

**Impacts of the human pharmaceutical diclofenac in the
aquatic environment**

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Abstract

An increasing number of pharmaceuticals have been found in the aquatic environment and the issue has become a human and environmental health concern. Many pharmaceuticals are not fully degraded in wastewater treatment plants (WWTPs) and are continuously released in the aquatic environment resulting in concentrations in the low $\mu\text{g/l}$ range in the receiving waters. Diclofenac is a widely used non-steroidal anti-inflammatory drug (NSAID) and is persistent in the aquatic environment. This pharmaceutical has been frequently reported in wastewater effluents, surface waters, groundwaters and even drinking water. NSAIDs are known to inhibit the cyclooxygenase activity, an enzyme present in many species of the animal kingdom responsible for the synthesis of prostanoids, and chronic exposure to environmental diclofenac may have detrimental effects on metabolism of non-target organisms including microbes and fish. In this thesis, microbiology, genomics and metabolomics approaches were used to investigate the effects of diclofenac on aquatic microbes and fish.

In the first study of the thesis (chapter 3), the biodegradation of selected NSAIDs was investigated, together with their potential toxicity to aquatic microbes. Aerobic biodegradation experiments were conducted using activated sludge and wastewater effluents as microbial inocula and diclofenac, ketoprofen or naproxen as sole carbon source (1-10 mg/l) in order to isolate and identify the bacterial degraders. Changes in the bacterial populations were monitored by optical density and PCR-DGGE. The analytical techniques solid phase extraction (SPE) and ultraperformance liquid chromatography-mass spectrometry (UPLC-TOF-MS) were optimised to quantify the pharmaceuticals in environmental samples. High recovery rates were obtained with 94% for diclofenac; 92% for ketoprofen and 85% for naproxen and with detection capabilities down to 3-7 ng/l. Results from the biodegradation experiments showed that ketoprofen and naproxen were eliminated at up to 99 and 55% respectively over a 40 days period. Consistently with previous studies, diclofenac showed no significant degradation. In all the enrichments, a significant decrease in the bacterial abundance was observed as a consequence of NSAIDs exposure and attempts to isolate the bacterial degrading populations were unsuccessful. Given the apparent micro-toxicity of these NSAIDs, the standardised test Microtox[®] was carried out with *Vibrio fischeri*. The EC₅₀ (15 min) estimated ranged from 13.5 mg/l \pm 2.3 for diclofenac to 42.1 mg/l \pm 3.9 for naproxen. Further toxicological tests were performed with diclofenac on bacterial strains isolated from activated sludge. Growth inhibitory effects were observed from 50-70 mg/l for *Micrococcus luteus*, *Zoogloea ramigera* and *Comamonas denitrificans*. *Pseudomonas putida* seemed more tolerant to diclofenac exposure and toxic effects were observed from 90 mg/l. These studies showed that diclofenac was the most toxic NSAID but toxicological effects in bacteria only occurred at concentrations at least 1,000 times higher than those found in the environment. However, chronic exposure to lower concentrations may cause similar interferences and affect the degradation potential of naturally occurring microbial populations.

The second study (chapter 4) investigated the biological effects of sub-chronic exposure to waterborne diclofenac (0.5, 1, 5 and 25 $\mu\text{g/l}$) in female juvenile rainbow trout *Oncorhynchus mykiss*. After 21-day exposure, mRNA expression levels of cytochrome p450 1a1 (*cyp1a1*), cyclooxygenase (*cox*) 1 and 2, and *p53* were investigated in the liver, kidney and gills using RT-PCR and QPCR. These genes were selected as they are likely targets for diclofenac in mammals. Histopathological investigations were carried out in the small intestine, liver and kidney because

diclofenac has been reported to induce toxicity responses in these tissues. Fish bile was also analysed by SPE and UPLC-TOF-MS to evaluate the bioconcentration potential of diclofenac and look for evidences of diclofenac metabolism. Results showed a significant reduction of both *cox1* and *cox2* expression in the liver, gills and kidney from 1 µg diclofenac/l. In contrast diclofenac induced an increase in mRNA levels for *cyp1a1* in the liver and gills but a significant reduction of *cyp1a1* expression in the kidney from 1 µg/l. There were no clear effects of diclofenac on the mRNA levels of *p53*. Diclofenac exposure caused tissue damages at exposure concentrations as low as 1 µg/l. Histopathological injuries included inflammation, hyperplasia and fusion of the villi in the small intestine and tubule necrosis in the kidney. There were no obvious changes in the liver of diclofenac-exposed fish. The analysis of bile revealed a bioconcentration potential between 509 ± 27 and 657 ± 25 . A reactive metabolite of diclofenac was also detected at the highest exposure concentration which may be responsible for the severe injuries found in those fish. Sub-chronic exposure to environmental concentrations of diclofenac altered gene expression and it is possible that long term exposure to environmental diclofenac lead to significant impacts on fish health.

In the final part of this thesis (chapters 5 and 6) effects on the metabolite composition of biofluids were analysed in diclofenac-exposed fish. This work entailed developing and validating appropriate methodologies to analyse fish bile and blood plasma. Methanol extraction and UPLC-TOF-MS were optimised to analyse the plasma metabolome but the methodologies were not suitable to detect low abundance molecules such as eicosanoids due to the interferences (ion suppression) in the samples matrix. Multivariate data analysis failed to detect the endogenous metabolites of the plasma affected by the chemical exposure. The only discriminating metabolite was found after analysis of the plasma samples from control vs. 25 µg/l treatment groups and identified as the exogenous compound diclofenac. To analyse the bile, the developed SPE methodology was carried out in order to separate the metabolites between a free steroids (fatty acids, eicosanoids, etc.) fraction and a conjugated steroids (bile salts) fraction. Due to high levels of taurocholic acid masking other metabolites in the conjugated fraction, some bile samples were hydrolysed to deconjugate these metabolites. The non-hydrolysed and hydrolysed bile fractions were analysed by UPLC-TOF-MS in positive and negative ionization. Multivariate data analysis using principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) revealed significant perturbations in the bile metabolite profile of diclofenac-exposed rainbow from the lowest exposure concentration (0.5 µg/l). Over 50 metabolites were elevated or reduced as a result of the 21-day exposure, suggesting that diclofenac affected several metabolic pathways. One metabolite was identified as a lipooxygenase product. This suggests that the inhibition of prostanoids synthesis can cause a shift in the arachidonic cascade and increase the synthesis of other eicosanoids. Most of the other discriminative metabolites remain unidentified and FT-MS analysis will be performed to obtain a structural identity. The metabolomics study further highlights the concern of environmental diclofenac in non-target organisms and the need to investigate the metabolic pathways affected.

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List of abbreviations

2D-PAGE	2-dimensional polyacrylamide gel electrophoresis
AHH	aryl hydrocarbon hydroxylase
ANOVA	analysis of variance
BLAST	Basic Local Alignment Search Tool
BC	bioconcentration
bp	base pair
BPI	base peak intensity
CAS	chemical abstract service
cDNA	complementary deoxyribonucleic acid
CE-MS	capillary electrophoresis - mass spectrometry
cm	centimetre
<i>cox</i>	cyclooxygenase
C_T	threshold cycle
<i>cyp</i>	cytochrome P450
<i>cyp1a1</i>	cytochrome P450 1a
Da	dalton
dATP	deoxyadenosine triphosphate
DCF	diclofenac
dCTP	deoxycytidine
dGTP	deoxyguanosine triphosphate
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTPs	deoxyribonucleotide triphosphates
dTTP	thymidine triphosphate
E_2	17 β -estradiol
EC_{50}	50% effect concentration
ECOSAR	Ecotoxicological Structure Activity Relationship
EDC	endocrine disrupting chemical
EE_2	17 α -ethinylestradiol
EPA	eicosapentaenoic acid
EROD	ethoxyresorufin- <i>O</i> -deethylase
ESI	electrospray ionisation
eV	electron volt
FT-MS	Fourier transform - mass spectrometry
g	gram
GC	gas chromatography
HEPES	hydroxyeicosapentaenoic acids
HETES	hydroxyeicosatetraenoic acids
HPLC	high performance liquid chromatography
hr	hour
IMS	industrial methylated spirit

IS	internal standard
Kg	kilogram
kV	kilo volt
l	litre
LC	liquid chromatography
LOD	limit of detection
LOEC	lowest observed effect concentration
M	mole per litre
M+H	protonated molecule
M-H	deprotonated molecule
MEC	measured environmental concentration
mg	milligram
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
mm	millimetre
mM	millimole per litre
M-MLV	moloney murine leukaemia virus
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSM	minimal salts medium
MW	molecular weight
<i>m/z</i>	mass to charge ratio
n	number of samples
ng	nanogram
nm	nanometre
nM	nanomole per litre
NMR	nuclear magnetic resonance
NSAIDs	non steroidal anti-inflammatory drugs
OD	optical density
OPLS	orthogonal partial least squares projection to latent structures
p	statistical probability
P450	cytochrome P450
<i>p53</i>	tumour protein 53
PAH	polycyclic aromatic hydrocarbon
PCA	principal component analysis
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PEC	predicted environmental concentration
pg	picogram
PIT	passive integrated transponder
PLS-DA	partial least squares projection to latent structures discriminant analysis
pmol	picomole
PNEC	predicted no effect concentration

ppm	part per million
Q ²	cumulative variation predicted by the PCA or PLS model
Q-PCR	real-time quantitative reverse transcription PCR
R ²	variation explained by the PCA or PLS model
RNA	ribonucleic acid
Rnase	ribonuclease
<i>rpl8</i>	ribosomal protein l8
rpm	rotation per minute
rRNA	ribosomal ribonucleic acid
RT	retention time
RT-PCR	reverse transcription polymerase chain reaction
SE	standard error
sec	seconds
SPE	solid phase extraction
TIC	total ion chromatogram
T _M	melting temperature
TOF	time of flight
UPLC-MS	ultraperformance-liquid chromatography - mass spectrometry
UV	ultraviolet
v/v	volume to volume ratio
V	volt
vs.	versus
WWTP	wastewater treatment plant
°C	degree Celsius
µg	microgram
µl	microlitre
µm	micrometre
µM	micromole per litre

List of abbreviated chemicals

11-HETE	±11-hydroxyeicosatetraenoic acid
CoCl ₂	phosgene
CTAB	cetyltrimethylammonium bromide
CuSO ₄	copper (II) sulphate
E ₂ - <i>d4</i> -S	[2,4,16,16- <i>d4</i>] 17β-estradiol sodium 3-sulfate
EDTA	ethylenedinitrilotetraacetic acid
H ₂ SO ₄	sulphuric acid
K ₂ HPO ₄	potassium phosphate dibasic
KH ₂ PO ₄	potassium dihydrogen phosphate
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
MnSO ₄	manganese sulphate
NaCl	sodium chloride
NaOH	sodium hydroxide
Na ₂ SO ₄	sodium sulphate
NH ₄ OH	ammonium hydroxide
(NH ₄) ₂ SO ₄	ammonium sulphate
P- <i>d9</i>	[2,2,4,6,6,17α-21,21,21- <i>d9</i>] progesterone
PGB ₂	prostaglandin B ₂
PGE ₂	prostaglandin E ₂
PGJ ₂	prostaglandin J ₂
TAE	Tris-acetate-EDTA
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
TxB ₂	tromboxane B ₂
ZnSO ₄	zinc sulphate

CHAPTER 1: General Introduction

1.1. Pharmaceuticals in the environment

The extensive use of pharmaceuticals in human and veterinary medicine has become a major environmental concern because pharmaceuticals are designed to be highly active or toxic to biological systems. Even though individual pharmaceuticals have been detected in low concentrations (ng- μ g/l) the occurrence of numerous pharmaceuticals with similar mechanisms of action may have significant effects on aquatic and terrestrial wildlife through additive exposures (Daughton and Ternes, 1999). Much research has been done on antibiotics and endocrine disrupting chemicals (EDCs) especially pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and synthetic oestrogens (D'Ascenzo et al., 2003). As a result pesticides are now some of the most highly regulated chemicals. A series of studies have shown that synthetic estrogens (e.g. 17β -estradiol (E_2) and 17α -ethinylestradiol (EE_2)) are ubiquitous in the aquatic environment and exposure to environmental concentrations (ng/l range) can affect the physiology and reproductive health of wildlife (Jobling et al., 1998; Filby et al., 2007). The increasingly high use of antibiotics has also led to the genetic selection of more resilient and harmful bacteria, which is of great concern for human and environmental health (Díaz-Cruz et al., 2003). Over the last decade, research focus has turned to the occurrence and fate of other therapeutic classes including anti-epileptics, lipid regulators and non-steroidal anti-inflammatory drugs (NSAIDs). Ecotoxicological investigations have demonstrated that these compounds can strongly interfere with metabolic activities of non target organisms and cause serious tissue damage (Ferrari et al., 2003).

1.1.1. Origin

Approximately 100,000 different pharmaceuticals are manufactured each year but there is no data on the worldwide use of these pharmaceuticals (Kümmerer, 2009). The annual consumption of individual pharmaceuticals can vary considerably from country to country. Some pharmaceuticals are only available with prescription whilst others can be purchased over the counter or on the internet, making it difficult to determine the actual amounts consumed. In Germany, 50,000 pharmaceuticals were registered in 2001 (Kümmerer, 2004). Data collected in Europe indicate that the most frequently used are often sold in the 100 tons per year (Fent et al., 2006). These include acetylsalicylic acid (e.g. 836 tons in Germany in 2001), paracetamol (e.g. 390 tons in England in 2000), carbamazepine (87 tons in Germany in 2001) and diclofenac (85 tons in Germany in 2001). Pharmaceuticals can enter the environment via several routes (Figure 1.1). After human application, pharmaceuticals are excreted, intact or metabolised, via urine and faeces. Pharmaceuticals and their metabolites enter wastewater treatment plants (WWTPs) where the compounds not readily degraded are discharged within the wastewater effluents leading to a continuous input of pharmaceuticals in the receiving waters. The sludge produced in WWTPs can be used as fertiliser in agricultural fields and contaminate the soil. Waste discharged from hospitals and pharmaceutical industries are also treated in WWTPs and contribute significantly to the amounts released in the environment (Bound and Voulvoulis, 2005). Pharmaceuticals used for veterinary purposes are introduced in the environment via animal excretions. Manure can be applied to landfill and subsequently leach in groundwaters or reach surface waters via run-offs (Koutsouba et al., 2003). In addition, the use of food additives such as antibiotics and growth promoters used in aquaculture has led to a direct input of pharmaceuticals in waters and soil (Díaz-Cruz et al., 2003).

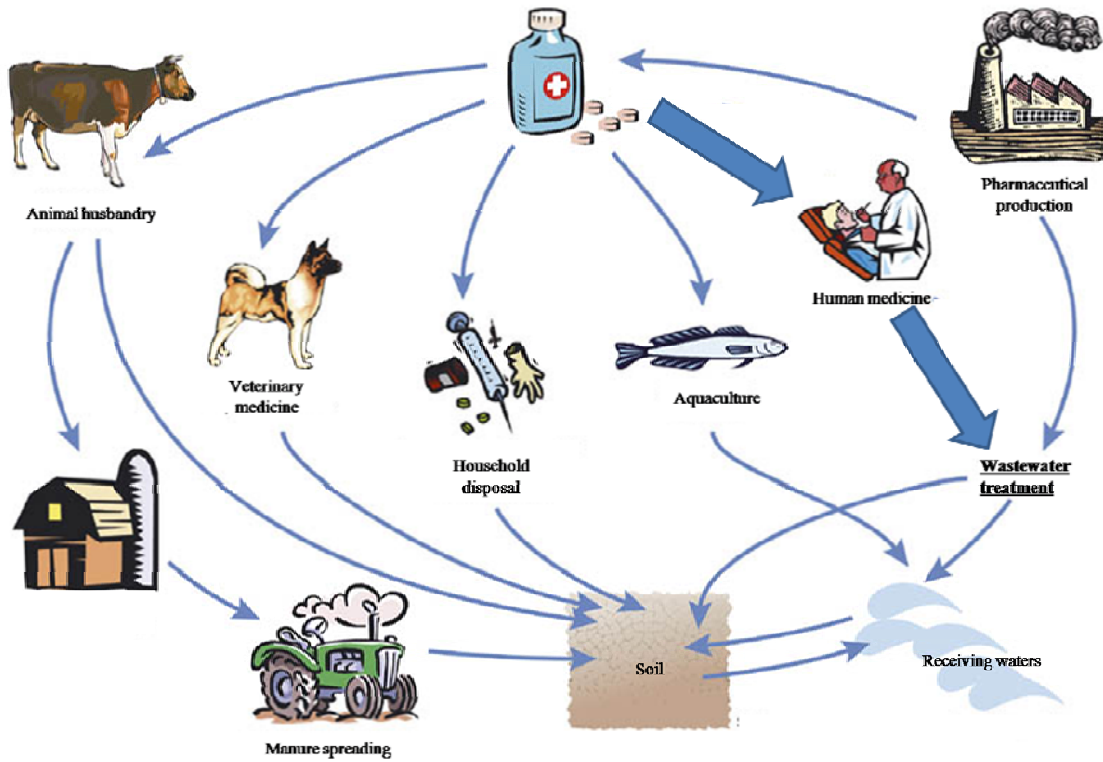


Figure 1.1 – Routes of entry for pharmaceuticals (adapted from Boxall, 2004).
 Pharmaceuticals used for human medicine are the main source of entry in the aquatic environment.

These various routes along with an inappropriate disposal of used medicines, contribute to an increased load of pharmaceuticals in surface and river waters and in exceptional cases pharmaceuticals have been found to reach drinking water (Jones et al., 2005).

1.1.2. Occurrence in the aquatic environment

The first compound of pharmaceutical origin, clofibric acid, was reported in the US in treated WWTP effluents at concentrations between 0.8 and 2 µg/l (Garrison et al., 1976). Clofibric acid is a metabolite of the lipid regulators clofibrate and etofibrate and has become the most widely detected pharmaceuticals. Subsequently, an increasing number of pharmaceuticals have been found in WWTP effluents, rivers, estuaries, seawater, groundwater and even drinking water (Richardson and Bowron, 1985; Stumpf et al., 1999; Thomas and Hilton, 2004; Zorita et al., 2009). Surveys of WWTP influents and effluents have concluded that the main therapeutic classes detected are β-blockers, analgesics, anti-epileptics and lipid regulators. Highest environmental concentrations have been found near WWTPs which suggests that conventional WWTPs are not well adapted for the complete removal of pharmaceuticals. The occurrence of 32 pharmaceuticals was reported in WWTP effluents in Germany with the anti-epileptic carbamazepine and the lipid regulator gemfibrozil detected at maximum concentrations of 6.3 and 4.6 µg/l respectively (Ternes, 1998). Metcalfe et al. (2003) found elevated levels of carbamazepine, naproxen and diclofenac (up to 2 µg/l) in Canadian treated wastewater. Castiglioni et al. (2005) detected up to 30 different pharmaceuticals in treated WWTP effluents across Italy. The concentrations measured ranged from 150-1081 ng/l for the antibiotic ofloxacin, 27-1168 ng/l for the β-blocker atenolol and 33-1318 ng/l for carbamazepine. A monitoring study carried out in the UK found propranolol (mean 75 ng/l) in all, diclofenac (mean 500 ng/l) in 86% and ibuprofen

(mean 3000 ng/l) in 84% of the WWTP effluents analysed (Ashton et al., 2004). In India, exceptionally high levels of antibiotics ciprofloxacin (up to 3100 µg/l) and losartan (up to 2500 µg/l) and β-blocker metoprolol (950 µg/l) were detected in the final effluents of WWTPs treating wastewaters from pharmaceuticals industries (Larsson et al., 2007).

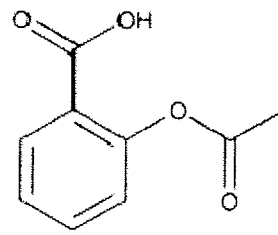
The receiving waters usually contain fewer compounds occurring at lower concentrations. A survey on the occurrence of pharmaceuticals in US streams showed that the pharmaceuticals ibuprofen, fluoxetine and triclosan found at low levels (ng/l range) in WWTP effluents were no longer detected in surface waters (Boyd et al., 2003). A similar contamination pattern had been reported by Ternes (1998). However, other studies have shown that some pharmaceuticals are ubiquitous and can be detected in the receiving waters. In the UK, pharmaceuticals were detected in estuaries at concentrations up to 111 ng/l for clofibric acid, 125 ng/l for diclofenac and 928 ng/l for ibuprofen (Thomas and Hilton, 2004). More recently Kasprzyk-Hordern et al., (2008) found more than 50 pharmaceuticals, EDCs and illicit drugs in UK surface waters. The anti-inflammatory compounds codeine (up to 813 ng/l), naproxen (up to 146 ng/l), ketoprofen (up to 14 ng/l), ibuprofen (up to 93 ng/l) and diclofenac (up to 261 ng/l) were amongst the most frequently detected. Certain pharmaceuticals are very recalcitrant and can reach groundwater and drinking water supplies (Heberer, 2002; Loraine and Pettigrove, 2006). The NSAID phenazone has been found in drinking waters at concentrations up to 400 ng/l (Jones et al., 2005). Concentrations of some pharmaceuticals the most frequently detected in the aquatic environment are listed in Table 1.1.

Table 1.1 – Concentrations of selected pharmaceuticals in the aquatic environment.

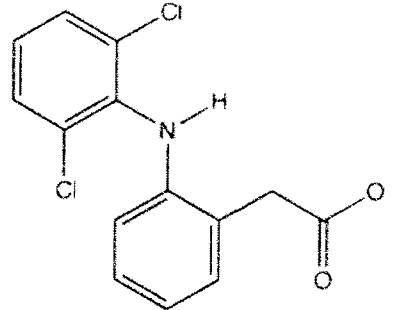
Compound therapeutic use	Concentration (ng/l)	Aquatic environment	Country	Reference
Bezafibrate	4600	wastewater effluent	Germany	Ternes (1998)
<i>lipid regulator</i>	27	drinking water	Germany	Jones et al. (2005)
Carbamazepine	2300	wastewater effluent	Canada	Metcalfe et al. (2003)
<i>antiepileptic</i>	100	surface waters	Sweden	Bendz et al. (2005)
	258	drinking water	US	Jones et al. (2005)
Clofibric acid	1600	wastewater effluent	Germany	Ternes (1998)
<i>lipid regulator metabolite</i>	4000	groundwater	Germany	Heberer (2002)
	270	drinking water	Germany	Heberer (2002)
Diclofenac	5450	wastewater effluent	Italy	Andreozzi et al. (2003)
<i>anti-inflammatory</i>	91	surface water	UK	Ashton et al. (2004)
	89	groundwater	Germany	Heberer (2002)
	6	drinking water	Germany	Heberer (2002)
Gemfibrozil	4760	wastewater effluent	Italy	Andreozzi et al. (2003)
<i>lipid regulator</i>	70	drinking water	Canada	Jones et al. (2005)
Ibuprofen	27300	wastewater effluent	UK	Ashton et al. (2004)
<i>anti-inflammatory</i>	2500	surface water	Spain	Farré et al. (2001)
	82	groundwater	Germany	Heberer (2002)
	3	drinking water	Germany	Jones et al. (2005)
Metoprolol	2200	wastewater effluent	Germany	Ternes (1998)
<i>β-blocker</i>	60	surface water	Sweden	Bendz et al. (2005)
Naproxen	33900	wastewater effluent	Canada	Metcalfe et al. (2003)
<i>anti-inflammatory</i>	110	surface water	Sweden	Bendz et al. (2005)
Paracetamol	6000	wastewater effluent	Germany	Heberer (2002)
<i>analgesic</i>	10000	surface water	US	Kolpin et al. (2002)

1.2. Non-steroidal anti-inflammatory drugs

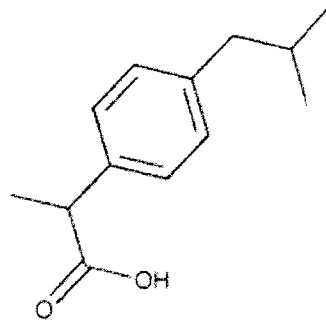
Non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed to reduce inflammation and manage pain in primary care such as back pain, migraine, fever or arthritis. Many can be purchased over the counter including acetylsalicylic acid, ibuprofen, naproxen, diclofenac and indomethacin. In Germany, it has been estimated that more than 80 tons of diclofenac are used each year (Zwiener and Frimmel, 2003). In 2001, naproxen, diclofenac and ibuprofen were amongst the top 25 pharmaceuticals used in the UK (Jones et al., 2002). After application, it has been shown that up to 15% of diclofenac and 8% of ibuprofen ingested is excreted from the human body (Jjemba, 2006). NSAIDs entering WWTPs are poorly removed and have been detected worldwide (Koutsouba et al., 2003; Löffler and Ternes, 2003; Carballa et al., 2004; Tauxe-Wuersch et al., 2005; Yamamoto et al., 2009). They are often found in the ng- $\mu\text{g}/\text{l}$ range in WWTP effluents, surface waters, rivers, estuaries and groundwaters (see Table 1.1). Stumpf et al. (1999) were the first to report the presence of diclofenac in the aquatic environment. Ibuprofen and diclofenac were even detected in drinking water in Germany at low ng/l concentrations (Heberer, 2002). Figure 1.2 illustrates some of the NSAIDs detected during environmental surveys. A monitoring study carried out in Canada showed that the NSAIDs naproxen, ibuprofen and diclofenac were amongst the most frequently detected in WWTP effluents at maximum concentrations of 1.2, 0.77 and 0.75 $\mu\text{g}/\text{l}$ respectively (Lishman et al., 2006). In the UK an increasing number of studies have reported the presence of NSAIDs in WWTP effluents and rivers (Thomas and Hilton, 2004; Jones et al., 2007; Kasprzyk-Hordern et al., 2008). Hilton et al. (2003) observed that diclofenac was the most frequently detected in WWTP effluents.



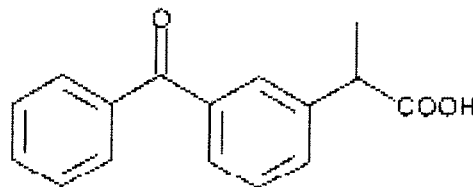
acetylsalicylic



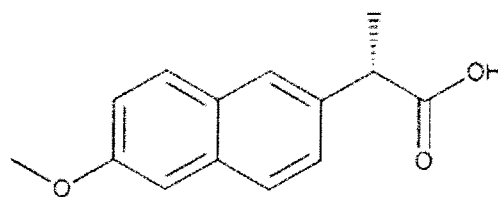
diclofenac



ibuprofen



ketoprofen



naproxen

Figure 1.2 – Chemical structure of selected NSAIDs.

1.2.1. Mode of action

The exact mode of action is not well understood but the primary action of NSAIDs is to inhibit the activity of the cyclooxygenase (*cox*) enzyme that oxygenates fatty acids such as arachidonic acid and eicosapentaenoic acid into prostanoids. In most vertebrates species studied, two isoforms have been found (Chandrasekharan and Simmons, 2004; Ishikawa and Herschman, 2007). The first cyclooxygenase, called *cox1*, is usually constitutively expressed in many tissues including kidney, intestine, brain; and is responsible for the maintenance of basal prostanoids synthesis (Cha et al., 2006). The second cyclooxygenase, *cox2*, is mainly inducible by a wide range of extracellular and intracellular stimuli. Prostanoids synthesised by *cox* activity, comprised the eicosanoids prostaglandins and tromboxanes and have been shown to play key roles in physiological functions of cells (Dubois et al., 1998). Figure 1.3 shows the pathway for prostanoids synthesis and their distribution in tissues. In addition arachidonic acid can serve as substrate for lipoxygenase enzymes to form lipoxins, leukotrienes and hydroxyeicosatetraenoic acids (HETEs) and for cytochrome P450 monooxygenase enzymes to produce epoxyarachidonic acid (Cha et al., 2006). Eicosanoids regulate a variety of physiological functions such as renal blood flow, platelets aggregation, synthesis of gastric mucus and blood clotting, embryonic implantation (Simmons et al., 2004). Toxicity caused by NSAIDs has been attributed to the non selective inhibition of both *cox*. The application of new NSAIDs (e.g. celecoxib, meloxicam, etoricoxib and rofecoxib) selectively inhibiting *cox2* is thought to reduce the adverse effects (Dorne et al., 2007).

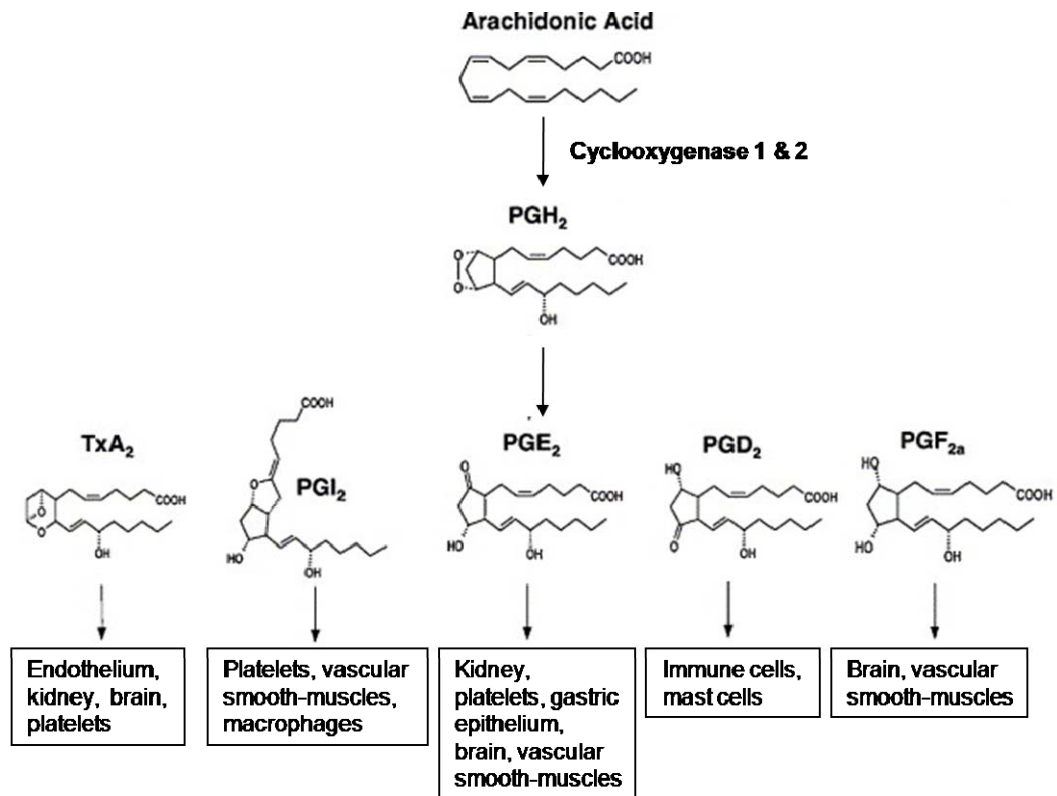


Figure 1.3 – Schematic diagram of the conversion of arachidonic acid to prostanoids.

Boxed are the tissues where these molecules are expressed in mammals. Diagram adapted from Cha et al. (2006).

1.3. Removal of NSAIDs in the environment

The fate of pharmaceuticals depends on the following key factors: adsorption potential, biodegradability and photosensitivity. Research on the removal of pharmaceuticals has not always established whether these compounds are completely degraded (mineralisation) or simply chemically altered (Kümmerer, 2009). As the rate of pharmaceutical discharge in the environment is often faster than the rate of removal, those poorly removed may accumulate in the aquatic environment and affect the aquatic wildlife.

1.3.1. Wastewater treatment plants

WWTPs are the major route of entry for NSAIDs via municipal and hospital wastewaters, and are designed to reduce the amount of suspended particles and chemicals in wastewaters before the release in receiving waters (Figure 1.4). Conventional plants treating municipal wastes often have a preliminary treatment used to remove inorganic matters from the wastewaters. Raw wastewater flows through screens to remove any large solids (e.g. plastics, toilet paper, etc.) that may block the machinery and then passed into grit removers. Once the flow contains only organic matter, it is transferred into primary settlement tanks to allow the suspended solid matters to settle out. The raw sludge formed at the bottom of the tanks will be further treated and used on landfill. The secondary treatment also called biological treatment is a critical stage for the removal of pharmaceuticals. Wastewater is kept for a prolonged duration in oxygenised tanks with sludge containing microorganisms; known as activated sludge. Compounds with a low polarity tend to be retained in the sludge and degraded by the microorganisms, whilst more polar compounds remain in the aqueous

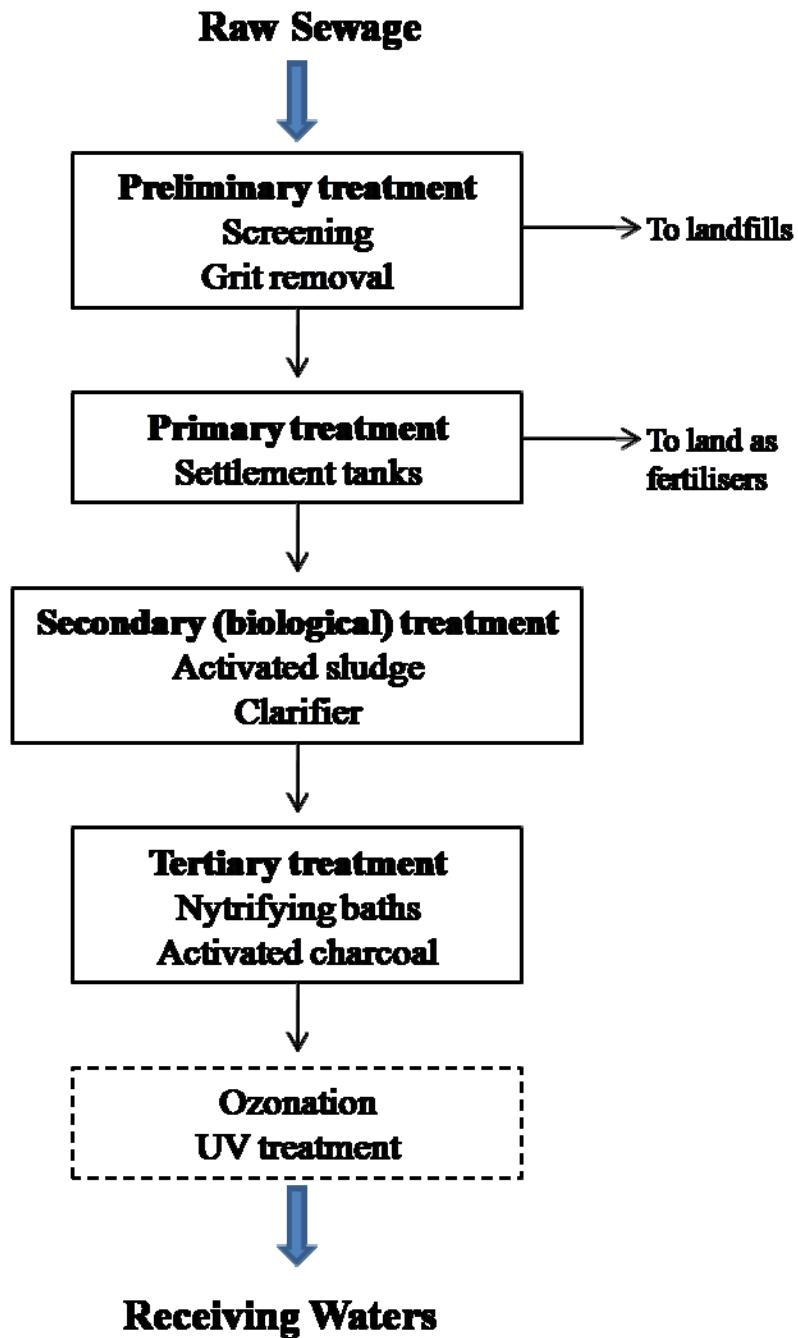


Figure 1.4 – Schematic diagram of a wastewater treatment plant. In some cases a fourth treatment can be applied such as ozonation or UV treatment to purify the final effluent before its release into the aquatic environment.

phase and will be discharged in the environment (Bendz et al., 2005). Activated sludge is then settled in order to clarify the wastewater flow and the final effluent is discharged into rivers, estuaries or seawater. To improve the removal efficiencies of pollutants WWTPs may use a tertiary treatment and UV disinfection.

1.3.2. Removal efficiency in wastewater treatment plants

The efficiency of WWTPs can be evaluated by measuring the concentration of pharmaceuticals in the influents and effluents. Removal efficiencies of NSAIDs have shown a great variability for the same compound depending on treatment technologies in the WWTPs. For example, the removal of ketoprofen has been reported between 8-53% in WWTPs in Sweden (Tauxe-Wuersch et al., 2005). Occasionally, negative removal efficiencies have been reported due to the degradation of conjugated metabolites leading to the release of the parent compound. Diclofenac has been found to increase in concentration from influent to effluent and removal as low as -105% have been measured (Zorita et al., 2009). In general ibuprofen has been removed by 90-100% during wastewater treatment whilst variable removal efficiencies have been reported for naproxen (15-78%) and ketoprofen (48-69%) (Stumpf et al., 1999). Diclofenac is one of the pharmaceuticals with the lowest average removal efficiency (17-23%) in WWTP (Heberer, 2002; Quintana et al., 2005).

1.3.3. Biodegradation

Microbial degradation is possibly the most important removal mechanism for NSAIDs because they remain in the aqueous phase. In WWTPs, bacteria and fungi can degrade partially or completely these compounds in the aerobic and anaerobic phases of the activated sludge. Fungal species have been reported to degrade pharmaceuticals.

Zhong et al. (2003) discovered three fungal species of *Cunninghamella* that were able to degrade naproxen. Microbial degradation of diclofenac has also been observed by the fungal species *Epiccocum nigrum* (Webster et al., 1998). Research has shown that biodegradation rates are difficult to predict and can be affected by the hydraulic retention time, age of the activated sludge and weather conditions (Daughton and Ternes, 1999). Diclofenac was found to be significantly biodegraded when the hydraulic retention time was at least 8 days (Fent et al., 2006). The rates of elimination of ibuprofen and naproxen appeared to be elevated in WWTPs with hydraulic retention times greater than 12 hr (Metcalfé et al., 2003). Aged activated sludge contains microbial populations that have been exposed to pharmaceuticals for a few years and are more effective to remove these compounds. González et al. (2006) found that the biodegradation rate of diclofenac was enhanced if microbes were acclimated to the compounds. However, the introduction of entirely new pharmaceuticals each year means that microbes may not be adapted enough and degradation can be challenging.

The removal of acidic pharmaceuticals by microbes in the activated sludge is a topic of research interest. Laboratory based experiments showed that ibuprofen and naproxen were fully mineralised by microbes present in activated sludge but only after addition of lactose as another carbon source, a mechanism known as co-metabolism (Quintana et al., 2005). The study also revealed that ketoprofen was used as sole carbon source by the microbes and transformed in 28 days producing stable metabolites, only diclofenac showed no degradation. Similarly, Yu et al. (2006) reported that diclofenac was poorly degraded with only 30% of the initial concentration removed, and ibuprofen, ketoprofen and naproxen were biodegraded in the activated sludge by more than 80% in 50 days. It has been established that microbial degradation can also occur in the receiving waters. Microcosm experiments using river water have shown that ibuprofen

can be rapidly degraded in 21 days (Winkler et al., 2001). Yamamoto et al. (2009) found that biodegradation can be slow and variable in river water with half-lives of 2-58 days for acetaminophen, 450-480 days for ibuprofen, 12-104 days for mefenamic acid and 410-430 days for indomethacin. A study on microbial degradation of pharmaceuticals in estuarine and seawater reported the fast biodegradation of acetaminophen (half-life 11 days) and a much slower degradation for ketoprofen with a half-life over 100 days (Benotti and Brownawell, 2009). However, most biodegradation studies have used NSAIDs at concentrations one or two orders of magnitude higher than the concentrations detected in the aquatic environment. It may be that environmental concentrations entering WWTPs are too low to trigger the appropriate enzymatic reactions for degradation of these compounds (Daughton and Ternes, 1999).

Little is known about the microbial communities actively degrading pharmaceuticals. Gröning et al. (2007) investigated the microbes degrading diclofenac and were able to identify bacteria at a group level. They observed that diclofenac degradation was restricted to Gram negative bacteria from the *γ-Proteobacteria* and the *Cytophaga-Flavobacterium* group. Kraigher et al. (2008) demonstrated that the exposure of activated sludge microbes to 5-500 µg/l of diclofenac, ibuprofen, naproxen and ketoprofen can lead to a shift in the community structure and inhibit the growth of bacteria (genus *Nitrospira*) that play a key role in nitrification during wastewater treatment. This, may in turn, reduce the removal efficiency of NSAIDs in wastewaters.

1.3.4. Sorption

Sorption is the physical sequestration of dissolved organic compounds onto filterable materials. In WWTPs this process facilitates the removal of poorly soluble pharmaceuticals from wastewaters into the sludge. NSAIDs are present in their ionised

form at pH of natural waters and remain dissolved, thus sorption may not be a relevant removal process (Benotti and Brownawell, 2009). A degradation study by Urase and Kikuta (2005) reported that acidic pharmaceuticals have a low sorption potential that may increase with lower pH conditions. Basic pharmaceuticals however, can adsorb to suspended solids to a considerable extent. Antibiotics and synthetic estrogens have a strong tendency for adsorption and are retained in the sludge (D'Ascenzo et al., 2003; Díaz-Cruz et al., 2003). Thus they are often found in soils and can leach to surface waters and groundwaters.

1.3.5. Abiotic processes

Abiotic degradation of pharmaceuticals is possible by photodegradation and hydrolysis. Pharmaceuticals are known to resist hydrolysis and this process is usually regarded as negligible for most pharmaceuticals found in the aquatic environment. Photodegradation of pharmaceuticals has been reported in the receiving waters and the aerated tanks of WWTPs. Using field measurements and laboratory experiments, Buser et al. (1998) identified photodegradation as the main removal mechanism of diclofenac in surface waters with a half-life of 1 hr. These results were supported by a study on the abiotic degradation of diclofenac exposed to sunlight (Andreozzi et al., 2003) and a rapid degradation rate was measured (half-life 5 days). Lin and Reinhard (2004) investigated the photodegradation rate of various pharmaceuticals by solar irradiation. They reported half-lives of 1.4 hr for naproxen and 15 hr for ibuprofen indicating that these compounds are photosensitive. Degradation of pharmaceuticals is important to reduce the bioavailability and minimise the adverse effects. Research has shown that in some cases the photoproducts may be toxic. DellaGreca et al. (2003) demonstrated that

some photoproducts of naproxen formed by irradiation were as toxic as or more than the parent compound.

1.4. Environmental impact of NSAIDs

Pharmaceuticals entering the aquatic environment can remain biologically active and as such can interact with the aquatic wildlife. Ecotoxicological testing has provided valuable information on the toxic effects of pharmaceuticals which can be used for ecological risks assessment (Henschel et al., 1997). Mathematical models such as ECOSAR have also been developed to predict the behaviour and potential toxicity of pharmaceuticals, but *in vivo* and *in vitro* studies have been more useful to understand the mechanism of toxicity and tissues affected (Fent et al., 2006). Because pharmaceuticals are continuously released in the environment aquatic populations are exposed to low concentrations of pharmaceuticals throughout their life which may affect their development, reproduction or survival rate. For example, cyclooxygenase enzymes are conserved in many vertebrates and invertebrates, thus NSAIDs may affect prostanooids biosynthesis and cause adverse effects in non target organisms.

1.4.1. Acute toxicity

Environmental risk assessments have been largely based on acute toxicity tests. Many standardised tests exist using invertebrates, bacteria, fish and algae with a range of endpoints including luminescence, growth, mobility and mortality (Crane et al., 2006). Farré et al. (2001) analysed the acute toxicity of NSAIDs on the luminescence of bacteria (*Vibrio fischeri*) and reported a 50% reduction in bioluminescence ($EC_{50} = 30$ min) of 13.5 mg/l for diclofenac, 19.2 mg/l for ibuprofen and ketoprofen and 35 mg/l for naproxen. Naproxen has been observed to cause acute toxic effects in invertebrate

species *Ceriodaphnia dubia* with EC₅₀ (24 hr) of 66.4 mg/l (Isidori et al., 2005). Jones et al. (2002) and Carlsson et al. (2006) have compiled the acute toxic effects of pharmaceuticals including diclofenac, naproxen, ibuprofen and ketoprofen. They concluded that diclofenac and ibuprofen are potentially dangerous to the environment. However, insufficient data are available for naproxen and ketoprofen to determine the risks of these compounds in the environment. Of all the NSAIDs diclofenac has shown the highest acute toxicity to aquatic species with lowest observed effect concentration often below 100 mg/l (LOEC) (Fent et al., 2006). The crustacean *C. dubia* [LOEC (7 days)= 1 mg/l] seems to be the most sensitive to acute diclofenac concentrations (Ferrari et al., 2003). Acute toxicity of diclofenac has also been reported for aquatic plant *Lemna minor* [EC₅₀ (7 days)= 7.5 mg/l (Cleuvers, 2003)] and rotifer *Brachionus calyciflorus* [LOEC (48 hr)= 25 mg/l (Ferrari et al., 2003)]. Little is known on the effects of acute exposure in fish. Laville et al. (2004) published one of the first *in vitro* study of acute toxicity of diclofenac in rainbow trout primary hepatocytes and observed effects on cell toxicity [EC₅₀ (24 h)= 420 µM] and cytochrome P450 enzymatic activity [EC₅₀ (24 hr)= 63 µM]. Recently, acute exposure of medaka fish (*Oryzias latipes*) to 1 µg/l and 8 mg/l diclofenac resulted in a significant increase of *cyp1a*, vitellogenin and *p53* gene expression in the liver, gills and intestine (Hong et al., 2007).

1.4.2. Chronic toxicity

An earlier study reported the toxicity of diclofenac in three *Gyps* vulture species with catastrophic declines in the populations in Pakistan and India (Oaks et al., 2004). Vultures that prey on dead carcasses had been eating diclofenac-treated cattle but they were not able to metabolise and excrete diclofenac efficiently. Low concentrations of diclofenac [LOEC= 0.007 mg/kg] found in the vultures resulted in renal failure. Since

then extensive research has been carried out on chronic toxicity of environmentally relevant concentrations of diclofenac especially in fish. In zebrafish (*Danio rerio*) diclofenac had no effects on the embryonic development except for a small delay in hatching time after 1 and 2 mg/l exposure (Hallare et al., 2004). Many chronic exposures to diclofenac have been done using salmonid species as they are easily maintained under laboratory conditions and can occur in freshwater and seawater environments. Rainbow trout (*Oncorhynchus mykiss*) exposed to diclofenac for 28 days presented renal lesions and alterations in the morphology of the gills from 5 µg/l as well as bioconcentration of diclofenac in the liver, kidney, gills and muscles (Schwaiger et al., 2004). The highest bioconcentration factor (2732) was found in the liver of fish at 1 µg/l. Subtle subcellular damages were even detected in the liver, gills and kidney of the fish from 1 µg/l (Triebkorn et al., 2004). A 21 day study with brown trout (*Salmo trutta*) reported the inflammation of the gills and kidney from 5 µg diclofenac/l due to the accumulation of granulocytes in these organs (Hoeger et al., 2005). A field study also reported that rainbow trout exposed to WWTP effluents bioconcentrated diclofenac in the plasma (Brown et al., 2007). It is a general consensus that chronic exposure to diclofenac can impair renal functions in fish. The kidney has also been found to be the target organ of diclofenac toxicity in many organisms such as birds (Oaks et al., 2004; Meteyer et al., 2005), mice (Hickey et al., 2001) and human (Ng et al., 2006). Other studies have observed side effects in the small intestine of diclofenac-exposed organisms (Seitz and Boelsterli, 1998; Hoeger et al., 2008).

1.5. Xenobiotic metabolism in fish

Many xenobiotics are lipophilic and must be biotransformed in order to produce more polar compounds and facilitate renal excretion. Fish have been shown to metabolise xenobiotics through two phase reactions (Goksøyr and Förlin, 1992). In phase I reaction, a functional group is added to the molecule. This involves the cytochrome P450 monooxygenase system. During phase II reaction, xenobiotics (or its phase I metabolite) bind to polar endogenous molecules such as glucuronic acid, sulphate, glutathione or amino acids and form conjugates with a higher water solubility and more easily excreted by the kidney (Daughton and Ternes, 1999). The metabolism can lead to detoxification or bioactivation into reactive intermediates more toxic than the parent compound (Sarasquete and Segner, 2000).

1.5.1. Cytochrome P450 monooxygenase system

Cytochromes P450 (P450s) are a super family of heme proteins involved in the oxidative metabolism of a broad range of substrates in mammals, aquatic vertebrates and bacteria (Hasler et al., 1999). P450s monooxygenases are located predominately in the endoplasmic reticulum membrane (microsomes) and mitochondria of the liver where they catalyse reactions including bile acid formation, aromatic hydroxylation and xenobiotics metabolism (Gonzalez, 1990). In fish, P450s have been found in the liver, intestine, kidney, gills and brain, and established as dominant enzymes during phase I reactions metabolism (Sarasquete and Segner, 2000). The main function of P450s is to bind and activate molecular oxygen to a substrate molecule. They can also utilise oxygen from peroxides for monooxygenations (Anzenbacher and Anzenbacherová, 2001). The main catalytic functions of cytochrome P450s are shown in Figure 1.5. P450s can catalyse other types of reactions including hydroxylation of an aliphatic or

aromatic carbon; epoxidation of a double bond; heteroatom oxygenation, oxidative group transfer, cleavage of esters and dehydrogenation (Testa et al., 2007).

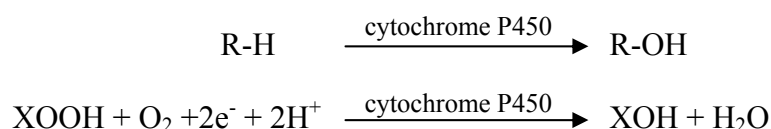


Figure 1.5 – Hydroxylation reactions catalysed by cytochrome P450s. R–H substrate; R–OH hydroxylated product; XOOH peroxide; XOH hydroxylated by-product.

1.5.2. Cytochromes P450 in fish and use as biomarker of aquatic pollution

There are multiple P450 isoforms classified into families and subfamilies according to their function or presence in a particular species. The activity of individual isoforms can vary from species to species and is influenced by water temperature, seasons, sex, developmental and reproductive stage (Andersson and Förlin, 1992). Members of *cyp1*, *cyp2*, *cyp3* and *cyp4* families play a key role in human xenobiotics metabolism and many of them have been characterised in fish (Stegeman, 1992; Thibaut et al., 2006). Isoforms of cytochrome P450 1a (*cyp1a*) subfamily have been found critical during phase I reaction metabolism of pharmaceuticals and as such have been extensively studied (Bucheli and Fent, 1995; van der Oost et al., 2003; Rudzok et al., 2009). Fish act as a lipophilic sink for many pharmaceuticals and they can build up to levels sufficient to induce *cyp1a* (Williams et al., 1998). The induction of *cyp1a*

expression and the related activities (ethoxyresorufin-*O*-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH)) are now well established as *in vivo* biomarkers of pharmaceutical exposure and uptake in fish (Tyler et al., 2008). Some pharmaceuticals have also been shown to inhibit *cypla* activity (Laville et al., 2004). However as mentioned previously, *cypla* activity can be affected by a number of endogenous and exogenous factors. This needs to be taken into account when using *cypla* as a biomarker of environmental exposure.

1.5.3. Bile as major excretory route of pharmaceuticals

The rate and route of pharmaceutical excretion depends on molecular size and lipid solubility of the compound. In fish, bile is the major route of excretion for pharmaceuticals and their metabolites (Gibson et al., 2005). Excretion can also occur by branchial and urinary route. Many endogenous and exogenous compounds as well as their phase I and phase II metabolites have been identified in the bile (Seitz and Boelsterli, 1998; Pettersson et al., 2006). Pharmaceuticals metabolised by the hepatocytes in the liver are concentrated in the bile and can transverse the intestine in order to be eliminated via the faeces. A study on diclofenac metabolism has shown that liver cells can bioactivate a major phase II metabolite into a highly reactive glucuronide conjugate. Lipophilic compounds and conjugates found in the bile have the potential to be reabsorbed by the intestinal cells in a process called enterohepatic recirculation, leading to morphological and functional damages in the intestine (Hoeger et al., 2008).

1.6. Analysis of pharmaceuticals in environmental samples

1.6.1. Sample preparation

The detection of pharmaceuticals in environmental samples at concentrations down to 1 ng/l is an analytical challenge. Therefore the success of the analysis relies on the quality of the extraction. Several techniques exist for sample preparation such as soxhlet extraction, pressurised liquid extraction, ultrasonic solvent extraction, stir bar sorptive extraction and more recently on-line solid phase extraction, liquid phase microextraction and solid phase microextraction have been developed for minimal solvent use (Zhang et al., 2009). Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are the most common techniques used to separate and concentrate pharmaceuticals from environmental aqueous matrices (Ridgway et al., 2007). LLE separates organic compounds by direct partitioning with an immiscible solvent. This technique can separate a wide range of molecules based on their relative solubility to the solvents (Silvestre et al., 2009). The main disadvantage is the large volumes of solvent required and samples may need to be further concentrated in order to detect trace molecules. SPE has become a technique of choice in environmental studies because it overcomes many of the disadvantages of LLE (Pavlovic et al., 2007). A range of SPE cartridges containing solid sorbents with different physico-chemical properties are available to extract specific compounds from complex matrices (e.g. wastewater effluent, urine, blood, tissue homogenates). The pH of the samples can be adjusted to favour the extraction of acidic or basic pharmaceuticals. SPE provides an improved selectivity, reproducibility and high recoveries of trace pharmaceuticals. SPE can also be automated and that can increase the sample throughput. However the properties of the matrices can affect the ability of the sorbent to retain molecules (Ridgway et al., 2007).

1.6.2. Detection techniques

A number of instrumental techniques have been employed for residue analysis of pharmaceuticals in environmental samples, with mass spectrometry (MS) being the most frequent. Various mass analysers are available including single or triple quadrupole, ion-trap, time-of-flight (TOF) and Fourier transform (FT). MS techniques are usually coupled with the separation technique gas chromatography (GC) or liquid chromatography (LC). Many pharmaceuticals lack volatility and must be derivatized in order to be compatible with GC analysis (Buchberger, 2007). Derivatization is a lengthy procedure that can introduce errors in the detection but GC is less affected by the interferences in the matrix and is a method well established for environmental studies (Koutsouba et al., 2003; Metcalfe et al., 2003). LC offers an improvement over GC since derivatization is avoided but limit of detection down to a single ng/l can still be achieved (Kot-Wasik et al., 2007). Many studies have employed LC-MS to analyse WWTP effluents and detect NSAIDs occurring in low concentrations (Farré et al., 2001; Löffler and Ternes, 2003; Lindqvist et al., 2005; Zorita et al., 2009). LC coupled with tandem MS (LC-MS-MS) has proven successful for the simultaneous determination of several pharmaceuticals from different therapeutic classes including NSAIDs (Castiglioni et al., 2005; Gómez et al., 2006). The recent development of ultraperformance liquid chromatography (UPLC) technology has provided an improved sensitivity to analyse environmental water samples. Using UPLC-TOF-MS, Kosjek et al. (2009) were able to detect diclofenac and its metabolites in WWTPs.

1.7. Ecotoxicogenomics to assess biological effects of pharmaceuticals in aquatic organisms.

Ecotoxicogenomics refer to the integration of the “omics” technologies genomics, proteomics and metabolomics into ecotoxicology. The purpose is to provide an insight into the physiological status of organisms and understand their interactions and responses to xenobiotics (Steinberg et al., 2008). Molecular changes occur at concentrations lower than changes at tissue or organism level. Therefore the analysis of gene expression, protein levels and metabolites fluxes in organisms exposed to a pollutant can help for the discovery of biomarkers more informative, sensitive and species/pollutant specific than the traditional biomarkers used (e.g. histopathology, mortality, reproductive success) (Poynton et al., 2008). “Omics” technologies have been applied in controlled laboratory experiments to identify time, concentration or tissue specific responses and determine the mode of action of pollutants. The results generated can be used to predict environmental effects of a pollutant in the aquatic environment. However, very few studies have applied ecotoxicogenomics in the field to assess the effects of pharmaceuticals on wild animals.

1.7.1. Genomics

Genomics is the study of the genome of an organism. In the context of ecotoxicogenomics, it is known as transcriptomics and has been defined as the analysis of mRNA levels in a pollutant-exposed tissue or organism compared with a steady state (Poynton and Vulpe, 2009). The aim is to identify and associate gene expression profiles with a toxicological outcome and to facilitate a better understanding of the mechanisms of toxicity (Farr and Dunn, 1999). Microarrays, either as cDNA or oligonucleotides, and more recently digital transcriptomics are the main techniques

employed. The application of transcriptomics to study pharmaceuticals exposure has provided greater insights into the biological effects of synthetic estrogens and their mimics in fish. For example, Santos et al. (2007) used a 17,000 oligonucleotide based microarray to analyse the expression of over 100 genes in zebrafish (*Danio rerio*) exposed to EE₂. The genes affected were mainly associated with protein metabolism in males and mitochondria organisation and biogenesis in females. Wintz et al. (2006) studied the gene expression profile of fathead minnow (*Pimephales promelas*) exposed to 2,4-dinitrotoluene and were able to link the genes affected with lipid metabolism and respiration (oxygen transport). Garcia-Reyero et al. (2008) used a 22,000 oligonucleotide microarray to compare gene expression profile of laboratory fish with the expression profile of fish caged upstream and downstream a WWTP effluent. They found that 48 hr exposure to the WWTP effluent was sufficient to affect metabolic pathways involved in cell communication, innate immune response and embryonic development. The use of genomics in environmental studies remains limited by the lack of sequenced and identified genes in ecologically relevant species (Finne et al., 2007).

1.7.2. Proteomics

Proteomics is the large-scale study of proteins in cells or tissues. Changes in gene expression affect protein levels but some proteins are modulated independently of any effects on the transcriptome (Poynton et al., 2008). The comparison of protein profiles from stressed conditions versus controls should provide information regarding the molecular mechanisms of response to xenobiotics. The separation techniques 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and the identification techniques LC-MS and Fourier Transform (FT)-MS have been used to assess differential protein expression profiles (Miracle and Ankley, 2005). Although

proteomics is a relatively new technology, it has been applied in environmental monitoring studies in vertebrate and invertebrate species (Iguchi et al., 2006). Albertsson et al. (2007) investigated proteins expression in the liver of rainbow trout (*O. mykiss*) following exposure to WWTP effluents. They observed significant alterations in the protein profiles of WWTP effluent exposed fish compared to control fish and reported that the exposure interfered with ATP synthesis in mitochondria. Although the biological implications are not always clear, this technique was able to identify the metabolic functions affected.

1.7.3. Metabolomics

Metabolomics refers to the analysis of metabolites fluxes in a cell or tissue. It is also known as metabonomics. Transcript levels of a gene are not always correlated to protein expression and translated proteins are not always enzymatically active. Metabolomics, however, aims to profile the end products (e.g. signalling molecules, hormones, lipids) of the cellular processes and hence is more closely related to the physiological state of an organism (Ankley et al., 2006). This approach is ideal for studying multiple species because it is not dependent on knowledge of an organism's genome (Viant, 2008). Analytical techniques used in metabolomics include nuclear magnetic resonance (NMR), LC-MS and GC-MS (Weckwerth and Morgenthal, 2005). Metabolomics technologies have been applied to the study of pharmaceuticals in aquatic organisms (Bundy et al., 2009). Many laboratory studies have investigated the effects of chemical exposure on the metabolic profile of fish to determine the metabolic pathways and physiological functions affected (Samuelsson et al., 2006; Ekman et al., 2007; Katsiadaki et al., 2009). Viant et al. (2006) used a metabolomics approach to determine the mode of action of the herbicide dinoseb on medaka fish embryo (*O. latipes*). They

reported changes in the levels of ATP and selected amino acids which resulted in reduced growth and abnormal development. Metabolomics is a promising approach but a large number of metabolites found remain unidentified and interpretation of the effects observed can be extremely challenging (Viant, 2003).

1.8. Bioinformatics

Ecotoxicogenomics studies involve the analysis of many components in biological samples and generate a large amount of data. Big changes in the gene expression, protein levels or metabolites composition are easy to identify, but subtle changes require statistical processing to determine the significance of the changes observed (Idborg et al., 2004). Multivariate analysis is generally applied to “omics” data using unsupervised and supervised projection methods to reduce the dimension of the data and allow the class separating metabolites to be distinguished (Ramadan et al., 2006).

The unsupervised method principal component analysis (PCA) is used to simplify complex datasets into interpretable models and display the interrelationship between the samples (observations) analysed. This method does not require any information on the samples (Eriksson, 2006). Data are represented as points in a multivariate K -dimensional space. PCA finds the line with maximum variance (called the first principal component, PC1) in the K -dimensional space of the whole data and each observation is projected onto this line. If the first PC is not enough for modelling the systematic variation of the data set then, another PC, orthogonal to the first one, is calculated which best explains the second greatest systematic variation of the data (Eriksson, 2006). PCA aims to explain as much variation as possible in as few

components as possible (van den Berg et al., 2006). PCA can also be applied to subgroups in order to discriminate amongst the samples and find potential biomarkers.

The supervised methods projection to latent structures (PLS), or partial least-squares discriminant analysis (PLS-DA) and orthogonal PLS (OPLS) require initial information about the classes, such as gender, concentrations, time, etc., to build a model and identify the class separating metabolites (Griffin et al., 2007). These techniques are applied to the data when PCA do not establish clearly the markers discriminating between sample groups (UMETRICS, 2007). In PLS the information about the different classes of observations is recorded in matrix X data (observations x predictors matrix) and the responses from PCA are kept in a matrix now called Y data (Eriksson, 2006). PLS then fits two PCA-like models at the same time, one for X and one for Y, and simultaneously aligns these models, creating a model to predict Y from X. Thus, PLS has been shown to enhance the separation between groups of observations by rotating PCA components facilitating the identification of the most important biomarkers (Wagner et al., 2007).

1.9. Aims of the thesis

As more pharmaceuticals are produced and released in the market, it is important to gather knowledge on existing pharmaceuticals and find suitable methods to predict the fate and behaviour of the new ones.

The hypotheses of this thesis were:

- 1) Diclofenac can be readily biodegraded by environmental microbes without altering their dynamics within the populations.
- 2) The toxicity of diclofenac reported in small mammals and humans is the same in fish.

In this thesis microbiology, animal physiology, genomics and metabolomics approaches were applied to investigate the effects of the NSAID diclofenac in the aquatic environment. This thesis is presented as a series of chapters. The experiments and aims of each study are described below:

Chapter 2 provides a detailed description of the protocols optimised, chemicals purchased and equipments used during this project.

In Chapter 3 microcosm experiments were conducted with river water and activated sludge. The environmental microbes were cultured under laboratory conditions with diclofenac and other NSAIDs (ketoprofen and naproxen) as sole carbon source in order to promote the growth of degrading bacteria. Cultures were routinely monitored to measure the degradation rate of NSAIDs and the bacterial growth rate. The toxicity of diclofenac was investigated and compared to other NSAIDs using a standardised microtoxicity test on *Vibrio fischeri*. Further toxicity analyses were carried out using bacteria isolated from activated sludge to determine the effects of diclofenac on microbial growth. The aims of this study were:

- To investigate the biodegradability of low concentrations of diclofenac.
- To isolate and identify the bacteria involved in the degradation process.
- To evaluate the toxic potential of diclofenac on microbial organisms using growth and luminescence as endpoints.

In Chapter 4, a laboratory based exposure was carried out using rainbow trout (*Oncorhynchus mykiss*). Fish were exposed to environmentally relevant concentrations of diclofenac via the water (0.5, 1, 5 and 25 µg/l) for 21 days. The effects of diclofenac exposure on rainbow trout were investigated in the gills, kidney, intestine and liver using histopathology and mRNA expression levels of *cox1*, *cox2*, *cyp1a1* and *p53* as endpoints. Bile analysis was carried out to study the bioconcentration potential of diclofenac. The aims were:

- To assess the ability of exposure to environmentally relevant concentrations of diclofenac to induce or inhibit the expression of selected genes in the kidney, gills and liver of rainbow trout.
- To determine the morphological alterations caused by diclofenac exposure in key organs (kidney, liver and small intestine).
- To gain a better understanding of the metabolism and fate (degradation and bioaccumulation) of diclofenac in rainbow trout.

Chapter 5 reports the various methods and parameters tested to analyse fish biofluids. The extraction methods solid phase extraction and methanol extraction were used for bile and blood plasma samples respectively. The parameters of the detection technique UPLC-TOF-MS were optimised to detect simultaneously various classes of signalling molecules. The methods were validated using samples spiked with known chemicals in order to assess the specificity, accuracy and reproducibility of the techniques to detect and identify a large number of metabolites at once. The aims were:

- To develop a methodology to extract and identify key molecules (eicosanoids, bile acids, fatty acids) in bile samples.
- To optimise methanol extraction and UPLC-TOF-MS methodologies in order to purify plasma samples and detect low abundance molecules such as eicosanoids in those samples.
- To analyse the plasma metabolome and find plasma markers affected by diclofenac exposure.

In Chapter 6 the methods developed in Chapter 5 were employed to analyse the bile metabolome of fish from the diclofenac exposure. Bile samples were treated by hydrolysis when required, fractionated and analysed by UPLC-TOF-MS. Multivariate data analysis was applied to the resulting metabolic profiles to probe for concentration related effects and identify key metabolites and biochemical pathways affected by exposure to diclofenac. The aims were:

- To determine the lowest concentration of diclofenac able to induce changes in the metabolic profile of rainbow trout after 21 day exposure.
- To identify the exogenous (diclofenac and its metabolites) and endogenous metabolites induced or inhibited.
- To discover new metabolic pathways affected by diclofenac exposure in fish and possibly link them to mammalian systems.

The final chapter (Chapter 7) provides a critical synthesis on the major findings from the thesis work and their implications for the discharge of diclofenac (and other NSAIDs) in the aquatic environment. Our research revealed that diclofenac is not readily biodegraded and that sub-chronic exposure to environmental concentrations of diclofenac can have significant effects on fish metabolism. These findings provide additional but valuable information for the environmental risk assessment of diclofenac in order to understand and predict the fate of this compound in the environment. This chapter further considers future research required to improve the removal of diclofenac in WWTPs and to fully understand the toxicity in aquatic organisms.

CHAPTER 2: General Materials and Methods

2.1. Biodegradation studies

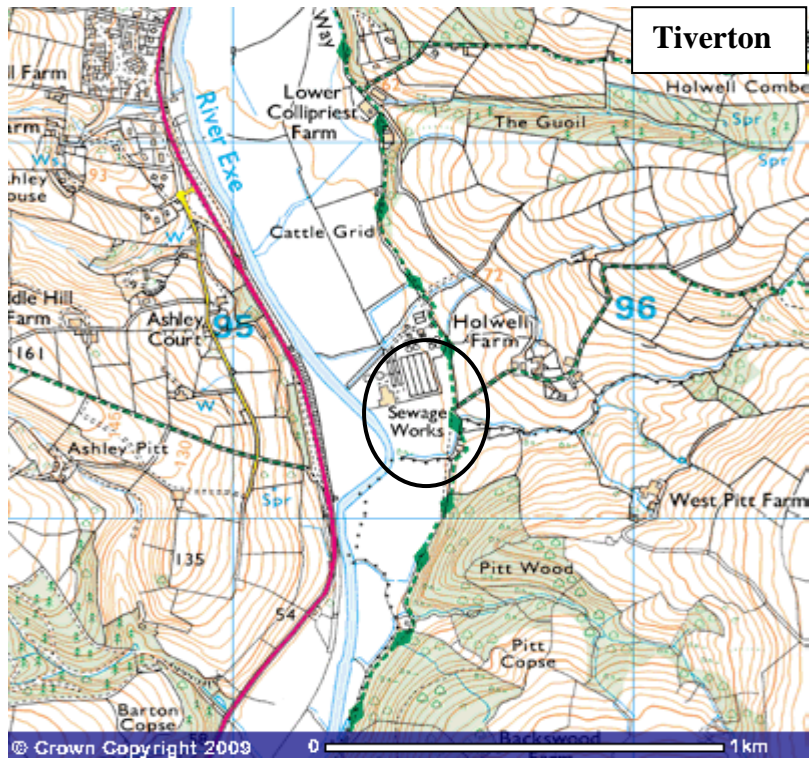
The objectives of this study were to enriched environmental samples with the non-steroidal anti-inflammatory drugs naproxen, diclofenac and ketoprofen and isolate and identify microbial communities able to degrade these compounds.

2.1.1. Sampling sites

Environmental sampling was carried out in the Exe catchment (Devon, United Kingdom) from December 2005 to February 2007. River Exe receives sewage effluents from eight different wastewater treatment plants treating domestic and industrial wastes. Water samples used in the biodegradation studies were taken from the two main wastewater treatment plants (WWTPs) in the area (see Figure 2.1). River water was collected 100 m downstream the Tiverton WWTP (SS 954 103 GB grid). Activated sludge, sewage effluent and river water were collected at Countess Wear (SX 948 891 GB grid). The samples were collected in 1 l amber bottles filled to the top and sealed. The bottles were previously acid washed and autoclaved at 121°C for 15 min. All samples were stored at 4°C. The sampling sites are described Figure 2.1. The samples were collected into sterile 1 l amber bottles filled to the top and sealed and stored at 4°C, until required.

2.1.2. Bacterial growth media

All glassware and culture media were autoclaved at 121°C for 15 minutes. The cultures were grown in minimal salts media (MSM) with pharmaceutical as sole carbon



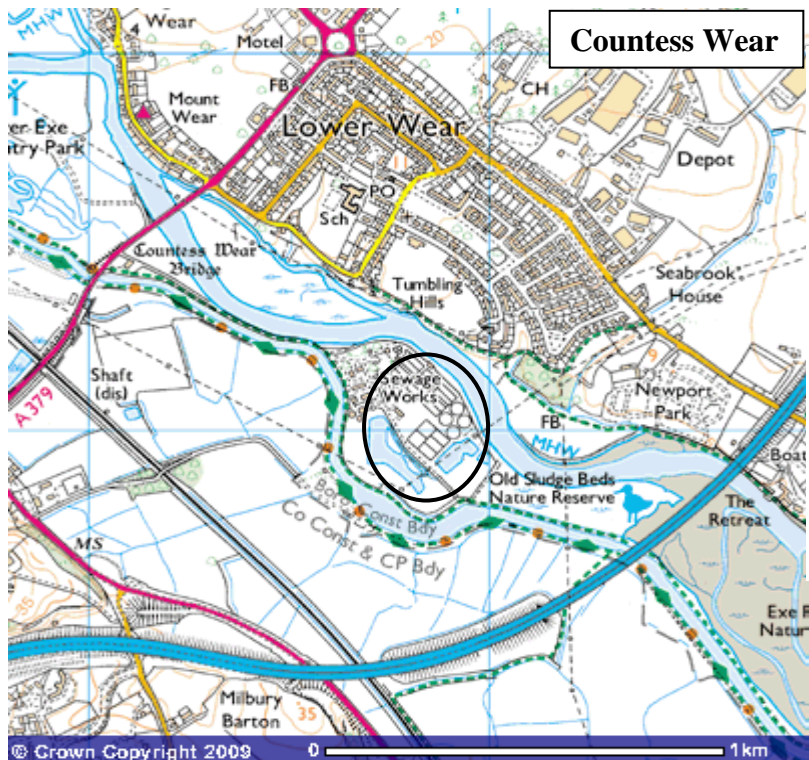
Tiverton

Domestic poulation
19233

Trade equivalent
11601

Primary treatment
Settlement

Secondary treatment
Filter bed



Countess Wear

Domestic poulation
19233

Trade equivalent
11601

Primary treatment
Settlement

Secondary treatment
Activated sludge

Tertiary treatment
Nitrifying BAFF

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Figure 2.1 – Maps of the sampling sites. Tiverton WWTP is located in a rural area and Countess Wear WWTP is located in an urban area.

source. The MSM was prepared as described by Tett et al., (1994) in deionised water using the following chemicals (g/l): MgSO_4 0.2, $(\text{NH}_4)_2\text{SO}_4$ 0.5, KH_2PO_4 0.5, Na_2EDTA 0.12, NaOH 0.02 and 0.5 ml of concentrated H_2SO_4 . A stock solution of trace elements was made up using the following chemicals (g/100 ml deionised water): ZnSO_4 0.4, CuSO_4 0.1, Na_2SO_4 0.01, Na_2MoO_4 0.1, CoCl_2 0.01, MnSO_4 0.04, and 1 ml of this solution was added per 1 l of MSM. The pH was adjusted to 7.0 using 0.5 M NaOH solution.

2.1.3. Microcosm enrichment cultures

Triplicate cultures were established in 250 ml Erlenmeyer flasks containing 100 ml MSM and spiked with ketoprofen, naproxen or diclofenac. The pharmaceuticals were previously dissolved in deionised water to a final concentration of 10 mg/ml. A volume of 1-5 ml of environmental sample (listed in Table 2.1) was added to the enriched media. The flasks were closed with porous foam and incubated statically for 48 hours then transferred to an orbital shaker at 200 rpm at 25°C. At each sampling time point all the cultures were analysed for optical density measurements and sampled for DNA extraction. The absorbance was read at 600_{nm} against an abiotic control. The concentrations of pharmaceuticals present in the enrichments were monitored using Solid Phase Extraction (SPE) to extract the pharmaceuticals and liquid chromatography/mass spectrometry for chemical quantification.

Control cultures were established in triplicate. Positive controls (biotic) were initiated using 100 ml MSM and 1 mg of environmental sample. Negative control cultures (abiotic) were set up using 100 ml MSM spiked with the pharmaceuticals in order to determine the rate of abiotic loss. All flasks were closed with porous foam and

incubated with enrichment cultures. Biotic controls were sampled at each time point as for the experimental samples.

2.1.4. Sub-culturing method

At day 5, 10, 15 and 40, the enrichments were sub-cultured in order to isolate the bacterial degraders. 1 ml of each culture was inoculated into fresh MSM with the relevant pharmaceutical. The flasks were closed and incubated at 25°C.

2.2. Nucleic acid extraction

2.2.1. 5% CTAB/phosphate buffer

The following stock solutions were required for the nucleic acid extractions and were made up as follows: 10% CTAB in 0.7 M NaCl (10 g CTAB and 4.09 g of NaCl in 1L deionised water), K_2HPO_4 (8.71 g of K_2HPO_4 in 50 ml deionised water) and KH_2PO_4 (1.36 g KH_2PO_4 in 10 ml deionised water). The potassium phosphate buffer 240 mM (pH 8) was prepared by mixing 22.56 ml of K_2HPO_4 with 1.44 ml of KH_2PO_4 stock solutions and making up to 100 ml with deionised water. The 5% CTAB/Phosphate buffer (120 mM pH 8) was prepared by mixing 50 ml of the 10% CTAB in 0.7 M NaCl with 50 ml of the potassium phosphate buffer.

2.2.2. Extraction procedure

Environmental samples were filtered through 0.22 μ m pore size membranes using a Watson-Marlow vacuum pump. The cells were recovered by introducing the filters in the barrel of a 5 ml syringe and washing them with 0.6 ml of CTAB buffer. The CTAB resuspension was transferred into a 2 ml screw-capped tube containing 0.5-1.0 g of zirconia/silica beads (0.1 mm diameter, Biospec Products). 0.5 ml of phenol

chloroform: isoamyl alcohol (25:24:1) was added and cells were lysed in a mini bead beater (Biospec Products) for 40 seconds at maximum rate. The tubes were then centrifuged at 13,000 rpm for 5 min. The supernatant was transferred into a sterile Eppendorf tube and an equal volume of phenol chloroform:isoamyl alcohol was added (about 0.5 ml). The samples were vortexed and centrifuged at 13,000 rpm for 5 min. The top aqueous layer was transferred to a new sterile Eppendorf tube. DNA was precipitated by adding 1 ml of 30% Polyethylene Glycol (PEG)/1.6 M NaCl solution and incubated overnight at room temperature (or a minimum of 4 hr). After incubation, the tubes were centrifuged at 13,000 rpm for 10 min to pellet the nucleic acids and the supernatant was discarded. The pellets were washed with 100% ice cold ethanol. The ethanol and any remaining liquid were removed. The pellets were then allowed to air dry. The DNA was resuspended in 40 µl ultrapure PCR quality water (Sigma) and stored at -20°C. Successful DNA extraction was confirmed by agarose gel electrophoresis

2.3. Agarose gel electrophoresis

50x TAE buffer was prepared by dissolving 242 g TRIS in 600 ml deionised water. 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA were added. The solution was sterilized by autoclaving at 121 °C for 15 min.

Electrophoresis was performed using a 1% agarose gel. 1 g of agarose was dissolved in 98 ml of deionised water and 2 ml of 50x TAE buffer. The solution was heated in a microwave until the agarose was fully dissolved. After cooling, 4 µl ethidium bromide (10 mg/ml) was added and the gel solution was gently mixed before pouring into a gel tank. Gels were left to set for 30 min, and then inserted into an electrophoresis tank containing 1x TAE buffer. Gels were loaded with 5 µl sample

mixed with 2 µl of loading dye (Promega). A DNA ladder (Hyperladder 1, Bionline) was loaded for each gel to indicate product size. Gels were run at 100 V for 30 minutes and visualised on a UV transilluminator (AutoChemiSystem) using LabWorks 46 Gel Pro Application Software.

2.4. Polymerase chain reaction amplification of 16S rRNA gene

Polymerase Chain Reaction (PCR) was used for subsequent identification of the bacterial species in the enrichments. This enzymatic process which amplifies genomic sequences was developed by Kary Mullis and co-workers in 1983. DNA amplification was carried out using the method described by Muyzer et al. (1993). The 16S rRNA gene of different bacterial species was amplified using primers corresponding to positions F341 (5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGG GCCTACGGG-AGGCAGCAG-3') and R534 (5'-ATTACCGCG GCTGCTGG-3') in *Escherishia coli*. These primers contain a 40 nucleotide GC rich sequence (GC-clamp). Each PCR reaction was performed in 50 µl volume and contained: 2 µl of each primer (10 pM/µl), 25 µl master mix (containing 50 units/ml Taq polymerase, 400 µM of each dNTPs, 3 mM Mg Cl₂, Promega), 19 µl of ultrapure water and 2 µl template DNA (50 ng). A negative control was also performed whereby no template DNA was added. All PCR reactions were performed with a ThermoHybaid® PCR thermocycler. The temperatures and cycling conditions were as follows: 1 cycle at 95°C for 1 min; denaturation step 30 cycles at 95°C for 1 min, annealing step at 55°C for 1 min, elongation step at 72°C for 2 min and a final elongation step at 72°C for 7 min. Products were visualized by agarose gel electrophoresis.

2.5. Microtox acute toxicity test

The toxicity of selected pharmaceuticals was evaluated using Microtox® toxicity test. The marine bacteria *Vibrio fischeri* naturally emit light as a result of their metabolic activity. Toxic substances changing their metabolic status would lead to a reduction of the bioluminescence. The bacteria freeze-dried (NRRL B-11177), reconstitution solution, diluent (2% NaCl) and an osmotic adjusting solution were purchased from SDI (Newark, USA). The pharmaceuticals were dissolved in deionised water for a final concentration of 50 mg/l. The bioassay was conducted on a Microtox Model 500 Analyzer and the '49.5% Basic Test' protocol was followed. The percent of inhibition (%I) was estimated against a saline control solution after 5 and 15 min of pharmaceutical exposure. Data obtained were analysed with the MicrotoxOmni® data reduction software (Azur Environmental). The inhibition curves produced were used to estimate the 50% effective concentration (EC₅₀) of each pharmaceutical.

2.6. Disc diffusion assay

The bioassay was optimised to assess the toxicity of diclofenac on specific bacteria naturally occurring in WWTPs. The minimum inhibitory concentration of diclofenac was calculated for each bacterial strain.

2.6.1. Bacterial strains

The following strains were purchased from DSMZ (Germany): *Pseudomonas putida* (5232), *Comamonas denitrificans* (17787), *Micrococcus luteus* (14235) and the filamentous bacteria *Zoogloea ramigera* (287). All bacteria were revived in nutrient broth and incubated at 28°C for 24 hr.

2.6.2. Toxicity test protocol

Diffusion discs were made with filter paper (Whatman grade 1) using a hole puncher and sterilised by autoclaving at 121°C for 15 min. 15 ml of nutrient agar was dispensed in plastic petri dishes. The plates were allowed to cool and 500 µl of bacterial inocula were spread over the agar. The discs were then placed onto the plates using tweezers. A serial dilution of diclofenac was prepared and 50 µl of the relevant solution was added to the discs for final concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/l of diclofenac. The plates were incubated for 16 hr at 28°C. After incubation, the inhibition zone around each disc was measured in 3 different directions to obtain the mean diameter.

2.7. Detection of pharmaceuticals in water samples

Analytical methods were optimised to isolate and detect diclofenac in water matrices. The technique was then applied to quantify diclofenac in water samples taken from the biodegradation study and the fish exposure tanks.

2.7.1. Solid phase extraction (SPE)

The cartridges Oasis HLB 6cc (Waters, Milford, USA) and Strata X (Phenomenex, Macclesfield, UK) were tested at acidic pH, and the cartridges Strata X-AW (Phenomenex, Macclesfield, UK) were tested at neutral pH. The extraction was performed using a SPE manifold (Alltech). The protocols are summarised Table 2.1. The internal standard (IS) tolfenamic acid was added to the elution solvents to quantify the extraction efficiencies of the target pharmaceuticals. The highest extraction efficiencies were obtained at acidic pH with Oasis HLB 6cc and these cartridges were subsequently used to analyse the aqueous samples of the biodegradation study and the

tank water of the fish exposure. All samples were filtered using GF/C paper (Whatman) and diluted to 5% methanol. The internal standard was added at 5% of the concentration of the tested pharmaceuticals prior to the extraction. The samples were loaded into the cartridges that were previously conditioned, then washed with ultrapure water and eluted in the same way as described in Table 2.2. The solvents collected sequentially were evaporated to dryness in a SpeedVac concentrator (Savant Instrument, NY, USA). The samples were reconstituted in 200 μl of methanol:water (1:1, v/v) and filter-centrifuged for 1 min at maximum speed using VectaSpin MicroAnapore (0.2 μm , Whatman). All samples were then transferred to HPLC vials and sealed using PTFE liner solid caps. All glassware used for the extraction were baked at 500°C for 2 hr to eliminate any organic contaminants.

2.7.2. Ultraperformance liquid chromatography time-of-flight mass spectrometry

The extracted samples were separated using an Acquity UPLC BEH C₁₈ column (100 mm x 2.1 mm, 1.7 μm particle size; Waters, Elstree, UK). An injection volume of 20 μl was used. The mobile phase consisted of 5% methanol and 0.2% formic acid in water (A) and 0.2% formic acid in methanol (B) at a flow rate of 0.1 ml/min. The mobile phase gradient was kept at 50% solvent A for the first 3 min followed by a linear gradient over 12 min to 100% solvent B. This was held for 4 min and re-equilibrated to 50% A for 7 min. The pharmaceuticals were detected on a Micromass QTOF-MS system (Manchester, UK) with electrospray ionization operated in either positive (+ESI) or negative (-ESI) ionization mode. The internal calibration (lockmass) of the mass spectrometer was performed with sodium formate and sulfadimethoxine infused at 40 $\mu\text{l}/\text{min}$ via lockspray interface to ensure accurate mass measurements. The internal lockmass m/z were 311.0814 (+ESI mode) and 309.0658 (-ESI mode).

Table 2.1 – Solid phase extraction protocol.

	Acidic pH	Neutral pH
Sample preparation	20 ml water with 200 µl acetic acid 500 ng of the relevant pharmaceutical	20 ml water with 30 mM sodium acetate buffer (pH 7) 500 ng of the relevant pharmaceutical
Cartridges	Oasis HLB 6cc (200 mg/6 ml) Strata X 33µM Polymeric Sorbent Patent (200 mg/6 ml)	Strata X-AW 33µM Polymeric Weak Anion (200 mg/6 ml)
Conditioning	4 ml ethyl acetate 4ml methanol 4 ml 1% acetic acid in HPLC water	4 ml ethyl acetate 4ml methanol 4 ml 30 mM sodium acetate buffer

Load the samples		
Wash with 3 ml of HPLC water		
Dry cartridges by vacuum aspiration		
Elution	4 ml ethyl acetate 4 ml methanol 4 ml 2% formic acid in methanol	

Nitrogen was used as nebulizing and desolvation gas and argon was used as collision gas. The MS parameters were collision energy 7 eV, capillary voltage 2.65 kV, cone voltage 35 kV, source temperature 100°C with a cone gas flow of 0 l/hr, desolvation gas 308 l/hr and desolvation temperature 250°C. Data were acquired with MassLynx 4.1 and a mass window of 50 ppm was used to extract signals from the total ion current (TIC). Full scan spectra of the compounds were recorded in positive and negative mode in order to select the most abundant m/z ion. The theoretical parent ion for each analyte was calculated by using the Molecular Weight Calculator software (MassLynx V 4.1) from the atomic mass of the most abundant isotope of each element.

2.7.3. Quantification of pharmaceuticals

Five samples with known concentration for each pharmaceutical were injected in the UPLC-TOF-MS using the conditions described in section 2.8.2. To determine the extraction efficiency, a method known as internal calibration was used. Calibration curves were constructed by plotting a linear trend line of the ratio of the pharmaceutical peak area to IS peak area against concentration. For the experimental samples, a calibration curve was constructed by plotting a linear trend line of the pharmaceutical peak area against concentration, a method known as external calibration. In order to estimate how closely the estimated values for the trend line corresponded to the actual data, the regression coefficient (R^2) was calculated. The sensitivity of the method was evaluated by the instrumental limit of detection (LOD) for the different compounds and this was estimated as 3 times the signal-to-noise ratio. Each sample was analysed in four replicates and the precision (reproducibility) was calculated by the relative standard deviation of the measurements.

2.8. Fish studies

Fish were exposed to low concentrations of the pharmaceutical diclofenac for 3 weeks. The tissues collected after the exposure were analysed to assess the effects of diclofenac on selected gene expression and tissues' health.

2.8.1. Supply and maintenance of the fish

Juvenile female rainbow trout (*Oncorhynchus mykiss*) (~200 g) were acquired from Houghton Springs Fish Farm in Dorset (UK) and acclimated for 6 weeks in the laboratory prior to exposure to diclofenac. Fish were maintained in tanks with dechlorinated aerated tap water at a temperature of 10°C under a 12:12 hr light:dark photoperiod. They were fed three times a week with standard trout pellets (2 mm pellets, Biomar Ecoline, Denmark). All fish were PIT (Passive Integrated Transponder) tagged to allow subsequent identification of individual fish. Fish were also weighed and measured.

2.8.2. Experimental set up

Exposure to diclofenac was carried out in 150 l aerated tanks equipped with a flow through water system (18 l/hr). The experimental set up for the exposure is illustrated Figure 2.2. Fish were placed in groups of 15 per replicate tank and exposed to diclofenac (98% purity, Sigma) for 21 days at nominal concentrations of 0, 0.5, 1, 5 and 25 µg/l. In order to monitor the concentration of diclofenac in the tanks, 1 l water samples were taken at day 0, 7, 14 and 21. The samples were fixed with 5% methanol and stored at 4°C for up to 24 hr before analysis by SPE and UPLC-TOF-MS (section 2.7). Feeding was withheld 5 days before the final sampling day.

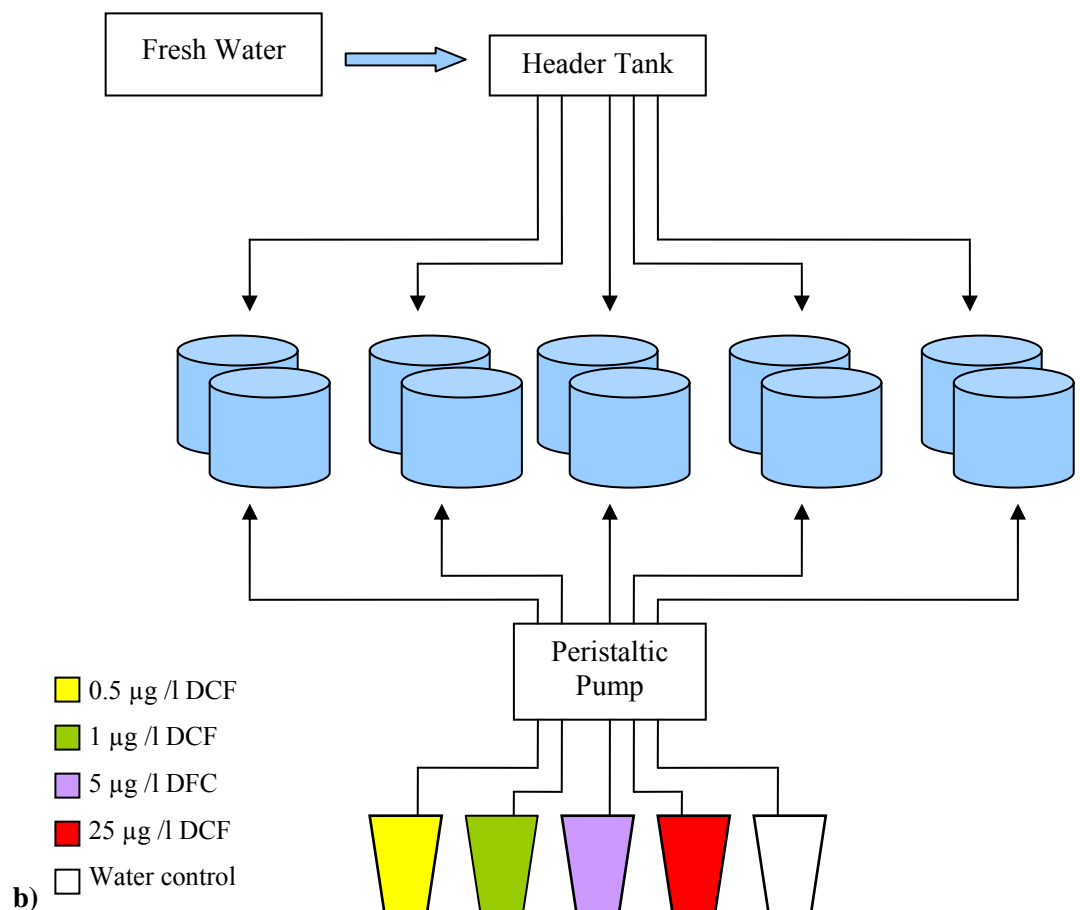
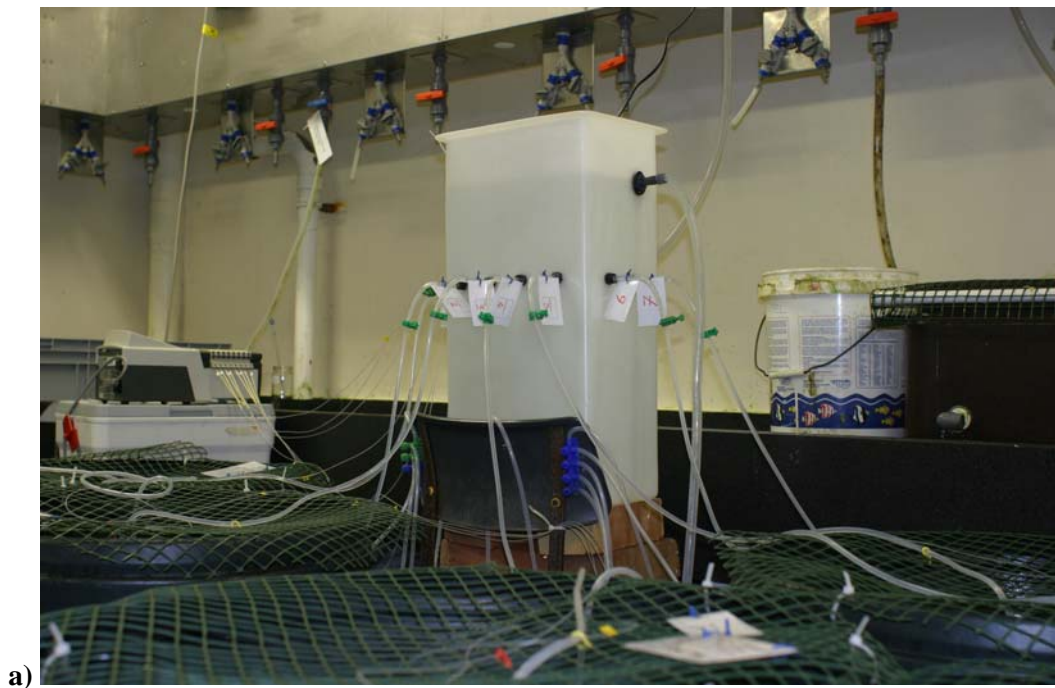


Figure 2.2 – Diclofenac (DCF) *in vivo* experimental set-up showing (a) header tank and peristaltic pump and (b) tank lay-out.

2.8.3. Dissection and tissue collection

Anaesthesia of the fish was carried out in accordance with Animals Act 1986 (Scientific Procedures). A solution of the anaesthetic benzocaine (ethyl 4-aminobenzoate) was prepared in ethanol (500 mg/l) and diluted in tap water (1:10, v/v). On day 21, fish were placed in a lethal dose of anaesthetic solution until movement of the operculum had ceased. The fish were then removed and the brain was destroyed. The length, weight and tag number of each fish were recorded. Liver, gills, intestine, kidney, blood and bile samples were excised from each fish.

Liver, gills and kidney samples collected for molecular studies were stored in 1.5 ml Eppendorf tubes and immediately frozen in liquid nitrogen. The samples were then stored at -70°C. Samples of liver, kidney and small intestine kept for histological analysis were placed in glass bottles and fixed with Bouin's solution. This solution contained a combination of fixative reagents (picric acid, formaldehyde and acetic acid) which prevent structural decomposition and excessive tissue hardening. After 4 hr, the tissues were transferred into 70% industrial methylated spirits (IMS). After 24 hr the tissues were placed into a fresh solution of 70% IMS and stored at room temperature until further analysis. Blood samples of 200 µl were taken from the caudal vein using heparinised syringes (23G 0.6 x 30 mm Microlance™ needle, Becton Dickinson, UK) and transferred to 1.5 ml Eppendorf tubes containing 5 µl of aprotinin. The tubes were centrifuged at 10,000 g for 15 min at 4°C (Biofuge fresco, Heraeus, Germany) and the plasma was transferred to methanol rinsed glass vials containing 400 µl of methanol for metabolomics studies. The bile and blood sampled for metabolomics analysis were stored in methanol rinsed glass vials containing two volumes of ice cold methanol.

2.9. Total RNA extraction and quantification

Total RNA was used to quantify the expression of specific genes using Q-PCR. Total RNA was extracted using Tri Reagent (Sigma) according to the manufacturer's protocol. This method is based on the RNA isolation technique developed by Chomczynski and Sacchi in 1986. Frozen samples (liver, kidney, gills) were immediately placed onto ice. In order to lyse the cells, the samples were homogenised with 1 ml of Tri Reagent using pellet pestles (Sigma-Aldrich) previously soaked in 0.1 M NaOH and rinsed with HPLC water. The homogenates were incubated for 5 min at room temperature to permit the dissociation of nucleoproteins. A volume of 200 μ l chloroform was added to each tube and the samples were shaken vigorously by hand for 15 sec. The tubes were incubated at room temperature for 15 min and centrifuged at 13,000 rpm for 15 min at 4°C. After centrifugation, the RNA remained in the colourless aqueous phase at the top. The DNA and proteins were present in the interphase and the lower organic phenol-chloroform phase respectively. The aqueous phase was transferred to a fresh tube and 1 ml of ice cold isopropanol was added. The samples were incubated overnight in the freezer at -20°C to precipitate the RNA. The following day the tubes were centrifuged at 13,000 rpm for 20 min at 4°C to pellet the RNA at the bottom. The supernatant was discarded and the pellets were washed with 1 ml of ice cold 75% ethanol, briefly vortexed and centrifuged at 7,500 rpm for 5 min at 4°C. Finally the RNA pellets were air dried and dissolved in 50 μ l HPLC water. The samples were stored at -80°C until used for Q-PCR. The isolated RNA was quantified with an ND-1000 Nanodrop spectrophotometer at 260 nm. The ratio of absorbance at 260/280 nm was used to assess the quality of the RNA. Samples with a ratio of \sim 2 were considered as pure. The ratio of absorbance at 260/230 nm was also used as a secondary measure of purity. Values between 1.8 and 2 were considered acceptable. Lower ratios indicated the

presence of contaminants such as proteins. All samples were diluted to a final concentration of 1 µg/µl using HPLC water.

2.10. Reverse transcription (RT)-PCR

RT-PCR was carried out to purify the RNA extracted and synthesise the first strand of cDNA. The procedure was conducted in a thermal cycler (MyCycler™ Bio-Rad) using DNase/RNase free PCR tubes (0.2 ml Thermo-tubes, Thermo Scientific). All reagents and RNA samples were kept on ice. First, the RNA samples were subjected to DNase treatment using RQ1 RNase-Free DNase (M6101, Promega, Southampton, UK). This initial stage uses an endonuclease to degrade any contaminating DNA. Each reaction contained 5 µl HPLC water, 1 µl RNase-free DNase 10x reaction buffer, 2 µg relevant RNA (~2 µl) and 2 µl DNase enzyme for a total volume of 10 µl. Samples were pulse spun briefly and incubated at 37°C for 30 min in the thermal cycler. Following the DNase digestion reaction, 1 µl of RQ1 stop solution was added. The samples were mixed well and incubated at 65°C for 10 min to deactivate completely the DNase. The samples were then returned on ice.

Reverse transcription was then performed to synthesize the first strand of cDNA forming a mRNA:cDNA hybrid. Random hexamers (10 pM/µl; 5'-NNNNNN-3') were ordered from MWG (Biotech, Germany) and 1 µl was added to each PCR tube. The samples were mixed well, incubated in the thermal cycler at 70°C for 5 min to melt the secondary structures within the RNA template and immediately chilled on ice to prevent the reformation of the secondary structures. RT-PCR reaction was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (M1701, Promega, Southampton, UK). 6µl of HPLC water, 1µl dNTP mix (10 mM each of dATP, dCTP, dGTP, dTTP; Invitrogen, UK), 1 µl M-MLV reverse transcriptase enzyme, and 5 µl 5x

M-MLV buffer were added. The samples were pulse spun briefly and incubated in the thermal cycler at 25°C for 15 min to allow the annealing of the random hexamers, then at 42°C for 50 min for cDNA synthesis and finally at 70°C for 10 min to inactivate the reaction. The tubes were then chilled at 4°C and stored in the freezer at -20°C.

2.11. Quantitative real-time PCR (Q-PCR)

Quantitative real-time PCR was used to quantify the expression of target mRNAs. The amplification reaction is monitored in real time using fluorescent reporters that bind to the PCR products (amplicons) formed. Data is acquired during the exponential phase of the reaction, where the doubling of amplicons occurs at every cycle. SYBR[®] Green was used as the fluorescent dye. The increase in fluorescence is proportional to the number of amplicons. Quantification is assessed by measuring the number of cycles required to reach above a fluorescent background, a parameter known as threshold cycle value (C_T value).

2.11.1. Primer design for Q-PCR

Beacon Designer software (Premier Biosoft International, Palo Alto, CA) was used to design SYBR Green PCR primer pairs. Sequences of the target genes were found in the genomic database available at NCBI and were aligned using program Clustal W (<http://www.ebi.ac.uk/clustalw>) to detect sequence similarities. The sequences were then inserted in the software to design primer specific to the target mRNAs. The design parameters were set as follow: melting temperature (T_M) of both primers 50-55°C, primer length 18-24 bp, amplicon length 80-140 bp and GC content 40-60%. Primer pair with a T_M close to 55°C and a low chance of hairpin loop, self or cross dimers were selected. These primers would be unlikely to form secondary structures

caused by inter- or intra-molecular interactions. Beacon Designer also automatically use Basic Local Alignment Search Tool (BLAST; NCBI) to confirm primer specificity to only one homologous sequence. The primers selected were purchased from MWG-Biotech.

2.11.2. Optimisation of primer-pair annealing temperature

The optimisation of the annealing temperature of a primer pair is important to maintain a high PCR product yield and specificity. A temperature gradient with 8 different annealing temperatures was run in triplicate for each primer pair. The range of temperatures tested was set at 1°C below the annealing temperature predicted by the primer design software up to 5°C above this temperature (range of 6°C). In this study the temperature gradient ranged from 56 to 62°C. The Q-PCR reactions were prepared as described section 2.12.4. A pool sample of cDNA from rainbow trout tissues was diluted 10-fold in HPLC water and 0.75 µl was added to each reaction. The cycling conditions were set as follow: 1 cycle at 95°C for 15 min followed by a denaturation step of 40 cycles at 95°C for 10 seconds and an annealing step at each of the 8 temperatures for 20 seconds. The temperature closest to 60°C with the lowest C_T value was selected.

2.11.3. Determination of Q-PCR amplification efficiency and melt curve

The amplification efficiency, linearity and specificity of the primers were evaluated by running a standard curve followed by a melt curve. Triplicate Q-PCR reactions (section 2.12.4) were run with 0.75 µl of 5 dilutions of cDNA (neat, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}). The cycling conditions were: 1 cycle at 95°C for 15 min followed by 50 cycles of denaturation at 95°C for 10 seconds and annealing at the optimised

temperature of the primer pair (section 2.12.2) for 45 seconds. During this stage the data are collected to determine the C_T value of each sample. The standard curve was generated using the iCycler software (iQTM Multicolour Real-Time PCR Detection System Optical Software Version 3.1, Bio-Rad) by plotting the logarithm of cDNA dilution against its corresponding C_T value (Figure 2.3). Primer efficiency should be close to 100% ($E = 2.0$) suggesting that the products are doubled at every cycle.

Because SYBR Green binds to double stranded DNA with no discrimination between the amplicons and other non specific amplification products, a melt curve was performed to determine the specificity of the primer pair (1 cycle at 95°C for 1 min, 1 cycle at 55°C for 1 min and 80 cycles for 10 seconds starting at 55°C with a temperature increase of 0.5°C for each subsequent 10 seconds cycle). In each sample the total fluorescence generated by SYBR green was recorded as the temperature changes and plotted in a graph (Figure 2.4). The amplicons for the targeted product have approximately the same melting point and produce a distinct single peak on the plot. Primer dimers melt at lower temperatures and additional PCR products have a higher melting point.

2.11.4. QPCR protocol for tissues

Real time PCR was performed on an iCycler iQTM Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) and data were acquired using iCycler software (as detailed above). For each reaction the PCR mastermix contained 7.5 µl 2x AbsoluteTM QPCR SYBR[®] Green Fluorescein Mix (ABgene, Epsom, UK), 6 µl HPLC water and 0.375 µl of the appropriate forward and reverse primer (10 µM). SYBR[®] Green I Fluorescein Mix contained a Thermo-Start[®] DNA polymerase, a hot-start Taq polymerase and the dye fluorescein used to normalise amplification data.

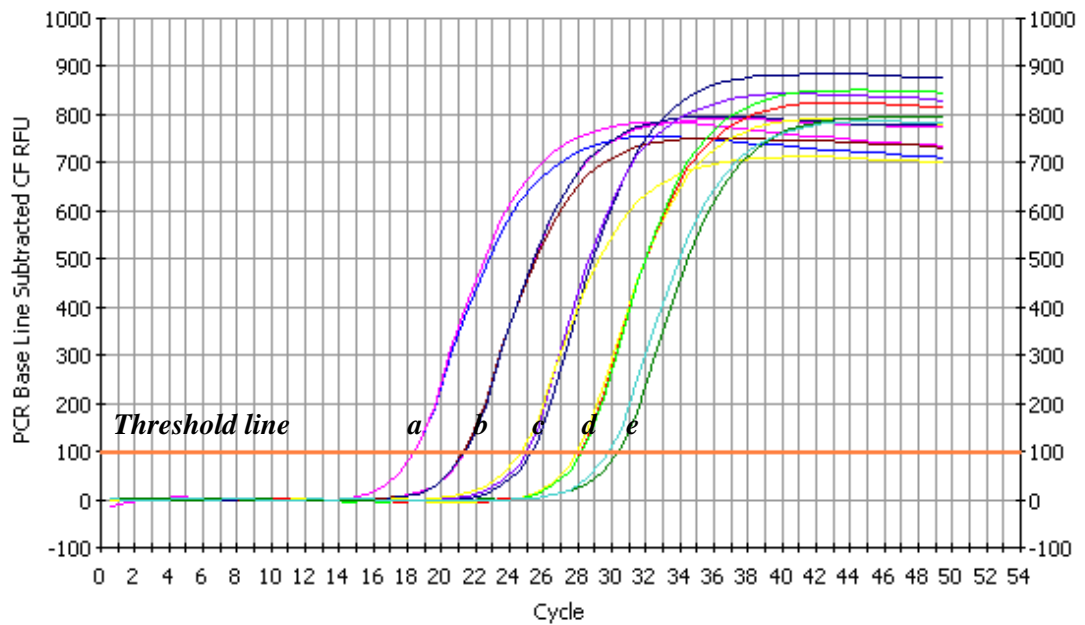


Figure 2.3 – Real-time PCR amplification graph. Each response curve corresponds to serial dilution of cDNA – a) neat, b) 10^{-1} , c) 10^{-2} , d) 10^{-3} , e) 10^{-4} . The points where the curves cross the threshold line are known as the threshold cycle (C_T) values. C_T values plotted against the relative fluorescence are directly proportional to the initial amount of template in the standards.

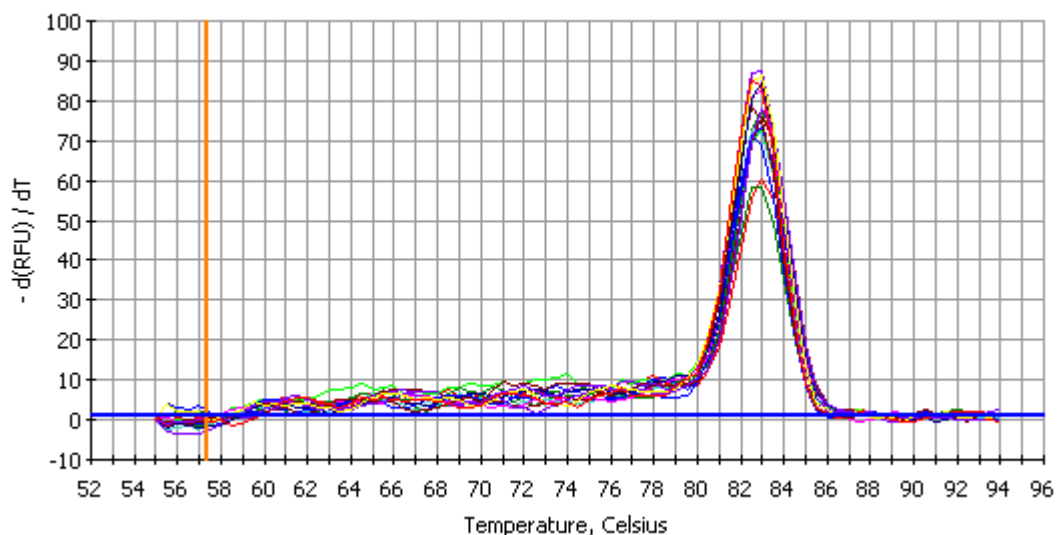


Figure 2.4 – Melt curve analysis. Fluorescence drops rapidly when the amplicons melt and produce a distinct peak.

Samples were run in triplicate in 96 well optical plates (ABgene, Epsom, UK). 14.25 µl of mastermix and 0.75 µl of the relevant cDNA were dispensed in each well. Each plate also had a negative control (0.75 µl of HPLC water instead of cDNA) to check for cDNA contamination and a pooled cDNA sample to assess inter-assay variability. The plates were sealed using optical quality plate seals (Bio-Rad). The Q-PCR cycling conditions were as follows: initial denaturation step at 95°C for 15 minutes, which activates the hot-start Taq polymerase, followed by 40 cycles of denaturation at 95°C for 10 seconds, and annealing at the appropriate optimised annealing temperature for the primer pair for 20 seconds. Following the amplification, a melt curve was performed (1 min at 95°C, 1 min at 55°C, then 80 cycles each of 10 seconds, beginning at 50°C, temperature increasing by 0.5°C at each cycle).

2.11.5. Data analysis

Relative gene expression levels were calculated based on the arithmetic comparative method of Livak and Schmittgen, (2001). Efficiency correction was added to the equation as it was not assumed that the efficiency of both the house keeping gene and the target gene were 100% ($E = 2$). Instead the efficiency values calculated with the standard curves were entered. Relative expression levels were calculated as follows:

$$RE = \frac{(E_{ref})^{C_T \text{ ref}}}{(E_{target})^{C_T \text{ target}}}$$

RE = relative gene expression

C_T = threshold cycle value

ref = housekeeping gene

$target$ = gene of interest

E = real time PCR amplification efficiency for each gene

2.12. Histopathology on fish tissues

The liver, small intestine and kidney of the rainbow trout were processed and stained to examine the histopathological alterations caused by diclofenac exposure.

2.12.1. Dehydration and embedding

Tissues were placed in labelled plastic cassettes and kept in 70% IMS until processing. Because fixed tissues are not firm enough to cut thin sections, they were embedded in paraffin wax. Fixative solutions and paraffin wax are not miscible. Thus the samples were placed in a tissue processor (Shandon Citadel 2000) in order to remove all fixative and ensure that the wax infiltrates completely the samples (Table 2.2). First the tissues were dehydrated through a series of alcohol solutions (step 1-7) to remove the fixatives and tissue water content. Then the tissues were transferred to Histo-clear® (Fisher Scientific), a clearing fluid miscible with alcohol and paraffin wax (step 8-10). Finally the tissues were impregnated with molten wax before the final embedding stage (step 11 and 12). Embedding was carried out in stainless steel moulds using a wax dispenser (Electrothermal Engineering Ltd., UK). The cassettes were incorporated to the blocks which were allowed to cool onto a cold plate (RA Lamb, London, UK).

2.12.2. Sectioning of the embedded tissues

Prior to sectioning, the blocks were put on ice to solidify the paraffin wax and produce better sections. The blocks were trimmed with a straight blade scalpel and sections of 5 µm were made with a rotary microtome (Shandon AS325 retraction). The sections were transferred in a bath of 30% IMS to reduce static charges and floated onto a warm water bath at 40°C to smooth the wax. The sections were then collected onto

Superfrost glass slides (BDH Laboratory Supplies), labelled and allowed to dry for 24 hours on a metal hot plate at 40°C.

2.12.3. Haematoxylin and Eosin (H&E) staining

To examine the tissue elements under light microscopy, the slides were routinely stained with haematoxylin and eosin, one of the most widely used histological stain (Bancroft and Stevens, 1982). Harris' haematoxylin is a basophilic dye which stains nucleic acids such as cell nucleus and ribosomes in blue/purple. Eosin is a counterstain that colours the cytoplasm and proteins in bright pink. Staining was completed using the protocol described Table 2.3. The slides were immersed in Histo-clear to be dewaxed and transferred through a series of graded IMS solutions to hydrate the tissue. After the haematoxylin staining, the slides were washed with tap water and 0.5 % acid alcohol. The sections were counterstained with eosin and progressively dehydrated in increasing concentrations of IMS. Finally the tissues were cleared in Histo-clear. Stained slides were mounted under glass coverslips (Fisherbrand) using the mounting medium histomount (National Diagnostics). The slides were allowed to dry for a few days at room temperature.

2.12.4. Analysis of fixed tissue sections

Stained slides were examined by light microscopy (Zeiss Axioskop 40 microscope; Carl Zeiss Ltd., Germany) coupled with an Olympus DP70 CCD camera (Olympus Optical, UK). The digital images were analysed using the software analysis 3.2 software (Soft Imaging System GmbH, Munster, Germany) for histopathological lesions.

Table 2.2 – Processing program for histological analyses.

Step	Solution	Immersion time
1	70% IMS	1 hr
2	70% IMS	1 hr
3	80% IMS	1 hr
4	90% IMS	1.5 hr
5	98% IMS	1.5 hr
6	100% ethanol	1.5 hr
7	100% ethanol	1.5 hr
8	Histo-clear	1.5 hr
9	Histo-clear	1.5 hr
10	Histo-clear	1 hr
11	Molten wax (~65°C)	1 hr
12	Molten wax (~65°C)	2 hr

Table 2.3 – H&E staining protocol for fixed sections.

Step	Solution	Immersion time
1	Histo-clear	5 min
2	Histo-clear	5 min
3	100% IMS	2 min
4	90% IMS	2 min
5	80% IMS	2 min
6	Tap water	2 min
7	Harris' haematoxylin non acidified (Thermoshandon, Pittsburg, US)	15 min
8	Tap water	2 min
9	0.5% of concentrated HCl in 70% IMS	2 min
10	Tap water	30 sec
11	70% IMS	30 sec
12	Tap water	30 sec
13	Eosin Y aqueous (Thermoshandon, US)	10 sec
14	Tap water	30 sec
15	80% IMS	30 sec
16	90% IMS	1 min
17	95% IMS	1 min
18	100% IMS	2 min
19	100% ethanol	2 min
20	Histo-clear	2 min
21	Histo-clear	3 min

2.13. Metabolomics studies

Blood and bile samples were analysed to determine their metabolite compositions and identify the metabolites affected by the chemical exposure. This study aimed to find biomarkers indicative of exposure to diclofenac.

2.13.1. Hydrolysis of bile samples

The following stock solutions were prepared: (A) sodium dihydrogen orthophosphate (0.2 M) and (B) disodium hydrogen orthophosphate (0.2 M). Phosphate buffer (0.1 M pH 6.05) was made by adding 16 ml stock A and 4 ml stock B to 20 ml HPLC grade water. Aliquots of 300 μ l bile samples were transferred into glass tubes and evaporated in a SpeedVac. The samples were reconstituted in 500 μ l of 0.1 M phosphate buffer. Deconjugation was carried out by adding 200 μ l of the following enzymes β -glucuronidase (type VII from *Escherichia coli*, 1000 units/ml) and sulphatase (type VI from *Aerobacter aerogenes*, 2 units/ml). A blank sample was prepared using 10 μ l of HPLC instead of bile. The top of each tube was covered with foil and closed. The samples were vortexed and incubated at 37°C for 2 hr and left overnight at room temperature. After incubation, the reaction was not stopped with acetic acid to maintain the pH neutral. Controls were set up to monitor the activity and specificity of both enzymes. Glucuronidase and sulphatase (20 μ l) were incubated separately with 50 μ l phosphate buffer, 20 μ l HPLC water and 10 μ l nitrophenol glucuronide (10 mg/ml) or potassium nitrophenyl sulphate (10 mg/ml). Working enzymes should turn the solution yellow. All glassware used were washed with detergent, rinsed with water and washed with acetone. They were then wrapped in foil and baked at 500°C for 2 hr.

2.13.2. Solid phase extraction of bile samples

Extraction of the bile samples was carried out using the cartridges Strata X-AW (60 mg, 6 ml, weak anion exchange) (Phenomenex, Macclesfield, UK). Samples were diluted in 7 ml sodium acetate buffer (300 mM, pH 7.0) and loaded into the cartridges previously conditioned with 3 ml ethyl acetate, 3 ml 2% formic acid in methanol, 2 ml HPLC water and 3 ml sodium acetate buffer. After sample loading, the cartridges were washed with 1 ml of HPLC water and dried under vacuum aspiration for 10 min. Bile extracts were eluted with 2 ml ethyl acetate and 2 ml 2% formic acid in methanol as one fraction. The cartridges were then rinsed with 1 ml HPLC water, dried for 10 min and further eluted with 2 ml 2% ammonium hydroxide in methanol. The fractions were evaporated to dryness in a SpeedVac and brought up in 100 μ l of methanol:water (1:1, v/v). The bile extracts were vortexed, centrifuged in 0.22 μ m ultrafiltration tubes (Millipore) for 1 min at maximum speed and transferred to HPLC vials using a Pasteur pipette. The extracts were stored at -20°C until UPLC-TOF-MS analysis.

2.13.3. Methanol extraction of blood plasma samples

Blood metabolites were separated using a methanol extraction procedure. Aliquots of 200 μ l plasma samples were mixed to 200 μ l of ice cold methanol in glass test tubes and 80 μ l of internal standard (IS) E₂-*d*4-S and P-*d*9 (working solution 10 pg/ μ l). A control sample was prepared with 200 μ l methanol instead of blood plasma. Samples were vortexed and kept on ice for 5 min then centrifuged for 10 min at 3,000 rpm. The supernatant was transferred to a new glass tube with a glass pipette and evaporated to dryness in a SpeedVac. Blood extracts were reconstituted in ice cold methanol:water (1:1, v/v), vortexed and kept on ice for 15 min. The extracts were then purified using an Extraction Plate Manifold and 96-well Sirocco™ Protein

Precipitation Plates (Waters, UK). The extraction plates were washed with methanol: water (1:1, v/v) and dried for 10 min by vacuum aspiration. The extracts were transferred to the 96-well plates, filtered by vacuum aspiration and placed in HPLC vials and stored at -20°C. The samples were then analysed using the UPLC-TOF-MS system detailed section 2.7.2.

2.13.4. Pre-processing of data

Chromatographic raw data were processed using MarkerLynx V 4.1 software package (Waters Corporation, Milford, USA). The chromatograms were aligned with the IS where applicable and normalized to spectral area. The parameters used for the detection of the spectral peaks were optimised to reduce the noise level.

Data collected were then exported to SIMCA-P software (Umetrics UK Ltd., Windsor Berkshire, UK) where it was centered by subtracting their averages and scaled. Principal Component Analysis (PCA) was performed and provided an overview of the data and to detect outliers. This projection method reduces the dimensionality of the dataset, usually 2-5 dimensions (components) to provide a general overview of the data. The components are uncorrelated variables transformed from the original variables of the data and they account for as much of the variability of the data as possible. Strong and moderate outliers were identified in the score plot and in the distance to the model X-space (DModX) respectively. Outliers were removed for subsequent analysis.

2.13.5. Multivariate analysis

A supervised analysis PLS (partial least-squares to latent structures) was performed to find class-separating differences amongst the treatments. A 7-fold cross validation was used to determine the significant components of the model. In order to

improve the PLS model findings, OPLS (orthogonal PLS) models were performed so that class separation is found only in the first predictive component. The potential markers were selected from the S-plot of the OPLS models, a plot of contribution of variables vs. confidence, where high confidence variables with large changes between groups are found at the extreme ends of the “S”. Variables represent the ion of a metabolite (e.g [M+H] or [M-H]) depending on the ESI mode. The accuracy of the models was evaluated using the explained variation (R^2X for PCA, and R^2Y for PLS and OPLS), and the predicted variation (Q^2). R^2 informs on how well the model fits to the data and Q^2 is the cross-validation parameter estimating the predictive ability of the model.

2.13.6. Model and data validation

To assess the risk of overfitting, a cross validation was performed on the PLS-DA models with a predicted variation (Q^2) higher than 0.4. Training sets were built leaving five observations of each treatment out in turn and remodelling the data until 60% of the observations were used to predict class membership (external validation).

The raw data (not normally distributed) of each potential metabolite marker was analysed using the non-parametric test Kruskal-Wallis followed by a Mann-Whitney test post hoc for pair-wise comparisons of the control vs. one the exposure. Post hoc tests can increase the number of false positives (Type I errors), thus a Bonferroni corrected critical value was calculated dividing 0.05 by the number of variables in each model. No observations were removed for this analysis.

CHAPTER 3: Biodegradation of non-steroidal pharmaceuticals in the aquatic environment and their toxicity to microbes

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Contributions of each author:

In this chapter I conducted all the experiments myself including the experimental design, sampling, water chemistry and data analyses.

Dr Hill provided a technical assistance to optimise the analytical techniques (solid phase extraction and UPLC-MS) used to analyse the water samples. Prof Tyler, Dr Burton and Prof Lappin-Scott supervised this work.

Abstract

The occurrence of pharmaceuticals in the aquatic environment has become an issue of growing concern. Non-steroidal drugs are some of the most frequently detected pharmaceuticals in the aquatic environment due to a combination of their recalcitrance and continuous input. Although the concentrations reported are generally too low to cause acute toxicity to vertebrates, effects of chronic exposure on naturally occurring microbial communities are unknown, with potentially major environmental consequences. This study investigated both the ability of aquatic microbes to degrade the non-steroidal anti-inflammatory drugs (NSAIDs) diclofenac, naproxen and ketoprofen, and subsequently the biological effects of these drugs on selected bacteria. Activated sludge and wastewater effluents collected from local wastewater treatment plants (WWTPs) were used in microcosm experiments in order to assess the biodegradation potential of NSAIDs. Solid phase extraction and liquid chromatography-mass spectrometry were used to detect the pharmaceuticals in the enrichments. Up to 60% of naproxen and 99% of ketoprofen was removed after 40 days of incubation, whereas diclofenac was not eliminated and reached up to 90% of its initial concentration. The test pharmaceuticals reduced the growth rate of these microbial communities, suggesting toxic impacts from a few $\mu\text{g/l}$. Assessment of the toxicity of the NSAIDs on *Vibrio fischeri* (Microtox® test) and bacteria isolated from activated sludge showed toxicological effects at concentrations 10,000 times higher than those detected in the environment. Further evaluations on the abilities of microbial communities to degrade pharmaceuticals and their direct effects on microbes are much needed to more fully assess the persistence and impacts of pharmaceuticals in freshwater ecosystems.

Keywords: Diclofenac, Ketoprofen, Naproxen, Microbial degradation

3.1. Introduction

The occurrence of pharmaceuticals in the aquatic environment has been known for some time (Richardson and Bowron, 1985) and has become an issue of international environmental concern. Many pharmaceuticals have been detected in wastewater effluents, surface waters and groundwaters, often at concentrations in the range of 0.1-10 µg/l (Halling-Sorensen et al., 1998). Traces of clofibric acid (5-270 ng/l), diclofenac (up to 6 ng/l), carbamazepine (24-258 ng/l), and a variety of other active ingredients have also been detected in drinking water (Jones et al., 2005). Several surveys have shown that globally, wastewater treatment plants (WWTPs) are the major route of entry for pharmaceuticals into the aquatic environment (e.g. UK, Ashton et al., 2004; continental Europe, Andreozzi et al., 2003; Canada, Lishman et al., 2006; US, Kolpin et al., 2002; Brazil, Stumpf et al., 1999), but other sources include via livestock, point sources (e.g. via agricultural effluents) or diffuse run-off (Boxall, 2004). Thus low levels of pharmaceuticals are continuously discharged in the aquatic environment and prolonged exposures could lead to effects for non target organisms, especially given that these drugs are specifically designed to modify biological processes, many of which are highly conserved across taxa. In India, exceptional levels of pharmaceuticals (e.g. antibiotic ciprofloxacin) enter the aquatic environment at concentrations exceeding levels toxic to bacteria (Larsson et al., 2007). This is potentially a major health hazard since the presence of antibiotics in the environment may predispose bacteria to develop drug resistance.

Most pharmaceuticals entering the aquatic environment via WWTPs are derived from human excretions and inappropriate disposals of prescription drugs and are thus found unmodified from the ingested drug, as metabolites and/or as conjugates. The primary elimination mechanisms for pharmaceuticals in the environment are via microbial degradation and sorption to filterable particulates (Daughton and Ternes,

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes (1999). Other abiotic transformations such as hydrolysis and photodegradation also contribute to the elimination process.

Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most frequently detected pharmaceuticals worldwide in aquatic environments (Bendz et al., 2005). In 2000, ibuprofen, naproxen, diclofenac and mefenamic acid were reported to be amongst the 25 pharmaceuticals most used drugs in the UK (Jones et al., 2002). NSAIDs are organic acids with a low sorption coefficient ($pK_a \sim 4$) which implies that microbial degradation plays an important role for their removal during wastewater treatment. Environmental studies on NSAIDs have reported limited rates of microbial degradation and have attempted to identify the metabolites produced (Zwiener and Frimmel, 2003; Quintana et al., 2005; Gonzalez et al., 2006; Gröning et al., 2007). These studies have concluded that ibuprofen is easily degraded in the environment, whereas diclofenac was usually described as recalcitrant. Photodegradation is currently considered the main pathway of environmental naproxen and diclofenac transformation (Tixier et al., 2003).

The recalcitrance of pharmaceuticals has been shown to induce toxic effects in wildlife populations. In Pakistan, the virtual extinction of three vulture populations has been ascribed to diclofenac-induced renal toxicity (Oaks et al., 2004). The occurrence of NSAIDs and other biologically active compounds in the aquatic environment has raised concerns about their adverse effects on the microbial communities, but there has been little study in this regard (Boxall, 2004). Ecotoxicological risk assessments have focussed mainly on aquatic organisms using data on pharmaceutical distribution, measured environmental concentration (MEC), predicted environmental concentration (PEC) and predicted no effect concentration (PNEC) (Yamamoto et al., 2009). Acute and chronic toxicities of pharmaceuticals were evaluated using standardised tests with bacteria, algae and crustaceans (Farré et al., 2001; Ferrari et al., 2003; Cleuvers, 2004).

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

These tests provided valuable information on the toxicity of single compound and initiated further investigations on the additive effects of pharmaceutical mixtures in the aquatic environment (Richards et al., 2004). More recently, research has considered the toxic effects of pharmaceutical metabolites in the environment. For example clofibrac acid, is a product converted from a pharmacologically inactive parent compound (Daughton and Ternes, 1999). Naproxen photoproducts have also exhibited higher toxicity than the parent compound (Isidori et al., 2005). These observations emphasise the need to consider the degradation pathway and products of pharmaceuticals in order to predict their potential effects in the environment. Degradation pathways can help in studies on the biodegradability and toxicity of chemicals. Several databases such as the BIOWIN software are now available to predict the biodegradation behaviour of pharmaceuticals in wastewater treatment and the receiving waters. However, the results do not always reflect the behaviour predicted. Yu et al. (2007) found that different databases give very different results.

Here we conducted a series of laboratory experiments to assess the biodegradability and toxicity of selected NSAIDs (diclofenac, naproxen and ketoprofen) in the aquatic environment. The objectives were to enrich and isolate bacterial communities able to degrade diclofenac, ketoprofen and naproxen and to gain a better understanding of the effects of these compounds on aquatic microbes. Furthermore, the identification of degrading microbes in mixed communities may provide beneficial information for the improvement of bioremediation for these NSAIDs.

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

3.2. Materials and methods

3.2.1. Chemicals and reagents

The pharmaceuticals used in this study are described in Table 3.1. They were purchased from Sigma-Aldrich (Poole, UK) at the highest purity available. All other chemicals were obtained from Fisher (UK). HPLC-grade solvents were supplied by Rathburn Chemicals Ltd (Walkerburn, Scotland).

3.2.2. Environmental sampling

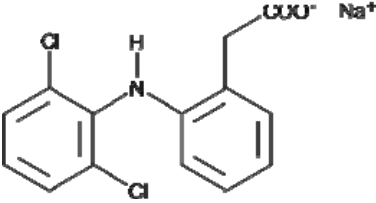
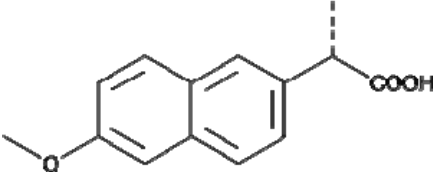
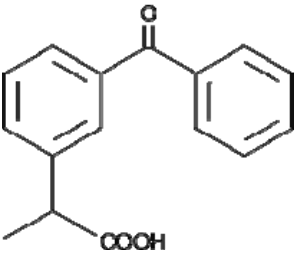
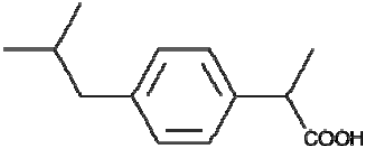
Microbial inocula used in the experiments were collected from the River Exe catchment (South Devon, UK). Countess Wear is the largest WWTP in the Exe catchment and receives wastewater from a domestic population of 130,658 people as well as discharges from a major hospital and local industries (SX 948 891 GB grid). Activated sludge and river water at the sewage outfall were collected from this site. The WWTP at Tiverton treats mainly domestic wastewater from about 19,233 people and is located in a more rural area compared with Countess Wear WWTP (SS 954 103 GB grid). River water was collected approximately 100 m downstream Tiverton WWTP. Samples were kept in autoclaved 1 l amber glass bottles filled completely and sealed.

3.2.3. Biodegradation study

To identify potential communities of degrading microbes, a first batch of enrichment cultures was set up with 1 ml of activated sludge as a microbial inoculum and spiked with 10 mg/l of diclofenac, ketoprofen or naproxen. A second batch of enrichments was conducted with 1 ml sludge or 5 ml collected water and 1 mg diclofenac/l as the sole carbon source. All the enrichments were initiated in triplicate in

Table 3.1 – Properties of NSAIDs used in this study

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

Compound (CAS no) MW	Structure	Excreted unchanged	Reference
Diclofenac (15307-86-5) 295.016		5-15%	a, b
Naproxen (22204-53-1) 230.094		<1%	b
Ketoprofen (22071-15-4) 254.094		<1%	b
Ibuprofen (15687-27-1) 206.130		1-8%	a, b

a- Bendz et al., (2005); b- Kasprzyk-Hordern et al., (2009)

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

250 ml Erlenmeyer flasks containing 100 ml of minimal salts growth medium.

Minimal salts medium was prepared as described in Tett et al. (1994) and contained: 0.2 g MgSO₄, 0.5 g (NH₄)₂ SO₄, 0.5 g KH₂PO₄, 1.5 g K₂HPO₄, 0.12 g Na₂EDTA, 0.02 g NaOH and 0.5 ml of concentrated H₂SO₄ per litre of deionised water. A 1 ml of trace elements solution was added (0.4 g Zn SO₄, 0.1 g CuSO₄, 0.01 g Na₂SO₄, 0.1 g Na₂O₄, 0.04 g MnSO₄, and 0.01 g CoCl₂ in 100 ml of deionised water). Biotic controls were set up without the addition of pharmaceuticals and abiotic controls contained an autoclaved bacterial inoculum. Activated sludge contains many carbon sources readily available for microbes. Thus the microbial growth of the enrichments was compared to biotic controls in order to determine whether the microbes were metabolising the pharmaceuticals or other carbon sources present in the environmental samples. The flasks were closed with porous foam to allow the oxygen transfer. The enrichments were incubated statically for 48 hr then transferred to an orbital shaker at 25°C. The turbidity of the medium was used as an indicator of microbial growth measured at 600 nm. To ensure that sufficient carbon source was available during the experiments and enhance the growth of the bacterial degraders, the enrichments were sub-cultured at day 5, 10, 15 and 40. 5 ml of each culture was inoculated into fresh MSM with the relevant pharmaceutical.

3.2.4. Solid Phase Extraction (SPE)

Preliminary experiments investigated the extraction efficiency of the NSAIDs using the cartridges Oasis HLB 6cc (200 mg, Waters, UK) and Strata X (200 mg, Phenomenex, UK). The recoveries were determined with 20 ml of tap-water spiked with 500 ng of each pharmaceutical (n=4). For this study, cartridges Oasis HLB 6cc were preferred as similar recovery rates were obtained for all chemicals. Prior to the extraction, the environmental samples were filtered using GF/C paper (Whatman, UK)

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes and diluted to 5% methanol. A volume of 50 µg/l of tolfenamic acid was added as an internal standard for subsequent quantification. The extraction protocol is described in Figure 3.1. During the procedure, the cartridges were not allowed to dry unless stated. Elution solvents collected sequentially in glass test tubes were evaporated to dryness in a SpeedVac concentrator (Savant Instruments Inc.- SpeedVac Plus) and made up to 500 µl with methanol: water (1:1, v/v). A thorough vortex was applied to the fractions and they were centrifuged in filter tubes (VectaSpin MicroAnapore, 0.22 µm pore size, Whatman). The filtered extracts were transferred to vials for chemical analysis.

3.2.5. Ultraperformance Liquid Chromatography/Electrospray ionisation time-of-flight- Mass Spectrometry

Chromatography was performed on a Waters Acquity UPLC autosampler equipped with an Acquity UPLC BEH C₁₈ column (1.0 x 100 µm, 1.5 µm particle size; Waters, Elstree, UK). The extracted samples were injected at a volume of 20 µl. The mobile phase was made of 5% methanol and 0.2% formic acid in water (A) and 0.2% of formic acid in methanol (B) at a flow rate of 0.1 ml/min. The elution gradient started with 50% eluent B for 3 min followed by a 12 min linear gradient to 100% eluent B. This was held for 4 min and re-equilibrated to 50% B in 4 min.

The LC system was coupled with a Micromass QTOF-MS system (Manchester, UK), with an electrospray source operating in positive ion mode. The parameters of the MS system were optimised and set as follow: collision energy 7 eV, capillary voltage 2.65 kV, cone voltage 35 kV, source temperature 100°C with a cone gas flow of 0 l/hr, desolvation gas 308 l/hr and desolvation temperature 250°C.

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

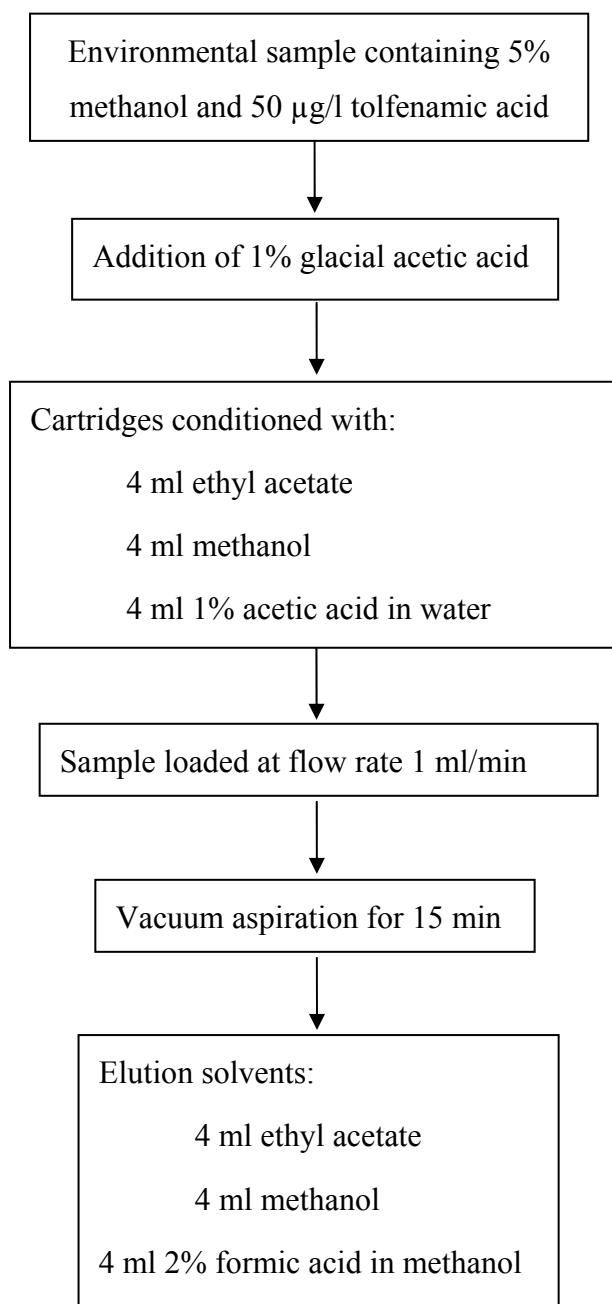


Figure 3.1 – Solid Phase Extraction protocol at acidic pH using Oasis HLB 6cc cartridges employed to analyse the water samples.

3.2.6. Quantification of NSAIDs

A 5-point calibration curve was constructed for each test pharmaceutical prior to sample analysis. Standards were prepared in methanol:water (1:1, v/v). Data were acquired and analysed using the software Masslynx V4.1 (Waters Inc 2005). The limit of detection of each pharmaceutical was estimated as three times the signal-to-noise ratio.

3.2.7. Bioluminescence assay

Based on evidence from the enrichment studies for toxicity of the selected NSAIDs, a standardised acute toxicity assay was performed with the bioluminescent bacteria *Vibrio fischeri* (NRRL B-11177). Light emission is directly linked to metabolic activity and toxic compounds affecting the metabolism would lead to a reduction of the light emitted. The toxicity of ketoprofen, naproxen and diclofenac was compared to a widely used drug of the same class, ibuprofen. A Microtox® kit containing the freeze-dried bacteria, reconstitution solution, diluent (2% NaCl) and an osmotic adjusting solution was purchased from SDI (Newark, USA). The test substances were dissolved in deionised water for a final concentration of 50 mg/l. The bioassay was conducted using the photometer Microtox Model 500 Analyzer. The '49.5% Basic Test' was followed. The natural bioluminescence of *Vibrio fischeri* was measured after 5 and 15 min incubation to determine the effective concentration (EC₅₀) of the compound that would reduce the bioluminescence by 50%. EC₅₀ from concentration response curves were estimated using the software Microtox Omni V1.18 (Azur Environmental, 1999).

3.2.8. Disc diffusion assay

The toxicity of diclofenac was evaluated further using bacterial species commonly found in activated sludge (Dias and Bhat, 1964). *Pseudomonas putida*

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes (DSMZ 5232), *Comamonas denitrificans* (DSMZ 17787), *Micrococcus luteus* (DSMZ 14235) and the filamentous bacteria *Zoogloea ramigera* (DSMZ 287) were selected to investigate the effects of diclofenac on colony growth. The bacteria were grown in nutrient broth overnight and 0.5 ml of inoculum was subsequently applied to nutrient agar plates in duplicate. Filter paper discs (Whatman grade 1) containing diclofenac in concentrations of 0 to 100 mg/l were placed on the agar of each plate. The plates were then incubated for 16 hr at 28°C and the diameter of the inhibition zone recorded.

3.2.9. Predicted pathway for the biodegradation of diclofenac

The biodegradation pathway of diclofenac was predicted using the University of Minnesota Biocatalysis/Biodegradation database (UM-BBD). This program, available online, predicts the chemical structures that can be formed under aerobic or anaerobic conditions. For this study only the aerobic pathways were considered. The database indicates the metabolic pathways that may occur in the environment and the metabolites produced as a result. For each metabolite selected, subsequent breakdown products are suggested, forming a putative biodegradation pathway.

3.3. Results

3.3.1. Recovery efficiencies for NSAIDs

Diclofenac, ketoprofen and naproxen were recovered from clean water samples with extraction efficiencies of $94\% \pm 6.4$, $92\% \pm 4.3$ and $85\% \pm 8.2$ respectively. The pharmaceuticals recovered were predominantly eluted in the ethyl acetate and the remaining amounts in the methanol fraction. The limit of detection (LOD) obtained from the ethyl acetate eluting fraction were 4.7, 5.9 and 7.3 ng/l for diclofenac, ketoprofen and naproxen respectively. These rates of recoveries and detection

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes capabilities suggest that the protocol applied here is suitable for the screening of pharmaceuticals in environmental water samples.

3.3.2. Biodegradation study

Ketoprofen enrichments grew beyond the growth of the biotic controls, suggesting that the microbial communities in those enrichments were able to metabolise the pharmaceutical (Figure 3.2a). A rapid biodegradation was observed with a half-life of 17-20 days. However, the growth was not sustained beyond 15 days and sub-cultures did not produce pure bacterial cultures. The chemical analysis of the enrichments showed that ketoprofen was transformed in 40 days; although the degradation metabolites were not detected. The majority of the compound was eliminated after 15 days, despite the fact that the microbial growth declined. Similar observations were made for the enrichments with naproxen. Between 45-58% of naproxen was recovered by day 40, indicating a relatively slow degradation rate (half-life of 39-45 days) (Figure 3.2b). But the loss of the parent compound did not coincide with the increase in the microbial growth rate. The biodegradation of diclofenac was very slow with an estimated half-life of over 100 days. Unlike the other enrichments and the controls, there was no bacterial growth observed.

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

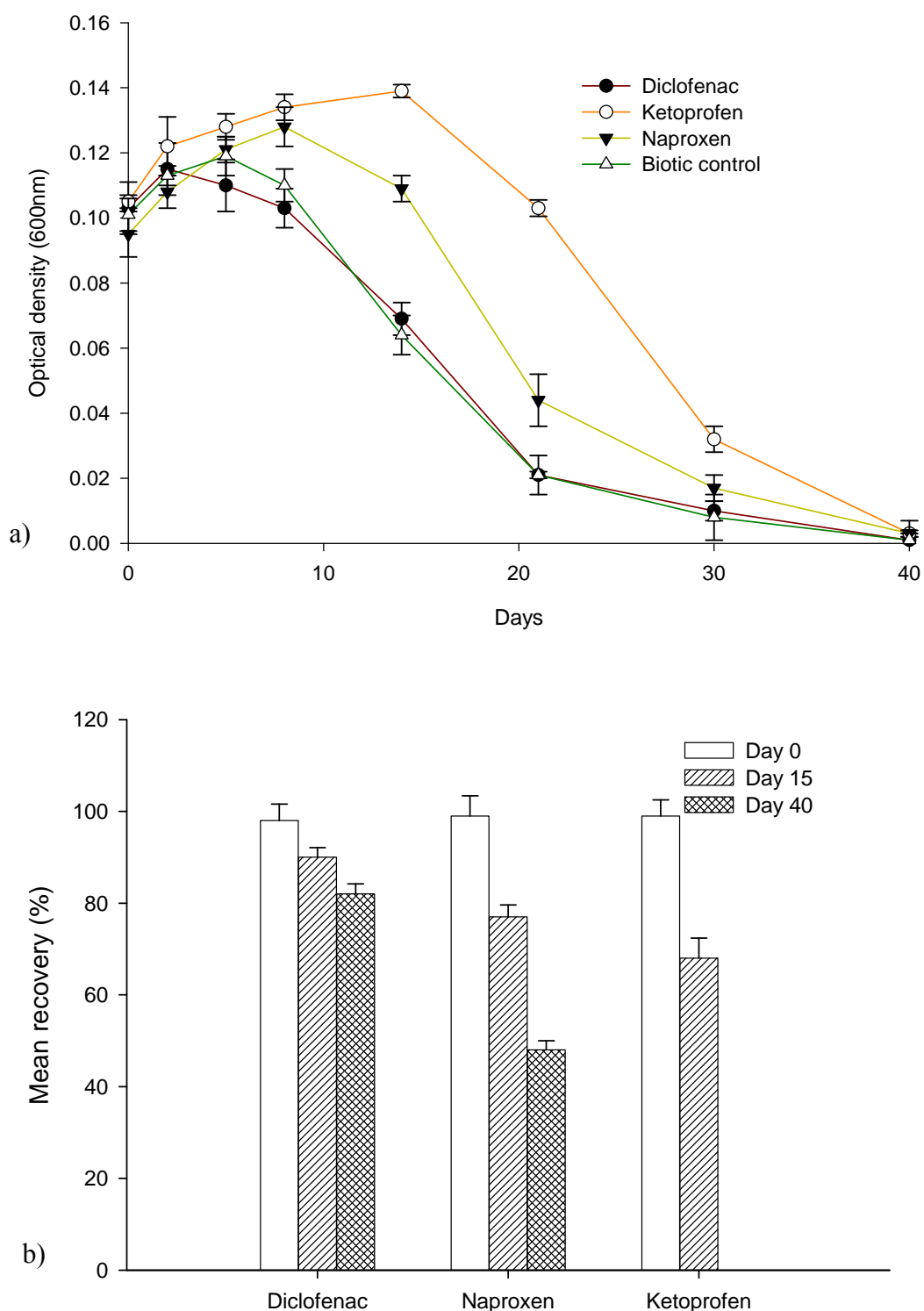


Figure 3.2 – Degradation of NSAIDs (conc. 10 mg/l) with sludge samples incubated in an orbital shaker at 25 °C. Data represented as mean \pm SE; n=3. Samples were collected between January and July 2006. a) microbial growth monitored by optical density at 600 nm, b) percentage of NSAIDs recovered in the enrichments using LC-MS.

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

3.3.3. Diclofenac degradation study

Further experiments were carried out to investigate the biodegradability of diclofenac using river water, sewage effluent and activated sludge samples as microbial inocula. In order to minimize the potential toxic effects of diclofenac, the concentration was reduced to 1 mg/l of growth medium. The analysis of the control cultures indicated that a small percentage of diclofenac can be lost by abiotic processes. The highest removal rate (half-life of 75 days) was measured in the enrichments with activated sludge (Figure 3.3b). A significant increase in microbial growth was recorded only in the enrichments with activated sludge, which contains other carbon sources readily available. Sub-culturing did not enhance the microbial growth and no pure cultures were isolated. The enrichments with river water exhibited a slow growth rate which declined after a few days of incubation. Biodegradation rates were slow in those enrichments and half-lives ranged between 100 and 150 days.

3.3.4. Bioluminescence assay

The environmental impact of diclofenac, naproxen and ketoprofen was assessed according to criteria of the European Community legislation on classification described by Carlsson et al. (2006). The pharmaceuticals had an acute toxicity ranging between 10 and 50 mg/l, they can be considered harmful to aquatic organisms. Diclofenac was the most toxic of the four NSAIDs with an EC_{50} ranging between 11.1 and 13.9 mg/l. Naproxen, ketoprofen and ibuprofen showed more moderate levels of toxicity on *Vibrio fischeri* (Table 3.2). The EC_{50} measured for all four NSAIDs did not vary significantly after 5 and 15 min exposure. The toxic concentrations estimated in this study are much higher than those detected in the environment. Thus these pharmaceuticals are unlikely to cause acute toxic effects.

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

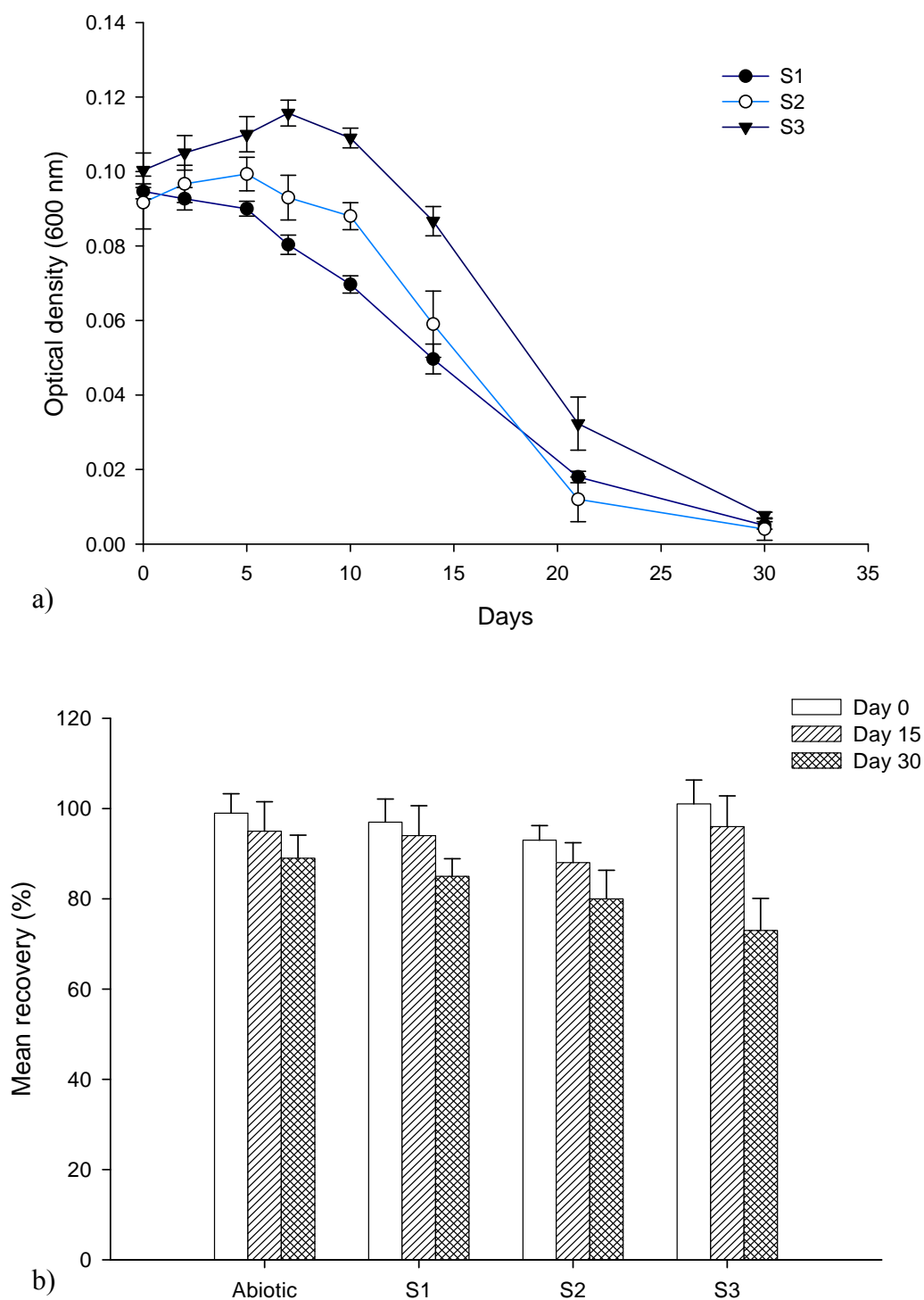


Figure 3.3 – Degradation of diclofenac (1 mg/l) using environmental samples. Samples were collected in February 2007 and the enrichments were incubated in an orbital shaker at 25 °C. Data represented as mean \pm SE, n=3. a) microbial growth monitored by optical density at 600 nm, b) percentage removal of diclofenac. S1- Tiverton WWTP effluent, S2- Countess Wear WWTP effluent, S3- activated sludge.

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

Table 3.2 – Acute toxicity testing (EC₅₀, 15 min) of NSAIDs using *Vibrio fischeri*.

	Diclofenac	Ketoprofen	Naproxen	Ibuprofen
EC ₅₀ (mg/l)	13.5	24.3	42.1	24.7
95% CI	11.11 – 14.01	23.08 – 25.44	33.64 – 48.7	20.08 – 29.38
R ²	0.989	0.983	0.955	0.975

CI: confidence interval.

3.3.5. Disc diffusion assay

Further investigations into the toxicity of diclofenac showed that both Gram positive and negative bacteria can withstand high concentrations of diclofenac. The minimum inhibitory concentration (MIC) for *P. putida* was estimated at 90 mg/l. Diclofenac exposure did not significantly affect the bacterial growth (Table 3.3). MICs for *Z. Ramigera*, *M. luteus* and *C. denitrificans* were between 50-70 mg/l. Even though the mode of action of diclofenac was not fully investigated, the results suggest that diclofenac is bacteriostatic. Colonies sampled from the agar plates containing 50-100 mg/l of diclofenac were sub-cultured onto fresh nutrient agar plates. After 24 hr incubation, the effects of diclofenac were reversed and the bacterial growth significantly higher. Diclofenac was able to impair the growth of both Gram-positive and Gram-negative bacteria but significantly higher concentrations were necessary to kill the bacteria.

3.6. Prediction of the biodegradation pathway for diclofenac

Although diclofenac biodegradation has been previously reported, this study found no evidence of microbial degradation. The investigations on the putative degradation pathway of diclofenac provided similar conclusions. The environmental samples used were oxygen-rich, thus only the aerobic degradation pathway was considered. Diclofenac structure was entered into the UM-BBD but a clear degradation pathway could not be defined. Four breakdown products were proposed but none of them were likely to occur naturally under aerobic conditions. One compound could be identified as hydroxydiclofenac, a common metabolite during diclofenac transformation. Hydroxydiclofenac was selected as the first possible step in diclofenac degradation. The transformation of this metabolite was also described as unlikely to

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

Table 3.3 – Mean diameter of the inhibition zone.*

Concentration of diclofenac (mg/l)	<i>Comamonas denitrificans</i>	<i>Micrococcus luteus</i>	<i>Pseudomonas putida</i>	<i>Zoogloea ramigera</i>
0	-	-	-	-
10	-	-	-	-
20	-	-	-	-
30	-	-	-	-
40	-	-	-	-
50	11.6	-	-	9.2
60	12.7	10.3	-	12.8
70	14.0	11.9	-	14.6
80	17.8	13.8	-	18.6
90	19.9	16.1	6.2	23.0
100	22.3	18.7	9.4	24.2

*This corresponds to the area surrounding the discs where bacterial growth was inhibited.

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes occur in the environment. This suggests that there are no known enzymes capable of successfully degrading the compound. A diagram of the proposed degradation of diclofenac is shown in Figure 3.4.

3.4. Discussion

There are approximately a hundred new biologically active compounds released each year into the environment (Kümmerer, 2004). Despite the hazard risk assessments that are carried out routinely for pharmaceuticals, some releases have had considerable adverse effects in the environment. In India and Pakistan the unusually high mortality rate of three *Gyps* vulture populations was caused by diclofenac (Fent et al., 2006). The presence of ethinylestradiol in rivers can impair the sexual development and function in fish (van Aerle et al., 2001). Surveys carried out worldwide have shown that some pharmaceuticals persist and accumulate to detectable levels in the receiving waters (Boxall, 2004).

Many analytical techniques are now available to monitor these pharmaceuticals in environmental samples. The most frequently used techniques are gas chromatography-mass spectrometry (GC-MS) which often require a derivatization step, and liquid-chromatography-mass spectrometry (LC-MS). LC-MS has an improved selectivity and sensitivity compared with GC-MS to detect polar pharmaceuticals and their metabolites in complex matrices (Kosjek et al., 2008). In our study we applied SPE to extract the pharmaceuticals followed by UPLC-TOF-MS in positive electrospray ionization for chemical detection and quantification. The recovery rates of the NSAIDs compared favourably with the most efficient extraction rates reported for environmental samples (Farré et al., 2001; Gómez et al., 2006). The optimised technique proved to be suitable for the screening and identification of the acidic pharmaceuticals studied

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

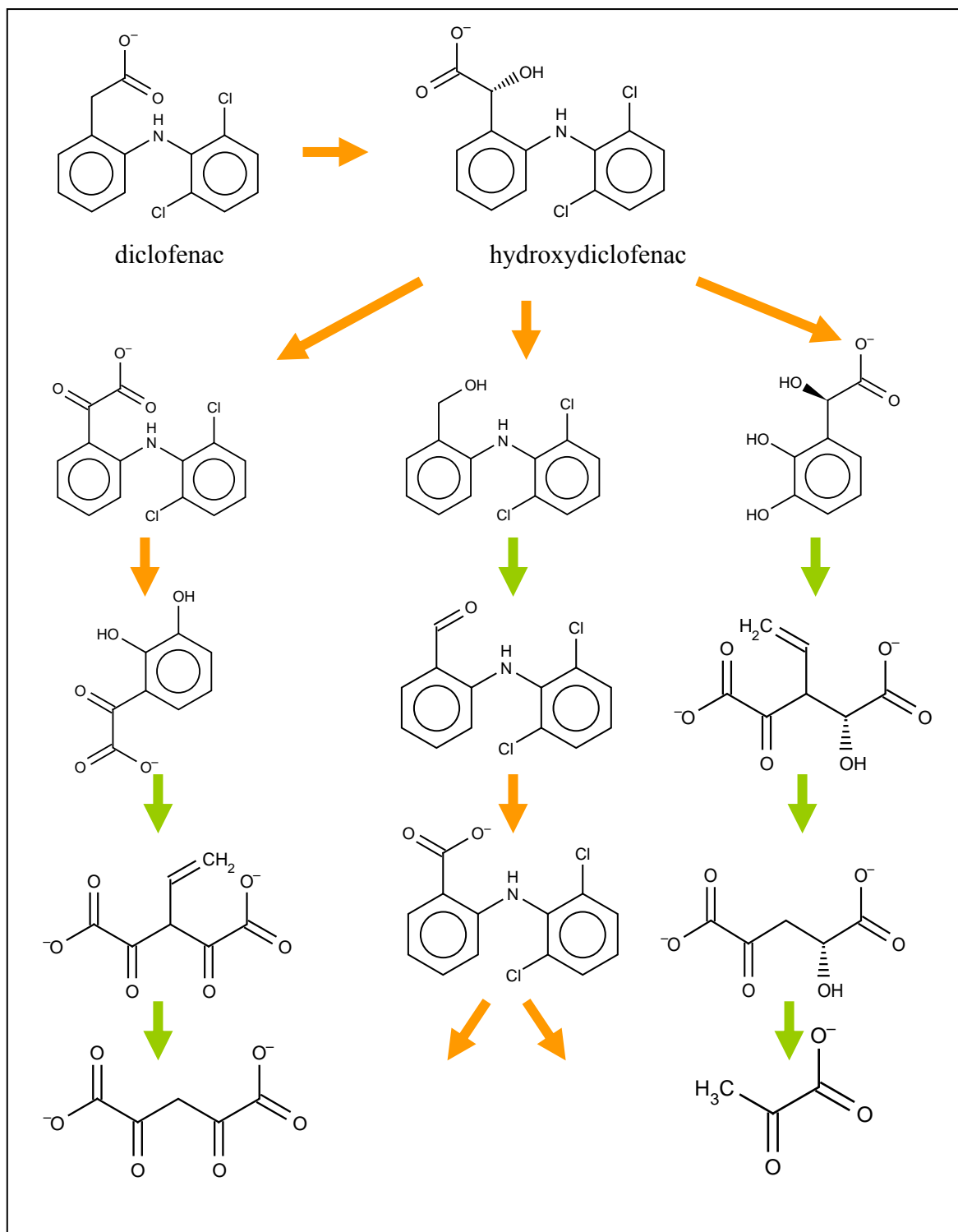


Figure 3.4 – Proposed degradation pathways of diclofenac. Data from UM-BBD was used to critically assess the biodegradation potential of diclofenac in the environment. Arrows indicate whether the metabolites are likely (green) or unlikely (orange) to occur under aerobic conditions.

3. Biodegradation of non-steroidal pharmaceuticals and their toxicity to microbes

at low concentrations (low ng/l range), and well within the range that they occur in the aquatic environment (Tixier et al., 2003).

Biodegradation of NSAIDs is known to occur in the activated sludge during wastewater treatment (Ternes, 1998). Here, the enrichments were designed to promote bacterial growth, give a selective advantage to the NSAIDs degraders and identify them. After 40 days, ketoprofen was found below the limit of detection and naproxen appeared to be partially degraded. Biodegradation was relatively rapid for both compounds with half-lives of less than 50 days. There was no evidence, however, for any microbial degradation of diclofenac. Previous biodegradation experiments comply with these findings (Quintana et al., 2005). The effects of NSAIDs on the enriched microbial communities observed had never been reported. The bacterial population declined after 10-15 days though the highest removal rates of naproxen and ketoprofen were reported between 15 and 30 days. The enrichments contained less than 0.5 g of carbon/l which may not be sufficient to sustain the bacterial growth. To overcome this issue the enrichments were frequently sub-cultured to ensure that the bacteria actively growing had enough carbon. Sub-culturing did not improve the growth rate and bacterial degraders could not be isolated and identified. The observations suggest that the transformation of naproxen and ketoprofen was limited to small bacterial populations that may not be culturable under laboratory conditions.

Abiotic transformation may have also played a role in the enrichments and contributed to the removal of these pharmaceuticals. Indeed, photodegradation has been shown to be an important process in the removal of diclofenac and other NSAIDs from surface waters (Buser et al., 1998; Tixier et al., 2003). Studies on naproxen degradation have also indicated that the compound is photosensitive and

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that several metabolites formed can be more toxic than the parent compound (Isidori et al., 2005). Our enrichments were incubated in the dark but exposed to sunlight on a daily basis (~1 hr per day) whilst sampling and taking measurements. Therefore photodegradation may account for a small percentage of NSAIDs removal. Previous studies have shown that NSAIDs do not tend to adsorb onto solid particulates, which suggests that sorption is not a relevant removal mechanism. However, investigations on the mobility of NSAIDs have demonstrated that diclofenac and ibuprofen had a much higher sorption potential in aquifer sediments than predicted (Scheytt et al., 2005). The comparative analysis of the enrichments and the abiotic controls indicates that sorption onto solid particulates was very minimal in our study.

The second batch of enrichments with diclofenac did not provide better insights in the biodegradation process of this compound. Diclofenac removal rate was comparable to the abiotic loss (Figure 3.3b), which implied that biodegradation did not occur. The results were supported by the putative biodegradation pathway in Figure 3.6. According to the UM-BBD, it is unlikely that diclofenac would be readily degraded under aerobic conditions. Other predictive studies have also reported diclofenac as not readily biodegraded (Carlsson et al., 2006; Yu et al., 2006). But whilst the recalcitrance of diclofenac has been established, the removal rates remain highly variable. Laboratory based experiments investigating the aerobic degradation of diclofenac reported very slow degradation rates with half-lives over 100 days (Zwiener & Frimmel, 2003, Quintana et al., 2005). Other studies have reported up to 60% of diclofenac biodegraded in 30 days with no indications of the microorganisms involved (Yu et al., 2006). Recently, Gröning et al. (2006) investigated the microbial degradation of diclofenac in biofilms of river sediments.

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They reported that diclofenac metabolism might be restricted to Gram negative bacteria from the γ -*Proteobacteria* and the *Cytophaga-Flavobacterium* group. A more precise identification of the degrading microbial communities (at species or genus level) can be challenging.

Bacteria make up to 95% of the activated sludge microbial communities but less than 1% of the bacterial biodiversity can be cultured under laboratory conditions (Dias and Bhat, 1964). Beside, other microbes such as fungi, protozoa and sludge worms are present in activated sludge and may contribute to the removal process. The conditions of our enrichments were optimised to enhance the growth of bacteria alone and did not take into account the other microbes. Indeed fungal species have been reported to degrade certain NSAIDs. Zhong et al. (2003) discovered three fungal species of *Cunninghamella* that were able to degrade naproxen. Microbial degradation of diclofenac has also been observed by the fungal species *Epicoccum nigrum* (Webster et al., 1998). Because of the complexity of the microbial communities, in situ experiments might be more appropriate.

One plausible hypothesis for the reduced bacterial growth is the toxicity of pharmaceuticals such as diclofenac on the bacterial populations. Recent studies demonstrated that NSAIDs at concentrations from 50 $\mu\text{g/l}$ reduced the bacterial diversity in activated sludge (Kraigher et al., 2008). This may interfere with the biodegradation potential of the bacterial populations. The toxic concentrations measured in this study were consistent with the data found in the literature (Farré et al., 2001). The results of the acute toxicity were also in accordance with the findings of our biodegradation experiments. The bacteria were more sensitive to diclofenac, which suggests that diclofenac may cause long-term adverse effects in the aquatic environment through disruption of microbial communities. Diclofenac also exhibited

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anti-bacterial properties and inhibited the growth rate of various bacterial strains. Other research further supports our findings. Dutta et al. (2004) have shown that DNA synthesis was inhibited after exposure to 10-20 mg diclofenac/l. The concentrations affecting the microbes were more than a 1,000 times higher than those detected in the environment.

Even though a single pharmaceutical did not have a high toxicity, the adverse effects might be increased in the environment by synergic action. Indeed, pharmaceuticals are present as a mixture in the aquatic environment and some substances may target the same pathways in biological systems. Thus the cumulative effects of these compounds could increase the toxicity response of non-target organisms (Richards et al., 2004). Cleuvers (2004) demonstrated that the acute exposure of a mixture of diclofenac, ibuprofen, naproxen, and acetylsalicylic acid had a higher toxic effect on the planktonic crustacean *Daphnia magna* than a single compound exposure. To fully evaluate the fate of pharmaceuticals and other pollutants in the aquatic environment, it is important to consider the effects of the metabolites produced but also the possible interactions that a mixture of chemicals may have on non-target organisms.

3.5. Conclusions

Our study showed that biodegradation of diclofenac, ketoprofen and naproxen is a complex process in the aquatic environment. The slow degradation rates suggest that these pharmaceuticals may accumulate in the receiving water. Enrichment cultures and other classical isolation methods may not be suitable to

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identify degrading communities. These techniques fail to culture and detect a great proportion of microbes present. The toxicity of these compounds also proved to be a limiting factor. Successful enrichments require concentrations of NSAIDs that exceed the toxic levels and impair the microbial metabolic activities. Further *in situ* research is necessary to understand the dynamics of microbial populations exposed to pharmaceuticals and other emerging pollutants.

CHAPTER 4: Uptake and biological effects of environmentally relevant concentrations of the non-steroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*)

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Contributions of the authors:

I conducted all the experiments and statistical analyses presented in this chapter including the fish exposure, dissection of the fish, all the molecular work (optimisation of the primers and QPCR assays), and histology as well as the chemical analysis of the tank water and the bile.

Dr Hill provided a technical assistance during the chemical analysis of the water and bile samples by UPLC-MS and Prof Tyler supervised this study.

Abstract

Diclofenac, a non-steroidal anti-inflammatory drug, is widely detected in surface waters and can potentially cause deleterious effects in fish. Here, we investigated the biological effects of 21-day exposure to waterborne diclofenac at environmentally relevant concentrations (0, 0.5, 1, 5 and 25 µg /l) in rainbow trout. Bioconcentration in the bile and effects in selected tissues were assessed via responses in the expression of genes (cytochrome P450 (*cyp*) 1a1, cyclooxygenase (*cox*) 1 and 2, and *p53*) involved in metabolism of xenobiotics, prostaglandin synthesis and cell cycle control respectively, together with histopathological alterations in these tissues. Bioconcentration of diclofenac in the bile ranged between 509 ± 27 and 657 ± 25 and various metabolites of diclofenac were putatively identified as hydroxydiclofenac, diclofenac methyl ester and the potentially reactive metabolite hydroxydiclofenac glucuronide. Expression levels of both *cox1* and *cox2* in liver, gills and kidney were significantly reduced by diclofenac exposure from only 1 µg /l. Expression of *cyp1a1* was induced in the liver and the gills, but inhibited in the kidney of exposed fish. Diclofenac exposure induced tubular necrosis in the kidney and hyperplasia and fusion of the villi in the gastrointestinal tract from 1 µg/l. This study demonstrates that sub-chronic exposure to environmental concentrations of diclofenac can interfere with the biochemical functions of fish and lead to tissue damage, highlighting further the concern about this pharmaceutical in the aquatic environment.

Keywords: diclofenac, fish, bioconcentration, QPCR, *cox*, *cyp1a1*, *p53*, histopathology.

4.1. Introduction

Pharmaceuticals are environmental pollutants of concern for human and environmental health and many enter the aquatic environment, continually via wastewater treatment plants (WWTPs) discharges. Pharmaceuticals are generally detected in wastewater effluents and surface waters in the ng- μ g/l concentration range (Halling-Sorensen et al., 1998; Farré et al., 2008; Kümmerer, 2009), but in some exceptional circumstances significantly higher concentrations can occur (e.g. β -blockers and antibiotics found in treated wastewaters from plants serving drug factories in India (Carlsson et al., 2009)). Antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), β -blockers and oral contraceptives are the most frequently detected in aquatic environments (Daughton and Ternes, 1999; Fent et al., 2006). Typically effect concentrations for individual pharmaceutical in aquatic wildlife are at least one order of magnitude higher than concentrations measured in surface waters. For example, Gagné et al. (2006) demonstrated that analgesics caused cellular oxidative damages at concentrations 10 to 100 times higher than those detected in wastewater effluents. However, certain pharmaceuticals can induce biological effects in the low ng/l range, as is the case for feminised responses in fish exposed to the synthetic estrogen, ethinylestradiol (Jobling et al., 1998; Filby et al., 2007).

The NSAID diclofenac is a widely used human and veterinary pharmaceutical prescribed to reduce inflammation and manage pain. In mammals it acts by inhibiting the cyclooxygenase (*cox*) enzymes which catalyse the synthesis of prostanoids. *Cox* genes are conserved in many vertebrate species and appear to serve similar functions to those in humans. Prostanoids are involved in many biochemical functions such as inflammation, blood flow regulation, platelets aggregation and secretion of gastric mucus (Phillis et al., 2006). In 2004, diclofenac was linked to the rapid decline of three

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Gyps vulture populations in sub-continental India and these populations are now at risk of extinction (Oaks et al., 2004). Environmental surveys have shown that diclofenac and its metabolites are poorly removed in conventional WWTP and often detected in wastewater effluents in concentrations up to a few $\mu\text{g/l}$ (Stülten et al., 2008; Letzel et al., 2009). A few studies have investigated the ecotoxicological effects of diclofenac on aquatic organisms, including fish and some of the side effects (e.g. an inhibition of prostaglandin synthesis) are consistent with side effects observed in humans treated with this pharmaceutical (Hoeger et al., 2005).

Research on diclofenac metabolism in rats and humans has shown that several *cyp* isoforms are involved in its biotransformation (Tang et al., 1999). In fish, *cyp1a* is dominant in the liver but can also be found in the kidney and intestine (Sarasquete and Segner, 2000). The expression of *cyp1a* and its related enzyme activity, ethoxyresorufin-*O*-deethylase (EROD) are regularly used as indicators of pharmaceutical exposure and uptake in fish (van der Oost et al., 2003) and various classes of human pharmaceuticals can interfere with EROD activity (Laville et al., 2004; Thibaut et al., 2006). Diclofenac has been shown both to enhance *cyp1a* expression and to induce the expression of the *p53* gene, suggesting effects on cell cycle control, in Japanese medaka (*Oryzias latipes*) (Hong et al., 2007).

In this study we investigated the uptake and exposure effects of waterborne diclofenac in the rainbow trout, with a focus on environmentally relevant concentrations. Fish bile was analysed to evaluate the bioconcentration factor (BCF) and metabolism of diclofenac in rainbow trout. Expression of *cox1*, *cox2*, *cyp1a1* and *p53* mRNA were used as biomarkers for responses in key biological process thought to be affected by diclofenac in fish. We further investigated for histopathological effects in

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some of the most likely target tissues, specifically, the liver, intestine and kidney using light microscopy.

4.2. Materials and methods

4.2.1. Diclofenac exposure

Juvenile female rainbow trout were obtained from Houghton Springs Farm (Dorset, UK). After acclimatisation for 6 weeks in the laboratory, the fish were placed in groups of 15 in duplicate (n=30 in total; for each treatment) 150 l aquaria under flow-through conditions (water flow rate 18 l/hr). Fish were exposed to nominal concentrations of 0.5, 1, 5, 25 µg diclofenac/l and water control for 21 days. Fish were fed three times a week with commercial trout pellets. Photoperiod was maintained under a 12:12 hr light:dark regime and the water temperature was 12°C.

4.2.2. Fish sampling

After 21 days, fish were anaesthetised using benzocaine (500 mg /l in ethanol) and killed by destroying the brain according to UK Home Office Animal Licence procedures. The head kidney, first gill arch and the anterior liver were excised from each fish, snap-frozen and stored at -80°C until required for the gene expression analysis. The posterior kidney, posterior liver and a sample of the small intestine were fixed in Bouin's solution for 24h and stored in 70% IMS for histological work. Bile samples were diluted with 2 volumes of ice cold methanol and kept at -80°C until required for chemical analysis to measure the concentration of diclofenac in the bile.

4.2.3. Condition factor

On days 0 and 21 of the exposure, total length and body weight of each fish were recorded. From this the Fulton's condition factor K was estimated using the formula:

$$K = \frac{\text{body weight (g)} \times 100}{\text{total length (cm)}^3}$$

4.2.4. Analysis of diclofenac in water and bile samples

On days 0, 7, 14 and 21, 1 l water samples were collected from each tank, fixed with 5% methanol and stored at 4°C for up to 24 hr before chemical extraction. Briefly, water samples were acidified with 1% acetic acid and the internal standard (IS) tolfenamic acid was added at 10% of diclofenac nominal concentration. Diclofenac was extracted on Oasis HLB 6cc solid phase extraction cartridges (200 mg, Waters, Milford, USA) and eluted in 4 ml of ethyl acetate. Bile samples (150 µl) from fish exposed to 0.5, 5 and 25 µg/l were diluted in sodium acetate buffer (300 mM, pH 7.0). SPE extraction was performed using Strata X-AW cartridges (60 mg, Phenomenex, Macclesfield, UK) and diclofenac and its metabolites were eluted with 2 ml of 2% formic acid in methanol and 2 ml 2% ammonium hydroxide in methanol.

The analysis of diclofenac was performed using ultraperformance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS) in +ESI and -ESI. The precision, recovery and detection limit of the methodology were optimised by spiking control water and fish bile samples (n=4) with diclofenac. Samples were quantified using a 6-point calibration curve. Diclofenac was recovered at 94% ± 6.4 in water and 79% ± 7.8 in bile. Data acquired were processed and analysed using the MassLynx software V4.1. Chromatographic data were searched to identify metabolites

4. Uptake and biological effects of diclofenac in rainbow trout of diclofenac. Structural identification was determined using the elemental composition tool in MassLynx.

4.2.5. RNA extraction and reverse transcription (RT) PCR

Total RNA was extracted from the liver, head kidney and gills using Tri Reagent (Sigma, UK) according to the manufacturer's instructions. The RNA concentration was calculated by measuring the absorbance at 260 nm with a Nanodrop spectrophotometer. Ratios of absorbance at 260/280 and 260/230 were used to assess the quality of the RNA isolated. Prior to cDNA synthesis, the RNA (~2 µg per sample) was treated with RQ1 DNase (Promega, UK) to remove any contaminants. RT-PCR was performed on the DNase treated RNA to synthesise the first strand of cDNA using random hexamers primers (5'-NNNNNN-3', MWG, Biotech) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, UK), according to the manufacturer's instructions.

4.2.6. Primer design and real-time PCR optimisation

Sequences for *cox1*, *cox2*, *cyp1a1*, *p53* genes were searched in NCBI GenBank database (www.ncbi.nlm.nih.gov) and specific primers for these target genes in the rainbow trout were designed using Beacon designer software 3.0 and synthesised by MWG-Biotech (see supplementary material S4.1). The annealing temperature of each primer pair was optimised on a temperature gradient. The amplification efficiency (E) and detection range of each primer pair were determined by running real-time PCR reactions in triplicate on a 10-fold serial dilution of rainbow trout cDNA. Standard curves were calculated as the threshold cycle (C_T value; numbers of PCR cycles required for fluorescence to increase above background level) against the logarithm of

the cDNA dilution. The specificity of the primer pairs was confirmed by melt curve analysis.

4.2.7. Real-time PCR

Real time PCR was performed on an iCycler iQ Real-time Detection System (Bio-Rad, Hercules, CA). Each PCR amplification was run in triplicate in 15 µl reaction volume using 7.5 µl 2x Absolute QPCR SYBR Green Fluorescein Mix (ABgene, Epsom, UK), 6 µl HPLC water, 0.375 µl of the appropriate forward and reverse primer (10 µM) and 0.75 µl cDNA. A negative control with water replacing the template cDNA was run for each plate. To assess intra- and inter-assay variability, a common pooled sample of cDNA was quantified on each plate. The temperatures and cycling conditions were as follows: initial denaturation step at 95°C for 15 min, which activates the hot-start Taq polymerase, then 40 cycles of denaturation at 95°C for 10 seconds and annealing at 59.9°C (*cox1*, *cyp1a1* and *p53*) or 57.3°C (*cox2*) for 20 seconds followed by a melt curve analysis. Normalisation of the target cDNA, expression was conducted using the housekeeping gene *rpl8* (sense 5'-ACAACCCAGAC ACCAAGAAG-3', anti-sense 5'-CAG CAACCACACCAACAAC-3'; annealing at 60°C) as reported previously by Filby and Tyler (2007). Relative expression (RE) levels were determined based on the arithmetic comparative method of Livak and Schmittgen (2001) with a correction in the differences of *E* between the target and housekeeping gene.

$$RE = (E_{ref})^{C_T_{ref}} / (E_{target})^{C_T_{target}}$$

where *ref* is the housekeeping gene, *target* is the gene of interest, *E* is PCR amplification efficiency for each gene and *C_T* is threshold cycle value.

4.2.8. Histology

Fixed tissues (10 samples per tissue per treatment) were embedded in paraffin wax and sections of 5 µm were cut, mounted onto slides and stained with haematoxylin and eosin (H&E) using standard procedures. Each section was examined for histological alterations on a Zeiss Axioskop microscope (Carl Zeiss Ltd., Germany). The lesions were semi-quantified using a rating system:

1 (normal) – All 8 sections examined for each sample are healthy with no apparent damage;

2 (mild) – Minor changes observed in less than 3 of 8 sections analysed. Lesions included partial fusion and mild hyperplasia in the villi and high number of developing nephrons compared to the control fish in the kidney;

3 (moderate) – Fusion, hyperplasia and proliferation of Goblet cells in the villi, numerous developing nephrons and reduction of Bowman's space surrounding the glomeruli in the kidney. Lesions found in 5 of 8 sections of each sample;

4 (severe) – Lesions (total fusion of villi, hyperplasia, enlarged Goblet cells, tubular necrosis, loss of Bowman's space) in at least 6 of 8 sections analysed.

4.2.9. Data analysis

Statistical analysis was performed using SigmaStat 2.03 (Jandel Scientific Software). Differences between experimental groups were determined by one-way analysis of variance (ANOVA) with Dunnett's post test against the water control groups. Non-normally distributed data were log-transformed prior to statistical analysis. Non-parametric data were analysed with Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons post hoc test. Statistical differences were considered at $p < 0.05$. All experimental data are represented as the mean \pm standard error (SE).

4.3. Results

4.3.1. Analysis of diclofenac concentrations in the tank water

UPLC-TOF-MS analysis of water samples taken each week revealed that the actual exposure concentrations were 90-105% of the nominal concentrations for both replicate tanks (Table 4.1).

4.3.2. Condition factor

Exposure of rainbow trout to diclofenac did not affect the body size (length or weight, data not shown). There were also no significant effects of diclofenac on K between day 0 and day 21 within a treatment group (see supplementary material S4.2).

4.3.3. Concentration of diclofenac and identification of its metabolites in fish bile

Diclofenac was detectable in the bile of fish from the lowest exposure concentration 0.5 µg/l. Mean measured concentrations and factor of concentration are shown in Table 4.2. Diclofenac accumulated in the bile by a factor of 622 to 780 at 0.5 µg/l, 499 to 601 at 5 µg/l and 406 to 600 at 25 µg/l exposure concentration. The highest level of diclofenac was found in the bile of fish exposed to 25 µg/l with a mean concentration of 12724 ng/ml.

The analysis of the chromatograms resulted in the putative identification of different metabolites of diclofenac in fish bile from 0.5 µg/l (Table 4.3). A reactive metabolite of diclofenac, hydroxydiclofenac glucuronide (m/z 486.032) was detected at the highest exposure concentration (25 µg/l).

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Table 4.1 – Mean measured diclofenac concentrations ($\mu\text{g/l} \pm \text{SE}$) in replicate tanks water.

Nominal concentration	Diclofenac concentration ($\mu\text{g/l}$) \pm SE			
	0.5	1	5	25
Measured concentration	0.54 ± 0.2	1.59 ± 0.45	6.28 ± 0.97	24.04 ± 2.6
	0.46 ± 0.81	1.55 ± 0.7	5.78 ± 1.9	22.51 ± 1.4

Table 4.2 – Measured diclofenac concentrations (ng/ml, mean \pm SE) and estimated concentration factor in rainbow trout bile after 21-day exposure.

Nominal concentration	Mean measured concentrations in bile	Factor of accumulation ^a
0.5	328 ± 13	657
5	2672 ± 73	534
25	12724 ± 640	509

^a calculated as measured diclofenac concentration/nominal exposure concentration.

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Table 4.3 – Putative metabolites of diclofenac identified in the bile of fish exposed to waterborne diclofenac for 21 days.

<i>m/z</i> value	RT	ESI mode	Putative elemental composition	Putative compound
310.003	4.39	-	C ₁₄ H ₁₀ NO ₃ Cl ₂	Hydroxydiclofenac
310.0402	9.29	+	C ₁₅ H ₁₄ NO ₂ Cl ₂	Diclofenac methyl ester metabolite
486.032	1.89	-	C ₂₀ H ₁₈ NO ₉ Cl ₂	Hydroxydiclofenac glucuronide

m/z: mass to charge ratio; RT: retention time; ESI: electrospray ionisation.

4.3.4. Target gene expression

Real-time PCR analysis of *rpl8* mRNA levels measured in the liver, gills and kidney showed no significant variations between any of the treatment groups for any of the target tissues studied (ANOVA, $p > 0.05$; Figure 4.1), consistent with previous studies confirming *rpl8* as a reliable “housekeeping” gene for quantifying diclofenac exposure effects (Filby and Tyler, 2007).

In control tissues, *cyp1a1* levels were the highest in liver and *cox1* and *cox2* mRNA highest in the gills and liver. Kidney tissues showed the highest mean expression levels of *p53*. Diclofenac exposure resulted in a decrease in the relative expression of *cox1* mRNA in the kidney and liver at exposure s of 0.5 $\mu\text{g/l}$ (Figure 4.2a) and 1 $\mu\text{g/l}$ in the gills (ANOVA, $p < 0.05$). Expression of *cox1* in the kidney of fish exposed to 25 $\mu\text{g/l}$, was highly suppressed ($p < 0.01$). Similarly *cox2* mRNA showed reductions in expression ($p < 0.05$) following diclofenac exposure in all three tissues (Figure 4.2b) with effective concentrations of 0.5 $\mu\text{g/l}$ for the liver, 1 $\mu\text{g/l}$ for the kidney and 5 $\mu\text{g/l}$ for the gill tissues. Levels of *cox2* mRNA expression seemed less affected than *cox1* mRNA. Relative expression of *cyp1a1* was up regulated in the gills and liver of diclofenac-exposed fish (Figure 4.2c) at exposure concentrations of 0.5 $\mu\text{g/l}$ and 1 $\mu\text{g/l}$, respectively ($p < 0.05$). The maximum up regulation however was less than a 2-fold increase. Contrary to the results obtained in the liver and gills, *cyp1a1* expression was down regulated in the kidney at a threshold concentration of 1 $\mu\text{g/l}$ ($p < 0.05$).

Response patterns for the expression of *p53* mRNA were more varied across the tissues studied (Figure 4.2d). There was no treatment effect on *p53* mRNA in the liver but a significantly increased expression in the gills at the lowest two exposure concentrations (0.5 and 1 $\mu\text{g/l}$) and a down regulation in the kidney from an effective concentration of 1 $\mu\text{g/l}$ ($p < 0.05$).

4. Uptake and biological effects of diclofenac in rainbow trout

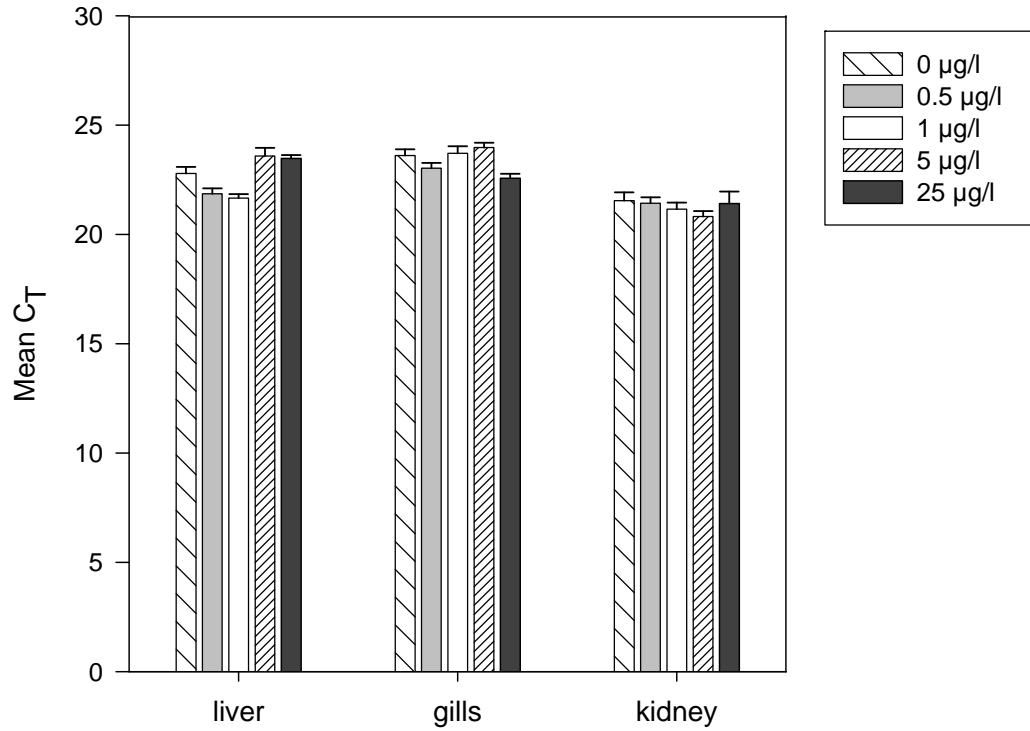


Figure 4.1 – Average threshold cycle (C_T) for *rpl8* amplification in liver, gills and kidney after 21-day exposure to diclofenac. Data represented as mean \pm SE, n=15 fish.

4. Uptake and biological effects of diclofenac in rainbow trout

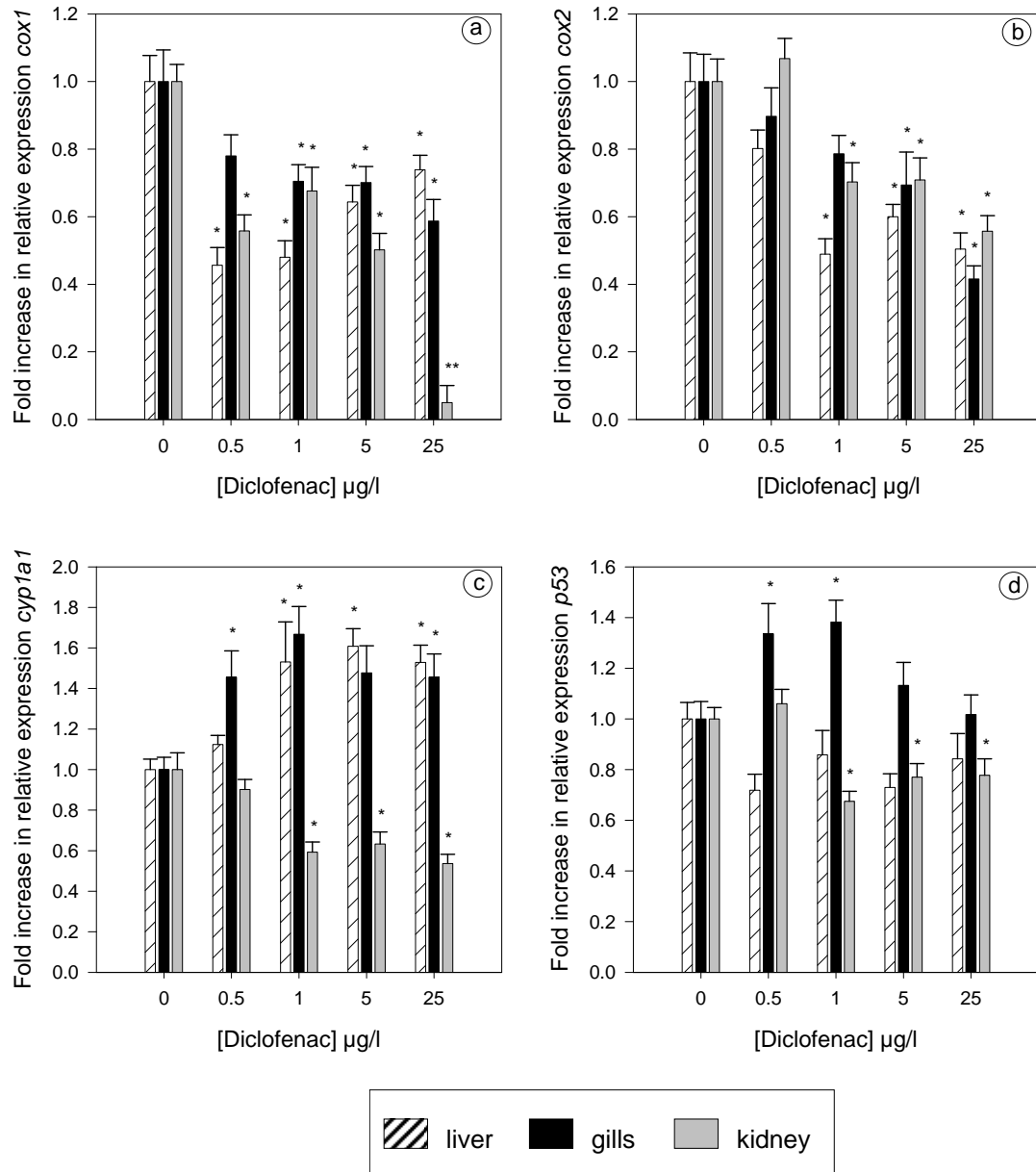


Figure 4.2 – Relative expression of *cox1* (a), *cox2* (b), *cyp1a1* (c) and *p53* (d) in rainbow trout after 21-day diclofenac exposure. Data are represented as mean \pm SE with n=15 fish. Statistical differences between treatment groups were determined by one-way ANOVA.

*p<0.05 and **p<0.01.

4.3.5. Histopathological findings

Histopathological examination of the liver indicated no visible lesions in any of the treatment groups (see supplementary material S4.3). In contrast, diclofenac induced morphological changes in both the small intestine and the kidney. In the small intestine, there was an increase of hyperplasia and fusion of the villi at a threshold concentration of 1 µg diclofenac/l and in concentration dependent manners (Figure 4.3). One fish from the 5 µg/l exposure tank and three fish from the 25 µg/l exposure tanks showed complete fusion of several villi (Figure 4.3b). Enlargement of goblet cells in the intestine occurred from a threshold concentration of 5 µg/l. In the posterior kidney, regressive changes were induced in the tubules and glomeruli (Figure 4.4). Diclofenac-exposed fish also presented greater numbers of developing nephrons compared with the control fish. A mild tubular necrosis and a significant loss of interstitial space surrounding the glomeruli were observed at the two highest exposure concentrations.

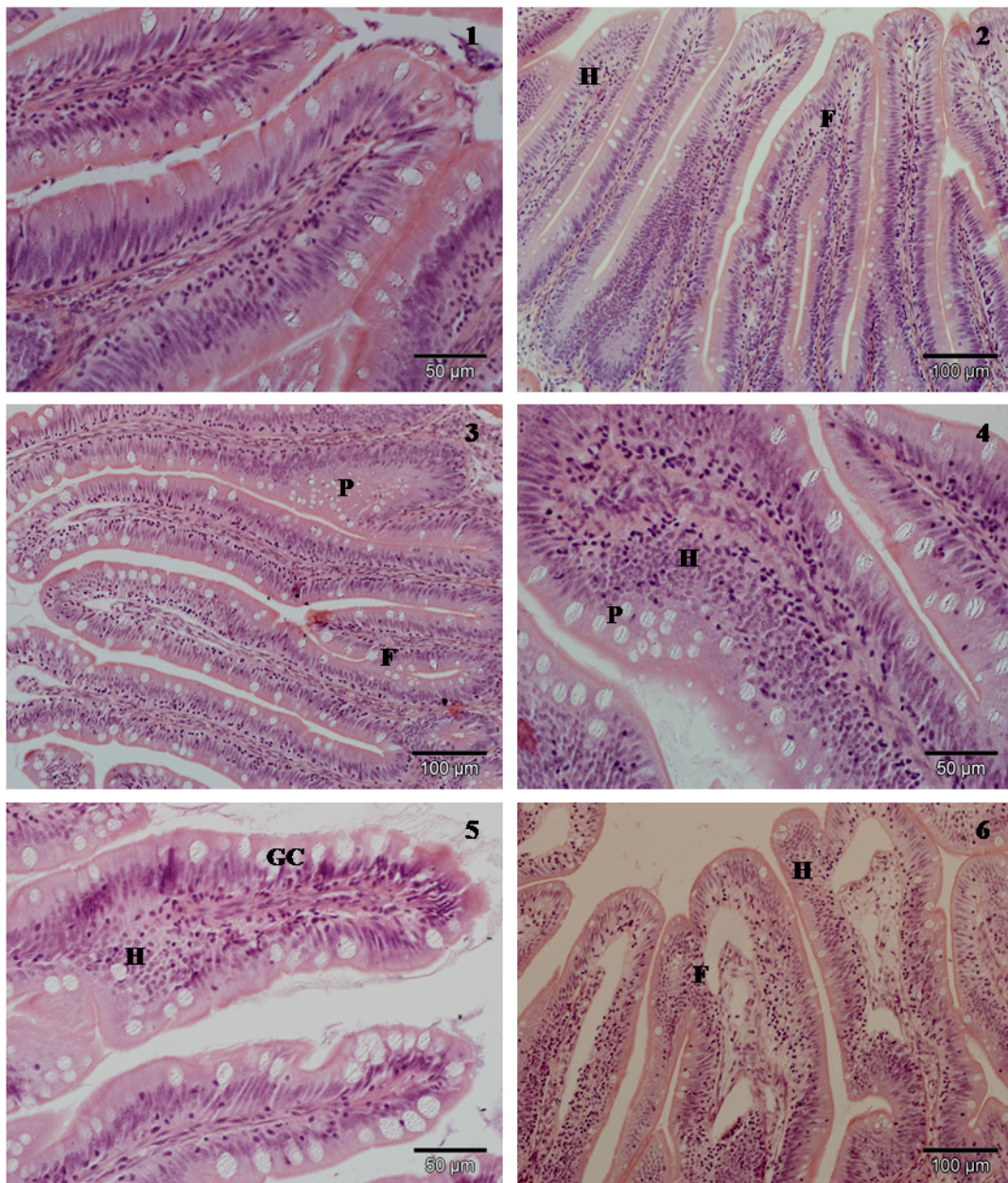


Figure 4.3a – Histopathological lesions in small intestine of rainbow trout induced by 21-day diclofenac exposure. 1) healthy villi of control fish with single cell layer; 2) hyperplasia (H) and fusion (F) observed at 0.5 $\mu\text{g/l}$; 3) small fusion and hyperplasia and proliferation of Goblet cells at 1 $\mu\text{g/l}$; 4) hyperplasia and proliferation of Goblet cells at 5 $\mu\text{g/l}$; 5) enlarged Goblet cells at 25 $\mu\text{g/l}$; and 6) hyperplasia, inflammation and fusion of the villi at 25 $\mu\text{g/l}$; H&E stain.

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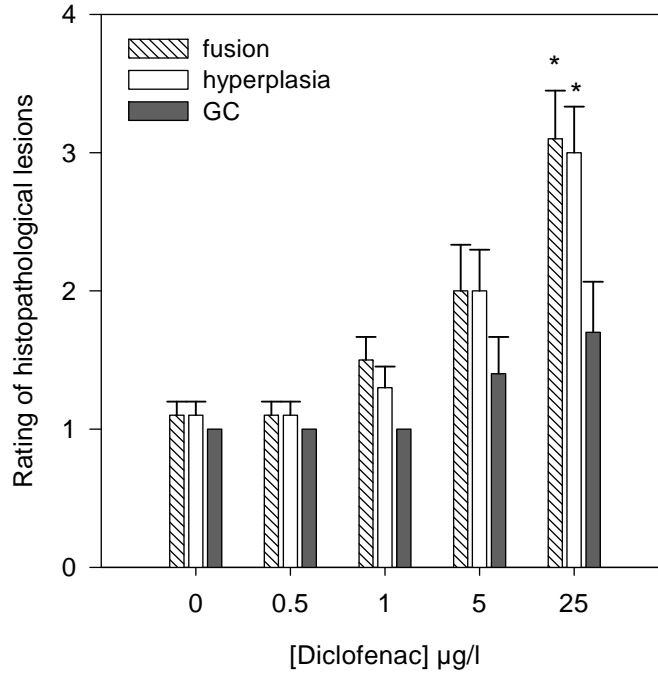


Figure 4.3b – Semi-quantitative assessment of histopathological lesions in the small intestine of rainbow trout exposed to diclofenac for 21 days. Data are represented as mean \pm SE, n=10 fish. Fusion, hyperplasia and enlarged Goblet cells (GC) were observed. Rating system: 1= normal, 2= mild/ moderate, 3= moderate/severe, 4= severe. Data analysed by Kruskal-Wallis ANOVA with Dunn's test post hoc.

* p<0.05.

4. Uptake and biological effects of diclofenac in rainbow trout

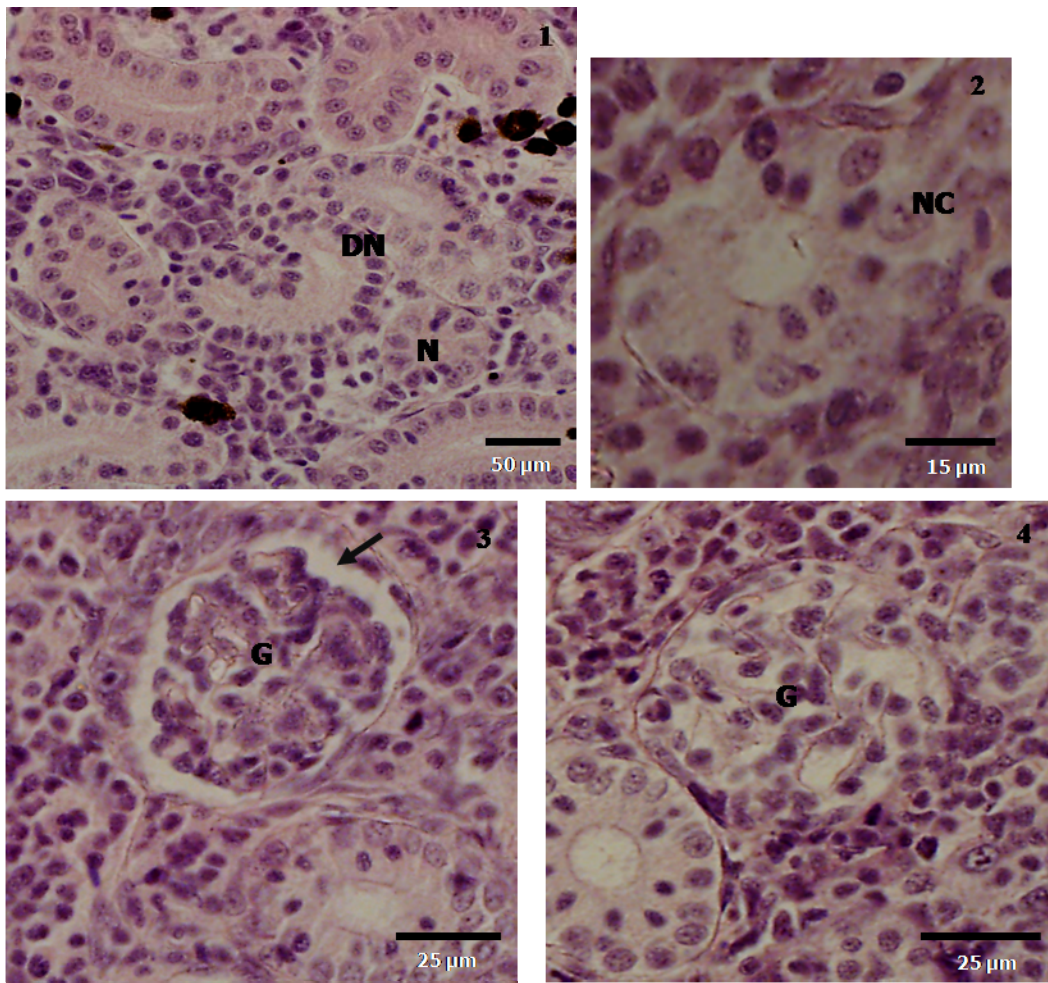


Figure 4.4a – Histopathological lesions in the kidney of diclofenac-exposed rainbow trout after 21 days. N: nephrons, DN: developing nephrons, G: glomeruli, NC: necrosis, black arrow: Bowman space. 1) kidney from water control tissue; 2) fish exposed to 0.5 µg/l; 3) increasing number of developing nephrons in fish exposed to 5 µg/l; 4) tubular necrosis and loss of Bowman space at 25 µg/l. H&E stain, magnification 40x.

4. Uptake and biological effects of diclofenac in rainbow trout

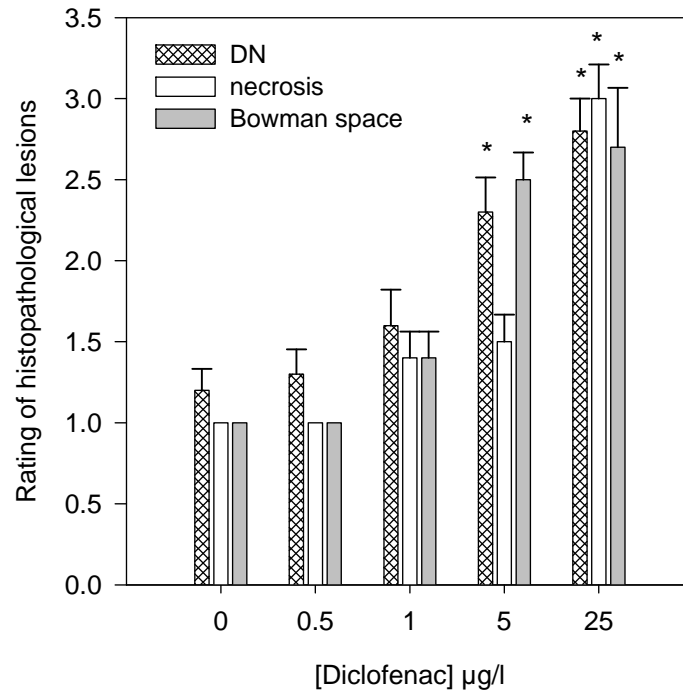


Figure 4.4b – Semi-quantitative assessment of histopathological lesions in the kidney of rainbow trout exposed to diclofenac for 21 days. Data are represented as mean \pm SE, n=10 fish. Tissue damages included an increased number of developing nephrons (DN), tubular necrosis and loss of Bowman space surrounding the glomeruli. Rating system: 1= normal, 2= mild/ moderate, 3= moderate/severe, 4= severe. Data analysed by Kruskal-Wallis ANOVA with Dunn's test post hoc.

* p<0.05.

4.4. Discussion

Since the discovery of a compelling link between diclofenac exposure and the extinction of some Asian *Gyps* vulture populations, an increasing body of research has investigated the ecotoxicological effects of diclofenac on non target organisms. Many studies conducted however have focused on acute effects at high exposure concentrations (Hallare et al., 2004; Laville et al., 2004; Hong et al., 2007). Here we show that chronic exposure to environmental concentrations of waterborne diclofenac can both induce disturbances in normal molecular processes controlling metabolism of xenobiotics and prostaglandin synthesis, induce adverse tissue pathologies in intestine and kidney. Diclofenac is presently measured in effluents and surface waters in low $\mu\text{g/l}$ concentrations. However its release from a prescription only usage, and thus ability to purchase it over the counter, is likely to result in an increased usage and disposal in the future, adding to the potential exposure problems for aquatic organisms. Our investigations showed that diclofenac was concentrated in the bile of rainbow trout with a factor ranging between 509 and 657 for the different exposure concentrations. Bile is a major excretory route of many xenobiotics, including diclofenac (Treinen-Moslen and Kanz, 2006). Our findings compare favourably with a previous study on diclofenac-exposed rainbow trout where BCFs of up to 2732, 971, 763 and 69 were reported in the liver, kidney, gills and muscles respectively for a 28 day exposure (Schwaiger et al., 2004). However our estimated accumulation factors in fish bile are lower compared to other pharmaceuticals. Nonylphenol bioconcentrated to much higher levels in roach after 14-day exposure to 5 $\mu\text{g/l}$ (Smith and Hill, 2004). More recently research by Brown et al. (2007) demonstrated that ibuprofen (BCF 18667) and gemfibrozil (BCF 199) bioconcentrated in the plasma of rainbow trout exposed to wastewater effluents at higher levels than diclofenac (BCF 11).

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Exposure to diclofenac appears to target similar pathways in fish as in mammals and both isoforms of the *cox* gene were down regulated in the kidney, gills and liver of the exposed fish. Prolonged inhibition of the *cox* activity has been shown to alter the prostaglandin-regulated mechanisms such as blood flow in the kidney (Fent et al., 2006). Impairment and the subsequent failure of renal functions was established as a major cause of the widespread mortality of Asian *Gyps* vultures exposed to diclofenac (Taggart et al., 2007), although the specific mechanisms for this effect are not yet known (Ng et al., 2006; Hoeger et al., 2008). Interestingly, in this study diclofenac had an inhibitory (rather than stimulatory) effect on *cyp1a1* expression in the kidney of rainbow trout. This might indicate a reduced capability for the metabolism of diclofenac in this tissue which could lead to an enhanced bioconcentration and greater likelihood for tissue damage. The histopathological analysis of the kidney reinforced this hypothesis. Concentration dependent effects were seen on the developing nephrons, tubules and glomeruli, structures vital for normal kidney functions. Signs of nephrotoxicity even occurred at the lowest diclofenac exposure concentration. The decreased levels of *p53* mRNA in the kidney (from 1 µg/l) could potentially result in reduced regeneration rates for damaged kidney tissues. Renal lesions caused by diclofenac have been reported in salmonids previously but only at higher concentrations (5 µg/l) exceeding those measured in the natural environment (Schwaiger et al., 2004; Hoeger et al., 2005). Using electron microscopy as a technique, thus with higher visual resolution than light microscopy, previous studies have shown induction of glomerulonephritis, necrosis of endothelial cells, and hyaline droplet degeneration in the kidney of diclofenac-exposed rainbow trout from 1 µg/l (Triebkorn et al., 2004).

In fish, gills are the primary route of entry of many xenobiotics making them vulnerable to contaminant exposure (Teh et al., 1997). In the gills of the diclofenac-

4. Uptake and biological effects of diclofenac in rainbow trout

exposed rainbow there was an up regulation of *cyp1a1* mRNA at the lowest exposure concentration (0.5 µg/l), in accordance with that shown in medaka (Hong et al., 2007) indicating possible metabolism of diclofenac in this tissue. The significant reduction in *cox1* and *cox2* in the gills implies that eicosanoids production was decreased in this tissue. Although the functional role of these molecules is not well understood, it is believed that the reduction in eicosanoids can affect blood coagulation, homeostasis and immune responses in the gills (Holland et al., 1999). Histopathological examinations were not carried out on the gills, but the existing studies have all concluded that exposure to diclofenac in the lower µg/l range can lead to severe structural damages in the fish gills, including pillar cell necrosis, epithelial lifting, hyperplasia and hypertrophy of epithelial chloride cells (Schwaiger et al., 2004) and in turn affect respiration and other gill physiological functions (Triebkorn et al., 2004; Hoeger et al., 2005). In our work, the gills of diclofenac-exposed trout also had an enhanced level of *p53* mRNA, a gene that plays a key role in cell cycle control, at exposure concentrations of 0.5 and 1 µg/l.

Diclofenac has been shown to induce *cyp* activity in the hepatocytes affecting metabolism (Bort et al., 1999; Rudzok et al., 2009). Here we found a threshold concentration for effects on *cyp1a1* mRNA at 1 µg/l, however, although changes in *cyp1a1*, and *cox1* and *cox2* mRNA were observed, no obvious tissue damage was evident in the liver of the diclofenac-exposed fish. Triebkorn et al. (2004) found evidence of cellular toxicity such as glycogen depletion and macrophage infiltration in the liver of diclofenac exposed fish rainbow trout from 1 µg/l. A previous study reported elevated *p53* mRNA levels in the liver, gills and intestine of medaka exposed to 1 µg diclofenac/l for 4 days (Hong et al., 2007) but we found no significant effects in

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the liver and a reduction in the kidney. *P53* mRNA levels were increased only in the gills of trout exposed to 0.5 µg diclofenac/l.

Diclofenac and the metabolites formed in the liver are excreted into the bile (Rudzok et al., 2009) and recently, it has been demonstrated in fish that these compounds can re-enter the liver via the intestine leading to damages of the intestinal tract (Hoeger et al., 2008). This enteric recirculation also occurs in mammals and has been demonstrated to result in injuries to the gastric epithelium in rats (Seitz and Boelsterli, 1998). This is thought to be caused by reactive metabolites of diclofenac particularly the glucuronide conjugates. In our study, the search for specific metabolites of diclofenac led to the detection of hydroxydiclofenac, hydroxydiclofenac glucuronide and methyl ester metabolite of diclofenac. The severe lesions observed in the intestine of the rainbow trout exposed to the highest concentration of diclofenac may have been caused by the reactive glucuronide metabolite found. The enteropathy observed was characterised by inflammation and lesions in the villi. These structural damages have been reported in humans and result in blood loss, increased permeability and bile salt malabsorption (Treinen-Moslen and Kanz, 2006). Our findings on the intestine contrast with Schwaiger et al. (2004) who found no significant damage in the intestine of rainbow trout exposed to 1 to 500 µg diclofenac/l. This may be because our exposure was conducted without any solvent. Previous work has shown that using a solvent carrier may modulate the toxic response in fish (Hutchinson et al., 2006).

In conclusion, exposure to environmentally relevant concentrations of diclofenac was shown to impair the health of exposed rainbow trout, altering the expression of selected genes linked with key functional processes controlling metabolism,

4. Uptake and biological effects of diclofenac in rainbow trout

prostaglandin synthesis and cell cycle, and inducing structural disruptions in the kidney and intestine. Our findings, together with other published studies, for effects of diclofenac on the gastrointestinal tract and kidney of fish, suggest modes of action, similar to those in mammals. Further work should be done to compare the plasma concentration of diclofenac in the exposed fish with human therapeutic plasma concentrations in order to predict the potential impact of diclofenac in fish (Huggett et al., 2003; Giltrow et al., 2009).

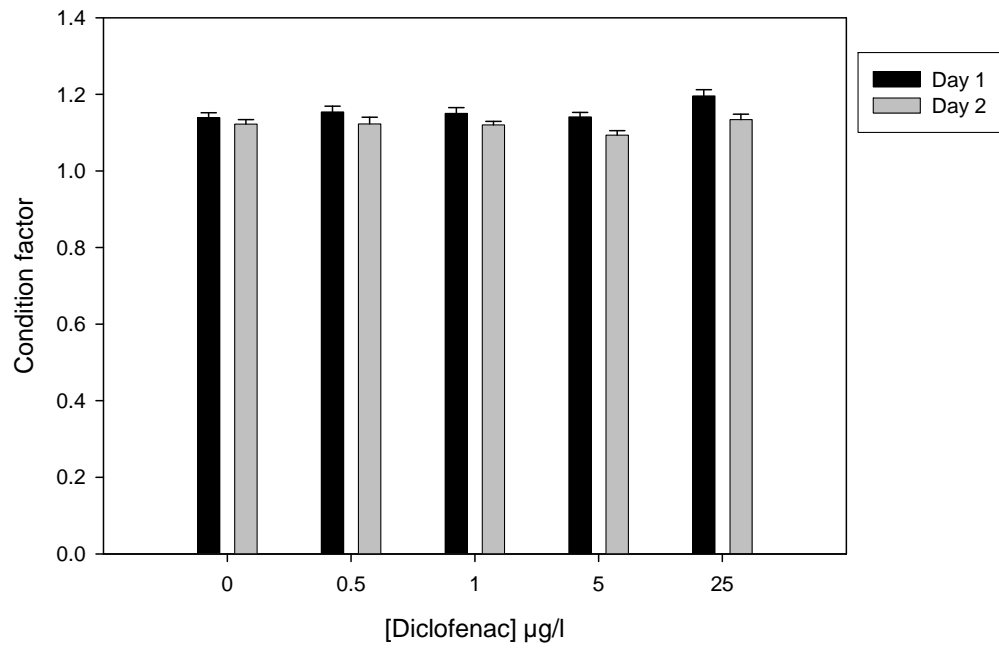
Supplementary materials

S4.1 – Primers designed and optimised for real-time PCR analysis.

Gene name	Accession num	Sequence
<i>cox1</i>	AJ299018	F 5'-CAAACGAGAGGTAGCATCAATC-3' R 5'- GTTCTTCAAATGTGTGGTAGGG-3'
<i>cox2</i>	AJ238307	F 5'-CCAGGACGATTAAACCAAACAG-3' R 5'- ACCGCTTCAACAACGATAGG-3'
<i>cyp1a1</i>	U62796	F 5'-GCACAATAACCCTCACCTCAG-3' R 5'- GCCACTCAGAACAACCACAG-3'
<i>p53</i>	M75145	F 5'-CCATCCTCACCATCATCACC-3' R 5'- TCCTCTGTCTTCCTGTCTCG-3'

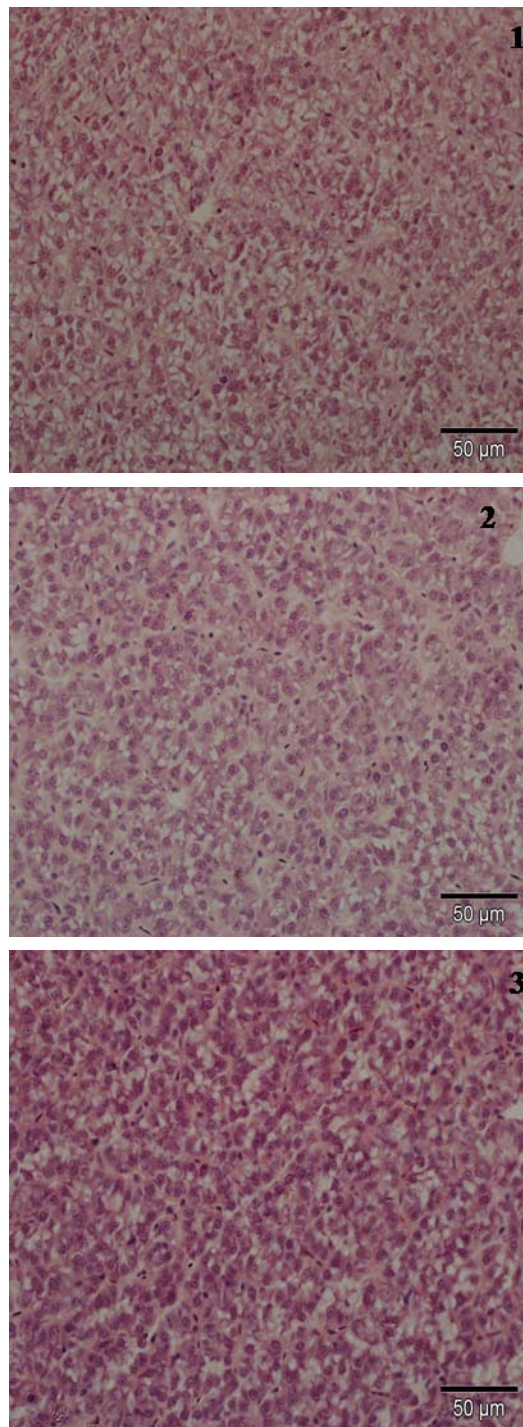
Gene sequences were found in NCBI GenBank database and designed using Beacon designer software 3.0.

4. Uptake and biological effects of diclofenac in rainbow trout



S4.2 – Condition factor of rainbow trout. Each column represents mean + SE (n=15). There were no significant differences in the treatment groups between day 0 and day 21 (one-way ANOVA, $p > 0.05$).

4. Uptake and biological effects of diclofenac in rainbow trout



S4.3 – Histological sections of liver tissue from control and diclofenac-exposed rainbow trout. 1) water control, 2) exposed to 1 μg/l and 3) exposed to 25 μg/l. There were no effects observed. Magnification 40x; H&E stain.

Technical limitations did not permit the capture of images at a higher magnification.

CHAPTER 5: Development of analytical techniques for metabolic profiling of biofluids in rainbow trout (*O. mykiss*)

5.1. Introduction

The “omics” technologies have become powerful tools to examine the changes in gene expression, protein and metabolite levels caused by diseases, toxic insults or environmental stimuli (Fiehn et al., 2000; Aardema and MacGregor, 2002). However, the information obtained from genomics and proteomics studies do not indicate how these changes relate to the biological functions of an organ or organism. Metabolomics, also known as metabonomics is a developing technology that measures all the small-molecule metabolites in a biological system (Goodacre et al., 2004; Weckwerth and Morgenthal, 2005). Analysing the subtle alterations of the metabolic profile can offer valuable insights into the physiological status of an organism. This approach has proved useful to elucidate gene function, pharmaceuticals impacts and has considerable potential for the discovery of biomarkers of chemical exposure (Fiehn, 2001).

Several analytical techniques exist for metabolomics analysis including nuclear magnetic resonance spectroscopy (NMR), liquid chromatography coupled to mass spectrometry (LC-MS), gas chromatography coupled to MS (GC-MS) and capillary electrophoresis coupled to MS (CE-MS). So far, a single technique has not been capable of extracting all the metabolites present in a biological sample (Moco et al., 2007). NMR spectroscopy is a technique of choice for analysis of biofluids and intact tissues. NMR requires minimal sample preparation, no chromatographic separation and offers a non destructive approach (Viant et al., 2003; Samuelsson et al., 2006; Ekman et al., 2007). LC-MS has been increasingly employed in metabolomics studies (Wilson et al., 2005; Bedair and Sumner, 2008). This technique is more sensitive than NMR and

5. Development of analytical techniques for metabolic profiling of biofluids provides more comprehensive data of the metabolome (Weckwerth and Morgenthal, 2005). However LC has some limitations including longer analysis times, need for chromatographic separation and extensive sample preparation. The recent development of ultraperformance liquid chromatography linked to time-of-flight mass analysers (UPLC-TOF-MS) has improved the chromatographic resolution and enabled the identification of a broad range of metabolites in complex matrices (Plumb et al., 2002).

Biofluids, especially urine and blood plasma, have been frequently used to assess the effects of xenobiotics exposure as they are easily collected and can be analysed with little preparation (Aresta et al., 2006; Lutz et al., 2008; Pasikanti et al., 2008). Fewer studies exist on bile profiling (Pettersson et al., 2006; Plumb et al., 2009). Bile is produced by liver cells and is critical for both digestion of fats and excretion of many endogenous compounds and xenobiotics (Farina et al., 2009). Thus the metabolic profiling of bile may offer crucial information on liver and intestinal malfunctions. However bile contains many compounds (e.g. bile salts) that may produce interferences in the matrix and affect the detection of low abundance molecules.

Here we describe the development of analytical methods to profile the metabolite composition of biofluids. The sample preparation techniques solid phase extraction (SPE) and methanol extraction were developed to extract a wide range of metabolites from the bile and plasma respectively. Analytical methods using UPLC coupled to electrospray time-of-flight mass spectrometry (UPLC-TOF-MS) were developed for metabolite profiling. Results from profiling of the plasma and bile samples from diclofenac-exposed rainbow trout are presented in this chapter and chapter 6 respectively.

5.2. Materials and methods

5.2.1. Chemicals

Diclofenac (purity $\geq 98\%$), cholic acid (purity $\geq 99\%$), taurocholic acid ($>97\%$), urea ($\geq 98\%$), uric acid ($\geq 99\%$), bilirubin ($\geq 98\%$), eicosapentaenoic acid (EPA, $\geq 99\%$), docosahexaenoic acid (DHA, $>98\%$), tripalmitate ($\geq 99\%$), L-thyroxine ($\geq 98\%$), L- α -phosphatidylcholine ($\geq 99\%$), creatinine (anhydrous) and prostaglandin B₂ (PGB₂, $\geq 98\%$) were purchased from Sigma-Aldrich (Gillingham, UK). The eicosanoids 11R-hydroxyeicosatetraenoic acid (11-HETE), prostaglandin E₂ (PGE₂) and thromboxane B₂ (TXB₂) (purity $\geq 98\%$) were purchased from Cayman Chemical (Michigan, USA). The deuterated internal standards [2,2,4,6,6,17 α -21,21,21-*d*9] progesterone (P-*d*9) and [2,4,16,16-*d*4] 17 β -estradiol sodium 3-sulphate (E₂-*d*4-S) (isotope purity $>98\%$) were obtained from C/D/N Isotopes (Quebec, Canada). Sodium acetate, sodium hydroxide (NaOH) and ammonium hydroxide (NH₄OH) were supplied by Sigma-Aldrich. Solvents and water (all HPLC grade) and formic acid were purchased from Rathburn (Walkerburn, UK).

Stock solutions of individual standard were prepared as described in Table 5.1 and stored at -20°C . Working standard solutions containing all the compounds were obtained by further dilution in methanol (1 ng/ μl and 10 pg/ μl).

5.2.2. Sample collection

Blood taken from the caudal vein and bile were sampled from juvenile female rainbow trout exposed to waterborne diclofenac (0.5, 1, 5 and 25 $\mu\text{g/l}$) and water control for 21 days. The fish maintenance and experimental set up are described section 2.10.1. Plasma samples were obtained by centrifugation of the blood. The biofluids were stored

Table 5.1: Preparation of standard stock solutions.

Compounds	Class	Stock solution (concentration)
11-HETE	eicosanoid	bought in ethanol (100 ng/ μ l)
Bilirubin	metabolite marker of liver toxicity	in water + 15% NH_4OH (10 $\mu\text{g}/\mu\text{l}$)
Cholic acid	bile acid	in ethanol:water (1:1) (10 $\mu\text{g}/\mu\text{l}$)
Creatinine	metabolite marker of kidney toxicity	in water (10 $\mu\text{g}/\mu\text{l}$)
DHA and EPA	fatty acid	in ethanol (10 $\mu\text{g}/\mu\text{l}$)
E_2 - <i>d4</i> -S and P- <i>d9</i>	internal standard	in ethanol (100 ng/ μ l)
PBG ₂	eicosanoid	bought in ethanol (10 ng/ μ l)
PGE ₂	eicosanoid	bought in ethanol (100 ng/ μ l)
Phosphatidylcholine	bile phospholipid	in ethanol:water (1:1) (10 $\mu\text{g}/\mu\text{l}$)
Taurocholic acid	bile salt	in ethanol:water (1:1) (10 $\mu\text{g}/\mu\text{l}$)
Thyroxine	metabolite marker of tissue toxicity	in acetone + 30% NH_4OH (10 $\mu\text{g}/\mu\text{l}$)
TXB ₂	eicosanoid	bought in ethanol (100 ng/ μ l)
Urea	metabolite marker of kidney toxicity	in water (10 $\mu\text{g}/\mu\text{l}$)
Uric acid	metabolite marker of kidney toxicity	in water + 3% NaOH (10 $\mu\text{g}/\mu\text{l}$)

5. Development of analytical techniques for metabolic profiling of biofluids in two volumes of methanol at -80°C and aliquots used to develop appropriate extraction and LC-MS methods for metabolomics analyses.

5.2.3. Preparation of bile samples

One of the major problems with profiling bile on LC-MS is the presence of high concentrations of bile acids which on electrospray ionisation (ESI), tend to suppress the ions of other metabolites. Therefore the aim was to develop a SPE method to separate conjugated bile acids such as taurocholic acid (major bile acid in fish and other vertebrates) from other metabolites of interest including eicosanoids that may be targeted by diclofenac action.

Samples of bile from control fish (n=4, containing 50 µl bile with 100 µl methanol) were diluted in 7 µl sodium acetate buffer solution (pH 7). Another group of 4 bile samples were evaporated to dryness, reconstituted in 500 µl of 0.1 M phosphate buffer (pH 6) and hydrolysed using the method described section 2.13.1. Hydrolysed, non-hydrolysed and blank samples (containing 50 µl water instead of bile) were extracted using 60 mg Strata X-AW SPE cartridges (Phenomenex, Macclesfield, UK). The cartridges were pre-conditioned as described 2.13.2. In order to collect a wide range of metabolites, while reducing ion suppression, elution was carried out with four solvents with different polarity: 2 ml ethyl acetate (EA; free metabolites fraction), 2 ml methanol (M; peptides fraction), 2 ml 2% formic acid in methanol (FA; eicosanoids fraction) and 2 ml 2% ammonium hydroxide in methanol (N; conjugated metabolites fraction). After vacuum drying the extracts were brought up in 100 µl methanol:water (1:1, v/v). Aliquots of 100 µl of untreated bile samples from control fish (n=4) along with the SPE extracts for the non-hydrolysed and hydrolysed bile samples were filtered using 0.22 µm ultrafiltration tubes (Millipore) and transferred to HPLC vials for chemical analysis.

5.2.4. SPE fractionation and recovery of standard metabolites

Four replicate samples of 10 ml sodium acetate buffer solution (pH 7.0) were spiked with 100 µl of both standards and IS working solutions at 10 pg/µl (see section 5.2.1). The samples were fractionated by SPE using the protocol described in the above section (section 5.2.3). However the standards were eluted using EA, FA and N solvents, because previous work showed that very few compounds were eluted in the M fraction. The standards were analysed by UPLC-TOF-MS and quantified using a calibration a 5-point calibration curve constructed for each standard.

5.2.5. Preparation of plasma samples

The plasma metabolome was extracted using a methanol extraction protocol adapted from Bruce et al. (2008). Plasma samples (n=4) were thawed on ice and aliquots of 200 µl were spiked with 80 µl of both a mixture of eicosanoids solution (PGE₂, TXB₂, PGB₂, 11-HETE and PGJ₂) and the IS solution at 10 pg/µl. To evaluate the efficiency of the extraction method, plasma samples were mixed with 3 different methanol solutions for final solvent percentages of 70, 80 and 90%. After centrifugation, the supernatant was transferred to clean glass vials, vacuum dried, brought up in 100 µl methanol:water (1:1, v/v) and filtered using SiroccoTM Protein Precipitation Plates (Waters, UK). The purified samples were placed in HPLC vials and stored at -20°C.

5.2.6. UPLC-TOF-MS analysis

Metabolomics analysis was carried out on a Waters Acquity UPLC system coupled to a Micromass QTOF mass spectrometer. Details of the UPLC column and MS parameters are provided in section 2.7.2. 10-µl aliquots of bile and 20-µl aliquots of

5. Development of analytical techniques for metabolic profiling of biofluids

plasma extracts were injected at a flow rate of 0.09 ml/min and run in either +ESI or –ESI mode to detect the protonated [M+H] and deprotonated [M-H] ions respectively. The following mobile phase was used to profile the bile: 95:5% water-methanol (A) and methanol (B) both containing 0.2% formic acid. The solvent gradient was initiated at 50% B up to 100% B in 12 min and maintained for 4 min before re-equilibration to 50% B for 4 min. To optimise the chromatographic detection and resolution, plasma samples were run using both an acidified mobile phase (5% acetonitrile and 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B)) and a neutral mobile phase (5% acetonitrile and 0.2% ammonium hydroxide in water (A) and 0.2% ammonium hydroxide in acetonitrile (B)). A linear gradient was used initiated at 10% B up to 100% B in 10 min. This was kept for 6 min and re-equilibrated to 10% B for 8 min.

5.2.7. Data handling

MassLynx raw data files were transformed and processed using MarkerLynx V 4.1 (Waters, Milford, USA). This application, which provides automated peak detection and integration, was used to estimate the number of markers in each sample. The chromatograms were aligned with the IS *E₂-d₄-S* in –ESI and *P-d₉* in +ESI. The parameters used for the peak detection were set as follows: mass tolerance or mass accuracy of the data 0.04 Da, width of an average peak at 20% height 20 sec, baseline noise between peaks 100, number of masses per RT 50, minimum intensity allowed for a spectral peak to be considered as a marker 1% of the base peak intensity; mass window tolerance in which a spectral peak was considered to be the same marker 0.04 Da and RT tolerance 0.1 min.

5.2.8. Multivariate data analysis of plasma samples

Multivariate data analysis was performed using 15 plasma samples from each replicate tank 0, 0.5, 1, 5 and 25 μg diclofenac/l. For this, all m/z x RT datasets were normalised to the maximum spectral area in each sample and exported to Simca-P (Umetrics Ltd, Bekshire, UK) where they were log-transformed and mean-centered to reduce skewness. Principal component analysis (PCA) was conducted to visualise the data and detect any outliers. The supervised method projections to latent structures discriminant analysis (PLS-DA) was then performed to identify the metabolites affected by diclofenac exposure.

5.3. Results

5.3.1. Method development for metabolic profiling of bile

5.3.1.1. Analysis of the chromatograms for the bile extracts

Bile was analysed in both + and –ESI and produced total ions chromatograms (TIC) with many ions present at saturation (Figure 5.1). The spectral analysis of the large saturated peaks indicates the presence of bile acids most likely taurocholic acid (mass accuracy 2.3 ppm) and other taurine related compounds (Figure 5.2). The chromatographic analysis also showed that more peaks were produced in –ESI than in +ESI. This is expected as most bile acids formed deprotonated ions $[\text{M}-\text{H}]$. SPE fractionation permitted the separation of conjugated bile acids and other conjugates in the N fraction, whilst other metabolites were eluted in the remaining fractions. Figure 5.3 shows the chromatograms of fractionated non-hydrolysed bile extracts. The TIC produced for the bile metabolites eluted in EA, M and FA fractions were similar to those of the blanks eluted with the same solvents. This indicates that few bile metabolites are present in those fractions and the peaks observed are impurities from

5. Development of analytical techniques for metabolic profiling of biofluids

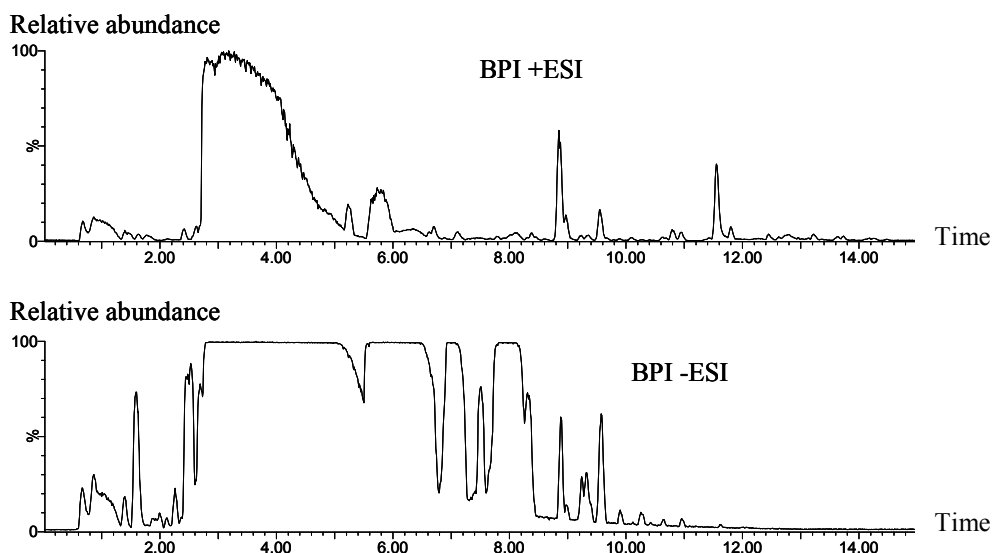


Figure 5.1 – Total ion chromatograms (as base peak intensity BPI) of untreated bile from water control fish in +ESI and –ESI modes. The saturated peaks observed in the chromatograms are caused by the high levels of bile acids. The parameters of the LC-MS were optimised but the sensitivity was not improved and the peaks remained saturated.

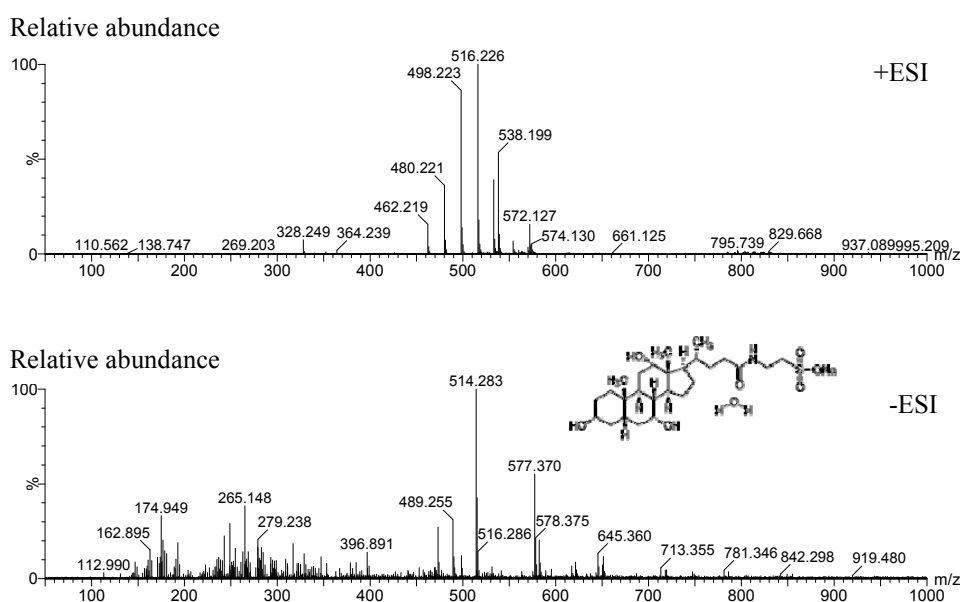


Figure 5.2 – Spectral ion chromatograms of the saturated peaks in untreated bile in +ESI and –ESI modes. The prevailing m/z ions 514 and 516 are most likely the bile salt taurocholic acid.

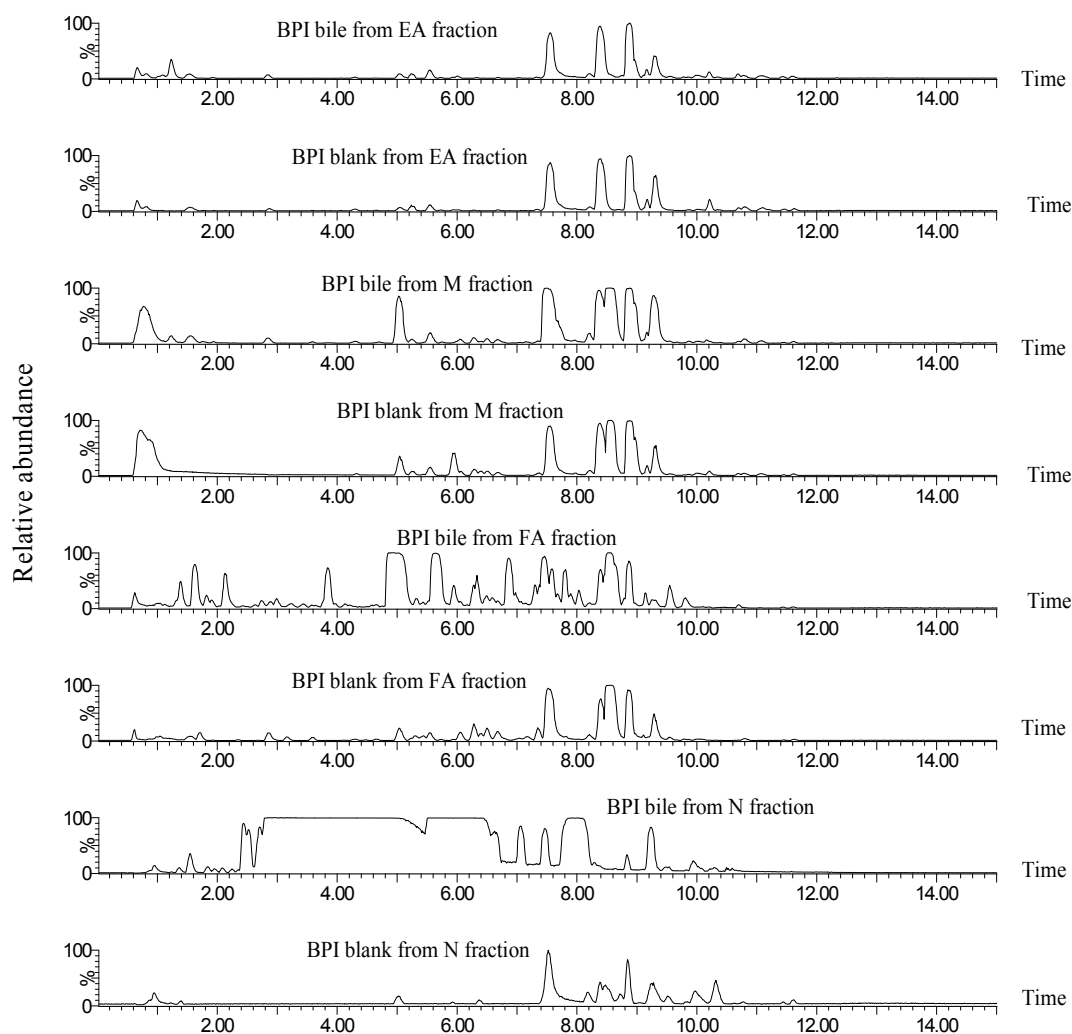


Figure 5.3 – Total ion chromatograms (as base peak intensity BPI) of fractionated non-hydrolysed bile from a control fish and a blank sample in –ESI mode. The chromatograms indicate that most compounds were eluted in the N fraction, which resulted in over saturated peaks. Eluting fractions EA: ethyl acetate, M: methanol, FA: formic acid in methanol, N: ammonium hydroxide in methanol.

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the cartridges. The chromatogram of the N fraction, however, had large saturated peaks caused by the presence of bile acids. To deconjugate these compounds and facilitate their detection, bile samples were hydrolysed. The chromatograms of fractionated hydrolysed bile are presented Figure 5.4. As observed in the non-hydrolysed fractionated samples, the chromatograms of the hydrolysed bile extracts in EA, M and N fractions were comparable to the blanks. Hydrolysis did reduce the number of saturated peaks but some taurine related compounds remained in the N fraction.

5.3.1.2. Quantitative analysis of the bile extracts

Chromatographic data were further analysed using MarkerLynx to estimate the numbers of metabolites detected in untreated bile, fractionated non-hydrolysed bile and fractionated bile (Table 5.2 and 5.3). MarkerLynx data indicated that some metabolites were lost during SPE fractionation. The untreated bile samples contained more metabolites than the fractionated non-hydrolysed and hydrolysed bile samples. It was also observed that SPE fractionation introduced impurities in the samples. The analysis of blank samples revealed the presence of up to 538 ions. To ensure that the metabolites eluted in the EA, M and FA fractions were accurately detected and identified, the injection volume was increased. Increasing the injection volume to 10 μ l provided more potential bile metabolites in the EA and FA fractions but did not improve the number of peaks detected in the M fraction.

5. Development of analytical techniques for metabolic profiling of biofluids

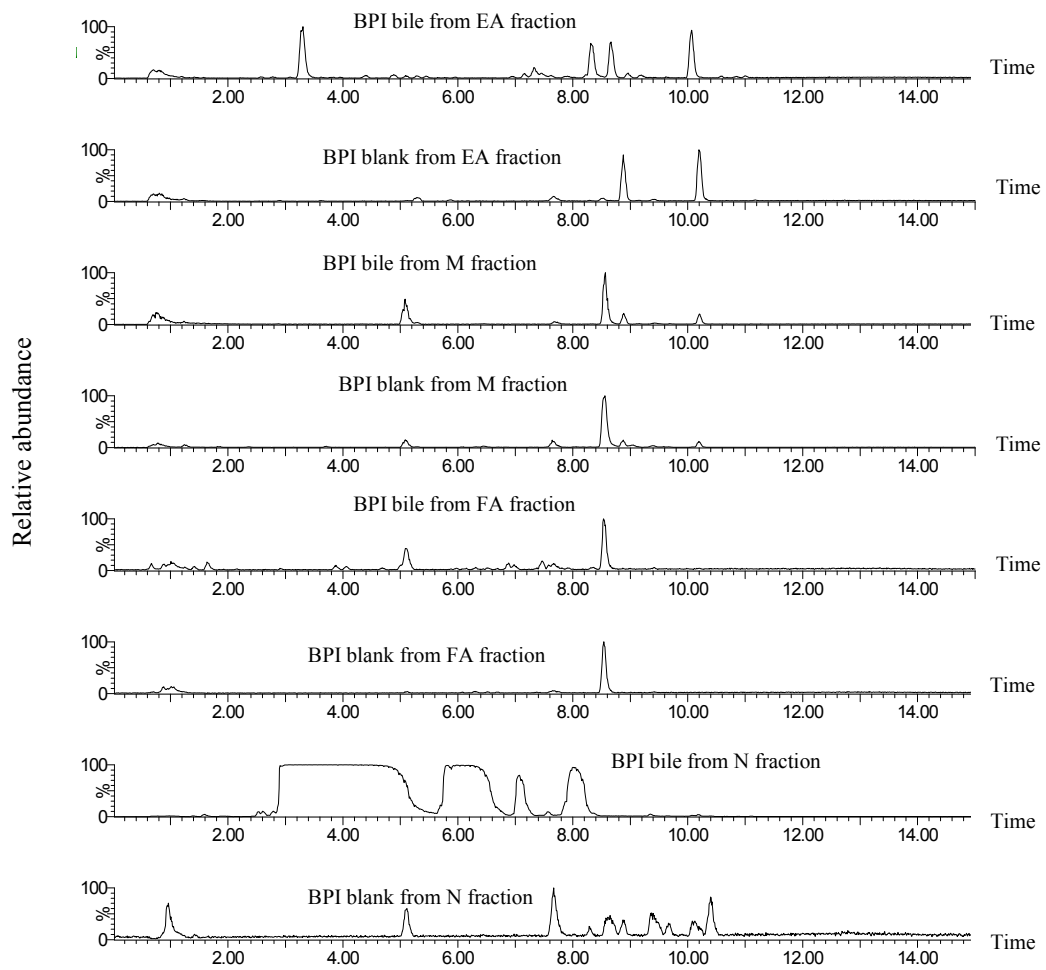


Figure 5.4 – Total ion chromatograms (as base peak intensity BPI) of fractionated hydrolysed bile and blank samples in –ESI mode. Eluting fractions EA: ethyl acetate, M: methanol, FA: formic acid in methanol, N: ammonium hydroxide in methanol.

Table 5.2 – Number of markers estimated by MarkerLynx for non-hydrolysed bile samples untreated and fractionated using SPE.

	Sample	Untreated	Fractionation				Total
		Bile	EA	M	FA	N	
Positive ESI	1	2252	-	-	77	453	530
			(134)	(-)	(1104)		
	2	1660	-	-	194	788	982
			(310)	(-)	(1081)		
	3	1526	-	-	56	643	699
			(355)	(-)	(974)		
	4	1613	-	-	68	503	571
			(178)	(-)	(1509)		
	mean± SE	1763 ± 330.8			696 ± 85		
Negative ESI	1	4121	8	-	17	2719	2744
			(158)	(398)	(3396)		
	2	3952	-	-	29	2839	2868
			(99)	(403)	(4135)		
	3	3485	-	-	23	2589	2623
			(112)	(336)	(3331)		
	4	3935	11	-	20	3225	3256
			(173)	(217)	(3874)		
	mean± SE	3873 ± 673			2873 ± 485		

ESI: electrospray ionisation, EA: ethyl acetate fraction, M: methanol fraction, FA formic acid in methanol fraction and N: ammonium hydroxide in methanol fraction. Numbers of markers obtained after subtracting the average number of markers detected in the blank samples for the same fraction. Rows highlighted indicate the average number of bile markers detected in 1 µl SPE extract. In brackets is the number of markers detected after injecting 10 µl SPE extracts.

Table 5.3 – Number of markers estimated by MarkerLynx for hydrolysed bile samples fractionated using SPE.

	Sample	Fractionation				Total
		EA	M	FA	N	
Positive ESI	1	-	5	10	1039	1054
		(67)	(55)	(649)		
	2	-	8	27	1171	1206
		(43)	(62)	(763)		
	3	-	7	25	1091	1123
		(58)	(71)	(684)		
	4	-	12	20	1162	1194
		(93)	(63)	(741)		
	mean \pm SE	1174 \pm 161				
Negative ESI	1	100	6	139	1558	1903
		(633)	(77)	(1447)		
	2	176	11	197	1572	1956
		(633)	(78)	(1186)		
	3	122	23	85	2128	2358
		(862)	(104)	(1101)		
	4	128	18	205	2131	2482
		(787)	(167)	(1254)		
	mean \pm SE	2175 \pm 356				

ESI: electrospray ionisation, EA: ethyl acetate fraction, M: methanol fraction, FA formic acid in methanol fraction and N: ammonium hydroxide in methanol fraction. Numbers of markers obtained after subtracting the average number of markers detected in the blank samples for the same fraction. Rows highlighted indicate the average number of bile markers detected in 1 μ l SPE extract. In brackets is the number of markers detected after injecting 10 μ l SPE extracts.

5.3.2. SPE fractionation and recovery of standard metabolites

The UPLC-TOF-MS analysis revealed that many of the target metabolites were successfully extracted and quantified using 60 mg SPE cartridges. The chromatographic peaks of these compounds are illustrated in Figure 5.5. The SPE recovery efficiency, elution solvent, retention time (RT) and m/z value of the ions formed in +ESI mode and –ESI mode are listed in Table 5.4. The IS steroids *E₂-d₄-S* and *P-d₉*, were detected in –ESI and +ESI modes respectively. Both compounds were recovered at levels greater than 80%.

Recoveries of the target metabolites from aqueous matrix ranged from 45% for 11-HETE to 91% for cholic acid. SPE method separated taurocholic acid from the other metabolites classes. The eicosanoids PGE₂, TXB₂, PGB₂ and 11-HETE were eluted in the FA fraction and were detected in –ESI mode forming only [M-H] ions. Despite the addition of formic acid in the mobile phase, TXB₂ did not produce a sharp peak and gave the most variable recovery efficiency. This compound may have been degraded. Thyroxine and phosphatidylcholine were only detected as protonated ions [M+H] in positive mode. Bilirubin was detected in both ionisation modes but was not recovered after SPE fractionation. The smaller molecules creatinine, urea and uric acid could not be detected under the UPLC conditions used in our study.

5. Development of analytical techniques for metabolic profiling of biofluids

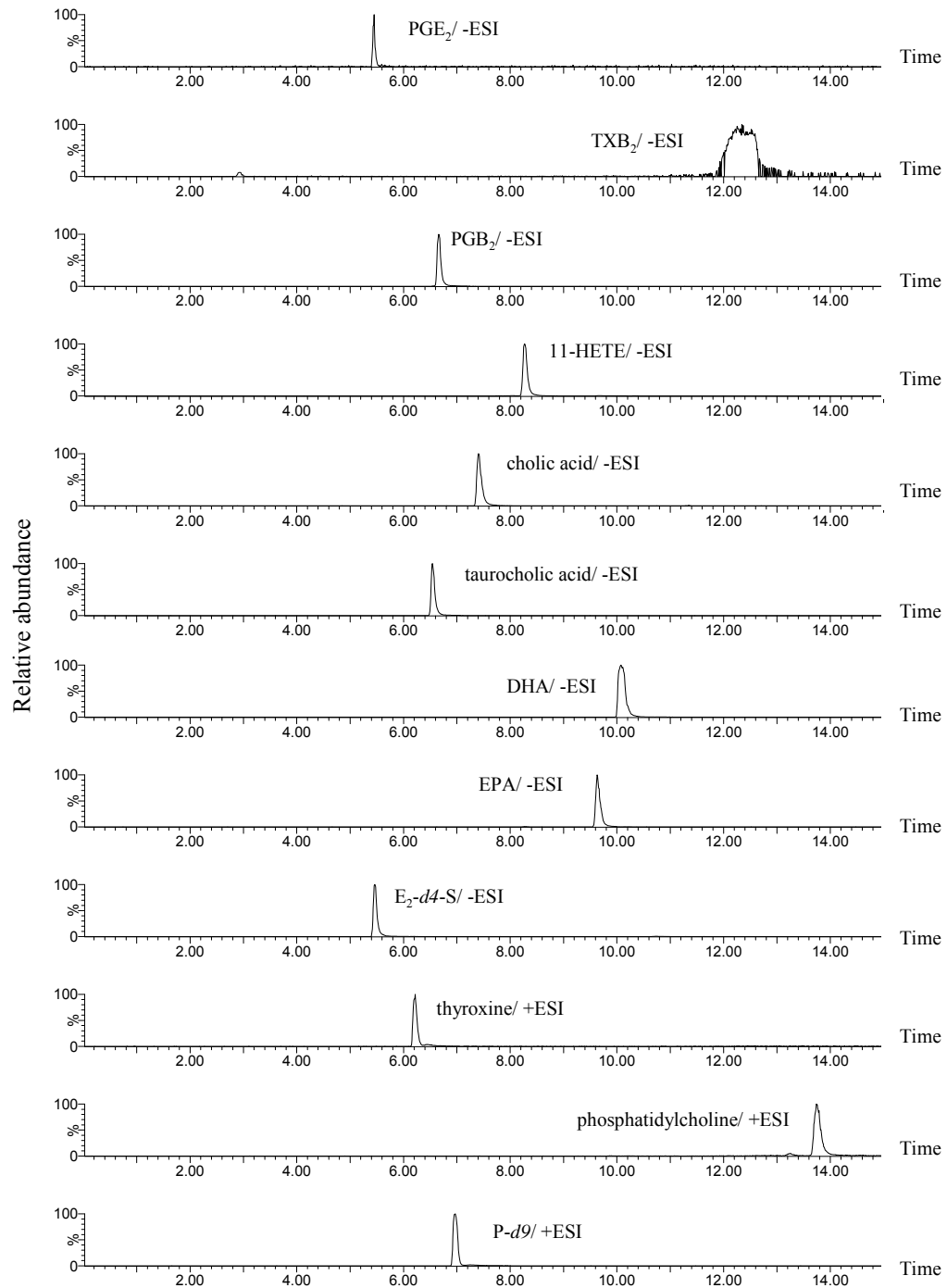


Figure 5.5 – Chromatograms of target metabolites usually found in fish bile and the internal standards used for quantification.

Table 5.4 – UPLC-TOF-MS analysis of target metabolites in aqueous samples: chemical formula, the calculated m/z of the ionic species formed in either +ESI or –ESI modes and SPE recovery efficiency.

Standards	Formula	[M+H]	[M-H]	RT	Recovery	UPLC-TOF-MS analysis*
11-HETE	C ₂₀ H ₃₂ O ₃	321.2430	319.2273	8.25	45 ± 2	FA/-ESI
Cholic acid	C ₂₄ H ₄₀ O ₅	409.2954	407.2797	7.39	91 ± 4	EA+FA/-ESI
DHA	C ₂₂ H ₃₂ O ₂	329.2481	327.2324	10.04	90 ± 6	EA+FA/-ESI
E ₂ - <i>d4</i> -S	C ₁₈ H ₂₀ D ₄ O ₅ S	357.1674	355.1518	5.47	93 ± 2	N/-ESI
EPA	C ₂₀ H ₃₀ O ₂	303.2324	301.2168	9.61	86 ± 4	EA+FA/-ESI
P- <i>d9</i>	C ₂₁ H ₂₁ D ₉ O ₂	324.2889	322.2733	6.98-	81 ± 1	EA/+ESI
PGB ₂	C ₂₀ H ₃₀ O ₄	335.2222	333.2066	6.67	80 ± 5	FA/-ESI
PGE ₂	C ₂₀ H ₃₂ O ₅	353.2328	351.2171	5.45	78 ± 2	FA/-ESI
Phosphatidylcholine	C ₄₂ H ₈₂ NO ₈ P	760.5856	758.5700	13.77	69 ± 5	FA/-ESI
Taurocholic acid	C ₂₆ H ₄₄ NO ₇ S	516.2995	514.2838	6.55	89 ± 3	N/-ESI
Thyroxine	C ₁₅ H ₁₁ I ₄ NO ₄	777.6945	775.6789	6.21	87 ± 5	FA/+ESI
TXB ₂	C ₂₀ H ₃₄ O ₆	371.2434	369.2277	11.99	70 ± 8	FA/-ESI

*Eluting fraction used to quantify the analytes – EA: ethyl acetate fraction; FA: formic acid in methanol fraction; N: ammonium hydroxide in methanol fraction; [M+H]: protonated ion; [M-H]: deprotonated ion; RT: retention time. Recoveries expressed in % mean ± SE.

5.3.3. Method development for metabolic profiling of plasma samples

The number of peaks displayed increased with increased methanol concentrations (Table 5.5). However the comparisons between blank and plasma samples extracted with the overall 90% methanol solution indicated that the 90% methanol extracts also contained many impurities. Therefore it was concluded that overall 80% methanol solution (assuming that plasma is 100% aqueous) was the best extraction method for the metabolic profiling of plasma samples.

Blood plasma were analysed in both +ESI and -ESI modes, TIC in both ionisation are shown Figure 5.6. The sensitivity and reproducibility of the UPLC-TOF-MS was assessed based on the analysis of chromatograms for control plasma samples and plasma spiked with a low concentration of eicosanoids (4 ng/ml of plasma). TIC produced for control and spiked samples were nearly identical (data not shown). This is to be expected as the eicosanoids spikes were very low and plasma contains many metabolites creating interferences. Thus the eicosanoids normally detected as deprotonated ions [M-H] in negative mode (Figure 5.7), were ion suppressed. This resulted in a reduced intensity signal, especially for PGB₂ and PGE₂.

The limit of detection (LOD) for the eicosanoids detected in water and plasma samples run with the acidic mobile phase are reported Table 5.6. Most eicosanoids had a LOD below 3 ng/ml in water samples. However the detection capabilities were reduced in the plasma samples and LOD varied from 4.9 ng/ml for PGE₂ to 8.7 ng/ml for PGJ₂.

Table 5.5 – Number of markers detected after methanol extraction of plasma samples.

Methanol solution	70%	80%	90%
Sample 1	2762	3348	3645
Sample 2	2459	3426	3541
Sample 3	2263	3247	3220
Sample 4	2647	3681	3918
Mean \pm SE	2533 \pm 420	3426 \pm 587	3581 \pm 615
Mean blank	193 \pm 45	270 \pm 48	904 \pm 36

Numbers of markers in this table were obtained after subtracting the average number of markers detected in the blank samples.

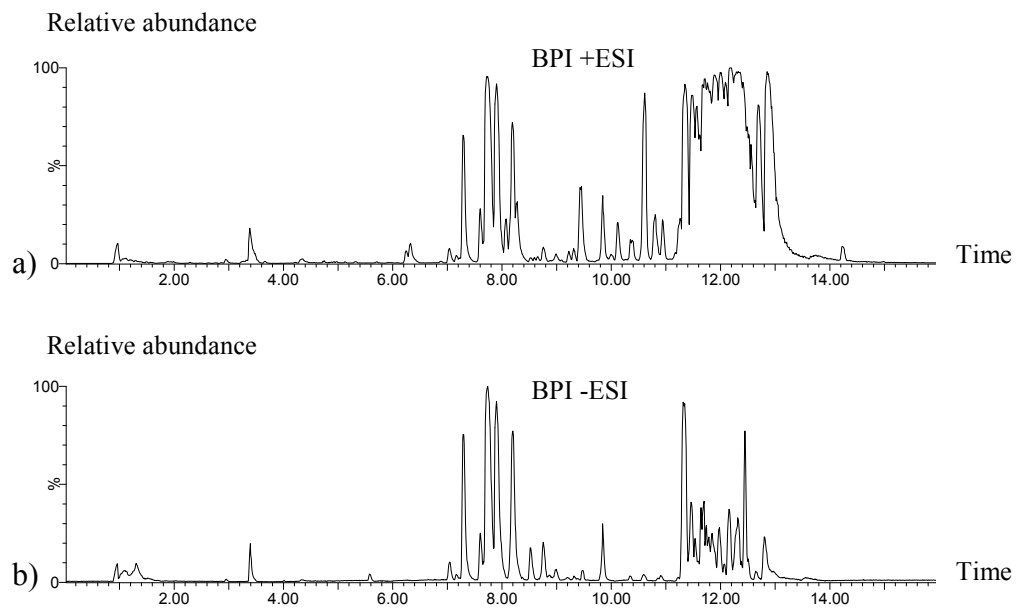


Figure 5.6 – Total ion current (as base peak intensity BPI) of plasma sample from a control fish in +ESI mode (a) and –ESI mode (b).

5. Development of analytical techniques for metabolic profiling of biofluids

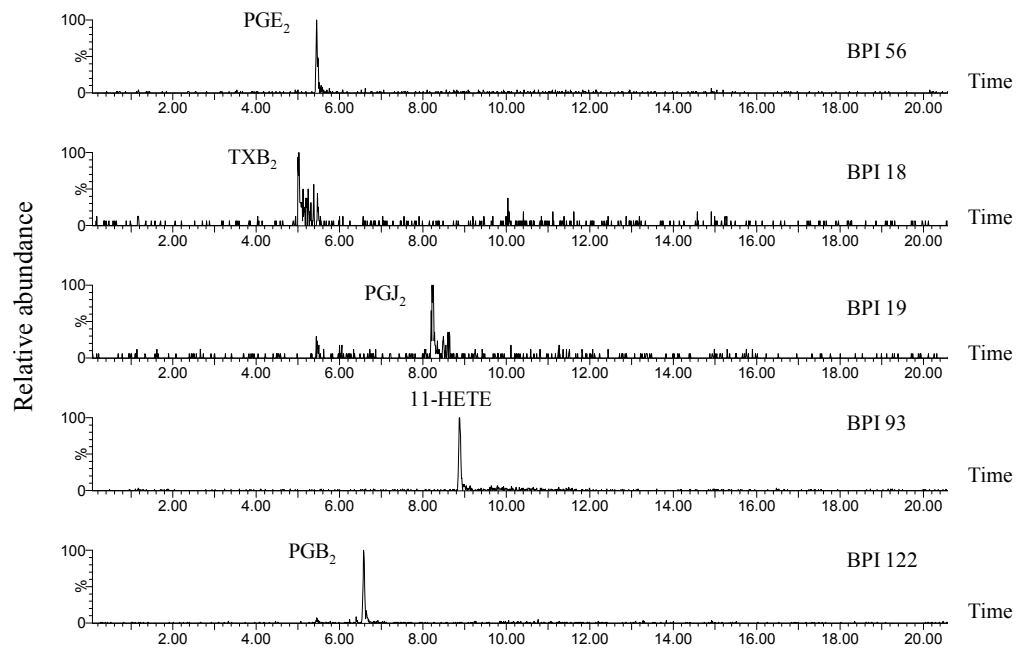


Figure 5.7 – Chromatograms of eicosanoids standards (4 pg/ μ l) in water samples run in –ESI mode with acidic mobile phase.

Table 5.6 – Limit of detection (LOD) of eicosanoids in water and plasma samples using UPLC-TOF-MS.

	LOD in water samples	LOD in plasma samples
	± SE (ng/ml)	± SE (ng/ml)
11-HETE	1.3 ± 0.8	3.2 ± 1.4
PGB ₂	1.5 ± 1.1	6.2 ± 1.6
PGE ₂	1.6 ± 0.9	4.9 ± 0.6
PGJ ₂	2.2 ± 0.4	8.7 ± 0.8
TXB ₂	4.7 ± 0.7	5.1 ± 1.3

LOD expressed as mean ± SE

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To improve the chromatographic detection, plasma samples were run in an acidic (formic acid) and neutral (ammonium hydroxide) mobile phase. The pH of the mobile phase affects the formation of ionic species and can enhance or reduce the signal intensity. In this study, the acidic mobile phase was preferred as it provided a sharper chromatographic peak and less background noise (Figure 5.8). It was noted that the m/z value of 11-HETE produced two peaks on the chromatogram. However, the mass spectra of the peaks showed that another eicosanoid was co-eluted with 11-HETE. This explains the increase in signal intensity in the spiked samples (BPI of 93 in water and up to 247 in spiked plasma samples).

5.3.4. PCA and PLS-DA analyses of plasma samples from diclofenac exposure

Plasma samples from fish exposed to diclofenac for 21 days and control fish were run in +ESI and –ESI modes using the acidic mobile phase and analysed using multivariate data analysis to identify the metabolites affected by diclofenac exposure. The projection method PCA was carried out to display the interrelationship between the plasma samples of exposed fish (0.5, 1, 5 and 25 μg diclofenac/l) and the water controls.

The supervised clustering method PLS-DA was then applied to identify the class separating variables. The resulting PCA and PLS-DA loadings gave poor models as shown by the low values of the explained (R^2X and R^2Y for PCA and PLS-DA respectively) and predicted variation (Q^2) in Table 5.7. This indicates that the models did not represent accurately the data. Many potential outliers were found in the PCA models indicating a great variability within the data set. Normally outliers must be removed for subsequent analysis by removing strong outliers resulted in the appearance of new outliers. Therefore it was decided to remove 5-10 of the stronger outliers and perform PLS-DA regardless. PLS-DA slightly improved the class separation compared with the PCA models (Figure 5.9).

5. Development of analytical techniques for metabolic profiling of biofluids

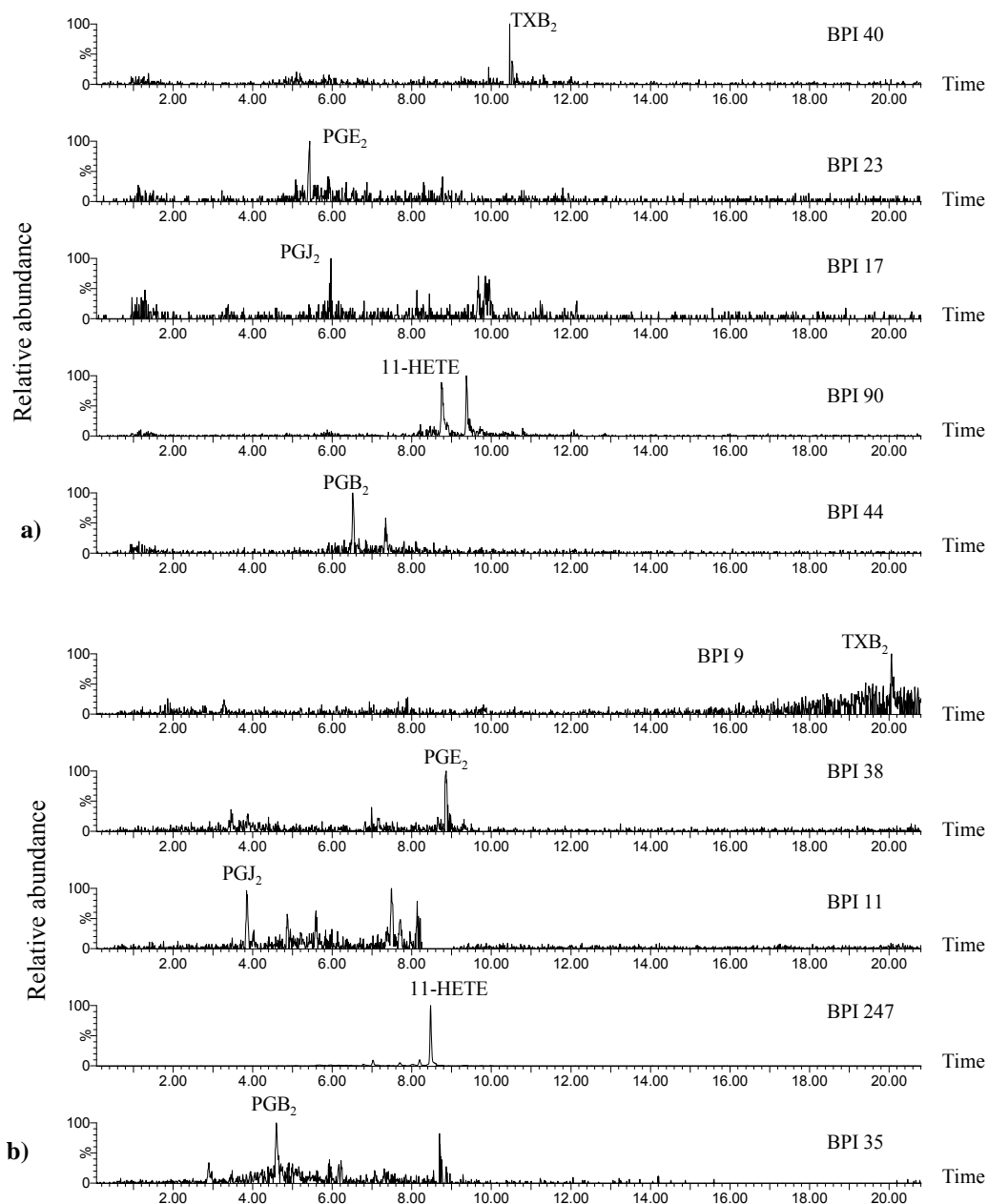


Figure 5.8 – Selected ion chromatograms (-ESI) of the eicosanoids recovered in the 80% methanol extracts of spiked plasma samples. a) acidic mobile phase, b) neutral mobile phase.

Table 5.7 – Performance parameters of multivariate discriminant models for the comparison of control and diclofenac exposed rainbow trout (0.5, 1, 5 and 25 µg/l).

UPLC- TOF-MS	Groups	Multivariate method	Components	R ² X	R ² Y	Q ²
-ESI	All	PCA	6	0.428		0.28
-ESI	All	PLS-DA	3	0.227	0.298	0.074
+ESI	All	PCA	6	0.566		0.438
+ESI	All	PLS-DA	2	0.533	0.955	0.0473
-ESI	C, 0.5, 1	PCA	5	0.332		0.157
-ESI	C, 0.5, 1	PLS-DA	2	0.118	0.737	0.118
+ESI	C, 0.5, 1	PCA	5	0.561		0.345
+ESI	C, 0.5, 1	PLS-DA	2	0.352	0.162	-0.0522
-ESI	C, 5, 25	PCA	5	0.34		0.154
-ESI	C, 5, 25	PLS-DA	2	0.095	0.624	-0.017
+ESI	C, 5, 25	PCA	4	0.352		0.187
+ESI	C, 5, 25	PLS-DA	2	0.179	0.426	0.123

ESI: electrospray ionisation; R²X: variation explained by the model; R²Y: cumulative variation explained by the model; Q²: cumulative variation predicted by the model. The groups C, 0.5, 1, 5 and 25 correspond to fish exposed to diclofenac at concentrations 0, 0.5, 1, 5 and 25 µg/l respectively.

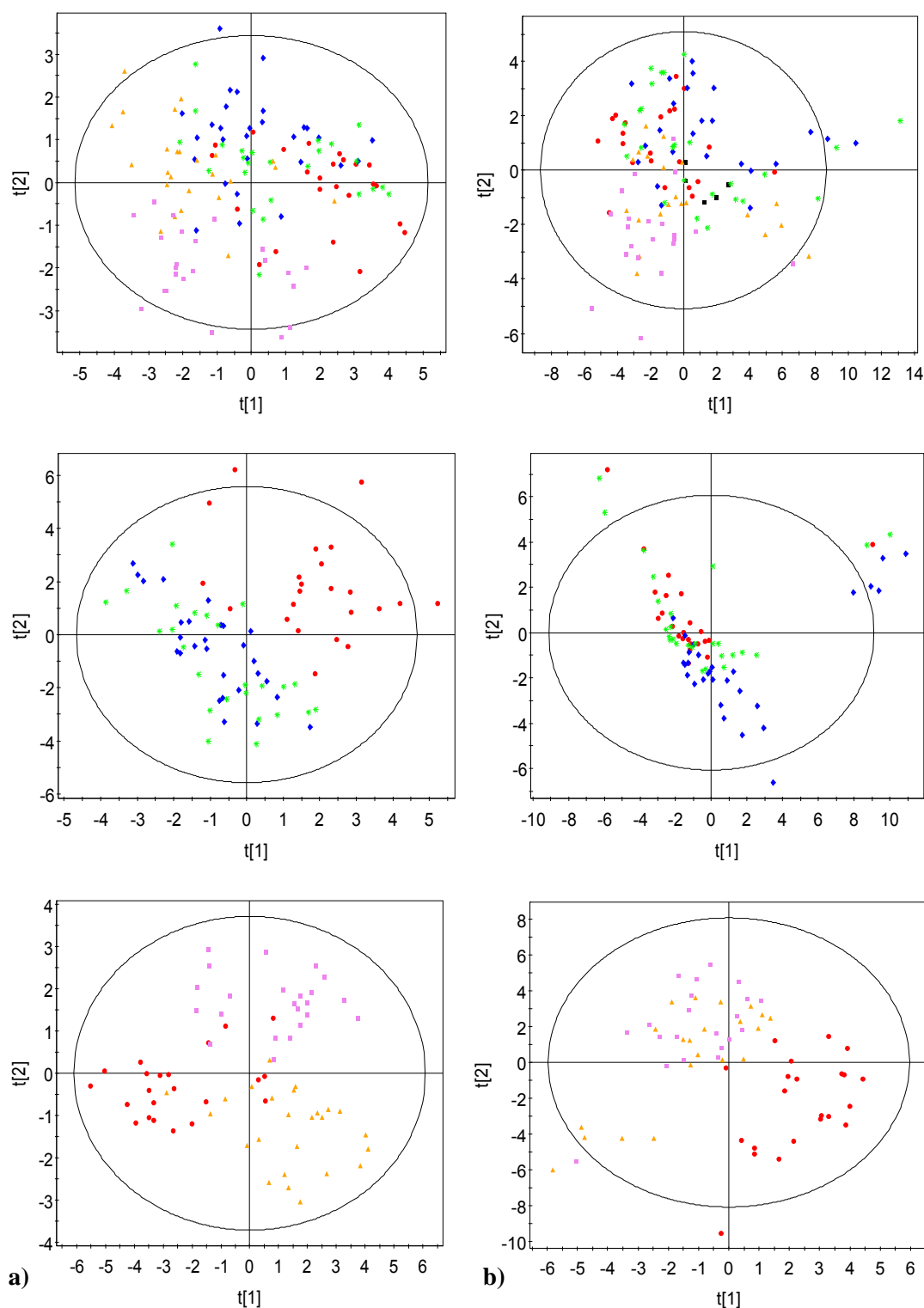


Figure 5.9 – Score plots from partial least squares discriminant analysis (PLS-DA) of fish plasma following diclofenac exposure (n=24 to 28). ●: control; ▲: 0.5 µg/l; ×: 1 µg/l; ▲: 5 µg/l and ■: 25 µg/l. Top two models show the relationship between all the control and exposed fish. Data were then remodelled to show the relationship for control vs low dose (0.5 and 1 µg/l) and control versus high dose (5 and 25 µg/l). Column **a)**: UPLC-TOF-MS analysis in -ESI mode and **b)**: in +ESI mode.

5.3.5. Identification of class-separating variables

No discriminative variables were found between the control and fish exposed to either 0.5 or 1 µg diclofenac/l. The stronger discrimination occurred between the control and the highest diclofenac exposure concentration (25 µg/l). Only one marker (m/z 298.004 in –ESI) was found responsible for the small class separation. Inspection of the raw data revealed that this compound was present at low levels in plasma samples from 5 µg/l exposure tanks and at higher levels in all the samples from 25 µg/l exposure tanks (Figure 5.10). The elemental composition tool calculated the theoretical mass formula of the m/z 294.008 marker and provided a theoretical formula of $C_{14}H_{11}NO_2Cl_2$ with a mass accuracy of 1 ppm. This elemental composition corresponds to the deprotonated ion of diclofenac. No other endogenous metabolites were found to be responsible for the class separation.

5.4. Discussion

Biofluids contain a great diversity of substances with varied chemical properties and concentration ranges (Pasikanti et al., 2008). In these complex matrices, high abundant molecules can interfere and mask the detection of low abundant molecules. Ideally, the methodologies employed for metabolomics studies must be able to detect free steroids, organic acids, sugar as well as other low abundant signalling molecules to observe many changes occurring as they can be easily collected without killing the organism (Dorado et al., 2003; Samuelsson et al., 2006; Wagner et al., 2007). However an increasing number of studies are now using analysing the metabolic profile of bile to find metabolite markers of xenobiotics exposure. Indeed, this biofluid has been found to contain high concentrations of xenobiotics and endogenous compounds that are later excreted from the body (Pettersson et al., 2006; Farina et al., 2009).

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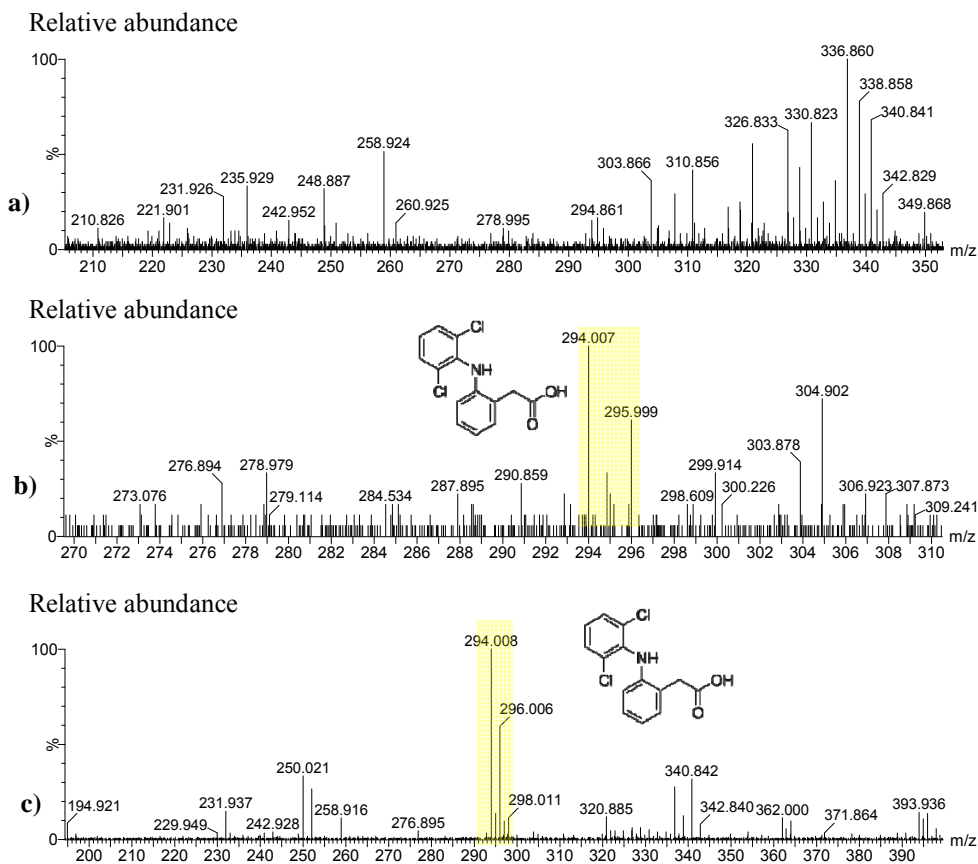


Figure 5.10 – Mass spectra of diclofenac ion (m/z 294.008) in $-ESI$ mode in plasma samples of a) control fish, b) exposed to 5 μg diclofenac/l and c) exposed to 25 $\mu\text{g/l}$.

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bile in mice and dogs (Plumb et al., 2009). Here, we demonstrated that SPE fractionation of bile can improve the chromatography by separating the conjugates from the other free molecules. Bijlsma et al. (2009) reported that SPE methods using acid in the conditioning step can decrease the interferences within the matrix and increased the recovery efficiency. The untreated bile samples contained more metabolites but many were co-eluted which resulted in large saturated peaks in the chromatograms. This may affect the detection of small abundance metabolites. Previous studies have reported that SPE and UPLC-TOF-MS technology are reliable tools to analyse conjugated and free molecules in biological samples (Flores-Valverde and Hill, 2008; Plumb et al., 2009). The efficiency of the extraction method was confirmed using a range of targeted metabolites likely to occur in bile. Satisfactory recoveries were obtained from spiked aqueous samples for most compounds (69-93%) except for 11-HETE recovered at less than 50%.

The targeted metabolites (e.g. eicosanoids, thyroxine, fatty acids) and the metabolites non-conjugated present in the bile samples were eluted in the EA and FA fractions. As the MarkerLynx analysis showed few peaks in both fractions and virtually nothing in the M fraction, the FA and EA fractions can be combined in subsequent studies to reduce the number of samples for analysis. The N fraction was used to extract the conjugated metabolites. Bile contains up to 90% of bile acids in a conjugated form as well as conjugated metabolites of drugs (Alnouti et al., 2008) and indeed the vast majority of bile metabolites were eluted in the conjugated N fraction. The chromatographic analysis showed that taurocholic acid and other taurine related compounds were present at saturation which may affect the chromatographic resolution. LC-MS based metabolomics require good quality chromatograms to ensure accurate identification of metabolites and the conjugated fraction may hold important information that is difficult to interpret. Hydrolysed bile samples produced TIC with

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less saturated peaks compared to the TIC of non-hydrolysed bile samples. Hydrolysis is used to cleave conjugates and produce free molecules that may be more easily detected and quantified using the technology platform reported upon here. Some limitations were observed in the UPLC-TOF-MS system as the smallest signalling molecules urea, uric acid, bilirubin and creatinine could not be detected even in the standards. The optimisation of the MS parameters did not overcome this problem.

Plasma is a matrix rich in proteins that may interfere with the detection of other signalling molecules through ion suppression. It is important to clean the samples and reduce the matrix interference effects prior to MS analysis. The extraction protocol published by Bruce et al. (2008) was adapted to our study and the methanol extracts were further purified by filtration in order to remove the protein precipitates and minimize ion suppression. Our conclusions were supported by their findings that the overall 80% methanol solution gave the best extraction efficiency. The method development was carried out with samples spiked with eicosanoids only. Diclofenac is known to act by inhibiting the cyclooxygenase interfering with the productions of eicosanoids. Research has shown that at environmental concentrations diclofenac can accumulate in fish organs and interfere with the metabolic activities (Schwaiger et al., 2004; Hoeger et al., 2005). Thus changes in the abundance of certain eicosanoids were expected. Eicosanoids are small signalling molecules present in various tissues (e.g. kidney, intestine, brain) in the low pg range and have been quantified successfully using GC or LC-MS technologies (Tsukamoto et al., 2002; Blewett et al., 2008). Using our analytical methodologies, the lower LOD were in the ng range. The mobile phase composition was investigated to optimise the detection of selected eicosanoids in the plasma. The addition of acids in the mobile phases are known to improve the resolution and shape of the chromatographic peaks in +ESI mode by assisting protonation of molecules (García, 2005). However, the metabolites in plasma samples were best

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detected in –ESI mode and less is known about the influence of acidic mobile phases in this ionization mode. Here, the addition of formic acid to acetonitrile provided a sharper chromatographic peak for the selected eicosanoids but the signal intensity was decreased. The observations are in accordance with the general conception that deprotonated ions are not easily formed under acidic pH conditions (Wu et al., 2004). The chromatographic analysis of target eicosanoids in water and spiked plasma samples indicated clearly that the detection of eicosanoids is mitigated by matrix interferences and the MS technology platform used is not sensitive enough for the identification of very low abundance signalling molecules. Other substances such as salts, ion pairing agents and other ionic endogenous compounds may still be interfering with the ionization process (Annesley, 2003).

The multivariate data analysis of plasma samples from diclofenac exposure indicated that the methodologies employed were not sensitive and accurate enough to allow the identification of potential metabolite markers of chemical exposure. There were no real differences between the metabolic profiles of control and diclofenac-exposed rainbow trout. The low coefficients for the explained variance of the PCA and PLS-DA models (R^2 between 0.09 and 0.5) imply that the variation observed in the data may not be related to diclofenac exposure. The difference between the explained (R^2Y) and the predicted (Q^2Y) coefficients indicate that the PLS-DA models are over-fitted and there was no true class separation between the samples. Thus the models could not be validated. As mentioned earlier eicosanoids are very low abundance molecules (pg range). However our metabolomics data were acquired from only 150 μ l plasma samples and it is possible that the volumes of used were too low to identify these unstable molecules. Larger volumes of plasma may be more appropriate to extract and identify eicosanoids. The limit of detection for the eicosanoids in water samples were low and ranged between 1.3 ng/ml \pm 0.8 and 4.7 ng/ml \pm 0.7 whilst eicosanoids are

5. Development of analytical techniques for metabolic profiling of biofluids often found in pg/ml or less. Matrix effects observed previously further reduced the detection capabilities of the UPLC-TOF-MS. Therefore, it is possible that low abundance endogenous metabolites were affected by diclofenac but could not be detected or identified. Class separation observed between the control fish and trout exposed to 5 and $\mu\text{g/l}$ was attributed to the pharmaceutical diclofenac. Surprising diclofenac was not detected at the lower exposure concentrations (0.5, 1 and 5 $\mu\text{g/l}$). Previous studies have demonstrated that chronic exposure of rainbow trout to sewage effluents containing 0.2 to 2 μg diclofenac/l could lead to bioaccumulation in the plasma with a bioconcentration factor up to 11 (Brown et al., 2007). This implies that the analytical techniques applied in this study to analyse the metabolic profile of plasma need to be further optimised.

5.5. Conclusions

Metabolomics is now recognised as a widely used technique for evaluating the toxicity of pharmaceuticals. Here, we demonstrated that bile is a suitable biofluid for metabolomics studies on fish and may provide key information on the uptake and toxic effects of xenobiotics in aquatic vertebrates. The SPE and UPLC-TOF-MS system developed were sensitive enough to detect and identify a wide range of bile metabolites (eicosanoids, fatty acids, bile acids). Therefore this methodology has the potential to be applied to investigate the effects of pharmaceuticals and other pollutants in fish. The hydrolysis treatment proved beneficial to reduce the amount of taurocholic acid present at saturation. Other deconjugating enzymes may be used to ensure that most of the conjugated metabolites present in the bile are cleaved. The methodologies employed to analyse the plasma metabolome require further optimisation in order to successfully detect low abundance metabolites and identify potential metabolites markers of diclofenac exposure.

CHAPTER 6: Identifying the biological effects of diclofenac in fish using metabolomics profiling

6.1. Introduction

Metabolomics has been dedicated to the study of metabolite composition and dynamics in a cell, organ and organism (Katajamaa and Oresic, 2007). This technology has been employed in toxicology, biomedical research, drug development and plant physiology (Clarke and Haselden, 2008; Tianniam et al., 2008; Wishart, 2008; Issaq et al., 2009). Over the past decade, metabolomics studies have shown some great potential as a diagnostic tool to discover biomarkers of diseases. Brindle et al. (2002) showed that metabolomics combined with pattern recognition techniques on human serum can be used to diagnose the presence and severity of coronary artery disease with high (90%) accuracy. Metabolic profiling of cerebrospinal fluid has also been employed to identify metabolites of microbial origin to distinguish bacterial meningitis from viral meningitis (Coen et al., 2005). In cancer research, the use of metabolomics has led to the identification of urinary nucleosides as markers for the early diagnosis of colorectal cancer (Feng et al., 2009).

The specificity of the markers identified in biomedical research suggests that laboratory-based metabolomics studies could be used to determine the exposure to a specific chemical in the environment. Recent research has demonstrated the ability of metabolomics application to assess the effects of environmental exposure to xenobiotics in aquatic wildlife (Samuelsson et al., 2006; Viant et al., 2006; Ekman et al., 2007; Flores-Valverde and Hill, 2008). Viant et al. (2006) studied the metabolic profile of Japanese medaka (*Oryzias latipes*) embryos exposed to the herbicide dinoseb and they were able to associate the metabolic changes with reduced growth rate and increased

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling embryotoxicity. However, very few metabolomics studies have been able to identify biomarkers of exposure and elucidate the mode of action of xenobiotics and their physiological implications in fish (Poynton et al., 2008).

Environmental surveys have shown that significant amounts of pharmaceuticals are continuously discharged in the aquatic environment via wastewater treatment plants (WWTPs) (Heberer, 2002; Larsson et al., 2007; Farré et al., 2008). These compounds remain biologically active and can interfere with the metabolism of non-target organisms (Thibaut et al., 2006; Carlsson et al., 2009). In the literature, however there are less than 100 publications that have reported the effects of human pharmaceuticals exposure (mainly synthetic estrogens) on the metabolic profile of aquatic organisms (Iguchi et al., 2006; Katsiadaki et al., 2009). The effects of non-steroidal pharmaceuticals on fish have received very little attention despite the fact that these compounds are frequently detected in the aquatic environment up to a few $\mu\text{g/l}$.

In this study, rainbow trout (*Oncorhynchus mykiss*) was used as a model species to evaluate how diclofenac might affect fish metabolism. Diclofenac is a non-steroidal anti-inflammatory known to affect the production of prostanoids, molecules controlling many physiological processes, by inhibiting the cyclooxygenase (*cox*) enzymes. Cellular metabolic pathways are highly conserved among species and side effects observed in mammalian systems (e.g. reduction of folate biosynthesis) may occur in fish resulting in deleterious physiological changes. This chapter reports the application of solid phase extraction (SPE) and UPLC-TOF-MS methodologies developed in Chapter 5 to profile the bile of rainbow trout from diclofenac exposure in Chapter 4. Multivariate data analysis was conducted using principal component analysis (PCA), partial least squares analysis discriminant analysis (PLS-DA) and orthogonal PLS (OPLS) in order to identify the endogenous metabolites affected by diclofenac and to infer possible biological pathways affected. This study also aimed to identify the

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling metabolites of diclofenac potentially responsible for the damage observed in the intestinal epithelium of exposed fish reported in Chapter 4.

6.2. Materials and Methods

6.2.1. Chemicals

Diclofenac, β -glucuronidase (type VII from *Escherichia coli*), sulphatase (type VI from *Aerobacter aerogenes*), sodium acetate and ammonium hydroxide were purchased from Sigma-Aldrich (Poole, UK). The internal standard (IS) steroids [2,2,4,6,6,17 α -21,21,21-*d*9] progesterone (P-*d*9) and [2,4,16,16-*d*4] 17 β -estradiol sodium 3-sulphate (E₂-*d*4-S) were obtained from C/D/N Isotopes (Quebec, Canada). All solvents and water were of HPLC grade and purchased from Rathburn (Walkerburn, UK).

6.2.2. Diclofenac exposure and sample collection

Exposure of juvenile female rainbow trout (*Oncorhynchus mykiss*) to diclofenac was conducted as described in Mehinto et al., (see Chapter 4, section 4.2.1). Briefly, fish (125-230 g body weight) were placed in groups of 15 per duplicate tank (n=30 per treatment) and exposed to waterborne diclofenac at nominal concentrations 0.5, 1, 5 and 25 μ g/l and a water control. After 21 days of exposure, fish were anaesthetised with benzocaine and killed by destroying the brain according to UK Home Office Animal Licence procedures. Bile samples were taken using a syringe and kept at -80°C in glass vials containing two volumes of methanol.

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6.2.3. Bile hydrolysis

In order to cleave the bile salts and facilitate the detection of other signalling molecules, fish bile (from water control, 0.5 and 5 µg/l treatment groups) was deconjugated by enzymatic hydrolysis adapted from the method described by Gibson et al. (2005). Bile samples of 300 µl were evaporated to dryness in a SpeedVac concentrator (Savant Instruments Inc.- SpeedVac Plus) and reconstituted with 500 µl of 0.1 M phosphate buffer (pH 6.0) and 200 µl of each β-glucuronidase (1000 units/ml) and sulphatase (2 units/ml) enzymes. The samples were then incubated at 37°C for up to 16 hr. The activity of each of the hydrolysis enzymes was monitored by incubation with standard substrates of 4-nitrophenol-β-glucuronide and 4-nitrophenol sulphate (as explained in section 2.13.1).

6.2.4. Metabolite extraction and UPLC-TOF-MS analysis

Non-hydrolysed bile (300 µl, n= 16 for water control and 25 µg/l tanks) and hydrolysed bile (900 µl, n=12-16 for water control, 0.5 and 5 µg/l tanks) was used for the metabolomics study. Each bile sample was diluted in sodium acetate buffer (300 mM, pH 7.0) to make up a volume of 8 ml and fractionated using the solid phase extraction (SPE) methodology optimised in Chapter 5. Briefly, SPE was performed with the pre-conditioned Strata X-AW cartridges (60 mg, Phenomenex, Macclesfield, UK). Bile metabolites were eluted with ethyl acetate and 2% formic acid in methanol, and both extracts were combined into 1 fraction (EA+FA fraction containing neutral and acidic compounds). The cartridges were then eluted with 2% ammonium hydroxide in methanol (N fraction containing conjugated metabolites and anionic compounds). The SPE extracts were dried to remove any solvent, reconstituted in 100 µl of methanol:water (1:1, v/v) and filtered with centrifuge tubes filters of 0.22 µm pore size (Millipore).

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Bile metabolites were separated on an Acquity UPLC BEH C18 column (100 mm x 2.1 mm, 1.7 μ m particle size; Waters, Elstree, UK) and analysed using a Micromass (Manchester, UK) TOF-MS system operated in +ESI mode and –ESI mode to detect the metabolites in their protonated or deprotonated form. The injection volume was of 5 μ l and each sample was run using the acidified UPLC mobile phase 95:5% water-methanol (A) and methanol (B). The flow rate was 0.09 ml/min and the solvent gradient started at 50% B (for non-hydrolysed bile) and 30% B for (hydrolysed bile) up to 100% B in 12 min and maintained for 4 min before re-equilibration to the initial percentage of solvent B for 4 min. The ionisation parameters used for this study are described in section 2.7.2. In order to avoid any instrument bias, samples were run in a random order.

6.2.5. Data transformation, pre-processing and pre-treatment

Raw data obtained from the UPLC-MS were pre-processed using MarkerLynx V4.1 (Waters, Milford, USA). The chromatograms were aligned with the IS P-*d9* in +ESI mode and E₂-*d4*-S to correct the retention time between runs and all *m/z* x RT data sets were normalised to the maximum spectral area observed in each sample. The parameters used for the detection of the spectral peaks were optimised to reduce the background noise to a minimum and set as follows: mass window and mass tolerance 0.04 Da, average peak width at 5% height 20 sec, peak to peak baseline noise 15, number of masses per retention time (RT) 50, minimum intensity allowed for a spectral peak was 1% of the base peak intensity; retention time window 0.1 min. Data were then exported into SIMCA-P software (Umetrics, UK) where they were log-transformed and mean-centered to reduce skewness.

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6.2.6. Multivariate data analysis

Chromatographic data from the fish bile analysis were subjected to multivariate analysis. The unsupervised method principal component analysis (PCA) was conducted to provide an overview of the dataset. Any strong and moderate outliers identified in the PCA score plots were removed prior further analysis (as described in section 2.13.4). The datasets were then processed using the supervised techniques partial least square (PLS) discriminant analysis. This projection method is used to produce predictive models by correlating the variations in the metabolic profile (matrix X) with exposure concentration of diclofenac (matrix Y). The number of significant principal components was determined by cross-validation. The models with predictive ability (Q^2) above 0.4 were subjected to orthogonal projection to latent structure discriminant analysis (OPLS-DA) to identify the differential metabolites. To assess the accuracy of the models PLS models ($Q^2 > 0.4$) response permutation testing was performed. For each treatment, 5 test observations were excluded from the dataset, and the data remodelled to assess for correct classification of the test observations and validate the model. This procedure was repeated until 60% (arbitrary measure) of the observations were tested for correct classification.

6.2.7. Identification of metabolites

The discriminative variables (RT x m/z signal) of diclofenac exposure were selected from the S-plot of the OPLS models, a plot of contribution of variables vs. confidence where high confidence variables with large changes between the groups are found at the extreme ends of the “S”. Raw data of the selected metabolites were not normally distributed, thus they were statistically analysed using the non parametric test Kruskal-Wallis analysis of variance with Mann Whitney U post hoc test for pair-wise comparison of the control vs. each exposure concentration. Using this approach it is

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling advised to use a correction method (e.g. Bonferroni correction) to reduce the risk of type I errors (i.e. metabolites falsely identified as discriminating between 2 groups). Metabolites of statistical significance ($p < 0.01$) were subjected to Bonferroni correction.

Elemental composition of the discriminative variables was calculated using MassLynx V4.1, giving a deviation between the predicted and the experimental m/z , in ppm. Theoretical formula with the best isotopic fit and a deviation < 5 ppm were selected. This information, together with any fragmentation data from the TOF analysis of the metabolites in each SPE fraction was used to attempt an identification of the metabolites markers of diclofenac exposure. The following databases were used to search the structural identity of the bile metabolites: human metabolome (<http://www.hmdb.ca/>), KEGG ligand (<http://www.genome.jp/ligand/>), PubChem (<http://pubchem.ncbi.nlm.nih.gov/>), and BiGG (<http://bigg.ucsd.edu/>).

6.3. Results

6.3.1. PCA overview of the free metabolites and conjugated metabolites fractions from non-hydrolysed bile in control and diclofenac-exposed (25 $\mu\text{g/l}$) fish

Multivariate data analyses were performed to determine the changes in the bile metabolic profile of juvenile rainbow trout exposed to diclofenac. None of the PCA analyses for the non-hydrolysed bile datasets analysed in both the $-$ ESI mode and $+$ ESI modes gave good models as shown by the low values obtained for the observed (R^2X) and predicted (Q^2) variation in these datasets (Table 6.1). However some clustering of samples was observed in the free metabolites fraction (EA+FA) analysed in $+$ ESI mode (data not shown). The PCA score plots of the conjugated metabolites fraction (N) showed 4 outliers in $-$ ESI mode and 2 outliers in $+$ ESI mode. Visual examination of the spectra for these 6 samples revealed very high levels of bile salts such as taurocholic

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling acid (m/z 514). Therefore these samples were removed for subsequent analysis but the PCA analyses of the modified data did not improve the models.

6.3.2. Overview of PLS-DA and OPLS models for non-hydrolysed bile

Class separation was achieved at PLS-DA level between the control and diclofenac-exposed fish. The high values for the predicted variation ($R^2Y > 0.99$) and observed variation ($Q^2 > 0.8$) indicated that good models were produced for the free metabolites fraction analysed in both ionisation modes (Table 6.2). The clear class separation of the control vs. 25 $\mu\text{g/l}$ treatment groups is illustrated in the PLS-DA score plots in Figure 6.1. The analysis of the conjugated metabolites fraction did not result in tight clusters for both treatment groups. High values were estimated for the explained variation with $R^2Y = 0.944$ in +ESI mode and 0.996 in –ESI mode, but the predictive ability values (Q^2) were lower (< 0.75) than those reported in the free metabolites fraction. Response permutation testing was performed to assess the validity of the PLS-DA models. The models had a classification success of $\geq 80\%$ except for the conjugated metabolites fraction (55%) analysed in +ESI mode and it is possible that this PLS-DA model was overfitted (Table 6.2).

In order to further investigate the class separation between control and 25 μg diclofenac/l fish, OPLS-DA was conducted. The resulting “S” plot for the free metabolites fraction indicated 21 discriminative variables (m/z) in +ESI mode and 5 discriminative variables in –ESI mode. Only 7 discriminative variables were detected in the conjugated metabolites fraction analysed in –ESI mode and none in the same fraction analysed in +ESI mode.

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Table 6.1 – Performance parameters of principal component analyses for the comparison of the metabolic profiles in non-hydrolysed bile extracts.

Fraction	ESI mode	Groups	Multivariate method	Principal components	R²X	Q²
EA+FA	+	C, 25	PCA	2	0.252	0.087
EA+FA	-	C, 25	PCA	2	0.217	0.0375
N	+	C, 25	PCA	3	0.408	0.221
N	-	C,25	PCA	2	0.28	0.101

EA+FA: ethyl acetate and formic acid in methanol fraction (free metabolites include steroids and eicosanoids); N: ammonium hydroxide in methanol fraction (conjugated metabolites); ESI: electrospray ionisation; Groups C and 25 correspond to fish exposed to water control and 25 µg diclofenac/l; R²X: variation explained by the PCA models; Q²: cumulative variation predicted by the model.

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Table 6.2 – Multivariate discriminant models for non-hydrolysed bile samples of control and diclofenac-exposed rainbow trout.

Fraction	ESI mode	Groups	Multivariate method	Principal components	R ² X	R ² Y	Q ²	External validation
EA+FA	+	C, 25	PLS-DA	2	0.248	0.991	0.907	89%
EA+FA	+	C, 25	OPLS	1+1	0.248	0.991	0.846	
EA+FA	-	C, 25	PLS-DA	2	0.168	0.993	0.831	85%
EA+FA	-	C, 25	OPLS	1+1	0.168	0.993	0.806	
N	+	C, 25	PLS-DA	2	0.28	0.944	0.73	55%
N	+	C, 25	OPLS	1+2	0.325	0.99	0.41	
N	-	C, 25	PLS-DA	4	0.365	0.996	0.716	80%
N	-	C, 25	OPLS	1+1	0.31	0.985	0.585	

EA+FA: ethyl acetate and formic acid in methanol fraction (free metabolites and eicosanoids); N: ammonium hydroxide in methanol fraction (conjugated metabolites); ESI: electrospray ionisation; Groups C and 25 correspond to fish exposed to water control and 25 µg diclofenac/l; R²X and R²Y: variation explained by the PCA and PLS models respectively; Q²: cumulative variation predicted by the model.

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling

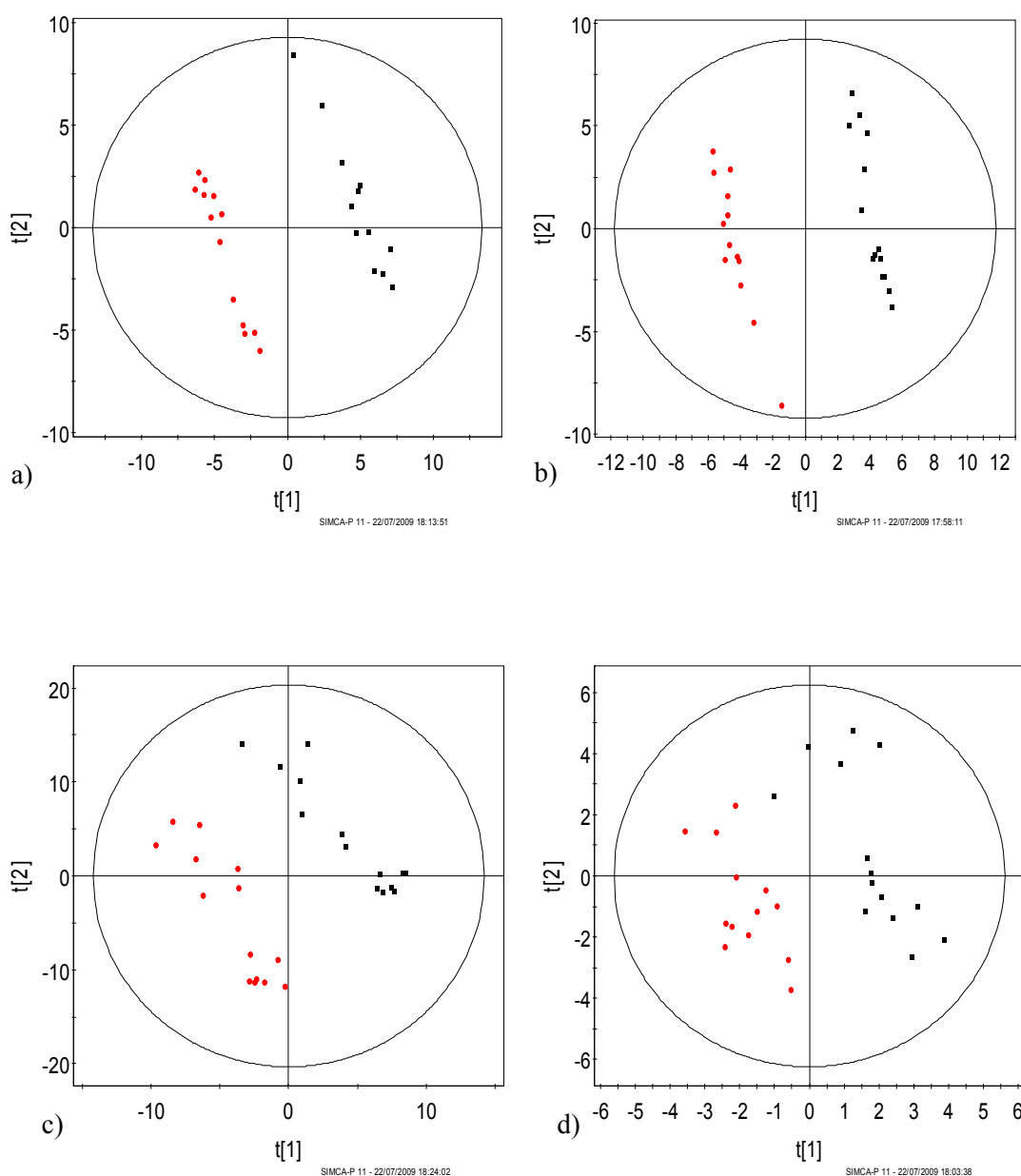


Figure 6.1 – PLS-DA score plots using the first 2 components of the non-hydrolysed bile fractions of control fish (■) and fish exposed to 25 µg diclofenac/l (●), n=16. Free metabolites fraction in +ESI mode (a) and –ESI mode (b); conjugated metabolites fraction in +ESI mode (c) and –ESI mode (d). Clustering indicate that many free metabolites and conjugates were affected by 21-day diclofenac exposure.

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6.3.3. PCA overview of the free metabolites (EA+FA) fraction from hydrolysed bile extracts.

As mentioned earlier, high levels of bile acids present in the conjugated metabolites fraction (N) may have interfered with the identification of many metabolites in this fraction, therefore hydrolysis was performed and the cleaved metabolites were eluted in the free metabolites fraction. Because of this UPLC-TOF-MS analysis was only carried out for the free metabolites fraction (EA+FA) of the hydrolysed bile samples. The PCA score plots obtained from the analysis of the 3 treatment groups (control, 0.5 and 5 µg/l) indicated a poor separation between the treatments in both ionization modes. This was shown by the low values for the explained (R^2X) variation (Table 6.3). Further PCA were performed on the control vs. 0.5 µg/l and control vs. 5 µg/l treatment groups. The models did not show any obvious clustering of the treatment groups. The values of the predicted variation (Q^2) were below 0.2 for all the PCA models of hydrolysed bile analysed which indicated that the models produced were of poor quality.

6.3.4. Overview of PLS-DA and OPLS models for hydrolysed bile extracts.

The supervised pattern recognition analysis (PLS-DA) of the hydrolysed bile samples depicted distinct clustering without any overlap in model space between the control samples and the samples from fish exposed to either 0.5 or 5 µg diclofenac/l (Figures 6.2). High values were obtained for the explained variation (R^2Y), however the predictive ability of the model was lower for the datasets analysed in –ESI mode ($Q^2=0.647$) compared with the dataset analysed in +ESI mode ($Q^2=0.858$) (Table 6.4). The UPLC-TOF-MS datasets were reanalysed using PLS-DA and OPLS to identify the class separating variables for just 2 of the treatment groups, i.e. the control vs. 0.5 µg/l groups

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Table 6.3 – Performance parameters of principal component analyses for the comparison of the metabolic profiles in hydrolysed bile extracts.

Groups	ESI mode	Multivariate method	Principal components	R²X	Q²
C, 0.5, 5	+	PCA	3	0.307	0.144
C, 0.5, 5	-	PCA	2	0.226	0.0701
C, 0.5	+	PCA	2	0.276	0.102
C, 0.5	-	PCA	2	0.287	0.0579
C, 5	+	PCA	2	0.256	0.101
C, 5	-	PCA	2	0.242	0.0401

Groups C, 0.5 and 5 correspond to fish exposed to water control, 0.5 and 5 µg diclofenac/l respectively; ESI: electrospray ionisation; R²X: variation explained by the model; Q²: cumulative variation predicted by the model.

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Table 6.4 – Multivariate discriminant models for the comparison of control and diclofenac exposed rainbow trout in hydrolysed bile.

Groups	ESI mode	Multivariate method	Principal components	R²X	R²Y	Q²	External validation
C, 0.5, 5	+	PLS-DA	5	0.372	0.987	0.858	80%
C, 0.5, 5	-	PLS-DA	6	0.38	0.988	0.647	83%
C, 0.5	+	PLS-DA	3	0.338	0.995	0.909	90%
C, 0.5	+	OPLS	1+1	0.248	0.983	0.771	-
C, 0.5	-	PLS-DA	4	0.369	0.996	0.663	76%
C, 0.5	-	OPLS	1+1	0.215	0.942	0.489	-
C, 5	+	PLS-DA	3	0.292	0.995	0.902	90%
C, 5	+	OPLS	1+1	0.23	0.975	0.823	-
C, 5	-	PLS-DA	2	0.228	0.968	0.79	100%
C, 5	-	OPLS	1+1	0.228	0.968	0.769	-

Groups C, 0.5 and 5 refer to fish exposed to water control, 0.5 and 5 µg diclofenac/l respectively; ESI: electrospray ionisation; R²X and R²Y: variation explained by the PCA and PLS models; Q²: cumulative variation predicted by the model.

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling

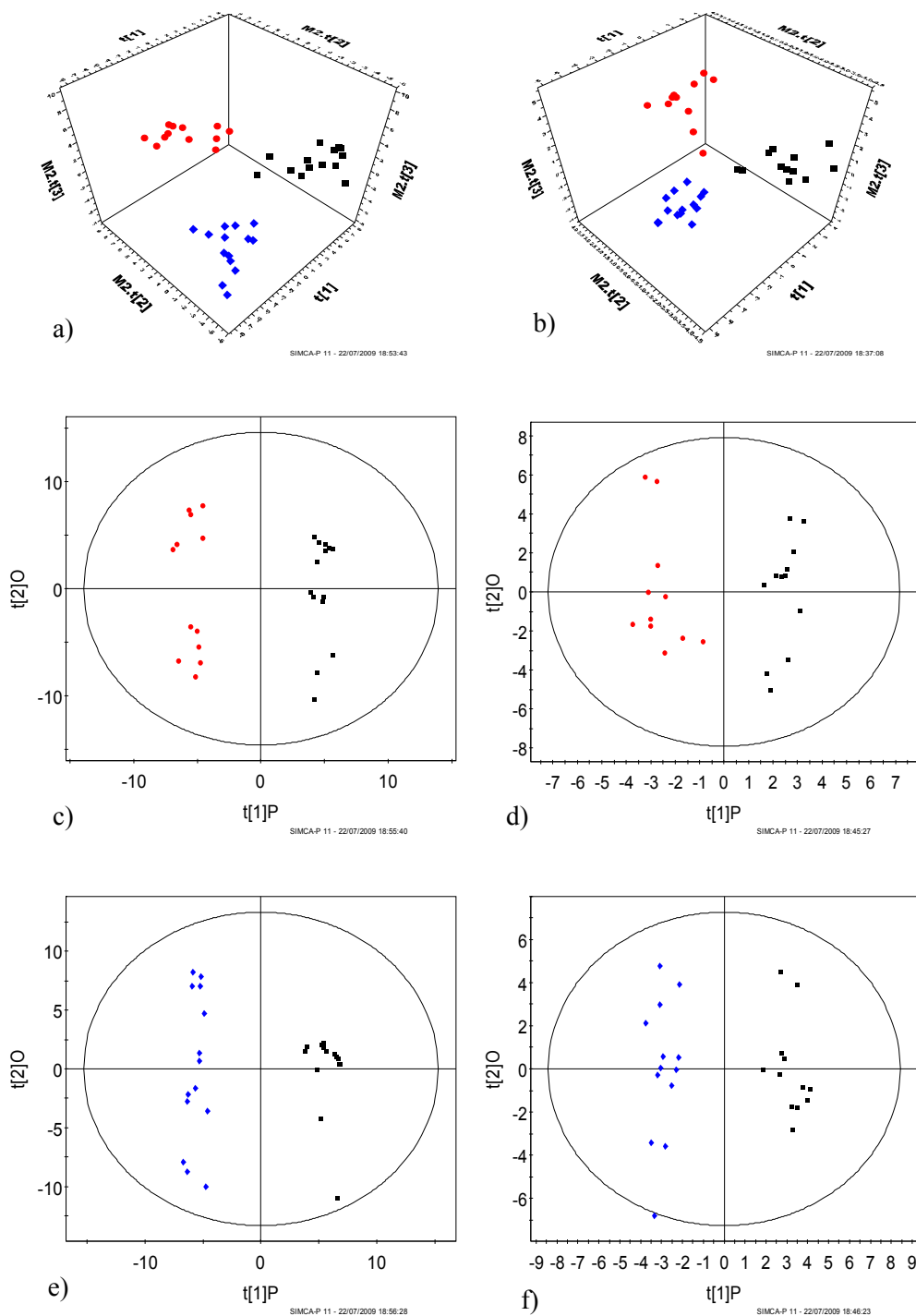


Figure 6.2 – Score plots of the multivariate discriminant analysis of the free metabolites (EA+FA) fraction from hydrolysed bile from 21-day diclofenac exposure. ■ control group; ● 0.5 µg diclofenac/l and ► 5 µg diclofenac/l. PLS-DA scores 3D plots of the 3 treatment groups analysed in +ESI mode (a) and –ESI mode (b); OPLS score plots for control vs. 0.5 µg/l in +ESI mode (c) and –ESI mode (d); OPLS score plots for control vs. 25 µg/l in +ESI mode (e) and –ESI mode (f). Clustering indicate that many free metabolites and conjugates were affected by 21-day diclofenac exposure.

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling and control vs. 5 µg/l groups. All the analyses resulted in good models as indicated by the explained (R^2Y) and predicted variation (Q^2) (Table 6.4). The analyses performed in –ESI mode for the control and 0.5 µg/l groups provided the weakest models and this was demonstrated by the lower predictive values in the PLS-DA ($Q^2= 0.663$) and OPLS ($Q^2= 0.489$) models. The classification success determined from the external validation testing varied from 76-100%. In the S-plot of the free metabolites fractions analysed in +ESI mode, 86 discriminative variables were found and in the same fractions analysed in –ESI mode, a total of 28 discriminative variables were selected for further analysis.

6.3.5. Identity of metabolites of diclofenac

The loading plots (S-plot) of the OPLS models are used to describe the relationship between the variables of the control fish and those of the diclofenac-exposed fish, thus highlighting the metabolites responsible for the class separation. The variables significantly elevated or reduced by diclofenac exposure are found on opposites sides of this plot (Figure 6.3) and represent the metabolites in a positively charged, [M+H] negatively charged [M-H] and/or sodium adduct [M+Na] form.

Amongst the variables selected in the S-plots, the ion 294.008 [M-H] and 296.025 [M+H] was detected in the free metabolites fraction of the fish exposed to diclofenac, from the lowest exposure concentration of 0.5 µg/l. This variable was identified as diclofenac using the elemental composition tool and comparison of retention time on UPLC with that of an authentic standard. Three potential metabolites of diclofenac were also found in the bile of exposed rainbow trout. Their putative chemical formula and structural identity is shown in Table 6.5. Figure 6.4 represents the ion chromatograms of diclofenac and hydroxydiclofenac, methyl ester metabolite and hydroxydiclofenac glucuronide in fish bile. Like diclofenac, the hydroxy-metabolite was detected as a protonated [M+H] and a deprotonated [M-H] ion in bile

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling

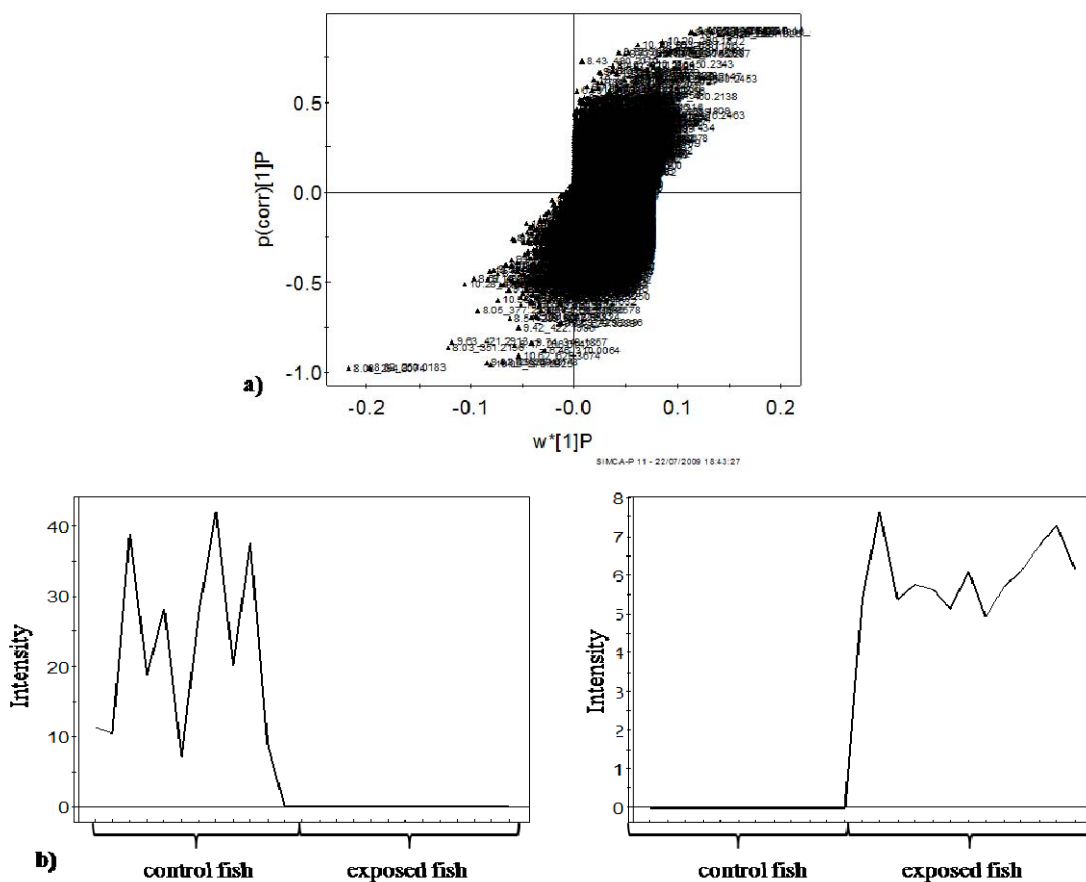


Figure 6.3 – a) S-plot from the OPLS analysis of hydrolysed bile of control and 5 μg diclofenac/l exposed fish in the free metabolites fraction analysed in $-$ ESI mode. b) trend view of some of the markers of diclofenac exposure identified by OPLS analysis of control and 5 μg /l exposed trout.

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling

Table 6.5 – Identification of diclofenac and putative metabolites.

<i>m/z</i>	Fraction/ESI	RT	Putative formula	ppm	Putative identity
294.008	EA+FA/-ESI	8.03	C ₁₄ H ₁₀ NO ₂ Cl ₂	0	Diclofenac
250.018	EA+FA/-ESI	8.03			fragment of 294: loss of CO ₂
298.004	EA+FA/-ESI	8.03			Cl isotope of 294
310.003	EA+FA/-ESI	4.39	C ₁₄ H ₁₀ NO ₃ Cl ₂	-0.9	Hydroxydiclofenac
230.038	EA+FA/-ESI	4.39			fragment of 310: loss of HCl
266.014	EA+FA/-ESI	4.39	C ₁₃ H ₁₀ NOCl ₂	0	fragment of 310: loss of CO ₂
314.000	EA+FA/-ESI	4.39			Cl isotope of 310
331.993	EA+FA/-ESI	4.38	C ₁₄ H ₁₀ NO ₃ Cl ₂ Na	3.9	sodium adduct of 310
310.0402	EA+FA/+ESI	9.29	C ₁₅ H ₁₄ NO ₂ Cl ₂	0.6	Methyl ester metabolite
486.032	N/-ESI	1.89	C ₂₀ H ₁₈ NO ₉ Cl ₂	1.3	Hydroxydiclofenac glucuronide

m/z: mass to charge ratio; EA+FA: free metabolites fraction; N: conjugated metabolites fraction; ESI: electrospray ionization; RT: retention time; ppm: part per million. Highlighted in grey are the compounds and in white the fragments formed by the UPLC-TO-MS.

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling from the lowest exposure concentration (0.5 µg/l). Mass spectral analysis indicated that the ions 250.03, 266.01 and 331.99 detected in –ESI mode are fragments and sodium adduct of the hydroxy-metabolite formed as a result of the UPLC-TOF-MS analysis (Figure 6.5). The methyl ester metabolite found as a protonated ion and the hydroxylated glucuronide metabolite found as a deprotonated ion, were only detected in the non-hydrolysed bile samples of fish exposed to 25 µg/l.

6.3.6. Identity of metabolite markers of diclofenac exposure

The variables (RT x *m/z* signals) representing diclofenac and the putative metabolites were removed from the models in order to detect metabolites of endogenous origin whose concentrations were affected by diclofenac exposure. The most discriminative variables between control and diclofenac-exposed trout are summarised in Table 6.6. Non parametric statistical analysis was conducted on the original (normalised but skewed) UPLC-TOF-MS data to confirm that all the variables selected in the OPLS loading plots were indeed altered by diclofenac exposure. In the different fractions of non-hydrolysed and hydrolysed bile, a total of 60 metabolites passed the Bonferroni correction analysis. The elemental composition tool was used on the mass spectra of these potential metabolites markers in order to assign a theoretical chemical formula (deviation <5 ppm). The elucidation of the structural identification of the metabolites using the UPLC-TOF-MS data (*m/z*) was not possible due to the lack of positive matches in any of the available databases. Any information collected on the metabolites (e.g. fragments and adducts formed) were recorded to facilitate their identification. Data will be analysed by FT-MS to give more accurate mass measurements which will reduce the number of candidate formulas for the metabolites. Due to time limitations these data are not yet available.

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling

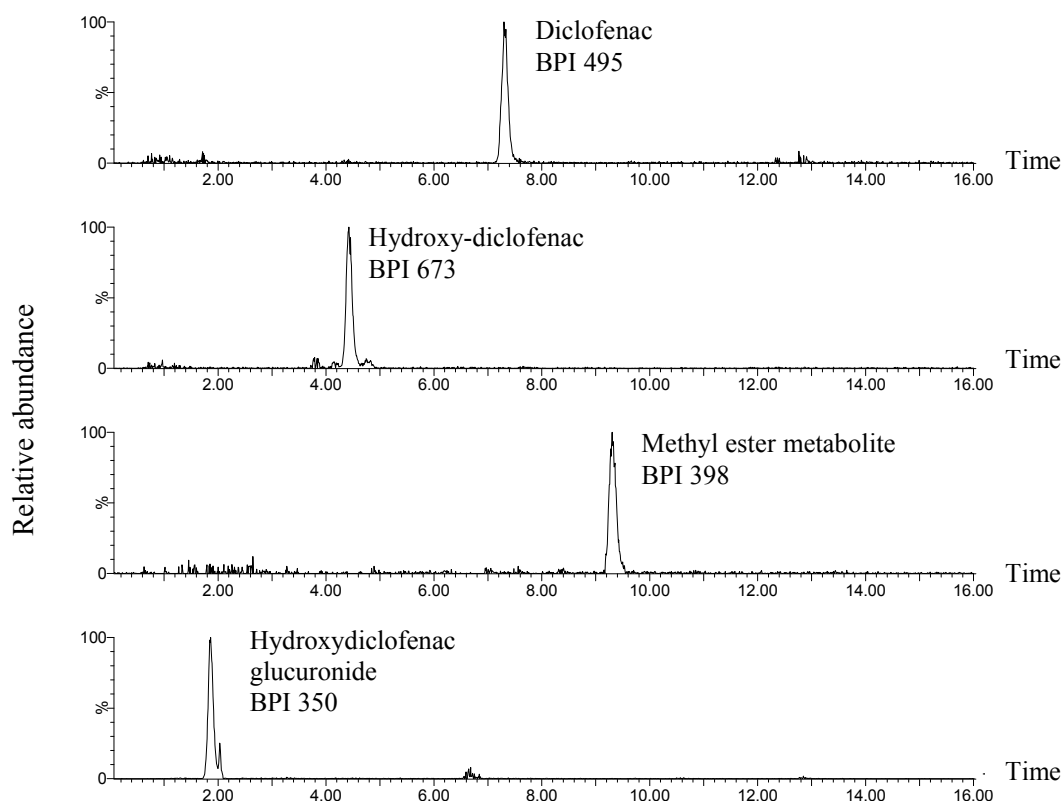


Figure 6.4 – Total ion chromatograms showing diclofenac and its metabolites in rainbow trout after 21-day exposure to diclofenac.

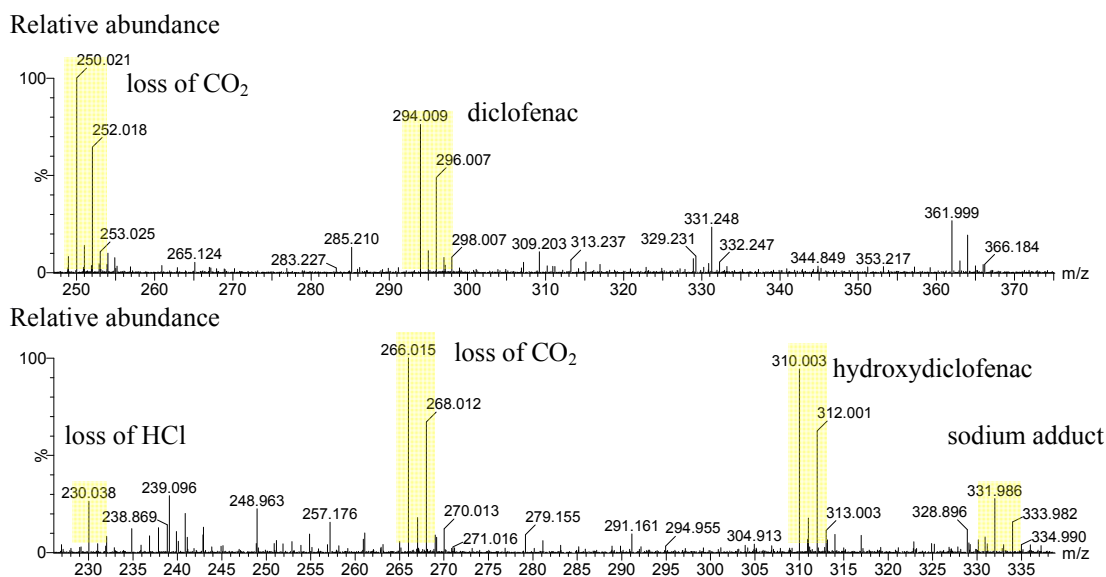


Figure 6.5 – Spectral ion chromatograms of diclofenac and hydroxydiclofenac fragments produced by UPLC-TOF-MS analysis.

Table 6.6a – Markers of diclofenac exposure identified in non-hydrolysed bile of rainbow trout.

Fraction/ ESI mode	Marker ion <i>m/z</i>	RT	Putative formula of ion	Theoretical mass	pp m	Effect of 25 µg DCF/l	p value	Putative compound/ comments
EA+FA/-ESI	287.193	2.79	C ₁₄ H ₂₇ N ₂ O ₄		-2.4	↑	0.000*	
EA+FA/-ESI	333.206	9.31	C ₂₀ H ₂₉ O ₄	333.2066	0	↑	0.000*	Lipoxygenase product
N/-ESI	171.014	1.01				↑	0.000*	
EA+FA/+ESI	402.197	6.38				↓	0.0005	
EA+FA/+ESI	414.994	2.52				↑	0.000*	
EA+FA/+ESI	573.298	12.70				↓	0.0005	
EA+FA/+ESI	627.309	8.92				↑	0.000*	Na adduct 649.279
EA+FA/+ESI	657.341	9.10				↑	0.0005	Na adduct 679.314
EA+FA/+ESI	685.326	10.09				↑	0.000*	Na adduct 707.296
EA+FA/+ESI	689.119	0.91				↓	0.000*	
EA+FA/+ESI	715.360	10.20				↑	0.000*	Na adduct 737.330
EA+FA/+ESI	743.342	11.13				↑	0.000*	Na adduct 765.313
EA+FA/+ESI	747.129	1.03				↑	0.000*	
EA+FA/+ESI	773.375	11.20				↑	0.00005	Na adduct 795.346
EA+FA/+ESI	831.391	12.01				↑	0.0005	Na adduct 853.360
EA+FA/+ESI	889.405	12.49				↑	0.0005	Na adduct 911.376
EA+FA/+ESI	917.385	12.73				↑	0.0004	
EA+FA/+ESI	975.400	12.96				↑	0.0005	Na adduct 997.371

Table 6.6b – Markers of diclofenac exposure identified in hydrolysed bile of rainbow trout.

ESI mode	Marker ion <i>m/z</i>	RT	Putative formula of ion	Theoretical mass	ppm	Effect of DCF /p value		Putative compound/ comments
						0.5 µg/l	5 µg/l	
-ESI	379.284	10.05	C ₂₃ H ₃₉ O ₉	379.2848	-1.1		↑ 0.000*	Glycerolipid?
-ESI	392.149	8.56					↓ 0.000*	loss of gluthamyl 263.116 2x 392 ion 785.308
-ESI	394.166	9.10					↓ 0.000*	loss of gluthamyl 265.129
-ESI	398.192	10.20					↓ 0.000*	loss of gluthamyl 269.157
-ESI	420.180	9.60					↓ 0.000*	loss of gluthamyl 291.141 2x420 ion 841.365
-ESI	442.223	8.11	C ₂₅ H ₃₂ NO ₆		0.2	↓ 0.0001	↓ 0.000*	?
-ESI	478.226	9.73					↑ 0.000*	loss of gluthamyl 349.185
-ESI	799.320	8.58					↓ 0.000*	gluthamate group?
-ESI	805.379	9.59					↓ 0.000*	
+ESI	320.235	11.68				↓ 0.0004		
+ESI	341.245	9.00				↑ 0.00003	↑ 0.000*	
+ESI	355.293	8.20					↓ 0.000*	
+ESI	457.353	10.20				↓ 0.0005	↑ 0.000*	endogenous?
+ESI	532.379	10.89					↑ 0.0004	

Table 6.6b continued

ESI mode	Marker ion <i>m/z</i>	RT	Putative formula of ion	Theoretical mass	ppm	Effect of DCF (p value)		Putative compound/ comments
						0.5 µg/l	5 µg/l	
+ESI	561.402	11.09				↓ 0.000*	↓ 0.000*	
+ESI	590.423	11.30				↓ 0.000*	↓ 0.000*	
+ESI	619.443	11.49				↓ 0.000*	↓ 0.000*	fragment 262.194
+ESI	621.409	8.12				↓ 0.000*		
+ESI	623.251	1.23				↓ 0.000*	↑ 0.000*	
+ESI	629.291	8.80				↓ 0.000*		fragment 317.156
+ESI	641.443	11.50				↓ 0.000*		
+ESI	659.388	10.10					↑ 0.0004	
+ESI	667.976	11.63				↓ 0.000*		
+ESI	679.449	8.68				↓ 0.000*		
+ESI	696.991	11.80				↓ 0.000*		
+ESI	715.509	9.09					↓ 0.00005	
+ESI	743.498	9.47				↓ 0.000*		
+ESI	747.300	1.48				↓ 0.000*	↓ 0.000*	
+ESI	765.486	9.47					↓ 0.000*	
+ESI	771.508	12.23				↓ 0.000*		

Table 6.6b continued

ESI mode	Marker ion <i>m/z</i>	RT	Putative formula of ion	Theoretical mass	ppm	Effect of DCF (p value)		Putative compound/ comments
						0.5 µg/l	5 µg/l	
+ESI	787.401	9.10					↓ 0.000*	fragment 737.491
+ESI	795.533	9.49				↓ 0.000*	↓ 0.000*	phospholipid?
+ESI	800.524	12.36				↓ 0.000*		phospholipid?
-ESI	823.528	9.80					↓ 0.000*	phospholipid?
-ESI	853.573	9.81				↓ 0.000*	↓ 0.000*	phospholipid?
-ESI	859.586	10.10				↓ 0.000*	↓ 0.000*	phospholipid?
-ESI	881.566	10.10				↓ 0.000*		fragment 829.538
-ESI	911.611	10.11				↓ 0.000*	↓ 0.000*	phospholipid?
-ESI	917.624	10.39					↓ 0.000*	phospholipid?
-ESI	935.691	10.19				↓ 0.000*		phospholipid?
-ESI	939.605	10.40				↓ 0.000*	↓ 0.000*	phospholipid?
-ESI	945.697	8.85				↓ 0.000*	↑ 0.000*	endogenous?

EA+FA: free steroid fraction; N: conjugated steroid fraction; ESI: electrospray ionization, *m/z*: mass to charge ratio; RT: retention time, ppm: part per million,

DCF: diclofenac

*p value below 1×10^{-6}

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling

Putative identities were obtained for only 2 markers, but FT-MS analysis is required for confirmation. The variable m/z 333.206 [M-H] found in non-hydrolysed bile was increased in fish exposed the highest concentration of diclofenac (25 $\mu\text{g/l}$). The putative chemical formula indicates that this ion may be an eicosanoid, possibly a lipoxygenase product. The variable m/z 379.284 [M-H] found in the hydrolysed bile was identified as a potential glycerolipid. The concentration of this metabolite was elevated in fish exposed to 5 μg diclofenac/l. In the hydrolysed bile analysed in –ESI mode, the metabolite marker m/z 442.223 was suppressed by diclofenac exposure from 0.5 $\mu\text{g/l}$. A theoretical mass was obtained using the elemental tool composition (<0.2 ppm) but no structural identity has been found yet.

6.4. Discussion

Metabolomics enables the study of all the metabolites present in a biological system (Moco et al., 2007). Hence, the characterisation of the primary and secondary endogenous metabolites can provide valuable insights in the cellular processes and their responses to pollutants. Nuclear magnetic resonance (NMR)-based and mass spectrometry (MS)-based systems are the most commonly analytical platforms in metabolomics. The development of ultra-performance liquid chromatography (UPLC)-MS has provided a high-resolution and high-mass accuracy technique to extract and identify metabolites (Moco et al., 2007). The main pitfall of MS techniques is the existence of matrix effects such as ion suppression that affect the detection of low abundance molecules (Antignac et al., 2005). Bile contains high levels of bile acids that can mask the presence of other metabolites by ion suppression and suitable techniques have to be used to detect the lower abundance signalling molecules. As reported in Chapter 5, SPE fractionation allowed the separation of conjugated metabolites (e.g.

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling (taurocholic acids) from other free metabolites (including fatty acids, eicosanoids, phospholipids, etc.). Pattern recognition techniques used to analyse non-hydrolysed bile samples resulted in poor models for the conjugated metabolites fraction and very few discriminative metabolites were detected due to interferences with taurocholic acid. Conjugation of endogenous and exogenous metabolites is a major process in detoxification (Bailey and Dickinson, 2003). During phase II reactions, glucuronic acid, sulphate and/or glutathione are conjugated onto pharmaceuticals in order to increase the solubility and facilitate renal excretion. Glucuronic acid also binds to fatty acids and bile acids by a glycosidic bond in the liver and these conjugates are transported to the bile before excretion from the body. In order to overcome the matrix effects in the conjugated fraction, fish bile was hydrolysed using glucuronidase and sulphatase enzymes, which aim to deconjugate the metabolites, linked to glucuronic acid and sulphate molecules. Previous studies have reported the benefits of hydrolysis to investigate the bile metabolome (Smith and Hill, 2004; Gibson et al., 2005).

The metabolomics datasets obtained from the UPLC-TOF-MS analysis had more variables (i.e. metabolites) than observations (i.e. samples), thus multivariate data analyses (PCA, PLS-DA and OPLS) were employed as graphical and statistical tools to extract meaningful information from the multi-dimensional datasets (Eriksson, 2006). The unsupervised projection method PCA was applied to visualise the general trend of the datasets without losing any important information. PCA models for our datasets did not show clustering tendency and this was confirmed by the low values (R^2X and Q^2) obtained by cross-validation. It may be that the low abundance of metabolites relating to diclofenac exposure and the presence of high abundance metabolites (e.g. bile acids) resulted in highly variable datasets. Research has shown that such complex data can reduce the performance of PCA and this analysis should be followed by a supervised analysis such as PLS-DA (Oldiges et al., 2007). PLS-DA carried out in this study

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling clearly improved the separation between the treatment groups. The main problem of a supervised projection method is the tendency to overfit the models (i.e. poorly represent the datasets). It has been reported that adequate cross-validation and external validation can overcome this problem (Broadhurst and Kell, 2006). The PLS-DA models for non-hydrolysed and hydrolysed bile were validated with more than 80% accuracy in the classification of treatment groups and OPLS analysis was able to discriminate the metabolites responsible for the class separation. The multivariate analysis revealed that exposure to diclofenac at concentrations as low as 0.5 $\mu\text{g/l}$ interfered with the metabolism of rainbow trout and many metabolites were found to be altered after the 21-day exposure. Bonferroni correction was applied to reduce the risk of type I errors (false discoveries). The drawback of this correction method is that true metabolites markers of exposure and effect may not pass the Bonferroni corrected levels leading to the inflation of type II errors (Broadhurst and Kell, 2006).

Bile is a major route for the excretion of diclofenac in mammals and the detection of this compound in all the exposed fish confirm the uptake of diclofenac from the water. Our findings are supported by previous studies that reported the bioaccumulation of diclofenac in fish plasma and bile following the exposure to environmentally relevant concentrations (see Mehinto et al. Chapter 4; Brown et al., 2007). Research in mammals has demonstrated that up to 5% of diclofenac entering the body is excreted as phase I metabolites produced by hydroxylation and methylation (Bort et al., 1999; Jjemba, 2006). Phase I metabolites were putatively identified as hydroxydiclofenac detected from 0.5 $\mu\text{g/l}$ exposure concentration and methyl ester metabolite detected at 25 $\mu\text{g/l}$ exposure concentration. Phase II metabolites, glucuronide diclofenac and glutathione diclofenac have also been identified in mammals and the bioactivation of these conjugates has been implicated in diclofenac-induced toxicity effects (Tang et al., 1999; Boelsterli, 2003). It is thought that the conjugated metabolites

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling

bind covalently to proteins and form diclofenac conjugate-protein adducts that are recognized by the immune system as foreign, leading to an immune response and toxic damage mainly in the liver (Grillo et al., 2003). The glucuronide and glutathione conjugates of diclofenac were not detected in the bile of diclofenac-exposed trout, but they may have been masked by taurocholic acid present in the conjugated metabolites fraction. However, the hydroxylated form of the glucuronide metabolite was detected in the conjugated fraction of non-hydrolysed bile in fish exposed to 25 µg/l. The presence of diclofenac and its reactive metabolites in bile has been recognized as an important factor in intestinal injuries observed in patients treated with the pharmaceutical (Treinen-Moslen and Kanz, 2006). The identification of diclofenac and a possible glucuronide metabolite could explain the morphological damages found in the small intestine of rainbow trout exposed to environmentally relevant concentrations (see section 4.3.5). The deleterious effects of diclofenac and its metabolites have been previously demonstrated by Hoeger et al. (2008). They observed that diclofenac and the metabolites accumulated in brown trout (*Salmo trutta*) and entered the enterohepatic circulation, resulting in a prolonged availability in the bile which may cause intestinal injuries.

This metabolomics study has revealed that many bile metabolites were affected by diclofenac exposure. Due to time restrictions, the metabolites have not yet been identified but research is undergoing using FT-MS in an attempt to do so. Metabolomics approaches generate large amounts of data and elucidation of the structure and name of the endogenous metabolites is often the most difficult and time consuming task (Goodacre et al., 2004). The databases available for metabolite recognition are presently predominated with metabolites of humans and other mammalian systems. Thus it is perhaps not surprising that the resulting putative molecular weights for the metabolites markers discovered in fish bile did not match the molecular weights of any metabolites

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling in these databases. A preliminary examination of the data indicated that more metabolites were reduced by diclofenac exposure than those induced and many of them seem to be phospholipids. This is surprising as phospholipids are converted into fatty acids and the inhibition of prostanoids synthesis would suggest an increase of arachidonic acid and phospholipids. The effects of diclofenac on phospholipids biosynthesis have not been previously reported and a definite identity for the phospholipids-like metabolites could provide further insight on the mode of action of diclofenac in fish. One variable (m/z 442.223) was found to be completely suppressed even at an exposure concentration of only 0.5 μg diclofenac/l and this may potentially be an effective physiological metabolite marker of diclofenac exposure.

Among the metabolites elevated as a result of diclofenac exposure, the variable m/z 333.206 was putatively identified as a lipoxygenase product in the non-hydrolysed bile fraction. This metabolite showed a significant increase in the 25 $\mu\text{g}/\text{l}$ treatment group compared with the controls but it was not discriminatory in the hydrolysed bile of trout exposed to 0.5 and 5 $\mu\text{g}/\text{l}$. Few data exist on the metabolic pathways affected by diclofenac in fish to make any meaningful conclusions on these observations. Most studies on diclofenac have used traditional endpoints such as toxicity testing (EC_{50}), histopathology or target gene expression to evaluate the effects of exposure (Hallare et al., 2004; Schwaiger et al., 2004; Triebskorn et al., 2004; Hoeger et al., 2005; Hong et al., 2007). The observations here on the metabolome of exposed rainbow trout compare favourably with these other studies (see Chapter 4). Indeed, we reported earlier that diclofenac can significantly inhibit the *cox* activity in the liver, gills and kidney of rainbow trout. Arachidonic acid serves as a substrate for the synthesis of other eicosanoids (Figure 6.5) and the inhibition of prostanoids synthesis pathway may lead to an enhanced activity of the lipoxygenase and epooxygenase enzymes. This may explain the increase of this putative lipoxygenase product.

6. Identifying the biological effects of diclofenac in fish using metabolomics profiling

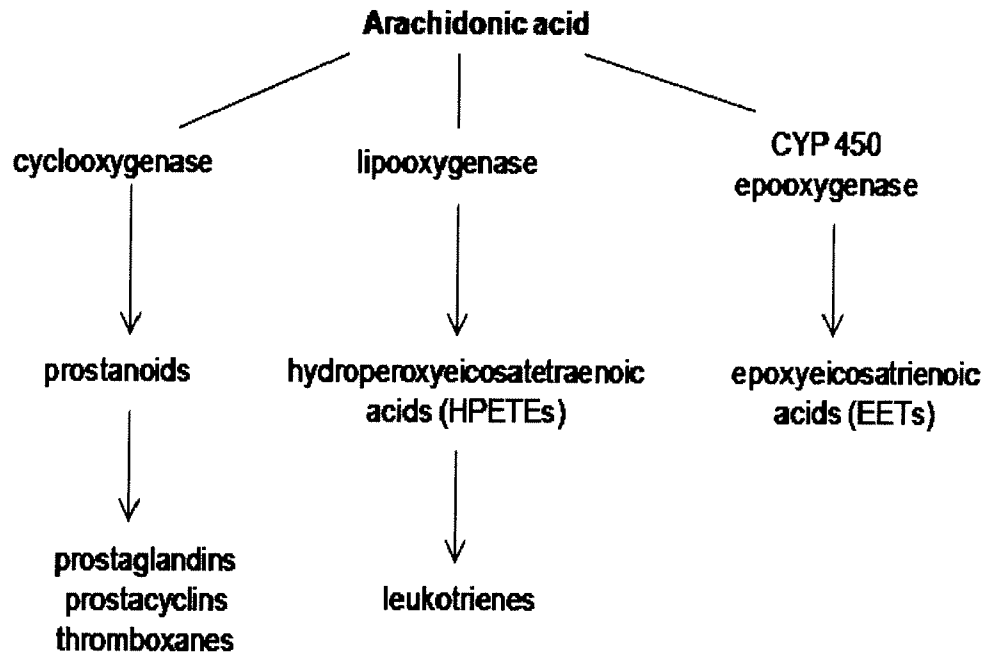


Figure 6.6 – Conversion of arachidonic acid into eicosanoids.

5. Conclusions

The present study has demonstrated that the exposure to diclofenac via the water lead to significant changes in the metabolic profile of bile in rainbow trout. This is of environmental significance as our lowest exposure concentration (0.5 µg/l) is within the range of concentrations detected in WWTP effluents (up to 1 µg/l) and surface waters in the UK (up to 0.2 µg/l) (Hilton and Thomas, 2003; Ashton et al., 2004; Kasprzyk-Hordern et al., 2008). Future research should focus on the disruption of the fish metabolome at lower concentrations. It has also been shown that a metabolomics approach is a useful approach for identifying metabolites of the test compound. The putative identification of hydroxydiclofenac and hydroxydiclofenac glucuronide suggests that diclofenac metabolism in fish is the same, or at least similar, to that occurring in mammals. However, the glutathione metabolite, another key conjugate of diclofenac was not detected and was possibly masked by taurocholic acid. Analytical methods need to be developed to detect these reactive metabolites in bile, especially at the lowest exposure concentration in order to determine any correlations with the pathology observed in the small intestine. Although most of the endogenous metabolites affected are, as yet, unidentified, this study further demonstrates the potential of metabolomics for the discovery of new biomarkers of chemical exposure and potentially provides valuable information on the genes or tissues affected by the exposure. The significant increase in the putative lipoxygenase product in exposed fish suggests that diclofenac not only inhibits prostanoids synthesis but may also interfere with the synthesis of other classes of eicosanoids affecting other physiological processes.

CHAPTER 7: General Discussion

In 2007, the global consumption of diclofenac was estimated at 877 tons per year (Zhang et al., 2008). The recent availability of diclofenac over the counter is likely to cause an increase in the amounts used and may result in higher concentrations in UK aquatic environments. There has been a series of studies indicating that diclofenac can induce harm in wildlife and this thesis sets out to determine the fate of diclofenac and its interactions with biological systems in the aquatic environment through the use of microbiology, molecular and metabolomics techniques.

The studies undertaken on biodegradation of diclofenac demonstrated that it was highly resistant to degradation by microbes in the activated sludge and the receiving waters (section 3.3). Previous enrichments studies with pharmaceuticals as sole carbon source have reported similarly low biodegradation rates for diclofenac (Quintana et al., 2005; Yu et al., 2006). Enrichment experiments, however, are based on the assumption that microbial populations can be sustained with only one carbon source, whereas in reality pharmaceuticals degradation often occurs through co-metabolism involving more than one microbial species and carbon source. Specific microbes degrade partially the parent compound and the resulting products are further degraded by other types of microbes. Thus other carbon sources are required to sustain the growth and diversity of different microbial populations that collectively allow for the degradation process of diclofenac to occur. In essence, therefore enrichment studies employing a single chemical (here diclofenac) as a sole carbon source do not necessarily reflect degradation activities that occur in natural environments. A further consideration in the biodegradation studies undertaken is that they were designed to promote the growth of bacteria, but other microorganisms may be involved in the degradation process (e.g.

fungi, rotifers). Many bacteria that play vital roles in biodegradation in natural environments have been found to be not culturable under laboratory conditions (Dias and Bhat, 1964), contributing further to the loss of microbial diversity and degradation potential in the cultures. The composition of the culture media can also influence the microbial growth. Minimal salts media (MSM) employed in this study had been previously optimised to culture and isolate bacteria from soil samples (Tett et al., 1994) and the nutrients requirements may be different for aquatic microbes, thus this medium may not have been optimal for the microbes of interest.

The removal of diclofenac in WWTPs has been estimated between 17 and 80%, with most studies reporting degradation below 40% in the activated sludge (Ternes, 1998; Stumpf et al., 1999; Lindqvist et al., 2005; Zorita et al., 2009). It is thought that biodegradation occurs predominantly in the aerobic part of the activated sludge, however adopting aerobic conditions for the enrichments resulted in less than 20% of the initial concentration of diclofenac being removed over a 30-40 days period and these results were supported by the putative degradation pathway of diclofenac under aerobic conditions (section 3.2.9). This indicates that diclofenac may not be readily degraded under aerobic conditions and that anaerobic biodegradation may account for the removal of pharmaceuticals. Previous studies on the biodegradation of pharmaceuticals in WWTPs have reported up to 35% of diclofenac removed by anaerobic degradation but only 10% by aerobic degradation (Zwiener and Frimmel, 2003). Other factors too, such as pH, can affect the diversity of the microbial populations and degradation rates. Urase and Kikuta (2005) have reported that the degradation rate of NSAIDs increased under acidic conditions. All of these factors illustrate the difficulties in mimicking accurately the conditions in the environment and in maintaining the microbial diversity that has often been reported to produce the highest degradation potential. Therefore

extrapolating between the biodegradation of environmental pollutants under laboratory conditions and what may occur in the natural environment must be done with care.

More advanced biodegradation studies are now using laboratory-scale fixed-bed bioreactors and membrane bioreactors, which imitate the tanks in WWTPs. Contrasting with enrichment studies, the microbes are cultured in their original medium and the dynamics of the microbial populations are less affected. Using these techniques, the degradation of diclofenac has been observed up to 60% (Gonzalez et al., 2006; Kraigher et al., 2008; Kosjek et al., 2009). Although the degradation rates are improved, laboratory-scale bioreactors, however, are not ideal for isolating the microbes actively degrading the pharmaceuticals. Formation of biofilms in those bioreactors is believed to be important for the degradation process (Zwiener and Frimmel, 2003; Gröning et al., 2007). Because the microbes in biofilms are protected from external stressors, co-metabolism and gene transfer are facilitated, thus enhancing the degradation potential of the microbial populations. Biofilms can be reproduced in laboratory and may be a more efficient way to culture complex degrading microbial populations.

The observations on microbial growth during the biodegradation study showed that diclofenac can be toxic effects to those microbial populations. In a standardised bioassay with *Vibrio fischeri* it was indeed confirmed that diclofenac was the most toxic NSAID tested ($EC_{50} \geq 10$) (section 3.3.4), concurring with previous research (Farré et al., 2001; Cleuvers, 2003). Although standardised acute toxicity assays have proven very useful for establishing environmental risk assessments for pharmaceuticals, the test organisms used are not necessarily found in the ecosystems where these pharmaceuticals are most prevalent. *Vibrio fischeri*, for example, is a marine species and although diclofenac has been reported in seawater, the highest concentrations are found in freshwater environments (Kümmerer, 2009). This argues for more studies on

the toxic effects of diclofenac on organisms occurring in the activated sludge and freshwater environments. Other acute toxicity tests using freshwater invertebrates and algae have reported EC_{50} for diclofenac well below 10 mg/l (Ferrari et al., 2003). Nevertheless, in this thesis, studies demonstrated that both some Gram positive and Gram negative bacteria from the activated sludge had a high tolerance for diclofenac (section 3.3.5). Minimum effective concentrations for growth inhibition ranged between 50 mg/l for *Zoogloea ramigera* and 90 mg/l for *Pseudomonas putida*, and illustrate that environmental concentrations of diclofenac appear harmless to some microbes. Molecular analysis of mixed microbial communities exposed to diclofenac provides a better insight into the effects of the pharmaceutical on the dynamics within the microbial communities in the environment. Kraigher et al. (2008) reported that exposure to NSAIDs from 50 μ g/l can indeed affect the diversity of the microbial communities in the activated sludge and reduce the growth of key microorganisms for degradation processes.

The apparent recalcitrance and microtoxicity of diclofenac suggest that this compound has the potential to accumulate in aquatic organisms with an enhanced likelihood to cause deleterious effects. Exposure and effect studies in rainbow trout confirmed this assumption, with altered expression of genes playing key roles in xenobiotic metabolism (*cyp1a1*), eicosanoids synthesis (*cox1* and *cox2*) and cell repair (*p53*) as well as histopathological lesions in the gills, kidney, liver and small intestine (Chapter 4). Diclofenac residues were found in the bile of exposed rainbow trout showing both its metabolism and active excretion from the body. This was supported by the increase expression of *cyp1a1* in the liver and gills of diclofenac-exposed fish, as reported previously (Hong et al., 2007). The bioconcentration of diclofenac measured in the bile (BC 509-657), however, indicates that it is not degraded completely and is bioavailable in fish tissues and biofluids. Research in mammals, and more recently in

fish, has demonstrated that diclofenac present in the bile can enter the enterohepatic circulation instead of being evacuated via the urine (Boelsterli, 2003; Hoeger et al., 2008). In the kidney of exposed rainbow trout, the significant reduction in *cyp11a1* expression implies that diclofenac bioaccumulated in this tissue (although this was not directly measured) and this may explain the nephrotoxicity observed in this tissue (see Figure 4.4a). A metabolomics analysis of fish urine may provide better insights into the physiological functions altered by diclofenac exposure and help to establish a link between the pathology observed and the functions impaired in the kidney.

Diclofenac is a non selective cyclooxygenase (*cox*) inhibitor and as such it can suppress the expression of both *cox* isoforms in mammals. Although *cox* genes are highly conserved in aquatic vertebrates, there is no previous work reporting the effects of diclofenac on *cox* gene expression in non mammalian organisms. Here we showed that sub-chronic exposure (21 days) to environmentally relevant concentrations of diclofenac resulted in a significant reduction of both *cox1* and *cox2* expression in the liver, gills and kidney (section 4.3.4). Histopathological examinations also indicated that exposure to diclofenac from 0.5 µg/l caused damages in the kidney and small intestine. This indicates that pharmaceuticals released in the environment remain bioactive and long term exposure may lead to a complete inhibition of *cox* expression resulting in severe morphological damages and functional impairment, and ultimately affecting the survival of the fish. Despite the significant effects on gene expression in the liver, there was no apparent structural damage in this tissue, as determined by light microscopy on the tissue. Triebkorn et al. (2004), however, have previously shown that electron microscopy is more suitable as a technique to determine the scope of damage in tissues after exposure to chemicals, and thus it is possible that there were effects in the exposed trout, but to detect them required a higher resolution microscopy technique. Very few studies have reported damage to the small intestine of fish. In mammals,

however, gastrointestinal injuries are common side effects of diclofenac (Seitz and Boelsterli, 1998; Treinen-Moslen and Kanz, 2006) and additional medicine has been prescribed to help protect the gut lining. This study presents clear evidence that diclofenac can damage the small intestine of fish (see figure 4.3a) and this may have subsequent effects on processes such as nutrient absorption. The findings support previous research suggesting that the presence of diclofenac in enterohepatic circulation can lead to intestinal damages (Hoeger et al., 2008). Histopathology on the gills was not undertaken in the fish exposure study, and this was a shortfall of this work as it may have provided visual evidence of the inhibitory effects of diclofenac on prostanoids synthesis; altered cellular structure of the gills including chloride cells and granulocytes, are often used as biomarkers of malfunctions in homeostasis and immune response.

The elevated gene expression of *p53* observed in the gills of diclofenac-exposed fish (1 and 5 $\mu\text{g/l}$) is in accordance with the histopathological lesions such as cell necrosis reported in previous work (Schwaiger et al., 2004; Hoeger et al., 2005). Some of the effects of diclofenac on *p53* gene expression, however, were unexpected. Hong et al. (2006) had shown that acute exposure of medaka (*O. latipes*) to diclofenac (1 $\mu\text{g/l}$ and 8 mg/l) resulted in a significant induction of *p53* expression in the liver, gills and small intestine. In this study, however, *p53* gene expression was not affected in the liver and although cellular necrosis was induced in the kidney we found no significant reduction of *p53* expression in this tissue. *P53* is involved in complex molecular cascades leading to the induction of apoptosis to discard damaged cells or cell cycle arrest to repair DNA. Analysing the expression of the genes induced by *p53* and responsible for cell death and cell repair may provide better insights of the effects caused by diclofenac on cellular programming. To our knowledge, this is the first study report the genotoxic effects of diclofenac in fish at environmentally relevant concentrations.

Metabolomics approach was applied to the diclofenac-exposed rainbow trout (plasma and bile) in order to identify the potential pathways implicated in the metabolome disruption. Recent metabolomics studies on fish have reported that the exposure to xenobiotics can lead to significant changes in their metabolite composition, indicating that pharmaceuticals can strongly interfere with the metabolism of non target organisms (Gibson et al., 2005; Samuelsson et al., 2006; Viant et al., 2006; Ekman et al., 2007; Katsiadaki et al., 2009). Biofluids have often been employed in metabolomics and several techniques are available to extract and identify the various classes of signalling molecules. So far a single analytical technique has not been able to extract all the metabolites and new methodologies need to be developed to do so. Initially, methanol extraction and UPLC-TOF-MS techniques optimised to analyse the fish plasma metabolome were capable of extracting a large number of metabolites but the low abundance metabolites (e.g. eicosanoids) seemed poorly detected. The techniques optimised were unable to detect changes in the metabolome of diclofenac-exposed fish even though changes were observed in the bile and there were clear effects on gene expression. The metabolomics data indicated that diclofenac accumulated to detectable levels in the plasma of fish exposed to 5 and 25 µg diclofenac/l without any apparent interference with the endogenous metabolic profile of the fish. The reasons for this were perhaps that the metabolites affected by diclofenac predominantly represented low abundance molecules and that the methodology used was unable to detect these molecules due to the high levels of proteins in the plasma that caused interferences in the matrix. The analytical techniques therefore still require optimisation to be able to successfully extract and detect endogenous metabolites in the plasma. The new SPE and UPLC-TOF-MS methodology developed to profile the bile was highly successful for extracting and detecting a range of signalling molecules including eicosanoids, fatty acids and other metabolites (free and conjugated) in response to diclofenac exposure.

Fractionation enabled the separation of the bile acids causing ion suppression from other metabolites and the recoveries of targeted bile metabolites were in most cases above 75% (section 5.3.2). Furthermore, additional treatment of the bile by hydrolysis permitted the deconjugation of bile salts (e.g. taurocholic acid) and certain conjugated metabolites which improved the detection of endogenous metabolites previously masked by taurocholic acid. Although conjugation is often done with a glycosidic bond, the glucuronidase and sulphatase enzymes did not cleave all the bile conjugates and other enzymes may be required to cleave those remaining conjugates. Using the optimised methodology, analysis of the bile metabolome resulted in significant differences in metabolic profiles of exposed rainbow trout compared with the control fish. The perturbation of over 60 endogenous metabolites was found in fish exposed to environmentally relevant concentrations of diclofenac (see Table 6.6).

The putative identification of hydroxydiclofenac, a methyl ester metabolite and hydroxylated glucuronide diclofenac indicate that the metabolism of diclofenac and side effects observed in mammals may also occur in aquatic vertebrates. Indeed, diclofenac-induced toxicity observed in a previous study (section 4.3.5) has been previously reported in mammals, birds and aquatic organisms and has often been related to the accumulation of diclofenac and the production of reactive metabolites (Seitz and Boelsterli, 1998; Tang et al., 1999; Hickey et al., 2001; Boelsterli, 2003; Grillo et al., 2003; Meteyer et al., 2005; Ng et al., 2006). Further investigations are needed on the glucuronide metabolite of diclofenac to determine if glucuronic acid is on the acyl group. Acyl-glucuronide is a highly reactive metabolite in mammals (Bailey and Dickinson, 2003) and this may explain the intestinal pathology observed in diclofenac-exposed rainbow trout. Many of the endogenous metabolites isolated in this study have not yet been identified yet, this is a time consuming process and the putative molecular weights do not match the masses for their equivalents in mammals that are in the

databases. Preliminary investigations suggest that diclofenac had strong inhibitory effects on a range of functional pathways at environmentally relevant concentrations; many resulting in a significant reduction of phospholipids synthesis (see Table 6.6b). Interestingly, one metabolite marker has been completely suppressed by diclofenac, even at the lowest exposure concentration (0.5 µg/l). It is envisaged that subsequent identification of this (and other) metabolites may reveal new pathways affected by diclofenac exposure and help to gain a better understanding of its mode of action in fish. One of the metabolite markers affected and identified as a possible lipooxygenase product, highlights the extent to which diclofenac can affect the arachidonic cascade. It seems that the inhibition of cyclooxygenase enzyme can enhance the activity of the lipooxygenase enzyme and increase the synthesis of other eicosanoids. This study has helped to demonstrate the potential of metabolomics for determining the effects and mechanisms of action of pharmaceuticals (especially diclofenac) in fish; however meaningful conclusions require further work on the identification of the discriminative metabolites in order to provide more definitive links to physiological functions.

Future research

There are many avenues for future research that would enhance our knowledge on the biological implications of exposure to NSAID in the environment. Here are some key areas that seem to be the most needed. In the first instance, if we are to understand the fate and persistence of NSAID in the environment there is the need for optimisation of microbial culture techniques to enable us to identify the microbial species and genes involved in diclofenac and other NSAID degradation. Biodegradation studies using

bioreactors have shown promising results and the use of stable isotope probing (radioactive labelled diclofenac) may be useful to help identify the microbes actively degrading diclofenac. To better understand the implications for exposure to NSAID on microbial populations, chronic exposure of mixed microbial communities are needed to assess the toxicity of diclofenac on metabolic processes other than growth (e.g ATP production, biofilm formation).

To assess the impacts of exposure to NSAIDs on fish and other aquatic organisms, longer term exposures to environmentally relevant concentrations are needed. These will provide us with more realistic assessments on the potential for these drugs to induce harm in wildlife. Expanding effects measurement to include genomics too will likely advance our understanding significantly on the pathways and processes affected (and thus potential to impair health).

There is little doubt that genomics has the potential to expand our knowledge significantly on the potential for harm of pharmaceuticals in the environment and develop a more thorough understanding on the mechanisms of action. Metabolomics techniques on biofluids, including blood plasma and bile hold very significant promise. These methods however, need to be further optimised to enable the detection of low abundance signalling molecules, such as eicosanoids affected by diclofenac in the plasma. The development of methodologies able to reduce the levels of bile salts and decrease the matrix effects may permit the detection of the glutathione metabolite of diclofenac. The analysis of fish urine should also be considered to further understand the mechanism of toxicity of diclofenac in the kidney.

Considering the effects of diclofenac specifically, obtaining the structural identity of the hydroxylated glucuronide of diclofenac will be valuable in determining if

this compound is the acyl-metabolite of diclofenac responsible for hepatotoxicity, renal injuries and gastrointestinal injuries reported in mammals.

Finally, the application of FT-MS to the metabolomics work would be better able than TOF-MS to identify the metabolites affected by diclofenac exposure. This technology platform provides a more accurate molecular weight, which will reduce the number of candidate metabolites proposed in the available databases.

Conclusions

Diclofenac proved to be persistent in the aquatic environment and toxic effects were observed in aquatic organisms. The recalcitrance of diclofenac to microbial degradation is of environmental concern, especially as its usage may increase in the coming years and that it is known to be capable of inducing harm in various wildlife species. Research is needed to better understand the biodegradation process of diclofenac in order to determine how WWTPs processes can be optimised for its removal. The identification of key microbial species and genes able to degrade pharmaceuticals could be beneficial for bioremediation. Information on the molecular mechanisms may be used to engineer microbial populations pre-disposed for the degradation of pharmaceuticals.

The toxic effects of sub-chronic exposure to environmentally relevant concentrations of diclofenac, reported in these studies, illustrate that diclofenac can act in fish in a similar manner than in mammals. This infers that long term exposure may result in side effects such as kidney failure and intestinal pathology which may be detrimental to the health and survival of aquatic vertebrates. The results from our studies further highlight the need for more suitable tests in chemical and environmental

risk assessments based on chronic exposure to low concentrations of pharmaceuticals rather than acute toxicity tests with sub-lethal concentrations. The changes in the metabolite profiling of fish bile from the lowest exposure dose suggest that environmental concentrations can alter fish physiology. The discovery of metabolites and functional pathways affected by diclofenac at environmental concentrations are critical to understand the potential threat of this compound in the ecosystem.

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