Mechanisms of trace metal and diclofenac toxicity in inanga (*Galaxias maculatus*): contextualising responses of a non-model native New Zealand species to standard fish models.

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“The more clearly we can focus our attention on the wonders and realities of the universe about us, the less taste we shall have for destruction”

– Rachel Carson

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Acknowledgements

What a ride…

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Abstract

Pharmaceuticals and trace metals are increasingly prevalent in the aquatic environment, due to anthropogenic pressures. The essential trace metal zinc (Zn) and the non-essential trace metal cadmium (Cd) are particularly enriched in New Zealand settings owing to factors such as galvanised roof runoff, and superphosphate fertiliser application, respectively. The emerging pharmaceutical contaminant diclofenac is increasing in waters worldwide due to heavy usage and lack of breakdown in waste water treatment. Although present at low concentrations, environmental persistence and high bioactivity of these contaminants results in toxicological impacts on aquatic biota. However, most toxicity studies in fish are conducted on model Northern Hemisphere species, and almost nothing is known regarding the sensitivity of widespread Southern Hemisphere fish such as the inanga (*Galaxias maculatus*). This species exhibits a number of unusual physiological traits that may alter their responses to environmental contamination. Furthermore, as one of the few amphidromous fish species they move freely through estuaries and near-coastal streams that are compromised by the presence of agricultural, urban and industrial effluents containing key contaminants such as pharmaceuticals and trace metals. In order to adopt regulations that adequately protect New Zealand's freshwater fish fauna, it is important to determine that mechanisms of toxicity and the biological foundations of regulatory modelling tools established in model species, still apply to fish such as inanga.

To investigate mechanisms of trace metal toxicity, inanga were exposed to graded concentrations of Zn or Cd for 96 h. Whole body metal accumulation, ionoregulation (calcium and sodium influx) oxidative stress (catalase and lipid peroxidation), and metabolism (respirometry) endpoints were examined. Zn exposure caused increases in catalase activity and lipid peroxidation, but only at 1000 µg L^{-1}, a concentration at which Zn also significantly inhibited calcium influx, and stimulated sodium influx. Cd induced lipid peroxidation and inhibited catalase in the liver after exposure to concentrations as low as 2.5 µg L^{-1}. Measures of ionoregulatory function were not impaired. In general, inanga was shown to be tolerant to waterborne metals, with mechanisms of toxicity conserved relative to better-studied Northern Hemisphere species. This suggests that mechanistic-based regulatory tools are applicable for the environmental protection of this species.

Further, the mechanisms of diclofenac toxicity to inanga were explored, by examining accumulation, and its effects on metabolic rate, ionoregulation, and oxidative stress at environmentally-relevant (0.17 µg L^{-1}) and elevated (763 µg L^{-1}) concentrations. Following an acute 96 h exposure, a bioconcentration factor of 87 was derived for the 0.17 µg L^{-1} exposure concentration, approaching values where transfer through the food chain may be important. Lipid peroxidation in inanga liver was significantly elevated at both exposure concentrations, but lipid peroxidation in kidney and gill decreased after diclofenac exposure. Catalase activity was also elevated in the liver of inanga, but activity decreased in the gill. There were no effects of diclofenac on metabolic rate or ion (sodium and calcium) influx rates. These data indicate toxicologically-relevant adverse outcomes and bioconcentration of diclofenac at environmentally-relevant levels.

The finding that oxidative stress was a major mode of diclofenac impact, led to an examination of whether this mode of action was also prevalent in the more traditional models from the Northern Hemisphere (zebrafish embryos, *Danio rerio*; larval fathead minnow, *Pimephales promelas*). Significant effects on lipid peroxidation were noted, but only at concentrations higher than those found in the environment (0.01, 1, 100 mg L^{-1}), and only in the fathead minnow. This research showed distinct species-specific effects, a finding that deserves additional consideration in the development of predictive approaches for the protection of aquatic biota from adverse outcomes elicited by pharmaceuticals.

Given the finding that Cd causes pro-oxidant effects, and diclofenac generally behaves as an antioxidant in inanga, the effects of binary mixtures of these two contaminants, which are likely to co-occur in wastewater effluents, were examined. Antioxidant defence (catalase, superoxide dismutase, glutathione S-transferase) and oxidative damage (protein carbonylation, lipid peroxidation) were assessed in exposures of Cd, diclofenac and these contaminants in combination, at concentrations previously shown to induce impacts on oxidative stress. Relative to singular exposures, mixtures of Cd and diclofenac caused significant changes in patterns of oxidative stress, indicating a clear interaction between the two toxicants. In particular, diclofenac exposure reduced Cd-induced impairment of antioxidant defence and the induction of oxidative damage, suggesting that where these two toxicants co-occur traditional models of predicting toxicity based on individual contaminants may be compromised.

The results from this thesis contribute significantly to a limited body of research regarding the impacts of environmental contaminants on an important

Southern Hemisphere fish species, and are among the first data looking at the effects of simple trace metal/pharmaceutical mixtures in any fish. This research also contributed significant new knowledge regarding the comparative effects of diclofenac in two important model species. As such, the results from this thesis will provide data that can be utilised by regulatory bodies in their adoption and/or development of regulatory tools for protection of freshwater fish fauna.

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List of Abbreviations and Acronyms

Acronym	Definition
ACN	Acetonitrile
ANOVA	Analysis of Variance
ANZECC	Australia/New Zealand Environment Conservation Council
ATPase	Adenosine Triphosphatase
BCF	Bioconcentration Factor
BLM	Biotic Ligand Model
C	Oxygen capacitance
CA	Carbonic Anhydrase
Ca^{2+}	Calcium
CCV	Continuing Calibration Verification
Cd	Cadmium
COX	Cyclooxygenase
Cu	Copper
DCF-*d4*	Diclofenac-*d4*
DMT1	Divalent Metal Transporter
DOC	Dissolved Organic Carbon
DTNB	5,5-Dithio-bis-2-nitrobenzoic acid (Ellman's reagent)
DW	Dry Weight
ECaC	Epithelial Ca Channel
FET	Fish Embryo Test
g	Grams
GC-MS	Gas Chromatography-Mass Spectrometry
GPx	Glutathione peroxidase
GSH	Total glutathione
GST	Glutathione S-Transferase
H^+	Proton
H_2O_2	Hydrogen peroxide
hpf	Hours post fertilisation
hph	Hours post hatch
HPLC	High Performance Liquid Chromatography
HSD	Honest Significant Difference
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
K^+	Potassium
K_{ow}	Octanol/water partition coefficient
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry

LC_{50}	Median lethal concentration
LOD	Limit of Detection
LOQ	Limit of Quantification
MDA	Malondialdehyde
MDL	Method Detection Limit
MO_2	Metabolic rate
MQ	Milli Q
MS-222	3-aminobenzoic acid ethylester
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
Na^+	Sodium
NKA	Na^+/K^+ ATPase
NOEC	No Observed Effect Concentration
NZ	New Zealand
O_2	Oxygen
O_2^-	Superoxide radical
OECD	Organisation for Economic Co-operation and Development
PO_2	O_2 partial pressure
ppm	Parts per Million
QA/QC	Quality Assurance/Quality Control
RIPA	Radio Immunoprecipitation
ROS	Reactive Oxygen Species
SD	Standard Deviation
SEM	Standard Error of the Mean
SIM	Selected Ion Mode
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TNB	5-Thio-2-nitrobenzoic acid
TRAP	Toxicology Research Analysis Program
US EPA	United States Environmental Protection Agency
WWTP	Waste Water Treatment Plant
Zn	Zinc

Chapter 1. General Introduction

1.1. Overview

Aquatic organisms are subject to a range of environmental stressors including physical (e.g. temperature, water flow), biological (e.g. competition for resources, predation), and chemical (e.g. dissolved oxygen (O_2), salinity) factors. Among the chemical factors influencing environmental health are those of a primarily anthropogenic origin, which includes toxicants such as pharmaceuticals and trace metals. These contaminants are increasingly prevalent in the aquatic environment due to anthropogenic pressures such as agricultural and industrial practices, population increases and urbanisation (Corcoran et al., 2009; Davis et al., 2001; McDowell, 2009; McGeer et al., 2012; O'Sullivan et al., 2012). Although trace metals and pharmaceuticals are present in waters only at low concentrations (Acuña et al., 2015; McDowell, 2009; O'Sullivan et al., 2012), their environmental persistence and high bioactivity results in toxicological impacts on aquatic biota (Lonappan et al., 2016; Niyogi et al., 2008; Santore et al., 2002). To date, however, our understanding of toxic impacts is limited, often to a few model species, which may have little real-world relevance to the contaminated ecosystems themselves. It is therefore important to develop an enhanced understanding of the impacts of these contaminants, particularly in species that inhabit contaminated environments. However, in the case of emerging contaminants such as pharmaceuticals, there is still a lack of research in model species that are used for the development of regulatory tools. Specifically, understanding of the mechanisms by which contaminants impact biological pathways is a key knowledge gap. Mechanistic data facilitate the development of tools that can be incorporated into environmental regulations, allow assessment of sensitivities between species, and permit prediction of the impact of contaminant mixtures.

1.2. Trace metals

Trace metals occur naturally at low concentrations in almost all waters, usually reflecting the ambient geology of the water body. However, anthropogenic activities such as agriculture, urbanisation, and mining have resulted in elevated levels of trace elements, such as zinc (Zn) and cadmium (Cd), in natural waters (Davis et al., 2001; McDowell, 2009; McGeer et al., 2012; O'Sullivan et al., 2012). These two trace elements are of particular toxicological importance in New Zealand (NZ), and will be discussed below (Section 1.2.2; Section 1.2.3).

1.2.1. Water chemistry and the BLM

The toxicity of metals depends not only on their concentration, but also on their speciation, which is driven by water chemistry parameters (e.g. pH, ionic strength, dissolved organic carbon (DOC); Di Toro et al., 2001; McGeer et al., 2012). There are a number of regulatory tools that account for water chemistry, and therefore predict the bioavailability, and eventual toxicity, of waterborne metals. The Biotic Ligand Model (BLM) is one such tool, providing a site-specific assessment of metal toxicity (Santore et al., 2002). BLM's have been developed for a number of metals and are in regulatory use worldwide (e.g. Bodar, 2005; United States Environmental Protection Agency, 2007). In Australia and NZ, the BLM approach is an approved method for water quality assessment, but it is not specifically mandated (ANZECC/ARMCANZ, 2000). BLM approaches have been developed using a few model species (e.g. rainbow trout and fathead minnows), and as such may not necessarily be applicable to other species, particularly if the mechanisms of metal toxicity differ (Niyogi and Wood, 2004). Consequently, more research is required to understand mechanisms of metal toxicity and to determine if they are conserved

between species. Such data will validate the use of BLM's in settings, and for species, outside of those that were used to develop and calibrate the models.

1.2.2. Zn

1.2.2.1. Zn concentrations in the NZ environment

Zn is enriched in the aquatic environment through sources such as corrosion of galvanised products, breakdown of car tire rubber and urban runoff (Davis et al., 2001; O'Sullivan et al., 2012; Veleva et al., 2010). Zn is of particular concern in NZ, as it has been recorded in urban streams at concentrations as high as 270 µg L^{-1} (O'Sullivan et al., 2012), and levels as high as 1280 µg L^{-1} have been reported in acid-mine impacted streams of the West Coast of NZ, known to be an important habitat for native aquatic species (Harley, 2015).

1.2.2.2. Zn pathways of uptake, and tissue distribution

Although toxic at high concentrations (see Section 1.2.2.3), Zn is an essential element playing an important role in many biochemical processes. For example, Zn-dependent proteins comprise around 10% of the proteome (Hogstrand, 2011; Watanabe et al., 1997). Because of its essentiality, there are dedicated Zn transporters located in the gills and gut of fish enabling them to acquire waterborne and dietary Zn, respectively (Bury et al., 2003).

Waterborne Zn uptake occurs through one of two main pathways, both of which are thought to only transport divalent Zn ion (Zn^{2+}). Dedicated Zn^{2+} (e.g. ZIP) or divalent cation (e.g. divalent metal transporter; DMT1) transporters are present on the apical gill epithelial surface and may achieve initial uptake (Bury et al., 2003). Absorption into the animal requires transport across the basolateral epithelial surface, which is achieved by transporters such as Zn Transporter-1 (ZnT-1; Bury et al.,

2003). However, Zn^{2+} may also be taken up through apical calcium (Ca^{2+}) channels such as ECaC (epithelial Ca channel). This uptake pathway is dependent on the relative concentrations of Ca^{2+} and Zn^{2+} present in the water, as they are competing for the transporter (Giardina et al., 2009). Uptake through this pathway is believed to be a significant mechanism of Zn toxicity in fish (Section 1.2.2.3).

Most Zn uptake, however, occurs via consumption of food sources that contain Zn. Dietary absorption of Zn, in general, contributes more towards Zn body burden than waterborne Zn^{2+} uptake, likely reflecting both the high concentrations of Zn that the fish is exposed to by the pathway, and the higher capacity of gastrointestinal Zn uptake relative to the gill (Glover and Hogstrand, 2002). However, the mechanisms of Zn uptake across the gut are believed to be similar to those in the gill (Bury et al., 2003). The exception to this is that some Zn is absorbed across the gut of fish liganded to organic nutrients such as amino acids (Glover et al., 2003). Although quantitatively the gut is the more important pathway of Zn uptake in fish, Zn taken up via the gut is thought to be of less toxicological significance than that taken up by the branchial pathway, owing largely to the main mode of toxicity of this element.

Once absorbed by the gut or the gill, Zn then enters the bloodstream and is transported to tissues such as the liver, where the metal-binding protein metallothionein facilitates Zn donation to metalloenzymes (Hogstrand, 2011; Valavanidis et al., 2006). Metallothionein can also act to sequester potentially toxic levels of Zn, and prevent them from interfering with sensitive cellular entities (Hogstrand, 2011; Valavanidis et al., 2006). However, at higher levels of exposure,

homeostatic regulation of Zn can be overwhelmed, and toxicity may result (Hogstrand et al., 1996; Loro et al., 2014; Spry and Wood, 1995).

1.2.2.3. Zn toxicity effects

The sharing of a branchial uptake pathway with Ca^{2+}, means that the presence of Zn^{2+} will interfere with Ca^{2+} uptake, at least in studied model species such as the rainbow trout (*Oncorhynchus mykiss*; Hogstrand et al., 1994, 1995, 1996, 1998). This effect, coupled with the ability of Zn to inhibit the basolateral Ca-ATPase (adenosine triphosphatase) and inhibit basolateral Ca transfer, results in hypocalcaemia and can eventually cause fish death (Hogstrand et al., 1996; Spry and Wood, 1995).

While interference with Ca homeostasis appears to be the main mode of Zn toxicity, effects on other biochemical and physiological processes have also been noted. For example, Zn has been shown to inhibit the basolateral Na^+/K^+-ATPase (NKA; Loro et al., 2014), the enzyme that is primarily responsible for the transport of ions across the fish gill, thus ensuring ionic and acid-base homeostasis (Evans et al., 2005; Section 1.6.3.2). Furthermore, at high exposure levels Zn is known to cause branchial mucus secretion, a mechanism of toxicity that increases diffusive distance and impairs both ion regulation, but also other vital gill-based processes such as O_2 uptake (Skidmore, 1970).

In addition to ionoregulatory and respiratory effects of Zn, impacts on oxidative stress markers have also been recognised. For example, killifish (*Fundulus heteroclitus*) exposed to Zn^{2+} exhibited a decrease in tissue catalase activity (an enzyme that degrades hydrogen peroxide (H_2O_2) formed through reactive oxygen species (ROS)), and an increase in lipid peroxidation (a marker of oxidative damage;

Loro et al., 2012). However, there is little information as to whether Zn involvement in oxidative stress is a widespread phenomenon in fish (Lushchak, 2011).

The median lethal concentrations (LC_{50}) for freshwater fish exposed to Zn range from 66 to 40 900 µg L^{-1} (Eisler, 1993), although sublethal effects have been noted in sensitive species, such as brown trout (*Salmo trutta*), at Zn exposure levels of 5 µg L^{-1} (Sayer et al., 1989). This is of concern as these concentrations are lower than those commonly measured in urban streams (O'Sullivan et al., 2012).

1.2.2.4. Regulatory context.

The manifestation of toxic impacts from exposure to Zn^{2+} has resulted in the development of guideline concentrations for aquatic ecosystems. Regulatory tools, such as the BLM (Section 1.2.1), have been developed to protect aquatic species. However, many jurisdictions also publish guidelines that are more prescriptive. In NZ and Australia, the Australia NZ Environment Conservation Council (ANZECC) use a trigger value, a concentration at which effects are expected to manifest within a certain percentage of aquatic species. For example, the 95% trigger value for Zn^{2+} in freshwater is 8 µg L^{-1}, a conservative value compared to other countries (Table 1.1; ANZECC/ARMCANZ, 2000). However, environmental concentrations of Zn^{2+} are regularly observed in exceedance of this value (Harley, 2015; O'Sullivan et al., 2012). Typically, these regulatory tools are developed from a few model species, which may not be species that reflect local fauna. For example, prior to this thesis there was no understanding of how sensitive NZ's native freshwater fish fauna were to Zn^{2+} toxicity. Therefore, knowledge of whether Zn toxicity mechanisms are conserved in species inhabiting local ecosystems is required to understand whether existing regulatory tools are likely to be protective.

Table 1.1. Zn^{2+} trigger values for 95% protection of freshwater species.

Country	Concentration	Reference
Great Britain	14.2 µg L^{-1}	Maycock et al., 2010
Canada	30 µg L^{-1}	Alberta Environment, 1999
United States	120 µg L^{-1}	United States Environmental Protection Agency, 2007
Australia and New Zealand	8 µg L^{-1}	ANZECC/ARMCANZ, 2000

1.2.3. Cd

1.2.3.1. Cd concentrations in the NZ environment

Cd is another important environmental contaminant found in aquatic environments. This trace metal most commonly ends up in the environment due to its use in batteries, pigments, stabilisers, coatings, some alloys, and it is also a contaminant in superphosphate fertilisers (McDowell, 2009). This latter source is a particular issue for NZ waters. Superphosphate fertilisers are liberally applied to NZ agricultural soils, resulting in Cd build-up and subsequent run-off into rivers (McDowell, 2009). Like Zn, another common source of Cd^{2+} contamination is from acid mine drainage, whereby the acidity mobilises naturally-occurring Cd. For example, downstream of the Tui Base-Metal Mine in Te Aroha waters may have Cd^{2+} concentrations as high as 286 µg L^{-1} (Sabiti et al., 2000).

1.2.3.2. Cd pathways of uptake, and tissue distribution

Any exposure to Cd^{2+} will be potentially toxic to aquatic species. Unlike Zn, Cd is a non-essential metal and therefore animals such as fish do not have dedicated Cd-specific uptake pathways. In its ionic form (Cd^{2+}), Cd is divalent and like Zn, can mimic Ca^{2+}. Consequently, waterborne Cd^{2+} has the capacity to be taken up via the epithelial Ca^{2+} channel present in the gills of fish (Almeida et al., 2009; Niyogi et al., 2008; Verbost et al., 1988). Cd may also be taken up by DMT1. Although this

transporter is thought primarily responsible for the uptake of essential trace metals such as ferrous iron and Zn^{2+}, it can also transport Cd^{2+} (Cooper et al., 2006; Komjarova and Bury, 2014). For example, Cd accumulation in zebrafish has been correlated with the transcription levels of DMT1 in the gills (Cooper et al., 2006).

As for Zn, the digestive tract is also an important source of Cd^{2+} uptake (McGeer et al., 2012). The mechanisms of gastrointestinal Cd uptake are believed to be conserved, relative to those in the gill, and as for Zn, liganded Cd may also offer an additional route of uptake (Kwong and Niyogi, 2012). Again, the gastrointestinal pathway of absorption is likely to contribute most significantly to overall Cd body burden, and this absorbed Cd is less significant in terms of toxicity, owing to the relatively greater importance of Ca uptake at the gill relative to the gut (McGeer et al., 2012). The handling of Cd is similar to that of Zn. Once absorbed, Cd is principally accumulated in the liver of fish, although the kidney also is an important sink (McGeer et al., 2012). Intracellularly, Cd will be primarily sequestered by metallothionein, which will bind to the metal, reducing its bioreactivity and toxicity (e.g. Wu et al., 2006). However, there are limited mechanisms available to excrete Cd once absorbed, meaning that there is a tendency for Cd to bioaccumulate in fish, although this occurs to a lesser extent than it does in aquatic invertebrates (Eisler, 1985).

1.2.3.3. Cd toxicity effects

One of the principle mechanisms of Cd toxicity in fish, is through the inhibition of Ca homeostasis, with this mechanism of action being similar to that described in Section 1.2.2.2, for Zn. Competition between the divalent Cd^{2+} and Ca^{2+}, coupled with Cd-induced inhibition of Ca-ATPase (Verbost et al., 1988), inhibits

branchial Ca^{2+} uptake, leading to hypocalcaemia and eventual mortality (McGeer et al., 2000a).

Exposure to Cd^{2+} also generates disturbances to ion transport processes other than those of Ca^{2+}. For example, Cd impairs carbonic anhydrase (CA), and thus perturbs sodium (Na^{+}) homeostasis (see explanation in Section 1.6.3.2). In a study in rainbow trout, McGeer et al. (2000a) demonstrated decreases in whole body Na after acute exposure to Cd, thought to be mediated through the effects of CA inhibition on Na^{+} uptake. Similarly, da Silva and Martinez (2014) showed that gill tissue of the freshwater fish *Prochilodus lineatus* displayed decreases in Na^{+} transport enzyme activities after exposure to Cd^{2+}, leading to a disturbance in Na^{+} homeostasis.

Ionoregulatory disruption is not the only mode of Cd^{2+} toxicity in freshwater fish. Oxidative damage (measured by lipid peroxidation, DNA damage, and protein carbonylation), has been noted following Cd exposure in several studies (Atli and Canli, 2007; Hisar et al., 2009; McGeer et al., 2012; Nunes et al., 2015). Oxidative damage occurs when there is an imbalance between the production of ROS that cause oxidative damage, and antioxidant defence mechanisms, which scavenge ROS (see Section 1.6.1; Lushchak, 2011). Cd has the ability to impair the activity of antioxidant enzymes through binding to the active site, and/or impairing appropriate enzyme folding (Wang et al., 2015). Cd can also increase ROS production through displacement of iron in the Fenton reaction, which may lead to oxidative damage (Nair et al., 2013). Likewise, if Cd is bound to metallothionein, then this reduces the capacity of this protein to scavenge ROS, further exacerbating ROS accumulation (Nair et al., 2013).

Disruptions to ionoregulation and the damage associated with oxidative stress may induce an increased metabolic output in fish (see Section 1.6.3.2). A number of studies have examined effects of Cd on metabolic rates in fish, but the outcomes of these studies are equivocal (McGeer et al., 2000a; Peles et al., 2012; Pistole et al., 2008; Rose et al., 2006). This is due to the length of time it can take for Cd to cause disruptions to homeostasis, meaning that the length of exposure plays a large role in the outcome of metabolic rate measurements. For example, Pistole et al. (2008) exposed fathead minnows to Cd for 24 and 96 h. Fish exposed for 24 h exhibited a decrease in metabolic rate, whereas fish exposed for 96 h experienced an increase.

Largely owing to its non-essential nature and ability to bioaccumulate, effect concentrations of Cd are lower than Zn. In terms of lethal effect concentrations, the LC_{50} values for freshwater fish exposed to Cd range from 0.5 to 73,500 µg L^{-1} (McGeer et al., 2012). For some species, these are concentrations well within those likely to be measured in the environment, which poses potential concern.

1.2.3.4. Regulatory situation.

The higher toxicity of Cd relative to Zn has resulted in relatively low environmental trigger concentrations for Cd. For example, the 95% trigger value for protection of freshwater species in NZ and Australia against Cd is 0.2 µg L^{-1} (ANZECC/ARMCANZ, 2000). As for Zn, the trigger values set by the ANZECC are lower than those of other jurisdictions (Table 1.2). Again, however, there are no data examining the sensitivity of NZ freshwater fish species to Cd, and thus the applicability of these regulatory limits to NZ settings is not known.

Table 1.2. Cd trigger values for 95% protection of freshwater species.

Country	Concentration	Reference
Europe	1.5 µg L^{-1}	European Union, 2013
Canada	1 µg L^{-1}	Alberta Environment, 1999
United States	1.8 µg L^{-1}	United States Environmental Protection Agency, 2007
Australia and New Zealand	0.2 µg L^{-1}	ANZECC/ARMCANZ 2000

1.3. Pharmaceuticals in the aquatic environment

In 1999, Daughton and Ternes identified pharmaceuticals as environmental toxicants of emerging concern (Daughton and Ternes, 1999). Pharmaceuticals are administered to humans and animals, to alter physiological function, and thus aid in the mitigation of homeostatic disturbances and disease states (Corcoran et al., 2010; Fent et al., 2006). Rates of pharmaceutical consumption are increasing as a result of increased populations and usage rates. Many pharmaceuticals are not fully metabolised in humans and animals, leading to the excretion of these chemicals into wastewater (Arnold et al., 2014). Furthermore, for many of these chemicals and their metabolic breakdown products, there is limited removal through wastewater treatment plant (WWTP) methodologies, leading to the appearance of biological active contaminants in receiving waters. This is a particular issue in developing countries with poor water treatment, and limited regulations regarding pharmaceutical manufacturing and disposal. However, even in developed nations, there has been an increased occurrence of pharmaceuticals in natural waters (Corcoran et al., 2009; Daughton and Ternes, 1999; Larsson, 2014; Zhang et al., 2008).

Although present at relatively low concentrations, pharmaceuticals generally exhibit high bioactivity, a characteristic that may cause a number of toxicological impacts in non-target species inhabiting contaminated systems (Acuña et al, 2015;

Memmert et al., 2013; Praskova et al., 2014). Non-target species (i.e. those accidentally exposed through the presence of pharmaceuticals in water) are susceptible to effects due to conserved biological pathways upon which pharmaceuticals act. Therefore, pharmaceuticals designed to alter physiological function in humans and agricultural animals, may have unintended effects on these same physiological pathways in other species (Corcoran et al., 2010). In response to the presence of pharmaceuticals in environmental samples and growing evidence of their toxic impacts on wildlife, in 2013 the European Union included three pharmaceuticals on its list of priority pollutants in the Water Framework Directive for the first time (European Union, 2013). One of these pharmaceuticals was diclofenac.

1.3.1. Diclofenac

Among the pharmaceuticals of potential environmental concern, diclofenac has received particular attention from the scientific and regulatory communities. Diclofenac is a non-steroidal anti-inflammatory drug (NSAID), available as either a prescription medicine, or an over-the-counter drug, depending on the country of sale. Diclofenac is used as both a human and veterinary medicine for the treatment of inflammation, typically arthritis (Brogden et al., 1980). The primary mechanism of action for diclofenac is through inhibition of prostaglandin synthesis, a key mediator of inflammation. This is achieved via the inhibition of cyclooxygenase (COX) enzymes (Gan, 2010).

1.3.1.1. Diclofenac in the NZ environment

Worldwide consumption of diclofenac is approximately 940 tons per year (Zhang et al., 2008). Partly due to these high consumption rates, diclofenac has been detected in environmental samples (surface, ground, and drinking water) from at least

50 countries (Aus der Beek et al., 2016). More specifically, diclofenac has been measured in global wastewater and surface waters at concentrations ranging from low ng L^{-1} to low µg L^{-1} with an average concentration of 0.2 µg L^{-1} (Acuña, 2015; Aus der Beek, 2016; Zhang et al., 2008). In NZ, however, there are only limited data regarding the presence and concentration of pharmaceuticals in marine and freshwater environments. Stewart et al. (2014) conducted a survey of contaminants (flame retardants, herbicides, pesticides, pharmaceuticals and metals) in the sediments of Auckland estuaries. They found 21 pharmaceuticals present in at least one sample location, with diclofenac being present in two samples. The average concentration of diclofenac was around 2 ng g^{-1} dry weight (DW) of sediment. Stewart et al. (2016) also conducted a study using passive sampling methods to monitor contaminants in water samples around Auckland, NZ. Diclofenac was not detected in any of the three sites they assessed (Stewart et al., 2016). To the best of my knowledge, there are no published studies examining the presence of pharmaceuticals in the NZ environment. However, given that diclofenac is among the most widespread environmental pharmaceutical contaminants worldwide (Zhang et al., 2008), it is likely that diclofenac is present, particularly in near-urban waters receiving WWTP effluents.

1.3.1.2. Regulatory context

Due to the presence of diclofenac in water samples throughout Europe, it has recently been placed on the European Union Water Framework Directive watch list (European Union, 2013), so that monitoring data can be collected to understand its environmental risk. There is, however, limited information regarding the concentrations that may elicit an effect on aquatic species inhabiting contaminated waters. Only recently, Kumar et al. (2016) developed a recommended guideline value for diclofenac (770 µg L^{-1}) for 95% protection of species. They developed this value

using high quality chronic toxicity data that examined endpoints of population-level relevance (e.g. development, growth, survival; Kumar et al., 2016). However, their analysis precluded examination of lower level impacts, such as changes in biochemistry and physiology, which could cause significant effects on biological health at an individual level. Therefore, this guideline value may not protect individuals against sublethal effects of diclofenac. NZ does not have any regulatory policies regarding the presence of pharmaceuticals in the environment. Monitoring programmes instead focus on the presence of metals and persistent organic pollutants (Stewart et al., 2014). It is clear that there is a significant research gap in NZ regarding the presence and concentration of pharmaceuticals in the environment and their impacts on local aquatic species.

1.3.1.3. Diclofenac uptake and bioaccumulation

The persistence of diclofenac in the environment has caused a number of effects in non-target species. The most notable impact reported to date, was the rapid decline of vulture populations in the Indian subcontinent, following accidental poisoning through consumption of carcasses of cattle recently treated with diclofenac (Oaks et al., 2004; Shore et al., 2015). Bioaccumulation of diclofenac occurred in the kidney, and vultures died from renal failure (Oaks et al., 2004; Schultz et al., 2004). Since the deaths of the vultures in the Indian sub-continent, studies have been conducted to assess the how other non-target species can be affected by exposure to diclofenac.

The presence of diclofenac in natural waters has led to concerns regarding potential adverse outcomes in aquatic ecosystems. With a moderately lipophilic log K_{ow} (octanol/water partition coefficient; 4.75; Table 1.3), diclofenac rapidly partitions

across epithelial surfaces, and consequently bioconcentrates in fish (Scheytt et al., 2005; Memmert et al., 2013). Bioconcentration factor (BCF) values of less than 10 in rainbow trout exposed for 14 days to diclofenac concentrations up to 18.7 µg L^{-1} have been reported, and it was therefore suggested that bioconcentration of diclofenac was likely to be of limited concern (Memmert et al., 2013). However, Memmert et al. (2013) performed their study at pH 7.5-8.4, which may underestimate bioconcentration in aquatic habitats with lower pH. Because diclofenac is a weak acid with a pKa of 4.15, surface water exposures at acidic pH may result in elevated accumulation. This is because the proportion of non-ionised, and more lipophilic, diclofenac increases with increased acidity (Erickson et al., 2006a, b; Nichols et al., 2015).

Table 1.3. Physico-chemical properties of diclofenac

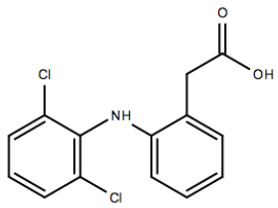
Properties	
Chemical structure	
CAS number	15307-79-6
Molecular weight	318.13
Log K_{ow}	4.75
pKa	4.15

Table modified from Lonappan et al., 2016

1.3.1.5. Toxic impacts of diclofenac

Because diclofenac accumulates in fish, there have been several studies examining the toxic effects of diclofenac exposure. One key endpoint that has been examined is oxidative stress. For example, diclofenac has been shown to cause oxidative stress in model aquatic species such as rainbow trout and zebrafish, at exposure concentrations approaching levels reported from environmental monitoring studies (Feito et al., 2012; Gröner et al., 2016; Memmert et al., 2013; Praskova et al., 2011; Saucedo-Vence et al., 2015; Schwaiger et al., 2004; Stepanova et al., 2013). Oxidative stress responses to diclofenac appear related to Phase I metabolism and antioxidant defence mechanisms (Islas-Flores et al., 2014). For example, during Phase I metabolism, diclofenac may generate a highly reactive superoxide anion (Hong, 2007), which can cause oxidative damage unless antioxidant enzymes can sufficiently scavenge. However, it is also important to note that diclofenac can also decrease oxidative damage. This may occur through increases in the rate of antioxidant enzymes (Stepanova et al. 2013; Praskova et al., 2014; Feito et al., 2012), or directly, as the inhibitory effect of diclofenac on COX enzymes reduces ROS production, an effect associated with a decline in oxidative damage (Mouithys-Mickalad et al., 2004). It is likely that differences in species sensitivities to diclofenac, is related, at least in part, to differences in antioxidant defence mechanisms and Phase I metabolism (Islas-Flores et al., 2014; Connors et al., 2013).

Effects of diclofenac are not restricted to those on oxidative stress, however. For example, chronic exposure to environmentally-relevant diclofenac concentrations has been shown to alter tissue integrity. Diclofenac altered histopathological measures in the kidney and gills of rainbow trout at an exposure concentration of 5 µg L^{-1} (Schwaiger et al., 2004), while cytological alterations to the same tissues occurred at

diclofenac exposure concentrations of 1 µg L^{-1} (Triebskorn et al., 2004). Rainbow trout gills exhibited damaged to the pillar cells and capillary wall after exposure to 5 µg L^{-1} of diclofenac (Schwaiger et al., 2004). Likewise, rainbow trout exposed to diclofenac (1000 µg L^{-1}) exhibited gill damage (Schwaiger et al., 2004). Owing to the importance of the gill in a variety of physiological processes, branchial damage is likely to have other consequences. For example, plasma Na concentrations increased in Indian carp exposed to 100 µg L^{-1} diclofenac for 96 h (Saravanan et al., 2011; Saravanan and Ramesh, 2013). This was likely a compensatory mechanism to deal with osmoregulatory imbalances which resulted from damage to the gill surface.

Although sublethal toxic impacts occur at low, environmentally-relevant concentrations, mortality does not occur at such levels. Early life-stages of zebrafish show LC_{50} values that range between 6 and 22 mg L^{-1} (Chen et al., 2011; Praskova et al., 2011), varying with exact age and length of exposure. Clearly developmental stage plays a significant role in LC_{50} determination, as more developed zebrafish (2-3 months) display a 96 h LC_{50} of 176 mg L^{-1} (Praskova et al., 2011), while common carp (3 months of age) have an LC_{50} of 71 mg L^{-1} (Saucedo-Vence et al., 2015). These concentrations are significantly in excess of those found in the environment, therefore mortality is unlikely to be a key outcome of environmental exposure to diclofenac.

1.4. Mixture toxicity

Anthropogenic releases of effluents into the environment rarely consist of single contaminants (Dethloff et al., 1999; Heys et al., 2016; Hinton and Aizawa, 2006). However, environmental quality guidelines such as those provided by ANZECC, and the European Water Framework Directive, only publish values for safe

environmental concentrations of individual contaminants, not those occurring in mixtures (Tables 1.1 and 1.2; Brack et al., 2017). Similarly, some of the key regulatory tools, which are designed to support these guidelines (e.g. BLM; Section 1.2.1), have been developed and validated only for single toxicants.

Standardised toxicity testing involves exposure to an individual chemical, allowing calculation of sensitivity of a given species, as described by parameters such as the LC_{50} or no-observed effect concentration (NOEC). Mechanistic toxicity testing is also commonly used to understand how contaminants interact with biochemical and physiological endpoints in biota. The problem with this form of regulatory development and testing is that it does little to inform researchers and regulators about the interactions between, and within, classes of chemicals (Hinton and Aizawa, 2006). As such, environmental concentrations may be within acceptable guidelines, but the combined effect of multiple "below criterion" toxicants may have severe consequences on the physiological processes of organisms that inhabit contaminated ecosystems. Understanding mixture toxicity is important as it improves knowledge related to toxicity effects on species, in a way that is more applicable to environmental situations.

As the regulation of contaminant concentrations in aquatic settings is focused on individual toxicants, the failure to account for other co-occurring contaminants has the potential to lead to over- or under-estimation of toxicity (Heys et al., 2016). This problem is well-recognised, and there is growing development of modelling tools, and supporting research, that seeks to account for toxic effects of mixtures (e.g. Nys et al., 2017; Brix et al., 2016). However, the majority of the research conducted on mixtures

focuses on chemicals of the same class (e.g. pharmaceuticals, metals, or pesticides; Barbee et al., 2014, Nava- Álvarez et al., 2014; Watanabe et al., 2016).

There is, however, significant attention given to examining the toxicity of whole effluent samples to aquatic biota (Olvera-Néstor et al., 2016). Although exposure to whole effluent samples is environmentally realistic, often the lack of mechanistic characterisation of the effects, and the complex chemistry of these mixtures, precludes the ability to identify which contaminant or contaminants is/are driving the toxicity (Heys et al., 2016). Consequently, while these approaches are useful for examining risk of a specific effluent at a specific time, they have limited utility for the prediction of toxicity, should the composition of the effluent change, and/or a limited capacity for facilitating the translation of impacts from one study organism to another. As such there is significant value in understanding how simple contaminant mixtures alter the biological responses of exposed biota, in order to build a mechanistic knowledge of effects that will facilitate predictive modelling.

Currently, one approach for assessing mixture toxicity is the use of additivity models. The basic concept underlying additivity modelling is that the effect of the mixture can be predicted from the sum of the effects of the individual contaminants (Heys et al., 2016). This approach assumes that effects are additive, and that non-additive effects (e.g. synergism and antagonism) are unlikely to occur (Rodea-Palomares et al., 2015). However, at least for mixtures of metals and pharmaceuticals, there is evidence that mixture toxicity may not always be simple to predict. Alsop and Wood (2013), conducted a study examining the toxicity of copper (Cu) in association with fluoxetine, 17-β oestradiol or 17-α ethinyloestradiol to zebrafish larvae for 96 h. Mortality in larvae occurred at a lower concentration in mixtures, compared to when

they were exposed to individual contaminants. The mechanism of effect was thought to be ionoregulatory, with depletion of body ions noted (Alsop and Wood, 2013). Although these effects were additive, additivity was not predicted. Cu is a well-known ionoregulatory toxicant (Glover et al., 2016), but prior to the current work there was limited evidence for effects of the tested pharmaceuticals on ionoregulation in fish. This demonstrates the difficulty in predicting mixture effects, especially across contaminant classes.

There are very few studies investigating the combined effects of both metals and pharmaceuticals even though they commonly occur in the same receiving environments. This is a consequence of both residential and industrial wastes being mixed at WWTPs. For example, work by Vystavna et al. (2012) showed that WWTPs input both pharmaceuticals and trace metals into the Udy River in the Ukraine. Andreu et al. (2016) investigated the presence of pharmaceuticals and trace metals in Mediterranean coastal wetlands, and found a strong correlation, indicative of a common source. The toxic effects of pharmaceuticals and their mixtures, has been recently highlighted as a key question in the field (Boxall et al., 2012).

1.5. Fish as model species for toxicology studies

Receiving environments for contaminants are commonly aquatic, with treated and raw effluent, and storm water draining into freshwater and marine water bodies (Ballatori and Villalobos, 2002). Therefore, aquatic biota, such as fish, are ideal study species for understanding the impacts of common environmental contaminants, in that they have direct relevance to the impacted environment. Including both freshwater and marine habitats, there are approximately 30,000 species of fish, constituting approximately 50% of the subphylum Vertebrata (Bolis et al., 2001). Fish span across

the food web representing primary, secondary, and tertiary trophic levels, making them a truly diverse and ecologically-important group (van der Oost et al., 2003; Ballatori and Villalobos, 2002). Because of their importance in aquatic settings, and the propensity of such settings to be impacted by toxicants, it is critical to gain an understanding of how fish species will be impacted by exposure to environmental contaminants (Nagel and Isberner, 1998). This importance is recognised by the inclusion of fish in regulatory guidelines.

However, that fish share a number of biological traits with higher vertebrates, also makes them a valuable model for understanding both human disease and toxicity. Indeed, where it would be unethical and uneconomical to subject humans or other higher vertebrates to toxicity testing, fish are ideal surrogates (Ankley and Johnson, 2004; Bolis et al., 2001; van der Oost et al., 2003). Many of the molecular mechanisms of toxicity are highly conserved across vertebrate species making data in fish, at least somewhat applicable to other groups (Ankley and Johnson, 2004).

There are also many practical benefits to using fish in toxicology studies. Fish are highly fecund, and their eggs are mostly transparent making developmental abnormalities easy to identify (Bolis et al., 2001; van der Oost et al., 2003). They are also easily cultured in laboratory environments (Bolis et al., 2001; van der Oost et al., 2003). Research that investigates the impacts of environmental contaminants is, however, generally limited to a few ‘model’ species (e.g., zebrafish, fathead minnows, rainbow trout, and common carp). However, it is important to understand whether these model fish are representative of other fish species.

1.5.1. Inanga

Galaxias maculatus (commonly known as inanga (NZ), jolly tail (Australia) or puye (South America)) is one of the world's most widely distributed freshwater fish species (McDowall, 1990), although it remains restricted to temperate Southern Hemisphere waters (McDowall, 2006). This species is of significant value, being the predominant component of the culturally- and economically-important NZ whitebait fishery (McDowall, 2006), and a potential aquaculture species in South America (Mardones et al., 2008).

Inanga are one of the few truly amphidromous fish (McDowall, 2007). They hatch on spring tides in estuarine nurseries, migrate out to the ocean as larvae, where they develop into juvenile fish through the winter, before migrating back to freshwater in the spring (McDowall, 1990; McDowall, 2007; Watanabe et al., 2014). They therefore inhabit near-coastal streams which are the likely sinks of agricultural, urban and industrial contamination.

Inanga display a number of physiological characteristics that are quite distinct from those of more commonly studied Northern Hemisphere fishes. For example, inanga are scaleless and in aquatic settings, the skin accounts for almost 40% of total O_2 uptake (Urbina et al., 2014a). The importance of the skin in transport processes usually associated with the gill means that the skin could act as an alternative locus of toxicity and/or a rescue pathway supplementing transport processes impacted by toxicant actions at the sensitive branchial epithelium (Urbina et al., 2014a).

Inanga have also been shown to inhabit waters that are highly acidic (Olsson et al., 2006). This tolerance has evolved over time to avoid predation from introduced species, such as trout. However, their habitation of low pH waters may expose them

to increased bioavailability of both trace metals and pharmaceuticals (Campbell and Stokes, 1985; Nicholls et al., 2015).

At the initiation of the research contained within this thesis, very little literature was available regarding the sensitivity of inanga to environmental contaminants. Studies of the toxicity of pulp and paper effluent (Stauber et al., 2001) and the fungicide chlorothalonil (Davies and White, 1985), have shown that relative to other tested species, inanga has a very similar sensitivity. However, expanding this work to a variety of different pesticides, Davies and colleagues asserted that toxicity data derived for the model species rainbow trout were not adequate for the prediction of toxicity to inanga (Davies et al., 1994). In contrast, when examining the relative sensitivity of inanga to the important contaminant ammonia, Richardson (1997) concluded that international regulations developed in the Northern Hemisphere would be adequate to protect this species. More recently, there has been growing interest in the sensitivity of inanga to trace metals. Data from Glover et al. (2016; waterborne Cu), Blewett et al. (2016; waterborne nickel) and Barbee et al. (2014; sediment metal mixtures), have led to novel insights into how metal toxicity may differ in inanga relative to other freshwater fish species. For example, inanga exposed to Cu exhibit elevated ammonia excretion, a finding distinct from the pattern commonly observed in fish (Glover et al., 2016). Together these studies suggest that toxic responses of inanga to environmental toxicants may not be conserved, highlighting the importance of understanding of how other common contaminants, such as diclofenac, Cd and Zn (for which no information is available), may alter physiological and biochemical responses in inanga.

1.5.2. Zebrafish

Zebrafish are a well-characterised species commonly used in environmental toxicology (Ballatori and Villalobos, 2002), but also for understanding human genetic diseases (Howe et al., 2013). Zebrafish have been in use since the 1930's and since then their physiology, genetics and biochemistry have become widely understood (Dai et al., 2014; Hill et al., 2005). Hill et al. (2005) suggested that more is known about "what is normal" about a zebrafish than any other species, making it an ideal model for understanding adverse physiological, biochemical and genetic changes as a result of toxic impacts (Hill et al., 2005). Zebrafish are excellent laboratory species due to their small size, which minimises housing, feeding and dosing costs. The small size and transparency of their embryos, allows for high throughput screening methods and easy identification of embryonic development and deformities (Ballatori and Villalobos, 2002; Hill et al., 2005). There are, however, a number of genes in the zebrafish that possess no other teleost fish orthologues. This may therefore lead to species sensitivities difference in response to exposure to environmental contaminants (Howe et al., 2013). Zebrafish are ideal species for fish embryo tests (FET) as they can spawn every second day (Ankley and Johnson, 2004).

Many studies have utilised zebrafish as a model organism for understanding the impacts of acute and chronic exposure to pharmaceuticals (Chen et al., 2014; Diniz et al., 2015; Hallare et al., 2004) and metals (Alsop and Wood, 2011; Hallare et al., 2005; Komjarova and Blust, 2009, 2014). These include studies that have examined diclofenac toxicity (see Section 1.3.1), and also the toxicity of Cd (see Section 1.2.3) and Zn (see Section 1.2.2).

1.5.3. Fathead minnows

The fathead minnow is the most commonly used fish for ecotoxicological studies in North America (Ankley and Villeneuve, 2006). It is widely distributed throughout North American waters, and thus has value as an environmental sentinel species, beyond its utility as a laboratory species. Its short life cycle, well characterised reproductive behaviours, and its ability to tolerate a wide range of water qualities makes it a suitable test organism (Ankley and Villeneuve, 2006).

Similar to zebrafish, the fathead minnow has been used in a significant number of pharmaceutical (Nallani et al., 2011; Nichols et al., 2015; Overturf et al., 2012; Parrott et al., 2009) and metal (Pistole et al., 2008; Santore et al., 2002; Zahner et al., 2006) toxicity studies. However, prior to the current thesis there were no studies that assessed the sensitivity of fathead minnows to diclofenac. Fathead minnows have, however, been used to study bioconcentration of the NSAID, ibuprofen (Nallani et al., 2011). Exposure to metals has been demonstrated to significantly impair physiological endpoints in fathead minnows (see Section 1.5.3).

1.6. Assessing toxicant effects

1.6.1. Markers of oxidative stress

As detailed in Sections 1.2.2.3, 1.2.3.3, and 1.3.1.4, oxidative stress is a likely response of fish to a wide range of toxicants. Oxidative stress occurs due to an imbalance between the accumulation of ROS, and the ability of antioxidant enzymes to detoxify them (Lushchak, 2011; Figure 1.1). Environmental contaminants are commonly seen to stimulate ROS production. There are three main mechanisms by which this occurs. First, the toxicants themselves are redox active, capable of initiating the generation of ROS and/or propagating reactions that eventually lead to damage (e.g. Cu; Luschak, 2011). Second, contaminants such as trace metals, may be

able to displace redox active metals (particularly iron) from enzyme active sites, and the free iron is then available to enter the Fenton reaction, which generates a ROS cascade. Third, a toxicant may indirectly induce ROS, for example as described for diclofenac in Section 1.3.1.4. The other mode by which contaminants can induce oxidative stress is by altering antioxidant levels and/or activities (Lushchak, 2011). Examining components of oxidative stress pathways can provide a mechanistic understanding of metal and pharmaceutical toxicity (Lushchak, 2011). Markers of both antioxidant capacity (catalase, glutathione-S-transferase (GST), superoxide dismutase (SOD)) and oxidative damage (lipid peroxidation, DNA damage, and protein carbonylation) can be utilised to provide an overview of the mechanisms by which oxidative stress is generated (Gonzalez-Rey and Bebianno, 2014; Vlahogianni et al., 2007; Vlahogianni and Valavanidis, 2007).

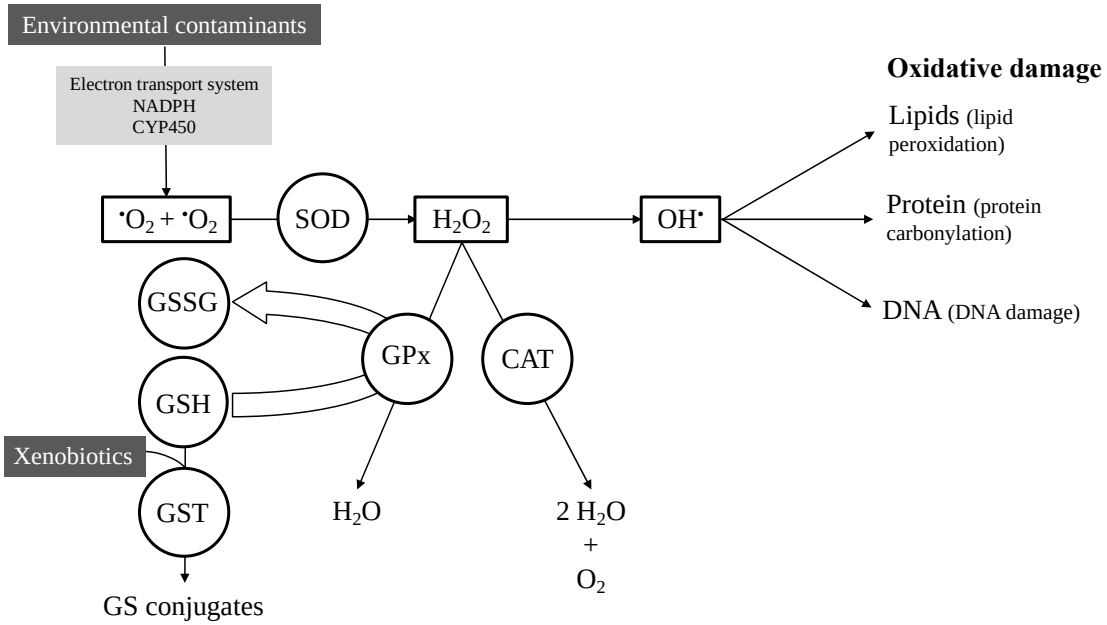


Figure 1.1. Schematic of the oxidative damage and defence system. Key antioxidant enzymes and molecules are indicated by circles, ROS are indicated by rectangles, and contaminants are indicated by dark grey, adapted from Binelli et al. (2011). See 'List of Acronyms', for definition of acronyms and abbreviations used here.

1.6.1.2. Antioxidant enzyme

Antioxidant enzymes are adaptive and have the ability to increase their activity in response to environmental contaminants (Vlahogianni et al., 2007). This decreases the amount of oxidative damage occurring in cellular membranes, DNA and proteins. Measurement of both the oxidative damage and the enzymes that respond to oxidative stress can function as important biomarkers when assessing environmental toxicity. Among the commonly studied antioxidants are SOD (Wang et al., 2015), catalase (Nava-Álvarez et al., 2014; Pretto et al., 2011) and GST (Praskova et al., 2014). SOD is responsible for the conversion of the superoxide radical (O_2^-) to O_2 or H_2O_2. H_2O_2 can then be further reduced by catalase to O_2 and H_2O (Lushchak, 2011), a role which is also performed by glutathione peroxidases (GPx). A key component of the function of GPx is glutathione (GSH). This is an important tripeptide involved in the detoxification of xenobiotics, as it acts as an electron donor in the GPx-mediated reduction of H_2O_2. GSH is also a co-substrate for GST, an enzyme that attaches GSH to electrophilic contaminants (often formed during Phase I metabolism), reducing their capacity to cause oxidative damage (see Figure 1.1; Lushchak, 2011).

1.6.1.3. Oxidative damage

If ROS production exceeds antioxidant defence, then oxidative damage is likely to result. This damage will manifest as lipid peroxidation (Stepanova et al., 2012), protein carbonylation (Blewett et al., 2016), and DNA damage (Ghelfi et al., 2016). All three of these disturbances can, and have been, used as an endpoint to assess the impacts of toxicants on the oxidative stress pathways in fish (see Lushchak, 2011 for review).

1.6.3. Markers of physiological impairment

1.6.3.1. O_2 consumption

Respiration rate is a measure of energy use, specifically the rate of O_2 consumption. Respiration rate is also a proxy for metabolic rate, a parameter that will change as a consequence of toxicant-induced inhibition of energy use or acquisition, or due to increased costs associated with toxicant exposure (e.g. increased cost of damage repair; Lighton and Halsey, 2011). As O_2 fuels all necessary costs to an animal, measuring metabolic rates following exposure to contaminants will therefore contribute towards an understanding of alterations to fitness, and ultimately, survival (Rose et al., 2006).

1.6.3.2. Ion transport

In freshwater, fish are more concentrated than their surrounds and thus lose ions via diffusion to the water. They must recoup these ions in order to maintain salt and water balance. The gill is the principal locus by which ion homeostasis is corrected. This is achieved by a complex network of membrane transporters and enzymes (Evans et al., 2005). Owing the absolute need for freshwater fish to maintain salt and water balance, toxicants that disturb this process are among the most toxic to fish (Wood, 2012).

Of particular importance is the transport of Na^+. The exact mechanism by which fish absorb Na^+ across the gill depends upon the species, however some principles remain conserved between competing models (Hwang et al., 2011). A key entity in Na^+ uptake is CA, which as described in Section 1.2.3.3, is an important target of metal toxicants. CA is a metalloenzyme involved in the generation of protons (H^+) through the conversion of carbon dioxide and water to bicarbonate, using

Zn as a cofactor (**Error! Reference source not found.**). The H^+ is then used, at least n some fish species, to help create generate a gradient favouring Na^+ uptake across the gill. Therefore, disruptions to CA will lead to impairment of Na^+ transport (da Silva and Martinez, 2014). The other key entity in Na^+ transport, which is also a target of toxicants (see Sections 1.2.2.3 and 1.2.3.3), is NKA. Translocating three Na^+ out of the cell in exchange for two potassium ions (K^+), the NKA generates an electrochemical gradient that favours the passage of Na^+ from the dilute freshwater, into the cell. In fact, the actions of NKA are critical for the cellular transport of most ions (Hwang et al., 2011), leading to deleterious consequences when inhibition of this enzyme by toxicants occurs (Wood, 2012).

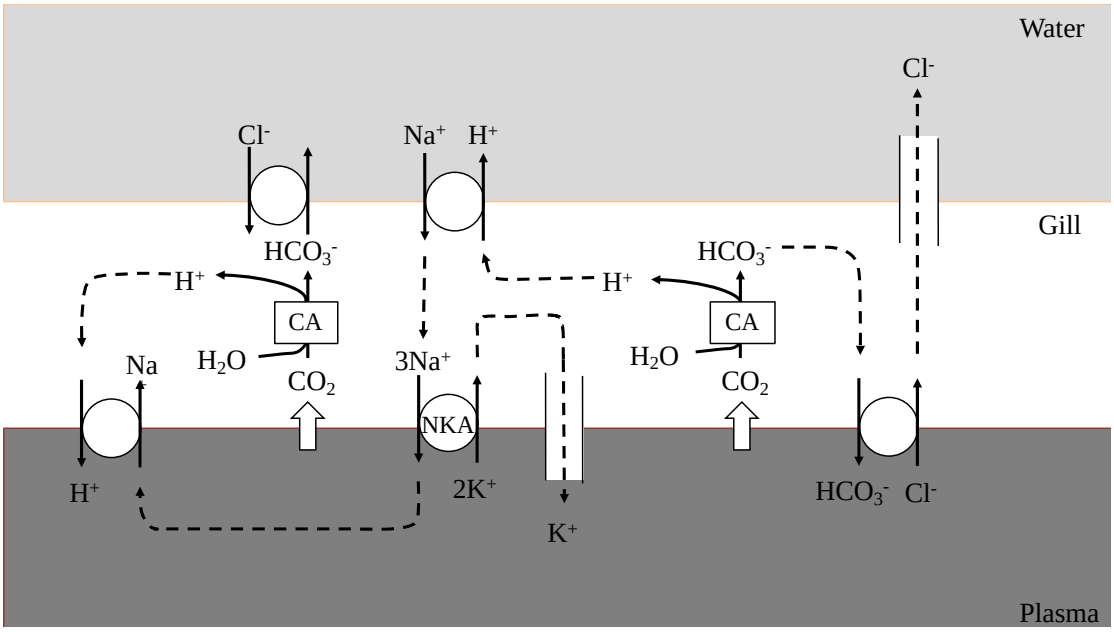


Figure 1.2. Diagram showing the interplay between transportation of sodium (Na^+) from the water into the blood and H^+ generation via carbonic anhydrase (CA). Figure adapted from Batlle et al. (2006).

1.6.4. Nomenclature

Throughout this thesis, different notation is used with respect to ions and elements. In scenarios where chemistry is known and the ionic form is most likely to occur, notation with ionic charge is used (i.e. X^+, X^{2+}). Under scenarios where

chemistry is unknown (i.e. inside a fish) notation without charge is used (i.e. X). This applies to both elements usually found as ions (e.g. Na, Ca), and trace metals (Cd, Zn).

1.7. Objectives

This thesis aims to determine the mechanisms by which the important environmental contaminants Zn, Cd and/or diclofenac affect a culturally- and economically-important native NZ fish species, inanga. To generate additional data for contextualising the effects of diclofenac on inanga, this work also seeks to examine the impacts of this pharmaceutical on standard Northern Hemisphere model species, zebrafish, and fathead minnow. This will be achieved by conducting acute laboratory experiments focussed on understanding whole body tissue contaminant burdens, and impacts on the biochemistry (oxidative stress) and/or physiology (metabolic rate, ion regulation) of the fish species of interest. This research will generate novel data of interest to environmental regulators, for integrating pharmaceutical and metal assessment for the protection of both ecosystems and human health (via food chain exposure).

The objectives of the thesis are outlined below:

1. Determine how exposure to environmentally-relevant concentrations of Zn affects biochemical and physiological sublethal endpoints in inanga.
2. Determine how exposure to environmentally-relevant concentrations of Cd affects biochemical and physiological sublethal endpoints in inanga.
3. Determine how exposure to environmentally-relevant concentrations of diclofenac affects biochemical and physiological sublethal endpoints in inanga.

4. Determine how exposures to simple binary mixtures of a trace metal (Cd) and pharmaceutical (diclofenac) impact oxidative stress biomarkers.
5. Determine how a pharmaceutical (diclofenac) impacts oxidative stress biomarkers in model North American fish species.

1.8. Thesis structure and chapter outlines

Chapters 2 to 6 address Objectives 1 through 5, in that order. Chapter 2 examines the mechanisms by which exposure to a graded series of Zn concentrations, encompassing environmental regulatory and effect levels, impacts Zn body burden, biochemical (catalase activity, lipid peroxidation), and physiological (ion influx, metabolic rate) endpoints in inanga. Chapter 3 is a similar study, where the impacts of Cd are examined in the same fish species, using a similar set of endpoints. A similar set of analytical, biochemical and physiological indicators are used in Chapter 4 to determine the effects of diclofenac exposure on inanga. Chapter 5 is a comparative study wherein the effects of diclofenac exposure on oxidative stress endpoints are examined in larval fathead minnow and embryonic zebrafish. Chapter 6 uses simple binary mixtures of Cd and diclofenac to assess the effects of these chemicals, with opposing putative modes of action, on oxidative stress endpoints. Finally, in Chapter 7, the importance of the study is contextualised, its environmental implications are considered, and future work is proposed.

Chapter 2. Mechanisms of zinc toxicity in the galaxiid fish, *Galaxias maculatus*

McRae, N. K., Gaw, S., Glover, C. N. 2016. Mechanisms of zinc toxicity in the galaxiid fish, *Galaxias maculatus*. Comparative Biochemistry and Physiology, Part C, 179, 184-190.

2.1. Introduction

The migration of juvenile inanga through estuaries is likely to expose them to high levels of environmental contaminants (see Section 1.5.1; Harley and Glover, 2014), and as adults, inanga inhabit near-coastal streams with significant potential for contamination by agricultural, urban or mining effluents. For example, levels of Zn as high as 270 µg L^{-1} have been recorded in urban streams of the Canterbury region of NZ (O'Sullivan et al., 2012), while concentrations as high as 1280 µg L^{-1} have been reported in acid-mine impacted streams of the West Coast, known to be an important inanga habitat (Harley, 2015). Although limited to certain metals, and life stages, previous research has shown that inanga are significantly impacted by exposure to metals (Barbee et al., 2014; Harley, 2015; Harley and Glover, 2014), but physiological mechanisms of metal toxicity remain unknown. Among other impacts such as altered land-use, introduced species, and overfishing, pollution is considered one factor responsible for the decline in inanga populations (Rowe et al., 1999; Rowe et al., 2007).

The goals of the current study were to investigate Zn toxicity (Section 1.2.2) to inanga (Section 1.5.1). Assessing the impacts metal toxicants have on inanga will provide insight into their sensitivity, thus contributing information vital for the monitoring and protection of this species in NZ and worldwide. It will also confirm that modelling approaches based on physiological mechanisms of uptake and toxicity are applicable to species outside those in which the models have been tested and calibrated. In the current study, fish were exposed for 96 h to concentrations of Zn representing a regulatory level (8 µg L^{-1}; value considered to be protective to 95% of freshwater biota; ANZECC/ARMCANZ, 2000), an elevated environmental level (270 µg L^{-1}; O'Sullivan et al., 2012), and an extreme environmental level (1000 µg L^{-1};

Harley, 2015). Endpoints examined included whole body Zn accumulation, markers of oxidative stress (catalase activity, lipid peroxidation), ionoregulatory dysfunction (Ca and Na influx), and respiratory toxicity (O_2 consumption).

2.2. Materials and Methods

2.2.1. Animal collection and holding

Late-stage juvenile inanga were caught using seine nets from natural spring-fed near-coastal streams, with no upstream effluent inputs, in the Canterbury region of the South Island of NZ. The average concentration of Zn at the collection sites was 1.9 (± 0.3) µg L^{-1} (mean (± standard error of the mean; SEM); n = 3). Fish were placed into aerated plastic containers and transported back to the aquarium facility at the University of Canterbury, before being housed in 500-L aquaria receiving flow-through freshwater and constant aeration. They were held under constant temperature (15°C) and light (12 h dark: 12 h light) conditions. Fish were acclimated for one month prior to experimentation and during this time were fed daily (Nutrafin® Max, USA). Feeding ceased 48 h prior to, and during, experimentation. The University of Canterbury Animal Ethics Committee approved all procedures.

2.2.2. Zn exposure

For biochemical and O_2 consumption analysis, a total of 32 inanga (mean ± SEM, 1.34 ± 0.20 g) were randomly distributed (n = 8) to one of four Zn exposures (nominally: control (no added Zn), 8, 270 or 1000 µg L^{-1}) for 96 h. Exposures were conducted in plastic containers (4.5 L) that were acid washed before exposures. Desired Zn levels were achieved by spiking chambers with stock solutions (1 or 10 g L^{-1} $ZnSO_4$) to 2 L of aquarium water (pH 6.7; total hardness 0.70 mmol L^{-1}; total alkalinity 0.519 mmol L^{-1}; electrical conductivity 18.8 mS m^{-1}; total Ca 0.57 mmol L^{-}

^{1}; total magnesium 0.14 mmol L^{-1}; total potassium 0.29 mmol L^{-1}; total Na 0.37 mmol L^{-1}; chloride 0.31 mmol L^{-1}; dissolved organic carbon <0.2 mg C L^{-1}). Waters were left for 24 h to equilibrate, before addition of fish (one fish per chamber). Exposure chambers were continually aerated, and maintained under constant temperature (15 ± 1°C) and light (12 h dark: 12 h light) regimes. A complete water change was performed at 48 h, with water that had been equilibrated for 24 h.

A second exposure was conducted for the Ca and Na influx experiments. This exposure was conducted in an identical manner to that described above, except in this instance just two concentrations were tested (control and 1000 µg L^{-1}). A total of 16 fish were exposed for each influx (mean ± SEM; Ca influx; 0.91 ± 0.05 g, Na influx; 0.51 ± 0.11 g; both n =8), with two fish per exposure chamber.

Water samples were taken for Zn analysis at four time points (fish addition, before and after the water change, and at the conclusion of the exposure). These values were averaged across each replicate, and then replicates were averaged to provide the measured Zn exposure concentration. Water was sampled by passing it through a Millex 0.45 µm filter (Millipore Ltd., Cork, Ireland) using a syringe (Chirana, Slovakia) without a rubber stopper to avoid Zn contamination. Water samples (15 mL) were acidified with 20 µL of ultrapure 70% nitric acid (HNO_3), and stored at 4°C before being analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as described below.

2.2.3. O_2 consumption

O_2 consumption was measured in fish via closed box respirometry (Urbina and Glover, 2013; Urbina et al., 2012). At cessation of the Zn exposure, fish were placed individually into 0.25 L Schott glass bottles and covered with plastic mesh so water

could flow in. Chambers were submerged in a controlled temperature water bath (15 ± 1°C) for the duration of the experiment. Fish were acclimated for 1 h prior to the chambers being sealed with a rubber bung. Attached to the bung was a syringe filled with water and a three-way tap to take samples. Fish naturally depleted O_2 in the chamber and measurement of this is a proxy of metabolic rate. Water samples were taken every 15 (± 1) minutes. An O_2 electrode was refurbished prior to each experiment and was attached to a temperature-controlled water jacket. The O_2 partial pressure (PO_2) of the water samples was read using an O_2 meter (Strathkelvin, Glasgow, Scotland) and was recorded via a Powerlab (ADInstruments, Waverly, Australia) data recording system. Respirometry continued until six samples were taken or PO_2 reached 60 mmHg. Prior to experiments, a calibration was performed with a zero (0.01M Na tetraborate) and air-saturated water. All values were corrected for atmospheric pressure. Blank respirometers (without fish) were run to account for any microbial respiration. Metabolic rate (MO_2; mg O_2 g^{-1} h^{-1}), was calculated as:

$$MO_2 = \frac{\Delta PO_2 \times C \times V}{W \times T}$$

where ΔPO_2 is the change in O_2 partial pressure, C is O_2 capacitance adjusted for temperature and salinity (2.01115), V is the volume of water in the respirometer (L), W is the mass of the individual fish (g), and T is the time (h). Random assortment of fish into treatment groups resulted in a significant size difference between groups. To account for this the scaling relationship between size and metabolic rate in inanga (from Urbina and Glover, 2013) was used to normalise all O_2 consumption values to a 1 g fish.

Following respirometry, fish were euthanised with an anaesthetic overdose (1 g L^{-1} 3-aminobenzoic acid ethylester; MS-222) followed by severing of the spinal cord. Fish were blotted dry, weighed, and dissected. Liver (catalase and lipid peroxidation, see below) and gills (for analyses not included here) were quickly removed and snap frozen in liquid nitrogen for later biochemical analysis. These and the remaining tissue (for Zn analysis) were stored at -80°C until further analysis.

2.2.4. Whole body Ca and Na influx

Methods for Ca and Na influx were based on those of Hogstrand et al. (1994) and Glover et al. (2012), respectively. These assays were performed separately, but using similar protocols. Fish were removed from exposure chambers at the end of the 96-h exposure, and transferred into influx exposure chambers (4-L plastic sealable bags) containing 2 L of aquarium water (up to 4 fish per bag; ion composition reported above). Bags were held in a water bath to maintain a constant temperature (15 ± 1°C). These chambers were spiked with Zn, at a level identical to that which they had been previously exposed (control or 1000 µg L^{-1}). To account for the effects of handling stress on ion transport (Harley and Glover, 2014), fish were left for 2 h prior to addition of radiolabelled Ca (^{45}Ca; 20 μC_i; Perkin-Elmer) or Na (^{22}Na; 20 μC_i; Perkin-Elmer). Water samples (1 mL) were taken for determination of specific activity. Ionic composition of the water was confirmed via ICP-MS, following protocols described below. After 1 h, fish were euthanised with an overdose of MS-222 (1 g L^{-1}), and rinsed in a high Ca (10 mM $Ca(NO_3)_2$) or Na (1 M NaCl) solution to displace any adsorbed but not absorbed ^{45}Ca or ^{22}Na. Inanga were then blotted dry and weighed.

Whole bodies from the Ca influx exposure were digested in 10 mL of 2N HNO_3 for 48 h at 65°C. Scintillation fluor (15 mL; UltimaGold) was added to subsamples of digests (2 mL), and counted on a liquid scintillation counter (TriCarb 2910 TR). Water samples had fluor added (5 mL UltimaGold) and were counted in a similar manner. Quench correction of tissue samples was applied using the external standards ratio method. Mass-specific Ca influx (J_{in}; nmol g^{-1} h^{-1}) was calculated as follows:

$$J_{in} = \frac{CPM}{SA \times W \times t}$$

where *CPM* is the quench-corrected whole body counts per minute, *SA* is the measured mean specific activity of ^{45}Ca in the water (cpm μM^{-1}), *W* is fish mass (g), and *t* is time (h). Whole body ^{22}Na activity and water samples were directly analysed by gamma counting (Wallac Wizard 1470; Perkin-Elmer), with specific activity and mass-specific Na influx (J_{in}; nmol g^{-1} h^{-1}), calculated in a similar manner to Ca influx.

2.2.5. Catalase activity

Liver tissue (~0.02 g) was homogenised in 800 µL buffer (100 mM Tris-HCl, 2 mM EDTA and 5 mM $MgCl_2$) using a plastic pellet homogeniser. Of this homogenate, 200 µL was removed for lipid peroxidation analysis (see below), and the remaining homogenate used for measurement of tissue catalase activity, using methods similar to those of Chandurvelan et al. (2013). The remaining homogenate was centrifuged at 30,000 g for 10 min at 4°C. A 50 µL sample of the supernatant was diluted 10x with the homogenisation buffer, and 50 µL of the resulting solution was added to a 96-well plate (UV star; Greiner Bio-One). H_2O_2 (200 µL) was then added to the plate, before being immediately placed in a plate reader set at 240 nm. Protein

concentration was determined via the Bradford assay (Bradford, 1976). Catalase activity was expressed as U mg protein^{-1} min^{-1}.

2.2.6. Lipid peroxidation

Lipid peroxidation was quantified using a Lipid Peroxidation Assay Kit (MAK085, Sigma Aldrich). Liver tissue was used to determine lipid peroxidation by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a coloured product, which was proportional to the MDA present. The assay was conducted by adding 300 µL MDA lysis buffer and 3 µL butylated hydroxytoluene to the homogenate (200 µL; see above) and the mixture was centrifuged at 13,000 g for 10 min. The resulting supernatant (200 µL) was placed into a microcentrifuge tube and 600 µL TBA solution (reconstituted with 7.5 mL glacial acetic acid and made up to 25 mL with milli Q (MQ) water) was added. The samples were then incubated for 60 min in a water bath at 90°C. Once samples cooled to room temperature, 200 µL was transferred to a 96-well plate and absorbance was read in a microplate reader at a wavelength of 532 nm. Lipid peroxidation was expressed as µmol MDA mg protein^{-1} (Chandurvelan et al., 2013), where the amount of protein was calculated via a Bradford assay (Bradford, 1976).

2.2.7. Tissue and water analysis by ICP-MS

Whole body Zn (tissue remaining after excision of gill and liver) was quantified using ICP-MS with methods similar to those of Gaw et al. (2012). Tissue was weighed and placed in a freeze drier (Lab Conco Freezone 2.5) for 1 week. Freeze-dried tissue was then placed into acid-washed polycarbonate vials and DW (mean ± SEM; 0.28 ± 0.05 g) was recorded. Tissue was then stored at room temperature until further analysis. Tissue was digested by adding 5 mL 10% ultrapure

HNO_3 and left for 24 h before refluxing at 85°C for 1 h. Volumes were adjusted to 20 mL using MQ water. Samples were diluted using 2% ultrapure HNO_3 and placed in acid-washed test tubes to be analysed by ICP-MS (Agilent 7500cx). Quality assurance/quality control (QA/QC) was achieved by using procedural blanks treated as described above (Gaw et al., 2012). Recoveries of Zn from the certified reference material (DORM-4; Sigma Aldrich) were acceptable. Detection limits were calculated as three standard deviations of the mean blank concentration (1.2 µg g^{-1}).

Acidified and filtered water samples taken from the Zn exposures were directly analysed by ICP-MS (Agilent 7500cx). As for tissue samples, QA/QC was achieved by using procedural blanks (Gaw et al., 2012). Detection limits were calculated as three standard deviations of the mean blank concentration (1.3 µg L^{-1}).

2.2.8. Statistical analysis

Data were tested for normality using the Shapiro-Wilk test, and any failing data were log-transformed. Data were then analysed by one-way ANOVA followed by a Tukey HSD (honest significant difference) post-hoc test. The exception was Ca and Na influx data, which were subjected to a t-test. All analysis was performed using RStudio (RStudio version 3.1.0). Statistical significance was set at $p < 0.05$ and all data are expressed as mean ± SEM.

2.3. Results

No mortalities were recorded during exposures. Analysis of dissolved Zn levels in the water showed that there was a significant level of Zn in the aquarium water (~6 µg L^{-1}; Table 1), leading to the regulatory exposure level (nominally 8 µg L^{-1}) being closer to 15 µg L^{-1} (Table 2.1.). Measured dissolved values for Zn are used from this point forth.

Table 2.1. Nominal and dissolved concentrations of Zn (µg L^{-1}) in 96 h exposures.

Zn concentrations (µg L^{-1})	
Nominal	Dissolved
0	5.8 ± 0.5
8	15 ± 1
270	232 ± 3
1000	1016 ± 32

Values expressed as mean ± SEM, n = 4.

2.3.1. Whole body Zn accumulation and O_2 consumption.

There was a significant increase in Zn accumulation in inanga (whole body minus gill and liver) with Zn exposure (p <0.0001; Figure 2.1). This effect was apparent at the lowest added Zn level (15 µg L^{-1}), but increases in Zn exposure level resulted in no further increase in accumulation. There were no significant changes in O_2 consumption (Figure 2.2) in Zn-exposed fish (p = 0.531).

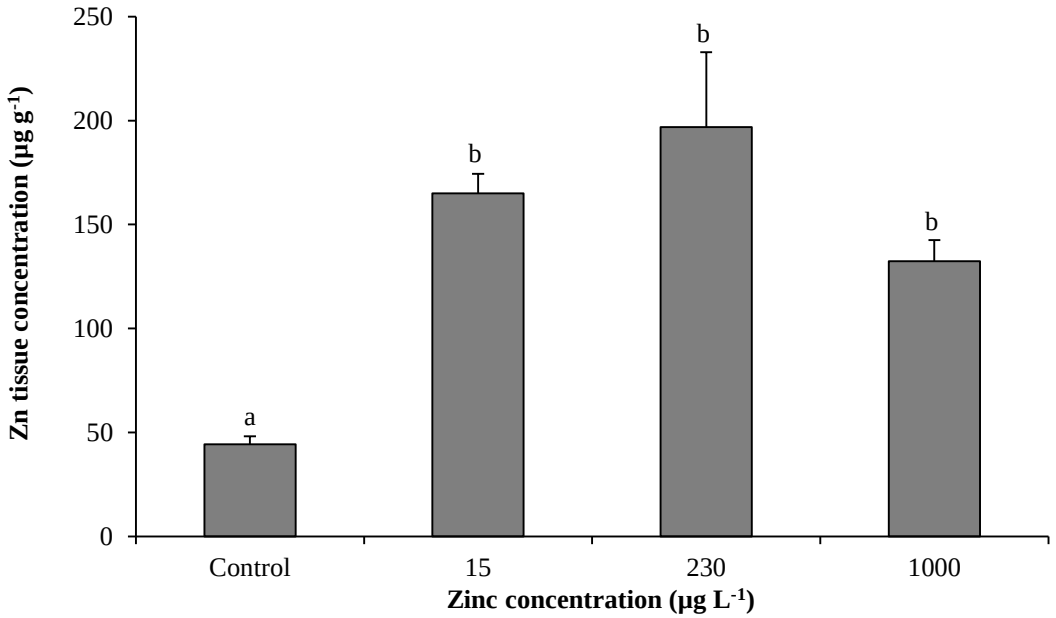


Figure 2.1. Zn accumulation (mean ± SEM) in inanga (whole body minus gills and liver; n= 8) after exposure to Zn for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

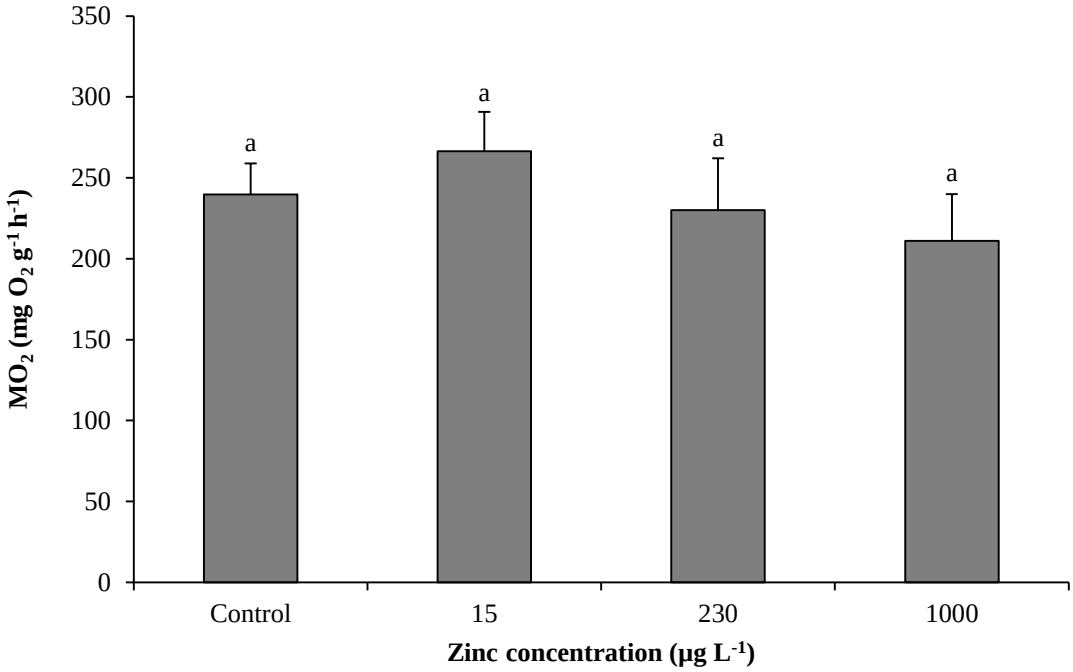


Figure 2.2. Inanga O_2 consumption (MO_2; mg O_2 g^{-1} h^{-1}) after exposure to Zn for 96 h. Plotted values represent means ± SEM (n = 8). Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

2.3.2. Ionoregulatory effects

Ca influx decreased significantly upon exposure to 1000 µg L^{-1} Zn (p = 0.033; Figure 2.3). There was, however, a significant increase in Na influx as a result of exposure to 1000 µg L^{-1} Zn (Figure 2.4; p = 0.003).

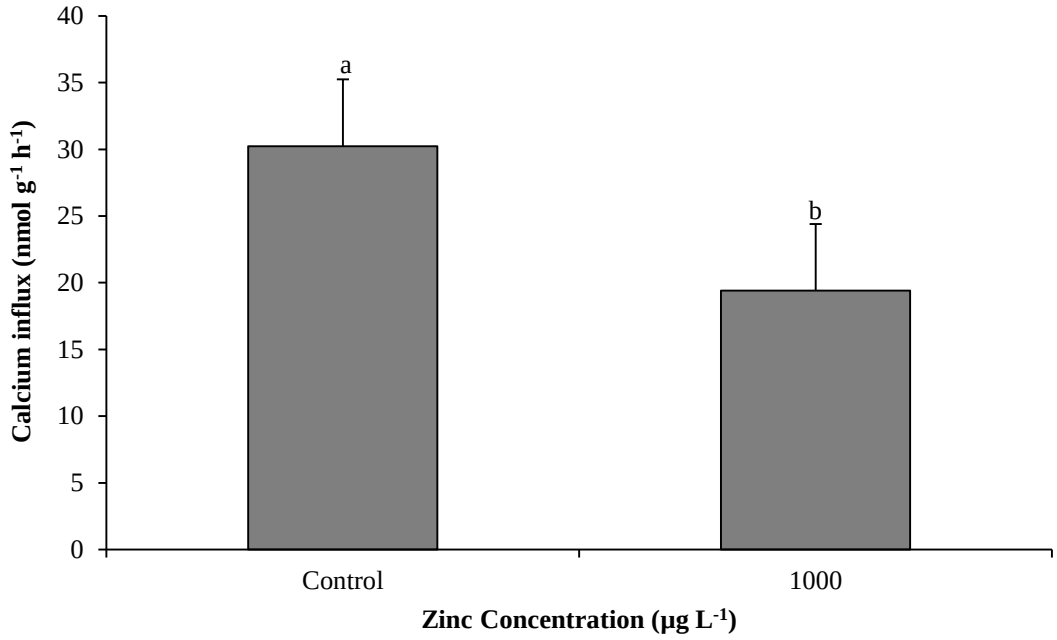


Figure 2.3. Unidirectional Ca influx (nmol g^{-1} h^{-1}; mean ± SEM) in inanga (n = 8) after exposure to control and 1000 µg L^{-1} Zn for 96 h. Bars sharing letters are not significantly different, as determined by t-test (α=0.05).

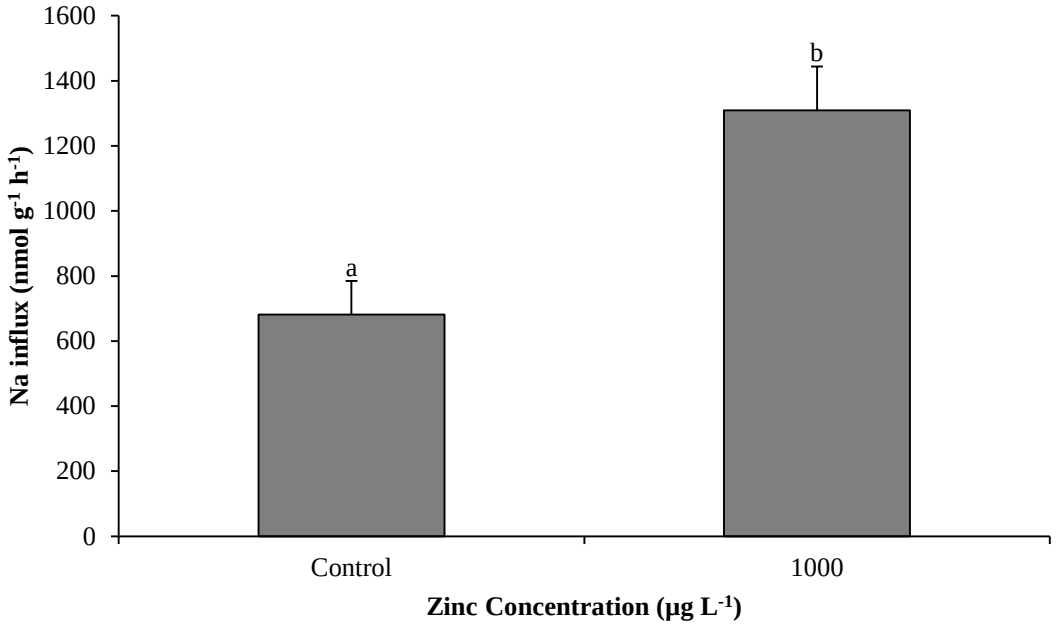


Figure 2.4. Unidirectional Na influx (nmol g^{-1} h^{-1}; mean ± SEM) in inanga (n = 8) after exposure to control and 1000 µg L^{-1} Zn for 96 h. Bars sharing letters are not significantly different, as determined by t-test (α=0.05).

2.3.3. Oxidative stress

There was a significant increase in catalase activity in the liver of inanga exposed to 1000 µg L^{-1} Zn (p = 0.003; Figure 2.5) compared to all other treatments. Lipid peroxidation in the liver tissue of inanga exposed to Zn, showed a significant increase at 1000 µg L^{-1} (p = 0.027; Figure 2.6) when compared to the control.

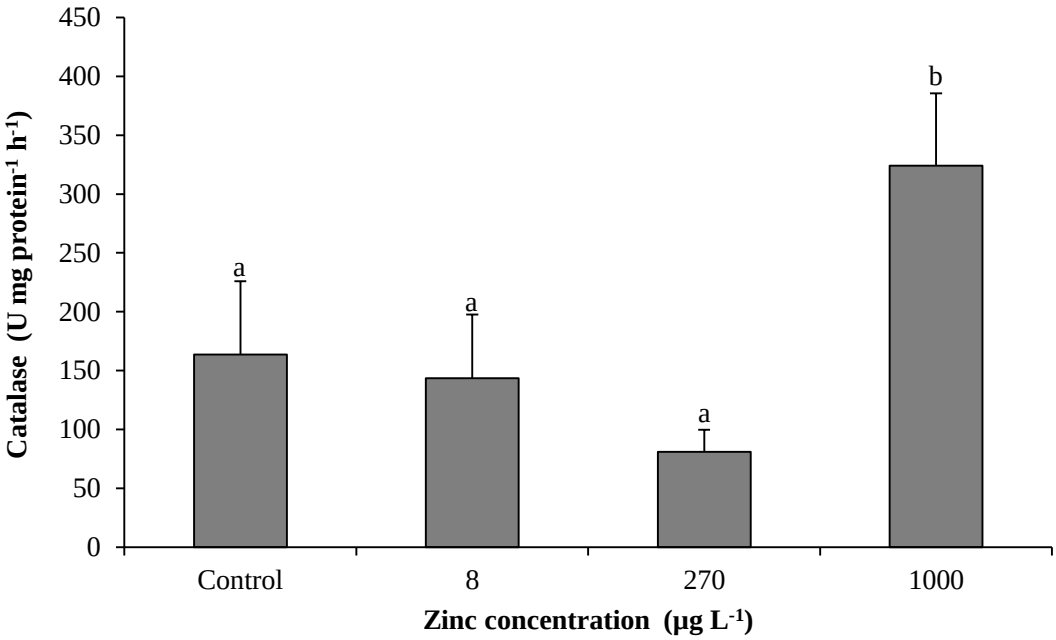


Figure 2.5. Catalase activity (U mg protein^{-1} h^{-1}; mean ± SEM) in inanga liver tissue (n = 8) after exposure to Zn (96 h). Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

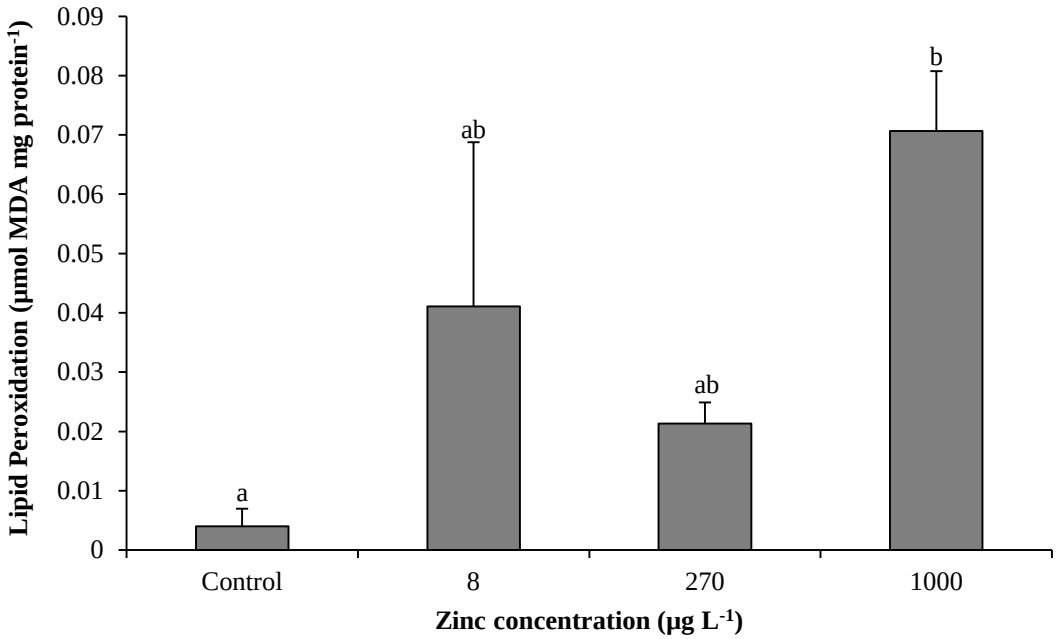


Figure 2.6. Lipid peroxidation (µmol MDA mg protein^{-1}; mean ± SEM) in inanga liver tissue (n = 8) after exposure to Zn (96 h). Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

2.4. Discussion

2.4.1. Ion transport effects

This study showed that Ca influx decreased significantly as a result of exposure to 1000 µg L^{-1} Zn for 96 h (Figure 2.3). Zn-induced impairment of Ca uptake has been shown previously in freshwater rainbow trout (Hogstrand et al., 1996) and zebrafish (*Danio rerio*; Alsop and Wood, 2011), and the finding of a similar effect in inanga suggests this to be a conserved mechanism of waterborne Zn toxicity in freshwater fish. Although the exact mechanism underlying this effect was not investigated in the current study, previous work indicates that Zn competes with Ca for entry into the gill via an apical ECaC (Qiu et al., 2007), and once inside the cell Zn inhibits the basolateral transport step by inhibiting Ca-ATPase (Hogstrand et al., 1996). The net effect of this inhibition is hypocalcaemia, which can eventually lead to death (Hogstrand et al., 1994).

A recent study investigating Zn toxicity to the killifish found similar effects of Zn on Ca metabolism, with reduction of plasma Ca and inhibited gill Ca-ATPase activity (Loro et al., 2014). These authors also noted an impact of Zn on Na metabolism, with a 30% decrease in plasma Na level observed, attributed to inhibition of the basolateral NKA (Loro et al., 2014). Although less commonly reported, there is other evidence suggesting Na uptake is impacted by Zn exposure in freshwater fish, with a transient decrease in plasma Na noted in rainbow trout (McGeer et al., 2000b). Similarly, Zn-exposed brook charr (*Salvelinus fontinalis*) have been shown to exhibit a net whole body Na loss (Grippo and Dunson, 1996). In the current study, Zn exposure was shown to stimulate, rather than inhibit, Na influx (Figure 2.4), suggestive of a unique response of Na metabolism to Zn exposure in inanga. This may

be explained by an impact of Zn on stress. Zn exposure is known to stimulate cortisol secretion in fish (Ibrahim et al., 2000), an effect in contrast to the inhibitory effects of metals such as Cd (Sandhu and Vijayan, 2011) and Cu (Oliveira et al., 2008). This effect of Zn is supported by strong molecular and physiological evidence for cortisol-Zn interactions in Zn metabolism and the cellular stress response (Bury et al., 2008). Inanga are highly sensitive to stress, and handling has been shown to increase Na influx 2-3 fold relative to rested controls (Harley and Glover, 2014). In the study of Harley and Glover it was proposed that cortisol mediated an increase in ventilation rate and/or epithelial permeability, exacerbating Na loss. In order to balance body ions, this induced a compensatory increase in Na influx. Thus in the current study it is hypothesised that Zn exposure may stimulate Na influx in inanga in an indirect manner, mediated by a specific effect of Zn on cortisol metabolism. Regardless of the mechanism of effect, the presented data indicate that while Zn impacts Na ion metabolism, in contrast to effects on Ca, the mechanisms may not be conserved between species.

2.4.2. Impacts of oxidative stress

One important mechanism of toxicity in fish exposed to waterborne trace metals is oxidative stress (Lushchak, 2011). For example, Zn exposure (500 µg L^{-1}) in freshwater killifish induced an increase in ROS, inhibited antioxidant defence mechanisms (e.g. catalase), and increased oxidative damage (e.g. lipid peroxidation) in liver and other tissues (Loro et al., 2012). In the current study, inanga exposure to Zn (1000 µg L^{-1}) led to an increase in liver catalase activity (Figure 2.5), and an increase in hepatic lipid peroxidation (Figure 2.6).

Catalase is an antioxidant enzyme that catalyses the breakdown of H_2O_2 (formed by O_2^-) to O_2 and water, thus protecting the cells from oxidative stress. The catalase results suggest that Zn stimulates ROS production in the liver of inanga, and in an attempt to scavenge these, catalase activity increases. While contrasting with the results of Loro et al. (2012), these data are consistent with other studies that showed an increase in catalase in Nile tilapia (*Oreochromis niloticus*) at Zn exposure levels of 500 µg L^{-1} (Atli et al., 2006). The variation in directionality of catalase activity changes does not correlate with exposure concentration, exposure duration (all 96 h), or basal level of catalase activity (low in inanga and killifish, high in tilapia). Instead it is likely that the response is species-specific and may relate to differences in trace element bioaccumulation or sub-cellular partitioning (Eyckmans et al., 2012), and/or activities of other antioxidant defence mechanisms (Forlin et al., 1995).

Enhanced lipid peroxidation was observed in the liver of inanga exposed to the highest level of Zn tested (1000 µg L^{-1}), suggesting that the increase in catalase was not able to successfully protect against Zn-induced oxidative stress. These results are consistent with previous research that has shown increased lipid peroxidation in Zn-exposed aquatic biota (Loro et al., 2012; Soto et al., 2011; Valavanidis et al., 2006). These data indicate that acute exposures to Zn are likely to contribute significantly to oxidative damage, which could be an important mechanism of toxicity, albeit only under high environmental exposure scenarios. Furthermore, the conserved lipid peroxidation response, relative to the variable directionality of catalase changes, indicates that oxidative damage measures may be a more reliable indicator of sublethal Zn toxicity compared to effects on antioxidant defence pathways. This is consistent with current recommendations regarding the choice of oxidative stress endpoints in studies of aquatic biota (Hellou et al., 2012).

2.4.3. Effect of Zn on metabolic rate

Metabolic rate is an integrated biomarker that allows determination of potential energetic costs of toxicant exposure (Sokolova et al., 2012), and has been successfully used to show impacts of trace metal exposure on fish (e.g. De Boeck et al., 1995; McGeer et al., 2000b). Furthermore, elevated waterborne Zn exposure has previously been shown to impair O_2 uptake (Skidmore, 1970). For these reasons, the effects of Zn exposure on inanga O_2 consumption rate were measured. However, no significant effects were noted (Figure 2.2). This indicates that despite impacts on ion transport and oxidative stress, there was no overall metabolic cost of Zn exposure. This finding is consistent with the lack of changes in metabolic rate in rainbow trout exposed for 30 days to 250 µg Zn L^{-1} (Alsop et al., 1999). This does not, however, account for the possibility that any extra costs were met by a diversion of metabolic resources from other, non-obligatory, physiological processes, such as growth and reproduction. If such an effect was occurring, then impacts on growth and reproduction may eventually result. Growth and reproduction are known endpoints affected by chronic Zn exposure in fish (Pierson, 1981).

These results also reinforce the hypothesis that impacts on Ca influx are at a specific locus, rather than an impact on branchial diffusion distance (i.e. Zn-stimulated mucus secretion; Skidmore, 1970). If such a scenario was occurring then an impaired metabolic rate would be expected, which was not observed. It is, however, important to note that inanga are known to take a large proportion (38%) of O_2 up across their scaleless cutaneous surface (Urbina et al., 2014a). Thus it remains possible that gill mucus secretion could be a toxic mechanism impacting Ca influx, but the presence of supplementary gas exchange across the skin may 'rescue' O_2 uptake.

2.4.4. Zn tissue burden

In the current study, all Zn exposure concentrations resulted in significantly higher amounts of Zn in the carcass, relative to the control (Figure 2.1). However, as Zn exposure concentration increased, tissue levels of Zn did not. Such a finding is consistent with the essentiality of Zn and the presence of mechanisms that act to regulate Zn body burden (Hogstrand, 2011; Komjarova and Blust, 2009). Patterns of whole body Zn content do not, therefore, correlate with changes in biochemistry and physiology, with stronger sublethal effects observed at the highest Zn exposure levels despite no increase in whole body Zn. This is inconsistent with the general concept of the BLM that predicts tissue accumulation will correlate with physiological and biochemical impacts (see Section 1.2.1; Niyogi and Wood, 2004; Santore et al., 2002). This is likely due to the fact that whole body, rather than tissue-specific, burdens were the measure of accumulation.

Chapter 3. Effects of waterborne cadmium exposure on metabolism, oxidative stress, and ion regulation in inanga (*Galaxias maculatus*)

3.1. Introduction

The majority of studies investigating the impact of Cd^{2+} on freshwater fish is limited to a few model species, such as trout, zebrafish, and tilapia (Hisar et al., 2009; McGeer et al., 2000a; Wang et al., 2015). However, very little research has examined whether the principles of Cd^{2+} toxicity established in these species, also holds for other, non-model fish (see Section 1.5). Previous work has shown that inanga exposed to Zn and Cu display different sublethal toxicity mechanisms, compared to well-studied species (Glover et al., 2016; McRae et al., 2016; Chapter 2). This is likely a consequence of their distinct physiology (see Section 1.5.1), which is thought to influence mechanisms of contaminant uptake and toxicity (see Section 2.3; McRae et al., 2016). It is therefore essential to build on this work, by extending our understanding of mechanisms of toxicant impact in inanga and how these compare to other fish species, by examining the effect of Cd exposure to this culturally important species. Cd is a contaminant of some concern in NZ waterways, largely owing to agricultural runoff associated with superphosphate fertiliser usage, and mining effluents (see Section 1.2.3.1).

Chapter 2 highlighted the impacts that Zn^{2+}, an essential metal, had on inanga. The current chapter seeks to investigate the impact that Cd^{2+}, a non-essential metal, has on the same species. Based on previous studies this research sought to delineate the mechanisms of Cd toxicity on metabolism (respiration rate; Peles et al., 2012), ionoregulation (unidirectional Na^{+} and Ca^{2+} influx; Atli and Canli, 2007; McGeer et al., 2000b) and oxidative stress (catalase activity and lipid peroxidation; Nunes et al., 2015; Pretto et al., 2011). Oxidative stress was measured in the kidney and liver of inanga, as these tissues are known to be the main sites of Cd accumulation, at least in other fish species (McGeer et al., 2000a; Hollis et al., 1999). Exposure concentrations

were chosen based on the ANZECC guideline for 95% protection of aquatic species (0.2 µg L^{-1}; ANZECC/ARMCANZ, 2000), an environmentally-relevant concentration (2 µg L^{-1}; Sabiti et al., 2000), and an effect concentration (10 µg L^{-1}; Hollis et al., 1999).

3.2. Methods

3.2.1. Animal collection and holding

Methods for fish collection and holding, as well as water chemistry and experimental conditions are outlined in Section 2.2.1. Fish were held for a minimum of 2 weeks in flow-through freshwater before being subjected to the manipulations described below. All work was approved by the University of Canterbury Animal Ethics Committee.

3.2.2. Cd exposure

For biochemical and O_2 consumption analysis, a total of 32 inanga (mean ± standard deviation (SD), 0.63 ± 0.24 g) were randomly distributed (n = 8) to one of four Cd exposure concentrations (nominally: control (no added Cd), 0.2, 2, or 10 µg L^{-1} for 96 h. Desired Cd levels were achieved by spiking chambers with stock solutions (1 g L^{-1} $CdSO_4$) to 2 L of aquarium water. Full protocols outlining exposure methods and water sampling regimes are described in Section 2.2.2.

Additional Cd exposures were conducted for the Ca^{2+} and Na^{+} influx experiments (Section 3.2.5). These exposures (two fish per exposure chamber) were conducted in an identical manner to those used for biochemical and O_2 consumption analysis, except in this instance just two concentrations were tested (control and 2 µg L^{-1} (Na flux) or 10 µg L^{-1} (Ca flux)). The Na^{+} influx exposure was conducted first,

using a 2 µg L^{-1} concentration. Since there was no effect the Ca^{2+} influx exposure was conducted using 10 µg L^{-1}. A total of 16 fish were exposed for each influx experiment (mean ± SD; Ca^{2+} influx; 0.84 ± 0.48 g, Na^{+} influx; 0.77 ± 0.29 g; both n = 8).

3.2.3. O_2 consumption

O_2 consumption was measured using the protocol described in Section 2.2.4. Briefly, fish were subjected to closed-box respirometry, where water samples were taken every 15 minutes, until six samples were taken or PO_2 reached 60 mmHg. The blank-corrected decline in water O_2 was used to calculate metabolic rate. Fish were euthanised at the completion of O_2 consumption measurements (see Section 2.2.5). Kidney and liver tissue were removed and used for biochemical measurements (see Sections 2.2.5 and 2.2.6), and the remaining tissue was used for analysis via ICP-MS.

3.2.4. Tissue and water analysis by ICP-MS

Water and tissue analysis methods are described in Section 2.2.7. Tissue constituted the remaining carcass (mean DW ± SD; 0.12 ± 0.04 g) after the removal of the kidneys and liver. Recoveries of Cd from the certified reference material (DORM-4) was 133% (n = 2). Detection limits for water analysis were calculated as three standard deviations of the mean blank concentration (0.05 µg L^{-1}). Detection limits for tissue analysis were calculated as three standard deviations of the mean blank concentration (0.03 µg g^{-1}).

3.2.5. Whole body Ca and Na flux

Methods for Ca^{2+} and Na^{+} influx are described in Section 2.2.4. Influx of these ions was determined in the presence of Cd, at a level identical to that which they had been previously exposed (control, 2 or 10 µg L^{-1}).

3.2.6. Oxidative stress

Catalase activity and lipid peroxidation were measured in liver and kidney tissue (~ 0.02 g). Methods were identical to those described in Sections 2.2.5 and 2.2.6.

3.2.7. Statistical analysis

Data were tested for normality using the Shapiro-Wilk test, and any failing data were log-transformed. Data were then analysed by one-way ANOVA followed by a Tukey HSD post-hoc test. The exception was Ca^{2+} and Na^{+} influx data, which were subjected to a t-test. All analysis was performed using RStudio (RStudio version 3.1.0). Statistical significance was set at $p < 0.05$ and all data are expressed as mean ± SD.

3.3. Results

There were no mortalities over the duration of the exposures. Measured concentrations of Cd^{2+} are outlined in

Table 3.1, and these values will be those referred to in the text from this point forth.

3.3.1. Accumulation of Cd in the whole body of exposed inanga

The concentration of Cd measured in the whole body tissue (excluding liver and kidney) of inanga exposed to 10 µg L^{-1} for 96 h was significantly higher than that of fish exposed to all other concentrations (ANOVA $p = 0.011$; Figure 3.1). There was, however, no significant difference in accumulation between these other concentrations (1 and 2.5 µg L^{-1}) and the control.

Table 3.1. Nominal and dissolved concentrations of Cd^{2+} (μg L^{-1}) in 96 h exposures.

Cd^{2+} concentrations (μg L^{-1})	
Nominal	**Measured dissolved**
0	0.05 ± 0.04
0.2	1.05 ± 0.79
2	2.45 ± 0.43
10	9.98 ± 0.67

Values expressed as mean ± SD, n = 4.

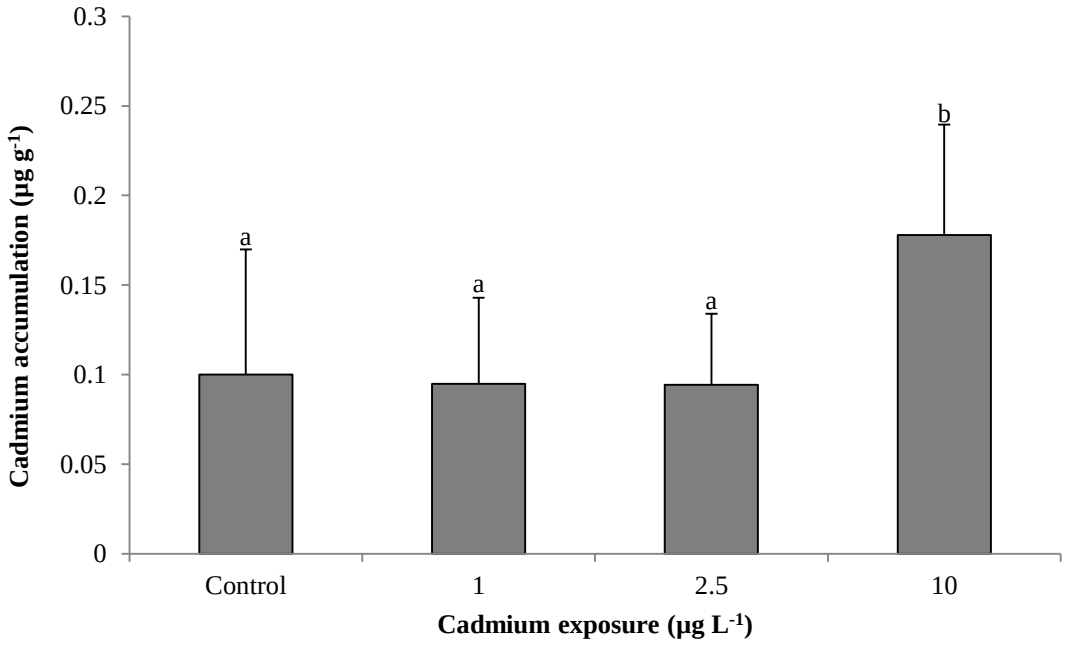


Figure 3.1. Cd accumulation (mean ± SD) in of inanga (whole body minus liver and kidney; n = 8) after exposure to Cd for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$)

3.3.2. O_2 consumption

Inanga exposed to 2.5 μg L^{-1} Cd^{2+} demonstrated a significant reduction in O_2 consumption compared to the control ($p = 0.043$; Figure 3.2). However, there was no significant difference in metabolic rates of fish exposed to the control, 1 μg L^{-1}, and 10 μg L^{-1} exposure concentrations.

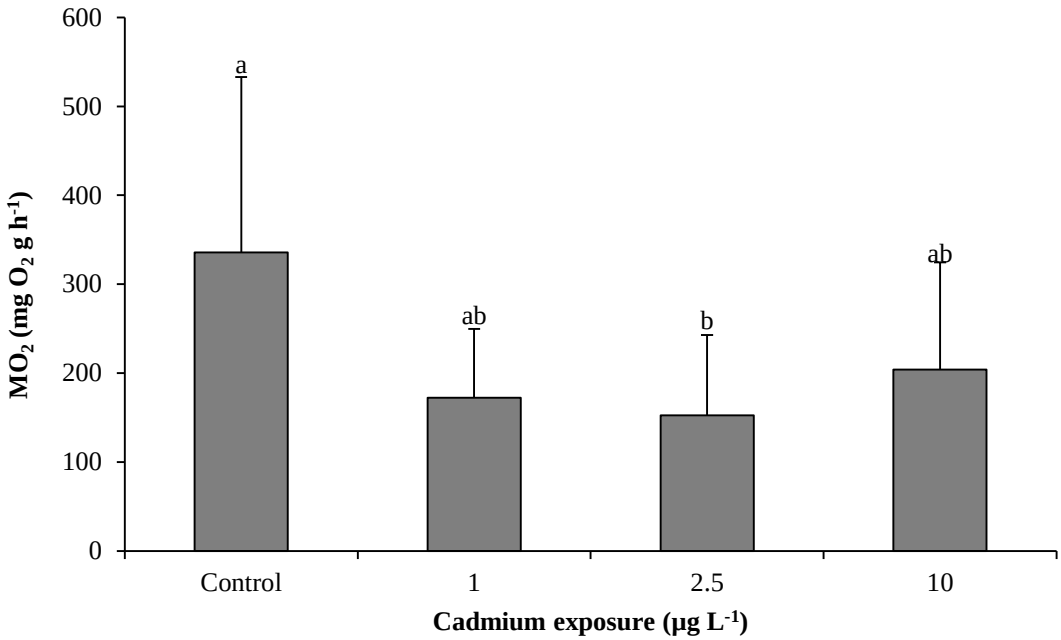


Figure 3.2. Inanga O_2 consumption (MO_2: mg O_2 g^{-1} h^{-1}) after exposure to Cd^{2+} for 96 h. Plotted values represent means ± SD (n = 8). Bars sharing letters are not significantly different (one way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

3.3.3. Ionoregulatory effects

There was no significant effect of Cd^{2+} on Ca^{2+} influx in inanga when comparing fish subjected to the control exposure (no added Cd^{2+}) and 2.5 µg L^{-1} Cd^{2+} ($p = 0.427$; Figure 3.3). Similarly, Cd^{2+} exposure (10 µg L^{-1}) did not significantly affect Na^{+} influx in fish, when compared to the control (Figure 3.4).

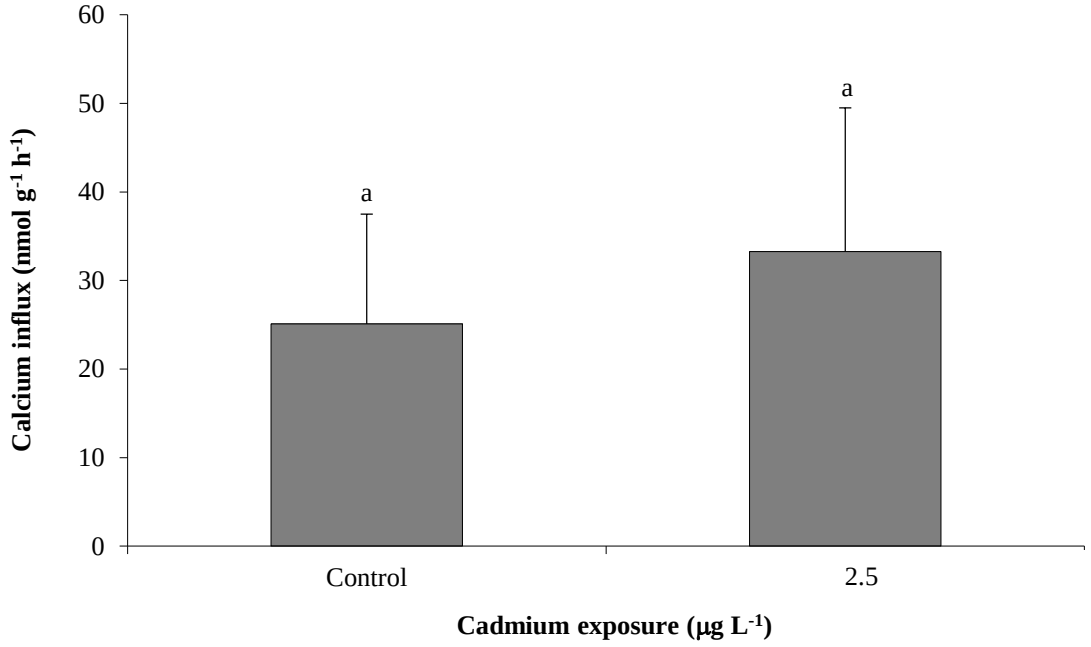


Figure 3.3. Unidirectional Ca influx (nmol g^{-1} h^{-1}; mean ± SD) in inanga (n = 8) after exposure to control and 2.5 µg L^{-1} Cd^{2+} for 96 h. Bars sharing letters are not significantly different, as determined by t-test (α = 0.05).

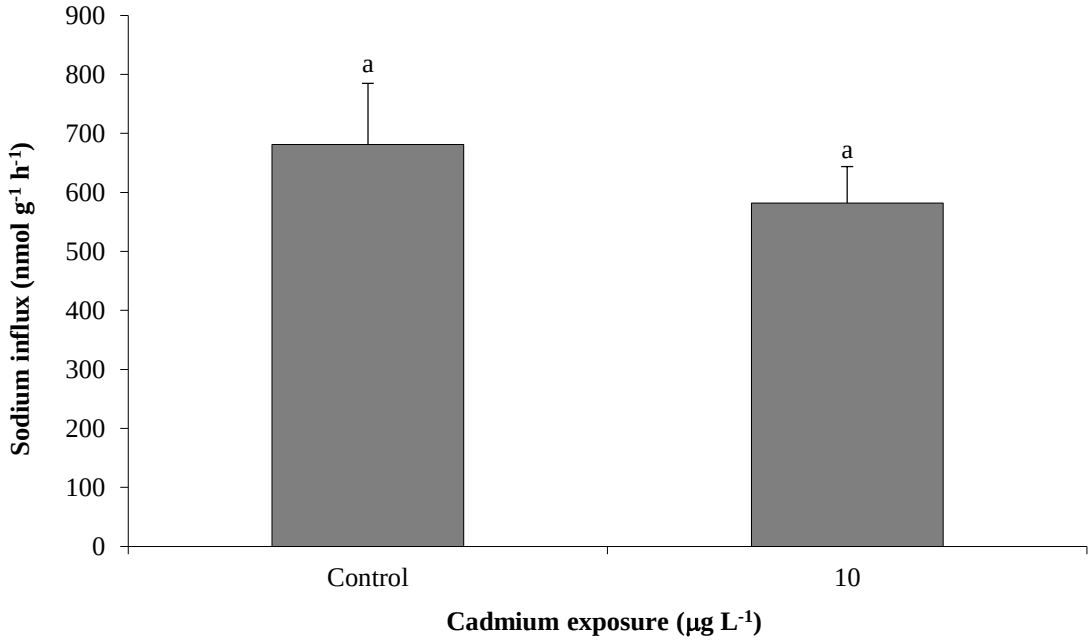


Figure 3.4. Unidirectional Na influx (nmol g^{-1} h^{-1}; mean ± SD) in inanga (n = 8) after exposure to control and 10 µg L^{-1} Cd^{2+} for 96 h. Bars sharing letters are not significantly different, as determined by t-test (α = 0.05).

3.3.4. Oxidative stress

Catalase activity in the kidney of inanga remained unaffected by exposure to Cd^{2+} (up to 10 µg L^{-1}; $p = 0.104$; Figure 3.5). Likewise, lipid peroxidation in the kidney was also unaltered by exposure to Cd^{2+} ($p = 0.130$; Figure 3.6)

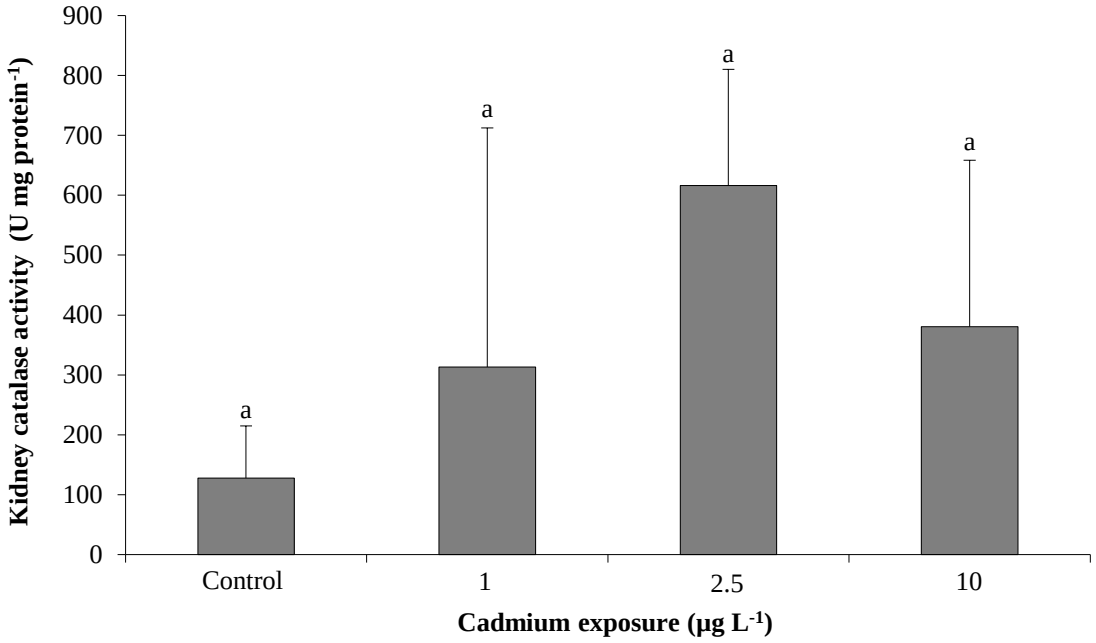


Figure 3.5. Catalase activity (mean ± SD) in the kidney of inanga (*Galaxias maculatus*; n = 5 - 8), after exposure to Cd^{2+} for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

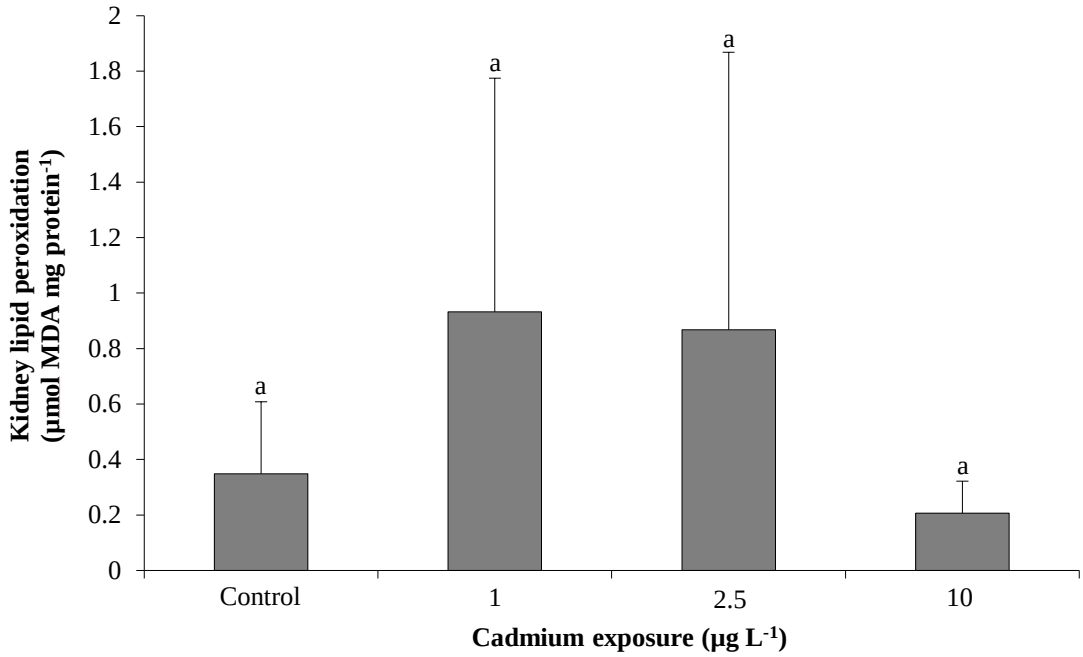


Figure 3.6. Lipid peroxidation (mean ± SD) in the kidney of inanga (*Galaxias maculatus*; n = 5 - 8), after exposure to Cd^{2+} for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

Exposure to Cd^{2+} (2.5 and 10 µg L^{-1}) caused a decrease in hepatic catalase activity in inanga, compared to the control ($p < 0.001$; Figure 3.7). In contrast, exposure to Cd^{2+} (2.5 and 10 µg L^{-1}) resulted in a significant increase in lipid peroxidation in the liver of inanga, compared to both the control and 1 µg L^{-1} exposures ($p < 0.0001$; Figure 3.8).

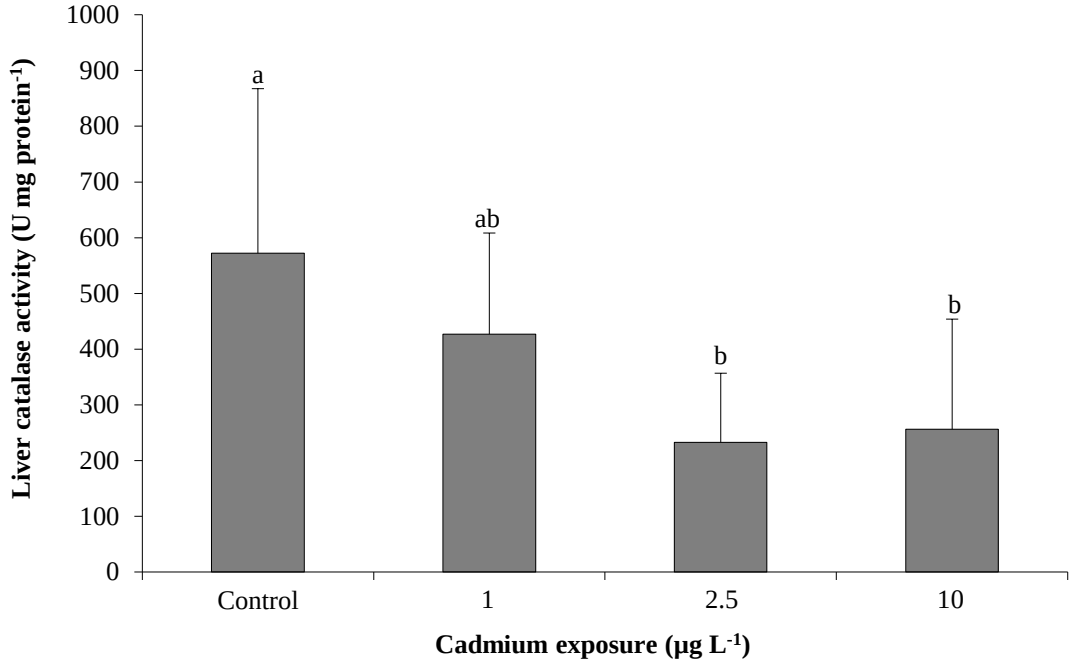


Figure 3.7. Catalase activity (mean ± SD) in the liver of inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd^{2+} for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

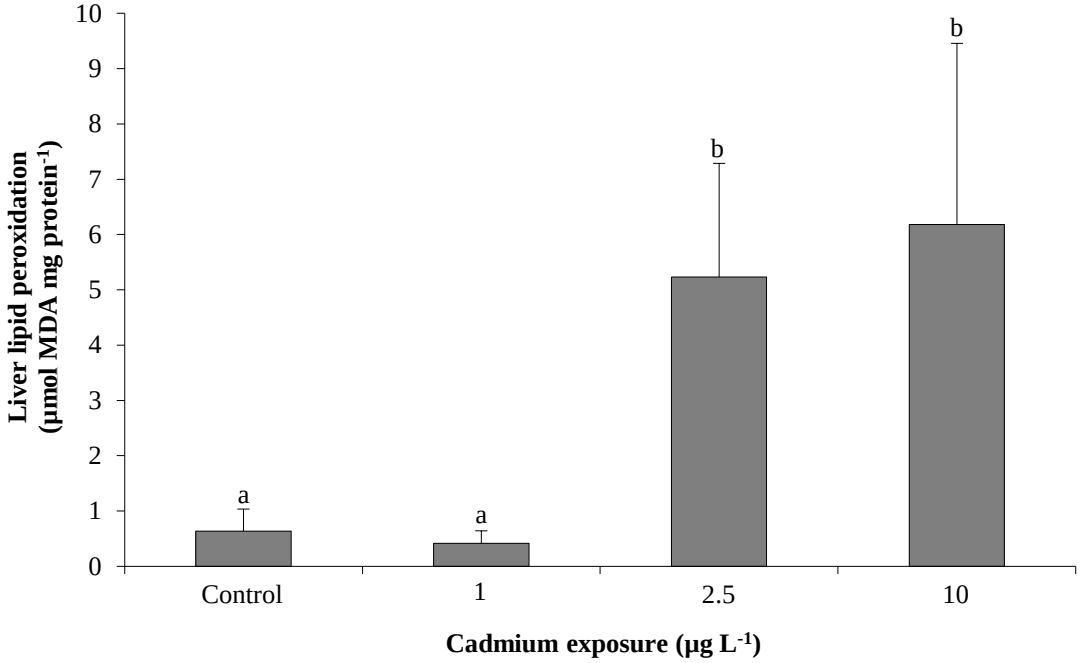


Figure 3.8. Lipid peroxidation (mean ± SD) in the liver of inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd^{2+} for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

3.4. Discussion

This study sought to investigate the impact that waterborne Cd^{2+} has on physiological and biochemical endpoints in the non-model fish species, inanga. Inanga were exposed to a concentration representative of the ANZECC 95% protection trigger value (nominally 0.2 µg L^{-1}, although measured concentration was 1 µg L^{-1}; ANZECC/ARMCANZ, 2000), an environmentally-relevant concentration (2.5 µg L^{-1}; Sabiti et al., 2000), and an effect concentration (10 µg L^{-1}; Hollis et al., 1999). Significant accumulation of Cd in the carcass of inanga relative to the unexposed control only occurred in the 10 µg L^{-1} exposure. No effects on ion influx (Ca^{2+} and Na^{+}) or on oxidative stress in the kidneys were observed. However, inanga exposed to 2.5 µg L^{-1} Cd^{2+} had an impaired metabolic rate, and at concentrations greater than 2.5 µg L^{-1} inanga displayed an altered hepatic oxidative stress response (decreased liver catalase activity and increased liver lipid peroxidation).

3.4.1. Tissue burden

The current study measured Cd concentration in the whole body of inanga (remaining carcass after removal of liver and kidney) exposed to 1, 2.5 and 10 µg L^{-1} for 96 h. A significant increase in Cd accumulation was observed only in those fish exposed to 10 µg L^{-1} after 96 h. These results are similar to those in a previous study conducted by Hollis et al. (1999). These authors exposed rainbow trout to Cd^{2+} and measured whole body accumulation over 10 days (Hollis et al., 1999). After 2 days, there was no significant accumulation in the carcass (after liver and gill removal) and whole body (no tissue removed) of fish exposed to 3 or 10 µg L^{-1}, however after 10 days there was significant accumulation at both exposure concentrations. These authors proposed that the gills are an effective barrier against Cd^{2+} uptake into the

fish, meaning that Cd only reached increased levels in internal tissues if exposure concentrations were relatively high, and/or exposure duration was relatively long. Supporting this idea, Hollis and colleagues (1999) measured Cd concentrations in the gills that were 7- and 16-fold higher than the whole body, at 3 and 10 µg Cd^{2+} L^{-1} respectively, after 10 days exposure. In the current study gill Cd burden was not specifically measured, but it is likely that the appearance of significantly elevated whole body Cd in inanga only at the highest exposure concentration (10 µg L^{-1}) after 4 days reflects branchial processes that minimise Cd^{2+} uptake. One such process is likely to be the production of branchial mucus. Studies have shown that mucus secretion is induced by waterborne metals (Handy and Eddy, 1990), and Cd^{2+} has been shown to bind to this mucus (Maunder et al., 2011), which would likely prevent its interaction with potential uptake pathways at the gill.

In the present study, Cd accumulation was measured in the carcass of inanga, after removal of the kidney and liver for measurement of oxidative stress parameters. Once absorbed, the kidney and liver are key sites for accumulation of Cd (McGeer et al., 2000a; Chowdhury et al., 2005). Therefore, the relatively lack of Cd accumulation measured in the current study at concentrations less than 10 µg L^{-1}, may also reflect that these tissues were removed prior to tissue burden analysis.

3.4.2. Effect of Cd^{2+} on metabolic rate

In the current study, Cd^{2+} exposure was shown to decrease metabolic rate (Figure 3.2). In contrast, McGeer et al. (2000b) observed the responses of adult rainbow trout to prolonged (up to 100 days) Cd^{2+} exposure and demonstrated that this chronic exposure did not have an impact on metabolic rate (McGeer et al., 2000b). This difference is likely due to the duration of exposure. For example, in a study on

fathead minnows exposed to Cd^{2+} for 24 h, a decrease in metabolic rate, similar to the response seen in the current study, was observed (Pistole et al., 2008). These authors attributed this effect to reduced ventilation. The reduction in the flow of water over the gills, is a mechanism that will also reduce the exposure of the gills to waterborne metal. In Cu-exposed inanga, this relationship between ventilation and metal uptake has been shown, wherein an increased ventilation rate led to an increase in metal accumulation (Harley and Glover, 2014). However, a consequence of reducing water flow across the gills, is a potential reduction in O_2 uptake (Pistole et al. 2008), leading to the decreased metabolic rate following short-term Cd^{2+} exposure in fathead minnows. This effect is, however, flexible with time. In the same study on fathead minnows, longer (96 h) exposures to Cd^{2+} resulted in increased metabolic rate (Pistole et al., 2008). It was suggested that as fish experienced a prolonged exposure, mechanisms limiting uptake become counterproductive. When coupled with an increased metabolic demand associated with enacting cellular mechanisms for Cd defence (e.g. metallothionein induction, antioxidant defence induction) and/or repair, then ventilation increases to levels equal to or exceeding control levels, and metabolic rate increases (Pistole et al., 2008). In the current study, the reduced metabolic rate after 96 h exposure to Cd^{2+} (2.5 µg L^{-1}) suggests inanga are capable of remaining in this "branchial water flow limiting" phase for relatively prolonged periods, at least at that exposure concentration. Higher Cd^{2+} exposure concentrations (10 µg L^{-1}), may limit the effectiveness of this strategy, hence the maintenance of a metabolic rate equivalent to control fish (Figure 3.2). This hypothesis is supported by data examining metabolic rate at higher Cd^{2+} exposure concentrations. Exposure of golden shiners to 200 µg Cd^{2+} L^{-1} results in observed increases in metabolic rate (Peles et al.,

2012). Further investigation evaluating the impact Cd^{2+} has on metabolic rate over time is warranted to gain further understanding of the patterns of impairment.

3.4.3. Ionoregulatory effects

In contrast to metabolic rate, ionoregulation remained unaffected by exposure to Cd^{2+} (Figures 3.3 and 3.4). In contrast to these results, Verbost et al. (1988) demonstrated the effect of Cd^{2+} on the transport of Ca^{2+} across the isolated basolateral membrane in the gill of rainbow trout. It was clear from their research that Cd^{2+} (0.056 µg L^{-1}) inhibited Ca-ATPase, and thus uptake of Ca^{2+}. It should, however, be noted that this was an *in vitro* experiment, and that *in vivo* effects of Cd on basolateral surfaces will be complicated by the presence of intracellular binding ligands such as metallothionein that may prevent the interaction of the metal with this membrane (Kamunde, 2009). However, there is also *in vivo* evidence for Cd^{2+} effects on Ca^{2+} metabolism in fish. McGeer et al. (2000a) showed a transient decrease in whole body concentrations of Ca^{2+} after acute exposure of rainbow trout to Cd^{2+} (3 µg L^{-1}). Other studies have shown that the presence of elevated Ca^{2+} in the water protects against Cd^{2+} toxicity to fish (Richards and Playle, 1999; Hollis et al., 2000). It is likely that this effect is mediated by increased competition between Ca^{2+} and Cd^{2+} for transport via the ECaC. For example, decreased uptake of Cd^{2+} into rainbow trout is associated with a decrease in expression of this transporter (Galvez et al., 2007; Franklin et al., 2005). The lack of effect of Cd^{2+} exposure on inanga, may be a consequence of the nature of the current study, with the most prominent effects of Cd^{2+} on Ca^{2+} homeostasis occurring in studies where Cd^{2+} exposure was longer in duration and/or at a higher concentration (e.g. McGeer et al., 2000a; Hollis et al., 2000). Species differences may also account for the lack of effect. For example, recent research has shown that the skin of inanga is capable of absorbing Ca^{2+} (Harley, 2015). If this

occurs via a pathway with a relatively lower affinity for Cd^{2+} than branchial uptake pathways, then it is possible that Ca^{2+} influx could occur relatively unimpeded by the presence of waterborne Cd^{2+}. This possibility requires further examination.

Exposure to Cd^{2+} can generate other ionoregulatory disturbances, such as that observed on Na^+ homeostasis. Impairment of Na^+ transport after exposure to Cd^{2+} is considered to be a consequence of Cd binding to the CA active site (see Section 1.6.2.2; **Error! Reference source not found.**; McGeer et al., 2000a). The activity of A is linked closely to Na^+ uptake, as CA maintains the concentration of protons in the cell, which are used to drive Na^+ transport via the Na/H^+ exchanger (Hwang et al., 2011). This Na^+ then acts as a substrate for NKA, which translocates Na^+ into the blood. This process enables freshwater fish, to take Na^+ up from dilute freshwaters, balancing the Na^+ lost to the environment via passive diffusion. Owing to the key role that NKA plays in Na^+ uptake, impairment of this enzyme can therefore also lead to disruptions in Na^+ transport (Atli and Canli, 2007). However, the results from the current study showed that Na^+ influx was not impaired by exposure to Cd^{2+} in inanga (Figure 3.4).

In contrast to the findings presented here, McGeer et al. (2000a) demonstrated disruptions to whole body Na concentrations after exposing rainbow trout to Cd^{2+}, while da Silva and Martinez (2014) showed that streaked prochilod gill tissue also displayed decreases in NKA and CA activity after exposure to Cd^{2+} (10 μg L^{-1}) for 96 h, thus leading to disturbances in Na^+ homeostasis. Atli and Canli (2007) measured a decrease in intestinal and gill NKA activity in Nile tilapia after waterborne exposure to Cd for 14 days. However, de la Torre et al. (2000) exhibited no alterations to gill NKA activity in the common carp after chronic (14 day) exposure to Cd (1.6 mg L^{-1}).

Likewise, Peles et al. (2012) showed no effect on NKA in golden shiners after exposure of Cd (200 - 1400 µg L^{-1}). Together, these studies show that effects of Cd^{2+} on Na^{+} transport pathways are variable and are likely to depend on exposure concentration and duration. Under longer exposure to higher concentrations there is a greater chance for Cd gill burdens to exceed intracellular binding capacity, leading to the spill over of Cd to sensitive cellular entities such as CA and NKA.

3.4.4. Impacts of Cd^{2+} on oxidative stress

Inanga exposed to Cd^{2+} showed no alteration to levels of catalase activity or lipid peroxidation in the kidney (Figure 3.5; Figure 3.6). However, in the liver catalase activity decreased, and lipid peroxidation increased after exposure to Cd^{2+} concentrations greater than 2.5 µg L^{-1} (Figure 3.7; Figure 3.8). Oxidative stress in aquatic animals is an important biomarker for sublethal toxicity in response to contaminant exposure (Lushchak, 2011). In response to Cd^{2+} exposure, ROS accumulation can occur due to depletion/inhibition of antioxidants and antioxidant enzymes, and also by a direct effect of the metal in generating ROS. For example, Cd has been shown to have a strong binding affinity for the active site of the SOD enzyme in zebrafish liver (Wang et al., 2015), and exposure to Cd has been shown to disrupt the protein structure of catalase (Wang et al., 2015). These actions lead to an increased accumulation of ROS, and result in oxidative damage.

The decrease in catalase activity in the liver of inanga (Figure 3.7), is likely directly related to the increase in oxidative damage (lipid peroxidation), as it represents an impaired ability to sequester ROS. These results differ to those observed by Nunes et al. (2015), who measured an increase in catalase activity, and an increase in lipid peroxidation, in the liver of Eastern mosquitofish. Under their exposure

conditions Cd likely induced an increase in activity, but this was not sufficient to prevent against oxidative damage. Pretto et al. (2011) measured the impact of a 7-d exposure to Cd^{2+} (0.44, 236 and 414 µg L^{-1}) on oxidative stress parameters in South American catfish. They also showed an increase in catalase activity in the liver, and similar to the current results, measured no change in catalase activity in the kidneys. Levels of oxidative damage in the liver and kidney were not impacted by exposure to Cd^{2+} indicating that catalase functioned effectively in scavenging ROS oxidative damage (Pretto et al., 2011). In this context, it appears the South American catfish has more robust antioxidant mechanisms for detoxification of Cd compared to those of inanga, especially since exposure concentrations (414 µg L^{-1}) were up to 4-fold higher (Pretto et al., 2011).

The current results indicate that the liver was the tissue most significantly impacted by Cd^{2+} exposure in inanga. Cd caused a decrease in antioxidant capacity, as measured by catalase activity (Figure 3.7), which resulted in an increase in lipid peroxidation (Figure 3.8). The kidney, however, was unaffected by exposure to Cd (Figure 3.5 and Figure 3.6). Previous research has also demonstrated the sensitivity of the liver to oxidative stress after exposure to Cd. Hisar et al. (2009) demonstrated that rainbow trout antioxidant enzymes (SOD, catalase and GST) in the liver were stimulated after only 1 day of exposure to Cd (1000 and 5000 µg L^{-1}). Atli and Canli (2007) also showed that the liver in adult Nile tilapia was the most sensitive tissue after exposure to Cd (916, 1833, and 3999 µg L^{-1}) for 14 days. Catalase activity increased across all exposures and had a stronger response in the liver compared to gills, muscle, and intestine. They noted that the liver is the site where ROS are most commonly generated. Although the current responses differed, in that there was a decrease in catalase activity, it is clear from the results of the present study, and those

of Atli and Canli (2007), Pretto et al. (2011), and Nunes et al. (2015), that the liver is the key tissue for measuring oxidative stress, as it the main site of Cd accumulation (McGeer et al., 2012), and thus the toxic impacts are likely to be greater, at least under conditions where Cd concentrations exceed the capacity of cellular defence mechanisms.

Chapter 4. Acute exposure to an environmentally-relevant concentration of diclofenac elicits oxidative stress in the culturally important galaxiid fish, *Galaxias maculatus.*

McRae, N. K., Glover, C. N., Burket, S. R., Brooks, B. W., Gaw, S. 2017. Acute exposure to an environmentally-relevant concentration of diclofenac elicits oxidative stress in the culturally important galaxiid fish, *Galaxias maculatus.* In review: Environmental Toxicology and Chemistry.

4.1. Introduction

To date, studies examining the effect of diclofenac on fish have been restricted to a few common model species from the Northern Hemisphere (Section 1.5). Whether other fish species, including those from the other geographic regions, respond differently to diclofenac is unknown. In the present study, inanga was selected for an exploratory study because it is a widespread Southern Hemisphere fish species (McDowall, 1990). The migration of juvenile inanga through estuaries is likely to expose them to high levels of environmental contaminants (Harley and Glover, 2014), and as adults, inanga inhabit near-coastal streams that receive WWTP discharges (See Section 1.5.1).

It is critically important to recognise a number of unusual physiological traits that may alter inanga responses to diclofenac (McDowall, 1990). For example, the skin of inanga is scaleless, bestowing it with transport functions (e.g. O_2, ammonia; Urbina et al., 2014a; Urbina et al., 2014b). This may reduce the capacity of the skin to act as a barrier for toxicant absorption, thus increasing bioavailability of organic contaminants. In fact, inanga have been reported to inhabit natural waters that may reach pH values as low as 4.1 (Olsson et al., 2006), which would further increase bioavailability of weak acids and alter speciation of trace metals. Furthermore, a recent study has shown that, in contrast to model fish species, inanga exposed to Cu do not exhibit impaired ammonia excretion, thought to be a key mechanism of Cu toxicity (Glover et al., 2016). These authors suggested that this was due to the capacity of inanga skin to act as a "rescue pathway", continuing to excrete ammonia and thus circumvent Cu-mediated inhibition of ammonia excretion at the gill. Similar important differences in toxic mechanisms relative to other freshwater fish have been observed for inanga in response to Zn exposure (Chapter 2).

Understanding of the effects of pharmaceuticals on non-target species has been identified as a significant knowledge gap (Brooks et al., 2009; Boxall et al. 2012; Rudd et al., 2014). The current study presents an initial exploratory attempt to address this need by investigating the biochemical and physiological responses of inanga, a culturally and commercially important species, to diclofenac. The specific aim of this study was to determine whether diclofenac significantly influences commonly measured toxicological endpoints, including important homeostatic processes such as O_2 uptake, ion transport, and common markers of oxidative stress (catalase activity, lipid peroxidation) in the liver, gills, and kidney. Physical disruption of the branchial epithelium following diclofenac exposure likely affects gill-based processes, such as respiration and ion transport (Schwaiger et al., 2004; Hoeger et al., 2005; Memmert et al., 2013). To test this hypothesis, we measured O_2 uptake (metabolic rate) and Na^+ and Ca^{2+} influx. It was anticipated that diclofenac could alter oxidative stress endpoints, as during its biodegradation through CYP450 enzymes it releases a reactive superoxide anion that generates oxidative damage if left unscavenged by antioxidant defences (Islas-Flores et al., 2014). At the end of the study, diclofenac body burdens in inanga were measured.

4.2. Methods

4.2.1. Animal collection and holding

Methods for fish collection and holding, as well as water chemistry and experimental conditions are outlined in Section 2.2.1. Fish were held for a minimum of 2 weeks in flow-through freshwater before being subjected to manipulations described below. All work was approved by the University of Canterbury Animal Ethics Committee.

4.2.2. Diclofenac experiments

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Prior to experimental use, glassware was rinsed three times with analytical grade methanol, dichloromethane, and acetonitrile (ACN). Diclofenac stock solutions (0.05 or 500 mg L^{-1} in MQ water (>18 MΩ) were freshly prepared before each exposure, and were stored at 4°C in glass amber bottles. Glass chambers (4 L) were used to house fish during experiments. Desired diclofenac exposure concentrations were achieved by dosing these chambers with stock solutions in 2 L of aquarium water to give nominal diclofenac concentrations of 0 (control), 0.2 (environmentally relevant concentration; Acuña et al., 2015), or 770 µg L^{-1} (proposed water quality guideline; Kumar et al., 2016). Additional treatment levels were not examined due to field sampling logistics for inanga. Water was left for 24 h to equilibrate before the addition of fish.

A total of 48 inanga (mean mass ± SD; 2.41 ± 1.40 g) were assigned randomly to one of 8 replicate chambers for each of the three treatment levels. Two fish were placed in each chamber, one for physiological and biochemical analysis, and one for tissue diclofenac analysis. Water was continually aerated throughout the experiment and maintained at a constant temperature (15 ± 1°C). To minimise potential photodegradation of diclofenac, studies were conducted in the dark, with occasional use of red light to monitor fish health and conduct water changes. The experiment was performed for 96 h, with renewal of 90% of the water every 24 h. Each water renewal was prepared at the appropriate diclofenac concentration 24 h prior to use.

At the conclusion of the study, fish selected for biochemical and physiological analyses were examined for O_2 consumption (see below), before being euthanised (0.1

g L^{-1} MS-222), and tissues collected for biochemistry analysis. The remaining fish were immediately euthanised and whole fish were collected for tissue diclofenac determination, which is further described below. Both tissues and whole fish were snap-frozen in liquid nitrogen, before being stored at -80°C.

A second study was conducted to explore Ca^{2+} and Na^{+} influx responses. This experiment was conducted in an identical manner as described above, though only one concentration of diclofenac was selected in addition to controls (770 µg L^{-1}). A total of 16 fish were included (mean mass ± SD; Ca^{2+} influx; 2.46 ± 2.19 g, Na^{+} influx; 0.64 ± 0.34 g; both n = 8) with two fish per experimental unit.

Water samples were taken throughout the studies both before and after water renewals. Samples (770 µg L^{-1}, 100 mL; 0.2 µg L^{-1} and control, 1000 mL) were taken from the chambers using a solvent-rinsed glass measuring cylinder, placed in solvent rinsed amber bottles (100 mL or 1 L). Samples were acidified to pH <2 with ultrapure 70% sulfuric acid (770 µg L^{-1}, 20 µL; 0.2 µg L^{-1} and control, 200 µL) so diclofenac was in its natural form and would bind to the cartridges (Ying et al., 2009). Samples were stored at 4°C for no more than 48 h before being extracted (see below). Water concentrations were calculated as a mean (± SD) across all time points.

4.2.3. Measurement of oxidative stress

Catalase activity and lipid peroxidation were measured in gill, liver and kidney tissue (~ 0.02 g). Methods were identical to those described in Sections 2.2.5 and 2.2.6.

4.2.4. O_2 consumption

O_2 consumption was measured using the protocol described in Section 2.2.4. Briefly, fish were subjected to closed-box respirometry, where water samples were taken every 15 minutes, until six samples were taken or PO_2 reached 60 mmHg. The blank-corrected decline in water O_2 was used to calculate metabolic rate. Fish were euthanised at the completion of O_2 consumption measurements (see Section 2.2.5).

4.2.5. Whole body Ca and Na flux

Methods for Ca^{2+} and Na^{+} influx are described in Section 2.2.4. Influx of Ca and Na were determined in the presence of diclofenac, at a level identical to that which they had been previously exposed (control, 0.17 or 763 µg L^{-1}).

4.2.6. Analytical chemistry

4.2.6.1. Water extraction and analysis

Water samples were extracted within 48 h of sampling, using methods similar to those of Ying et al. (2009). Water samples were passed, under vacuum, through water cartridges (Strata™ -X 300 µm Polymetric Reversed Phase, 500 mg/6 mL sorbent), which were preconditioned using 5 mL of methanol and 5 mL of MQ water. QA/QC was achieved by having a MQ blank (100 mL MQ), MQ spike (100 mL MQ; either 500 µg L^{-1} or 0.1 µg L^{-1} diclofenac), cartridge spike (5 mL MQ; either 500 µg L^{-1} or 0.1 µg L^{-1} diclofenac), cartridge blank (5 mL MQ), and comparative standard (1 mL of either 500 µg L^{-1} or 0.1 µg L^{-1} diclofenac in methanol). Cartridges were dried completely before diclofenac was eluted into clean glass vials using 3 x 4 mL aliquots of methanol. Samples were then evaporated under a stream of nitrogen gas at 55°C, before being re-dissolved in 1 mL of ACN and stored at 4°C until analysis (Ying et al., 2009).

Diclofenac water samples from the 0.2 µg L^{-1} exposure concentration were measured by GC-MS (Gas Chromatography-Mass Spectrometry) using a Shimadzu GC-2010 Gas Chromatograph, interfaced to a Shimadzu AOC-20i Auto injector and a Shimadzu GCMS-QP2010Plus detector (Ying et al., 2009). Before analysis, samples (100 µL) were spiked with hydroxypyrene (25 µL; 0.1 µg L^{-1}) to act as internal standard. Samples were converted to their trimethylsilyl derivatives using MSTFA (100 µL; N-methyl-N-(trimethylsilyl)trifluoroacetamide) at 80°C for 1 h.

Instrument control, data acquisition, and data processing were performed using Shimadzu GC-MS Solution software (Version 2.70). The instrument was equipped with a Restek Rxr®-5SILMS w/Integra-Guard® column (30 m × 0.25 mm ID, 0.25 µm df). Helium was used as the carrier gas at a flow rate of 1.02 mL min^{-1}. The programming of the oven was as follows: 80°C (1 min) to 150°C (10°C min^{-1}), to 215°C (3°C min^{-1}), to 280°C (10°C min^{-1}) for 10 min. The injector temperature was set at 280°C, and the ion source temperature were set at 230°C. Ions were quantified using selected ion mode (SIM). The target ions were characterised by a mass/charge (*m/z*) ratio of 214 *m/z* for diclofenac, and 290 *m/z* for hydroxypyrene. Retention times were 20.54 (diclofenac), and 22.17 (hydroxypyrene) min. The limit of quantification (LOQ) for the water samples in the method was 20.55 µg L^{-1}. Diclofenac spike recoveries were 75.65% (n = 2). Samples were not corrected for recoveries. Calibration standards (0, 1, 10, 50, 100, 150, 200 µg L^{-1}) were run prior to, during (0, 10, and 100 µg L^{-1}; every 10 samples), and after sample analysis. Linear regression of the standards resulted in $r^2 = 0.99912$. MQ water blank, control water and cartridge blank samples showed no contamination during the sampling and extraction process.

Diclofenac water samples from the 770 µg L^{-1} treatment level were measured by HPLC (High Performance Liquid Chromatography; Dionex Ultimate 3000 LC system; ThermoFisher) equipped with a Phenomenex Prodigy column (250 × 4.60 mm 5 micron) at 240 nm. The method was adapted from Shu et al. (2013). A 100 µL sample was diluted with 400 µL ACN (5x dilution) prior to analysis. The mobile phase was ACN and 10 µM NaH_2PO_4 adjusted to pH 3 by 85% H_3PO_4. The injection volume was 20 µL for each sample. Analytical methods were as follows: 0 to 8 min (40% ACN and 60% phosphate buffer), 8.1 to 14 min (60% ACN and 40% phosphate buffer), 14.1 to 15 min (40% ACN and 60% phosphate buffer). The flow rate was set to 1 mL min^{-1} (Shu et al., 2013). Calibration standards (0, 0.1, 10, 50, 100, 250 mg L^{-1}) were run prior to, during (0, 10, and 100 µg L^{-1}; every 10 samples), and after sample analysis. The LOQ in the water samples for the method was 282 µg L^{-1}. Recoveries were 85.46% (n = 4), and a linear regression of the standards resulted in r^2 = 0.9998.

4.2.6.2. Tissue extraction and analysis

Fish tissue was transported frozen from the University of Canterbury to Baylor University for tissue analysis of diclofenac. Tissue extraction methods in this study were similar to Ramirez et al. (2007) and Du et al. (2012). Frozen fish were homogenised and approximately 1 g was transferred to a 20-mL borosilicate glass vial and weighed. Homogenising solution (8 mL of a 1:1 mixture of 0.1 M acetic acid and methanol) was added, along with 50 µL of the surrogate, diclofenac-*d4* (DCF-*d4;* 100 µg L^{-1}). Samples were shaken and spun on a rotary extractor for 20 min at 25 ± 0.1°C, transferred into 50 mL polypropylene copolymer round-bottomed centrifuge tubes (Nalgene Co., Nalgene Brand Products, Rochester, NY) and centrifuged at 12000 rpm for 55 min at 4°C. Once complete, supernatant was transferred to 18 mL borosilicate

glass culture tubes (VWR Scientific), and solvent evaporated under nitrogen gas at 45°C in a Turbovap evaporator. Once dried (approx. 4 h), samples were reconstituted with 1 mL 95:5 0.1% (w/v) formic acid:methanol before analysis (Du et al., 2012; Ramirez et al., 2007). The method detection limit for diclofenac was 2.7 µg kg^{-1}.

Diclofenac tissue concentrations were measured via isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) on a Varian Prostar system with model 210 binary pumps, model 410 autosampler, and model 1200 L triple quadrupole mass analyser. Details pertaining to chromatography, ionisation mode, monitored transition, and limit of detection for diclofenac were previously reported (Du et al., 2012; Du et al., 2014). A nine-point calibration curve for diclofenac, ranging from 0.1-1500 µg L^{-1}, was prepared with analytical grade standard obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Calibration standards were spiked with 100 µg L^{-1} d4-diclofenac obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). Standards were prepared in 95:5 0.1% (v/v) aqueous formic acid:methanol. Linear regression of the standards resulted in r^2 = 0.998.

Continuing calibration verification (CCV) samples were used to monitor instrument calibration, with an acceptability criterion of ± 20%. Sample batches also included blanks (methanol) and duplicate matrix spikes (clean samples spiked with d4-diclofenac and diclofenac identical to the CCV concentration; Du et al., 2012).

4.2.7. BCF and calculation of human exposure

Non-kinetic BCFs were estimated from the measured water concentrations via HPLC or GC-MS, and the concentration of diclofenac in the whole body of the fish determined via LC-MS/MS:

$$BCF = \frac{C_B}{C_W}$$

where *BCF* is BCF, C_B is diclofenac accumulation in the fish tissue, and C_W is concentration of diclofenac in the exposure water. The environmentally-relevant and analytically-verified treatment level of diclofenac was used to estimate human consumption:

$$d = a \times s$$

where *a* is the accumulation of diclofenac in inanga exposed to 0.17 µg L^{-1} over 96 h (µg kg^{-1}), *s* is the serving size of inanga (0.286 kg $serving^{-1}$; US EPA, 1989), *d* is the amount of diclofenac per serving (µg $serving^{-1}$).

4.2.8. Statistical analysis

Data were tested for normality using the Shapiro-Wilk test, and any failing data were log-transformed. All data were then analysed by one-way ANOVA followed by a Tukey HSD post-hoc test. The exception to this was the ion flux data, which were analysed using an unpaired Student's t-test. All analysis was performed using RStudio (RStudio version 3.1.0). Statistical significance was set at $p < 0.05$ and all data are expressed as mean ± SD.

4.3. Results

No mortalities were recorded during the exposures. Diclofenac was measured at 0.17 ± 0.16 µg L^{-1} for the 0.2 µg L^{-1} treatment level, while the 770 µg L^{-1} exposure concentration was measured at 763 ± 43 µg L^{-1}. Diclofenac was not detected in the control water samples. These analytically-verified values are referred to hereafter.

4.3.1. Oxidative stress

Catalase activity in the gills of inanga was significantly reduced by the 0.17 (p = 0.0169) and 763 (p = 0.0097) µg L^{-1} treatment levels of diclofenac when compared to controls (Figure 4.1). Further, an elevated concentration of diclofenac (763 µg L^{-1}) significantly decreased (p = 0.0083) lipid peroxidation in the gills of inanga (Figure 4.2), though exposure to an environmentally relevant level (0.17 µg L^{-1}) did not have an effect significantly different from controls (p = 0.998).

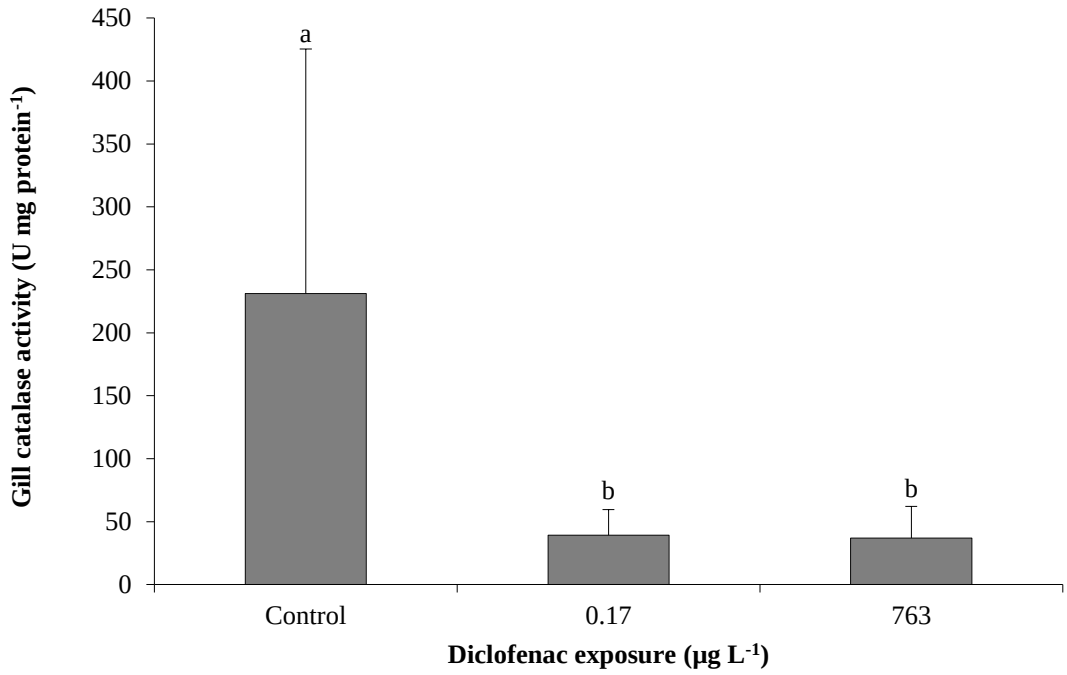


Figure 4.1. Catalase activity (mean ± SD) in the gill of inanga (*Galaxias maculatus*; n = 5-8), after exposure to diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

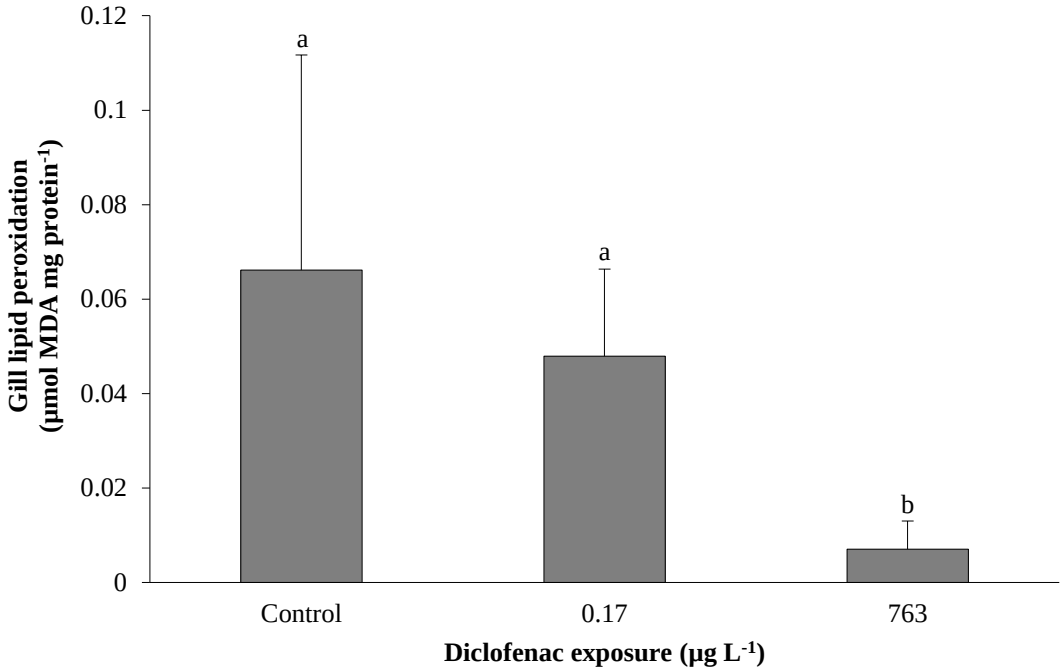


Figure 4.2. Lipid peroxidation (mean ± SD) in the gill of inanga (*Galaxias maculatus*; n = 5-8), after exposure to diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

Consistent with observations in gill tissues, only the highest treatment level of diclofenac (763 µg L^{-1}) significantly decreased lipid peroxidation in the kidney of inanga when compared to the control exposure ($p = 0.0163$; Figure 4.7). In contrast to lipid peroxidation, there was no significant change in catalase activity in the kidney after exposure to either diclofenac treatment level ($p = 0.3605$; Figure 4.3).

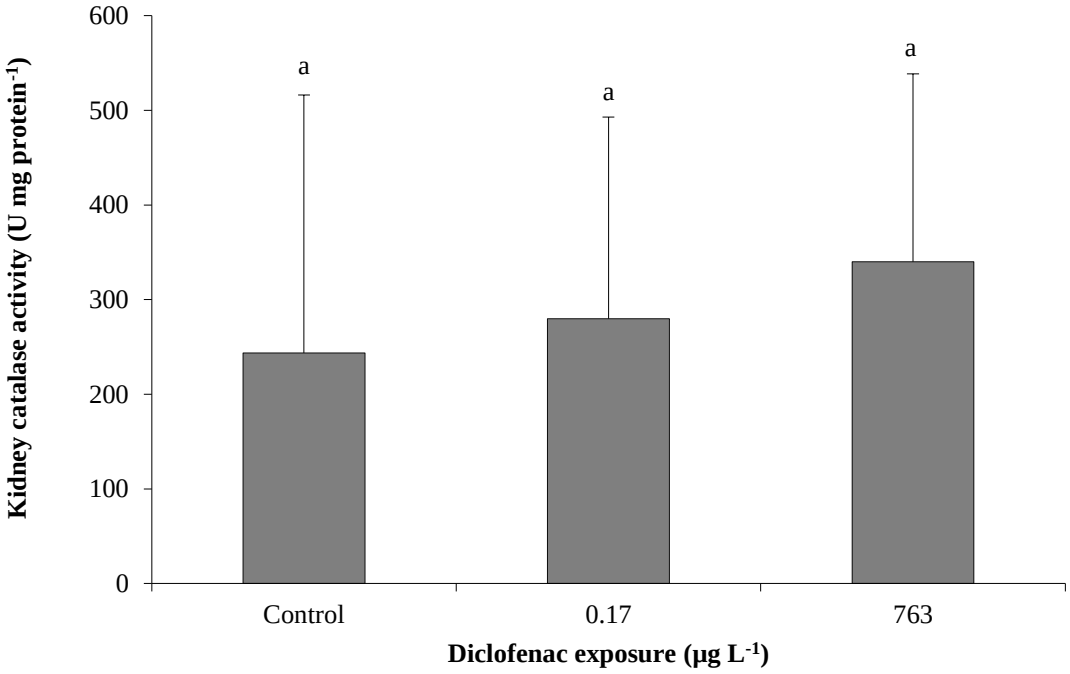


Figure 4.3. Catalase activity (mean ± SD) in the kidney of inanga (*Galaxias maculatus*; n = 5-8), after exposure to diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

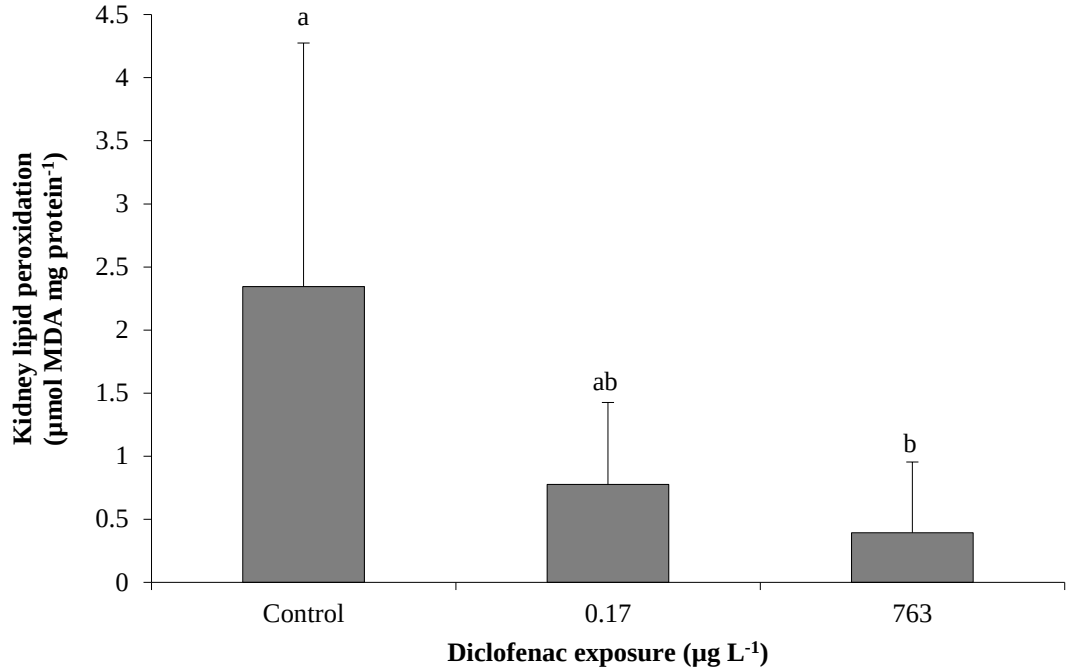


Figure 4.4. Lipid peroxidation (mean ± SD) in the kidney of inanga (*Galaxias maculatus*; n = 5-8), after exposure to diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

The treatment levels of diclofenac (0.17 and 763 µg L^{-1}) exhibited an increase of 11-fold and 19-fold, respectively, in catalase activity in the liver of inanga ($p > 0.001$; Figure 4.5). There was no significant difference between the 0.17 µg L^{-1} and

763 µg L^{-1} treatment levels with respect to hepatic catalase activity (p = 0.7677; Figure 4.5). A significant increase (10-fold; p = 0.0092) in lipid peroxidation in the liver of inanga was elicited by a 96 h exposure to 763 µg L^{-1}, but not by the 0.17 µg L^{-1} treatment level (p = 0.0962), when compared to the controls (Figure 4.6).

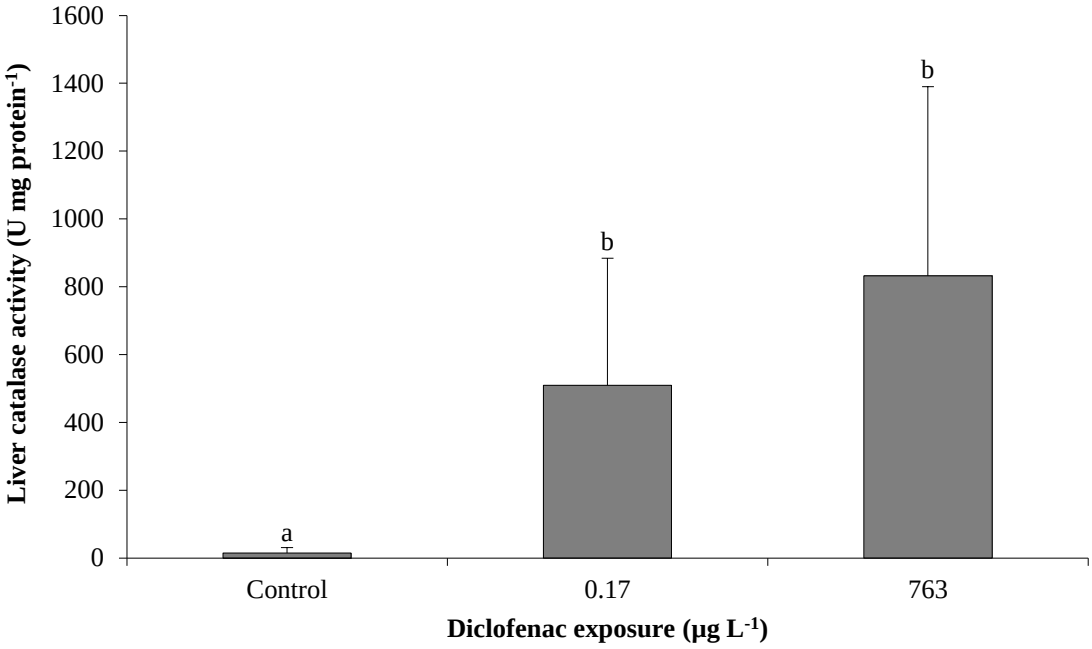


Figure 4.5. Catalase activity (mean ± SD) in the liver of inanga (*Galaxias maculatus*; n = 5-8), after exposure to diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

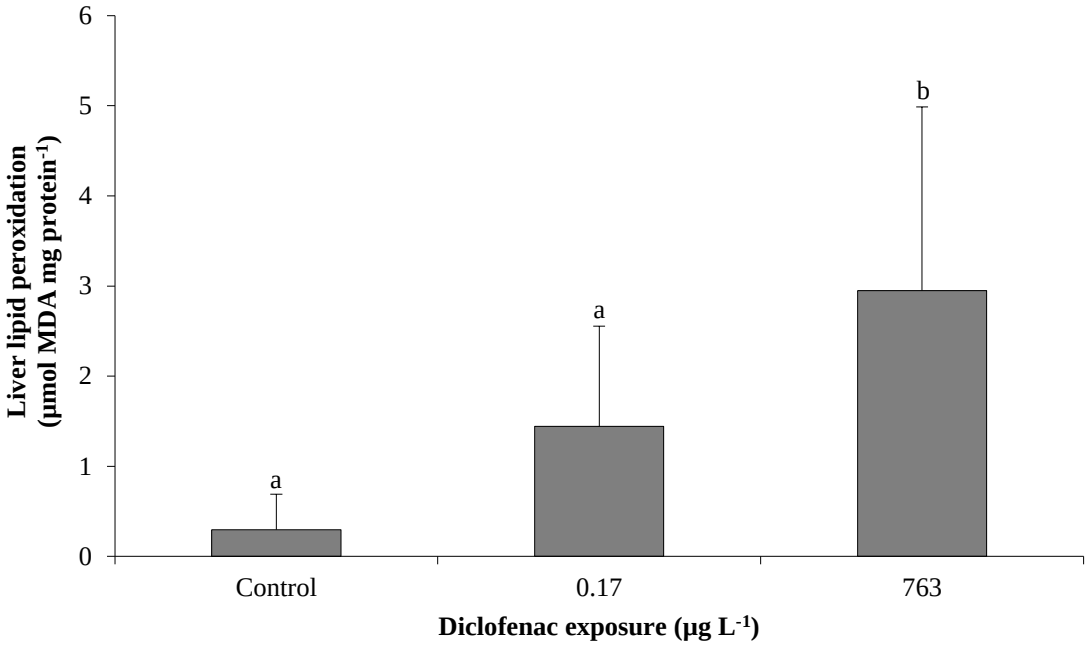


Figure 4.6. Lipid peroxidation (mean ± SD) in the liver of inanga (*Galaxias maculatus*; n = 5-8), after exposure to diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

4.3.2. Whole body O_2 consumption

Inanga O_2 consumption was not significantly ($p = 0.5021$) affected by either treatment level of diclofenac (Figure 4.7). Similarly, there were no significant effects of diclofenac on either Ca^{2+} ($p = 0.9529$; Figure 4.8) or Na^{+} influx ($p = 0.2073$; Figure 4.8) influx in comparison to controls.

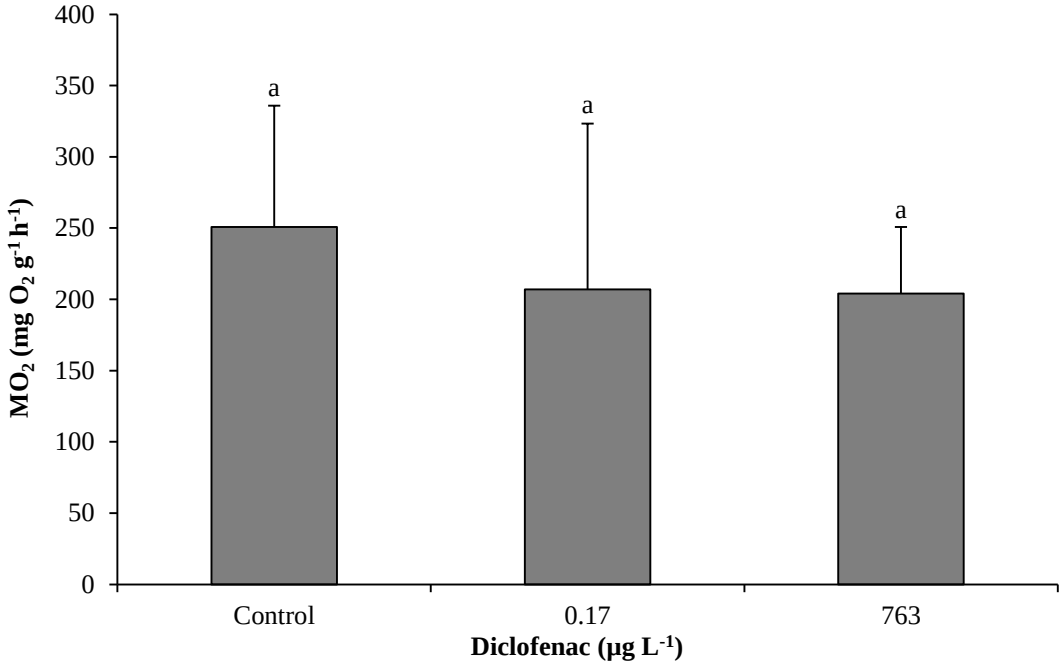


Figure 4.7. Mean (± SD; n = 8) O_2 consumption ($MO_{2;}$ mg O_2 g^{-1} h^{-1}) of inanga (*Galaxias maculatus*) after exposure to diclofenac for 96 h. Bars sharing letters are not significantly different as determined by one-way ANOVA followed by post-hoc Tukey test; α = 0.05.

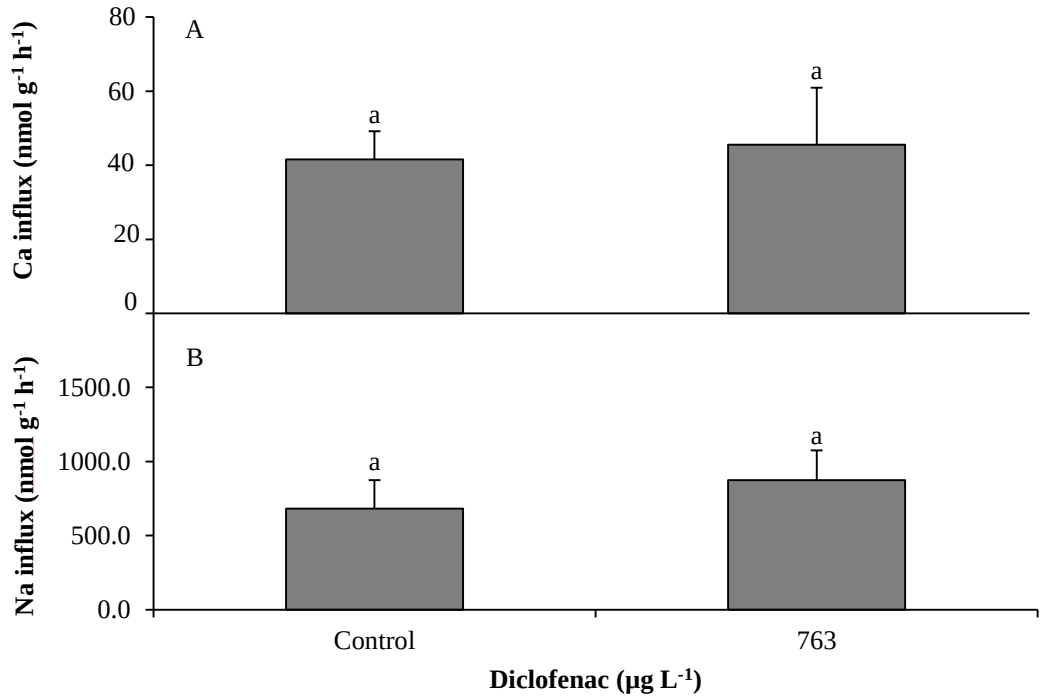


Figure 4.8. Mean (±SD) unidirectional Ca^{2+} (A) and Na^{+} (B) influx (nmol g^{-1} h^{-1}) in inanga (*Galaxias maculatus*) (n = 8) after exposure to control and 760 µg L^{-1} diclofenac for 96 h. Bars sharing letters are not significantly different, as determined by t-test (α=0.05).

4.3.3. Bioconcentration of diclofenac

There was a dose-dependent increase in the bioconcentration of diclofenac in inanga following a 96 h exposure. Accumulation in the highest treatment level (763 µg L^{-1}) was significantly higher (1811 µg kg^{-1}) than that in fish exposed to 0.17 µg L^{-1} (14.9 µg kg^{-1}) or controls in which no diclofenac was detected ($p < 0.0001$; Figure 4.9). From these observations and analytically-verified water treatment levels, a BCF value of 2.1 (± 1.2) from the highest treatment level (763 µg L^{-1}) was calculated, a value significantly lower ($p = 0.0002$) than the mean BCF (87 ± 55) determined for the 0.17 µg L^{-1} treatment (Figure 4.10). Based on observed bioconcentration in the 0.17 µg L^{-1} treatment level, human consumption would result in 4.25 µg of diclofenac per serving.

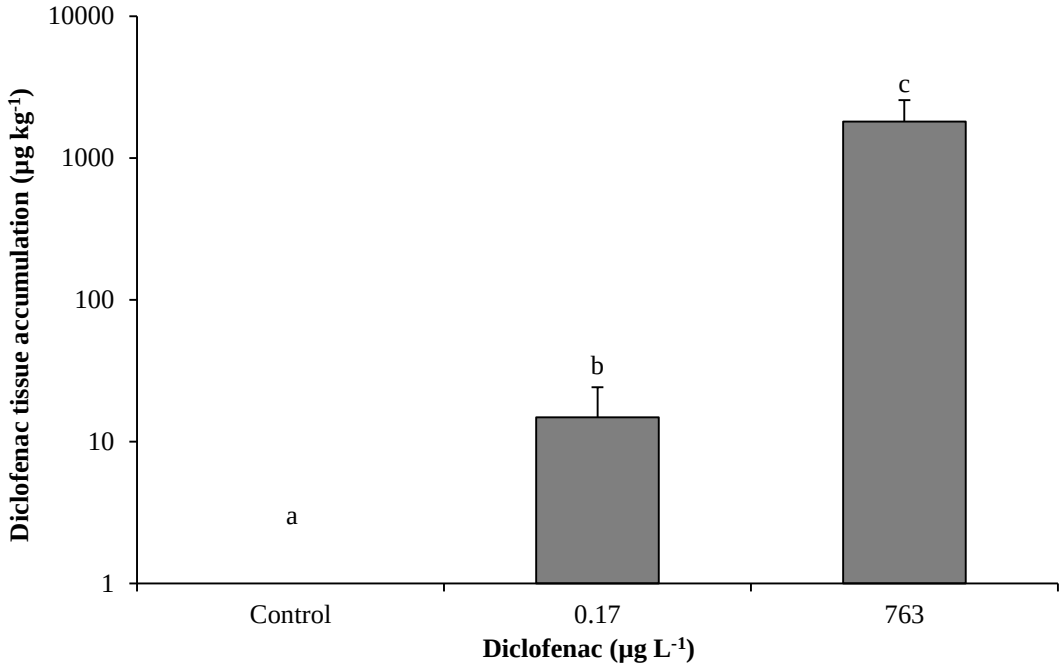


Figure 4.9. Whole body diclofenac accumulation (mean ± SD in inanga (*Galaxias maculatus*; whole body; n = 8) after exposure to diclofenac for 96 h. Bars sharing letters are not significantly different as determined by one-way ANOVA followed by post-hoc Tukey test; α = 0.05. n.d. = Not detected.

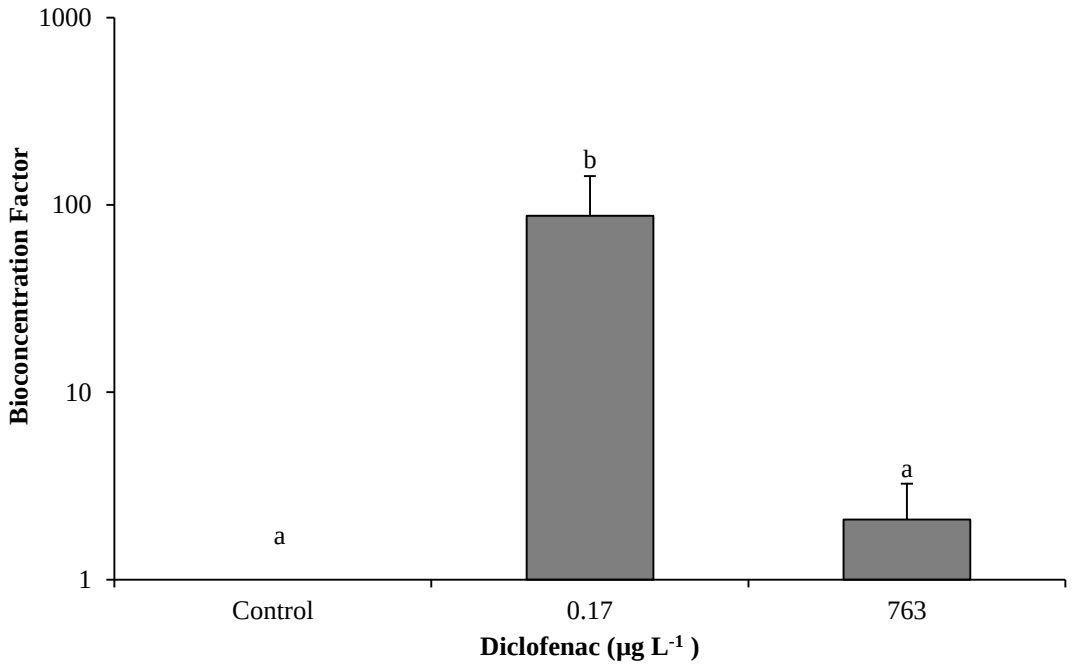


Figure 4.10. Mean (n = 8; ± SD) non-kinetic bioconcentration factor (BCF) based on whole body determination of inanga (*Galaxias maculatus*) exposed to diclofenac for 96 h. Bars sharing letters are not significantly different as determined by one-way ANOVA followed by post-hoc Tukey test; α = 0.05. n.d. = Not detected.

4.4. Discussion

The primary objective of the present study was to examine whether diclofenac induces sublethal oxidative stress indicators of toxicity in a culturally important and commonly observed fish species in the Southern Hemisphere. Herein, inanga were exposed to two concentrations of diclofenac (0.17 and 763 µg L^{-1}). One treatment level represented a concentration within the range of those measured in the environment (median worldwide level: 0.02 ± 0.72 µg L^{-1}, Acuña et al., 2015), while a higher treatment level was selected to exceed previously reported NOEC for diclofenac toxicity in trout (0.5-320 µg L^{-1}; Schwaiger et al., 2004; Hoeger et al., 2005; Mehinto et al., 2010; Memmert et al., 2013). In addition, this treatment level (nominally 770 µg L^{-1}) was recently proposed as a water quality guideline for diclofenac based on a review of existing literature regarding the biological effects of this pharmaceutical (Kumar et al., 2016).

4.4.1. Impact of diclofenac on oxidative stress

Oxidative stress is the result of the accumulation of ROS overwhelming the cells capacity to detoxify ROS. There are two general mechanisms that lead to oxidative stress. Either a toxicant generates increased ROS, which in turn can stimulate antioxidant pathways, or the toxicant inhibits antioxidant defence pathways (Lushchak, 2011). There is evidence that diclofenac exerts effects through both of these mechanisms. For example, it is thought that the metabolism of diclofenac through mixed-function oxidases (CYP450) generates superoxide anions (Islas-Flores et al. 2013), while a decrease in the antioxidant enzyme catalase, has been noted in the liver and gill of carp exposed to diclofenac (Nava-Álvarez et al., 2014).

In the current study, gill catalase activity decreased following diclofenac exposure (Figure 4.1). This suggests that diclofenac may have impaired catalase activity in this tissue. Under such circumstances an increase in lipid peroxidation might be expected, owing to the reduced ability to scavenge ROS. However, lipid peroxidation was reduced in the gill at the highest exposure concentration in the current study. Conversely, this instead suggests a down-regulation of catalase. This may be due to the upregulation of other oxidative defence pathways, such as SOD. This exact pattern (a decrease in catalase activity, associated with an increase in SOD activity) has been shown in the gill of carp exposed to diclofenac (Nava-Alvarez et al., 2014). Similarly, Feito and colleagues (2012) also demonstrated a reduction in lipid peroxidation in zebrafish embryos that were exposed to diclofenac (0.03 µg L^{-1}, 90 min), an effect they also attributed to an upregulation in SOD activity. In contrast, Saucedo-Vence et al. (2015) demonstrated an increase in gill catalase activity and lipid peroxidation in common carp exposed to diclofenac (7 mg L^{-1}) for 4 days. This exposure level is 10 times higher than that used in the current experiment, and thus

could have generated ROS that exceeded the ability of SOD to effectively scavenge ROS. This lack of SOD effectiveness may have induced an increased catalase activity, which was also insufficient to offset the ROS generated, leading to increased lipid peroxidation. It is also important to note that functional differences exist between key components of the oxidative stress pathways in fish, and these are likely to contribute towards species differences (Goldstone et al., 2010; Connors et al., 2013).

In terms of the oxidative damage endpoint (lipid peroxidation), the results observed in the kidney were similar to those of the gill. The results of this study showed that the kidney of inanga exhibited a decrease in lipid peroxidation after exposure to 763 µg L^{-1} diclofenac (Figure 4.2). The mechanism of effect here is therefore likely to be the same as that in the gill, whereby the upregulation of the antioxidant pathway leads to an enhanced scavenging of ROS, and an improved oxidative damage status. In the kidney of inanga this occurs in the absence of any significant change in catalase activity, suggesting the involvement of alternative ROS scavenging pathways, such as SOD. This overall positive effect of diclofenac on cellular oxidative damage has also been observed in other systems. For example, Petersen et al. (2005) exposed human lens epithelial cells to diclofenac and H_2O_2. At low diclofenac concentrations, there was a significant protective effect against oxidative damage in comparison to when the cells were exposed to H_2O_2 alone, presumably mediated by the "priming" effect of diclofenac on antioxidant enzyme pathways.

In the liver of inanga there was an increase in catalase activity in the 0.17 and 763 µg L^{-1} exposures relative to the control (Figure 4.3). The high hepatic catalase

activity may be a response to an increase in ROS. Diclofenac has been shown to be biotransformed in the liver, by CYP450, which increases ROS in the form of superoxide anion (Islas-Flores et al., 2013). In the current study, the increase in catalase activity appears insufficient to adequately protect against damage, with an increase in lipid peroxidation in the 763 µg L^{-1} treatment level (Figure 4.6). The liver is the main site of diclofenac accumulation in trout (Memmert et al., 2013; Schwaiger et al., 2004), and is also the site with the highest concentration of CYP450 activities. Because the liver is exposed to increasing concentrations of the toxicant, CYP450 activities are likely to increase, thus generating more ROS, and inducing increased antioxidant defence. This scheme has support in the literature with evidence of increased transcription of cyp1a1 with increasing diclofenac exposure concentration in the liver of rainbow trout (Gröener et al., 2015). In the case of inanga in the current study, exposure to 763 µg L^{-1} diclofenac is likely sufficient to produce levels of ROS that exceed antioxidant scavenging activity. This suggests that the liver is the most likely site of toxic effects following acute diclofenac exposure in this fish species.

4.4.2. Impact of diclofenac on metabolic rate

Metabolic rate reflects the ability of an organism to extract O_2 from its environment and/or the costs of homeostasis. In the current study, the effect of diclofenac on metabolic rate was investigated owing to previous reports that diclofenac exposure can cause physical damage to the gill (Schwaiger et al., 2004; Hoeger et al., 2005; Memmert et al., 2013). These changes induced by diclofenac included necrosis of pillar and respiratory epithelial cells (Schwaiger et al., 2004), and thickening of lamellae (Memmert et al., 2013). As the branchial epithelium is the primary locus for O_2 uptake, diclofenac exposure might have been expected to impair O_2 consumption. However, such an observation was not made in the current study.

Although gill histology would be required to confirm whether gill damage occurs, this may be a function of the much shorter exposure interval (4 d) in the current study, relative to the studies referenced above (> 21 d). Alternatively, it is possible that the significant contribution that the skin of inanga makes towards O_2 uptake (Urbina et al., 2014a) minimises the significance of any gill-based effects of diclofenac.

The gill is also the key locus for ion uptake in freshwater fish. By virtue of a higher osmolality than their surroundings, fish in freshwater are faced with constant diffusive loss of ions to the water, and in order to achieve ion homeostasis they must take these ions up via the gill (Hwang et al., 2011). Previous studies have shown that ion influx in inanga is highly sensitive to environmental stressors (Harley and Glover, 2014; McRae et al., 2016). However, similar to our observations for metabolic rate, there were no significant effects of diclofenac exposure on Na^+ and Ca^{2+} influx in inanga. These data are the first to specifically examine effects of diclofenac on ion transport processes. However, in *Cirrhinus mrigala* (Indian carp) exposed to diclofenac (1, 10, 100 µg L^{-1}) for 96 h, a significant increase in Na plasma levels has been observed (Saravanan et al., 2011; Saravanan and Ramesh, 2013). Saravanan and Ramesh (2013) explained that the increase plasma Na levels might be a compensatory response due to osmoregulatory imbalances, resulting from the effect of diclofenac on metabolism and active transport (Saravanan and Ramesh, 2013). In our experiment, there were no changes to Na flux or metabolic rate, suggesting that there is species-specificity in the sublethal mechanisms of diclofenac toxicity.

4.4.3. Bioconcentration of diclofenac in inanga

In addition to examining inanga oxidative stress responses, inanga were observed to bioconcentrate diclofenac at both treatment levels, though performing a kinetic BCF study following regulatory guidelines was not within the scope of the present research. Despite increasing use of fish models in biomedical research and reports of pharmaceutical bioaccumulation in the environment, an understanding of comparative pharmacokinetics is lacking across the developmental stages of specific fish models (Kristofco et al., 2016), and among fish species and other aquatic organisms (Connors et al., 2013, Brooks, 2014, Nichols et al., 2015). For example, BCF values for diclofenac in aquatic organisms are limited, and have only been reported by two studies in rainbow trout. Memmert et al. (2013) employed a standard kinetic OECD (Organisation for Economic Co-operation and Development) experimental design with a 14 d uptake period followed by a depuration period. These authors observed kinetic BCF values of less than 10 at both exposure concentrations (2.1 or 18.7 µg L^{-1}). Such findings are routinely observed during BCF studies performed at concentrations lower than those eliciting toxicity in aquatic organisms. In contrast, Schwaiger et al. (2004) did not employ a depuration period and reported muscle BCFs in rainbow trout that ranged from 0.3 (at 500 µg L^{-1}) to 69 (at 1 µg L^{-1}) after a 28 d exposure. Results from the present study, which was also not intended to examine depuration, observed an inverse relationship between increasing exposure concentration and BCF values (Figure 4.10), which were simply estimated at the end of the 96 h study (see Gobas and Morrison, 2000). As noted above, the present study was not intended to derive a kinetic BCF value for regulatory purposes; however, the resulting data are generally similar to those reported by Schwaiger et al. (2004). Whether the depuration stage associated with deriving a kinetic BCF that was

employed by Memmert et al. (2013) contributed to differences in observations by Schwaiger et al (2004) and the present study is not known, though elimination of some contaminants represents an important factor influencing BCF determinations (Connors et al., 2013). It is also important to note that the body burden concentrations in the present study are unlikely to accurately reflect exposure across diverse environmental scenarios (Veith et al., 1979). Thus, developing a comparative understanding of biotransformation differences among aquatic organisms remains a critical research need; however, in the case of diclofenac, intrinsic clearance occurs, but appears relatively limited at least according to *in vitro* data from rainbow trout (Connors et al., 2013).

There are several other differences among these previous efforts reporting diclofenac bioconcentration by rainbow trout and the current study. For example, though size of fish can influence the uptake of organic chemicals (Sijm and van der Linde, 1995), patterns of BCF did not correspond to patterns of fish mass by Memmert et al. (2013) (1.2 g) and Schwaiger et al. (2004) (167 g). Whether the significant age differences (e.g., juvenile vs. 1.8 years) between organisms employed by Memmert et al. (2013) and Schwaiger et al. (2004) differentially influenced bioconcentration is not known because potential uptake or elimination differences of pharmaceuticals across the life history of fish species have not been determined (Kristofco et al. 2016). In addition, duration of exposure was shorter in the current study because inanga was exposed over 4 d, consistent with recent research examining uptake of an ionisable base pharmaceutical (Nichols et al., 2015). This duration was shorter than in previous rainbow trout studies (14 d, Memmert et al., 2013; 28 d, Schwaiger et al., 2004). However, no direct influence of exposure

duration on kinetic or non-kinetic BCF estimates appears to exist among the three studies, though this question deserves additional attention.

Differences in pH between the present study and those conducted previously in rainbow trout are also likely to have influenced the experimental outcomes. Because surface water pH strongly influences bioavailability and toxicity of acids and bases, the US EPA (1985; 1986; 1999) has developed water quality criteria that specifically account for pH (e.g., for the base ammonia, and the acid pentachlorophenol). Erickson et al. (2006a, b) previously demonstrated the significant influence of pH gradients on uptake of weak acids by rainbow trout. Because diclofenac is an acid with a pKa of 4.18, subtle changes in exposure pH may influence bioavailability and uptake. Because the pH in the present study was 6.7 compared to pH 7.4 in the Schwaiger et al. (2004) study, and ranged from pH 7.5-8.4 in the study of Memmert et al. (2013), a greater proportion of diclofenac would have been present unionised. Diclofenac would therefore be expected to accumulate to a greater degree in the present study than the previous work with rainbow trout. Future studies are needed to identify influences of pH on diclofenac uptake and elimination by fish, including those species like inanga which inhabit surface waters with lower pH than usually employed in toxicology and bioaccumulation studies with common laboratory models.

Observations of BCFs among previous rainbow trout research and the present study with inanga suggest species differences may also have influenced diclofenac accumulation. For example, inanga are known to utilise the skin as an uptake surface, with evidence of O_2 uptake and ammonia excretion across this epithelium (Urbina et al., 2014a). Consequently, a higher BCF of inanga at environmentally-relevant

exposure concentrations may reflect increased uptake of diclofenac across the skin. Whether this route of exposure is an important contributor towards diclofenac body burden requires further investigation.

Inanga are an important fishery species in the Southern Hemisphere (Mardones et al., 2008; McDowall, 2006). Juveniles (whitebait) returning to freshwaters are seine-netted at the mouths of streams and rivers, and are traditionally fried whole, in batter. For this reason, measures of whole body diclofenac, as in the current study, may be relevant for estimating exposure during human consumption and to high trophic level predators. Using fish consumption guidelines for human health risk assessment from the US EPA, it was calculated that there would be, on average, 4.25 µg of diclofenac per serving of whitebait (US EPA, 1989). Diclofenac tablets contain 25-150 mg of diclofenac, with the adult recommended maximum daily dose of 50 to 1100 mg (Actavis UK Ltd, 2015; Medsafe, 2014). Therefore, adult human consumption would need to be in excess of 11,000 servings of whitebait to exceed recommended diclofenac intake. While recommended doses for children are smaller (100 mg) or even zero (children under 14) (Actavis UK Ltd, 2015; Medsafe, 2014), it is unlikely that the consumption of whitebait will generate significant effects on human consumers related to diclofenac accumulation. In addition to human exposure, observations in the present study may be of importance for other species that utilise inanga as a prey item. Inanga are fed upon by other fish species such as trout (McIntosh, 2000), and are also found in the stomach contents of coastal birds (Falla and Stokell, 1945). However, ionisable pharmaceuticals are not expected to appreciably biomagnify in aquatic systems (Du et al., 2014, 2016), so when combined with the relatively low BCF values estimated here, and those reported in other studies with rainbow trout, the risk of secondary poisoning appears low. Future field studies

are needed to examine bioaccumulation and trophic transfer in aquatic systems from the Southern Hemisphere and those with lower pH.

Chapter 5. Comparative diclofenac toxicology to two model freshwater fish, zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*)

5.1. Introduction

Zebrafish and fathead minnows are among the most commonly used species for understanding the impacts of environmental contaminants on aquatic biota (Section 1.5). However, while widely employed as model species, there is only a limited amount of information regarding the sensitivity of zebrafish to diclofenac (Hallare et al., 2012; Praskova et al., 2014), and no studies that examine the sensitivity of fathead minnows to this important environmental pharmaceutical contaminant. Given their importance for the development of environmental regulations (Ankley and Villeneuve, 2006; Embry et al., 2010), there is, therefore, a need to conduct research on the impacts of pharmaceuticals on these species. Furthermore, delineating differences in sensitivity between species and developmental stages is of critical importance to ensure that the development of regulatory tools is protective of a range of species (Brooks et al., 2009; Rudd et al., 2014; Kristofco et al., 2016; Connors et al., 2013).

The objective of this study was to understand how two commonly-used aquatic toxicology model species differ in their sensitivity to diclofenac. This was achieved by exposing fathead minnow and zebrafish to graded concentrations of diclofenac, following standardised OECD and US EPA methods. Exposure concentrations ranged from environmentally-relevant (0.001 mg L^{-1}; Acuña et al., 2015) to effect concentrations (10 mg L^{-1}; Praskova et al., 2014). A suite of cellular and biochemical endpoints (DNA damage, lipid peroxidation, and GSH; Section 1.6.1) were used to assess toxicity. The focus on these as indicators of biological impact stemmed from previous research within this thesis, showing that endpoints of oxidative stress are highly sensitive to diclofenac exposure in inanga. This sublethal study, followed an acute lethal study to establish overall sensitivity.

5.2. Methods

5.2.1. Fish culture

All work in this chapter was conducted in the Environmental Chemistry laboratories at Baylor University, Texas, USA. All culture and experimental conditions followed Institutional Animal Care and Use Committee protocols approved at Baylor University.

5.2.1.1. Fathead minnow

Fathead minnows were maintained under standard culture conditions at Baylor University. Fish were housed in a flow-through system supplied with aged, dechlorinated tap water (alkalinity: 107 mg L^{-1} $CaCO_3$, hardness: 137 mg L^{-1} $CaCO_3$, conductivity: 317 µS, pH: 7.6, dissolved O_2: 8.27 mg L^{-1}) at a constant temperature of 25 ± 1°C under a 16:8 light/dark photoperiod. Fish were fed twice daily with brine shrimp (*Artemia* sp. nauplii; Pentair AES, Apopka, FL) and TetraMin® Tropical Flakes (Pentair AES, Apopka, FL, USA). Individuals were aged to at least 120 d, at which time they were placed in tanks in a 1:4-5 male to female ratio for breeding. Embryos were collected, and within 24 h of hatching, larvae were used for toxicity studies.

5.2.1.2. Zebrafish

Tropical 5D wild type zebrafish were maintained under standard culture conditions at Baylor University as previously described (Usenko et al., 2011; Kristofco et al., 2014). Briefly, adult fish were kept at a density of <4 fish per litre in a z-mod recirculating system (Marine Biotech Systems, Beverly, MA, USA) with water (pH 7.0, 260 parts per million (ppm) Instant Ocean®, Cincinnati, OH, USA) at 26–28°C, 16:8 light-dark cycle. Zebrafish were fed twice daily with brine shrimp

(*Artemia* sp. nauplii) and once per day with TetraMin® Tropical Flakes. Sexually mature fish were bred to produce embryos for toxicity studies.

5.2.2. LC_{50} rangefinder test

An initial study was conducted to establish a 48 h LC_{50} value for diclofenac for each species. This study was conducted over 48 h for logistical purposes, but previous work has shown that there is minimal difference between the 48 h and 96 h mortality in zebrafish (Kovrižnych et al., 2013). Fathead minnow larvae (24 hours post-hatch (hph); n = 2 replicate LC_{50} tests; 10 larvae per concentration) and zebrafish embryos (30% epiboly stage; 4 hours post-fertilisation (hpf); n = 2 replicate LC_{50} tests; 15 embryos per concentration) were loaded into exposure chambers containing diclofenac (stock prepared fresh daily; 0.039, 0.39, 3.9, 7.8, 15.6, 31.2, 156 mg L^{-1}) added to water of chemistry identical to that of their holding water, for 48 h. Mortalities were counted daily via observation of the heart rate. Water was changed daily for zebrafish. Water chemistry was monitored at the beginning, before and after water changes, and at the end of the exposure, and measured via LC-MS/MS (see Section 5.2.5).

5.2.3. Zebrafish embryo and fathead minnow larvae diclofenac exposure

Standardised toxicology experimental designs from the US EPA were used for fathead minnows and the OECD (FET OECD no. 236) for zebrafish (US EPA, 2002; OECD, 2013). Test media were the same as described as above (Section 5.2.1). Minor modifications to these guidelines were necessary to allow for the collection of the minimum amount of fish tissue needed for each of the sublethal oxidative stress endpoints (Corrales et al., 2016). The diclofenac treatment levels are outlined in Table 5.2. The stock solution for each chemical was the same for both species and the stock

concentration was approximately three times the highest 48h LC_{50} concentration. All zebrafish and fathead minnow experiments were performed in climate controlled incubators with backup power supply. Fathead minnow larvae (24 hph; n = 8, containing 10 larvae) and zebrafish embryos (4 hpf; n = 12, containing 15 embryos) were placed into exposure chambers (fathead minnow, 100 mL; zebrafish, 20 mL) containing diclofenac. Following a 96-h exposure, five fathead minnows or ten zebrafish were pooled to represent each replicate (n = 3 for each biochemical analysis). Three common biochemical biomarkers of oxidative stress (lipid peroxidation, DNA damage, GSH) were then measured in the pooled whole fish. Remaining larvae were used for behavioural and genetic analysis not reported here. Hatching rate was counted for zebrafish larvae daily.

Throughout all exposures general water chemistry parameters were measured (alkalinity, hardness, dissolved O_2, temperature). Water samples (10 mL) were taken at the beginning, during water exposures, and the end of the exposures for analytical verification of diclofenac concentrations by LC-MS/MS (Section 5.3.5).

5.2.4. Oxidative stress biomarkers

DNA oxidative damage was measured using a commercially available kit (kit 589320; Cayman Chemical Company, Ann Arbor, MI, USA). Prior to performing the DNA oxidative damage immunoassay, DNA was extracted from the whole body of fathead minnow or zebrafish by homogenising tissue in DNAzol (Molecular Research Center, Cincinnati, OH, USA). After DNA was quantified using the NanoDrop2000 (Thermo Scientific, Wilmington, DE, USA), 5 µg DNA per sample were used to determine DNA damage following the manufacturer's instruction (Cayman Chemical Company). The basis of this assay is that the oxidatively-damaged guanine bases in

DNA compete with an added tracer (8-OH-dG-acetylcholinesterase conjugate) for a monoclonal antibody that recognises both of these substrates. The more oxidatively-damaged DNA in the sample, the less tracer that binds to the antibody, leading to a reduced signal. The tracer is measured by the addition of Ellmans reagent. This contains an acetylcholine esterase substrate, which results in a yellow colour that can be measured by absorbance at 412 nm.

GSH was also determined using a Cayman Chemical kit (kit 703002; Cayman Chemical Company, Ann Arbor, MI, USA). Homogenised and centrifuged (10,000 x g for 15 min at 4 °C) whole body samples were first deproteinated with 1.25 M metaphosphoric acid and 0.2 M triethanolamine. DTNB (5,5-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) was then added. The sulfhydryl group of GSH present in the fish homogenates reacts with DTNB to produce 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production was measured, which is directly proportional to the amount of GSH in a sample. Protein concentration was measured using a Bradford assay (Bradford, 1976). Total GSH was expressed as µmol µg protein^{-1}.

Lipid peroxidation was determined using a Thiobarbituric Acid Reactive Substances (TBARS) assay (kit 705002; Cayman Chemical Company, Ann Arbor, MI, USA). This assay measures the amount of malondialdehyde (MDA), a reactive carbonyl compound which is a natural product of lipid peroxidation in living organisms. Samples were homogenised in radio immunoprecipitation (RIPA) buffer, then centrifuged at 1,600 x g for 15 min at 4°C. TBA was then added to homogenised whole body samples of zebrafish and fathead minnows. Where lipid peroxidation occurred, an MDA-TBA adduct was formed, which was measured at 530 nm (BioTek Synergy^{TM} HT plate reader, Vermont, USA; Gen5 2.05 software). Protein

concentration was measured using a Bradford assay (Bradford, 1976), and lipid peroxidation was expressed as µmol µg protein^{-1}.

5.2.5. Analytical verification by LC-MS/MS

Water samples (n = 6) were taken at the beginning, before and after the water change, and at the cessation of the exposure. In the LC_{50} exposure, samples were taken from at the beginning and end of the exposures. In the sublethal exposure, samples were taken from the control, two lowest (0.001 and 0.1 mg L^{-1}) and highest (10 mg L^{-1}) exposures for logistical reasons. Analytical verification was also carried out for the stock (100 mg L^{-1}) which was used to dose the exposure chambers, to verify the concentrations of diclofenac.

Water samples from the sublethal exposure and reference test were analytically verified following previously reported methods (Du et al., 2014). All water samples were immediately frozen and stored at -20°C until analysis. Samples were later thawed, and aliquots of experiment water were transferred to 18 mL borosilicate glass culture tubes (VWR Scientific). Each sample was spiked with 50 µL of diclofenac-*d*4 internal standard (100 µg L^{-1}) obtained from Toronto Research Chemical (Toronto, Ontario, Canada). Samples were evaporated under N_2 gas at 45°C in a Turbovap evaporator. Next, samples were reconstituted in 1 mL of 5:95 methanol:0.1% formic acid and syringe filtered through a 0.2 µm filter prior to analysis via LC-MS/MS (Du et al., 2012).

5.2.5.1. Instrumental analysis

Diclofenac concentrations were analytically verified using isotope-dilution LC-MS/MS with an Agilent Infinity 1260 autosampler with a quaternary pumping system, Agilent jet stream thermal gradient electrospray ionisation source, and model

6420 triple quadrupole mass analyser. Chromatography was performed using a 10 cm × 2.1 mm Poroshell 120 SB-AQ column (120Å, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA) preceded by a 5 mm × 2.1 mm Poroshell 120 SB-C18 attachable guard column (120Å, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA). The ionisation mode, monitored transition, and retention time for diclofenac, and diclofenac-*d4* (DCF-*d4*) were as follows: ESI - diclofenac 294.0 > 249.8, retention time = 4.8 min; ESI – DCF-*d4* 299.0 > 254.8, retention time = 4.8 min.

The limit of detection (LOD) and LOQ were determined by running several method blanks. LOD was defined as the concentration that yielded a value 3 times the signal-to-noise ratio, while LOQ was defined as the concentration that yielded a value 10 times the signal-to-noise ratio. The LOD and LOQ for diclofenac were determined to be 0.17 ng L^{-1} and 1.7 ng L^{-1}, respectively. Eight standards, ranging in concentration from 0.5 to 500 ng L^{-1}, were used to construct a linear calibration curve ($r^2 \geq 0.99$). Instrument calibration was monitored over time via analysis of CCV samples, with an acceptability criterion of ± 20%. Method detection limits (MDLs) represented the lowest concentrations of an analyte, where there is 99% confidence that the concentration is different from zero, for a given matrix (water MDL = 4.74 ng L^{-1}, tissue MDL = 2.31 µg kg^{-1}).

5.2.6. Statistical analysis

Acute mean 48 h LC_{50} values were calculated using the US EPA Toxicology Research Analysis Program (TRAP). Values obtained were then analysed by a Fisher's exact test (Sigma Plot). Biochemical biomarkers were analysed initially for normality by the Kolmogorov-Smirnov test, then by one-way ANOVA and Dunnett's post-hoc test, hatch rate was calculated using a two-way ANOVA followed by

Dunnett's post-hoc test (Sigma Plot). Statistically significant differences between LC_{50} values were determined using a modification of the Motulsky method, as described by Glover et al. (2003). Data that did not meet assumptions of normality were transformed. Statistical significance was set at $p < 0.05$.

5.3. Results

5.3.1. Measured exposure concentrations of diclofenac

Concentrations of diclofenac in exposure chambers were quantified using LC-MS/MS. Results from the lethal (Table 5.1) and sublethal (Table 5.2) water measurements are outlined below. Nominal concentrations are used throughout the text, hereafter.

Table 5.1. Measured exposure concentrations of diclofenac for the lethal exposure

	Diclofenac concentration (mg L^{-1})	
Nominal exposure	Mean measured concentration	± SD
Control	<LOD	
0.039	0.03	0.01
0.39	0.37	0.08
3.9	3.57	0.36
7.8	7.77	0.97
15.6	14.58	1.11
31.2	34.08	4.67
156	147	18

<LOD indicates value was below the limit of detection

Table 5.2. Measured exposure concentrations of diclofenac for the sublethal exposure

	Diclofenac concentration (mg L^{-1})	
Nominal exposure	Mean measured concentration	± SD
Control	<LOD	
0.001	0.002	0.001
0.01	n.d	
0.1	0.113	0.021
1	n.d	
10	6.765	0.456
100 (stock)	71.904	0.248

<LOD indicates value was below the limit of detection. n.d indicates not determined

5.3.2. LC_{50} values

Zebrafish (4 hpf) exposed to diclofenac displayed a mean (± SD) 48 h LC_{50} of 46 ± 21 mg L^{-1}. For fathead minnows (24 hph) the 48 h LC_{50} for diclofenac was 66 ± 9 mg L^{-1} (mean ± SD). There was no significant difference between the LC_{50} values (p = 0.335; Figure 5.1).

Figure 5.1. Mean LC_{50} (n = 2) of zebrafish and fathead minnow after exposure to diclofenac for 48 h. Results were analysed using TRAP software and presented as mean ± SD.

5.3.3. Hatching rate

There was a significant decrease in hatching rate of zebrafish larvae after exposure to diclofenac for 96 h, when comparing effect of time ($p < 0.0001$), effect of concentration ($p < 0.0001$) and the interaction (Figure 5.2; $p < 0.0001$), relative to the control. Hatching started at 24 h and was > 97% complete by 96 h across all treatments.

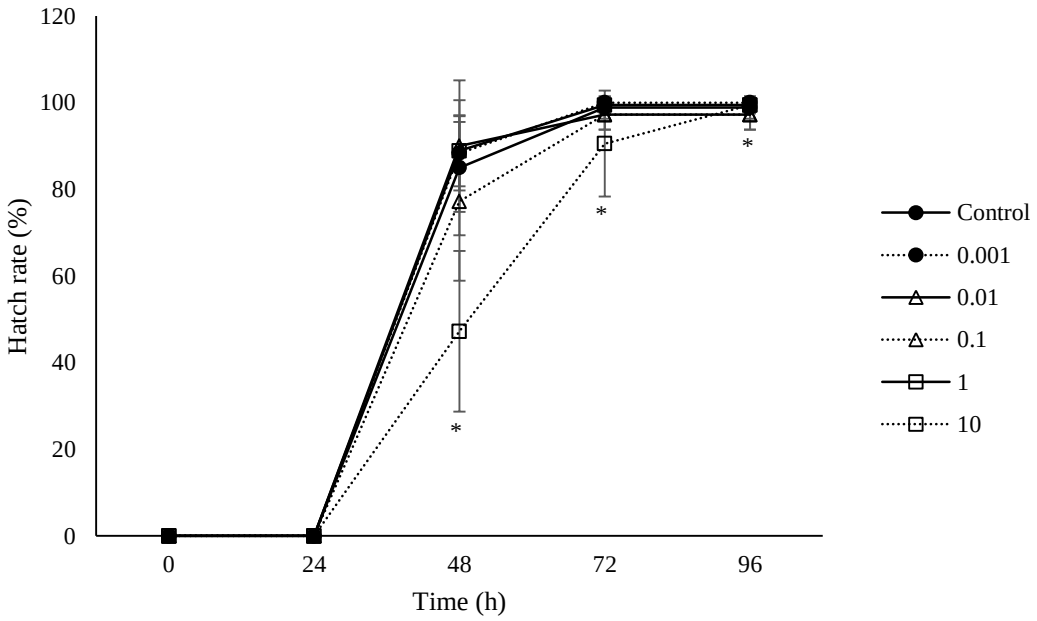


Figure 5.2. Mean hatching rate of zebrafish embryos during exposure to diclofenac (control, 0.001, 0.01, 0.1, 1 or 10 mg L^{-1}) for 96 h. Results were analysed using Sigma Plot and presented as mean ± SD. Statistical significance (*) was tested by two-way ANOVA followed by Dunnett's post-hoc test (n = 3, $p < 0.05$).

5.3.4. Mortality in sublethal exposures

There was less than 10% mortality in control exposures of zebrafish embryos (Figure 5.2), and larval fathead minnows (Figure 5.3). This in line with OECD and US EPA guidelines. However, survival (%) was significantly decreased in zebrafish exposed to 10 mg L^{-1} diclofenac, relative to the control ($p < 0.0001$). In contrast, fathead minnows showed no significant change in survival, in any of the diclofenac exposure concentrations ($p > 0.05$).

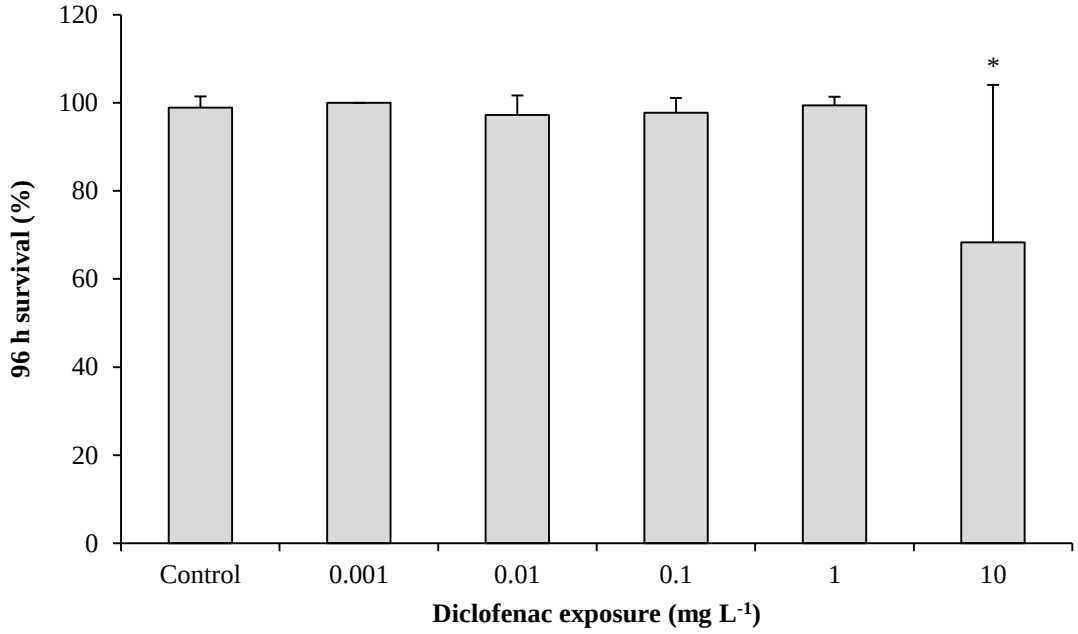


Figure 5.3. Mean (± SD) 96 h survival (%) of zebrafish larvae exposed to diclofenac for 96 h. Results were analysed using Sigma Plot and presented as mean ± SD. Statistical significance (*) was tested by one-way ANOVA followed by Dunnett's post-hoc test (n = 12, $p < 0.05$).

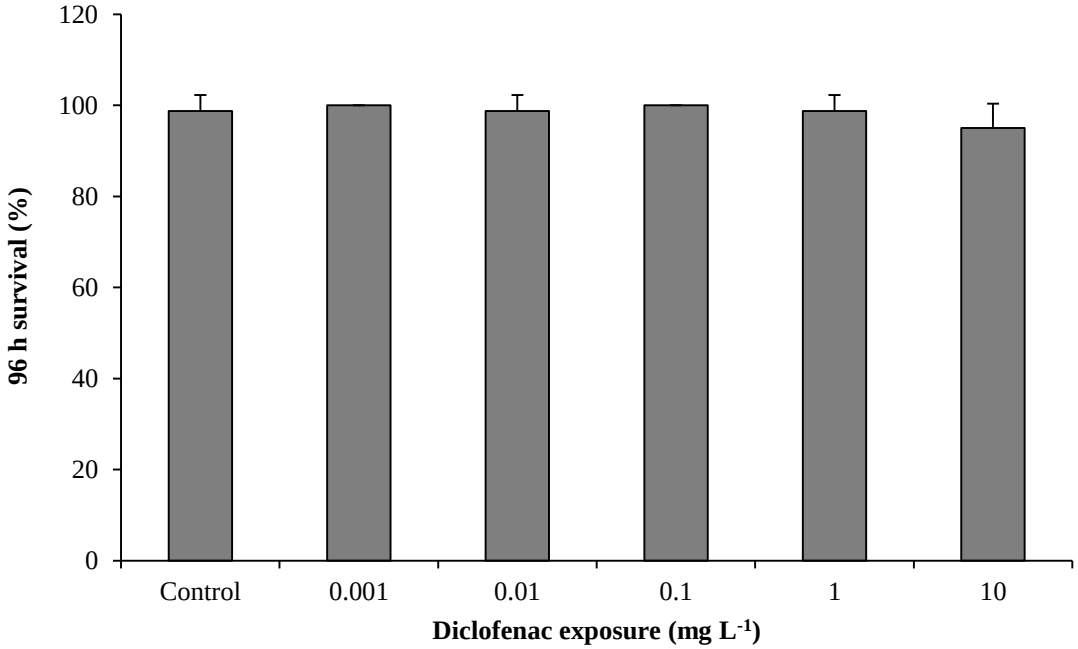


Figure 5.4. Mean (± SD) 96 h survival (%) of fathead minnow larvae exposed to diclofenac for 96 h. Results were analysed using Sigma Plot and presented as mean ± SD. Statistical significance was tested by one-way ANOVA followed by Dunnett's post-hoc test (n = 8, $p < 0.05$).

5.3.5. DNA Damage

Diclofenac exposures caused no significant DNA damage in zebrafish (p = 0.509; Figure 5.5). There was, however, insufficient tissue to measure DNA damage at the 10 mg L^{-1} exposure due to the number of mortalities and tissue prioritisation for the other biomarkers. Fathead minnows exposed to diclofenac exhibited DNA damage in the 10 mg L^{-1} exposure when compared to the control (p = 0.032; Figure 5.6).

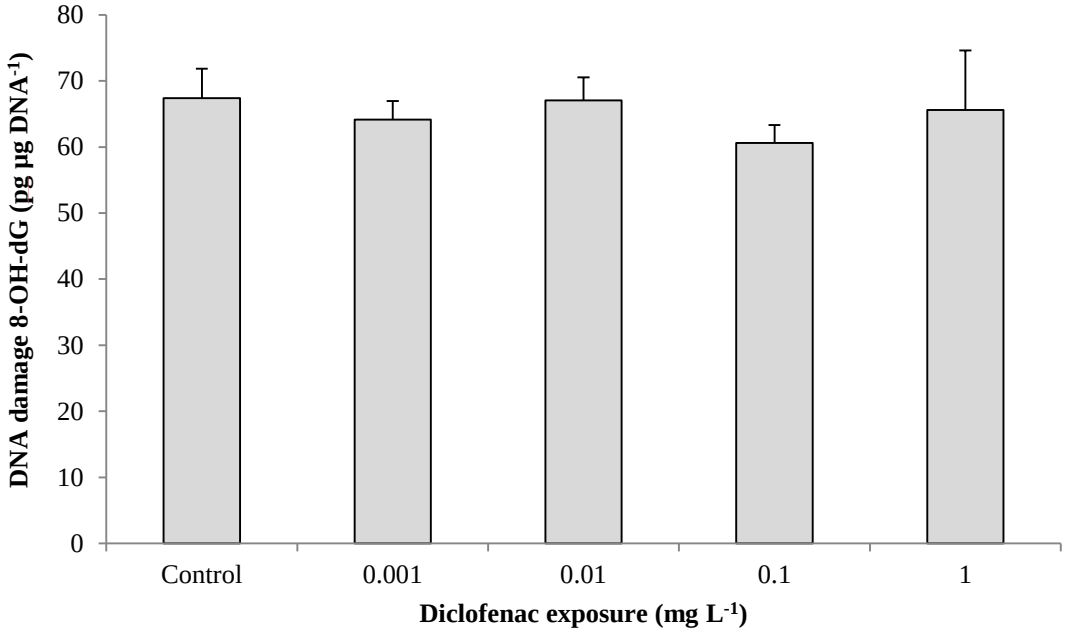


Figure 5.5. DNA damage measured as the amount of free 8-OH-dG (8-hydroxy-2-deoxyguanosine) was determined in zebrafish following a 96 h exposure to diclofenac. Results were analysed using Sigma Plot and presented as mean ± SD. Statistical significance was tested by one-way ANOVA followed by Dunnett's post-hoc test (n = 3, $p < 0.05$).

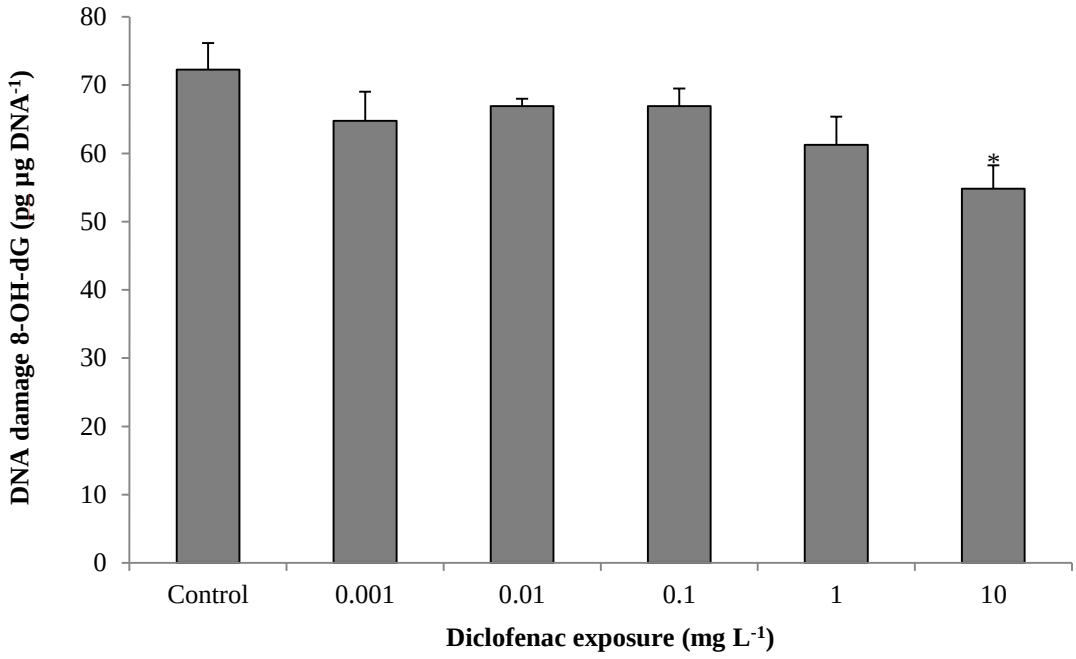


Figure 5.6. DNA damage measured as the amount of free 8-OH-dG (8-hydroxy-2-deoxyguanosine) was determined in fathead minnow following a 96 h exposure to diclofenac. Results were analysed using Sigma Plot and presented as mean ± SD. Statistical significance (*) was tested by one-way ANOVA followed by Dunnett's post-hoc test (n = 3, $p < 0.05$).

5.3.6. Oxidative stress

Zebrafish exposed to diclofenac exhibited no changes to total GSH ($p = 0.422$; Figure 5.7). Similarly, there was no effect of diclofenac on GSH in fathead minnows ($p = 0.753$; Figure 5.8).

Diclofenac exposure had no effect on lipid peroxidation ($p = 0.355$; Figure 5.9) in zebrafish. However, lipid peroxidation in fathead minnow was significantly reduced at diclofenac exposure concentrations of 0.01 (2 fold), 1 (4 fold), and 10 (1058 fold) mg L^{-1} compared to the control ($p < 0.001$; Figure 5.10).

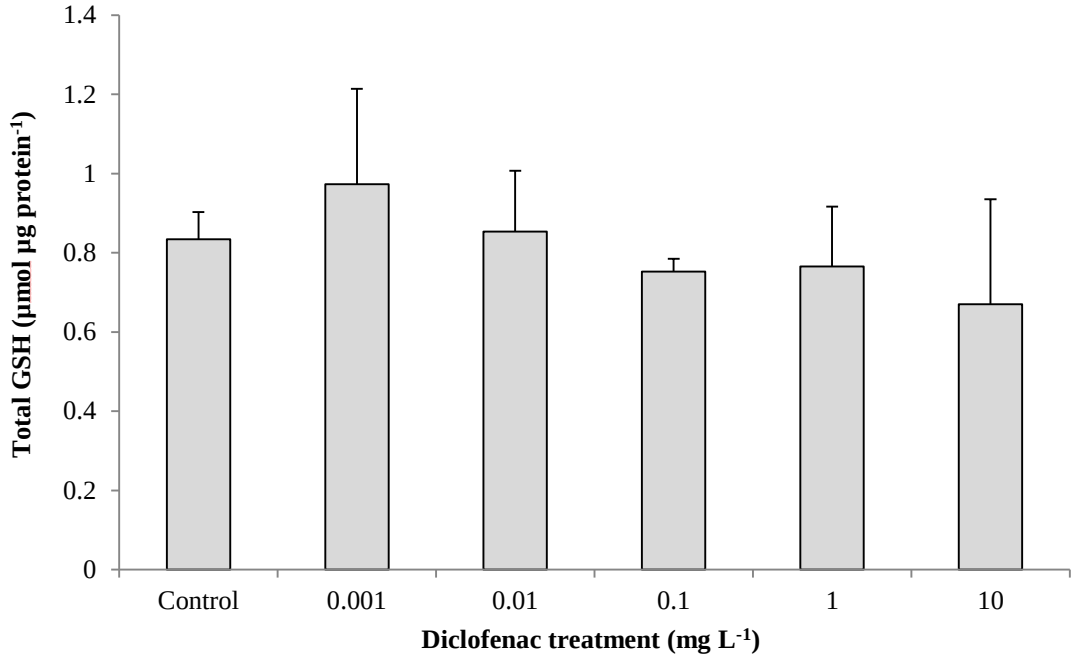


Figure 5.7. Glutathione (µmol µg protein^{-1}) was determined in zebrafish (A) following a 96 h exposure to diclofenac. Results were analysed using Sigma Plot and presented as mean ± SD. Statistical significance was tested by one-way ANOVA followed by Dunnett's post-hoc test (n = 3, $p < 0.05$).

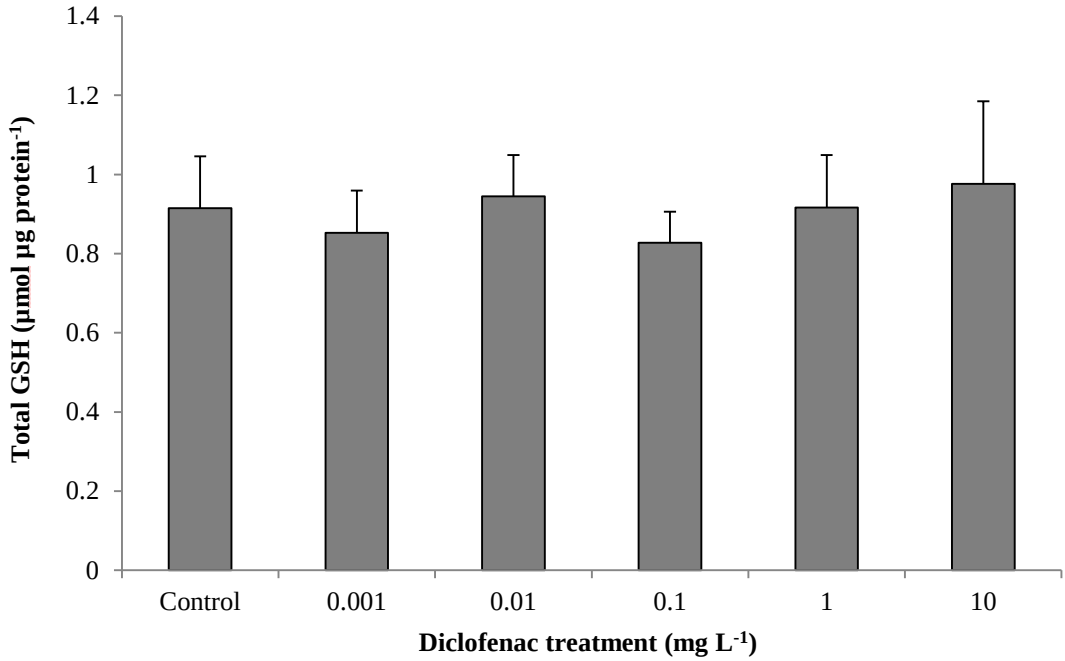


Figure 5.8. Glutathione (µmol µg protein^{-1}) was determined in fathead minnow following a 96 h exposure to diclofenac. Results were analysed using Sigma Plot and presented as mean ± SD. Statistical significance was tested by one-way ANOVA followed by Dunnett's post-hoc test (n = 3, $p < 0.05$).

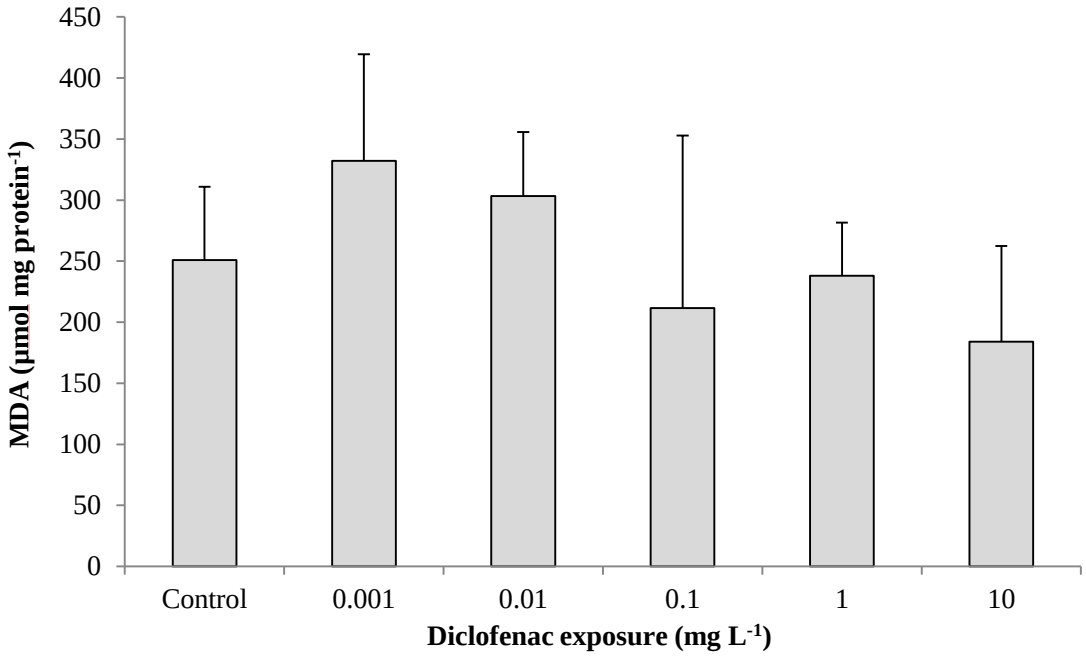


Figure 5.9. Lipid peroxidation measured as MDA concentration (µmol µg protein^{-1}) was determined in zebrafish following a 96 h exposure to diclofenac. Results were analysed using Sigma Plot and presented as mean ± SD. Statistical significance was tested by one-way ANOVA followed by Dunnett's post-hoc test (n = 3, $p < 0.05$).

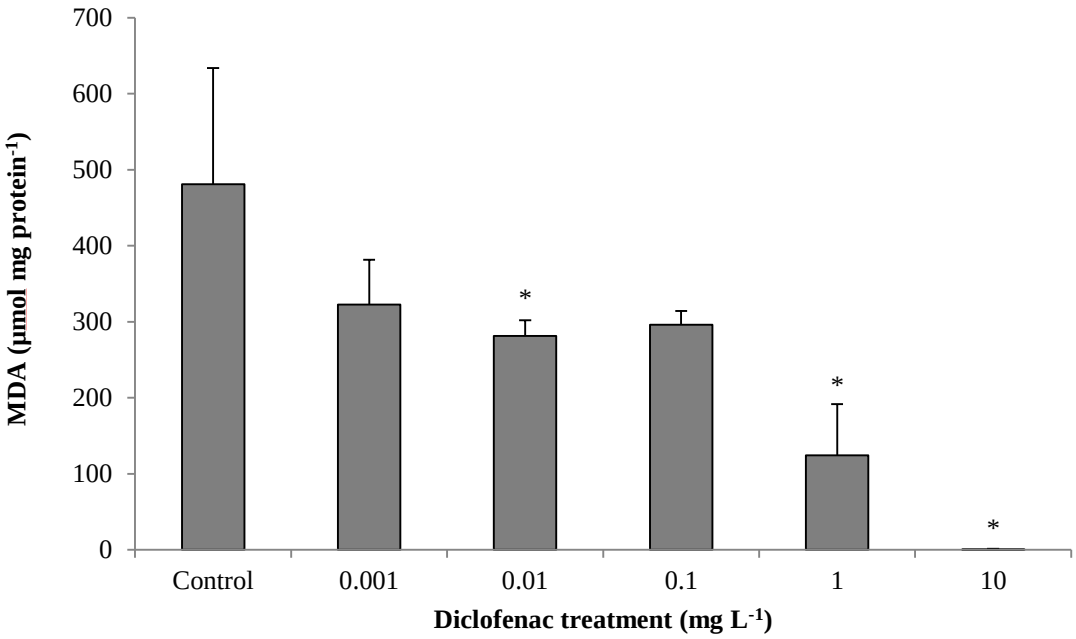


Figure 5.10. Lipid peroxidation measured as MDA concentration (µmol µg protein^{-1}) was determined in fathead minnows following a 96 h exposure to diclofenac. Results were analysed using Sigma Plot and presented as mean ± SD. Statistical significance (*) was tested by one-way ANOVA followed by Dunnett's post-hoc test (n = 3, $p < 0.05$).

5.4. Discussion

5.4.1. Overview

The effects of diclofenac on fish have been investigated in only a limited number of studies utilising only a few selected species (Feito et al., 2012; Gröener et al.; Memmert et al., 2013; Praskova et al., 2011; Saucedo-Vence et al., 2015; Schwaiger et al., 2004; Stepanova et al., 2013). To date, little consideration has been given to differences in sensitivities across species. The current study, the first of its kind, was designed to directly compare sensitivity of two common freshwater fish models, the fathead minnow and zebrafish, to diclofenac. Once their acute sensitivities had been established via an LC_{50} analysis, both species were exposed to graded concentrations of diclofenac (0.001 – 10 mg L^{-1}). Overall, the results of the current study showed that concentrations up to 10 mg L^{-1} had no effect on oxidative stress endpoints (DNA damage, lipid peroxidation, GSH) in zebrafish. However, hatch rate and survival were impacted by exposure to diclofenac at 10 mg L^{-1}. In contrast to zebrafish, fathead minnows exposed to diclofenac exhibited a decreased lipid peroxidation at concentrations greater than 0.01 mg L^{-1}, and decreased DNA damage at 10 mg L^{-1}. Survival, and GSH were not affected by exposure to diclofenac in the fathead minnow.

5.4.2. LC_{50} values

Table 5.3. LC_{50} values for fish exposed to diclofenac

Species	**Exposure duration (h)**	**Diclofenac LC_{50} concentration (mg L^{-1})**	**Stage/Age**	**Reference**
African catfish	96	25.12	Adult	Ajima et al., 2014
Common carp	96	70.98	Juvenile (3 months)	Saucedo Vence et al., 2015
Fathead minnow	48	66	24 hph	Current study
Japanese medaka	96	10.1	Adult	Nassef et al., 2009
Medaka	96	8	Juvenile	Hong et al., 2007
Zebrafish	60	1.42	12 hpf	Chen et al., 2011
Zebrafish	144	6.11	8 hpf	Praskova et al., 2011
Zebrafish	48	21.75	12 hpf	Chen et al., 2011
Zebrafish	48	46	4 hpf	Current study
Zebrafish	24	93.03	12 hpf	Chen et al., 2011
Zebrafish	96	166.6	2-3 months	Praskova et al., 2011
Zebrafish	96	176.4	2-3 months	Praskova et al., 2011
Zebrafish	12	227.21	12 hpf	Chen et al., 2011

Zebrafish (4 hpf) exposed to diclofenac for 48 h exhibited an LC_{50} of 46 ± 21 mg L^{-1}. This value is within the range of other studies that have examined the lethal effects of diclofenac in zebrafish (see Table 5.3). These data show that LC_{50} values in this species vary from 1.4 to 227 mg L^{-1}, depending on duration of exposure and age of the study animals.

The LC_{50} value for fathead minnows was slightly higher (66 ± 9 mg L^{-1}) than that of the zebrafish (44 ± 20 mg L^{-1}), but not significantly so (p = 0.335; Figure 5.1). Previous research has demonstrated differences in species sensitivities (see Table 5.3). For example, common carp (aged 3 months) exposed to diclofenac had an LC_{50} value of 70.98 mg L^{-1} (Saucedo-Vence et al., 2015), whereas zebrafish of the same age exposed to diclofenac for 96 h have an LC_{50} more than 2 times higher (176.4 mg L^{-1}; Praskova et al., 2011). It is clear from the results of the current study, and those

of other researchers, that developmental stage, duration of exposure, and species can all play a role in determining the sensitivity of a species to diclofenac.

5.4.3. Total GSH

Species differences were also observed in response to sublethal exposures. There were no changes in the total amount of GSH in either fathead minnows or zebrafish exposed to diclofenac. Praskova et al. (2014) exhibited no changes to either whole body GST, or glutathione reductase after exposing 20-day old zebrafish to diclofenac (0.02 – 60 mg L^{-1}) for 28 days. GSH is an important cofactor for the activity of these enzymes (Lushchak, 2011), and thus the lack of change in GSH suggests that this may not be a sensitive endpoint for the investigation of diclofenac effects. This may relate to the key mechanism by which diclofenac is believed to generate oxidative stress. The Phase I metabolism of diclofenac generates a superoxide anion (Hong, 2007), and thus the most sensitive endpoint for diclofenac exposure is likely to be SOD, responsible for the dismutation of this ROS (Feito 2012; Nava-Alvarez et al., 2014). This is supported by previous research indicating that SOD is an important antioxidant involved in the detoxification of ROS generated from diclofenac exposure (Hong, 2007; Islas-Flores et al., 2013). Examination of a suite of antioxidant enzymes that are likely involved in the detoxification process, would be a beneficial next step in this research, allowing a more defined understanding of the mechanism by which diclofenac may influence antioxidant defences.

5.4.4. DNA damage

Exposure of zebrafish to diclofenac for 96 h had no significant impacts on the levels of DNA damage in this species, irrespective of exposure concentration.

However, at the highest diclofenac exposure concentration (10 mg L^{-1}) fathead minnow larvae exhibited a decrease in the DNA damage. Ghelfi et al. (2016) exposed juvenile South American catfish to diclofenac (0, 0.2, 2 and 20 µg L^{-1}) for 96 h. They too reported a decrease in the total amount of DNA damage. In their case fish exposed to 20 µg L^{-1} diclofenac showed a decrease in renal DNA damage, but levels of DNA damage in both the liver and blood were unaffected. They attributed this decrease in DNA damage to a decrease ROS, supported by further evidence of a reduction in lipid peroxidation in the kidneys (Ghelfi et al., 2016). The pharmacological mode of action of diclofenac is to inhibit the synthesis of prostaglandins, via inhibition of the COX enzymes, resulting in an anti-inflammatory effect (Gan, 2000). However, COX enzyme activity is associated with the production of ROS, and thus the presence of diclofenac may result in a decline in ROS production and a decrease in oxidative damage (Mouithys-Mickalad et al., 2004). In the current study, it is likely that the overall reduction in ROS protected against genotoxicity, albeit only at high exposure concentrations and only in fathead minnows.

The data presented here is in contrast to that produced by Rocco et al. (2011). These authors showed a loss of DNA integrity and significant increases in DNA damage in the whole body of adult zebrafish exposed to an environmentally-relevant concentration of diclofenac (0.18 µg L^{-1}) after 3 days. However, over time this damage decreased from 57% damage at 7 days, to 33% damage at 15 days, both values which were higher than the unexposed control. Nevertheless, this suggests that the DNA was able to undergo repair after prolonged exposure (Rocco et al., 2011). There is the possibility that DNA damage may have occurred in zebrafish exposed to diclofenac in the present study, however the duration of the study was long enough for repair to occur.

5.4.5. Lipid peroxidation

Lipid peroxidation, a consequence of oxidative damage to lipids, decreased as a result of exposure to diclofenac at concentrations higher than 0.01 mg L^{-1} in fathead minnows. These findings are similar to those seen for DNA damage in the current study, but are also supported by Stepanova et al. (2012) who described a decrease in oxidative damage in common carp in response to diclofenac exposure (3 mg L^{-1}). Although the Stepanova et al. (2013) study was conducted over a longer period of time (30 days), developmental stage and diclofenac exposure concentrations were similar to those of the current study. As described above for DNA damage, this decline in lipid peroxidation is likely to be the result of decreased ROS accumulation through inhibition of COX enzymes.

In the current study zebrafish displayed no significant changes in the oxidative damage. This contrasts with a previous study in this species, where a decrease in lipid peroxidation at diclofenac concentrations greater than 0.02 mg L^{-1} was observed (Praskova et al., 2014). It is likely that developmental stage explains these differences (see Section 5.4.6, below).

In another previous study, Feito et al. (2012) showed a decrease in lipid peroxidation in zebrafish embryos, again contrasting with the current study where no effect was noted. These authors conducted a 90-minute exposure and saw effects only at the lowest exposure concentration (0.03 µg L^{-1}). It is possible that this difference in response is due to the low concentrations of diclofenac used by Feito and colleagues. At low concentrations, an induction in antioxidant enzymes may have occurred, which elicited a hormetic effect, whereby antioxidant enzymes were triggered to be protective against oxidative damage (Feito et al., 2012). In agreement with this,

Petersen et al. (2005) demonstrated the protective effect of diclofenac again oxidative damage in human epithelial tissue. At low concentrations of diclofenac, there was less oxidative damage, which they attributed to a "priming" effect on antioxidant enzymes (Petersen et al. 2005).

5.4.6. Developmental differences

Fathead minnows displayed significantly different responses to diclofenac exposure, when compared to zebrafish. One explanation for this may be that zebrafish (4 hpf) and fathead minnows (24 hph) were exposed to diclofenac at different developmental stages. Zebrafish were in the embryonic developmental stage at test initiation, whereas fathead minnow were in the larval developmental stage for the duration of the study. Hallare et al. (2004) exposed zebrafish to diclofenac for 96 h, and did not see any impairment to development or induction of stress proteins in the embryos. They attributed this to the fact that the chorion provided a protective barrier, which reduced the uptake of diclofenac.

Overall, the decreases in oxidative damage (lipid peroxidation and DNA damage) may be attributed to diclofenac eliciting a pharmacological effect on fathead minnows. This has led to an overall improvement in health of the fish exposed to diclofenac. Sublethal exposure of diclofenac to the fathead minnow may therefore be beneficial. However, in recent years there is increasing interest in the roles that ROS may play as signalling molecules for normal physiological function (Weidinger and Kozlov, 2015). Consequently, there could be unintended physiological consequences associated with a diclofenac-mediated decrease in ROS.

Prior to this work there were no studies that directly compared toxic responses to diclofenac in two common model fish species. In the current chapter the use of

standardised testing was employed to generate direct comparisons between the two fish models. By conducting these studies simultaneously, differences such as those occurring as a result of seasonality and subtle differences in laboratory protocols were accounted for. However, one limitation associated with employing standardised US EPA (fathead minnow) and OECD (zebrafish) tests is that the exposures are initiated at differing developmental stages. Previous research has indicted that fish at later developmental stages are less sensitive to environmental contaminants (Kristofco et al., 2016), while in the current study there were notable differences in responses between the two test models that could be attributed to developmental stage. These differences in responses between fathead minnows and zebrafish pose difficulties in the development of regulatory tools for pharmaceuticals, as species sensitivities and developmental stages need to be accounted for.

Chapter 6. The effects of binary mixtures of cadmium and diclofenac on oxidative stress in the galaxiid fish, *Galaxias maculatus*

6.1. Introduction

Currently, tools for assessing water quality, and water regulations themselves, are mostly developed from toxicity data arising from exposures of individual contaminants (see Section 1.4). However, rarely does exposure occur in this manner in the natural environment, where instead mixtures of contaminants are most prevalent (Dethloff et al., 1999; Heys et al., 2016; Hinton and Aizawa, 2006). Therefore, it is important to gain an understanding of how common environmental toxicants interact, and the impacts that these mixtures have on aquatic biota (Hinton and Aizawa, 2006). Among the aquatic biota most at risk of toxicity resulting from mixtures are those species that inhabit water bodies that act as receiving environments for agricultural, industrial and urban waste water. Inanga is one such species (Section 1.5.1; McDowell, 2009; Harley, 2015).

In the current study, inanga was exposed for 96 h to diclofenac and Cd^{2+}, either as individual contaminants or in simple binary mixtures. Previous research in this thesis has shown that these toxicants individually impact oxidative stress endpoints in this important fish (Cd, Chapter 3; diclofenac, Chapter 4). Cd^{2+} concentrations were chosen to be representative of an environmental level (2.5 µg L^{-1}) and an effect concentration (10 µg L^{-1}; see Section 3.4), while the exposure concentration of diclofenac (770 µg L^{-1}) was the recommended environmental quality guideline derived by Kumar et al. (2016; Section 4.3). This is the first work examining the effects of binary mixtures of Cd^{2+} and diclofenac in any fish species. Given previous research (Sections 3.4 and 4.3) identified changes in oxidative stress parameters in inanga exposed to both of these toxicants, a suite of oxidative stress-related endpoints was examined, in inanga gill, liver and kidney. These endpoints included antioxidant defences (catalase, SOD and GST) and markers of oxidative

damage (protein carbonylation and lipid peroxidation). In Chapter 3 it was shown that Cd^{2+} acts as a pro-oxidant, while data in Chapter 4 showed that diclofenac acts as an antioxidant. Two hypotheses were therefore generated: 1. The presence of diclofenac will act to offset pro-oxidant effects of Cd; 2. The presence of Cd will act to offset antioxidant effects of diclofenac.

6.2. Methods

6.2.1. Animal collection and holding

Methods for fish collection and holding, water chemistry and environmental conditions are outlined in Section 2.2. The University of Canterbury Animal Ethics Committee approved all procedures.

6.2.2. Mixed exposures

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Experimental conditions are outlined in Section 3.2.2 and 4.2.2 . Desired exposure concentrations were achieved by dosing exposure chambers with stock solutions in 2 L of aquarium water. Exposures consisted of a control (no added Cd or diclofenac), diclofenac alone (nominally 770 µg L^{-1}), low Cd alone (2 µg L^{-1}), high Cd alone (10 µg L^{-1}), low Cd + diclofenac (2 µg L^{-1} Cd and 770 µg L^{-1} diclofenac), and high Cd + diclofenac (10 µg L^{-1} Cd and 770 µg L^{-1} diclofenac). Water was left for 24 h to equilibrate before the addition of fish. A total of 96 inanga (mean mass ± SD; 0.35 ± 0.17 g) were assigned randomly to one of 8 replicate chambers for each of the 6 treatment levels. After 96 h, fish were euthanised (0.1 g L^{-1} MS-222), weighed, and tissues (gill, liver and kidney) were collected. These tissues were snap-frozen in liquid nitrogen, before being stored at -80 °C until biochemical analysis. Each tissue sample represented that pooled from two fish.

Water samples (n = 3) were taken throughout the exposure using the same methods as Sections 3.2.2 and 4.2.2. Analysis via ICP-MS (Cd) and HPLC (diclofenac) was conducted using methods outlined in Sections 2.2.7 and 4.2.6. The LOQ for diclofenac in the water samples for the method was 209 μg L^{-1}. Recovery for diclofenac was 84% (n = 3). Linear regression for diclofenac standard curve (1, 10, 50, 100, 250, 500, 1000 μg L^{-1}) resulted in $r^2 = 0.997$. Detection limits for Cd were calculated as three standard deviations of the mean blank concentration (0.003 μg L^{-1}).

6.2.3. Measurement of oxidative stress

Gill, kidney and liver tissue were homogenised using 1600 μL buffer (100 mM Tris-HCl, 2 mM EDTA and 5 mM $MgCl_2$) using a plastic homogeniser. This homogenate was then divided among the various assays (GST, 200 μL; SOD, 200 μL; catalase, 200 μL; lipid peroxidation, 200 μL; protein carbonylation, 400 μL; protein assay, 30 μL) and stored at -80°C until assays were conducted. Catalase activity and lipid peroxidation were measured using methods described in Section 2.2.5 and 2.2.6.

GST activity was quantified in gill, kidney, and liver tissue using a GST kit, according to manufacturer's instructions (CS0410, Sigma-Aldrich). GST activity was determined by the rate of conjugation of the thiol group in GSH to the 1-chloro-2, 4-dinitrobenzene (CDNB) substrate, via the measurement of the conjugated product (GS-DNB) at 340 nm. The rate of increase in absorption is directly proportional to the GST activity in the sample. GST activity was expressed as μmol mg $protein^{-1}$ min^{-1}, where protein was determined as described in Section 2.2.5.

SOD was quantified in gill, kidney and liver tissue using a SOD kit, according to manufacturer's instructions (19160, Sigma-Aldrich). SOD activity was determined

by measuring the rate that O_2 was reduced, which is linearly related to the xanthine oxidase activity which is inhibited by SOD. The assay was conducted by adding Dojindo's highly water-soluble tetrazolium salt (WST-1; 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to the tissue, incubating at 37°C for 20 min and reading immediately on a plate reader at 450 nm. The activity of SOD was expressed as U SOD mg protein $^{-1}$, where protein was determined as described in Section 2.2.5.

Protein carbonylation was determined in gill, kidney and liver tissue using a commercially-available assay kit according to manufacturer's instructions (MAK094, Sigma-Aldrich). Protein carbonyl content was determined by the derivatisation of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of stable dinitrophenyl (DNP) hydrazone adducts, which were measured spectrophotometrically at 375 nm. This was achieved by adding DNPH (100 µL) to each sample and incubating at room temperature for 10 min. 100% trichloroacetic acid was then added to the solution and incubated on ice for 5 min before being centrifuged at 13,000 x *g* for 2 minutes. The supernatant was removed and ice cold acetone was then added to the pellet, sonicated for 30 s, and incubated at -20°C for 5 min, followed by centrifugation for a further 2 min. The acetone was removed and 200 µL of 6 M guanidine solution was added to the pellet and sonicated briefly to solubilise. Protein carbonyl content was expressed as nmol carbonyl mg protein^{-1}, where protein was determined as described in Section 2.2.5.

6.2.4. Statistical analysis

Data were tested for normality using the Shapiro-Wilk test, and any failing data were log-transformed. All data were then analysed by one-way ANOVA

followed by a Tukey HSD post-hoc test. All analysis was performed using RStudio (RStudio version 3.1.0). Statistical significance was set at $p < 0.05$ and all data are expressed as mean ± SD.

6.3. Results

There were no mortalities recorded during the exposures. Measured concentrations of Cd^{2+} and diclofenac are outlined in Table 6.1, and were close to nominal values. There was no diclofenac, and only low Cd, detected in the controls. Measured concentrations for both Cd and diclofenac are used from this point forth.

Table 6.1. Nominal and measured concentrations of diclofenac and cadmium.

	Nominal (μg L^{-1})		Measured (μg L^{-1})	
Treatment name	Diclofenac	Cadmium	Diclofenac	Cadmium
Control	0	0	<LOD	0.03 ± 0.01
$Diclofenac_{770}$	770	0	859.7 ± 154.9	0.04 ± 0.01
Cd_2	0	2	<LOD	1.8 ± 0.4
Cd_9	0	10	<LOD	9.2 ± 0.3
Cd_2 /$diclofenac_{770}$	770	2	752 ± 230.7	2.1 ± 0.03
Cd_9 /$diclofenac_{770}$	770	10	656.9 ± 211.6	9.3 ± 0.06

Values are expressed as mean ± SD (μg L^{-1}; n = 3). <LOD = below limit of detection

6.3.1. Antioxidant enzymes

There was no significant change in the catalase activity in the gill of inanga exposed to Cd^{2+} or diclofenac individually, or to mixtures of these contaminants (ANOVA $p = 0.1598$; Figure 6.1). There was, however, a significant decrease in catalase activity in the kidney after exposure to both the Cd_9 treatment ($p = 0.002$) and the Cd_9/$diclofenac_{770}$ treatment ($p = 0.03$), relative to the control exposure (Figure 6.2). There was a significant increase in the catalase activity in liver of inanga exposed to Cd_9 ($p = 0.02$), $diclofenac_{770}$ ($p = 0.003$), and Cd_9/ $diclofenac_{770}$ treatment ($p = 0.007$; Figure 6.3).

There was no significant effect of any of the exposure treatments on GST activity in the gills of inanga (ANOVA $p = 0.764$; Figure 6.4). GST activity in the kidney was significantly decreased after being exposed to Cd_2 and Cd_9/diclofenac$_{770}$ treatments, when compared to the control ($p < 0.005$ and $p = 0.009$, respectively; Figure 6.5). There was no significant difference between any of the treatments for GST activity in the liver of inanga (ANOVA $p = 0.7032$; Figure 6.6).

Gills of inanga exposed to the Cd_2 treatment had impaired SOD activity in comparison to the Cd_2/diclofenac$_{770}$ treatment ($p = 0.03$; Figure 6.7). None of the other exposures significantly affected branchial SOD activity. Cd_2 exposure resulted in the impairment of SOD activity in the kidney of inanga, relative to the control ($p = 0.01$), diclofenac$_{770}$ ($p = 0.02$), Cd_9 ($p = 0.007$), and Cd_2/diclofenac$_{770}$ ($p = 0.04$) treatments (Figure 6.8). There was no significant effect of any exposure regime on hepatic SOD activity (ANOVA $p = 0.18$; Figure 6.9).

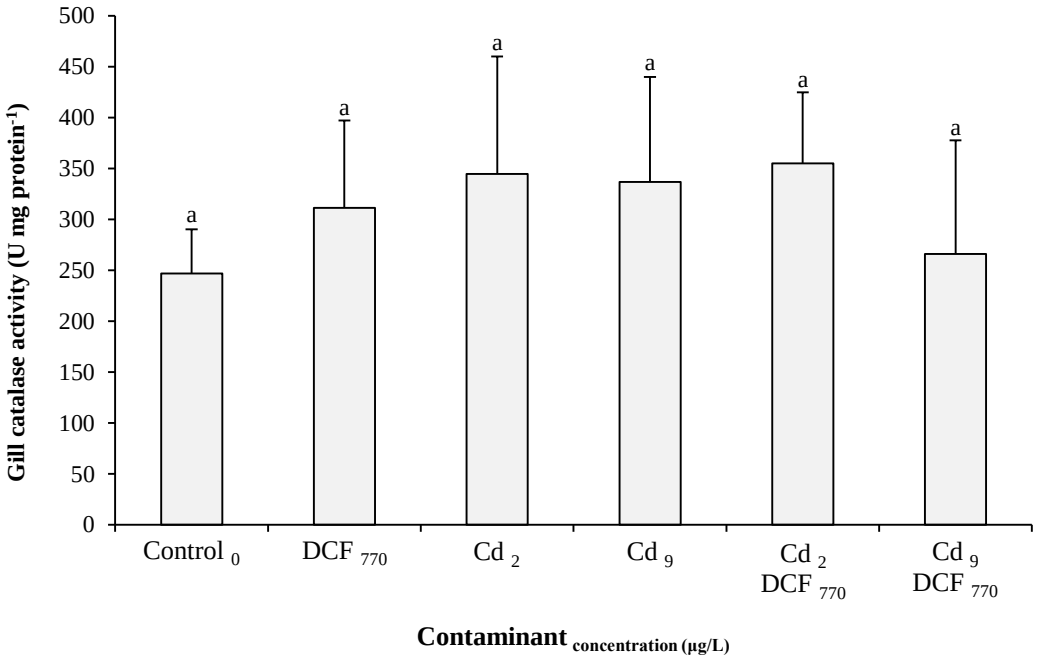


Figure 6.1. Gill catalase activity (U mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/or diclofenac (DCF) for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

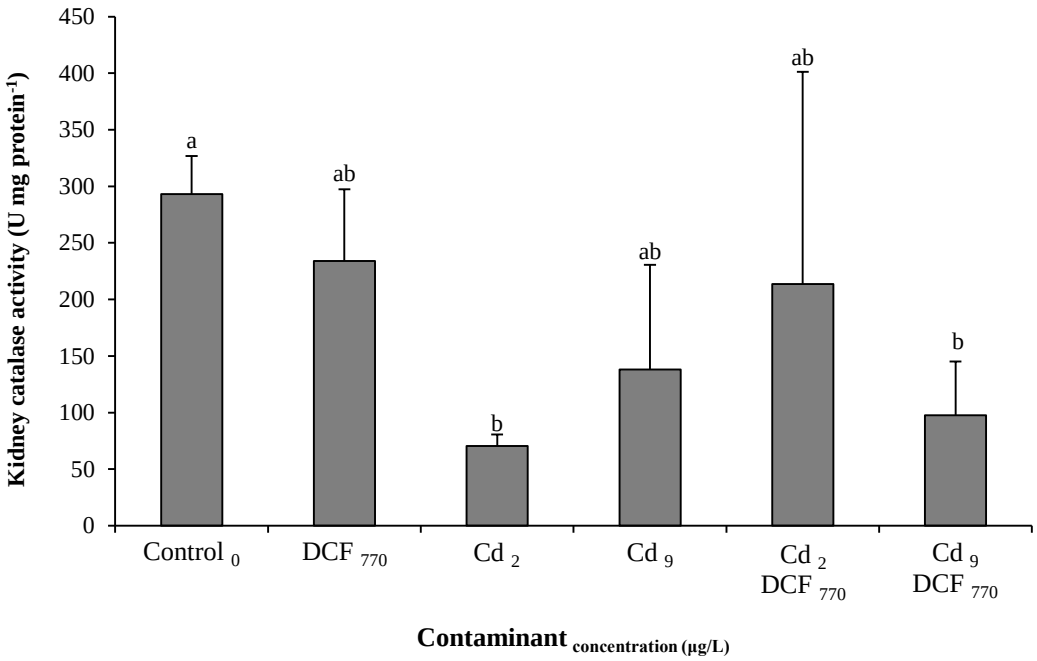


Figure 6.2. Kidney catalase activity (U mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/or diclofenac (DCF) for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

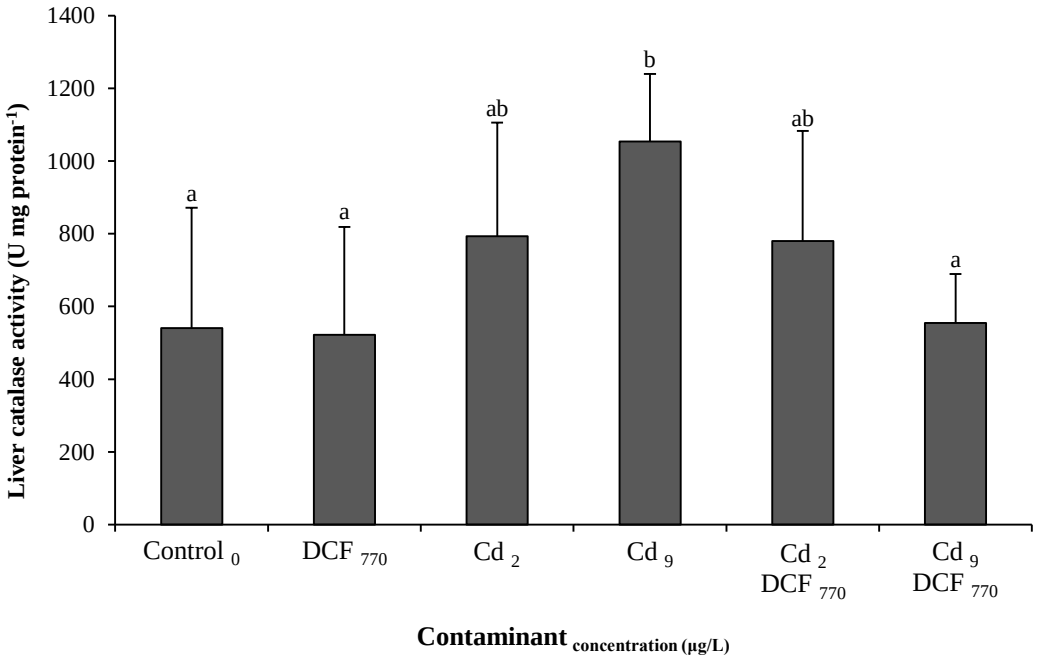


Figure 6.3. Liver catalase activity (U mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/or diclofenac (DCF) for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

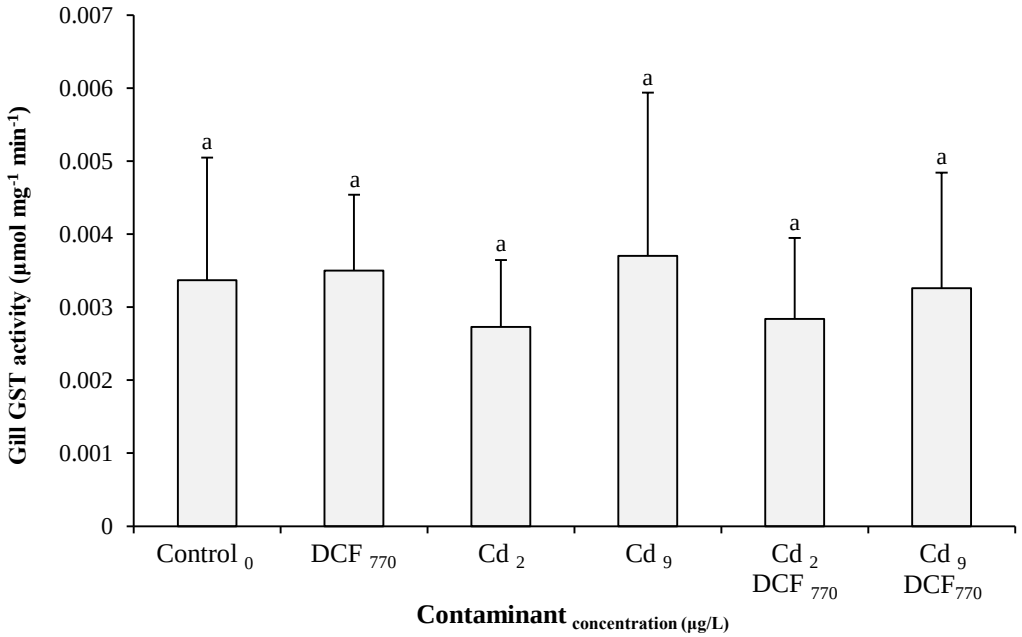


Figure 6.4. Gill glutathione S-transferase (GST) activity (µmol mg^{-1} min^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac (DCF) for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

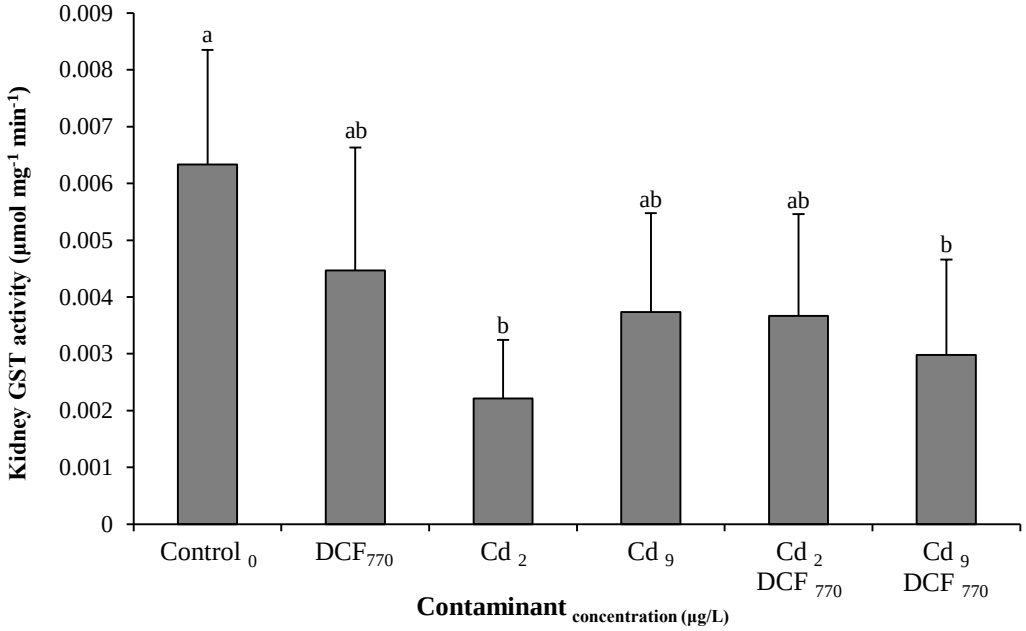


Figure 6.5. Kidney glutathione S-transferase (GST) activity (µmol mg^{-1} min^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac (DCF) for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

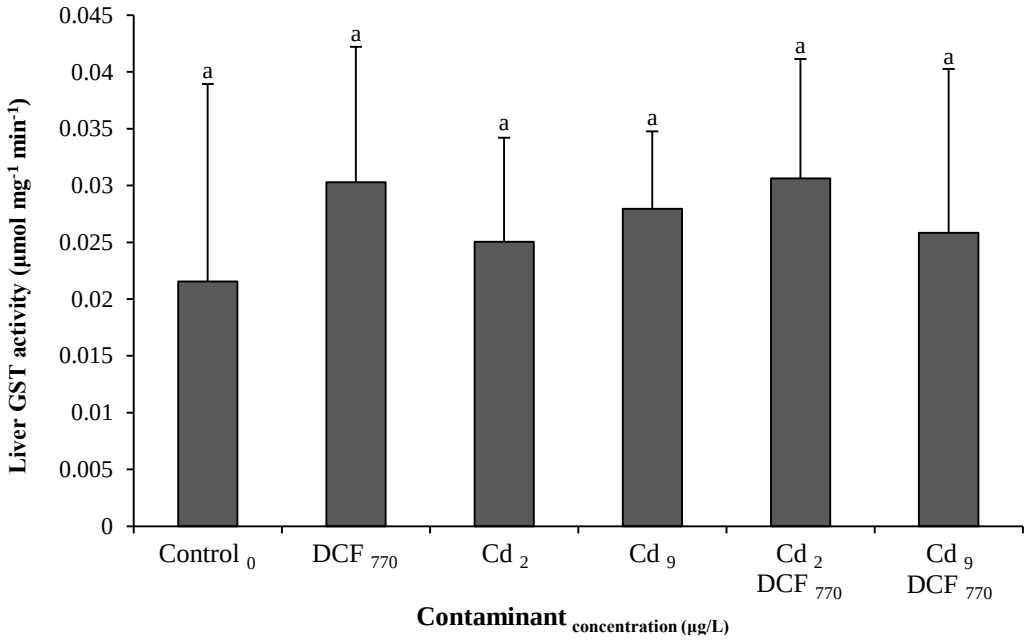


Figure 6.6. Liver glutathione S-transferase (GST) activity (µmol mg^{-1} min^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac (DCF) for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

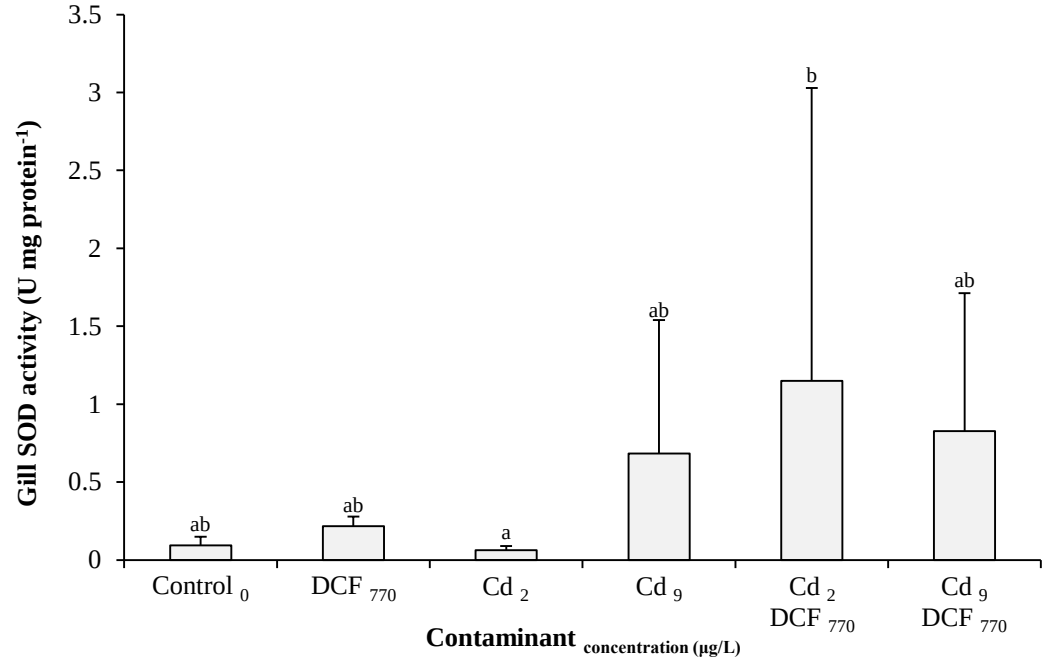


Figure 6.7. Gill superoxide dismutase activity (U mg $protein^{-1}$; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

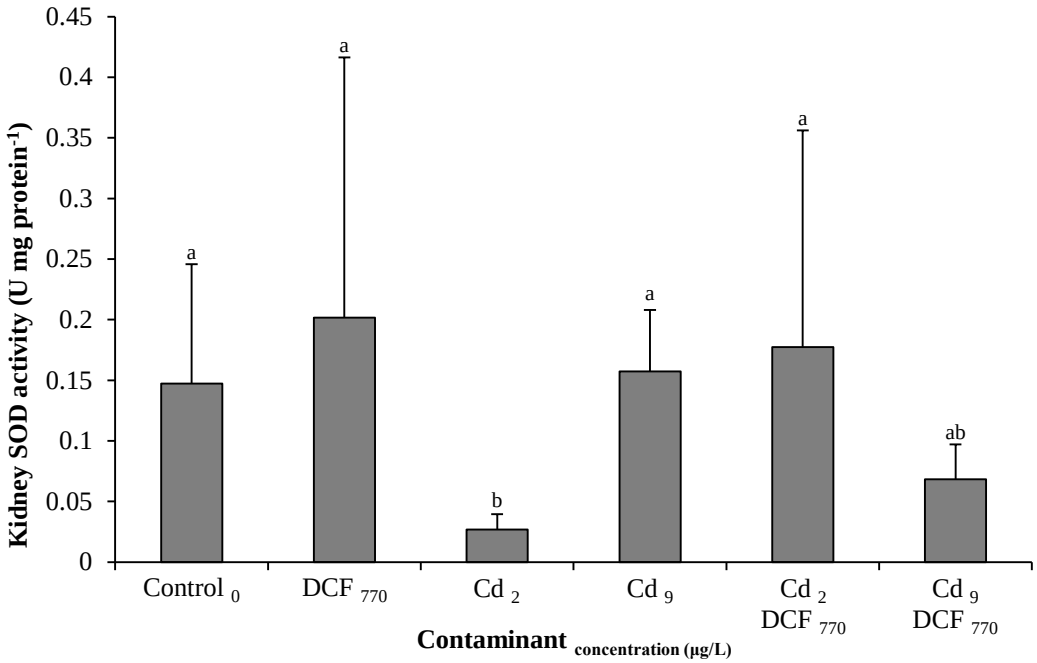


Figure 6.8. Kidney superoxide dismutase activity (U mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

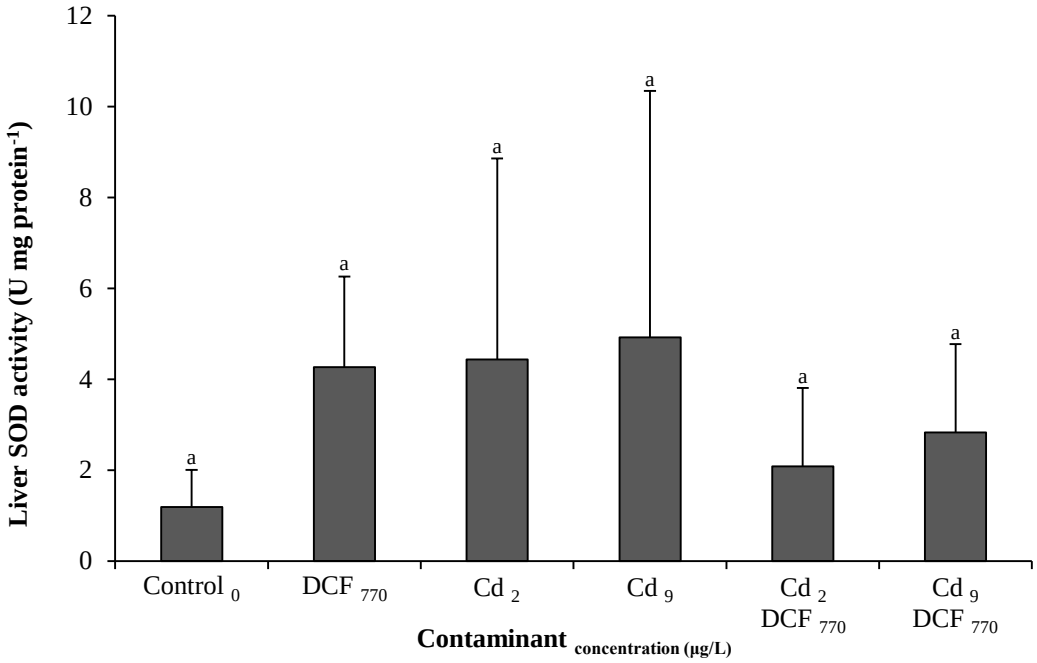


Figure 6.9. Liver superoxide dismutase activity (U mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

6.3.2. Oxidative damage

There were no changes in lipid peroxidation in the gills of inanga exposed to Cd^{2+} and/or diclofenac (ANOVA $p = 0.350$; Figure 6.10). There was, however, a significant decrease in lipid peroxidation in the kidney after exposure to the diclofenac$_{500}$ ($p = 0.01$), Cd_2 ($p = 0.02$), and Cd_9/diclofenac$_{770}$ ($p = 0.02$) relative to the Cd^{2+} and diclofenac-free control (Figure 6.11). There was a significant increase in lipid peroxidation in the liver of fish exposed to Cd_9, when compared to the control ($p = 0.03$), Cd_2 ($p = 0.02$), Cd/diclofenac$_{770}$ ($p = 0.004$), and Cd_9/diclofenac$_{770}$ ($p = 0.006$; Figure 6.12).

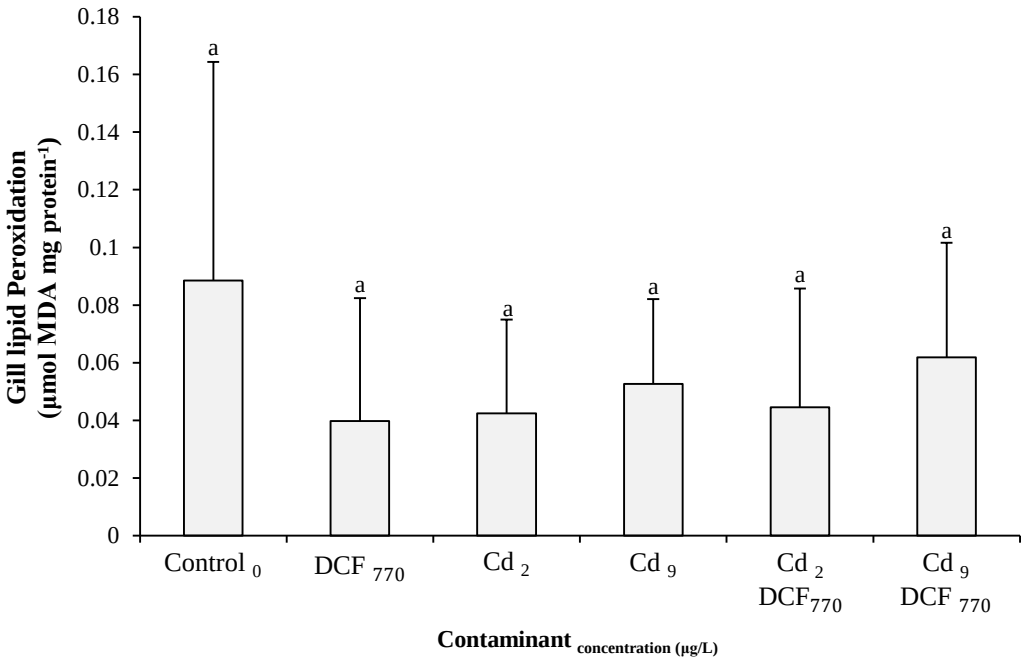


Figure 6.10. Gill lipid peroxidation (µmol MDA mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

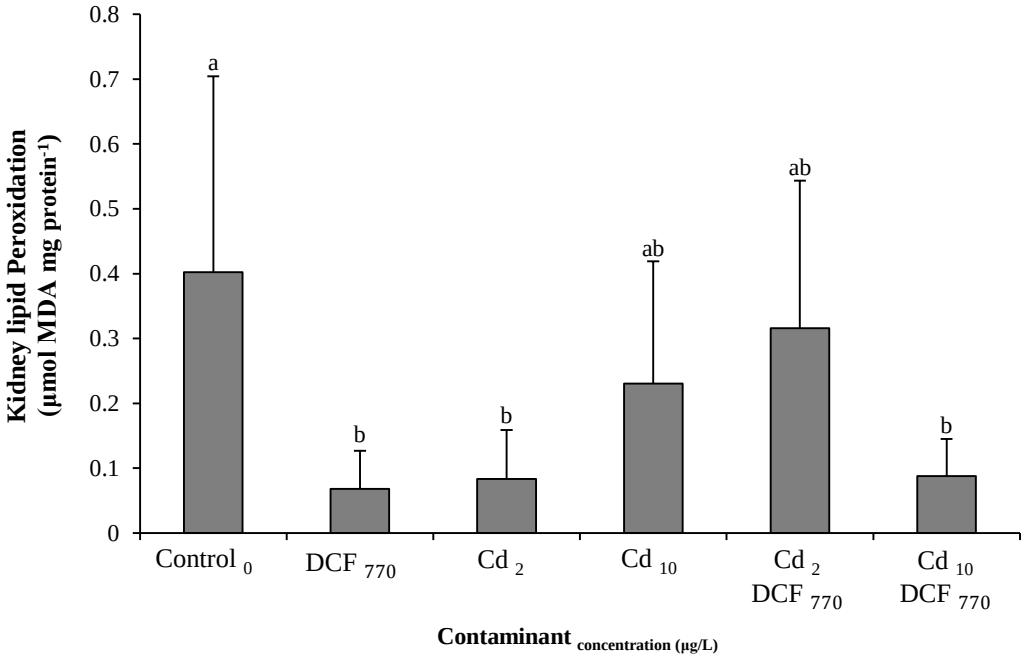


Figure 6.11. Kidney lipid peroxidation (µmol MDA mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

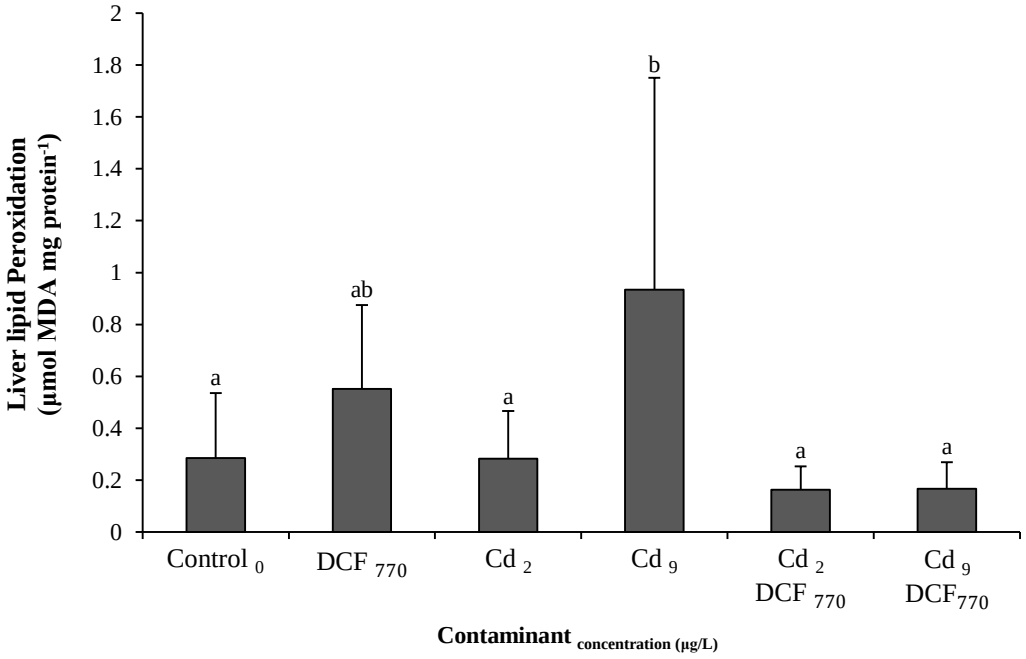


Figure 6.12. Liver lipid peroxidation (µmol MDA mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; $\alpha = 0.05$).

There was a significant increase in protein carbonylation in the gills of inanga exposed to Cd_9, relative to diclofenac$_{770}$ ($p = 0.04$), and Cd_2 ($p = 0.05$; Figure 6.13).

However, protein carbonylation was not altered after exposure to any of the treatments in the kidney and the liver (ANOVA p = 0.109 and 0.098 respectively; Figure 6.14; Figure 6.15).

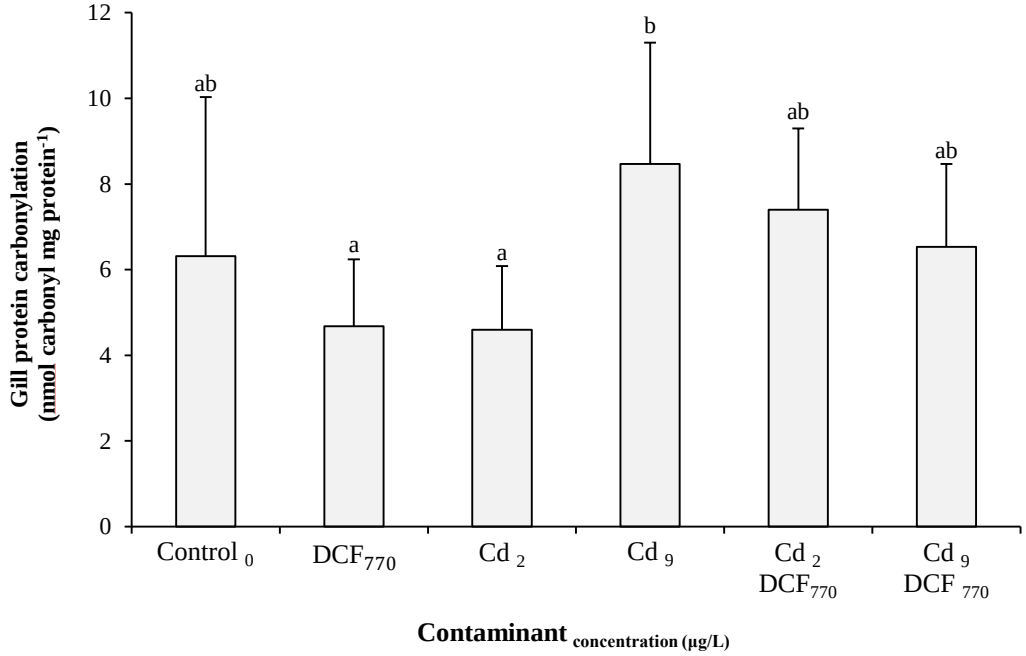


Figure 6.13. Gill protein carbonylation (nmol carbonylation mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

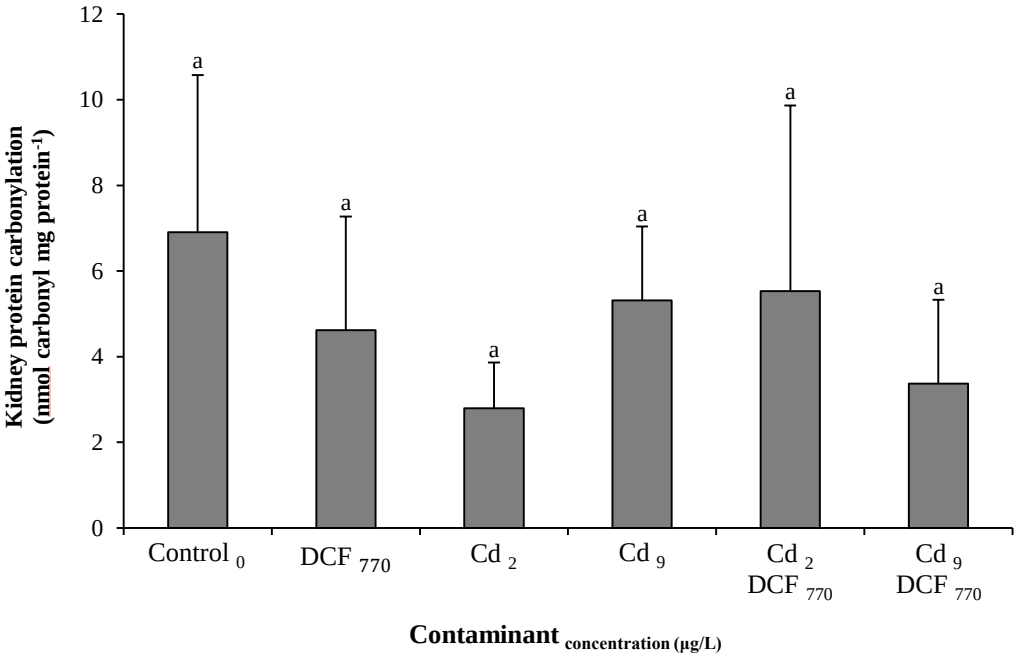


Figure 6.14. Kidney protein carbonylation (nmol carbonylation mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

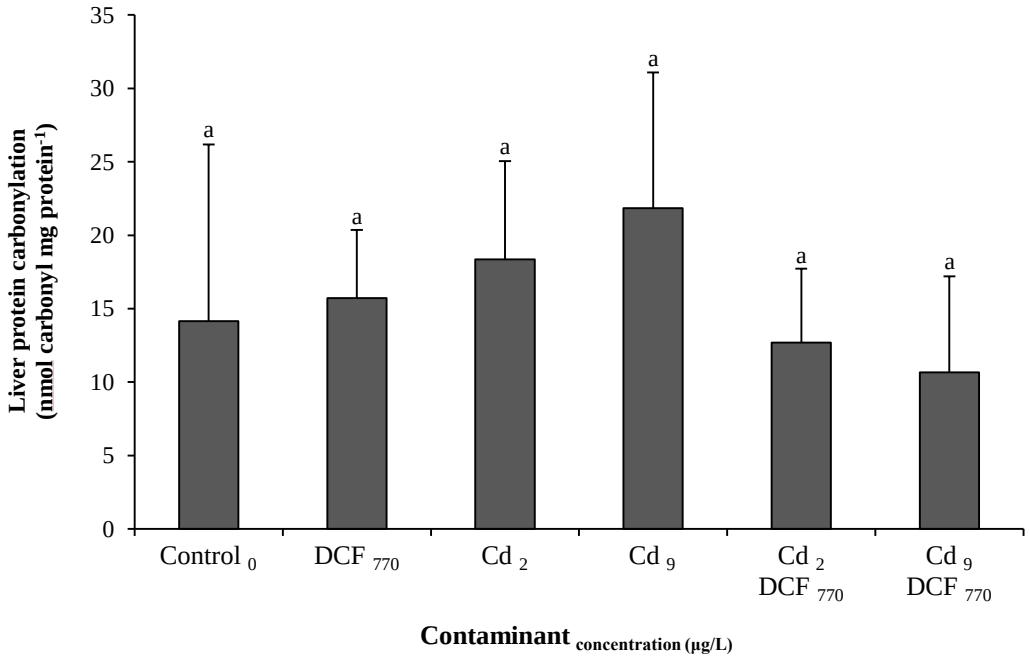


Figure 6.15. Liver protein carbonylation (nmol carbonylation mg protein^{-1}; mean ± SD) in inanga (*Galaxias maculatus*; n = 5-8), after exposure to Cd and/ or diclofenac for 96 h. Bars sharing letters are not significantly different (one-way ANOVA followed by post-hoc Tukey test; α = 0.05).

6.4. Discussion

6.4.1. Effect of diclofenac on Cd toxicity

Cd^{2+} has been shown to cause an increase in oxidative damage in inanga, at the concentrations used in the current study (Section 3.3.4). In contrast, diclofenac is an antioxidant, as shown by the previous work, whereby diclofenac reduced lipid peroxidation in both fathead minnows (Section 5.4.5) and inanga (Section 3.4.4). The results from the current study show that diclofenac exposure clearly offsets Cd-induced impairment of antioxidant enzymes and the induction of oxidative damage. This supports the first hypothesis: that the presence of diclofenac will act to offset pro-oxidant effects of Cd.

In the current chapter, diclofenac reduced the Cd-mediated increase in catalase activity (Figure 6.2; Figure 6.3). Diclofenac also reduced the induction of damage (lipid peroxidation) caused by Cd in the liver (Figure 6.12), and increased SOD activity back to control levels in the kidney (Figure 6.8) and gill (Figure 6.7). In fact, diclofenac stimulated an increase in branchial SOD above control levels at the higher Cd^{2+} (9 µg L^{-1}) exposure concentration. The improvement in antioxidant status in the presence of diclofenac likely offsets the lipid peroxidation induced by Cd.

Cd has commonly been seen to induce oxidative damage in fish by binding to the active site of antioxidant enzymes and through the generation of ROS by displacing iron in the Fenton reaction (see Section 1.2.3.3; Atli and Canli, 2007; Hisar et al., 2009; McGeer et al., 2012; Nunes et al., 2015). Results from Sections 3.3.4, 4.3.1 and the current chapter strongly suggest that the accumulation of ROS decreases significantly when fish are exposed to diclofenac. This can occur through two mechanisms (Section 1.3.1.4). Either diclofenac directly decreases the amount of ROS produced, or it increases the activity and/or amount of antioxidant pathways present. The first option is most likely. The main mechanism of action for diclofenac results in inhibition of ROS production through inhibition of the COX enzymes (Gan, 2010; Mouithys-Mickalad et al., 2004). The current results show that Cd alone inhibits SOD activity, most likely through direct inhibition (Valko et al., 2005). However, when exposed at the same time as diclofenac, activity of SOD is restored to control levels in the kidney and gills. One explanation for this observation is that diclofenac may bind to Cd^{2+} (see Section 6.4.2., below). If binding occurs then this may prevent Cd from interacting with SOD, accounting for the restored activity.

Catalase activity increased in the liver after exposure to Cd^{2+}, suggesting this was an induction response, designed to increase defence in light of increased ROS. Although this response differs from that previously observed for Cd^{2+} exposures in this species (see Chapter 3 and Section 6.4.3, below), this increase in catalase activity has been observed previously in fish (Nunes et al., 2015; Pretto et al., 2011). However, when Cd^{2+} exposure occurred in the presence of diclofenac, catalase activity was restored to control levels. This could be achieved by diclofenac reducing COX-induced ROS generation to a level whereby the additional ROS generated by Cd is sufficient for catalase activity to cope without the need for induction.

The antioxidant effects of diclofenac have been previously observed in inanga, but have also been seen in other species (Stepanova et al., 2013; Praskova et al., 2014; Fieto et al., 2012; see Sections 4.4 and 5.4.2.). However, these results are the first to show that the co-exposure of an antioxidant pharmaceutical in diclofenac, has the capacity to offset the pro-oxidant effects of metal toxicity.

6.4.2. Effect of Cd on diclofenac impacts

In contrast to the effect of diclofenac on Cd, Cd appeared to have no effect on the oxidative stress changes induce by diclofenac exposure. None of the endpoints displayed a significant difference between diclofenac exposure alone, versus the same parameter in the presence of diclofenac and Cd^{2+}. This was despite significant effects of diclofenac compared to control exposures (decrease in kidney lipid peroxidation; Figure 6.11). These results argue against the second proposed hypothesis: that the presence of Cd will act to offset antioxidant effects of diclofenac.

The lack of effect of Cd on oxidative stress status in diclofenac exposures likely relates to the molar concentration of diclofenac (1.7 μM) being significantly

greater than that of Cd^{2+} (89 nM at 9 µg L^{-1}). Diclofenac has the ability to bind Cd^{2+} (Tabrizi et al., 2015). As such the binding of Cd^{2+} by diclofenac would alter the speciation of Cd^{2+} in the water. It is considered that only ionic Cd^{2+} is bioavailable to fish (McGeer et al., 2012), and thus the presence of diclofenac in the water is likely to significantly decrease Cd^{2+} bioavailability and toxicity (Niyogi et al., 2008; Verbost et al., 1988). Even if a Cd-diclofenac species was bioavailable, it may be a relatively inert species. There is, for example, evidence that Cd-diclofenac species have a distinct bioreactivity to either Cd or diclofenac alone, at least in mammalian cell lines (Tabrizi et al., 2015).

6.4.3. Responses of oxidative stress to individual exposures

A key finding of the current study is the variation in oxidative stress responses depending on the timing of exposures. As exposure concentrations in the current chapter were derived directly from previous work (Cd, Chapter 3; diclofenac, Chapter 4), and with an overlap in some of the measured endpoints (catalase, lipid peroxidation), comparisons in responses between different batches of wild-caught fish can be examined. For example, inanga exposed to Cd^{2+} for 96 h in Chapter 3 demonstrated decreased catalase activity in the liver after exposure to both 2.5 and 10 µg L^{-1} (Figure 3.5). However, fish exposed to Cd for 96 h in the same facility in identical water chemistry in the current study exhibited an increase in catalase activity in the liver after exposure to 9 µg L^{-1}, and no change at the lower exposure concentration (2 µg L^{-1}). Similarly, inanga exposed to 763 µg L^{-1} diclofenac in Chapter 4 exhibited a decrease in gill catalase activity, and an increase in liver catalase activity. However, in the current study, catalase activity in both the gills and liver were unaffected by diclofenac exposure.

Lipid peroxidation also demonstrated differing response patterns between this chapter and previous work. For example, fish exposed to 10 μg L^{-1} Cd in Chapter 3 exhibited no change in kidney lipid peroxidation and an increase in liver lipid peroxidation. Conversely, in the current study exposure to Cd (2 μg L^{-1}) caused a decrease in kidney lipid peroxidation (Figure 6.10), and exposure to Cd (both 2 and 9 μg L^{-1}) did not induce changes to lipid peroxidation levels. Diclofenac exposure in the previous study (Chapter 4) resulted in decreased lipid peroxidation in the gills (Figure 4.2) and kidney (Figure 4.4) of inanga, and liver lipid peroxidation levels increased in response to diclofenac exposure. However, in the current study lipid peroxidation levels remained unchanged compared to the control after exposure to diclofenac.

Differences in catalase activity and lipid peroxidation are not likely due to exposure concentration differences, as there is no distinct pattern to explain the responses. Exposures were also conducted with time-matched controls so differences in basal activities can be accounted for. It is therefore likely that variations in the responses to Cd and diclofenac relate to seasonal effects. Inanga used in all chapters in this thesis were obtained from natural populations sourced from the same streams, and then housed for a period of time in the laboratory. All animals used in exposures were juveniles, but were caught at different times of the year, and thus differed in terms of environmental exposure history and season of collection. Furthermore, inanga sex cannot be determined by external examination, and even following dissection juvenile fish are difficult to sex. As such, exposures carried out for different chapters may have been impacted by these sources of variation. There is literature support for these factors (sex, season, exposure history) playing a role in differential responses. Sanchez et al. (2008) conducted a survey looking at how stickleback oxidative stress endpoints changed throughout the year, and as a function

of fish sex. Their results showed that GPx was significantly lower in males compared to females, while Phase 1 metabolism, GPx, and total GSH varied throughout the year, also in a sex-dependent manner.

Chapter 7. General Discussion

7.1. Summary of findings

The first objective of this thesis was to determine how exposure to environmentally-relevant concentrations of Zn, Cd, and diclofenac affects biochemical (catalase, lipid peroxidation) and physiological (metabolic rate, ion regulation) endpoints in inanga. Once initial studies identified oxidative stress as an important mechanism of action for all of these toxicants, this endpoint became the focus of the research. Subsequent objectives therefore sought to: determine how exposure to trace metal (Cd) and pharmaceutical (diclofenac) mixtures impact oxidative stress; and establish how a diclofenac affects oxidative stress in model North American fish species. To achieve this, an expanded suite of biochemical oxidative stress endpoints (including SOD, catalase, GST, GSH, lipid peroxidation, DNA damage, protein carbonylation) was used.

7.1.1. Impact of Zn and Cd on inanga

The sensitivity of inanga to an essential (Zn) and a non-essential (Cd) metal were explored in Chapters 2 and 3. Zn^{2+} exposure caused increases in catalase activity and lipid peroxidation, but only at the highest exposure level tested (1000 μg L^{-1}; Section 2.2.3). Zn^{2+} also significantly inhibited Ca^{2+} influx (Section 2.3.2). The sublethal changes induced by Zn^{2+} exposure in inanga appeared to be conserved relative to other, better-studied, fish species, however there were some notable exceptions. For example, the effect of Zn on Na^{+} influx was the opposite of that predicted, and the mechanism underlying this effect may require further investigation. These data were the first to explore the sensitivity of any galaxiid fish to Zn, information that will be critical to ensuring adequate environmental protection of this important species.

Inanga demonstrated similar mechanisms of sublethal Cd toxicity compared to other fish species, but again there were subtle differences in effects. Significant accumulation of Cd in the whole body of inanga only occurred after exposure to 10 μg L^{-1} (Section 3.4.1). Accumulation did not, however, correlate to toxic impacts. For example, oxidative damage occurred in the liver at Cd concentrations in excess of 2.5 μg L^{-1} (Section 3.4.4). However, tissue-specific Cd burdens were not able to be determined in this study, and such data may provide a better indicator of the relationship between accumulation and effect. Furthermore, subcellular fractionation approaches, that assign Cd to biologically active and biologically inert fractions (e.g. Wallace and Lopez, 1997), may offer a clearer picture of how accumulation relates to toxicity. Physiological (ion transport and metabolic rate) and biochemical (catalase and lipid peroxidation) endpoints in the kidney, were not impacted by exposure to Cd, likely due to the low exposure concentrations and acute nature of the study. These tissue-specific responses highlight the importance of the liver in Cd toxicity, at least in acute exposures. A notable finding in this study was the lack of effect of Cd^{2+} on Ca^{2+} influx, given the large volume of literature suggesting an interaction between these ions (e.g. Richards and Playle, 1999; Hollis et al., 2000; Franklin et al., 2005). This was attributed to the low level, acute exposure conducted in the current study. Future work examining impacts over longer time-frames may be of value.

7.1.2. Impact of diclofenac on inanga, fathead minnows and zebrafish

The sensitivity of inanga to the pharmaceutical, diclofenac, was examined in Chapter 4. At an environmentally-realistic exposure concentration (0.17 μg L^{-1}), a BCF of 87 was calculated, approaching values where transfer through the food chain could be important (Section 4.4.3). Lipid peroxidation was tissue-specific, with increases in the liver but decreases in kidney and gill tissue (Section 4.4.1). Catalase

activity was also elevated in the liver of inanga, but activity decreased in the gill. There were no effects of diclofenac on metabolic rate or ion influx rates (Section 4.4.2). These data identified oxidative stress as a key endpoint of diclofenac exposure, with clear tissue-specific differences in responses. These could relate to the role of the liver in detoxification of organic toxicants, and the potential generation of superoxide anions through Phase I metabolism (i.e. generating oxidative stress; Hong, 2007), and other tissues where diclofenac may have pharmacological effects (i.e. inhibition of ROS associated with inflammation pathways; Mouithys-Mickalad et al., 2004).

Following on from the finding of oxidative stress as a key variable altered during diclofenac exposure in fish, work was initiated to examine this endpoint in more detail. Chapter 5 therefore focussed on the impact of diclofenac on two model Northern Hemisphere species (zebrafish and fathead minnow). Preliminary toxicity testing showed that zebrafish are not likely to be impacted by environmental concentrations of diclofenac, as their LC_{50} value was beyond concentrations likely to be found in aquatic environments (Acuña et al., 2015), and similarly sublethal endpoints were also not impacted by exposure to diclofenac concentrations up to 7 mg L^{-1} (measured value; Section 5.4). Fathead minnows displayed characteristics more similar to those "pharmacological" effects of diclofenac seen in inanga, with decreased lipid peroxidation after exposure to 0.01 mg L^{-1} of diclofenac (Section 5.3.6). This study highlighted different responses in two key model fish species in near-identical exposure conditions, and suggests that future regulations may need to account for species differences and/or differences in developmental stage.

7.1.3. Impact of diclofenac and Cd mixtures on inanga

Inanga were utilised to determine the impacts of simple binary mixtures of a pro-oxidant (Cd) and an antioxidant (diclofenac) on oxidative stress endpoints (Chapter 7). These were the first data to investigate mixtures of diclofenac with metals. Clear interactions between Cd and diclofenac were observed, with the ability of diclofenac to offset Cd-induced oxidative stress being the most notable effect (Section 6.4.1). Another key finding of this study was that basal toxic responses differed with the timing of experiments, with responses of inanga to diclofenac and Cd alone in this mixture study, sometimes distinct from those in previous chapters. Future work examining the sources of variation in these responses would be of significant value.

7.2. Environmental implications

The results outlined in Chapter 2 indicate that regulatory tools that rely on conserved mechanisms of toxicity between species, such as the BLM, are likely to be of general applicability to inanga, a widespread and important Southern Hemisphere fish. Significant effects of Zn^{2+} on inanga were restricted to an exposure concentration of 1000 µg L^{-1} (Section 2.3). This is a level found only in extreme environmental exposure scenarios, such as those associated with acid mine drainage-contaminated waters on NZ's West Coast (Harley, 2015). Although highly contaminated, these streams still provide potential habitats for inanga and other galaxiid species (Harley, 2015). Zn toxicity could, therefore, be an issue for galaxiid fish inhabiting these water bodies, and could be a factor driving population decline. Currently, the 95% trigger value for Zn^{2+} in NZ freshwaters is 8 µg L^{-1} (ANZECC/ARMCANZ, 2000). This is the value thought protective for 95% of freshwater species. The results of the

current study show that this value is sufficiently low to protect inanga from acute sublethal Zn^{2+} toxicity.

In contrast, exposure to Cd^{2+} (Chapter 3) at low, environmentally relevant concentrations (2.5 µg L^{-1}) induced oxidative damage and decreased antioxidant capacity in the liver of inanga (Section 3.3.6). Therefore, even at very low concentrations, Cd had the ability to significantly impact inanga biology. Although inanga are likely to be protected from Cd toxicity by the ANZECC guideline concentration (0.2 µg L^{-1}; 95 % protection), the results from this study clearly show that inanga may be negatively impaired by exposure to Cd in contaminated water bodies (Section 3.5). As NZ soils have high concentrations of Cd, due to the use of superphosphate fertilisers (Section 1.2.3; McDowell et al, 2009), the presence of Cd in inanga habitats may be an issue of concern.

It is, however, important to highlight that there are key mechanistic differences in the responses of inanga to both Zn^{2+} and Cd^{2+}, relative to model species (e.g. the effect of Zn^{2+} of Na^{+} influx, and the lack of effect of Cd^{2+} on Ca^{2+} influx). Tools such as the BLM rely on conserved mechanisms of uptake and toxicity for accurate prediction of "safe" water metal concentrations (Santore et al., 2002). For species that do not conform to the established paradigm, such as inanga, then there may have to be additional research to calibrate the model (Niyogi and Wood, 2004).

In simple mixtures, diclofenac reversed Cd-induced oxidative damage (Section 6.3). While the outcomes, even from simple mixture studies, are complex, these data showed that mechanisms of interactions can begin to be elucidated by this approach. The presence of contaminant mixtures is a likely environmental scenario (Cedergreen, 2014), and this study has shown that using lethal and sublethal data from

individual contaminants in regulatory tools may not be representative of toxic effects occurring in the environment. However, the large range of different contaminants, and fluctuations in their concentration over time and space, provides a challenge that can only be addressed by mechanistic approaches to understanding how contaminants interact to impact biological health (McCarty and Borgert, 2006).

7.3. Future research

This thesis clearly identified the lack of knowledge regarding the concentration of pharmaceuticals in the NZ environment as an important information gap. Currently there is only one study that has investigated the presence of diclofenac in the NZ environment, and this study was limited to a single environmental matrix (sediment), in a single geographical location (Auckland; Stewart et al., 2014). Expanding environmental occurrence data would help to guide regional councils to develop effective management strategies, by allowing the data presented in the current thesis to act as an initial guideline for ensuring that populations of inanga are not impacted by the effects of diclofenac.

Additional studies examining the sensitivity of NZ's native aquatic fauna to common aquatic toxicants is warranted. The studies in this thesis have shown that inanga may not always respond to toxicants in a manner similar to species used in the development of regulations. This is likely to be a consequence of the different physiology of these fish (e.g. Urbina et al., 2014a), but ecological factors may also play a role. For example, inanga is known to inhabit acidic DOC-enriched waters, which they use as a refuge against predation by introduced trout species (Olsson et al., 2006). The unique chemistry of these waters may alter toxicant behaviour and bioavailability (Campbell and Stokes, 1985; Nicholls et al., 2015), and represents an

exposure scenario that regulatory models may not be able to account for (Al-Reasi et al., 2013).To this end, additional work not included in this thesis, has started to examine the mechanisms of diclofenac bioaccumulation in inanga in waters of very low pH, where diclofenac is present in a more lipophilic form (McRae, Glover, Burket, Gaw, Brooks, unpublished).

7.4. Conclusion

Overall, the findings from this study will allow the responses of inanga to key environmental contaminants to be contextualised relative to more well-studied, model species. This will permit researchers and regulatory bodies to adopt environmental regulations incorporating the unique biological and ecological factors faced by NZ's important freshwater fish fauna. Importantly, this thesis also highlighted that key model species, such as the zebrafish and fathead minnow, can differ in their responses to environmental contaminants, questioning the value of relying on such a small sample of the many thousands of different fish species, to dictate environmental regulatory levels. Most importantly, it is hoped that this work will contribute towards environmental protection of a treasured key Southern Hemisphere fish species, inanga.

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