Effects of Ibuprofen on Rainbow Trout (Oncorhynchus mykiss) Following Acute and Chronic Waterborne Exposures

by

Monique Robichaud

A Thesis Submitted in Partial Fulfillment of the Requirements of the Degree of:
Master of Science in Applied Bioscience

In
The Faculty of Science
University of Ontario Institute of Technology

August, 2011

© Monique Robichaud, 2011
Abstract

Pharmaceuticals and personal care products are a growing concern in the aquatic environment. Compounds from the class of non-steroidal anti-inflammatory drugs are commonly detected in surface waters and have the potential to negatively affect aquatic organisms. The purpose of this experiment was to determine the acute and chronic effects of ibuprofen on rainbow trout (*Oncorhynchus mykiss*). Cyclooxygenase (COX) activity, vitellogenin (VTG) concentration and ethoxyresorufin-O-deethylase (EROD) activity were evaluated following waterborne ibuprofen exposure of trout to 1 and 10 mg/L in the acute exposure and 1, 32 and 1000 µg/L in the chronic exposure, along with an experimental control, E2 control of 1000 µg/L and an E2-ibuprofen mixed treatment. Ibuprofen did not inhibit COX enzyme activity in either gill or kidney tissue. To evaluate the estrogenic effects of ibuprofen, VTG concentrations were measured; by the end of the 56 day chronic exposure VTG concentrations significantly increased in all of the ibuprofen treatments relative to the controls. EROD activity may have been inhibited by ibuprofen but definitive conclusions could not be made. These findings indicate that more research needs to be done studying ibuprofen in aquatic systems.

**Key Words:** Rainbow trout (*Oncorhynchus mykiss*), NSAIDs, Ibuprofen, Cyclooxygenase (COX), Vitellogenin (VTG), Ethoxyresorufin-O-Deethylase (EROD)
Acknowledgements

I would like to thank all of the people who have helped me complete this thesis during my time at UOIT:

My supervisor Dr. Douglas Holdway for his assistance, expertise and encouragement throughout my project and also for taking a chance on a student who had absolutely no background in aquatic toxicology.

My committee members Dr. Julia Green-Johnson and Dr. Dario Bonetta for their insightful discussions during the planning of my experiments.

Dr. Rodrigo Orrego for his enthusiasm, extensive knowledge and for having the patience to teach and assist me with almost every aspect of my project.

John Guchardi for his continual technical support and for helping design the system necessary for my project to be completed.

Mike Allison for assisting with the chemical analysis of my water samples.

My fellow labmates, Lindsay Beyger, Zacharias Pandelides, Richard Nesbitt and Christopher Bourdages, whom I shared countless hours with in our windowless office and lab. I thank you for helping in whatever way you could and also for your friendship.

Alex, who supported me during every rough patch I hit and listened to all my complaints even though all he thought I did was ‘work with fish.’ I love you.

And my family, for their unconditional love and understanding through all of the weekend lab shifts, rants and tears.
# Table of Contents

Abstract..............................................................................................................................ii

Acknowledgements...........................................................................................................iii

Table of Contents..............................................................................................................iv

Index to Figures..............................................................................................................viii

Index to Tables...................................................................................................................x

Index to Appendices.........................................................................................................xi

List of Abbreviations.......................................................................................................xii

1.0 Literature Review.........................................................................................................1

1.1 Introduction..............................................................................................................1

1.2 Pharmaceuticals in the Environment........................................................................1

1.3 Wastewater Treatment Plants..................................................................................4

   1.3.1 Treatment Processes....................................................................................4

   1.3.2 Factors Influencing Removal......................................................................7

1.4 Environmentally Relevant PPCP Concentrations..................................................8

1.5 Endocrine Disruptors...............................................................................................12

   1.5.1 What are Endocrine Disruptors?..................................................................12

   1.5.2 PPCPs as EDCs............................................................................................13

   1.5.3 Knowledge Gaps in EDC Research............................................................14

1.6 Non-Steroidal Anti-Inflammatory Drugs (NSAIDS).............................................16

   1.6.1 Brief History and Introduction....................................................................16

   1.6.2 Prostaglandins and Cyclooxygenase Enzymes..........................................17

   1.6.3 Mode of Action............................................................................................21
1.6.4 Ibuprofen .................................................................24
1.7 Exposure Techniques .........................................................25
1.8 Biomarkers of PPCP Toxicity in Aquatic Environments ...............27
  1.8.1 Condition Factor ..........................................................28
  1.8.2 Organo-somatic Indices .................................................29
  1.8.3 COX Inhibition .............................................................29
  1.8.4 Vitellogenin (VTG) Concentration ...................................30
  1.8.5 Ethoxyresorufin-O-deethylase (EROD) Activity ..................31
2.0 Purpose of Study ..................................................................34
3.0 Methodology .......................................................................35
  3.1 Test Organisms ..................................................................35
  3.2 Chemicals and Reagents ....................................................35
  3.3 Flow-Through Dose Controlled System ..................................36
  3.4 Experimental Design .........................................................39
    3.4.1 Acute Exposure ..........................................................39
    3.4.2 Chronic Exposure ......................................................41
  3.5 Water Sampling ..................................................................42
  3.6 Sampling ............................................................................43
  3.7 Analysis ..............................................................................43
    3.7.1 Ultra Performance Liquid Chromatography – Mass Spectroscopy .... 43
    3.7.2 Condition Factor .........................................................44
    3.7.3 Liver Somatic Index ......................................................44
    3.7.4 Gonad Somatic Index ....................................................44
3.7.5 Cyclooxygenase Activity Assay....................................................44
3.7.6 Bradford Protein Analysis..............................................................45
3.7.7 Enzyme Linked Immunosorbent Assay for Fish Vitellogenin....................................................................................46
3.7.8 Ethoxyresorufin-O-deethylase Activity Assay....................................................47

3.8 Statistical Analysis...........................................................................................49

4.0 Results.........................................................................................................................50

4.1 Acute Exposure................................................................................................50
   4.1.1 Water Analysis...............................................................................50
   4.1.2 Mortality Data................................................................................50
   4.1.3 K, GSI, LSI....................................................................................53
   4.1.4 Total COX Activity........................................................................55
   4.1.5 Total Plasma Protein & VTG Concentrations................................75
   4.1.6 EROD Activity...............................................................................58

4.2 Chronic Exposure.............................................................................................62
   4.2.1 Water Analysis...............................................................................62
   4.2.2 Mortality Data................................................................................62
   4.2.3 K, GSI, LSI....................................................................................68
   4.2.4 Total COX Activity........................................................................71
   4.2.5 Total Plasma Protein & VTG Concentrations................................75
   4.2.6 EROD Activity...............................................................................80

5.0 Discussion.................................................................................................................83

5.1 Mortality..........................................................................................................83
5.2 K, GSI, LSI.....................................................................................................86
5.3 COX Activity........................................................................................................89
5.4 VTG Concentration..........................................................................................92
5.5 EROD Activity.................................................................................................95

6.0 Conclusions.......................................................................................................99

References...............................................................................................................101

Appendices..............................................................................................................116
Index to Figures

Figure 1: General schematic of WWTPs including treatment phases.................................6

Figure 2: Relative percentages of PPCPs measured in the environment based
on summary of published data...........................................................................................11

Figure 3: Conversion pathway of arachidonic acid to various prostaglandins...............20

Figure 4: Structural configuration of the active sites of the two COX isoforms..............23

Figure 5: Set up of flow-through dose controlled system.................................................38

Figure 6: Mortality from the acute ibuprofen exposure experiment..................................52

Figure 7: Liver somatic index (LSI) from the acute ibuprofen exposure experiment......54

Figure 8: Total COX activity measured from the gill and kidney tissue from the
acute ibuprofen exposure experiment...............................................................................56

Figure 9: Comparison of total COX activity between the gill and kidney tissue
from the acute ibuprofen exposure experiment......................................................................57

Figure 10: Total plasma protein concentrations from the acute ibuprofen exposure
experiment............................................................................................................................59

Figure 11: Vitellogenin (VTG) concentrations from the acute ibuprofen exposure
experiment.............................................................................................................................60

Figure 12: Liver ethoxyresorufin-O-deethylase (EROD) activity data from the
acute ibuprofen exposure experiment...............................................................................61

Figure 13: Jaw damage observed during the chronic exposure...........................................66

Figure 14: Complete LSI data from the chronic ibuprofen exposure experiment..........69

Figure 15: LSI data from the chronic ibuprofen exposure broken down by
sampling time.......................................................................................................................70
Figure 16: Complete kidney COX activity data from the chronic ibuprofen exposure experiment..........................................................................................................72

Figure 17: Kidney COX activity data from the chronic ibuprofen exposure broken down by sampling time..................................................................................................73

Figure 18: Kidney COX activity data from the chronic ibuprofen exposure broken down by sampling times and represented as fold of control.................................................74

Figure 19: Complete plasma protein concentrations from the chronic ibuprofen exposure experiment..........................................................................................................76

Figure 20: Plasma protein concentrations from the chronic ibuprofen exposure broken down by sampling time..................................................................................................77

Figure 21: Complete VTG concentrations from the chronic ibuprofen exposure experiment..........................................................................................................................78

Figure 22: VTG concentrations from the chronic ibuprofen exposure broken down by sampling time..................................................................................................................79

Figure 23: Complete EROD activity data from the chronic ibuprofen exposure experiment..........................................................................................................................81

Figure 24: EROD activity data from the chronic ibuprofen exposure broken down by sampling time ..................................................................................................................82
Index to Tables

**Table 1:** Ibuprofen water concentrations from the acute exposure measured using UPLC-MS..........................................................51

**Table 2:** Ibuprofen water concentrations from the chronic exposure from select treatments measured using UPLC-MS..............................................64

**Table 3:** Mortality data and instances of jaw damage recorded from the chronic exposure experiment..........................................................65

**Table 4:** Number of fish sampled for each treatment during each sampling period for the chronic exposure experiment...........................................67
Index to Appendices

Appendix 1: Settings used for the UPLC-MS system ...................................................115

Appendix 2: Calibration standards prepared for the Bradford protein assay.................118

Appendix 3: Calibration standards prepared for the VTG ELISA................................119

Appendix 4: Calibration standards prepared for the EROD activity assay...............120

Appendix 5: Condition factor (K) calculated for the acute ibuprofen exposure experiment........................................................................................................................121

Appendix 6: Gonad somatic index (GSI) calculated from the acute ibuprofen exposure experiment................................................................................................................122

Appendix 7: Condition factor (K) calculated from the chronic ibuprofen exposure experiment................................................................................................................123

Appendix 8: Gonad somatic index (GSI) calculated from the chronic ibuprofen exposure experiment...........................................................................................................125
List of Abbreviations

7ER = 7-ethoxyresorufin
AA = arachidonic acid
COX = cyclooxygenase
DI = deionized
DMSO = dimethyl sulfoxide
E2 = 17 – β – estradiol
EDCs = endocrine disrupting compounds
FU = Fluorescence units
GSI = Gonad Somatic Index
Ibu = Ibuprofen
K = Condition Factor
LSI = Liver Somatic Index
MS = Mass spectroscopy
NADPH = β – nicotinamide adenine dinucleotide 2’ – phosphate reduced tetrasodium salt hydrate
OTCs = Over the counter drugs
PCR = Polymerase chain reaction
PIE = Pharmaceuticals in the environment
PPCPs = Pharmaceuticals and personal care products
UPLC = Ultra performance liquid chromatography
WWTPs = wastewater treatment plants
1.0 Literature Review

1.1 Introduction

Chemicals used for the prevention and treatment of illness, as well as for individual hygiene, can be collectively described as pharmaceuticals and personal care products, or PPCPs (Corcoran et al., 2010). The frequency and modes of their use makes it likely that PPCPs will ultimately enter water systems and thus PPCPs have become a growing concern in aquatic environments (Bound & Voulvoulis, 2004). Research has shown that there is a direct correlation between the types of pharmaceuticals commonly utilized in society and their presence within the environment (Corcoran et al., 2010). One such class of PPCPs are non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs have the ability to pass through wastewater treatment plants (WWTPs) and are the most documented pharmaceutical in water systems (Santos et al., 2010). While it is accepted that these chemicals are present in the environment, further research is needed to elucidate their effects on non-target aquatic organisms.

1.2 Pharmaceuticals in the Environment

Pharmaceuticals and personal care products have been detected in the environment since the 1970s but it was not until recently that growing concerns about potential environmental effects made them the focus of research. Researchers discussed the effects of estrogenic compounds found in activated sludge on organisms in the mid 1960s but this was largely ignored (Marsalek, 2007). Now, as more work goes into
studying these chemicals a new area of study is emerging known as ‘Pharmaceuticals in the Environment’, or PIE (Khetan & Collins, 2007).

Discharge of PPCPs into the environment occurred unregulated for decades even though it was known that the active compounds may affect non-target organisms (Daughton & Ternes, 1999). Paralleling the emergence of new, sophisticated analytical instrumentation and techniques to measure these drugs, more information is being gathered on the appearance, persistence and activity of PPCPs in the environment (Daughton & Ternes, 1999). It has been estimated that there are approximately 2000 to 3000 different compounds used as human pharmaceuticals (Marsalek, 2007). This number is unquestionably going to grow as the pharmaceutical industry increases the effectiveness of the biologically active ingredients. Work is continuously being done to increase the potency, bioavailability and degradation resistance of pharmaceuticals (Khetan & Collins, 2007). While these characteristics make the pharmaceutical more desirable as a treatment method, it is these same characteristics that increase the potential toxicity in the environment.

Before the effects on the environment can be discussed in relation to PPCPs, it must first be understood how these chemicals make their way into natural systems. For drugs to make their way into the environment, they first need to be used. When analyzing worldwide sales of pharmaceuticals, North America dominates the market with 45%, which is not closely followed by Europe (13%) and Japan (10%) (Corcoran et al., 2010). Global pharmaceutical sales for 2010 were estimated at almost 800 billion US dollars (IMS Health, 2010a). Focusing on Canada, Intercontinental Medical Statistics (IMS) Health stated that when studying the top 10 dispensed therapeutic classes in 2010
over 504 million prescriptions were filled (IMS Health, 2010b). While this number may seem large it is significantly increased when all of the drugs available without prescriptions, called over-the-counter drugs, or OTCs, are considered, making these values greatly underestimated (IMS Health, 2010b; Daughton & Ternes, 1999; Ternes, 1998).

The sheer volume of PPCPs used in society is directly linked to their entrance into the environment. Many of the chemicals detected in water systems are compounds from classes of pharmaceuticals linked to problems associated with Western society, such as pain, anxiety and depression management and cholesterol regulation (Corcoran et al., 2010). This is due to the fact that many pharmaceuticals are excreted as the parent compound or active metabolites through urine and feces. Thirty to ninety percent of the administered dose is excreted as the active chemical, depending on the drug (Halling-Sørensen et al., 1998).

Treated wastewater is the main route for pharmaceuticals to enter the environment (Kotchen et al., 2009). When a person takes a medication, prescribed or illicit, most of the drug passes through the body and is introduced into the sewage system (Bound & Voulvoulis, 2004). The chemical may remain as the active parent compound or may be metabolized into a form which is more or less biologically active than its original state.

In addition to excretion, PPCPs are introduced into the sewage system by improper disposal techniques. A study by Kotchen et al. (2009) found that many people improperly disposed of their pharmaceuticals by flushing them down the toilet or sink. Through the use of a survey, the researchers not only discovered this fact but also that many people did not think of the consequences of this practice. Another minor route of
PPCP entry is from washing and bathing. Once in the system, the pharmaceuticals make their way to the treatment plants.

1.3 Wastewater Treatment Plants

1.3.1 Treatment Processes

As stated previously, WWTP effluents are the main route of PPCPs into the environment. Upon excretion via urine and feces, these chemicals are able to enter municipal sewage treatment plants (Choi et al., 2006). A schematic of the general treatment phases of a standard WWTP can be found in Spellman (2009) (Figure 1). The general treatment stages can be defined as pretreatment, primary treatment, secondary treatment, tertiary treatment and sludge treatment (Spellman, 2009).

An initial pretreatment step is the first stage for incoming waters (Carballa et al., 2004). Large contaminant material, such as garbage and other waste products, are physically removed from the influent waters by the use of coarse bar screens (Carballa et al., 2004). This process may be supplemented by other removal techniques, including shredding and grit removal (Spellman, 2009). Once the physical removal of large materials is accomplished, the partially cleaned influent waters move into primary treatment.

In primary treatment, influent waters are pumped into large tanks called clarifiers, or sedimentation tanks (Carballa et al., 2004). In these circular holding basins, the water is held to allow for separation of heavier particulate material. Some material will settle to the bottom, such as dirt, and some will float to the top, such as lipids. Once this has
occurred, mechanical arms sweep the tanks and remove the particulate matter as sludge (Spellman, 2009). This sludge is collected for further processing.

Secondary treatment is the next stage in the purifying process (Carballa et al., 2004). From the clarifiers, the wastewater moves into the biological reactor. The biological reactors are specialized tanks where degradation takes place using controlled bacterial populations (Spellman, 2009). Under either aerobic or anaerobic conditions, the bacteria degrade or sequester contaminant material before the water moves to secondary sedimentation tanks (Caraballa et al., 2004; Fent et al., 2006). This second sedimentation tank is to allow for any bacteria that may have escaped the biological reactor to be recollected as sludge and added back into the treatment system. Excess sludge from this stage is combined with sludge collected from the primary treatment and processed for other uses, such as dispersal on agricultural fields (Santos et al., 2010).

Depending on the type of WWTP and its location, the treatment water may be released back into the environment or may go through a final treatment stage, known as tertiary treatment. This generally consists of final disinfection, such as ozonation or chlorination, as well as treatment steps to remove nutrients through metal flocculation (Daughton & Ternes, 1999). The United States commonly uses chlorine as a disinfectant whereas European countries, and Canada, generally use ozonation (Synder et al., 2003). Once this stage is completed effluent waters are released into receiving environmental systems.
Figure 1: General schematic of WWTPs including treatment phases. Treatments include pretreatment, primary treatment, secondary treatment and tertiary treatment for influent waters. Sludge treatment processes the solid material collected from the water treatment phases for other uses. Modified from Spellman (2009).
1.3.2 Factors Influencing Removal

PPCPs are removed through two main processes involved in sewage treatment: adsorption and biological degradation (Fent et al., 2006; Santos et al., 2010). Removal of pharmaceuticals with suspended particulate material is highly dependent upon the chemical structure of the individual compounds. Their hydrophobic and electrostatic interactions with particulate material are essential for removal. Compounds with low adsorption coefficients will tend to stay in the aqueous phase (Carballa et al., 2004). This is also the case with acidic pharmaceuticals, such as NSAIDs, with low pKa values. At neutral pH, these compounds will be in their ionic form and will have a greater tendency to stay within the water column and not precipitate out (Fent et al., 2006). When compounds are mainly found in the dissolved phase, biological degradation becomes the main removal method. Removal is often imperfect due to the fact that WWTPs were not designed to handle pharmaceuticals (Christen et al., 2010; Khetan & Collins, 2007).

Microorganisms in the activated sludge systems are most efficient at removing carbon, nitrogen and other microbial contaminants. Many PPCPs are synthetic chemicals which are not natural to the environment (Carballa et al., 2004). Because they are not naturally found they may not be effectively removed. There are two main schools of thought about this reduction in removal efficacy. First, it is believed that removal is low due to the tendency for the chemicals to be present in trace amounts (Christen et al., 2010). Also, with the pharmaceutical industry booming, new pharmaceuticals are continuously being introduced to the ever expanding market. This means that more and more pharmaceuticals are passing through WWTPs that the bacteria in the reactors have never encountered previously (Daughton & Ternes, 1999). Since the bacteria have never
‘seen’ the new chemicals before, there is no selective pressure for them to have the mechanisms necessary for their degradation.

In addition to the actual mechanisms within the WWTPs that facilitate PPCP removal, there are other external factors that influence the degree to which the drugs are removed. The construction of the treatment facility itself, along with the types of treatment steps performed, affect removal. One very significant factor is retention time, or the length of time it takes influent waters to pass through the treatment stages (Fent et al., 2006). The longer the influent waters remain in each treatment step the greater the potential for degradation. Longer retention times may help to counteract the low concentration of the PPCPs and help to increase biological degradation. Another fact is seasonality, which can greatly alter removal efficiency in the biological reactors, by which variation in light intensity and temperature are key factors in the removal process (Khetan & Collins, 2007). A study conducted in Finland measured the concentrations of a number of drugs, including ibuprofen, in effluent waters over 3 seasons. It was found that in winter months there was a significant reduction in drug removal which resulted in, depending on the pharmaceutical, up to a 5 fold increase in their concentrations in the effluent waters (Khetan & Collins, 2007). It is a combination of all of these factors that leads to pharmaceuticals and other personal care products entering environmental systems at biologically relevant concentrations.

1.4 Environmentally Relevant PPCP Concentrations

Once through the WWTPs, pharmaceuticals can be detected in effluent waters, and in surface waters, in the ng/L to µg/L range (Corcoran et al., 2010). The factors
influencing these concentrations have already been discussed (ie. use in society, human metabolism and excretion, variations in WWTP removal efficiencies) but it should be noted how extensively these chemicals can be found within the environment. Pristine environmental areas, far from urban centers with large populations, have been sampled and pharmaceutical drugs detected (Daughton & Ternes, 1999). Any place that humans visit has the potential to be contaminated with these drugs, unlike other pollutants such as pesticides. Pristine national parks, for example, commonly have aging sewage systems. If there is enough human traffic which use these systems, PPCPs can leach into the surrounding environment and through rainfall and flooding be carried away (Daughton & Ternes, 1999). Also, the reuse of sludge from WWTPs can increase environmental PPCP concentrations. Some chemicals become adsorbed onto particulate material during the treatment process and therefore become associated with the sludge. When this sludge material is used on agricultural fields as a fertilizer, the highly concentrated PPCP content can run off into nearby surface and ground water sources (Santos et al., 2010) It appears that as these compounds become more common in everyday life no area will be completely free of their influence.

Research into PPCPs in the environment has increased significantly. A summary by Santos et al. (2010) showed that the most detected pharmaceuticals in environmental systems are non-steroidal anti-inflammatory drugs (NSAIDs) (Figure 2). This is expected because common types of NSAIDs, such as ibuprofen and naproxen, are readily available as OTCs, such as Advil® and Aleve® respectively. The more these drugs are taken, the more readily they will be detected in water systems. The remainder of this work will focus on NSAIDs and their effects in aquatic environments.
Previously, most of the research done on PPCPs was focused in Europe (Metcalfe et al., 2003b). In recent years, more researchers have been looking at Canadian water systems and analyzing which PPCPs are commonly detected. The main studies done are by Metcalfe et al. (2003a,b) and Lishman et al. (2006). These articles detected the presence of NSAIDs in Canadian regions.

While some chemicals can be readily broken down in surface waters through photodegradation, such as naproxen, and others persist for relatively short periods of time, they still pose an ecological threat (Isidori et al., 2005). This is because PPCPs are classified as being ‘pseudo-persistent’, meaning that while they may not persist in the environment they still may cause effects from the continuous addition (Durán-Alvarez et al., 2009). It is this pseudo-persistence that can lead to the endocrine disrupting effects of PPCPs.
Figure 2: Relative percentages of PPCPs measured in the environment based on summary of published data. Figure from Santos et al. (2010).
1.5 Endocrine Disruptors

1.5.1 What are Endocrine Disruptors?

One of the main communication systems in the body is the endocrine system (Vogel, 2004). It is responsible for sending hormone signals and regulating body functions necessary for growth, reproduction and behaviour (EPA, 1997; Vogel, 2004). Therefore, any compound that disrupts or causes an imbalance in the natural homeostasis of the body can be described as an endocrine disruptor (Labelle, 2000). Endocrine disrupting compounds (EDCs) are any chemical agent that interferes with the synthesis, secretion, transport, binding, function or elimination of hormones (EPA, 1997). Any malfunctions of these body systems can lead to disease (Vogel, 2004).

There has been some debate over how these chemicals should be classified because they are usually grouped by action rather than by chemical structure. The general consensus is that there are 3 classes of EDCs; estrogenic chemicals, androgenic chemicals and thyroidal chemicals, although anti-estrogenic and anti-androgenic chemicals are also common (Synder et al., 2003). Regardless of class, all EDCs act in a few select ways. They can act as mimics, blockers or triggers (Labelle, 2000). Agents that mimic hormones trick the organism into perceiving that they are the natural compound, eliciting effects that would arise from normal hormonal interaction (Labelle, 2000). EDCs may also inhibit the activity of certain enzymes (Vogel, 2004). Without the activity of specific enzymes, metabolic pathways may be disrupted. Lastly, these chemicals may also act as triggers, abnormally initiating pathways by modifying hormone receptor levels or altering synthesis patterns (Labelle, 2000). These effects are the reasons why the body possesses mechanisms to excrete these chemicals.
1.5.2 PPCPs as EDCs

As discussed previously, PPCPs may enter the aquatic system as the parent compound or as metabolites, because most compounds are metabolized before removal from the body (Halling-Sørensen et al., 1998). The mechanisms of metabolism for xenobiotic compounds are referred to as Phase I and Phase II reactions (Daughton & Ternes, 1999). Collectively, these two phases successively increase the polarity of the compound, enhancing its excretion. Monooxygenases, such as the cytochrome P450 enzymes, are utilized in Phase I reactions (Daughton & Ternes, 1999). These enzymes add reactive functional groups to the PPCP molecules while Phase II reactions use conjugation with glucuronic acid, also called glucuronidation, to increase the hydrophilicity of the molecules (Daughton & Ternes, 1999). The more hydrophilic the compound is, the more readily it will be eliminated from the body.

The reason PPCPs are such a concern in the aquatic environment as endocrine disruptors is that these chemicals are designed to target specific metabolic and molecular pathways (Christen et al., 2010). Humans take these drugs for specific biological reasons and these same effects can be caused in non-target organisms (Bound & Voulvoulis, 2004). Similar pharmacodynamic effects can be seen in aquatic wildlife at higher trophic levels, such as fish and amphibians, because many possess similar metabolic pathways, receptors and biomolecules (Brown et al., 2007; Santos et al., 2010). For example, if a pharmaceutical is designed to inhibit an enzyme in humans, fish which possess that same enzyme may also be affected upon exposure to that chemical.

Complexity arises from the fact that not all human pharmaceuticals on the market have well defined modes of action (Daughton & Ternes, 1999; Christen et al., 2010).
Some drugs, like ibuprofen, have been well studied and the exact metabolic target is known but this is not always the case with newer pharmaceuticals. Also, aquatic organisms may experience effects from PPCPs that are not expected. Human pharmaceuticals commonly have listed side-effects but these side-effects may be more severe in non-target species because of slight differences in physiology.

The impact of PPCPs in the environment is also not solely based upon the concentration of the parent compound but also upon the presence of any metabolites or transformation products. It was previously mentioned that the NSAID naproxen undergoes photodegradation in surface waters. Toxicity studies utilizing small invertebrate species have shown that some of these photoproducts are in fact more toxic than that of the parent compound (DellaGreca et al., 2004; Isidori et al., 2005).

1.5.3 Knowledge Gaps in EDC Research

While there is a significant knowledge base discussing the potential effects of PPCPs in the environment as endocrine disruptors, there is little evidence showing effects to aquatic organisms on the population level (Corcoran et al., 2010). This is because the field is dominated by acute studies. Most research is based on short term laboratory studies performed at high concentrations (Corcoran et al., 2010). This does not represent what occurs in nature and would only be relevant to discuss spills where large concentrations of PPCPs are accidentally introduced into the environment (Santos et al., 2010).

Chronic studies, as well as those that look at the bioconcentration of PPCPs in organisms, are lacking (Brown et al., 2007; Santos et al., 2010). Chronic exposures are
defined as experiments in which organisms are exposed to chemicals over long periods of
time or a substantial portion (generally > 10%) of their life cycle (Crane et al., 2006).
With PPCPs being continuously discharged into the environment at low levels, aquatic
organisms are potentially exposed over their entire life span. Although emitted in low
concentrations, drugs have the ability to bioaccumulate in the tissues of wildlife.

Brown et al. (2007) proposed a model to calculate the degree to which different
pharmaceuticals accumulate in fish species. Concentration in the tissues of the fish
depends not only on the physicochemical properties of the compounds but also their
bioavailability. In relation to those dissolved drugs which are taken up across the gills,
drugs that are associated to particulate material through electrostatic interactions are less
available to the fish and therefore will be present in lower concentrations within the
organism (Brown et al., 2007).

A final factor that needs to be analyzed in relation to the endocrine disrupting
ability of PPCPs is the fact that in the environment these chemicals are not present in
isolation. In laboratory settings, pharmaceuticals are generally evaluated as individual
chemicals but in wastewater effluents they are present as part of complex mixtures.
Research into how these chemicals interact within mixtures needs to be undertaken
(Santos et al., 2010). Some chemicals have the ability to modify the toxicity of others, so
the expected effects of one pharmaceutical may be different when that chemical is in the
presence of another. There have been only a few studies published looking specifically at
mixture effects. Studies by Cleuvers (2003, 2004) using small invertebrate organisms
demonstrated that a mixture of PPCPs caused an increased effect on the test subjects in
comparison to when the individual chemicals were exposed in isolation.
While the gaps in the field of environmental pharmaceutical research are large, small steps need to be taken in order to fill them. This can be done by first studying those compounds which are detected with the highest frequency, which are the non-steroidal anti-inflammatory drugs.

1.6 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

1.6.1 Brief History and Introduction

The use of chemical compounds to treat pain and inflammation is not a modern notion. It can be dated back to ancient Egypt, where elders applied treatments of dried myrtle leaves to those with ailments (Vane & Botting, 1998). In the body, inflammation is the response of living tissue to injury (Vane & Botting, 1995). This initiates a cascade of events including enzyme activation, extravasation of fluids, cell migration and tissue breakdown and repair (Vane & Botting, 1995). It was this notion of pain and inflammation management that caused researchers to study the physiological effects that take place, leading to J. R. Vane’s 1971 discovery of a class of pharmaceuticals that can inhibit these reactions, known as non-steroidal anti-inflammatory drugs (Cha et al., 2006). In summary, Vane demonstrated that NSAIDs inhibit the cyclooxygenase (COX) enzymes responsible for prostaglandin (PG) production necessary for the inflammatory response. Although this breakthrough did not occur until the 1970s, inquiry into the phenomena had been occurring since the 1930s (Cha et al., 2006).
1.6.2 Prostaglandins and Cyclooxygenase Enzymes

Before a discussion of how NSAIDs act to inhibit the inflammatory response in the body, the response itself needs to be discussed. The starting point for this concept is understanding prostaglandins. Prostaglandins play a variety of biological functions. In addition to inflammation and pain, PGs are necessary for homeostasis in a variety of pathways such as maintaining the gastric mucosa, regulating kidney blood flow and various reproductive mechanisms (Fent et al., 2006). PGs are part of a larger group of biomolecules called prostanoids. This group then falls under the larger category of eicosanoids (Cha et al., 2006). Eicosanoids are evolutionarily conserved bioactive lipid molecules consisting of 20 carbon fatty acid molecules (Cha et al., 2006). Any altered expression of these compounds can lead to pathophysiological conditions (Ishikawa & Herschman, 2007).

PGs are present in most cells, although these compounds are rapidly synthesized in response to stimuli rather than being stored (Ruggeri & Thoroughgood, 1985). They are produced through the activity of the cyclooxygenase enzymes. COX enzymes, also called prostaglandin endoperoxide synthases (PGHS), are present in two isoforms that are responsible for PG production. COX are bifunctional enzymes that convert arachidonic acid (AA) into PGH\(_2\), the common intermediate to all PG molecules (Figure 3) (Vane & Botting, 1995). AA is released from the cellular membrane by the action of phospholipase A2 (Cha et al., 2006). Once available, the cyclooxygenase activity of the COX enzyme cyclises AA and converts it to PGG\(_2\) through the addition of a 15-hydroxyl group (van Anholt et al., 2003). PGG\(_2\) is then reduced to PGH\(_2\) by the peroxidase activity of the enzyme (Vane & Botting, 1995). It is PGH\(_2\) that other prostaglandin
peroxidases act upon to produce tissue specific PGs, such as PGI$_2$ (smooth muscle control), PGE$_2$ (gastric epithelium, kidney) and PGF$_{2\alpha}$ (reproductive tissues, brain) (Cha et al., 2006).

With AA being released from the plasma membrane, the enzymes are also membrane bound. COX are membrane bound haemoproteins found in high concentration in the endoplasmic reticulum (Vane & Botting, 1995). COX is oriented towards the lumen of the endoplasmic reticulum and integrates into only 1 layer of the plasma membrane (Knights et al., 2010). The homodimers consist of 3 domains; an N-terminal epidermal growth factor-like domain, a membrane binding motif and a C-terminal enzymatic domain (Knights et al., 2010; Vane & Botting, 1995). It is thought that the growth factor-like domain is responsible for the association of the two enzymatic monomers (Knights et al., 2010).

COX are present in 2 isoforms; COX-1 and COX-2. COX-1 is the constitutive form of the enzyme, responsible for maintaining background PG levels required for normal body function (Vane & Botting, 1995). The inducible form, COX-2, is responsible for the production of PGs necessary for the inflammatory response. COX-2 can be activated by the presence of cytokines, mitogens or endotoxins (Mitchell et al., 1994). In human COX enzymes there is a 60% sequence homology between the two isoforms, with each being approximately 70 kD in size (Vane & Botting, 1995). COX enzymes are not only found in mammals, but have also been detected in other vertebrate species. Zou et al. (1999) demonstrated that there were inducible forms of COX present in fish species. Ishikawa and Herschman (2007) elaborated on this point using the rainbow trout (Oncorhynchus mykiss) genome and discovered there were actually two
different types of inducible COX isoforms; COX-2a and COX-2b. Depending on the species, there is a 65 – 84% DNA sequence homology between fish and human isoforms (van Anholt et al., 2003). For rainbow trout in particular, there is an 83-84% homology between COX-1 and 77% homology between COX-2 when compared to human cyclooxygenase enzymes (Fent et al., 2006). This further shows that COX genes are conserved across many species and that they perform similar functions to those in humans (Mehinto et al., 2010).
Figure 3: Conversion pathway of arachidonic acid to various prostaglandins. The enzymes phospholipase A2, COX and various prostaglandin peroxidases assist in the conversion process. Figure modified from Knights et al. (2010).
1.6.3 Mode of Action

NSAIDs are used as therapeutic agents due to their ability to inhibit COX enzymes (Vane & Botting, 1998). In general, they are racemic mixtures of weak acids that either reversibly or irreversibly bind to the active site of the enzymes, rendering them inactive (Khetan & Collins, 2007; Santos et al., 2010). This can be achieved in one of three ways. NSAIDs can inhibit by rapid, competitive reversible binding, such as the case of ibuprofen. They can also use rapid low-affinity binding followed by high-affinity time dependent binding, like diclofenac. Lastly, in the method used by aspirin, they can inhibit COX activity by rapid reversible binding followed by covalent modification (Knights et al., 2010). All of these methods are dependent on drug interactions with the active site of the enzyme.

The active site of COX is a long, hydrophobic channel designed to accommodate arachidonic acid in the first step of the enzymatic process (Vane & Botting, 1998). Within this cavity, the amino acids tyrosine 385 and serine 530 are essential for the conversion of AA to PGG₂, but these are not the most important residues to consider when discussing NSAID inhibition (Vane & Botting, 1998). The main difference between the actives sites of COX-1 and COX-2 is the size of their active sites themselves. The active site of COX-2 is larger than that of the other isoform, allowing it to accommodate compounds of a larger molecular size (Vane & Botting, 1998). The difference in cavity space of the active site is due to one amino acid substitution in particular. In the active site of COX-2, the larger isoleucine residue at site 523 is substituted for a smaller valine (Knights et al., 2010). The substitution significantly opens the active site by forcing the surrounding amino acids into a different configuration.
(Figure 4). It is this physical difference that allows for the selectivity amongst the pharmaceuticals.

As Vane theorized during his work with NSAIDs, it is possible to design PPCPs preferentially that target one isoform over another (Vane & Botting, 1995). By creating molecules that target only COX-2, some of the negative side effects associated with NSAID use may be reduced (Mitchell et al., 1994). If the COX-2 isoform, responsible for the PGs that cause pain and inflammation, is inhibited while the constitutive form necessary for homeostasis is unaffected, fewer biological functions will be disturbed.
Figure 4: Structural configuration of the actives sites of the two COX isoforms. (A) The active site of COX-1 has isoleucine at position 523, creating a smaller entrance into the cavity whereas (B) COX-2 has valine at position 523, forcing the surrounding amino acids into a different configuration, allowing for a wider opening. This substitution allows for the selectivity in NSAIDs, where smaller molecules (ie. ibuprofen) can fit into the active sites of both whereas larger molecules (ie. naproxen) cannot. Figure modified from Knights et al. (2010).
1.6.4 Ibuprofen

There are numerous OTCs currently on the market that act as COX inhibitors. The PPCPs that are most used are ibuprofen and, more recently, naproxen. Ibuprofen is considered a non-selective COX inhibitor since it acts on both COX-1 and COX-2. Naproxen, on the other hand, is COX-2 selective (Vane & Botting, 1998). The ratio of inhibition between COX-2/COX-1 is what decides selectivity. The ratios are calculated using IC$_{50}$ values, defining the concentration at which that particular pharmaceutical inhibits 50% of either isoform (Knights et al., 2010). Naproxen has a larger structure, which can fit into the wide active site of COX-2 but not as easily into that of COX-1 (Figure 4). The remainder of this discussion, as well as the research undertaken, will focus on ibuprofen.

Ibuprofen is the NSAID produced in the highest quantity globally, which is in part due to the World Health Organization including it as a core medicine on its “Essential Drug List” (Han et al., 2010). Commonly ingested orally, ibuprofen is transported through the body via the circulatory system, where up to 99% of the compound is bound to plasma albumin (Thomas, 2007). In addition to being bound to plasma proteins, ibuprofen can also be found within tissues at significantly high concentrations. A possible reason for its inclusion on the essential drug list is the fact that ibuprofen is very effective. Although it only possesses a half life of approximately 2 hours, being non-selective in its COX inhibition allows ibuprofen to prevent symptoms caused by pain and inflammation (Thomas, 2007; Vane & Botting, 1998). Upon excretion, approximately 10% is still present as the parent compound where the rest is metabolized through Phase I and Phase II metabolism (Khetan & Collins, 2007). It is first oxidized and then
conjugated with glucuronic acid prior to excretion (Halling-Sørensen et al., 1998). While this may suggest that only a small percentage of the active compound is entering aquatic systems, it is possible for the reconversion of metabolites into the parent compound through enzymatic reactions or chemical hydrolysis (Daughton & Ternes, 1999). With its high use worldwide, quick removal rate from the body and the fact that it remains in the biologically active compound in water systems makes ibuprofen an ideal NSAID to study in relation to toxicity.

1.7 Exposure Techniques

When designing toxicity experiments, standardized guidelines are routinely consulted, such as those produced by the U.S. EPA (Fent et al., 2006). While such tests are standard procedure, generic guidelines may not be sufficient when evaluating PPCP toxicity in aquatic environments. Mortality is an endpoint commonly used to evaluate toxicity but such an endpoint is generally not relevant to pharmaceuticals given their low environmental concentrations elicit mainly sub-lethal effects (Fent et al., 2006). When sub-lethal effects have been evaluated, they are mainly based on acute rather than chronic exposures.

Acute experiments are classified as exposures lasting no longer than 96 hours (Huggett et al., 2003). Although acute tests are commonly performed they are not entirely relevant when it comes to determining the safety of PPCPs in aquatic systems. This is because acute testing is normally completed using abnormally high chemical concentrations that greatly exceed those found in the environment (Huggett et al., 2003). Because of this lack of environmental realism, the actual impact of these chemicals to
aquatic organisms may be masked by limiting methodologies (Fent et al., 2006). Another issue associated with acute experiments is that the exposure techniques may vary greatly. Acute studies have been completed using injections as the exposure route for pharmaceuticals. For example, Hoeger et al. (2008) studied how the NSAID diclofenac distributed within the tissues of brown trout (Salmo trutta f. fario) through the use of intraperitoneal injections. While this research is important to build the knowledge base on how PPCPs move through tissues, the exposure route may not be environmentally relevant.

More chronic PPCP exposure studies, a minimum of 1-3 months in length, are urgently needed (Huggett et al., 2003). Aquatic organisms, living near WWTP discharge sites, are continuously exposed to PPCPs and other chemicals over their entire lifespans. Aquatic toxicological research needs to mimic this occurrence. Undertaking chronic experiments, however, is significantly more difficult, encouraging the use of mathematical models to estimate chronic exposure, but such a theoretical approach also has its limitations (Fent, 2008). Ragugnetti et al. (2011) recently conducted a chronic exposure using tilapia (Oreochromis niloticus), although the exposure only lasted 10 days. One of the longest exposures reported for ibuprofen involved Japanese medaka (Oryzias latipes) exposed to waterborne ibuprofen over 6 weeks (Flippin et al., 2006).

For chronic toxicity testing, 3 main areas should be considered when designing experiments. First, the target specificity should be determined (Fent, 2008). The researchers should know which specific biomolecules, tissues and organs should be analyzed depending on the chemical used. Second, evaluation of side-effect specificity is required (Fent, 2008). Side effect specificity will consider effects that are expected but
since some PPCPs do not have well defined modes of action, physiological mechanisms in aquatic organisms may differ from those of humans. Lastly, species specificity needs to be taken into consideration (Fent, 2008). This last point will cover any general chronic effects that account for differences in physiology. These factors, in combination, provide the basis for well designed chronic exposure experiments.

1.8 Biomarkers of PPCP Toxicity in Aquatic Environments

Ecotoxicology is based on the study of endpoints which may indicate processes leading to disease (Ward & Henderson, 1996). These endpoints are called biomarkers. Specifically, biomarkers can be defined as any measurement of body fluids, cells or tissues that indicate biochemical or cellular alterations due to the presence of pollutants (van der Oost et al., 2003). These markers of toxicity can be subdivided into three main categories: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility (Ward & Henderson, 1996). While some biomarkers span categories, the main purpose of these endpoints is to determine if potentially harmful chemicals have entered the environment and are causing effects (van der Oost et al., 2003; Ward & Henderson, 1996). Although these tests have their limitations, such as not taking into consideration any physicochemical effects that occur while these chemicals are in the environment that may modulate toxicity, they still provide a good representation of the interaction of the chemical and the biological system (van der Oost et al., 2003). For complex research, a mixture of different biomarkers should be selected to achieve a greater understanding of the processes occurring (Ward & Henderson, 1996).
Fish are commonly used as the test organisms for biomarker research. Fish are found in most aquatic environments and are of ecologic importance. They play a significant role in the energy transfer between lower and higher tropic levels and are an important food resource for humans (van der Oost et al., 2003). While there are differences between fish species, which ever species is chosen should have a relationship to the chemical or region being tested (van der Oost et al., 2003). For pharmaceutical research in Canada, rainbow trout (Oncorhynchus mykiss) are good test organisms. Not only are they relevant to the Canadian environment, it has been previously discussed how trout possess similar enzymatic pathways to humans making them susceptible to PPCPs, such as NSAIDs (see section 1.6.2 Prostaglandins and Cyclooxygenase Enzymes).

1.8.1 Condition Factor

Condition factor (K) is a parameter widely used to gauge the overall quality of fish. Proposed by Fulton in 1904, K is the relationship between the weight and length of a fish (Barnham & Baxter, 1998; Nash et al., 2006). How ‘fat’ or ‘skinny’ a fish is can be made by the visual comparison of its weight to length, whereas K is a mathematical representation of the same concept. The condition factor of a fish can be affected by many factors, such as the sex of the fish, the season measured or its developmental stage (Barnham & Baxter, 1998). Using this concept, condition factor can be applied as a biomarker for toxicity. If a chemical was altering the metabolic processes in a fish, its weight may significantly decrease. If a population of trout start out in good health, with K values exceeding 1.0, and over the exposure time the K values decrease, this is an indicator that the chemical is eliciting a deleterious effect. Condition factor can be
applied to follow the health of individual fish or to assess fish population health as a whole.

1.8.2 **Organo-somatic Indices**

Like condition factor, other indices can be used to determine general health qualities of fish, collectively known as organo-somatic indices. Use of these indices is standard practice in fish physiology studies (Schmitt & Dethloff, 2000). The indices are ratios of the weight of individual organs to the total weight of the fish (Schmitt & Dethloff, 2000). Two commonly used are the liver somatic index (LSI) and gonad somatic index (GSI). By measuring LSI and GSI, a view into the overall health of the organ system can be determined. Changes in LSI may represent problems with metabolism or energy storage, whereas changes in GSI provide insight into the structural conditions (maturity) of the gonads rather than their functionality (Schmitt & Dethloff, 2000). It should be noted that numerous factors can also affect these index values so their use needs to be evaluated carefully before definitive conclusions are made (Schmitt & Dethloff, 2000).

1.8.3 **COX Inhibition**

An in depth discussion about cyclooxygenase and COX inhibition by NSAIDs, and how this can be used as biomarkers of toxicity, can be found in 1.6.2 *Prostaglandins and Cyclooxygenase Enzymes* and 1.6.3 *Mode of Action*. 

29
1.8.4 Vitellogenin (VTG) Concentration

Vitellogenin (VTG) is an estrogen-dependent yolk protein precursor that is synthesized in the liver of non-mammalian female vertebrates (Allner et al., 2000; Schmitt & Dethloff, 2000). In fish, oogenesis is the process by which VTG is produced by the liver and taken up by the oocytes (Nicolas, 1999). More specifically, vitellogenesis is the physiological process by which VTG is taken up by the maturing oocytes to accumulate yolk (Nicolas, 1999). This whole process is heavily dependent upon hormonal regulation.

VTG production is regulated by the hypothalamus-pituitary-gonadal axis. The hypothalamus begins by releasing gonadotropin releasing hormones (GnRH) which will act upon the pituitary gland (Nicolas, 1999). Upon stimulation, the pituitary gland then releases gonadotropins (Nicolas, 1999). Gonadotropins are responsible for regulating the functional activity of the gonads as well as promoting reproductive events, such as meiosis and ovulation. When the ovaries are stimulated, they begin to grow and their follicles release newly synthesized estradiol (Nicolas, 1999; Sumpter & Jobling, 1995). Estradiol enters the blood stream and stimulates the liver to produce VTG (Hansen et al., 1998; Nicolas, 1999). Once in the ovary, VTG is then enzymatically cleaved to produce the desired yolk proteins, lipovitellin and phosvitin (Schmitt & Dethloff, 2000).

For analysis of VTG it should be noted that it is species specific (Hansen et al., 1998). Specific antibodies are required for analyzing VTG from differing fish species, but what makes it an important biomarker is that its production can be induced by external estrogens. Factors that mimic naturally occurring hormones can cause VTG induction in male fish, or female fish that have not yet reached reproductive age (Hansen
et al., 1998). If VTG can be induced in immature female fish this may be a significant biomarker in NSAID research. If natural PG levels are reduced by COX inhibition, VTG synthesis may be inhibited even if an external hormone, such as 17-β-estradiol, is applied. Ibuprofen may also have the opposite effect by acting as an estrogenic chemical and inducing VTG production. It is this concept of mixture chemical exposure that will help to elucidate pharmaceutical toxicity to non-target aquatic organisms.

1.8.5 Ethoxyresorufin-O-Deethylase (EROD) Activity

Another biomarker that may be useful in the determination of how NSAIDs affect fish is ethoxyresorufin-O-deethylase (EROD) activity. In fish studies, it is widely accepted to measure EROD activity as an in vivo indicator of CYP1A activity (Schmitt & Dethloff, 2000; Whyte et al., 2000).

The family of cytochrome P450 (CYP) enzymes are heme-containing proteins that play a role in the transformation of lipophilic substances (Carlsson et al., 1999). These proteins, which are part of Phase I metabolism reactions, oxidize, hydrolyze or reduce compounds to increase their hydrophilicity for excretion (Carlsson et al., 1999; Schmitt & Dethloff, 2000). The enzymes are concentrated mainly in the liver of fish and can be located in the smooth endoplasmic reticulum (Schmitt & Dethloff, 2000). The subfamily responsible for these reactions is the CYP1A group. These enzymes are similar in fish as they are in mammals. In trout, CYP1A1 and CYP1A3 are the enzymes responsible for metabolism of xenobiotics chemicals, but for the purposes of this discussion they will be grouped as CYP1A (Carlsson et al., 1999).
CYP1A induction is a biomarker of exposure for certain chemicals, such as polyaromatic hydrocarbons (PAHs) and similarly structured planar compounds (Schmitt & Dethloff, 2000). Upon exposure, xenobiotic compounds bind to the aryl hydrocarbon receptor (AhR) (Whyte et al., 2000). This receptor is a ligand-activated transcription factor. When bound to a ligand, AhR binds DNA and initiates transcription of specific genes, such as those for CYP1A and other enzymes required for Phase II metabolism (Whyte et al., 2000). Therefore, the increased presence of compounds able to activate AhR will lead to increased amount and activity of CYP1A.

An indirect measure of this is through measuring the activity of EROD. EROD activity is a highly sensitive indicator of contaminant uptake in fish since it is related to receptor mediated induction of CYP-dependent monooxygenases (Schmitt & Dethloff, 2000). EROD measures the conversion of 7-ethoxyresorufin (7ER) to resorufin, which can be measured fluorometrically (Whyte et al., 2000). When using EROD activity as a biomarker, it should be understood that it is a better indicator of exposure rather than effect (Schmitt & Dethloff, 2000). Research is still being done to determine the link between CYP1A induction and how this may relate to toxicity within organisms (Whyte et al., 2000). It is believed that increasing CYP1A activity alters the state of homeostasis within the cell which can lead to detrimental effects (Schmitt & Dethloff, 2000). Measuring EROD activity is useful because it shows that AhR active compounds are present, and represents the cumulative impacts of all such chemicals, even if they are below measurable detection limits (Schmitt & Dethloff, 2000).

Care needs to be taken when evaluating EROD activity because it may be influenced by abiotic and biotic factors such as water temperature, the age of the test
organisms and that organism’s reproductive age (Schmitt & Dethloff, 2000). Results from EROD activity assays need to be evaluated critically to ensure that these external factors are not skewing the results obtained. While some chemicals may increase CYP1A activity at one concentration, the same compound may inhibit activity at higher concentrations (Whyte et al., 2000). Also, some chemicals, either in isolation or in mixtures, may inhibit EROD activity (Whyte et al., 2000). While extremely useful, discretion needs to be used when commenting on results.
2.0 Purpose of Study

The purpose of this research was to study the effects of NSAIDs, specifically the non-selective COX inhibitor ibuprofen, on rainbow trout (*Oncorhynchus mykiss*) following acute and chronic exposures. Using a flow-through dose controlled system for waterborne exposure, a 96 hour and an 8 week (56 day) experiment were developed to help augment the body of literature for this particular PPCP and test organism. The main research goals were to:

(1) Determine if COX activity in rainbow trout was affected following acute and chronic ibuprofen exposure and if this activity varied between tissues.

(2) Determine if plasma VTG levels were altered during exposure to ibuprofen and a mixture of the hormone 17-β-estradiol (E2) when compared to plasma samples from fish exposed to the hormone alone; and

(3) Determine if exposure to ibuprofen altered EROD activity in the trout liver tissue.
3.0 Methodology

3.1 Test Organisms

Juvenile female rainbow trout (Oncorhynchus mykiss) weighing approximately 50 grams were obtained from Linwood Acres Trout Farm Ltd. (Campbellcroft, Canada) on May 28th, 2010. After being transported to the aquatic toxicology wet laboratory at the University of Ontario Institute of Technology (UOIT, Oshawa, Ontario, Canada), the fish were allowed to acclimate prior to experimentation in 1500 L tanks. During the acclimation period, the fish were fed a ration of 0.4% body weight and the temperature was maintained at 12.3 ± 1.2º C and a pH of 7.1 ± 0.4.

3.2 Chemicals and Reagents

The chemicals required for exposure and analysis of tissue samples were purchased from Sigma Aldrich (Oakville, Canada), Fisher Scientific (Ottawa, Canada) and BioShop Canada Inc. (Burlington, Canada). The exception to this was the polyclonal antibody necessary for the vitellogenin enzyme-linked immunosorbent assay (ELISA), which was purchased from the Biosense Laboratory (Thormøhlenstgt, Norway). Individual chemicals were purchased and standard protocols were followed for all biomarker assays, except total COX activity. To complete the COX analysis, COX Activity Assay Kits (Catalogue No. 760151) were purchased from the Cayman Chemical Company (Michigan, USA) and performed following the manufacturers recommendations.
3.3 Flow-Through Dose Controlled System

Before establishing the dose system for experimentation, the peristaltic dose pump that was to be used (multi-channel cartridge pump, Watson Marlow Bredel Pumps, Massachusetts, USA) was calibrated to ensure that a constant volume of water was accurately dispensed. This was done by setting up a mock system and collecting the water that was run through the pump for a 24 hour period at a set speed (rpm) and tube diameter. At the end of the 24 hour period, the volume of water dispensed was weighed and the flow rate calibrated to ensure that it matched that specified by the manufacturer. Once this was verified, the conditions necessary for each exposure were tested.

A constant supply of clean water was provided to each of the exposure tanks. The flow of water was set at 4 turnovers of water each day to achieve 99% molecular renewal every 24 hours (Sprague, 1969). Depending on the flow of water into the tank (2.08 mL/sec for the acute exposure and 3.24 mL/sec for the chronic exposure) which varied due to the different tank volumes used, the dose pump was set to replenish the amount of drug lost from the tank each second using the ibuprofen stock solutions prepared. Stock solutions were prepared in concentrations higher than the desired treatment concentrations to allow for dilution upon mixing with the clean water entering the tank.

Once the system had been calibrated on the bench top, the pump was set up in the wet laboratory. Nalgene bottles (1 L) were used to house the stock solutions. Holes were drilled in the lids of the bottles and plastic rods were inserted, coming to rest just above the bottom of the bottle (Figure 5a). Flexible plastic tubing of a set diameter (1.42 mm) was connected to the plastic rods, which then were fed through individual channels on the peristaltic pump (Figure 5b). The stock bottles, along with the pump itself, were
positioned on a shelf built above the treatment tanks. The tubing then exited the pump and was connected into the water supply tube flowing into each individual tank (Figure 5c). This allowed for mixing of the highly concentrated stock solution and clean water prior to entering the tank.

The dose pump was set to deliver a dose of 120 µL/min for the acute exposure and 80 µL/min for the chronic exposure. The dose rate was altered so that the 1 L stock solution bottles would need to be replenished only once a week during the course of the chronic experiment.
Figure 5: Set up of flow-through dose controlled system. (A) 1 L nalgene stock solution bottles. (B) Peristaltic dose pump. (C) Connection of dose tubing with water system.
3.4 Experimental Design

3.4.1 Acute Exposure

The acute exposure, set at 96 hours, was designed to test if exposure to ibuprofen, using concentrations higher than what is found in the environment, could act as an endocrine disruptor on juvenile rainbow trout in isolation and in combination with the hormone E2. To do this, 5 different treatments were run in duplicate. Two different ibuprofen concentrations were selected, 10 mg/L and 1 mg/L, along with a control containing no drug, a positive control tank containing E2 at a concentration of 1 mg/L and a E2-ibuprofen mixture tank containing the highest ibuprofen treatment, 10 mg/L, and 1 mg/L E2.

To achieve the desired ibuprofen concentration within the treatment tanks, stock solutions were prepared using ibuprofen sodium. Ibuprofen sodium was used instead of ibuprofen in the pure form due to potential solubility issues. As previously described, the stock solutions were prepared at a higher concentration to allow for dilution within the tanks. It should also be noted that the concentrations refer to the amount of anionic ibuprofen and not the concentration of the salt. To achieve a concentration of 10 mg/L and 1 mg/L in the treatment tanks, using a flow rate of 120 µL/min, stocks of 10.4 g/L and 1.04 g/L ibuprofen were prepared. These solutions were then placed into the nalgene bottles in the dose system. Each tank had a stock bottle associated with it, even if it did not receive a continuous dose of ibuprofen. The stock bottles associated with the control and E2 control tanks were filled with DI water, to ensure that each tank was treated in the same manner.
Seventy litre glass aquaria were used as the exposure tanks but were only filled to 45 L. The reduced volume of water was selected due to the fact that only 4 fish were being used per treatment, as well as to reduce the amount of ibuprofen required to prepare the concentrated stock solutions. The tanks were filled with clean water to 45 L and the dosing system was started. The initial running of the system before the fish were introduced allowed the tanks time to reach the appropriate concentration. After 24 hours had passed, 4 fish were moved from the 1500 L acclimation tanks and placed into each of the 10 exposure tanks, which signified the beginning of the experiment. A total of 44 fish were used in the experiment, 4 in each of the 10 treatment tanks plus 4 fish that were sampled from the 1500 L acclimation tanks to serve as background levels for all analyses.

Unlike ibuprofen which was exposed continuously, a pulse exposure was used for E2. The high cost of the chemical prevented a continuous exposure. To prepare the E2, 180 mg was dissolved in 4 mL of acetone. This allowed 1 mL of the solution to be added to each of the four E2 exposure tanks, creating a concentration of 1 mg/L.

After the fish had been placed into the treatment tanks, the water system and dose pump were turned off, creating a static system for the E2 pulse exposure. Again, all the tanks were treated in the same manner even if they were not receiving a hormone dose. A 1 L beaker was used to remove approximately 1 L of water from each of the tanks receiving the E2 dose and 1 mL of the E2 solution was added. This was to allow mixing before the water was added back to the treatment tanks. With the E2 solution added, the tanks were kept in a static state for 2.5 hours. When the flow through system was resumed, the concentration of E2 in the tanks slowly decreased over the next 24 hours.
All tanks were vacuumed each morning although the fish were not fed during the course of the experiment. The average temperature of the treatment tanks was 16.3 ± 1.1°C with a pH of 7.1 ± 0.4. At the end of the 96 hours, the system was shut off and all the fish were sampled.

### 3.4.2 Chronic Exposure

The chronic exposure lasting 8 weeks (56 days) was designed to study the same end points as the acute exposure, but completed using concentrations focused around those measured in the Canadian environment. A logarithmic scale was used for the three ibuprofen treatment concentrations (1, 32 and 1000 µg/L), along with an experimental control, E2 control of 1000 µg/L and an E2-ibuprofen mixed treatment of 1000 µg/L E2 and 1000 µg/L ibuprofen. It should be noted that the E2 concentrations, along with one of the ibuprofen treatment concentrations, are the same in both the acute and chronic exposures (ie. 1 mg/L and 1000 µg/L). The 6 treatments were run in duplicate, with the replicates being staggered by 1 day.

To achieve the desired concentrations of ibuprofen in the aquaria, stock solutions of 2.43 g/L, 77.78 mg/L and 2.43 mg/L ibuprofen were prepared. The stock bottle set-up was the same as the acute exposure.

Prior to the start of the experiment, the fish were acclimated in the full 70 L glass aquaria without any drug exposure for 4 days due to the higher density of fish in the tanks. Fifteen fish were placed in each treatment tank to allow for 5 fish to be sampled on each sampling time (day 14, day 28 and day 56). A total of 186 fish were used in the experiment, 15 in each of the 10 treatment tanks and 6 fish that were used as background
control samples. At the start of the experiment the dose system was turned on to allow the ibuprofen in the tank to gradually increase to the desired concentration.

The E2 pulse exposure was also done in the same fashion as the acute experiment. On the first day of every week the water and dose system were turned off to allow for a 2.5 hour static E2 exposure. Upon completion the water and dose pump were turned back on. This was completed every week for a total of 8 exposures.

Each tank was cleaned every morning and the fish were fed a daily ration of 0.4% body weight divided into two feedings (morning and afternoon). The temperature was maintained at 13.1 ± 0.6º C over the 8 weeks with a pH of 6.7 ± 0.2.

3.5 Water Sampling

While the exposures were occurring, water samples from the treatment tanks were taken to determine the actual ibuprofen concentration. Once during the acute experiment, and 4 times during the chronic exposure (weeks 2, 4, 7 and 8) water was removed from select treatment tanks using a syringe and filtered directly into a 2 mL ultra performance liquid chromatography (UPLC) vial (LCMS certified amber screw top vials, Waters, Mississauga, Canada). A 0.2 µm syringe filter (Puradisc PES filter media, Whatman Ltd., Mississauga, Canada) was used to ensure all particulate material was removed from the water samples prior to analysis. All water samples were stored at 4º C.
3.6 Sampling

All fish were anesthetized in MS-222 at a concentration of 100 mg/L before dissection. Blood, liver, gonad, kidney, brain and gill samples were collected from all fish, with the exception of the gills from the fish used in the chronic exposure experiment. Blood was collected using 4 mL lithium heparin vacutainers (BD, New Jersey, USA) and stored on ice until the end of the sampling day. Tissue samples were placed directly in labelled cryovials and flash frozen using liquid nitrogen before being stored at -80º C. After all the fish had been sampled, the blood samples were centrifuged at 3000 rpm for 10 minutes and the plasma transferred into 1.5 mL microcentrifuge tubes and stored at -80º C.

3.7 Analysis

3.7.1 Ultra Performance Liquid Chromatography – Mass Spectroscopy

Water analysis was performed using an ultra performance liquid chromatography – mass spectroscopy system (Waters Acquity UPLC, Micromass Quattro micro API). Settings used were based on the information provided in Miao et al. (2002) with a Waters Acquity UPLC BEH C18 1.7 µm 2.1 x 100 mm column (Appendix 1). Calibration standards were prepared using the same ibuprofen sodium as in the treatments, diluted using Milli-Q water and filtered through 0.45 µm syringe filters. Integration values obtained from the standards were used to prepare calibration curves. Using these curves the actual concentration of ibuprofen in the treatment tanks was determined.
3.7.2 Condition Factor

The condition factor (K) was calculated using the weight of the whole fish and the total length based on the equation:

\[
K = \frac{\text{Body Weight}}{\text{Total Length}^3} \times 100
\]

3.7.3 Liver Somatic Index

The liver somatic index was calculated based on the relationship between liver weight to whole body weight using the equation:

\[
\text{LSI} = \frac{\text{Liver Weight}}{\text{Body Weight}} \times 100
\]

3.7.4 Gonad Somatic Index

The gonad somatic index (GSI) was calculated based on the relationship between gonad weight to whole body weight using the equation:

\[
\text{GSI} = \frac{\text{Gonad Weight}}{\text{Body Weight}} \times 100
\]

3.7.5 Cyclooxygenase Activity Assay

The total COX activity present in the trout tissue samples were evaluated by measuring the peroxidise activity of the COX-1 and COX-2 isoforms on a colourimetric substrate. To complete the COX Activity assay in accordance with the manufacturers’ instructions, frozen gill and kidney samples were weighed out to 80 mg and 400 µL of Grinding Buffer (0.1 M Tris-HCl, pH 7.8, 1 mM EDTA) was added and homogenized. After homogenization, samples were centrifuged at 10 000 g for 15 minutes at 4 °C. The supernatant was collected and stored at -80° C until analysis.
When analysis was to be performed, tissue supernatant samples were removed and thawed. Each sample was divided into two aliquots, one which remained the active sample and the other was boiled for 5 minutes to destroy all enzyme activity to produce inactive background values. After boiling, the background samples were centrifuged for 1 minute at 8 000 g.

To prepare the microplate, 120 µL of Assay Buffer (100 mM Tris-HCl, pH 8.0) was added to each well, except the COX standard wells where 150 µL were added. 10 µL of heme, prepared by diluting 88 µL of the provided heme solution with 1912 µL of assay buffer, was added to each well. Forty microliters of the background sample were added to the plate in duplicate and 40 µL of the active sample were added to the plate in triplicate. 10 µL of the provided COX standard was added to the standard wells. The plate was shaken slightly to mix and incubated at 25º C for 5 minutes. After the incubation period, 20 µL of colourimetric substrate (N,N,N’,N’-tetramethyl-p-phenylenediamine) was added to each well, along with 20 µL of arachidonic acid, prepared by dissolving 100 µL of the provided arachidonic acid solution with 100 µL of the provided potassium hydroxide and diluting to 2 mL with Milli-Q water. After this last addition, the plate was slightly shaken to mix, incubated for 5 minutes at 25º C and read at 590 nm using a microplate reader (Bio-Tek Synergy HT microplate reader).

3.7.6 Bradford Protein Analysis

The assay used to determine total protein concentration in tissue samples is a microplate protocol modified from Bradford (1976). Total protein concentrations were determined for all liver and plasma samples using the liver homogenate prepared for the
ethoxyresorufin-O-deethylate (EROD) activity assay and the plasma samples for the fish vitellogenin enzyme linked immunosorbent assay (ELISA).

To begin, a 1 mg/mL solution of bovine serum albumin (> 96%) was prepared by dissolving 1 mg in 1 mL of Milli-Q water. This stock solution was then used to prepare a 6 point calibration curve (Appendix 2). The tissue samples were diluted to 1/100 using Milli-Q water. Ten microlitres of each calibration standard and tissue sample were added to the microplate in triplicate. Two hundred twenty five microlitres of Bradford Reagent were added to every well and the plate vortexed at 400 rpm for 1 minute. The plate was incubated in the dark for 45 minutes and the absorbance read at 595 nm.

3.7.7 Enzyme Linked Immunosorbent Assay for Fish Vitellogenin

The microplate ELISA assay for fish vitellogenin was a 3 day protocol. On day 1, trout plasma samples were allowed to thaw and diluted either 1/1000 or 1/1500 in coating buffer (50 mM carbonate/ bicarbonate buffer, pH 9.6) to bring the total protein concentration for each sample to approximately 50 µg/mL. A 7 point calibration curve was prepared using the VTG standard and coating buffer (Appendix 3). Once all the standards and samples were prepared, 50 µL of each were added to the plate in triplicate and an additional 50 µL of coating buffer were added to the wells. The plate was covered, placed in a moistened plastic container and allowed to incubate overnight at 4º C.

After the incubation period, the plate was rinsed with a wash solution (Tween-Phosphate buffered saline, TPBS) and a blocking solution of bovine serum albumin in PBS (BSA 2% in PBS) was added to each well in a volume of 100 µL. The plate was then left to incubate at room temperature for 1 hour. The plate was again washed with
TPBS and 50 µL of primary antibody (polyclonal antibody rabbit anti-sea bream PO-2) was added to each well and was allowed to incubate overnight at 4º C to ensure binding to the VTG proteins.

On day 3, the plate was washed with TPBS before 50 µL of secondary antibody (secondary antibody peroxidase conjugated sheep anti-IgG Rabbit) was added to each well and allowed to incubate for 1 hour to allow the secondary antibody to bind to the primary antibody. During this incubation, the developing solution of 0.04% O-phenylenediamine with 0.012% hydrogen peroxide was prepared so 50 µL could be added to each well upon completion of the incubation. Once the developing solution was added, the plate was incubated in a humid container at 37º C for 30 minutes. The oxidation of the O-phenylenediamine by the peroxidase enzyme during this incubation allowed for the colourimetric reaction to occur. The reaction was then stopped using 1.8 M sulfuric acid and the absorbance read at 492 nm.

3.7.8 Ethoxyresorufin-O-Deethylase Activity Assay

The protocol used to measure EROD activity in the trout liver samples was adapted from Efler et al. (1998). Prior to analysis, the S9 fraction from the liver tissue had to be prepared. Fifty milligrams of liver tissue was weighed and 250 µL of HEPES grinding buffer (pH 7.5) was added and the sample homogenized. After homogenization, the samples were centrifuged at 9000 g for 20 minutes at 4º C. The supernatant was removed, along with the microsomal layer, and was stored at -80º C until analysis.

Before the analysis could be completed, a number of reagents were prepared. A resorufin super stock solution was prepared by dissolving 5 mg of resorufin per 1 mL
dimethyl sulfoxide (DMSO, > 99.7%). The resorufin needed to be completely dissolved so the solution was allowed to sit on a stir plate for 24 hours to ensure complete mixing. This solution was used to prepare a working stock solution by diluting 1 mL of super stock in 9 mL of DMSO. The 7-ethoxyresorufin (7ER) was also prepared in advance. A solution of 0.022 mg/mL 7ER in DMSO was prepared and the absorbance checked to ensure it read 1.6 and 1.7 absorbance units. When the resorufin super stock solution and 7ER were not being used they were stored at -20° C.

When the assay was to be completed, the liver samples were removed from the freezer and allowed to thaw. During this time the assay standards were prepared. A 5 point calibration curve was prepared using the resorufin working solution, further diluted in HEPES grinding buffer, and HEPES grinding buffer (Appendix 4). A solution of 7ER/HEPES buffer (pH 7.8) was prepared by diluting 550 µL 7ER in 4550 µL HEPES buffer. Once the solutions were prepared 50 µL of the standards and samples were added to the plate in triplicate. Fifty microlitres of 7ER/HEPES was then added to each well on the plate. The plate was placed in darkness and allowed to incubate at room temperature for 10 minutes. During this time a solution of NADPH (β – nicotinamide adenine dinucleotide 2'- phosphate reduced tetrasodium salt hydrate, 93.3%) was prepared by dissolving 20 mg in 1 mL of Milli-Q water. Before the end of the 10 minute incubation period, the plate was brought to the microplate reader so 10 µL of NADPH could be added to each well immediately before the fluorescence of the plate was read. The plate reader was set with an excitation filter of 530 nm (30 nm bandwidth) and an emission filter of 590 nm (35 nm bandwidth), and a sensitivity of 25. Each well of the plate was
read once a minute for 15 minutes. This allowed for the fluorescence units/min (FU/min) to be calculated for each sample as well as the slope of resorufin calibration curve.

3.8 Statistical Analysis

All statistics were run using Statistica 9.0 (Statsoft, Inc., Oklahoma, USA). All data sets were checked for normality using Shapiro-Wilks test and homogeneity of variance and transformed accordingly prior to running parametric analyses. All data sets, except those from the Bradford protein assay and VTG ELISA for the chronic experiment met the requirements for parametric analysis. All of the data were tested at α ≤ 0.05. Data from the acute experiments were analyzed using a 1-way ANOVA and Tukey HSD tests. Data from the chronic experiments, except total plasma protein concentration and VTG ELISA data, were analyzed using a factorial ANOVA and Tukey HSD. For the data sets that did not conform to parametric analysis, a Kruskal-Wallis ANOVA was used to test individual factors for significance. If significance was determined, that factor was then tested using a Mann-Whitney U test. All data was then graphed using SigmaPlot 11.0 (Systat Software, Inc., Germany).
4.0 Results

For both the acute and chronic exposure experiments, the results were analyzed initially by comparison of the treatments, including the E2 control, to the experimental control. If a significant difference was determined ($p \leq 0.05$), an asterisk (*) was denoted on the corresponding figure. Letters were also used to distinguish similarity or significant difference between treatments where appropriate.

4.1 Acute Exposure

At the conclusion of the exposure, the average fish weight was $76.4 \pm 19.5$ g.

4.1.1 Water Analysis

Water samples were taken from all treatment tanks, measured using UPLC-MS and the results pooled (Table 1). The remainder of this work will reference the nominal concentrations.

4.1.2 Mortality Data

During the 96 hour exposure, there was a total mortality of 3 fish. One dead fish was found in each of the E2 control (replicate 1), 1 mg/L ibuprofen (replicate 1) and the mixed E2-ibuprofen treatment (replicate 1) tanks. All three fish showed discoloration on their caudal fin (Figure 6). For analysis, it should be noted that all treatments except those mentioned above have $n = 8$, while those with mortality had $n = 7$. 
Table 1: Ibuprofen water concentrations from the acute exposure measured using UPLC-MS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mg/L ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND*</td>
</tr>
<tr>
<td>E2 Control</td>
<td>ND</td>
</tr>
<tr>
<td>Ibu 1 mg/L</td>
<td>1.23 ± 0.02</td>
</tr>
<tr>
<td>Ibu 10 mg/L</td>
<td>4.66 ± 0.17</td>
</tr>
<tr>
<td>Mixed**</td>
<td>4.87 ± 0.43</td>
</tr>
</tbody>
</table>

*ND = Not Detected

** Mixed Treatment = 1 mg/L E2 + 10 mg/L Ibu
Figure 6: Mortality from the acute ibuprofen exposure experiment (mixed E2-ibuprofen treatment). Note the discolouration on the caudal region of the fish.
4.1.3 K, GSI, LSI

The condition factor showed that the fish used were in good condition upon completion of the experiment. The K values for all treatments were close to, or over 1.0 (Appendix 5). There was also no difference between fish in the various treatment tanks and controls based on GSI (Appendix 6).

There were significant differences in LSI found between the treatments and control (Figure 7). The LSI for the E2 control and the E2-ibuprofen mixed treatment were significantly higher than the control, while the two ibuprofen treatments did not vary from each other.
Figure 7: Liver somatic index (LSI) from the acute ibuprofen exposure experiment. Bars represent mean ± SD. * denotes statistically significant difference (p ≤ 0.05) with respect to the control treatment. n=8 for all treatments except E2 control, Ibu 1 mg/L and mixed 1 mg/L E2 + 10 mg/L Ibu treatments where n=7.
4.1.4 Total COX Activity

Total COX activity was measured in the gill, as well as the kidney, for all fish in the acute experiment. This was due to the availability and condition of the samples, as well as the lack of relevant COX activity data for these tissues present in the literature. When looking at the gill, there were no significant differences as compared to the control (Figure 8a). While the control was similar to all of the treatments, there were other variations detected. The COX activity of the E2 control was significantly higher than the E2-ibuprofen mixed treatment. This was the only difference, with both ibuprofen treatments being similar to all of the other experimental groups.

Similar trends were observed when analyzing the kidney data. Again, the control was not different from any of the treatment groups, with one difference being detected amongst treatments. Kidney COX activity in the E2 control and 1 mg/L ibuprofen treatment were significantly different, with the E2 control having lower COX activity (Figure 8b).

When the tissues were compared in terms of treatment, the only difference observed was between the E2 control groups (Figure 9).
**Figure 8:** Total COX activity measured from the (A) gill and (B) kidney tissue from the acute ibuprofen exposure experiment. Bars represent mean ± SD. Different letters denote treatments belonging to groups with statistically significant difference (p ≤ 0.05). n=8 for all treatments except E2 control, Ibu 1 mg/L and mixed 1 mg/L E2 + 10 mg/L Ibu treatments where n=7.
Figure 9: Comparison of the total COX activity between the gill and kidney tissue from the acute ibuprofen exposure experiment. Bars represent mean ± SD. Different letters denote statistically significant difference between tissues within a treatment (p≤0.05). n=8 for all treatments except E2 control, Ibu 1 mg/L and mixed 1 mg/L E2 + 10 mg/L Ibu treatments where n=7.
4.1.5 Total Plasma Protein & VTG Concentrations

Blood samples were collected from all fish except those in the 1 mg/L ibuprofen exposure so total plasma protein concentrations and VTG concentrations were not measured for that treatment. After the 96 hour exposure, there were no significant differences in total plasma protein concentration between any of the treatments and the controls (Figure 10).

The VTG concentrations between the treatments were similar to one another and there were no significant differences in VTG between treatment groups (Figure 11).

4.1.6 EROD Activity

There were no significant differences in EROD between any treatments, possibly due to the large variability of the control group (Figure 12).
**Figure 10:** Total plasma protein concentrations from the acute ibuprofen exposure experiment. Bars represent mean ± SD. n=8 for all treatments except E2 control and mixed 1 mg/L E2 + 10 mg/L Ibu treatment where n=7.
Figure 11: Vitellogenin (VTG) concentrations from the acute ibuprofen exposure experiment. Bars represent mean ± SD. n=8 for all treatments except E2 control and mixed 1 mg/L E2 + 10 mg/L Ibu treatment where n=7.
Figure 12: Liver ethoxyresorufin-O-deethylase (EROD) activity data from the acute ibuprofen exposure experiment. Bars represent mean ± SD. n=8 for all treatments except E2 control, Ibu 1 mg/L and mixed 1 mg/L E2 + 10 mg/L Ibu treatment where n=7.
4.2 Chronic Exposure

Average fish weight was 89.9 ± 31.4 g upon completion of the 56 day chronic exposure. This value did not vary greatly over the 3 sampling periods, with the average weights for sampling times 1, 2 and 3 being 84.8 ± 30.0 g, 95.6 ± 30.9 g and 89.7 ± 33.5 g, respectively.

4.2.1 Water Analysis

Water samples were taken from a subset of the exposure tanks from both replicates. Samples were collected and analyzed from the control, 1000 µg/L ibuprofen exposure and mixed E2-ibuprofen treatments (Table 2).

4.2.2 Mortality Data

During the chronic 8 week exposure, there was a combined experimental mortality of 17 fish (Table 3). No mortality was experienced in any of the control, E2 control or 1 µg/L ibuprofen treatments. The greatest mortality occurred in the mixed E2-ibuprofen treatment, with each tank experiencing a mortality of 5 fish. Deaths were first observed during week 2 and continued for the remainder of the experiment.

In addition to recording mortality, fish that died were individually checked for physical damage. Jaw damage was observed in most dead fish, initially seen on day 30 and which progressed for the remainder of the experiment (Table 3). Beginning as just abrasions around the mouth of the fish, damage advanced to deterioration of the complete lower jaw (Figure 13). It should be noted that only dead fish were examined for this
damage. Other fish in the treatment tanks may have displayed this tissue degradation but if they survived to the sampling periods they were not included with this data.

Due to the significant experimental mortality that occurred, especially near the completion of the experiment, the number of fish in each treatment needed to be considered when evaluating the data. For all analyses, replicates were pooled, allowing for a maximum of 10 fish to be included for each treatment at each time period. The control, E2 control and 1 µg/L ibuprofen treatments had no mortality and n = 10 for each sampling period while other treatments had reduced sample numbers (Table 4). These sample sizes relate to all of the chronic exposure figures.
**Table 2:** Ibuprofen water concentrations from the chronic exposure from select treatments measured using UPLC-MS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentrations per Sampling Time (µg/L)</th>
<th>Ave ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 4</td>
</tr>
<tr>
<td><strong>Rep – 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Ibu 1000 µg/L</td>
<td>1030</td>
<td>973</td>
</tr>
<tr>
<td>Mixed**</td>
<td>979</td>
<td>993</td>
</tr>
<tr>
<td><strong>Rep – 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ibu 1000 µg/L</td>
<td>984</td>
<td>1010</td>
</tr>
<tr>
<td>Mixed</td>
<td>909</td>
<td>984</td>
</tr>
<tr>
<td><strong>Combined</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Ibu 1000 µg/L</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Mixed</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

*ND = Not Detected

** Mixed Treatment = 1000 µg/L E2 + 1000 µg/L Ibu
**Table 3:** Mortality data and instances of jaw damage recorded from chronic exposure experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th># Deaths</th>
<th></th>
<th># Fish with Jaw Damage*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicate 1</td>
<td>Replicate 1</td>
<td>Replicate 1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E2 Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ibu 1 µg/L</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ibu 32 µg/L</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Ibu 1000 µg/L</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mixed**</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

*Refers only to observations made on fish that died during the course of the exposure and not those that were sampled.

** Mixed Treatment = 1000 µg/L E2 + 1000 µg/L Ibu
**Figure 13:** Jaw damage observed during the chronic exposure. (A) initial damage observed which progressed into (B) full jaw degradation.
Table 4: Number of fish sampled for each treatment during each sampling period for the chronic exposure experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 14</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>E2 Control</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Ibu 1 µg/L</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Ibu 32 µg/L</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Ibu 1000 µg/L</td>
<td>5</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Mixed*</td>
<td>4</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td><strong>Day 28</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>E2 Control</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Ibu 1 µg/L</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Ibu 32 µg/L</td>
<td>5</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Ibu 1000 µg/L</td>
<td>5</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Mixed</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td><strong>Day 56</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>E2 Control</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Ibu 1 µg/L</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Ibu 32 µg/L</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Ibu 1000 µg/L</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Mixed</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

* Mixed Treatment = 1000 µg/L E2 + 1000 µg/L Ibu
4.2.3 K, GSI, LSI

Over the 56 day experimental exposure, none of the treatments showed significant difference in condition factor (K) from the control, each other or between sampling periods (Appendix 7). All of the treatments, at all of the sampling times, had values close to 1.0.

The same trend was observed with the gonad somatic index (GSI). None of the treatments varied significantly from the control at each sampling period. When combined for the whole experiment, again, no differences were observed (Appendix 8).

Similar to the results found in the acute exposure, differences were detected in the liver somatic index (LSI) (Figure 14). Overall, there were significant differences in LSI between treatments. While the LSI for all three ibuprofen exposures were similar to the control, they varied from one another. The 1 µg/L treatment LSI was significantly lower than the LSI for the 1000 µg/L treatment. Also, the E2 control and mixed E2-ibuprofen treatment LSIs were different from all other treatments, including the control, and to one another. The mixed E2-ibuprofen treatment had a higher LSI value than the E2 control.

To see the trends clearly, the data were broken into their respective sampling times (Figure 15). On Day 14, the E2 control and the mixed treatment LSIs were significantly greater than the control LSI. Although differing from the control treatment, the two were similar to the 1000 µg/L ibuprofen treatment LSI. Across the remaining sampling times, day 28 and day 56, the E2 control and mixed E2-ibuprofen treatment LSIs remained significantly higher than the controls as well as the remainder of the other treatment groups.
**Figure 14:** Complete LSI data from the chronic ibuprofen exposure experiment. Data from all 3 sampling periods (day 14, 28, 56) have been pooled. Bars represent mean ± SD. Different letters denote statistically significant difference between treatments ($p \leq 0.05$). $n=30$ for all treatments except Ibu 32 µg/L ($n=25$), Ibu 1000 µg/L ($n=28$) and mixed 1000 µg/L E2 + 1000 µg/L Ibu treatments ($n=20$).
Figure 15: LSI data from the chronic ibuprofen exposure broken down by sampling time. Bars represent mean ± SE. * denotes statistically significant difference (p ≤ 0.05) with respect to the control treatment. Letters denote similarity between groups. n=10 for all treatments except 32 µg/L (n=10, n=9, n=6), 1000 µg/L (n=9, n=11, n=8) and mixed 1000 µg/L E2 + 1000 µg/L Ibu (n=9, n=8, n=3) treatments for day 14, day 28 and day 56, respectively.
4.2.4 Total COX Activity

Unlike the acute exposure experiment, total COX activity in the chronic exposure experiment was only measured in kidney tissue of the fish because of the availability and condition of the tissue. Also, only a subset was analyzed. The control, E2 control, 1000 µg/L ibuprofen and E2-ibuprofen mixed treatments were assayed over the 3 sampling periods. When all the data was combined, there were no differences between the mean COX activity values for any of the treatments, with the only difference being reduced error associated with the ibuprofen exposures (Figure 16). No statistical significance was detected in COX activity between any of the treatments and control for the 3 sampling periods (Figure 17). Although no difference was determined, biological significance may have been overshadowed by variation in the control groups. An issue arose due to the average COX activity in the control group decreasing between day 14 and day 56.

To control for changing control values over time, the COX activity for each treatment was graphed as a fold of the control for each respective sampling period (Figure 18). Ibuprofen treatments on day 14 showed approximately 25% inhibition of activity compared to the control. Two weeks later, the activity increased to levels similar to the control but by day 56 the activity was almost 50% greater than controls. Similarly, the E2 control, 1000 µg/L ibuprofen and mixed E2-ibuprofen treatments from the first sampling period were significantly different from the E2 control and ibuprofen treatments for the last sampling period.
Figure 16: Complete kidney COX activity data from the chronic ibuprofen exposure experiment. Data from all 3 sampling periods (day 14, 28, 56) have been pooled. Bars represent mean ± SD. n=30 for all treatments except Ibu 32 µg/L (n=25), Ibu 1000 µg/L (n=28) and mixed 1000 µg/L E2 + 1000 µg/L Ibu (n= 20) treatments.
Figure 17: Kidney COX activity data from the chronic ibuprofen exposure broken down by sampling time. Bars represent mean ± SE. Different letters denote statistically significant difference between groups (p≤ 0.05). n=10 for all treatments except 32 µg/L (n=10, n=9, n=6), 1000 µg/L (n=9, n=11, n=8) and mixed 1000 µg/L E2 + 1000 µg/L Ibu (n=9, n=8, n=3) treatments for day 14, day 28 and day 56, respectively.
Figure 18: Kidney COX activity data from the chronic ibuprofen exposure broken down by sampling time and represented as fold of control. Bars represent mean ± SE. Different letters denote statistically significant difference between groups (p ≤ 0.05). n=10 for all treatments except 32 µg/L (n=10, n=9, n=6), 1000 µg/L (n=9, n=11, n=8) and mixed 1000 µg/L E2 + 1000 µg/L Ibu (n=9, n=8, n=3) treatments for day 14, day 28 and day 56, respectively.
4.2.5 Total Plasma Protein & VTG Concentrations

Overall, the E2 control and mixed E2-ibuprofen treatments had significantly higher total plasma protein concentrations over the course of the entire chronic experiment (Figure 19). When sampling periods are analyzed separately, it can be seen that the two treatments were consistently different from the controls and remaining treatments over each time point (Figure 20).

The results of the VTG ELISAs mimic that of the total plasma protein concentration. When the entire experiment is analyzed, three treatments show significant difference in VTG concentration. The E2 control, the 1000 µg/L ibuprofen and mixed E2-ibuprofen treatments had significantly higher VTG relative to the controls (Figure 21). This relationship was clarified when the data were analyzed by sampling time. While the E2 control and mixed E2-ibuprofen treatment have VTG concentrations that were significantly higher than the controls, some of the other ibuprofen exposures also varied (Figure 22). On day 14, the 1000 µg/L ibuprofen treatment VTG concentration was significantly lower than the control. On day 28, this treatment was no longer different while the 1 µg/L treatment was significantly lower than controls. By the last sampling period on day 56, every treatment had VTG concentrations that were significantly greater than the control.
Figure 19: Complete plasma protein concentration from the chronic ibuprofen exposure experiment. Data from all 3 sampling periods (day 14, 28, 56) have been pooled. Bars represent mean ± SD. * denotes statistically significant difference (p ≤ 0.05) with respect to the control treatment. n=30 for all treatments except Ibu 32 μg/L (n=25), Ibu 1000 μg/L (n=28) and mixed 1000 μg/L E2 + 1000 μg/L Ibu (n= 20) treatments.
Figure 20: Plasma protein concentrations from the chronic ibuprofen exposure broken down by sampling time. Bars represent mean ± SE. * denotes statistically significant difference (p ≤ 0.05) with respect to the control treatment. n=10 for all treatments except 32 µg/L (n=10, n=9, n=6), 1000 µg/L (n=9, n=11, n=8) and mixed 1000 µg/L E2 + 1000 µg/L Ibu (n=9, n=8, n=3) treatments for day 14, day 28 and day 56, respectively.
**Figure 21:** Complete VTG concentrations from the chronic ibuprofen exposure experiment. Data from all 3 sampling periods (day 14, 28, 56) have been pooled. Bars represent mean ± SD. * denotes statistically significant difference (p ≤ 0.05) with respect to the control treatment. n=30 for all treatments except Ibu 32 µg/L (n=25), Ibu 1000 µg/L (n=28) and mixed 1000 µg/L E2 + 1000 µg/L Ibu (n= 20) treatments.
Figure 22: VTG concentration data from chronic ibuprofen exposure broken down by sampling time. Bars represent mean ± SE. * denotes statistically significant difference (p ≤ 0.05) with respect to the control treatment. n=10 for all treatments except 32 µg/L (n=10, n=9, n=6), 1000 µg/L (n=9, n=11, n=8) and mixed 1000 µg/L E2 + 1000 µg/L Ibu (n=9, n=8, n=3) treatments for day 14, day 28 and day 56, respectively.
4.2.6 EROD Activity

EROD activity over the entire experiment in every treatment except the 32 µg/L ibuprofen exposure was significantly lower than the control (Figure 23). Analysis by sampling period, however, revealed temporal differences (Figure 24). On day 14, the 1 µg/L ibuprofen treatment was the only group with significantly lower EROD activity than controls. By day 28, the EROD activity of the lowest ibuprofen treatment had increased whereas the activity of the highest exposure, 1000 µg/L ibuprofen, was now significantly reduced relative to controls. By day 56, two groups showed significant reduction in EROD activity; the E2 control and the E2-ibuprofen mixed treatment. The mixed E2-ibuprofen treatment EROD activity was also significantly lower than EROD activity in the 1 µg/L and 1000 µg/L treatments.
Figure 23: Complete EROD activity data from the chronic ibuprofen exposure experiment. Data from all 3 sampling periods (day 14, 28, 56) have been pooled. Bars represent mean ± SD. Different letters denote statistically significant difference between groups (p ≤ 0.05). n=30 for all treatments except Ibu 32 µg/L (n=25), Ibu 1000 µg/L (n=28) and mixed (1000 µg/L E2 + 1000 µg/L Ibu) (n= 20).
Figure 24: EROD activity data from chronic ibuprofen exposure broken down by sampling time. Bars represent mean ± SE. * denotes statistically significant difference (p ≤ 0.05) with respect to the control treatment. Different letters denote statistically significant difference between groups (p ≤ 0.05). n=10 for all treatments except 32 µg/L (n=10, n=9, n=6), 1000 µg/L (n=9, n=11, n=8) and mixed 1000 µg/L E2 + 1000 µg/L Ibu (n=9, n=8, n=3) treatments for day 14, day 28 and day 56, respectively.
5.0 Discussion

5.1 Mortality

Mortality occurred during both the acute and chronic ibuprofen exposure experiments. The dead fish observed were distinct due to the apparent physical differences. During the 96 hour exposure experiment, the fish that died had discolouration of their caudal region. One reason for this may be stress. The fish were held prior to experimentation in 1500 L circular tanks and were moved to 70 L tanks, filled to 45 L, without acclimation. This stress to the fish may have resulted from the change in tank conditions. In circular tanks, water enters tangentially, which not only creates a current for the fish to swim against but also results in homogeneous water conditions (Oca & Masaló, 2007). This allows water parameters, such as dissolved oxygen content, to be uniform throughout the entire tank. Also, the water current aids in self-cleaning of the tank, forcing the solid material into the center outflow pipe (Oca & Masaló, 2007). Moving from the stable conditions of the large holding tanks to the smaller experimental aquaria would likely increase the stress levels of the fish. In the glass exposure tanks, the low flow and rectangular aquarium shape does not allow for a current to be created. However, while low flow could have formed stagnant areas, the presence of an air stone in the tanks helped maintain oxygen levels as well as circulate water.

Another possible reason for the observed colour change was physical trauma. A loss of scales may account for the apparent ‘white colour’ in the caudal region. With nothing abrasive in the exposure aquaria except the air stones, which were covered, the
damage was probably caused by other fish. In both nature and the laboratory, salmonid fish form dominance hierarchies (Gilmour et al., 2005). Fish are able to assume dominant positions over other more subordinate fish through antagonistic interactions (Gilmour et al., 2005). This generally occurs through aggression (Gilmour et al., 2005). Confined to smaller experimental tanks the subordinate fish would have nowhere to escape the aggression of the dominant fish. Chasing and fin nipping may cause a loss of scales, producing the light colour. The lack of feeding during the 96 hour exposure may have also increased this activity.

A combination of these factors may have lead to the discolouration observed on the fish that died during the acute exposure. It is assumed that the fish that died were the subordinate fish in the tank that became weakened due to aggression asserted by the dominant fish. To reduce the possibility of this occurring in future experiments, fish should be fed to satiation prior to being placed in the experimental tanks and an acclimation period should occur prior to the start of the exposure. The use of smaller fish would have also reduced stress levels significantly.

The acute experiment was run before the chronic exposure, which allowed for experimental design changes to be made. In the chronic exposure the fish were allowed to acclimate in the treatment tanks before the exposure began. This allowed the fish to become accustomed to their new environment, presumably reducing stress. Although dominance did occur in the tanks due to the increased number of fish (n = 15), the fish were fed twice daily and the density was reduced with each sampling period. While the overall conditions were improved compared to the acute exposure, mortality was
significantly higher in the ibuprofen and mixed treatments, likely due to the long exposure period.

While some of the mortality experienced can be attributed to the reasons already outlined for the acute experiment, such as aggression, the physical damage observed requires further explanation. A large number of the dead fish showed significant jaw damage. The jaw damage observed in the two highest ibuprofen treatments (32 µg/L and 1000 µg/L ibuprofen), as well as the mixed E2-ibuprofen treatment, may be a treatment effect.

Prostaglandins play a crucial role in the complex regulatory network of inflammation and tissue damage (Tilley et al., 2001). Not only do they act to promote inflammation and initiate the healing process, they also play a role in the termination of the inflammatory response (Tilley et al., 2001). With this knowledge studies began to focus on the effectiveness of wound healing in the presence of reduced PG levels caused by NSAID exposure. Using mouse epithelium as a model system, Müller-Decker et al. (2002) showed that exposure to NSAIDs did not retard normal skin healing. Mice fed an oral diet of selective and non-selective COX inhibitors did not show a difference in incision wound healing compared to the controls (Müller-Decker et al., 2002). Other studies, however, have demonstrated that the regenerative properties of different tissues, such as cartilage and muscle, can be affected upon NSAID use (Bondesen et al., 2004; Chang et al., 2006). Using epiphyseal-articular cartilage obtained from fetal rats, Chang et al. (2006) found that NSAIDs altered the activity of chondrocytes, critical for cartilage maintenance and bone repair. It was discovered that at therapeutic concentrations the pharmaceuticals inhibited thymidine incorporation, arresting the cell cycle (Chang et al.,
This suppressed proliferation and induced cellular death, but it should be noted that COX-2 selective NSAIDs demonstrated non-significant effects on cytotoxicity (Chang et al., 2006). In addition to structural tissue, Bondesen et al. (2004) investigated the role of PGs in myogenesis. Using mice chronically treated with potent NSAIDs specific for COX-1 and COX-2 they determined that following a localized freeze injury COX-2 was crucial for muscle regeneration. By following myofiber regeneration for up to 5 weeks post injury, the group determined that PGs produced from the COX-2 isoform were essential during the early stages of muscle regeneration (Bondesen et al., 2004).

Although these pathways are still not well defined, it is understood that inflammation is necessary for repair. Inflammation causes fluid exudation, bringing cellular agents to the site to act in tissue repair, which is modulated by PGs (Tilley et al., 2001). With chronic exposure to ibuprofen, the inflammatory response in the exposed fish may have been inhibited or completely prevented. If the fish were damaged through aggressive behaviours that were taking place in the tank, the lack of PGs in their system may have caused the small facial damage to progress into the large wounds where muscle and cartilage damage was apparent. To the best of the author’s knowledge this kind of tissue damage in relation to ibuprofen exposure has not been documented previously in rainbow trout and further research into wound healing in aquatic organisms exposed to NSAIDs needs to occur.

5.2 K, GSI, LSI

All of the fish from each treatment in both of the experiments had condition factor (K) values close to, or over 1. While according to some scaling guidelines these fish
might be considered in ‘fair’ or ‘poor’ condition, this endpoint indicated that overall population health did not vary significantly during the course of the experiments (Barnham & Baxter, 1998). The general condition of each fish did not significantly improve nor decrease, showing that conditions were stable. The fish were not fed to satiation so their weight should not have increased as compared to length. Also, the fish were not on a starvation ration so they should not have lost weight compared to their length. While condition factor does not provide a great deal of information on the effects of acute and chronic ibuprofen exposure, by remaining constant throughout the experiment it removed one potential confounding factor.

Like condition factor, gonad somatic index (GSI) did not show any significant changes over the exposure periods. No changes were anticipated during the acute experiment because although some treatments were dosed with E2, 96 hours was not sufficient to see such changes at an organ level. Changes in GSI, however were expected during the chronic ibuprofen experiment due to the increased exposure period. Although the fish used were sexually immature, the hormone treatment may have initiated development of the gonads.

One potential reason for this not occurring was the nature of the exposure. The fish were not treated with the hormone continuously. Although exposed for 8 weeks, the fish were only exposed to E2 on the first day of every week. This concentration of E2 was sufficient to increase plasma protein concentrations, such as VTG, but may not have been enough to alter the gonad itself. Werner et al. (2003) continuously exposed immature lake trout to various concentrations of the hormone 17-α-ethynylestradiol, ranging from 4 ng/L to 400 ng/L for 21 days. They found a significant increase in GSI
for all of the treatment groups (Werner et al., 2003). In the present study, E2 was added to the water at a concentration of 1 mg/L so changes should have been observed if the fish were continually exposed. In future studies, the concentration of E2 used in a waterborne exposure could be reduced from 1 mg/L to allow for the continual addition of the hormone to the treatment tanks.

Both experiments showed differences between treatments with respect to liver somatic index (LSI). Elevated LSIs indicate a large liver relative to the overall body weight. There are many factors that can affect liver weight in fish. Change in liver size is most often associated with toxicant exposure (Schmitt & Dethloff, 2000). Research has demonstrated increased levels of xenobiotic detoxification caused increased size rather than increased amounts of detoxification enzymes present including the class of CYP1A enzymes (Schmitt & Dethloff, 2000). In contrast, other work has shown that liver size can decrease when exposed to certain types of chemicals (Schmitt & Dethloff, 2000).

In the acute exposure experiment, the E2 control and mixed E2-ibuprofen treatment had LSI values that were significantly higher than the control group. The chronic experiment also displayed a significant increase in LSI of those two treatments compared to the control group. In the chronic experiment, the mixed E2-ibuprofen treatment had a significantly higher LSI than the LSI of the E2 control group overall. This may be misleading because when the data were separated into sampling times, the treatments did not differ from each other at any of the time points. The E2-ibuprofen mixed treatment LSI on day 56 was the largest of any treatment but only had n = 3 due to mortality, so the small sample size may have skewed the analysis.
There are few reasons to explain the increase in LSI observed. One reason is the presence of E2. With the addition of the hormone the immature female trout would begin sexual maturation processes, such as vitellogenesis (Schmitt & Dethloff, 2000). The liver in fish undergoing vitellogenesis increases in size due to the production of VTG (Werner et al., 2003). This correlates with the results of the VTG ELISA data from the chronic exposure. Even though no change in VTG was detected in the acute exposure experiment, the process of VTG production may have been initiated in the liver, with the duration of exposure not being long enough for the concentrations in the blood to be at measurable levels. As mentioned previously, liver size can increase due to detoxification processes, so ibuprofen exposure may have contributed to the increase in LSI.

Flippin et al. (2007) exposed Japanese medaka (Oryzias latipes) to ibuprofen for 21 days and calculated the LSI. They found that there was no change in LSI from the control in the ibuprofen exposure treatments, but did state that the trend for females was to have increased LSI (Flippin et al., 2007). Parrott and Bennie (2009) conducted a life cycle study utilizing fathead minnows (Pimephales promelas) exposed to a mixture of pharmaceuticals, including ibuprofen, and also did not find a significant change in LSI. From these findings it can be said that the increase in LSI observed in both the acute and chronic experiment was likely the result of the hormone treatment initiating vitellogenesis and not from the liver detoxification of ibuprofen.

5.3 COX Activity

There was no inhibition of total COX activity measured in either the acute or chronic ibuprofen experiments. The results from the 96 hour exposure showed no
significant difference in COX activity between any of the treatments and the control group for gill or kidney tissue. The only difference observed was the E2 control in the gill had a higher activity than the mixed E2-ibuprofen treatment, whereas the E2 control had a lower COX activity than the 1 mg/L ibuprofen treatment in the kidney tissue. When the tissues were compared, this difference in the E2 control was the only significant variation detected.

Research conducted by Akarasereenont et al. (2000) using human endothelial tissue demonstrated that 17-β-estradiol can alter COX activity. By dosing the tissue with E2, they showed that there was an increase in 6-keto-PGF$_{1α}$ production caused by the increased activity of COX-2 (Akarasereenont et al., 2000). In addition to humans, E2 was also proven to modify COX-2 activity in rat oviduct tissue when the rats were injected with the hormone (Pérez Martínez et al., 2006). This trend was not apparent in this study. In gill tissue, there was a significant difference in trout COX activity between the two treatments that were exposed to E2. The reason for this difference may be that the ibuprofen present in the mixed E2-ibuprofen treatment inhibited the COX activity induced by the hormone. This cannot be definitively stated though because although COX activity differed between treatments, there was no difference in activity from the controls. Also, there was no sign of this trend in COX activity in kidney tissue so no definitive conclusions can be made.

When analyzing the overall COX activity for the chronic exposure even less difference was seen between the tissues. There was no difference between the mean COX activities for any of the treatments measured. Although ibuprofen is a well studied COX inhibitor, other published works have commented on the lack of inhibition observed
in fish. Cavallaro and Burnside (1988) studied the effect of PGs on retinomotor movement in teleosts to see if reduced PG levels could affect these motor functions. Fish were treated with a number of COX inhibitors, including ibuprofen and it was found that while the potent COX inhibitor indomethacin reduced COX enzyme activity, ibuprofen showed no effects (Cavallaro & Burnside, 1988).

This was recently corroborated by Lister and van der Kraak (2008). Using zebrafish (*Danio rerio*) to elucidate the role of PGs on oocyte maturation and ovulation, they observed no change in COX activity when the fish were exposed to the NSAID indomethacin up to 100 µg/L for 16 days (Lister & van der Kraak, 2008). COX activity in ovary and whole body homogenates showed no changes (Lister and van der Kraak, 2008). However, levels of PGE$_2$ were significantly lower and no reasons were provided for this observation (Lister and van der Kraak, 2008).

Similarly, Flippin et al. (2007) exposed Japanese medaka to ibuprofen for 6 weeks at environmentally relevant concentrations and measured liver COX activity. They observed high variability in the COX activity in the controls and reduced variability was the primary effect of the ibuprofen exposure (Flippin et al., 2007). This effect was found in overall COX activity in the present chronic exposure experiment. Both measured treatments, 1000 µg/L ibuprofen and mixed E2-ibuprofen, had reduced variability in COX activity relative to the controls. Control variability in COX activity was large likely due to changing activity over the 8 week experiment. From day 14 to day 56, total COX activity in the control decreased significantly. When plotted as fold of control to remove this effect, COX activity continually increased from day 14 to day 56 for the E2 control, 1000 µg/L and E2-ibuprofen mixed treatments.
COX activity in trout did not appear to be inhibited by ibuprofen exposure, however, it is possible that inhibition occurred prior to sampling since even for the acute exposure, the first sampling did not occur until 96 hours. Since later time periods were analyzed (day 28 and day 56) and no inhibition was found, one possible outcome was that inhibition occurred before 96 hours. With the longer exposure, the fish may have recovered their COX activity. The fish may also have increased ibuprofen metabolism to lessen its effects, or the tissues may have increased synthesis of the COX enzymes in an attempt to counteract reduced PG levels (Flippin et al., 2007). Another possible explanation may be that the natural variation in COX activity hides any inhibition that may have occurred (Flippin et al., 2007). If the activity between samples varies naturally by a significant amount, even if the activity in one particular sample was reduced, the reduced activity of another sample may differ so much that the effect is lost in the overall variability of the treatment.

To definitively determine if ibuprofen inhibits COX activity in rainbow trout, more research needs to be completed. Acute studies, with more sampling times and higher sample sizes may show that ibuprofen inhibits COX activity but only in a limited time window.

5.4 VTG Concentration

Acute ibuprofen exposure showed no change in total plasma protein concentrations, and subsequently, VTG concentrations. It should be noted that the total plasma protein concentration and VTG concentration are linked. Each plasma sample was diluted accordingly to bring the total protein concentration within the optimal range.
for the ELISA to be run. Samples with higher total protein concentrations had higher VTG levels. In the acute exposure experiment, no measurable VTG concentrations were expected. Although vitellogenin is known to be induced by external estrogens such as E2, 96 hours of waterborne hormone exposure is not sufficient time to induce its production (Hansen et al., 1998). E2 treatments involved exposure to 1 mg/L, but this concentration would not have been completely taken up by the organism. These results showed that 96 hours of waterborne exposure to 1 mg/L E2 was not sufficient to induce VTG in female rainbow trout. It is possible that if the potent hormone E2 is not able to induce estrogenic effects in 96 hours, any potential effects caused by ibuprofen would not be detected either.

To determine potential estrogenic effects of ibuprofen on rainbow trout, the chronic exposure experiment was undertaken. Overall total plasma protein concentrations indicated that treatments exposed to E2, including the E2 control and the mixed E2-ibuprofen treatment, had significantly greater plasma protein concentrations than the controls and the other ibuprofen treatments. This trend was apparent over all 3 sampling periods and directly correlated to the VTG results. Overall VTG data showed that the E2 control, 1000 µg/L ibuprofen treatment as well as the mixed E2-ibuprofen treatment showed significantly greater VTG concentrations than the control group. In relation to the sampling time, it is important to recognize that the E2 control and E2-ibuprofen mixed treatments always displayed greater VTG concentrations, and it was not until day 56 that all three ibuprofen treatments were significantly greater than the control.

With all of the ibuprofen treatments showing increased VTG concentrations, this suggests the possible estrogenic activity of ibuprofen. The NSAID diclofenac increased
VTG expression levels in male Japanese medaka following 96 hours of exposure to concentrations as low as 1 µg/L with sampling occurring at 12 hours, 1, 2, and 4 days (Hong et al., 2007). This study thus suggests that non-steroidal anti-inflammatory drugs are capable of endocrine disruption via estrogenic effects (Hong et al., 2007).

A recent study by Han et al. (2010) investigated the relationship between ibuprofen and steroidogenesis in Japanese medaka and a human adrenocortical carcinoma cell line (H295R). This research made two main observations. First, through the use of the cell line, they found that ibuprofen increased E2 production in a dose-dependent manner (Han et al., 2010). Secondly, they found that ibuprofen increased VTG concentrations in male fish (Han et al., 2010). They also showed that in addition to increased E2 concentrations in the ibuprofen exposed treatments, there was also increased aromatase activity (Han et al., 2010).

Aromatase is a cytochrome P450 enzyme (CYP19) that is responsible for the biosynthesis of estradiols from androgens (Brueggemeier et al., 2005). This finding contradicted other published research investigating the link between aromatase and COX (Brueggemeier et al., 2006). Localized production of PGs can affect aromatase activity. PGE2 can influence estrogen synthesis by increasing the concentration of cyclic AMP (cAMP) (Brueggemeier et al., 2006). It is believed that without the presence of PGs, caused by COX inhibition, the activity of aromatase would also be decreased (Brueggemeier et al., 2006).

The research presented here has similar trends to that of Han et al. (2010). The ibuprofen exposures may have caused increased E2 levels in the fish, which could stimulate the production of VTG, increasing the levels compared to the controls. To
confirm this trend, the E2 levels as well as the aromatase activity in the ibuprofen treated samples should be measured. The exact mechanism by which ibuprofen increases E2 and VTG concentrations is not known and more research needs to be done in this field.

5.5 EROD Activity

Measured EROD activity from the acute exposure showed that there were no significant differences between the treatments and the controls. Changes in EROD activity were only observed in the chronic exposure experiment.

At the end of the chronic exposure, it was determined that all of the treatment groups, other than the 32 µg/L ibuprofen exposure, had EROD activity that was significantly lower than the controls. It would appear that ibuprofen was able to inhibit EROD activity in trout liver tissue.

In addition to the ibuprofen treatments, the E2 control also showed significant reduction in EROD activity. It has been well studied that E2 has the ability to inhibit CYP1A activity (Elskus, 2004; Navas & Segner, 2000; Whyte et al., 2000). Navas & Segner (2000), using cultured rainbow trout hepatocytes, demonstrated that maturing female fish, or fish exposed to E2, suppress hepatic CYP1A protein levels and EROD activity. Although the mechanism is unknown, it is believed that there is a relationship between the E2 receptors and estrogen responsive elements in the CYP1A gene (Navas & Segner, 2000). They also found that levels of E2 capable of suppressing EROD activity were similar to those able to induce VTG production (Navas & Segner, 2000). This concept was backed up by Elskus (2004). Again using rainbow trout primary
hepatocytes, they showed that E2 could inhibit EROD activity in cells that were treated with a potent EROD inducer (Elskus, 2004).

This trend was supported in the current study, although not until day 56 when a significant reduction in activity was measured in the E2 control as well as the mixed E2-ibuprofen treatment. This is different from published data where inhibition was apparent in acute studies, but the designs of the experiments were different. In those works, inhibition was measured using tissue cultures allowed to soak in the hormone directly, whereas this experiment used *in vivo* exposures to waterborne E2 concentrations. The concentration received by the fish would be less than the concentrations in the tank (1 mg/L), so this may be responsible for the delay in observed inhibition.

On the other hand, there are conflicting reports about the effects of NSAIDs on EROD activity. Work published on the NSAID diclofenac show the drug induces as well as inhibits EROD. Laville *et al.* (2004) demonstrated that diclofenac was able to inhibit EROD activity in rainbow trout hepatocytes using tissue cultures. In contrast, acute work done with Japanese medaka utilizing real-time PCR showed diclofenac was able to induce CYP1A gene expression in liver tissue (Hong *et al.*, 2007). A 9.3-fold induction was measured at 1 µg/L whereas a 64-fold induction was measured at a diclofenac concentration of 8 mg/L (Laville *et al.*, 2004). This indicated that at low concentrations the NSAID is a weak inducer while it is a moderate inducer at higher concentrations (Whyte *et al.*, 2000).

When focusing on ibuprofen specifically, there are also conflicting reports. A 48 hour exposure of rainbow trout hepatocytes to ibuprofen showed no change in measured EROD activity (Gagne *et al.*, 2006). This was the same result as published by Thibaut *et
Ibuprofen showed no inhibition of EROD activity in carp (Cyprinus carpio) liver (Thibaut et al., 2006). When Thibaut and Porte (2008) measured the effects of ibuprofen on a cell line from topminnow (Poeciliopsis lucida) for a time study they found that ibuprofen induced EROD activity. EROD activity was measured at 3, 6 and 12 hours at ibuprofen concentrations of 0.1, 1, 10 and 20 µM and ibuprofen induced EROD activity at a concentration of 10 µM at 6 hours only (Thibaut & Porte, 2008). No other induction was observed at any other concentration at any other sampling period.

The results of the chronic experiment show that there was inhibition of EROD activity in some of the ibuprofen treatments. The 1 µg/L ibuprofen exposure showed significant inhibition of EROD compared to the control on day 14, but the activity levels increased to similar to the control by day 28. Also, the 1000 µg/L treatment showed inhibition of activity on day 28 but not on day 14 or 56. The 32 µg/L treatment did not show inhibition at any of the sampling times measured.

The mixed E2-ibuprofen treatment followed a pattern similar to the E2 control by only being significantly different from the control at day 56. As stated previously, the 1000 µg/L treatment did not show reduced EROD activity on the last sampling period and the E2-ibuprofen mixed treatment did, but the small sample size for the mixed treatment on day 56 (n = 3) may have skewed the results. To get a better idea of the mixture effects of E2 and ibuprofen on EROD activity, the experiment would need to be repeated to ensure enough samples were measured from the mixed E2-ibuprofen treatment.

The lack of apparent trends in the data may be due to the fact that ibuprofen may act as both an inhibitor as well as an inducer of EROD activity, depending on the
concentration and length of exposure (Thibaut & Porte, 2008). To definitively determine the effects of ibuprofen on rainbow trout in vivo, more experiments need to be completed. Acute studies measuring EROD activity more frequently using a wide range of concentrations may shed light on whether ibuprofen is an inducer or inhibitor of EROD activity.
6.0 Conclusions

The purpose of this research was to evaluate the effects of ibuprofen on rainbow trout following acute and chronic exposures. The main objectives were to evaluate if COX activity was inhibited in various tissues, if ibuprofen was able to elicit estrogenic effects by measuring plasma VTG concentrations and if EROD activity was altered following exposure. Upon analysis, it was found that there were no changes in gill and kidney tissue COX activities following a 96 hour and 8 week exposure to ibuprofen. VTG concentrations were measured and although no change was determined in the acute exposure, slight estrogenic effects were detected in the chronic experiment. By day 56 of the chronic exposure, all of the ibuprofen treatments showed significantly greater VTG concentrations when compared to the controls. Lastly, no change in EROD activity was observed in the acute experiment, but every treatment in the chronic exposure except the 32 µg/L ibuprofen treatment displayed significantly reduced EROD activity. There are many conflicting reports in the literature regarding ibuprofen’s effect on EROD, indicating that this endpoint needs more research to evaluate its utility as a biomarker of exposure to ibuprofen.

Many aspects of the effects of ibuprofen on aquatic organisms, including rainbow trout, still need to be evaluated. It has been well studied that ibuprofen is an inhibitor of the COX enzyme, but research has shown that there is an apparent lack of inhibition in fish (Cavallaro & Burnside, 1988; Flippin et al., 2007; Lister & van der Kraak, 2008). In regards to VTG, the mechanisms by which ibuprofen can increase these levels are not understood. It is believed that ibuprofen can increase E2 levels, leading to an increase in vitellogenin, but the pathway for this hormonal increase has not been elucidated (Han et
The effect on EROD activity is also poorly understood. In addition to ibuprofen, other pharmaceuticals from the class of non-steroidal anti-inflammatory drugs, such as diclofenac, have had conflicting data published. Both ibuprofen and diclofenac have been stated to inhibit as well as induce EROD activity (Gagne et al., 2006; Hong et al., 2007; Laville et al., 2004; Thibaut & Porte, 2008). The results of EROD activity from the chronic exposure show that ibuprofen may act as an inhibitor of activity, but more work needs to be completed before definitive conclusions can be made. It is these inconsistencies regarding the effects of ibuprofen in the aquatic environment that necessitate more work be focused in this field.

With North America dominating the market on pharmaceutical sales, the concern about PPCPs in the environment is not going to diminish (Corcoran et al., 2010). NSAIDs are one of the most prescribed drug classes and are commonly found in WWTP effluents and surface waters (Christen et al., 2010). Because of their presence in the environment, more work needs to go into studying ibuprofen and its interactions with fish. The effects of ibuprofen appear to be different in various aquatic organisms so well designed experiments are necessary to build the knowledge base (Han et al., 2010). The results of the outlined experiment are just one step towards understanding the effects of ibuprofen in the aquatic environment.
References


Appendix 1

Settings used for the UPLC-MS system. These parameters were used for (A) the chromatography and (B) mass spectroscopy instruments.

(A)

Waters Acquity SDS Method

Comment:
Solvent Selection A: A2
Solvent Selection B: B2
Low Pressure Limit: 0 psi
High Pressure Limit: 10000 psi
Solvent Name A: Methanol
Solvent Name B: Ammonium Formate
Switch 1: No Change
Switch 2: No Change
Switch 3: No Change
Seal Wash: 1.0 min
Chart Out 1: System Pressure
Chart Out 2: $V$
System Pressure Data Channel: No
Flow Data Data Channel: No
$\%A$ Data Channel: No
$\%B$ Data Channel: No
Primary A Pressure Data Channel: No
Accumulator A Pressure Data Channel: No
Primary B Pressure Data Channel: No
Accumulator B Pressure Data Channel: No
Degasser Pressure Data Channel: No
(Gradient Table)
Time(min) Flow Rate (mL/min) $\%A$ $\%B$ Curve
1. Initial 0.200 40.0 60.0 Initial
2. 3.00 0.200 85.0 15.0 6
3. 6.00 0.200 85.0 15.0 6
Run Events: yes

Waters Acquity Autosampler Method

Comment:
Load Ahead: Disabled
Loop Option: Full Loop
Loop offline: disable
Weak Wash Solvent Name: Water
Weak Wash Volume: 600 mL
Strong Wash Solvent Name: Acetonitrile
Strong Wash Volume: 200 mL
Target Column Temperature: 25.0 $\circ$C
Column Temperature Alarm Band: Disabled
Target Sample Temperature: 4.0 $\circ$C
Sample Temperature Alarm Band: Disabled
Pull Loop Overfill Factor: Automatic
Syringe Draw Rate: Automatic
Needle Placement: 2.0
Pre-Aspirate Air Gap: 4.0
Post-Aspirate Air Gap: 4.0
Column Temperature Data Channel: No
Ambient Temperature Data Channel: No
Sample Temperature Data Channel: No
Sample Organizer Temperature Data Channel: No
Sample Pressure Data Channel: No
Switch 1: No Change
Switch 2: No Change
Switch 3: No Change
Switch 4: No Change
Chart Out: Sample Pressure
Sample Temp Alarm: Disabled
Column Temp Alarm: Disabled
Run Events: yes

Waters Acquity 2996

116
Start Wavelength (nm) 253.00
End Wavelength (nm) 295.40
Resolution (nm) 2.4
Sampling Rate (spectra/s) 20,000
Filter Response 1
Exposure Time (ms) Automatic
Interpolate 656 Yes
Acquisition stop time (mins) 6.00
Save to disk: Yes

Waters Acquity 1996 Analog Channel 1
Output Mode Off

Waters Acquity 1996 Analog Channel 2
Output Mode Off

End Of Report
<table>
<thead>
<tr>
<th>Experiment Report</th>
<th>MassLynx 4.1</th>
<th>Page 1 of 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>File:</td>
<td>e:\instrumental\analgesic\analgesic.prolacquدبilumaproxttest05.exp</td>
<td></td>
</tr>
<tr>
<td>Printed:</td>
<td>Wednesday, June 15, 2011 14:50:48 Eastern Daylight Time</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Default Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creation Time</td>
<td>Thu 27 May 2010 14:49:19</td>
</tr>
<tr>
<td>Instrument Identifier</td>
<td></td>
</tr>
<tr>
<td>Version Number</td>
<td>1.0</td>
</tr>
<tr>
<td>Duration (min)</td>
<td>2.6</td>
</tr>
<tr>
<td>Calibration Filename</td>
<td>E:\Instrumental Chem\May 10 2011 cal.cal</td>
</tr>
<tr>
<td>Solvent Delay Divert Valve Enabled</td>
<td>0</td>
</tr>
<tr>
<td>Number Of Functions</td>
<td>2</td>
</tr>
</tbody>
</table>

**Function 1 : MRM of 229.00->184.90, Time 3.00 to 4.60, ES-**

<table>
<thead>
<tr>
<th>Type</th>
<th>MRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion Mode</td>
<td>ES-</td>
</tr>
<tr>
<td>Inter Channel Delay (sec)</td>
<td>0.020</td>
</tr>
<tr>
<td>InterScan Time (sec)</td>
<td>0.020</td>
</tr>
<tr>
<td>Span (Da)</td>
<td>0.2</td>
</tr>
<tr>
<td>Start Time (min)</td>
<td>3.0</td>
</tr>
<tr>
<td>End Time (min)</td>
<td>4.5</td>
</tr>
<tr>
<td>Repeats</td>
<td>10.0</td>
</tr>
<tr>
<td>Prnt(Da) Dau(Da) Dwell(s) Cone(V) Coll(eV) Delay(s)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>229.00 184.90 0.200 Tune 7.00 0.020</td>
</tr>
</tbody>
</table>

**Function 2 : MRM of 205.00->161.00, Time 4.60 to 5.60, ES-**

<table>
<thead>
<tr>
<th>Type</th>
<th>MRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion Mode</td>
<td>ES-</td>
</tr>
<tr>
<td>Inter Channel Delay (sec)</td>
<td>0.020</td>
</tr>
<tr>
<td>InterScan Time (sec)</td>
<td>0.020</td>
</tr>
<tr>
<td>Span (Da)</td>
<td>0.2</td>
</tr>
<tr>
<td>Start Time (min)</td>
<td>4.6</td>
</tr>
<tr>
<td>End Time (min)</td>
<td>5.6</td>
</tr>
<tr>
<td>Repeats</td>
<td>10.0</td>
</tr>
<tr>
<td>Prnt(Da) Dau(Da) Dwell(s) Cone(V) Coll(eV) Delay(s)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>205.00 161.00 0.200 Tune 8.00 0.020</td>
</tr>
</tbody>
</table>
Appendix 2

Calibration standards prepared for the Bradford protein assay. The bovine serum albumin (BSA) solution used was prepared by diluting 1 mg of BSA in 1 mL of Milli-Q water, creating a 1 mg/mL BSA solution. All standards were prepared in 1.5 mL microcentrifuge tubes and used immediately.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume (µL)</th>
<th>Concentration BSA (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BSA Solution</td>
<td>Milli-Q Water</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>225</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>5</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
<td>50</td>
</tr>
</tbody>
</table>
Appendix 3

Calibration standards prepared for the VTG ELISA. The VTG standard was prepared by diluting 10 μg of VTG obtained from the distributor in 1000 μL 1x phosphate buffered saline (PBS). This solution was then divided into aliquots of 80 μL, which were thawed and used to prepare the calibration curve.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume (μL)</th>
<th>Concentration VTG (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VTG Standard</td>
<td>Coating Buffer</td>
</tr>
<tr>
<td>1</td>
<td>0.7</td>
<td>332.6</td>
</tr>
<tr>
<td>2</td>
<td>1.4</td>
<td>331.9</td>
</tr>
<tr>
<td>3</td>
<td>2.8</td>
<td>330.6</td>
</tr>
<tr>
<td>4</td>
<td>6.9</td>
<td>326.4</td>
</tr>
<tr>
<td>5</td>
<td>13.9</td>
<td>319.4</td>
</tr>
<tr>
<td>6</td>
<td>20.8</td>
<td>312.5</td>
</tr>
<tr>
<td>7</td>
<td>27.8</td>
<td>305.6</td>
</tr>
</tbody>
</table>
Appendix 4

Calibration standards prepared for the EROD activity assay. Ten microlitres of the resorufin working solution were diluted in 4990 µL of HEPES grinding buffer to prepare the working stock/HEPES solution. All solutions were prepared in 1.5 mL microcentrifuge tubes and were kept in the dark until use.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume (µL)</th>
<th>Concentration Resorufin (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Working Stock/HEPES</td>
<td>HEPES Grinding Buffer</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>3</td>
<td>400</td>
<td>600</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
<td>400</td>
</tr>
<tr>
<td>5</td>
<td>800</td>
<td>200</td>
</tr>
</tbody>
</table>
Appendix 5

Condition factor (K) calculated from the acute ibuprofen exposure experiment. Bars represent mean ± SD. All treatments n=8, except E2 control, Ibu 1 mg/L and mixed E2-ibuprofen treatment where n=7.
Appendix 6

Gonad somatic index (GSI) calculated from the acute ibuprofen exposure experiment. Bars represent mean ± SD. All treatments n=8, except E2 control, Ibu 1 mg/L and mixed E2-ibuprofen treatment where n=7.
Appendix 7

Condition factor (K) calculated from the chronic ibuprofen exposure experiment. (A) Data from all 3 sampling periods (day 14, 28, 56) have been pooled. Bars represent mean ± SD. n = 30 for all treatments except Ibu 32 µg/L (n=25), Ibu 1000 µg/L (n=28) and mixed 1000 µg/L E2 + 1000 µg/L treatment (n=20). (B) Bars represent mean ± SE. n = 10 for all treatments except 32 µg/L (n=10, n=9, n=6), 1000 µg/L (n=9, n=11, n=8) and mixed 1000 µg/L E2 + 1000 µg/L (n=9, n=8, n=3) treatments for day 14, day 28 and day 56, respectively.

(A)
Sampling Time

- Day 14
- Day 28
- Day 56

Condition Factor (K)

- Control
- E2 Control
- Ibu 1 ug/L
- Ibu 32 ug/L
- Ibu 1000 ug/L
- Mixed

Y-axis: Condition Factor (K)

X-axis: Sampling Time

Day 14
Day 28
Day 56
Appendix 8

Gonad somatic index (GSI) calculated from the chronic ibuprofen exposure experiment. 

(A) Data from all 3 sampling periods (day 14, 28, 56) have been pooled. Bars represent mean ± SD. n = 30 for all treatments except Ibu 32 µg/L (n=25), Ibu 1000 µg/L (n=28) and mixed 1000 µg/L E2 + 1000 µg/L treatment (n=20). (B) Bars represent mean ± SE. n = 10 for all treatments except 32 µg/L (n=10, n=9, n=6), 1000 µg/L (n=9, n=11, n=8) and mixed 1000 µg/L E2 + 1000 µg/L (n=9, n=8, n=3) treatments for day 14, day 28 and day 56, respectively.

(A)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>E2 Control</th>
<th>ibu 1 ug/L</th>
<th>ibu 32 ug/L</th>
<th>ibu 1000 ug/L</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonad Somatic Index (GSI)</td>
<td>0.00</td>
<td>0.02</td>
<td>0.04</td>
<td>0.06</td>
<td>0.08</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Bar graph showing GSI values for different treatments with error bars indicating mean ± SD or SE.](image)
(B)

Sampling Time

Day 14  Day 28  Day 56

Gonad Somatic Index (GSI)

Control  E2 Control  Ibu 1 ug/L  Ibu 32 ug/L  Ibu 1000 ug/L  Mixed

Gonad Somatic Index (GSI)

Sampling Time

Day 14  Day 28  Day 56