al., 2008).

Les échantillons des queues à tache noire dans les remous l'effluent MWWE dans le fleuve Saint-Laurent ont montré une spermatogenèse retardée, une augmentation des taux de VTG, des caractéristiques intersexuels et une motilité de sperme réduite (Aravindakshan et al., 2004b). De plus, les ombles de fontaine (Salvelinus fontinalis) exposés au MWWE ont démontré que l'induction de la VTG apparait seulement suivant une exposition à long terme, suggérant que les composés chimiques responsables des effets ostrogéniques doivent s'accumuler chez les poissons (de Montgolfier et al., 2008). Puisque le MWWE contient un grand nombre de composés chimiques, il est probable que l'accumulation à court terme de ceux-ci dans l'effluent peut inhiber les effets ostrogéniques des xenoestrogènes et des hormones naturelles dans l'effluent. Suite à l'exposition à long terme, la bioaccumulation de certains composés chimiques peut être suffisante pour faire pencher la balance vers les effets ostrogéniques et l'induction de la VTG.

1.3 Jonctions lacunaires

Les jonctions lacunaires (GJ) sont des amas de canaux qui passent à travers les membranes, permettant aux cellules voisines de communiquer par l'échange de petites molécules incluant: des messagers secondaires (Ca^{2+}), des nucléotides, des ions, des siARNs et des métabolites (Goodenough et al., 1996; Sohl et al., 2004; Cruciani et al., 2006; Plante et al., 2007). Les GJ se réunissent typiquement en groupe de 10 à 10.000 canaux sur la surface de la membrane du plasma, que l'on nomme des plaques (Vinken et al., 2006). Les canaux GJ sont composés de deux demi-canal, un de chaque cellule (Cruciani et al., 2006; de Montgolfier et al., 2007). Six connexines (Cx) s'amalgament pour former un hémi-canal. Un hémi-canal peut être homologue, composé de la même Cx, ou peut être composé de deux ou plusieurs Cx différentes. (Cruciani et

al., 2006). La différence structurelle parmi les hémi-canaux composés de différentes Cxs déterminera la perméabilité des canaux aux molécules (Echevarria and Nathanson, 2004).

Les Cxs sont une famille de protéines passant à travers les membranes divisée en sous-groupes α, β, ε, y, et σ, en ajoutant un chiffre indiquant l'ordre de leur découverte, comme par exemple la jonction lacunaire alpha 1 (GJA1) (Vinken et al., 2006; Li et al., 2010). Une deuxième nomenclature plus commune est basée sur le poids moléculaire (en kDa) de chaque Cx. Par exemple, Cx43 est 43 kDa (ou GJA1) (Vinken et al., 2006; Li et al., 2010).

Différents types de cellules peuvent exprimer plusieurs Cxs (Rackauskas et al., 2010). Trois Cx se trouvent dans le foie des mammifères, Cx43 (GJA1) se trouve dans les cellules épithéliales biliaires et non-parenchymes, Cx32 (GJB1), et Cx26 (GJB2) se trouvent dans les hépatocytes (Yang et al., 2003; Plante et al., 2007; Vinken et al., 2008; Fukumasu et al., 2010; Rackauskas et al., 2010; Vinken et al., 2011). Cx32 se trouve à travers les lobules et on pense qu'elle est le composant majeur de la GJ du foie (Yang et al., 2003). Cx26 se trouve dans la les régions périportales des lobules hépatiques (Yang et al., 2003). Par conséquent, chaque Cx fait partie de la création des GJ ayant des fonctions physiologiques uniques.

La fluidité des molécules à travers le canal GJ se nomme « la communication de la jonction lacunaire intercellulaire » (GJIC) et est importante pour le contrôle de l'homéostasie cellulaire (Vinken et al., 2006). GJIC est responsable des processus comme la transcription du gène Cx et la dégradation du GJ (Vinken et al., 2006). La formation déficiente ou impropre du GJ est associée à un nombre de maladies, incluant des formes de neuropathies, la surdité héréditaire, les cataractes, les maladies de la peau, les maladies du cœur et le cancer (Willecke et al. 2002). Les sites de phosphorylation qui se trouvent sur la carboxy-terminal, et la composition du connexon, détermineront les caractéristiques de perméabilité des canaux (Warn-Cramer and Lau, 2004).

Chez les mammifères, incluant le rat, la souris, la vache et l'humain, on a identifié des Cx43, Cx32 et Cx26 dans le foie à l'aide d'un microscope électronique à balayage (Paul, 1986; Chen et al. 2012; Parthasarthi, 2012). A ce jour, Cx43 est le seul Cx identifié dans le foie des poissons et dans le dard-perche (Christie et al., 2004; Cruciani et al., 2006). Les GJs jouent un rôle important dans la régulation de la fonction du foie en régulant l'émission de glucose, la sécrétion de la bile,

la croissance hépatocellulaire et la suppression de la formation des tumeurs (Echevarria and Nathanson, 2004). Bien qu'on ait identifié seulement un seul type de Cx dans le foie des poissons, il est possible que ces mêmes fonctions soient contrôlées par le GJIC et peut-être par des Cxs inconnus.

Cx43 est un Cx ubiquiste se trouvant chez les mammifères et a été identifié chez quatre espèces de poissons: dard-perche, truite arc-en-ciel, omble de fontaine et carpe commune (Van der Heyden et al., 2004; de Montgolfier et al., 2007, 2008). C'est un homologue du Cx43 chez la carpe, truite arc-en-ciel et GJA1 chez le rat et l'humain (Chatterjee et al., 2001; Cheng et al., 2003; Christie et al., 2004; Chatterjee et al., 2005; Cruciani et al., 2006; de Montgolfier et al., 2007).

L'analyse immunohistochimique a déterminé que chez les mammifères, le Cx32 est le Cx dominant dans les hépatocytes (Bruzzone et al.1996; Bone et al., 1997; Echevarria and Nathanson, 2004; Laird et al., 2005). Durant le différentiation hépatocyte, les taux du Cx32 mRNA augmentent considérablement et jouent un rôle en soutenant la structure normale et l'organisation des hépatocytes (Yang et al., 2003; Echevarria and Nathanson, 2004). Le Cx32 est présent dans le foie en quantité approximativement 10 fois plus importante que le Cx26. Il est suggéré que le Cx32 joue un rôle dans la stabilisation des taux Cx26 (Plante et al., 2002; Echevarria and Nathanson, 2004). Le Cx32 joue un rôle important en formant une fusion entre les hépatocytes voisines pour une bonne distribution des messagers secondaires faisant partie des fonctions uniques du foie (Yang et al., 2003). Les canaux formés entre les hépatocytes peuvent être homotypiques, contenant seulement le Cx32, ou hétérotopique pouvant contenir le Cx26, permettant la transmission spécifique des messages (Yang et al., 2003).

Des altérations dans les taux de Cx32 et l'induction des iso-enzymes CYP P450 peuvent être importantes à la biotransformation des xénobiotiques (Vinken et al., 2008). Une deuxième fonction importante du foie, étant un élément du GJIC, est l'excrétion de la bile du foie (Vinken et al., 2008). L'excrétion de la bile est un processus d'élimination pour la dégradation des xéno-biotiques (Di Giulio et al., 2008). L'écoulement de la bile s'amorce au niveau des hépatocytes et du conduit de la bile épithéliale, et nécessite le GJIC dépendant du signal Ca^{2+}. L'action

sécrétoire de la bile dans le foie est contrôlée par des fluctuations des taux de Ca^{2+} (Echevarria and Nathanson, 2004).

Le Cx26, qui est aussi présent dans les hépatocytes, suit un trajet unique durant la formation du GJ alors qu'il contourne l'appareil Golgi durant la translocation de la membrane plasma (Garcia-Rodriquez et al., 2011). Le Cx26 est le seul Cx mentionné qui n'est phosphoré, peut-être à cause de sa carboxy-terminal plus courte (Traub et al., 1989; Laird et al., 2005). On en connaît peu à propos du rôle que le Cx26 joue dans la fonction du foie, cependant des études ont démontré que la surexpression du Cx26 et Cx32 peut servir de fonction anti-cancéreuse (Echevarria and Nathanson, 2004).

Le foie est la cible de plusieurs xénobiotiques à cause de son rôle dans le processus de détoxification. Plusieurs fonctions spécifiques du foie nécessitent le GJIC pour régler la sécrétion de l'albumine, la détoxification de l'ammoniac, la glycogénolyse, la sécrétion de bile et la biotransformation de la phase I des xénobiotiques (Vinken et al., 2008). Les enzymes métaboliques xénobiotiques sont chargés de modérer la toxicité des composés chimiques et de protéger les organismes en métabolisant les contaminants rapidement pour les éliminer (Vinken et al., 2008). Les modifications des éléments de ce système, soit par la présence ou la phosphorylation du Cx, soit par la modification d'une des protéines kinases impliquées dans la phosphorylation du Cx, peut avoir un effet important sur le GJIC et les fonctions qu'il contrôle. Ainsi, les contaminants peuvent modifier le GJIC en modifiant les taux de Cx et sa phosphorylation. Des études précédentes ont démontré que les contaminants individuels et les mélanges peuvent modifier le GJIC. Pour comprendre les mécanismes fondamentaux de ces voies complexes, il faut considérer une approche plus globale et génomique.

Les contaminants ciblant le foie peuvent altérer la manifestation du Cx, ce qui peut détériorer le GJIC et ainsi avoir des conséquences négatives sur les conditions homéostatiques (Habeebu et al. 1998; Vinken et al., 2008). L'exposition grave au Cd peut induire la prolifération des cellules et endommager foie (Habeebu et al. 1998). Les souris adultes mâles ont reçu une injection de 0.5 mg/L $CdCl_2$ et des échantillons ont été prélevés à 1.5, 3, 6, 9, 14, 24 et 48 heures pour déterminer les délais des réactions. (Jeong et al., 2000). Une seconde expérience a été menée à

des fin de comparaison (Jeong et al., 2000). Les souris mâles ont reçu une injection de 0.9 µg/L, 1.83 µg/L, 3.6 µg/L, 5.5 µg/L, 7.2 µg/L et 11 µg/L et des échantillons du foie on été prélevés 9 heures plus tard (Habeebu et al., 1998; Jeong et al., 2000). L'activité GJIC a été mesurée en utilisant un colorant fluorescent; Lucifer Yellow CH (Jeong et al., 2000). On a conclu que le Cd a causé une inhibition de GJIC dépendante du temps et de la dose de Cd (Jeong et al., 2000). Les taux de mRNA Cx26 et Cx32 se sont auto-régulés également selon la dose et le temps (Jeong et al., 2000). Les découvertes suggèrent que le Cd provoque une réduction des protéines Cx26 et Cx32 et du nombre de canaux GJ par cellule (Jeong et al., 2000). Cette inhibition s'est produite avant l'apoptose provoquée par le Cd, suggérant que le GJIC peut jouer un rôle de régularisation dans l'induction de l'apoptose par le Cd (Jeong et al., 2000). La toxicité cellulaire du Cd a été attribuée en partie à l'induction du stress oxydatif, qui a démontré l'inhibition du GJIC (Upham et al., 1997; Shaikh et al., 1999). Le niveau d'inhibition le plus élevé est atteint à 6h, tandis que le niveau maximum de l'apoptose s'est produit à 9h (Habeebu et al., 1998; Jeong et al., 2000). Le GJIC réduisait et l'apoptose augmentait au cours du temps alors que la dose augmentait (Jeong et al., 2000). Ces découvertes suggèrent que le GJIC joue un rôle dans l'initiation de l'apoptose provoquée par le Cd (Jeong et al., 2000).

1.4 Génomiques

Les toxico-génomiques fournissent les ressources pour examiner un nombre important de gènes, de protéines et de métabolites physiologiques des organismes suivant l'exposition à un stress environnemental ou un produit toxique (Ankley et al., 2006). Alors que la technologie et les ressources ont évolué, l'étendue de l'analyse a également évolué. L'étude se portait auparavant sur de centaines de gènes s'attachant aux puces à ADN, et désormais à des milliards de gènes (Ankley et al., 2006). La technologie des puces à ADN actuelle permet au génome partiel ou entier d'un organisme de s'attacher à un substrat, permettant ainsi la comparaison de plusieurs traitements lors d'une seule et même expérience (Hook et al., 2006; Ankley et al., 2006). Avant la technologie génomique, on étudiait les gènes individuellement, ignorant les interactions complexes et les cascades de voies qui peuvent désormais être mis en lumière avec les puces à ADN (Ankley et al., 2006; Hook et al., 2006). L'examen de ces voies est nécessaire, en particulier pour évaluer les effets des petites doses qui peuvent causer des effets cellulaires et moléculaires subtils, mais cruciaux.

L'analyse par puce à ADN peut aider à identifier les profils individuels des contaminants dans un mélange qui agissent individuellement ou en synergie (Monosson, 2005; Sexton and Hattis, 2007). Garcia-Reyero et al. (2009) ont employé une approche génomique pour comparer les profils des hormones stéroïdes individuelles avec celles d'un mélange d'estrogènes et un antagoniste d'ER, appelé ZM-182780 (ZM) (Garcia-Reyero et al., 2009). La composition du mélange a démontré que le ZM a la capacité de bloquer les effets de quelques gènes activés par l'estrogène (Garcia-Reyero et al., 2009). Cependant, il ne bloque pas les effets d'estrogènes pour tous les gènes, indiquant deux modèles différents dans les conditions individuelles ou de mélange (Garcia-Reyero et al., 2009). Grâce à cette étude, on a aussi découvert que les gènes ont été affectés par le mélange mais pas par les contaminants individuels (Garcia-Reyero et al., 2009). L'analyse des voies a isolé la voie de la synthéase prostaglandine-endopéroxyde 2 (PTGS2) affectée par le mélange de l'EE2 et du ZM (Garcia-Reyero et al., 2009). Le PTGS2 est causé presque deux fois plus par l'EE2 uniquement, mais le mélange n'avait aucun effet (Garcia-Reyero et al., 2009). Le PTGS2 est impliqué dans la synthèse des prostaglandines de l'acide arachidonic et est influencé par l'E2 dans le tissu mammalien (Shah, 2005). Le PTGS2 active le signal transducteur et l'activateur de la transcription 1 (STAT1) qui sont utilisés dans la voie kinase Janus (JAK) JAK-STAT, importante pour l'immunité et l'apoptose. En général, ces découvertes laissent supposer une réaction cellulaire de survie, en augmentant l'immunité et l'apoptose et en réduisant la prolifération cellulaire pour empêcher le développement du cancer.

Ces études mettent l'accent sur l'importance d'utiliser des analyses par puce à ADN afin identifier les mécanismes de toxicité, et de comprendre les effets des perturbateurs de l'endocrine. Ensemble, les indicateurs cellulaires (telles que les jonctions lacunaires) et le profilage de l'expression des gènes, devraient fournir des outils puissants pour comprendre les mécanismes de toxicité et pour prédire les conséquences pathologiques associées à l'exposition aux mélanges complexes comme le MWWE.

4.0 Objectifs

On en connait peut sur les xénobiotiques et comment elles affectent la communication moléculaire dans le foie. Le premier objectif de cette étude est d'identifier quels Cx sont présents

dans le foie du l'omble de fontaine. Le second objectif est de déterminer si les taux de Cx dans le foie sont altérés suivant l'exposition aux concentrations classées du MWWE.
Une deuxième étude à pour objectif d'identifier les altérations des voies principales régulatrices dans le foie du mené à grosse tête exposé à 20 % de MWWE, et ce, en utilisant des génomes pour évaluer les changements potentiels dans la communication du foie.

5.0 Résultats

5.0.1

Identification des connexines hépatiques

L'amplification de l'ARNm hépatique par le RT-PCR avec les amorces d'ADN F1R1 a produit deux amplicons, 605 bp et 425 bp. Ce produit résultant, l'ADNc amplifié, est séquencé et une séquence homologue a été identifiée par BLAST (GenBank, National Center for Biotechnology Information, Bethesda, MD). Il a été confirmé que les deux amplicons étaient le Cx43. Bien que l'on ait observé deux amplicons pour Cx43, l'amplicon à 425 bp fait partie d'un segment d'un amplicon plus large trouvé à 605 bp. L'amplification des amorces d'ADN F2R2 a produit un amplicon de 450 bp. Cette séquence et ses homologues ont indiqué que cet amplicon de DNAc était le Cx30.

En utilisant des outils de séquençages multiples, on a pu déterminer que le Cx43 et le Cx30 sont alignés avec les séquences connues des autres mammifères. Le Cx43 dans le foie de l'omble de fontaine a démontré une homologie de 100 % au celui du dard-perche, de 75 % à celui de la truite arc-en-ciel et de 30 % avec la GJA1 de souris et du rat. L'alignement de la séquence du Cx30 du foie de l'omble de fontaine a démontré une homologie de 38 % avec la jonction lacunaire beta 6 (GJB6) de la souris et de 33 % avec celle du rat.

L'effet de MWWE sur les connexines hépatiques

Après 12 semaines d'exposition à 0 % (témoin), 1 %, 10 % et 20 % de MWWE, les taux d'ARNm de Cx30 et Cx43 chez l'omble de fontaine ont démontré des modifications, comparées aux groupes témoin. Les taux d'ARNm Cx43 du groupe témoin n'ont pas démontré une différence notable. Les taux d'ARNm Cx43 dans le foie des poissons exposés à 10 % et 20 %

étaient un peu moins élevés que dans le groupe témoin mais ce n'était cependant pas significatif. Les taux d'ARNm Cx30 hépatiques étaient moins élevés dans le groupe exposé à 1 % que dans celui du groupe témoin. En revanche, les taux étaient considérablement plus élevés dans les groupes exposés à 10 % et 20 % que dans ceux du groupe témoin.

5.0.2

L'analyse par puces à ADN

La puce à ADN du méné à grosse tête utilisée dans cette étude contenait 15,208 gènes. Un total de 309 gènes différents sont apparus dans les tissus hépatiques des poissons exposés avec un taux deux fois plus élevés que celui de l'ARNm témoin. Des 309 gènes différents, 118 ont démontré une croissance de gènes et 191 ont démontré une décroissance de gènes.

Immunotransfusion

Une analyse d'immunotransfusion a été faite pour déterminer si le taux de CTNNB1, le médiateur principal dans la voie de signalisation Wnt, était affecté par l'exposition au MWWE. Les taux de protéine CTNNB1 ont été mesurés selon des groupes cytosolique et des fractions nucléaires du foie chez le méné à grosse tête dans le groupe témoin et le groupe de vairons exposés. Les taux de protéine CTNNB1 dans le groupe cytosol et du groupe de fractions nucléaires des vairons exposés étaient moins élevés que ceux dans le groupe témoin, mais ce n'était cependant pas significatif.

6.0 Discussion

6.0.1

Connexines hépatiques

Dans l'étude présentée, nous avons démontré qu'il y avait deux Cx présents dans le foie de l'omble de fontaine: Cx43 et Cx30. Une étude précédente a indiqué la présence du Cx43 dans le foie du dard-perche (Cheng et al., 2003), et la présence du Cx43 est clairement établie dans le foie des mammifères (Echevarria and Nathanson, 2004; Cruciani et al., 2006; Vinken et al.,

2008). Lorsque l'on a comparé le Cx43 chez l'omble de fontaine à la séquence du GJA1 mammalien chez la souris et le rat, on a trouvé une homologie de 30 %.

La présence de Cx30 dans le foie de l'omble de fontaine (et chez les poissons en général) est originale puisque le Cx30 n'a jamais été détecté dans le foie des êtres humains et des rongeurs auparavant. Lorsque l'on a analysé le taux de Cx30 avec la technique BLAST, on a identifié une homologie de 30.3 % avec le GJB6 chez la souris et 30.3 % également avec Cx26 chez les êtres humains. Il est intéressant de voir que le GJB6 chez la souris n'est pas présent dans le foie, mais il est localisé dans l'oreille. On a cependant trouvé du Cx26, Cx32 et Cx43 dans le foie des rongeurs (Vinken et al., 2008). Le Cx26 chez les êtres humains se trouve dans la peau, l'oreille et dans les hépatocytes (Plante et al., 2002; Echevarria and Nathanson, 2004). Les écarts sont probablement attribués aux différences d'évolution entre les poissons et les mammifères. Cependant, des recherches complémentaires sont nécessaires afin de localiser le Cx43 et le Cx30 dans le foie des poissons et pour élucider leur rôle.

Les résultats de cette étude ont démontré une augmentation du Cx30 dans le foie de l'omble de fontaine exposé aux deux concentrations d'effluent les plus élevées après 12 semaines. Les études précédentes ont démontré que l'exposition au MWWE contenant une grande variété de xéno-estrogènes, peut altérer le taux de Cx dans les testicules des poissons. Ceci crée une spermatogenèse retardée, en plus d'une motilité et d'une concentration de spermes réduites (Aravindakshan et al., 2004a,b; de Montgolfier et al., 2007). Selon nos recherches, il n'y a aucune publication portant sur la présence du Cx dans le foie de l'omble de fontaine et il n'existe aucun rapport explicitant les effets de l'effluent sur le foie des poissons.

Les résultats de cette étude suggèrent que l'omble de fontaine exposé aux concentrations du MWWE, pendant une période de 12 semaines, montrent des taux de Cx altérés. Des études semblables ont montré que les taux de Cx altérés peuvent causer l'interruption du GJIC qui est essentiel pour maintenir l'homéostasie dans les d'organes.

L'analyse par puce ADN

L'exposition des poissons au MWWE génère une variété de changements physiologiques incluant la reproduction et le dysfonctionnement immunitaire. Peu d'études ont examiné les

implications des xénobiotiques dans le foie, malgré son emplacement dans le corps et son rôle dans la biotransformation des produits toxiques (Vinken et al., 2008). La signalisation cellulaire est requise pour que le foie puisse effectuer ses fonctions primaires incluant le glycogénolyse, la sécrétion de la bile et la biotransformation xénobiotique (Vinken et al., 2008). Des programmes d'analyse des voies ont identifié deux voies altérées incluant les voies anti-estrogènes et Wnt/CTNNB1.

Voie anti-estrogène

L'étude présentée a mis au jour que 13 gènes réglés par l'E2 ont été altérés suivant l'exposition au MWWE à court-terme. Dans cette étude, quatre gènes directement régulés par l'E2 ont été altérés, incluant le NELL2 qui s'est manifesté comme protection contre l'apoptosis via la voie de signalisation ERK (Choi et al., 2010). Trois autres gènes ont démontré une décroissance incluant le HSPA5, qui est régulé par l'E2 en deux directions (Guzel et al., 2011; Watanabe et al., 2003), SPOCK2 qui est positivement régulé par l'E2 dans l'utérus de la souris (Ivanga et al., 2007), et BNIP2 qui se trouve dans les cellules neurales induites sélectivement par l'estrogène (Belcredito et al., 2001).

Huit gènes en aval de l'E2 et indirectement régulés par l'E2 ont été altérés suivant l'exposition au MWWE dans le foie du méné à grosse tête. Ces gènes ont un taux surélevé d'ENPEP impliqué dans la régulation du processus de développement. Il a été précédemment démontré que DNAJC5 augmente en fonction de l'exposition aux températures élevées et d'autres produits toxiques, que PLXNA3 augment lors de l'apoptosis, et que UBQLN4 est impliqué dans la dégradation des protéines. On a globalement observé une inhibition de l'E2, en adéquation avec les résultats précédents, et qu'une exposition au MWWE à court-terme n'est pas suffisante pour induire l'E2. Les taux de VTG ont été mesurés par le qPCR durant la validation des puces à ADN et ont été comparés aux résultats de ces puces à ADN. Il n'y a pas eu d'augmentation notable de VTG mesurée par la puce à ADN ou l'analyse qPCR. Globalement, 13 gènes et la voie anti-estrogène ont été altérés suivant l'exposition à court-terme au MWWE. Dans une étude précédente, l'omble de fontaine a été exposé pendant 4 et 12 semaines, et on a découvert que l'induction ARNm de la VTG chez les mâles et les jeunes poissons s'est produite seulement après 12 semaines d'exposition (de Montgolfier et al. 2008). Les effets anti-estrogènes initiaux

observés dans cette étude défendent la théorie que l'effluent n'a pas d'influence sur les effets d'estrogènes suivant l'exposition à court-terme. On observe des effets seulement suivant l'exposition à long-terme dus à la bioaccumulation de certains contaminants ostrogéniques.

Voie de signalisation de Wnt/CTNNB1

Plusieurs gènes ont été impliqués dans la voie Wnt/CTNNB1, différents de ceux chez le méné à grosse tête exposé. Les résultats de l'étude présentée ont démontré l'induction de la glycoprotéine Wnt4 chez les vairons exposés au MWWE. La voie Wnt est un réseau de protéines conservées qui règlent les signaux entre les cellules durant l'embryogenèse, la différentiation cellulaire, l'adhésion des cellules et le cycle des cellules, en activant les gènes ciblés Wnt (Hendriks and Reichmann, 2002; Monga and Michalopoulos, 2005). Le Wnt est la seule molécule connue à être impliquée dans la détermination du sexe (Liu et al., 2009). Le foie des méné à grosse tête exposés au MWWE a démontré des taux d'ARNm et de LRP6 élevés. Le LRP6 peut activer l'auto-phosphorylation et peut récupérer l'axin et l'APC, ce qui inhibe le GSK3 (Metcalfe and Bienz, 2011). Une inhibition de GSK3 peut déclencher une accumulation de CTNNB1 dans le cytosol et le noyau. L'accumulation de CTNNB1 dans le noyau accroit l'activité de transcription du gène. (Fang et al., 2000).

La voie Wnt peut être activée lors de la formation du complexe Fz-LRP. Le signal du complexe traverse des protéines intermédiaires et et active la molécule dishevelled (dsh). Dsh inhibe la dégradation de la CTNNB1 en récupérant le GSK3β, et rend l'ubiquitination de la CTNNB1 inactive (Lade and Monga, 2011; Monga and Michalopoulos, 2005; Veeman et al., 2003). Le Dkk-1 interagit avec le LRP5/6 et le corécepteur KREMEN1, et empêche la formation du complexe LRP5/6-Wnt-Frizzled (Cadoret et al., 2002; Davidson et al., 2002; Mao et al., 2002; Osada et al., 2006). Le DACT1 s'attache au dsh et empêche la dégradation de la CTNNB1, et améliore l'activité de transcription des gènes ciblés par la voie Wnt. Dans l'étude présente, le méné à grosse tête exposé au MWWE a démontré une décroissance du DACT1, laissant supposer qu'un des mécanismes régulant le taux de la CTNNB1 est inhibé. La signalisation dsh à travers la voie Wnt est possible lors de la transduction par une des deux branches, et est dépendante du gène cible et sa fonction.

En l'absence de signal Wnt, APC, axin et GSK3, la CTNNB1 subit une phosphorylase, ce qui provoque sa dégradation par une protéine E3 ubiquitaire. Cette étude a démontré que les poissons exposés au MWWE ont démontré une augmentation des trois protéines ubiquitaires E3, ARIH2, la classe d'enzymes topoisomérase type 1 sérine-arginine riche (TOPORS) et ubiquitaire 4 (UBQLN4). Un manque de la signalisation Wnt dans le noyau peut agir comme un inhibiteur de la transcription du gène cible en conjonction avec le co-inhibiteur Groucho/AES (Monga and Michalopoulos, 2005). Deux inhibiteurs de la voie Wnt, AES et phosphatase 2A (PPP2R5E) ont démontré une un taux surélevé chez les poissons, laissant supposer une inhibition de la voie Wnt. La réduction de la signalisation Wnt est confirmée par nos observations quant à l'immunotransfusion où il y a une réduction constante des taux des deux protéines.

Les résultats de cette étude laissent penser que l'exposition au MWWE à court-terme démontre une inhibition des signaux estrogènes et des signaux de la voie Wnt. Des études récentes ont démontré que la présence d'E2 inhibe dkk1, permettant la dérégulation des voies Wnt/CTNNB1 (Scott et al., 2012). Des études supplémentaires ont examiné si la privation à long-terme d'E2 peut produire l'affaiblissement du mécanisme de régulation des voies Wnt/CTNNB1(Scott et al., 2012). Les rats femelles ont été privé d'E2 pendant 10 semaines pour déterminer si l'affaiblissement du mécanisme de régulation du dkk1 et de la voie Wnt était évident (Scott et al., 2012). Après 10 semaines, il est évident que l'E2 est responsable de la régulation de la voie Wnt à travers les niveaux basals du dkk1, causant la décroissance de la CTNN1 (Scott et al., 2012). On pense que cette régulation se produit à travers la voie de signalisation p53, qui est réduite durant la privation d'E2 (Scott et al., 2012).

7.0 Conclusions

Il y a deux Cx évidents dans le foie de l'omble de fontaine, Cx30 et Cx43. Suivant l'exposition à court terme au MWWE, il n'y a pas eu changement notable au Cx43. A l'inverse, le Cx30 a augmenté considérablement durant les deux expositions les plus élevées.

L'usage des techniques de la puce à ADN est essentiel pour identifier les voies de signalisation principales pendant l'exposition au MWWE à court-terme. Deux voies ont été repérées par le

programme d'analyse des voies ayant une grande proportion de gènes qui subissent une altération chez les poissons exposés. Ces voies incluent les voies anti-estrogènes et les voies de signalisation Wnt.

Nos résultats laissent supposer que l'exposition au MWWE peut avoir des effets notables sur les voies principales dans le foie, provoquant une interruption de l'homéostasie. Selon nos recherches, c'est la première fois que le Cx a été repéré dans le foie de l'omble de fontaine.

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