ANALYTICAL DETERMINATION AND BIOCHEMICAL EFFECTS OF WATERBORNE DICLOFENAC EXPOSURE TO THE COMMON BULLY (*GOBIOMORPHUS COTIDIANUS*)

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Abstract

Diclofenac, a non-steroidal anti-inflammatory drug used as a human pharmaceutical and a veterinary medicine has been identified as a priority emerging organic compound. Diclofenac has been detected within waterways globally at low ng-µg/L concentrations. While diclofenac has been shown to be toxic to aquatic organisms, there is currently a lack of data on the toxicity of diclofenac to endemic New Zealand fish species. A current limitation of ecotoxicity studies is the cost and workload associated with determining exposure concentrations. Cheap and robust alternative extraction methods need to be developed. This study developed a method of passive sampling using nylon filters to extract diclofenac from water samples. Extraction recoveries of diclofenac were concentration dependent and there was no effect of pH on recovery. Average recoveries for diclofenac for 0.05 and 500 µg/L were 103% and 58% respectively. The toxicity of diclofenac on the common bully was investigated in a concentration-dependent and a salinity dependent manner. Exposure concentrations for diclofenac were 0, 0.1, and 1000 µg/L and the salinities tested were 0, 6, and 20‰. The biochemical endpoints used for determination of toxicity were ethoxyresorufin-O-deethylase, glutathione-S-transferase and protein carbonylation. Overall, there was no effect of diclofenac observed on the common bully.

Abbreviations

4'-OHD 4-hydroxy diclofenac

5'-OHD 5-hydroxy diclofenac

AcN Acetylnitrile

BCA Bicinchoninic acid

BSA. Bovine serum albumin

CAT Catalase

CDNB 2,4-Dinitrochlorobenzene

COX Cyclooxygenase

CTR Control temperature room

CYP450 Cytochrome P450

DCM Dichloromethane

DNPH 2,4-Dinitrophenylhydrazine

EC_{50} Effective concentration 50%

EDTA Ethylenediaminetetraacetic acid

EE2 Ethinyl estradiol

EIS Electron impact spectra

EME Electromembrane extraction

EOCs Emerging organic contaminants

EROD Ethoxyresorufin-O-deethylase

GC-MS Gas chromatography mass-spectrometry

GPX Glutathione peroxidase

GSH Glutathione

GST Glutathione-S-transferase

H_2O_2 Hydrogen peroxide

H_2SO_4 Sulphuric acid

HC HEPEs Cortland buffer

HPLC High performance liquid chromatography

LC_{50} Lethal concentration 50%

LD_{50} Lethal dose 50%

LLE. Liquid-liquid extraction

LPME. Liquid-phase micro-extraction

MeOH Methanol

MSTFA N-Trimethylsilyl-N-methyl

trifluoroacetamide

NSAIDs Non-steroidal anti-inflammatory drugs

O_2^- Oxide ion

OH· Hydroxyl ion

PAHs Polycyclic aromatic hydrocarbons

PCBs Polychlorinated biphenyl

PDA Photodiode array

PFTBA Perfluorotributylamin

PPCPs Pharmaceuticals and personal care products

QA/QC. Quality assurance/Quality control

ROS Reactive oxygen species

SBME. Solid bar micro-extraction

SCX Strong cation exchange

SIM Selected ion mode

SOD Superoxide dismutase

SPE. Solid phase extraction

SPME. Solid phase micro-extraction

SUPRAS. Supramolecular solvent

TCA Trichloroacetic acid

UV. Ultra violet

WWTPs Waste water treatment plants

Chapter 1: Introduction

1.1 Introduction

Emerging organic contaminants (EOCs) are compounds not routinely monitored for in the environment and include chemicals that can be classified as "pharmaceuticals and personal care products (PPCPs)" (Richardson et al. 2005). These are products designed for human or veterinary use but which find their way into the environment primarily through waste water. To a large degree their presence in the environment is due to waste water treatment plants (WWTPs) not adequately removing these chemicals from sewerage. Unfortunately, even though the majority of the compounds have short chemical half-lives, they are permanently present within waterways because of continual entry into the environment (Boyd et al. 2003). Given that these are chemicals designed to be biologically active, their pseudo-persistence in the environment raises concerns regarding their impact on aquatic species (Jiang et al. 2014) .

While the presence of EOCs in the aquatic environment is of growing concern, but our understanding of environmental concentrations, and especially impacts on aquatic biota, is limited. Aquatic organisms such as fish are continuously exposed to the contaminants, and owing to their biology are especially at risk. Fish utilise their gills for a multitude of critical functions, including oxygen uptake, nitrogenous waste excretion and osmoregulation (Evans et al. 2005). To facilitate these processes, the gill has a very high surface area, a very thin diffusive distance, and a flow of water is constantly passed over the gill to maintain the diffusive gradients that drive its functions. However, these properties also exacerbate its exposure to waterborne contaminants. A higher exposure will lead to greater accumulation, and enhanced toxicological impact (Blewett et al. 2013). This property makes fish particularly sensitive to environmental toxicants and therefore makes them ideal organisms to study the effects of EOCs. As such fish play an important role in regulatory settings, where impacts on fish can be used to determine safe levels of contaminants in the environment (Lin et al. 2015).

1.2 Emerging organic contaminants

The EOCs are a group of compounds composed primarily of anthropogenic chemicals that are present in the environment. Examples of EOCs include, but are not limited to, veterinary products, pesticides, and PPCPs such as analgesics/anti-inflammatories, antibiotics, cardiovascular pharmaceuticals (β-blockers/diuretics), psycho-stimulants, oestrogens and hormonal compounds, and anti-epileptic drugs (Kanda et al. 2003). These are chemicals that have not been routinely monitored or tested for in the environment. With advances in analytical chemistry and emerging evidence of their impacts on non-target biota, EOCs are increasingly the focus of research to provide new information on their entry into the environment as well as their toxicity (Kennish 2002).

The presence of EOCs within the environment is of concern due to the potential for adverse effects on non-target species through prolonged exposure at low concentrations (Lee et al. 2012). However, despite the growing awareness of EOC's and their potential risk, there is only limited data regarding environmental concentrations and biological impacts. For example, knowledge of EOC levels are largely restricted to the United States (Boyd et al. 2004), Canada (Lissemore et al. 2006), the United Kingdom (Kasprzyk-Hordern et al. 2008), China (Peng et al. 2008), and small European countries considered popular holiday destinations (Kuster et al. 2008; Oliva et al. 2014). That leaves large regions of the globe under- or un-represented in terms of knowledge of the levels of EOCs present. Furthermore, it is important to highlight that many of these studies focus in on a small subset of the total EOCs that may be present. It is even more difficult to find data that has examined the biological impacts of these contaminants on aquatic biota. While some studies will assess the presence of EOC's in a given aquatic setting, they will not follow up on possible toxicological effects (Hao et al. 2007; Wu et al. 2008). Further work is required to identify

the presence and concentrations of EOCs, including PPCP's, in other areas of the world, as well as looking at their impact on endogenous species.

One EOC for which there is growing knowledge of both environmental concentrations and biological impacts is ethinyloestradiol (EE2), an estrogenic compound used as an oral contraceptive (Endrikat et al. 1997). EE2 appears in the aquatic environment in part through improper disposal of pills. Alternatively, EE2 may be metabolised and excreted from the human body appearing in a conjugated form in WWTP effluents. However, in the environment it is converted back to EE2 by microbial activity (Ying et al. 2002). Many studies have reported findings supporting the negative impact of EE2 on aquatic species. For example, increased vitellogenin (a precursor of yolk protein), a lack of sexual differentiation, and feminisation of male fish have been observed (Lange et al. 2001; Örn et al. 2003; Parrott et al. 2005). These changes can have devastating impacts on populations. In one study experimental dosing of low levels of EE2 resulted in the complete elimination of fish from an EE2 treated lake within a few years (Nash et al. 2004; Filby et al. 2007; Kidd et al. 2007).

EE2 is one of the best-studied PCPP's, but there is increasing recognition of the importance of other such chemicals in aquatic environments. One group of interest are the non-steroidal anti-inflammatory drugs (NSAIDs). These are widely used in human and veterinary health as pain relief medications, but they also represent an important class of EOC that present in increasing concentrations in the environment (Angela Yu-Chen et al. 2005; Murray et al. 2010). The NSAIDs include drugs such as aspirin (salicylate) and ibuprofen, the most common pain relief medication worldwide. However, one NSAID, diclofenac, is of particular interest given its known impacts in the environment.

1.3 Diclofenac

1.3.1 Diclofenac as a pharmaceutical

Diclofenac (2-[-2′,6′-(dichlorophenyl)amino]phenyl acetic acid), is a NSAID that is used worldwide as a human pharmaceutical and a veterinary medicine (Green et al. 2004). It is primarily used to treat inflammatory diseases, but is also recommended as pain relief for menstrual cramps, migraines, as well as a variety of muscle aches and pains (Tiwari et al. 2015). In humans, severe side effects of diclofenac include an increased chance of serious cardiovascular thrombotic events, myocardial infarction, stroke, stomach ulceration and renal failure (Chan et al. 2002).

Diclofenac causes its pharmacological effects through the inhibition of the cyclooxygenase (COX) enzyme. This enzyme is responsible for the synthesis of prostaglandins that are signalling molecules used for a wide variety of biological functions, including inflammation and the induction of pain (Vane et al. 1998). Diclofenac binds to both COX-1 and COX-2 isoforms, preventing the conversion of arachidonic acid to pro-inflammatory prostaglandins and thereby reducing inflammation (Kirchheiner et al. 2003). The inhibition of COX-2 results in a reduction of inflammation and pain relief, whereas inhibition of COX-1 results in an increased chance of gastrointestinal distress (Silverstein et al. 2000). Diclofenac is thought to bind more favourably to COX-2 than other NSAID's resulting in it being a more commonly dispensed NSAID for inflammatory illnesses (Mancy et al. 1999).

Diclofenac is metabolised in the liver and has two primary metabolites. The major metabolite is 4'-hydroxy-diclofenac (4'-OHD) while 5-hydroxy-diclofenac (5-OHD) is also produced but at lower concentrations (Lee et al. 2012). Studies have shown the cytochrome

P450 enzymes CYP2C9 and CYP3A4 to be primarily responsible for the conversion of diclofenac to 4'-OHD and 5-OHD, respectively (Yasar et al. 2001; Thörn et al. 2011).

Diclofenac is sold under a number of trade names (Voltaren, Cataflam, Zorvolex, and Zipsor) with the most well-known being Voltaren. Within New Zealand, diclofenac is dispensed in two forms: a tablet for general pain relief, and a gel for targeted pain relief and it the 18th most frequently prescribed drug in New Zealand (PHARMAC). Diclofenac is able to be obtained without prescription in most parts of the world, and thus is sold over-the-counter in supermarkets and pharmacies. The exception to this is the USA and the UK where diclofenac is a prescription drug (Moore 2007; Hug et al. 2012). Due to its accessibility, diclofenac is commonly used in households to treat bumps and bruises, sports injuries, as well as on-going muscular pains. The gel is preferred for long term use as there are fewer adverse side effects associated with it, in comparison to the oral dose (Mester et al. 2002). This is reflected in reported usage rates across countries where it is used and sold in large volumes (Table 1.1).

Table 1. 1: Comparison of diclofenac consumption and diclofenac sold between 1998 and 2008 in a variety of countries

Country	**Year**	**Diclofenac consumption[1]**	**Diclofenac sold (tonnes)**	**Reference**
Serbia	2005	46.4		Mijatovic et al. (2011)
	2006	43.1		
	2007	49.4		
	2008	42.1		
Croatia	2005	16.5		
	2006	20.3		
	2007	14.8		
	2008	13.5		
Denmark	2005	19.9		
	2006	20.7		
	2007	21.4		
	2008	22.3		
Australia	1998	-	4.4	Khan et al. (2004)
England	2000	-	26.1	Jones et al. (2002)
Germany	1999	-	250	Scheytt et al. (2005)[1]

[1] The number of defined daily doses per 1000 inhabitants per day.

1.3.2 Diclofenac as an EOC

The two forms of diclofenac have different routes of entry into aquatic environments. When taken orally. 65% of the dose, as well as metabolites, are excreted. While some of this will be retained within the WWTP system, most enters the environment (Lienert et al. 2007). The topical form of diclofenac enters the environment as the pure compound owing to the mechanism of application. The gel is often rubbed onto the site of pain with excess gel being washed off and entering the household waste water (Kasprzyk-Hordern et al. 2008). Diclofenac is not fully removed during wastewater treatment, resulting in entry into the environment via sewerage (Hartmann et al. 2008). Diclofenac has been recently placed on a watch list for regulation under the European Water Framework Directive due to its extensive presence in waterways and the reported toxicity on aquatic organisms (Acuña et al. 2015).

Research has shown diclofenac is detected in waterways across Europe, Asia, and the Americas (Table 1.2) at concentrations up to 1800 ng/L. Concentrations vary from study to study, likely owing to factors such as: analytical techniques used to determine diclofenac, population densities and rates of diclofenac consumption, the specific WWTP processes at each location, and water body dilution.

With the growing interest in the environmental impacts of PCPPs in the aquatic environment, there has been a need to develop new methods that facilitate the measurement of these chemicals in water (Cleuvers 2003; Ferrari et al. 2003; Santos et al. 2010). Many studies use solid phase extraction (SPE), liquid-liquid extraction or their micro counterparts (Farré et al. 2007; Zhou et al. 2014; Toledo-Neira et al. 2015). These are valid techniques that result in high extraction rates as they have been refined for pharmaceutical extraction (Wu et al. 2008). The issue with these techniques is that they require a laboratory setting for analysis

of samples. Passive sampling using filters is a technique that is being refined and modified as it allows for direct sampling of water sources allowing for in-field extraction (Nyoni et al. 2010). This reduces the need for transporting of water samples to the laboratory in which time degradation is occurring, instead giving accurate real time concentrations (Wille et al. 2011). This thesis develops a method for passive sampling using nylon filters to extract diclofenac from exposure water samples to determine whether it is a viable method for extracting diclofenac from water samples generated in ecotoxicity testing.

Table 1. 2: Diclofenac concentrations present in global water sources

Country	**Diclofenac concentration (ng/L)**	**Water source**	**Reference**
Germany	1300-3300	WWTP	Stülten et al. (2008)
Pakistan	400-1800	River	Scheurell et al. (2009)
Slovenia	9-282	River	Kosjek et al. (2005)
Canada	2-5 140-190	Estuary WWTP	Comeau et al. (2008)
Taiwan	2	Surface seawater	Jiang et al. (2014)
USA	2.5	WWTP	Vidal-Dorsch et al. (2012)
UK	<8-195	Estuary	Thomas et al. (2004)
Portugal	0.18	Sea	Paíga et al. (2015)
Spain	6000 4000	River Sea	Noche et al. (2011)
Ireland	110-550	Sea	McEneff et al. (2014)

In New Zealand, no studies have been carried out to detect the presence of diclofenac in local waters. Given that diclofenac is one of the most frequently prescribed drugs in New Zealand and is also available over the counter it would be expected that diclofenac would be present in waterbodies receiving WWTP effluents. Due to the absence of research within New Zealand, studies need to be carried out to investigate the presence of diclofenac in the aquatic environment and its impact on local aquatic species (refer to Section 1.5).

1.3.3 Environmental chemistry of diclofenac

1.3.4 Diclofenac and biochemical effects

1.3.4.1 Effects on cytochrome P450 activity

Cytochrome P450s (CYP450) are a class of more than 50 different enzymes, with well-characterised endogenous roles in the production of steroids, cholesterol, bile acids and eicosanoid signalling chemicals (Miners et al. 1998). CYP450 enzymes are also well known for their role in drug metabolism as Phase 1 detoxification enzymes (Guo et al. 2014). Consequently, as described above, CYP450s are responsible for the breakdown of diclofenac (Bort et al. 1999). Upon exposure to this, and other organic chemicals, CYP activity can be induced. This occurs via a mechanism that involves a xenosensor, such as the aryl hydrocarbon receptor (Bass et al. 2009). Once activated this sensor will induce CYP activity, so that the organism can deal with the influx of drug or toxicant. Induction of CYP activity by diclofenac has been noted in the literature (Lauer et al. 2009).

The induction of CYP450 is a useful biomarker of exposure to organic contaminants, especially within aquatic species (Lopes et al. 2011; Burkina et al. 2012). Such contaminants include chlorinated dibenzo-p-dioxins, dibenzofurans, polycyclic aromatic hydrocarbons (PAHs), and planar polychlorinated biphenyls (PCBs). All these contaminants induce

CYP450 activity in fish tissues following environmental exposure (Ricciardi et al. 2006). Diclofenac has also been shown to induce CYP activity in fish (Mehinto et al. 2010).

1.3.4.2 Diclofenac and effects on Phase II metabolism.

Once a drug or toxicant has undergone oxidation by the CYP enzymes, it is common for Phase II metabolism to occur. This phase involves the conjugation of the metabolite with a chemical group, making the metabolite more soluble, and able to be excreted (Zamek-Gliszczynski et al. 2006). There are a series of different enzymes that are responsible for Phase II metabolism, including UDP glucuronosyltransferases, N-acetyltransferases, and glutathione S transferase (GST) (Willett et al. 2000). Like for Phase I enzymes, Phase II pathways can be upregulated by exposure to toxicants. For example, GST has been shown to be induced by a number of pharmaceuticals and organic toxicants (Xu et al. 2005), including diclofenac (Khanduja et al. 1997).

1.3.4.3 Oxidative stress markers

One common mechanism of toxicity shared among a variety of different toxicant classes is the induction of oxidative stress (Lushchak 2011). Oxidative stress is the result of an imbalance between reactive oxygen species (ROS) and the antioxidant systems of the body. Metabolic processes are responsible for the formation of certain ROS such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and the hydroxyl radical ($OH\cdot$) anion (Malanga et al. 2004). Reactive oxygen species cause toxicity through binding to proteins, lipids and DNA/RNA. Due to their reactive nature, they bind to the DNA bases causing structural alterations that go on to affect translation and transcription resulting in inhibition of protein and enzyme formation (Wiseman et al. 1996; Valko et al. 2005). Diclofenac has been known to cause oxidative damage through binding to lipids resulting in an increase in lipid peroxidation (Gomez-Olivan et al. 2014)

Countering the ROS are a number of anti-oxidant mechanisms that act to scavenge ROS before they can cause damage. These include the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX; Oviedo-Gomez, 2009). Consequently, oxidative stress can be measured as either an increase in ROS that cause effects, an increase in oxidative damage, or as a change in the activity of anti-oxidant defence mechanisms. Diclofenac has been known to react with glutathione (GSH) indicating it is metabolised to prevent damage to cells. Hepatic protein adducts have been detected in liver cells in mice resulting in the diclofenac-GSH conjugate becoming a useful biomarker for the hepatotoxicity of diclofenac (Grillo et al. 2003).

1.3.5 Diclofenac toxicity

1.3.5.1 General toxicity

The ability of diclofenac to act as an environmental toxicant was first shown by studies that showed severe impacts of this drug on vultures. From 1992 until 2007, there was a significant decline in vulture numbers in Asia. Among the affected species, the white-rumped vulture (*Gyps bengalensis*) showed a 99.9% population decline while populations of the Indian vulture (*Gyps indicus*) and the slender-billed vulture (*Gyps tenulrostris*) declined by 95% (Swan et al. 2006a). Analysis of vulture carcasses found that a major factor in this decline was diclofenac. The diclofenac was used as veterinary pain relief on farmed cattle. When the cattle died, they were left in the fields to be consumed by the vultures (Cuthbert et al. 2011). The LD_{50} (dose that causes 50% mortality in the population) of diclofenac for vultures is considered to be between 98 and 225 µg/kg, making diclofenac a Class 1 (the most severe class) toxic compound for vultures. This lead to diclofenac manufacturing being banned in India, Pakistan, and Nepal in 2006 (Taggart et al. 2009).

Studies have investigated the toxicity of diclofenac on differing fish species to determine the median lethal concentration (LC_{50}), median effect concentration (EC_{50}) and the median lethal dose at 50% (LD_{50}). Diclofenac has been recorded as having an LC_{50} of 166.6 ± 9.8 mg/L in juvenile zebrafish (*Danio rerio*) and an LC_{50} of 6.11 ± 2.48 mg/L in embryos of the same species (Praskova et al. 2011). The LC_{50} of diclofenac for the African catfish (*Clarias gariepinus*) was lower than that for the zebrafish at 25.12 mg/L (Ajima et al. 2015). Regarding the $EC_{50,}$ a dose of 6 ng egg^{-1} was found to affect half of the population in medaka fish (*Oryzias latipes*) embryos (Nassef et al. 2010). The EC_{50} was determined for two cell lines of rainbow trout (*Oncorhynchus mykiss*) hepatocytes with concentrations of 19 µM and 420 µM effecting half of the cells exposed (Laville et al. 2004). The EC_{50} of diclofenac in zebrafish embryos was recorded as 5.3 mg/L (van den Brandhof et al. 2010). In summary, diclofenac is known to be toxic to fish species, however toxicity differs between species as well as age. Hence further studies need to be carried out to determine the toxicity of diclofenac on the common bully as the values cannot often be compared across species.

1.3.5.2 Effect of salinity on toxicity

Salinity is an environmental factor that exerts significant influence over the function of aquatic biota. Osmoregulating organisms such as fish, must maintain an internal osmotic concentration in spite of fluctuations in external osmotic concentrations. In freshwater, fish are more concentrated than their surrounds are thus constantly faced with diffusive ion loss, and water gain, and thus to maintain salt and water balance must actively take up ions and excrete excess water (Evans et al., 2005). These tasks are principally achieved by the gills and the kidneys, respectively. In marine settings the opposite occurs, with diffusive ion gain and water loss (Evans et al., 2005). In seawater the gill is again the principal tissue associated with regulating ion transport, and fish also drink in order to balance water loss. Because of the differences in physiology, there can be significant differences in the toxicological impacts

of a contaminant, depending on whether the organism is in freshwater or seawater. For example, drinking in seawater will expose the gut to waterborne contaminants, potentially increasing the sites of absorption and thus toxicity. Such an effect has been observed in fish exposed to the organic toxicant naphthalene (Levitan et al. 1979). It is also important to note that the different water chemistry between freshwaters and seawaters may also influence the chemical form of a toxicant, and thus salinity may also impact toxicity in this manner (Glover et al. 2016).

Because of the important potential influence of salinity on toxicant effect, research is now investigating the effect of salinity on toxicity of pharmaceutical compounds. For example, one study has examined the impact of ibuprofen and salicylate exposure on the hyposmoregulatory capacity of rainbow trout. Salicylate, and to a lesser degree, ibuprofen both disrupted corticosteroidogenesis, which impacts the acclimation of fish to seawater (Gravel et al. 2009). Understanding how environmental variables such as salinity impact uptake and toxicity of diclofenac is crucial to protecting aquatic organisms. Such data are of particular relevance as many of the receiving environments where diclofenac will eventually end up will be impacted by salinity.

1.4 Methods for determining toxicity

When determining the effects of environmental contaminants on aquatic species there are two main approaches used: field-based biomonitoring and laboratory-based exposures. Biomonitoring is where organisms are obtained from the wild and analysis of animal health and/or body burdens of contaminants provides results directly relevant to environmental levels of contaminants. Biomonitoring can therefore provide information of the impact of pollutants on natural populations (Lu et al. 2013). Exposures involve laboratory experiments where organisms are exposed to contaminants of known concentration under controlled

environmental conditions (e.g. temperature, flow rate, pH, salinity; (Bostrom et al. 2015). In these experiments acute and chronic toxicities are able to be tested, and mechanisms of toxicant effect can be isolated from confounding influences of the environment (Lee et al. 2011; Galus et al. 2013). These studies therefore provide data that can be used to predict environmental effects and help determine the impact of compounds of concern (Munoz et al. 2008; Kosma et al. 2014). Eventually such data can be used by regulators to place restrictions on the level of contamination of specific compounds in the environment (Sayeed et al. 2003).

1.5 New Zealand fish

In New Zealand there are approximately 66 freshwater species, over 1,000 marine species and 270 coastal species of fish, with approximately 40%, 11% and 25% of the species being endemic, respectively. Some of the species have required conservation care in the form of marine reserves to maintain population numbers and diversity (Davidson et al. 2002). A high rate of endemism means that many of the fish species at risk from environmental contamination in New Zealand do not feature in overseas studies as they are only found in New Zealand where there is a notable lack of research regarding diclofenac as a contaminant. As regulatory tools are based on model Northern hemisphere species (Stockin et al. 2007), it means that if there are significant differences in sensitivity between well-studied overseas species and New Zealand fish, then New Zealand fish may not be protected by regulations.

1.5.1 Common bully

The common bully is a freshwater fish species endemic to New Zealand and is a member of the Gobiidae family. It is one of seven species of the *Gobiomorphus* genus found across New Zealand. The other 6 are the giant bully (*G. gobioides*), the Cran's bully (*G. basalis*), the upland bully (*G. breviceps*), the redfin bully (*G. huttoni*), Tarndale bully (*G. alpinus*), and the bluegill bully (*G. hubbsi*) (Department of Conservation 2016). The common bully is a

small fish, reaching a maximum length of 15 cm and weighing no more than 16 g at maturity. It has a blunted head, a solid body and two dorsal fins. Their body colour ranges from light- to mid-brown depending on habitat and light exposure, with males obtaining a black colouration during mating season. During spawning, the females lays eggs that are orange in colour, with the eggs being laid on any hard surface. The males will fertilise the eggs forming territories that they then protect (Rowe 1996).

The common bully is amphidromous. This is a type of diadromy that involves hatching in estuarine waters, followed by a migration from fresh to salt water during the larval stage, before a return to freshwater (Michel et al. 2008). The migratory period during the larval stage is not obligatory, meaning some fish will reach maturity in freshwater sources (Hicks 2012). Studies show that these non-migratory fish are more commonly found in inland water sources, while the diadromous members of the species are found closer to the sea. Due to its amphidromous nature, the common bully can be found in marine, brackish, and freshwater environments making it an ideal organism in which to examine the impacts of salinity on toxicity.

The common bully is a demersal species, being commonly found in the water column just above, or on, the river or sea bed where it feeds on invertebrates. It prefers rivers and streams that are shallow with a swift current and cover (Jowett et al. 1995). These waterways are likely to come in contact with diclofenac as they are often found near estuaries and wastewater outputs. The common bully is also present in many urban streams, which may be subjected to domestic runoff (Kostarelos et al. 2010).

1.5.2 Previous toxicology studies using the common bully

The common bully has previously been used New Zealand toxicology studies. For example, van den Heuvel et al. (2007) used the common bully to monitor the impacts of pulp and paper effluent in the Tarawera River in New Zealand. It was determined that there was an

impact on spawning season, where spawning occurred in winter rather than spring. It was concluded that this was a genetic impact rather than one relating to effluent exposure. However, these authors attributed the genetic impact to effluent exposure, by suggesting that the effluent exposure was high enough in early years of pulp milling to diminish the downstream population causing a genetic bottleneck

However, some studies have shown that the bully is impacted by environmental pollution. For example, exposure to pulp and paper effluents was shown to result in changes in reproductive physiological function in this species (Bleackley 2008). Specifically, a depletion of carotenoid pigments and a disruption of follicular steroid biosynthetic capacity in females was observed (Bleackley 2008).

1.6 Research rationale

. There is a lack of knowledge regarding the impact of diclofenac on native fish species in New Zealand. The common bully is saline tolerant and is found in locations where diclofenac could occur (coastal streams, urban waterways). Because of this, developing a method for practical sampling in the field is essential. Passive sampling can be used to obtain real-time values present in the environment as well as useful in lab practices regarding cost efficiency. Based on previous research it is known that exposure of fish to diclofenac results in changes in Phase I and II metabolism, and that oxidative stress is an impacted end-point. Changes in these end-points over short-term exposures can be indicative of long-term toxic impacts (Scholz et al. 2008). Diclofenac is an important contaminant to consider due to it proving to be toxic to aquatic species and being prevalent in waterways. It has already greatly impacted the vulture species in India and more work is being carried out to determine its impact on aquatic species to prevent another species decline.

1.7 Aims

The aims of this thesis were to;

- Develop an extraction technique that is cost- and time-effective for extracting diclofenac from water samples collected from ecotoxicity assays.
- To test for a range of biochemical endpoints to determine the effect of diclofenac on the common bully.

1.8 Thesis structure

This thesis is broken down into five chapters. Following this general Introduction (Chapter 1), Chapter 2 describes the chemical and biological methods used throughout the following chapters. Chapter 3 outlines the development of a method for determining diclofenac in water. This passive filter-based method, coupled with advanced chemical analysis is shown to be an effective method for detection of low level, environmental, concentrations of diclofenac in water. Chapter 4 describes a study examining mechanisms of diclofenac toxicity to the common bully looking at the effects of dose and water salinity. It shows that the common bully displays changes in biochemistry that trend towards those observed in other studied fish species. A general discussion and conclusion to this thesis can be found in Chapter 5 that summarises the experimental chapters and places this work in a literature and environmental context.

Chapter 2 : Methods

2. Overview

This chapter outlines the methods used in this thesis, including fish capture and acclimation, glassware cleaning procedures, diclofenac exposures, biochemical assays, analysis of water samples, and statistical analysis. Each sub-section outlines the preparation of reagents and provides an overview of the method. All procedures involving fish carried out were approved by the University of Canterbury Animal Ethics Committee.

2.1. Sampling sites and method capture

Collections of the common bully (*Gobiomorphus cotidianus*) were carried out between the months of July and September 2015 from Lake Ellesmere. Fish were collected at a site just off of Timber Yard Road (-43.797404, 172.370574). Lake Ellesmere is a large brackish lake south of Christchurch, which at the time of collection had an average salinity of 6.9‰. Approximately 70 fish were caught using a 6 m long drag net. The fish were transported back to the university in lidded buckets with bubblers inserted to keep the water oxygenated. The buckets were secured in the truck to prevent excess movement. The weights and lengths of the fish caught were 5.83 ± 2.55 g (mean ± standard error of the mean; SEM) and 8.05 ± 1.34 cm (mean ± SEM), respectively.

2.2. Acclimation

At the University of Canterbury, fish were held at 15°C, under a light regime of 14 h light: 10 h dark, with dawn and dusk transitions. Fish were subjected to three distinct salinity conditions through the course of the thesis (freshwater, 6‰, and 20‰). Fish used in the freshwater exposures were held in plastic 80 L tanks with approximately 24 fish per tank. These tanks were supplied with flow-through freshwater a flow rate of approximately 10 mL/min. The freshwater had the following water chemistry: pH 7.1; total hardness 0.7

mmol/L; total alkalinity 0.519 mmol/L; electrical conductivity 18.8 mS/m; Ca 0.57 mmol/L; Mg 0.14 mmol/L; K 0.29 mmol/L; Na 0.37 mmol/L; Cl 0.31 mmol/L; dissolved organic carbon (DOC) <0.2 mg C/L. The tanks were checked daily to monitor the flow of water, cleanliness of the tanks, and the health of the fish. Fish were fed to satiation every three days using Nutrafin Max fish food flakes, with excess food removed the day after feeding. Fish were not fed two days prior to, nor for the duration of, exposures. These freshwater fish were held in the tanks for a minimum of 7 days before use in an exposure.

Following collection, two groups of fish were immediately subjected to salinity acclimation. These fish (collected at 6.9‰), were held in tanks under conditions identical to those described above, but with static-renewal water flow conditions. The group to be used at 6‰ were immediately transferred to water with a salinity of approximately 6‰, and held for 21 days. Complete water changes were conducted every 3 days. For the second group, after three days being held at 6‰, water in the tank was completely replaced with water at a salinity of 10‰. This process of water changes and salinity increases (3 to 4‰ per change) was repeated every 3 days until a final salinity of 20‰ was attained. Fish in both salinity-acclimation groups were held for a minimum of two weeks before use in an exposure. Salinity was adjusted by addition of natural seawater (collected from Lyttelton Harbour at sites seen in Figure 2 below). At a salinity of 6‰ the water chemistry was: pH 7.2; Na 98 mmol/L; Ca 3 mmol/L; Mg 11 mmol/L; K 2 mmol/L; DOC <0.2 mg C/L, while at 20‰ the water chemistry was: pH 7.6; Na 325 mmol/L; Ca 9 mmol/L; Mg 37 mmol/L; K 8 mmol/L; DOC 0.3 mg C/L. The feeding regime for salinity-acclimated fish was the same as for those kept in freshwater.

2.3. Glassware preparation

The glassware used for the exposures (tanks used to hold fish during experimentation, bottles containing diclofenac solutions, and lidded jars used to hold water samples) were acid

washed in 14% nitric acid for a minimum of 24 hours to minimise contamination. They were then rinsed clean using deionised water and left to air dry for approximately 24 hours. Once dry, they were then rinsed in triplicate with each of High Performance Liquid Chromatography (HPLC) grade dichloromethane (DCM), methanol (MeOH), and acetonitrile (AcN) resulting in a total of 9 rinses. These were left to dry overnight before use.

All glassware used for water analysis, HPLC, and Gas Chromatography- Mass Spectrometry (GC-MS) sample preparation (including Schott bottles, measuring cylinders, volumetric flasks, funnels, storage bottles and vials) were left overnight in a detergent bath and then rinsed using tap water and left overnight to air dry. They then underwent three MeOH rinses, followed by three DCM rinses, and three AcN rinses, with any residual solvent left to evaporate off in a fume hood overnight before use. Glassware was cleaned in this manner in between every use. GC-MS glassware had to be MeOH free before use to prevent interference with the derivatisation agent (N-Methyl-N-(trimethylsilyl) trifluoroacetamide; MSTFA).

2.4. Diclofenac exposures

2.4.1. Diclofenac dosing

Stock solutions were used to dose the water used in the exposures. Three dosing solutions were prepared. A 500 mg/L solution was prepared by dissolving 0.1345 g of diclofenac in 250 mL of Milli-Q water (> 18 MΩ). The 500 mg/L stock was diluted with Milli-Q to prepare 10 mg/L and 50 µg/L dosing solutions. The 500 mg/L and the 50 µg/L solutions were added to 2 L of water in 4 mL volumes to obtain final concentrations of 1000 µg/L and 0.1 µg/L respectively. The 0 µg/L control was obtained by using a clean measuring cylinder (Section 2.3) to measure the 2 L of exposure water (freshwater, 6 or 20‰).

2.4.2. Concentration-dependent effects of diclofenac

Exposures were carried out in a controlled temperature room (CTR) set to 12 ± 1 °C in 3 L amber glass tanks, cleaned as described above (Section 2.3). To minimise diclofenac degradation, exposures were conducted in the dark, with red light used when necessary. Air lines were inserted into each exposure chamber to facilitate aeration. Exposures were carried out in 2 L of aquarium freshwater. Three diclofenac concentrations were used: 0 µg/L (control), 0.1 µg/L (environmental mimic), and 1000 µg/L (effect level). Diclofenac was added to exposure waters for 24 hours before fish were added to allow equilibration. The stock solutions of diclofenac (see Section 2.4.1) were stored in the CTR in 4-L lidded amber bottles for 24 hours before use. Each exposure concentration had eight replicates (fish), with 1 fish per exposure tank.

Exposures were conducted for 96 h, with water changes at 48 h using solutions that had been equilibrated for 24 h, as above. Water samples were taken from the exposure tanks at four time points for chemistry analysis (see Sections 2.6.2 and 2.6.4): 0 h, 48 h before the water change, 48 h after the water change, and 96 h. Samples were taken by filling a 100 mL amber lidded jar to the brim with water from the exposure tank. Gloves were worn while carrying out water sampling to minimise contamination. Samples were processed immediately and kept covered to avoid potential photodegradation (refer to Section 2.6.1).

2.4.3. Effect of salinity on diclofenac toxicity

These experiments were conducted under the same environmental conditions as those described in Section 2.4.2, with the exception of the salinity of the exposure water (0‰, 6‰ and 20‰) and that just two diclofenac concentrations were examined (0 and 1000 µg/L). The 96 h exposure was conducted identically to those described for concentration-dependent

exposures, with stock solution handling, water pre-equilibration, water changes, and water sampling for diclofenac conducted as previously detailed.

2.4.4. Tissue sampling protocol

At the end of exposures, fish were euthanised by anaesthetic overdose (1 g/L tricaine methanosulfate, Sigma Aldrich), followed by severing of the spinal cord. Each fish was individually measured for body weight (± 0.01 g) and total length (± 1.0 mm). Gills were removed immediately after euthanasia and used directly for ethoxyresorufin O-deethylation **(**EROD) analysis (Section 2.5.1). The liver was dissected, wrapped in aluminium foil, flash frozen in liquid nitrogen, and then stored at -80°C. The liver tissue was later used in the analysis of oxidative stress markers (glutathione S-transferase assays and protein carbonylation; Sections 2.5.2 and 2.5.3).

2.5. Biochemical assays

2.5.1. Ethoxyresorufin O-deethylation assay

The EROD assay was based on that described by Jönsson et al. (2003), which utilises measurement of the colour change from clear to pink when ethoxyresorufin undergoes O-deethylation to form resorufin. Briefly, freshly dissected gills were rinsed with HEPES-Cortland (HC) buffer and then placed into a 12-well cell culture plate containing 2 mL of HC buffer per well. The HC buffer composition was as follows: 0.38 g/L KCl, 7.74 g/L NaCl, 0.23 g/L $MgSO_4 \cdot 7H_2O$, 0.23 g/L $CaCl_2 \cdot 2H_2O$, 0.41 g/L $NaH_2PO_4 \cdot H_2O$, 1.43 g/L HEPES, 1 g/L glucose, pH 7.7. Approximately 8-10 gill filaments were cut into 2 mm pieces with fine scissors to maximise exposure to the buffer. The HC buffer was removed from the wells and replaced with 500 µL of reaction buffer. The reaction buffer was prepared by adding 0.5 µL of 10^{-6} M 7-ethoxyresorufin and 0.5 µL of 10^{-5} M dicumarol to 0.5 mL of HC buffer. The volumes of each reagent were adjusted depending on the total volume of reaction buffer

required. The gills were left to incubate at room temperature for 10 minutes. After incubation, 200 µL duplicate samples of reaction buffer were transferred to a Greiner Bio-one non-binding black bottomed 96-well microplate. The remaining reaction buffer was replaced with 700 µL of fresh reaction buffer and left to incubate at room temperature for a further 30 minutes, at which point a further 200 µL sample was transferred to the 96-well plate. Duplicates of resorufin standards (obtained from Sigma-Aldrich) ranging from 0 to 250 nm were added to generate a calibration curve. This was carried out for every run. Duplicates of reaction buffer, and Milli-Q water were added in 200 µL volumes to acts as controls and blanks respectively. The samples were run on a plate reader (SpectraMax M5) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

To calculate EROD activity, a scatter graph was generated using picomoles of the resorufin standards on the x-axis and the fluorescence of the standards on the y-axis. A trend line was inserted and the corresponding equation was used to convert fluorescence to an amount of resorufin formed (pmol). From the resulting value, the mean 'blank' was subtracted from each sample to correct for background, and corrected for dilution factor. This value was divided by the time period it was incubated for (10 or 30 minutes), and the number of filament tips, to give activity values as pmoles of resorufin per filament tip per minute (Jonsson et al. 2003).

2.5.2. Glutathione S-transferase assay

The activity of glutathione S-transferase (GST) was determined by an assay that utilises the GST-mediated conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB), and measures the resulting absorbance change at 340 nm (Wilce et al. 1994). GST activity was determined via an assay kit purchased from Sigma-Aldrich (CS0410; Sigma-Aldrich). Before commencing the assay, reagents were prepared. A 10 mL master mix was

prepared by mixing 14.7 mL of Dulbecco's phosphate buffered saline, 0.1 mL of 200 mM reduced L-glutathione and 0.1 mL of CDNB together, which was sufficient for 75 assays carried out in a 96-well plate. The CDNB and the Dulbecco's phosphate buffered saline were warmed to 25°C before being mixed.

Liver tissue (approximately 0.09 g) was homogenised for 30 seconds using a plastic centrifuge tube homogenisation tool in cold buffer (100 mM Tris-base, 2 mM EDTA, 5 mM $MgCl_2.6H_2O$, pH 7). Into a 96-well plate, duplicates 4 µL controls of provided GST, triplicate 20 µL samples of homogenised liver, and duplicate 200 µL blanks of master mix were added to individual wells. Master mix was added to the samples and controls to make the total volume up to 200 µL. The plate was gently shaken to mix the components before being run on the plate reader. The absorbance was set to detect wavelengths at 340 nm. The samples ran for 6 minutes while shaking, with readings taken every minute.

A trend line was fitted to a calibration curve and the equation obtained from it produced the absorbance per minute value (ΔA_{340}/min) for each sample. To obtain the ΔA_{340}/min value for the blank, the highest A_{340} obtained over the six minutes was subtracted from the initial A_{340} (the lowest absorbance value), and this was then divided by the time difference between these two values. The ΔA_{340}/min of the blank was then subtracted from the ΔA_{340}/min of each of the samples. The corrected ΔA_{340}/min was then used in Equation 2.1:

Equation 2.1

$$\frac{\Delta A_{340}/\text{min} \times V\,(\text{mL}) \times \text{dil}}{\varepsilon_{\mu M} \times V_{enz}\,(\text{mL})} = \mu mol/mL/\text{min}$$

where V (mL) was the reaction volume used (0.2 mL), *dil* was the dilution factor of the sample, $\mathcal{E}_{mM}$ (µM/cm) was the extinction coefficient for CDNB conjugate at 340 nm (5.3 µM), and V_{enz} (mL) was the volume of the enzyme tested (20 µL). The equation produced GST specific activity in µmoles per mL/min.

2.5.3. Protein carbonylation assay

The protein carbonylation assay measures the production of stable carbonyl groups that form as a result of protein oxidation (Colombo et al. 2016). To determine carbonyl content, the assay works on the basis of derivatising protein carbonyl groups using 2, 4-dinitrophenylhydrazine (DNPH). This results in a colour change from a vivid yellow to a colourless solution, which can be monitored spectrophotometrically at 375 nm. The assay protocol used was from a kit obtained from Sigma Aldrich (MAK094).

Before commencing the assay, the vials containing the reagents were centrifuged before being opened. A 10 mL vial of acetone was placed in the freezer at -20°C whilst bringing the following reagents to room temperature: DNPH solution, streptozocin solution, and 6 M guanidine solution. Homogenised liver samples that were prepared for the GST assay were bought to room temperature and centrifuged to remove insoluble material. The samples were then diluted with Milli-Q water to reach a protein concentration of 10 mg/mL. To ensure there was no interference from nucleic acids, 10 µL of the 10% streptozocin solution was added for every 100 µL of sample. Upon addition of the streptozocin, samples were left to incubate at room temperature for 15 minutes before being centrifuged at 13,000 x g for 5 minutes. The supernatant was transferred to a new tube. For the DNPH assay reaction, 100 µL of the DNPH solution was added to each supernatant sample. The sample was vortexed for approximately 5 seconds before being left to incubate at room temperature for 10 minutes. After incubation, 30 µL of ice-cold 10% trichloroacetic acid (TCA) solution was added to each sample to precipitate the proteins. The sample was vortexed for approximately 5 seconds before being left to incubate on ice for 5 minutes. Samples were then centrifuged at 13,000 x g for 2 minutes. The supernatant was carefully removed and discarded. A 500 µL volume of ice-cold acetone was added to each pellet to remove excess DNPH before

sonicating in a bath for 30 seconds. Post sonication, the sample was left to incubate at -20°C for 5 minutes before centrifuging at 13,000 x g for two minutes. The acetone was discarded gently using a Pasteur pipette to prevent disturbance of the TCA pellet, removing any free DNPH remaining. To the TCA pellet, 200 µL of 6 M guanidine solution was added before sonicating for 30 seconds. Once the pellet had re-solubilised, 100 µL of each sample was transferred in triplicate to the 96-well plate provided with the kit. Absorbance was measured at a wavelength of 375 nm using a microplate reader.

To determine the amount of carbonyl in the sample well, the following equation was used:

Equation 2.2 $$C = (A_{375} / \varepsilon_{mM} \times \lambda) \times V$$

where *C* is the amount of carbonylation per well (nmole/well), A_{375} is the corrected sample absorbance, ε_{mM} is the millimolar extinction coefficient (6.364 mM/cm) multiplied by the path length in a well (λ; 0.2893 cm) and the 100 represents the total volume (*V*) in the well (100 µL). To then determine the amount of carbonyl per mg of protein, Equation 2.2:

Equation 2.3 $$CP\ (\text{nmol carbonyl/mg protein}) = (C/P) \times 1{,}000 \times D$$

Where *C* is the result of Equation 2.2, *P* is the amount of protein in the well, D is the dilution factor, and 1,000 is the conversion factor (µg to mg). The end result is the concentration of protein carbonylation in nmol per mg of protein.

2.5.4. Bradford assay

The (Bradford 1976) assay is a commonly used protein determination assay. It works on the principle of a colour change from brown to blue upon the dye, Brilliant Blue G, binding to the protein in a concentration-dependent manner (Bradford, 1976). Samples of

liver homogenates and standards (bovine serum albumin (BSA); Sigma-Aldrich) were sub-sampled onto a non-UV clear bottomed 96 well plate in 10 µL aliquots. To each well, 200 µL of Bradford reagent (Sigma-Aldrich) was added, and after a 10-minute incubation, absorbance was measured on a plate reader at 595 nm. A calibration curve was constructed, and protein concentrations of the homogenised liver samples were calculated. This assay was used for all protein concentration measurements except those used to standardise the protein carbonyl assay (see Section 2.5.5). The Bradford assay was not suitable for protein determination in the protein carbonyl assay due to interference from the guanidine in the test samples.

2.5.5. Bicinchoninic acid assay

To determine protein concentration of the samples run during the protein carbonyl assays, a bicinchoninic acid (BCA) assay was performed (BCA1-1KT; Sigma Aldrich). The BCA assay works on the basis of a colour change from pale blue/green to purple upon proteins reducing Cu (II) to Cu (I) (Huang et al. 2010). Using a 96 well plate, 5 µL of sample was transferred along with 200 µL of BCA working reagent. Standards were made from BSA as in Section 2.5.4. Milli-Q water and the buffer used to prepare the homogenised tissue (Section 2.5.2) were used as blanks for the standards and samples respectively. The plate was left to incubate at 37°C for 30 minutes. The plate was then read at 562 nm using a microplate reader. Protein concentrations were determined from a calibration curve.

2.6. Analytical protocols

2.6.1. Diclofenac extraction

Diclofenac was extracted from water samples using a filter absorption method as described in detail in Chapter 3. Water samples of 100 mL volume were transferred to Schott bottles, acidified to a pH of 2 using approximately 3 drops of H_2SO_4, and had a Sartorius Biolab 4.8 cm diameter, 0.2 µm nylon membrane filter added. The sample was then covered in aluminium foil to protect against photo-degradation and put on a shaker table for 24 hours at medium speed. The filter was removed and transferred to a 15 mL amber vial, which was then placed on a heating block at 65-100°C to dry. The filter was rinsed three times using 4 mL of MeOH, and was vortexed during every rinse to ensure maximum extraction of diclofenac from the filter and the aliquots of MeOH were combined in a 15 mL amber glass vial. The MeOH was then evaporated off under nitrogen at 65°C until dry. The vial was rinsed three times with MeOH (500 µL, 250 µL, 250 µL) and the aliquots of MeOH were combined in a 15 mL amber glass vial..

Each extraction batch (n = 24 samples) included a spiked sample and a Milli-Q blank. Two spike concentrations (0.1 and 200 µg/L) were used dependent on the expected sample concentration.

2.6.2. High performance liquid chromatography analysis

HPLC is an analytical method that separates compounds dissolved in a solvent, based on their structure and size due to their interactions with a solid adsorbent column (Kupiec, 2004). HPLC was used in this project as it is an affordable method of sample detection that works well for high environmental concentrations of diclofenac (Nováková et al. 2006).

2.6.3. High performance liquid chromatography protocol

Samples were analysed on a Dionex UltiMate 3000 HPLC fitted with a photodiode array (PDA) detector, quaternary pump, mobile phase degasser, and autosampler. The column used was a Phenomenex Luna 5u C18 (2) column (150 x 4.60 mm). The mobile phase was 60:40 NaH_2PO_4 buffer, pH 3: acetonitrile, run isocratically at 1 mL per minute. Injection volume of the test solution was 20 µL with a total analysis time of 31 minutes per sample. Ultra-violet PDA detection of diclofenac was at 240 nm with a retention time of 13.2 minutes.

A calibration curve of diclofenac standards was prepared for each run with a concentration range of 0 to 2000 µg/mL. The calibration standards were run before and after each batch of samples. The 100 and 1000 µg/mL standards, and a blank of AcN were analysed after every 10 samples, while every 20 samples a sample was injected in duplicate to monitor variability. These samples confirmed the stability and reproducibility of the calibration. Calibration curves were linear ($R^2 = 0.9995$) over the calibration concentration range.

2.6.4. Gas chromatography–mass spectrometry analysis

GC-MS is an analytical method that combines two commonly-used techniques to successfully separate volatile and semi-volatile compounds while also selectively detecting them. Compounds are separated via GC, before being pumped into the MS where they are ionised. Upon ionisation, the individual compounds are exposed to a beam of electrons that induces fragmentation which results in a determination of mass that can be divided by the charge (Fialkov et al. 2007). This allows determination of the molecular weight. Because of this specificity, the GC-MS detects compounds at levels as low as sub-nanograms (Sneddon et al. 2007). The GC-MS is a popular technique for qualitative and quantitative determination of low concentration compounds (Lee et al. 2005).

2.6.5. Derivatisation

The samples prepared in Section 2.6.1 were evaporated until dry under nitrogen at 80°C and then had 100 µL of AcN added. Standards were made, ranging in concentration from 0 to 100 µg/L. To both the standards and the samples, 25 µL of hydroxypyrene (internal standard) and 100 µL of MSTFA (derivatisation agent) were added, before being vortexed and placed on a heating block at 80°C for 1 hour (Farré et al. 2007).

2.6.6. Analysis of derivatised samples

The derivatised sample extracts and calibration standards were analysed by GC-MS using a Shimadzu GC-2010 Gas Chromatograph, interfaced to a Shimadzu AOC-20i Auto Injector and a Shimadzu GCMS-QP2010Plus detector. Instrumental control, data acquisition and data processing were performed using the Shimadzu GCMS Solution software (Version 2.70). Analytes were separated on a Rxi-5Sil column (5% diphenyl/95% dimethyl polysiloxane) 30 m x 0.25 mm ID, 0.25 µm film thickness, with an integrated guard column (10 m, Integra-Guard) (Restek, Belleftone USA). Derivatised samples and calibration standards were injected into the injection port in splitless mode at a temperature of 280°C in 1 µL volumes. The initial oven temperature of 80°C was held for 1 minute, then increased at a rate of 10°C/min to 150°C, then increased at 8°C/min to 215°C, and then increased by 10°C/min until it reached the final temperature of 280°C. The total run time was 32.63 minutes. Helium was used as the carrier gas at a flow rate of 5.5 mL/min.

The ion source was held at 230°C and the GC-MS interface at 280°C. Electron Impact Spectra (EIS) were obtained at 70 eV in selected ion mode (SIM). The MS was calibrated against perfluorotributylamine (PFTBA) before each sample run using the mass spectrometry auto tune function.

Retention times and mass-to-charge ratios used for detection and quantification of the individual compounds were as follows: diclofenac quantifying ion 214, qualifying ions 73, 367, and 352, retention time 20.91 minutes; hydroxypyrene (the internal standard) quantifying ion 290, qualifying ions 275 and 259, retention time 22.65 minutes.

2.6.7. Quality assurance/quality control

To give quality control, controls were run with every exposure. This was to ensure there was no contamination of the water used and to make sure the cleaning of the tanks was being carried out properly. To provide quality assurance, exposure samples were run in duplicate for the first exposure and in triplicate for the following two exposures. Blanks were also carried out in duplicate or triplicate respective of the exposure week.

A water sample spiked with diclofenac was included in every batch to determine method recovery. Water blanks were spiked with either 2 mL or 1 mL of diclofenac standard (Section 2.4.1) depending on the expected concentration (200 µg/L and 0.1 µg/L respectively). These samples then underwent the same extraction process as experimental samples.

Duplicates of samples, high and low standards, and a blank were run during both HPLC and GC-MS analyses to ensure stability and reproducibility of results. Standards were run before the samples to ensure there was no interference with the readings such as early elution or detection of the compound, or excess background noise. The blanks were used to detect contamination from the machines and to act as a background reference when analysing samples.

2.7 Statistical analysis

Statistical analysis was carried out using SigmaPlot (ver. 11.2; Systat). Normality of data was assessed using Kolmogorov-Smirnov test. Homogeneity of variance was determined using Levene's test. Where necessary, square root and log transformations were performed on data so that the assumptions were met for analysis using analysis of variance (ANOVA). In Chapter 4, concentration-dependent data underwent parametric one-way ANOVAs and the salinity-dependent data underwent two-way ANOVA (with diclofenac concentration and water salinity the two factors). For some of the assay data reported in Chapter 4, high variability in one of the tested groups was noted. In this scenario, a Student t-test was performed in order to reduce the impact of this variability on all comparisons. This test was performed as an indicative test only, with the statistical analysis of record being the ANOVA. All data are presented as the mean ± SEM. A probability level of $p < 0.05$ was considered significant.

Chapter 3: The development of a passive sampling technique for the recovery of diclofenac from water

3.1 Introduction

With the growing interest in the impacts of PCPPs in the aquatic environment, there has been a need to develop new methods that facilitate the measurement of these chemicals in water (Cleuvers 2003; Ferrari et al. 2003; Santos et al. 2010). Such methods are important not only for determining levels in natural waters, but are also critical for laboratory testing, in order to confirm levels of exposures, and to ensure that factors such as photodegradation do not impact exposure concentrations. The requirement for laboratory studies to report measured rather than nominal concentrations can generate large numbers of samples all requiring extraction within a relatively short time-frame. There is therefore a need to develop cost- and time-efficient methods of extracting and analysing compounds from water samples generated in ecotoxicity tests. A range of techniques have been used to extract PPCPs from water samples including: liquid-liquid extraction (LLE), liquid phase microextraction (LPME), solid-phase extraction (SPE), micro-solid phase extraction (μ-SPE/SPME), and solid bar microextraction (SBME).

Liquid-liquid extraction is a technique that uses an aqueous solution and an immiscible organic solvent to extract compounds (Raydo et al. 2015). The water sample is shaken with an immiscible solvent in a separating funnel. The resulting mixture is left to stand and separate into various layers before the organic solvent is removed and analysed. This technique is time intensive and requires large amounts of solvent and equipment (Wells 2003). Liquid phase microextraction uses a drop of water-immiscible solvent to extract the analytes. Exposure of the solvent to the sample can occur directly by dropping the solvent into a stirred sample solution, or by stabilising the drop in a hollow fibre (Pedersen-Bjergaard et al. 2002; Ouyang et al. 2007). LPME therefore works on a similar principle to LLE but is faster and incurs a greatly reduced solvent cost (Larsson et al. 2009).

Solid phase extraction involves passing a water sample through a cartridge containing an appropriate sorbent under vacuum, followed by elution with organic solvents. This technique allows for the concentration of large volumes of water samples. It is commonly used as it is a simple, cost-effective and convenient method, however the drawbacks are the time intensive sample preparation, low compound selectivity, the impracticality of large batches of samples, and the cost of commercial sorbents such as C18 and strong cation exchange (SCX; (Vaananen et al. 2000; Gilart et al. 2012; Rezaei et al. 2013). Micro-solid phase extraction (also known as solid phase microextraction; µ-SPE or SPME) works along the same principles of SPE but with a fused silica fibre that has a stationary phase on the outside. This is used to trap the analyte between itself and the matrix of other compounds in the sample, increasing specificity beyond that which can be achieved by SPE (Balakrishnan et al. 2006).

Solid bar micro-extraction is a technique that is more time- and solvent-efficient than SPE that has been developed for the extraction of pharmaceuticals. It works by packing sorbent materials into a small sealed tube. The tube is then placed within the aqueous sample and the compounds permeate through the membrane of the tube and bind to the sorbent material within. Permeation is increased by the stirring of the solid bar which increases contact of the compound to the sorbent. The compounds can then be eluted using solvents (Al-Hadithi et al. 2011).

Multiple techniques have been previously used to extract diclofenac from water samples. Of greatest methodological concern is the low environmental levels at which diclofenac occurs. SPME has been used successfully to extract diclofenac from a range of matrices and such methods have a detection limit as low as 4 ng/L (Wang et al. 2011). This is a time- and cost-effective method with good recovery rates (Pebdani et al. 2015). Electromembrane extraction (EME) is another method used that is similar to LPME, where

the organic phase is held within the wall of a fibre so that is immobilised. Two electrodes produce a charge that induces migration through the wall of the fibre. This technique allows for high sensitivity and selectivity of diclofenac (Ramos Payan et al. 2011). Both of these techniques are faster and more solvent efficient than SPE (Stuelten et al. 2008; Rezaei et al. 2013).

Passive samplers are a form of sampling that occurs *in situ* in water bodies, and which relies on sorption of the analyte to the sampler over time. They are used for analysis of large samples of water due to their cost- and time-effectiveness. Once the sampler has been placed in the sample, it can be left for any length of time to obtain maximum sorption (Nyoni et al. 2011). A disadvantage of this technique is the low specificity and sensitivity due to the large volumes it is used for (Nyoni et al. 2010). Passive samplers are used to obtain time-weighted average concentrations of pollutants in field settings and are also used to obtain bioavailable levels of pollutants by mimicking biological equilibrium uptake (Mazzella et al. 2010). Passive samplers are also used to provide comparison between passively-accumulated contaminants and tissue concentrations in aquatic biota collected from similar sites, thus providing information on the ability of such organisms to regulate contaminant accumulation (Joyce et al. 2015).

Filter papers have the potential to be used as passive samplers for ecotoxicity tests, as laboratory studies have shown that these can remove PPCPs from water samples. For example, one study investigated the absorption of three oestrogen compounds to a range of filters to determine the optimal filtration technique to prevent losses due to sorption (Walker et al. 2010). The basis of this proposed method is that the compound of interest, in this case diclofenac, adsorbs onto a filter while in an aqueous phase. Once adsorbed, the compound can be extracted off the filter using a solvent that can be evaporated down, increasing the concentration of the compound and thus facilitating analysis. This approach offers a number

of significant benefits such as: reduced sorbent cost, *in situ* application, and time efficiency (Vrana et al. 2005). Furthermore, the use of filter s as passive samplers is a relatively inexpensive approach, and allows high through-put of samples.

Passive sampling methods are being investigated to replace or supplement SPE as cheaper and faster techniques (Nyoni et al. 2010). Although high recovery can be achieved by SPE, there are many steps to prepare one sample for analysis. In comparison, the filter-based method being developed is cost-, and time-efficient by removing the need for cartridges using only filters instead, as well as reducing the amount of solvent required (Kibbey et al. 2010). The limitation on the number of samples that can be extracted is based on glassware availability and lab space for shaker tables and sample preparation.

In the current study a filter-based extraction technique for diclofenac was developed, with a specific emphasis on how incubation time, pH, and diclofenac concentration affected recovery. For the first of these factors (incubation time), three extraction periods were tested: 4, 8, and 24 hours. These periods were chosen as being convenient periods given normal operating hours within a laboratory setting. There were two pH conditions tested, pH 5.7 and pH 2. Diclofenac has a pKa of 4.2 and at a pH 6.0 or greater, diclofenac will be present as an anion. As the pH increases, the ionic form becomes more prevalent (Cheng et al. 2015). Thus these two pH's allowed assessment of the effect of diclofenac adsorption while in different ionic states. There were two diclofenac concentrations tested within this study, 0.1 µg/L and 1000 µg/L. These were chosen as environmental and effect levels, respectively and also represent the two concentrations used in the concentration-dependent exposures to common bullies (Chapter 4).

The adapted method used nylon filters supplied by Sartorius Biolab, as this material was the most effective in adsorbing the three hormones studied in Walker's research (Walker et

al. 2010). Nylon filters have hydrophilic properties and are resistant to organic solvents, making them suitable for sorbing compounds from aqueous solutions in preparation for analysis (Han et al. 2010).

3.2 Objective

The objective of this chapter was to;

- Develop a technique for extracting diclofenac from water samples collected from ecotoxicity assays that is cost- and time-effective.

3.3 Method development

3.3.1 Materials

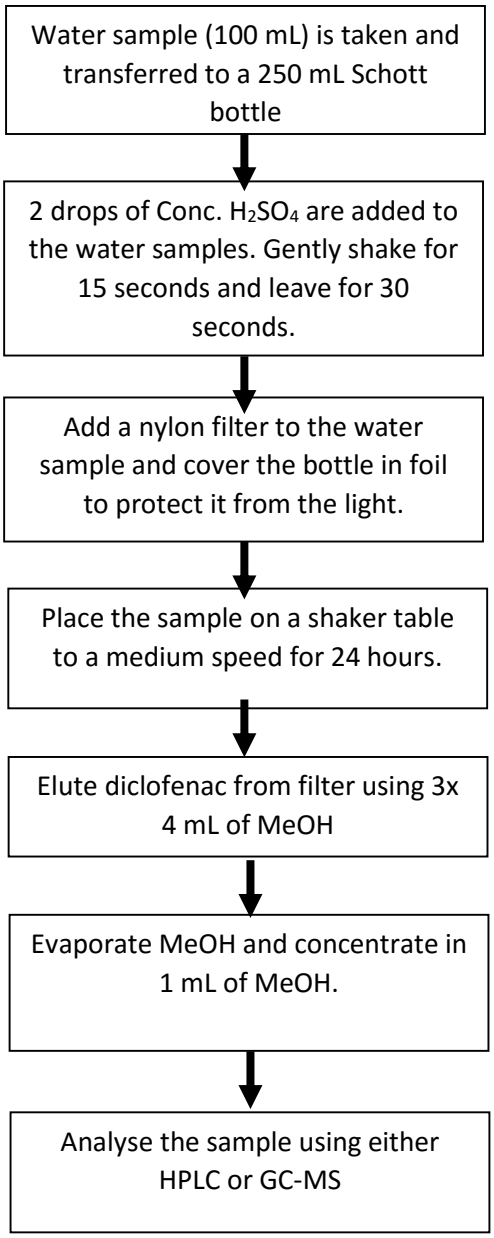
The filters used in this study were obtained from Sartorius Biolab Products, Germany. The filters were made of a nylon membrane that was 4.9 cm in diameter and had a pore size of 0.2 µm. The filters were hydrophilic and had an adsorption rating for BSA of 100 µg/cm^2. Milli-Q water (>18 MΩ) was used for all blanks, spiking solutions and buffers. Diclofenac sodium salt was obtained from Sigma-Aldrich. The solvents (MeOH, DCM, and AcN) used were all HPLC grade.

3.3.2 Method outline

The method used to extract diclofenac using nylon filters is outlined in Figure 3.1. The samples used in the method development were 100 mL aliquots of Milli-Q water that had been spiked with diclofenac standards obtained from Sigma-Aldrich to obtain concentrations of 500 and 0.5 µg/L. All samples were assayed in triplicate and were placed in Schott bottles, into which one nylon filter was added, before the bottles were covered in aluminium foil to protect samples from photodegradation. During incubation, samples were kept on a shaker table at a moderate speed. After incubation, the filters were removed and placed into an

amber vial and eluted using three 4-mL aliquots of MeOH which were combined. The resulting MeOH was evaporated to dryness at 65˚C under nitrogen and made up to a final volume of 1 mL in MeOH. It was then quantitatively transferred to a pre-weighed HPLC vial. The newly concentrated sample was then analysed using HPLC (Section 2.6.3). Results were statistically analysed using ANOVA (Section 2.7).

Figure 3.1: A flow chart outlining the filtration extraction method.



3.3.3 QA/QC

For quality control and assurance, blanks were used. The blanks were 100 mL aliquots of Milli-Q water. The blanks were non-acidified in the method development and the filters were not dried. A blank was run with every incubation sample. The blanks were used to ensure there was no contamination of the water and to confirm cleaning standards were being met to avoid cross contamination from previous experiments. For quality assurance, replicates were run for each condition in triplicate. This allowed for determination of accuracy and ensured higher reliability in the statistical testing.

3.4 Extraction study

3.4.1 Incubation time

Three different incubation periods (4, 8, and 24 hours) were investigated to identify the most suitable time to allow for maximum adsorption of diclofenac to the filter. The filters were not dried and samples were analysed at a pH of 5. The concentration of diclofenac in the samples was 500 µg/L and samples were prepared in triplicate as described in Section 3.3.2.

3.4.2 Effect of concentration

Concentration is important when developing an extraction method as often it can affect extraction efficiency. Two concentrations of diclofenac were tested in this study: 500 and 0.5 µg/L. These concentrations were chosen because they were half of the concentration that was going to be used in the final method, meaning that if degradation occurred the method would still be valid for detecting lower than nominal concentrations. Triplicate samples of Milli-Q water were spiked (500 and 0.5 µg/L) and acidified before being incubated for 24 hours.

3.4.3 Filter condition

The condition of the filter is another factor that could influence recovery of diclofenac. Of specific interest was the moisture content of the filter (wet versus dry). If the dry filter did not yield recoveries equal to or significantly greater than the wet filters then the method would be limited to extracting concentrations detectable at HPLC level only. This is because water damages the column and affects the efficiency of derivatisation (Sugaya et al. 2001). Triplicate samples were 500 µg/L in concentration and were incubated for four hours. The saturated condition was leaving the filter wet for the elution phase as outlined in Section 3.3.2. For the dry condition the filter was dried at 65°C for 3 hours before carrying out the elution phase.

3.4.4 Effect of pH

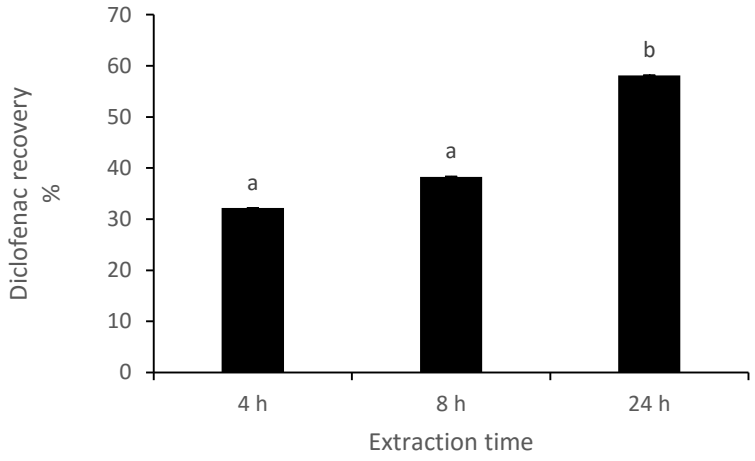
Two different pH conditions were investigated: pH 5.7 and pH 2. pH 5.7 was obtained by using pure Milli-Q water in equilibrium with air, and pH 2 was obtained with the addition of 3 drops of concentrated H_2SO_4. After addition of the acid, samples of 100 mL volume were left to equilibrate for 30 seconds after being thoroughly mixed by hand, before the addition of the filter. The remaining method was exactly as outlined in Section 3.3.2. The effect of pH was only investigated for the 24 hour incubation period. All samples were run in triplicate. Two concentrations were tested: 500 and 0.5 µg/L.

3.5 Results

3.5.1 Effect of incubation time

Diclofenac recovery increased with time (Figure 3.2). A recovery of 32% after four hours, increased to 38% after 8 hours and to 58% after 24 hours. The 24 hour extractions were significantly greater than the four and eight hour treatments ($p = 0.0007$ and 0.008 respectively).

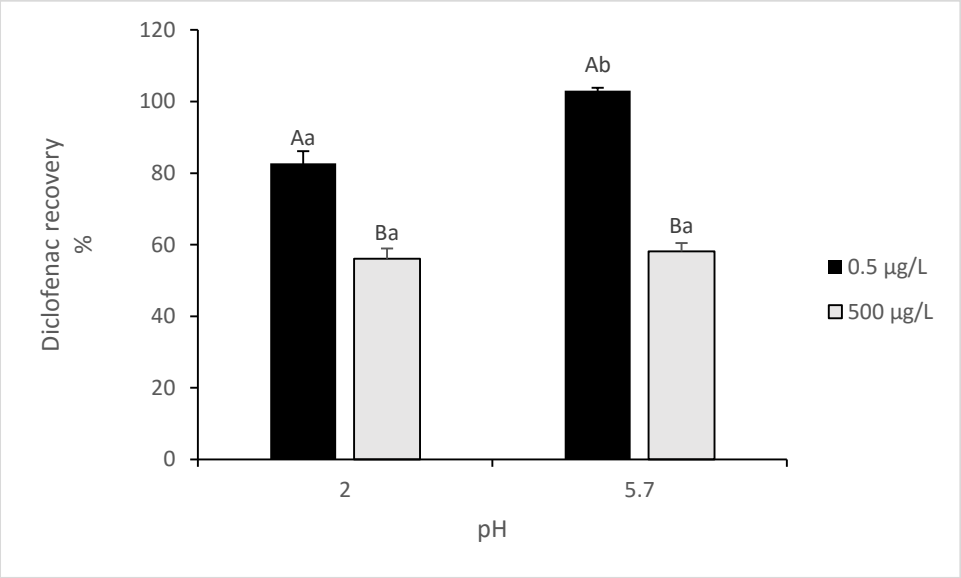
Figure 3.2: Recovery of diclofenac at pH 5.7 at differing incubation times (4, 8, and 24 hours). The spike concentration was 500 µg/L (n = 3; mean ± SEM). Time periods sharing letters are not significantly different.



3.5.2 Effect of pH and concentration

As seen in Figure 3.3, the percent recovery was greater for the non-acidified samples for the 0.05 µg/L spike in comparison to the 500 µg/L treatment ($p = 0.0017$). There was no significant difference in recovery rates when comparing concentration and acidification ($p = 0.4442$). The recovery of diclofenac was concentration-dependent. The average recovery was 93% for the 0.05 µg/L spike and 57% for the 500 µg/L spike.

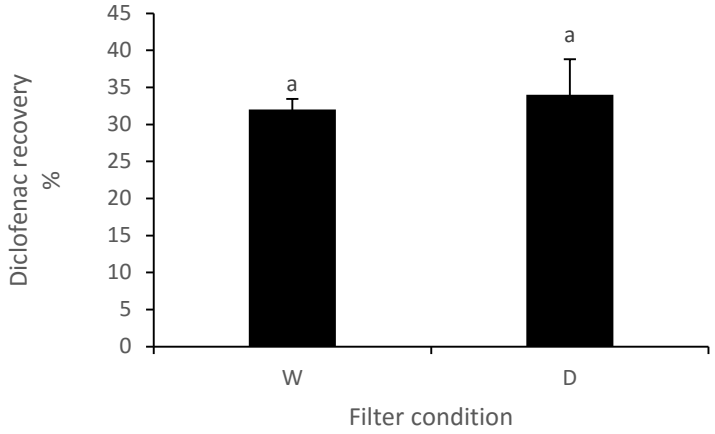
Figure 3.3: Recovery of diclofenac at pH 2 (acidified) and pH 5.7 (non-acidified) with an incubation period of 24 hours. The spike concentrations were 500 and 0.5 µg/L (n = 3; mean ± SEM). Samples sharing a capital letter are not significantly different in relation to concentration. Samples sharing a lower case letter were not significantly different in relation to pH.



3.5.3 Effect of filter condition

There was no significant difference in diclofenac recovery between wet and dry filters ($p = 0.7063$, Figure 3.4). The recovery rates for the wet filters was 32.19% (±) 1.43% and the recovery rate for the dry filters was similar at 34.22% (±) 4.81%

Figure 3.4: Recovery of diclofenac at pH 5.7 at differing filter conditions (wet and dry). The spike concentration was 500 µg/L (n = 3; mean ± SEM). Time periods sharing letters are not significantly different.



3.6 Discussion

3.6.1 Extraction method

The application of the passive sampler method to diclofenac extraction within this study yielded recovery rates of 56-104% over 24 hours. There were greater recoveries observed at the lower concentrations which is likely due to saturation of binding sites being reached at a concentration lower than that of the highest tested diclofenac concentration (Section 3.6.5). The recovery values obtained for the 500 µg/L-spiked samples are consistent with those observed in literature (Table 3.1). Diclofenac has been studied intensively with many extraction and analytical techniques being employed to measure its concentration in a range of samples. The use of passive sampling with nylon filters works within the environmental range of concentrations (Section 1.3.2), meaning it has utility for environmental monitoring. This method does not, however, improve upon selectivity of compounds extracted as the filters used have a broad selectivity (Satorious Biolab Products, Germany). Another drawback is that the volume of sample required for the extraction process remains high. More work needs to be carried out to refine passive sampling using nylon

filters to determine recovery rates at higher concentrations if this technique is to be used for laboratory exposures testing high concentrations.

Table 3.1: Comparison of extraction and analytical techniques used when studying diclofenac. Recovery concentrations obtained and recovery percentages are included as a reference point for values obtained in this study.

Technique	**Compounds**	**Recovery concentrations**	**Recovery percentage**	**Reference**
Passive sampling, HPLC, High resolution MS	Pharmaceuticals	0.2 ng/L	87.9%	Wille et al. (2011)
		20 ng/L	105.2%	
SPE, LC-MS	Diclofenac	0.055 µg/L	81%	Stülten et al. (2008)
SPE, HPLC-UV	Diclofenac	100 µg/L	86.5%	Zhou et al. (2014)
		10 µg/L	85.7%	
SPE, SUPRAS[2], HPLC-UV	Diclofenac	10 µg/L	102%	Rezaei et al. (2013)
		25 µg/L	96.4%	
		50 µg/L	93.8%	
Passive sampler, HPLC, GC-MS	Diclofenac	500 µg/L	56-58%	This study
		50 µg/L	82.7-103.5%	

[2] Supramolecular solvent-based microextraction

3.6.2 Effect of incubation time

Of the three time points tested (4, 8, and 24 hours), the 24 hour incubation period yielded the greatest recovery. This was anticipated as there was more time for the diclofenac to adsorb to the filter. This was also the most practical time-period in terms of carrying out the extraction. This is because the samples can be put on to incubate in the morning, meaning there is an entire day to carry out the drying and rinsing of the filters, and the extraction of the diclofenac. The four-hour incubation is manageable, however it would require a 10-12-hour session in the laboratory to have the sample incubated, extracted, and ready for analysis. The 8-hour incubation is the least manageable and requires the samples to be put on to incubate late at night so they are ready to be treated in the morning. More importantly the 4- and 8-h time intervals yielded poor recoveries making them impractical form the perspective of both time and effectiveness.

The incubation times for this study were longer than those used in other studies where incubation times were 10 minutes static and 30 minutes dynamic, and 15 minutes static and 45 minutes dynamic from spiked glass matrices and tablets respectively (Yamini et al. 2002). This is due to the fact this study used a passive sampler, a technique which is applicable for long term monitoring of environmental levels in aquatic settings. Passive samplers can be left in the sample environment for weeks or months (Vroblesky et al. 2001; Bao et al. 2012; Page et al. 2014). Due to this study investigating the concentration present in a small aliquot of water, the sample periods were shorter than those reported in most passive sampler studies.

3.6.3 Effect of pH

There was no significant difference between acidified and non-acidified samples at a tested diclofenac concentration of 500 µg/L. Diclofenac can exist in two forms: a neutral and a charged species. Lowering the pH ensures that diclofenac, which has a pKa of 4.2, is

present in the more hydrophobic neutral species thereby enhancing sorption (Kole et al. 2011; Ramos Payan et al. 2011; Racamonde et al. 2015). Acidification is included in many methods for diclofenac recovery found in the literature (Sun et al. 2008).

Other studies have investigated the optimal pH for extraction of diclofenac from water. Al-Hadithi et al. (2011) determined the optimal pH for extracting diclofenac samples is pH 2, as once the pH increases, diclofenac becomes ionised and has stronger interactions with the water, thereby reducing extraction efficiency. At least in the small volume water samples in the present study, it appears that acidification is unnecessary at low concentrations. More research examining recovery of low concentration diclofenac samples at varying pH will need to be carried out to determine whether acidification of the sample has no impact on diclofenac recovery at higher sample volumes. If this is the case, in future tests it would be interesting to determine at what concentration acidification becomes detrimental to the recovery.

3.6.4 Effect of drying

The effect of drying the filter prior to extraction was investigated. There was no significant difference between drying the filters and leaving them saturated (Figure 3.4). Removing water is advantageous as it enables samples extracted using nylon filters to be derivatised and analysed by GC-MS, which has lower detection limits than HPLC-UV. There is little research that has investigated the impact of drying filters on the total recovery on organic contaminants in general. This is likely due to environmental concentrations being in the ng/L to µg/L range (Table 3.1), resulting in GC-MS analysis being the practical choice. Peralta et al. (2010) used nylon membrane filters to extract amiloride and furosemide from a pharmaceuticals mixture. The filters were dried prior to extraction of the compounds from the filter with no impact on total recovery. Caban et al. (2015) used a Speedisk extraction disk to

extract PCPPs from raw and treated drinking water. The compounds successfully extracted were ibuprofen, paracetamol, flurbiprofen, naproxen, ketoprofen, diclofenac, diethylstilboestrol, 17β-oestradiol, and oestriol. The filters were dried before extraction and resulted in a recovery of 80% or above for most of the pharmaceuticals extracted. Drying filters before extraction is a common practice when using GC-MS analysis for pharmaceuticals. There does not appear to be a negative impact on recovery rates due to this process.

3.6.5 Effect of concentration

The percentage recovery decreased with increasing concentration suggesting saturation of binding sites on the filter (Figure 3.3). Therefore, by increasing the number of filters used, an increase in recovery would be expected owing to the increase in available binding sites. Alternatively, the decrease in recovery with concentration increase could be due to a weakening in the diffusion gradient because of the concentration of diclofenac already present on the filter. Passive samplers such as the nylon filter used here, work on the principle of diffusion driven by a concentration gradient (Vrana et al. 2005). Equilibration of the compound between aqueous phase and the filter in a static test (i.e. no movement of concentration into or out of the solution) will impact the recovery (Booij et al. 2015). If equilibration is reached between the filter and sample, there is the possibility of a plateau being reached in terms of sample recovery. Regarding the current study, it is possible the higher concentration reached equilibration due to sorption capacity being reached, whereas the 500 µg/L test solutions did not, due to sorption capacity being large enough to deal with the relatively lower concentrations of diclofenac.

For samples that are higher in diclofenac concentration, a longer incubation period may be required. It is known that higher concentrations produce linear uptakes for a longer

period of time, due to there being a greater amount of the compound to bind to the filter (Allan et al. 2010). A possible solution to this would be increasing the number of filters present to minimise the lack of binding sites. This is a minor issue, as this concentration (1000 µg/L) is well above environmental levels and is only likely to be an issue with high laboratory exposures (Table 1.2). This issue is solved with access to other extraction methods available in the laboratory such as SPE.

3.7 Conclusion

A nylon filter-based method was developed for extracting diclofenac from water samples collected from ecotoxicity experiments. It was concluded that recovery was more effective at lower concentrations with recovery percentages of 83-103% for the 0.5 µg/L samples, compared to 58% for the 500 µg/L samples. It was concluded that these recovery values were suitable for use in the exposure study outlined in Section 2.4. However, further work is needed to ensure maximal recovery of the analyte from the filter.

Chapter 4: Effect of diclofenac exposure on the biochemistry of the common bully

4.1 Introduction

Due to its continuous entry into the environment, diclofenac accumulates within waterways and yet little is known about its environmental impact (Ort et al. 2009). Diclofenac is a high risk of generating environmental impacts. This is due to its relatively high environmental concentration, its persistence, and its high biological activity, which has already been linked to non-target species toxicity (Swan et al. 2006b).

As a biologically-active chemical, with target effects on human gastrointestinal, renal, and cardiovascular systems (Emberson 2013), there is interest in determining biological

effects on non-target species (Gonzalez-Rey et al. 2014). To determine the effects on non-target species, laboratory exposures examining mechanisms of effects over both acute and chronic time-frames are commonly used (Mehinto et al. 2010; Stepanova et al. 2013; Saucedo-Vence et al. 2015). Many studies have investigated the impact of diclofenac on cells and embryos (van den Brandhof et al. 2010; Ribas et al. 2014), however such studies cannot account for whole organism factors that will influence absorption, distribution, tissue-dose, biotransformation, and other factors associated with complex whole animal physiology (Parasuraman 2011).At present, only a few species have been studied in terms of diclofenac impacts on non-target aquatic biota, and the results vary from study to study. Much work remains to build a comprehensive view of the impact of diclofenac on aquatic organisms.

There are a number of key factors that will influence diclofenac toxicity. Exposure concentration is likely of greatest importance, but water chemistry may also play a role. For example, salinity is an environmental factor that exerts significant influence over the function of aquatic biota. As detailed in Section 1.3.5.2, salinity influences the physiology of osmoregulating animals such as fish, thus affecting the pathways of contaminant uptake, and the sensitivity of different toxic endpoints e.g. (Levitan et al. 1979). Therefore, accounting for factors such as salinity will be important for determining the impact of diclofenac on biochemical function in fish.

There is limited information available regarding diclofenac in New Zealand waters and its impacts on New Zealand's aquatic species (Hughes et al. 2013). This chapter examined the effect of diclofenac concentration and the effect of salinity on biochemical endpoints of toxicity in the common bully. For the study of concentration-dependent toxicity two exposure levels, 0.1 and 1000 µg/L, were used to simulate an environmental and effect level, respectively. The environmental concentration (0.1 µg/L) is well within the range of diclofenac concentrations measured in waters (see Table 1.2). Importantly, this concentration

is also within the range where it could be easily detected via GC-MS. The effect concentration (1000 µg/L) was a concentration which, according to previous research, is known to impact biochemical function in fish (Nava-Alvarez et al. 2014), but without causing lethality (e.g. LC_{50} for carp is 70.98 mg/L; (Islas-Flores et al. 2013).

Three key biochemical pathways were examined: a marker of oxidative damage (protein carbonylation); and the activity of a Phase 1 biotransformation enzyme (CYP), and the activity of a Phase II biotransformation enzyme, which is also strongly implicated as an anti-oxidant (GST) (Section 1.3.4.2).To assess the CYP activity, the EROD assay was used. This assay is commonly used in ecotoxicological studies when determining exposure to contaminants (Carlsson et al. 1999; Kammann et al. 2005; Van Soest et al. 2007). The EROD assay is capable of measuring the increase in CYP induction, hence it's utility as a marker of organic contaminant exposure. CYP enzymes are present wherever toxicants may accumulate, so are predominant in the liver, but also at sites of contaminant uptake such as in the gills, and kidneys (Liu et al. 2008; Burkina et al. 2012). Protein carbonylation is a marker of oxidative damage (Osório et al., 2013, and has been commonly examined used in ecotoxicological studies to determine the effect of exposure of organisms to contaminants (Driessen et al. 2015; Magni et al. 2016). As a marker of damage protein carbonylation can represent either an increase in ROS, or an inhibition of anti-oxidant defence mechanisms. Diclofenac has been recorded as increasing protein carbonylation in the common carp (*Cyprinus carpio*) with an increase observed in blood and muscle after exposure (Gonzalez-Gonzalez et al. 2014). Activity of glutathione-S-transferase is another commonly used assay when determining the effect of xenobiotic compounds (Donham et al. 2005). The GST family is a set of detoxification enzymes that are used in Phase II cellular detoxification (Glisic et al. 2015). They are used within the organism to conjugate glutathione (GSH) to electrophiles to detoxify them until the oxidative stress has been reduced or overcome (McDonagh et al.

2005). Detoxification often occurs in the liver, hence the liver tissue is most commonly examined as it has the largest increase in concentration of detoxification enzymes (Padmini et al. 2009). A study investigating the impact of the NSAID naproxen saw an upregulation of GST after exposure to 100 µg/L of naproxen in zebrafish (Stancová et al. 2015). Exposure of diclofenac to the wolf fish (*Hoplias malabaricus)* saw an increase in GST levels in a dose-dependent manner with exposure levels of 4×10^{-9}, 4×10^{-8} and 4×10^{-7} M diclofenac (Gröner et al. 2015). The GST assay was used as a marker opposed to SOD or CAT so that it could be determined as to whether diclofenac increased enzyme production or merely caused oxidative damage.

4.1.1 Objective

- Test for a range of biochemical endpoints to determine the effect of diclofenac on the common bully.

4.2 Methods

4.2.1 General methods

Fish (n = 70; mass = 5.83 g ± 2.55 g) were collected in mid-August/late September, during late winter, early spring (spawning season), as per the protocol outlined in Section 2.1. Fish were allocated to one of two exposure scenarios. The first tested the effects of increased concentration of diclofenac in freshwater and was performed in mid-August/early September. The second examined the effect of salinity on diclofenac toxicity and was carried out late September/early October. Owing to the timing of the exposures, some of the fish used were pregnant females.

4.2.1.1 Concentration-dependent exposure

The concentration-dependent exposure examined the effects of two diclofenac concentrations (0.1 µg/L, environmental mimic; 1000 µg/L, effect level) relative to a control (0 µg/L). Three stock solutions were prepared. A 500 mg/L solution was prepared by dissolving 0.1345 g of diclofenac in 250 mL of Milli-Q water. The 500 mg/L stock was diluted with Milli-Q water to prepare 10 mg/L and 50 µg/L spiking solutions. The 500 mg/L and the 50 µg/L solutions were added to 2 L of water in 4 mL volumes to obtain final concentrations of 1000 µg/L and 0.1 µg/L respectively. The stock solutions were prepared at the start of the concentration dependent exposures and used throughout until the end of the salinity dependent exposures. Diclofenac was added to exposure waters 24 hours before fish were added to ensure equilibrium. Each concentration had an n = 8 (n is the number of fish),

with 1 fish per exposure tank. Full details of the experimental methods are reported in Section 2.2.

4.2.1.2 Salinity-dependent exposure

These experiments were conducted under the same environmental conditions to those described in Section 2.4.2, with the exception of the salinity of the exposure water (0‰, 6‰ and 20‰) and diclofenac concentrations examined (0 and 1000 µg/L). The water chemistry and salinity acclimation protocol are outlined in Section 2.2. The 96 h exposure was conducted identically to those described for concentration-dependent exposures (Section 2.4.2), with stock solution handling (Section 2.4.1), water pre-equilibration, water changes, and water sampling for diclofenac conducted as previously detailed (Section 2.4.2).

4.2.2 Tissue sampling

At the conclusion of the exposures, fish were euthanised and dissected as per the protocol in Section 2.4.4. For analysis of the effect of diclofenac on the common bully, two tissue samples were used in the biochemical assays: gill and liver. The gill tissue was used to measure the rate of CYP activity in units of pmol resorufin/filament tip/h as per the protocol outlined in Section 2.5.1. The liver was used to measure protein carbonylation in units of nmol carbonyl/mg protein and GST activity in units of µmol/mg protein/minute. Protocols for both the protein carbonylation and the GST assays can be found in Sections 2.5.3 and 2.5.2, respectively.

4.2.3 Statistical analysis

For some assays some samples did not record any activity for the biochemical endpoint being measured. Where this occurred, these values were discarded, on the basis that even in control tissues all of these assays should provide a measurable value. Thus a non-detectable value (which occurred evenly among exposure concentrations) likely represented an assay

failure, rather than an actual zero value. Furthermore, any outliers that were more than two standard errors away from the mean, were excluded from analysis. All final n values for biochemical assays are reported in Table 4.1.

Table 4.1 Final n values for fish used in biochemical assays (EROD, PC, GST) at varying salinities and diclofenac concentrations. The mean weight (±) SEM of the fish is included.

Salinity (ppt)	**Concentration (µg/L)**	**Mean**	**SEM**	**n**
0	0	4.51	1.26	7
	0.1	7.99	1.48	6
	1000	6.23	3.30	6
6	0	5.46	2.12	7
	1000	4.90	3	8
20	0	5.09	1.13	7
	1000	5.91	1.52	8

Statistics were run using Sigmaplot (ver.11.2; Systat). Data were tested for normality using the Kolmogorov-Smirnov test and homogeneity of variance using Levene's test. Any data that failed one of these tests were transformed via either log- or square root-transformation. This transformation resulted in the assumptions of parametric analysis being fulfilled. All data were then subjected to a one-way ANOVA (concentration-dependence) or a two-way ANOVA (salinity-dependence; with salinity and concentration as the two factors), with a post-hoc Tukey's test. Some data-sets (specifically EROD activity and protein carbonylation in the concentration-dependent study) showed high variability in the highest exposure concentration. In these cases, in addition to the ANOVA, an unpaired Student's t-

test was performed to act determine significance between specific exposure concentrations. This was used only as a guide, and was not considered the definitive statistical approach.

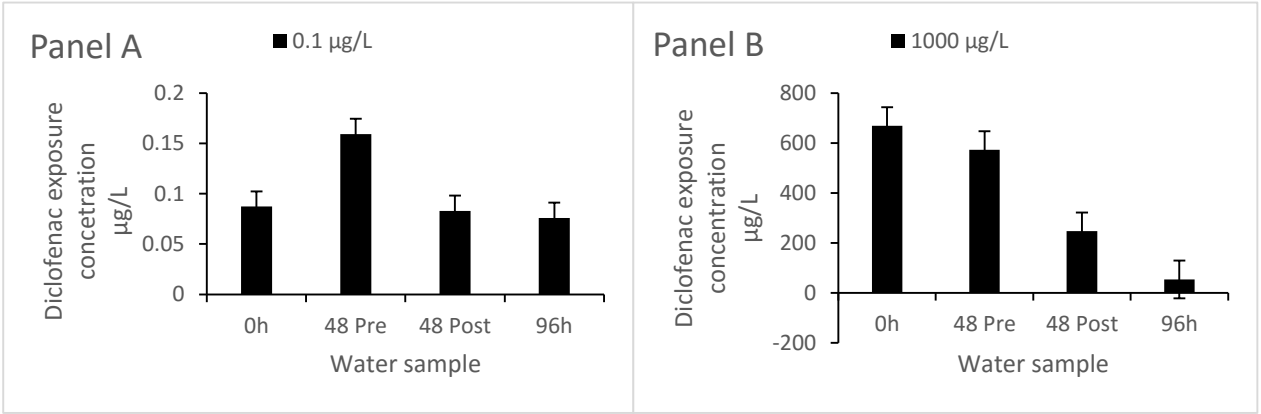
4.3 Results

4.3.1 Exposures

4.3.1.1 Concentration-dependent exposure

Figure 4.1 represents the measured diclofenac exposure concentrations from the concentration-dependent exposures. There was an average recovery of 0.10 µg/L for the 0.1 µg/L exposure group and an average recovery of 380 µg/L for the 1000 µg/L group. Degradation is observed across the 1000 µg/L test group with higher concentrations observed at 0 and 48 hours (after the water change), and lower recoveries observed at 48 hours (before the water change) and 96 hours. The 0.1 µg/L test showed consistent recoveries at each time point with the exception of a spike at 48 hours (before the water change). There was no detectable diclofenac in the control exposures (data not shown).

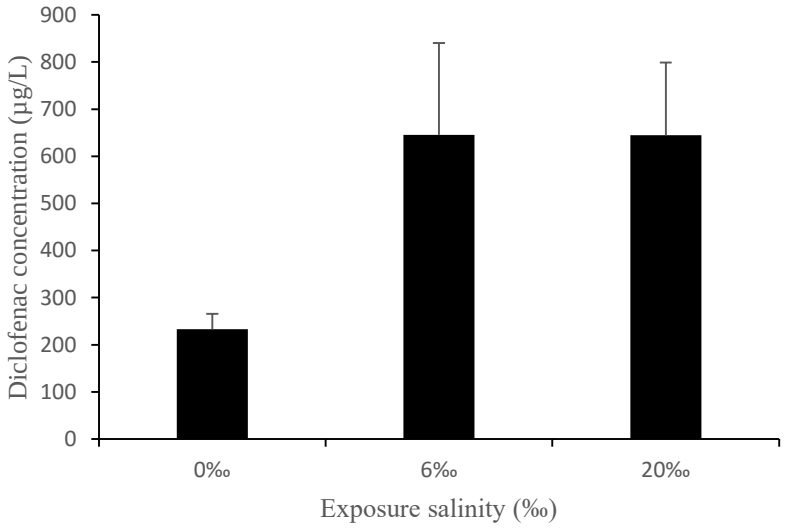
Figure 4.4: Measured diclofenac concentrations in exposure water over the course of 96 hour exposures with spiking concentrations of 0.1(a) and 1000 µg/L (b). Plotted points represent mean values (± SEM) of n = 5-8 replicates, where each replicate represents the mean of four samples taken at various time points



4.3.1.2 Salinity-dependent exposure

Figure 4.2 shows the measured diclofenac exposure concentrations from the salinity-dependent exposures. There was a mean recovery of 232 µg/L, 645 µg/L, and 644 µg/L for the 0‰, 6‰, and 20‰ salinities respectively. A one-way ANOVA showed there was no significant difference in exposure concentrations between salinities ($p = 0.146$). There was no detectable diclofenac in the control exposures (data not shown).

Figure 4.5: Measured diclofenac concentrations in waters of varying salinity over the course of 96 h exposures at a nominal exposure concentration of 1000 µg/L. Samples represent mean (± SEM) of the average of four samples taken at various times from three replicate exposure chambers at each salinity.



4.3.2 Biochemical assays

4.3.2.1 Glutathione-S-transferase assay

Diclofenac had no significant effect on liver GST activity as determined by a one-way ANOVA ($p = 0.349$). However, the large variability noted in the 1000 µg/L exposure group likely precluded significance. Therefore, an unpaired Student's t-test was performed which re-enforced the one-way ANOVA resulting in no significant difference between the control and the 0.1 µg/L exposure concentrations ($p = 0.115$).

Figure 4.6: Effect of diclofenac exposure concentration on GST activity in the liver of common bullies exposed for 96 h. Plotted points represent means (± SEM) of 5-7 replicates.

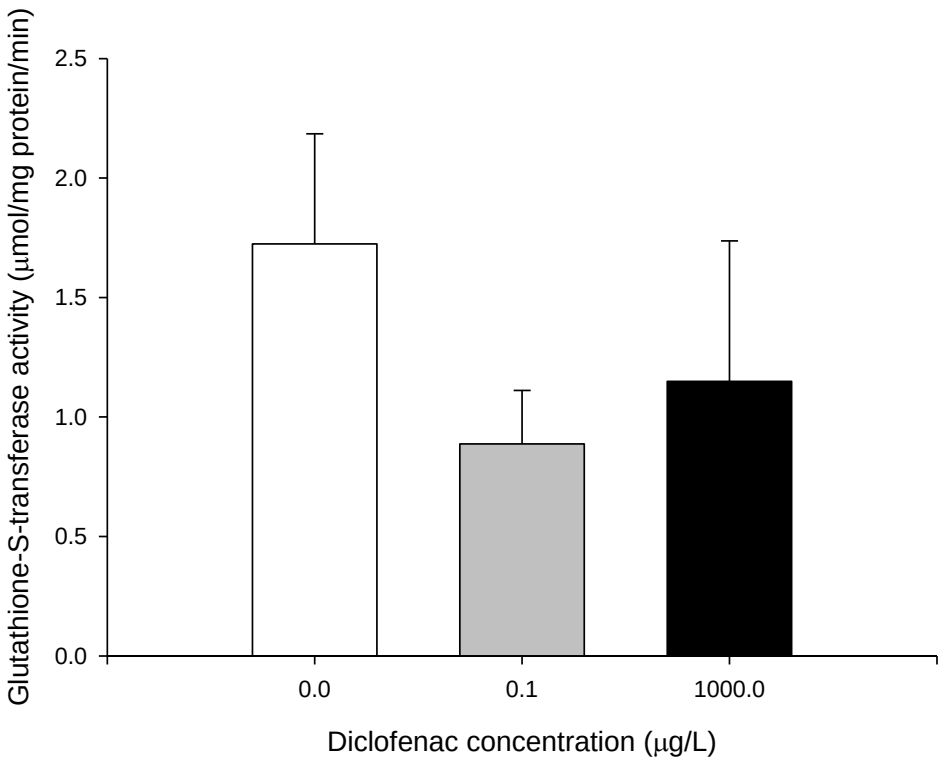
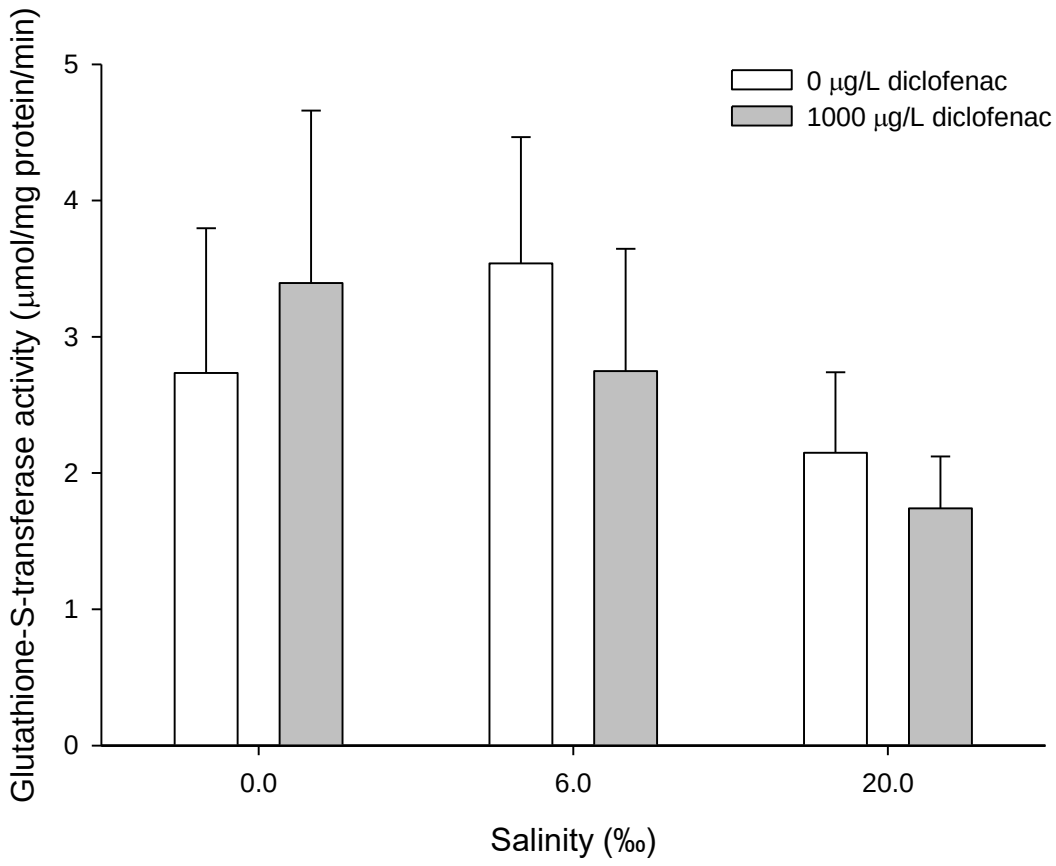


Figure 4.4 shows the impact of salinity and diclofenac concentration on GST activity. The outcome of the two-way ANOVA showed that there was no overall significant effect of

salinity on GST activity (p = 0.733) and there was no overall significant effect of diclofenac exposure concentration on activity (p = 0.829). Similarly, no effect of the interaction between these two factors was observed (p = 0.505).

Figure 4.7: Effect of salinity on GST activity in the liver of common bullies exposed to diclofenac for 96 h. Plotted points represent means (± SEM) of 8 replicates.



4.3.2.2 EROD assay

Figure 4.5 shows the impact of increasing diclofenac concentration on EROD activity in the gills of the common bully. Diclofenac had no significant effect on gill CYP activity as determined by a one-way ANOVA ($p = 0.151$). However, there was a large variability noted in the 1000 µg/L exposure group, which may have precluded significance. Therefore, an unpaired Student's t-test was performed, which did highlight a significant difference between the control and 0.1 µg/L exposure group ($p = 0.021$). The t-test did not detect any significant difference between the control and the 1000 µg/L group ($p = 0.145$), nor between the 0.1 and 1000 µg/L groups ($p = 0.482$).

Figure 4.8: Effect of diclofenac exposure concentration on EROD activity in the gills of common bullies exposed for 96 h. Plotted points represent means (± SEM) of 3-5 replicates.

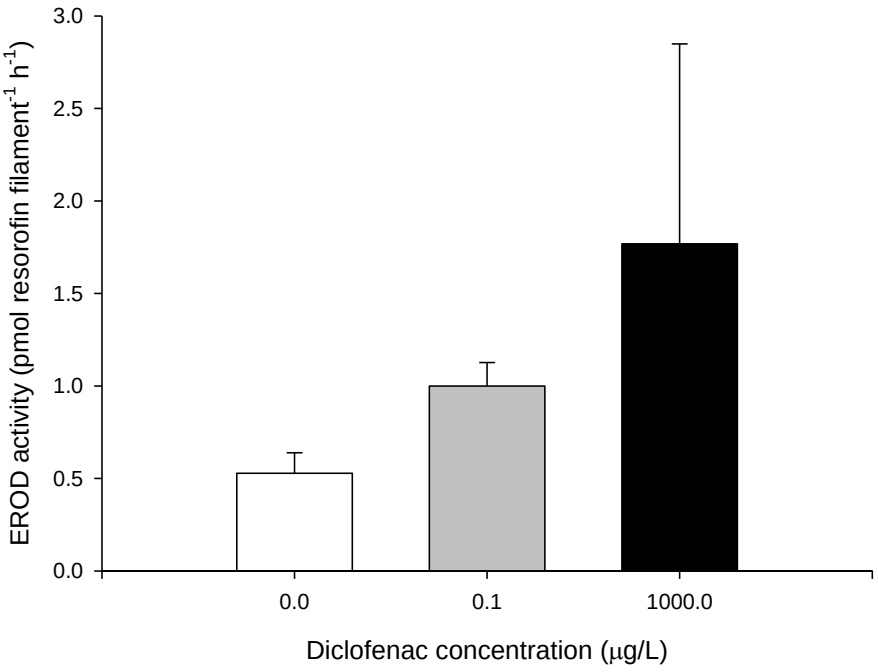
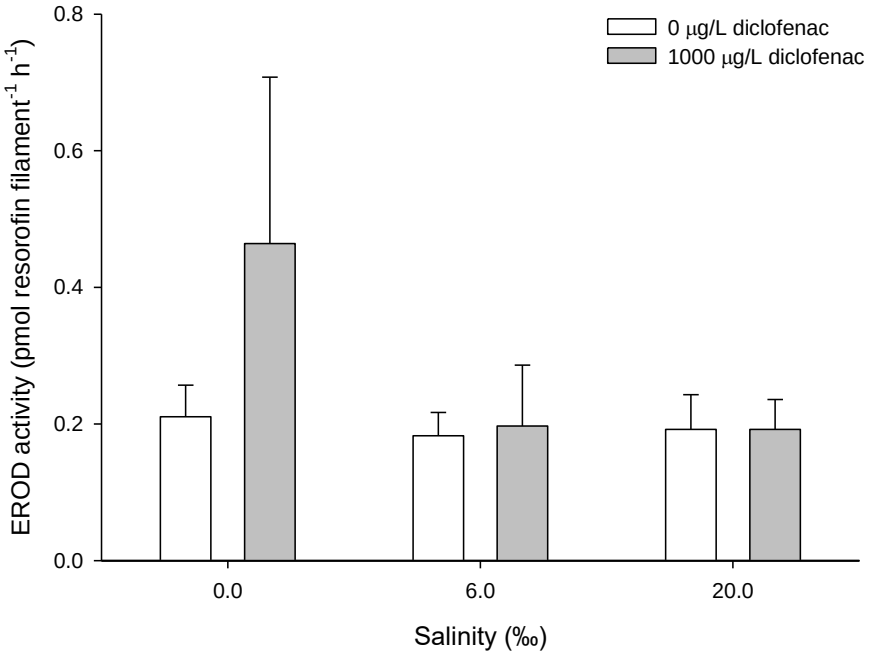


Figure 4.6 shows the relationship between salinity and diclofenac effects on EROD activity. There was no significant overall effect of salinity ($p = 0.782$) or diclofenac exposure

concentration (p = 0.543) on activity. Similarly, no effect of the interaction between these two factors was observed (p = 0.843).

Figure 4.9: Effect of salinity n on EROD activity in the gills of common bullies exposed to diclofenac for 96 h. Plotted points represent meanss (± SEM) of 6-7 replicates.



4.3.2.3 Protein carbonylation assay

Figure 4.7 shows the relationship between diclofenac concentration and protein carbonylation in liver tissue. Statistical testing via one-way ANOVA did not yield a significant outcome (p = 0.199), but as with the EROD results, there was a high variance. A t-test did produce a significant difference between the 0 and 1000 µg/L groups with a p value of 0.040.

Figure 4.10: Effect of diclofenac exposure concentration on protein carbonylation in the liver of common bullies exposed to diclofenac for 96 h. Plotted points represent means (± SEM) of 5-7 replicates.

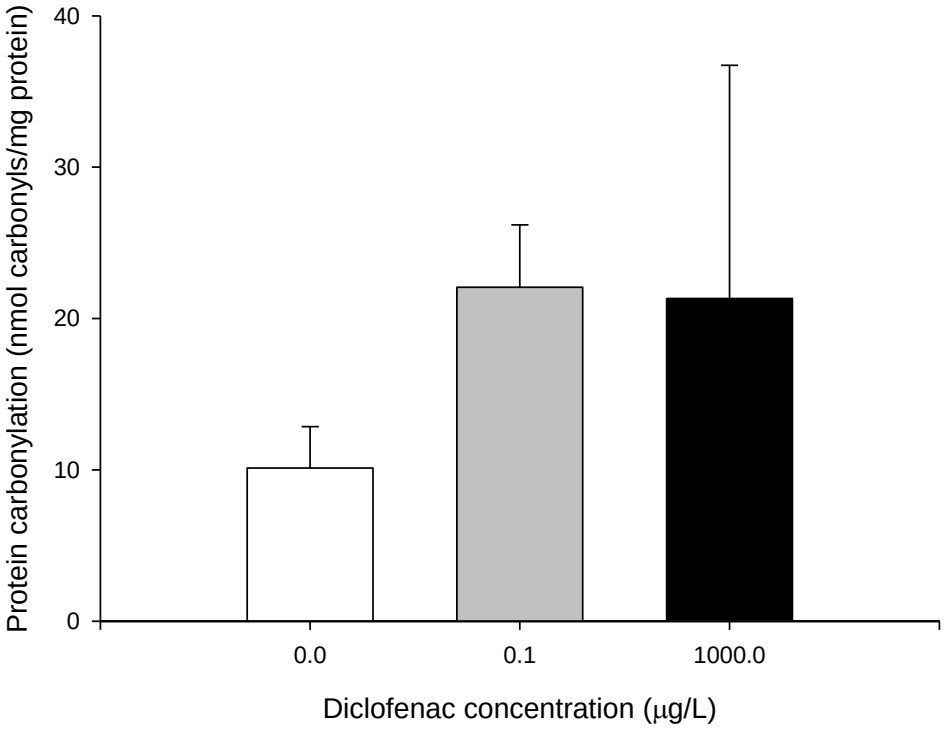
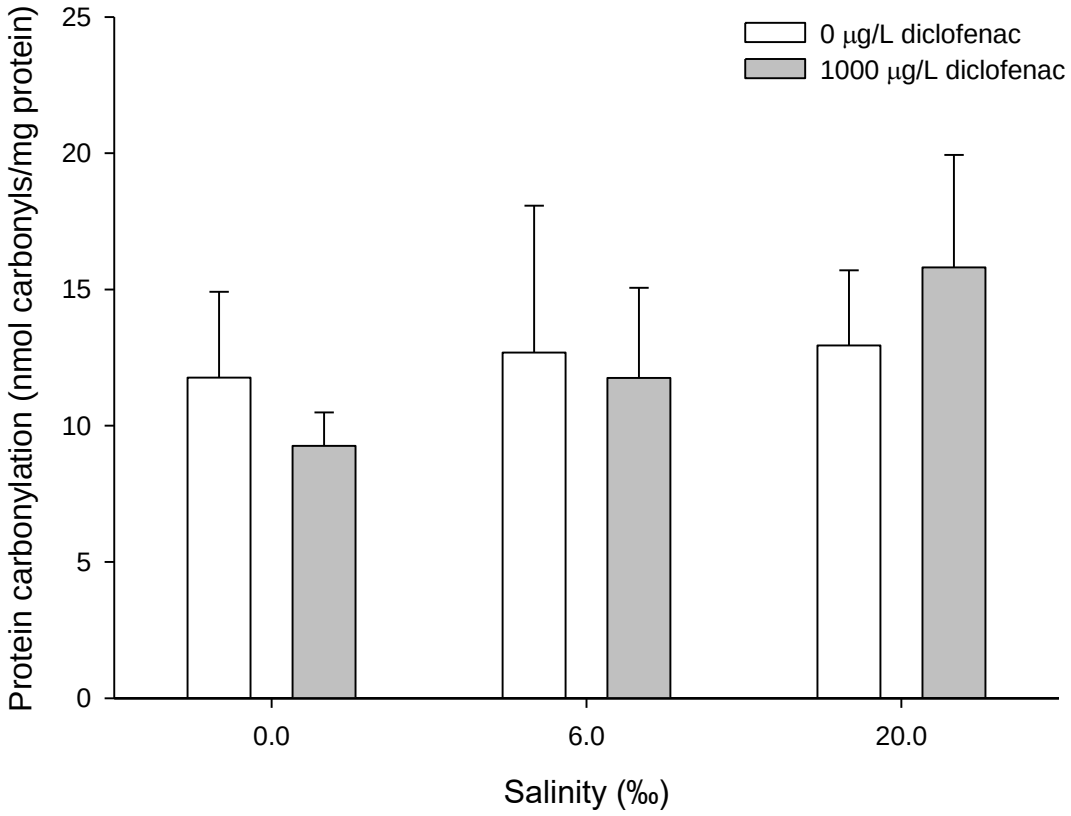


Figure 4.8 highlights the effect of salinity on the impacts of diclofenac on protein carbonylation in liver tissue. Statistical analysis did not yield any significant effects of diclofenac concentration ($p = 0.448$), salinity ($p = 0.442$) or the interaction between salinity and concentration ($p = 0.903$).

Figure 4.11: Effect of salinity on protein carbonylation in the liver of common bullies exposed to diclofenac for 96 h. Plotted points represent means (± SEM) of 8 replicates.



4.4 Discussion

4.4.1 Diclofenac exposure concentrations

In the concentration-dependent study there was an average recovery of 0.10 µg/L for the 0.1 µg/L exposure group and an average recovery of 380 µg/L for the 1000 µg/L. The much lower than targeted exposure concentration in the high exposure group could reflect one of three possibilities. The first is that the fish themselves took up diclofenac leading to a decrease in exposure concentration. This is unlikely as the same pattern was not observed at the lower concentration, and previous work has shown that at lower exposure levels, relative more diclofenac is taken up by fish (Schwaiger et al. 2004). The second possibility is

photodegradation. Diclofenac is known to degrade upon exposure to light (Buser et al. 1998), but under the current exposure conditions this was minimised by conducting the experiments in the dark. The third possibility is that the exposure concentrations were relatively accurate, but the recovery method used to measure these provided a lower-than-dosed concentration. As detailed in Chapter 3, the passive filter-based method of assaying diclofenac used in the current study, is relatively poor at recovering diclofenac from waters with high concentrations (such as the 1000 µg/L exposure concentration used here; see Table 4.1). This is the most likely explanation for the relatively low measured diclofenac concentration in the current study.

4.4.2 Salinity-dependent exposure

Diclofenac has an environmental presence in both fresh and saline waters (Table 1.2). There is much literature examining the influence of salinity on toxicant effects in aquatic biota. For example, salinity generally has a significant protective effect on trace metal toxicity (Bielmyer et al. 2010). This is in part due to the change in metal ion speciation (from their toxic, ionic forms to forms that are less bioavailable), but also because of differences in the physiology of biota at different salinities (Bielmyer et al. 2010). One key difference in physiology is drinking. In high salinity settings, where the external osmolality is greater than internal osmolality, fish drink in order to regain water lost by diffusion (Evans et al. 2004). This means that the gut could be a potential uptake pathway for diclofenac, increasing body burden, and thus increasing the toxic impacts of this drug. A previous study examining the organic toxicant naphthalene, showed this exact pattern, with increased tissue levels and toxic effects in killifish at higher exposure salinities (*Funduls heteroclitus)*; Levitan and Taylor 1979). However, in the current study, there was no evidence of this effect. Future studies to examine tissue diclofenac burden would be required in order to determine whether the lack of

response was due to a lack of additional tissue uptake, or a lack of toxicological impact of the additional body burden.

There has been little previous work examining the impact of salinity on diclofenac toxicity. However, the research that has been done found similar outcomes to those described here. For example, there was no effect of salinity on diclofenac toxicity (survival, development) to the estuarine shrimp *Palaemon longirostris* (González-Ortegón et al. 2016), while in the crab *Carcinus maenas*, salinity did not alter the outcome of a diclofenac-mediated impact on osmoregulatory capacity (Eades et al. 2010).

Future work should consider examining ionoregulatory effects of diclofenac exposure on common bullies. Exposure of other NSAIDS (ibuprofen, salicylate) has been shown to impair corticosteroidogenesis in rainbow trout, an effect which will impact the acclimation of this species to seawater (Gravel et al. 2009). Furthermore, the exposure of fish to diclofenac leads to gill damage (Schwaiger et al. 2004; Hoeger et al. 2005; Memmert et al. 2013) which could also impair the ability of fish to osmoregulate.

It is worth noting that salinity had no effect on biochemical measures even in control tissues. There is evidence that EROD activity, GST activity, and protein carbonylation can all be influenced by salinity alone, independent of the presence of a toxicant (Donham et al. 2006; Amutha et al. 2010; Blewett et al. 2015). However, this depends on the species tested e.g. (Jönsson et al. 2003; Fonseca et al. 2011). It appears that enzyme measurements in common bully are relatively resistant to salinity. This is positive from the perspective of environmental monitoring, as it means that measurements will not be impacted by environmental factors, a characteristic of a good biomarker (van der Oost et al. 2003). However, the lack of toxicant-responsiveness suggests that the common bully may not be the most sensitive fish species. This is consistent with previous data that show the common bully

was among the most tolerant of tested native New Zealand freshwater fish species to hypoxia (Landman et al. 2005).

4.4.3 Effects of diclofenac on GST

Glutathione S-transferase has two key roles in toxicology. It is a biotransformation enzyme that is used to detoxify both endogenous and exogenous compounds (Kim et al. 2010), but is also an important anti-oxidant by virtue of the fact that it detoxifies pro-oxidant contaminants, and pro-oxidant products formed by Phase I metabolism (Sharma et al. 2004). For this reason GST is often used as an indicator of an organism's exposure to a toxic compound (Kim et al. 2010). GST has been shown to play a role in diclofenac metabolism (Dragovic et al. 2013), and thus diclofenac exposure would be expected to increase GST activity in common bully, as has been previously reported for other fish (e.g. common carp; Stepanova et al. 2013). In the current chapter, no effect of diclofenac on GST was observed.

There are a number of possible explanations for this. The levels of diclofenac taken up by the common bully may not have been high enough to elicit a response. Further work examining diclofenac in common bully tissue would be useful in determining the effectiveness of diclofenac uptake by this fish. It is also possible that other components of the oxidative stress cascade may be assisting in the scavenging of ROS preventing upregulation of GST. This is supported by a study that showed GST activity actually decreases in the liver of wolf fish (*Holplias malabaricus*) exposed to diclofenac (Guiloski et al., 2015). Although GST activity went down, there was an increase in the activity of superoxide dismutase, total glutathione and glutathione peroxidase activity. This implies that the ROS scavenging of these three important antioxidant components, may have been sufficient to have caused a decrease in GST activity.

It is clear, however, that there are contrasting outcomes regarding the effect of diclofenac exposure on GST activity. As mentioned above, a study on early life-stages of the common carp (*Cyprinus carpio*) showed induction of GST activity (Stepanova et al. 2013), while in wolf fish GST activity decreased (Guiloski et al., 2015). Praskova et al. (2014) described diclofenac at a concentration of 0.2 mg/L, as having no effect on GST activity in the zebrafish (*Danio rerio*), while Ghelfi et al. (2016), did not observe any significant effect up to a concentration of 20 µg/L in catfish (*Rhamdia quelen*). It seems likely that there are species differences in GST responsiveness to diclofenac, although differences in exposure concentration, life-stage of the exposed animal, and exposure duration, may also be important.

4.4.4 The effects of diclofenac on CYP activity

The exposure of *Gobiomorphus cotidianus* to diclofenac, was expected to result in an increase in CYP activity. Diclofenac binds to the aryl-hydrocarbon receptor activating it and inducing CYP1A in tissues including the gills (Jönsson et al. 2003). The results of the ANOVA suggested that there was no statistical significance effect of diclofenac concentration on EROD activity in the current study. However, the large variation in EROD activity values in the highest exposure group likely precluded significance. Subsequently, a Student's t-test showed that at the environmental exposure level (0.1 µg/L) a significant increase in EROD activity existed, relative to the control. Conducting multiple t-tests instead of an ANOVA is not statistically valid, unless there is a multiple comparisons correction. When a Bonferroni correction was applied (alpha level/number of comparisons = new alpha level of 0.017), the p value for the comparison still narrowly eluded significance (0.021). Nevertheless, there is sufficient evidence to suggest that there is a non-significant trend of increased EROD activity with diclofenac exposure in the gills of common bully.

It is possible this variation within groups was due to the timing of fish collection. The fish used in the exposures were caught just prior to mating season. There is evidence suggesting male fish have higher EROD activity than females (Wunderlich et al. 2015), and that female EROD levels increase in late Spring/early Summer (Sole et al. 2002; Gungordu et al. 2011). This suggests that a mixture of male and female fish in the highest exposure group may have contributed to the variability. It is also worth noting that fish in the salinity-dependent exposure had EROD activity levels that differed from the identical exposure concentration in the concentration-dependent study. This may have been related to the small differences in the timing of these two exposures as it relates to spawning season. These differences highlight the importance of time-matched controls (as used in the current study), to ensure that factors such as reproductive status are accounted for within each experiment.

The finding of an upwards trend in EROD activity is consistent with the bulk of fish literature regarding diclofenac effects. After exposure to 1 µg/L of diclofenac for 14 days and 2 mg/L of diclofenac for 24 hours, induction of EROD activity occurred in the three-spined stickleback (Prokkola et al. 2015). Diclofenac exposure at a concentration of 100 µM also induced EROD activity in European seabass (*D. labrax*; (Ribalta et al. 2014). However, there are some data that suggests diclofenac causes an inhibition of EROD activity. This has been observed in rainbow trout hepatocytes at concentrations of 22-940 µM (Laville et al. 2004), and following whole animal exposures to 100 µM diclofenac in the Mediterranean fish species *Trachyrynchus scabrus*s (Ribalta et al. 2014). Inhibition of EROD activity in these studies is thought to be due to excess formation of ROS that inhibit CYP enzymes at the transcriptional or post-transcriptional level through inhibition and degradation of mRNA (Smutny et al. 2013). It is therefore possible that the lack of a truly significant EROD effect in the current study may be a consequence of mixed stimulatory and inhibitory effects on gill EROD activity in the common bully.

4.4.5 Effect of diclofenac on protein carbonylation

The results of a one-way ANOVA showed that there was no effect of diclofenac on protein carbonylation in the liver of the common bully. When conducting a t-test a p value of 0.040 was obtained when comparing the 0 and 0.1 µg/L groups but this value was again above the Bonferroni corrected alpha value of 0.017. Nevertheless, there was a statistical trend towards an increase in protein carbonylation with diclofenac exposure.

An increase in protein carbonylation has been reported in previous studies examining diclofenac toxicity in fish. For example, (Saucedo-Vence et al. 2015) measured a significant increase in protein carbonylation across five tissue types in the common carp (*Cyprinus carpio*) at diclofenac concentrations of 7 mg/L. Diclofenac exposure also resulted in increased protein carbonylation in the aquatic sediment-dwelling bioassay amphipod *Hyalella azteca* (Oviedo-Gomez et al. 2010), and also in the common carp (Nava-Alvarez et al. 2014). Coupled with the nearly significant results in common bully, protein carbonylation in response to diclofenac exposure appears to offer the most consistent results of all the tested endpoints in the current study. As such protein carbonylation is likely to be the endpoint with the most utility as a biomarker of diclofenac effect in aquatic organisms.

4.5 Conclusion

In summary, there were no statistically valid significant effects of diclofenac on any of the measured biochemical end-points in the common bully. This was probably due to high variability in some exposure groups, likely a result of having mixed sex fish, and variability in reproductive status. The trends in the data do, however, agree with the bulk of existing literature, and suggest that mechanism of diclofenac toxicity are likely to be conserved in the common bully. However, further work on fish outside of spawning season would be required to confirm this.

Chapter 5: General discussion and conclusion

5 Overview

Diclofenac is a widespread contaminant within global waterways, generating concern regarding its toxicity to aquatic species (Fent et al. 2006). However, prior to this thesis there was no research that explored the possibility of diclofenac generating toxic effects in New Zealand bullies. Consequently, this thesis had two aims. The first was to develop a simple and cost-effective method for extracting diclofenac from water that would facilitate the measurement of environmental and laboratory concentrations of diclofenac (Chapter 3). The second was to examine diclofenac toxicity on the native New Zealand fish species, the common bully, by investigating changes in key biochemical pathways (Chapter 4).

Chapter 3 details the development of a passive sampling technique for the extraction of diclofenac from water samples from ecotoxicity experiments. Many studies use a form of solid phase extraction or liquid-liquid extraction (Rezaei et al. 2013; Ji et al. 2014; Zhou et al. 2014; Toledo-Neira et al. 2015), however passive sampling is an upcoming technique for monitoring environmental levels of pollutants in large bodies of water (Joyce et al. 2015). Passive sampling techniques allow for real-time collection of environmental concentrations, and critically are both time- and cost-effective (Nyoni et al. 2010). In this thesis, the developed method involved the placement of a simple nylon filter in a collected water sample, which was left for 24 h for adsorption to occur. Thereafter, the sample could be extracted and analysed via either HPLC or GC-MS, depending on the requisite detection limit. This technique was found to be highly effective for recovering diclofenac from waters where it was present at low concentrations. At a concentration of 0.05 µg/L, recovery rates for diclofenac were 103%. This result indicates that this technique could have significant value for environmental monitoring of diclofenac concentrations.

A key drawback to the method was its inability to adequately deal with high diclofenac concentrations, such as those used as the "effect level" in the diclofenac exposures conducted in Chapter 4. It was likely that the filter was saturated by the high concentrations, meaning that the recovery was poor (58%). However, this is a problem that could likely be overcome by increasing the adsorptive surface, by adding filters to the water sample.

Overall, therefore the developed technique was successful. It offered a proof-of-concept for nylon-based filter extraction of diclofenac for water samples from ecotoxicity assays. It was a time- and cost-effective method, and facilitated high throughput of samples. Importantly it was a technique that was compatible with both HPLC and GC-MS.

It is, however, worth noting that there were issues regarding contamination when using the GC-MS for analysis of 0.1 µg/L water samples. Because each sample had to be run for approximately 22 minutes before any peak could be observed, a high concentration standard was run beforehand to ensure correct positioning of the peak and reference ions. This resulted in carry-over from the standard to the first batch of samples. This carry-over meant the samples were unable to be used, reducing the total number of usable samples. Once the issue had been identified, the arrangement of standards and samples were corrected and the final concentrations obtained were in line with the hypothesis. It did mean that for the 0.1 µg/L samples the total sample group was smaller than originally planned, which may have impacted statistical significance.

To investigate the possibility of diclofenac causing toxic effects in New Zealand aquatic species, the common bully was chosen as an experimental organism. This fish species is native to New Zealand, has a widespread geographical distribution (Vanderpham et al. 2013), and has been previously used in studies for assessing environmental impact of toxicants (van den Heuvel et al. 2007). In addition to examining the effect of diclofenac concentration, the

effect of salinity on toxicity was also investigated. The common bully is also an ideal species in which to examine this environmental factor as it is a facultatively diadromous species living part of its lifecycle in freshwater and part in salt water, and as such is tolerant to salinity changes (Bleackley 2008). However, in contrast to expected changes, biochemical analysis of the common bully after exposure to varying concentrations of diclofenac (0, 0.1, and 1000 µg/L) and varying salinities (0, 6, and 20‰) yielded no significant results with respect to EROD activity, GST activity and protein carbonylation. This was due in part to high variability, at least in the case of EROD activity and protein carbonylation. This variability likely resulted from the presence of mixed sex exposures, and the coincidence of the exposure with reproductive cycles, which are known to impact these measures (Sole et al. 2002; Gungordu et al. 2011; Wunderlich et al. 2015). Pairwise statistical analysis did show non-significant trends towards increases in EROD activity and protein carbonylation. Coupled with literature reports of biochemical changes in fish and other aquatic biota exposed to diclofenac, the results of Chapter 4 suggest that the mechanism of diclofenac toxicity is likely to be conserved in the common bully. This analysis also suggests that protein carbonylation is likely to be the most informative biomarker of diclofenac exposure, as it is the endpoint that changes the most consistently, in contrast to the other two biochemical end-points that vary in terms of the directionality of the effects (i.e. both increases and decreases of activity have been reported).

That mechanisms of toxicity appear to be conserved in common bully indicates that, in general, regulatory tools for diclofenac that have been developed to protect biota internationally will be applicable to this species. Importantly, the end-points of toxicity measured in this study seem to be unimpacted by salinity, suggesting that this will not be an important factor that could give “false positives”. However, the statistical outcomes make it difficult to determine the true sensitivity of the common bully to diclofenac. It is possible that

they are not a good biomonitoring or sentinel species as they may be relatively tolerant to diclofenac. Future work should examine the impacts of diclofenac across a range of other native aquatic biota. Of particular interest will be the galaxiid fish. Like the common bully, this group of fish species are native to New Zealand, and occupy many of the same waters as do common bullies (McDowall 2006). However, in some studies conducted to date, they appear to be more sensitive to environmental stressors (Landman et al. 2005). Previous studies (Section 1.5.2) support the use of the common bully for environmental toxicology research. The common bully has a proven history of responding to environmental toxicants and inhabits areas that have exposure to pollutants. This makes it a useful test subject for the research questions addressed in this thesis.

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