THE APPLICABILITY OF THE “READ-ACROSS HYPOTHESIS” FOR ASSESSING THE EFFECTS OF HUMAN PHARMACEUTICALS ON FISH

A thesis submitted for the degree of Doctor of Philosophy

by

Alpa Patel

Department of Life Sciences
College of Health and Life Sciences
Brunel University London
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DECLARATION

The work submitted in this thesis was conducted between 2010 and 2014 at Brunel University London and AstraZeneca’s Brixham Environmental Laboratory. This work was carried out independently, except where otherwise specified, and has not been submitted for any other degree.

Alpa Patel
ABSTRACT

The presence of human pharmaceuticals in the environment has raised concerns regarding their potential adverse effects on non-target aquatic organisms. Pharmaceuticals are designed to target specific molecular pathways in humans in order to produce known pharmacological and physiological responses, before toxicological effects are seen. The “Read-Across Hypothesis” stipulates that pharmaceuticals can produce similar biological effects in fish, as in humans, if the molecular target is conserved, and the internal (blood plasma) concentrations are similar. The read-across hypothesis was tested using ibuprofen, a non-steroidal anti-inflammatory drug, and the model fish test species, the fathead minnow (Pimephales promelas), to determine if ibuprofen can cause similar target-mediated effects in teleost fish and humans, at comparable blood plasma concentrations. Fathead minnows were exposed, using continuous flow-through systems, for ≤96 hours to a range of ibuprofen water concentrations (100, 270, 370 and 500 µg/L) to determine if plasma concentrations similar to human therapeutic plasma concentrations (H7PCs, or Cmax) could be established in fish blood plasma. The mode of action of ibuprofen was used to identify relevant endpoints (i.e. cyclooxygenase (COX) enzyme) in order to examine target-mediated effects following drug exposure. The water and plasma ibuprofen concentrations were determined using LC-MS/MS. The measured ibuprofen plasma concentrations in individual fish were linked to target-mediated effects on COX gene expression, COX enzyme activity and prostaglandin E2 (PGE2) synthesis (products of COX activity), which were quantified using molecular (QPCR) and biochemical (colourimetric and enzyme immunoassay) assays, and linked with the Cmax of ibuprofen. It was demonstrated that in fish with a mean ibuprofen plasma concentration 1.8-fold below the Cmax, PGE2 concentrations (the most robust endpoint) was significantly inhibited following ibuprofen exposure. However, in fish exposed to an ibuprofen concentration closer to (2 to 3-fold above) environmentally relevant water concentrations (i.e. 9 µg/L), when the mean plasma concentration was 224-fold below the Cmax, fish did not respond to ibuprofen exposure. This study provides qualitative and quantitative evidence for the applicability of the “read-across hypothesis”, and highlights its potential utility for prioritising pharmaceuticals for environmental risk assessment.
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<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>B0</td>
<td>Maximum binding</td>
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<td>Bioconcentration factor</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Cmax</td>
<td>Maximal (or peak) plasma concentration</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase (protein)</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase (gene)</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DWC</td>
<td>Dilution Water Control</td>
</tr>
<tr>
<td>E₂</td>
<td>17-β-oestradiol</td>
</tr>
<tr>
<td>EBI</td>
<td>European Bioinformatics Institute</td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine-disrupting chemical</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EE₂</td>
<td>17-α-ethinylestradiol</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
</tr>
<tr>
<td>ERA</td>
<td>Environmental Risk Assessment</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FHM</td>
<td>Fathead minnow</td>
</tr>
<tr>
<td>FPM</td>
<td>Fish Plasma Model</td>
</tr>
<tr>
<td>F₅₀PC</td>
<td>Fish steady-state Plasma Concentration</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSI</td>
<td>Hepatosomatic Index</td>
</tr>
<tr>
<td>H₇PC</td>
<td>Human Therapeutic Plasma Concentration</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% Inhibitory Concentration</td>
</tr>
<tr>
<td>K</td>
<td>Condition factor</td>
</tr>
<tr>
<td>LB medium</td>
<td>Luria Broth medium</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>50% Lethal Concentration</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography-Mass spectrometry/Mass spectrometry</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>LOEC</td>
<td>Lowest Observed Effect Concentration</td>
</tr>
<tr>
<td>Log D</td>
<td>Distribution coefficient</td>
</tr>
<tr>
<td>Log Kₗw</td>
<td>Octanol-water partition coefficient</td>
</tr>
<tr>
<td>Log P</td>
<td>Partition coefficient</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MEC</td>
<td>Measured Environmental Concentration</td>
</tr>
<tr>
<td>mg/L</td>
<td>Milligram per Litre</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>MoA</td>
<td>Mode of Action</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>ND</td>
<td>Non-Detected</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NOEC</td>
<td>No Observed Effect Concentration</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
</tr>
<tr>
<td>NSB</td>
<td>Nonspecific binding</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OECD</td>
<td>Organisation for Economic Co-operation and Development</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEC</td>
<td>Predicted Environmental Concentration</td>
</tr>
<tr>
<td>PGEM</td>
<td>Prostaglandin E metabolite</td>
</tr>
<tr>
<td>PGₓ</td>
<td>Prostaglandin x</td>
</tr>
<tr>
<td>pg/mL</td>
<td>Picogram per millilitre</td>
</tr>
<tr>
<td>pKa</td>
<td>Acid Dissociation Constant</td>
</tr>
<tr>
<td>PNEC</td>
<td>Predicted No Effect Concentration</td>
</tr>
<tr>
<td>PTGS</td>
<td>Prostaglandin-endoperoxide synthase</td>
</tr>
<tr>
<td>QPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SC</td>
<td>Solvent Control</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective Serotonin Reuptake Inhibitor</td>
</tr>
<tr>
<td>STP</td>
<td>Sewage Treatment Plant</td>
</tr>
<tr>
<td>TA</td>
<td>Total activity</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TPMD</td>
<td>N,N,N',N'-tetramethyl-p-phenylenediamine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WFD</td>
<td>Water Framework Directive</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater Treatment Plant</td>
</tr>
<tr>
<td>μg/L</td>
<td>Microgram per Litre</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>±</td>
<td>Plus or minus</td>
</tr>
</tbody>
</table>
CHAPTER 1: GENERAL INTRODUCTION
1.1 PHARMACEUTICALS IN THE ENVIRONMENT

Pharmaceuticals are a large and diverse group of compounds designed to cure, treat and prevent disease. The importance of pharmaceuticals to modern society is reflected by their widespread usage in human and veterinary medicine, agriculture and aquaculture (Boxall, 2004; Fent et al., 2006). According to Intercontinental Medical Statistics (IMS) Health (Market Prognosis, 2013), expenditure on global pharmaceutical sales is expected to reach US $1 trillion in 2014 and exceed US $1.2 trillion by 2017, forecasting increased spending on worldwide health care. A growing total population and an ageing demographic are contributory factors to the increasing global use of human pharmaceuticals. Within the European Union (EU) alone, there are over 3,000 different active pharmaceutical ingredients (APIs) licensed for use in human medicines (Fent et al., 2006). An inevitable consequence of the extensive use of pharmaceuticals is their increased discharge into the environment. Around 160 pharmaceuticals have now been detected in sewage effluents, surface and ground waters, and even some drinking waters worldwide, and many of them are reported to be present at low concentrations, in the ng/L to µg/L range (Daughton and Ternes, 1999; Halling-Sørensen et al., 1998; Heberer, 2002; Kümmerer, 2010; Santos et al., 2010). Human pharmaceuticals have highly specific biological activities within the body, they are designed to resistant degradation and they can exert their intended therapeutic effects at concentrations much lower than the ones of other environmental pollutants (such as pesticides) (Kümmerer, 2009). The presence of pharmaceuticals in the environment has become an issue of scientific and political concern, because of their potential impact on aquatic and terrestrial wildlife, and human health.

1.1.1 Pharmaceutical consumption

The consumption of human pharmaceuticals in the UK is substantial. In 2013, over 1 billion prescription items were dispensed within the community (England and Wales only), costing a reported GDP £8.6 billion (NHS, 2013). This figure, however, only represents prescription medications and does not include those that are purchased over-the-counter, and so actual usage would be considerably higher. Consumption patterns can vary between different countries and over time, depending on regulations and approvals, prescribing practices and health care systems (Watts et al., 2007). IMS holds
data on all APIs sold in the UK, which can provide a better estimation of prescription and over-the-counter usage. Table 1, shows that the active ingredient sold in the highest quantities was paracetamol, at nearly three and a half thousand tonnes. Metformin, an antidiabetic drug used to treat type 2 diabetes, was ranked second at almost one thousand tonnes. Ibuprofen, amoxicillin and aspirin were also sold in high quantities, all of which exceeded more than one hundred tonnes. The large-scale consumption of pharmaceuticals, particularly of over-the-counter medicines, can contribute to their increased release into the environment, through patient use, and influence their presence in the aquatic environment.

Table 1. The amount (tonnes) of the top five active pharmaceutical ingredients (APIs) sold in the UK in 2011 (based on IMS data obtained by AstraZeneca).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Therapeutic class</th>
<th>Amount of active ingredient (tonnes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol (acetaminophen)</td>
<td>Analgesic</td>
<td>3,472</td>
</tr>
<tr>
<td>Metformin</td>
<td>Antidiabetic</td>
<td>967</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Non-steroidal anti-inflammatory</td>
<td>258</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>Antibiotic</td>
<td>198</td>
</tr>
<tr>
<td>Aspirin (Acetylsalicylic acid)</td>
<td>Analgesic</td>
<td>130</td>
</tr>
</tbody>
</table>

1.1.2 Sources and fate of human pharmaceuticals in the environment

Human pharmaceuticals are discharged into the aquatic environment through a number of different routes (Figure 1). The principal route is following patient consumption and excretion. After ingestion, pharmaceuticals can undergo incomplete or extensive metabolism, before they are excreted from the body in an unchanged form, as conjugates, and/or as metabolites. The degree to which pharmaceuticals are metabolised is highly variable; the antibiotic amoxicillin is largely unmetabolised and 60% of the drug is excreted in the parent form (Gordon et al., 1972), whereas only 1-2% of the antiepileptic drug carbamazepine is excreted unaltered (Houeto et al., 2012). Hepatic metabolism changes the physicochemical properties of the compound through Phase I reactions, which involve oxidation, reduction or hydrolysis of the drug, and Phase II which involves conjugation (for example the addition of glucuronic acid, sulphate,
acetic acid or amino acid) to produce more polar, water-soluble metabolites that can be more easily excreted in the urine and faeces (Daughton and Ternes, 1999). However, after undergoing metabolism, some APIs including aspirin, diclofenac, carbamazepine and sulphonamides produce bioactive metabolites which can persist in the environment (Celiz et al., 2009).

After excretion, the APIs are transported through the wastewater system to treatment plants, where they undergo a series of biological and chemical sewage treatment processes. The majority of APIs are removed from wastewater through biodegradation by microorganisms, and abiotic removal by adsorption to suspended solid sludge particles (Jelic et al., 2011). The removal of pharmaceuticals with suspended particulate material is dependent upon their hydrophobic and electrostatic interactions. Compounds with low adsorption coefficients, for example a low octanol-water partition coefficient ($\log K_{ow}$), are less likely to adsorb to organic matter and will tend to stay in the aqueous phase (Carballa et al., 2004). However, wastewater treatment plants (WWTPs) are not specifically designed to eliminate APIs and therefore the removal rates can vary depending on the nature of the pharmaceutical, the technology and performance of the treatment plant (for example the retention time or the length of time it takes influent waters to pass through the treatment stages), temperature and loading differences due to seasonal variations (Fent et al., 2006). Many pharmaceuticals are insufficiently removed, particularly hydrophilic APIs, and these will be discharged into the surface waters through the treated wastewater effluents. It is not surprising that many pharmaceuticals resist degradation, considering that most of these drugs are designed to be persistent, so that they can be retained for long enough within the body in order to exert their therapeutic effects.

Active pharmaceutical substances can also be discharged into the environment through improper disposal of unused or expired medicines, and through discharges from pharmaceutical manufacturing plants (which can include accidental spills during production or distribution) (Figure 1) (Monteiro and Boxall, 2010). The manufacture of pharmaceuticals is a controlled process in the UK adhering to Good Manufacturing Practice regulations, and therefore the release of APIs from these sites into the environment should be minimal (Kümmerer, 2009). However, these strict regulatory
practices may be lacking in developing countries, and where pharmaceutical manufacturing has been moved in order to reduce costs, the discharge of APIs into the environment presents more of an issue. For example, in India concentrations of up to several mg/L for single compounds have been reported in the effluents of WWTPs serving manufacturing sites (Larsson et al., 2007). The improper disposal of pharmaceuticals in household waste is another contributory source of the release of APIs into the environment. Pharmaceuticals that are available over-the-counter can be purchased in large quantities, which can make them prone to becoming expired and disposed of improperly. These activities can result in the deposition of large amounts of pharmaceutical substances at the WWTP. Similarly, disposal through household refuse can lead to contamination of landfill sites and leachate could contaminate the surrounding groundwater. The environmental impact of improper disposal could be significantly reduced by improving patient education (Kümmerer, 2009).

Once pharmaceuticals have been released into the surface waters, their fate and transport largely depend on their physiochemical properties and the environmental conditions (Pal et al., 2010). For example, the acid dissociation constant (pKa) can be used to estimate how much a pharmaceutical will dissociate at different environmental pH levels, and the Log $K_{ow}$, which is a measure of lipophilicity, can be used to estimate how likely a compound is to accumulate in organic matter. Pharmaceuticals with a Log $K_{ow}$ greater than 5 can more readily adsorb to organic particles and settle in soil or sediment (Mompelat et al., 2009), or they may be taken up in the fatty tissue of animals and plants, where they may cause biological effects. Some APIs undergo natural attenuation through dilution or further structural changes through a variety of biotic and non-biotic processes. For example, biodegradation is an important elimination mechanism for ibuprofen in surface waters (Buser et al., 1999) and many pharmaceuticals, including diclofenac, are abiotically removed through photodegradation (Andreozzi et al., 2003). Despite degradation processes in the environment, many APIs are classed as being “pseudo-persistent”, meaning that while they are not intrinsically persistent (i.e. they can be degraded to various extents), they are continuously being introduced into the aquatic ecosystem, thereby increasing their potential for adverse effects in exposed organisms (Daughton and Ternes, 1999).
Figure 1. Routes of entry of human pharmaceuticals into the aquatic environment. The most common route of entry is via consumption (image adapted and redrawn from Monteiro and Boxall, 2010).
1.1.3 Concentrations of human pharmaceuticals in the aquatic environment

The subtle connection between anthropogenic activities and the release of pharmaceuticals into the natural environment was made over three decades ago, when the prescription drug clofibric acid (the active metabolite of blood lipid regulators) and salicylic acid (a metabolite of aspirin) were reported in treated wastewater effluents (Garrison et al., 1976; Hignite and Azarnoff, 1977). However, following this initial documentation, relatively few studies were published until the 1990’s, and since then, there has been a proliferation in studies reporting trace levels of pharmaceuticals in sewage effluents, surface and ground waters and drinking waters (Daughton and Ternes, 1999; Halling-Sorensen et al., 1998; Heberer, 2002; Kolpin et al., 2002; Kümmerer, 2009; Ternes, 1998). Undoubtedly, one of the main reasons for this apparent increased detection is through the development of highly sensitive analytical methods that have enabled more accurate and reliable quantification of pharmaceuticals in different environmental matrices at low concentrations (ng to µg/L). Most analytical methods used for determination of APIs are mainly based on solid phase extraction (SPE), followed by liquid or gas chromatography (LC or GC), combined with mass spectrometry (MS), or for increased sensitivity and specificity, tandem mass spectrometry (MS/MS) (Richardson and Ternes, 2011). Due to their polarity, low volatility and labile thermal stability, LC-MS/MS has become the most common method for API determination in environmental water samples (Fatta-Kassinos et al., 2011). Furthermore, instrumental developments have enabled lower limits of quantification (LOQ) to be achieved using LC-MS/MS, which can be in the low ng/L range.

The measured environmental concentrations of some pharmaceuticals, covering a range of therapeutic classes, are shown in Table 2. This, however, is not a comprehensive list of all the pharmaceuticals identified, and is simply a representation of some of the pharmaceuticals that have been measured in parts of Europe, North America and Asia, highlighting their widespread occurrence. Limited information is available regarding the concentrations and occurrence of APIs in other parts of the world (Hughes et al., 2013). In most cases, the highest concentrations of pharmaceuticals are found in effluent, which were in the high ng/L to µg/L range. A very high concentration of ibuprofen...
(between 1.27-55 μg/L) was detected in water effluents in Spain, which could be attributed to the number (four) of WWTPs that serve this particular river. The estimated discharge of ibuprofen into this river was between 40 to 2,236 g/day (Santos et al., 2009), which is the equivalent to 5,590 therapeutic (400 mg) doses of ibuprofen. Paracetamol was also reported at very high concentrations in the UK and China (25 and 37 μg/L, respectively). This is not surprising considering its high over-the-counter sales and consumption rate, which is in excess of 3,000 tonnes per year (Table 1).

The concentrations of pharmaceuticals in surface waters were lower (low ng/L range) than in effluents. The river and surface water concentrations of pharmaceuticals can vary greatly, depending on the amount of dilution of effluent in receiving waters, regional usage of APIs and the efficiency of the wastewater treatment processes (Pal et al., 2010). However, consistent with the reported concentrations of paracetamol in effluents, the surface water concentrations were found to be the highest in the UK (1.5 μg/L). Other pharmaceuticals with high ng/L surface water concentrations were bezafibrate (lipid regulator) which had the second highest reported concentration (667 ng/L in the UK), and carbamazepine (antiepileptic) (647 ng/L in the UK), the third highest concentration. Both of these pharmaceuticals were reported at concentrations around 10-fold higher in the UK, than in Europe (Spain), whereas other pharmaceuticals such as paracetamol was reported at concentrations between 10-21-fold higher in UK, than in Spain and Asia (South Korea), respectively. Naproxen was reported at similar concentrations in the UK and North America, but higher than in Spain. These apparent differences between countries may reflect the prescribing practices in individual countries, the population density within a region, or the varying efficiencies of WWTPs, and therefore making comparisons between the reported concentrations in different regions can be challenging.
Table 2. Pharmaceuticals measured in wastewater effluents and surface waters in the UK and worldwide. * denotes unusually high concentration found in effluent. (a) Kasprzyk-Hordern et al., 2009; (b) Santos et al., 2009; (c) López-Serna et al., 2010; (d) Comeau et al., 2008; (e) Zhang et al., 2007; (f) Kim et al., 2007; (g) Lin et al., 2008. ND means non-detected. – denotes not measured.

<table>
<thead>
<tr>
<th>Therapeutic Class</th>
<th>Mode of Action (MoA)</th>
<th>Pharmaceutical</th>
<th>Location</th>
<th>Concentration (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Effluent</td>
<td>Surface Water</td>
</tr>
<tr>
<td><strong>Anti-inflammatory drugs</strong></td>
<td>Cyclooxygenase enzyme inhibitors</td>
<td>Ibuprofen</td>
<td>UK</td>
<td>491 (a)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Spain</td>
<td>1,270-55,000* (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>North America</td>
<td>220 (d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diclofenac</td>
<td>UK</td>
<td>496 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spain</td>
<td>ND (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>South Korea</td>
<td>9-127 (f)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Naproxen</td>
<td>UK</td>
<td>703 (a)</td>
</tr>
<tr>
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<td>Spain</td>
<td>2,100 (b)</td>
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<td>North America</td>
<td>5,100 (d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aspirin</td>
<td>UK</td>
<td>13 (a)</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Ketoprofen</td>
<td>37 (a)</td>
</tr>
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<td></td>
<td></td>
<td>Spain</td>
<td>840 (b)</td>
</tr>
<tr>
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<td></td>
<td>Mefenamic acid</td>
<td>UK</td>
<td>222 (a)</td>
</tr>
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<td></td>
<td></td>
<td>UK</td>
<td>24,525* (a)</td>
</tr>
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<td></td>
<td></td>
<td>Paracetamol</td>
<td>Spain</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>China, South Korea</td>
<td>36,950* (g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trimethoprim</td>
<td>UK</td>
<td>3,052 (a)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>China, South Korea</td>
<td>321 (g)</td>
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<tr>
<td><strong>Antibiotics</strong></td>
<td></td>
<td>Erythromycin</td>
<td>UK</td>
<td>2,841 (a)</td>
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<tr>
<td></td>
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<td></td>
<td>Spain</td>
<td>677 (c)</td>
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### Table 2. Continued…

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<tr>
<th>Therapeutic Class</th>
<th>Mode of Action (MoA)</th>
<th>Pharmaceutical</th>
<th>Location</th>
<th>Concentration (ng/L)</th>
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<td>Effluent</td>
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<td><strong>β-blockers</strong></td>
<td>β-adrenergic receptor inhibitors</td>
<td>Propranolol</td>
<td>UK</td>
<td>523 (a)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Spain</td>
<td>51 (c)</td>
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<td></td>
<td></td>
<td>China</td>
<td>132 (g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Atenolol</td>
<td>UK</td>
<td>7,602 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>China</td>
<td>1,607 (g)</td>
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<tr>
<td><strong>Lipid regulators</strong></td>
<td>Activation of nuclear receptors</td>
<td>Bezafibrate</td>
<td>UK</td>
<td>90 (a)</td>
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<tr>
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<td></td>
<td></td>
<td>Spain</td>
<td>217 (c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clofibric acid</td>
<td>UK</td>
<td>75 (a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spain</td>
<td>22 (c)</td>
</tr>
<tr>
<td><strong>Psychoactive compounds</strong></td>
<td>Blockage of voltage-dependent sodium ion channels</td>
<td>Carbamazepine</td>
<td>North America</td>
<td>33 (d)</td>
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<td></td>
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<td>UK</td>
<td>4,596 (a)</td>
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<td></td>
<td>Spain</td>
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<td>Diazepam</td>
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</tbody>
</table>
1.1.4 Effects of human pharmaceuticals in the environment

The presence of human pharmaceuticals in the environment has led to concerns regarding their potential impact on aquatic and terrestrial organisms. In order to determine the type of effects that may be elicited, an understanding of how these contaminants are designed to work is required. For a chemical to exert pharmacological or biological activity as an orally active drug in humans, it must be relatively small and lipophilic enough to effectively penetrate biological membranes within the body in order to reach its intended pharmacological target, according to “Lipinski’s rule of five” (Lipinski et al., 1997) which evaluates the “drug-likeness” of a chemical.

1.1.4.1 Mode of action of human pharmaceuticals

Pharmaceuticals achieve their therapeutic effects by binding to a molecular drug target, which initiates a specific physiological response within an organism. The majority of drug targets are proteins, which are classed into four major groups: receptors, ion channels, enzymes and transporters (Figure 2) (Rang et al., 2003). Pharmaceuticals are designed to have high-specificity, high-affinity interactions with their intended molecular drugs targets in humans in order to alter specific biochemical processes and biological pathways at low concentrations, through their specific modes of action (MoA). The realisation that many human pharmaceuticals are present in the environment has highlighted the potential for unwanted effects on aquatic and terrestrial wildlife, as many human drug targets have been conserved throughout evolution (Gunnarsson et al., 2008; Seiler, 2002) and therefore, they may be able to alter biological pathways or physiological responses in non-target organisms, by interacting with the same or similar molecular drug targets, as they do in humans (Christen et al., 2010). The characterisation of the MoAs of different pharmaceuticals that are found in the environment could prove useful in determining the risks posed by these contaminants, as the potential effects they may elicit (at environmentally relevant concentrations) are related to the MoA of individual or classes of pharmaceuticals.
1.1.4.2 Evidence for the effects of pharmaceuticals on wildlife

To date, there have been two clear cases documenting the causal link between exposure and adverse effects of human pharmaceuticals on wildlife. These are the “feminisation” of wild fish through exposure to endocrine-disrupting chemicals, such as the synthetic oestrogen 17-α-ethinylestradiol (EE₂), a component of the female contraceptive pill (Jobling et al., 1998; Sumpter, 1995), and the dramatic crash of a population of vultures through ingestion of the non-steroidal anti-inflammatory drug (NSAID) diclofenac (Oaks et al., 2004). The impact of both of these pharmaceuticals has been widely studied, and there are considerable data linking them to measurable effects in the environment.
1.1.4.2.1 Feminisation of male fish

Endocrine-disrupting chemicals (EDCs) can mimic natural hormones by binding to and activating oestrogen receptors, which can induce physiological effects on reproduction and development. Evidence for endocrine disruption was first reported in the early 1990’s in the UK, when caged male fish located downstream from a sewage treatment plant (STP) were found with elevated plasma concentrations of the female specific egg-yolk protein precursor vitellogenin, indicating exposure to oestrogenic compounds present in effluents (Purdom et al., 1994). Further field studies confirmed that there was a high incidence of “intersex” fish (characterised by the presence of oocytes and/or oviducts in the testes of otherwise male fish) in wild populations living downstream from STPs (Jobling et al., 1998). Sewage effluents were found to contain several compounds with oestrogenic activities, including natural steroid hormones such as 17-β-oestradiol (E$_2$) and synthetic oral contraceptive hormones such as EE$_2$ (Desbrow et al., 1998), as well as industrial phenols such as nonylphenol and bisphenol A (Harries et al., 1997; Jobling et al., 1996). The exact causative agent of feminisation was not identified; however laboratory studies have since provided convincing evidence for causality between exposure to steroidal oestrogens, especially EE$_2$, and reproductive disruption in fish (Tyler and Routledge, 1998). EE$_2$ can cause effects on fish reproduction at concentrations as low as 1 ng/L (Parrott and Blunt, 2005) and population-level effects at concentrations only slightly higher (~5 ng/L) (Kidd et al., 2007; Länge et al., 2001; Nash et al., 2004), indicating the potential for EE$_2$ to cause dramatic effects on fish, at environmentally relevant concentrations. However, whether there is a long-term population-level impact in wild populations as a result of intersexuality through feminisation of males is still uncertain (Harris et al., 2011). Although intersexuality in aquatic organisms was not foreseen, it is important to highlight that the MoA for EE$_2$ is through the oestrogen receptor, which is highly conserved between human and other vertebrate species, including fish (Christen et al., 2010).

1.1.4.2.2 Acute poisoning of vultures

The most well-documented example of a pharmaceutical causing acute, population-level effects through the food chain is exemplified by diclofenac, which has been
implicated in the catastrophic decline of over 99% of the *Gyps* species of vultures in India and Pakistan (Green et al., 2004). Diclofenac was once widely used as a veterinary drug for the treatment of inflammatory diseases in domestic cattle. Exposure to diclofenac was found to be the principal cause of death in a large number of scavenging birds that had been feeding on the carcasses of cattle treated with a normal veterinary dose of the drug. The *Gyps* vultures were found to be highly sensitive to diclofenac, and exposure to the drug induced acute kidney failure and abdominal gout in these birds (Oaks et al., 2004). The median lethal dose ($LC_{50}$) of diclofenac in this species is low (0.1-0.2 mg/kg) (Green et al., 2004), and therefore exposure to lethal doses could be easily attained through ingestion of the tissues of cattle that were treated with the drug, consistent with diclofenac poisoning. Diclofenac is an inhibitor of the cyclooxygenase (COX) enzyme, which is involved in the synthesis of prostanoids (Vane, 1971). It has been postulated that diclofenac may have induced renal failure, which is a known side effect of diclofenac overdose in humans (Hickey et al., 2001), in these vultures through the inhibition of the modulating effects of prostanoids in the kidneys (Meteyer et al., 2005). It is estimated that somewhere between 10 and 40 million vultures were poisoned, and as a consequence the *Gyps* species of vultures have now been listed as critically endangered across the Indian subcontinent (Sumpter, 2010). The manufacture of diclofenac for veterinary use has since been banned in some countries (Pain et al., 2008). This unprecedented decline of vulture populations due to diclofenac poisoning has highlighted a major issue in our current knowledge concerning the potential effects of pharmaceuticals present in the environment and in non-target organisms.

### 1.1.4.3 Laboratory evidence for the effects of pharmaceuticals

It is often very difficult to identify the causal link between pharmaceutical exposure and adverse effects in the wildlife. This is particularly true for aquatic wildlife, which are exposed to a highly complex mixture of chemicals in the water, and therefore identifying the specific chemical(s) of concern, and linking them to the measurable effects reported (for example, intersexuality in fish) is challenging. Nevertheless, the link is much easier to establish in laboratory (*in vivo* exposures) studies, and numerous studies have documented the effects of individual chemicals on aquatic
species at low concentrations, including anti-inflammatories and analgesics, antibiotics, β-blockers, steroidal hormones and psychoactive compounds (reviewed in Corcoran et al., 2010; Fent et al., 2006; Santos et al., 2010). These studies have demonstrated that human pharmaceuticals can affect a wide range of non-target aquatic species, and fish in particular, appear to be more sensitive to human drugs than other (non-vertebrate) organisms. This is most likely due to the high level of evolutionary conservation between human drug targets in fish (Gunnarsson et al., 2008; Seiler, 2002) (discussed further in Section 1.3.1.1). Some of the reported effects of pharmaceuticals seen in fish are comparable to the effects that would be expected in humans based on the MoA (Christen et al., 2010). For example, the synthetic oestrogen EE₂, induces oestrogenic effects in fish, such as vitellogenin synthesis (see earlier, Section 1.1.4.2.1), the synthetic glucocorticoid beclomethasone, used for the treatment of asthma and allergies in patients, can produce anti-inflammatory effects in fish, through an increase in plasma glucose concentration (Kugathas and Sumpter, 2011) and the antidepressant, fluoxetine (generic name “Prozac”) used to modulate behaviours in humans, can also affect the behaviour of fish (Dzweczynski and Hebert, 2012; Weinberger II and Klaper, 2014). Although the extrapolation of effects observed in laboratory studies to the field, and wild fish, are not always directly comparable, these studies form a fundamental component of the environmental risk assessment of pharmaceuticals, as discussed below.

1.2 ENVIRONMENTAL RISK ASSESSMENT (ERA)

In both the EU and North America, there are regulations governing the environmental risk assessment (ERA) of human pharmaceuticals (Holm et al., 2013). The ERA procedure outlined in the EU by the European Medicines Agency is mandatory for all new pharmaceutical products prior to their approval for entry onto the market (EMEA, 2006). In addition, the EU has policies on water quality and sustainability that are enforced through legislation under the Water Framework Directive (WFD) (2000/60/EC). A recent proposal was made during the revision of the WFD (2000/60/EC) to include, for the first time, three human pharmaceuticals (EE₂, E₂ and diclofenac) as “priority substances” due to their widespread use and
detection in surface waters. However, it was decided that these substances would instead be included on an environmental monitoring “watch list” under the WFD. These substances will be monitored in the surface waters of EU member states using established environmental quality standards to ensure adequate protection of the aquatic environment and human health (European Union, 2000).

1.2.1 ERA within the EU

The ERA is a tiered stepwise process consisting of two phases: the first phase (Phase I) estimates the maximal concentration of the pharmaceutical expected to occur in the environment, and the second phase (Phase II) assesses their potential fate and effects in the environment (Bound and Voulvoulis, 2004). During Phase I the predicted environmental concentration (PEC) in surface water (PEC_{surface water}) is calculated using information based on usage data, and a worst-case scenario is assumed regarding exposure, for example, no metabolism occurred in the patient and there was no removal of the API during sewage treatment processes (Holm et al., 2013). If the estimated PEC_{surface water} value exceeds the environmental threshold limit of 0.01 µg/L, the environmental fate and effects of the API is evaluated in Phase II. In general, if the threshold limit is not exceeded, this implies that the API is present at such low concentrations that an environmental risk is unlikely. However, in some cases the action limit is not applicable, and further testing is carried out in Phase II, irrespective of whether the threshold limit is exceeded or not. For example, if the API is known to bioaccumulate, or if it is a suspected EDC, then it may be able to adversely affect reproduction of lower vertebrates at concentrations lower than 0.01 µg/L (for example, Länge et al., 2001). In such cases, a tailored risk assessment strategy that addresses the specific mechanism or MoA of the API is performed.

Phase II screening is split into tier A and B. Phase II tier A involves evaluating the risk quotient, the ratio between the PEC_{surface water} and a predicted no-effect concentration (PNEC) (Winter et al., 2010). The PNEC value is used to determine the environmental concentration of a substance that is unlikely to have any adverse effect on the environment. The PNEC is derived from experimental studies generated through a set of recommended standard toxicity tests issued by the European Commission, the Organisation for Economic Co-operation and Development
(OECD) or the International Organisation for Standardisation (ISO), using aquatic test-species from three trophic levels; one plant (the 72-hour algal growth inhibition test [OECD test guideline 201]), one invertebrate (the 21-day Daphnia reproduction test [OECD test guideline 211]) and one vertebrate (the fish early-life stage toxicity test [OECD test guideline 210]). An “assessment factor” (generally of 1000 to the lowest toxicity value) is applied when deriving the PNEC, which accounts for the uncertainty of the data that may arise when extrapolating toxicity test datasets, such as inter-species variability (Holm et al., 2013). If the PEC:PNEC ratio is greater than 1, further refinement using additional testing is carried out in Phase II Tier B, as an ecological risk may be suspected in the aquatic environment.

1.2.1.1 General limitations of ERA

Since the introduction of the ERA, its usefulness and applicability for the assessment of pharmaceuticals has been challenged, particularly in terms of the characterisation of their effects in the species tested (Schmitt et al., 2010). Some of the general limitations of the current ERA approach for human pharmaceuticals are discussed below.

The standard toxicity tests used to derive the PNEC usually involve using apical endpoints such as survival, growth and reproduction, at concentrations that can be several orders of magnitude higher than reported environmental concentrations. Pharmaceuticals present in the environment are not expected to produce acute toxic effects because their concentrations are generally considered to be low. However, because they are highly potent, they can elicit biological effects at concentrations much lower than those causing acute toxicity. For most APIs, the PNEC is generated through endpoints that do not reflect the mechanism, or MoA of the chemical, nor do they address the effects mediated through chronic, low-level exposure, which may result in long-term effects (Crane et al., 2006). For example, the antidepressant fluoxetine, can alter feeding and mating behaviours (Weinberger II and Klaper, 2014), resulting in subtle changes that can potentially lead to changes in the population or community structure (Gaworecki and Klaine, 2008).
Secondly, current risk assessments examine individual pharmaceutical toxicity on test-organisms under standard laboratory conditions to determine their ecotoxicity. However, APIs present in the environment are one component of a complex mixture of chemicals and therefore this is not environmentally realistic (Arnold et al., 2013). Aquatic organisms may be exposed to mixture of APIs and other chemicals, which can have synergistic, additive or antagonistic effects, yet such mixture effects are not currently taken into account. Several pharmaceuticals with the same or similar modes of action, which can affect comparable metabolic pathways or biochemical processes in non-target organisms may be present in the environment at the same time (Christen et al., 2010).

Thirdly, the standard toxicity tests incorporate a limited number of test-species (usually one plant, one invertebrate and one vertebrate), and relies upon the extrapolation of toxicological responses in these species, to the responses that may, or may not, be seen in a vast number of other aquatic species that are present in the environment. A more mechanistic approach to identifying the molecular responses using MoA approaches can provide the basis for more accurate extrapolation of effects in the same species, and across species. For example, cross-species extrapolation of some endpoints, such as reproduction-related data obtained in invertebrates, may not be as informative for fish species, as the number and similarity of conserved human drug targets in invertebrates is much lower than in fish (Gunnarsson et al., 2008). This is the likely explanation for why fish are much more sensitive to effects of EE2 than invertebrates (Caldwell et al., 2008).

As a final point, current ERA requirements only apply to medicines that were authorised after 2006 and therefore those that were registered before this time have not undergone the same risk assessment process. There are currently over 3,000 active substances licenced for use in human medicines (Fent et al., 2006) and many were authorised long before this was a requirement, which has resulted in a lack of ecotoxicity data, from which robust PNECs can be derived. However, with so many pharmaceuticals in common use, performing a full battery of tests for all new and old APIs is not practical (Caldwell et al., 2014). This has led scientists to seek alternative methods for the retrospective risk assessment of APIs in the environment, and
strategies for the prioritisation of pharmaceuticals for further ecotoxicity testing (Boxall et al., 2012). A number of prioritisation schemes have already been explored for individual or groups of pharmaceuticals, based on their therapeutic class or MoA, physiochemical properties or prescription sales/usage data (Roos et al., 2012; Berninger and Brooks, 2010; Caldwell et al., 2014); however, the latter will not take account of pharmaceuticals that are sold over-the-counter or on the internet. Prioritisation using MoA approaches could help to identify the species of aquatic wildlife that may be more susceptible to effects of particular pharmaceuticals. In view of this, one way to utilise the MoA to more accurately determine the risks posed by human pharmaceuticals in the current ERA process, could be to extrapolate information based on their pharmacology and toxicity in humans and mammals (generated during drug discovery and development) in order to predict their potential pharmacological or toxicological effects in environmentally relevant non-target species (Winter et al., 2010). Special consideration should be given to those species showing conserved molecular drug targets and biological pathways, as a means to link potential pharmaceutical MoA-related effects to physiological responses (Ankley et al., 2007).

1.3 THE “READ-ACROSS” APPROACH

The extrapolation of biological and toxicological responses to chemicals between species is often referred to as “read-across”. The powers of cross-species predictive extrapolation are well established, primarily through the use of animal models (for example, rodent models) in the drug development process. During the pre-clinical safety assessment of a pharmaceutical candidate, numerous in vitro and animal studies are performed, from which data may be extrapolated to humans (implying “read-up”), using appropriate scaling factors that take into consideration known differences and “uncertainties” in physiology, genetics and biochemistry (Berninger and Brooks, 2010; Winter et al., 2010). Advances in DNA sequencing and bioinformatics have led to the elucidation of several genomes, increasing our understanding of the conservation of molecular drug targets among species (Perkins et al., 2013), including humans and aquatic species.
The reliable and accurate extrapolation of toxicity data is also a fundamental aspect in the ERA of chemicals to wildlife. Human pharmaceuticals represent one class of environmental contaminants where vast amounts of data on their pharmacology and toxicology have been generated during drug discovery and development (Berninger and Brooks, 2010; Winter et al., 2010). The potential use of this data (generated in animal models and humans), to predict the environmental impact of human pharmaceuticals on aquatic species (which would presumably imply cross-species extrapolation in the form of “read-down” from higher vertebrates to lower vertebrates and invertebrates) has provided the basis for the “Read-Across Hypothesis” (Huggett et al., 2003; Rand-Weaver et al., 2013). The read-across hypothesis encompasses the MoA of pharmaceuticals (assuming that there has been the evolutionary conservation of molecular targets) in aquatic organisms (fish), and uses the internal concentrations in fish and in humans, to predict the risk of a pharmacological (or toxicological) effect occurring in that particular organism. Therefore the risk assessment process could be made more powerful by incorporating the internal concentrations of pharmaceuticals.

1.3.1 “Read-Across Hypothesis”

The read-across hypothesis stipulates that human pharmaceuticals will elicit the same target-mediated pharmacological response(s) in fish, as in humans, and that these effects will occur at comparable internal concentrations (Rand-Weaver et al., 2013). The formulation of the so-called read-across hypothesis was first articulated by Huggett et al., (2003), who proposed the link between the external (exposure) and internal concentrations of a pharmaceutical in fish, through the development of the theoretical “Fish Plasma Model” (FPM) (Huggett et al., 2003). Determining the concentration of a given pharmaceutical inside the fish (i.e. the “internal” concentration) from the environmental water concentration (i.e. the “exposure” concentration), is fundamental to the read-across hypothesis, since a direct extrapolation between the environmental (exposure) concentration of a pharmaceutical to the molecular drugs targets it may interact with in a particular organism is not appropriate or strictly correct. Indeed, it is the internal concentration(s) of a pharmaceutical (for example, the concentration(s) in the blood
plasma), and not the environmental water concentration, that can result in, if any, target-mediated pharmacological (or toxicological) responses in the exposed aquatic organism (Rand-Weaver et al., 2013).

According to the read-across hypothesis, the relationship between the internal concentration (estimated or experimentally measured) in the exposed aquatic species (i.e. fish) and in humans, can be used to determine the likelihood of an adverse effect occurring. In humans, the effective internal concentration of a given pharmaceutical is referred to as the “human therapeutic plasma concentration” (HTPC) i.e. the concentration(s) found in the blood plasma of patients taking the drug. The HTPC value(s) are determined during the drug development process, and can also be expressed as the “maximum concentration” (Cmax) or “area under the curve” (AUC). These values describe the presence of a drug in the systemic circulatory system at either a single point in time (Cmax) or as a function of time (AUC) (Huggett et al., 2003). The closer the internal concentration(s) of a given pharmaceutical are between fish and humans, the greater the likelihood that a pharmacological response will occur. If the read-across hypothesis is correct, then its application could be used to guide future risk assessments of human pharmaceuticals, for example through the identification of more robust endpoints or effective concentrations required for target activation (or inhibition), where there may be pre-existing gaps of knowledge through a lack of relevant ecotoxicity data (Ankley et al., 2007; Gunnarsson et al., 2008).

Both the conservation of molecular targets and the internal concentration(s) of a pharmaceutical underpin the “read-across hypothesis”, and will be discussed in further detail below.

1.3.1.1 Target conservation

It is widely accepted that the presence of a specific molecular drug target (for example, a receptor or enzyme) within an organism suggests that there is the potential for a specific drug-target interaction to occur, which may result in a pharmacological (or toxicological) effect in that particular organism. The evolutionary (and functional) conservation of human targets in a given species can
potentially increase the risk of eco-toxicological effects (Ankley et al., 2007; Christen et al., 2010; Gunnarsson et al., 2008; Huggett et al., 2003). Huggett et al. (2003) first reported the conservation of enzymes and receptors between humans and teleosts. Further work by Gunnarsson et al., (2008) assessed the degree of conservation of 1,318 human drug targets across sixteen species, including some species that are used in current ecotoxicity testing (Figure 3), as a means to identify orthologs (common ancestral proteins derived at the time of speciation) and prioritise those species potentially sensitive to human pharmaceuticals. Human drugs targets were found to be conserved by up to 86% in zebrafish (*Danio rerio*), while only 61% of targets were conserved in *Daphnia magna* and 35% in green algae, indicating that targets were most conserved in aquatic vertebrates and less so in invertebrates and plants (Gunnarsson et al., 2008).

![Figure 3](image.png)

*Figure 3. The predicted conservation (%) of human drug targets in vertebrate and invertebrate species. Targets were classified according to the gene ontology in five categories (enzyme, receptor, ion channel, transporter, or other) (image taken from Gunnarsson et al., 2008).*
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The degree of conservation suggests that vertebrates may show greater sensitivity to the effects of certain human pharmaceuticals than invertebrates. Nevertheless, human molecular targets have been identified in invertebrates, for example the rotifer *Brachionus manjavacas* contains a proposed progesterone receptor in its reproductive organs (Stout et al., 2010) and *Daphnia magna* contain a putative pathway for eicosanoid biosynthesis (Heckmann et al., 2008), the target for NSAIDs. These findings suggest that these phyla may still be susceptible to the effects elicited by human pharmaceuticals. Enzymes were the most conserved molecular drug target across all phyla investigated (Figure 3), implying that pharmaceuticals that interact with these drug targets may have more widespread effects than those that interact with receptors, which were less well conserved (Gunnarsson et al., 2008).

The conservation of human molecular targets has enabled MoA-related specific effects to be identified in fish (as outlined in Section 1.1.4.3). However, the complexity of predicting biological effects based on cross-species target conservation should not be underestimated. The presence of a conserved drug target alone does not guarantee that a functional interaction will occur. This is because a pharmaceutical that is designed to act efficaciously on a specific target in humans may interact with a different target in another species (Rand-Weaver et al., 2013; LaLone et al., 2013). For example, some secondary sexual effects observed in fish exposed to synthetic progestins (Runnalls et al., 2013) have been linked to their interaction with the androgen receptor, an off-target, rather than the progesterone receptor (the intended target) (Caldwell et al., 2014). Similarly, caution must also be applied when deducing functions, or effects, based on the interaction between a human drug and targets present in other species (Perkins et al., 2013). For example, some species, such as the zebrafish have undergone genome duplications, resulting in multiple orthologs for 15% of human genes (Howe et al., 2013). These duplicated genes are potentially free to gain new, or lose their existing functionalities. This is the case for the cyclooxygenase genes, of which there are three isoforms present in some teleost fish (Ishikawa et al., 2007), but only two isoforms in humans. In some cases, a pharmaceutical may interact with an unclassified drug target if the correct target has not been conserved, or it may interact with a drug target that fulfils the role of several others in that particular species. In any of these scenarios, the possibility
that the MoA is not conserved cannot be discounted (Rand-Weaver et al., 2013). Furthermore, the conservation of secondary targets in the biological pathway downstream of the primary molecular target must be given some consideration, although the lack of the primary target itself would indicate the biological pathway has not been conserved.

1.3.1.2 Internal concentrations and theoretical Fish Plasma Model (FPM)

Currently, the ERA relies upon the surface (or river) water concentration of pharmaceuticals to predict their toxicity in aquatic species. One of the fundamental aspects that the read-across hypothesis addresses is whether or not the internal concentrations inside aquatic organisms (fish) are likely to be sufficient to induce biological (or toxicological) responses. A reliable measure of the internal concentration is imperative in predicting the risk of an effect in fish, since some pharmaceuticals can bioconcentrate, thereby producing concentrations inside the exposed organism that are much higher than the exposure concentrations. For example, some pharmaceuticals present in sewage effluents were found to bioconcentrate more than 50-fold in fish blood plasma (Fick et al., 2010). A number of studies have analytically determined the internal (blood plasma) concentrations of pharmaceuticals in fish exposed in the laboratory (Bartram et al., 2012; Cuklev et al., 2011, 2012; Garcia et al., 2012; Giltrow et al., 2009; Lahti et al., 2011; Mimeault et al., 2005; Nallani et al., 2011, 2012; Owen et al., 2009, 2010; Valenti et al., 2012; Winter et al., 2008), and in the field (Brown et al., 2007; Fick et al., 2010; Lahti et al., 2012). However, in some cases, where the internal blood plasma concentrations cannot be analytically determined, the theoretical Fish Plasma Model (FPM) (Huggett et al., 2003) provides an alternative framework for predicting the uptake of drugs into fish blood plasma. This model can be used to estimate the stabilised fish blood plasma concentration i.e. the “fish steady-state plasma concentration” (FSSPC) of a compound from the surrounding water, based on its pharmacokinetic properties and a given predicted or measured environmental concentration (PEC or MEC) (Huggett et al., 2003). Based on the model’s prediction of uptake, the closer the internal concentration(s) of a given pharmaceutical are between fish and humans, the
greater the likelihood of a pharmacological response to occur in that particular organism.

The lipophilicity (Log $K_{ow}$) of a compound is used as the main indicator of uptake into the blood of fish from the surrounding water (Huggett et al., 2003). The Log $K_{ow}$ is used to estimate the partitioning of a compound between the aqueous phase and the arterial blood (Log $P_{blood:water}$) in fish (Fitzsimmons et al., 2001) (Equation 1):

$$\text{Log } P_{\text{blood:water}} = 0.73 \times \text{Log } K_{ow} - 0.88$$

Equation 1. The Log $P_{\text{blood:water}}$ in fish (Fitzsimmons et al., 2001).

Using this relationship, the $F_{SSPC}$ can be estimated (Huggett et al., 2003) (Equation 2):

$$F_{SSPC} = \text{PEC (or MEC)} \times (P_{\text{blood:water}})$$

Equation 2. The “Fish steady-state Plasma Concentration” ($F_{SSPC}$) (Huggett et al., 2003).

The $F_{SSPC}$ is usually determined using the Log $K_{ow}$ (Fitzsimmons et al., 2001). However, as pharmaceuticals are amenable to ionisation at different pH’s, including the pH of blood plasma, the distribution coefficient Log D, measured at physiological pH (7.4), has also been identified as another, potentially more realistic, predictor of uptake in fish (Owen et al., 2009). The $F_{SSPC}$ is compared with $H_{TPC}$ and if the model predicts that a pharmaceutical present in the water can bioconcentrate in fish plasma, and reach concentrations that are similar to the $H_{TPC}$ ($H_{TPC} = F_{SSPC}$), there is a risk that the drug could exert a pharmacological response (Figure 4). The FPM has been proposed as one method for prioritising pharmaceuticals for further environmental risk assessment (Roos et al., 2012; Schreiber et al., 2011). However, experimental validation of the FPM is required before the model can be widely used to assess the risks posed by pharmaceuticals in the environment, by evaluating how well the concentration of a pharmaceutical in fish blood plasma can be theoretically modelled for a given water concentration.
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Figure 4. Application of the Fish Plasma Model (FPM) proposed by Huggett et al., (2003) to two pharmaceuticals, 17-α-ethinylestradiol (EE₂) and atenolol. The FPM compares the “human therapeutic plasma concentration” (H₇PC) and the predicted “fish steady-state plasma concentration” (F₉SSPC). If H₇PC = F₉SSPC, the risk of a pharmaceutical having a pharmacological effect in fish is high. EE₂ using the predicted environmental concentrations (PEC) will produce F₉SSPC > H₇PC, indicating a high risk of pharmacological effects occurring. Atenolol, which is highly hydrophilic, does not bioconcentrate to a significant extent, resulting in F₉SSPC < H₇PC and therefore no biological effect is expected (image taken from Rand-Weaver et al., 2013).

1.3.2 Current evidence for the “Read-Across Hypothesis”

The read-across hypothesis takes into account the measurement of the exposure and internal concentrations of a pharmaceutical, relevant MoA endpoints (based on the conservation of human drug targets) and relates target-mediated (pharmacological) effects to the H₇PC (hereafter also referred to as the Cmax), in order to predict likelihood of an adverse effect occurring in aquatic organisms (Rand-Weaver et al., 2013). A number of studies assessing the impact of pharmaceuticals present in the environment have addressed aspects of the read-across hypothesis, and Table 3,
proposes a classification of these studies according to how well they utilise the hypothesis.

Table 3. Classification of studies according to how well they utilise the “Read-Across Hypothesis” (taken from Rand-Weaver et al., 2013). “Mode of action (MoA)-related effects and \(^6\)HTPC denotes the human therapeutic plasma concentration.

<table>
<thead>
<tr>
<th>Level</th>
<th>Exposure concentration</th>
<th>Endpoints</th>
<th>Internal concentration</th>
<th>Specific pharmacological effects</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Measured</td>
<td>MoA-related(^a)</td>
<td>Measured</td>
<td>Seen only at (^6)HTPC(^b)</td>
<td>Integration of mammalian data</td>
</tr>
<tr>
<td>3</td>
<td>Measured</td>
<td>MoA-related</td>
<td>x</td>
<td>Cannot be related to (^6)HTPC</td>
<td>Independent of mammalian data</td>
</tr>
<tr>
<td>2</td>
<td>Measured</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>X</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

The strongest support for the read-across hypothesis is represented by a level 4 study. In order for a study to be ranked as a level 4; the administered dose of a pharmaceutical must be confirmed by measuring the water concentration(s), the internal concentration(s) must be obtained by measuring the blood plasma concentration(s), the exposure must be linked to a specific biological effect by measuring MoA-relevant endpoints, these effects must be comparable with those expected at the \(^6\)HTPC (or Cmax), and it must be demonstrated that these effects were seen only at concentrations similar to those in a human taking the drug (Rand-Weaver et al., 2013). Currently, there is no published study that has addressed all aspects of the read-across hypothesis. Therefore, evidence for the applicability of the read-across hypothesis for the assessment of human pharmaceuticals is fundamentally lacking.

The highest level of support for the read-across hypothesis has been demonstrated in a study where behavioural effects were observed in fathead minnows, exposed to sertraline, a selective serotonin reuptake inhibitor (SSRI), used to modify behaviour in human, which resulted in measured plasma concentrations that were similar, or above, the \(^6\)HTPC (Valenti et al., 2012). However, as none of the exposure water
concentrations tested produced plasma concentrations that were below the human therapeutic range, which is a requirement necessary to fully validate the read-across hypothesis, the study cannot be classified as a level 4 study. In another study, two-week exposure of rainbow trout (*Oncorhynchus mykiss*) to (measured) water concentrations of diclofenac resulted in plasma concentrations that were approximately 1.5-88% of the Cmax, which affected molecular endpoints (hepatic gene expression) associated with inflammation and the immune response (Cuklev et al., 2011), which appear to be consistent with the MoA of NSAIDs.

In other studies (classed as level 3), the read-across hypothesis has been used to confirm that MoA-related effects similar to those seen in humans occur in aquatic organisms, however, a measure of the internal blood plasma (actual) concentration is not present. For example, EE2 and levonorgestrel present in oral contraceptives, have been shown to inhibit reproduction in fathead minnows (*Pimephales promelas*) at exposure concentrations in the ng/L range (Länge et al., 2001; Runnalls et al., 2013), psychiatric drugs used to modulate behaviours in humans, can alter the behaviour of fish (Gaworecki and Klaine, 2008; Weinberger II and Klaper, 2014), and ibuprofen an anti-inflammatory, can inhibit prostanoid (PGE2) synthesis involved in pain perception in zebrafish (Morthorst et al., 2013). In these studies, exposure water concentrations were measured, however plasma concentrations were not determined and therefore it is difficult to determine the extent of uptake into fish, or if the drug was able to bioconcentrate and so it is not possible to correlate these effects to the H7PC. In such studies, it is possible to estimate the steady state plasma concentrations (FSSPC) of pharmaceuticals based on the measured exposure concentrations using the FPM (Huggett et al., 2003) which may strengthen the support for the read-across hypothesis. In some cases, the observed effects are difficult to interpret because tissue concentrations, instead of the plasma concentration have been measured, for example, for antidepressants in the brain of fathead minnows (Schultz et al., 2011) and the NSAID diclofenac in the gill, kidney, liver and muscle tissues of rainbow trout (Schwaiger et al., 2004). In other studies, the exposure water and plasma concentrations of the pharmaceutical have been measured, but the endpoints measured do not reflect the MoA in humans. For example, whole fish growth effects (length and weight) were observed in rainbow
trout exposed to the β-blocker, propranolol, at plasma concentrations above the Cmax, however cardiac effects were not measured (Owen et al., 2009).

The studies that are ranked as levels 1 and 2 provide no support for the read-across hypothesis, or contain very little relevant information. In these studies, generally, there is no measure of the exposure water or plasma concentration, or any specific effect relevant to the MoA. For example, the potential for ibuprofen to change behavioural responses (decreased activity from 65% in control to 30% in treated groups) in the invertebrate amphipod, *Gammarus pulex*, following exposure to environmentally relevant concentrations of 1 and 10 ng/L (De Lange et al., 2006) is questionable at best. In other studies, the tested exposure concentrations were very high (mg/L) for an effect to be elicited, and therefore cannot be related to the MoA. For example, in one study examining chronic effects on population dynamics in *Daphnia magna* exposed for 14-days to (measured) ibuprofen concentrations of 0-80 mg/L, reproduction was affected at 13.4 mg/L and was completely inhibited at 80 mg/L (Heckmann et al., 2007). Considering the concentrations used, it is most likely that the observed effects are related to non-specific narcosis, or toxicity, rather than the MoA of the drug. However, no plasma concentrations were measured to confirm this, and a clear lack of reference to the mammalian data indicates that the design of the study was independent of this information.

There are, of course, some studies that appear to not support, or disprove, the read-across hypothesis. The best case example is diclofenac poisoning in *Gyps* vultures that had been scavenging on the carcasses of cattle treated with the drug, that resulted in renal failure and subsequent death in these birds (Oaks et al., 2004) (Section 1.1.4.2.2). It seemed on initial reflection that these birds were highly sensitive to diclofenac, however renal failure is a known side effect of this drug in humans (Hickey et al., 2001). The read-across hypothesis assumes that potential toxicological effects, will occur when the internal blood plasma concentration inside the exposed organism exceeds the Cmax. Upon closer examination, and interpretation of the results, a comparison of body-weight, plasma volume, and ingested diclofenac dose between humans and vultures (assuming no metabolism) indicated that the ingested diclofenac dose in these vultures was very close to the human toxic dose (Rand-
Weaver et al., 2013), and therefore, is in fact in some level of agreement with the hypothesis that pharmacological effects are likely to precede toxicological effects.

These findings demonstrate that there is a large number of studies that are in agreement, or show some level of support for the read-across hypothesis. However, the data from different exposure studies is highly variable, from study to study, due to differences in species selection, exposure length or study duration, and inconsistencies in exposure water or internal blood plasma concentration measurements. Therefore, more standardised exposure studies may help to strengthen the support (or lack of) for the read-across hypothesis. For those studies that show an apparent lack of support for the hypothesis, in general, the design of these studies has not taken into consideration any available human/mammalian data.

In this study, the applicability of the read-across hypothesis will be tested using a (level 4) designed study with the non-steroidal anti-inflammatory drug, ibuprofen, and the ecologically relevant fish species, the fathead minnow.
1.4 NON-STERoidal ANTI-INFLammatory DRUGs

The NSAIDs are a structurally diverse group of compounds (Figure 5) with a similar MoA; the inhibition of cyclooxygenase (COX), and therefore they are also known as COX inhibitors. NSAIDs are commonly prescribed for the treatment of inflammatory conditions, such as rheumatoid arthritis and osteoarthritis, and to alleviate the symptoms of low to moderate pain conditions such as back pain and migraine (Vane and Botting, 1995). Many NSAIDs can be purchased over-the-counter, including aspirin, ibuprofen, diclofenac and naproxen. NSAIDs are amongst the most widely produced and used pharmaceuticals in the world, and the overall production is around 50,000 tonnes per year (Dannhardt and Kiefer, 2001). Due to their prevalent use, many NSAIDs have been detected in the aquatic environment, raising concerns over their potential impacts on non-target aquatic species, such as fish.

![Classification and structure of some representative NSAIDs](image_taken_from_Pereira-Leite_et_al._2013)

Figure 5. Classification and structure of some representative NSAIDs (image taken from Pereira-Leite et al., 2013).
Some of the NSAIDs that have been measured in surface waters (and in effluents) in
the UK and globally are shown in Table 2, and most are in the ng/L to µg/L range.
Field studies have shown that aquatic organisms can readily uptake NSAIDs from
the environment. Brown et al., (2007) found that several NSAIDs, including
ibuprofen, diclofenac and naproxen, could be detected in the blood plasma of caged
rainbow trout exposed to sewage effluents. Following a two-day exposure (at one
effluent site), FssPCs of up to 84 µg/L (18,667-fold higher), 14 µg/L (56-fold higher)
and 12 µg/L (5-fold higher) were reported in fish exposed to measured effluent
concentrations of 0.0045 µg/L ibuprofen, 0.25 µg/L naproxen and 2.32 µg/L
diclofenac, respectively. However ketoprofen, which was also present in the effluent
(0.28 µg/L) was not detected in blood plasma (Brown et al., 2007), which was
attributed to its lower capacity to bioconcentrate. Similarly, in another field study,
Fick et al., (2010) demonstrated that out of 25 examined pharmaceuticals, ibuprofen,
diclofenac, naproxen, as well as ketoprofen and tramadol, could be detected in the
blood plasmas of rainbow trout exposed to effluents (Fick et al., 2010). In this study,
the NSAIDs had the highest concentrations in fish blood plasmas compared to any of
the other pharmaceuticals measured. This was a direct result of the high
concentrations in the effluent (for example, ibuprofen, ketoprofen and naproxen were
detected at a concentration 103 times higher than Levonorgestrel). Since several
NSAIDs exhibit the same or a similar MoA it is plausible that the internal
concentrations required to elicit pharmacological effects may be more easily reached.

1.4.1 Evidence for the effects of NSAIDs in aquatic organisms

A number of studies have documented the effects produced by NSAIDs in aquatic
organisms. Exposure to diclofenac has resulted in histopathological changes in the
gills and kidney of fish (Hoeger et al., 2005; Schwaiger et al., 2004; Triebskorn et al.,
2004), at concentrations as low as 1 µg/L (Schwaiger et al., 2004), which could be
explained by the significant bioconcentration of diclofenac in some fish tissues.
Ibuprofen can induce haematological changes (Saravanan et al., 2012), alter
spawning behaviours (Flippin et al., 2007), delay hatching (Han et al., 2010), and
affect ion regulation and stress responses (Gravel and Vijayan, 2007; Gravel et al.,
2009) in fish. Fewer studies have examined the effects of naproxen and ketoprofen,
which is most likely due to their less frequent use. However, in all the reported studies, the internal concentrations (except Schwaiger et al., 2004) were not measured, indicating that the amount of drug required to elicit the reported observed effects is unknown. Nevertheless, these findings demonstrate that NSAIDs may disrupt several physiological systems in aquatic organisms.

1.5 IBUPROFEN

Ibuprofen was first introduced in the UK in 1969 as a prescription-only medicine for the treatment of rheumatoid arthritis (Davies and Avery, 1971). It belongs to the propionic acid derivative subgroup of NSAIDs (Figure 5). The chemical structure of ibuprofen contains an asymmetric (chiral) α-carbon (Figure 6) that produces S or R enantiomers and therefore ibuprofen is marketed as a racemic mixture (Davies, 1998). The pharmacological activity of ibuprofen is almost exclusively through the S enantiomer, which is about 160 times more potent than the R enantiomer (Adams et al., 1976).

![Figure 6. Chemical structure of ibuprofen. The structure of ibuprofen contains a benzene ring, attached to a propionic acid side-chain with a chiral α-carbon (highlighted with a blue spot) which is adjoined to the carboxylic acid (COOH) functional group (image adapted from Davies, 1998).](image)

Ibuprofen is manufactured as “normal” (400 mg three times a day) or “sustained” release (800-1,600 mg once a day) preparations which are available over-the-counter or on prescription, respectively (Wood et al., 2006). The effects of ibuprofen are dose- and duration dependent, and following a therapeutic dose of 400 mg, the peak plasma concentrations (Cmax) range between 15,000-30,000 µg/L (Schulz et al., 2012). Ibuprofen is readily absorbed in the gastrointestinal tract and pharmacokinetic studies have shown that maximal analgesic efficacy can be achieved within 1-2 hours.
after oral administration (Bienert et al., 2006; Laska et al., 1986). Ibuprofen is metabolised in the liver (over 70-80%) and is excreted in the urine, in the form of metabolites conjugated with glucuronic acid i.e. glucuronide-conjugated hydroxy-ibuprofen and carboxy-ibuprofen (Davies, 1998). The physicochemical properties of ibuprofen are indicated in Table 4. Ibuprofen is largely insoluble in water (21 mg/L at 25°C), weakly acidic (pKa of 4.91) and has a relatively high Low K\text{ow} (3.80) and therefore has the potential to accumulate within aquatic organisms.

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{13}H_{18}O_{2}</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>206.28</td>
</tr>
<tr>
<td>Partition Coefficient (Octanol-Water) (Log K\text{ow})</td>
<td>3.80 (\textsuperscript{a})</td>
</tr>
<tr>
<td>Distribution-coefficient (Log D) at pH 7.4</td>
<td>0.8 (\textsuperscript{b})</td>
</tr>
<tr>
<td>Acid dissociation constant (pKa)</td>
<td>4.91</td>
</tr>
<tr>
<td>Water Solubility at 25°C (mg/L)</td>
<td>21.0 (\textsuperscript{c})</td>
</tr>
</tbody>
</table>

1.5.1 Ibuprofen Mode of Action

In 1971, Sir John Vane demonstrated that the primary action of NSAIDs was the inhibition of the COX enzyme, officially known as prostaglandin-endoperoxide synthase (PGHS) (EC 1.14.99.1) (Vane, 1971). COX is involved in the biosynthesis of prostanoids, which exhibit a wide range of physiological functions within the body, including mediation of pain, swelling, inflammation, blood coagulation, vasodilation, regulation of vascular permeability, maintaining the gastric mucosa and kidney function, as well as various reproductive functions (Vane and Botting, 1995). The therapeutic and adverse effects of NSAIDs are due to their inhibition of prostanoids via the COX enzyme.

1.5.2 Cyclooxygenase (COX) and prostanoid synthesis

The COX enzyme, catalyses the rate-limiting step in the conversion of arachidonic acid to prostanoids, a subclass of the eicosanoid group of biologically active lipid
mediators (Vane et al., 1998). There are two isoforms of the COX enzyme; COX 1 and COX 2 that are encoded by different genes, prostaglandin-endoperoxide synthase 1 (PTGS1) and prostaglandin-endoperoxide synthase 2 (PTGS2), respectively (colloquially known, and hereafter referred to, as the COX 1 and COX 2 genes). COX 1 was first characterised and isolated from sheep seminal vesicles in the late 1980’s (DeWitt and Smith, 1988; Merlie et al., 1988; Yokoyama and Tanabe, 1989). The second isoform (COX 2) was later isolated from mouse and chicken fibroblast cell cultures in the early 1990’s (Xie et al., 1991; Kujubu et al., 1991).

Both enzymes are involved in the conversion of arachidonic acid to prostaglandin H2 (PGH2), a common intermediate for the prostanoids, which include prostaglandins, prostacyclin and thromboxane (Simmons et al., 2004). Arachidonic acid, a 20-carbon fatty acid, is a basic constituent of all cell types, which is liberated from the phospholipid membrane by the action of phospholipase enzymes, primarily phospholipase A2 (PLA2), in response to physiological stimuli, such as acute injury, phagocytic particles or tissue damage (Vane et al., 1998). In addition to the eicosanoids generated through COX, arachidonic acid can also serve as substrate for lipooxygenase enzymes to form to lipoxins, and cytochrome P450 (CYP) monooxygenase enzymes that generate epoxyeicosatrienic acids (Stables and Gilroy, 2011).

The COX enzymes are membrane-bound proteins located on the lumenal surfaces of the endoplasmic reticulum (ER) and the nuclear membrane. The COX 1 and COX 2 enzymes are homodimers of 599 and 604 amino acids respectively, with a molecular mass of 70 kDa (Rouzer and Marnett, 2009). Both proteins share 60% identity between their amino acid sequences, similar three-dimensional structures, and identical reactions in prostanoid synthesis (Xie et al., 1991; Kujubu et al., 1991; O’Banion et al., 1991). Each monomer of the dimer consists of three structural domains: an N-terminal epidermal growth factor-like domain, a membrane binding domain, and a C-terminal catalytic domain which contains two adjacent, but spatially distinct catalytic sites; the cyclooxygenase and the peroxidase active sites, on either side of a haem prosthetic group (Figure 7) (Knights et al., 2010). The catalytic domain constitutes the majority of the COX monomer, and contains several functionally important residues required for catalysis. The COX monomers dimerise
through their epidermal growth factor-like domains, and are held together by hydrophobic interactions, hydrogen bonding, and salt bridges, which is necessary for their structural integrity and catalytic functions (Simmons et al., 2004).

Figure 7. Structure of the COX 1 homodimer. The functional domains of COX include; an epidermal growth factor-like (EGF-like) domain (blue), a membrane binding domain (red), a globular catalytic domain (grey). The catalytic domain contains the cyclooxygenase and the haem group (brown) of the peroxidase active site (not shown). The binding site for the primary substrate of COX, arachidonic acid is also highlighted (yellow). This is the site for NSAID binding, including ibuprofen (image adapted from Theoretical and Computational Biophysics Group, 2012).

COX is a bifunctional protein that sequentially catalyses two reactions. In the first reaction, the cyclooxygenase activity of the COX enzyme, oxygenates arachidonic acid to form the unstable hydroperoxy endoperoxide, prostaglandin G₂ (PGG₂). In the second reaction, the peroxidase activity reduces the hydroperoxy group of PGG₂ to produce prostaglandin H₂ (PGH₂). PGH₂ diffuses from COX and is further transformed by different cell-specific prostaglandin synthases to five primary prostanoids, which include the prostaglandins PGE₂, PGD₂, PGF₂α; prostacyclin; PGI₂, and thromboxane A₂; TxA₂ (Figure 8) (Rouzer and Marnett, 2009).
Prostanoids are biologically active lipid mediators that act locally in an autocrine or paracrine manner by binding to membrane receptors that belong to the G protein-coupled receptor (GPCR) family, or in some cases, nuclear receptors (Simmons et al, 2004). The receptors for the ligand PGE\textsubscript{2} (termed EP1, EP2, EP3, and EP4, see Figure 8), can directly modulate intracellular levels of cyclic adenosine monophosphate (cAMP) and inositol phosphate, which are secondary messengers involved in signal transduction pathways. The effects mediated by prostanoids within the body are widespread, and some of the processes they regulate are shown in Table 5.

The main therapeutic effects of NSAIDs are through their inhibition of COX 2-derived prostanoids (Vane et al., 1998). The mechanism of NSAID inhibition can vary, for example, aspirin is the only clinically used NSAID that can irreversibly bind to the COX enzymes, whereas all others act non-covalently, but most NSAIDs are competitive reversible active site inhibitors that compete with arachidonic acid (substrate). Tissue injury and inflammation are associated with increased local PGE\textsubscript{2} and PGI\textsubscript{2}, prostanoid synthesis and pain hypersensitivity. NSAIDs are weak organic acids with hydrophobic properties, which facilitates their ability to reach the inflamed tissues where the pH is lower (due to local acidosis) to produce anti-inflammatory effects. Inhibition of PGE\textsubscript{2} and PGI\textsubscript{2} increases the activation threshold of nociceptors, thereby resulting in a decrease in their terminal membrane excitability, thus reducing pain signals (Ricciotti and FitzGerald, 2011). The adverse effects associated with NSAID therapy is due to their suppression of COX 1-mediated prostanoid production, which exhibit cytoprotective effects on the gastric mucosa, for example, by stimulating mucus production and inhibiting gastric acid secretion (Cha et al., 2006), thereby causing a predisposition to gastric ulceration and bleeding. Therefore, it has been suggested that side effects caused by NSAIDs are from the inhibition of COX 1, while the anti-inflammatory actions are a result of COX 2 inhibition (Mitchell et al., 1993).
Figure 8. Pathway for prostanoid synthesis mediated by the COX enzymes (image adapted from Rahnamai et al., 2012). Arachidonic acid is released from the cell membrane in resting cells by phospholipase A2 (PLA2) and is catalysed by the COX enzymes (COX 1 or COX 2), to the highly unstable endoperoxides PGG2 (not shown) and PGH2. PGH2 is the intermediate for all prostanoids and is further transformed by prostaglandin synthases to prostaglandins PGE2, PGD2, and PGF2α, prostacyclin (PGI2) and thromboxane A2 (TXA2). The prostanoids bind to membrane receptors, which include the PGE2 receptors (EP1-4), PGD2 receptors (DP1 and DP2) and PGF2α receptors (FP2), prostacyclin (IP) and thromboxane (TP) receptors, to mediate widespread effects within the body. NSAIDs such as ibuprofen, aspirin and diclofenac exert their anti-inflammatory, analgesic and antipyretic effects by inhibiting the COX 1 and COX 2 enzymes leading to a peripheral inhibition of prostanoids.
Table 5: Prostanoids and their effects within the body (Cha et al., 2006).

<table>
<thead>
<tr>
<th>Biological System</th>
<th>Prostanoid</th>
<th>Effects mediated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproductive</td>
<td>PGE$<em>2$, PGF$</em>{2a}$</td>
<td>Induction of labour; uterine contraction, oxytocic action</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>TXA$_2$, PGI$_2$,</td>
<td>Platelet aggregation, vascular permeability, arterial vasodilation &amp; vasoconstriction</td>
</tr>
<tr>
<td></td>
<td>PGE$<em>2$, PGF$</em>{2a}$</td>
<td></td>
</tr>
<tr>
<td>Respiratory</td>
<td>PGE$<em>2$, PGF$</em>{2a}$</td>
<td>Bronchodilation &amp; constriction</td>
</tr>
<tr>
<td></td>
<td>TXA$_2$</td>
<td></td>
</tr>
<tr>
<td>Renal</td>
<td>PGE$_2$, PGI$_2$</td>
<td>Regulation of renal blood flow, glomerular filtration, renin release</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>PGE$_2$, PGI$_2$</td>
<td>Cytoprotection; maintenance of the gastric mucosa</td>
</tr>
<tr>
<td>Immune</td>
<td>PGE$_2$, PGI$_2$</td>
<td>Leukocyte activation and proliferation, local vasodilation at sites of inflammation</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>PGE$_2$, PGD$_2$,</td>
<td>Fever, sleep regulation, pain sensitisation, thermoregulation</td>
</tr>
<tr>
<td>(CNS)</td>
<td>PGE$_2$, PGI$_2$</td>
<td></td>
</tr>
</tbody>
</table>

1.5.3 Regulation of COX

The mammalian COX enzymes are encoded by the \textit{COX 1} (\textit{PTGS1}) and \textit{COX 2} (\textit{PTGS2}) genes which are mapped to chromosomes 9(q32–q33.3) and 1(q25.2–q25.3), respectively. The \textit{COX 1} gene is approximately 22 kb in length with 11 exons and is transcribed as a 2.8 kb mRNA product, which is relatively stable. In contrast, the \textit{COX 2} gene is smaller, and is 8 kb in length with 10 exons and is transcribed as a 4.5 kb mRNA product. The \textit{COX 2} gene is an immediate early gene that is activated by a wide variety of inflammatory and proliferative stimuli (Rouzer and Marnett, 2009). \textit{COX 1} is constitutively expressed in many tissues and cells, including the vascular endothelia, monocytes, platelets and renal collecting tubules, and is involved in maintaining basal prostanoid levels required for “housekeeping” functions, particularly in the kidney and gastric mucosa (Vane and Botting, 1995). A third COX enzyme, termed “COX 3”, which is a splice variant of a retained intron (intron 1) in the \textit{COX 1} gene, has been identified in canines as an acetaminophen-sensitive isoform (Chandrasekharan et al., 2002). COX 3 (also termed COX 1b) has
been identified in the rat (Snipes et al., 2005; Kis et al., 2003) and in humans (Reinauer et al., 2013) although a functional protein is not produced.

COX 2 is expressed in fewer cell types, and is mostly undetectable in the tissues under physiological conditions, but its expression can be rapidly and transiently induced during pain and inflammation. Under physiological states, COX 2 gene regulation is controlled at various levels including gene transcription and post-transcriptional events (Harper et al., 2008). The transcriptional activation of COX 2 can be stimulated by the binding of growth factors, mitogens and cytokines to the numerous regulatory transcription factors located within its 5′-untranslated region, including; activating protein-1 (AP-1), nuclear factor κB (NFκB), nuclear factor interleukin-6 (NF-IL-6) and response elements, such as cyclic AMP-response elements (CREs), and as a consequence up-regulate COX 2 gene expression. Therefore, COX 2 is considered to be an “inducible” enzyme (Morita, 2002). The expression of COX 2 is also regulated at the post-transcriptional level through changes in its mRNA stability. The mRNA of COX 2 contains several AU-rich elements (AREs) and several mRNA instability sequences (i.e. ‘AUUUA’ motifs) (Shaw and Kamen, 1986) located in the 3′-untranslated region (Chandrasekharan and Simmons, 2004), which is characteristic of immediate-early genes (genes that are transiently activated and rapidly degraded). The COX 2 response to pro-inflammatory stimuli usually leads to a large sustained increase in prostanoids at the sites of inflammation (Samad et al., 2001). However, there are some exceptions to the original “constitutive” versus “inducible” paradigm of COX; for example, COX 1 expression can be induced in some pathological conditions, such as spinal cord injury (Schwab et al., 2000), and COX 2 has been found to be constitutively expressed in the brain (Breder et al., 1995), and in the kidneys (Harris and Breyer, 2001) of some mammals.

At present, it is unclear whether or not NSAIDs can directly modulate COX gene transcription. In some cells that express COX 2, NSAIDs (naproxen) can inhibit both enzymatic activity and gene expression (Zyglewska et al., 1992). However, in another study, in macrophages pre-treated with acetylsalicylic acid, indomethacin and naproxen, and stimulated with lipopolysaccharide, there was a marked inhibition
in PGE\textsubscript{2} production, but not on \textit{COX 1} or \textit{COX 2} mRNA and protein expression (Barrios-Rodiles et al., 1996). The latter findings suggest that the action of NSAIDs on human macrophages is not directed towards the transcription (or translation) of the \textit{COX} genes, but only to the enzymatic activity of the proteins. Perplexing the issue further, in one study investigating mitogen-induced changes in \textit{COX} gene transcription, \textit{COX} mRNA and \textit{COX} enzyme expression in mouse embryonic fibroblast (3T3) cells, serum stimulation led to a sequential increase in \textit{COX 2} gene transcription, \textit{COX 2} mRNA, and \textit{COX 2} enzyme levels. Subsequent treatment with an anti-inflammatory steroid, dexamethasone (glucocorticoid), resulted in a concomitant reduction in \textit{COX 2} transcription, \textit{COX 2} mRNA, and enzyme level, providing support for the notion that the control of transcription is one primary mechanism for regulating \textit{COX 2} expression (DeWitt and Meade, 1993). However, in the same study, although an increase in \textit{COX 1} gene transcription occurred following serum stimulation, coincident with an increase in \textit{COX 1} mRNA, subsequent treatment with dexamethasone reduced the serum-stimulated increases in \textit{COX 1}, however, changes in \textit{COX 1} mRNA were not accompanied by detectable changes in \textit{COX 1} protein (DeWitt and Meade, 1993), suggesting that the \textit{COX 1} and \textit{COX 2} genes are differentially regulated.

1.6 \textbf{COX AND PROSTANOIDS IN AQUATIC VERTEBRATES}

The \textit{COX} proteins and genes have been identified in many aquatic vertebrates, and cloned in several fish species such as the zebrafish (\textit{Danio rerio}) (Grosser et al., 2002; Ishikawa et al., 2007), rainbow trout (\textit{Oncorhynchus mykiss}) (Zou et al., 1999; Ishikawa and Herschman, 2007), brook trout (\textit{Salvelinus fontinalis}) (Roberts et al., 2000), spiny dogfish (\textit{Squalus acanthias}) (Yang et al., 2002) and sea bass (\textit{Dicentrarchus labrax}) (Buonocore et al., 2005). There are many indications that \textit{COX} in fish are functionally homologous to their mammalian counterparts. \textit{COX 1} is the predominant platelet isoform in both zebrafish and humans (Grosser et al., 2002) indicating it may have a similar role in platelet aggregation, thereby supporting the role that \textit{COX 1} is involved in maintaining homeostasis in fish. Similarly, as in mammals, \textit{COX 2} is an inducible enzyme that can be stimulated by mitogens and cytokines in fish (Zou et al., 1999; Roberts et al., 2000; Buonocore et al., 2005),
indicating it may play similar roles during pathological conditions. These studies provide evidence that COX is evolutionarily and functionally conserved in fish.

A number of prostanoids have also been identified in fish tissues and cells, including red blood cells, macrophages, and oocytes (Busby et al., 2002). Prostanoids have been implicated in several roles in fish reproduction, such as stimulating ovulation (Fujimori et al., 2011; Sorbera et al., 2001) and modulating sexual behaviours (Gonçalves et al., 2014; Laberge and Hara, 2003). These findings suggest that prostanoids could play a crucial role in fish reproduction. Prostanoids have also been linked to cortisol synthesis in fish, which can be disrupted by NSAIDs (Gravel and Vijayan, 2007). The occurrence of prostanoids and other eicosanoids has also been reported in invertebrates, such as corals and crustaceans (Heckmann et al., 2008), suggesting that COX is conserved across several phyla, although their roles in these species are not fully understood.

1.7 AIMS AND OBJECTIVES

The read-across hypothesis stipulates that a pharmaceutical will elicit the same pharmacological response(s) in fish, as in humans, only if there has been conservation of the molecular targets, and the internal (blood plasma) concentrations are similar. Despite the general acceptance of the hypothesis, experimental evidence for the applicability of the read-across hypothesis for the assessment of human pharmaceuticals is fundamentally lacking. This is primarily due to the limited number of studies that have measured exposure water and internal (blood plasma) concentrations, and linked specific pharmacological effects using relevant endpoints with human therapeutic plasma concentrations (classified as a level 4 study design).

The read-across hypothesis was tested using ibuprofen, a COX inhibitor and the model fish test-species; the fathead minnow. In order to test the core hypothesis, the following objectives were proposed:

1. To determine whether internal (blood plasma) concentrations of ibuprofen similar to human therapeutic plasma concentrations (Cmax) could be established in fathead minnows.
2. To identify whether COX, the target of ibuprofen in humans, is present in the fathead minnow.

3. To investigate the pharmacological effects of ibuprofen exposure on fathead minnows, using molecular and biochemical endpoints, relevant to the mode of action.

4. To link the effects of ibuprofen in fathead minnows to the blood plasma concentrations of the drug, and in relation to human therapeutic concentrations.

This thesis consists of seven additional chapters, a brief outline of each chapter is provided below.

Chapter 2: details the general experimental methods, including the fish exposure studies conducted at AstraZeneca’s Brixham Environmental Laboratory (BEL) (Freshwater Quarry, Brixham, Devon, UK), and describes some of the main molecular techniques used for the analysis of fish tissues.

Chapter 3: evaluates the concentrations of ibuprofen in the exposure water and fathead minnows, and establishes the exposure water concentration(s) of ibuprofen required to reach internal (blood plasma) concentrations in fish similar to human therapeutic plasma concentrations (Cmax). The accuracy of the theoretical FPM as a means to estimate the uptake of ibuprofen into fish is also examined.

Chapter 4: addresses whether or not the mode of action of ibuprofen is conserved in fish, by establishing if the target of ibuprofen, COX, is present in the fathead minnow. Molecular techniques were used to identify the genes that encode for the COX enzymes in fathead minnow tissues.

Chapter 5: investigates the effects of ibuprofen exposure on a molecular endpoint (gene expression) relevant to the mode of action, in fathead minnow tissues.

Chapter 6: examines the effects of ibuprofen exposure on biochemical endpoints relevant to the mode of action, in fathead minnow tissues.
Chapter 7: links the molecular and biochemical effects of ibuprofen, to the blood plasma concentrations in fathead minnows, and in relation to the human therapeutic plasma concentration (Cmax).

Chapter 8: summarises the major findings and discusses the broader implications of the reported findings in terms of using the read-across hypothesis to assess the impact of human pharmaceuticals present in the environment to aquatic systems. Limitations of the study and recommendations for further research in the area are also proposed.

The work presented in this thesis forms part of a larger study that investigated the read-across hypothesis using four different classes of human pharmaceuticals, including a selective serotonin reuptake inhibitor (SSRI) (fluoxetine), an angiotensin converting enzyme (ACE) inhibitor and a synthetic glucocorticoid (beclomethasone), in addition to the COX inhibitor used in this study. Collectively, these findings may be used to evaluate the read-across approach as a potential tool for the environmental risk assessment of human pharmaceuticals.
CHAPTER 2: MATERIALS AND METHODS
Chapter 2 Materials and Methods

This chapter covers the experimental methods and is divided into two main sections. The first covers the fish exposure studies, including the analytical method used for quantification of ibuprofen in exposure waters and fish blood plasmas at AstraZeneca’s BEL (Freshwater Quarry, Brixham, Devon, UK) and the second section describes some of the main molecular techniques used for the analysis of fathead minnow tissues.

2.1 FISH EXPOSURE STUDIES

All exposure studies were conducted at AstraZeneca’s BEL between March 2011 and January 2014. This study was carried out under project and personal licences granted by the Home Office under the UK Animals (Scientific Procedures) Act 1986, and in accordance with AstraZeneca's local and global ethical policies. Although the exposure studies were not conducted under strict OECD guidelines, the studies did follow the test acceptance criteria in OECD test guidelines for fish, such as those used in the “fish acute toxicity test” [203] (OECD, 1992) and the “fish early-life stage toxicity test” [210] (OECD, 2013). These criteria included <10% mortality in the controls, maintenance of water quality (for example, air saturation and temperature maintained to more than 60% dissolved oxygen (DO) and acceptable deviations (± 1.5 ºC) of the stated temperature during exposures, respectively), and analytical determinations of the test concentrations. Appropriate measures were undertaken to monitor and maintain stable conditions during fish exposures, including alarmed systems for the mains power to the exposure laboratory, the automated water flow system and the water trough levels (in the event of power failure). The water temperature in the test vessels was also continuously monitored (using a probe positioned in at least one test vessel per study) using a “parameter monitoring system”.

2.1.1 Test-species: the fathead minnow (Pimephales promelas)

The fathead minnow (Pimephales promelas) is a commonly used small fish test-species used in eco-toxicological research (Ankley and Johnson, 2004). The fathead minnow is representative of the ubiquitous and ecologically important Cyprinidae family of freshwater fish, which is widely distributed across North America (Ankley...
and Villeneuve, 2006). The term fathead minnow is compounded together through the Greek derivatives of word *Pimephales*, meaning “fat head;” and *promelas*, meaning *pro*, as in “before or in front”, and *melas*, meaning “black”, in reference to the black head of breeding males. Characteristically, fathead minnows are small, ray-finned bony fish (average length of 50 mm), and males and females are easily distinguishable. Typically, males exhibit nuptial tubercles on the snout and an elongated, prominent fleshy fatpad on top of the head which extends in a narrow line from the nape to the dorsal fin, which are not normally seen in females. They are also larger in size (3-5 g) and have a darker body colouration except for two wide, light-coloured vertical bands. In contrast, females are smaller (2-3 g) with a dull olive/silver body colouration (Figure 9) (Ankley and Villeneuve, 2006).

The fathead minnow is routinely used in short-term 48-96 hour tests evaluating lethality as an endpoint, through to complex partial and full life-cycle tests involving a battery of apical endpoints and mechanistic endpoints (Ankley and Villeneuve, 2006). The only shortcoming of using the fathead minnow as a model species, is that the whole genome sequence is not publically available. However, the fathead minnow was selected in this study due to its slightly larger size than the zebrafish, which facilitated individual blood plasma and tissue sampling.

![Figure 9. Male and female fathead minnow, *Pimephales promelas* (image taken from http://aquaticpath.phhp.ufl.edu/fhm/intro.html).](image-url)
2.1.2 Fish husbandry

Adult fathead minnows were bred and kept in communal holding tanks under a flow-through system. The water temperature was maintained at 25 ± 1 °C, pH 7.4 and DO at ≥80% air saturation. Fish were subject to a 16:8 hour (light:dark) photoperiod with a 30 minute transition period between dawn and dusk. Fish were fed twice daily with a combination of food pellet (Biomar, Brande, Denmark) and frozen adult brine shrimp (Artemia sp.).

2.1.3 Test substance and preparation of stock solutions

The test substance, ibuprofen (CAS No.15687-27-1) (MW 206.3), was purchased from Sigma-Aldrich (Dorset, UK) with ≥98% purity and stored at room temperature. Ibuprofen has low water solubility and therefore all stock solutions were prepared in solvent, acetone (CAS No. 67-64-1, ≥99% purity) (Fisher Scientific, Loughborough, UK). The solvent concentration was maintained at 0.016 mL/L (0.0016%) in all stock test solutions, which was well below the recommended OECD guideline of ≤0.1 mL/L (0.01%). Ibuprofen stock solutions were prepared in acetone for each of the following nominal test concentrations; 5, 10, 32, 100, 270, 350, 370 and 500 µg/L.

2.1.4 Dilution water

The dilution water was mains tap water, which had been coarsely filtered to remove particulate material, passed through activated carbon and dechlorinated using a sodium thiosulphate solution. Mineral salts were added, as required, to maintain hardness levels of ≥140 mg/L as calcium carbonate (CaCO₃) (Länge et al., 2001). The treated water was passed through an ultraviolet steriliser and was filtered to 10 µm and then fed into a holding tank where it was thermostatically heated to 25 ± 1 °C. The water was finally filtered to 5.0 µm before distribution into the test vessels.

2.1.5 Static and continuous flow-through systems

Fish were exposed to ibuprofen using either a static or continuous flow-through system (Table 6). In the static exposures, ibuprofen test solutions were administered
directly into the test vessel containing dilution water at the beginning of the exposure period, and remained unchanged throughout the duration of the study. The water was gently aerated at the surface using a glass pipette if there was depletion of DO below the recommended limit. The static system was simpler in design than the flow-through and therefore was adopted for the preliminary and final exposures (Table 6, ‘Exposures 1 and 6’) where time constraints had applied. The majority of exposure studies were however, conducted using the flow-through system (Table 6, ‘Exposures 2-5’). In these studies, ibuprofen test solutions were replenished in the test vessel by the flow of incoming dilution water and through removal of metabolic waste products in the outflow water. This system permitted the water temperature and DO concentrations to be maintained more easily, and allowed a more stable test concentration to be achieved in the vessel. Section 2.1.6 (below) describes the flow-through system in further detail.
Table 6. Summary of fathead minnow exposure studies to ibuprofen. Exposure studies (total of 6) were conducted using either a static or a continuous flow-through system to nominal ibuprofen concentrations ranging from 5, 10, 32, 100, 270, 350, 370, 500 µg/L. The duration of, fish sampling occasions (hours), number (n) of fish used in total and per treatment (including dilution water control (DWC) (not present in ‘Exposure 6’), solvent control (SC) (x 2 tanks in ‘Exposure 4’) and ibuprofen exposure treatments, fish sex; male (M) or female (F) and the tissues sampled for each study are specified. Further details on the endpoints measured or analysis carried out can be found in the individual chapters (3-6).

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Exposure type</th>
<th>Nominal ibuprofen (µg/L)</th>
<th>Duration (hours)</th>
<th>Sampling points (hours)</th>
<th>No. of fish (n)</th>
<th>No. of fish per treatment (n)</th>
<th>Sex (M/F)</th>
<th>Tissues sampled</th>
<th>Further details provided in chapter(s):</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure 1</td>
<td>Static</td>
<td>10, 32 &amp; 100</td>
<td>≤24</td>
<td>24 only</td>
<td>n = 25</td>
<td>10 µg/L 32 µg/L 100 µg/L</td>
<td>5 5 5</td>
<td>Brain, gill, gonad (ovary), gut, heart, liver, muscle</td>
<td>4</td>
<td>Preliminary study</td>
</tr>
<tr>
<td>Exposure 2</td>
<td>Continuous</td>
<td>100, 500</td>
<td>≤96</td>
<td>3, 24, 48, 96</td>
<td>n = 64</td>
<td>100 µg/L 500 µg/L</td>
<td>16 16 16 16</td>
<td>Brain, gill, gonad (testis), gut, heart, liver</td>
<td>3 &amp; 5</td>
<td></td>
</tr>
<tr>
<td>Exposure 3</td>
<td>Continuous</td>
<td>270, 370</td>
<td>≤144 (≤96 exposure + ≤72 depuration)</td>
<td>24, 48, 72, 96 (exposure) + 24, 72 (depuration)</td>
<td>n = 100</td>
<td>270 µg/L 370 µg/L</td>
<td>25 25 25 25</td>
<td>Brain, gill, liver</td>
<td>3 &amp; 5</td>
<td></td>
</tr>
<tr>
<td>Exposure 4</td>
<td>Continuous</td>
<td>270</td>
<td>≤120</td>
<td>72, 96, 120</td>
<td>n = 60</td>
<td>SC (x 2) 270 µg/L</td>
<td>38 M &amp; 46 F</td>
<td>Gill, liver, muscle</td>
<td>6</td>
<td>Tail-fin clipping procedure</td>
</tr>
<tr>
<td>Exposure 5</td>
<td>Continuous</td>
<td>350</td>
<td>72</td>
<td>72 only</td>
<td>n = 60</td>
<td>SC 350 µg/L</td>
<td>58 M &amp; 2 F</td>
<td>Gill</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Exposure 6</td>
<td>Static</td>
<td>5, 350</td>
<td>≤72</td>
<td>24, 48, 72</td>
<td>n = 45</td>
<td>SC 5 µg/L 350 µg/L</td>
<td>M</td>
<td>Gill</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
2.1.6 Flow-through system

2.1.6.1 Test apparatus

The flow-through system was comprised of a dilution water supply, test vessels and dosing apparatus (syringe pump). All test apparatus was constructed of glass where possible. Test vessels consisted of glass tanks \((L 610 \times W 305 \times H 310 \, \text{mm})\), with a working water volume of 45 L and each one was connected to a 2 L glass mixing vessel that was positioned on top of a magnetic stirrer, as shown in Figure 10.

Figure 10. A continuous flow-through system used to expose fish to ibuprofen. Water flowed from an overhead trough into a 45 L glass tank \((a)\) through a 2 L glass mixing vessel with an outlet \((b)\). The mixing vessel was positioned on top of a magnetic stirrer \((c)\) to ensure continuous mixing of test solutions before delivery into the respective tank. Water flowing through the tanks went to waste.
The dilution water flowed (via water flow control devices) from an overhead water trough into each tank from the mixing vessel outlet at an overall rate of 250 mL/minute, allowing the water in the tank to be renewed approximately every three hours (eight tank changes in 24 hours). Water flowing through the tank (controlled by outlet devices at the back of the vessel) went to waste. The tanks were randomly distributed to receive dilution water only (dilution water control, DWC), acetone only (solvent control, SC) or ibuprofen test solution.

2.1.6.2 Dosing of test solutions to tanks

Each ibuprofen stock test solution was dosed using a syringe pump dosing system, into its respective glass mixing vessel (prior to delivery into its adjoining tank) using a 50 mL glass syringe (SGE Analytical Science, Milton Keynes, UK) connected via a “toxin” line (teflon tubing), as shown in Figure 11. Each syringe (and plunger) was used to withdraw and hold one test solution over the exposure period; this was secured on to a multiple syringe infusion motor pump (Harvard Apparatus, Kent, UK) which drove the plunger of the syringe in, causing the test solution to be dispensed through the toxin line at a constant rate. All ibuprofen stock test solutions were dosed at 0.004 mL/minute (4 µL/minute) in order to achieve the nominal concentration in the exposure tanks. Test solutions were dosed into the mixing vessel to ensure complete mixing between the concentrated ibuprofen stock solution (dosed at 0.004 mL/minute, equivalent to 5.76 mL per 24 hours) and the dilution water (flow rate of 250 mL/minute, equivalent to 360 L per 24 hours) before delivery to exposure tanks (to obtain a nominal dilution ratio of ibuprofen in the tank equivalent to 62,500). To account for possible solvent effects, acetone was dosed into the SC tank at the same rate as the ibuprofen test solutions (0.004 mL/minute), such that the solvent concentration in the water and test solutions was the same (0.0016%).

Dosing to tanks was initiated at least 48 hours prior to the introduction of fish in order to allow the exposure tanks to equilibrate with the nominal ibuprofen test concentrations and to monitor the function of the dosing system. Calibration of the dosing system was made by recording the volume of the test solutions in the syringe over known periods of time, and the dilution water flow rates (maintained to ± 10%)
were taken daily throughout the exposure period to ensure correct operation of the dosing system.

Figure 11. Dosing system used for ibuprofen in a continuous flow-through system. Ibuprofen test solutions were dosed from syringes into their respective glass mixing vessels via “toxin” lines (i) to allow mixing with dilution water before delivery into the adjoining exposure tank. A close up of the dosing system (ii) showing the 50 ml syringe/plungers used to hold the test solution over the exposure period. The syringe/plungers were secured onto an infusion motor pump, which drove the syringes in, causing the test solution to be continually expelled into the mixing vessel at a constant rate of 4 µl/minute.
Chapter 2 Materials and Methods

2.1.6.3 Exposure conditions

On Day 0, prior to the fish being introduced into the tanks, the dosing system was checked to ensure normal operation and the water temperature, pH, DO, alkalinity, hardness and conductivity was measured (in an aliquot of water taken from each of the tanks) to assess the water quality. Fish were collected from the holding tanks in the husbandry unit and randomly distributed into the tanks. The health of the fish was recorded after three hours for any signs of stress, and thereafter on each exposure day. The duration and the number (n) of fish used for each exposure study and each treatment are shown in Table 6. During exposures, the water temperature was maintained at 25 ± 1 °C, pH 7.4 (± 1) and DO ≥80% air saturation (equivalent to 6.4 mg/L at 25 °C). These parameters were measured at least twice (in an aliquot of water taken from each tank) over the exposure period. Fish were subjected to a 16:8 hour (light:dark) photoperiod with a 20 minute transition period between dawn and dusk. The fish were not fed during exposure periods except in ‘Exposure 3’, when the study period exceeded 96 hours. These fish were fed once with a combination of pellet and brine shrimp (Artemia sp.).

2.1.7 Fish blood plasma and tissue sampling

After the exposure period, fish were terminally anaesthetised using 500 mg/L of MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich, Dorset, UK) buffered with 1 M sodium bicarbonate (NaHCO₃) (pH 7.4) (Sigma-Aldrich, Dorset, UK). The operculum was carefully monitored until movement had ceased. Once unconscious, the fish was wet weighed (to the nearest 0.01 g) and measured in length (standard length to the nearest 0.1 mm). Each fish was terminated by removal or destruction of the brain using a seeker, followed by removal of the tail-fin using a scalpel and collection of the blood into a 75 µL heparinised micro-capillary tube (Fisher Scientific, Loughborough, UK). The micro-capillary tubes were sealed at one end using soft plasticine and centrifuged at 10,000 rpm for 4 minutes at room temperature (210 Haematocrit Microcentrifuge, Hettich, NC, USA) to separate the plasma from the red blood cells. The plasma volume was recorded and then transferred into a 0.5 mL micro-centrifuge tube, using a 20 µL pipette and a fine tip (Microloader™ tip, Eppendorf, Stevenage, UK). Analysis of ibuprofen
concentrations in the plasma samples was performed immediately; otherwise, the samples were stored at -20 °C.

After blood sampling, fish were dissected and tissues were collected. Each tissue was split into two samples and each sample was weighed (to the nearest 0.01 g) and placed into a pre-labelled 2.0 mL micro-centrifuge tube and flash frozen using liquid nitrogen. Tissues were stored at -80 °C and subsequently transported to Brunel University London (on dry ice) for molecular and biochemical analysis.

2.1.8 Exposure water sampling

Water samples (5 mL) were collected once per day from each treatment tank before and subsequently, on the same day as the fish were sampled during the exposure study. The waters were sampled using a 5 mL pipette (Eppendorf, Stevenage, UK) from the centre of each tank to allow determination of actual ibuprofen exposure concentrations. The samples were analysed immediately or stored at -20 °C until required.

2.1.9 Quantification of ibuprofen in exposure waters and blood plasma

The quantification of ibuprofen in exposure waters and fish blood plasmas was conducted by Mr. Henry Trollope at AstraZeneca’s BEL. The analytical method was previously optimised for the detection and quantification of ibuprofen in water and fish blood plasma samples. Quantification of ibuprofen was performed using reversed-phase Liquid Chromatography coupled with tandem Mass Spectrometry (LC-MS/MS). The parameters used for the LC-MS/MS method are shown in Table 7. Initial chromatographic separation was achieved using a Dionex Ultimate 3000 instrument at 50 °C using a Gemini® NX C18 (50 × 2.0 mm) analytical column (Phenomenex, CA, USA). The mobile phase consisted of 0.1% ammonia in water (solvent A) and 0.1% ammonia in methanol (solvent B) at a flow rate of 500 µL/minute. The gradient conditions were 90% solvent A followed by a linear gradient to 100% solvent B over 3 minutes, held at 100% (solvent B) for 1 minute and re-equilibrium to the initial conditions (90% solvent A and 10% solvent B) over 1 minute (total of 5 minutes). An injection volume of 10 µL was used. Ibuprofen was
detected using an Ion Trap mass spectrometer (LTQ, Thermo Scientific, UK) with heated electrospray ionisation in the negative ionisation mode. For increased MS/MS sensitivity, selected reaction monitoring (SRM) was used for the detection of deprotonated ibuprofen (m/z 205>161 [loss of carbon dioxide]). Data were acquired and processed using Xcalibur™ software (Thermo Scientific, UK). An internal standard, ibuprofen-d₃ (CAS No. 121662-14-4, ≥98.0% purity) (Sigma-Aldrich, Dorset, UK) was used for the detection of ibuprofen in blood plasma samples in SRM mode (m/z 208>164). Due to time restraints, it was not possible to measure ibuprofen metabolites in the exposure waters or in plasmas. An example of an ion chromatogram of ibuprofen and ibuprofen-d₃ in fish blood plasma is shown in Figure 12.

Table 7. Parameters used for LC-MS/MS analysis of ibuprofen in water and blood plasma samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC instrument</td>
<td>Dionex Ultimate 3000</td>
</tr>
<tr>
<td>Analytical column</td>
<td>Gemini® NX C18 (50 × 2.0 mm internal diameter) (Phenomenex, CA, USA) (3.0 μm particle size)</td>
</tr>
<tr>
<td>Column Pre-filter</td>
<td>0.5 μm (Supelco, USA)</td>
</tr>
<tr>
<td>Column temperature</td>
<td>50 °C</td>
</tr>
<tr>
<td>Flow rate</td>
<td>500 μL/min</td>
</tr>
<tr>
<td>Injection volume</td>
<td>10 μL</td>
</tr>
<tr>
<td>Detection system</td>
<td>Quadrupole Ion Trap (LTQ, Thermo) mass spectrometer</td>
</tr>
<tr>
<td>Ionisation mode</td>
<td>Negative</td>
</tr>
<tr>
<td>Sheath gas flow</td>
<td>Nitrogen at 60 arbitrary units</td>
</tr>
<tr>
<td>Auxiliary gas flow</td>
<td>Nitrogen at 20 arbitrary units</td>
</tr>
<tr>
<td>Sweep gas flow</td>
<td>Nitrogen at 0 arbitrary units</td>
</tr>
<tr>
<td>Ion spray voltage</td>
<td>2.5 kV</td>
</tr>
<tr>
<td>Vaporiser temperature</td>
<td>350 °C</td>
</tr>
<tr>
<td>Capillary temperature</td>
<td>350 °C</td>
</tr>
<tr>
<td>Data acquisition and processing</td>
<td>Xcalibur™ software</td>
</tr>
</tbody>
</table>
2.1.9.1 Preparation of standards & samples

For quantification of ibuprofen in water samples, a set of standards was prepared from a stock solution of ibuprofen test material (100 mg/L) in HPLC-grade acetonitrile (ACN) by serial dilution using HPLC-grade water (Sigma-Aldrich, Dorset, UK). Water samples (5 mL) from the exposure tanks were diluted (1:4) with HPLC-grade water and an aliquot (~1 mL) was transferred to an autosampler vial for analysis. Water samples (5 mL) collected from the DWC and SC tanks required no dilution and an aliquot (~1 mL) was directly transferred to a vial for analysis. An 8-point calibration curve was constructed by plotting the ibuprofen peak area, against standard concentrations ranging from 0.625-160 µg/L (see Appendix). The calibration curve was used to determine the ibuprofen concentration in the exposure tanks.

For quantification of ibuprofen in blood plasma samples, a second set of standards was prepared from the stock solution (100 mg/L) by serial dilution using HPLC-grade water and HPLC-grade ACN containing an internal standard, ibuprofen-d₃.

Figure 12. Example of an ion chromatogram of ibuprofen (A) and internal standard, ibuprofen-d₃ (B) in blood plasma of a fish exposed to 370 µg/L for 72 hours.
(500 µg/L) (Sigma-Aldrich, Dorset, UK). A dilution ratio of (84:16) HPLC-grade water:ibuprofen-d₃ was used to provide an internal standard concentration of 80 µg/L. An 8-point calibration curve was constructed by plotting the ratio of ibuprofen to internal standard peak area, against standard concentrations ranging from 0.625-160 µg/L (see Appendix). Plasma samples (µL) from control and exposed fish were spiked (1:4) with HPLC-grade ACN containing ibuprofen-d₃ (80 µg/L). The samples were transferred to a 96-well plate and thoroughly mixed using a plate shaker for 15 minutes at room temperature to facilitate protein precipitation. The plate was centrifuged at 3,220 x g for 30 minutes at 20 ºC to pellet the proteins. The maximum allowable volume of supernatant (up to 100 µL) was transferred to a fresh well and diluted (1:5) with HPLC-grade water to produce an overall dilution x 25 of the plasma sample. The calibration curve was used to determine the ibuprofen concentration in the plasma samples. Plasma sample readings that were outside of the calibration range were diluted and re-analysed.

2.1.9.2 Method validation

The limit of detection (LOD) and limit of quantification (LOQ) are two important performance characteristics used in the method validation of analytical measurements. The LOD is the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, and the LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy. In this study, the sensitivity of the method was determined by the concentration of the lowest (acceptable) calibration standard. Each sample was analysed in two replicates.
2.2 MOLECULAR TECHNIQUES

2.2.1 Total RNA isolation

Total RNA was isolated from fish tissues using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich, Dorset, UK), according to the manufacturer’s protocol. Briefly, fish tissues (up to 40 mg) were promptly disrupted in lysis solution containing 2-mercaptoethanol (2-ME) to denature proteins, using a tissue lyser (Tissue Lyser II, QIAGEN, Manchester UK). The tissue/lysis solution mixture was pipetted onto a filtration column and centrifuged at 16,000 x g for 2 minutes at room temperature to shear the DNA. Ethanol (70%) was added to filtrated lysate to facilitate RNA binding to the silica membrane in the binding column. The membrane containing bound RNA was washed twice with wash solution and centrifuged at 16,000 x g for 15 seconds at room temperature to remove any remaining DNA, proteins and salts. The membrane was centrifuged one final time at 16,000 x g for 2 minutes at room temperature to allow it to dry and to remove residual ethanol. The RNA was eluted with 50 µL of elution solution into a clean micro-centrifuge tube. The quantity of the isolated RNA was determined by spectrophotometry at an absorbance of 260 nm (NanoDrop 1000, Thermo Scientific, LabTech, East Sussex, UK). The ratio of absorbance at 260/280 nm was used to assess the purity of the RNA. A ratio of ~2.0 was acceptable for RNA purity. The RNA integrity was assessed on an agarose gel stained with ethidium bromide and visualised under ultraviolet (UV) light. RNA samples were immediately treated with deoxyribonuclease I (DNase I) or stored at -80 °C.

2.2.2 Deoxyribonuclease I (DNase I) treatment of RNA

Isolated RNA samples were treated with Deoxyribonuclease I (DNase I) (DNase I, Amplification Grade, Sigma-Aldrich, Dorset, UK) to minimise genomic DNA contamination. One unit of DNase I completely digests 1 µg of plasmid DNA to oligonucleotides in 10 minutes at 37 °C. RNA (2 µg) was diluted with nuclease-free water (8 µL) and incubated with 1 µL of 10 x Reaction Buffer (200 mM Tris-HCl [pH 8.3] and 20 mM MgCl₂) and 1 µL of DNase I (1 U/µL) for 15 minutes at room temperature. The enzyme was inactivated by adding 1 µL of Stop Solution (50 mM
EDTA) and denatured by heating to 70 °C for 10 minutes. The treated RNA samples were prepared for reverse transcription or stored at -80 °C.

2.2.3 Reverse transcription complementary DNA (cDNA) synthesis

Total RNA was reverse transcribed to complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Paisley, UK). cDNA synthesis was primed using oligo(dT)_{20} primers that utilise the poly-A tails found at the end of eukaryotic mRNAs. RNA (2 µg) was diluted with nuclease-free water (up to 8 µL) and mixed with 1 µL of 50 µM oligo(dT)_{20} primers and 1 µL of 10 mM deoxyribonucleotide triphosphate (dNTP) mix (10 mM each of dATP, dGTP, dCTP,dTTP) in a 0.5 mL micro-centrifuge tube. The mixture was incubated at 65 ºC for 5 minutes to allow the RNA, primers and dNTPs to denature, and then placed on ice for 1 minute. A subsequent mixture containing 2 µL of 10 x RT buffer, 4 µL of 25 mM MgCl₂, 2 µL of 0.1 M DTT, 1 µL of RNaseOUT (40 U/µL) and 1 µL of SuperScript III RT (200 U/µL) were combined in a separate 0.5 ml micro-centrifuge tube. A volume of 10 µL of this second mixture was added to each RNA sample. The mixture was incubated at 50 ºC for 50 minutes using a heat block to create the cDNA product. The reactions were terminated by heating at 85 ºC for 5 minutes and then chilled on ice for 2 minutes. Residual RNA was removed by adding 1 µL of RNase H (2 U/µL) to the sample and incubating at 37 ºC for 20 minutes.

For quantitative real-time PCR (qPCR) analysis (see Chapter 5) treated RNA was reverse-transcribed to cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hertfordshire, UK). This kit utilises a mixture of both oligo(dT) and random hexamer primers, that can allow greater coverage of a transcript pool since random primers can prime from anywhere across the length of long transcripts. RNA (1 µg) was diluted with nuclease-free water (up to 15 µL) and mixed with 4 µL of 5 x iScript Reaction Mix and 1 µL of iScript Reverse Transcriptase (1 U/µL) in a 0.5 ml micro-centrifuge tube. The cDNA mixture (20 µL) was incubated at 25 ºC for 5 minutes, 42 ºC for 30 minutes to allow annealing between the primers and RNA template, 85 ºC for 5 minutes to terminate the reactions and held at 4 ºC for 5
minutes. The newly synthesised cDNA samples were used immediately in a PCR reaction or stored at -20 ºC.

2.2.4 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is an in vitro technique that is used for the amplification of a specific DNA sequence within a heterogeneous mixture of DNA sequences, within a micro-centrifuge tube. During PCR, double-stranded DNA containing the target sequence is separated and specific primers, which have been designed to complement either end of the target sequence, anneal to the DNA flanking the sequence to be amplified. Under optimal conditions, DNA polymerase extends the complementary sequence in the presence of dNTPs allowing a new complementary DNA strand to be synthesised. This newly synthesised strand serves as a template for subsequent cycles of DNA synthesis, and with every cycle the amount of DNA is exponentially amplified (Figure 13) (Alberts et al., 2008).

![Diagram of PCR](image)

Figure 13. Diagrammatic image of the amplification of a specific region of DNA by polymerase chain reaction (PCR) (image adapted from Alberts et al., 2008). Double-stranded DNA is separated into single stands by thermal denaturation. Once the DNA has cooled, primers can anneal to the target regions (at a temperature specific to the primers). A DNA polymerase enzyme is used to extend the primers in the presence of other components including dNTPs and buffer, and the target strands re-anneal. The newly formed double-stranded DNA serves as a template for subsequent PCR cycles.
2.2.4.1 Factors affecting PCR

Several factors can affect the PCR process and the resultant success of amplification including the design of primers, the DNA polymerase enzyme used, and the cycling conditions employed which are discussed further.

2.2.4.1.1 Primer design

The National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), and ENSEMBL (http://www.ensembl.org/index.html) were used to search for the prostaglandin-endoperoxide synthase 1 (PTGS 1) and prostaglandin-endoperoxide synthase 2 (PTGS 2) genes (colloquially referred to as the cyclooxygenase (COX 1 and COX 2) genes) in human and teleost fish, and in particular in the fathead minnow. However, no mRNA sequences for the COX genes were available in the fathead minnow. Therefore, published data on COX gene sequences in zebrafish (Grosser et al., 2002; Ishikawa et al., 2007) and rainbow trout (Zou et al., 1999; Ishikawa and Herschman, 2007) were used to search for homologues in other teleost fish. The NCBI database was screened using the “Basic Local Alignment Search Tool” (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) which retrieved both full length and partial mRNA sequences for up to six COX gene isoform sequences in zebrafish, rainbow trout, Atlantic salmon (Salmo salar), mummichog (Fundulus heteroclitus) and one frog species (Xenopus laevis). These gene sequences were used as templates for designing generic primers to isolate the COX 1 and COX 2 genes in the fathead minnow. The accession numbers for the template sequences from the NCBI database are shown in Table 8.
Table 8. Accession numbers for COX (*PTGS*) gene sequences from the NCBI database used in the multiple alignment analysis.

<table>
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<tr>
<th>Species</th>
<th>Latin name</th>
<th>COX gene isoform</th>
<th>Accession numbers</th>
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<tr>
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<td>COX 1</td>
<td>NM_000962.3</td>
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<tr>
<td></td>
<td></td>
<td>COX 2</td>
<td>NM_000963.3</td>
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<td>Zebrafish</td>
<td><em>Danio rerio</em></td>
<td>COX 1</td>
<td>NM_153656.1</td>
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<td>COX 2b</td>
<td>NM_001025504.2</td>
</tr>
<tr>
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<td><em>Oncorhynchus mykiss</em></td>
<td>COX 1</td>
<td>NM_001124361.1</td>
</tr>
<tr>
<td></td>
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<td>COX 2</td>
<td>NM_001124348.1</td>
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<td></td>
<td></td>
<td>COX 2b</td>
<td>NM_001124667.1</td>
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<tr>
<td>Atlantic salmon</td>
<td><em>Salmo salar</em></td>
<td>COX 1</td>
<td>NM_001140374.1</td>
</tr>
<tr>
<td>Mummichog</td>
<td><em>Fundulus heteroclitus</em></td>
<td>COX 1a</td>
<td>EU703782.1</td>
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<tr>
<td></td>
<td></td>
<td>COX 1b</td>
<td>EU703781.1</td>
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<td>African clawed frog</td>
<td><em>Xenopus laevis</em></td>
<td>COX 1</td>
<td>NM_001097920.1</td>
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</table>

Template sequences for the COX 1 and COX 2 sequences were aligned using the Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) multiple sequence alignment tool, to examine regions of highly conserved nucleotide bases between the template sequences for each of the genes. Primers were designed to target the COX 1 and COX 2 genes in the fathead minnow based on the highly conserved regions identified in the template sequences, according to the primer conditions outlined below. Two pairs of generic primers (one ‘external’ and one ‘internal’ set) were designed to isolate the COX 1 gene, as shown in Figure 14 (highlighted in blue and green boxes, respectively) and the COX 2 gene, as shown in Figure 15 (highlighted in purple and pink boxes, respectively).

All primers were 20-22 nucleotides in length, with a guanine and cytosine (G and C) content of 40-60%. This is because guanine and cytosine bind to each other using three covalent bonds, whereas adenine and thymine (A and T) bind to each other using only two covalent bonds, and therefore a higher G and C content was preferable for stronger bonds to be formed between the DNA template and primer. Both the length and G and C content of the primer sequences influence the primer melting temperature (Tm) which affects the stability of DNA template-primer duplex and therefore both primers (for each primer set) were designed to have the same, or a
similar Tm (between 58-60 °C). The Tm of the primers was calculated using the following formula: \( Tm = 2 \times [A + T] + 4 \times [G + C] \). The Tm was used to calculate the annealing temperature for each primer pair, which was generally 2-5 °C below the lowest Tm of the two primers. The characteristics of each primer including the Tm, likelihood of secondary structure formations such as heterodimers, self-dimers and hairpins were assessed using primer analysis tools such as ‘Primer 3 Plus’ (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Primers for the fathead minnow COX genes were synthesised from Sigma Genosys Ltd (Suffolk, UK) and reconstituted with nuclease-free water to a concentration of 100 μM. Primer stocks were diluted with nuclease-free water to a working concentration of 10 μM and stored at -20 °C until required.
Chapter 2 Materials and Methods

Clustal Omega multiple sequence alignment (Page 1 of 3)
Rainbow trout COX 1
Atlantic salmon COX 1
Zebrafish COX 1
Mummichog COX 1a
Mummichog COX 1b
Human COX 1
African clawed frog COX 1

GCCTCACCATGTATGCCACCCTGTGGCTGAGAGAACACAACCGCGTTTGT
GCCTCACCATGTACGCCACCCTGTGGCTGAGAGAACACAACCGCGTCTGT
GGCTCGGCATGTACGCTACCTTGTGGCTTCGTGAGCACAACCGTGTGTGT
GCCTCACCGTGTATGCCACCATCTGGCTGAGGGAGCACAACAGAGTGTGT
GTTTGAGTTTGTACGCCACGCTGTGGCTCAGGGAGCATAACAGAGTCTGT
GGCTCATGCTGTATGCCACGCTCTGGCTACGTGAGCACAACCGTGTGTGT
GATTGATGATGTATGCAACCCTTTGGCTCCGCGAACACAATAGGGTTTGT
* *
**** ** ** * ***** * ** ** ** * ** ***
‘External’ COX 1 F
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GACATCCTGAAGGCAGAGCACCCCACCTGGGGGGATGAGCAGCTCTTTCA
GAAATCCTGAAACAAGAACATCCAACCTGGGGTGATGAGCAGCTCTTCCA
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GACATCCTGAAAGCAGAGCATCCCACCTGGGACGATGAGCAGCTTTTCCA
GACCTGCTGAAGGCTGAGCACCCCACCTGGGGCGATGAGCAGCTTTTCCA
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** * *****
** ** ** ** **** ** ******** ** **

1177
990
1065
510
1048
1071
964

Rainbow trout COX 1
Atlantic salmon COX 1
Zebrafish COX 1
Mummichog COX 1a
Mummichog COX 1b
Human COX 1
African clawed frog COX 1

GACCGCCAGGCTCATCGTAATAGGTGAGACCATCCGGATAGTGATCGAGG
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** * * ** ** * ** ** **** **
** * ** ** *

1277
1090
1165
610
1148
1171
1064

Rainbow trout COX 1
Atlantic salmon COX 1
Zebrafish COX 1
Mummichog COX 1a
Mummichog COX 1b
Human COX 1
African clawed frog COX 1

AGTACGTGCAGCACCTGAGTGGCTACCTGTTGGATCTGAAGTTTGACCCA
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** ** ***** ** ** ** **
** * ** * * ** **

1327
1140
1215
660
1198
1221
1114

Rainbow trout COX 1
Atlantic salmon COX 1
Zebrafish COX 1
Mummichog COX 1a
Mummichog COX 1b
Human COX 1
African clawed frog COX 1

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* ** **
***** ***
** ** ** * * **
‘Internal’COX 1 F
GTTCAAACAGCTGTACCACTGGCACCCCCTGATGCCAGACAGCTTCCACA
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***
* * ** ******** ** ** ***** **
**

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1248
1271
1164

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*
*
*
**
** *** * * ** ** **

1475
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1363
808
1346
1369
1262

Rainbow trout COX 1
Atlantic salmon COX 1
Zebrafish COX 1
Mummichog COX 1a
Mummichog COX 1b
Human COX 1
African clawed frog COX 1

Rainbow trout COX 1
Atlantic salmon COX 1
Zebrafish COX 1
Mummichog COX 1a
Mummichog COX 1b
Human COX 1
African clawed frog COX 1
Rainbow trout COX 1
Atlantic salmon COX 1
Zebrafish COX 1
Mummichog COX 1a
Mummichog COX 1b
Human COX 1
African clawed frog COX 1

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1121
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1214

65


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<td>African clawed frog</td>
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Rainbow trout COX 1       | TGGAAGCCCAGCAGATTCGGCAGACAGGGCTTGTATAGATCACTC 1920                                                                 |
| Atlantic salmon COX 1    | TGGAAGCCCAGCAGATTCGGCAGACAGGGCTTGTATAGATCACTC 1733                                                                         |
| Zebrafish COX 1          | TGGAAGCCCAGCAGATTCGGCAGACAGGGCTTGTATAGATCACTC 1808                                                                           |
| Mummichog COX 1a         | TGGAAGCCCAGCAGATTCGGCAGACAGGGCTTGTATAGATCACTC 1253                                                                           |
| Mummichog COX 1b         | TGGAAGCCCAGCAGATTCGGCAGACAGGGCTTGTATAGATCACTC 1791                                                                           |
| Human COX 1              | TGGAAGCCCAGCAGATTCGGCAGACAGGGCTTGTATAGATCACTC 1814                                                                           |
| African clawed frog COX 1| TGGAAGCCCAGCAGATTCGGCAGACAGGGCTTGTATAGATCACTC 1707                                                                           |

Figure 14. Clustal Omega (partial) alignment of template COX 1 gene sequences (5’-3’) used to design COX 1 primers in the fathead minnow. Identical nucleotide bases between sequences are indicated by an asterisk (*). The ‘external’ and ‘internal’ forward (F) and reverse (R) primers used to isolate COX 1 in the fathead minnow are shown in blue and green boxes, respectively.
### Clustal Omega multiple sequence alignment (Page 1 of 2)

#### 'External' COX 2 F

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<th>Zebrafish COX 2a</th>
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#### 'Internal' COX 2 F

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<td>TTGCTGTGGGTCATGAGGCCTTTGGGCTGGTTCCCGGACTTATGATGTAC</td>
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</tbody>
</table>

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**Note:** The sequence alignment includes multiple sequence comparisons of COX 2 proteins from different species, highlighting conserved and variable regions. The alignments are color-coded to indicate similarities and differences across the sequences.
Continued (Page 2 of 2)

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession</th>
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<td>Human COX 2</td>
<td>CTTAGTGGAATCTATTATTTAGCTCAAGTTTGATCCTCCGAACCTACCTCTCTTCAA 1105</td>
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<td>Rainbow trout COX 2b</td>
<td>TTTGATGGGCCTACCTTCAACACTGAACCTTCCCTTTTCAA 1198</td>
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</table>

Figure 15. Clustal Omega (partial) alignment of template COX 2 gene sequences (5’-3’) used to design COX 2 primers in the fathead minnow. Identical nucleotide bases between sequences are indicated by an asterisk (*). The ‘external’ and internal’ forward (F) and reverse (R) primers used to isolate COX 2 in the fathead minnow are shown in purple and pink boxes, respectively.
The exact sequences for the forward and reverse primers used to amplify the COX genes in the fathead minnow are shown in Table 9. Both of the COX 1 primer sets contained a degenerate (forward) primer. Degenerate primers contain positions within the sequence that can be substituted with one or more different nucleotide bases. These primers are useful when template sequences from related species have been used to design the primers for genes of interest.

Table 9. Primers sets used to isolate the COX 1 and COX 2 genes in the fathead minnow. An ‘external’ and ‘internal’ primer set was designed to isolate each of the COX 1 and COX 2 genes. Degenerate primers were used to isolate COX 1, these primers contain base positions (underlined) that can be substituted for other known nucleotide bases. R positions can be substituted with A or G, B with G or T or C and H with A or T or C bases.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer ID</th>
<th>Primer Sequence 5’- 3’</th>
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<tr>
<td>COX 1</td>
<td>External COX 1 F</td>
<td>ACCTGGGGRBGATGAGCAGCT</td>
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<td></td>
<td>External COX 1 R</td>
<td>CCAAATGTGCTGGGCTTCCA</td>
</tr>
<tr>
<td></td>
<td>Internal COX 1 F</td>
<td>CACTGGCACCCHCTBATGCC</td>
</tr>
<tr>
<td></td>
<td>Internal COX 1 R</td>
<td>AATGTGCTGGGCTTCCAGTA</td>
</tr>
<tr>
<td>COX 2</td>
<td>External COX 2 F</td>
<td>ACTTCACCCACCAGTTTTC</td>
</tr>
<tr>
<td></td>
<td>External COX 2 R</td>
<td>ATCAGTGGGTGCCAGTGTA</td>
</tr>
<tr>
<td></td>
<td>Internal COX 2 F</td>
<td>TCTGGCTGAGAGACACAC</td>
</tr>
<tr>
<td></td>
<td>Internal COX 2 R</td>
<td>ATGC GGTTCTGTGATACTTGGA</td>
</tr>
</tbody>
</table>

To increase the specificity of the primers during COX gene amplification, ‘nested PCR’ was also used. The ‘external’ COX gene primers were used in the first round of amplification, to flank a larger region of the target COX gene sequence and then the ‘internal’ (nested) primers (located within the external primers) were used in a subsequent round of amplification, to further amplify the product obtained from the first round of PCR (Figure 16), to reduce the amplification of non-specific products.
2.2.4.1.2 DNA polymerase enzyme

Several different Taq DNA polymerase enzymes, such as REDTaq® DNA Polymerase (Sigma-Aldrich, Dorset, UK), AmpliTaq Gold® (Applied Biosystems, Life Technologies, Paisley, UK) and the Taq DNA polymerase (Invitrogen Life Technologies, Paisley, UK) were tested in PCR reactions during this study, with varying levels of success. The Taq DNA polymerase (Invitrogen Life Technologies, Paisley, UK) performed most consistently yielding a greater number of PCR products when compared to the other polymerases, and therefore was selected for all subsequent PCR reactions used for the isolation of the COX genes.

2.2.4.1.3 Cycling conditions

The PCR process is achieved through several cycles of repeated heating and cooling to allow DNA melting and enzymatic DNA replication. The annealing temperature of the primer to the DNA strand is particularly important and specific to each primer set. The annealing temperature and number of cycles used for the primers varied according to the ‘conventional’ or ‘touch-down’ PCR programmes employed as
shown in Figure 17. During ‘conventional’ PCR, amplification was achieved using one specific primer annealing temperature only, which was generally 2-5 °C below the lowest Tm of the primer pair. This programme consisted of 25-30 cycles with one annealing temperature between 55-60 °C (Figure 17 A). A modified ‘touch-down’ PCR programme was also used to increase the specificity of the primers during amplification. During touch-down PCR, the initial cycles of amplification were performed using an annealing temperature that was equal to or above the Tm of the primers used (between 58-60 °C). The temperature was subsequently lowered in decrements of 0.5 °C with every cycle (for 30 cycles) until the desired ‘touch-down’ annealing temperature (45 °C) was reached. Amplification was then continued at this temperature for the remaining cycles (15 cycles) (Figure 17 B). The cycling range of temperatures changes the stringency of the primers; the high temperatures at the earlier cycles ensure increased stringency of the primers allowing for greater primer specificity however, at the lower temperatures the risk of non-specific products being amplified is increased. Following thermal cycling, PCR reactions were visualised on an agarose gel and the resultant PCR products were examined under UV light.
Figure 17. Conventional (A) and touch-down (B) PCR cycling parameters used for the amplification of COX genes in the fathead minnow.
2.2.5 **Agarose gel electrophoresis**

Agarose gel electrophoresis was used to visualise the PCR reactions and determine whether the product(s) of interest had been amplified. This simple and effective technique is used for the separation of RNA and DNA based on their size. Separation is achieved by the migration of such molecules through a highly cross-linked agarose gel matrix in response to an electrical current. As RNA and DNA are negatively charged, they will migrate from the negative cathode toward the positive anode separating products by size in a series of bands. The PCR products were visualised on an agarose gel made with 1.5 g of certified biological agarose and 100 mL of TBE buffer (1 x) (90 mM Tris, 90 mM Boric acid, 2 mM EDTA). The solution was heated in a microwave until the agarose powder had completely dissolved, cooled to 60 °C and then 5 µL of ethidium bromide (10 mg/mL) was added to the gel solution. EtBr forms fluorescent complexes with nucleic acids and is used to stain the PCR products so that they can be visualised under UV light. The solution was decanted into a plastic mould with a comb inserted, in order to create empty wells. The gel was left to cool and solidify for approximately 30–45 minutes. The colourless PCR reactions were mixed with 2 µL of 10 x gel-loading buffer (0.3% (w/v) bromophenol blue, 65% (w/v) sucrose, 10 mM Tris-HCl [pH 7.5] and 10 mM EDTA) to add density and to allow tracking of the samples. A 1 Kb+ DNA ladder (Invitrogen Life Technologies, Paisley, UK) was used for size determination in base pairs (bp) of the PCR products. The samples were run at 80 V for 45 minutes and visualised under UV light using a gel documentation system (Gel Doc XR, Bio-Rad, Hertfordshire, UK).

2.2.6 **Extraction and purification of PCR products (DNA) from gel**

Following examination under UV light, PCR products of the correct expected size (in bp) were extracted from the agarose gel and the DNA was recovered using the MinElute™ Gel Extraction kit (QIAGEN, Manchester, UK), according to the manufacturer’s protocol. Briefly, the PCR product was carefully excised from the gel using a sharp scalpel, weighed and mixed with three volumes of digestion buffer and incubated at 50 °C for 10 minutes to ensure that the gel had been completely dissolved. One gel volume of isopropanol was added to the sample to facilitate DNA
binding to the silica membrane in the MinElute column. The membrane containing bound DNA was washed twice with an ethanol-containing wash buffer. After the second wash buffer had been added the column was left to stand for 3 minutes and centrifuged at 10,000 x g for 1 minute at room temperature one final time to remove any residual ethanol. The column was transferred into a clean 1.5 mL micro-centrifuge tube and the DNA was eluted with 10 µL of DEPC-treated water. DNA samples were collected, quantified by spectrophotometry (NanoDrop 1000, Thermo Scientific, LabTech, East Sussex, UK) and stored at -20 ºC until required for recombinant cloning.

2.2.7 Recombinant DNA cloning

PCR is a simple and effective technique used to produce millions of copies of a DNA sequence that can be subsequently visualised as a DNA band by gel electrophoresis. However, a DNA band of the correct expected size may contain several DNA sequences, all of which have the same size. This can be particularly problematic if the primers have not been optimised or when examining for an unknown DNA sequence with primers that are not specific to the sequence, such as the generic primers that were designed in this study. Recombinant DNA cloning presents an additional method of separating and identifying all potential DNA sequences that may be found within a single DNA band of interest and subsequently amplifying the desired DNA product. This multi-step procedure fundamentally involves inserting the (gel-extracted and purified) DNA product into a bacterial plasmid vector, through a process of “ligation”. The plasmid is then introduced into a suitable bacterial host cell through a process of “transformation” and the host cells are subsequently grown on agar plates to amplify the desired DNA product.

2.2.7.1 Ligation

Ligation of extracted PCR (DNA) products was carried out using the QIAGEN PCR Cloning Kit (Manchester, UK), which utilises the pDrive cloning vector (3.85 Kb in size), according to the manufacturer’s protocol. DNA products generated by Taq DNA polymerases contain a single adenine (A) overhang at each end referred to as a “sticky end” which can hybridise with high specificity to the uracil (U) overhang.
found at either side of the linearised cloning vector, as shown in Figure 18. Only one single DNA product can be inserted into a plasmid vector, thus separating different DNA products of the same size. Up to 4 µL (26 ng) of the purified DNA product was mixed with 1 µL of pDrive cloning vector (50 ng/µL) (using a 10:1 DNA product to vector molar ratio) and 5 µL of 2 x Ligation Master Mix. The solution was made up to 10 µL using distilled water, gently mixed and incubated for two hours at 4 °C. During this incubation time, the adenine (5’ end) and uracil (3’ end) residues in the DNA product and cloning vector, respectively, hybridised together to produce a complete plasmid (Figure 18). The plasmid also contains a multiple cloning site located within the lacZ gene, and a drug-resistant gene marker (AmpR), which confers resistance to the antibacterial compound ampicillin, therefore allowing screening of the bacterial cells (with an inserted plasmid) during “transformation” (see next section).

Figure 18. Schematic diagram of ligation of a PCR (DNA) product into a plasmid vector to form a “recombinant DNA” molecule. An adenine (A) residue from the PCR product hybridises with high specificity to the uracil (U) in the cloning vector. The plasmid contains a multiple cloning site located within the lacZ gene where the exogenous DNA is inserted and an ampicillin-resistant gene marker (AmpR), which allows selective screening of cells during “transformation”.
2.2.7.2 Transformation

Immediately following the ligation incubation period, 2 µL of ligation mix was added to quickly thawed QIAGEN EZ Competent (E.coli) cells (provided in the QIAGEN PCR Cloning Kit) and incubated on ice for 5 minutes. The cell mixtures were subsequently heat-shocked at 42 °C for 30 seconds and then immediately returned to the ice, allowing the permeated cells to take up the plasmid vectors. Only one plasmid vector is usually taken up by one cell. The transformed cells were incubated on ice for 2 minutes; this was followed by the addition of 250 µL of room temperature nutrient-rich SOC medium, used to stabilise the cells and maximise uptake into the competent cells. The transformed cell mixtures were grown on agar medium (1.5% (w/v) agar, 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl, autoclaved and cooled to 50 °C) with the following added antibiotics; ampicillin (100 µg/mL), X-gal (5-Bromo-4-Chloro-3-Indolyt-betagalactopyranoside) (50 mg/mL) and IPTG (isopropyl-beta-D-thiogalactopyranoside) (0.1 M stock). The cell mixtures were spread on to agar-containing plates using a sterilised glass spreader and incubated overnight at 37 ºC.

X-gal is used as a differentiation marker, which allows selective screening of transformed bacterial cells using the blue/white screening method. Bacterial cells transformed with plasmids containing the DNA insert will produce white colonies (i.e. recombinant plasmids), whereas cells transformed with the plasmid only (without the DNA insert) will result in blue colonies (i.e. non-recombinant). The ampicillin allows screening of transformed bacterial cells (i.e. an inserted plasmid with ampicillin resistance), as only these cells can colonise the ampicillin-containing agar.

After overnight incubation, the plates were incubated at 4 ºC for 2 hours to facilitate colour development (i.e. blue and white colonies) which was further enhanced by IPTG, allowing easier determination of the white-coloured recombinant colonies and blue-coloured non-recombinant colonies. The white colonies were selected for further assessment using PCR.
2.2.7.3 Assessment of colonies using PCR

White colonies were amplified using PCR with M13 primers (Forward 5’ GTAAAACGACGGCCAGT 3’) and (Reverse 5’ AACAGCTATGACCATG 3’) to identify which colonies contained the correct-sized DNA inserts of interest. These primers amplify the region in between the T7 and SP6 promoter sites (located on the cloning vector) which contains the exogenous PCR (DNA) product insert. A total of 20 colonies were selected and placed into individual PCR reactions and visualised on a 1.5% agarose gel. Positive colonies containing the correctly-sized DNA inserts were subsequently cultured in Luria Broth (LB) medium supplemented with ampicillin (100 µg/mL) and incubated overnight at 37 ºC with vigorous shaking to increase bacterial growth. After incubation, the cultures were mini-prepped to purify the plasmid DNA.

2.2.7.4 Plasmid DNA purification

Plasmids were isolated from bacterial cell cultures using the QIApREP Miniprep Kit (QIAGEN, Manchester, UK) according to the manufacturer’s protocol. All steps were carried out at room temperature. Bacterial cells were centrifuged at 6,000 x g for 15 minutes to produce a cell pellet. The supernatant was disposed of into previously prepared multi-purpose (Virkon) disinfectant solution. The pellet was firstly lysed in 250 µL of an alkaline solution containing RNase A, followed by 250 µL of a second lysis buffer containing an optimised salt concentration for DNA binding. 350 µL of neutralisation buffer was then added and the tube was thoroughly mixed by inverting several times to promote the precipitation of proteins and genomic DNA. The mixture was centrifuged at 17,900 x g for 10 minutes which produced a white pellet and the supernatant (containing the plasmid) was pipetted onto a QIA prep spin column and centrifuged at 17,900 x g for 1 minute. The spin column was washed with 750 µL of ethanol-containing wash buffer and centrifuged to allow the plasmid DNA to adsorb onto the silica membrane. The column was centrifuged one final time to remove any residual ethanol. The DNA was eluted with 50 µL of elution buffer and the DNA yield was quantified using the NanoDrop spectrophotometer. The plasmid DNA samples were stored at -20 ºC.
2.2.7.5 Restriction digestion

The purified plasmids were examined using a restriction digestion to identify which colonies contained the correct-sized DNA inserts, before they were sent for DNA sequencing. Restriction enzymes recognise short, specific nucleotide sequences on double-stranded DNA molecules (“restriction sites”) that can be cleaved into smaller fragments, allowing determination of the size of the inserted DNA fragments. Plasmid DNA samples were digested using the EcoRI restriction enzyme that cleaves DNA in a staggered manner to produce “sticky ends”. A restriction digestion was performed using 1 µg of plasmid DNA, 2 µL of 10 X restriction buffer (10 U/µL), 1.5 µL of EcoRI enzyme and an adjustable volume of distilled water to make a 20 µL sample mixture. Samples were incubated at 37 °C for one hour and the digested products were separated on a 1% agarose gel and visualised under UV light.

2.2.8 DNA sequencing

The purified DNA products of the correct size were diluted to a concentration of 600 ng in 30 µL using distilled water. The forward and reverse primers used during amplification (or alternately if plasmid DNA was used, the M13 primers) were also prepared at a concentration of 32 picomoles in 10 µL using distilled water. The samples were sequenced using the DNA Sequencing Service, University of Dundee, Scotland (www.dnaseq.co.uk). The results from the sequencing were provided as a chromas plot indicating the nucleotide sequence of the DNA product, as shown in Figure 19. The quality of the sequencing was indicated by the height and definition of the peaks. The beginning of the sequencing reaction generally produced low quality peaks. A sequence was considered reliable only when the nucleotide peaks had become sharp and well defined.
Figure 19. Example of a chromatogram file showing the nucleotide sequence of a partial COX 2 gene fragment expressed in fathead minnow gill tissue (analysed without cloning). The quality of the peaks was indicated by the histograms (grey bars) above each DNA sequence peak and the quality ‘threshold’ (blue line). Generally, the first 20-50 sequenced bases showed low quality peaks. The arrow indicates where the quality of sequence peaks was deemed to be reliable. The direct sequencing method was used to confirm that the amplified DNA was the target.

2.2.9 Computational analysis of sequences

Nucleotide sequences were analysed for similarity to other COX gene sequences and annotation using the NCBI database. The nucleotide sequences were analysed in their sense (5’-3’) direction, and where the sequence product was in the anti-sense direction, the nucleotide sequence was reverse-complemented using an automated online tool (http://www.bioinformatics.org/sms/rev_comp.html).

The sequenced DNA products were queried against the sequences listed within the NCBI database using the BLAST tool to ensure the correct sequences had been amplified, and to help identify regions of similarity between the proposed fathead minnow COX gene sequences. Sequences were listed as significantly similar to the query sequence (proposed fathead minnow COX sequence); somewhat similar or dissimilar in rank order, starting with the most similar. Alongside each listed sequence, a ‘score (max and total)’ value was provided, that is the numerical value of the overall ‘quality’ of the alignment between the query sequence and the matched database sequence segment, a ‘query cover’ value, that is the query sequence length
that is included in the aligned segments, an ‘expect (E)’ value, which is the threshold for the number of matches (or ‘hits’) in the database that occur by chance (the lower the E value, or the closer it was to zero, the more significant the match) and an ‘identity’ value, that is the extent to which two or more sequences are related by comparison. All obtained sequences were aligned using the Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) pairwise or multiple sequence alignment tool. The nucleotide sequences were subsequently translated into their amino acid sequences using the ExPASy proteomics server of the Swiss Institute of Bioinformatics (www.expasy.ch/tools/dna.html) and searched in protein databases. The nucleotide sequence was translated into six possible reading frames; frame 1 was obtained the first translated nucleotide, frame 2 from the second and frame 3 from the third. Frames 4 to 6 were the same as frames 1 to 3, but in the reverse direction. The longest reading frame containing no stop codons was selected for further analysis. Protein sequences were analysed using BLAST and ‘InterPro’ (EMBL-EBI) (https://www.ebi.ac.uk/interpro/), an online bioinformatics tool used for the classification and annotation of proteins (Hunter et al., 2012). The protein sequences were also aligned against other COX protein sequences using Clustal Omega to identify regions of similarity and conserved sites.

2.2.10 Statistical analyses

The results are presented as mean ± standard deviation (mean ± SD) unless otherwise stated. Statistical analysis and graphs were created using GraphPad Prism 6 (GraphPad Software, Inc). Data were analysed for normality and variance of homogeneity (D'Agostino-Pearson normality test). Where assumptions of normality and homogeneity were met, statistical significance was tested using t-tests or one-way analysis of variance (ANOVA). If equal variance was not met, then a non-parametric test (Mann-Whitney test) was carried out (comparing the median). Statistical significance was set at a level of $p < 0.05$, unless otherwise indicated. Any variations in the statistical analyses conducted are covered in individual chapters.
CHAPTER 3: ESTABLISHING HUMAN THERAPEUTIC CONCENTRATIONS OF IBUPROFEN IN FATHEAD MINNOW BLOOD PLASMA
3.1 INTRODUCTION
The widespread detection of human pharmaceuticals in the aquatic environment has raised concerns over their potential impact on aquatic organisms. There is now a growing interest in the use of mammalian pharmacological (and toxicological) data to assess the effects of pharmaceuticals present in the environment in exposed non-target organisms (Huggett et al., 2003; Winter et al., 2010; Berninger and Brooks, 2010). The read-across hypothesis stipulates that it is possible to use the relationship between internal (blood plasma) concentrations of a given pharmaceutical in the intended target (i.e. human) and non-target (i.e. fish) to determine the risk of a pharmacological effect occurring in that organism (Huggett et al., 2003; Rand-Weaver et al., 2013). The human therapeutic plasma concentration (Cmax) is utilised as an anchor to establish the relationship between the internal concentrations in the exposed non-target organism. In humans, the therapeutic concentrations of ibuprofen in the blood plasma (following a normal therapeutic dose of 400 mg) can range between 15,000-30,000 µg/L (Schulz et al., 2012), and therefore this range will be used as the reference range in this study.

There are a limited number of studies where the exposure water, and internal concentrations of ibuprofen (Brown et al., 2007; Fick et al., 2010; Nallani et al., 2011) and other pharmaceuticals (for example, Cuklev et al., 2011, 2012, Giltrow et al., 2009; Lahti et al., 2011; Owen et al., 2009; Valenti et al., 2012), have been analytically measured in aquatic organisms. However, as it is not feasible to measure the internal concentrations of every single one of the 3,000 pharmaceuticals that are licenced for use (Fent et al., 2006), predictive modelling may provide an alternative approach. The theoretical FPM (Huggett et al., 2003) may be used to estimate the “fish steady-state plasma concentration” (FSSPC) of a pharmaceutical from a given exposure water concentration (Huggett et al., 2003) (refer to Chapter 1, Section 1.3.1.2 for further details). The FPM was used to predict the uptake of ibuprofen into fish blood, and the accuracy of the model was evaluated by comparing the estimated and experimental values, in order to validate the use of the FPM as a potential tool for estimating internal concentrations of pharmaceuticals in fish. The factors that can influence the uptake of a drug from the surrounding water into the blood plasma
compartment are largely determined by its pharmacokinetic properties, such as the lipophilicity (Log \( K_{\text{ow}} \)). Ibuprofen has a relatively high Log \( K_{\text{ow}} \) of 3.80 (Brown et al., 2007) and in field studies, ibuprofen has been shown to bioconcentrate into rainbow trout blood plasma by up to 18,667-fold from environmentally relevant effluent concentrations (0.0045 \( \mu \)g/L) (Brown et al., 2007). However, in one laboratory exposure study, conducted using higher exposure concentrations (i.e. 250 \( \mu \)g/L), ibuprofen was found to only weakly bioconcentrate (by 1.4 fold) into the blood plasma of channel catfish (*Ictalurus punctatus*) (Nallani et al., 2011). The results from these reported studies suggest that variation exists in the uptake of ibuprofen.

The first aim of this study was to determine whether or not ibuprofen could bioconcentrate into fathead minnow blood plasma and reach human therapeutic plasma concentrations (Cmax), and to establish the exposure water concentration of ibuprofen at which the Cmax is reached (if possible) in fish blood plasma. The second aim is to evaluate the accuracy of the FPM.

### 3.2 METHODS

A brief overview of the exposure details used in this chapter are provided below, full details of the materials and methods can be found in Chapter 2.

#### 3.2.1 \( \leq 96 \) hour flow-through exposure

To determine whether or not therapeutic concentrations of ibuprofen could be established in fathead minnow blood plasma, fish were exposed (in a range-finder study) to four exposure concentrations of ibuprofen; 100, 270, 370 and 500 \( \mu \)g/L for \( \leq 96 \) hours, using continuous flow-through systems (Chapter 2, Section 2.1.5, Table 6; ‘Exposure 2’ and ‘Exposure 3’). In the first study, adult male fathead minnows (n=64) were exposed to DWC, SC (acetone) and nominal ibuprofen exposure water concentrations of 100 and 500 \( \mu \)g/L (n=16 per treatment) (Chapter 2, Section 2.1.5, Table 6; ‘Exposure 2’). In a subsequent follow-up study, adult male fathead minnows (n=100) were exposed to DWC, SC and nominal ibuprofen exposure water concentrations of 270 and 370 \( \mu \)g/L (n=25 per treatment, including depuration) (Chapter 2, Section 2.1.5, Table 6; ‘Exposure 3’). Prior to the exposure period, fish
were randomly distributed into 45 L tanks. All exposure conditions were maintained as described in Chapter 2, Section 2.1.6.3. The fish were not fed throughout the exposure period (except in ‘Exposure 3’ see below).

3.2.2 Depuration in clean water (‘Exposure 3’ only)

Following the 96 hour exposure period, the remaining fish that had been exposed to DWC, SC, 270 and 370 µg/L (n=9 per treatment) were fed once with food pellet and brine shrimp (*Artemia sp.*). Two hours after feeding, the fish were transferred into tanks containing dilution water only for ≤72 hours to allow clearance of the test substance/depuration.

3.2.3 Fish blood plasma & tissue sampling

After the exposure and depuration period, all fish were terminated and blood plasma and tissues (gill, liver and brain) were collected and stored according to the method described in Chapter 2, Section 2.1.7. Fish exposed to DWC, SC and ibuprofen concentrations of 100 and 500 µg/L were sampled after 3, 24, 48 and 96 hours (n=4 fish at each time point) and fish exposed to DWC, SC and ibuprofen concentrations of 270 and 370 µg/L were sampled after 24, 48, 72 and 96 hours (n=4 fish at each time point). Depurated fish were sampled after 24 (n=4) and 72 hours (n=5). The weight and length measurements were used to calculate the condition factor (K) (Equation 3). The liver (and body) weights were used to calculate the hepatosomatic index (HSI) (Equation 4).

3.2.3.1 Condition factor (K)

Fulton's condition factor (K) is indicative of the overall well-being of fish. K was calculated using the fish body weight (W) (in g) and standard length (L) (in cm) and a factor of 100 to bring K closer to unity, using Equation 3 (Froese, 2006):

\[
K = \frac{W}{L} \times 100
\]

*Equation 3. Fulton's condition factor (K).*
3.2.3.2 Hepatosomatic Index (HSI)

The hepatosomatic index (HSI) was calculated based on the relationship between the liver weight (in g) to whole body wet weight (in g) using Equation 4:

\[ \text{HSI} = \frac{\text{Liver weight (g)}}{\text{Fish weight (g)}} \times 100 \]

Equation 4. Hepatosomatic index (HSI).

3.2.4 Exposure water sampling

Water samples (5 mL) were collected from the DWC, SC and 100, 270, 370 and 500 μg/L ibuprofen exposure tanks prior to Day 0 (-72 hours) and subsequently after 3, 24, 48, 96 hours (100 and 500 μg/L) and 24, 48, 72, 96 hours (270 and 370 μg/L) following exposure. Water samples were collected from the depuration tanks containing dilution water before fish were introduced. The exposure waters were collected at the same time each day, or just before fish were sampled.

3.2.5 Quantification of ibuprofen in exposure waters and blood plasma

The concentration of ibuprofen in exposure waters and control and ibuprofen-treated fish blood plasmas were analysed (two analytical measurements per sample) using the LC-MS/MS method, as described in Chapter 2, Section 2.1.9.

3.2.6 Estimating plasma ibuprofen concentrations using the Fish Plasma Model (FPM)

The FPM model can be used to predict the FSSPC of a pharmaceutical compound from the surrounding water. To evaluate the accuracy of the model, the uptake of ibuprofen into fathead minnow blood plasma was modelled using the FPM and compared to the experimentally measured values. The partitioning of ibuprofen between the aqueous phase and the arterial blood (\( \log \text{P}_{\text{blood:water}} \)) in fathead minnows was estimated using Equation 1 (see Chapter 1, Section 1.3.1.2). The \( \log \text{P}_{\text{blood:water}} \) is usually determined using the \( \log \text{K}_{\text{ow}} \) (Fitzsimmons et al., 2001), however, as pharmaceuticals are amenable to ionisation at different pH’s, including
the pH of blood plasma, the FPM was also used to estimate uptake using the
distribution coefficient Log D, measured at physiological pH (7.4). The Log
\( P_{\text{blood:water}} \) was used to derive the \( F_{\text{SSPC}} \) using Equation 2 (see Chapter 1, Section
1.3.1.2). MEC refers to the measured environmental (exposure water) concentration
of ibuprofen i.e. 100, 270, 370 and 500 \( \mu \text{g/L} \).

### 3.2.7 Statistical analysis

The results are presented as mean ± SD unless otherwise stated. Data collected were
analysed for normality and variance of homogeneity (D'Agostino-Pearson normality
test). Where assumptions of normality and homogeneity were met, statistical
significance of treatment effects on standard length (mm), body weight (g), K and
HSI between control and ibuprofen-treated groups over 96 hours was tested using
one-way analysis of variance (ANOVA) followed by a multiple comparison test
(Holm-Sidak or Tukey's test). Statistical significance was set at a level of \( p < 0.05 \).
3.3 RESULTS

3.3.1 Water concentrations of ibuprofen

To determine the actual exposure concentrations, in the continuous flow-through system, the water concentration of ibuprofen in each exposure tank was quantified over 96 hours and compared to the nominal test concentrations (Table 10).

Table 10. Measured ibuprofen (µg/L) in tank waters over the 96 hour exposure period. Ibuprofen measurements in the waters of the DWC and SC tanks were below the LOD (<2.5 µg/L).

<table>
<thead>
<tr>
<th>Nominal exposure concentration µg/L</th>
<th>No. of exposure hours</th>
<th>Measured water concentration µg/L</th>
<th>% Nominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>3</td>
<td>107</td>
<td>107</td>
</tr>
<tr>
<td>100</td>
<td>24</td>
<td>107</td>
<td>107</td>
</tr>
<tr>
<td>100</td>
<td>48</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td>100</td>
<td>96</td>
<td>102</td>
<td>102</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>105 ± 2</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>270</td>
<td>24</td>
<td>285</td>
<td>105</td>
</tr>
<tr>
<td>270</td>
<td>48</td>
<td>179</td>
<td>66</td>
</tr>
<tr>
<td>270</td>
<td>72</td>
<td>343</td>
<td>127</td>
</tr>
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<td>270</td>
<td>96</td>
<td>307</td>
<td>114</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>278 ± 70</td>
<td>103</td>
<td></td>
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<tr>
<td>370</td>
<td>24</td>
<td>422</td>
<td>114</td>
</tr>
<tr>
<td>370</td>
<td>48</td>
<td>416</td>
<td>113</td>
</tr>
<tr>
<td>370</td>
<td>72</td>
<td>428</td>
<td>116</td>
</tr>
<tr>
<td>370</td>
<td>96</td>
<td>371</td>
<td>100</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>409 ± 26</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>539</td>
<td>108</td>
</tr>
<tr>
<td>500</td>
<td>24</td>
<td>419</td>
<td>84</td>
</tr>
<tr>
<td>500</td>
<td>72</td>
<td>518</td>
<td>104</td>
</tr>
<tr>
<td>500</td>
<td>96</td>
<td>532</td>
<td>106</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>502 ± 56</td>
<td>103</td>
<td></td>
</tr>
</tbody>
</table>

The mean measured (± SD) water concentrations of ibuprofen over 96 hours were 105 ± 2, 278 ± 70, 409 ± 26 and 502 ± 56 µg/L in the 100, 270, 370 and 500 µg/L exposure tanks, respectively. The mean measured ibuprofen concentration (over 96 hours) was significantly different in each exposure tank ($p < 0.05$, using one-way
ANOVA). Overall, the time-weighted average ibuprofen concentration in water samples were 103, 103, 111 and 103% of the nominal exposure concentrations, respectively. The concentrations of ibuprofen in water across all exposure hours were within an acceptable range (± 20% of the expected ‘nominal’ concentration), with the exception of the 270 µg/L exposure tank. The measured concentrations at 48 and 72 hours were outside the expected range, and were 66% and 127% of the nominal, respectively. This fluctuation in the measured concentrations was accounted for by a build-up of test substance at the end of dosing line. After the issue had been rectified, normal dosing was resumed and the ibuprofen concentration in the tank was within the expected range by 96 hours. During the exposure period, ibuprofen measurements in the water samples collected from the DWC and SC tanks were below the LOD of 2.5 µg/L, which was determined to be the lowest acceptable standard concentration for which ibuprofen could be reliably measured in water with the analytical method used. This was therefore interpreted as no ibuprofen being present in these tanks. After the exposure period, fish that had been exposed to nominal concentrations of 270 and 370 µg/L for 96 hours were placed into depuration tanks, containing dilution water only, for either 24 or 72 hours. In order to confirm that no ibuprofen was present in these tanks, water samples were measured prior to the introduction of fish, and these measurements were also found to be below the LOD of 2.5 µg/L, indicating that there was no contamination of the depuration tanks with ibuprofen.

For simplicity, the nominal concentrations were used for the presentations of results in this chapter, unless otherwise stated. Graphical representation of the measured water concentrations before (-72 hours) and during the 96 hour exposure period following exposure to water concentrations of 100, 270, 370 and 500 µg/L are shown in Figure 20. Measured water concentrations were stable at 100 and 370 µg/L over the exposure period, whereas fluctuations were seen at 270 and 500 µg/L. However, only two measurements reported in the 270 µg/L exposure tank were outside of the expected range (± 20% of the nominal). Ibuprofen measurements were taken daily over the exposure period, or just before fish were sampled, to ensure that fish were exposed to consistent water concentrations of the drug throughout the exposure.
Figure 20. Measured ibuprofen concentrations (µg/L) in exposure waters before (-72 hours) and during the 96 hour exposure period. Water in the exposure tanks were sampled after 3 (100 and 500 µg/L only), 24, 48, 72 (270 and 370 µg/L only) and 96 hours of exposure. Ibuprofen measurements in the waters of the control (DWC and SC) tanks were below the LOD (<2.5 µg/L). The asterisks highlight the exposure concentrations outside of the ‘acceptable’ range (± 20% of the nominal).

3.3.2 Fish plasma concentrations of ibuprofen

3.3.2.1 Fish plasma ibuprofen and human therapeutic concentrations

Ibuprofen concentrations were quantified in blood plasmas to allow assessment of the uptake of ibuprofen from the exposure waters into fathead minnows, and to determine whether internal concentrations in fish similar to human therapeutic plasma concentrations (Cmax) of 15,000-30,000 µg/L could be established. Fish were exposed to water concentrations of 100, 270, 370 and 500 µg/L ibuprofen for ≤96 hours. Fish were firstly exposed to DWC, SC, 100 and 500 µg/L for 3-96 hours, and in a follow-up study, fish were exposed to DWC, SC, 270 and 370 µg/L for 24-96 hours. Blood plasma samples collected from fish exposed to 100 µg/L (3-96 hours) and 500 µg/L (3-72 hours) were pooled (4 fish at each time point for each concentration) for ibuprofen measurement, in order to establish whether the plasma ibuprofen concentrations were in the human therapeutic range. The 96 hour samples from the fish in the 500 µg/L exposure tank were used to determine whether it was
possible to measure ibuprofen in individual plasma samples, and subsequently, all samples from the 270 and 370 μg/L exposure tanks (24-96 hours) were analysed individually. Plasma samples collected from fish in control (DWC and SC) tanks were pooled as ibuprofen was not expected in these fish.

The mean plasma ibuprofen concentration in fish exposed to 100, 270, 370 and 500 μg/L ibuprofen over 3-96 hours is shown in Figure 21. The uptake of ibuprofen into fish was rapid, and following three hours of exposure (the earliest time point examined) to the lowest and highest water concentrations (i.e. 100 and 500 μg/L, respectively) the measured plasma ibuprofen concentrations were 8 and 111-fold higher than their measured water concentrations (Table 11). The increasing plasma concentration in fish exposed to 270 μg/L ibuprofen between 72-96 hours suggests that the plasma concentrations had not stabilised, and therefore it is possible that beyond the 96 hours of exposure, the plasma ibuprofen concentrations in these fish may have continued to increase. However, due to Home Office regulations, it was not possible to continue the exposure after 96 hours, without feeding the fish (this was avoided to facilitate fish tissue sampling). In relation to the human therapeutic range (i.e. 15,000-30,000 μg/L), the mean blood plasma concentration in fish exposed to 100 and 500 μg/L (over 96 hours) was 660 (±180) μg/L and 106,000 (±71,290) μg/L, respectively. Therefore, these two water concentrations resulted in plasma ibuprofen concentrations that were either too low (23-fold below the lowest Cmax value of 15,000 μg/L) or too high (3.5-fold above the highest Cmax value of 30,000 μg/L) to attain human therapeutic concentrations (Figure 21 and Figure 22 A & D). The mean blood plasma concentration in fish exposed to 270 and 370 μg/L (over 96 hours) was 14,409 (±22,084) μg/L and 40,504 (±36,100) μg/L, respectively. Therefore, these intermediate water concentrations produced plasma concentrations in fish that were within, or similar to, the Cmax (Figure 21 and Figure 22 B & C), indicating that human therapeutic concentrations can be established in fathead minnows. Ibuprofen concentration in the control fish were below the LOD of 62.5 μg/L (Figure 21 and Figure 22), which was determined to be the lowest acceptable standard concentration for which ibuprofen could be reliably measured in blood plasmas with the analytical method used. This indicated that there was no contamination of the control tanks with ibuprofen.
Figure 21. Plasma ibuprofen (µg/L) in fathead minnows exposed to 100, 270, 370 and 500 µg/L over 96 hours. Blood plasmas were sampled after 3 (100 and 500 µg/L only), 24, 48, 72 (270 and 370 µg/L only) and 96 hours of exposure. Plasma samples collected from fish exposed to 100 µg/L (3–96 hours) and 500 µg/L (3–72 hours) were pooled (n=1). The mean (±SD) of individual fish (n=3-4 at each time point) exposed to 270, 370 and 500 µg/L (at 96 hours only) is shown. Ibuprofen measurements in the plasmas of fish in the control (DWC and SC) tanks were below the LOD (<62.5 µg/L). Cmax denotes the human therapeutic plasma concentrations of ibuprofen.
Figure 22. Plasma ibuprofen (µg/L) in fathead minnows after 3-96 hours of exposure to nominal water concentrations of 100 (A), 270 (B), 370 (C) and 500 (D) µg/L. Plasma samples collected from fish exposed to 100 µg/L (A) after 3-96 hours and 500 µg/L (D) after 3-72 hours were pooled (n=1). The mean (±SD) is shown for plasma collected from individual fish (n=3-4 at each time point) exposed to 270 (B) and 370 (C) µg/L after 24-96 hours and 500 µg/L (D) at 96 hours only. Cmax denotes the human therapeutic plasma concentrations of ibuprofen. Ibuprofen measurements in the plasmas of control fish were below the LOD (<62.5 µg/L).
3.3.2.2 Inter-individual variability in plasma ibuprofen concentrations and depuration

As previously stated, ibuprofen was successfully quantified in individual fish (n=4) exposed to 500 µg/L at 96 hours, and in fish exposed to 270 and 370 µg/L for 24-96 hours (n=3-4 at each time point), to allow assessment of the inter-individual variation in drug plasma concentrations, and its relationship with biological effects (see Chapter 7).

The mean plasma ibuprofen concentrations in fish exposed to 500 µg/L at 96 hours was 179,739 (± 126,138) µg/L but the lowest and highest measured plasma concentrations ranged between 60,940 and 352,903 µg/L, demonstrating the variability in plasma ibuprofen concentrations in fish, exposed to the same tank, for the same length of time. To further dissect the observed variation in plasma concentrations, ibuprofen was measured in individual fish after 24, 48, 72 and 96 hours (n=3-4 at each time point) of exposure to 270 and 370 µg/L (Figure 23). The difference between the lowest and highest measured plasma concentrations in fish exposed to 270 µg/L after 48 and 72 hours was below 10-fold, indicating similar plasma concentrations in fish at these two time points. However, there was a 275-fold difference between the lowest and highest measured plasma concentrations in fish in the 270 µg/L exposure tank after 96 hours, suggesting greater variability in uptake, metabolism or excretion with increasing exposure length. Surprisingly, only one fish in this exposure tank (at 96 hours) had a plasma concentration that was within the Cmax, suggesting that the mean plasma concentration can be misleading. This is clearly exemplified by the plasma concentrations measured in individual fish in the 370 µg/L exposure tank after 96 hours, as none of these exposed fish had a plasma concentration within the therapeutic range. At this exposure time point, n=3 fish had a plasma concentration that was determined to be ≤12,500 µg/L. It was not possible to accurately determine the plasma concentrations due to over-dilution of the samples; and therefore a value of 6,250 µg/L, representing 50% of the calculated value was assigned as a conservative estimate of ibuprofen in these samples.

To determine the removal rate of ibuprofen, fish were placed into depuration tanks containing dilution water only for 24 (n=4) or 72 (n=5) hours. Ibuprofen was rapidly
eliminated from fish and within 24 hours, blood plasma concentrations were at, or close to the LOD (<62.5 µg/L) (Figure 23) although one fish in the 370 µg/L exposure tank had a plasma concentration 4-fold higher than the LOD, indicating variability in fish metabolism and/or excretion.
Figure 23. Variation in plasma ibuprofen (µg/L) in individual fish (n=3-4) exposed to 270 and 370 µg/L over 24-96 hours. After 96 hours of exposure, fish were fed and after two hours were placed into depuration tanks containing dilution water for 24 (n=4) or 72 (n=5) hours. Ibuprofen measurements in the plasmas of control fish were below the LOD (<62.5 µg/L). Cmax denotes the human therapeutic plasma concentrations. Brackets denote number of fish with the same concentration.
3.3.2.3 Bioconcentration of ibuprofen

The plasma concentrations of ibuprofen exceeded the exposure water concentrations, demonstrating that ibuprofen is able to bioconcentrate in fish. The proportional bioconcentration factor (BCF) of ibuprofen was determined using the measured blood plasma concentrations (at each exposure hour) over the measured water concentration. Measured plasma concentrations were between 5-8, 7-158, 7-325 and 76-338-fold higher than the measured water concentrations in the 100, 270, 370 and 500 µg/L exposure tanks, respectively, over 96 hours (Table 11). The BCF values indicate that ibuprofen can strongly bioconcentrate in fathead minnow blood plasma.

Table 11. Ibuprofen plasma concentrations in fathead minnows exposed to water concentrations of 100, 270, 370 and 500 µg/L over 3-96 hours. The bioconcentration factor (BCF) was calculated using the mean measured water concentration in the exposure tanks and mean measured (mean ± SD) blood plasma concentrations in individual exposed fish (n=3-4), except at 100 µg/L after 3-96 hours and 500 µg/L after 3-72 hours where plasma samples were pooled (n=1).

<table>
<thead>
<tr>
<th>Nominal Conc. µg/L</th>
<th>No. of exposure hours</th>
<th>Measured water concentration µg/L</th>
<th>Measured plasma concentration µg/L (n)</th>
<th>BCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>3</td>
<td>107</td>
<td>897 (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>107</td>
<td>550 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>105</td>
<td>697 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>102</td>
<td>485 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>105 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>270</td>
<td>24</td>
<td>285</td>
<td>2000 ± 1732 (3)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>179</td>
<td>2620 ± 1170 (3)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>343</td>
<td>4429 ± 2555 (4)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>307</td>
<td>48590 ± 82,880 (4)</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>278 ± 70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>370</td>
<td>24</td>
<td>422</td>
<td>2944 ± 1386 (4)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>416</td>
<td>2942 ± 1594 (4)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>428</td>
<td>139,104 ± 121,065 (4)</td>
<td>325</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>371</td>
<td>17,170 ± 20,362 (4)</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>409 ± 26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>539</td>
<td>59,935 (111)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>419</td>
<td>31,800 (76)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>518</td>
<td>152,714 (295)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>532</td>
<td>179,739 ± 126,138 (4)</td>
<td>338</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>502 ± 56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.3 Accuracy of the Fish Plasma Model (FPM)

The accuracy of the FPM for estimating the $F_{SSPC}$ of ibuprofen in fathead minnow blood plasma was evaluated. The $F_{SSPC}$ was calculated using $\log K_{ow}$ of 3.80 (Brown et al., 2007) and $\log D_{7.4}$ of 0.8 as predicted using the Advanced Chemistry Development/PhysChem Suite (ACD, 2006) to enable comparison of the most suitable constant for estimating ibuprofen uptake. The FPM (calculated using $\log K_{ow}$) over-estimated the $F_{SSPC}$ of ibuprofen by 12-fold in fish exposed to 100 µg/L and under-estimated the $F_{SSPC}$ by 3-fold in fish exposed to 500 µg/L over 96 hours, compared to the measured concentrations (Figure 24). The FPM accurately estimated the $F_{SSPC}$ in fish exposed to 270 and 370 µg/L (after 96 hours). The $F_{SSPC}$ calculated using the $\log D$ (pH 7.4) severely under-estimated the uptake of ibuprofen across all water concentrations, suggesting that this was not a suitable constant. These results suggest that the FPM can be used to accurately determine ibuprofen concentrations in fathead blood plasma, and identify the exposure water concentration required to establish the Cmax of ibuprofen in blood plasma.
Figure 24. Relationship between measured plasma concentration and predicted $F_{SS\,PC}$ of ibuprofen in fathead minnows exposed to mean measured ($\pm$SD) water concentrations of 105 ($\pm$ 2), 278 ($\pm$ 70), 409 ($\pm$ 26) and 502 ($\pm$ 56) µg/L over 96 hours. The $F_{SS\,PC}$ was calculated using Log $K_{ow}$ and Log $D_{7.4}$. The predicted $F_{SS\,PC}$ values were based on the equation described by Fitzsimmons et al., (2001) and the FPM proposed by Huggett et al., (2003). $F_{SS\,PC}$ = ‘fish steady-state plasma concentration. $C_{max}$ denotes the human therapeutic plasma concentrations of ibuprofen.
3.3.4 Physiological responses to ibuprofen exposure

3.3.4.1 Fish mortality

During both 96 hour exposure studies, there were no fish mortalities. It was expected that the highest exposure concentration used (500 µg/L) would not adversely affect fish, based on the 96-hour LC50 of ibuprofen reported in bluegill sunfish (*Lepomis macrochirus*) (173 mg/L) (Halling-Sorensen et al., 1998).

3.3.4.2 Condition factor (K) and Hepatosomatic index (HSI)

The mean standard length (mm), body weight (g), K and HSI in fish in the control (DWC and SC) tanks and fish exposed to 100, 270, 370 and 500 µg/L of ibuprofen over 96 hours is shown in Figure 25. The standard length, body weight and K, which is an indicator of the overall well-being of fish, did not differ significantly (*p* > 0.05, using one-way ANOVA) between the fish in the exposure tanks and the fish in the control (DWC or SC) tanks (n=16 per treatment) (Figure 25 A-F) or the depurated fish (n=9 per treatment) (data not shown). The K value can highlight a fish’s ability to adapt to drug exposure, and the lack of response in exposed fish indicates that the exposure levels and duration did not induce obvious chemical stress in the fish. The physiological status was also assessed using somatic indices, such as the HSI, which also did not differ significantly (*p* > 0.05, using one-way ANOVA) between the fish in the exposure tanks and the fish in the control (DWC or SC) tanks (n=16 per treatment) (Figure 25 G-H) or the depurated fish (n=9 per treatment) (data not shown). No solvent effects were observed.
Figure 25. Standard length (mm), weight (g), condition factor (K) and hepatosomatic index (HSI) in control (DWC and SC) fish and ibuprofen-treated fathead minnows. Fish were exposed to water ibuprofen concentrations of 100 and 500 µg/L (A, C, E and G, respectively) and 270 and 370 µg/L (B, D, F and H, respectively) over 96 hours. Boxes represent mean (full line) with 25th and 75th percentile. The bars extend to the 10th and 90th percentiles, with outliers represented as dots. The sample size per treatment group (n=16).
3.3.4.3 Blood haematocrit

The major side effects of ibuprofen use and NSAID therapy in patients are related to common haematological disorders, such as anaemia. To examine whether there were any changes in blood parameters in fathead minnows following ibuprofen exposure, the haematocrit was assessed, which is a measure of the amount of total blood (mm) that is occupied by red blood cells. In this study, the haematocrit (%) did not differ significantly ($p > 0.05$, using one-way ANOVA) between fish in the ibuprofen exposure tanks, and the fish in the control (DWC or SC) tanks (n=14-16 per treatment) (Figure 26). These findings suggest that the exposure water concentrations used in this study did not induce haematological changes in fathead minnows.

![Figure 26. Haematocrit (%) values for fathead minnow in control (DWC and SC) tanks and in fish exposed to 270 and 370 µg ibuprofen/L over 96 hours. Boxes represent mean (full line) with 25th and 75th percentile. The bars extend to the 10th and 90th percentiles, with outliers represented as dots. The sample size per treatment group (n=14-16).]
3.4 DISCUSSION

The aim of this chapter was to establish if human therapeutic concentrations of ibuprofen could be reached in fathead minnow blood plasma, and to identify the exposure water concentration (and duration of exposure) required to reach these concentrations. Mean ibuprofen concentrations in fathead minnow blood plasmas similar to the human therapeutic range (Cmax) (15,000-30,000 µg/L) (Schulz et al., 2012) were established in fish that had been exposed to water concentrations of 270 and 370 µg/L for 96 hours.

3.4.1 Blood plasma concentrations of ibuprofen in fathead minnows

Measurements of the ibuprofen concentration in the exposure tanks confirmed that fish were exposed to water concentrations close to the nominal, therefore validating the flow-through drug delivery system used in this study. A measure of the exposure water concentration, over regular intervals, is imperative in order to determine whether stable concentrations of a drug can be maintained, since fluctuations, for example from drug solubility (Hutchinson et al., 2006), can potentially result in the inaccurate assessment of drug bioconcentration, and/or the potential risk to aquatic organisms. However, there are many difficulties in both measuring, and maintaining stable exposure concentrations using dosing systems, highlighting the complexity of recreating realistic exposure scenarios for fish.

The uptake of ibuprofen into fish was rapid, and ibuprofen accumulated in fathead minnows at all tested water concentrations. The pharmacokinetic properties of a pharmaceutical can facilitate its partitioning into the plasma compartment of blood, and ibuprofen has a relatively high Log Kow (3.80) (Brown et al., 2007) indicating that it is lipophilic enough to bioconcentrate. There was a high level of variability in the reported BCF values in this study. The measured ibuprofen concentrations in fish plasmas ranged between 5-8, 7-158, 7-325 and 76-338-fold higher than the measured water concentrations in the 100, 270, 370 and 500 µg/L exposure tanks, respectively, over 96 hours. These findings demonstrate that the plasma concentrations of ibuprofen were highly variable, and at higher exposure concentrations, there was more variability in plasma concentrations, which could have been attributed to changes in ibuprofen uptake (absorption through the gills and/or skin), metabolism
and/or elimination of the drug within fish. The BCF values reported for ibuprofen in channel catfish plasma after 7-day exposure to similar measured water concentrations (314 ±55 µg/L) (Nallani et al., 2011) was much lower (1.4-fold), indicating that, in contrast to the findings in this study, ibuprofen did not bioconcentrate above the exposure water concentrations. However, physiological differences between the species may have been a reason for the low bioconcentration values, as catfish have a higher lipid content possibly resulting in wider distribution of drug than in fathead minnows.

Fathead minnows that had been exposed to ibuprofen water concentrations of 270 (n=4) and 370 (n=4) µg/L over 96 hours had mean blood plasma concentrations that were within or similar to the human therapeutic range. However, upon closer examination, it was apparent that only one fish in the 270 µg/L exposure tank had a plasma concentration within the Cmax, and in the 370 µg/L tank, none of the fish had a plasma concentration within the Cmax. These findings demonstrate the high level of variability in ibuprofen plasma concentrations, and its potential implication in the risk assessment of pharmaceuticals to fish.

Fathead minnows exposed to 100 and 500 µg/L ibuprofen had plasma concentrations that were below or above the Cmax, respectively. Fish in the 500 µg/L exposure tank had plasma concentrations that consistently exceeded the Cmax over the exposure period and the mean plasma concentration in these fish (106,000 µg/L) was determined to be half of the reported plasma concentration defined as being ‘toxic’ in humans (200,000 µg/L) (Schulz et al., 2012). The exposure concentrations used in this study reflect those that are used to produce pharmacological or MoA-related effects in fish, rather than those associated with toxicity. Hence, there were no adverse symptoms or signs of toxicity observed in fish with these plasma concentrations (or any other tested water concentration), which was confirmed by the lack of physiological effects, indicative of chemical stress (as assessed by K and HSI in fish), in ibuprofen-exposed fish compared to the control groups (using one-way ANOVA).

The human therapeutic plasma concentrations (Cmax) range is fundamental to the read-across hypothesis, as the risk for a pharmacological (or toxicological) effect to
occur in the non-target species (i.e. fish) is predicted based on the relationship between internal blood plasma concentrations in the intended target, humans, and in fish. For most pharmaceuticals, the Cmax values are determined during the drug development phase (Huggett et al., 2003). The Cmax range of ibuprofen, 15,000-30,000 µg/L reported by Schulz et al., (2012) (following a normal 400 mg dose) has been compiled from clinical trials and toxicology reports (Forsyth et al., 1988; Dewland et al., 2009), however, other reported ibuprofen Cmax values range between 6,800-10,000 µg/L (Mehlisch and Sykes, 2013) and 10,000-50,000 µg/L (Regenthal et al., 1999). These findings suggest that the “therapeutic window” for ibuprofen is relatively high in patients, and the difference between the minimum and maximum plasma concentrations at which analgesic effects occur in humans, is most likely due to individual sensitivity.

The quantification of ibuprofen in individual fish revealed that there was a high level of inter-individual variability in plasma ibuprofen concentrations in fish. However, the results from the depuration phase showed that some fish that had been in dilution water for 72 hours, had plasma ibuprofen concentrations that were above the LOD, which suggests variability in fish metabolism and/or excretion, as well as uptake. In humans and mammals, ibuprofen is primarily metabolised in the liver by the cytochrome P450 (CYP) CYP2C8 and CYP2C9 enzymes, which contribute to the formation of hydroxy metabolites (Rainsford, 2009). Although no orthologs to these CYP isoforms have been identified in fish, 2-hydroxy ibuprofen has been identified as a major metabolite in fish (Jones et al., 2012; Gomez et al., 2011), suggesting that fish may also be able to metabolise ibuprofen in a similar manner to mammals. The variability in drug plasma concentrations and differences in drug responses between individuals has been linked to genetic polymorphisms in the genes coding for CYP2C8 and CYP2C9 enzymes (García-Martín et al., 2004), which may also be a contributing factor to the variability in drug (ibuprofen) metabolism in fish.
3.4.2 Validation of the Fish Plasma Model (FPM)

The second aim of this chapter was to evaluate the predictive FPM. Variations in analytical methods, instrumentation and cost implications can result in non-standardised quantification of pharmaceuticals, and therefore predictive modelling can provide an alternative method for estimating internal concentrations of compounds. The FPM has been proposed as one method for estimating the FSSPC of a pharmaceutical from the surrounding water, which is a prerequisite for determining the risk for a pharmacological (or toxicological response) to occur within an organism. We compared the predicted FSSPC and measured plasma concentration for ibuprofen in fathead minnows. The estimated FSSPCs were similar to the measured plasma concentration after 96 hour exposure to water concentrations of 270 and 370 µg/L. Therefore the FPM was highly accurate at predicting the plasma concentration of ibuprofen at these concentrations. These data suggest that it is possible to use the FPM to identify water concentrations of pharmaceuticals that are able to produce plasma concentrations of a drug similar to the Cmax range, which is a requirement of the read-across hypothesis. These findings are in agreement with other studies that have used the FPM to accurately (within an order of magnitude) predict the plasma concentrations of human pharmaceuticals in fish (Fick et al., 2010; Valenti et al., 2012).

The main parameters that drive the uptake of a pharmaceutical compound into fish are the Log K_{ow}, which is a measure of lipophilicity and Log D_{7.4} at physiological pH. The FSSPC calculated using Log K_{ow} was more accurate at predicting ibuprofen plasma concentrations than Log D_{7.4}, which under-estimated the plasma concentrations at all exposure concentrations. However, the accuracy of the FPM is linked to the input of the constant data used. Therefore misinterpretation of, or potential inaccuracies of the model will be reflected by the input data, for example the Log K_{ow} of ibuprofen can range between 3.80 (Brown et al., 2007) and 3.97 (Avdeef et al., 1998). The experimental value for the Log D_{7.4} of ibuprofen is not available in the literature and therefore a predicted value (0.8) (ACD, 2006) has been used, however, this value has not been experimentally verified.
Furthermore, the FPM does not take into account active excretion, metabolism, or drug plasma protein binding kinetics in fish, and therefore other possible sources of misinterpretation of the model may arise from differences in these pharmacokinetic properties. For example, the presence of steroid hormone binding globulins (plasma glycoproteins) in teleost fish gills (Miguel-Queralt and Hammond, 2008), may increase the uptake rate of steroid pharmaceuticals, suggesting that further experimental studies that consider all factors are required.

In summary, human therapeutic concentrations of ibuprofen can be reached in fathead minnow blood plasma after 96 hours of exposure to water concentrations of 270 and 370 µg/L. However, large inter-individual variation in plasma concentrations were observed.
CHAPTER 4: IDENTIFYING THE 
CYCLOOXYGENASE (COX) GENE IN THE 
FATHEAD MINNOW
4.1 INTRODUCTION

Pharmaceuticals have highly specific modes of action (MoA) that arise from their interactions with specific proteins (i.e. enzymes/receptors) (Christen et al., 2010). Advances in molecular biology, including DNA sequencing, has enabled the genomes of several different organisms to be characterised, and online bioinformatics resources such as the NCBI database, which contains thousands of nucleotide and protein sequences, has allowed human orthologs and conserved proteins in other species to be identified. The powers of cross-species sequence (in silico) analysis has already been demonstrated in one study by Gunnarsson et al., (2008), where a number of conserved human proteins were identified across sixteen different species including aquatic vertebrates, suggesting that aquatic organisms may also be potentially affected by pharmaceuticals present in the environment (Ankley et al., 2007; Huggett et al., 2003; Seiler, 2002). As pharmaceuticals mediate targeted effects based on their MoA (rather than non-specific effects), the presence of a conserved target in a species could be used as a first step in identifying whether the MoA is conserved. MoA-specific biological effects similar to those seen in humans have been identified in fish. For example, synthetic oestrogens (such as EE2) can interact with the oestrogen receptor (Jobling et al., 1996) and progestins (such as Levonorgestrel) can interact with the progesterone receptor (Runnalls et al., 2013) to regulate reproductive functions in fish, and β-blockers, such propranolol may act through beta-adrenergic receptors to alter cardiovascular functions (Owen et al., 2007, 2009). These findings suggest that the effects elicited by human drugs are most likely to be mediated through specific proteins (Owen et al., 2007). The evolutionary conservation of specific human proteins in a given species could be used to link the MoA of a particular pharmaceutical to the potential physiological or biological effects that are likely to be seen, and identify those organisms that are most sensitive to their effects following drug exposure (Ankley et al., 2007).

In all studied mammals (including human, sheep, mouse) there are two isoforms of the COX enzyme (1 and 2) that are encoded by the prostaglandin-endoperoxide synthase 1 and 2 (PTGS1 and PTGS2) genes (referred to as the COX 1 and COX 2 genes). A third isoform, referred to as COX 3 or COX 1b (Chandrasekharan et al.,
2002; Snipes et al., 2005) has also been identified in some mammals, although a functional COX 1b protein is not produced (Reinauer et al., 2013).

In humans, COX 1 and COX 2 mRNA is expressed in several tissues including lung, uterus, testis, brain, pancreas, kidney, liver, thymus, prostate, mammary gland, stomach and small intestine, with the highest levels of COX transcripts being found in the prostate (O’Neill and Ford-Hutchinson, 1993). Both enzymes are differentially regulated and have different physiological functions, despite the fact that they catalyse identical reactions in prostanoid biosynthesis (Rouzer and Marnett, 2009). It is not fully understood why there are two isoforms of COX in mammals and in higher vertebrates, or when the duplication of the COX gene occurred (Järving et al., 2004). It is hypothesised that gene duplications arise from evolutionary whole-genome duplication events (Ohno et al., 1968). Evidence suggests that two successive rounds of whole-genome duplication occurred early in vertebrate evolution (Meyer and Schartl, 1999), and a third genome duplication event occurred in the stem lineage of teleost fishes (teleost-specific genome duplication), that resulted in the diversification of teleosts and their divergence from ray-finned fish (Jaillon et al., 2004; Meyer and Van de Peer, 2005). Therefore, the last common ancestor of fish and mammals is likely to be the origin of the vertebrate lineage.

4.1.1 COX in Fish

COX proteins have been identified in several fish species using bioinformatics databases (Table 12). However, the COX genes that code for these proteins have only been cloned and characterised in zebrafish (Grosser et al., 2002; Ishikawa et al., 2007), rainbow trout (Ishikawa and Herschman, 2007), brook trout (Roberts et al., 2000), spiny dogfish (Yang et al., 2002) and sea bass (COX 2 only) (Buonocore et al., 2005). Database screening revealed that the zebrafish and rainbow trout contain one COX 1 and two COX 2 enzyme isoforms, whereas some other species, such as the mummichog, possess two COX 1 (named COX 1a and 1b) and one COX 2 isoform. Other species, such as brook trout contain one COX 1 and one COX 2 (Table 12), similar to the mammalian isoforms. This suggests alternate duplication of chromosomal regions and differential retention of the COX genes during teleost
evolution (Havird et al., 2008). The identification of COX proteins and COX genes in several fish species suggest that COX is evolutionarily conserved in fish.

The evolutionary (and functional) conservation of molecular drug targets in fish is a pre-requisite of the read-across hypothesis, and one that is required to link the MoA of ibuprofen in humans, with fish. Therefore, the aim of this chapter was to identify whether the gene(s) coding for the target of ibuprofen in humans (i.e. COX enzymes), was present in the fathead minnow, to establish if the MoA is also conserved.
Table 12. Summary of COX proteins present in fish. Sources include the NCBI (accession numbers provided) and Ensemble databases (protein IDs provided). * denotes fish species where the COX genes have been cloned and characterised in the laboratory.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Latin name</th>
<th>COX Isoform</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zebrfish*</td>
<td>Danio rerio</td>
<td>COX 1, COX 2a, COX 2b</td>
<td>NP_705942, NP_705943, NP_001020675.1</td>
</tr>
<tr>
<td>Rainbow trout*</td>
<td>Oncorhynchus mykiss</td>
<td>COX 1, COX 2a, COX 2b</td>
<td>NP_001117833.1, NP_001117820.1, NP_001118139.1</td>
</tr>
<tr>
<td>Brook trout*</td>
<td>Salvelinus fontinalis</td>
<td>COX 1, COX 2</td>
<td>AAF14529.1, AAD45896.1</td>
</tr>
<tr>
<td>European Seabass*</td>
<td>Dicentrarchus labrax</td>
<td>COX 2</td>
<td>AAQ22672.1</td>
</tr>
<tr>
<td>Spiny dogfish*</td>
<td>Squalus acanthias</td>
<td>sCOX</td>
<td>AAL37727.1</td>
</tr>
<tr>
<td>Mummichog</td>
<td>Fundulus heteroclitus</td>
<td>COX 1a, COX 1b, COX 2</td>
<td>ACH73266.1, ACH73265.1, AAS21313.2</td>
</tr>
<tr>
<td>Japanese medaka</td>
<td>Oryzias latipes</td>
<td>COX 1a, COX 1b, COX 2</td>
<td>ENSORLP0000000625, ENSORLP0000006426, ENSORLP0000011863</td>
</tr>
<tr>
<td>Three-spined stickleback</td>
<td>Gasterosteus aculeatus</td>
<td>COX 1a, COX 1b, COX 2</td>
<td>ENSGACP0000018397, ENSGACP0000021267, ENSGACP000009851</td>
</tr>
<tr>
<td>Longhorn sculpin</td>
<td>Myoxocephalus octodecemspinosus</td>
<td>COX 1a, COX 1b, COX 2</td>
<td>ACO34913.1, ACH73272.1, ACH73267.1</td>
</tr>
<tr>
<td>Spotted green puffer</td>
<td>Tetraodon nigroviridis</td>
<td>COX 1a, COX 1b, COX 2</td>
<td>ENSTNIP0000009226, ENSTNIP000002081, ENSTNIP0000021290</td>
</tr>
<tr>
<td>Pufferfish</td>
<td>Takifugu rubripes</td>
<td>COX 1a, COX 1b, COX 2</td>
<td><a href="http://ensembl.fugu-sg.org">http://ensembl.fugu-sg.org</a> ENSTRUP0000042693, ENSTRUP0000046266, ENSTRUP0000010041</td>
</tr>
<tr>
<td>Atlantic croaker</td>
<td>Micropogonias undulates</td>
<td>COX 1, COX 2</td>
<td>BAF52621.1, BAF52620.1</td>
</tr>
<tr>
<td>Coelacanth</td>
<td>Latimeria chalumnae</td>
<td>COX 1, COX 2</td>
<td>ENSLACP0000001481, ENSLACP0000021975</td>
</tr>
</tbody>
</table>
4.2 METHODS

A brief overview of the methods used is provided below, full details can be found in Chapter 2.

4.2.1 Tissue acquisition

Fathead minnow tissues including the brain, gill, gonad (ovary), gut, heart, liver and muscle were collected from control (DWC) fish only (n=5) from the ≤24 hour static exposure (preliminary study, Chapter 2, Section 2.1.5, Table 6; ‘Exposure 1’) to examine the expression of the COX genes in tissues at basal levels (exposed fish tissue samples were not used).

4.2.2 Total RNA isolation, DNase I treatment and cDNA synthesis

Briefly, total RNA was extracted from each tissue (up to 40 mg) using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Dorset, UK) and RNA preparations were treated with Deoxyribonuclease I (DNase I, Amplification Grade, Sigma-Aldrich, Dorset, UK) to minimise genomic DNA contamination. Treated RNA (2 µg) was reverse-transcribed to cDNA using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Paisley, UK) and stored at -20 °C, as described in Chapter 2, Sections 2.2.1-2.2.3.

4.2.3 Polymerase Chain Reaction (PCR), recombinant DNA cloning and sequencing analysis

PCR was carried out using the primers used to isolate COX genes in the fathead minnow (Table 9), as identified in Chapter 2, Section 2.2.4.1.1. Primers were used in different combinations and in nested reactions to increase the probability of isolating one (or both) isoforms of the COX genes. The primer combinations used to successfully isolate the COX 1 and COX 2 genes in the fathead minnow, and expected product size in bp for each primer set, are summarised in Table 13.

PCR reaction mixtures were combined in a 0.2 mL micro-centrifuge tube. Each 20 µL PCR reaction consisted of 1 µL of cDNA (250 ng/µL), 2 µL of 10 x PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 0.1 µL of Tag DNA polymerase (5 U/µL) (Invitrogen Life Technologies, Paisley, UK), 0.4 µL of 10 mM dNTP mix (10 mM
each of dATP, dCTP, dGTP, dTTP), 1 µL of MgCl₂ (25 mM), 1 µL of forward and reverse COX primers (0.5 µM each) and 14.5 µL of nuclease-free water. For nested PCR reactions, 0.5 µL of the amplified DNA (from the first round of PCR) was used, as the target DNA had already been exponentially amplified in the first round. PCR reaction mixtures were incubated on a thermal cycler (Tetrad 2 Thermal Cycler, Bio-Rad, Hertfordshire, UK) and were amplified using ‘touch-down’ cycling conditions. These conditions included; one cycle at 94 °C for 5 minutes (initial denaturation), 30 cycles of touch-down PCR with denaturation at 94 °C for 30 seconds, annealing starting at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. The annealing temperature was subsequently reduced in decrements of 0.5 °C per cycle until the touch-down annealing temperature was reached (45 °C). Amplification was then performed at this temperature over 15 cycles, with denaturation at 94 °C for 30 seconds, annealing at 45 °C for 30 seconds, and extension at 72 °C for 30 seconds. A final extension step was performed at 72 °C for 10 minutes and the reactions were held at 4 °C.

PCR reactions were visualised on a 1.5% agarose gel. Product bands of the correct expected size (in bp) were then excised from the agarose gel and purified using the MinElute™ gel extraction kit (QIAGEN, Manchester, UK). The DNA samples were stored at -20 °C until required for direct sequencing, or recombinant cloning, which was carried out according to the method described in Chapter 2, Section 2.2.7. DNA products were sequenced using the DNA Sequencing Service, University of Dundee, Scotland (www.dnaseq.co.uk) and analysed using online nucleotide and protein sequences bioinformatics tools such as BLAST and InterPro databases, as described in Chapter 2, Sections 2.2.8-2.2.9.
Table 13. Forward (F) and Reverse (R) primer combinations used to isolate $COX\ 1$ and $COX\ 2$ in the fathead minnow and their expected product size (bp).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer ID</th>
<th>Primer combinations used to isolate $COX$</th>
<th>Expected product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX 1</td>
<td>External $COX\ 1\ F$</td>
<td>External $COX\ 1\ F\ &amp;\ R$</td>
<td>653</td>
</tr>
<tr>
<td></td>
<td>External $COX\ 1\ R$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Internal $COX\ 1\ F$</td>
<td>Internal $COX\ 1\ F\ &amp;\ R$</td>
<td>454</td>
</tr>
<tr>
<td></td>
<td>Internal $COX\ 1\ R$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX 2</td>
<td>External $COX\ 2\ F$</td>
<td>External $COX\ 2\ F\ &amp;\ R$</td>
<td>522</td>
</tr>
<tr>
<td></td>
<td>External $COX\ 2\ R$</td>
<td>Internal $COX\ 2\ F\ &amp;\ R$</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>Internal $COX\ 2\ F$</td>
<td>Internal $COX\ 2\ F\ &amp;\ External\ COX\ 2\ R$</td>
<td>222</td>
</tr>
<tr>
<td></td>
<td>Internal $COX\ 2\ R$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3 RESULTS

4.3.1 Assessment of RNA quality

RNA isolated from fathead minnow gill, liver and brain tissues was visually assessed on a 1.5% agarose gel to give an indication of RNA quality. Intact RNA is characterised by two distinct bands (28S and 18S), the 28S band should be approximately twice as strong as the 18S, as shown in the gill and liver samples (Figure 27). The RNA extracted from the brain produced weak bands, indicating low RNA quantity. Therefore, the brain was not selected for further analysis. There was no indication of DNA contamination or RNA degradation (smeared bands).

Figure 27. Assessment of RNA integrity by agarose gel electrophoresis. RNA isolated from fathead minnow gill and liver showed clean, distinct bands corresponding to the 28S (upper bands) and 18S (lower bands) ribosomal RNA indicating intact RNA. Samples were separated on a 1.5% agarose gel. A 1kb+ DNA ladder (Invitrogen) was used for size determination.

4.3.2 Identifying a putative fathead minnow COX 1 transcript

To examine whether the COX 1 gene was expressed in the fathead minnow, a first round of PCR (using the COX 1 external primers) was conducted with cDNA obtained from gill, gut, heart and muscle tissues. A subsequent nested round of amplification (using the COX 1 internal primers) was performed to increase the specificity of the reaction. The expected product size for the external and internal primers was 653 bp and 454 bp, respectively (Figure 28).
Amplification using the external COX 1 primers resulted in no visible products, indicating that these primers were not specific enough or did not generate sufficient products to be visualised. The internal COX 1 primers used in the nested round yielded one product in the gill, gut and muscle tissues, all corresponding to the expected size of 454 bp (no PCR product was generated in the heart). The product bands were excised from the gel, ligated into the pDrive cloning vector method and transformed into E.coli competent cells. Several colonies were assessed using PCR with M13 primers to determine if the resulting colonies contained the correct-sized inserts (data not shown). Nine positive colonies were cultured in LB medium followed by plasmid purification. The plasmid DNA samples were digested using the EcoRI restriction enzyme to confirm the size of the insert (Figure 29).
Figure 29. Restriction digest of purified plasmid containing PCR product inserts. Digested fragments of ~380 bp were observed in the gill and muscle tissues. Two additional weak fragments at 500 bp were also seen in the gill (indicated by arrow). No digested fragments were seen in the gut. Samples were separated on a 1.5% agarose gel. A 1kb+ DNA ladder (Invitrogen) was used for size determination.

One large fragment of approximately 4,000 bp was observed, indicative of the cleavage of the pDrive cloning vector (3.85 Kb). Cleaved fragments of around 380 bp were seen in the gill and muscle, and a further weak fragment at 500 bp was also seen in the gill. The 380 bp fragments were smaller than the expected product size of 454 bp, whereas the 500 bp fragment was near to the expected size when the location of the EcoR1 restriction site was considered. However, all six plasmid samples were sent for DNA sequencing. No fragments were observed in the gut, but one high molecular weight band was seen indicating that the enzyme had not cleaved the sample properly.

The sequencing analysis revealed that a restriction site was located within the amplified products (shown for gill, in Figure 30), which resulted in fragments of 360 bp and 150 bp. The 360 bp product was observed on the gel, whereas the 150 bp product was too small to be seen. The fragment at 500 bp was from the cleavage site.
just outside of the DNA insert, and therefore was slightly larger than the PCR product size.

The nucleotide sequences were determined for both sense and anti-sense strands of the plasmid inserts, and the identity of the DNA sequences was confirmed using BLAST. The sequenced products generated in the gill and muscle tissues were all identified as being putative COX 1 fragments, indicating that these primers were successful in isolating fathead minnow COX 1 gene sequences.

Figure 30. 5'-3' nucleotide sequence of a putative fathead minnow COX 1 gene fragment from a cloned PCR product generated from gill RNA. The PCR product (454 bp in size, shown in red) was generated using the COX 1 forward and reverse primers (bold and highlighted in yellow). The boxes represent the EcoRI restriction sites (GAATTTC), including the one site that was located within the sequence. The double-underlined sequence indicates the (reverse-complemented) M13 reverse primer used to ampl the plasmid.

The BLAST analysis and pairwise alignment confirmed that the putative COX 1 transcript obtained in the fathead minnow shared 85% identity to zebrafish COX 1 (Figure 31).
Figure 31. Clustal Omega alignment of the putative fathead minnow (FHM) COX 1 (454 bp) and partial zebrafish (Zf) COX 1 nucleotide sequences. Identical residues are indicated by *. 

Identity score = 85%
4.3.3 Identifying putative fathead minnow COX 2 transcripts

To examine whether the COX 2 gene(s) were expressed in the fathead minnow, a first round of PCR amplification (using the external, internal and a combination of the internal forward and external reverse COX 2 primers) was performed using cDNA obtained from liver, ovary and gill tissues (Figure 32). The expected product size for the external primers was 522 bp, for internal primers 180 bp, and the combination internal forward and external reverse primers was 222 bp. Figure 32 shows the results following the first round of PCR amplification using the various COX 2 primers.

First round of PCR amplification

Figure 32. PCR products generated in the first round of PCR using primers designed to isolate a COX 2 gene in fathead minnow tissues. Three different primer set combinations (external, internal and external/internal) were used to isolate COX 2 in the fathead minnow, which yielded PCR products (numbered in the boxes) of approximately 250 bp (2&3), 300 bp (4), 350 bp (1) and 650 bp (5) in the tissues. Samples were separated on a 1.5% agarose gel. A 1kb+ DNA ladder (Invitrogen) was used for size determination. Excess primers resulted in primer dimers (indicated by arrow).
Amplification using the external COX 2 primers resulted in one weak product band in the gill corresponding to a size of approximately 650 bp (product 5), which was slightly larger than the expected size (522 bp) for this primer pair. Amplification using the internal primer set showed two product bands in each tissue, one at 350 bp (product 1) and one at 250 bp (product 2), both of which were larger than the expected size of 180 bp. The presence of additional product bands was anticipated considering that these generic primers were designed against template COX gene sequences from human, zebrafish and rainbow trout species and the lower stringency cycling conditions could have encouraged non-specific amplification. Finally, the internal forward and external reverse combination primer set yielded one weak product in the liver at 300 bp, one weak product in ovary at 250 bp (product 3) and one clear product band in the gill at 300 bp (product 4), all of which were larger than the expected product of 222 bp for this primer pair.

Product bands 1-5 (Figure 32) were extracted from the gel and purified. The purified DNA products 1-4 were further amplified in a second round of PCR, using the same respective COX 2 primer sets and cycling conditions to increase the amount of DNA for direct sequencing (Figure 33). The second round of amplification yielded a new smaller product of 250 bp in the liver in addition to original 350 bp product, which was closer to the expected product of 180 bp. The purified products from the ovary and gill samples, yielded the same sized products as the first round of PCR amplification. The product bands (indicated by the arrows) were extracted from the gel, purified and sent for direct sequencing.
The sequenced DNA products were analysed using BLAST and the results are shown in Table 14. The amplified product obtained in the liver (product 1) showed sequence similarity with guinea pig (78%) and human (76%) COX 1 nucleotide sequences. Conversely, the second product generated in the liver (product 2) showed sequence similarity with COX 2 in the Japanese medaka (85%), rainbow trout COX 2 and COX 2b (81% and 82%) and brook trout COX 2 (82%) nucleotide sequences. Although these results suggest there was some identity between the fathead minnow sequences with COX sequences in human and fish species, there was a low sequence coverage overall, indicating that only small fragments of the fathead minnow sequences and the COX sequences listed in the NCBI database were similar. The sequenced product generated in the ovary (product 3) did not show any similarity with COX sequences. The sequenced product generated in the gill (product 4) however, showed the greatest identity to zebrafish COX 2b (Table 14) confirmed by BLAST analysis, with 78% identity between the nucleotide sequences (Figure 34).
Table 14. Nucleotide BLAST analysis of amplified DNA sequences obtained in the fathead minnow using COX 2 primers.

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Nucleotide database results</th>
<th>% Identity and sequence coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Guinea pig COX 1</td>
<td>78% (43/55)</td>
</tr>
<tr>
<td></td>
<td>Homo sapiens COX 1</td>
<td>76% (45/59)</td>
</tr>
<tr>
<td>2</td>
<td>Japanese Medaka COX 2</td>
<td>85% (70/82)</td>
</tr>
<tr>
<td></td>
<td>Rainbow trout COX 2</td>
<td>81% (79/98)</td>
</tr>
<tr>
<td></td>
<td>Rainbow trout COX 2b</td>
<td>82% (67/82)</td>
</tr>
<tr>
<td></td>
<td>Brook trout COX 2</td>
<td>82% (72/88)</td>
</tr>
<tr>
<td>3</td>
<td>Zebrafish T1R taste receptor gene</td>
<td>81% (125/154)</td>
</tr>
<tr>
<td>4</td>
<td>Zebrafish COX 2b</td>
<td>88% (192/218)</td>
</tr>
<tr>
<td></td>
<td>Zebrafish COX 2a</td>
<td>81% (172/212)</td>
</tr>
<tr>
<td></td>
<td>Zebrafish COX 1</td>
<td>71% (146/207)</td>
</tr>
</tbody>
</table>

Figure 34. Clustal Omega alignment of the putative fathead minnow (FHM) COX 2b (237 bp) and partial zebrafish (Zf) COX 2b nucleotide sequences. Identical residues are indicated by *.
The purified DNA product obtained from the gill sample (product 5) using the external COX 2 primer set was re-amplified using nested PCR (with the internal COX 2 primers) (Figure 35). Three bands were observed including one strong product band at 400 bp and two weaker products at around 750 bp, and 200 bp. The product band at 200 bp was similar to the expected size (180 bp) for this primer pair, however all three product bands were extracted from the gel, purified and sent for direct sequencing.

**Nested PCR amplification**

![Nested PCR amplification](image)

Figure 35. PCR products generated from the nested round of PCR amplification using COX 2 primers with purified fathead minnow gill DNA. All three bands were extracted from the gel and purified for direct DNA sequencing. One strong product band was observed at 400 bp and two weaker products were observed at around 750 bp, and 200 bp. Samples were separated on a 1.5% agarose gel. A 1kb+ DNA ladder (Invitrogen) was used for size determination.

The amplified DNA product that was similar to the expected size (180 bp) was analysed using BLAST and pairwise alignment, which showed 89% identity with the zebrafish COX 2a sequence (Figure 36).
Figure 36. Clustal Omega alignment of the putative fathead minnow (FHM) COX 2a (381 bp) and partial zebrafish (Zf) COX 2a nucleotide sequences. Identical residues are indicated by *.

To confirm the identity of two putative COX 2 gene transcripts in fathead minnows, pairwise alignment was performed. The identity between fathead minnow COX 2a and COX 2b sequences was 73% (Figure 37), suggesting that the two gene isoforms are similar, but not the same. In zebrafish, the COX 2a and COX 2b isoforms share 68% identity (Table 15). However due to the limited amount of sequencing data obtained in this study, the sequence coverage was much lower in the fathead minnow than the zebrafish COX 2a and COX 2b sequences.
Chapter 4 Results

Figure 37. Clustal Omega alignment of the putative fathead minnow (FHM) COX 2a (381 bp) and COX 2b (237 bp) nucleotide sequences. Identical residues are indicated by *.

| FHM COX 2a | CTTGCTCTATTTAAAAAGATGGAAGCCAAAGTTACAGGTGGTGGGTGGTGAGGTGACCT 60 |
| FHM COX 2b | ----------------------------------------------- | 0 |
| FHM COX 2a | CCGTGGTGAAAGATGTGGACTCCAGTTGTGAAGGTGTACCCT | 120 |
| FHM COX 2b | ----------------------------------------------- | 0 |
| FHM COX 2a | AAAATTGGCTGTGGCAGGCTTCGGTCTGGTCCCAGGTTTGATGATGTATGCAACC | 180 |
| FHM COX 2b | ----------------------------------------------- | 0 |
| FHM COX 2a | ATTTGGCTCCGGGAACACTGTGTGGGTATGTTATCATGAAAGCAAGCAGATCCGCAGACTTG | 240 |
| FHM COX 2b | GGGGTGTCTTACGACAGCTCCAGACTGG 30 |
| FHM COX 2a | GTAACGAGGACCGTTCCTTCGGTCTGGTCCCAGGTTTGATGATGTATGCAACC 300 |
| FHM COX 2b | GTAACGAGGACCGTTCCTTCGGTCTGGTCCCAGGTTTGATGATGTATGCAACC 300 |
| FHM COX 2a | GTGATTGAGGACTACGTTCAGCATCTGAGTGGCTACAACTTCAAGCTCAAGTTTGACCCA 360 |
| FHM COX 2b | GTGATTGAGGACTACGTTCAGCATCTGAGTGGCTACAACTTCAAGCTCAAGTTTGACCCA 360 |
| FHM COX 2a | GAGCTTCTTCTCAATCAACGC --------------------------------------- 381 |
| FHM COX 2b | GAGCTTCTTCTCAATCAACGC --------------------------------------- 381 |
| FHM COX 2a | CTTCACACTGGGACCCCCACGTCAGTCAAAA 237 |
| FHM COX 2b | CTTCACACTGGGACCCCCACGTCAGTCAAAA 237 |

Identity score = 73%

A summary of the pairwise nucleotide sequence identities between the putative fathead minnow COX gene sequences and the zebrafish, rainbow trout and human COX genes is shown in Table 15. The putative fathead minnow COX 1 transcript shared greater identity with zebrafish and rainbow trout COX 1 sequences, compared to the COX 2 sequences in these species. Likewise, the putative fathead minnow COX 2 transcripts shared greater identity with COX 2 sequences in the zebrafish and rainbow trout, compared to the COX 1 sequences. The putative fathead minnow COX 1 transcript was similar to both human COX 1 and COX 2 gene sequences, whereas the putative fathead minnow COX 2 transcripts were more similar to the human COX 2 gene. However, it is difficult to confirm this due to the limited transcript information that was obtained from the fathead minnow.
Table 15. Pairwise nucleotide (bp) sequence alignment (%) between the putative fathead minnow (FHM) COX gene sequences with zebrafish (Zf), rainbow trout (Rt) and human COX gene sequences (NCBI accession numbers are provided) using Clustal Omega alignment. The putative FHM COX sequences had the greatest identity to their homologues in the zebrafish (shown in red).

<table>
<thead>
<tr>
<th>Gene sequence</th>
<th>FHM COX 1</th>
<th>FHM COX 2a</th>
<th>FHM COX 2b</th>
<th>Zf COX 1</th>
<th>Zf COX 2a</th>
<th>Zf COX 2b</th>
<th>Rt COX 1</th>
<th>Rt COX 2</th>
<th>Rt COX 2b</th>
<th>Human COX 1</th>
<th>Human COX 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHM COX 1 (454bp)</td>
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<td></td>
<td></td>
<td></td>
<td>47</td>
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<td></td>
<td></td>
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<td>72</td>
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<tr>
<td>FHM COX 2a (381bp)</td>
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<tr>
<td>FHM COX 2b (237bp)</td>
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<td>68</td>
<td>61</td>
<td>70</td>
<td>73</td>
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<tr>
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<td>Human COX 2 NM_000963.3</td>
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<td></td>
<td></td>
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<td>57</td>
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</table>

128
4.3.4 Analysis of the putative COX transcripts

To determine if the COX protein sequences were encoded by the COX gene transcripts, the nucleotide sequences were translated into their amino acid (aa) sequences using ExPASy (www.expasy.ch/tools/dna.html) and analysed using InterPro and BLAST. The translation predicted a 129 amino acid of the fathead minnow COX 1 gene fragment, a 98 amino acid sequence of the fathead minnow COX 2a gene fragment and a 79 amino acid sequence of the fathead minnow COX 2b gene fragment.

The ‘InterPro’ database is an online bioinformatics tool (that consists of 14 different protein databases) that uses “protein signatures” to allow the functional characterisation of proteins by classifying sequences at superfamily, family and subfamily levels, predicting the occurrence of functional domains, repeats and important sites from different protein databases (Hunter et al., 2012). The putative fathead minnow COX amino acid sequences were characterised by the presence of a functional domain typical of an “animal haem peroxidase” (using PFAM database), and a “haem-dependent peroxidase” superfamily, and the PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system recognised specifically the domains typical of “prostaglandin G/H synthases/cyclooxygenases” (Figure 38). These results confirm the presence of the functional domains of COX in the fathead minnow, suggesting that the gene products encode for functional proteins that can potentially be targeted by NSAIDs like ibuprofen.
Figure 38. DNA fragments amplified in the fathead minnow using the COX gene primers were translated into their amino acid sequences and analysed using the ‘InterPro’ bioinformatics database, which uses “protein signatures” to search for functional domains from 14 member protein databases. The putative fathead minnow COX 1 fragment (shown as an example) was characterised by the presence of a functional domain typical of an “animal haem peroxidase” (using PFAM database), and a “haem-dependent peroxidase” superfamily, and the PANTHER (Protein Analysis Through Evolutionary Relationships) database revealed similarity to the cyclooxygenase enzyme.
The pairwise sequence alignment of the putative fathead minnow COX amino acid sequences with zebrafish, rainbow trout and human COX sequences is shown in Table 16. The putative fathead minnow COX 1 amino acid sequence showed greater identity with the zebrafish (86%), rainbow trout (85%) and human (70%) COX 1 amino acid sequences, compared to the COX 2 sequences. Similarly, the putative fathead minnow COX 2a and COX 2b amino acid sequences also shared greater identity with zebrafish, rainbow trout and human COX 2 sequences, compared to COX 1. The sequence identity between the putative fathead minnow COX 1, COX 2a and COX 2b proteins with the zebrafish COX sequences were 86%, 93% and 80%, respectively.

The putative fathead minnow COX amino acid sequence were aligned with the human COX 1 (599 aa) (Figure 39) and COX 2 (604 aa) sequences (Figure 40). The sequence identity between the proposed fathead minnow COX 1 with the human COX 1 was 70%, and the proposed fathead minnow COX 2a and COX 2b with the human COX 2 was 81% and 72%, respectively (Table 16). Some conserved structural and functional amino acids were identified in the putative fathead minnow COX-like proteins, for example, the putative fathead minnow COX 1 protein contained an aspirin acetylation site (Ser-530), which is functionally conserved in mammalian COX (Figure 39). Two amino acids similar to those found in the substrate-binding domain of COX in humans (His-513 and Ile-523) were also identified in the proposed COX 1 protein sequence (Figure 39). In mammals, His-513 and Ile-523 determine conformational differences in the substrate binding channel between mammalian COX 1 and COX 2 enzymes (Wong et al., 1997; Gierse et al., 1996). In zebrafish COX enzymes, the His-513 and Ile-523 positions are substituted with Arg-513 and Val-523 (Grosser et al., 2002), and substitutions at these positions were confirmed in fathead minnow COX 1.

The putative fathead minnow COX 2a and COX 2b protein sequences contained an active site tyrosine (Tyr-355) which is required for enzyme catalysis and a nine amino acid haem-coordination binding motif (Figure 40), which is present in human COX 2 protein sequences. These findings suggest that the fathead minnow may contain functionally active COX proteins.
Table 16. Pairwise amino acid (aa) alignment (%) between the putative fathead minnow (FHM) COX amino acid sequences with zebrafish (Zf), rainbow trout (Rt) and human COX amino acid sequences using Clustal Omega alignment. The putative FHM COX sequences had the greatest identity to their homologues in the zebrafish (shown in red).

<table>
<thead>
<tr>
<th>Protein sequence</th>
<th>FHM COX 1</th>
<th>FHM COX 2a</th>
<th>FHM COX 2b</th>
<th>Zf COX 1</th>
<th>Zf COX 2a</th>
<th>Zf COX 2b</th>
<th>Rt COX 1</th>
<th>Rt COX 2</th>
<th>Rt COX 2b</th>
<th>Human COX 1</th>
<th>Human COX 2</th>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>FHM COX 2a</td>
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<td>70</td>
<td>93</td>
<td>90</td>
<td>63</td>
<td>89</td>
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</tr>
<tr>
<td>FHM COX 2b</td>
<td>65</td>
<td>75</td>
<td>80</td>
<td>60</td>
<td>73</td>
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</tbody>
</table>
Figure 39. Alignment of the putative fathead minnow (FHM) COX 1 (129 amino acid) with the human COX 1 (599 amino acids) protein sequence. Identical residues are indicated by * and similar residues are represented by the dots. One critical residue for aspirin acetylation (Ser-530) (highlighted in red) was identified in the sequence. Amino acids at positions 513 and 523 (His-513 and Ile-523) are postulated to determine conformational differences in the substrate binding domain between mammalian COX 1 and COX 2 enzymes (highlighted in blue). In zebrafish, positions at 513 and 523 are substituted by Arg and Val (Grosser et al., 2002) changes which are also evident in the fathead minnow.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Human COX 1</th>
<th>FHM COX 1</th>
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</thead>
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<tr>
<td>MSRSLLWLPLLFLLLLPPPLPVLADPGAPTVPNCCYYPQGHGICVRFGLDRCDCDTR</td>
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</tr>
<tr>
<td>TGYSGPNTIGPLLWTWLRSRLPSFSFTHFLLTHGRWFEWFEVNATIREMLRIVLTVRS</td>
<td>120</td>
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</tr>
<tr>
<td>NLIPSEQYNHAYISWEFSNVSYYTRILPSVKNDCPTMGTGKKQLPDAQQLLARFF</td>
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</tr>
<tr>
<td>LLRRKFPDPQGTNLMAFFAQQHTQKFQKSMGKGTPKALHGVDLGHLAYGDNLREQ</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>YQLRLFKDQLKLYQVLDGEMYPSPVEAPVLMHYPRIPQPSQAVGQEVFGLPLML</td>
<td>300</td>
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<tr>
<td>ATWLRHENNVRCDDKAHPTWDEQLFQTRILIGETIKIVIEYQVQSLGFLQLKF</td>
<td>360</td>
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</tr>
<tr>
<td>DPELLFQFQYRNRAMEFHLHYWHLMPSFVVGSQOYFLQFIQLQYVEA</td>
<td>420</td>
<td></td>
</tr>
<tr>
<td>LVDAFSAOYIAGDRGGNNMDHLHVDVRESREMRLQFNEYKRRFGMKPYSQYL</td>
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</tr>
<tr>
<td>VGEKMAPEELEELYGIDALEFPVLLLEKCHGPSIFGEMIEGAPFFKGLLMGMPICS</td>
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</tr>
<tr>
<td>PEYWKPSLFMEGFDVFIVKTATLKLCLNTKTPYVSFRVDPASQGDGPAVERPTEL</td>
<td>599</td>
<td></td>
</tr>
</tbody>
</table>

Identity score = 70%
Figure 40. Alignment of the putative fathead minnow (FHM) COX 2a (98 amino acids) and COX 2b (79 amino acid) with the human COX 2 (604 amino acids) protein sequences. Identical residues are indicated by * and similar residues are represented by the dots. One critical residue for catalysis (Tyr-355) (highlighted in red) was identified in the sequence. Residues involved in haem-coordination are identified in the box.

<table>
<thead>
<tr>
<th>Human COX 2</th>
<th>FHM COX 2a</th>
<th>FHM COX 2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLARALLLCAVLALSHTANPPCCSHPCQNRGVCMSVGFQYKCDCTRGTGYPGENCSTPEFL</td>
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<td></td>
</tr>
<tr>
<td>TRIKLFKPTPNTVHYILTFFKGFNVVNNIFLRAIMSYVTLSRSHLDOSPTYNADY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GYKSWEAFSNLSSYTRALPPVDCPTPLGVKQKQLPSNEIHEVKLLLDRRKFIPDPQGS</td>
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<tr>
<td>NNMFAFFAQHHTHQQKTIDHGRPAFTNLGHVDLNHGYGETLARQKRLLRKDFGRKMKY</td>
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<tr>
<td>QIIDGEMYPPTVKTQAEIYPPQVPEHLRFAVQGVFQLVPMHAMMTIQLREAHENRVD</td>
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<td></td>
<td></td>
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<td>STEL 604</td>
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</table>

Identity score: Human COX 2 / FHM COX 2a = 81% / Human COX 2 / FHM COX 2b = 72%
4.4 DISCUSSION

The purpose of this chapter was to establish whether the genes coding for the target of ibuprofen in humans (COX enzymes) were present in the fathead minnow. The expression of three putative COX gene transcripts were identified in the fathead minnow, and the translated sequences contained some functionally conserved amino acid residues, such as Ser-530, the site for aspirin acetylation, and Tyr-355 required for enzyme catalysis suggesting that the fathead minnow COX proteins may be functionally active. Therefore, ibuprofen (or other NSAIDs that act through COX inhibition) may bind to, and cause inactivation of COX, suggesting that MoA-related effects may also be seen in fish (see Chapters 5 and 6 for target-mediated effects following ibuprofen exposure).

4.4.1 COX gene expression in the fathead minnow

The expression of COX transcripts was examined in different fathead minnow tissues including gill, muscle, gut, liver, ovary and heart, using PCR, and generic primers used to isolate and amplify COX 1 and COX 2 genes in the fathead minnow (designed from template COX gene sequences from zebrafish, rainbow trout, human, Atlantic salmon, mummichog and clawed frog). Due to time restraints, the expression of the COX genes was examined in a selection of tissues only, as the purpose of this work was not to determine the expression of the COX genes in all the sampled tissues. COX 1 gene fragments were amplified in the gill and muscle tissues, whereas COX 2 gene fragments were amplified in the gill only. Putative transcripts for two isoforms of the COX 2 gene were identified in the fathead minnow, indicating that this species contains three putative COX genes (which we have termed as FHM COX 1, FHM COX 2a and FHM COX 2b), based on their similarity to the zebrafish genes (Ishikawa et al., 2007). The fathead minnow and zebrafish are both members of the Cyprinidae family and therefore share a high degree of evolutionary conservation, which support the findings of one putative COX 1 and two COX 2 sequences that we have identified in fathead minnow tissues. The expression of two COX 2 genes in some fish species corresponding to one COX 2 ortholog in higher vertebrates indicates that genome duplication occurred in the teleost lineage after its divergence from the vertebrate lineage (Meyer and Van de
Peer, 2005). Both the zebrafish and rainbow trout genomes contain two functional, inducible \textit{COX} 2 genes (Ishikawa et al., 2007; Ishikawa and Herschman, 2007), that are coded for by different gene products, suggesting that paralogs within a species can be functionally equivalent, or in some cases, they may evolve to gain or lose functionality. In mammals, \textit{COX} 1 is constitutively expressed and is relatively stable under basal conditions, whereas \textit{COX} 2 is rapidly inducible and contains several instability sequences, or ‘AUUUA’ motifs (Shaw and Kamen, 1986) located within its 3’ un-translated (3’UTR) region which is characteristic of immediate-early genes (genes that are transiently activated and rapidly degraded) (Chandrasekharan and Simmons, 2004). In zebrafish, the proximal 3’UTR regions of \textit{COX} 2a and \textit{COX} 2b possess AUUUA repeats, which suggest that these are both inducible genes, whereas in contrast, these repeats are not present in mammalian or zebrafish \textit{COX} 1 (Ishikawa et al., 2007), indicating functional conservation of human orthologs in fish.

In this study, the expression of the putative \textit{COX} 1 gene was identified in the gill and muscle fathead minnow tissues, whereas the expression of the putative \textit{COX} 2a and \textit{COX} 2b genes were identified in the gill only. Grosser et al., (2002) found that \textit{COX} 1 and \textit{COX} 2 transcripts were expressed in a range of zebrafish tissues, but \textit{COX} 2 was more highly expressed in the gill, when compared with the gut, testes, heart, skeletal muscle and brain (Grosser et al., 2002). Confounding this, Ishikawa et al., (2007) reported that \textit{COX} 1, \textit{COX} 2a and \textit{COX} 2b transcripts were all highly, but equally expressed in the zebrafish gill (Ishikawa et al., 2007). Nevertheless, these findings suggest that the \textit{COX} genes are most robustly expressed in the zebrafish gill, which most likely explains why the putative \textit{COX} transcripts were identified in this tissue in the fathead minnow too. The expression of \textit{COX} 1 (at basal levels) in the muscle could be linked to prostanoid functions in fish, such as smooth muscle contraction (Coleman and Sheldrick, 1989), whereas \textit{COX} 2 expression in the gills has been linked to osmoregulation and ion transport in killifish (Choe et al., 2006).

In the other examined tissues, there was no amplification in the heart (using the \textit{COX} 1 gene primers) (Figure 28), which may have been due to the smaller tissue weight and/or lower \textit{COX} transcript levels in this tissue and therefore a sufficient amount of product may not have been generated for visualisation on the gel. Amplification in
the ovary (using the COX 2 gene primers) (Figure 32) did not result in the isolation of a COX gene fragment, but unexpectedly, showed most similarity to a zebrafish taste receptor (Taste receptor, type 1, member 3) (81% identity). Furthermore, both Grosser et al., (2002) and Ishikawa et al., (2007) reported that COX 1, but not COX 2 was expressed in zebrafish liver, and although a PCR product was generated in the liver, it was not possible to confirm the identity of the COX gene isoform from the initial sequence results. This was not pursued any further given that a putative COX transcript was confirmed in products generated from the gill.

4.4.2 Conservation of COX between human and fish species

Bioinformatics analysis confirmed that the putative fathead minnow COX transcripts coded for COX proteins that were characterised by a functional domain typical of a “haem peroxidase”. The haem peroxidases are a group of enzymes that use various peroxides as electron acceptors to catalyse a number of oxidative reactions and it includes the myeloperoxidases, eosinophil peroxidases, lactoperoxidases, thyroid peroxidase; and prostaglandin H synthases (COX). Therefore, the integration of a bioinformatics approach was effectively applied to identify the targets based on the MoA of ibuprofen. It is plausible that this functional domain was identified in the fathead minnow because of the conserved amino acid residues that were present in the COX protein sequences, such as an active site tyrosine (Tyr-355) (Figure 40) which is required for enzyme catalysis, an aspirin acetylation site (Ser-530) (Figure 39), and haem-coordination binding motif. Collectively, these findings indicate that NSAIDs like ibuprofen can potentially bind to fathead minnow COX, and cause inactivation of the protein, since the binding sites for ibuprofen in humans have been conserved in the fathead minnow. This implies that the MoA of ibuprofen may also be conserved in fish.

The putative COX 1 amino acid sequence in the fathead minnow shared 70% identity to human COX 1, and the COX 2a and COX 2b sequences shared 81% and 72% identities to human COX 2, covering highly conserved regions of the sequence. However, the isolated putative COX transcripts in the fathead minnow represent only 22%, 16% and 13% respectively, of the entire COX 1 and COX 2 human protein sequences, and therefore overall the sequence coverage is very low. Therefore, the
degree of similarity between humans and fathead minnows can only be determined for a relatively small part of the COX gene, and as a result the degree of similarity between these two species, outside of the most highly conserved region is unclear.

In summary, the expression of three putative COX transcripts was identified in fathead minnow tissues (at basal levels), indicating that the target for ibuprofen in humans, is present in fish. The presence of some functionally conserved residues in the translated nucleotide sequences suggests that the MoA of ibuprofen may be conserved, and through the identification of the putative COX transcripts, further investigation on the effects of ibuprofen exposure mediated through the primary (or secondary) targets can be conducted.
CHAPTER 5: THE EFFECT OF IBUPROFEN EXPOSURE ON CYCLOOXYGENASE (COX) GENE EXPRESSION
5.1 INTRODUCTION

The application of ‘-omic’ technologies, including genomics, proteomics and metabolomics in ecotoxicology provides important opportunities to improve our understanding of the molecular mechanisms (and modes) of action of environmental stressors (Snape et al., 2004). Chemical-induced molecular changes may involve a cascade of gene interactions, a change in a single gene or a few genes (Hook et al., 2006), and advances in functional genomics have enabled the simultaneous global expression profiles of thousands of genes to be examined at one time (Nuwaysir et al., 1999).

The current ERA procedures are heavily dependent upon changes in apical endpoints such as growth, survival and reproduction. However, gene expression changes are more reflective of the initial responses, and therefore could be more useful as “biomarkers” of toxicant exposure and/or effects (Van Der Oost et al., 2003). For example, the vitellogenin genes have been used as a biomarker for exposure to oestrogen or oestrogen-like chemicals (Bowman et al., 2000; Celius et al., 2000; Denslow et al., 2001), which is indicative of reproductive and endocrine disruption in fish (Sumpter and Jobling, 1995) and the metallothionin gene has been used as a biomarker for heavy metal exposure (Knapen et al., 2007). Therefore, gene expression endpoints could potentially allow for increased sensitivity, earlier detection and measurement of toxicant effects at more environmentally relevant concentrations. Furthermore, chemicals that act through distinct MoAs can induce unique gene expression profiles or “fingerprints” which in some cases can be linked to phenotypic effects (Hamadeh et al., 2002). For example, the anti-androgen flutamide, and oestrogens such as EE$_2$ can induce phenotypic effects indicative of feminisation in fish (i.e. induction of plasma vitellogenin, reduced gonadosomatic index, and reduced secondary sex characteristics) through distinct (and common) gene pathways, thereby allowing differentiation of the two contaminants (Filby et al., 2007). However, further experimental validation is required before genomic approaches can be fully implemented into the ERA process (Hook et al., 2006). The use of genomic approaches for predictive risk assessment has already been well-established in species such as human and mouse. However, from an eco-
toxicological perspective, very few genomes have been characterised for aquatic species, and therefore gene expression biomarkers are currently of limited use for ERA (Hogstrand et al., 2002). Nevertheless, despite there being a lack of whole genome datasets, the expression of individual genes that have been identified and characterised in aquatic species have been examined using genomic approaches, such as quantitative real-time PCR (qPCR). This technique allows the simultaneous detection and quantification of DNA, where the amount of DNA is measured after each cycle of PCR amplification and the products (amplicons) are visualised by fluorescence.

Ibuprofen is a non-selective inhibitor of the COX 1 and 2 enzymes, that are coded for by the mammalian \textit{COX 1} and \textit{COX 2} genes. We have identified putative COX transcripts in fathead minnow tissues (as described in Chapter 4), and therefore the purpose of this chapter was to investigate the effect of ibuprofen exposure on COX gene expression (as a potential “biomarker” indicative of MoA effects).

There are currently limited data of the effects of NSAIDs on COX gene expression in fish; with some studies reporting no transcriptional changes in COX genes (using qPCR), following ibuprofen exposure to 21, 201 and 506 μg/L (for 7 days) (Morthorst et al., 2013) or at more environmentally relevant concentrations of ibuprofen (0.1 and 1 μg/L) (Ji et al., 2013), indicating that in the present literature, there is no clear evidence for transcriptional-level effects of NSAIDs (ibuprofen) on fish. In molecular biology, DNA (gene) is transcribed into messenger RNA (mRNA), which is the template from which proteins are synthesised, and has long been referred to as the “Central Dogma of Molecular Biology” (Crick, 1970). According to this hypothesis, gene expression is directly correlated to the amount of mRNA that is translated into functional protein. Therefore, it could be hypothesised that (transient) inactivation of the COX enzyme (following ibuprofen exposure) may result in a concomitant down-regulation of COX transcripts at the gene expression level. However, as ibuprofen inhibits the COX enzymes by substrate competition, a potential response to overcome this inhibition could also be increased COX enzyme synthesis and altered (up-regulated) gene expression, through a compensatory feedback mechanism.
5.2 METHODS

The full details of the materials and methods used are provided in Chapter 2, and the following contains details relevant to this chapter that are not covered elsewhere.

5.2.1 Tissue acquisition

Adult fathead minnows (n=16 per treatment) were exposed to nominal ibuprofen concentrations of 100, 270, 370 and 500 µg/L (mean measured ± SD, 105 ± 2, 278 ± 70, 409 ± 26 and 502 ± 56 µg/L, respectively) for ≤96 hours, using continuous flow-through systems (Chapter 2, Section 2.1.5, Table 6; ‘Exposure 2’ and ‘Exposure 3’). Tissues (including brain, gill, testis, gut, heart, liver) were sampled from fish exposed to DWC, SC (acetone), 100 and 500 µg/L ibuprofen after 3, 24, 48 and 96 hours, and fish exposed to DWC, SC, 270 µg/L ibuprofen after 24, 48, 72 and 96 hours (tissues collected from fish exposed to 370 µg/L were not analysed in this chapter). Four fish were sampled at each time point.

5.2.2 Quantitative real-time PCR (qPCR)

5.2.2.1 Primer design for putative fathead minnow COX transcripts and house-keeping genes

Primers for qPCR were designed using the putative fathead minnow COX 1, COX 2a and COX 2b gene sequences that were identified in Chapter 4. Primers were designed to be 18-30 nucleotides in length (to minimise non-specific binding), with a 40-60% GC content and a Tm of 60 °C. The Tm was calculated using the following formula: 

\[ Tm = 2 \times [A + T] + 4 \times [G + C] \]

The primers sets were designed to amplify amplicons of 100-200 bp in length. The primer sequences, expected amplicon sizes and Tm of the primers, termed FHM COX primers are shown in Table 17. Primers for the putative fathead minnow COX transcripts were synthesised by Sigma Genosys Ltd (Suffolk, UK) and reconstituted with nuclease-free water to a concentration of 100 µM. Primer stocks were diluted with nuclease-free water to a working concentration of 10 µM and stored at -20°C until required. The specificity of the primers was confirmed by PCR (to determine the size of the amplicons) and the generated amplicons were cloned and sequenced using the recombinant cloning method as described in Chapter 2, Section 2.2.7.
Table 17. Forward (F) and reverse (R) primer sequences for putative fathead minnow (FHM) COX 1, COX 2a and COX 2b gene transcripts, melting temperatures (Tm) (°C) and expected amplicon size (bp).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence: 5' -3'</th>
<th>Tm (°C)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHM COX 1 F</td>
<td>TCACTGAATCGAGGGAGCTT</td>
<td>60</td>
<td>159</td>
</tr>
<tr>
<td>FHM COX 1 R</td>
<td>ACACGACCTGGTGCGATATT</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>FHM COX 2a F</td>
<td>CGAAAGAATCTTCCAAACCCT</td>
<td>62</td>
<td>107</td>
</tr>
<tr>
<td>FHM COX 2a R</td>
<td>TCAACGCTTCAGTATCAGAA</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>FHM COX 2b F</td>
<td>GAGAGGCTCTTTCAACCGTA</td>
<td>62</td>
<td>107</td>
</tr>
<tr>
<td>FHM COX 2b R</td>
<td>CGACCGTTTCCAGTACCAGA</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

The putative fathead minnow COX genes (target genes) were normalised to a housekeeping (reference) gene, which was quantified in parallel to the target gene, to account for template DNA variation in the qPCR reaction. These genes are usually cellular maintenance genes that regulate the basic and ubiquitous functions of the cell. β-actin has been widely used as a housekeeping gene in several vertebrate studies, as it is highly conserved. Partial sequences for β-actin (GenBank: EU195887) and 18S RNA genes (GenBank: AY855349.1) have been characterised in the fathead minnow (Filby and Tyler, 2005, 2007). The suitability of three housekeeping genes, β-actin and two 18S ribosomal RNA genes; C18S (no exon boundary) and 3R18 (exon/intron boundary) were tested in this study. The primer sequences and expected amplicon size (bp) are shown in Table 18.
Table 18. Forward (F) and reverse (R) primer sequences for β-actin, C18S and 318S housekeeping genes and expected amplicon size (bp).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence: 5’ -3'</th>
<th>Tm (°C)</th>
<th>Amplicon size (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin F</td>
<td>GAATCCCAAAGCCAACAG</td>
<td>60</td>
<td>148</td>
<td>Filby and Tyler, 2007 (GenBank: EU195887)</td>
</tr>
<tr>
<td>β-actin R</td>
<td>AACACCATCACCAGAGTC</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18S F</td>
<td>AATGTCTGCCCTATCAACTTTTC</td>
<td>60</td>
<td>117</td>
<td>Filby and Tyler, 2007 (GenBank:AY855349)</td>
</tr>
<tr>
<td>C18S R</td>
<td>TGGATGTGGTAGCCGTTTC</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>318S F</td>
<td>CGTCGCCGCTGAATACCGCA</td>
<td>60</td>
<td>160</td>
<td>Primer 3 software (GenBank: AY855349)</td>
</tr>
<tr>
<td>318S R</td>
<td>CTCTCGTCCGTTGTCCGCGG</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.2.3 Performing the Quantitative real-time PCR (qPCR) assay

Quantitative real-time PCR (qPCR) was carried out using the (2 x) QuantiFast SYBR Green PCR kit (QIAGEN, Manchester, UK). Each 25 µL reaction contained 1 µL cDNA (50 ng/µL), 12.5 µL of SYBR Green master mix (containing Taq DNA polymerase, PCR Buffer, dNTP mix and a passive reference dye), 1 µL (0.4 µM) of forward and reverse primers and 9.5 µL nuclease-free water. The reactions were assayed in duplicates (unless otherwise stated) in a 96-well plate (MicroAmp™ Fast optical 96-well reaction plate, Applied Biosystems). As a negative control, nuclease free-water was added to the no-template control (NTC) wells. The plate was sealed using an adhesive film (MicroAmp® Optical adhesive film, Applied Biosystems, UK) and centrifuged at 1,000 x g for 30 seconds at room temperature to collect the components at the bottom of the wells. The plate was then loaded onto an ABI Prism® 7900 HT real-time PCR instrument (Applied Biosystems) and amplification and was carried out using the cycling conditions shown in Figure 41. After the PCR run had been completed, the data were analysed using the configured SDS 2.1 Software (Applied Biosystems). During real-time PCR, the SYBR Green emits fluorescence when bound to double-stranded DNA, which increases with each cycle of amplification. The cycle where the fluorescence (Rn) signal meets a set arbitrary threshold, is known as the ‘cycle threshold’ (Ct) value. Each Ct value is inversely
correlated to the amount of DNA; therefore a high Ct value corresponds to a lower proportion of nucleic acid in the sample.

![Quantitative real-time PCR cycle conditions](image)

**Figure 41.** Quantitative real-time PCR cycle conditions. The Taq polymerase is heat-activated during an initial PCR activation step and template DNA is separated in subsequent cycles of denaturation, followed by combined cycles of primer annealing and extension. A dissociation/melting curve was also performed to determine product specificity.

The SYBR Green dye can generate fluorescence signals when bound to any type of double-stranded DNA, including primer dimers or non-specific products, which can distort the resultant fluorescence readings. A dissociation/melting curve analysis was therefore performed after each PCR run (Figure 41) to determine if the desired product had been amplified. The dissociation/melting curve measures the fluorescence in each well as the temperature is slowly increased from 60-95 °C, allowing the laser of the machine to detect the loss of fluorescence signal as SYBR Green detaches from the denatured DNA. When the temperature reaches the Tm of a DNA product (which is dependent on its length, sequence and GC content), this indicates where there is 50% denaturing of the DNA product and is indicated by a peak. If several peaks are present, this denotes the presence of non-specific products in the sample, as each product has a specific Tm. Therefore, the dissociation/melting curve was used to differentiate between contaminant and product amplification.
5.2.3.1 Data analysis

There are two analytical methods that can be used for the quantification of gene expression in samples: absolute quantification and relative quantification. Both allow quantification of low quantities of input RNA and are equally sensitive. The difference between the two is how the measurements are quantified. In absolute quantification the exact copy number per cell is determined and therefore the expression level is in absolute numbers. In relative quantification, a comparison is made between the expression level of a target gene between two samples, for example a treated sample and an untreated control (Livak and Schmittgen, 2001). These quantitative differences require normalisation with a standard curve (of known mRNA concentration) or another reference (housekeeping) gene, respectively. The latter method was used in this study.

To allow comparison between COX expression in control and exposed fish tissues, relative quantification using the comparative ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001) method was employed. Using this method, firstly the difference between the Ct values ($\Delta Ct$) of the gene of interest (COX) and the house-keeping gene was calculated for each control and exposed tissue sample (A) (‘nomalised’ values). The difference between the normalised $\Delta Ct$ values between the exposed and the control (average of) samples was then used to calculate $\Delta\Delta Ct$ (B). Normalised values were then used to calculate the degree of induction or inhibition in gene expression between the exposed and control samples, expressed as “fold change” ($2^{-\Delta\Delta Ct}$) (C) using Equation 5:

(A) $\Delta Ct = Ct$ (gene of interest) – $Ct$ (house-keeping gene)

(B) $\Delta\Delta Ct = \Delta Ct$ (exposed sample) – $\Delta Ct$ (control sample)

(C) Fold change $= 2^{-\Delta\Delta Ct}$

Equation 5. Calculation used for relative gene quantification using $2^{-\Delta\Delta Ct}$ method.
5.2.3.2 Amplification efficiency (E)

The amplification efficiency (E) of the COX genes and house-keeping gene (β-actin) was determined using the Ct slope method. A 10-fold dilution series (0.1 to 100 ng) of cDNA obtained from fathead minnow gill tissues of SC and ibuprofen-exposed fish were amplified in qPCR reactions using each of the gene primer sets. The Ct values obtained were plotted against the log cDNA concentration (ng). The slope of the linear regression was used to calculate E (expressed as %) using Equation 6:

\[
\text{Amplification efficiency (E) (\%)} = 10^{-\frac{1}{\text{slope}}} - 1 \times 100
\]

Equation 6. Amplification efficiency (E).

5.2.3.3 Statistical Analysis

The results are presented as mean ± SD unless otherwise stated. Data for COX gene expression between ibuprofen treatments were analysed for normality and variance of homogeneity (D'Agostino-Pearson normality test). Where assumptions of normality and homogeneity were met, statistical significance was tested using t-tests or one-way analysis of variance (ANOVA). If equal variance was not met, then a non-parametric test (Mann-Whitney test) was carried out (comparing the median). Statistical significance was set at a level of \( p < 0.05 \), unless otherwise indicated.
5.3 RESULTS

5.3.1 qPCR assay optimisation

5.3.1.1 House-keeping gene expression in fathead minnow tissues

In order to identify a suitable house-keeping gene that was unaffected by treatment, three house-keeping genes, β-actin and two ribosomal 18S RNAs (C18S and 318S) genes were examined in a panel of tissues collected from one control and ibuprofen-treated (500 µg/L for 96 hours) fathead minnow (Figure 42).

Figure 42. Expression of C18S (A), 318S (B) and β-actin (C) house-keeping genes in fathead minnow tissue cDNA (50 ng/µL). Tissues were sampled from one SC and one ibuprofen-exposed (500 µg/L for 96 hours) fish. Ct denotes threshold cycle. Mean (±SD) of two replicates.
The Ct values, for \textit{C18S}, \textit{318S} and \textit{\(\beta\)-actin} did not differ significantly \((p > 0.05,\) using unpaired t test) between control and exposed tissues, demonstrating that these genes were not affected by ibuprofen treatment. As expected, the Ct values for the two ribosomal 18S RNAs genes were much lower than those for \textit{\(\beta\)-actin}, indicating that these genes are abundantly expressed in fathead minnow tissues. However, as the expected Ct values for the target genes were more similar to those of \textit{\(\beta\)-actin} (Ct values \(\geq 20\)), this gene was selected as the most appropriate house-keeping gene for this study. Therefore, \textit{COX} gene expression in control and ibuprofen-treated tissues were normalised to \textit{\(\beta\)-actin} expression.

5.3.1.2 Selection of tissues for qPCR analysis

Due to time constraints and cost-implications of analysing all the sampled tissues, it was decided to select the gill, liver and brain tissues for gene expression analysis only. The reasons for selecting these tissues were: the gill is the primary site for drug uptake from the water; the liver is the main site of metabolism and detoxification of contaminants; and the role of the COX isoforms in brain has not been clearly established, with the recent suggestion that COX 1 is inducible and COX 2 is constitutively expressed in the brain (Breder et al., 1995).

5.3.1.3 \textit{COX} gene primer optimisation and validation

Before proceeding with the gene expression analysis, it was necessary to ensure that the newly designed fathead minnow \textit{COX} gene primer pairs amplified the correct targets of interest. Firstly, the generated amplicons were analysed by agarose gel electrophoresis to ensure that the size corresponded to the product of interest (as described in Section 5.3.1.3.1); a dissociation/melting curve analysis was performed to ensure that a single product was formed (as described in Section 5.3.1.3.2); the identity of the amplicon was assessed by cloning and sequencing (as described in Section 5.3.1.3.3) and the target (and house-keeping) gene primer efficiencies were determined (as described in Section 5.3.1.3.4).

5.3.1.3.1 Determination of amplicon size

To determine if the \textit{COX} primers amplified the correct-sized amplicons in fathead minnow tissues, the qPCR reactions were run on a 1.5% agarose gel and visualised
under UV light. One amplicon was generated in the gill, liver and brain tissues with each of the FHM COX primers sets, corresponding to the correct expected size of 159 bp for FHM COX 1 primers and 107 bp for FHM COX 2a and COX 2b primers (Figure 43).

![Figure 43](image)

**Figure 43.** Amplicons generated from fathead minnow gill, liver and brain tissue cDNA using FHM COX primers. One product was amplified in the cDNA from all three tissues, of the correct expected sized of 159 bp using the FHM COX 1 primers and 107bp for the FHM COX 2a and COX 2b primers. Samples were separated on a 1.5% agarose gel. A 1kb+ DNA ladder (Invitrogen) was used for size determination.

### 5.3.1.3.2 Dissociation/melting curve analysis of amplicons

To confirm that one product had been amplified with each of the FHM primers, a dissociation/melting curve analysis was performed. The analysis revealed, as expected, the amplification of one DNA product with each of the primer sets, as shown in Figure 44. One distinct well-resolved peak was obtained for each of the primers sets, corresponding to a Tm of 80.5 °C for the FHM COX 1 and COX 2b primers, and 82 °C for FHM COX 2a primers. The Tm indicated that the 107 bp products generated by the COX 2a and COX 2b primers were different.
Figure 44. Dissociation/melting curve analysis of the amplified products (in the gill) using the FHM COX 1, COX 2a and COX 2b gene primers. The Tm (°C) of the amplified products were determined to be 80.5 using the COX 1 and COX 2b primers and 82.0 using COX 2a primers (red arrows) as indicated by each distinct peak. There was no indication of non-specific amplification, including primers dimers or secondary structures.

5.3.1.3.3 Confirmation of amplicon identity
To confirm that the primers had amplified the COX genes in the FHM, the amplicon product bands were extracted, cloned and sequenced. The sequenced amplicon products were analysed using BLAST on the NCBI database. The amplicons showed 99%, 100% and 98% identity, respectively with the putative fathead minnow COX 1, COX 2a and COX 2b transcripts (as identified in Chapter 4) as shown in Figure 45. These results demonstrate that the primers had amplified the correct fathead minnow target sequences.
Clustal Omega alignment of generated amplicon sequences obtained using the fathead minnow (FHM) COX gene primers against the putative fathead minnow COX 1, COX 2a and COX 2b gene fragment sequences. Identical residues are indicated by the lines.

**Identity score = 99 %**

**FHM COX 1 Amplicon**

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Sequence</th>
<th>Identity score</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHM COX 1</td>
<td>CTTCTCTAGAAGAAGAGCCTGCGTGAATCTAGTGAATATCGAGGCTGCTC</td>
<td>99%</td>
</tr>
<tr>
<td>FHM COX 1</td>
<td>TAGTTCTTAGGCTTTCTCTCTGTGAAATTCAGAGAGATGTGTTCTCAG</td>
<td>99%</td>
</tr>
<tr>
<td>Amplicon</td>
<td>ATTGAATCTCTCTTAGGCTAATCATTGGAAGGCCTGAGTCG</td>
<td>99%</td>
</tr>
</tbody>
</table>

**FHM COX 2a Amplicon**

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Sequence</th>
<th>Identity score</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHM COX 2a</td>
<td>TTGAAGAGAGGATCCTGGGTCAAATCTGGATGATTGCTGAGATGCTGAG</td>
<td>100%</td>
</tr>
<tr>
<td>FHM COX 2a</td>
<td>TAGTCCTACATCACAGTTTTTAGTTGTGTCTGACCAATCGGATGAG</td>
<td>100%</td>
</tr>
</tbody>
</table>

**FHM COX 2b Amplicon**

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Sequence</th>
<th>Identity score</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHM COX 2b</td>
<td>TTGAAGAGAGGATCCTGGGTCAAATCTGGATGATTGCTGAG</td>
<td>98%</td>
</tr>
<tr>
<td>FHM COX 2b</td>
<td>TAAACCTTCATCCAAATTTTAGTTGTGTCTGACCAATCAGGATGAG</td>
<td>98%</td>
</tr>
</tbody>
</table>

Figure 45. Clustal Omega alignment of generated amplicon sequences obtained using the fathead minnow (FHM) COX gene primers against the putative fathead minnow COX 1, COX 2a and COX 2b gene fragment sequences. Identical residues are indicated by the lines.

5.3.1.3.4 Amplification Efficiency (E) of target and house-keeping gene primers

When using relative gene quantification methods, it is necessary to ensure that the primer efficiencies of the target genes and house-keeping gene are similar. The primer E for each of the target genes and house-keeping gene was determined by plotting the Ct value for each gene against a 10-fold dilution series of fathead minnow gill cDNA (0.1-100 ng) obtained from SC (n=4) and ibuprofen-exposed fish.
(n=4) (Figure 46). Ideally, the expected slope for a 10-fold dilution series of template DNA is -3.32 when the efficiency of the primers is 100%, indicating a doubling of the target DNA with each cycle of PCR. However, in reality, primers are not 100% efficient. The primer efficiencies for the fathead minnow COX genes and β-actin ranged between 83-99% in the control samples (Figure 46. A) and between 88-98% in the exposed samples (Figure 46. B) indicating that there was similar efficiency between the target genes and house-keeping gene, albeit not at 100%. Nevertheless, these findings show that there was very little interference from inhibitors or limiting reaction substrates in the cDNA obtained from fathead minnow tissues.
Figure 46. Linear regression curves and amplification efficiencies (E) of fathead minnow (FHM) COX genes and β-actin primers in gill cDNA from control (A) and ibuprofen-exposed (B) fathead minnow. The Ct value (mean±SD of duplicates) for each primer set were plotted against the log concentration of cDNA (0.1-100 ng). The slope of the curve was used to calculate the E (%) for each gene.
5.3.2 **COX gene expression in fathead minnow tissues**

5.3.2.1 **Ibuprofen exposure to 100, 270 and 500 µg/L for 96 hours**

COX gene expression was examined in the tissues of fathead minnows that had been exposed to SC and ibuprofen water concentrations of 100, 270 and 500 µg/L (for 96 hours). These exposure concentrations were examined because they resulted in mean fish blood plasma concentrations that were below (100 µg/L), above (500 µg/L) or similar to the human therapeutic plasma concentration (Cmax) (270 µg/L) after 96 hours as identified in Chapter 3. Therefore, from the ibuprofen MoA perspective, it was interesting to establish the effects on gene expression at these concentrations.

The mean gene expression level (compared to the SC) for COX 1 was significantly up-regulated \((p < 0.05)\) in the gill at 100 µg/L, in the liver at 270 µg/L and in gill, liver and brain at 500 µg/L. COX 2a was significantly up-regulated \((p < 0.05)\) in the gill at 270 µg/L and in gill, liver and brain at 500 µg/L, whereas COX 2b was significantly up-regulated \((p < 0.05)\) in gill and liver at 100 µg/L, in the gill at 270 µg/L and in gill, liver and brain at 500 µg/L. (Figure 47). Overall, there was a general trend of COX gene up-regulation in exposed tissues; however this was statistically significant \((p < 0.05, \text{ using one tailed non-parametric test})\) in all three tissues in fish exposed to 500 µg/L ibuprofen. These findings demonstrate that at the highest exposure water concentration tested, COX gene expression was consistently elevated above the levels seen in SC fish. To ensure there were no solvent (acetone) effects, the gene expression dataset (of the exposed fish) was also analysed using the DWC fish (data not shown), which also showed a similar pattern of COX gene up-regulation (although the absolute Ct values were not the same).

Although COX gene expression in the tissues of exposed fish appeared to be largely up-regulated (compared to the control group) following ibuprofen exposure, large inter-individual variation was observed (Figure 48). There was down-regulation of COX 1 in the gill at 270 µg/L, COX 2a in the gill and brain at 100 µg/L, in liver and brain at 270 µg/L and COX 2b in the liver at 100 µg/L. The variation in gene expression levels was more apparent in the tissues of fish that had been exposed to 100 and 270 µg/L ibuprofen, compared to 500 µg/L, perhaps explaining why a statistically significant up-regulation was observed at this concentration. Due to the
observed variation it was difficult to establish if there was a concentration-dependent effect following exposure. Therefore, to determine if there was a time-dependent effect, COX gene expression was examined in fish (gill tissue only) exposed to 270 µg/L ibuprofen for 24 and 96 hours. Exposure for 24 hours (at 270 µg/L) did not result in human therapeutic concentrations (Cmax) of ibuprofen (refer to Chapter 3) in fathead minnow blood plasma, however as gene expression may be a sensitive biomarker of exposure, it was interesting to establish if the effects seen at 96 hours were also seen at 24 hours.
Figure 47. COX expression levels (mean ±SD) in the Gill (A), Liver (B) and Brain (C) of fathead minnows (n=4) exposed to 100, 270 and 500 µg/L ibuprofen for 96 hours. Expression levels of the target genes in control and exposed samples were normalised to β-actin, and the exposed samples (n=4) were expressed as fold change relative to the average of the (normalised) SC samples (n=4) (represented as 1). Asterisks indicate a significant difference between exposed samples and control group (p < 0.05 using one tailed non-parametric test).
Figure 48. COX expression levels in the Gill (A), Liver (B) and Brain (C) of individual fish (n=4) exposed to 100, 270 and 500 µg/L ibuprofen for 96 hours. Expression levels of the target genes in control and exposed samples were normalised to β-actin, and the exposed samples were expressed as fold change relative to the average of the (normalised) SC samples (n=4) (represented as 1). The black line within each group indicates the mean.
5.3.2.2 Ibuprofen exposure to 270 µg/L for 24 and 96 hours

COX gene expression in the gill tissues of fish (n=4) exposed to 270 µg/L ibuprofen was up-regulated (except COX 1) compared to the SC group, after 24 hours (Figure 49). COX 1 was significantly down-regulated, whereas COX 2b was significantly up-regulated (both p < 0.05 using one tailed non-parametric test) from the SC fish following exposure. COX 2a was up-regulated at 24 hours, but was not statistically different from the SC group. COX expression levels were also elevated in the gills of fish exposed to 270 µg/L ibuprofen after 96 hours, although statistically significant only for COX 2a and COX 2b genes (both p < 0.05). A clear time-dependent effect was observed between gene expression levels at 24 and 96 hours (Figure 49). However, only COX 2b was statistically significantly up-regulated (p < 0.05) in the gill between 24 and 96 hours. These results suggest that the length of exposure has an effect on COX gene expression levels.

![Gill 24 & 96 Hours](image_url)

Figure 49. COX expression levels (mean ±SD) in the gill of fathead minnows (n=4) exposed to 270 µg/L ibuprofen for 24 and 96 hours. Expression levels of the target genes in control and exposed samples were normalised to β-actin, and the exposed samples (n=4) were expressed as fold change relative to the average of the (normalised) SC samples (n=4) (represented as 1). The letter a) indicates a significant difference between exposed samples and control group and b) indicates a significant difference between exposed samples at 24 and 96 hours (p < 0.05 using one tailed non-parametric test).
5.4 DISCUSSION

In this chapter, the effect of ibuprofen exposure on putative COX transcripts was examined using quantitative real-time PCR (qPCR). The main finding from this study was that ibuprofen could alter the transcriptional level of the putative COX genes (identified in Chapter) in fathead minnow tissues following exposure to 100, 270 and 500 µg/L (after 96 hours).

5.4.1 COX gene expression

The COX genes code for the COX enzymes which are competitively inhibited by ibuprofen. The mean gene expression level of three putative COX genes were upregulated in fathead minnow tissues following exposure to 100, 270 and 500 µg/L (Figure 47), however, the expression levels were highly variable in individuals within the same exposure group (Figure 48). The putative COX genes were significantly elevated ($p < 0.05$) in the gill, liver and brain tissues of fish exposed to 500 µg/L ibuprofen, compared to SC group (after 96 hours). In contrast to the findings in this study, no transcriptional changes in COX gene expression levels were reported in zebrafish exposed to comparable water ibuprofen concentrations of 21, 201 and 506 μg/L (after 7 days) (Morthorst et al., 2013) and COX gene expression levels were not altered in fish exposed to environmentally relevant concentrations (0.1 and 1 µg/L) of ibuprofen (Ji et al., 2013), indicating that the data in the present study are contrary to the findings in the literature. As a possible explanation for the elevated transcriptional levels of the putative COX genes in the fathead minnow, we therefore propose that a compensatory feedback mechanism (gene up-regulation) may be occurring as a response to COX enzyme inhibition, following ibuprofen exposure to 100, 270 and 500 µg/L (after 96 hours). This proposed explanation is rather speculative, however at a recent SETAC Europe 24th Annual Meeting (Basel, Switerland) there were reports of elevated COX gene expression levels in inland silversides (Menidia beryllina) exposed to ibuprofen concentrations of 0.025 mg/L, 0.25 mg/L and 2.5 mg/L (after 14 days) (personal communication).

In the studies conducted by Morthorst et al., (2013) and Ji et al., (2013), the gene expression analysis was conducted in the sex (ovary) tissues, which may have differential COX expression to the tissues (gill, liver and brain) examined in this
study. The primary site of drug uptake into fish is via the gills (Owen et al., 2007), and in this study the COX genes were significantly up-regulated in this tissue at all three tested exposure concentrations, indicating that the metabolic activity in the gills may be different to that in the liver and brain tissues (Gomez et al., 2010). Whitehead and Crawford (2005) found that over 48% of 192 metabolic genes examined in brain, heart and liver in three populations of the teleost fish mummichog were differentially expressed (Whitehead and Crawford, 2005) suggesting that tissue-specific patterns of gene expression can be highly variable within the same species.

There was no clear concentration-dependent effect between ibuprofen exposure and elevated COX gene expression levels in exposed fish, due to the large inter-individual variation observed. Gene expression is a highly sensitive response biomarker, and therefore a variety of factors can influence transcriptional levels among individuals raised under controlled laboratory conditions. Possible explanations for the observed variability in this study could have been due to genetic variation, for example, variations in drug-metabolising (CYP) enzymes and changes in stress responses (i.e. cortisol synthesis) may also affect drug metabolism in fish (Gravel et al., 2009; Gravel and Vijayan, 2007; Heckmann et al., 2008). The gene expression levels were more variable in the fish that had been exposed to 100 and 270 µg/L ibuprofen (for 96 hours), compared to 500 µg/L. One possible explanation for this could be that at lower ibuprofen concentrations the fish may be able to metabolise the drug, whereas at 500 µg/L the fish may have been “overcome” by the high exposure concentration. Therefore, the compensatory up-regulation would most likely be dependent on individual sensitivity to ibuprofen and/or ability to metabolise the drug. Furthermore, the relatively small sample size (n=4 for each tissue) could also have contributed to the variation, which could be reduced by increasing the sample size.

Another factor that can contribute to variation is the time point at which expression levels are examined. A time-dependent effect was seen in the gill tissues of fish exposed to 270 µg/L after 24 and 96 hours. COX 2b expression levels were significantly up-regulated in the gills of exposed fish between 24 and 96 hours. In Atlantic salmon, high levels of COX 2b gene expression has been reported in the gill
after acute stress (Olsen et al., 2012). Considering that in this study, the fish were not fed during the exposure period, it is possible that by 96 hours the fish were under a substantial amount of stress, and the elevated COX 2b expression following ibuprofen exposure could be related to a potential role for COX 2b in stress mechanisms following toxicant exposure, although this has not been confirmed.

Transcriptomic changes identified using microarray datasets have shown that the hepatic expression of the COX 1 and COX 2 genes were also significantly down-regulated in the rainbow trout exposed 1.6 µg/L diclofenac (after 14 days) (Cuklev et al., 2011). Genes can be differentially regulated after the onset of continuous drug exposure and down-regulation of the COX genes reported by Cuklev et al., 2011 may have been due to a stabilised long-term response, whereas in this study, gene expression levels after 4-days of drug exposure may be more indicative of an early phase response. Similarly, a down-regulation of the COX genes was also reported in rainbow trout exposed to (0, 0.5, 1, 5, and 25 µg/L) diclofenac (after 21 days) (Mehinto et al., 2010), however, the findings from this study could not be verified as the primers used in the study, when analysed using BLAST, did not show any similarity to the cyclooxygenase genes, but instead showed a high level of similarity to cytochrome c oxidase gene sequences, also abbreviated as “cox”. However, it is unknown if the primer sequences were incorrectly printed in the supplementary information provided. Nevertheless, this issue could have been avoided if the identity of the amplicons generated had been confirmed by cloning and sequencing methods. This highlights a serious issue with the use of highly sensitive gene expression analysis studies and the potential for misinterpretation of the results.

These findings suggest that gene expression, of individual genes at least, may not be the most appropriate endpoint for elucidating the molecular mechanisms of toxicant exposure in fish, due to the difficulties in interpreting gene expression data. Nevertheless, the potential for genomic approaches in predictive ecotoxicology has already been demonstrated, primarily through the identification of gene targets and regulatory pathway representing mode of action-specific patterns for endocrine disruptors in fish (Schiller et al., 2013; Scholz and Mayer, 2008; Wang et al., 2010). However, often chemicals may display multiple modes of action, or they can have
several interactions at the gene level, which can produce differential effects that can vary even from tissue to tissue (Wang et al., 2012). Therefore, further knowledge is required to understand the full extent of xenobiotic exposures and their impact on the aquatic fauna and flora.

### 5.4.2 qPCR Validation

A fundamental element in the interpretation of gene expression data is the validation of the assay. Aside from biological variability, technical variability can also contribute to highly variable results, for example, through inadequate sample preparation, poor selection of the target gene and house-keeping (reference) gene primers leading to inefficient and sub-optimal assay performance; and inappropriate analysis of the data leading to misinterpretation of the findings, as addressed by the MIQE guidelines, that provides the ‘minimum information for publication of quantitative real-time PCR experiments’ (Bustin et al., 2009). In this study, the primers used for amplification of the COX target genes in fathead minnows were designed using the nucleotide sequences of the putative fathead minnow COX gene fragments that were identified as described in Chapter 4 (as the genome sequence for this species has not been published). To ensure that these primers had amplified the correct targets, the amplicons were cloned and sequenced, to verify the identity of the product, which is one method that could have been used by Mehinto et al., (2010) to confirm that the target COX genes had been differentially regulated in rainbow trout following diclofenac exposure (as opposed to the cytochrome c oxidase genes, based on the primer sequences provided in the study). There was no significant difference \((p > 0.05, \text{using unpaired t test})\) between the expression levels of the three house-keeping genes \((\beta\text{-actin}, C18S \text{ and } 318S)\) in the control and ibuprofen-treated tissues. \(\beta\text{-actin}\) is highly conserved protein involved in cell motility, structure and integrity in vertebrates and aquatic species, and the suitability of this gene as a house-keeping gene for normalisation (following ibuprofen exposure) has been demonstrated elsewhere (Gravel et al., 2009; Gravel and Vijayan, 2007; Heckmann et al., 2006; Ji et al., 2013). However, there are several conflicting studies on the suitability of different house-keeping genes in gene expression studies using fish (McCurlery and Callard, 2008; Filby and Tyler, 2007; Jorgensen et al., 2006; Olsvik et al., 2005;
Tang et al., 2007), and therefore some caution must be applied when using reference genes that have not been correctly validated.

In summary, the transcription of the COX genes in fathead minnow tissues was elevated following ibuprofen exposure, however, the gene expression levels in exposed fish tissues were highly variable. This was most likely due to the sensitivity of this endpoint, and therefore more biological replicates are required to allow further interpretation of these data.
CHAPTER 6: THE EFFECT OF IBUPROFEN EXPOSURE ON CYCLOOXYGENASE (COX) ENZYME ACTIVITY AND PROSTAGLANDIN E₂ CONCENTRATIONS
6.1 INTRODUCTION

The MoA of ibuprofen is through the inhibition of the COX 1 and 2 enzymes, which are the key enzymes involved in the conversion of arachidonic acid to prostanoids (Vane, 1971). COX 1 is constitutively expressed in a variety of cell types and is involved in maintaining “homeostatic” functions, whereas COX 2 can be induced in response to mitogenic stimuli, such as phorbol esters, lipopolysaccharides, and cytokines (Simmons et al., 2004; Xie et al., 1991). During pain and inflammation, COX 2 can be rapidly induced which increases the biosynthesis of prostanoids, primarily, prostaglandin E$_2$ (PGE$_2$). PGE$_2$ has been identified as a major prostanoid subtype in a number of teleost fish including zebrafish (Grosser et al., 2002; Ishikawa et al., 2007), rainbow trout (Knight et al., 1995) and bluntnose minnows (Bhandari and Venables, 2011), suggesting that human drug targets are conserved in aquatic species. Prostanoids and their receptors have been implicated in several physiological functions in fish, such as reproduction (Fujimori et al., 2011; Sorbera et al., 2001), stress responses and cortisol synthesis (Gravel and Vijayan, 2007), osmoregulation and ion transport (Choe et al., 2006; Gravel et al., 2009), suggesting that changes in prostanoid levels may have widespread effects in fish. Prostanoid inhibitors, such as ibuprofen and indomethacin can inhibit the PGE$_2$ concentrations in zebrafish (Lister and Van Der Kraak, 2008; Morthorst et al., 2013).

Ibuprofen is frequently detected (typically ng to low µg/L range) in rivers and surface waters, suggesting that there is potential for COX inhibitors to perturb physiological processes in aquatic vertebrates, presumably through the inhibition of COX and prostanoids. According to the read-across hypothesis, target-mediated pharmacological responses similar to those seen in humans, may occur in non-target organisms, provided that there has been sufficient drug exposure and similar blood plasma concentrations are reached. The purpose of this chapter was to examine the effect of ibuprofen exposure on fathead minnows using endpoints that are relevant to the MoA of ibuprofen in humans i.e. COX enzyme activity and prostanoids, to determine if these are sensitive endpoints for ibuprofen exposure in fish.

COX enzyme activity was previously measured in a preliminary study (data not shown) in the tissues of fathead minnow that had been exposed to SC and ibuprofen.
The outcome of this work showed that it was difficult to determine COX enzyme activity in “healthy” fish, because COX activity under normal physiological conditions is generally low (at basal levels, assuming no underlying disease) in fish. However, COX (2) is an inducible enzyme, which can be rapidly up-regulated in response to pain stimulus and local inflammation (Simmons et al., 2004), and is believed to be the target enzyme for the anti-inflammatory activity of NSAIDs (Vane, 1971). Therefore, the first aim of this chapter was to design an exposure study whereby COX enzyme activity in fish could be elevated above a basal level, and changes in enzyme activity could then be more easily quantified in SC or ibuprofen-treated fish. This exposure study incorporated a “tail-fin clipping” procedure as a means to induce COX activity above basal levels, and the fish were then treated with SC or ibuprofen to determine the effect of exposure on fish tissues. Fathead minnows were exposed to a water concentration of 270 µg/L as this exposure concentration resulted in a mean plasma concentration in fish that was similar to the Cmax (as identified in Chapter 3).

The second aim of this chapter was to examine the effect of ibuprofen exposure on the products of COX enzyme activity, primarily prostaglandin E2. In order to address this aim, one larger exposure study was designed (n=60) where fathead minnows were exposed to one exposure water concentration of 350 µg/L (instead of 370 µg/L, as identified in Chapter 3), as it was expected that plasma ibuprofen concentrations in fish closer to the Cmax would be reached, and is a requirement in order to test the read-across hypothesis. As any potential effects elicited in fish are expected to occur only at drug (ibuprofen) plasma concentrations similar to human therapeutic concentrations, a smaller follow-up (preliminary) exposure study was also conducted where fish were exposed to 5 and 350 µg/L ibuprofen, in order to determine if the read-across hypothesis could be validated by using a more environmentally-relevant exposure water concentration. A maximal ibuprofen surface water concentration of 5 µg/L has been previously reported in the UK (Ashton et al., 2004) and therefore this concentration was used as a guide in this study. At 5 µg/L, it was not expected that human therapeutic plasma concentrations of ibuprofen could be reached in fish and therefore, no effects were expected at this water concentration.
6.2 METHODS
The full details of the materials and methods used are provided in Chapter 2, and the following contains details not covered elsewhere. This section is divided into three main subsections; firstly covering the fish exposure studies conducted, including the design of experiment used to “induce” COX enzyme activity in tissues, and the two exposure studies used to determine the effect of ibuprofen exposure on prostaglandins (see Section 6.2.1). The second and third sections cover assays used for the measurement of COX enzyme activity (Section 6.2.2) and measurement of prostaglandin metabolites in fathead minnow tissues (Section 6.2.3).

6.2.1 Fish exposures

6.2.1.1 “Tail-fin clipping” and continuous ≤120 flow-through exposure to 270 µg/L ibuprofen

Adult fathead minnows (n=60) were exposed for ≤120 hours, using a continuous flow-through system to (DWC, n=15) SC (x 2) (acetone) (n=30) and a nominal ibuprofen water concentration of 270 µg/L (n=15) (Chapter 2, Section 2.1.5, Table 6; ‘Exposure 4’). Fathead minnows were exposed for 72 hours to two SC treatments (depicted as SC-tank 1 and SC-tank 2 in Figure 50) or 270 µg/L ibuprofen (depicted as 270 µg/L-tank 1 in Figure 50). After 72 hours of exposure, n=5 fish from SC-tank 1, SC-tank 2 and 270 µg/L-tank 1 were terminated and tissues (gill, liver and muscle) were collected and stored according to the method described in Chapter 2, Section 2.1.7. Exposure water samples were collected from each tank, just before the fish were sampled. The blood plasmas of control and treated fish were also collected before and after tail-clipping, but measured plasma concentrations are not presented in this chapter (see Chapter 7).
Figure 50. Experimental design of the 120 hour continuous flow-through study for analysing the effect of “tail-fin clipping” on COX enzyme activity in fathead minnows. Fish (n=15) were exposed for 72 hours to solvent control tanks (SC-tank 1 and SC-tank 2) or ibuprofen (270 µg/L-tank 1). After 72 hours n=5 fish were sampled (red markers). After 92 hours of exposure, n=10 fish were removed, anesthetised and a small surgical incision was made to upper tail-fin of each fish (green markers). The fish in SC-tank 1 and 270 µg/L-tank 1 were placed back into their respective tanks. However, the fish in SC-tank 2 were transferred into a fresh ibuprofen tank (270 µg/L-tank 2). After tail-clipping, fish n=5 were sampled at 96 and 120 hours from SC-tank 1, 270 µg/L-tank 1 and 270 µg/L-tank 2 (black markers).

After 92 hours of exposure, the remaining fish (n=10) from SC-tank 1, SC-tank 2 and 270 µg/L-tank 1 were removed one at a time, and transferred into a large beaker containing a non-lethal dose of MS-222 (250 mg/L, buffered with NaHCO₃ to pH 7.4) and each fish was carefully monitored until loss of balance was seen. Each fish was then removed one at a time, placed on a dissection board and a small section (<1/3rd) of the upper caudal fin was surgically removed using a scalpel in order to cause injury and subsequently induce COX activity in the fish (without impairing complete movement). Each fish was then placed into a bucket of aerated dilution water and monitored until balance was regained. Fish from the SC-tank 1 and 270 µg/L-tank 1 treatments were subsequently returned to their respective tanks. However, the fish in the SC-tank 2 were transferred to fresh tank also containing a nominal water concentration of 270 µg/L ibuprofen (depicted as 270 µg/L-tank 2 in
Chapter 6 Results

Figure 50). After tail-clipping had been performed, fish were further exposed to SC or 270 µg/L ibuprofen, and n=5 were subsequently terminated after 4 and 24 hours (depicted as 96 and 120 hours, respectively in Figure 50), and the blood plasmas and gill, liver and muscle tissues were collected, as described in Chapter 2, Section 2.1.7. Water samples from the SC and ibuprofen tanks were collected and analysed after 72, 96 and 120 hours of exposure, as described in Chapter 2, Sections 2.1.8-2.1.9.

6.2.1.2 Continuous 72 hour exposure to 350 µg/L ibuprofen
Adult fathead minnows (n=60) were exposed for 72 hours, using a continuous flow-through system, to (DWC, n=10), SC (n=10) and a nominal ibuprofen water concentration of 350 µg/L (n=40) (Chapter 2, Section 2.1.5, Table 6; ‘Exposure 5’). After 72 hours exposure all fish were terminated and the gill tissues were collected according to the method described in Chapter 2, Section 2.1.7. Exposure water samples were collected from each tank, just before the fish were sampled. The blood plasmas of control and treated fish were also collected (after 72 hours), but the measured plasma concentrations are not presented in this chapter (see Chapter 7, for plasma ibuprofen concentrations and target-mediated effects).

6.2.1.3 Static ≤72 hour exposure to 5 and 350 µg/L ibuprofen
In a smaller static exposure, adult male fathead minnows (n=45) were exposed for ≤72 hours to SC (n=9) and nominal ibuprofen water concentrations of 5 (n=18) and 350 µg/L (n=18) (Chapter 2, Section 2.1.5, Table 6; ‘Exposure 6’). After 24, 48 and 72 hours of exposure, fish (n=3 from SC, and n=6 from 5 and 350 µg/L treatments) were terminated and the gill tissues were collected according to the method described in Chapter 2, Section 2.1.7. Exposure water samples were collected from each tank, just before the fish were sampled. The blood plasmas of control and treated fish were also collected (after 24 and 72 hours), but the measured plasma concentrations are not presented in this chapter (see Chapter 7, for plasma ibuprofen concentrations and target-mediated effects).

6.2.2 Measurement of cyclooxygenase (COX) enzyme activity in fathead minnow tissues
COX is a bifunctional enzyme that exhibits both cyclooxygenase and peroxidase activities. Enzyme activity was measured in fish tissues using the COX Activity
Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer’s protocol. This assays utilises the peroxidase activity of COX, which can be easily quantified through an oxidation reaction of a colourimetric substrate, N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD). TMPD produces a yellow chromophore when oxidised that can be measured by spectrophotometry at 590 nm (Kulmacz and Lands, 1983).

6.2.2.1 Sample preparation
Fish tissues (up to 20 mg of each) were placed into pre-labelled 2.0 mL micro-centrifuge tubes and were promptly disrupted in cold buffer (0.1 M Tris-HCl, pH 7.8, 1 mM EDTA) using a tissue lyser (Tissue Lyser II, QIAGEN, Manchester, UK). The lysed samples were centrifuged at 10,000 x g for 15 minutes at 4 °C and the supernatants were transferred into clean 1.5 mL micro-centrifuge tubes and kept on ice. The supernatant from each tissue was divided into two aliquots. One aliquot was used as the ‘active’ sample, whilst the other was boiled in water for 5 minutes to destroy all enzyme activity, to produce an ‘inactive’ sample. After boiling, the samples were centrifuged at 8,000 x g for 1 minute at room temperature and the supernatant was used to generate a background value for each sample. The background value generated from the ‘inactive’ sample was used as a corresponding control for the ‘active’ tissue sample.

6.2.2.2 Performing the colourimetric assay
All reagents were either supplied in a ready-to-use format or were diluted according to the protocol on the day of the assay. All reactions were assayed in triplicate using the supplied 96-well plate. Firstly, 120 μL of assay buffer (100 mM Tris-HCl, pH 8.0) used as a dilution buffer and 10 μL heme (prepared by diluting 88 μL of the provided heme solution with 1912 μL of assay buffer) were added to each well. This was followed by the addition of 40 μL of the ‘active’ sample and 40 μL of the ‘inactive’ sample (for each tissue) to their respective wells. The plate was carefully shaken and incubated at 25 °C for 5 minutes. After incubation, 20 μL of TMPD (colourimetric substrate) was added to each sample and the reactions were initiated by the addition of 20 μL of arachidonic acid solution (primary substrate). The final volume in each well was 210 μL. The plate was carefully shaken and then incubated
at 25 ºC for 5 minutes. The optical density (OD) was determined by spectroscopy at 590 nm (xMark Microplate Absorbance Spectrophotometer, Bio-Rad, Hertfordshire, UK). Ovine COX 1 was supplied in the assay as a positive control, which produces an absorbance of 0.28 (at 590 nm) under standard assay conditions. The LOD of total COX activity was specified to be between 13-63 Units/mL (U/mL) using this assay. Wild-type mouse lung tissue obtained from Dr Pook’s group, Brunel University, was used as an additional positive control to ensure that the sample preparation and assay had been correctly performed.

### 6.2.2.3 Data analysis

Total COX activity for each tissue was initially calculated in Units/mL (U/mL) based on the reaction rate at 590 nm, as determined by the extinction coefficient of TMPD (0.00826 µM⁻¹). However, these units were normalised to the protein content (mg) in the homogenised tissue supernatants, as described in Section 6.2.4, and the results were presented in U/mg of protein. One unit of COX activity was defined as the amount of enzyme required to oxidise 1 nmole of TMPD per minute at 25 ºC, assuming a stoichiometry 2 molecules of TMPD oxidised per every molecule of PGG₂ reduced to PGH₂ (common intermediate formed from arachidonic acid, and the precursor for prostanoids). Total COX activity was calculated using Equation 7:

\[
\text{Total COX Activity} = \frac{\Delta A_{590} / 5 \text{ minutes}}{0.00826 \mu M} \times \frac{0.21 \text{ mL}}{0.04 \text{ mL}} \div 2^* = \text{nmol/min/mL (U/mL)}
\]

*It takes two molecules of TMPD to reduce PGG₂ to PGH₂.*

Where:
- \(\Delta A_{590}\) = Corrected ('inactive' - 'active' sample) absorbance (nm) for the oxidised substrate (TMPD)
- 5 minutes = Incubation period
- 0.00826 µM⁻¹ = Extinction coefficient of TMPD
- 0.21 mL = Total volume in well (210 µL)
- 0.04 mL = Sample volume (40 µL)

**Equation 7. Total COX Activity.**
6.2.3 Measurement of Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) metabolites (PGEM) in fathead minnow tissue

Prostanoids are highly unstable biological mediators, and often human and animal tissues contain low amounts of circulating PGE\textsubscript{2}, as this prostanoid is rapidly converted \textit{in vivo} to its 13,14-dihydro-15-keto PGE\textsubscript{2} metabolite (Ferreira and Vane, 1967), which can further undergo variable amounts of degradation to form other metabolites such as 13,14-dihydro-15-keto PGA\textsubscript{2}. Therefore, the determination of PGE\textsubscript{2} metabolites in a sample is potentially a more reliable method than measuring actual circulating PGE\textsubscript{2} (Figure 51). In this study, PGE\textsubscript{2} metabolites (PGEM) were quantified by enzyme immunoassay (EIA) using the Prostaglandin E Metabolite EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA). This assay converts both 13,14-dihydro-15-keto PGE\textsubscript{2} and 13,14-dihydro-15-keto PGA\textsubscript{2} metabolites into a single, stable derivative (bicyclo PGE\textsubscript{2}) that can be more easily quantified, to provide an estimate of the PGE\textsubscript{2} present in the sample. This assay has 100% cross-reactivity with 13,14-dihydro-15-keto PGE products and 38% with bicyclo prostaglandin E.

The PGEM enzyme immunoassay utilises the enzyme acetylcholinesterase (AChE). PGEM is covalently attached to a molecule of AChE to form a PGEM-AChE conjugate, which serves as the tracer (PGEM tracer) in the EIA. Briefly, the principle of this assay is based on the competition between the amount of PGEM in a sample and the PGEM tracer, for a limited number of PGEM-specific rabbit antiserum binding sites. The concentration of the PGEM tracer is constant, whilst the concentration of PGEM varies, and therefore the amount of PGEM tracer that is able to bind to the rabbit antiserum is inversely proportional to the concentration of PGEM in the sample. This rabbit antiserum-PGEM (either free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG antibody that has been attached to each well of the 96-well plate. Finally, Ellman’s reagent that contains the substrate to AChE is added to each well, which can be measured by spectrophotometry at 415 nm (Cayman Chemical Company, Ann Arbor, MI, USA).
Figure 51. Schematic diagram of the metabolism of PGE\(_2\) to a single stable derivative that can be quantified using the Prostaglandin E\(_2\) (PGE\(_2\)) metabolite (PGEM) enzyme immunoassay (EIA) assay (adapted and redrawn from the protocol provided by Cayman Chemical Company, Ann Arbor, MI, USA).
6.2.3.1 Sample preparation

Gill tissues (20 mg) were placed into pre-labelled 2.0 mL micro-centrifuge tubes and were promptly lysed in buffer (0.1 M phosphate, pH 7.4, 1 mM EDTA containing 10 μM indomethacin to prevent ex vivo formation of prostaglandins) using a tissue lyser (Tissue Lyser II, QIAGEN, Manchester, UK). The lysed samples were centrifuged at 8,000 x g for 10 minutes at 4 °C. The supernatants were transferred into clean 1.5 mL micro-centrifuge tubes and were purified by acetone precipitation. Four volumes of ice-cold acetone (Sigma-Aldrich, Dorset, UK) were added to each sample, which were then incubated at -20 °C for 60 minutes. The mixtures were centrifuged at 400 x g for 5 minutes at room temperature to pellet the proteins and the supernatants were subsequently transferred to clean 2.0 mL micro-centrifuge tubes. The acetone was evaporated under a stream of nitrogen to concentrate the sample. The samples were re-suspended in the supplied EIA buffer (1 x) (0.1 M phosphate solution, 0.1% bovine serum albumin [BSA], 0.4 M NaCl2, 1 mM EDTA and 0.01% sodium azide). The samples were then prepared for derivatisation.

6.2.3.2 Derivatisation to yield metabolites

The PGEM standard solution (supplied in the assay), and tissues samples were mixed with 1 M carbonate buffer and incubated at 37 °C overnight, to allow PGE2 metabolites to be converted to one single stable derivative that could be more easily measured. After incubation, 1 M phosphate buffer and EIA buffer was added to the samples and the standard (1000 pg/mL). The average curve regression coefficient (r2) was 0.9559.

6.2.3.3 Performing the enzyme immunoassay

Prior to the start of the assay, an 8-point standard curve was prepared by serial dilution of the standard using EIA buffer to provide a calibration curve ranging from 0.39-50 pg/mL. The average curve regression coefficient (r2) was 0.9559. From a preliminary assay with different dilutions of samples, it was decided that the SC tissue samples would be diluted 10-fold and the ibuprofen-treated samples diluted by 5-fold, using an appropriate amount of PGEM assay buffer (prepared by adding 13 mL of EIA buffer, 3 mL carbonate buffer [1 M] and 4 mL phosphate buffer [1 M]) in order to keep the absorbance values within the working range of the assay.
All standards and samples were assayed in duplicate. For each plate, wells were assigned for non-specific binding (NSB) (used to determine the binding of the tracer to the well), total activity (TA) (used to determine the total enzymatic activity of the AChE-linked tracer), maximum binding (B0) (used to determine the maximum amount of the tracer that the antibody can bind with) and blanks (used to determine the absorbance caused by Ellman’s reagent alone). 50 μL of the prepared standards (1-8) and diluted samples were pipetted into the designated wells (except for NSB, TA, B0 and blank wells). 50 μL of EIA buffer was pipetted into the NSB wells only and then 50 μL of PGEM assay buffer was pipetted into NSB and B0 wells. This was followed by the addition of 50 μL of PGEM tracer to each well except the TA and blank wells and then 50 μL of antiserum was pipetted into each well, except the TA, NSB and blank wells. The plate was sealed with an adhesive film and incubated for 18 hours at room temperature. After the incubation period, the contents of the wells were emptied, and the plate was washed five times with wash buffer (5 mL of 400 x concentrate, diluted to 2 L with water and 1 mL of Polysorbate-20 was added). After the final wash, the wells were emptied, and the plate was firmly tapped on a lint-free paper towel to remove any remaining wash buffer. 200 µL of the substrate Ellman’s reagent was added to each well and 5 µL of PGEM tracer was added to the TA well. The plate was sealed and placed on an orbital shaker (in the dark) for 90 minutes at room temperature to facilitate colour development. The OD was determined by spectrophotometry at 415 nm (xMark Microplate Absorbance Spectrophotometer, Bio-Rad, Hertfordshire, UK).

6.2.3.4 Data analysis
The average absorbance value of the blank wells was subtracted from the average absorbance values (of duplicate) samples and standards to account for background values caused by the Ellmans’ reagent. The average NSB value (amount of tracer bound to the well) was subtracted from the B0 (maximum amount of the tracer available) to obtain the corrected B0. For the standard wells and sample wells, the standard bound or sample bound tracer, over the maximum amount of tracer available (B/B0) was calculated. A standard curve was prepared using a logit transformation of B/B0 versus log concentration of the PGEM standard (pg/mL). A linear regression was performed through the calibration points, and the PGEM
concentration in the samples was determined from the standard curve. These units were normalised to the protein content (mg) in the homogenised tissue supernatants, as described in Section 6.2.4, and the results were presented in pg/mg of protein.

6.2.4 Protein determination in tissues
Total protein concentration in tissue samples was determined using the QuantiPro™ bicinchoninic acid (BCA) assay (Sigma-Aldrich, Poole, UK), according to the manufacturer’s protocol. This assay depends on the reduction of Cu$^{2+}$ to Cu$^{1+}$ by proteins under alkaline conditions, and the subsequent formation of a stable bicinchoninic acid and Cu$^{1+}$ colourimetric complex that can be measured using a spectrophotometer. For protein quantification, a standard curve was prepared using bovine serum albumin (BSA) standard (1.0 mg/mL) by serial dilution using nuclease-free water to produce a calibration range between 0.5-30 μg/mL. BCA assay working reagent sufficient for a 96-well plate were prepared. Tissues samples were diluted (1:20 using nuclease-free water) and assayed in duplicate using a 1:1 ratio of protein solution to BCA assay working reagent. The plate was incubated at 37 °C for 2 hours and the OD was determined by spectrophotometry at 562 nm (xMark Microplate Absorbance Spectrophotometer, Bio-Rad, Hertfordshire, UK). A linear regression was performed through the calibration points, and protein concentration (mg/mL) in the tissue samples were determined from the standard curve.

6.2.5 Statistical analysis
The results are presented as mean ± SD, unless otherwise stated. Data for COX activity and PGEM were analysed for normality and variance of homogeneity (D'Agostino-Pearson normality test). Where assumptions of normality and homogeneity were met, statistical significance was tested using t-tests or one-way ANOVA followed by a multiple comparison test (Holm-Sidak or Tukey’s test). Statistical significance was set at a level of $p < 0.05$, unless otherwise indicated.
6.3 RESULTS

6.3.1 Water concentrations of ibuprofen

Fathead minnows were exposed to ibuprofen in three separate exposure studies, two were conducted using continuous flow-through systems to 270 µg/L (over 120 hours) and 350 µg/L ibuprofen (over 72 hours), and one smaller static exposure study was conducted for 5 and 350 µg/L ibuprofen (over 72 hours). To compare the dosing systems used, the actual water concentrations of ibuprofen was quantified and compared to the nominal test concentrations (Table 19).

The mean measured (± SD) water concentrations of ibuprofen (over 120 hours) in 270 µg/L-tank 1 was 214 ± 102 µg/L and in tank 2 (that received fish after the tail-clipping procedure) was 227 ± 57 µg/L. In the 350 µg/L exposure tank (over 72 hours), the mean measured concentration was 368 ± 4 µg/L. The time-weighted average ibuprofen concentration in water samples in these tanks were 80, 84 and 105% of the nominal exposure concentrations, respectively, indicating that overall the exposure concentrations achieved using the continuous flow-through systems were within an acceptable range (± 20% of the expected ‘nominal’ concentration). However, the measured concentration in 270 µg/L- tank 1 at 120 hours was much lower than expected i.e. 36% of nominal, and in the 270 µg/L- tank 2 at 72 hours the measured concentrations was 66% of nominal. This very low measured concentration at 120 hours was due to a blockage at the end of dosing line, caused by build-up of test substance toward the end of the exposure period. The mean measured water concentrations following static exposures to 5 and 350 µg/L (over 72 hours) were 9 ± 1 µg/L and 473 ± 9 µg/L, and these concentrations were 181 and 135% of the nominal exposure concentrations, respectively. The measured concentrations at 5 µg/L were consistently high and were approximately double the concentration of the nominal. These findings show that ibuprofen dosed using continuous flow-through systems were closer to the nominal than those achieved using the static system, although it is unclear why this is the case. The measured concentrations in the 350 µg/L exposure tanks were 368 ± 4 µg/L and 473 ± 9 µg/L over 72 hours, using the continuous flow-through and static systems, respectively. For these reasons, in this chapter, the measured water concentrations of ibuprofen of 220 µg/L (average of
tank 1 and tank 2), 370 µg/L, 9 µg/L and 470 µg/L will be used (instead of the nominal concentrations).

Table 19. Measured concentrations of ibuprofen (µg/L) in exposure tank waters in three different exposure studies, using continuous flow-through (270 and 350 µg/L) and static systems (5 and 350 µg/L). Ibuprofen measurements in the waters of the DWC and SC tanks were below the LOD (<2.5 µg/L).

<table>
<thead>
<tr>
<th>Nominal exposure concentration µg/L</th>
<th>No. of exposure hours</th>
<th>Measured water concentration µg/L</th>
<th>% Nominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous ≤120 flow-through exposure to 270 µg/L ibuprofen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>270 (tank 1)</td>
<td>72</td>
<td>276</td>
<td>102</td>
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<tr>
<td>270 (tank 1)</td>
<td>96</td>
<td>271</td>
<td>100</td>
</tr>
<tr>
<td>270 (tank 1)</td>
<td>120</td>
<td>96</td>
<td>36</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>214 ± 102</td>
<td>80</td>
<td></td>
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<tr>
<td>Continuous 72 hour exposure to 350 µg/L ibuprofen</td>
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<td></td>
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<tr>
<td>350</td>
<td>24</td>
<td>363</td>
<td>104</td>
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<td>350</td>
<td>48</td>
<td>370</td>
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<tr>
<td>350</td>
<td>72</td>
<td>370</td>
<td>106</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>368 ± 4</td>
<td>105</td>
<td></td>
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<tr>
<td>Static ≤72 hour exposure to 5 and 350 µg/L ibuprofen</td>
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<td></td>
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<tr>
<td>5</td>
<td>24</td>
<td>10</td>
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<td>5</td>
<td>48</td>
<td>9</td>
<td>180</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>8</td>
<td>165</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>9 ± 1</td>
<td>181</td>
<td></td>
</tr>
</tbody>
</table>

During the exposure period, ibuprofen measurements in the water samples collected from the DWC and SC tanks were below the LOD of 2.5 µg/L, which was determined to the lowest acceptable standard concentration for which ibuprofen
could be reliably measured in water with the analytical method used. This was therefore interpreted as no ibuprofen being present in these tanks.

6.3.2 Effect of “tail-fin clipping” on COX activity in fish tissues

The total COX activity (normalised to mg of tissue protein) was measured in the gill, muscle and liver (pooled, the reason for which is explained later in this section) tissues of fish. In the muscle, total COX activity was low and ranged between 0-2 U/mg (equivalent to 0-5 U/mL) in fish exposed to SC (before tail-clipping). After 4 and 24 hours post tail-clipping, COX activity was mostly non-detectable, in both SC and ibuprofen-treated groups, indicating that tail-clipping had no measurable effect on altering COX activity levels (Figure 52. A). The LOD specified in this assay was 13-63 U/mL, which was over 2-fold higher than the total activity calculated in this study, suggesting that the measured activity was outside of the detection range.

In the gill, total COX activity was mostly non-detectable in exposed fish (Figure 52. B). Some activity was observed in fish exposed to ibuprofen, after 4 and 24 hours post tail-clipping, however, the measured levels were below the detection range specified in the assay. Therefore, it was not possible to make any further conclusions from the data obtained. The lack of measurable COX activity in the gill may have been due to this tissue being located further away from the site of injury, compared to the muscle tissue, that was sampled in closer proximity to the site where the trauma was inflicted.

Overall, these findings show that tail-fin clipping had no measurable effect on inducing COX activity above basal levels in the muscle or gill, at the time points examined (i.e. 4 and 24 hours), suggesting that either COX was not induced (to a measurable level) or that the amount of time that had elapsed following tail-clipping was inappropriate to see any observable effect. As the COX levels were non-measurable or very low before tail-clipping, it was not possible to determine if ibuprofen exposure had any effect on modulating COX activity, after injury to fish. To ensure that the assay was in correct working order, mouse (muscle and lung) tissues were used as an additional positive control when the assays were performed. The measured COX activity levels for the mouse tissues ranged between 42-58 U/mL in the muscle, and 70-88 U/mL in the lung (above the specified LOD for the
assay), suggesting that the assay may be more sensitive to mammalian COX, and less sensitive to fish COX enzymes. However, another possibility for the higher measured COX levels could have been the amount of starting tissue mouse (~40 mg) material used in the assay. It was speculated that the limited tissue material available from individual fish (~20 mg) might have contributed to the low measured levels; therefore, liver tissues from five fish were pooled. COX activity was detected between 1.5-5.5 U/mg (ranging from 32-42 U/mL, which was within the LOD for the assay) in pooled liver samples in all treatments (Figure 52. C). Tail-clipping appeared to induce COX activity in fish exposed to ibuprofen, 4 hours after tail-clipping, which then fell at 24 hours in the ibuprofen tank only, suggesting that this may have been an effect of ibuprofen exposure (in fish exposed to ibuprofen only). However, it was not possible to make any firm conclusions based on one pooled tissue sample.
Figure 52. Total cyclooxygenase (COX) activity (normalised to the mg of sample protein) in fathead minnow muscle (A) (n=5), gill (B) (n=5) (individual data points are shown, along with the mean ±SD) and liver (C) (pooled sample) tissues before and (4 and 24 hours) after tail-clipping. COX activity was measured in fish exposed to solvent control (SC) before and after tail-clipping, SC (before) and 220 µg/L ibuprofen (after tail-clipping) and fish exposed to ibuprofen (before and after tail-clipping). N.D denotes non-detectable.
6.3.3 Effect of ibuprofen exposure on prostaglandin E metabolite (PGEM) concentration

As we were unsuccessful in quantifying COX activity at basal levels in fathead minnows tissues (exposed to SC only), it was not possible to determine if ibuprofen exposure had any effect on altering COX enzyme activity. The MoA of ibuprofen is through its inhibition of prostanoid synthesis, via the inhibition of the COX enzymes. To examine if ibuprofen exposure could modulate prostanoids synthesis, prostaglandin E metabolite (PGEM) concentrations were measured in gill tissues. Fish were exposed to SC (n=10) or a measured ibuprofen water concentration of 370 μg/L (n=40) over 72 hours. A larger sample size of 40 fish was used as high inter-individual variability had previously been observed in both ibuprofen uptake (see Chapter 3) and gene expression (Chapter 5). PGEM concentrations in the gill tissues of fathead minnows exposed to 370 μg/L ibuprofen were significantly decreased (p < 0.001, unpaired t test) compared to the SC group (Figure 53), indicating that ibuprofen can alter PGEM concentrations in fish at this exposure concentration.
Figure 53. Prostaglandin E metabolite (PGEM) concentration in gill tissues of fathead minnows exposed to solvent control (SC) (n=10) and ibuprofen (n=40) (mean measured concentration (±SD) of 368 ± 4 μg/L) after 72 hours. Individual data points are shown, along with the mean ±SD. Asterisks (*** indicate a significant difference between SC group and exposed samples (p < 0.001 using unpaired t test).

To address whether or not these effects, at a similar magnitude, could be replicated in fish at exposure concentrations closer (2 to 3-fold above) to environmentally relevant ibuprofen concentrations, another group of fish were exposed in one further 72 hour static study to measured water concentrations of 9 (± 1) and 470 (473 ± 9) μg/L ibuprofen.

PGEM concentrations in the gill tissues of fathead minnows exposed to 9 μg/L ibuprofen (over 72 hours) were not significantly different (p > 0.05, one-way ANOVA) to the SC group (Figure 54). PGEM concentrations in the gills of fish exposed to 470 μg/L ibuprofen were significantly decreased (p < 0.05, one-way ANOVA followed by Tukey’s comparison test) compared to the SC group (Figure 54). These findings demonstrate that ibuprofen exposure can decrease PGEM concentrations in fish at 470 μg/L, but not at 9 μg/L (when compared to the SC group). Furthermore, the mean PGEM concentration in exposed fish in the 470 μg/L...
exposure group was significantly decreased ($p < 0.001$, one-way ANOVA followed by Tukey’s comparison test), when compared to the fish in the 9 μg/L exposure group. To examine if the results observed at 470 μg/L were comparable to the previous results (i.e. at 370 μg/L), the PGEM concentrations in fish at both concentrations were compared (data not shown). There was no significant difference in PGEM concentrations in fish between these two exposure concentrations, possibly due to these being more similar concentrations, and the large inter-individual variation in both groups.

To determine if there was a time-dependent effect on PGEM inhibition following ibuprofen exposure at 9 and 470 μg/L, PGEM concentrations were measured in fish after 24 and 72 hours of exposure (Figure 55). PGEM concentrations in fish exposed to 9 μg/L after 24 and 72 hours were not significantly different ($p > 0.05$, using unpaired t test). PGEM concentrations in fish exposed to 470 μg/L after 24 and 72 hours were significantly different ($p < 0.001$, using unpaired t test). PGEM concentrations were also significantly different ($p < 0.05$) between the 9 and 470 μg/L ibuprofen exposure groups at 24 and 72 hours.
Figure 54. Prostaglandin E metabolite (PGEM) concentration in gill tissues of fathead minnows exposed to solvent control (SC) (n=9) and ibuprofen concentrations of 9 (± 1) μg/L (n=12) and 470 (473 ± 9) μg/L (n=12) after 72 hours. Individual data points are shown, along with the mean ±SD. The letter a) indicates no significant difference between SC group and exposed group, b) indicates a significant difference (p < 0.05) between SC group and exposed group and c) indicates a significant difference (p < 0.001) between exposed groups (using one-way ANOVA followed by Tukey’s comparison test).
Figure 55. Prostaglandin E metabolite (PGEM) concentration in gill tissues of fathead minnows exposed to 9 (± 1) μg/L and 470 (473 ± 9) μg/L after 24 (n=6) and 72 (n=6) hours. Individual data points are shown, along with the mean ±SD. The letter a) indicates no significant difference, b) indicates a significant difference at $p < 0.05$ and c) at $p < 0.001$, between 24 and 72 hours (using unpaired t test).
6.4 DISCUSSION

In this chapter, the effect of ibuprofen exposure on COX enzyme activity, and one of its downstream products, prostaglandin E₂, was examined. The main finding from this study was that ibuprofen can significantly decrease prostaglandin E₂ concentrations in fathead minnows after 72 hours of exposure to 370 and 470 μg/L, but not at exposure concentrations more similar to environmentally relevant ibuprofen concentrations (i.e. 9 μg/L). (Note that the plasma ibuprofen concentrations in fish exposed to 9, 370, and 470 μg/L confirmed that ibuprofen was taken up into fish, however the relationship between plasma concentrations (in individual fish) and target-mediated effects will be discussed in Chapter 7).

6.4.1 COX enzyme activity

From the preliminary work, it was shown that COX enzyme activity at basal levels were too low to be accurately quantified in fish that were considered to be “healthy” (data not shown). However, similar to our previous findings, in this study COX activity in muscle and gill tissues were low in both SC and ibuprofen-treated fish (at basal levels i.e. before tail-clipping), and in most instances, the measured COX activity levels were below the LOD specified in the assay (Figure 52 A & B). In tissue-injury models, basal expression of COX 1 is constitutive, whereas an up-regulation of COX 2 is seen during the acute tissue injury phase (Dupouy et al., 2006). In an attempt to elevate COX activity in fathead minnows, injury was inflicted on fish through removal of a section of the tail-fin. Subsequently, the effect of this injury was examined by measuring COX activity in tissues after 4 and 24 hours, in fish exposed to SC only, and in fish exposed to 220 μg/L ibuprofen to investigate if ibuprofen treatment could alter (decrease) COX activity in fish (following tissue injury). However, after injury, COX activity in tissues were mostly non-detectable, therefore it was not possible to determine if a) injury had measureable effect on inducing COX activity in fish (after for 4 and 24 hours) and b) whether ibuprofen exposure could subsequently alter the induction that was expected (after injury). COX activity was detected in mouse muscle and lung tissues (which were used as an additional positive control in the assay), and some activity (within the LOD range) was observed in the pooled liver samples (Figure 52 C). This suggests that either the
assay was not sensitive enough for fish COX enzymes, or more likely, that the amount of (gill and muscle) tissue from individual fish was a limiting factor.

To date, only one study has demonstrated the inhibition (although not significantly) of total COX activity in fish following ibuprofen exposure (Flippin et al., 2007). Total COX activity was measured in Japanese medaka that had been exposed for 6-weeks to ibuprofen concentrations of 0, 1, 10 and 100 µg/L. The measured COX activity in liver tissues (1.5-4.5 U/mg) in Japanese medaka was comparable to those measured in pooled liver tissues in fathead minnows from this study (0.5-5.5 U/mg), when normalised to tissue protein content. Following the 6-week exposure, a reduction in the variability of COX activity between control and exposed groups was reported, which the authors concluded was a primary effect of ibuprofen (Flippin et al., 2007). Considering that only weak inhibition of COX activity was observed after 6-weeks of ibuprofen exposure, suggests that it may not have been possible to detect any changes in COX activity in this study, where fish had been exposed to ibuprofen for a maximal duration of 120 hours. Furthermore, with longer exposures, the potential for there to be a carry-over of ibuprofen in exposed fish tissues may be increased, which could influence COX activity levels. Therefore, in some instances, it may be useful to have a measure of the tissue ibuprofen concentration.

Another explanation could be that ibuprofen and other NSAIDs may lack the capacity to effectively inhibit COX enzyme activity in fish. For example, no changes in COX enzyme activity were reported following ibuprofen exposure in either the gill or kidney tissues of rainbow trout (Robichaud, 2011). However, these findings may also indicate that COX activity in the liver (as reported in Japanese medaka) may be differentially expressed to that in the gill (or muscle) tissue. Similarly, in green sunfish (*Lepomis cyanellus*) that had been treated with a number of COX inhibitors, including ibuprofen, drug treatment had no effect on inhibiting COX enzyme activity (Cavallaro and Burnside, 1988). In all of these instances, it cannot be ruled out that the assay was not sensitive or specific enough for fish COX enzymes, as in all cases mammalian total COX activity assays were used. For these reasons, the products of COX activity, prostaglandins were also examined in this
study to determine if this endpoint was more sensitive than COX enzyme activity in fish.

6.4.2 Prostaglandin E2 metabolite (PGEM) concentrations

The PGEM concentrations in the gill tissues of fathead minnows exposed to ibuprofen water concentrations of 370 and 470 μg/L (after 72 hours) were significantly decreased, compared to their respective SC groups. Large inter-individual variation was observed in the SC group (24-fold) and in fish exposed to 370 μg/L (155-fold) (Figure 53), and in the follow-up exposure study, in the SC group (13-fold), and in fish exposed to 470 μg/L (49-fold). Interestingly, less variation was observed in fish exposed to 9 μg/L (6-fold) (Figure 54). The variation observed in the SC groups indicates that factors other than drug exposure can modulate PGEM concentrations in “healthy” fish. In mammals, the conversion of PGH2 to PGE2 in the COX pathway is partially regulated by microsomal (mPGES-1 and mPGES-2) and cytosolic (cPGES) prostaglandin E2 synthases (Jakobsson et al., 1999; Tanioka et al., 2000; Watanabe et al., 1999). The orthologues of two human microsomal synthases have now been characterised in zebrafish (Pini et al., 2005) suggesting a similar mechanism of PGE2 regulation may also be present in fish. However, whether the variation in PGEM concentrations in SC fish can be explained by differences in the presence and activities of microsomal and cytosolic synthases, remains to be confirmed.

Despite the larger sample size in the 370 μg/L exposure group (n=40), compared to 470 μg/L exposure group (n=12), the PGEM concentrations in fish in these exposure groups (over 72 hours) was not significantly differently (data not shown). This suggests that the propensity for ibuprofen to inhibit PGE2 at these two concentrations is comparable. The potency of NSAIDs against COX 1 and COX 2 can be compared using the IC50 values. The estimated reported IC50 values for ibuprofen in humans range between 3.3-4.75 μM for COX 1, and over 30 μM for COX 2 based on whole blood assays of endotoxin-induced PGE2 production (Brideau et al., 1996; Gierse et al., 1995). The IC50 for ibuprofen inhibition of PGE2 production in the gill tissue of bluntnose minnow has been estimated to be 0.4 μM (Bhandari and Venables, 2011) suggesting that PGE2 in fish is approximately 10-75-fold more sensitive to ibuprofen
inhibition than in humans (based on IC$_{50}$ estimates). This indicates that the potency of ibuprofen in fish is greater than in humans, as a lower ibuprofen concentration can lead to greater magnitude of effect in fish. Therefore, some caution must be applied when using mammalian data to predict target-mediated pharmacological effects in aquatic species.

Ibuprofen and other NSAIDs are non-selective inhibitors of COX, which can result in the non-specific inhibition of prostanoids, and the disruption of several physiological functions in fish. NSAID inhibition of prostanoids can affect reproduction, for example in zebrafish indomethacin exposure (100 μg/L for 16 days) significantly inhibited PGE$_2$ which resulted in disruption of oocyte maturation and ovulation (Lister and Van Der Kraak, 2008) and ibuprofen exposure has been shown to alter the pattern of spawning in Japanese medaka (Flippin et al., 2007). In the former study, COX activity was not altered in the ovary and whole body homogenates following indomethacin exposure, confirming that PGE$_2$ is more sensitive to NSAID inhibition than COX enzyme activities.

Ibuprofen exposure did not affect PGEM concentrations in fish exposed to 9 µg/L (compared to the SC group) which was, respectively, 41-fold and 52-fold below the 370 and 470 µg/L ibuprofen exposure concentrations. Therefore, in this study the “no observed effect concentration” (NOEC) was determined to be 9 µg/L ibuprofen.

In summary, ibuprofen exposure can produce target-mediated effects in fathead minnows based on the MoA of ibuprofen in humans. However, COX enzyme activity was not suitable as an endpoint for MoA-relevant effects. Contrary to expectation, tissue injury did not elevate COX enzyme activity to measurable levels, and this was mostly likely due to a lack of assay sensitivity. The PGEM concentration was a sensitive terminal endpoint of the COX pathway, reflective of the MoA in humans. Ibuprofen exposure did not affect PGEM concentration in fish at 9 µg/L (the NOEC), but only at 370 and 470 µg/L ibuprofen, indicating that in this study, exposure concentrations approximately 2 to 3-fold above environmentally relevant concentrations did not adversely affect adult fathead minnows.
CHAPTER 7: LINKING BLOOD PLASMA IBUPROFEN CONCENTRATIONS WITH TARGET-MEDIATED EFFECTS IN FATHEAD MINNOWS
7.1 INTRODUCTION

The read-across hypothesis assumes that similar target-mediated effects will occur in fish, as in humans, if the molecular targets have been conserved and only if the plasma concentrations in fish are similar to the human therapeutic concentrations. In this study so far, it has been demonstrated that therapeutic concentrations of ibuprofen can be reached in fathead minnow blood plasma, and that an exposure water concentration of 270-370 µg/L ibuprofen (for 96 hours) is required to achieve the Cmax range of 15,000-30,000 µg/L (Schulz et al., 2012) in fathead minnows (as described in Chapter 3). It has been shown that the primary molecular target for ibuprofen, the COX enzymes, that are encoded for by the COX genes, are present in fathead minnows (as identified in Chapter 4) (albeit variations of the isoforms), demonstrating the MoA of ibuprofen may be conserved in fish. Ibuprofen exposure can alter COX gene expression in fathead minnows (as shown in Chapter 5). The downstream products of COX enzyme activity, prostaglandins (as determined by PGEM) are also affected by ibuprofen following exposure to water concentrations of 370 (and 470) µg/L, but not at concentrations similar (2 to 3 times higher i.e. 9 µg/L) to environmentally-relevant concentrations (as shown in Chapter 6). However, large inter-individual variation was observed in ibuprofen uptake, COX gene expression levels and PGEM concentrations. Therefore, the purpose of this chapter was to correlate the measured ibuprofen blood plasma concentrations with the observed target-mediated biological effects (on COX gene expression and PGEM) in fathead minnows, in order to determine the relationship between effective internal concentrations in fish with therapeutic concentrations in humans (Cmax).

7.2 METHODS

The measured plasma ibuprofen concentrations were correlated with the target-mediated effects on COX gene expression (refer to Chapter 5) and PGEM concentrations (refer to Chapter 6). Data on the measured plasma ibuprofen concentrations were previously collected from fathead minnows that had been exposed to 270 µg/L (n=4) (mean ± SD, 278 ± 70 µg/L) and 500 µg/L (n=4) (502 ± 56 µg/L) after 96 hours (refer to Chapter 3), and to 370 µg/L (n=40) (368 ± 4 µg/L), 9 µg/L (n=9) (9 ± 1 µg/L) and 470 µg/L (n=11) (473 ± 9 µg/L) after 72 hours (refer to Chapter 6).
gene expression and PGEM was examined using linear regression analysis, using GraphPad Prism 6 (GraphPad Software, Inc).

7.3 RESULTS

7.3.1 COX gene expression levels and plasma ibuprofen concentrations

COX gene expression was previously examined in gill, liver and brain tissues of fathead minnows (Chapter 5), and the fold change in gene expression level (compared to the SC group) was correlated with the plasma ibuprofen concentration of the exposed (n=4) fish. The blood plasma concentrations of the fish (n=4) exposed to 270 µg/L were 625, 4,400, 16,800 and 172,500 µg/L ibuprofen (mean of 48,590 ± 82,880 µg/L, see Chapter 3, Table 11), indicating that the plasma concentrations were below or within the Cmax, except for one fish that was above the Cmax. The blood plasma concentrations of the fish (n=4) exposed to 500 µg/L were 60,940, 352,900, 84,530 and 220,580 µg/L ibuprofen (mean of 179,739 ± 126,138 µg/L, see Chapter 3, Table 11), indicating that the plasma concentrations in these fish were all above the Cmax.

COX 1 gene expression was up-regulated in the tissues of fathead minnows with plasma concentrations below, within and above the Cmax. Down-regulation of COX 1 was observed in the gill, liver and brain tissues of one fish exposed to 270 µg/L, which had the highest plasma ibuprofen concentration in this group (Figure 56. A). However, up-regulation was observed in all the tissues of fish exposed to 500 µg/L, all of which had a plasma ibuprofen concentration above the Cmax. COX 2a was up-regulated in the tissues of fathead minnows with plasma concentrations below, within and above the Cmax, except in the liver of one fish (that a had a plasma concentration below the Cmax), and in the brain and liver tissues of another fish (that had a plasma concentration above the Cmax), and in all tissues of fish exposed to 500 µg/L (Figure 56. B). COX 2b was up-regulated in the tissues of fathead minnow exposed to 270 and 500 µg/L, except in the liver one fish that had a plasma concentrations below the Cmax, and in another fish that had a plasma concentration above the Cmax (Figure 56. C). These findings demonstrate that there was no clear trend between COX gene expression levels and increasing plasma ibuprofen concentration as up-regulation was seen in fish with plasma concentrations below
(24-fold below the lower Cmax value i.e. 15,000 µg/L), similar to, and above (12-fold above the upper Cmax value i.e. 30,000 µg/L) the Cmax.

Closer examination of gene expression in the gill, liver and brain tissues confirmed that there was no clear correlation between plasma ibuprofen concentration and COX gene expression in fathead minnows (Figure 57).
A. **COX 1**

![Graph showing fold change in Plasma Ibuprofen levels across different organs and treatments.]

- **Gill**
- **Liver**
- **Brain**

- **270 µg/L Ibuprofen**
- **500 µg/L Ibuprofen**

B. **COX 2a**

![Graph showing fold change in Plasma Ibuprofen levels across different organs and treatments.]

- **Gill**
- **Liver**
- **Brain**

- **270 µg/L Ibuprofen**
- **500 µg/L Ibuprofen**
Figure 56. COX 1 (A), COX 2a (B) and COX 2b (C) gene expression in gill, liver and brain tissues and plasma ibuprofen concentrations in fathead minnows exposed to 270 µg/L (mean ± SD, 278 ± 70) (n=4) (black) and 500 µg/L (mean ± SD, 502 ± 56) (n=4) (red) (for 96 hours). Expression levels of the COX genes in control and exposed tissue samples were normalised to β-actin, and the exposed samples (for each ibuprofen exposure group) were expressed as fold change relative to the average of the SC samples (n=4 in each group) (represented as 1). Shaded area denotes the human therapeutic plasma concentrations of ibuprofen (Cmax). N.D denotes non-detectable.
Figure 57. COX gene expression in gill (A, D & G), liver (B, E & H) and brain (C, F & I) tissues and plasma ibuprofen concentrations in fathead minnows (n=8) exposed to 270 µg/L (mean ± SD, 278 ± 70) and 500 µg/L (mean ± SD, 502 ± 56) ibuprofen for 96 hours. Expression levels of the COX genes in control and exposed tissue samples were normalised to β-actin, and the exposed samples (for each ibuprofen exposure group) were expressed as fold change relative to the average of the SC samples (n=4 in each group) (represented as 1). Shaded area denotes the human therapeutic plasma concentrations of ibuprofen (Cmax). r² values denote the linear regression values, however there was no significant (p < 0.05) correlation between plasma ibuprofen concentration and gene expression. N.D denotes non-detectable.
To determine if there was a time-dependent effect between COX gene expression and plasma ibuprofen concentrations, gene expression was examined in the gills of fish exposed to 270 µg/L after 24 (n=3) and 96 hours (n=4) (Figure 58). The plasma concentrations in fish exposed for 24 hours were determined to be 62.5, 3,396 and 2,543 µg/L (mean of 2,000 ± 1,732 µg/L), which were all below the Cmax. The fold change in COX gene expression was less evident at 24 hours, compared to 96 hours (Figure 58). COX 1 levels after 24 hours were down-regulated compared to the solvent control group, there was no change in COX 2a expression levels between control and ibuprofen-exposed fish, however COX 2b was up-regulated after 24 hours. However, due to the small sample size at 24 and 96 hours, no further conclusions can be made.

Figure 58. COX gene expression in gill tissues, and plasma ibuprofen concentrations in fathead minnows exposed to 270 µg/L (mean ± SD, 278 ± 70) after 24 (n=3) and 96 (n=4) hours. Expression levels of the COX genes in control and exposed samples were normalised to β-actin (house-keeping gene), and the exposed samples (n=4) were expressed as fold change relative to the average of the (normalised) solvent control samples (n=4) (represented as 1). Shaded area denotes the human therapeutic plasma concentrations of ibuprofen (Cmax). N.D denotes non-detectable.
7.3.2 Prostaglandin E\textsubscript{2} Metabolite (PGEM) and drug plasma concentrations of ibuprofen

Prostaglandin E\textsubscript{2} metabolite (PGEM) concentrations in the gill tissues was correlated with plasma ibuprofen concentrations (Figure 59). Fish were exposed to SC (n=8-10) and ibuprofen water concentrations of 370 (n=40), 9 (n=9) and 470 (n=11) µg/L ibuprofen, respectively. The mean measured (± SD) plasma ibuprofen concentration in these fish was 8,370 (± 5,456), 67 (± 22) and 2,680 (± 1,605) µg/L, respectively, indicating that the mean concentrations were 1.8-fold, 224-fold and 5.6-fold below the lowest Cmax value (i.e. 15,000 µg/L). There were no plasma concentrations that exceeded the Cmax range.

PGEM concentrations were significantly inhibited in fish exposed to 370 µg/L. However, there was no clear correlation between PGEM concentrations and blood plasma concentrations (Figure 59. A). Large inter-individual variability was observed between PGEM concentrations and plasma ibuprofen concentrations in both the control and exposed fish groups. In the exposed group, only 18% of the sample population had a plasma concentration within the Cmax range, and the rest were below the Cmax, demonstrating that ibuprofen can elicit effects over a wide range of concentrations in fish. PGEM inhibition was seen in fish with plasma concentrations up to 21-fold below (lowest measured plasma concentration was 710 µg/L in this group) the Cmax (15,000 µg/L), highlighting that PGEM is a highly sensitive endpoint. There was a weak trend between PGEM inhibition and plasma concentration in fish exposed to 470 µg/L (Figure 59. B), although the plasma concentrations in these fish were below the Cmax only. However, PGEM concentrations and plasma ibuprofen concentrations in fish exposed to 9 µg/L were similar to the SC group, indicating that there was no inhibition of PGEM in fish at this exposure concentration. The mean plasma concentration (67 µg/L) in this group was found to be 224-fold below the Cmax (15,000 µg/L), clearly demonstrating that PGEM inhibition only occurred at plasma concentrations similar to the Cmax range. Therefore, in this study, the NOEC for PGEM in fathead minnows was deduced as being 9 µg/L ibuprofen, which was 42-fold below 370 µg/L ibuprofen, which represents the lowest-observed effect concentration (LOEC).
However, the mean measured plasma concentration of fish in the 9 µg/L exposure group (67 µg/L, n=9) was only the 10-fold below the lowest measured plasma concentration (710 µg/L) in the 370 µg/L exposure group, suggesting that there was a relatively narrow margin between the plasma concentrations at which no PGEM inhibition was observed, and at which significant ($p < 0.001$ using unpaired t test, see Chapter 6) PGEM inhibition was observed (compared to their respective SC groups).

PGEM concentrations and plasma ibuprofen concentration in fish exposed to 9, 370 and 470 µg/L were collated, as shown in Figure 59. C. There was some overlap observed between the PGEM and plasma concentrations in fish exposed to 370 and 470 µg/L ibuprofen. The variability in PGEM concentrations in the SC groups (fish with plasma ibuprofen concentrations below the LOD of 62.5 µg/L), was more evident when these datasets were combined (from ~12-fold to 20-fold), suggesting that PGEM at basal levels may be influenced by other factors.
Figure 59. Prostaglandin E metabolite (PGEM) concentration in gill tissues, and plasma ibuprofen concentrations in fathead minnows exposed to SC (n=8-10) and 370 µg/L (n=40) (A), and 9 (n=9) and 470 (n=11) (B) µg/L ibuprofen after 72 hours, along with the combined datasets (C). Cmax denotes the human therapeutic plasma concentrations of ibuprofen.
To determine if there was a time-dependent effect, PGEM concentration in the gill was correlated with plasma ibuprofen concentrations in fish exposed to 9 and 470 µg/L after 24 and 72 hours. At 9 µg/L, there was no discernable difference between PGEM and plasma ibuprofen concentration in exposed fish after 24 and 72 hours (mean plasma ibuprofen concentrations of 66 and 71 µg/L, respectively) (Figure 60). At 470 µg/L, PGEM was significantly inhibited (see Figure 54), however there was no significant correlation between PGEM and plasma ibuprofen concentration with increasing exposure hours in exposed fish, after 24 and 72 hours (mean plasma ibuprofen concentrations of 1,765 and 2,680 µg/L) (Figure 60). These findings show that at 470 µg/L when the mean plasma ibuprofen concentration in fish exposed for 24 and 72 hours was similar, the level of PGEM inhibition was variable.

Figure 60. Prostaglandin E metabolite (PGEM) concentration in gill tissues, and plasma ibuprofen concentrations in fathead minnows following exposure to 9 (n=9) and 470 (n=11) µg/L ibuprofen for 24 and 72 hours. Shaded area denotes the human therapeutic plasma concentrations of ibuprofen (Cmax).
7.4 DISCUSSION
The purpose of this chapter was to correlate the measured blood plasma concentrations of ibuprofen with target-mediated biological effects observed in fathead minnows, as large inter-individual variation was observed in both plasma ibuprofen uptake, and the MoA-relevant endpoints examined in fish tissues (i.e. COX gene expression and PGEM; the surrogate marker of PGE₂ concentrations). The internal (blood plasma) concentration of a drug determines whether or not pharmacological response(s) will occur in an exposed organism, and according to the read-across hypothesis, it is possible to use the relationship between the internal blood plasma concentrations in fish and the effective concentrations in humans i.e. the human therapeutic plasma concentration (Cmax), in order to predict the likelihood of an effect (Huggett et al., 2003; Rand-Weaver et al., 2013). In this study, PGEM inhibition was observed in the gills of fish exposed to 370 µg/L ibuprofen (after 72 hours), when the mean blood plasma concentration (8,370 µg/L) was 1.8-fold below the lowest Cmax value for ibuprofen (15,000 µg/L) (Schulz et al., 2012), however PGEM inhibition was not observed in fish exposed to 9 µg/L, when the mean plasma concentration was 224-fold below the lowest Cmax value, clearly demonstrating that these effects only occur at plasma concentrations similar to the Cmax range. The Cmax range was not exceeded in fish exposed to 370 (or 470) µg/L therefore it was not possible to determine whether or not PGEM inhibition would have also occurred at higher concentrations, although it is likely.

Large inter-individual variation (by 15-fold) was observed in plasma ibuprofen concentration and PGEM inhibition in fish exposed to 370 µg/L (after 72 hours) and therefore, there was no clear correlation between the level of PGEM inhibition and plasma ibuprofen concentrations. The variability in a drug response in individuals at a specific dose (or exposure) is largely influenced by the pharmacokinetics of a drug, for example, variations in plasma protein binding can alter the distribution of bound and unbound (free) fractions in the body (Lin et al., 1987), and the pharmacodynamics (for example, enzyme/receptor binding and chemical interactions) (Derendorf et al., 2000; Reigner et al., 1997; Sheiner and Steimer, 2000) thus contributing to variable plasma concentrations and magnitude of effect(s) in humans. In fish, the drug-binding kinetics to plasma proteins is relatively unknown.
(Owen et al., 2007); however, differences in drug distribution may result in higher fractions of the unbound drug in some fish, that can inhibit PGEM at lower concentrations than others. Another possible explanation that may account for the variation in response in exposed fish is the differential saturation of the COX protein active sites following NSAID treatment, which has been observed in rat models (Satterwhite and Boudinot, 1991). Ibuprofen is weak, competitive non-selective inhibitor of the COX enzymes (Gierse et al., 1999) and therefore after 72 hours of continuous exposure, an increase in the plasma ibuprofen concentration beyond a certain level of exposure (i.e. where all available active sites have been occupied) would have no further effect on PGEM inhibition. There was a time-dependent effect observed between PGEM inhibition and plasma concentrations in fish exposed to 470 µg/L (compared to 9 µg/L), after 24 and 72 hours, indicating that the effects of ibuprofen in fish may also be duration-dependent, as in humans.

PGEM inhibition was observed in exposed fish over a range of plasma ibuprofen concentrations (from 710-22,000 µg/L, the lowest and highest measured plasma concentrations). In humans, variability between individuals in their response to drugs is well recognised (Wood, 2001), and therefore drugs are designed to exert their therapeutic effects over a range of concentrations (before adverse drug reactions occur) (Brune et al., 2010). The wide therapeutic index of ibuprofen ranges between 10,000-50,000 µg/L (following a normal therapeutic dose) (Mehlisch and Sykes, 2013; Regenthal et al., 1999; Schulz et al., 2012). However, the Cmax reference range used in this study was between 15,000-30,000 µg/L as reported by Schulz et al., (2012). High inter-individual variability in patients has been observed between ibuprofen plasma concentrations and the onset of analgesia (by 7-fold in patients who had taken a normal 400 mg therapeutic dose) (Mehlisch and Sykes, 2013). Although a direct extrapolation between PGEM in fish with PGEM in humans cannot be made, perceptible pain relief (using a dental pain model) in patients, could be interpreted as COX-mediated inhibition of prostaglandin E2.

The relationship between plasma ibuprofen concentrations and COX gene expression in fathead minnows exposed to 270 and 500 µg/L ibuprofen after 96 hours was examined. There was no clear dose-response between plasma concentration and the
up-regulation of the COX genes in exposed fish tissues. In a similar study, examining the correlation between blood plasma concentrations of diclofenac and molecular effects, Cuklev et al., (2011) demonstrated that following a two-week exposure of rainbow trout to 1.6–81.5 μg/L diclofenac (when the plasma concentration was approximately 1.5-88% of the Cmax of diclofenac), global hepatic gene expression changes were observed. At plasma concentrations close to the Cmax, a number of genes functionally associated with inflammation and the immune response were differentially regulated in the liver, which is consistent with the MoA of diclofenac, however the expression of the COX 1 and COX 2 genes were found to be down-regulated (Cuklev et al., 2011). In contrast, the findings in the present study showed that the COX genes were up-regulated in fish tissues at concentrations, below (24-fold below the lowest Cmax value), within, and above (12-fold above the highest Cmax value) the Cmax. However the exposure concentrations used in this study were higher (270 and 500 μg/L), and the length of exposure at which these effects were observed (96 hours) was considerably shorter.

There are relatively few studies that have examined the internal blood plasma concentrations of human pharmaceuticals in fish, and related them to target-mediated effects at concentrations below, and similar, to human therapeutic plasma concentrations. However, Valenti et al., (2012) did demonstrate that behavioural effects could be observed in fathead minnows exposed to (2.8-28.1 μg/L) sertraline, a SSRI used to modify behaviour in humans, at fish plasma concentrations of 305 μg/L (at the lowest exposure concentration) (Valenti et al., 2012), which resulted in plasma concentrations similar to the human therapeutic range of 50-250 μg/L (Schulz et al., 2012). However, the exposure water concentrations tested produced plasma concentrations that were similar, or above the human therapeutic range, and in order to fully validate the read-across hypothesis, biological effects in relation to plasma concentrations below, and similar to the human therapeutic range are required. At the highest exposure concentration of sertraline tested (28.1 μg/L), the mean measured plasma concentration was reported to be 1,927 μg/L, which was in the reported toxic range (1,680-3,000 μg/L) in humans (Schulz et al., 2012), however, no (further) adverse effects were reported in fish (Valenti et al., 2012).
In summary, there was no clear correlation between plasma ibuprofen concentration and PGEM or COX gene expression in fathead minnows. This was largely due to large inter-individual variation observed in both ibuprofen uptake and the endpoints examined. Unfortunately in this study, the scope for examining both parameters (i.e. PGEM concentrations and COX gene expression levels) in the tissues of the same individual fish was not possible, although it would have been interesting to see the level of variation in both parameters analysed in the same fish tissues. Ibuprofen exposure did not affect PGEM concentrations in the fish that were exposed to the lowest tested exposure concentration (9 μg/L), which had a mean plasma concentration 224-fold below the Cmax of ibuprofen in humans (lower value of range). However, in the fish that were exposed to 370 μg/L, when the mean plasma concentration was 1.8-fold below the Cmax (and more similar to the human therapeutic range), PGEM concentrations were decreased, therefore providing support (and validation) for the read-across hypothesis. Most importantly, these findings demonstrate that the present environmental concentrations (typically within the ng to low μg/L range) of ibuprofen (alone) are unlikely to pose an immediate risk to wild (adult) fish. However, further work to investigate the potential effects of ibuprofen exposure on PGEM concentrations (at 9 μg/L) in fish over a range of developmental stages, for example larvae, embryos and sexually immature fish is still required as the NOEC and LOEC may be lower at these stages.
CHAPTER 8: GENERAL DISCUSSION
8.1 CONCLUSIONS

In this study, we have demonstrated the validity of the “Read-Across Hypothesis” by providing, for the first time, both qualitative and quantitative evidence for its applicability, using ibuprofen and the model fish species, the fathead minnow. We also highlight the potential use of the FPM as a viable tool for estimating the internal concentration(s) of pharmaceutical drugs in fish, thereby strengthening the utility of the read-across hypothesis for ERA.

The major findings were that MoA-relevant pharmacological effects, on PGE$_2$ synthesis (the terminal endpoint of COX enzyme activity), were observed in “healthy” fish exposed to ibuprofen, when the mean blood plasma concentration was close to (1.8-fold below) the human therapeutic range (Cmax). At exposure concentrations closer to (2 to 3-fold above) environmentally relevant ibuprofen concentrations (i.e. 9 μg/L), when the mean plasma concentration was 224-fold below the Cmax, fish did not respond to ibuprofen exposure. These findings provide support for the read-across hypothesis and demonstrate that pharmaceuticals can exert the same target-mediated pharmacological effects in fish, as in humans, at similar blood plasma concentrations. These findings are consistent with the only one other published study by Valenti et al., (2012) where plasma concentrations of sertraline similar to the Cmax resulted in MoA-relevant behavioural effects in fathead minnows. However, all the water concentrations tested produced plasma concentrations that were above the Cmax, whereas this study provides experimental evidence for the read-across hypothesis using water concentrations that resulted in plasma concentrations within fish that were below (i.e. 9 μg/L) and similar (i.e. 370 μg/L) to the Cmax. As such, in fish exposed to 9 μg/L, there were no measurable target-mediated effects on PGE$_2$, therefore, the NOEC for PGE$_2$ inhibition in fathead minnows was deduced as being 9 μg/L ibuprofen, which was 42-fold below 370 μg/L ibuprofen, which represents the LOEC in this study.

The findings in this study indicate that ibuprofen (by itself, at least) is unlikely to pose an immediate threat to wild (adult) teleost fish at environmental exposure concentrations. However, in realistic environmental scenarios, pharmaceuticals exist as a complex mixture (Sumpter, 2009), and there may be several COX inhibitors
present in the ng to µg/L range, that can exert similar MoAs (or even multiple MoA)
Therefore, the potential for additive or synergistic effects to occur may result in the
threshold required for MoA (target) effects (i.e. PGE₂ inhibition) to be easily reached
or surpassed in wild fish. Table 20. shows the measured environmental concentraton
(MEC) of some COX inhibitors in UK surface waters (data obtained from Kasprzyk-
Hordern et al., 2009) and the reported NOEC and LOEC values obtained in this
study. The total measured concentrations of the COX inhibitors (2,143 ng/L) is 4-
fold below the NOEC (9 µg/L) and 126-fold below the LOEC (270 µg/L). However,
this is not a comprehensive list of all COX inhibitors, the impact of these compounds
of different life-stages may produce lower NOEC or LOEC values.

Table 20. Measured surface water concentrations of COX inhibitors in UK surface water (ng/L)
and the NOEC (9 µg/L) and LOEC (270 µg/L) values (for PGEM).

<table>
<thead>
<tr>
<th>COX inhibitor</th>
<th>Surface Water (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen</td>
<td>74</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>261</td>
</tr>
<tr>
<td>Naproxen</td>
<td>146</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>12</td>
</tr>
<tr>
<td>Aspirin</td>
<td>85</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>1,534</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>2,143</td>
</tr>
</tbody>
</table>

MEC vs. NOEC/LOEC
| NOEC (9 µg/L) | Total MEC= 4-fold below NOEC |
| LOEC (270 µg/L) | Total MEC= 126-fold below LOEC |

8.2 IMPLICATIONS OF THE “READ-ACROSS HYPOTHESIS” IN THE
ENVIRONMENTAL RISK ASSESSMENT (ERA) OF
PHARMACEUTICALS

There have been two clear examples of the causal link between exposure and adverse
effects of human pharmaceuticals on wildlife; which are the “feminisation” of wild
fish through exposure to the synthetic oestrogen EE₂ (Jobling et al., 1998; Sumpter,
1995), and the dramatic crash of a population of vultures through accidental
ingestion of diclofenac (Oaks et al., 2004). Although the extrapolation of effects
observed in laboratory studies to the field, and wild fish, are not always directly comparable, these studies form a fundamental component of the ERA of pharmaceuticals. The read-across hypothesis provides an “intelligent testing approach” that incorporates mammalian pharmacology and toxicity data, to predict the likelihood of pharmacological (or toxicological) responses in exposed species. In both of the scenarios where adverse effects have occurred, no or little consideration was given to the mammalian data. Although it was not known at the time, the oestrogenic effects of EE$_2$ observed in fish (Jobling et al., 1998; Parrott and Blunt, 2005; Purdom et al., 1994; Tyler and Routledge, 1998) may well have been predicted using mammalian data, based on the high degree of conservation of the oestrogen receptor between humans and fish (Gunnarsson et al., 2008; Huggett et al., 2003).

Furthermore, although the conservation of the target alone could not have predicted the population-level effects seen in vultures as a result of diclofenac poisoning, certainly an understanding of the toxicity of diclofenac in humans would have highlighted that the ingested dose in these vultures (assuming no metabolism) was very close to the human toxic range (Rand-Weaver et al., 2013).

### 8.2.1 Biomarker selection

The criteria for the read-across hypothesis was used to guide the experimental design of this study, and the MoA of ibuprofen was used to identify relevant molecular and biochemical endpoints, such as COX gene expression and PGEM (surrogate marker for PGE$_2$) as potential “biomarkers” to examine the effects of drug (ibuprofen) exposure on fish. Pharmaceuticals are designed to target specific proteins/biological pathways, which may be conserved in non-target species, and a higher level of evolutionary conservation between human and a given species may potentially increase the risk for eco-toxicological effects (Ankley et al., 2007; Christen et al., 2010; Gunnarsson et al., 2008; Huggett et al., 2003). The use of bioinformatics resources can greatly enhance our knowledge of cross-species target sequence conservation, and possibly species sensitivity, which could be utilised in the risk assessment of human pharmaceuticals. Traditional ERA is based on *in vivo* toxicity testing using apical endpoints that measure survival, reproductive failure, or developmental dysfunction (Villeneuve and Garcia-Reyero, 2011), however,
biomarkers can be more indicative of pharmacological or sub-chronic effects and are therefore more likely to precede toxicological effects (Rand-Weaver et al., 2013).

The response to biomarkers can be variable, and therefore challenges relating to the interpretation of the data can present as a major issue. In this study, COX gene expression was up-regulated in exposed fish (compared to the SC group) and therefore a feedback mechanism was proposed to account for COX enzyme inhibition following ibuprofen treatment. However, due to the complexity of COX gene regulation in humans, and the sensitivity of gene expression as an endpoint, this study highlights the difficulty in extrapolating drug effects in fish, based on their potential effects in humans. A clearer understanding of the expected findings on pharmacological targets following drug exposure (in humans) are required, before the mammalian data can be reliably used to predict pharmaceutical effects in fish. It is known, for example, that some secondary sexual effects observed in fish exposed synthetic progestins (Runnalls et al., 2013) have been linked to their interaction with the androgen receptor, an off-target, rather than the progesterone receptor (the intended target) (Caldwell et al., 2014) therefore, highlighting the complexity of cross-species extrapolation, as well as identifying the need for a wider understanding of fish physiology before molecular endpoints (alone) can be used to assess the impact of ibuprofen (or any other pharmaceuticals) on fish. Nevertheless, PGEM was found to be a relatively robust marker in this study, consistent with the MoA of ibuprofen in humans, although large inter-individual variation was observed (as discussed later). This demonstrates that there is potential for MoA biomarkers to be used alongside apical endpoints to prioritise those species that are potentially sensitive to human pharmaceuticals, and to predict the type of effects that they may elicit in exposed species. A lower degree of target conservation between humans and aquatic species indicates that generally, there is a lack of the biochemical “machinery” required to produce an effect in that particular species. This potentially explains why fish are much more sensitive to the effects of EE2 than invertebrates (Caldwell et al., 2008) which lack, or are not responsive, to oestrogens (Puinean et al., 2006). Therefore, if the MoA of a drug is known, this could be used to more informatively guide toxicological studies, as well as potentially reducing the number
of vertebrate (fish) studies, which are more complex, expensive and time-consuming and replace them with invertebrate studies (Winter et al., 2010).

8.2.2 Internal concentrations

The current ERA process relies heavily upon the exposure water concentration of pharmaceuticals, however, ultimately it is the internal (blood plasma) concentration of a drug at the target that can induce pharmacological or toxicological responses in the exposed organism. The read-across hypothesis is fundamentally driven by the internal exposure concentration(s) of a drug and therefore ibuprofen concentrations were experimentally determined in fish blood plasmas. Although a number of studies have successfully determined the internal (blood plasma) concentrations of pharmaceuticals in fish studies (Bartram et al., 2012; Brown et al., 2007; Cuklev et al., 2011, 2012; Fick et al., 2010; Garcia et al., 2012; Giltrow et al., 2009; Lahti et al., 2011; Mimeault et al., 2005; Nallani et al., 2011, 2012; Owen et al., 2009, 2010; Valenti et al., 2012; Winter et al., 2008), the high cost implications, and lack of instrumentation and technical resources for quantifying pharmaceuticals in aquatic organisms can be a major disadvantage in the applicability of the read-across hypothesis as viable tool for risk assessment. Therefore, an alternative approach for estimating the internal plasma concentrations in fish is the conceptual FPM (Huggett et al., 2003).

8.2.2.1 Applicability of the Fish Plasma Model (FPM)

The FPM can be used to estimate the uptake of a given pharmaceutical into fish, from the surrounding water. A comparison of the modelled and measured plasma ibuprofen concentrations showed that the FPM was most accurate at estimating the FSSPC following exposure to 270 and 370 μg/L ibuprofen (using the Log Kow) (Fitzsimmons et al., 2001; Huggett et al., 2003) which resulted in mean plasma concentrations that were similar to the Cmax. Therefore, highlighting the potential for the FPM to guide future studies where it is necessary to identify the exposure water concentrations required to produce human therapeutic concentrations of a drug in fish, therefore allowing mammalian pharmacological data to be more easily integrated into ERA. Certainly, studies have already demonstrated that there is merit in using the FPM as a potential tool to prioritise pharmaceuticals for further risk
assessment (Roos et al., 2012; Schreiber et al., 2011). However, as observed in this study, different parameters (i.e. Log $K_{\text{ow}}$ and Log $D_{7.4}$) may cause dramatic differences in the estimated $F_{\text{SS}}$PCs, and therefore further experimental validation of the FPM is required, which takes into consideration all potential factors that can influence drug plasma concentrations (i.e. ionisation state, plasma protein binding, sex differences, metabolism and/or excretion of the parent compound and its metabolites). More work is required to overcome the “uncertainties” of Huggett’s theoretical model such as the inter-individual variation in plasma drug (ibuprofen) concentrations observed (discussed below), and the potential role of genetic variability in wild fish, for example, through polymorphisms in the enzymes (e.g. CYP) involved in drug metabolism, which may influence steady-state drug plasma concentrations. The applicability of the FPM may also be limited to estimating the steady-state concentrations of single chemicals from “clean” water (Owen et al., 2007), as variations can exist in the uptake of pharmaceuticals in mixtures, particularly in the environment, as demonstrated in field studies (Brown et al., 2007; Fick et al., 2010; Lahti et al., 2012).

8.3 LIMITATIONS OF THE STUDY

8.3.1 Inter-individual variation

A high level of inter-individual variation was observed in both ibuprofen plasma concentrations, and the molecular and biochemical ($COX$ gene expression and PGEM) endpoints examined in healthy fish. In particular, considerable variation was observed in ibuprofen plasma concentrations, which made it more difficult to relate drug plasma concentrations with target-mediated effects. Therefore, it was not easy to establish what the effective plasma concentrations in fish were, nor the relationship between these effective concentrations with the range of concentrations defined as being effective in humans. Certainly, ibuprofen has a high therapeutic index, ranging from 10,000-50,000 μg/L (following a 400 mg therapeutic dose) (Mehlisch and Sykes, 2013; Regenthal et al., 1999; Schulz et al., 2012), although the Cmax range used for reference in this study was between 15,000-30,000 μg/L (Schulz et al., 2012).
COX gene expression was highly variable in the examined gill, liver and brain tissues, both within and between exposure groups (100, 270 and 500 μg/L) in fish. Gene expression levels can vary considerably between individuals (Whitehead & Crawford, 2005), which must be taken into consideration when performing and interpreting the data from gene expression studies. However, a relatively small sample size (n=4 at each examined concentration) may have contributed to the biological variability observed. In contrast, PGEM was found to be more robust as an endpoint, but despite the larger sample size (n=40 at 370 μg/L), large inter-individual variation was still observed in fish in both the SC (15-fold) and exposure (20-fold) groups. Although it is difficult to pinpoint the exact cause of such variation, it is most likely to be genetic, because even though the fish used in this study were bred from the same genetic strain, they are not identical clones, and therefore, variation may exist between individuals within the same population. To minimise variability from controllable factors, the fish used in exposure studies were of the same age, fish were not fed during any treatment (except in the depuration study, ‘Exposure 3’), the same handling and anaesthetisation procedures were used, and males were used (where possible) to avoid sex being a confounding factor. In further work, it would be useful to gain insight into the role (if any) of sex differences following exposure to ibuprofen (or other NSAIDs), particularly on PGEM concentrations following exposure, as prostanoid functions may vary between male and female fish. A deeper understanding of drug-specific pharmacological effects and fish physiology may help to elucidate where such variation arises from. In humans, both pharmacokinetic and pharmacodynamic factors are sources of inter- and intra-individual variability (Derendorf et al., 2000; Reigner et al., 1997; Sheiner and Steimer, 2000, as well as genetic polymorphisms (García-Martín et al., 2004).

8.3.2 Test-species
The fathead minnow, *Pimephales promelas*, has been used extensively as a model test-species by several different regulatory agencies, including the US Environmental Protection Agency (US EPA), the Environment Agency (EA) and the Organisation for Economic Cooperation and Development (OECD), and more recently to investigate the effects of EDCs (Ankley et al., 2003; Harries et al., 2000; Länge and Dietrich, 2002; Leino et al., 2005; Tyler et al., 1999). Much of the body of literature
has focused on the impact of the environmental oestrogens on reproductive functions, mediated through the oestrogen receptor and, the development of risk assessment geared toward reproduction-related endpoints (Segner et al., 2013). However, the diverse signalling pathways that can be activated through the oestrogen receptor suggest that these chemicals may have much broader functions in fish, other than just reproductive functions (Filby et al., 2006). So far, our knowledge of the impact of even the most-well studied pharmaceuticals (i.e. natural and synthetic oestrogens) is probably one-dimensional, because retrospectively, rather little is known about the physiology of fish species (or any other aquatic species). For example, further information is required about the metabolism of human drugs in fish i.e. do fish possess similar drug-binding plasma proteins to those found in mammals, and do these proteins have similar or different binding kinetics for human drugs? Secondly, a broader understanding of the inter-individual variation observed in fish sample populations in response to humans drugs is required, for example, what level of genetic variation is present in fish populations bred from the same genetic strain i.e. are genetic polymorphisms present between and within fish species, and is such variation only observed at certain levels of exposure, for example, at non-lethal doses only?

Another potential issue with using the selected test-species is that, in some cases, translating the effects of drugs designed to act in humans, to fish (and other aquatic species) is not straightforward. For example, in this study, we have demonstrated PGE\(_2\) inhibition in fish exposed to ibuprofen, which consistent with its MoA in humans. Ibuprofen is widely used to treat pain and inflammation, which in patients is usually measured by analgesic dose-responses and pain perception, which can be highly subjective. However, our knowledge of pain perception in fish, and even “healthy” fish is rather limited, and therefore the expected outcome may not be as obvious as, for instance, behavioural responses in fish that have been exposed to psychoactive compounds (Valenti et al., 2012), or to changes in reproductive functions in fish that have been exposed to endocrine disruptors (Jobling et al., 1998). Until further information about the physiology of the test-species is acquired, it may not be possible to understand the full extent and diversity of the potential effects that may be elicited by human pharmaceuticals in fish.
Another limitation of using the fathead minnow species, specifically, is the lack of genomic sequencing information available, as the genome has not been published as of yet. This made it more difficult to identify the functionally conserved regions of the putative COX gene sequence in the fathead minnow. Nevertheless, for the purpose of this study (and due to time restraints), it was not necessary to amplify the whole of the COX gene in the fathead minnow, as the amount of sequence obtained was justified in order to design primers to perform qPCR, of which the amplicons were cloned and sequenced to verify their identity.

### 8.3.3 Experimental design

All fish exposure studies were conducted at AstraZeneca’s BEL (Industry) and therefore there were a number of practical limitations due to time and resource availability. For instance, more time points were examined (at regular intervals) versus fewer individuals (n=4 or 5 at each time point) in ‘Exposures 2, 3 and 4’ whereas in ‘Exposure 5’ more fish (n=40) were used in the treatment tank at one time point, but no replicate tanks were included, and none of the exposure studies were repeated more than once (usually included to account for inter- and intra-population variation).

### 8.4 RECOMMENDATIONS FOR FUTURE WORK

Ibuprofen can inhibit PGE$_2$ synthesis in fathead minnows, and therefore, one recommendation for further work would be to explore the wider implications of PGE$_2$ inhibition in fish. Indeed, as in humans, PGE$_2$ has been implicated in several “homeostatic” functions in fish, including reproduction (Fujimori et al., 2011; Sorbera et al., 2001), stress responses (Gravel and Vijayan, 2007) and ion transport (Gravel, et al., 2009; Choe et al., 2006), and therefore, further work is required to demonstrate the consequences (if any) of PGE$_2$ inhibition in both male and female fish.

When assessing the potential effects of drug exposure on aquatic organisms, an understanding of the “expected level” of the biological variation within a sample population could be useful in guiding the experimental design (i.e. sample size, $n$), in order to reduce the uncertainty in the data. Therefore, the experimental design of the study is fundamental to the process of obtaining robust, reliable and reproducible
data that can be used by the ERA to more accurately ascertain the effects of human pharmaceuticals on fish. As such, another recommendation for future work, is to propose a systematic and consortium based approach for research groups conducting drug-exposure studies, whereby the experimental conditions, such as the exposure length, test-species are consistent, relevant endpoints based on the MoA of the drug, and information on the exposure and internal (blood plasma) concentrations are included, to allow easier interpretation of the data when elucidating the potential effects of human pharmaceuticals on exposed aquatic organisms.

The read-across hypothesis was tested in this study using ibuprofen, although a larger study was undertaken to test the applicability of the read-across hypothesis, using other drugs such as fluoxetine and beclomethasone. The outcome from this wider study has shown that these pharmaceuticals provide at least qualitative support for the read-across hypothesis. However, further work would be to confirm the validity of the read-across approach with other human pharmaceuticals that utilise different MoA.
REFERENCES


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Calibration curves used to quantify ibuprofen in exposure water and blood plasma samples using LC-MS/MS. The calibration curves were constructed using 1/concentration (1/x) weighting of the area of ibuprofen (1) ($r^2 = 0.9980$) or area ratio (ibuprofen/internal standard ibuprofen-d$_3$) (2) ($r^2 = 0.9982$) against the concentration of ibuprofen standard. The nominal internal standard concentrations was 80 µg/L ibuprofen.