Impacts of the human pharmaceutical diclofenac in the aquatic environment

Submitted by Alvine Coralie Mehinto

To the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, October 2009

This thesis is available for Library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgment.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been published and approved for the award of a degree by this or any other University.

Signature ...A. MEHINTO....
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 µg/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 EC_{50} (15 min) estimated ranged from 13.5 mg/l ± 2.3 for diclofenac to 42.1 mg/l ± 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 µg/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 \( \text{cox1} \) and \( \text{cox2} \) expression in the liver, gills and kidney from 1 µg diclofenac/l. In contrast diclofenac induced an increase in mRNA levels for \( \text{cyp1a1} \) in the liver and gills but a significant reduction of \( \text{cyp1a1} \) 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 \( 509 \pm 27 \) and \( 657 \pm 25 \). 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 lipoxygenase 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.
Acknowledgements

First of all I would like to thank my supervisors Professor Charles Tyler, Dr Sara Burton and Professor Hilary Lappin-Scott for their support and encouragement throughout this study. I am eternally grateful to my unofficial supervisor Dr Elizabeth Hill from University of Sussex for teaching me metabolomics and for her invaluable help with the chemical analyses.

Special thanks to Peter Splatt and Jan Shears for the vast technical support. I would like to thank Morley Williams from Southwest Water from (Countess Wear) for its help during sampling. I would also like to thank Dr Eduarda Santos, Dr Amy Filby, Dr Patrick Hamilton, Dr Lisa Bickley, and Dr Anke Lange for their precious help in the lab and during the writing process. I am bery grateful to all the members of the former EMERGE group and the EMFB group at University of Exeter and my colleagues at University of Sussex for their friendship and support. Special mentions must be made to Viv, Rhys, Tess, Jenny and Luanne who helped me during the fish exposure but also Rachel, Max, Theresa, Laura and Okhyun. Big thank you to Dalia and Nicola for their precious advice to “carry on regardless” and support. A huge thank you to Elena for guiding me through the metabolomics, and being such a good friend.

Thank you to Marco, Hervé, tante Colette, tante Arlette, my family and friends who have supported me during the PhD. Special thank you to my cousin Dr Bobo for everything…

Finally a huge thank you to my parents for their unconditional love, patience, support and believing in me.

This thesis is dedicated to my aunts Madina Kelani (1952-2009), Yvette Paraïso (1963-2008) and my cousin Titi Bouraima (1969-2008).
Table of contents

Title page and declaration .............................................................................................. i

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

Acknowledgements ........................................................................................................ iv

Table of contents .............................................................................................................. v

List of figures .................................................................................................................. xii

List of tables .................................................................................................................... xv

List of abbreviations ..................................................................................................... xvii

List of abbreviated chemicals ....................................................................................... xx

Chapter 1: General Introduction

1.1. Pharmaceuticals in the environment........................................................................... 1

1.1.1. Origin ................................................................................................................... 2

1.1.2. Occurrence in the aquatic environment ............................................................... 4

1.2. Non-steroidal anti-inflammatory drugs ...................................................................... 7

1.2.1. Mode of action ..................................................................................................... 9

1.3. Removal of NSAIDs in the environment ................................................................. 11

1.3.1. Wastewater treatment plants .............................................................................. 11

1.3.2. Removal efficiency in wastewater treatment plants ............................................ 13

1.3.3. Biodegradation .................................................................................................. 13

1.3.4. Sorption ............................................................................................................. 15

1.3.5. Abiotic processes ............................................................................................... 16

1.4. Environmental impact of NSAIDs ........................................................................... 17

1.4.1. Acute toxicity .................................................................................................... 17

1.4.2. Chronic toxicity ................................................................................................. 18

1.5. Xenobiotic metabolism in fish ................................................................................. 20
1.5.1. Cytochrome P450 monooxygenase system

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

1.5.3. Bile as major excretory route of pharmaceuticals

1.6. Analysis of pharmaceuticals in environmental samples

1.6.1. Sample preparation

1.6.2. Detection techniques

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

1.7.1. Genomics

1.7.2. Proteomics

1.7.3. Metabolomics

1.8. Bioinformatics

1.9. Aims of the thesis

Chapter 2: General Materials and Methods

2.1. Biodegradation studies

2.1.1. Sampling sites

2.1.2. Bacterial growth media

2.1.3. Microcosm enrichment cultures

2.1.4. Sub-culturing method

2.2. Nucleic acid extraction

2.2.1. 5% CTAB/phosphate buffer

2.2.2. Extraction procedure

2.3. Agarose gel electrophoresis

2.4. Polymerase chain reaction amplification of 16S rRNA gene

2.5. Microtox acute toxicity test

2.6. Disc diffusion assay

2.6.1. Bacterial strains

2.6.2. Toxicity test protocol
2.7. Detection of pharmaceuticals in water samples ....................................................... 40
  2.7.1. Solid phase extraction (SPE) ............................................................................. 40
  2.7.2. Ultraperformance liquid chromatography time-of-flight mass spectrometry ... 41
  2.7.3. Quantification of pharmaceuticals ................................................................. 43
2.8. Fish studies ............................................................................................................... 44
  2.8.1. Supply and maintenance of the fish ............................................................... 44
  2.8.2. Experimental set up ....................................................................................... 44
  2.8.3. Dissection and tissue collection ................................................................. 46
2.9. Total RNA extraction and quantification ............................................................... 47
2.10. Reverse transcription (RT)-PCR ............................................................................ 48
2.11. Quantitative real-time PCR (Q-PCR) .................................................................. 49
  2.11.1. Primer design for Q-PCR ............................................................................. 49
  2.11.2. Optimisation of primer-pair annealing temperature ...................................... 50
  2.11.3. Determination of Q-PCR amplification efficiency and melt curve ............... 50
  2.11.4. QPCR protocol for tissues ......................................................................... 51
  2.11.5. Data analysis ............................................................................................... 53
2.12. Histopathology on fish tissues ............................................................................. 54
  2.12.1. Dehydration and embedding ....................................................................... 54
  2.12.2. Sectioning of the embedded tissues ............................................................ 54
  2.12.3. Haematoxylin and Eosin (H&E) Staining ............................................... 55
  2.12.4. Analysis of fixed tissue sections ................................................................. 55
2.13. Metabolomic studies ............................................................................................ 58
  2.13.1. Hydrolysis of bile samples ........................................................................ 58
  2.13.2. Solid phase extraction of bile samples ..................................................... 59
  2.13.3. Methanol extraction of blood plasma samples ......................................... 59
  2.13.4. Pre-processing of data ............................................................................... 60
  2.13.5. Multivariate analysis ................................................................................. 60
  2.13.6. Model and data validation ....................................................................... 61
Chapter 3: Biodegradation of non-steroidal pharmaceuticals in the aquatic environment and their toxicity to microbes

Contribution of each author ................................................................. 63
Abstract ............................................................................................. 64
3.1. Introduction ..................................................................................... 65
3.2. Materials and methods ................................................................. 68
  3.2.1. Chemicals and reagents ............................................................ 68
  3.2.2. Environmental sampling .......................................................... 68
  3.2.3. Biodegradation study ............................................................... 68
  3.2.4. Solid Phase Extraction (SPE) .................................................... 70
  3.2.5. Ultraperformance Liquid Chromatography/Electrospray ionization time-of-flight- Mass Spectrometry ......................................................... 71
  3.2.6. Quantification of NSAIDs ....................................................... 73
  3.2.7. Bioluminescence assay ............................................................ 73
  3.2.8. Disc diffusion assay ............................................................... 74
  3.2.9. Predicted pathway for the biodegradation of diclofenac ............ 74
3.3. Results .......................................................................................... 74
  3.3.1. Recovery efficiencies for NSAIDs ........................................... 74
  3.3.2. Biodegradation study ............................................................. 75
  3.3.3. Diclofenac degradation study .................................................. 77
  3.3.4. Bioluminescence assay ......................................................... 77
  3.3.5. Disc diffusion assay ............................................................. 80
  3.6. Prediction of the biodegradation pathway for diclofenac .............. 80
3.4. Discussion ..................................................................................... 82
3.5. Conclusions .................................................................................. 88

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

Contribution of each author ................................................................. 90
Abstract ........................................................................................................................... 91
4.1. Introduction .............................................................................................................. 92
4.2. Materials and methods............................................................................................ 94
  4.2.1. Diclofenac exposure .......................................................................................... 94
  4.2.2. Fish sampling ..................................................................................................... 94
  4.2.3. Condition factor ................................................................................................. 95
  4.2.4. Analysis of diclofenac in water and bile samples ............................................. 95
  4.2.5. RNA extraction and reverse transcription (RT) PCR ........................................ 96
  4.2.6. Primer design and real-time PCR optimisation ................................................. 96
  4.2.7. Real-time PCR ................................................................................................... 97
  4.2.8. Histology ........................................................................................................... 98
  4.2.9. Data analysis ...................................................................................................... 98
4.3. Results ...................................................................................................................... 99
  4.3.1. Analysis of diclofenac concentrations in the tank water ................................... 99
  4.3.2. Condition factor ................................................................................................. 99
  4.3.3. Concentration of diclofenac and identification of its metabolites in fish bile... 99
  4.3.4. Target gene expression .................................................................................... 102
  4.3.5. Histopathological findings ............................................................................. 105
4.4. Discussion .............................................................................................................. 110

Supplementary materials ............................................................................................... 115

Chapter 5: Development of analytical techniques for metabolic profiling of biofluids in rainbow trout (O. mykiss)
5.1. Introduction ............................................................................................................ 118
5.2. Materials and methods............................................................................................ 120
  5.2.1. Chemicals ........................................................................................................ 120
  5.2.2. Sample collection ............................................................................................. 120
  5.2.3. Preparation of bile samples ............................................................................. 122
  5.2.4. SPE fractionation and recovery of standard metabolites .................................. 123
5.2.5. Preparation of plasma samples ................................................................. 123
5.2.6. UPLC-TOF-MS analysis .......................................................................... 123
5.2.7. Data handling .......................................................................................... 124
5.2.8. Multivariate data analysis of plasma samples .......................................... 125
5.3. Results .......................................................................................................... 125
5.3.1. Method development for metabolic profiling of bile .............................. 125
5.3.1.1. Analysis of the chromatograms for the bile extracts ......................... 125
5.3.1.2. Quantitative analysis of the bile extracts .............................................. 128
5.3.2. SPE fractionation and recovery of standard metabolites ....................... 132
5.3.3. Method development for metabolic profiling of plasma samples .......... 135
5.3.4. PCA and PLS-DA analyses of plasma samples from diclofenac exposure ........................................................................................................ 140
5.3.5. Identification of class-separating markers .............................................. 144
5.4. Discussion .................................................................................................... 144
5.5. Conclusions .................................................................................................. 149

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

6.1. Introduction .................................................................................................. 150
6.2. Materials and Methods ............................................................................. 152
6.2.1. Chemicals ................................................................................................ 152
6.2.2. Diclofenac exposure and sample collection .......................................... 152
6.2.3. Bile hydrolysis ......................................................................................... 153
6.2.4. Metabolite extraction and UPLC-TOF-MS analysis .............................. 153
6.2.5. Data transformation, pre-processing and pre-treatment ...................... 154
6.2.6. Multivariate data analysis ..................................................................... 155
6.2.7. Identification of metabolites .................................................................. 155
6.3. Results .......................................................................................................... 156
6.3.1. PCA overview of the free metabolites and conjugated metabolites fractions from non-hydrolysed bile in control and diclofenac-exposed fish .......... 156
6.3.2. Overview of PLS-DA and OPLS models for non-hydrolysed bile ..............157
6.3.3. PCA overview of the free metabolites (EA+FA) fraction from hydrolysed bile extracts..................................................................................................................161
6.3.4. Overview of PLS-DA and OPLS models for hydrolysed bile extracts ..........161
6.3.5. Identity of metabolites of diclofenac..............................................................165
6.3.6. Identity of metabolite markers of diclofenac exposure ................................168
6.4. Discussion .............................................................................................................174
5. Conclusions ...............................................................................................................180

Chapter 7: General Discussion

Discussion ......................................................................................................................181
Future research ..............................................................................................................189
Conclusions ...................................................................................................................191

References .......................................................................................................................193
List of figures

Figure 1.1: Routes of entry for pharmaceuticals........................................................3

Figure 1.2: Chemical structure of selected NSAIDs...............................................8

Figure 1.3: Schematic diagram of arachidonic acid conversion to prostanoids..........10

Figure 1.4: Schematic diagram of a wastewater treatment plant............................12

Figure 1.5: Hydroxylation reactions catalysed by cytochrome P450s.....................21

Figure 2.1: Maps of the sampling sites.....................................................................34

Figure 2.2: Diclofenac in vivo experimental set-up...............................................45

Figure 2.3: Real-time PCR amplification graph.......................................................52

Figure 2.4: Melt curve analysis..............................................................................52

Figure 3.1: Solid phase extraction protocol at acidic pH using Oasis HLB 6cc cartridges.................................................................72

Figure 3.2: Degradation of NSAIDs (conc. 10 mg/l) with sludge samples incubated in orbital shaker at 25 °C.........................................................76

Figure 3.3: Degradation of diclofenac (1 mg/l) by environmental samples..............78

Figure 3.4: Proposed degradation pathways of diclofenac using UMBBD...............83

Figure 4.1: Average threshold cycle (C_T) for rpl8 amplification in liver, gills and kidney after 21 day exposure to diclofenac.................................103

Figure 4.2: Relative expression of coxl (a), cox2 (b), cyp1a1 (c) and p53 (d) in rainbow trout after 21-day diclofenac exposure.................................104

Figure 4.3a: Histopathological lesions in small intestine of rainbow trout induced by diclofenac exposure.........................................................106

Figure 4.3b: Semi-quantitative assessment of histopathological lesions in the small intestine of diclofenac-exposed rainbow trout.................................107

Figure 4.4a: Histopathological lesions in the kidney of diclofenac-exposed rainbow trout after 21 days.................................................................108
Figure 4.4b: Semi-quantitative assessment of histopathological lesions in the kidney of diclofenac-exposed rainbow trout

Figure S4.1: Primers designed and optimised for QPCR assay

Figure S4.2: Condition factor of rainbow trout

Figure S4.3: Histological sections of liver tissue from control and diclofenac treated rainbow trout

Figure 5.1: Total ion chromatograms (as base peak intensity BPI) of untreated bile in +ESI and –ESI modes

Figure 5.2: Spectral ion chromatograms of the saturated peaks in untreated bile in +ESI and –ESI modes

Figure 5.3: Total ion chromatograms (as base peak intensity BPI) of fractionated non-hydrolysed bile and blank samples in –ESI mode

Figure 5.4: Total ion chromatograms (as base peak intensity BPI) of fractionated hydrolysed bile and blank samples in –ESI mode

Figure 5.5: Chromatograms of target metabolites and the internal standards

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

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

Figure 5.8: Selected ion chromatograms (-ESI) of the eicosanoids recovered in the 80% methanol extracts of spiked plasma samples

Figure 5.9: Score plots from partial least squares discriminant analysis (PLS-DA) of rainbow trout plasma following diclofenac exposure

Figure 5.10: Mass spectra of diclofenac ion (m/z 294.008) in –ESI mode in plasma

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 (●)

Figure 6.2: Score plots of the multivariate discriminant analysis of the free metabolites (EA+FA) fraction for hydrolysed bile from diclofenac exposure
Figure 6.3: a) S-plot from the OPLS analysis of hydrolysed bile of control and 5 µg/l groups 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.................................166

Figure 6.4: Total ion chromatograms showing diclofenac and its metabolites in diclofenac-exposed rainbow trout............................................................169

Figure 6.5: Spectral ion chromatograms of diclofenac and hydroxydiclofenac fragments produced by UPLC-TOF-MS analysis........................................169

Figure 6.6: Conversion of arachidonic acid into eicosanoids........................................179
List of tables

Table 1.1: Concentrations of selected pharmaceuticals in the aquatic environment......6

Table 2.1: Solid phase extraction protocol.................................................................42

Table 2.2: Processing program for histological analyses............................................56

Table 2.3: H&E staining protocol for fixed sections..................................................57

Table 3.1: Properties of NSAIDs used in this study.................................................69

Table 3.2: Acute toxicity (EC50, 15 min) of NSAIDs using Vibrio fischeri.............79

Table 3.3: Mean diameter of the inhibition zone.......................................................81

Table 4.1: Mean measured diclofenac concentrations (µg/l ± SE) in replicate tanks water.............................................................................................................100

Table 4.2: Measured diclofenac concentrations (ng/ml, mean ± SE) and estimated bioconcentration in rainbow trout bile after 21-day exposure.................100

Table 4.3: Putative metabolites of diclofenac identified in fish bile.......................101

Table S4.1: Primers designed and optimised for real-time PCR analysis.................116

Table 5.1: Preparation of standard stock solutions..................................................121

Table 5.2: Number of markers estimated by MarkerLynx for non-hydrolysed bile samples untreated and fractionated using SPE............................................130

Table 5.3: Number of markers estimated by MarkerLynx for hydrolysed bile samples fractionated using SPE.................................................................131

Table 5.4: UPLC-TOF-MS analysis of target metabolites in aqueous samples........134

Table 5.5: Number of markers detected after methanol extraction of plasma samples.............................................................................................................136

Table 5.6: Limit of detection (LOD) of eicosanoids in water and plasma samples using UPLC-TOF-MS.................................................................139

Table 5.7: Performance parameters of multivariate discriminant models for the comparison of control and diclofenac exposed rainbow trout.................142
Table 6.1: Performance parameters of principal component analyses for the comparison of the metabolic profiles in non-hydrolysed bile extracts.................................158

Table 6.2: Multivariate discriminant models for non-hydrolysed bile samples of control and diclofenac-exposed rainbow trout.........................................................159

Table 6.3: Performance parameters of principal component analyses for the comparison of the metabolic profiles in hydrolysed bile extracts..............................162

Table 6.4: Multivariate discriminant models for the comparison of control and diclofenac exposed rainbow trout in hydrolysed bile.............................................163

Table 6.5: Identification of diclofenac and putative metabolites........................................167

Table 6.6a: Markers of diclofenac exposure identified in non-hydrolysed bile of rainbow trout.................................................................................................170

Table 6.6b: Markers of diclofenac exposure identified in hydrolysed bile of rainbow trout.............................................................................................................170-172
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
cox cyclooxygenase
C_T threshold cycle
cyp cytochrome P450
cyp1a1 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 17β-estradiol
EC_{50} 50% effect concentration
ECOSAR Ecotoxicological Structure Activity Relationship
EDC endocrine disrupting chemical
EE_2 17α-ethinylestradiol
EPA eicosapentaenoic acid
EROD ethoxyresorufin-O-deethylase
ESI electrospray ionisation
eV electron volt
FT-MS Fourrier 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
m/z 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
p53 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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppm</td>
<td>part per million</td>
</tr>
<tr>
<td>( Q^2 )</td>
<td>cumulative variation predicted by the PCA or PLS model</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>real-time quantitative reverse transcription PCR</td>
</tr>
<tr>
<td>( R^2 )</td>
<td>variation explained by the PCA or PLS model</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Rnase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>( rpl8 )</td>
<td>ribosomal protein l8</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>retention time</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion chromatogram</td>
</tr>
<tr>
<td>( T_M )</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>UPLC-MS</td>
<td>ultraperformance-liquid chromatography - mass spectrometry</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume to volume ratio</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>WWTP</td>
<td>wastewater treatment plant</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>micrometre</td>
</tr>
<tr>
<td>µM</td>
<td>micromole per litre</td>
</tr>
</tbody>
</table>
### List of abbreviated chemicals

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-HETE</td>
<td>±11-hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>phosgene</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>copper (II) sulphate</td>
</tr>
<tr>
<td>E$_2$-$d^4$-S</td>
<td>[2,4,16,16-d4] 17β-estradiol sodium 3-sulfate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenedinitrilotetraacetic acid</td>
</tr>
<tr>
<td>H$_2$SO$_4$</td>
<td>sulphuric acid</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>potassium phosphate dibasic</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>potassium dihydrogen phosphate</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>magnesium sulphate</td>
</tr>
<tr>
<td>MnSO$_4$</td>
<td>manganese sulphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>sodium sulphate</td>
</tr>
<tr>
<td>NH$_4$OH</td>
<td>ammonium hydroxide</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>ammonium sulphate</td>
</tr>
<tr>
<td>P-$d^9$</td>
<td>[2,2,4,6,6,17α-21,21,21-d9] progesterone</td>
</tr>
<tr>
<td>PGB$_2$</td>
<td>prostaglandin B2</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PGJ$_2$</td>
<td>prostaglandin J2</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-Amino-2-hydroxymethyl-propane-1,3-diol</td>
</tr>
<tr>
<td>TxB$_2$</td>
<td>tromboxane B$_2$</td>
</tr>
<tr>
<td>ZnSO$_4$</td>
<td>zinc sulphate</td>
</tr>
</tbody>
</table>