LC-MS/MS Analysis of Pharmaceuticals in the Irish Aquatic Environment and the Potential for Human Exposure

Gillian McEneff, B.Sc.

Student No.: 10109552

Under the supervision of:

Dr. Brian Quinn

Dr. Brian Kelleher

Prof. Brett Paull

School of Chemical Sciences

Dublin City University

A thesis submitted for consideration for the award of:

Doctor of Philosophy

January 2014

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of **Doctor of Philosophy** is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ (Candidate) ID No.: _____

Date: _____

Table of Contents

Abstract

Reports concerning the quantitative analysis of pharmaceuticals in marine ecosystems are somewhat limited. It is necessary to determine pharmaceutical fate and assess any potential risk of exposure to aquatic species and ultimately, seafood consumers. However, in Ireland very little research has been carried out to determine the presence of pharmaceutical residues in the aquatic environment.

The research carried out investigates the occurrence of pharmaceuticals in the Irish aquatic environment and their potential to bioaccumulate in aquatic organisms and pose a risk to human health via dietary intake. Pharmaceutical residues were determined in liquid matrices, such as wastewater effluent, marine surface water (MSW) and artificial seawater (ASW), using solid phase extraction (SPE) in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Pharmaceutical extraction from marine mussels and fish tissues required an additional pressurised liquid extraction (PLE) step prior to SPE and LC-MS/MS.

The results of an *in situ* study, in which caged *Mytilus* spp. were deployed at three sites on the Irish coastline over a 1-year period are presented. All of the selected pharmaceuticals were quantified in wastewater effluent and marine surface waters and three out of the five monitored pharmaceuticals were detected in environmentally exposed mussel tissue.

The potential for pharmaceutical bioaccumulation in fish via trophic level transfer was investigated. A 28-day *in vivo* experiment was carried out in a flow-through system in which rainbow trout were fed wild marine mussels sampled from one of the most contaminated sites in Ireland. Although low-level residues of trimethoprim were detected in the mussel tissues, no bioaccumulation was reported for this drug or any of the other selected compounds in the liver of the exposed fish.

The effect of steaming on the concentrations of five pharmaceutical residues in exposed mussel tissue was investigated in an attempt to assess the potential risk of exposure to humans via ingestion of contaminated seafood. An *in vivo* experiment was carried out exposing marine mussels to pharmaceutical concentrations via direct injection and water uptake. A selection of water-exposed mussels were cooked (via steaming) resulting in a significant increase of parent pharmaceutical concentrations in the bivalves.

Acknowledgements

This research was carried out as part of the Science, Technology, Research and Innovation for the Environment (STRIVE) Programme which is funded by the Irish Government under the National Development Plan 2007-2013 and administered on behalf of the Department of the Environment, Heritage and Local Government by the Environmental Protection Agency. This research was also partly funded by the Science Foundation of Ireland (Grant Number 08/SRC/B1412).

I would like to thank Dr. Brian Quinn and Prof. Brett Paull for providing me with the opportunity to be a part of such great research groups such as the ICET and the ISSC and for the opportunity to travel to many domestic and international conferences throughout my studies. I would also like to thank Dr. Brian Kelleher and Dr. Leon Barron for their input and their much appreciated support and advice. Dr. Wiebke Schmidt is also acknowledged for her help and advice throughout this work.

Kind thanks to Lynn Vanhaecke and Julie Kiebooms for their hospitality expressed when visiting Ghent University in Belgium. Thanks to Iarfhlaidh Connellan, Edward Grant, Thomas Cleary, Robert Hernan, Liam Morrison, Kathleen O'Rourke, Martina Mulligan and Rachel McArdle for their help with experimental set-up and sample collection.

A big thank you to all the ISSC, School of Chemical Sciences and NCSR staff members and technicians, especially Stephen Fuller and Maurice Burke.

Thanks to everyone who has come and gone through the ISSC - Patrick, Orla, Amy, Ruth, Sara, Sinead, Ali, Disha, Mercedes, Damian, Katya, Dave, Lily, Ugis, Aymen and finally Nicola, who was a great support and friend throughout. I would also like to thank all those in the School of Chemical Sciences and Biotechnology.

Finally, a special thank you to all my great friends and my entire family especially my Mum, Johnny, Dad, Katie and Al for their constant love, support and encouragement.

List of Publications and Conference Presentations

Publications

- Schmidt, W., Rainville, L.C., **McEneff**, **G.**, Sheehan, D., Quinn, B. (2013) A proteomic evaluation of the effects of the pharmaceuticals diclofenac and gemfibrozil on marine mussels (*Mytilus* spp.): evidence for chronic sublethal effects on stress-response proteins. *Drug Testing and Analysis*. DOI: 10.1002/dta.1463.

- **McEneff**, **G.**, Barron, L., Kelleher, B., Paull, B., Quinn, B. (2013) The determination of pharmaceutical residues in cooked and uncooked marine bivalves using pressurised liquid extraction, solid phase extraction and liquid chromatography–tandem mass spectrometry', *Analytical and Bioanalytical Chemistry*. **405**, 9509-9521.

- **McEneff**, **G.**, Barron, L., Kelleher, B., Paull, B., Quinn, B. A year-long study of the spatial occurrence and relative distribution of pharmaceutical residues in sewage effluent, receiving marine waters and marine bivalves, *Science of the Total Environment.* Accepted December 2013.

Oral presentations

- **McEneff**, **G.**, Barron, L., Kelleher, B., Paull, B., Quinn, B. LC-MS/MS analysis of pharmaceuticals in the aquatic environment. The 22nd Irish Environmental Researchers Colloquim, University College Dublin, Ireland, 7th-9th March 2012.

- **McEneff**, **G.**, Barron, L., Kelleher, B., Paull, B., Quinn, B. LC-MS/MS analysis of pharmaceuticals in the aquatic environment. RSC/ChromSoc Advances in Clinical Analysis, Guy's Hospital Tower, London, UK, 30th October 2012.
  (Invited Speaker)

- **McEneff**, **G.**, Barron, L., Kelleher, B., Paull, B., Quinn, B. LC-MS/MS analysis of pharmaceuticals in the aquatic environment. Irish Mass Spectrometry Society (IMSS) Conference, Red Cow Hotel Dublin, Ireland, 1st May 2013.

Poster Presentations

- **McEneff**, **G.**, Schmidt, W., Quinn, B. A comparative *in situ* study on ecotoxicological effects of pharmaceuticals in Ireland. 8th Annual EPA Postgraduate Seminar, The Conference Centre Dublin, Ireland, 11th November 2010.

- **McEneff**, **G.**, Schmidt, W., Barron, L., Paull, B., Quinn, B. Solid phase extraction and LC-MS/MS analysis of pharmaceutical and personal care products in the Irish aquatic environment and their potential to bioconcentrate. Conference on Analytical Sciences, Ireland (CASi), Dublin City University, Ireland, 21st-22nd February 2011.

- **McEneff**, **G.**, Schmidt, W., Barron, L., Paull, B., Kelleher, B., Quinn, B. Solid phase extraction and LC-MS/MS analysis of pharmaceutical and personal care products in the Irish aquatic environment. Instrumental Methods of Analysis – Modern Trends and Applications (IMA), MAICh Conference Centre, Chania, Crete, Greece, 18th-22nd September 2011.

- **McEneff**, **G.**, Kellleher, B., Barron, L., Paull, B., Quinn, B., Solid phase extraction and LC-MS/MS analysis of pharmaceuticals in the Irish aquatic environment. 9th Annual EPA Postgraduate Seminar, The Conference Centre Dublin, Ireland, 17th November 2011.

- **McEneff**, **G.**, Schmidt, W., Barron, L., Paull, B., Kelleher, B., Quinn, B. Solid phase extraction and LC-MS/MS analysis of pharmaceuticals in the Irish aquatic environment. 6th Society of Environmental Toxicology and Chemistry (SETAC) World Congress, Estrel Hotel Berlin, Germany, 20th-24th May 2012.

- **McEneff**, **G.**, Barron, L., Kelleher, B., Paull, B., Quinn, B. LC-MS/MS analysis of pharmaceuticals in the Irish aquatic environment. 39th International Symposium of High Performance Liquid Chromatography and Related Techniques (HPLC), RAI Conference Centre Amsterdam, Netherlands, 16th-20th June 2013.

List of Figures

List of Tables

List of Equations

List of Abbreviations

Al_2O_2: Aluminium oxide

AOP: Advanced oxidation processes

APCI: Atmospheric pressure chemical ionisation

API: Atmospheric pressure interface

APPI: Atmospheric-pressure photoionisation

ASW: Artificial seawater

BAF: Bioaccumulation factor

BCF: Bioconcentration factor

BOD: Biological oxygen demand

BMF: Biomagnification factor

$C_{aqueous}$: Chemical concentration in the aqueous phase

C_B: Chemical concentration in the organism

C_D: Chemical concentration in the diet of the organism

CEC: Chemical of emerging concern

COD: Chemical oxygen demand

C_{solid}: Chemical concentration in the solid

C_{WD}: Freely dissolved chemical concentration in water

DAD: Diode array detection

DMSO: Dimethyl sulfoxide

DO: Dissolved oxygen

DOM: Dissolved organic matter

D_{ow}: Octanol-water distribution coefficient

DW: Dry weight

EDC: Endocrine disrupting compound

EFSA: European food safety authority

EI: Electron impact

EIC: Extracted ion chromatogram

EMEA: European Medicines Evaluation Agency

EPA: Environmental Protection Agency

ESI: Electrospray ionisation

GC: Gas chromatography

GST: Glutathione S-transferase

H_2O_2: Hydrogen peroxide

HBCD: Hexabromocyclododecane

HCB: Hexachlorobenzene

HCT: High capacity ion trap

HPLC: High performance liquid chromatography

IPHA: Irish Pharmaceutical Healthcare Association

K_D: Solid-water distribution coefficient

K_{oc}: Normalised organic carbon sorption coefficients

K_{ow}: Octanol-water partition coefficient

LC: Liquid chromatography

LLE: Liquid-liquid extraction

LOD: Limit of detection

LOEC: Lowest observed effect concentration

LOQ: Limit of quantification

LPME: Liquid phase micro-extraction

MAE: Microwave-assisted extraction

MAX: Mixed-mode anion exchange

MBR: Membrane bioreactor

MFO: Mixed function oxidase

MIP: Molecularly imprinted polymer

MRL: Maximum residue limit

MS: Mass spectrometry

MSW: Marine surface water

MXC: Mixed-mode cation exchange

NADPH: Nicotinamide adenine dinucleotide phosphate

NSAID: Non-steroidal anti-inflammatory drug

OA: Oxolinic acid

OECD: Organisation for Economic Co-operation and Development

OSPAR: Oslo-Paris Convention for the Protection of the Marine Environment of the North-East Atlantic

OTC: Oxytetracycline

PAH: Polycyclic aromatic hydrocarbon

PCB: Polychlorinated biphenyl

PE: Population equivalent

PEC: Predicted environmental concentration

PFC: Perfluorinated compound

pK_a: Acid dissociation constant

PLE: Pressurised liquid extraction

PNEC: Predicted no effect concentration

POP: Persistent organic pollutant

PPCP: Pharmaceutical and personal care product

PVDF: Polyvinylidene fluoride

QqLIT: Triple quadrupole linear ion trap

QqQ: Triple quadrupole

QSAR: Quantitative structural activity relationship

QTOF: Quadrupole time of flight

ROS: Reactive oxygen species

RSD: Relative standard deviation

SBSE: Stir-bar sorptive extraction

SPE: Solid phase extraction

SPM: Suspended particulate material

SPME: Solid phase micro-extraction

SRM: Selected reaction monitoring

SRT: Solid retention time

SSRI: Selective serotonin reuptake inhibitor

STRIVE: Science, Technology, Research and Innovation for the Environment

S/N: Signal-to-noise

TOC: Total organic carbon

TOF: Time of flight

UDP: Uridine-diphosphate

UDPGA: Uridine-diphosphate-glucuronic acid

UGT: Uridine-diphosphate-glucuronosyl transferase

UPLC: Ultra performance liquid chromatography

UV: Ultra-violet

WW: Wet weight

WWTP: Wastewater treatment plant

“One of the major problems is what we do to the soil, the air and the water. Everything we do, we take in our food.”

-Charlotte Gerson, Food Matters

1.0 General Introduction

1.1 Pharmaceuticals as Environmental Pollutants

Over the last three decades, public awareness of environmental health issues has soared in Ireland due to the increase in funding for environmental research from global policy makers such as the Irish Environmental Protection Agency (EPA) and the European Union (EU) (Colgan and Donlon, 2010; EUR-OP, 2012). In particular, the assessment and monitoring of environment exposure was found to be a leading research topic, accounting for 20.8 % of all environmental publications released between the years 1995 and 2005 (Tarkowski, 2007). Initially, the emphasis of environmental exposure research was on the so called 'priority pollutants' such as pesticides and industrial intermediates. As environmental interest grew and research progressed, so too did the technologies employed, resulting in the development of more advanced techniques and the discovery of a new group of emerging contaminants, collectively referred to as 'chemicals of emerging concern (CECs)' (Bhandari, 2009). These compounds include pharmaceuticals and personal care products (PPCPs), endocrine disrupting compounds (EDCs), perfluorinated compounds (PFCs), surfactants, gasoline additives, disinfection by-products, algal and cyanobacterial toxins, organometallic compounds, brominated and organophosphate flame retardants, plasticisers and nanoparticles (Gros *et al.*, 2006a).

For the purpose of this thesis, the array of compounds encompassed in the scope of PPCPs will be defined to include over-the-counter/prescription pharmaceuticals for human and animal use, excluding hormones. Pharmaceuticals have a specific structure, mode of action and biological effect which determines their function and therapeutic class. Pharmaceuticals can be classified into numerous therapeutic classes including anti-inflammatories, antibiotics, antipsychotics, antihypertensives, antidiabetics, antihistamines, lipid regulators, anticonvulsant, β-blockers, stimulants and statins. Pharmaceuticals were first introduced as environmental contaminants by Richardson and Bowron (1985) but, their negative environmental effect was only later acknowledged in the late nineties when they were described as 'agents of subtle change' (Daughton and Ternes, 1999). As previously mentioned, pharmaceutical concern in the environment is as a result of the rapid technological advances in recent years which have enabled the

improvement of analytical performance in terms of resolution and sensitivity and the detection of these now termed CECs.

Ireland is a leading location for the pharmaceutical industry, housing 9 of the 10 largest pharmaceutical companies in the world. The Irish market for pharmaceutical products in 2010 was valued at just under €2.3 billion by the Irish Pharmaceutical Healthcare Association (IPHA), indicating a high level of pharmaceutical consumption in Ireland (IPHA, 2012). Lacey *et al.* (2008) previously reported the presence of pharmaceuticals in Irish wastewater effluent at low $\mu g.L^{-1}$ concentrations. The release of pharmaceuticals in this concentration range has been shown to impact on the quality of the surrounding aquatic environment in other European countries and America (Corcoran *et al.*, 2010; Huerta *et al.*, 2012). Although hormones are not within the scope of this thesis, it is worth mentioning that the release of the hormone medroxyprogesterone acetate in Irish industrial wastewaters consequently resulted in the infertility of pigs in Holland. This hormone was detected in the pig feed and traced back to contaminated wastewater, sourced from a large U.S. pharmaceutical company based in Ireland, which was exported to Holland and Belgium to be recycled and processed in pig feed (Van Leengoed *et al.*, 2002). The most well-known example confirming the negative effects of pharmaceuticals on wildlife resulted in the decline of three species of vultures in Asia over the last 20 years. The anti-inflammatory, diclofenac, was administered to livestock and was reported to have caused acute renal failure in vultures preying on their carcasses (Oaks *et al.*, 2004).

In Europe, a number of policies and directives have been enforced in order to protect the environment from exposure to harmful chemicals. The EU Water Framework Directives list 33 priority pollutants and 8 other pollutants which must be regulated and monitored in all European wastewaters (EU Directives 2000/60/EC; 2008/105/EC). Pharmaceuticals have remained outside the scope for regulation and monitoring under this directive but in the past year, the EU Commission has revised the list of priority pollutants and following recent findings, has included the regulation of 12 new substances and the monitoring of 3 pharmaceutical compounds including the anti-inflammatory, diclofenac and the hormones, 17α-ethinylestradiol and 17β-estradiol (EU Directive 2013/39/EU). These compounds have been added to a 'watch list' which are not subject to EU standards but are instead closely monitored in EU surface waters for possible future

addition to the priority list. The Oslo-Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) is the only regulatory body to have considered pharmaceuticals as a threat to the environment as early as 2002. This convention monitors environmental conditions and issues standards on the release of hazardous and radioactive materials, eutrophication and marine biodiversity. Under this legislation, clotrimazole, an antifungal agent, is listed for priority action with a number of other pharmaceuticals listed as chemicals of possible concern (Table 1.1).

Table 1.1: OSPAR list of pharmaceuticals of possible concern to the marine environment

Pharmaceutical compound	Class	CAS no.
Chloroquine	Antimalarial	54-05-7
Chloroquine diphosphate	Antimalarial	50-63-5
Chlorpromazine	Antipsychotic	50-53-3
Mitotane	Antineoplastic	53-19-0
Prochlorperazine	Antipsychotic/Antiemetic	58-38-8
Fluphenazine	Antipsychotic	69-23-8
Fluphenazine dihydrochloride	Antipsychotic	146-56-5
Trifluoperazine dihydrochloride	Antipsychotic	440-17-5
Trifluperidol	Antipsychotic	749-13-3
Prochlorperazine edisylate	Antipsychotic/Antiemetic	1257-78-9
Pimozide	Antipsychotic	2062-78-4
Dimetacrine tartrate	Antidepressant	3759-07-7
Niflumic acid	Anti-inflammatory	4394-00-7
Dimetacrine	Antidepressant	4757-55-5
Niclofolan	Anthelmintic	10331-57-4
Miconazole nitrate	Antifungal	22832-87-7
Timiperone	Antipsychotic	57648-21-2
Midazolam	Anxiolitic	59467-70-8
Diammonium N-ethylheptadecafluoro -N-[2-(phosphonatooxy)ethyl] octanesulfonamidate	Chemical auxiliary agent	67969-69-1
Penfluridol	Antipsychotic	26864-56-2
Terofenamate	Anti-inflammatory	29098-15-5
Flunarizine	Antihypertensive	52468-60-7

The current list of pharmaceutical compounds deemed harmful to the aquatic environment is compiled based on ecotoxicology test results showing acute toxicity in the exposed organism. Pharmaceuticals exerting biological effects on organisms over time i.e. chronic exposure, are not currently regulated but should be considered as just as much of a threat to the aquatic environment.

1.2 Sources of Pharmaceuticals in the Aquatic Environment

Human actions, termed as 'involuntarily' and 'purposefully', are primarily responsible for the release of pharmaceuticals into the environment (Daughton, 2007). Involuntary actions include pharmaceutical excretion through the body or washing of topical medicines down the drain. Human pharmaceuticals are excreted into the sewage system as a mixture of the parent compound and metabolites, comprising mostly of transformation products and conjugated glucuronides (Heberer, 2002). Conjugated compounds have been previously shown to be easily cleaved during wastewater treatment, releasing the parent compound into the treated wastewater, and subsequently into the environment (Jelic *et al.*, 2011; Ternes, 1998). In contrast, purposeful actions include the disposal of unused or out of date medicines down the drain or into refuse waste. Medicines disposed of inappropriately into refuse waste enter landfill sites where the unchanged bioactive compounds can leach into the soil. Agricultural medicines administered to farmed animals are also a source of pharmaceutical pollution. Excreted manure, containing the metabolite/unchanged pharmaceutical mix, is often used as a fertilizer resulting in further exposure of these compounds to soil. Sludge from wastewater treatment plants (WWTPs) is also used as a soil fertilizer and may also be a source of pharmaceutical contamination in the environment. Soil leaching and groundwater recharge, caused by heavy precipitation, are the main modes of transportation for pharmaceuticals through the soil and into the aquatic environment. Other sources of pharmaceutical pollution of the aquatic environment include industrial spills and aquaculture. The origins and pathways of pharmaceuticals into the aquatic and terrestrial environment are depicted in Figure 1.1 (Boxall, 2004).

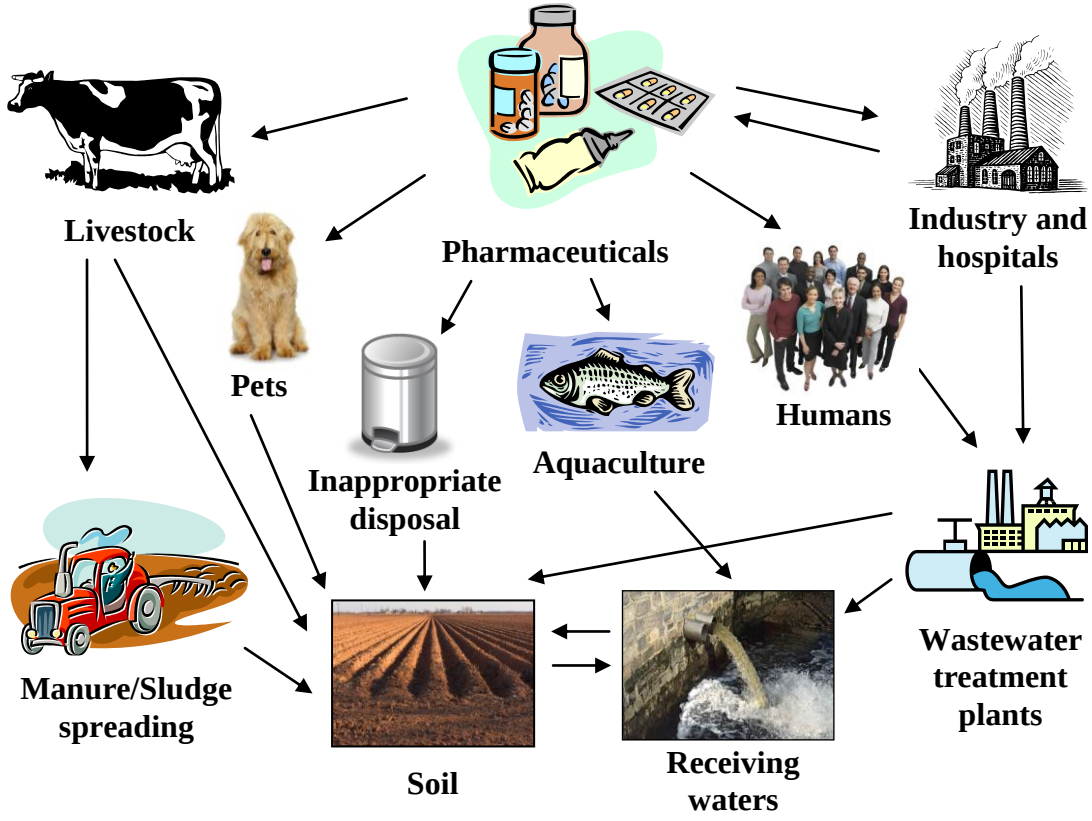


Figure 1.1: Sources of pharmaceuticals in the environment (Boxall, 2004)

1.3 Drug Metabolism

The fate of pharmaceuticals in the aquatic environment depends on numerous factors, such as the degree of transformation of the parent drug, the structure of the newly formed metabolites and the quantity of parent drug and metabolites excreted. Pharmacokinetics is the study of the processes by which a drug is absorbed, distributed, modified and excreted by the body (Rosenbaum, 2011). Currently, pharmaceuticals are designed in such a way that the active ingredient can be released at a targeted site to give the required pharmacological effect. In order to reach specific sites, pharmaceuticals must possess lipophilic properties to pass through the cell membranes of the body. Metabolism is an enzymatic process necessary for the transformation of lipophilic compounds to more polar metabolites suitable for elimination (Gumbleton, 2005). It is often considered as a deactivating process for drugs but for some compounds, known as 'prodrugs', metabolism is required to release the active parent compound and produce a pharmacological

effect (Rautio *et al.*, 2008). Drug metabolism mainly takes place in the liver but other organs such as the intestine, lungs and kidneys have the ability to metabolise drugs also (Gumbleton, 2005).

There are two reaction processes involved in the metabolism of compounds within the body; Phase I reactions involve the addition or exposure of a reactive functional group on the parent molecule and Phase II reactions conjugate the parent compound and/or the Phase I metabolite to a highly polar moiety. Phase I reactions include oxidation, reduction, hydrolysis, hydration, dethioacetylation and isomerisation (Gibson and Skett, 2001). Oxidation is the most common Phase I reaction as it performs numerous different types of functionalisation reactions. It is mostly controlled by cytochrome P450 enzymes, present in the endoplasmic reticulum of liver tissue. Cytochrome P450-catalysed mixed function oxidase (MFO) reactions occur in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and an oxidisable drug substrate. Phase II reactions include glucuronidation, glycosidation, sulfation, methylation, acetylation, condensation and amino acid, glutathione and fatty acid conjugation (Gibson and Skett, 2001). The large and diverse group of enzymes involved in Phase II reactions can only act on drug substrates in the presence of specific cofactors. For example, glucuronidation is the most common Phase II reaction due to the constant presence of the uridine-diphosphate (UDP)-glucuronosyl transferase enzyme (UGT), and its individual cofactor, UDP-glucuronic acid (UDPGA). The result of Phase I and Phase II reactions is the production of highly hydrophilic drug conjugates which are soluble in urine and easily eliminated from the kidneys via urine. There are various metabolic pathways for drugs, some more dominant than others and some equally as important. Phase II conjugation can take place directly for compounds containing reactive hydroxyl, carboxyl, amino and sulfhydryl groups (Faed, 1984). The lipid regulator, gemfibrozil, is a carboxylic acid containing compound and is primarily metabolised by Phase II glucuronidation to form the acyl glucuronide, gemfibrozil 1-*O*-β-glucuronide, shown in Figure 1.2 (Ogilvie *et al.*, 2006). Acyl glucuronides are highly reactive electrophiles, derived from carboxylic acids (Horng *et al.*, 2013). Further oxidation of gemfibrozil 1-*O*-β-glucuronide by hydroxyl groups has been reported at positions R_1, R_2 or R_3, also shown in Figure 1.2 (Ogilvie *et al.*, 2006).

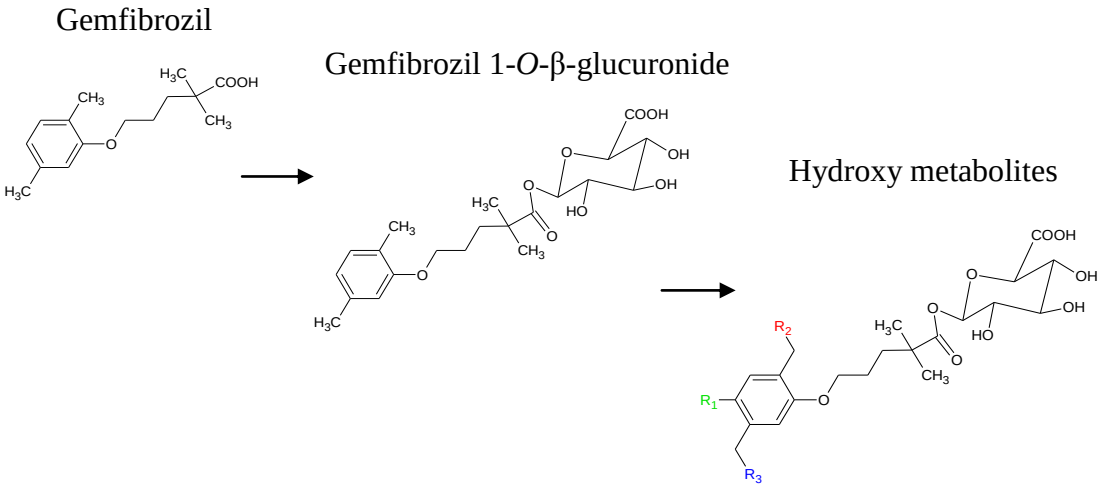


Figure 1.2: The primary metabolic pathway of gemfibrozil (Ogilvie *et al.*, 2006)

Metabolites of pharmaceuticals cannot be rendered unreactive or harmless as in the cases of paracetamol and amitriptyline which are partly metabolised to highly reactive compounds (Graham *et al.*, 2013; Rudorfer and Potter, 1997). Under experimental conditions, water-exposed rainbow trout were found to metabolise pharmaceuticals readily with higher concentrations of metabolites detected in fish bile and plasma than the parent drug (Lahti *et al.*, 2011). Besides the toxicological concerns, the possibility of uptake and metabolism of pharmaceutical compounds in exposed aquatic organisms still warrants further investigation.

1.4 The Wastewater Treatment Process

Municipal wastewater consists of the discharge from domestic and industrial sources and is made up of approximately 99.9 % water and 0.1 % dissolved and suspended solid material (Gray, 2004). The concentration of solid wastewater components varies greatly depending on the extent of industrial and agricultural influence on the catchment area. The main constituents include micro-organisms, biodegradable organic material and non-biodegradable organic material (oil, solvents and persistent organic pollutants (POPs)), nutrients, metals and inorganic materials (Henze *et al.*, 2002).

The collection, treatment and discharge of wastewater is legally required in Ireland under the EU directive 91/271/EEC (EPA Ireland, 1997). The main objective of WWTPs is to prevent or minimise the risk of ecological impact on effluent receiving waters and its surrounding environments by reducing suspended, organic and inorganic matter. The functions of individual WWTPs vary and are very much dependent on the nature of the receiving waters (Forster, 1985). The efficiency of the receiving WWTP is protected under an EU directive concerning industrial emissions, which demands the monitoring of industrial and agricultural wastewater prior to sewage discharge (EU Directive 2010/75/EU).

The treatment of municipal wastewater is a sequential process of mechanical, biological and chemical processes. A typical layout of a wastewater treatment plant is shown in Figure 1.3 (Mjalli *et al.*, 2007). Physical and mechanical processes are carried out during preliminary and primary treatment. Preliminary treatment aims to prevent plant operational problems by screening large objects, debris, wood, grit and oil and by facilitating storm overflow (EPA Ireland, 1997). Primary treatment involves the sedimentation of suspended solids in large clarifier tanks for sufficient lengths of time. After settling, suspended solids and floating organic matter are physically removed from the tank and the wastewater is ready for secondary treatment. Biological processes are carried out during secondary treatment to remove biodegradable and trace organic constituents, colloidal solids and nutrients present in municipal wastewaters (Tchobanoglous *et al.*, 2003). Activated sludge treatment is the most common type of secondary treatment. This type of treatment consists of two phases, aeration and sludge settlement, and aims to reduce the biological oxygen demand (BOD) of the wastewater. In suspended growth processes, such as activated sludge treatment, this process exposes wastewater to a diversified range of micro-organisms i.e. bacteria, protozoa, rotifiers and nematodes, each with distinct trophic levels, forming a complete ecosystem (Gray, 2004). These various micro-organisms form ‘flocs’ or active microbial biomasses which feed on organic matter and absorb colloidal and suspended materials (Horan, 1996). Oxygen diffusers or mechanical aerators, such as paddles, encourage high rates of microbial growth and respiration by providing aeration and help to keep flocs in suspension enabling maximum contact with organic matter in the wastewater (Gray, 2004). The final stage of secondary treatment is clarification of the wastewater by settlement. The formation of flocs is

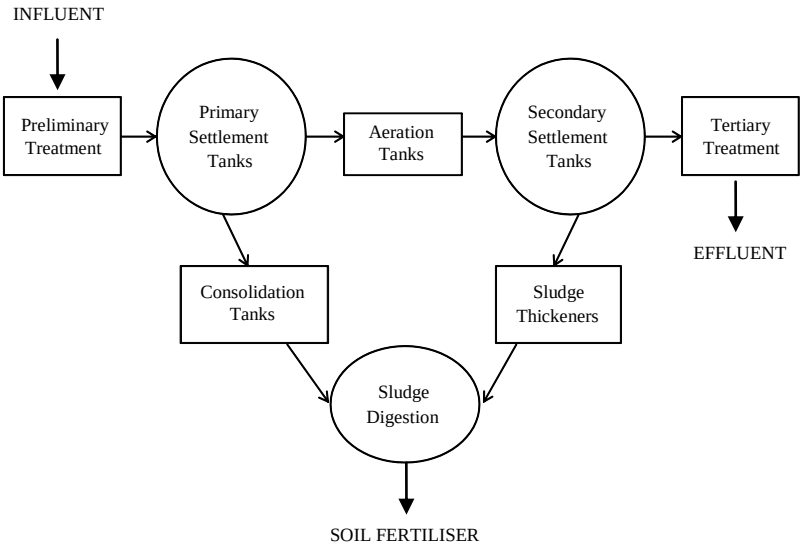


Figure 1.3: Schematic diagram of the basic overview of an activated sludge wastewater treatment plant (Mjalli *et al.*, 2007)

also useful in yielding a clarified effluent as the well-formed flocs settle rapidly into a sludge form which is recycled back into the aeration tanks for further BOD removal (Horan, 1996). The remainder of solids in the settlement tank exits the WWTP and can be used as soil fertiliser or be disposed of in landfills or by incineration. At this stage, the treated water is released from the WWTP as effluent or undergoes tertiary treatment (EPA Ireland, 1997).

Where discharges are made to more sensitive waters such as bathing waters or shellfish growing areas, more stringent treatments are necessary (EPA Ireland, 1997). Tertiary treatments are mainly physical in action and are utilised for the removal of pathogens, residual suspended solids, dissolved organic matter (DOM) and eutrophication causing nutrients such as nitrates and phosphates. Nitrogen, primarily removed during the activated sludge treatment due to nitrification and denitrification processes, can be removed by the air stripping of ammonia. This involves highering the pH of the wastewater to form volatile ammonia gas, which is then eliminated following aeration of the tank. Chemical removal of phosphorous is a low cost technique which requires the addition of metal salts/lime and the subsequent removal of precipitates from the water. Disinfection via ultra-violet (UV) treatment is usually the most common final step of the wastewater treatment process and is important for the removal of harmful pathogens (Tchobanoglous *et*

al., 2003). The removal of DOM has become an important issue in recent years due to the exposure of their negative biological effects on aquatic biota (Farre *et al.*, 2008b). Advanced wastewater treatments are carried out for the removal of dissolved organic and inorganic pollutants using both physical and chemical methods. Techniques for their removal include activated carbon, membrane filtration and advanced oxidation processes (AOPs) such as ozonation (Tchobanoglous *et al.*, 2003).

1.5 Pharmaceuticals in the Wastewater Treatment Process

There are three possible outcomes for pharmaceuticals travelling through the WWTP process; persistence throughout the process and release into receiving waters, transformation to more hydrophobic compounds and sorption to solids/activated sludge and removal after settlement or degradation to less harmful compounds. The degree of hydrophobicity of a compound is important when assessing their fate in the WWTP as more non-polar compounds adsorb onto activated sludge and more polar compounds remain in the water phase, passing through the system unchanged and travelling into receiving waters.

Previous studies have reported low removal efficiencies of pharmaceuticals in the conventional activated sludge process by comparing pharmaceutical concentrations in the influent to those released in the effluent (Jelic *et al.*, 2011; Ternes, 1998). A list of commonly used pharmaceuticals and their removal efficiencies in the biological wastewater treatment process has been compiled in Table 1.2. From the selected literature, it is clear that removal efficiencies can vary as a function of the type of compound being removed. Removal efficiencies for antibiotics ranged from 40 to 86 %, anti-inflammatories between 66 and 95 %, with the exception of diclofenac, β-blockers between 30 and 95 % and lipid regulators generally between 50 and 83 % but for one measurement of gemfibrozil, removal efficiencies of <10 % were recorded. Amitriptyline was removed efficiently during secondary treatment (>95 % efficiency) unlike carbamazepine and diclofenac, which increased in concentration, suggesting the cleavage of glucuronised metabolites and conversion back to their original form. The reconversion of

Table 1.2: Removal efficiencies of selected pharmaceuticals in the conventional activated sludge process

Pharmaceutical class	Compound	% removal	Reference
Antibiotic	Trimethoprim	40-50, 60-70	(Jelic *et al.*, 2011; Kasprzyk-Hordern *et al.*, 2009)
	Ciprofloxacin	86	(Vieno *et al.*, 2007)
Antidepressant	Amitriptyline	>95	(Kasprzyk-Hordern *et al.*, 2009)
Antiepileptic	Carbamazepine	7, −44	(Ternes, 1998; Vieno *et al.*, 2007)
Anti-inflammatory	Diclofenac	−34-40	(Kasprzyk-Hordern *et al.*, 2009; Lishman *et al.*, 2006)
	Ibuprofen	90, 95	(Lishman *et al.*, 2006; Ternes, 1998)
	Naproxen	66, 85, 93	(Kasprzyk-Hordern *et al.*, 2009; Lishman *et al.*, 2006; Ternes, 1998)
β-blocker	Atenolol	80-95, 63	(Kasprzyk-Hordern *et al.*, 2009; Vieno *et al.*, 2007)
	Propranolol	96, 30-75	(Kasprzyk-Hordern *et al.*, 2009; Ternes, 1998)
Lipid regulator	Gemfibrozil	69, <10, 66	(Jelic *et al.*, 2011; Lishman *et al.*, 2006; Ternes, 1998)
	Bezafibrate	83, 50	(Stumpf *et al.*, 1999; Ternes, 1998)

glucuronised compounds was previously demonstrated by Ternes *et al.* (1999) while studying the behaviour of estrogens during the activated sludge process.

Removal efficiencies of WWTPs are also dependent on the treatment technology in place, wastewater retention time in each phase of treatment, solid retention time (SRT) and weather conditions such as rainfall (Vieno *et al.*, 2007). Of all of these parameters, SRT is the most critical in the wastewater treatment process as it has been proved that longer SRTs greatly improve the removal efficiencies of pharmaceuticals (Clara *et al.*, 2005; Lishman *et al.*, 2006). A longer period of activated sludge treatment allows for the growth of diverse bacteria capable of further reducing the concentration of persistent pharmaceuticals (Clara *et al.*, 2005).

Carballa *et al.* (2004) focused upon sampling wastewater at each stage of the treatment process in an attempt to ascertain which treatment step provided the highest rate of removal for selected pharmaceuticals. Preliminary and primary treatments revealed no reduction in concentrations for the anti-inflammatories, ibuprofen and naproxen, and the antibiotic, sulfamethoxazole, however, removal efficiencies of between 40-70 % were achieved following biological treatment. Joss *et al.* (2006) carried out a comprehensive study investigating the biodegradation of PPCPs after biological treatment and found that only 4 out of 35 compounds were degraded by >90 % while 17 compounds were removed by <50 %. The main removal mechanism of some persistent pharmaceuticals in biological WWTPs is sorption on activated sludge, rather than biodegradation (Kim *et al.*, 2008) Previous studies have reported the sorption of pharmaceuticals, such as triclosan, sertraline and citalopram, on digested sludge collected from several WWTPs worldwide at up to $\mu g.g^{-1}$ concentrations (Barron *et al.*, 2008; Heidler and Halden, 2007; Jelic *et al.*, 2011). Other types of biological treatments in operation have not shown removal efficiencies for pharmaceuticals significantly greater than those produced after activated sludge treatment. Membrane bioreactor (MBR) systems use a suspended growth bioreactor, similar to the activated sludge process, but with micro-/ultrafiltration as an alternative to gravity sedimentation (Harper Jr. *et al.*, 2008). With longer SRTs and higher mixed liquor suspended solids concentrations, recent reports have only shown slightly higher efficiencies, if any, in comparison to similarly operated conventional systems (Clara *et al.*, 2004). A trickling filter did not perform as well at removing pharmaceuticals with efficiencies approximately 12-66 % lower than those yielded by activated sludge treatment (Stumpf *et al.*, 1999).

A comprehensive study, carried out by Snyder *et al.* (2008) evaluated advanced WWTP processes individually and in combination for their removal efficiency of selected pharmaceuticals. Studies revealed that UV treatment alone was not enough to give sufficient removal rates for pharmaceuticals but, when in combination with AOPs such as hydrogen peroxide (H_2O_2), reductions of >90 % were reported for the majority of the selected pharmaceuticals. Ozonation was another treatment found to be highly effective for the removal of most target pharmaceuticals with average removal efficiencies of >90 %.

1.6 Pharmaceutical Fate in the Environment

Designed to be robust and stable, most pharmaceuticals have a polar and non-volatile nature, making it difficult for their removal in the aquatic environment. Pharmaceuticals are predominately released into the aquatic environment with treated wastewater effluent but can also be leached through the soil from landfills and contaminated sewage sludge and manure used as fertiliser (Boxall, 2004). Knowledge concerning the fate of pharmaceuticals is essential when attempting to assess the potential risk posed by these micro-pollutants. The regular use and continuous introduction of pharmaceuticals into the aquatic environment confers some degree of pseudo-persistence (Hernando *et al.*, 2006). To date, over 80 pharmaceuticals have been detected at substantial concentrations in various environmental matrices such as surface waters (Ollers *et al.*, 2001; Roberts and Thomas, 2006; Ternes, 1998; Wille *et al.*, 2010), groundwaters (Lapworth *et al.*, 2012) and soils (Barron *et al.*, 2008). Several processes can occur on compounds in the aquatic environment including photochemical degradation, sorption onto solid matrices and, of particular importance in this work, dilution and transportation within the aquatic environment with potential uptake in biological species, which will be covered in more detail in the next section.

Pharmaceuticals are mainly administered orally and are therefore considered resistant to hydrolysis, with previous studies confirming this assumption (Buser *et al.*, 1998b; Lam *et al.*, 2004). Photodegradation is the primary pathway for the abiotic transformation of pharmaceuticals in the aquatic environment. Two types of photolytic reactions can occur; direct absorption of light by the compound, known as direct photolysis, or interaction of a reactive intermediate of another species produced by its absorption of light, known as indirect photolysis (Andreozzi *et al.*, 2003). Both reactions cause instability within the compound causing it to degrade into numerous photoproducts. With only a small number of published papers on this subject to date, research on the photochemical fate of pharmaceutical chemicals is limited. Buser *et al.* (1998b) investigated the photochemical fate of the non-steroidal anti-inflammatory drug (NSAID), diclofenac, in fortified natural lake water and found it was rapidly degraded to <1 % of the initial concentration after four days of exposure to natural sunlight. High rates of photodegradation were observed in similar experiments where diclofenac was exposed to natural sunlight

(Poiger *et al.*, 2001; Schmitt-Jansen *et al.*, 2007). Other pharmaceuticals which readily underwent phototransformation included naproxen, sulfamethoxazole, ofloxacin, propranolol and fluoxetine (Andreozzi *et al.*, 2003; Lam *et al.*, 2005; Packer *et al.*, 2003). In comparison, carbamazepine and clofibric acid were observed to have almost negligible rates of phototransformation upon irradiation (Andreozzi *et al.*, 2003). The antibiotics, oxolinic acid and ciprofloxacin, were also found to degrade at slow rates with oxolinic acid degrading much slower than ciprofloxacin (Turiel *et al.*, 2005). This highlights the persistence of antibiotics in the aquatic environment and the risk of microbial resistance posed by quinolone antibiotics in particular. Photoproducts derived from pharmaceuticals such as ciprofloxacin, naproxen and furosemide, have been found to contain the active group of the pharmaceutical and may have the potential to be more toxic than the parent molecules. Both the parent compound and their degradation products should be considered when carrying out environmental risk assessments of pharmaceuticals (Isidori *et al.*, 2005; Isidori *et al.*, 2006; Turiel *et al.*, 2005).

As previously mentioned, sludge from WWTPs and animal manure are often applied as agricultural fertiliser over farmed land, introducing both human and veterinary pharmaceuticals into the environment. Barron *et al.* (2009) investigated the occurrence of PPCPs in sludge and sludge enriched agricultural soils in Ireland. Significant levels of triclosan and carbamazepine were identified in dried sludge samples, while lower but still relevant concentrations of triclosan and nimesulide were found in the fertilised soil. Pharmaceuticals enter and persist within the soil depending on their capacity for sorption, resistance to photodegradation and affinity for water, which if high will cause leaching from the solid into water systems (Diaz-Cruz *et al.*, 2003). The distribution of pharmaceuticals between the aqueous phase and the solid phase is an equilibrium process, represented by the solid-water distribution coefficient (K_D) whereby;

$$K_D = C_{solid} / C_{aqueous} \qquad \text{Eqn. 1.1}$$

C_{solid} and $C_{aqueous}$ are the pharmaceutical concentrations in the solid and water phases, respectively. K_D values predict whether a substance will show appreciable adsorption or if they will remain in the aqueous phase. Compounds with higher K_D values are considered more of a possible threat to the soil environment. A previous

study on the sorption and fate of 49 pharmaceuticals in biosolid enriched soil revealed over two thirds of all selected pharmaceuticals showed low affinity to the soil matrix with K_D values ≤50 L.kg^{-1} (Barron *et al.*, 2009). Compound properties such as K_D, octanol-water partition coefficients (logK_{ow}) and normalised organic carbon sorption coefficients (K_{oc}) have been previously used to interpret sorption behaviours of pharmaceuticals, but can only be used as an approximate guide due to the complexity of solid matrices and the nature of pharmaceuticals (Carballa *et al.*, 2008). A more recent solids column study reported the leaching of salbutamol, sulfamethoxazole, sulfamethazine, ketoprofen and bezafibrate from sludge and spiked soil. Carbamazepine was mostly retained in the soil with low levels leached into the aqueous phase. The majority of the other pharmaceuticals, including β-blockers and trimethoprim, were also highly retained in the soil but, in the case of indomethacin, residues were mostly retained in the sludge. Interestingly, the majority of pharmaceuticals retained in the sludge or soil were detected at overall concentrations approximately 75 % less than those originally spiked suggesting high levels of transformation within the solid matrices (Barron *et al.*, 2010). Oppel *et al*. (2004) also reported the high retention of carbamazepine in soil along with diazepam and ibuprofen, while clofibric acid was found to seep out with the leachate.

1.7 Pharmaceutical Occurrence in the Aquatic Environment

1.7.1 Surface water

In recent years, monitoring studies have focused on the presence of pharmaceuticals in freshwater environments with comparatively little knowledge regarding the occurrence, distribution and fate of pharmaceuticals in marine or estuarine environments. This may be due to the difficulty of working with a more complex matrix or the assumption that pharmaceutical residues are diluted to negligible concentrations in the marine environment. Considering the vast number of studies on pharmaceutical occurrence in freshwater environments and the scope of this thesis, this section will specifically focus on the monitoring of pharmaceuticals in marine surface waters only.

In a study carried out by Thomas and Hilton (2004), 14 pharmaceuticals were monitored in British estuaries of the Thames, the Tyne, the Mersey, the Tees and Belfast Lough. The selected pharmaceuticals were chosen based on priority lists of the UK Environmental Agency and the OSPAR Commission (Hilton *et al.*, 2003). From the targeted list, 9 pharmaceuticals were detected in the estuarine water samples collected. Ibuprofen and trimethoprim were detected at the highest concentrations, measuring at 928 $ng.L^{-1}$ and 569 $ng.L^{-1}$, respectively, and clotrimazole was the most frequently detected pharmaceutical with a median concentration of 7 $ng.L^{-1}$. A number of other studies have analysed marine water samples collected from the North Sea, its estuaries and harbours. In two separate studies, clofibric acid was the only pharmaceutical detected in the North Sea at concentrations between 1-2 $ng.L^{-1}$ but, further into the River Elbe estuary clofibric acid was detected at concentrations of 18 $ng.L^{-1}$, along with diclofenac and ibuprofen measuring at 6.2 $ng.L^{-1}$ and 0.6 $ng.L^{-1}$, respectively (Buser *et al.*, 1998a; Weigel *et al.*, 2002). A more recent study, carried out by Wille *et al.* (2010), frequently detected residues of salicylic acid, carbamazepine and propranolol in marine surface water collected from several Belgian harbours and estuaries, with lower and less frequent residues of the antibiotics, trimethoprim and sulfamethoxazole, also detected. Further offshore, salicylic acid and carbamazepine were detected up to concentrations of 237 $ng.L^{-1}$ and 12 $ng.L^{-1}$, respectively, in the Belgian coastal area of the North Sea. Residues of ibuprofen and its metabolites were detected in effluent receiving marine waters in Norway at concentrations up to 7.7 $ng.L^{-1}$ (Weigel *et al.*, 2004). Langford and Thomas (2011) also reported the presence of the active metabolites, carbamazepine-10,11-epoxide and simvastatin hydroxy carboxylic acid, in marine coastal waters from Oslofjord at concentrations greater than their parent compounds, highlighting the need for environmental risk assessments of active metabolites.

Monitoring studies in the Mediterranean Sea revealed the presence of pharmaceuticals in the low $ng.L^{-1}$ range. Eight pharmaceuticals, including verapamil, atenolol and metolol, were detected in marine surface water collected from several bays around the island of Mallorca (Rodriguez-Navas *et al.*, 2013). Munaron *et al.* (2012) deployed passive samplers in French Mediterranean coastal waters for a period of 14-28 days and detected the presence of carbamazepine, theophilline and terbutaline. Outside of Europe, Comeau *et al.* (2008) investigated

pharmaceutical residues in Canadian marine surface water collected downstream from a WWTP. Although low residues of naproxen, gemfibrozil, ibuprofen and salicylic acid were detected in the effluent receiving waters, none of these acidic compounds were detected in the marine surface waters sampled at an estuary further downstream. Recently, a list of 108 PPCPs and alkylphenols were targeted in a monitoring study carried out in San Francisco Bay, California. From this list, 10 pharmaceuticals were detected in the marine waters of all 5 sampling locations with carbamazepine, gemfibrozil, sulfamethoxazole, and valsartan among the highest concentrations, measuring between 38-92 $ng.L^{-1}$ (Klosterhaus *et al.*, 2013). The detection of pharmaceuticals and their metabolites in marine surface waters suggests a high level of persistence of these compounds in the aquatic environment.

1.7.2 Aquatic biota

One of the main concerns surrounding pharmaceutical release into surface waters is their potential to bioaccumulate in aquatic organisms. Bioconcentration is the concentration of a compound in or on an organism through water exposure alone whereas bioaccumulation is the uptake of a chemical by an organism through a combination of water, food, sediment and air, as occurs in the natural aquatic environment (Arnot and Gobas, 2006). There are several elements that can influence the bioaccumulation of a pharmaceutical including its physicochemical nature, its bioavailability in the aquatic system, biotic factors relating to the exposed aquatic organism and the temperature, pH, flow and quality of its residing waters (Bremle *et al.*, 1995; Nakamura *et al.*, 2008; Rendal *et al.*, 2011). K_{ow} values determine the partition of a compound between octanol and aqueous phases, however, due to the ionisation of pharmaceuticals, the octanol-water distribution coefficient (D_{ow}) is a more reliable measure for bioaccumulation potential as both the neutral and ionised fractions of the compound are considered at a given pH (Cunningham, 2008).

Several studies have investigated the occurrence of pharmaceutical residues in wild aquatic species, focusing mainly on accumulation in wild fish species. The first of such work was carried out by Brooks *et al.* (2005) whereby numerous antidepressants were screened for in the tissues of wild fish residing in two separate effluent dominated streams in the US. Two antidepressants, fluoxetine and

sertraline, and their two respective metabolites, norfluoxetine and desmethylsertraline, were detected at levels greater than 0.1 $ng.g^{-1}$ wet weight (WW) in all tissues, with highest concentrations measured in the liver and brain. Additional selective serotonin reuptake inhibitors (SSRIs) and their metabolites have also been detected in similar studies carried out more recently in the US, again at low $ng.g^{-1}$ concentrations (Chu and Metcalfe, 2007; Schultz *et al.*, 2010). In 2009, Ramirez *et al.* (2009) led a national pilot study, to investigate the occurrence of PPCPs in fish fillet and liver from effluent-dominated rivers throughout the United States. All fish tissues tested positive for norfluoxetine, sertraline, diphenhydramine, diltiazem and carbamazepine, with the addition of gemfibrozil and fluoxetine detected in the liver. At one particular exposure site, sertraline was measured at concentrations up to 545 $ng.g^{-1}$ WW in the liver of the wild white sucker fish species. In Sweden, studies on juvenile rainbow trout sampled from several sewage effluent outfall sites revealed the presence of ibuprofen, diclofenac, naproxen, ketoprofen and gemfibrozil in the fish plasma with gemfibrozil detected at the highest concentrations (Brown *et al.*, 2007). More recently, Huerta *et al.* (2013) collected 11 wild fish species from 4 Mediterranean rivers in Spain for pharmaceutical and metabolite analysis. From the 20 selected compounds, 9 pharmaceuticals were detected in fish homogenates and liver tissues. Diclofenac was the most recurring compound, detected in 9 % of all samples collected, and carbamazepine measured highest at a concentration of 18 $ng.g^{-1}$ in fish liver. Bivalves, such as mussels, are natural filter feeders which have been widely utilised in POP monitoring programmes because of their high bioaccumulation capacities, fixed conditions and high populations in marine waters (Hunt and Slone, 2010; Monirith *et al.*, 2003). The uptake of pharmaceuticals, such as antibiotics, has been previously observed in mussel species collected from the Bohai Sea in China over a period of four years (Li *et al.*, 2012). Most recently, wild ribbed horse mussels sampled from five near shore sites in San Francisco Bay were found to contain low residues of carbamazepine and sertraline at concentrations up to 2.4 $ng.g^{-1}$ and 0.3 $ng.g^{-1}$ WW, respectively (Klosterhaus *et al.*, 2013).

The use of caged sample studies involves the caging of uncontaminated species at effluent outfall sites for a number of days. Fick *et al.* (2010) exposed cages of rainbow trout at three different effluent exposure sites for a period of 14 days. Out of the 25 pharmaceuticals present in the effluent, 16 were detected in the

fish plasma. In a similar study, two antidepressants were detected at high $ng.L^{-1}$ levels in the bile of rainbow trout exposed downstream to a Canadian WWTP for 14 days (Togunde *et al.*, 2012). With regard to pharmaceutical exposure studies using caged mussels, a recent study deployed five cages of blue mussels off the Belgian coast. Over a six-month period, five pharmaceuticals were detected in mussel tissues including salicylic acid residues, measuring up to 490 $ng.g^{-1}$ dry weight (DW) (Wille *et al.*, 2011). In a separate study in North Carolina, the antidepressant, fluoxetine, was detected in caged mussel tissues up to 79 $ng.g^{-1}$ WW after 14 days exposure in a wastewater effluent channel (Bringolf *et al.*, 2010).

1.8 Environmental Risk Assessment and Ecotoxicity of Pharmaceuticals

The European Medicines Evaluation Agency (EMEA) is responsible for the licensing and registration of medicinal products in the EU. Under the EU Directive 93/39/EEC, an environmental risk assessment is required prior to drug licensing, to determine any significant toxicological risks associated with a drug (Straub, 2002). The EMEA risk assessment procedure is carried out over three sections with the first involving the calculation of a predicted environmental concentration (PEC) value. PEC values are calculated by taking into account the defined daily dose in mg per person per day, population size treated, volume of wastewater produced per person per day and dilution factor in receiving waters (Huschek *et al.*, 2004). Calculated PEC values must be <0.01 $\mu g.L^{-1}$ to complete the risk assessment, otherwise the investigation proceeds to the next section where the predicted no effect concentration (PNEC) value is calculated. This value is based on acute toxicity data generated from standardised testing on algae, *daphnia* or fish. The assessment is complete if the PEC/PNEC ratio is <1 but if it exceeds this value, completion of the third section is required which may include further testing and safety measures such as chronic toxicity testing, modeling environmental fate, field studies, specific product labeling and restricted use (Straub, 2002).

Pharmaceuticals are designed to target specific receptors in humans and animals, and thus are extremely potent, even at low concentrations. As previously

mentioned, the main concern surrounding pharmaceutical release into surface waters is the potential for pharmaceuticals to target similar receptors in non-target aquatic species, causing similar effects or side-effects (Fent *et al.*, 2006). Under the current regulatory guidelines, environmental toxicity testing of pharmaceuticals only requires for standard acute toxicity tests to be carried out unless the PEC/PNEC ratio is >1 and further testing is required. Standardised toxicity tests on aquatic organisms are generally limited to algae, *daphnia* and/or fish from freshwater environments. This approach increases the uncertainty of the environmental impact of chemicals on other freshwater and marine aquatic species and even on the tested aquatic species due to intra species variations, such as age and gender, resulting in different sensitivities (Jones *et al.*, 2007). Toxic effects observed upon exposure of aquatic organisms to environmentally relevant pharmaceutical concentrations are listed in Table 1.3. The main effects observed in exposed aquatic biota were increased oxidative stress conditions and alterations in fish organs, mainly the kidneys.

Reactive oxygen species (ROS) are naturally produced in biological systems but, in the presence of chemical contaminants, their production is increased (Romeo and Giamberini, 2013). In response, a number of antioxidant enzymes are induced which have the potential to cause damage to biological tissue resulting in biochemical lesions. Oxidative stress is caused by an imbalance in favour of the production of ROS instead of their neutralisation by antioxidant defence mechanisms (Romeo and Giamberini, 2013). Due to the relationship between environmental pollutants and molecular responses of oxidative stress, the use of antioxidant enzymes, such as glutathione S-transferase (GST) and glutathione, as biomarkers of exposure has been reported to successfully highlight oxidative stress in exposed organisms (Hellou *et al.*, 2012). Biomarkers are a more sensitive endpoint than standard toxicity tests however, they can lack sensitivity and selectivity as response can vary depending on several biotic and abiotic factors.Hence, proteomics has recently come forward as a more robust method of assessing stress in exposed organisms and potential contamination (Campos *et al.*, 2012; Schmidt *et al.*, 2013).

Table 1.3: Effects of pharmaceuticals at environmentally relevant concentrations on non-target species following chronic exposure studies

Compound	Organism	Exposed concentration	Exposure period	Toxicity	Reference
Antidepressants	Fish	WWTP effluent	3 mth	Inhibited activity of the ATP-dependent Na/K-ATPase	(Lajeunesse *et al.*, 2011)
Carbamazepine	Mollusc	1-1000 nM	7 d	Increased oxidative stress	(Contardo-Jara *et al.*, 2011)
	Cnidarian	0.1-5 µM	48 h	Increased oxidative stress >30 nM	(Quinn *et al.*, 2004)
	Fish	1-100 $\mu g.L^{-1}$	28 d	Alterations to kidney >1 $\mu g.L^{-1}$	(Triebskorn *et al.*, 2007)
Diclofenac	Fish	1-500 $\mu g.L^{-1}$	28 d	Kidney lesions and alterations in gills >5 $\mu g.L^{-1}$	(Schwaiger *et al.*, 2004)
	Fish	1-500 $\mu g.L^{-1}$	28 d	Alterations to liver, kidney, gills & intestine >1 $\mu g.L^{-1}$	(Triebskorn *et al.*, 2004)
	Fish	0.5-25 $\mu g.L^{-1}$	21 d	Kidney & intestinal lesions >1 $\mu g.L^{-1}$	(Mehinto *et al.*, 2010)
	Fish	1-100 $\mu g.L^{-1}$	14 d	Effects on hepatic gene expression	(Cuklev *et al.*, 2011)
	Mollusc	1 $\mu g.L^{-1}$	96 h	Increased oxidative stress	(Quinn *et al.*, 2011)
	Mollusc	1 $\mu g.L^{-1}$	14 d	Increased oxidative stress	(Schmidt *et al.*, 2013)
Gemfibrozil	Mollusc	1 $\mu g.L^{-1}$	96 h	Increased oxidative stress	(Quinn *et al.*, 2011) (Schmidt *et al.*, 2011)
	Mollusc	1 $\mu g.L^{-1}$	14 d	Increased oxidative stress	(Schmidt *et al.*, 2013)
Ibuprofen	Mollusc	1-1000 nM	7 d	Increased oxidative stress	(Contardo-Jara *et al.*, 2011)
	Amphipod Crustacean	1-1x10^{6} $ng.L^{-1}$	2 h	Behavioural activity changes >10 $ng.L^{-1}$	(De Lange *et al.*, 2006)
Metaprolol	Fish	1-100 $\mu g.L^{-1}$	28 d	Alterations to liver >1 $\mu g.L^{-1}$	(Triebskorn *et al.*, 2007)

Computerised models using quantitative structural activity relationships (QSARs) have also been employed to assess the potential risk posed by pharmaceuticals. QSARs compare pharmaceuticals to similar, previously tested, compounds based on their structure and composition to predict toxicity data (Perkins *et al.*, 2003). Due to the lack of ecotoxicological data, QSAR models have proved useful by providing reasonable environmental risk data on the chronic toxicity of pharmaceuticals in surface waters (Sanderson *et al.*, 2003). A previous study ranked 2986 different pharmaceuticals relative to the risk they pose to algae, daphnids and fish using a QSAR model. Cardiovascular pharmaceuticals ranked the most hazardous therapeutic class with daphnids and algae found to be the most and least susceptible species to change following pharmaceutical exposure (Sanderson *et al.*, 2004).

1.9 Pharmaceutical Exposure to Humans

Unbeknownst to us, human health can also be exposed to pharmaceuticals through food and drinking water. The widespread use of veterinary medicines in aquaculture and livestock has generated great concern of developing antibiotic resistance. This may be the reason as to why antibiotics are the most investigated compounds when it comes to assessing the potential risk of pharmaceutical exposure to humans via trophic level transfer. In Canada, a 'Total Diet Study' was carried out to test the presence of veterinary drugs in fresh and canned fish and shrimp produced for human consumption. Results confirmed the exposure of humans to low $ng.g^{-1}$ concentrations of some banned and upapproved veterinary drug residues via consumption of these farmed seafoods (Tittlemier *et al.*, 2007). The European Food Safety Authority (EFSA) conducts an annual report on the monitoring of veterinary pharmaceutical residues in live animals and animal products from 27 member states (EFSA, 2013). The most recent report detailed the results from the year 2011 with tested animals and animal produce including bovines, pigs, sheep and goats, horses, poultry, rabbit, farmed game, wild game, aquaculture, milk, eggs and honey. For antibiotics, 0.2 % of just over 400,000 tested samples were deemed non-compliant, with the highest reoccurrence in honey. Other drugs such as NSAIDs and steroids were also detected in less than 0.7

% and 0.1 % of tested samples, respectively. The potential for human health risks due to exposure to contaminated seafood and animal produce are not confined geographically to one location due to global trade, making this a worldwide issue (Sapkota *et al.*, 2008).

Pharmaceutical residues found in drinking water are mostly polar compounds with a weak binding affinity to soil particles which enable them to pass through the soil to the groundwater. The presence of pharmaceuticals in groundwater is a cause for concern as groundwater is often reused and recycled as potable water. As previously mentioned, pharmaceuticals have been detected in drinking water supplies at low $ng.L^{-1}$ concentrations providing direct entry for pharmaceuticals into the human body (Benotti *et al.*, 2009; Focazio *et al.*, 2008; Heberer *et al.*, 2000; Stan and Heberer, 1997). The extent of exposure to humans has been deemed as negligible in most cases, citing 'no appreciable risk to humans exist' but the risk of chronic exposure to pharmaceuticals needs to be further assessed (Cunningham *et al.*, 2009; Schwab *et al.*, 2005).

1.10 Pharmaceutical Analysis of Environmental Samples

Since the initial detection of pharmaceuticals in the environment, instrumentation has advanced considerably, enabling the quantification of pharmaceuticals at part per trillion levels in very complex matrices. Within this review, the objective of this section was to highlight the most recent advances and current approaches in the methods carried out for the analysis of pharmaceuticals in the natural aquatic environment, covering both water and aquatic biota samples.

Most recently, the need for more efficient sample preparation and instrumental methods of analysis has resulted in the development of numerous multi-class detection methods for pharmaceuticals (Ferrer *et al.*, 2010; Gracia-Lor *et al.*, 2011; Gros *et al.*, 2006b; Petrovic *et al.*, 2006; US EPA, 2007). These methods vary in procedure and technique but the basic sample preparation steps, depicted in Figure 1.4, are fundamental to all methods of analysis for pharmaceuticals in solid and aqueous samples (Comerton *et al.*, 2009; Reemtsma and Quintana, 2006).

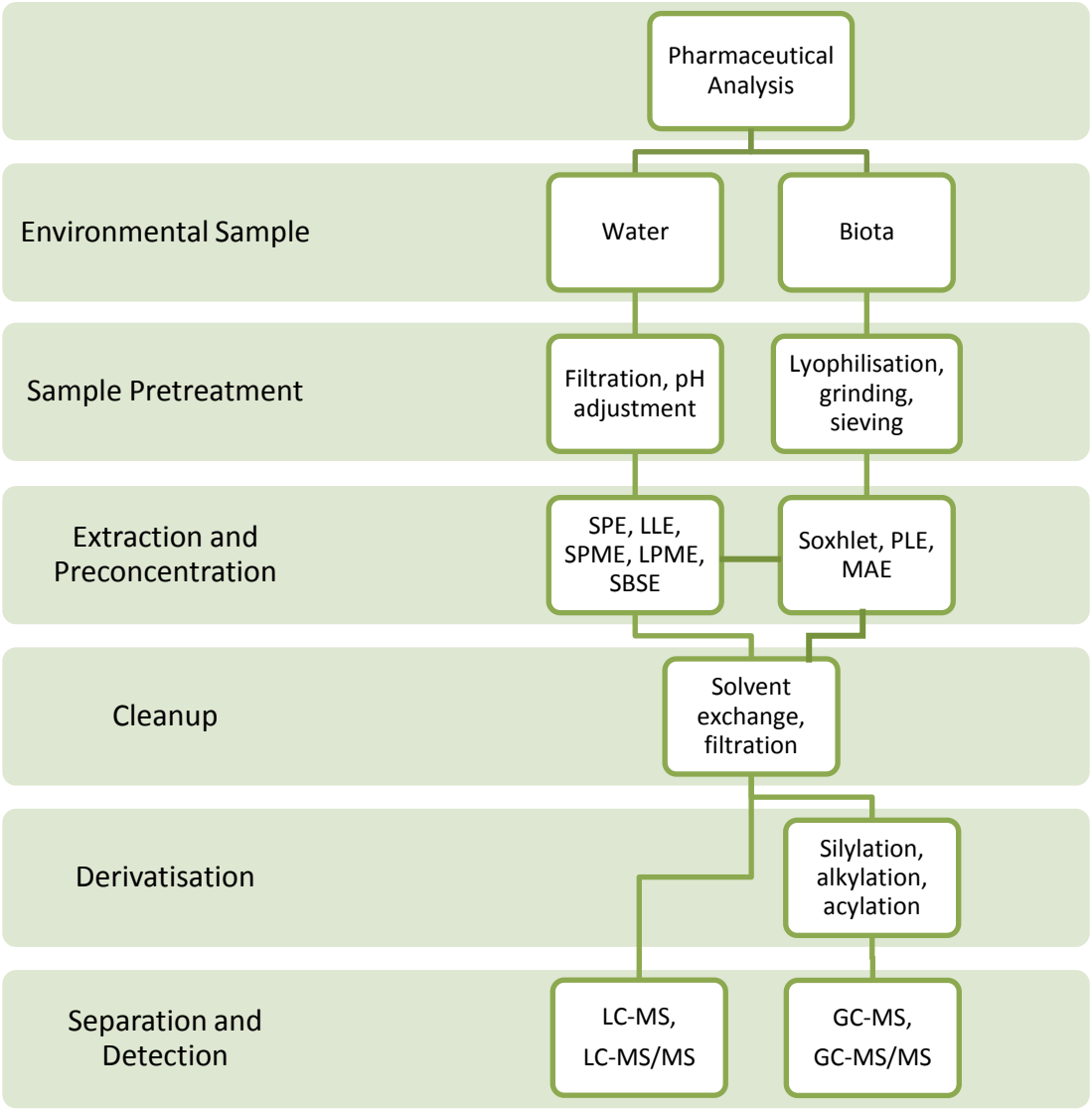


Figure 1.4: Sample preparation and analysis of pharmaceuticals in aqueous and biotic matrices (Reemtsma and Quintana, 2006)

Water samples include wastewater influent and effluent, marine water, freshwater groundwater and drinking water. Solid samples include biological tissue, soil and sludge but, within this review, we will discuss the techniques carried out to date on aquatic biological tissues only.

1.10.1 Water analysis

In order to minimise any initial losses of analytes, certain approaches are taken for sample collection and pre-treatment. Water samples are filtered soon after collection, typically with glass fibre filters (0.2-1.2 µm), to remove suspended particulate matter (SPM) present in the sample and reduce the clogging of extraction sorbents (Jjemba, 2008). Adjustment of sample pH is carried out to determine analyte speciation and promote interactions with the solid phase extraction (SPE) sorbent, enhancing analyte recovery (Comerton *et al.*, 2009).

For complex environmental samples, extraction is required to isolate and pre-concentrate target analytes, reduce or remove unwanted matrix components and overall, increase method sensitivity. A wide range of multi-class pharmaceuticals have been found to be reasonably extracted from aqueous samples and pre-concentrated using a technique known as SPE (Gros *et al.*, 2006a; Li *et al.*, 2006). Currently the most common sample preparation technique in environmental analysis, SPE is less time consuming, less wasteful and more sensitive than the traditionally used liquid-liquid extraction (LLE) (Pichon, 2000; Wu *et al.*, 2010). Two basic approaches to SPE are off-line i.e. extraction is separate to the analyte separation step and the SPE sorbent is usually packed in disposable cartridges, and on-line i.e. extraction is coupled with the analyte separation step by means of packing the sorbent into a pre-column in the injection loop of a high performance liquid chromatography (HPLC) system (Pichon, 2000). Off-line SPE can be time consuming and laborious but, in comparison to on-line SPE, the risk of contamination from sorbent reuse is eliminated and costs are somewhat reduced. Both off-line and on-line SPE techniques yield comparable values for precision and sensitivity (Trenholm *et al.*, 2009) but, for the vast majority of methods, off-line SPE is the method of choice (Gros *et al.*, 2009; Petrovic *et al.*, 2010).

A large variety of off-line SPE sorbents are commercially available with selection based on the nature of the analyte and the sample matrix type. Mixed-mode SPE sorbents have emerged onto the market with the advantage of displaying both hydrophobic and ion exchange properties and allowing for multi-class analysis of pharmaceuticals in aqueous samples. The most popular of these new polymeric sorbents are Oasis HLB, a copolymer of divinylbenzene and vinylpyrrolidone, manufactured by Waters, and Strata-X, a polydivinylbenzene resin containing piperidone groups, manufactured by Phenomenex. These sorbents have shown the

highest extraction efficiencies for numerous pharmaceuticals of varied classes (Cahill *et al.*, 2004; Gomez *et al.*, 2007; Lacey *et al.*, 2008; Roberts and Bersuder, 2006). Other mixed-mode cartridges used for pharmaceutical extraction include Oasis MXC (mixed-mode cation exchange) (Batt *et al.*, 2008; Castiglioni *et al.*, 2005; Kasprzyk-Hordern *et al.*, 2008) and Oasis MAX (mixed-mode anion exchange) (Laven *et al.*, 2009; Sousa *et al.*, 2011) both manufactured by Waters. Following extraction, analytes retained on SPE sorbents will remain stable until further analysis, once stored at −20 °C (Baker and Kasprzyk-Hordern, 2011).

More recently, molecularly imprinted polymers (MIPs) were developed to overcome the problem of poor recovery of polar analytes. This highly selective extraction technique only recognises molecules matching the shape and functional group position of the template in the polymer (Buszewski and Szultka, 2012). Although there have been several studies which have utilised MIPs for pharmaceutical extraction from environmental water samples (Beltran *et al.*, 2007; Gros *et al.*, 2008; Sun *et al.*, 2008; Zorita *et al.*, 2008), this is not a practical method for the analysis of multi-class pharmaceuticals. Sorptive extraction methods have also been used for the extraction of low-level pharmaceutical residues from complex water samples and include solid phase micro-extraction (SPME) (Araujo *et al.*, 2008; Rodriguez *et al.*, 2004; Wen *et al.*, 2006) and stir-bar sorptive extraction (SBSE) (Luo *et al.*, 2011; Quintana *et al.*, 2007; Van Hoeck *et al.*, 2009). Low detection limits were also determined for NSAIDs using liquid phase micro-extraction (LPME) in conjunction with LC-MS/MS and gas chromatography-mass spectrometry (GC-MS) analysis (Es'haghi, 2009; Quintana *et al.*, 2004). Although alternative techniques to SPE may be speedier, more cost efficient and less wasteful, higher sensitivity and precision values are achieved when using SPE for the extraction of multi-class pharmaceuticals (Fatta *et al.*, 2007).

Highly sensitive methods are required to determine the low levels of contaminants present in complex environmental samples. Hyphenated techniques, such as GC-MS and LC-MS, combine chromatographic separation with spectrometric detection and produce a highly sensitive analytical technique with high specificity. The traditional GC-MS approach is suitable for the analysis of thermally stable and volatile analytes, hence, a lengthy derivatisation procedure is required prior to pharmaceutical analysis. Such an approach is unfavourable as not only is it time consuming but, it also increases the level of variability of the method

(Fatta *et al.*, 2007). For these reasons, LC-MS has been extensively employed for the identification and quantification of a wide range of multi-class pharmaceuticals in environmental samples (Buchberger, 2011; Wu *et al.*, 2010).

LC is most commonly used in reverse phase with the mobile phase consisting of an aqueous phase (water) and an organic phase (usually acetonitrile or methanol). The aqueous phase often has the addition of a volatile additive such as ammonium acetate or formic acid to enhance ionisation efficiencies of basic and acidic pharmaceuticals, respectively. Ultra performance liquid chromatography (UPLC) is a new technique which exploits sub 2 µm particle-packed columns to produce faster and more resolved separations of pharmaceuticals (Wille *et al.*, 2012). Numerous analytical methods have been developed using UPLC and separations of up to 70 pharmaceutical residues in less than 7 mins have been reported (Batt *et al.*, 2008; Farre *et al.*, 2008a; Gracia-Lor *et al.*, 2011; Kasprzyk-Hordern *et al.*, 2007; Kasprzyk-Hordern *et al.*, 2008; Petrovic *et al.*, 2006). Limits of detection (LODs) were in the low $ng.L^{-1}$ range when combined with an MS detector, similar to conventional HPLC analysis.

Mass spectrometry has been the detection technique of choice for the analysis of pharmaceutical residues in aqueous samples for many years. Over the past two decades, LC-MS technologies have greatly advanced in reliability, sensitivity and selectivity with detection limits in the $ng.L^{-1}$ range and lower. Electrospray ionisation (ESI) is the most commonly used atmospheric pressure ionisation interface for the coupling of LC with mass spectrometry, compared to atmospheric pressure chemical ionisation (APCI) and atmospheric-pressure photoionisation (APPI) (Krauss *et al.*, 2010). A drawback associated with LC is the co-elution of unwanted matrix constituents with analytes of interest resulting in a decrease in ionisation efficiencies. The use of internal standards has somewhat overcome this problem but, depending on costs, availability and variability of the matrix, standard addition has proved to be just as effective (Botitsi *et al.*, 2007; Van De Steene *et al.*, 2006).

Quadrupole mass analysers set up in tandem are most frequently used for routine target analysis of complex environmental samples due to their relatively low cost and ability to fragment ions necessary for the accurate identification of analytes (Comerton *et al.*, 2009). The most common types of quadrupole mass analysers used are the triple quadrupole (QqQ) mass analyser and the ion trap, with

typical limits of detection for pharmaceuticals in contaminated waters measuring in the low $ng.L^{-1}$ concentration range (Petrovic *et al.*, 2005). These analysers offer high sensitivity and selectivity in selected reaction monitoring (SRM) mode for target analysis (Zwiener and Frimmel, 2004). For the accurate mass screening of both known and unknown compounds, the time-of-flight (TOF) and orbitrap mass analysers are applied due to their high resolving power, high mass accuracy and high sensitivity, down to the femtogram range (Krauss *et al.*, 2010). However, these techniques are much more expensive to run and have not been carried out as often in environmental analysis (Nurmi and Pellinen, 2011; Radjenović *et al.*, 2007). Recently, a new type of hybrid instrument combining the detection and identification capabilities of two different mass spectrometers has emerged. Triple quadrupole linear ion trap (QqLIT) (Bueno *et al.*, 2007; Gros *et al.*, 2012; Huerta-Fontela *et al.*, 2010; Jelic *et al.*, 2009), quadrupole time of flight (QTOF) (Farre *et al.*, 2008a; Ibanez *et al.*, 2009; Magner *et al.*, 2010; Petrovic and Barceló, 2006) and linear ion trap orbitrap (Cahill *et al.*, 2012; Hogenboom *et al.*, 2009) have the abilities to unequivocally identify pharmaceuticals in complex environmental matrices due to their full-scan product-ion spectrum and high resolution exact mass measurement of both precursor and product ions. In order to avoid the reporting of false positives of pharmaceutical residues in complex environmental matrices, the European Union Commission Decision 2002/657/EC requires the detection of at least four identification points for LC-MS/MS analysis. This can be achieved with the detection of one precursor ion and two daughter ions or two precursor ions each with one daughter ion (EU Decision 2002/657/EC, 2002).

LC in tandem with spectrophotometric detection, such as UV detection, diode array detection (DAD) and fluorescence are less expensive techniques which have been also previously utilised in the analysis of pharmaceuticals in environmental waters (Benito-Pena *et al.*, 2006; Garcia *et al.*, 2009; Kim *et al.*, 2013; Seifrtova *et al.*, 2008). However, the need for higher sensitivities in more complex environmental matrices is required, and the wider availability of mass spectrometers has resulted in a decrease in studies utilising these techniques.

A combination of both LC-MS and GC-MS techniques is recommended for the reliable confirmation and measurement of a wider range of compound properties (Comerton *et al.*, 2009). GC-MS is generally carried out using electron impact (EI) ionisation with previous studies reporting LODs for pharmaceuticals in

the low $ng.L^{-1}$ concentration range in environmental waters (Reddersen and Heberer, 2003; Togola and Budzinski, 2008).

1.10.2 Biota analysis

Prior to clean-up and extraction, biotic samples are de-shelled/deboned, if required, and dissected. Tissues are usually separated and pooled before homogenisation. Depending on the method, extraction can then be carried out or frozen samples can be freeze-dried, ground down to a powder and sieved prior to extraction. In comparison to studies on water, sediment and food, relatively fewer studies have investigated the presence of pharmaceutical residues in aquatic species. In most cases, the methods developed for sediment and food matrices can be easily adapted for the analysis of aquatic biota. In solid samples, soxhlet extraction has been replaced by alternative methods which use significantly less volumes of organic solvent. The most common extraction techniques carried out on aquatic biota include LLE (Brooks *et al.*, 2005; Dussault *et al.*, 2009; Klosterhaus *et al.*, 2013; Nallani *et al.*, 2011) followed by less solvent consuming techniques such as pressurised liquid extraction (PLE) (Chu and Metcalfe, 2007; Ramirez *et al.*, 2007; Schultz *et al.*, 2010; Wille *et al.*, 2011) and SPME (Togunde *et al.*, 2012; Zhou *et al.*, 2008). Microwave-assisted extraction (MAE) with micellar media is another extraction technique recently developed and previously employed for the quantification of six pharmaceuticals in mussel tissue (Cueva-Mestanza *et al.*, 2008). Following extraction of pharmaceuticals from solid samples, further clean-up of the aqueous extract may be required using extraction methods such as SPE.

A recent review, carried out by Huerta *et al.* (2012), compiled a list of previously applied analytical techniques for pharmaceutical analysis in biological tissues. From this list, LC-MS/MS was the most widely applied technique with the QqQ mass analyser utilised for the majority of studies and the QqLIT also utilised in two of the reported studies for the analysis of pharmaceuticals in both fish and molluscs (Contardo-Jara *et al.*, 2011; Schultz *et al.*, 2010). Most of the LODs reported using LC-MS/MS techniques were at concentrations <1 $ng.g^{-1}$. More recent studies not included in the review have analysed wild marine mussels for up to 104 PPCPs by LC-QqQ analysis (Klosterhaus *et al.*, 2013), combined UPLC with a QqQ mass analyser for the quantification of 11 pharmaceuticals in caged blue mussels (Wille *et al.*, 2011), detected a range of multi-class pharmaceuticals in

the tissues of wild fish analysed by LC-QqLIT (Huerta *et al.*, 2013; Togunde *et al.*, 2012) and quantified 5 pharmaceuticals in wild marine mussels from the Mediterranean Sea using an orbitrap mass analyser (Bueno *et al.*, 2013). Besides MS detection, other techniques previously used in tandem with LC for the detection of pharmaceuticals in biota include UV, DAD and fluorescence detection (Cueva-Mestanza *et al.*, 2008; Fernandez-Torres *et al.*, 2011; Schroder and Machetzki, 2007). Although these methods of analysis measured LODs at low $ng.g^{-1}$ concentrations, these measurements were still slightly higher than those produced by LC-MS/MS analysis. GC-MS has also been utilised in the detection of diclofenac and antidepressants in fish tissues (Brooks *et al.*, 2005; Nakamura *et al.*, 2008; Schwaiger *et al.*, 2004). There are a number of recently developed analytical techniques for the detection of pharmaceuticals in aquatic biota, however, there is still a need for more sensitive, reproducible and transferable methods which can be applied to a range of aquatic species.

1.11 Conceptual Framework

The research carried out in this thesis is encompassed in a large scale EPA Science, Technology, Research and Innovation for the Environment (STRIVE) project entitled 'The assessment and potential human impact of exposure to environmental contaminants on marine and freshwater bivalves'. The objective of this project was to combine chemical and biological analysis in an attempt to assess with an integrated approach the extent and effects of pharmaceutical pollution in the aquatic environment with the ultimate aim of developing a simple bioassay for fast, reliable identification and toxicological assessment of pharmaceuticals in environmental samples. An overview of the project in its entirety is presented in Figure 1.5, with the work undertaken in this thesis outlined under the chemical analysis section.

Pharmaceuticals have recently gained more attention and concern due to their possible toxicity and potential to cause adverse effects in marine organisms. Additionally, the potential for direct human exposure via ingestion of contaminated seafood needs to be investigated further, given the limited evidence thus far.

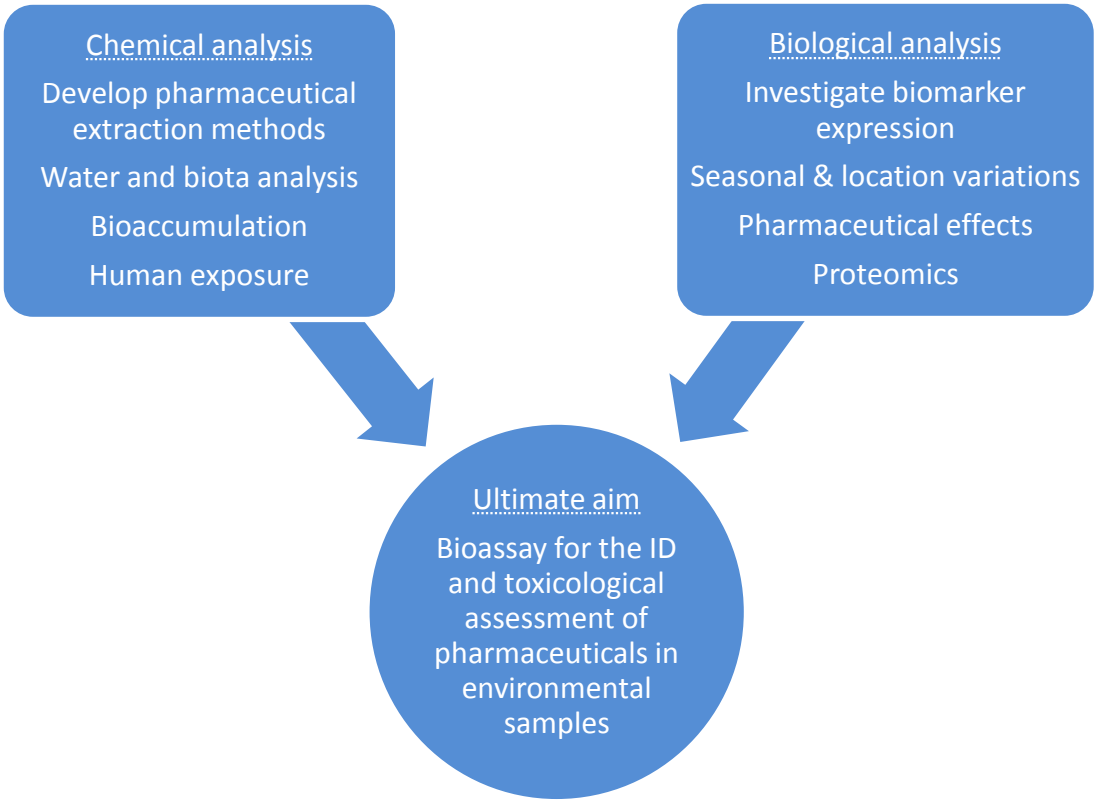


Figure 1.5: Overview of the objectives and ultimate aim of the research project including both chemical and biological analysis

Information regarding the presence of pharmaceuticals in aquatic ecosystems is urgently required in order to assess their fate and effects and the risks posed to the marine environment and human health. Therefore, the main objectives of this thesis were as follows:

- To optimise and validate analytical methods for the quantification of pharmaceuticals in aqueous samples, such as wastewater effluent and marine surface waters, and aquatic biota samples from the Irish aquatic environment.
- To investigate the occurrence and distribution of selected pharmaceuticals in the Irish marine environment.
- To assess the potential for pharmaceuticals to bioaccumulate in an aquatic food web via trophic level transfer.
- To determine the potential for human exposure to pharmaceuticals via dietary intake of contaminated seafood.

References

Andreozzi, R., Marotta, R., Paxeus, N. (2003) Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere* **50**, 1319-1330.

Araujo, L., Wild, J., Villa, N., Camargo, N., Cubillan, D., Prieto, A. (2008) Determination of anti-inflammatory drugs in water samples, by *in situ* derivatization, solid phase microextraction and gas chromatography-mass spectrometry. *Talanta* **75**, 111-115.

Arnot, J.A., Gobas, F. (2006) A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environmental Reviews* **14**, 257-297.

Baker, D.R., Kasprzyk-Hordern, B. (2011) Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography-mass spectrometry. *Journal of Chromatography A* **1218**, 8036-8059.

Barron, L., Tobin, J., Paull, B. (2008) Multi-residue determination of pharmaceuticals in sludge and sludge enriched soils using pressurized liquid extraction, solid phase extraction and liquid chromatography with tandem mass spectrometry. *Journal of Environmental Monitoring* **10**, 353-361.

Barron, L., Purcell, M., Havel, J., Thomas, K., Tobin, J.M., Paull, B. (2009) *STRIVE report 34: Occurrence and fate of pharmaceuticals and personal care products within sewage sludge and sludge-enriched soils*. Environmental Protection Agency, Ireland.

Barron, L., Nesterenko, E., Hart, K., Power, E., Quinn, B., Kelleher, B., Paull, B. (2010) Holistic visualisation of the multimodal transport and fate of twelve pharmaceuticals in biosolid enriched topsoils. *Analytical and Bioanalytical Chemistry* **397**, 287-296.

Batt, A.L., Kostich, M.S., Lazorchak, J.M. (2008) Analysis of ecologically relevant pharmaceuticals in wastewater and surface water using selective solid-phase extraction and UPLC-MS/MS. *Analytical Chemistry* **80**, 5021-5030.

Beltran, A., Caro, E., Marce, R.M., Cormack, P.A.G., Sherrington, D.C., Borrull, F. (2007) Synthesis and application of a carbamazepine-imprinted polymer for solid-phase extraction from urine and wastewater. *Analytica Chimica Acta* **597**, 6-11.

Benito-Pena, E., Partal-Rodera, A.I., Leon-Gonzalez, M.E., Moreno-Bondi, M.C. (2006) Evaluation of mixed mode solid phase extraction cartridges for the preconcentration of beta-lactam antibiotics in wastewater using liquid chromatography with UV-DAD detection. *Analytica Chimica Acta* **556**, 415-422.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A. (2009) Pharmaceuticals and endocrine disrupting compounds in US drinking water. *Environmental Science & Technology* **43**, 597-603.

Bhandari, A. (2009) *Introduction*. In: Bhandari, A., *et al.* (eds.), *Contaminants of Emerging Environmental Concern*. American Society of Civil Engineers, Reston, VA, 1-6.

Botitsi, E., Frosyni, C., Tsipi, D. (2007) Determination of pharmaceuticals from different therapeutic classes in wastewaters by liquid chromatography-electrospray ionization-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* **387**, 1317-1327.

Boxall, A.B.A. (2004) The environmental side effects of medication - How are human and veterinary medicines in soils and water bodies affecting human and environmental health? *Embo Reports* **5**, 1110-1116.

Bremle, G., Okla, L., Larsson, P. (1995) Uptake of PCBS in fish in a contaminated river system - bioconcentration factors measured in the field. *Environmental Science & Technology* **29**, 2010-2015.

Bringolf, R.B., Heltsley, R.M., Newton, T.J., Eads, C.B., Fraley, S.J., Shea, D., Cope, W.G. (2010) Environmental occurence and reproductive effects of the pharmaceutical fluoxetine in native freshwater mussels. *Environmental Toxicology and Chemistry* **29**, 1311-1318.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J. (2005) Determination of select antidepressants in fish from an effluent-dominated stream. *Environmental Toxicology and Chemistry* **24**, 464-469.

Brown, J.N., Paxeus, N., Forlin, L., Larsson, D.G.J. (2007) Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. *Environmental Toxicology and Pharmacology* **24**, 267-274.

Buchberger, W.W. (2011) Current approaches to trace analysis of pharmaceuticals and personal care products in the environment. *Journal of Chromatography A* **1218**, 603-618.

Bueno, M.J.M., Aguera, A., Gomez, M.J., Hernando, M.D., Garcia-Reyes, J.F., Fernandez-Alba, A.R. (2007) Application of liquid chromatography/quadrupole-linear ion trap mass spectrometry and time-of-flight mass spectrometry to the determination of pharmaceuticals and related contaminants in wastewater. *Analytical Chemistry* **79**, 9372-9384.

Bueno, M.J.M., Boillot, C., Fenet, H., Chiron, S., Casellas, C., Gomez, E. (2013) Fast and easy extraction combined with high resolution-mass spectrometry for residue analysis of two anticonvulsants and their transformation products in marine mussels. *Journal of Chromatography A* **1305**, 27-34.

Buser, H.R., Muller, M.D., Theobald, N. (1998a) Occurrence of the pharmaceutical drug clofibric acid and the herbicide mecoprop in various Swiss lakes and in the North Sea. *Environmental Science & Technology* **32**, 188-192.

Buser, H.R., Poiger, T., Muller, M.D. (1998b) Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: rapid photodegradation in a lake. *Environmental Science & Technology* **32**, 3449-3456.

Buszewski, B., Szultka, M. (2012) Past, present, and future of solid phase extraction: a review. *Critical Reviews in Analytical Chemistry* **42**, 198-213.

Cahill, J.D., Furlong, E.T., Burkhardt, M.R., Kolpin, D., Anderson, L.G. (2004) Determination of pharmaceutical compounds in surface- and ground-water samples by solid-phase extraction and high-performance liquid chromatography-electrospray ionization mass spectrometry. *Journal of Chromatography A* **1041**, 171-180.

Cahill, M.G., Dineen, B.A., Stack, M.A., James, K.J. (2012) A critical evaluation of liquid chromatography with hybrid linear ion trap—Orbitrap mass spectrometry for the determination of acidic contaminants in wastewater effluents. *Journal of Chromatography A* **1270**, 88-95.

Campos, A., Tedesco, S., Vasconcelos, V., Cristobal, S. (2012) Proteomic research in bivalves towards the identification of molecular markers of aquatic pollution. *Journal of Proteomics* **75**, 4346-4359.

Carballa, M., Omil, F., Lema, J.M., Llompart, M., Garcia-Jares, C., Rodriguez, I., Gomez, M., Ternes, T. (2004) Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Research* **38**, 2918-2926.

Carballa, M., Fink, G., Omil, F., Lema, J.M., Ternes, T. (2008) Determination of the solid-water distribution coefficient (K_D) for pharmaceuticals, estrogens and musk fragrances in digested sludge. *Water Research* **42**, 287-295.

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., Zuccato, E. (2005) A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. *Journal of Chromatography A* **1092**, 206-215.

Chu, S., Metcalfe, C.D. (2007) Analysis of paroxetine, fluoxetine and norfluoxetine in fish tissues using pressurized liquid extraction, mixed mode solid phase extraction cleanup and liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **1163**, 112-118.

Clara, M., Strenn, B., Ausserleitner, M., Kreuzinger, N. (2004) Comparison of the behaviour of selected micropollutants in a membrane bioreactor and a conventional wastewater treatment plant. *Water Science and Technology* **50**, 29-36.

Clara, M., Kreuzinger, N., Strenn, B., Gans, O., Kroiss, H. (2005) The solids retention time - a suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants. *Water Research* **39**, 97-106.

Colgan, S., Donlon, B. (2010) *Science and sustainability: research based knowledge for environmental protection*. Environmental Protection Agency, Ireland. http://www.epa.ie/downloads/pubs/research/48781%20EPA%20Science%20and%20Sustainability%20Report%20(Screen).pdf, Accessed November 2011.

Comeau, F., Surette, C., Brun, G.L., Losier, R. (2008) The occurrence of acidic drugs and caffeine in sewage effluents and receiving waters from three coastal watersheds in Atlantic Canada. *Science of the Total Environment* **396**, 132-146.

Comerton, A.M., Andrews, R.C., Bagley, D.M. (2009) Practical overview of analytical methods for endocrine-disrupting compounds, pharmaceuticals and personal care products in water and wastewater. *Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences* **367**, 3923-3939.

Contardo-Jara, V., Lorenz, C., Pflugmacher, S., Nutzmann, G., Kloas, W., Wiegand, C. (2011) Exposure to human pharmaceuticals carbamazepine, ibuprofen and bezafibrate causes molecular effects in *Dreissena polymorpha*. *Aquatic Toxicology* **105**, 428-437.

Corcoran, J., Winter, M.J., Tyler, C.R. (2010) Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. *Critical Reviews in Toxicology* **40**, 287-304.

Cueva-Mestanza, R., Torres-Padron, M.E., Sosa-Ferrera, Z., Santana-Rodriguez, J.J. (2008) Microwave-assisted micellar extraction coupled with solid-phase extraction for preconcentration of pharmaceuticals in molluscs prior to determination by HPLC. *Biomedical Chromatography* **22**, 1115-1122.

Cuklev, F., Kristiansson, E., Fick, J., Asker, N., Forlin, L., Larsson, D.G.J. (2011) Diclofenac in fish: blood plasma levels similar to human therapeutic levels affect global hepatic gene expression. *Environmental Toxicology and Chemistry* **30**, 2126-2134.

Cunningham, V.L. (2008) *Special characteristics of pharmaceuticals related to environmental fate*. In: Kummerer, K. (ed.) *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*. 3rd edn. Springer, Berlin, 23-34.

Cunningham, V.L., Binks, S.P., Olson, M.J. (2009) Human health risk assessment from the presence of human pharmaceuticals in the aquatic environment. *Regulatory Toxicology and Pharmacology* **53**, 39-45.

Daughton, C.G., Ternes, T.A. (1999) Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environmental Health Perspectives* **107**, 907-938.

Daughton, C.G. (2007) *Pharmaceuticals in the environment: sources and their management*. In: Barceló, D. (ed.) *Wilson & Wilson's Comprehensive Analytical Chemistry: Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle*. Elsevier, Amsterdam, 1-58.

De Lange, H.J., Noordoven, W., Murk, A.J., Lurling, M., Peeters, E. (2006) Behavioural responses of *Gammarus pulex* (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. *Aquatic Toxicology* **78**, 209-216.

Diaz-Cruz, M.S., de Alda, M.J.L., Barceló, D. (2003) Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. *Trac-Trends in Analytical Chemistry* **22**, 340-351.

Dussault, E.B., Balakrishnan, V.K., Solomon, K.R., Sibley, P.K. (2009) Matrix effects on mass spectrometric determinations of four pharmaceuticals and personal care products in water, sediments, and biota. *Canadian Journal of Chemistry* **87**, 662-672.

EFSA (2013) *Report for 2011 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products*. European Food Safety Authority. http://www.efsa.europa.eu/en/supporting/doc/363e.pdf, Accessed September 2013.

EPA Ireland (1997) *Waste water treatment manuals: primary, secondary and tertiary treatment*. Environmental Protection Agency, Ireland.

Es'haghi, Z. (2009) Determination of widely used non-steroidal anti-inflammatory drugs in water samples by *in situ* derivatization, continuous hollow fiber liquid-phase microextraction and gas chromatography-flame ionization detector. *Analytica Chimica Acta* **641**, 83-88.

EU Decision 2002/657/EC (2002) *EU commission decision 2002/657/EC concerning the performance of analytical methods and the interpretation of results*. Official Journal of the European Communities OJL221.

EU Directive 2000/60/EC (2000) *European Union water framework directive 2000/60/EC*. Official Journal of the European Communities, OJL327.

EU Directive 2008/105/EC (2008) *European Union water framework directive 2008/105/EC*. Official Journal of the European Communities, OJL348.

EU Directive 2010/75/EU (2011) *EU industrial emissions directive 2010/75/EU*. Official Journal of the European Communities, OJL334.

EU Directive 2013/39/EU (2013) *European Union water framework directive 2013/39/EU*. Official Journal of the European Communities, OJL226.

EUR-OP (2012) *EU research for the environment 2007-2013*. Publications Office of the European Union. http://ec.europa.eu/research/environment/pdf/research_for_the_environment.pdf, Accessed March 2012.

Faed, E.M. (1984) Properties of acyl glucuronides - implications for studies of the pharmacokinetics and metabolism of acidic drugs *Drug Metabolism Reviews* **15**, 1213-1249.

Farre, M., Gros, M., Hernandez, B., Petrovic, M., Hancock, P., Barceló, D. (2008a) Analysis of biologically active compounds in water by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* **22**, 41-51.

Farre, M., Perez, S., Kantiani, L., Barceló, D. (2008b) Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. *Trac-Trends in Analytical Chemistry* **27**, 991-1007.

Fatta, D., Nikolaou, A., Achilleos, A., Meric, S. (2007) Analytical methods for tracing pharmaceutical residues in water and wastewater. *Trac-Trends in Analytical Chemistry* **26**, 515-533.

Fent, K., Weston, A.A., Caminada, D. (2006) Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology* **76**, 122-159.

Fernandez-Torres, R., Lopez, M.A.B., Consentino, M.O., Mochon, M.C. (2011) Simultaneous determination of selected veterinary antibiotics and their main metabolites in fish and mussel samples by high-performance liquid chromatography with diode array-fluorescence (HPLC-DAD-FLD) detection. *Analytical Letters* **44**, 2357-2372.

Ferrer, I., Zweigenbaum, J.A., Thurman, E.M. (2010) Analysis of 70 Environmental Protection Agency priority pharmaceuticals in water by EPA Method 1694. *Journal of Chromatography A* **1217**, 5674-5686.

Fick, J., Lindberg, R.H., Parkkonen, J., Arvidsson, B., Tysklind, M., Larsson, D.G.J. (2010) Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents. *Environmental Science & Technology* **44**, 2661-2666.

Focazio, M.J., Kolpin, D.W., Barnes, K.K., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Barber, L.B., Thurman, M.E. (2008) A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States - II) Untreated drinking water sources. *Science of the Total Environment* **402**, 201-216.

Forster, C.F. (1985) *Biotechnology and Wastewater Treatment*. Baddiley, J., *et al.* (eds.), Cambridge University Press, New York.

Garcia, M.D.G., Canada, F.C., Culzoni, M.J., Vera-Candioti, L., Siano, G.G., Goicoechea, H.C., Galeral, M.M. (2009) Chemometric tools improving the determination of anti-inflammatory and antiepileptic drugs in river and wastewater by solid-phase microextraction and liquid chromatography diode array detection. *Journal of Chromatography A* **1216**, 5489-5496.

Gibson, G., Skett, P. (2001) *Pathways of drug metabolism*. In: *Introduction to Drug Metabolism*. 3rd edn. Nelson Thornes Company, Cheltenham, 1-34.

Gomez, M.J., Aguera, A., Mezcua, M., Hurtado, J., Mocholi, F., Fernandez-Alba, A.R. (2007) Simultaneous analysis of neutral and acidic pharmaceuticals as well as related compounds by gas chromatography-tandem mass spectrometry in wastewater. *Talanta* **73**, 314-320.

Gracia-Lor, E., Sancho, J.V., Hernandez, F. (2011) Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **1218**, 2264-2275.

Graham, G.G., Davies, M.J., Day, R.O., Mohamudally, A., Scott, K.F. (2013) The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. *Inflammopharmacology* **21**, 201-232.

Gray, N.F. (2004) *Biology of Wastewater Treatment*. Oxford University Press, London.

Gros, M., Petrovic, M., Barceló, D. (2006a) Multi-residue analytical methods using LC-tandem MS for the determination of pharmaceuticals in environmental and wastewater samples: a review. *Analytical and Bioanalytical Chemistry* **386**, 941-952.

Gros, M., Petrovic, M., Barceló, D. (2006b) Development of a multi-residue analytical methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. *Talanta* **70**, 678-690.

Gros, M., Pizzolato, T.M., Petrovic, M., de Alda, M.J.L., Barceló, D. (2008) Trace level determination of beta-blockers in waste waters by highly selective molecularly imprinted polymers extraction followed by liquid chromatography-quadrupole-linear ion trap mass spectrometry. *Journal of Chromatography A* **1189**, 374-384.

Gros, M., Petrovic, M., Barceló, D. (2009) Tracing pharmaceutical residues of different therapeutic classes in environmental waters by using liquid chromatography/quadrupole-linear ion trap mass spectrometry and automated library searching. *Analytical Chemistry* **81**, 898-912.

Gros, M., Rodriguez-Mozaz, S., Barceló, D. (2012) Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. *Journal of Chromatography A* **1248**, 104-121.

Gumbleton, M. (2005) *Processes of drug handling by the body*. In: Smith, J. and Williams, H. (eds.), *Introduction to the Principles of Drug Design and Action*. 4th edn. Lewis Publishers, Boca Raton, 1-31.

Harper Jr., W.F., Floyd-Smith, T., Yi T. (2008) *Chemical processes during biological wastewater treatment*. In: Aga, D.S. (ed.) *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems*. Lewis Publishers, Boca Raton, 363-382.

Heberer, T., Fuhrmann, B., Schmidt-Baumler, K., Tsipi, D., Koutsouba, V., Hiskia, A. (2000) Occurrence of pharmaceutical residues in sewage, river, ground-, and drinking water in Greece and Germany. *Abstracts of Papers of the American Chemical Society* **219**, U623-U623.

Heberer, T. (2002) Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology Letters* **131**, 5-17.

Heidler, J. and Halden, R.U. (2007) Mass balance assessment of triclosan removal during conventional sewage treatment. *Chemosphere* **66**, 362-369.

Hellou, J., Ross, N.W., Moon, T.W. (2012) Glutathione, glutathione S-transferase, and glutathione conjugates, complementary markers of oxidative stress in aquatic biota. *Environmental Science and Pollution Research* **19**, 2007-2023.

Henze, M., Harremoes, P., la Cour Jansen, J., Arvin, E. (2002) *Wastewater, volumes and composition*. In: Forstner, U., *et al.* (eds.), *Wastewater Treatment: Biological and Chemical Processes*. 2nd edn. Springer, Berlin, 11-36.

Hernando, M.D., Mezcua, M., Fernandez-Alba, A.R., Barceló, D. (2006) Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. *Talanta* **69**, 334-342.

Hilton, M., Thomas, K.V., Ashton, D. (2003) *Targeted monitoring programme for pharmaceuticals in the aquatic environment*. UK Environmental Agency.

Hogenboom, A.C., van Leerdam, J.A., de Voogt, P. (2009) Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography-hybrid linear ion trap orbitrap mass spectrometry. *Journal of Chromatography A* **1216**, 510-519.

Horan, N.J. (1996) *Biological Wastewater Treatment Systems: Theory and Operation*. Wiley & Sons, Sussex.

Horng, H., Spahn-Langguth, H., Benet, L.Z. (2013) *Mechanistic role of acyl glucuronides*. In: Kaplowitz, N. and DeLeve, L.D. (eds.), *Drug-Induced Liver Disease*. 3rd edn. Elsevier Science, San Diego, 35-70.

Huerta-Fontela, M., Galceran, M.T., Ventura, F. (2010) Fast liquid chromatography quadrupole-linear ion trap mass spectrometry for the analysis of pharmaceuticals and hormones in water resources. *Journal of Chromatography A* **1217**, 4212-4222.

Huerta, B., Rodriguez-Mozaz, S., Barceló, D. (2012) Pharmaceuticals in biota in the aquatic environment: analytical methods and environmental implications. *Analytical and Bioanalytical Chemistry* **404**, 2611-2624.

Huerta, B., Jakimska, A., Gros, M., Rodriguez-Mozaz, S., Barceló, D. (2013) Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A* **1288**, 63-72.

Hunt, C.D., Slone, E. (2010) Long-term monitoring using resident and caged mussels in Boston Harbor yield similar spatial and temporal trends in chemical contamination. *Marine Environmental Research* **70**, 343-357.

Huschek, G., Hansen, P.D., Maurer, H.H., Krengel, D., Kayser, A. (2004) Environmental risk assessment of medicinal products for human use according to European commission recommendations. *Environmental Toxicology* **19**, 226-240.

Ibanez, M., Guerrero, C., Sancho, J.V., Hernandez, F. (2009) Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry. *Journal of Chromatography A* **1216**, 2529-2539.

IPHA (2012) *Pharmaceutical Healthcare Facts and Figures*. Irish Pharmaceutical Healthcare Association. http://www.ipha.ie/alist/healthcare-facts-and-figures.aspx?article=dc6d83aa-9f1a-4f01-aaa1-6b4429c189b0, Accessed July 2012.

Isidori, M., Lavorgna, M., Nardelli, A., Parrella, A., Previtera, L., Rubino, M. (2005) Ecotoxicity of naproxen and its phototransformation products. *Science of the Total Environment* **348**, 93-101.

Isidori, M., Nardelli, A., Parrella, A., Pascarella, L., Previtera, L. (2006) A multispecies study to assess the toxic and genotoxic effect of pharmaceuticals: furosemide and its photoproduct. *Chemosphere* **63**, 785-793.

Jelic, A., Petrovic, M., Barceló, D. (2009) Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry. *Talanta* **80**, 363-371.

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sanchez, R., Ventura, F., Petrovic, M., Barceló, D. (2011) Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. *Water Research* **45**, 1165-1176.

Jjemba, P. (2008) *Detection and occurence of PPCPs in the environment*. In: *Pharma-Ecology: The Occurrence and Fate of Pharmaceuticals and Personal Care Products in the Environment*. Wiley, Hoboken, NJ, 81-116.

Jones, O.A.H., Voulvoulis, N., Lester, J.N. (2007) *Ecotoxicity of pharmaceuticals*. In: Barceló, D. (ed.) *Wilson & Wilson's Comprehensive Analytical Chemistry: Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle*. Elsevier, Amsterdam, 387-424.

Joss, A., Zabczynski, S., Gobel, A., Hoffmann, B., Loffler, D., McArdell, C.S., Ternes, T.A., Thomsen, A., Siegrist, H. (2006) Biological degradation of pharmaceuticals in municipal wastewater treatment: proposing a classification scheme. *Water Research* **40**, 1686-1696.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J. (2007) Multi-residue method for the determination of basic/neutral pharmaceuticals and illicit drugs in surface water by solid-phase extraction and ultra performance liquid chromatography-positive electrospray ionisation tandem mass spectrometry. *Journal of Chromatography A* **1161**, 132-145.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J. (2008) Multiresidue methods for the analysis of pharmaceuticals, personal care products and illicit drugs in surface water and wastewater by solid-phase extraction and ultra performance liquid chromatography-electrospray tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* **391**, 1293-1308.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J. (2009) The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. *Water Research* **43**, 363-380.

Kim, S., Weber, S., Batt, A., Aga, D.S. (2008) *Removal of pharmaceuticals in biological wastewater treatment plants.* In: Aga, D.S. (ed.) *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems*. Lewis Publishers, Boca Raton, 349-361.

Kim, D., Han, J., Choi, Y. (2013) On-line solid-phase microextraction of triclosan, bisphenol A, chlorophenols, and selected pharmaceuticals in environmental water samples by high-performance liquid chromatography-ultraviolet detection. *Analytical and Bioanalytical Chemistry* **405**, 377-387.

Klosterhaus, S.L., Grace, R., Hamilton, M.C., Yee, D. (2013) Method validation and reconnaissance of pharmaceuticals, personal care products, and alkylphenols in surface waters, sediments, and mussels in an urban estuary. *Environment International* **54**, 92-99.

Krauss, M., Singer, H., Hollender, J. (2010) LC-high resolution MS in environmental analysis: from target screening to the identification of unknowns. *Analytical and Bioanalytical Chemistry* **397**, 943-951.

Lacey, C., McMahon, G., Bones, J., Barron, L., Morrissey, A., Tobin, J.M. (2008) An LC-MS method for the determination of pharmaceutical compounds in wastewater treatment plant influent and effluent samples. *Talanta* **75**, 1089-1097.

Lahti, M., Brozinski, J.M., Jylha, A., Kronberg, L., Oikari, A. (2011) Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout. *Environmental Toxicology and Chemistry* **30**, 1403-1411.

Lajeunesse, A., Gagnon, C., Gagne, F., Louis, S., Cejka, P., Sauve, S. (2011) Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment - Evidence of biological effects. *Chemosphere* **83**, 564-571.

Lam, M.W., Young, C.J., Brain, R.A., Johnson, D.J., Hanson, M.A., Wilson, C.J., Richards, S.M., Solomon, K.R., Mabury, S.A. (2004) Aquatic persistence of eight pharmaceuticals in a microcosm study. *Environmental Toxicology and Chemistry* **23**, 1431-1440.

Lam, M.W., Young, C.J., Mabury, S.A. (2005) Aqueous photochemical reaction kinetics and transformations of fluoxetine. *Environmental Science & Technology* **39**, 513-522.

Langford, K., Thomas, K.V. (2011) Input of selected human pharmaceutical metabolites into the Norwegian aquatic environment. *Journal of Environmental Monitoring* **13**, 416-421.

Lapworth, D.J., Baran, N., Stuart, M.E., Ward, R.S. (2012) Emerging organic contaminants in groundwater: a review of sources, fate and occurrence. *Environmental Pollution* **163**, 287-303.

Laven, M., Alsberg, T., Yu, Y., Adolfsson-Erici, M., Sun, H.W. (2009) Serial mixed-mode cation- and anion-exchange solid-phase extraction for separation of basic, neutral and acidic pharmaceuticals in wastewater and analysis by high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry. *Journal of Chromatography A* **1216**, 49-62.

Li, K.M., Rivory, L.P., Clarke, S.J. (2006) Solid-phase extraction (SPE) techniques for sample preparation in clinical and pharmaceutical analysis: a brief overview. *Current Pharmaceutical Analysis* **2**, 95-102.

Li, W.H., Shi, Y.L., Gao, L.H., Liu, J.M., Cai, Y.Q. (2012) Investigation of antibiotics in mollusks from coastal waters in the Bohai Sea of China. *Environmental Pollution* **162**, 56-62.

Lishman, L., Smyth, S.A., Sarafin, K., Kleywegt, S., Toito, J., Peart, T., Lee, B., Servos, M., Beland, M., Seto, P. (2006) Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. *Science of the Total Environment* **367**, 544-558.

Luo, Y.B., Zheng, H.B., Wan, J.X., Gao, Q., Yu, Q.W., Feng, Y.Q. (2011) An anionic exchange stir rod sorptive extraction based on monolithic material for the extraction

of non-steroidal anti-inflammatory drugs in environmental aqueous samples. *Talanta* **86**, 103-108.

Magner, J., Filipovic, M., Alsberg, T. (2010) Application of a novel solid-phase-extraction sampler and ultra-performance liquid chromatography quadrupole-time-of-flight mass spectrometry for determination of pharmaceutical residues in surface sea water. *Chemosphere* **80**, 1255-1260.

Mehinto, A.C., Hill, E.M., Tyler, C.R. (2010) Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). *Environmental Science & Technology* **44**, 2176-2182.

Mjalli, F.S., Al-Asheh, S., Alfadala, H.E. (2007) Use of artificial neural network black-box modeling for the prediction of wastewater treatment plants performance. *Journal of Environmental Management* **83**, 329-338.

Monirith, I., Ueno, D., Takahashi, S., Nakata, H., Sudaryanto, A., Subramanian, A., Karuppiah, S., Ismail, A., Muchtar, M., Zheng, J.S., Richardson, B.J., Prudente, M., Hue, N.D., Tana, T.S., Tkalin, A.V., Tanabe, S. (2003) Asia-Pacific mussel watch: monitoring contamination of persistent organochlorine compounds in coastal waters of Asian countries. *Marine Pollution Bulletin* **46**, 281-300.

Munaron, D., Tapie, N., Budzinski, H., Andral, B., Gonzalez, J.L. (2012) Pharmaceuticals, alkylphenols and pesticides in Mediterranean coastal waters: results from a pilot survey using passive samplers. *Estuarine Coastal and Shelf Science* **114**, 82-92.

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T., Hirai, N., Tatarazako, N. (2008) The effects of pH on fluoxetine in Japanese medaka (*Oryzias latipes*): acute toxicity in fish larvae and bioaccumulation in juvenile fish. *Chemosphere* **70**, 865-873.

Nallani, G.C., Paulos, P.M., Constantine, L.A., Venables, B.J., Huggett, D.B. (2011) Bioconcentration of ibuprofen in fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*). *Chemosphere* **84**, 1371-1377.

Nurmi, J., Pellinen, J. (2011) Multiresidue method for the analysis of emerging contaminants in wastewater by ultra performance liquid chromatography-time-of-flight mass spectrometry. *Journal of Chromatography A* **1218**, 6712-6719.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., Mahmood, S., Ali, A., Khan, A.A. (2004) Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **427**, 630-633.

Ogilvie, B.W., Zhang, D.L., Li, W.Y., Rodrigues, A.D., Gipson, A.E., Holsapple, J., Toren, P., Parkinson, A. (2006) Glucuronidation converts gemfibrozil to a potent, metabolism-dependent inhibitor of CYP2C8: implications for drug-drug interactions. *Drug Metabolism and Disposition* **34**, 191-197.

Ollers, S., Singer, H.P., Fassler, P., Muller, S.R. (2001) Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low-ng/1 level in surface and waste water. *Journal of Chromatography A* **911**, 225-234.

Oppel, J., Broll, G., Loffler, D., Meller, M., Rombke, J., Ternes, T. (2004) Leaching behaviour of pharmaceuticals in soil-testing-systems: a part of an environmental risk assessment for groundwater protection. *Science of the Total Environment* **328**, 265-273.

Packer, J.L., Werner, J.J., Latch, D.E., McNeill, K., Arnold, W.A. (2003) Photochemical fate of pharmaceuticals in the environment: naproxen, diclofenac, clofibric acid, and ibuprofen. *Aquatic Sciences* **65**, 342-351.

Perkins, R., Fang, H., Tong, W.D., Welsh, W.J. (2003) Quantitative structure-activity relationship methods: perspectives on drug discovery and toxicology. *Environmental Toxicology and Chemistry* **22**, 1666-1679.

Petrovic, M., Hernando, M.D., Diaz-Cruz, M.S., Barceló, D. (2005) Liquid chromatography-tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review. *Journal of Chromatography A* **1067**, 1-14.

Petrovic, M., Barceló, D. (2006) Application of liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-QqTOF-MS) in the environmental analysis. *Journal of Mass Spectrometry* **41**, 1259-1267.

Petrovic, M., Gros, M., Barceló, D. (2006) Multi-residue analysis of pharmaceuticals in wastewater by ultra-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry. *Journal of Chromatography A* **1124**, 68-81.

Petrovic, M., Farre, M., de Alda, M.L., Perez, S., Postigo, C., Kock, M., Radjenovic, J., Gros, M., Barceló, D. (2010) Recent trends in the liquid chromatography-mass spectrometry analysis of organic contaminants in environmental samples. *Journal of Chromatography A* **1217**, 4004-4017.

Pichon, V. (2000) Solid-phase extraction for multiresidue analysis of organic contaminants in water. *Journal of Chromatography A* **885**, 195-215.

Poiger, T., Buser, H.R., Muller, M.D. (2001) Photodegradation of the pharmaceutical drug diclofenac in a lake: pathway, field measurements, and mathematical modeling. *Environmental Toxicology and Chemistry* **20**, 256-263.

Quinn, B., Gagne, F., Blaise, C. (2004) Oxidative metabolism activity in *Hydra attenuata* exposed to carbamazepine. *Fresenius Environmental Bulletin* **13**, 783-788.

Quinn, B., Schmidt, W., O'Rourke, K., Hernan, R. (2011) Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (Dreissena polymorpha) and their comparison with standardised toxicity tests. *Chemosphere* **84**, 657-663.

Quintana, J.B., Rodil, R., Reemtsma, T. (2004) Suitability of hollow fibre liquid-phase microextraction for the determination of acidic pharmaceuticals in wastewater by liquid chromatography-electrospray tandem mass spectrometry without matrix effects. *Journal of Chromatography A* **1061**, 19-26.

Quintana, J.B., Rodil, R., Muniategui-Lorenzo, S., Lopez-Mahia, P., Prada-Rodriguez, D. (2007) Multiresidue analysis of acidic and polar organic contaminants in water samples by stir-bar sorptive extraction-liquid desorption-gas chromatography-mass spectrometry. *Journal of Chromatography A* **1174**, 27-39.

Radjenović, J., Petrović, M., Barceló, D. (2007) Advanced mass spectrometric methods applied to the study of fate and removal of pharmaceuticals in wastewater treatment. *TrAC Trends in Analytical Chemistry* **26**, 1132-1144.

Ramirez, A.J., Mottaleb, M.A., Brooks, B.W., Chambliss, C.K. (2007) Analysis of pharmaceuticals in fish using liquid chromatography-tandem mass spectrometry. *Analytical Chemistry* **79**, 3155-3163.

Ramirez, A.J., Brain, R.A., Usenko, S., Mottaleb, M.A., O'Donnell, J.G., Stahl, L.L., Wathen, J.B., Snyder, B.D., Pitt, J.L., Perez-Hurtado, P., Dobbins, L.L., Brooks, B.W., Chambliss, C.K. (2009) Occurence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States. *Environmental Toxicology and Chemistry* **28**, 2587-2597.

Rautio, J., Kumpulainen, H., Heimbach, T., Oliyai, R., Oh, D., Jarvinen, T., Savolainen, J. (2008) Prodrugs: design and clinical applications. *Nature Reviews Drug Discovery* **7**, 255-270.

Reddersen, K., Heberer, T. (2003) Multi-compound methods for the detection of pharmaceutical residues in various waters applying solid phase extraction (SPE) and gas chromatography with mass spectrometric (GC-MS) detection. *Journal of Separation Science* **26**, 1443-1450.

Reemtsma, T., Quintana, J.B. (2006) *Analytical methods for polar pollutants*. In: Reemtsma, T. and Jekel, M. (eds.), *Organic Pollutants in the Water Cycle: Properties, Occurrence, Analysis and Environmental Relevance of Polar Compounds*. Wiley, Weinheim, 1-40.

Rendal, C., Kusk, K.O., Trapp, S. (2011) Optimal choice of pH for toxicity and bioaccumulation studies of ionizing organic chemicals. *Environmental Toxicology and Chemistry* **30**, 2395-2406.

Richardson, M.L., Bowron, J.M. (1985) The fate of pharmaceutical chemicals in the aquatic environment. *Journal of Pharmacy and Pharmacology* **37**, 1-12.

Roberts, P.H., Thomas, K.V. (2006) The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. *Science of the Total Environment* **356**, 143-153.

Rodriguez-Navas, C., Bjorklund, E., Bak, S.A., Hansen, M., Krogh, K.A., Maya, F., Forteza, R., Cerda, V. (2013) Pollution pathways of pharmaceutical residues in the aquatic environment on the island of Mallorca, Spain. *Archives of Environmental Contamination and Toxicology* **65**, 56-66.

Rodriguez, I., Carpinteiro, J., Quintana, J.B., Carro, A.M., Lorenzo, R.A., Cela, R. (2004) Solid-phase microextraction with on-fiber derivatization for the analysis of anti-inflammatory drugs in water samples. *Journal of Chromatography A* **1024**, 1-8.

Romeo, M., Giamberini, L. (2013) *History of biomarkers*. In: Amiard-Triquet, C., *et al.* (eds.), *Ecological Biomarkers: Indicators of Ecotoxicological Effects*. Lewis Publishers, Boca Raton, 15-44.

Rosenbaum, S. (2011) *Basic Pharmacokinetics and Pharmacodynamics: An Integrated Textbook and Computer Simulations*. Wiley & Sons, Hoboken, NJ.

Rudorfer, M.V., Potter, W.Z. (1997) The role of metabolites of antidepressants in the treatment of depression. *CNS Drugs* **7**, 273-312.

Sanderson, H., Johnson, D.J., Wilson, C.J., Brain, R.A., Solomon, K.R. (2003) Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. *Toxicology Letters* **144**, 383-395.

Sanderson, H., Johnson, D.J., Reitsma, T., Brain, R.A., Wilson, C.J., Solomon, K.R. (2004) Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. *Regulatory Toxicology and Pharmacology* **39**, 158-183.

Sapkota, A., Sapkota, A.R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., Lawrence, R. (2008) Aquaculture practices and potential human health risks: current knowledge and future priorities. *Environment International* **34**, 1215-1226.

Schmidt, W., O'Rourke, K., Hernan, R., Quinn, B. (2011) Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus spp.*) and their comparison with standardized toxicity tests. *Marine Pollution Bulletin* **62**, 1389-1395.

Schmidt, W., Rainville, L.-C., McEneff, G., Sheehan, D., Quinn, B. (2013) A proteomic evaluation of the effects of the pharmaceuticals diclofenac and gemfibrozil on marine mussels (*Mytilus spp.*): evidence for chronic sublethal effects on stress-response proteins. *Drug Testing and Analysis*, DOI: 10.1002/dta.1463.

Schmitt-Jansen, M., Bartels, P., Adler, N., Altenburger, R. (2007) Phytotoxicity assessment of diclofenac and its phototransformation products. *Analytical and Bioanalytical Chemistry* **387**, 1389-1396.

Schroder, U., Machetzki, A. (2007) Determination of flumequine, nalidixic acid and oxolinic acid in shrimps by high-performance liquid chromatography with fluorescence detection. *European Food Research and Technology* **225**, 627-635.

Schultz, M.M., Furlong, E.T., Kolpin, D.W., Werner, S.L., Schoenfuss, H.L., Barber, L.B., Blazer, V.S., Norris, D.O., Vajda, A.M. (2010) Antidepressant pharmaceuticals in two US effluent-impacted streams: occurrence and fate in water and sediment, and selective uptake in fish neural tissue. *Environmental Science & Technology* **44**, 1918-1925.

Schwab, B.W., Hayes, E.P., Fiori, J.M., Mastrocco, F.J., Roden, N.M., Cragin, D., Meyerhoff, R.D., D'Aco, V.J., Anderson, P.D. (2005) Human pharmaceuticals in US surface waters: a human health risk assessment. *Regulatory Toxicology and Pharmacology* **42**, 296-312.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R.D. (2004) Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part 1: histopathological alterations and bioaccumulation in rainbow trout. *Aquatic Toxicology* **68**, 141-150.

Seifrtova, M., Pena, A., Lino, C.M., Solich, P. (2008) Determination of fluoroquinolone antibiotics in hospital and municipal wastewaters in Coimbra by liquid chromatography with a monolithic column and fluorescence detection. *Analytical and Bioanalytical Chemistry* **391**, 799-805.

Snyder, S.A., Lei, H., Wert, E.C. (2008) *Removal of endocrine disruptors and pharmaceuticals during water treatment*. In: Aga, D.S. (ed.) *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems*. Lewis Publishers, Boca Raton, 229-260.

Sousa, M.A., Goncalves, C., Cunha, E., Hajslova, J., Alpendurada, M.F. (2011) Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE-LC-MS/MS. *Analytical and Bioanalytical Chemistry* **399**, 807-822.

Stan, H.J., Heberer, T. (1997) Pharmaceuticals in the aquatic environment. *Analusis* **25**, M20-M23.

Straub, J.O. (2002) Environmental risk assessment for new human pharmaceuticals in the European Union according to the draft guideline/discussion paper of January 2001. *Toxicology Letters* **131**, 137-143.

Stumpf, M., Ternes, T.A., Wilken, R.D., Rodrigues, S.V., Baumann, W. (1999) Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. *Science of the Total Environment* **225**, 135-141.

Sun, Z., Schussler, W., Sengl, M., Niessner, R., Knopp, D. (2008) Selective trace analysis of diclofenac in surface and wastewater samples using solid-phase extraction with a new molecularly imprinted polymer. *Analytica Chimica Acta* **620**, 73-81.

Tarkowski, S.M. (2007) Environmental health research in Europe - bibliometric analysis. *European Journal of Public Health* **17**, 14-18.

Tchobanoglous, G., Burton, F.L., Stensel, H.D. (2003) *Wastewater Engineering: Treatment and Reuse*. 3rd edn. McGraw-Hill, Boston.

Ternes, T.A. (1998) Occurrence of drugs in German sewage treatment plants and rivers. *Water Research* **32**, 3245-3260.

Ternes, T.A., Kreckel, P., Mueller, J. (1999) Behaviour and occurrence of estrogens in municipal sewage treatment plants - II. Aerobic batch experiments with activated sludge. *Science of the Total Environment* **225**, 91-99.

Thomas, K.V., Hilton, M.J. (2004) The occurrence of selected human pharmaceutical compounds in UK estuaries. *Marine Pollution Bulletin* **49**, 436-444.

Tittlemier, S.A., Van de Riet, J., Burns, G., Potter, R., Murphy, C., Rourke, W., Pearce, H., Dufresne, G. (2007) Analysis of veterinary drug residues in fish and shrimp composites collected during the Canadian Total Diet Study, 1993-2004. *Food Additives and Contaminants* **24**, 14-20.

Togola, A., Budzinski, H. (2008) Multi-residue analysis of pharmaceutical compounds in aqueous samples. *Journal of Chromatography A* **1177**, 150-158.

Togunde, O.P., Oakes, K.D., Servos, M.R., Pawliszyn, J. (2012) Determination of pharmaceutical residues in fish bile by solid-phase microextraction coupled with liquid chromatography-tandem mass spectrometry (LC/MS/MS). *Environmental Science & Technology* **46**, 5302-5309.

Trenholm, R.A., Vanderford, B.J., Snyder, S.A. (2009) On-line solid phase extraction LC-MS/MS analysis of pharmaceutical indicators in water: a green alternative to conventional methods. *Talanta* **79**, 1425-1432.

Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Kohler, H.R., Schwaiger, J. (2004) Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* **68**, 151-166.

Triebskorn, R., Casper, H., Scheil, V., Schwaiger, J. (2007) Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). *Analytical and Bioanalytical Chemistry* **387**, 1405-1416.

Turiel, E., Bordin, G., Rodriguez, A.R. (2005) Study of the evolution and degradation products of ciprofloxacin and oxolinic acid in river water samples by HPLC-UV/MS/MS-MS. *Journal of Environmental Monitoring* **7**, 189-195.

US EPA (2007) *Method 1694: Pharmaceuticals and personal care products in water, soil, sediment, and biosolids by HPLC/MS/MS*. US Environmental Protection Agency. http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/2008_01_03_methods_method_1694.pdf, Accessed March 2011.

Van De Steene, J.C., Mortier, K.A., Lambert, W.E. (2006) Tackling matrix effects during development of a liquid chromatographic-electrospray ionisation tandem mass spectrometric analysis of nine basic pharmaceuticals in aqueous environmental samples. *Journal of Chromatography A* **1123**, 71-81.

Van Hoeck, E., Canale, F., Cordero, C., Compernolle, S., Bicchi, C., Sandra, P. (2009) Multiresidue screening of endocrine-disrupting chemicals and pharmaceuticals in aqueous samples by multi-stir bar sorptive extraction-single desorption-capillary gas chromatography/mass spectrometry. *Analytical and Bioanalytical Chemistry* **393**, 907-919.

Van Leengoed, L., Kluivers, M., Herbes, R., Langendijk, P., Stephany, R., van den Berg, M., Seinen W., Grinwis G., van der Lugt J., Meulders F., Geudeke J., Verheijden J. (2002) The weakest link: medroxyprogesterone acetate in pig feed. *Tijdschrift voor Diergeneeskunde* **127**, 516-519.

Vieno, N., Tuhkanen, T., Kronberg, L. (2007) Elimination of pharmaceuticals in sewage treatment plants in Finland. *Water Research* **41**, 1001-1012.

Weigel, S., Kuhlmann, J., Huhnerfuss, H. (2002) Drugs and personal care products as ubiquitous pollutants: occurrence and distribution of clofibric acid, caffeine and DEET in the North Sea. *Science of the Total Environment* **295**, 131-141.

Weigel, S., Berger, U., Jensen, E., Kallenborn, R., Thoresen, H., Huhnerfuss, H. (2004) Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromso/Norway with emphasis on ibuprofen and its metabolites. *Chemosphere* **56**, 583-592.

Wen, Y., Wang, Y., Feng, Y.Q. (2006) Simultaneous residue monitoring of four tetracycline antibiotics in fish muscle by in-tube solid-phase microextraction coupled with high-performance liquid chromatography. *Talanta* **70**, 153-159.

Wheatley, A.D. (1985) *Wastewater treatment and by-product recovery*. In: Sidwick, J.M. (ed.) *Topics in Wastewater Treatment*. Blackwell, Oxford, 68-106.

Wille, K., Noppe, H., Verheyden, K., Vanden Bussche, J., De Wulf, E., Van Caeter, P., Janssen, C.R., De Brabander, H.F., Vanhaecke, L. (2010) Validation and application of an LC-MS/MS method for the simultaneous quantification of 13 pharmaceuticals in seawater. *Analytical and Bioanalytical Chemistry* **397**, 1797-1808.

Wille, K., Kiebooms, J.A.L., Claessens, M., Rappe, K., Vanden Bussche, J., Noppe, H., Van Praet, N., De Wulf, E., Van Caeter, P., Janssen, C.R., De Brabander, H.F., Vanhaecke, L. (2011) Development of analytical strategies using U-HPLC-MS/MS and LC-ToF-MS for the quantification of micropollutants in marine organisms. *Analytical and Bioanalytical Chemistry* **400**, 1459-1472.

Wille, K., De Brabander, H.F., De Wulf, E., Van Caeter, P., Janssen, C.R., Vanhaecke, L. (2012) Coupled chromatographic and mass-spectrometric techniques for the analysis

of emerging pollutants in the aquatic environment. *Trac-Trends in Analytical Chemistry* **35**, 87-108.

Wu, J.M., Zhang, L.F., Yang, Z.G. (2010) A review on the analysis of emerging contaminants in aquatic environment. *Critical Reviews in Analytical Chemistry* **40**, 234-245.

Zhou, S.N., Oakes, K.D., Servos, M.R., Pawliszyn, J. (2008) Application of solid-phase microextraction for *in vivo* laboratory and field sampling of pharmaceuticals in fish. *Environmental Science & Technology* **42**, 6073-6079.

Zorita, S., Boyd, B., Jonsson, S., Yilmaz, E., Svensson, C., Mathiasson, L., Bergstrom, S. (2008) Selective determination of acidic pharmaceuticals in wastewater using molecularly imprinted solid-phase extraction. *Analytica Chimica Acta* **626**, 147-154.

Zwiener, C., Frimmel, F.H. (2004) LC-MS analysis in the aquatic environment and in water treatment - a critical review - Part I: Instrumentation and general aspects of analysis and detection. *Analytical and Bioanalytical Chemistry* **378**, 851-861.

2.0 A Year-Long Study of the Spatial Occurrence and Relative Distribution of Pharmaceutical Residues in the Aquatic Environment using PLE and SPE with LC-MS/MS

Published as:

McEneff, G., Barron, L., Kelleher, B., Paull, B., and Quinn, B. A year-long study of the spatial occurrence and relative distribution of pharmaceutical residues in sewage effluent, receiving marine waters and marine bivalves. *Science of the Total Environment.* Accepted December 2013.

Abstract

Reports concerning the quantitative analysis of pharmaceuticals in marine ecosystems are somewhat limited. It is necessary to determine pharmaceutical fate and assess any potential risk of exposure to aquatic species and ultimately, seafood consumers. In the work presented herein, analytical methods were optimised and validated for the quantification of five pharmaceutical residues in wastewater effluent, receiving marine waters and marine mussels (*Mytilus* spp.). Selected pharmaceuticals included two non-steroidal anti-inflammatory drugs (diclofenac and mefenamic acid), an antibiotic (trimethoprim), an antiepileptic (carbamazepine) and a lipid regulator (gemfibrozil). This chapter also presents the results of an *in situ* study in which caged *Mytilus* spp. were deployed at three sites on the Irish coastline over a one-year period. For aqueous samples, pharmaceutical residues were determined using solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The extraction of pharmaceuticals from mussel tissues used an additional pressurised liquid extraction (PLE) step prior to SPE and LC-MS/MS. Limits of quantification (LOQs) $\leq$225 ng.L^{-1} were achieved in wastewater effluent, $\leq$38 µg.L^{-1} in marine surface water and $\leq$29 ng.g^{-1} in marine mussels. Method linearity was achieved for pharmaceuticals in each matrix with correlation coefficients of $R^2 \geq 0.976$. All five selected pharmaceuticals were quantified in wastewater effluent and marine surface waters. This work has demonstrated the susceptibility of the *Mytilus* spp. to pharmaceutical exposure following the detection of pharmaceutical residues in the tissues of this mussel species at measurable concentrations.

Aims and Objectives

- Develop and validate analytical methods for the quantification of selected pharmaceuticals in sewage effluent, marine surface water and marine mussels using pressurised liquid extraction (PLE) and solid phase extraction (SPE) techniques with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. These pharmaceuticals include two non-steroidal anti-inflammatory drugs (diclofenac and mefenamic acid), an antibiotic (trimethoprim), an antiepileptic (carbamazepine) and a lipid regulator (gemfibrozil).
- Set up an *in situ* experiment exposing caged mussels to three sites (two impacted sites and one control site) along the Irish coastline to investigate the occurrence of the selected pharmaceuticals in marine mussels.
- Apply developed methods to samples of 24-h composite effluent, marine surface water and marine mussels, collected monthly over a 12-month period, to determine the prevalence and relative distribution of selected pharmaceuticals in the marine environment.

2.1 Introduction

Current knowledge on the distribution pathways and fate of pharmaceuticals in the aquatic environment is somewhat limited and has emerged as an environmental issue. Unlike other environmental contaminants, pharmaceuticals have many physicochemical and biological properties which must be taken into account when predicting or assessing their fate in the environment. Human pharmaceuticals are excreted into the sewage system as a mixture of the parent compound and metabolites, comprising mostly of transformation products and conjugated glucuronides (Heberer, 2002). Conjugated compounds have been found to be easily cleaved during wastewater treatment, releasing the parent compound into the treated wastewater, and subsequently into the environment (Jelic *et al.*, 2011; Ternes, 1998). Veterinary medicines also enter the environment, mainly via medicated fish feed and agricultural soil leaching (Boxall, 2004; Heberer, 2002). Although susceptible to further degradation or transformation in the environment, their continuous introduction into receiving waters in reality confers some degree of pseudo-persistence (Hernando *et al.*, 2006). The seemingly ubiquitous presence of pharmaceuticals in the aquatic environment has been reported worldwide over the past decade or so, with over 80 pharmaceuticals and their metabolites detected at $\mu g.L^{-1}$ concentrations in municipal sewage effluent and surface waters and at $ng.L^{-1}$ concentrations in groundwater and drinking water (Fatta-Kassinos *et al.*, 2011; Heberer *et al.*, 2000; Lapworth *et al.*, 2012; Roberts and Thomas, 2006; Stan and Heberer, 1997; Thomas and Hilton, 2004). In order to investigate their fate, the quantitative determination of pharmaceuticals in aquatic ecosystems is necessary.

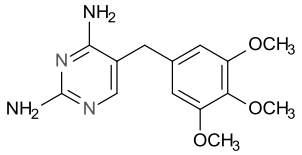
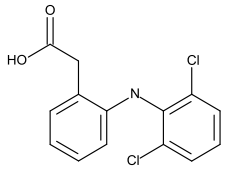
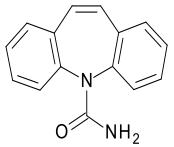
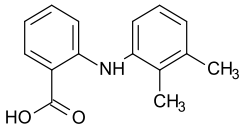
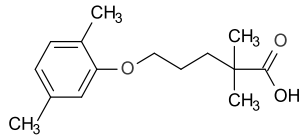
One of the main concerns surrounding pharmaceutical release into surface waters is their potential for bioaccumulation in aquatic organisms. The polar nature of most pharmaceuticals make them directly bioavailable to filter feeding organisms. Bivalves, such as mussels, are natural filter feeders which have been previously utilised in POP monitoring programmes because of their high bioaccumulation capacities, fixed location and high populations in marine waters (Hunt and Slone, 2010; Monirith *et al.*, 2003). The uptake of pharmaceuticals has been previously observed in wild mussel species collected from the Mediterranean Sea, San Francisco Bay and the Bohai Sea in China (Bueno *et al.*, 2013; Klosterhaus *et al.*, 2013; Li *et al.*, 2012). The use of caged sample studies allows

for the measurement of exposure levels as a function of time, making it easier to assess and compare the extent of pollution between contaminated sites. With regard to pharmaceutical exposure studies using caged mussels, recent studies carried out by Bringolf *et al.* (2010) and Wille *et al.* (2011) have involved the exposure of caged *Mytilus* spp. to effluent contaminated surface waters and the detection of salicylic acid, carbamazepine and fluoxetine in exposed mussel tissues.

There is comparatively larger knowledge of the fate of pharmaceuticals in the human body and during wastewater treatment processes, (Debska *et al.*, 2004; Fent *et al.*, 2006) but little research has been performed regarding pharmaceutical fate studies after effluent release into surface waters, particularly in the marine environment. Pharmaceuticals in the environment need to be quantified by means of *in situ* studies in order to assess the prevalence of pharmaceutical residues present in 'real' environmental matrices. Numerous effects on the reproduction and growth of non-target aquatic species have been observed following toxicity studies of pharmaceuticals at environmentally relevant concentrations (Huerta *et al.*, 2012; Quinn *et al.*, 2011; Schmidt *et al.*, 2011). Besides toxicity to aquatic species, trace pharmaceutical concentrations have been previously detected in drinking water in Greece and the US (Benotti *et al.*, 2009; Heberer *et al.*, 2002) and in packaged seafood (Tittlemier *et al.*, 2007; EFSA, 2013). The presence of pharmaceuticals in water and seafood may potentially act as risk to the consumer either through direct effect or indirectly through potential antimicrobial resistance (Cabello, 2006). In order to study the possible environmental and human health risks posed by these contaminants at environmentally relevant concentrations, information regarding their occurrence in the aquatic environment, particularly in aquatic species, is urgently required.

Based on pharmaceutical sales data from Ireland (HSE, 2010) and the UK (NHS, 2010) and the results from previous monitoring studies carried out in wastewater effluent (Hilton and Thomas, 2003; Lacey *et al.*, 2008; Lacey *et al.*, 2012), five pharmaceuticals were chosen from four different therapeutic classes: an antiepileptic (carbamazepine); two NSAIDs (diclofenac and mefenamic acid); a lipid regulator (gemfibrozil); and an antibiotic (trimethoprim). The chemical structures of these compounds and their physicochemical properties are given in Table 2.1. To the author's knowledge, this study was the first to quantify a range of pharmaceuticals in effluent, marine surface waters and marine mussels across a one-year period.

Table 2.1: Chemical structure, class and physicochemical properties (Bones *et al.*, 2006; Brown *et al.*, 2007) of selected pharmaceutical compounds

Trimethoprim	Diclofenac	Carbamazepine	Mefenamic acid	Gemfibrozil
				
Antibiotic	*Anti-inflammatory*	*Antiepileptic*	*Anti-inflammatory*	*Lipid regulator*
M_r 290.32	M_r 296.15	M_r 236.27	M_r 250.34	M_r 241.28
pK_a 6.60	pK_a 4.15	pK_a 13.90	pK_a 4.20	pK_a 4.80
LogK_{ow} 0.65	LogK_{ow} 3.91	LogK_{ow} 2.30	LogK_{ow} 4.16	LogK_{ow} 3.56
pH 7.5 pH 8.0	pH 7.5 pH 8.0	pH 7.5 pH 8.0	pH 7.5 pH 8.0	pH 7.5 pH 8.0
LogD_{ow} 0.55 0.63	LogD_{ow} 0.56 0.06	LogD_{ow} 2.30 2.30	LogD_{ow} 0.86 0.36	LogD_{ow} 0.86 0.36

2.2 Experimental

2.2.1 Reagents and chemicals

LC-MS grade acetonitrile and water, and analytical grade acetone, acetonitrile, ethyl acetate and methanol, were purchased from Fisher Scientific (Cheshire, UK). Dichloromethane, dichlorodimethylsilane, ammonium hydroxide solution, acetic acid and sulphuric acid were purchased from Aldrich (Gillingham, UK). Analytical grade carbamazepine (≥98 %), diclofenac sodium salt (≥98 %), gemfibrozil (≥99 %) and mefenamic acid (≥99 %) were obtained from Sigma-Aldrich (Steinheim, Germany) and trimethoprim (≥98 %) was ordered from Fluka (Buch, Switzerland). Ultra-pure water was obtained from a Millipore Milli-Q water purification system (Bedford, MA, USA).

Stock solutions (1000 $mg.L^{-1}$) of individual analytes were prepared in methanol and stored in a freezer at –20 °C and in the dark, for optimum stability. Working mixed standards were prepared weekly in either water or, where required, in 80:20 v/v 13 mM ammonium acetate in water/acetonitrile.

2.2.2 Sampling locations, procedures and experimental conditions

Blue mussels (*Mytilus* spp.) were sourced in the west of Ireland (Lettermullen, Co. Galway), from a Class A bivalve mollusc production area, designated by the Sea-Fisheries Protection Authority of Ireland under EC Regulation 854/2004. Animals chosen for this study were of the same size class (4-6 cm) and were collected in March pre-spawning. Mussels were placed on wet seaweed and transported to the laboratory in a cooler box. Shells were wiped free of debris and seaweed and the animals were depurated over seven days in a large tank of artificial seawater (ASW). ASW consisted of Peacock Seamix (NaCl 65.5 %, $MgSO_4$ 8.25 %, $MgCl_2$ 6 %, $CaCl_2$ 3 %, KCl 1.6 %, insolubles 0.05 %, H_20 15.6 %) dissolved in dechlorinated tap water to a salinity of 33 $g.kg^{-1}$ at 13 °C (±1 °C). Mesh cages (1 m x 0.5 m) were constructed and divided into two sections to reduce the loss of mussels in the event of the cage tearing (images shown in Figure 2.1 (a) & (b)).

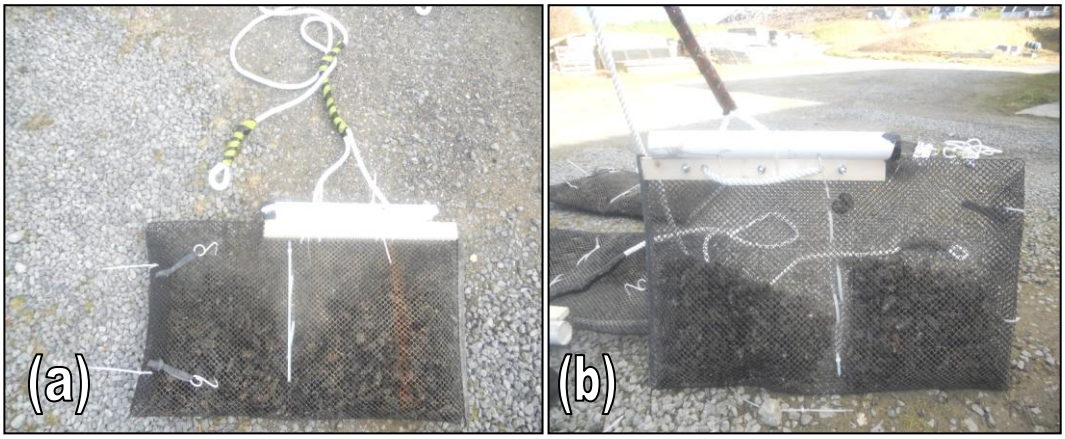


Figure 2.1 (a) & (b): Images of the constructed mesh cages filled with *Mytilus* spp. mussels before deployment

Each cage was filled with 2×300 mussels, which, over time, were found to attach themselves to the inside wall of the cage. As shown in Figure 2.2, cages were deployed at a control site (CON) and two effluent exposure sites off the east (EXP1) and west (EXP2) coasts of Ireland.

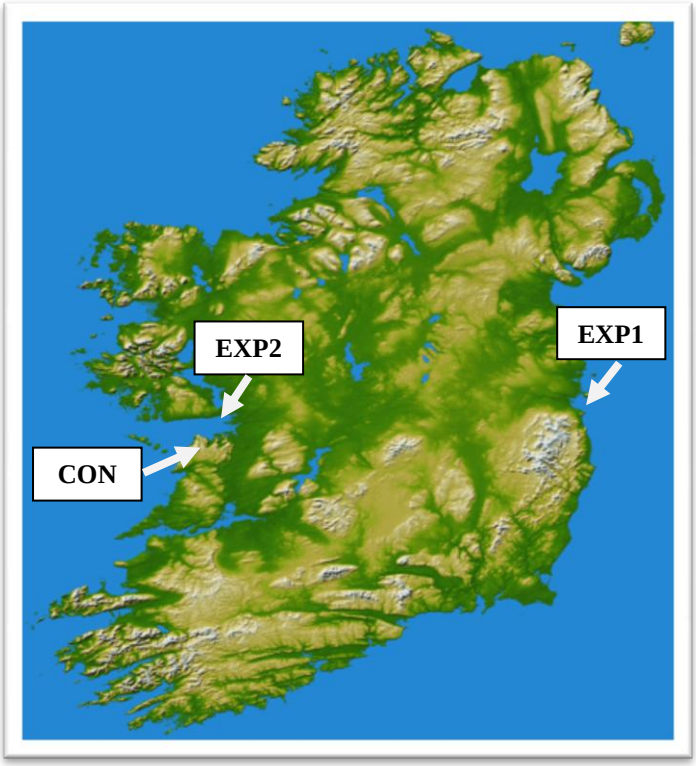


Figure 2.2: Map of Ireland showing sampling locations CON, EXP1 and EXP2

CON was located in a Class A defined enclosed bay, approximately 0.3 km off the west coast of Ireland. EXP1 was set up on the east coast of Ireland, approximately 100 m downstream from a wastewater effluent outfall pipe which discharges into a lower river estuary (see Figure 2.3 (a) & (b)). WWTP1 is a secondary treatment facility that also carries out tertiary treatment i.e. UV disinfection, from the months of May to September. It has a population equivalent (PE) of approximately 1.7 million and an average outflow of 500,000 $m^3.day^{-1}$.

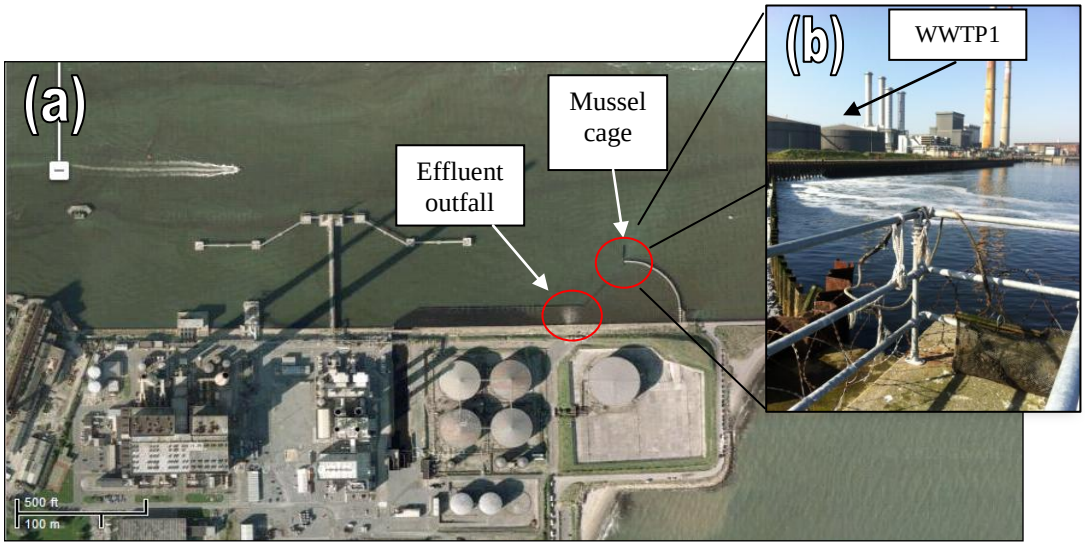


Figure 2.3 (a): Satellite view of EXP1 with locations of effluent outfall and mussel cage marked (GoogleMaps, 2012) **(b):** Mussel cage tied to bridge and WWTP1 and effluent outfall observed in background

EXP2 was located in a shallow bay on the west coast of Ireland, above a wastewater effluent diffuser pipe, approximately 0.4 km south of WWTP2 (see Figure 2.4 (a) & (b)). WWTP2 is a secondary treatment facility with a PE of approximately 110,000 and an average outflow of 49,000 $m^3.day^{-1}$. A year-long cage experiment was carried out from March 2011 to March 2012. Mussels (n=45) were sampled monthly alongside grab samples of surrounding marine surface waters and 24-h composite effluent samples from each WWTP.

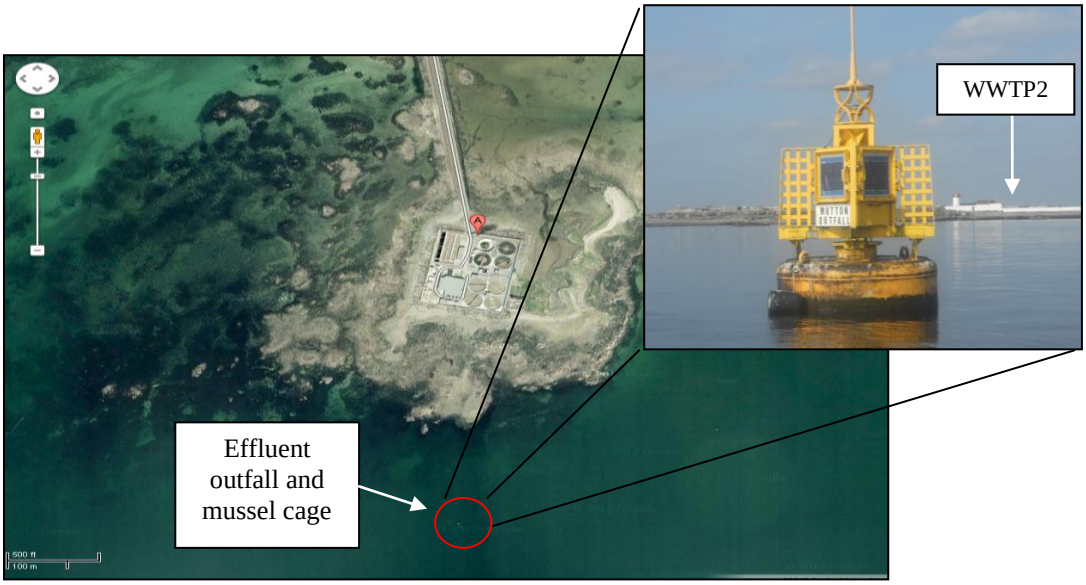


Figure 2.4 (a): Satellite view of EXP2 and the effluent outfall pipe where the mussel cage hangs from (GoogleMaps, 2012) **(b):** Buoy from which mussel cage hangs and under which lies the effluent outfall pipe for WWTP2 (observed in background)

Silanised amber Winchester glass bottles (2.5 L) were used to collect marine surface waters and effluent samples. When it was not possible to use this approach such as in the case of marine surface water from EXP2, samples were first collected in a pre-washed stainless steel bucket and then transferred into a silanised, amber Winchester glass bottle. All samples were transported to the laboratory in a cooler box. Mussels were de-shelled, pooled and frozen at −80 °C. Aqueous samples were stored at 4 °C and extracted onto SPE cartridges within 72 h of collection.

2.2.3 Sample pre-treatment, extraction and clean-up

2.2.3.1 Glassware preparation and silanisation

All glassware was pre-cleaned with 50:50 (v/v) methanol/water solution and silanised using a 10 % (v/v) dichlorodimethylsilane solution in dichloromethane. This was followed by two rinses with dichloromethane, methanol and ultra-pure water respectably in that order. Glassware was cleaned with 50:50 solution of methanol/water followed by 100 % ultra-pure water between silanisation washes.

2.2.3.2 Effluent and marine surface water

The analytical method previously established for WWTP influent and effluent (Lacey *et al.*, 2008) was re-optimised for the determination of pharmaceutical residues in effluent and also applied to marine surface water. Effluent and marine surface water samples (2.5 L) were first filtered through a 1.2 µm glass fibre filter (GF/C diameter 70 mm, Whatman, Kent, UK) and adjusted to pH 4 using dilute sulphuric acid. Samples were split into 500 mL aliquots and any necessary spiking was carried out at this stage (one sample left unspiked, three samples spiked at 1, 2 and 3 $\mu g.L^{-1}$, respectively, with a 1 $mg.L^{-1}$ pharmaceutical mix). For the extraction of pharmaceuticals, Strata-X SPE cartridges (6 mL, 200 mg, Phenomenex, Cheshire, UK) were first conditioned with 6 mL methanol and 6 mL ultra-pure water before loading of the sample. Cartridges were then rinsed with 6 mL of ultra-pure water and dried for 30 min. Sample extracts were eluted with 3x3 mL ethyl acetate/acetone (50:50) and dried under nitrogen while heated to 40 °C with a Turbovap LV (Zymark Corporation, Hopkington, MA, USA). Samples were reconstituted in 250 µL of 13 mM ammonium acetate with 20 % acetonitrile and filtered with 0.2 µm polyvinylidene fluoride (PVDF) syringe filters (Whatman, Kent, UK) before LC-MS/MS analysis. All samples were stored at –20 °C before analysis.

2.2.3.3 Marine mussels

For marine mussels, the same method as above was carried out with the addition of a PLE step prior to SPE. Previously developed methods for solids extraction were investigated and tailored further for the analysis of pharmaceuticals in marine bivalves (Lacey *et al.*, 2008; Wille *et al.*, 2011). Prior to extraction, mussels were frozen at –80 °C, freeze-dried, homogenised and sieved to 125 µm. PLE was performed on a Dionex ASE® 200 (Dionex Corp., Sunnyvale, CA, USA). A cellulose filter (19.8 mm, Dionex Corp.) was placed on the bottom of a 33 mL stainless steel extraction cell. Each cell was filled with 20 g of activated neutral aluminium oxide (Al_2O_3) (Sigma Aldrich, Steinheim, Germany). A mixture of 1 g of freeze-dried biotic sample with 10 g of ottawa sand (20-30 mesh, Fisher Scientific, Cheshire, UK) was placed on top of the Al_2O_3 and any necessary spiking was carried out at this stage (for method performance and external calibration curves). The remaining dead volume of the cell was filled with sand. A

combination of acetonitrile/water (3:1) was used as the extraction solvent. Extraction was carried out at 60 °C for three cycles of each 5 min. The solvent extracts (≈55 mL) were dried under nitrogen while heated to 40 °C with a Turbovap LV to a final volume less than 10 mL and further diluted to 200 mL with ultra-pure water. The diluted extract was further extracted by SPE, reconstituted and analysed by LC-MS/MS following the procedure described above for effluent and marine surface water samples in Section *2.2.3.2*.

2.2.4 Instrumental conditions

Separations were carried out with an Agilent 1200 LC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary solvent manager, autosampler, UV detector and Waters Sunfire C_{18} column (3.5 µm, 150 mm×2.1 mm, Waters Corp., MA, USA) and guard column (3.5 µm, 2.1 mm×10 mm, Waters Corp., MA, USA). Mobile phases were 80:20 v/v 13 mM ammonium acetate in water (pH 6.2) /acetonitrile (A) and 100 % acetonitrile (B) with a flow rate of 0.3 $mL.min^{-1}$ and a gradient profile as follows: 0-2 min was 100 % A; 2-3 min B was raised to 50 %; 3-9 min B was raised to 80 % and then adjusted to 100 % for a further 5 mins. Re-equilibration time was 15 min and injection volumes were 10 µL. A Bruker Daltonics Esquire HCT Ion Trap mass spectrometer equipped with an atmospheric pressure interface-electrospray ionisation (API-ESI) source was used for pharmaceutical identification and quantification. Nitrogen, used as a nebulising and desolvation gas, was provided by a high purity nitrogen generator, available on site through tap, and helium (99.999 %) (Air Products Plc, Crewe, UK) was used as a collision gas. The LC-MS/MS system was controlled using Bruker Compass HyStar version 3.2. Mass spectrometric analysis was carried out in SRM mode, measuring the fragmentation of the ions produced from each analyte. The optimal mass spectrometric conditions (Table 2.2) were determined by direct infusion using a Cole-Parmer 74900 Series syringe pump set to deliver 300 $\mu L.h^{-1}$ of analyte solution from a 500 µL glass syringe. The nebuliser pressure and drying gas flow were increased slightly after optimisation to account for the higher flow rates set for sample analysis.

Table 2.2: Electrospray ionisation tandem mass spectrometric conditions

Electrospray ionisation conditions		
	Negative ion mode	Positive ion mode
Capillary (V)	3150	2825
End plate offset (V)	−500	−500
Dry gas flow (N_2, $L.min^{-1}$)	20	20
Nebuliser pressure (psi)	8	8
Dry gas temperature (°C)	300	300
Mass spectrometric conditions		
	Negative ion mode	Positive ion mode
Skimmer (V)	−15	36
Capillary exit (V)	−70	130
Trap drive	20	24
Oct RF	100	200
Lens 1	2.3	−1.5
Lens 2	35	−32

2.2.5 Method performance

Quantities of linearity, LOD, limit of quantification (LOQ), percentage recovery, ion suppression and precision (both instrumental and method) were investigated for all target ions allowing for reliable confirmation of analytes. Instrumental linearity was evaluated using linear regression analysis measuring peak height versus concentration. LOD was determined by the lowest concentration of an analyte which produced a signal-to-noise (S/N) ratio of approximately 3:1. LOQ was determined as the analyte concentration to give a S/N ratio of 10:1. Both LOD and LOQ were calculated using the S/N ratios of three low-level spiked samples (n=9). Instrumental retention time precision was carried out for n=11 replicate injections of a 1 $mg.L^{-1}$ standard and n=6 replicates of the same standard used to determine peak height precision. Reproducibility studies were carried out by spiking sample matrices (n=6) pre-extraction to concentrations of 0.5 $\mu g.L^{-1}$ for ultra-pure water, marine surface water and wastewater effluent (500 mL) and 0.25 $\mu g.g^{-1}$ for marine mussels (1 g). Percentage recoveries were calculated using this data by comparison to final extracts of the unspiked matrix (n=3) reconstituted in 250 µL of mobile

phase A containing the expected 100 % recovery concentration. Ion suppression and enhancement was also investigated by comparing aqueous and mussel sample extracts spiked post-extraction to an analyte mixture of the same concentration prepared in mobile phase A. Calibration curves (n=12 in triplicate) were constructed in each matrix to assess method performance.

2.3 Results and Discussion

2.3.1 Sample preparation

Few studies to date have investigated the presence of pharmaceutical concentrations in marine species such as blue mussels (Cueva-Mestanza *et al.*, 2008; Klosterhaus *et al.*, 2013; Wille *et al.*, 2011). Soxhlet extraction methods have been replaced by alternative methods which use significantly less volumes of organic solvent such as PLE and microwave-assisted extraction (MAE). Several recent studies have utilised PLE to investigate the presence of pharmaceuticals in soil, sediment and aquatic biota (Barron *et al.*, 2008; Huerta *et al.*, 2013; Jelic *et al.*, 2009; Wille *et al.*, 2011). This technique was selected and used in conjunction with a modified SPE method previously developed for pharmaceutical extraction from effluent (Lacey *et al.*, 2008).

Prior to PLE, the freeze-dried biotic tissue was ground with a pestle and mortar and sieved to a particle size of approximately 125 µm. Smaller particle size allows for greater surface area exposure and shorter diffusion distances of analytes from sample to extraction solvent (Bjorklund *et al.*, 2000). Larger sample remains (≤20 %), unable to pass through the sieve, were discarded. For PLE optimisation, various extraction solvents were tested initially (1:1 methanol/water, 1:1 methanol/water with 1 % formic acid, 1:1 acetonitrile/water, 3:1 acetonitrile/water, 3:1 acetonitrile/water with 1 % formic acid). A mixture of 3:1 acetonitrile/water provided the best recovery. Second, the optimal temperature (60, 80 and 100 °C), static time (5 and 10 min), and number of cycles (2 and 3) were investigated. Three cycles of 5 min were found to be optimal for the extraction of the target analytes. Slightly higher recoveries were observed for all compounds, particularly at 60 °C compared to 100 °C. The flush volume was also evaluated starting at the

manufacturers recommendation of 60 % and increasing to 100 % which showed higher recoveries overall. Next, the addition of Al_2O_3 to the extraction cell was evaluated. Al_2O_3 inhibits the co-extraction of lipids and other hydrophobic matrix constituents and was tested by addition of several different quantities (10, 15 and 20 g) to the cell. Cleaner abstracts were obtained with the higher quantity of 20 g of Al_2O_3 added to the cell. For fine powdery samples, such as freeze-dried biotic tissue, it is recommended to mix the sample with dispersion agents, such as diatomaceous earth and sodium sulphate, to prevent clogging and allow for improved contact between the analyte and solvent (Runnqvist *et al.*, 2010). Therefore, various quantities of Ottawa sand (2.5, 5 and 10 g) were pre-mixed with the sample and transferred to the cell which was then filled to the top with sand. The higher the quantity of sand mixed through the sample, the slightly higher recovery of analytes observed, therefore, 10 g of sand was mixed with the samples. The sample mass itself was tested by analysing the freeze-dried and sieved biotic tissue (0.5, 1 and 2 g) spiked at 250 $ng.g^{-1}$. Golet *et al.* (2002) found that increasing the sample size of soil and sewage sludge samples resulted in lower analyte recoveries and turbid extracts, explaining why cleaner extracts with higher analyte recoveries were achieved with 1 g of sample in comparison to 2 g. Due to the complexity of biotic samples, further clean-up was required following PLE in order to achieve low matrix interference. Extraction of pharmaceuticals from the diluted PLE extract is most commonly carried out using SPE.

Strata-X SPE cartridges have previously been used for the extraction of acidic, basic and neutral pharmaceutical mixes in effluent, surface waters and marine mussels (Hilton and Thomas, 2003; Lacey *et al.*, 2008; Wille *et al.*, 2011). The analytical method previously established by Lacey *et al.* (2008) was re-validated here for the determination of pharmaceutical residues in all aqueous and marine mussel samples. Minor adjustments were made to assess any improved method performance including SPE eluting/reconstitution solvent, LC run time, mobile phase composition and gradient conditions. Higher recoveries have been reported for saline samples such as marine surface water when using larger post-load washing volumes (Wille *et al.*, 2010; Wu *et al.*, 2010) but, when evaluated, there was no overall increase in recovery with washing volumes greater than 6 mL. Eluting solvents were also re-investigated here as a means of increasing percentage recovery of analytes from a more complex matrix and in comparison to methanol, 50:50 (v/v) ethyl acetate/acetone exhibited marginally higher recoveries on average.

2.3.2 Analytical method development

Due to the limited volatility of the investigated analytes and their polar nature, which would require derivatisation prior to analysis with GC-MS, LC-MS/MS was the method of analysis chosen for this study. For pharmaceutical analysis using reversed-phase HPLC, water and acetonitrile are commonly used mobile phase solvents. Additives (in this case ammonium acetate) are normally used to facilitate efficient ESI through increasing ionic strength. A 13 mM ammonium acetate solution in a mixture of acetonitrile and water was selected as a suitable mobile phase. Separate analyses using negative and positive ionisation modes were employed to maximise peak definition and quantitative reliability. Direct infusion of each standard was carried out initially in full scan mode to determine an abundant precursor ion. In negative mode ESI-MS, $[M–H]^-$ precursor ions were produced for diclofenac, gemfibrozil and mefenamic acid and $[M+H]^+$ precursor ions were produced for carbamazepine and trimethoprim in positive mode ESI-MS. SRM was carried out under both positive and negative ion modes. Selected transitions, shown in Table 2.3, were monitored for the quantification and confirmation of each analyte. The fragmentation amplitude was set at 0.5 in negative mode and 0.6 in positive mode with isolation width set at 2.0 m/z for all analytes. A selection of EICs of each pharmaceutical, occurring at their highest concentrations in either effluent, marine surface waters or marine mussels, are shown in Figure 2.5.

Table 2.3: Precursor, quantification and confirmation ions (m/z) for each pharmaceutical and their corresponding fragmentation amplitudes

Compound	Precursor ion	ESI mode	Quantification ion	Confirmation ion	Fragmentation amplitude
Carbamazepine	237	+	194	192	0.6
Diclofenac	294	−	250	236	0.5
Gemfibrozil	249	−	121	127	0.5
Mefenamic acid	240	−	196	223	0.5
Trimethoprim	291	+	123	230	0.6

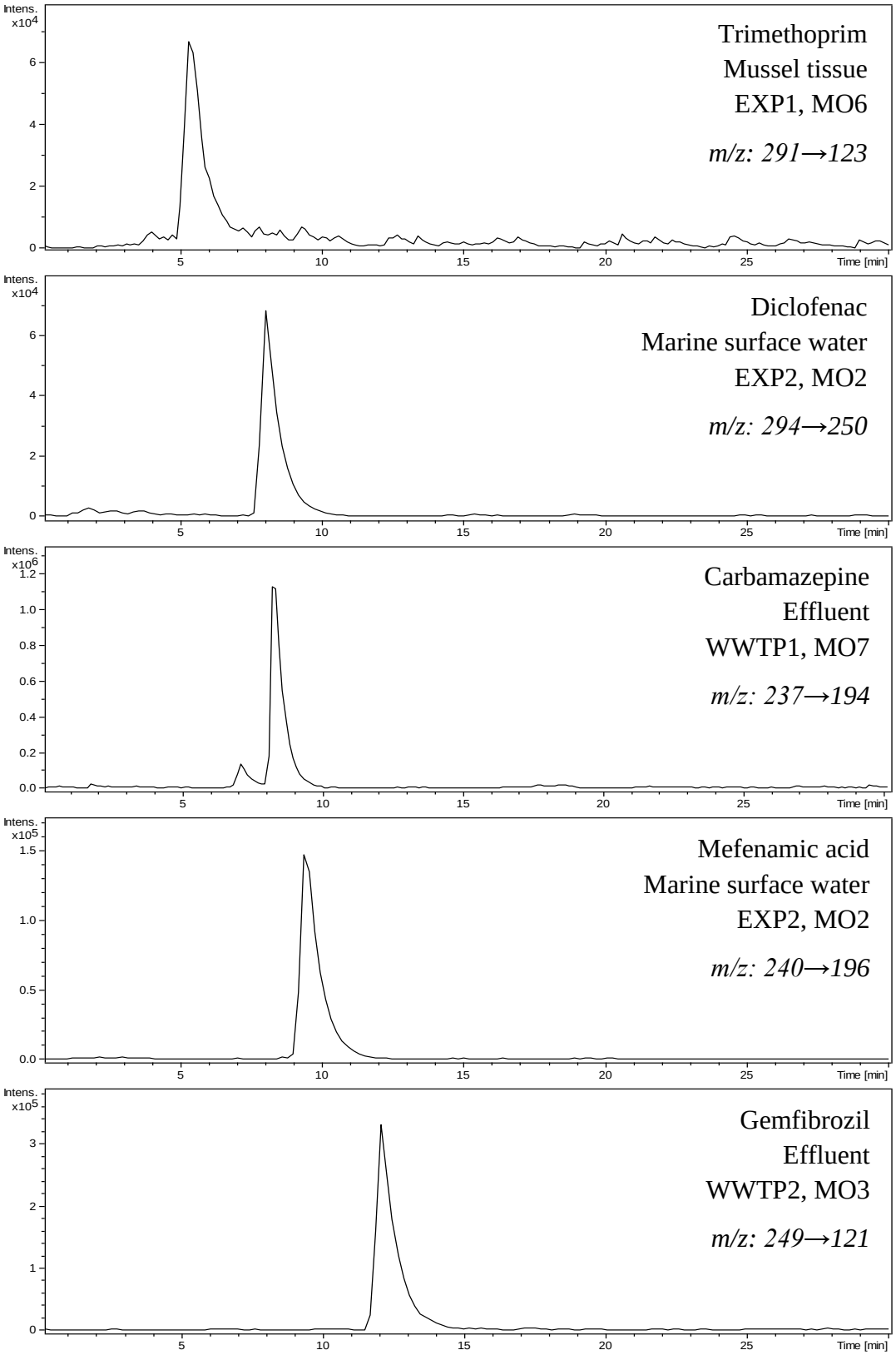


Figure 2.5: EICs of the most concentrated occurrence of selected pharmaceuticals in samples of wastewater effluent, marine surface water and marine mussels collected from exposure sites

2.3.3 Instrumental and method performance

Method performance results for individual pharmaceuticals in ultra-pure water, effluent, marine surface water and mussel tissue are shown in Table 2.4. Upon investigation of instrumental precision, retention times varied ≤1.3 % for all analytes in each matrix. For aqueous samples, correlation coefficients of ≥0.987 (n≥8) were achieved for most analytes, except mefenamic acid (R^2≈0.98), over the ranges of 0.025 to 5 µg.L^{-1} using negative ESI-MS/MS mode and 0.005 to 2.5 µg.L^{-1} using positive ESI-MS/MS mode. LOQs were as low as 15 to 225 ng.L^{-1} and 3 to 38 ng.L^{-1} in effluent and marine surface water, respectively. Due to the complexity and high salt content of marine surface water, pharmaceutical detection in this matrix is difficult and has not been reported to a wide extent. Method performance results correlated with previous data reported for the analysis of 13 pharmaceuticals in natural sea water (Wille *et al.*, 2010). Recoveries of analytes ranged from 62 to 99 % in effluent and 56 to 110 % in marine surface water for n=6 replicates of a 1 µg.L^{-1} standard mix with relative standard deviations (RSDs) ≤11 %. Signal suppression was observed for all analytes in marine surface water and for all analytes except gemfibrozil in effluent. The signal enhancement of gemfibrozil in effluent is in stark contrast to the signal suppression of gemfibrozil in marine surface water. At 56 %, gemfibrozil underwent the highest matrix induced suppression out of the five pharmaceuticals in marine surface water but, signal was not greatly reduced as recoveries >100 % were achieved. Most noticeable in the effluent matrix was the signal suppression observed for the pharmaceuticals in positive ESI-MS mode, i.e. trimethoprim and carbamazepine, at 63-65 %. In contrast, these compounds were the least suppressed analytes in the marine surface water matrix. The high salt content of marine surface waters suggests that salt residues may still be present in the sample extract and co-eluting with the selected analytes. As can be seen from Table 2.4, the method developed allows for quantification of the target analytes in ultra-pure water also. Recoveries between 79 and 98 % were achieved for all pharmaceuticals in ultra-pure water, highlighting the influence of complex matrices, such as effluent and marine surface water, on ion suppression or enhancement.

Table 2.4: Method performance data for pharmaceuticals in ultra-pure water (500 mL), wastewater effluent (500 mL), marine surface water (500 mL) and marine mussel tissue (1 g)

Compound		Ultra-pure water			Effluent		
	t_r* (min) n=12	LOQ ($ng.L^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6	LOQ ($ng.L^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6
Trimethoprim	5.9	2	0.993	84±3	49	0.993	88±6
Diclofenac	8.1	5	0.997	98±8	225	0.991	70±9
Carbamazepine	8.2	2	0.991	93±9	15	0.995	99±7
Mefenamic acid	9.0	6	0.999	96±10	219	0.976	62±8
Gemfibrozil	11.5	8	0.999	79±7	35	0.995	66±11
Compound		Marine surface water			Marine mussels (DW)		
		LOQ ($\mu g.L^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6	LOQ ($ng.g^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6
Trimethoprim		3	0.987	56±4	4	0.985	91±9
Diclofenac		22	0.995	110±11	29	0.990	83±8
Carbamazepine		4	0.995	86±5	6	0.987	100±5
Mefenamic acid		29	0.977	91±7	23	0.990	104±12
Gemfibrozil		38	0.995	108±5	18	0.993	100±20

*Average time recorded for each pharmaceutical in marine mussel tissues

[a] Three data points carried out in triplicate

[b] Eight data points carried out in triplicate

Marine mussels (1 g, n=12 in triplicate) were spiked in the extraction cell, after mixing with sand, with a standard mixture resulting in final concentrations of 5-5000 $ng.g^{-1}$ of each pharmaceutical (solvent volume added ≤250 μL). Linearity was achieved for analytes in negative mode from 10 to 5000 $ng.g^{-1}$ (R^2≥0.990) and in positive mode from 5 to 2500 $ng.g^{-1}$ (R^2≥0.985). All analytes were quantifiable at concentrations of between 4 and 29 $ng.g^{-1}$. These performance results are comparable to previously reported results for the same and similar pharmaceuticals investigated in marine mussels (Cueva-Mestanza *et al.*, 2008; Wille *et al.*, 2011) and other solid matrices such as sludge (Barron *et al.*, 2008) and fish (Ramirez *et al.*, 2007) (Table 2.5). Method recoveries were in the range of 83 to 104 % with precision varying ≤20 % for n=6 replicates of a 250 $ng.g^{-1}$ standard mix. Signal suppression ranged from 25 to 44 % for analytes in marine mussel tissue with the signal for gemfibrozil again exhibiting the greatest suppression.

In order to minimise the matrix effect on the quantification of ions using external calibration, standard addition (n=4, unless otherwise stated) was performed in effluent and marine surface water samples. However, for the quantification of pharmaceuticals in marine mussels, external calibration curves were prepared in matrix-matched samples to avoid lengthy processing times for larger amounts of samples. For quality control of the method, an injection of starting mobile phase was run between each sample with no carry over observed.

Table 2.5: Comparison of method performance results for pharmaceuticals in solid matrices from selected literature using LC-MS analysis

	Mussel tissue (DW)		Mussel tissue (DW)		Fish tissue (WW)		Sludge (DW)	
	Results		Wille *et al.* (2011)		Ramirez *et al.* (2007)		Barron *et al.* (2008)	
Compounds	Linearity	LOQ ($ng.g^{-1}$)	Linearity	LOQ ($ng.g^{-1}$)	Linearity	LOQ ($ng.g^{-1}$)	Linearity	LOD ($ng.g^{-1}$)
Carbamazepine	>0.98	6	>0.99	1	>0.99	0.1	>0.99	3
Diclofenac	>0.99	29	>0.99	25			>0.99	7
Gemfibrozil	>0.99	18			>0.99	0.9	>0.98	2
Mefenamic acid	>0.99	23						
Trimethoprim	>0.98	4	>0.99	1	>0.99	2.6	>0.98	50

2.3.4 Application to wastewater effluent, marine surface waters and caged *Mytilus* spp. exposed along the Irish coast

The developed methods were applied to 24-h composite effluent samples (WWTP1 and WWTP2), marine surface water grab samples and marine mussel samples (CON, EXP1 and EXP2). Samples from CON, EXP1 and WWTP1 were collected over a 12-month period. Due to rough sea weather and the consequential loss of a cage, a shorter exposure was carried out at EXP2, where marine mussels were sampled for three months and effluent and marine surface water samples were collected from WWTP2 and EXP2 for four months.

2.3.4.1. Wastewater effluent

As can be deduced from Figures 2.6 and 2.7, pharmaceutical residues were quantified in Irish wastewater effluent entering Irish surface waters. All five of the selected pharmaceuticals were detected in >85 % of effluent samples collected from each exposure site. Effluent discharged from WWTP2 was found to contain the highest concentrations of pharmaceutical residues, except for carbamazepine, with >1 $\mu g.L^{-1}$ measured for at least one sampling point over the four month period. Diclofenac and mefenamic acid were measured at environmentally relevant concentrations of 2.63 $\mu g.L^{-1}$ and 2.80 $\mu g.L^{-1}$, respectively, from WWTP2 and carbamazepine measured at concentrations up to 3.16 $\mu g.L^{-1}$ from WWTP1 (See Appendix). In comparison, WWTP1 caters for 15-fold more people than WWTP2 but pharmaceutical concentrations detected over the same four months (May to August 2011) were found to be slightly lower from WWTP1. This may be as a result of the tertiary treatment carried out on the wastewater at WWTP1 from May to September. UV treatment has been shown to partially remove some antibiotics in drinking water but this is at a UV dose approximately 100 times greater than that typically supplied for effluent disinfection (Adams *et al.*, 2002) therefore, pharmaceutical residues in UV treated effluent may be slightly reduced but not completely eliminated. When compared to the concentrations of pharmaceutical residues present in the effluent throughout the year, no obvious reduction in pharmaceutical concentrations was observed for UV treated effluent collected from WWTP1.

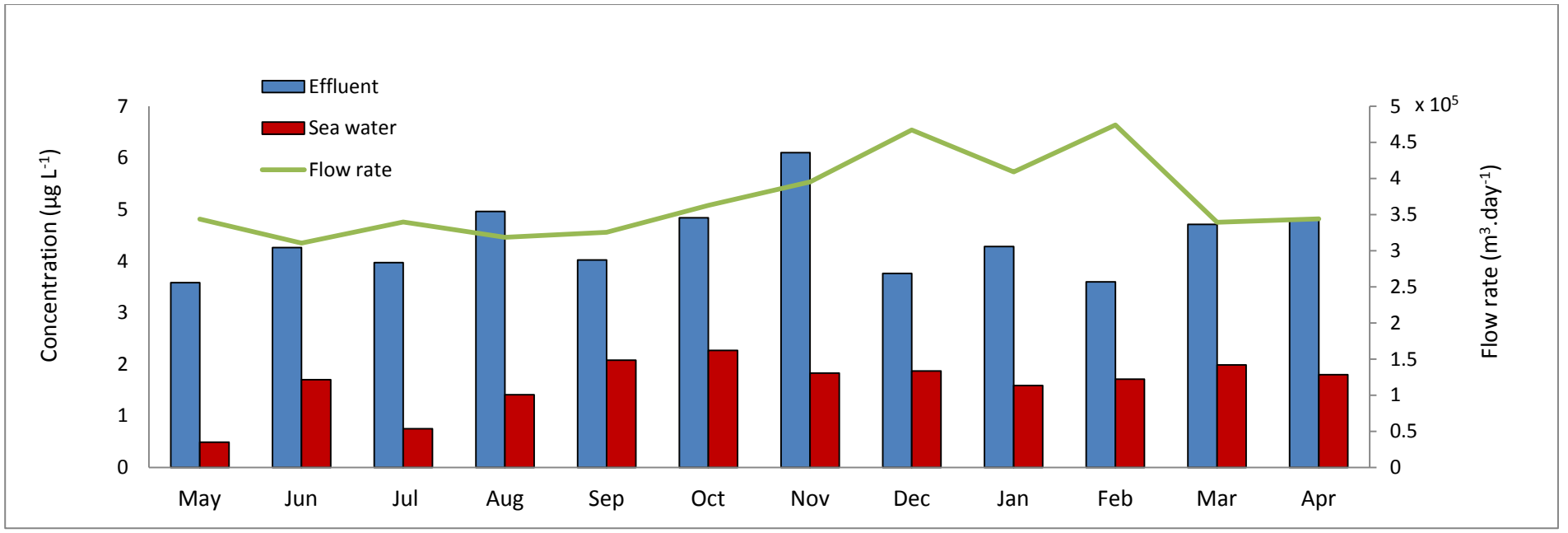


Figure 2.6: Cumulative pharmaceutical concentrations (µg.L^{-1}) detected in effluent from WWTP1 and marine surface water from EXP1 and WWTP flow rates (May 2011-April 2012)

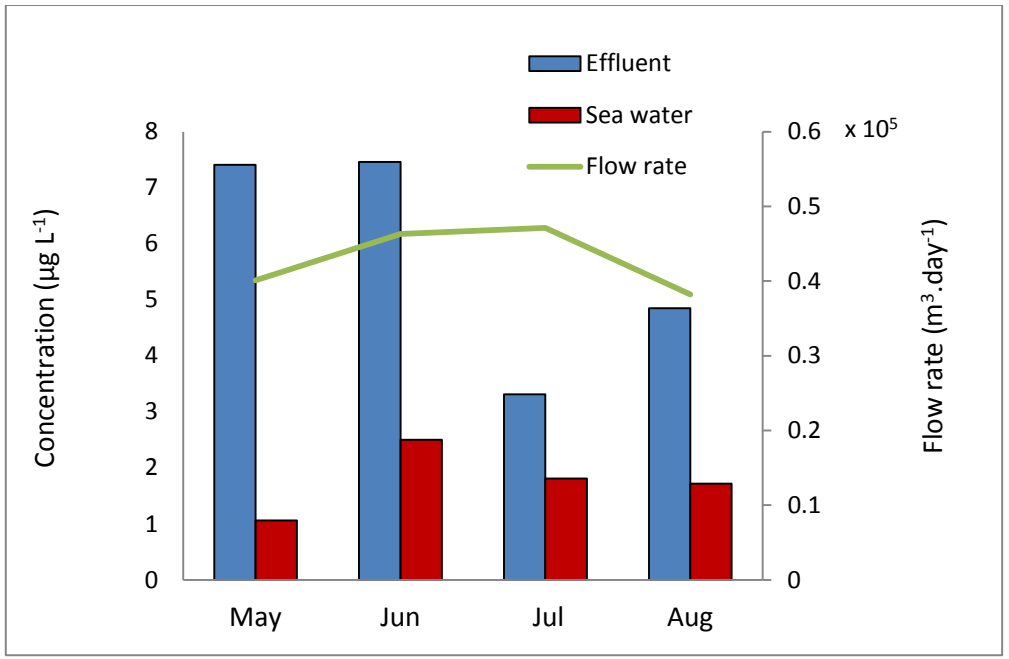


Figure 2.7: Cumulative pharmaceutical concentrations (µg.L^{-1}) detected in effluent from WWTP2 and marine surface water from EXP2 and WWTP flow rates (May 2011-August 2011)

In comparison to pharmaceutical concentrations detected in effluent from WWTPs worldwide, slightly higher levels were reported in the effluent from the selected Irish WWTPs in this study, in line with observations from previous Irish wastewater studies. Lacey *et al.* (2012) reported the presence of diclofenac, mefenamic acid, trimethoprim and carbamazepine in the effluent from WWTP1 at concentrations up to 0.5 $\mu g.L^{-1}$, 9.1 $\mu g.L^{-1}$, 0.6 $\mu g.L^{-1}$ and 6.5 $\mu g.L^{-1}$, respectively. Furthermore, gemfibrozil was measured up to 0.1 $\mu g.L^{-1}$ in influent but was not detected in the treated effluent. This study revealed similar orders of concentration for the same compounds in effluent sampled from the same WWTP however, gemfibrozil concentrations were detected in the effluent and measured from <LOD-0.65 $\mu g.L^{-1}$. Although population equivalents of the other secondary WWTPs studied by Lacey *et al.* (2012) were approximately half that of WWTP2, diclofenac residues were recorded in their effluents at concentrations up to 3 $\mu g.L^{-1}$.

2.3.4.2. Marine surface water

The pseudo-persistence of pharmaceuticals in the aquatic environment was observed with the continuous detection of pharmaceutical residues in marine surface waters, at slightly lower concentrations than those detected in effluent. The antiepileptic, carbamazepine, had the highest concentration in marine surface waters, measuring at 1.41 $\mu g.L^{-1}$ at EXP1. Trimethoprim was repeatedly detected at both exposures with much higher concentrations of up to 0.87 $\mu g.L^{-1}$ detected at EXP2. Residues of diclofenac and mefenamic acid varied in their concentrations each month but measured highest at EXP2 with concentrations of 0.55 $\mu g.L^{-1}$ and 0.61 $\mu g.L^{-1}$, respectively. The lipid regulator, gemfibrozil, was the least detected pharmaceutical in marine surface water but was measured at concentrations of up to 0.64 $\mu g.L^{-1}$ at EXP2. The highest pharmaceutical concentrations in marine surface waters were detected at EXP2, with the exception of carbamazepine (See Appendix). None of the selected analytes were detected in marine surface water samples from the CON site.

A selection of monitoring studies has been performed previously in European estuarine and marine surface waters. In a study carried out by Thomas and Hilton (2004), 14 pharmaceuticals were monitored in British estuaries of the Thames, the Tyne, the Mersey, the Tees and Belfast Lough. From the targeted list, nine pharmaceuticals were detected in the estuarine water samples collected. Ibuprofen and trimethoprim were determined at the highest concentrations,

measuring at 928 ng.L^{-1} and 569 ng.L^{-1}, respectively, and clotrimazole was the most frequently detected pharmaceutical with a median concentration of 7 ng.L^{-1}. A number of other studies have analysed marine water samples collected from the North Sea, its estuaries and harbours (Buser *et al*., 1998; Langford and Thomas, 2011; Weigel *et al.*, 2002; Weigel *et al.*, 2004). Eight pharmaceuticals were previously determined in Belgian coastal waters, with concentrations of salicylic acid and carbamazepine measured most frequently at concentrations up to 860 ng.L^{-1} (Wille *et al*., 2010). Monitoring studies in the Mediterranean Sea revealed the presence of eight pharmaceuticals, including verapamil, atenolol and metolol in the low ng.L^{-1} range. In comparison to previous monitoring studies, pharmaceutical residues determined in marine surface waters from EXP1 and EXP2 here are therefore similar to those previously detected in British and Belgian estuaries but, overall, are higher than the average pharmaceutical concentrations detected in other European marine surface waters.

The presence of high pharmaceutical concentrations in Irish WWTP effluent is likely to be the reason for the higher than average pharmaceutical concentrations measured in Irish marine surface waters. The cumulative pharmaceutical concentrations in effluent and marine surface water from both EXP1 and EXP2 exposure sites are shown in Figures 2.6 and 2.7, respectively. Most notably, higher concentrations of pharmaceutical residues in marine surface waters were determined during periods of higher effluent outflow. Insufficient data does not allow for the determination of the dilution factor at each sampling site, but, as observed from the results, the short distances of the sites (outlined in Section *2.2.2*) from the effluent outfall pipes did not allow for significant dilution of the pharmaceutical concentrations. Other factors may also explain these high pharmaceutical concentrations in marine waters including the restriction of access to sampling sites at high tide, requiring sample collection to be carried out at low tide, and the continuous flow of effluent from the outfall pipe, regardless of tidal patterns. That said, in some cases, pharmaceuticals in marine surface waters were determined at higher concentrations than those in the WWTP effluent. Pharmaceutical measurements were based on one sample of effluent and marine surface water collected from each site over 1-2 days each month, depending on weather conditions. The pharmaceutical concentrations in the effluent should not be taken as the exact same concentrations exposed to the marine surface water

sampled during the same month. Higher loads of certain pharmaceuticals may occur over time depending on human consumption.

Effluent and marine surface water analysis was carried out previously on samples collected approximately 700 m downstream of the effluent outfall pipe for WWTP1 between the months of July to September 2010. In comparison to the samples collected at EXP1, the pharmaceutical residues measured in the marine surface water samples were almost 100 times lower. These results reveal the extent of dilution of the pharmaceuticals at less than 1 km from the effluent outfall pipe. Effluent outfall pipes built further out at sea encourages dilution of environmental contaminants and reduced risk of chronic exposure to non-target aquatic organisms.

2.3.4.3. Marine mussels

The accumulation of a large number of pharmaceuticals in solid media such as sludge was the focus of recent work in our laboratory. These studies support some of the findings shown here for pharmaceutical occurrence in wastewaters and their potential for uptake in mussels (Barron *et al.*, 2008; Barron *et al.*, 2009; Barron *et al.*, 2010). Besides the physicochemical properties of pharmaceutical compounds, the potential for pharmaceuticals to bioaccumulate in aquatic biota also depends on several biological factors such as age, species, diet, habitat and reproductive cycle (Meredith-Williams *et al.*, 2012). The uptake of pharmaceuticals in biological tissue may be possible due to a small portion of unionised species of the compounds remaining in the aqueous phase, which has a greater affinity for lipophilic matter. With a pK_a of 6.6, trimethoprim is the only pharmaceutical of those selected not to be completely ionised in marine surface water at pH 8.0. This may be the reason why a very low but constant concentration of trimethoprim was detected in exposed mussel tissue. Residues of trimethoprim in the *Mytilus* spp. from EXP1 were quantified at concentrations of 9.22 $ng.g^{-1}$ and 7.28 $ng.g^{-1}$ DW (See Table A.2 of Appendix) for the respective months of October and November, during which time tertiary treatment was not carried out on the wastewater. Carbamazepine and mefenamic acid were also detected in mussel tissues from both exposure sites, but at <LOQ levels. Although mefenamic acid and carbamazepine have slightly higher LOQ values, their concentrations in surrounding marine waters were on average slightly higher than those detected for trimethoprim and thus, their presence in mussel tissues may be as a result of the uptake of residual levels,

although this may also be the case for trimethoprim. It is important to note that the mussels were not depurated before analysis, therefore, remains in the mussel digestive tract were included in the measurements. Selected pharmaceuticals had a broad range of temporal variability and particularly in the cases of gemfibrozil and mefenamic acid, residues dropped below detectable levels in marine surface waters. This may be due to the higher salt content in marine waters causing these pharmaceuticals to become less soluble, and ultimately, 'salt out' of solution. None of the selected analytes were detected in marine mussel samples collected from the CON site.

In a similar recent study, five cages of blue mussels were deployed off the Belgian coast. Over a six-month period, five pharmaceuticals were detected in mussel tissues including salicylic acid residues, measuring up to 490 $ng.g^{-1}$ DW (Wille *et al.*, 2011). Whilst salicylic acid was determined in the mid $ng.g^{-1}$ range, mostly lower and fluctuating concentrations of all other pharmaceuticals including carbamazepine, ofloxacin, propranolol and paracetemol were observed. In a separate study in North Carolina, the antidepressant, fluoxetine, was detected in caged mussel tissues up to 79 $ng.g^{-1}$ WW after 14 days exposure in a wastewater effluent channel. Fluoxetine was also detected in mussels caged approximately 100 m downstream from the effluent outfall pipe at concentrations of 9.8 $ng.g^{-1}$ WW where residues in surrounding waters measured up to 7.3 $ng.L^{-1}$ (Bringolf *et al.*, 2010). The presence of oestrogens in Irish seawater and their potential for uptake in marine mussels was only recently investigated. Although low $ng.L^{-1}$ levels of oestrone were detected in marine surface waters, no uptake was determined in exposed mussel tissues due to high levels of interference from the co-extraction of matrix components (Ronan and McHugh, 2013). Most recently, wild marine mussels were collected from several diversely impacted areas in the Mediterranean Sea with traces of carbamazepine measuring up to 3.5 $ng.g^{-1}$ DW in mussels residing from wastewater exposed waters (Bueno *et al.*, 2013). Similarly, wild ribbed horse mussels were collected from five near shore sites in San Francisco Bay and analysed for the presence of up to 104 PPCP residues. Carbamazepine and gemfibrozil were detected in the surrounding marine surface waters at concentrations between 38-92 $ng.L^{-1}$ but, mussels collected from the same sites were found to contain only very low residues of carbamazepine at concentrations up to 2.4 $ng.g^{-1}$ WW (Klosterhaus *et al.*, 2013). Uptake of pharmaceuticals has been previously confirmed in other wild aquatic species such as fish (Brooks *et al.*, 2005;

Fick *et al.*, 2010; Huerta *et al.*, 2013; Togunde *et al.*, 2012) with glucuronised parent compounds measuring at higher concentrations to the unmetabolised compounds in one particular study (Lahti *et al.*, 2011). The cost and limited availability of glucuronised pharmaceutical compounds did not allow for further investigation into the bioaccumulation of metabolites within the mussel.

Wild mussels were observed at EXP1 and a considerable number of crab and oyster pots were observed in close proximity to the effluent outfall pipe at EXP2. The presence of pharmaceuticals in the caged mussels suggests a possibility for pharmaceutical uptake in wild species and potential exposure to humans via ingestion. The measurement of pharmaceuticals in aquatic species that are important in terms of human consumption, such as mussels, is very useful in estimating the human exposure and dietary intake of these pharmaceuticals.

2.4 Conclusions

For the first time, the spatial occurrence of five targeted pharmaceuticals in the aquatic environment was monitored over a 12-month period. Analytical techniques such as PLE, SPE and LC-MS/MS were combined, optimised and applied to wastewater effluent, marine surface water and *Mytilus* spp. samples collected from two impacted sites and a control site on the Irish coastline. The presence of all five targeted pharmaceuticals was confirmed in the low $\mu g.L^{-1}$ in effluent and in the high $ng.L^{-1}$ in exposed marine surface water. Residues of carbamazepine measured highest in exposed marine surface water at concentrations up to 1.41 $\mu g.L^{-1}$. Three of the five detected pharmaceuticals in marine surface waters were also found to occur in exposed *Mytilus* spp., with residues of trimethoprim measuring at concentrations up to 9.22 $ng.g^{-1}$ DW. This study has confirmed the uptake of pharmaceuticals in marine bivalves at measurable quantities and also highlights the inability of mussels to act as reliable bioindicators of pollution for the selected pharmaceuticals due to temporal variations observed in the data. These findings will contribute to pharmaceutical fate studies and aid in the assessment of ecological and health risks posed by these contaminants in the natural aquatic environment.

References

Adams, C., Wang, Y., Loftin, K., Meyer, M. (2002) Removal of antibiotics from surface and distilled water in conventional water treatment processes. *Journal of Environmental Engineering-Asce* **128**, 253-260.

Barron, L., Tobin, J., Paull, B. (2008) Multi-residue determination of pharmaceuticals in sludge and sludge enriched soils using pressurized liquid extraction, solid phase extraction and liquid chromatography with tandem mass spectrometry. *Journal of Environmental Monitoring* **10**, 353-361.

Barron, L., Havel, J., Purcell, M., Szpak, M., Kelleher, B., Paull, B. (2009) Predicting sorption of pharmaceuticals and personal care products onto soil and digested sludge using artificial neural networks. *Analyst* **134**, 663-670.

Barron, L., Nesterenko, E., Hart, K., Power, E., Quinn, B., Kelleher, B., Paull, B. (2010) Holistic visualisation of the multimodal transport and fate of twelve pharmaceuticals in biosolid enriched topsoils. *Analytical and Bioanalytical Chemistry* **397**, 287-296.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A. (2009) Pharmaceuticals and endocrine disrupting compounds in US drinking water. *Environmental Science & Technology* **43**, 597-603.

Bjorklund, E., Nilsson, T., Bowadt, S. (2000) Pressurised liquid extraction of persistent organic pollutants in environmental analysis. *Trends in Analytical Chemistry* **19**, 434-445.

Bones, J., Nesterenko, P., Thomas, K., Paull, B. (2006) Dual gradient LC method for the determination of pharmaceutical residues in environmental samples using a monolithic silica reversed phase column. *International Journal of Environmental Analytical Chemistry* **86**, 487-504.

Boxall, A.B.A. (2004) The environmental side effects of medication - how are human and veterinary medicines in soils and water bodies affecting human and environmental health? *Embo Reports* **5**, 1110-1116.

Bringolf, R.B., Heltsley, R.M., Newton, T.J., Eads, C.B., Fraley, S.J., Shea, D., Cope, W.G. (2010) Environmental occurence and reproductive effects of the pharmaceutical fluoxetine in native freshwater mussels. *Environmental Toxicology and Chemistry* **29**, 1311-1318.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J. (2005) Determination of select antidepressants in fish from an effluent-dominated stream. *Environmental Toxicology and Chemistry* **24**, 464-469.

Brown, J.N., Paxeus, N., Forlin, L., Larsson, D.G.J. (2007) Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. *Environmental Toxicology and Pharmacology* **24**, 267-274.

Bueno, M.J.M., Boillot, C., Fenet, H., Chiron, S., Casellas, C., Gomez, E. (2013) Fast and easy extraction combined with high resolution-mass spectrometry for residue analysis of two anticonvulsants and their transformation products in marine mussels. *Journal of Chromatography A* **1305**, 27-34.

Buser, H.R., Muller, M.D., Theobald, N. (1998) Occurrence of the pharmaceutical drug clofibric acid and the herbicide mecoprop in various Swiss lakes and in the North Sea. *Environmental Science & Technology* **32**, 188-192.

Cabello, F.C. (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology* **8**, 1137-1144.

Cueva-Mestanza, R., Torres-Padron, M.E., Sosa-Ferrera, Z., Santana-Rodriguez, J.J. (2008) Microwave-assisted micellar extraction coupled with solid-phase extraction for preconcentration of pharmaceuticals in molluscs prior to determination by HPLC. *Biomedical Chromatography* **22**, 1115-1122.

Debska, J., Kot-Wasik, A., Namiesnik, J. (2004) Fate and analysis of pharmaceutical residues in the aquatic environment. *Critical Reviews in Analytical Chemistry* **34**, 51-67.

EFSA (2013) *Report for 2011 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products*. European Food Safety Authority. http://www.efsa.europa.eu/en/supporting/doc/363e .pdf, Accessed September 2013.

Fatta-Kassinos, D., Meric, S., Nikolaou, A. (2011) Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. *Analytical and Bioanalytical Chemistry* **399**, 251-275.

Fent, K., Weston, A.A., Caminada, D. (2006) Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology* **76**, 122-159.

Fick, J., Lindberg, R.H., Parkkonen, J., Arvidsson, B., Tysklind, M., Larsson, D.G.J. (2010) Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents. *Environmental Science & Technology* **44**, 2661-2666.

Golet, E.M., Strehler, A., Alder, A.C., Giger, W. (2002) Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. *Analytical Chemistry* **74**, 5455-5462.

GoogleMaps (2012) *Satellite map of selected exposure sites*. https://google.ie/maps, Accessed Aug 2012.

Heberer, T., Fuhrmann, B., Schmidt-Baumler, K., Tsipi, D., Koutsouba, V., Hiskia, A. (2000) Occurrence of pharmaceutical residues in sewage, river, ground-, and drinking water in Greece and Germany. *Abstracts of Papers of the American Chemical Society* **219**, U623-U623.

Heberer, T. (2002) Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology Letters* **131**, 5-17.

Heberer, T., Reddersen, K., Mechlinski, A. (2002) From municipal sewage to drinking water: fate and removal of pharmaceutical residues in the aquatic environment in urban areas. *Water Science and Technology* **46**, 81-88.

Hernando, M.D., Mezcua, M., Fernandez-Alba, A.R., Barceló, D. (2006) Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. *Talanta* **69**, 334-342.

Hilton, M.J., Thomas, K.V. (2003) Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquid chromatography-electrospray tandem mass spectrometry. *Journal of Chromatography A* **1015**, 129-141.

HSE (2010) *Primary care reimbursement service: statistical analysis of claims and payments*. Irish Health Service Executive. http://www.hse.ie/eng/staff/PCRS/PCRS_Publications/claimsandpayments2010.pdf, Accessed 2 Aug 2010.

Huerta, B., Rodriguez-Mozaz, S., Barceló, D. (2012) Pharmaceuticals in biota in the aquatic environment: analytical methods and environmental implications. *Analytical and Bioanalytical Chemistry* **404**, 2611-2624.

Huerta, B., Jakimska, A., Gros, M., Rodriguez-Mozaz, S., Barceló, D. (2013) Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A* **1288**, 63-72.

Hunt, C.D., Slone, E. (2010) Long-term monitoring using resident and caged mussels in Boston Harbor yield similar spatial and temporal trends in chemical contamination. *Marine Environmental Research* **70**, 343-357.

Jelic, A., Petrovic, M., Barceló, D. (2009) Multi-residue method for trace level determination of pharmaceuticals in solid samples using pressurized liquid extraction followed by liquid chromatography/quadrupole-linear ion trap mass spectrometry. *Talanta* **80**, 363-371.

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sanchez, R., Ventura, F., Petrovic, M., Barceló, D. (2011) Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. *Water Research* **45**, 1165-1176.

Klosterhaus, S.L., Grace, R., Hamilton, M.C., Yee, D. (2013) Method validation and reconnaissance of pharmaceuticals, personal care products, and alkylphenols in surface waters, sediments, and mussels in an urban estuary. *Environment International* **54**, 92-99.

Lacey, C., McMahon, G., Bones, J., Barron, L., Morrissey, A., Tobin, J.M. (2008) An LC-MS method for the determination of pharmaceutical compounds in wastewater treatment plant influent and effluent samples. *Talanta* **75**, 1089-1097.

Lacey, C., Basha, S., Morrissey, A., Tobin, J.M. (2012) Occurrence of pharmaceutical compounds in wastewater process streams in Dublin, Ireland. *Environmental Monitoring and Assessment* **184**, 1049-1062.

Lahti, M., Brozinski, J.M., Jylha, A., Kronberg, L., Oikari, A. (2011) Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout. *Environmental Toxicology and Chemistry* **30**, 1403-1411.

Langford, K., Thomas, K.V. (2011) Input of selected human pharmaceutical metabolites into the Norwegian aquatic environment. *Journal of Environmental Monitoring* **13**, 416-421.

Lapworth, D.J., Baran, N., Stuart, M.E., Ward, R.S. (2012) Emerging organic contaminants in groundwater: a review of sources, fate and occurrence. *Environmental Pollution* **163**, 287-303.

Li, W.H., Shi, Y.L., Gao, L.H., Liu, J.M., Cai, Y.Q. (2012) Investigation of antibiotics in mollusks from coastal waters in the Bohai Sea of China. *Environmental Pollution* **162**, 56-62.

Meredith-Williams, M., Carter, L.J., Fussell, R., Raffaelli, D., Ashauer, R., Boxall, A.B.A. (2012) Uptake and depuration of pharmaceuticals in aquatic invertebrates. *Environmental Pollution* **165**, 250-258.

Monirith, I., Ueno, D., Takahashi, S., Nakata, H., Sudaryanto, A., Subramanian, A., Karuppiah, S., Ismail, A., Muchtar, M., Zheng, J.S., Richardson, B.J., Prudente, M., Hue, N.D., Tana, T.S., Tkalin, A.V., Tanabe, S. (2003) Asia-Pacific mussel watch: monitoring contamination of persistent organochlorine compounds in coastal waters of Asian countries. *Marine Pollution Bulletin* **46**, 281-300.

NHS (2010) *Prescriptions dispensed in the community: England statistics for 1999 to 2009*. The National Health Scheme Information Centre of England. http://www.ic.nhs.uk/webfiles/publications/prescriptionsdispensed/Prescriptions_Dispensed_1999_2009%20.pdf, Accessed 2 Aug 2010.

Quinn, B., Schmidt, W., O'Rourke, K., Hernan, R. (2011) Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (*Dreissena polymorpha*) and their comparison with standardised toxicity tests. *Chemosphere* **84**, 657-663.

Ramirez, A.J., Mottaleb, M.A., Brooks, B.W., Chambliss, C.K. (2007) Analysis of pharmaceuticals in fish using liquid chromatography-tandem mass spectrometry. *Analytical Chemistry* **79**, 3155-3163.

Roberts, P.H., Thomas, K.V. (2006) The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. *Science of the Total Environment* **356**, 143-153.

Ronan, J.M., McHugh, B. (2013) A sensitive liquid chromatography/tandem mass spectrometry method for the determination of natural and synthetic steroid estrogens in seawater and marine biota, with a focus on proposed Water Framework Directive Environmental Quality Standards. *Rapid Communications in Mass Spectrometry* **27**, 738-746.

Runnqvist, H., Bak, S.A., Hansen, M., Styrishave, B., Halling-Sorensen, B., Bjorklund, E. (2010) Determination of pharmaceuticals in environmental and biological matrices using pressurised liquid extraction-Are we developing sound extraction methods? *Journal of Chromatography A* **1217**, 2447-2470.

Schmidt, W., O'Rourke, K., Hernan, R., Quinn, B. (2011) Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus* spp.) and their comparison with standardized toxicity tests. *Marine Pollution Bulletin* **62**, 1389-1395.

Stan, H.J., Heberer, T. (1997) Pharmaceuticals in the aquatic environment. *Analusis* **25**, M20-M23.

Tittlemier, S.A., Van de Riet, J., Burns, G., Potter, R., Murphy, C., Rourke, W., Pearce, H., Dufresne, G. (2007) Analysis of veterinary drug residues in fish and shrimp composites collected during the Canadian Total Diet Study, 1993-2004. *Food Additives and Contaminants* **24**, 14-20.

Thomas, K.V., Hilton, M.J. (2004) The occurrence of selected human pharmaceutical compounds in UK estuaries. *Marine Pollution Bulletin* **49**, 436-444.

Togunde, O.P., Oakes, K.D., Servos, M.R., Pawliszyn, J. (2012) Determination of pharmaceutical residues in fish bile by solid-phase microextraction coupled with liquid chromatography-tandem mass spectrometry (LC/MS/MS). *Environmental Science & Technology* **46**, 5302-5309.

Weigel, S., Kuhlmann, J., Huhnerfuss, H. (2002) Drugs and personal care products as ubiquitous pollutants: occurrence and distribution of clofibric acid, caffeine and DEET in the North Sea. *Science of the Total Environment* **295**, 131-141.

Weigel, S., Berger, U., Jensen, E., Kallenborn, R., Thoresen, H., Huhnerfuss, H. (2004) Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromso/Norway with emphasis on ibuprofen and its metabolites. *Chemosphere* **56**, 583-592.

Wille, K., Noppe, H., Verheyden, K., Vanden Bussche, J., De Wulf, E., Van Caeter, P., Janssen, C.R., De Brabander, H.F., Vanhaecke, L. (2010) Validation and application of an LC-MS/MS method for the simultaneous quantification of 13 pharmaceuticals in seawater. *Analytical and Bioanalytical Chemistry* **397**, 1797-1808.

Wille, K., Kiebooms, J.A.L., Claessens, M., Rappe, K., Vanden Bussche, J., Noppe, H., Van Praet, N., De Wulf, E., Van Caeter, P., Janssen, C.R., De Brabander, H.F., Vanhaecke, L. (2011) Development of analytical strategies using U-HPLC-MS/MS and LC-ToF-MS for the quantification of micropollutants in marine organisms. *Analytical and Bioanalytical Chemistry* **400**, 1459-1472.

Wu, J.M., Qian, X.Q., Yang, Z.G., Zhang, L.F. (2010) Study on the matrix effect in the determination of selected pharmaceutical residues in seawater by solid-phase extraction and ultra-high-performance liquid chromatography-electrospray ionization low-energy collision-induced dissociation tandem mass spectrometry. *Journal of Chromatography A* **1217**, 1471-1475.

3.0 The Potential for Bioaccumulation of Pharmaceuticals in Juvenile Rainbow Trout via Dietary Exposure using PLE and SPE with LC-MS/MS

Abstract

The potential for pharmaceuticals to bioaccumulate in aquatic species, such as fish, via trophic level transfer was investigated. Selected pharmaceuticals included two non-steroidal anti-inflammatory drugs (diclofenac and mefenamic acid), an antibiotic (trimethoprim), an antiepileptic (carbamazepine) and a lipid regulator (gemfibrozil). An *in vivo* experiment was carried out in a flow-through system in which juvenile rainbow trout were fed marine mussels (*Mytilus* spp.) collected from a highly polluted site exposed to wastewater effluent for a period of 28 days. The analytical methods previously developed for ultra-pure water and marine mussels were applied to carbon filtered municipal water and mussel feed samples, respectively. The pharmaceutical extraction method applied to marine mussels was further optimised for fish liver samples with limits of quantification (LOQs) ≤53 $ng.g^{-1}$ and correlation coefficients of $R^2 \geq 0.988$ achieved. Trimethoprim was the only pharmaceutical to be quantified in the wild *Mytilus* spp. at concentrations of 6.68 $ng.g^{-1}$ DW. After feeding, none of the selected pharmaceuticals were detected in the fish liver samples collected from the exposed rainbow trout. Hence, the potential for pharmaceuticals, in particular trimethoprim, to biomagnify via trophic level transfer is unlikely, however, bioaccumulation of pharmaceutical residues may have occurred at concentrations measuring below the method detection limits.

Aims and Objectives

- Develop and validate an analytical method for the detection and quantification of selected pharmaceuticals in fish tissues using pressurised liquid extraction (PLE) and solid phase extraction (SPE) techniques with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. These pharmaceuticals include an antiepileptic (carbamazepine), two non-steroidal anti-inflammatory drugs (NSAIDs) (diclofenac and mefenamic acid), a lipid regulator (gemfibrozil) and an antibiotic (trimethoprim).
- Set up an *in vivo* experiment exposing juvenile rainbow trout to pharmaceuticals via ingestion of effluent exposed marine mussels over a 28-day period.
- Apply new and previously developed methods for pharmaceutical extraction and analysis to carbon filtered municipal supply water, marine mussels and fish tissues collected from this *in vivo* exposure.
- Investigate the bioaccumulation of pharmaceuticals in juvenile rainbow trout through trophic level transfer via dietary exposure to contaminated mussel tissue feed.

3.1 Introduction

One of the many concerns surrounding pharmaceutical release into surface waters is their potential to bioaccumulate in aquatic biota via trophic level transfer. Bioaccumulation is the uptake of a chemical by an organism through a combination of water, food, sediment and air. As shown in Chapter 2, uptake of pharmaceuticals in aquatic biota can occur at low, environmentally relevant concentrations and the question remains if bioaccumulation of pharmaceuticals through the food chain is also a possibility. The best known occurrence of pharmaceutical bioaccumulation and exposure through the food chain caused the mass mortality of three vulture species in Asia. Following the consumption of dead livestock containing the anti-inflammatory pharmaceutical, diclofenac, thousands of vultures suffered renal failure leading to their unfortunate demise (Oaks *et al.*, 2004).

There have been few studies that have addressed the issue of bioaccumulation of pharmaceuticals in aquatic biota. Bioaccumulation can be expressed in terms of a bioaccumulation factor (BAF), which is the ratio of the chemical concentration in the organism (C_B) to the concentration of the freely dissolved chemical in the water column (C_{WD}), shown in Equation. 3.1 (Gobas and Morrison, 2000);

$$\text{BAF} = C_B / C_{WD} \qquad \text{Eqn. 3.1}$$

However, bioaccumulation of a chemical in an organism can also occur via dietary exposure. When this route of uptake is assessed, a biomagnification factor (BMF) is used as the endpoint (OECD, 2012) and is calculated as the ratio of the chemical concentration in the organism (C_B) to the concentration in the organisms diet (C_D), shown in Equation. 3.2 (Gobas and Morrison, 2000);

$$\text{BMF} = C_B / C_D \qquad \text{Eqn. 3.2}$$

Biomagnification occurs when the chemical concentration in an organism is greater than that in the organisms diet, due to dietary uptake (Gobas and Morrison, 2000). Low biomagnification factors cannot be disregarded as they can often highlight

low-level bioaccumulation of a compound in an organism which may be of significance at higher trophic levels.

The earliest study on the bioaccumulation of pharmaceuticals through the food chain involved the feeding of juvenile fish and shrimp with medicated brine shrimp (*Artemia franciscana*) containing the popular aquaculture antibiotics, sulfamethazole and trimethoprim (Chair *et al.*, 1996). Bioaccumulation of these antibiotics was observed in both fish and shrimp tissues after feeding with trimethoprim detectable up to 80-h post-dosage. Konwick *et al.* (2006) exposed juvenile rainbow trout to nine triazole fungicides (dual-use pesticides and pharmaceuticals) via commercial feed spiked at concentrations of 23-35 $\mu g.g^{-1}$ WW. Bioaccumulation in fish occurred after the first day of feeding however, due to high rates of elimination in fish, no triazole residues were detected after two days of depuration and BMF values were determined as <0.1. Another study, carried out by Nie *et al.* (2008) and translated by Daughton and Brooks (2011), compared the concentrations of ciprofloxacin in carp via water uptake and dietary intake. Interestingly, feeding alone resulted in the highest concentrations of the antibiotic accumulated in the fish in comparison to water uptake and dual exposure. The few studies carried out to date have focused on the accumulation of pharmaceuticals over one trophic level. Vernouillet *et al.* (2010) investigated the increase of the antiepileptic drug, carbamazepine, at successive trophic levels in the food chain. The green algae, *Pseudokirchneriella subcapitata*, were exposed to 150 $mg.L^{-1}$ carbamazepine and fed to the crustacean, *Thamnocephalus platyurus*, which was in turn fed to the cnidarians, *Hydra attenuata*. The results revealed trophic level transfer of carbamazepine was highest between the algae and crustacean with only trace residues of the antiepileptic detected in the exposed cnidarian. Considering bioaccumulation studies of other water pollutants at more environmentally relevant concentrations, Haukas *et al.* (2010) investigated the bioavailability of the brominated flame retardant, hexabromocyclododecane (HBCD), to ragworm by exposure through naturally contaminated sediment and mussel feed. Both sediment and mussel tissues used in this experiment were collected from a highly impacted area with concentrations measuring up to 85 $\mu g.g^{-1}$ DW and 7 $ng.g^{-1}$ WW, respectively. After 28 days of exposure, no HBCD residues were detected in ragworm exposed to contaminated sediment but, HBCD residues measuring between 7-12 $ng.g^{-1}$ were detected in the ragworm exposed to contaminated mussel tissue feed. Despite the higher levels of HBCD contamination in the sediment,

trophic transfer was found to be a more important mechanism for HBCD bioaccumulation in the benthic food web.

Thus far, indirect exposure of humans to pharmaceuticals via drinking water (Benotti *et al.*, 2009; Focazio *et al.*, 2008; Heberer *et al.*, 2000; Stan and Heberer, 1997) and dietary intake (Tittlemier *et al.*, 2007; EFSA, 2013) have been shown to be possible but, the extent to which drugs can travel through the food chain and ultimately pose as a threat to human health remains relatively unknown. From the five pharmaceuticals selected in this study, carbamazepine and trimethoprim are the only pharmaceuticals previously investigated for their potential to bioaccumulate in aquatic organisms via dietary exposure. Previous studies confirmed high bioaccumulation of carbamazepine at lower trophic levels but low risk of exposure to organisms higher up in the food chain (Vernouillet *et al.*, 2010). Trimethoprim was found to bioaccumulate in exposed fish and shrimp species with residues persisting for longer than three days post-dosage, which is of concern with respect to human consumption (Chair *et al.*, 1996). To the author's knowledge, this study was the first to investigate the potential for bioaccumulation of pharmaceuticals in fish via dietary exposure to wild bivalves collected from an impacted site.

3.2 Experimental

3.2.1 Reagents and chemicals

LC-MS grade acetonitrile and water, and analytical grade acetone, acetonitrile, ethyl acetate and methanol, were purchased from Fisher Scientific (Cheshire, UK). Dichloromethane, dichlorodimethylsilane, ammonia solution, acetic acid and sulphuric acid were purchased from Aldrich (Gillingham, UK). Analytical grade carbamazepine (≥98 %), diclofenac sodium salt (≥98 %), gemfibrozil (≥99 %) and mefenamic acid (≥99 %) were obtained from Sigma-Aldrich (Steinheim, Germany) and trimethoprim (≥98 %) was ordered from Fluka (Buch, Switzerland). Ultra-pure water was obtained from a Millipore Milli-Q water purification system (Bedford, MA, USA).

Stock solutions (1000 $mg.L^{-1}$) of individual analytes were prepared in methanol and stored in a freezer at −20 °C, in the dark, for optimum stability.

Working mixed standards were prepared weekly in either water or, where required, in 80:20 v/v 13 mM ammonium acetate in water/acetonitrile.

3.2.2 Sampling and experimental design

The facilities at Shannon Aquatic Toxicology Laboratory were used for the set-up of this exposure. Juvenile rainbow trout were sourced from a pond system fish farm facility in Roscrea, Co.Tipperary. Fish (approx. 50±15 g) were acclimatised for 13 days in one large tank of carbon filtered municipal supply water and fed commercial fish feed, in the form of pellets, at 1-2 % of total fish weight daily. A flow-through system was set up for nine 70 L aerated and covered tanks, using the same water supply set at a flow rate of 0.2 $L.min^{-1}$ (see images in Figure 3.1 (a) & (b)). This set-up included three tanks for control, three tanks for mussel control and three tanks for contaminated mussel exposure, organised randomly, as shown in Figure 3.2. Organisation for Economic Co-operation and Development (OECD) Guideline No. 305 was followed for the set-up and running of this exposure with any exceptions noted (OECD, 2012).

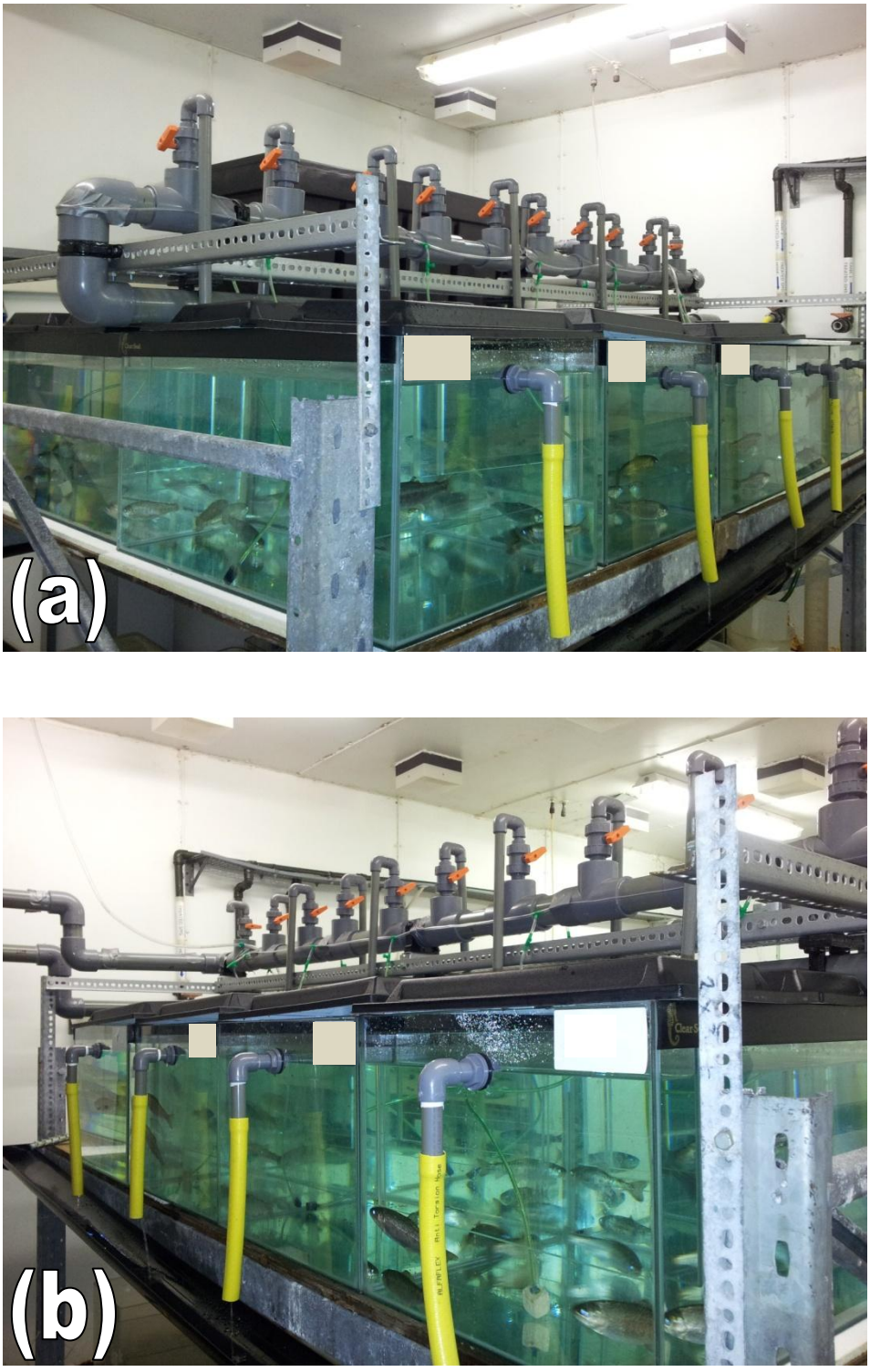


Figure 3.1 (a) & (b): Flow-through system connected to 9 x 70 L tanks. Fish and air stones visible in each labelled tank.

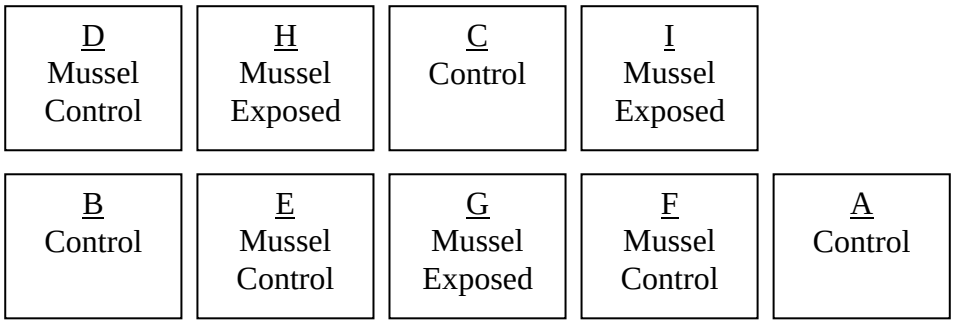


Figure 3.2: Set-up of labelled fish tanks for *in vivo* exposure

Six fish were weighed (see Table 3.1 for individual weights) and transferred into each tank to acclimatise for a further 24 h to reduce stress levels before exposure initiation. Water (500 mL) and fish (n=3) were sampled from the acclimation tank before the first day of exposure (t_0) as a control and from each of the nine exposure tanks after 14 days of feeding (t_{14}) and after 28 days of feeding (t_{28}). An additional nine fish were sampled from the acclimation tank for method performance studies. Silanised amber Winchester glass bottles (500 mL) were used to collect water samples from each tank which were then stored in the fridge at 4 °C. Fish were caught with a net and sampled individually. Fish were sacrificed, length and weight measurements were recorded and blood (≈1 mL), sampled from

Table 3.1: Weights (g) of juvenile rainbow trout in each tank measured pre-exposure

Tank	Fish weights (g)	Mean weight (g)
A	47, 53, 46, 58, 52, 49	51
B	52, 60, 54, 46, 49, 50	52
C	51, 54, 54, 60, 60, 52	55
D	46, 54, 50, 57, 44, 50	50
E	46, 44, 46, 46, 58, 61	50
F	43, 41, 43, 48, 47, 58	47
G	48, 48, 41, 44, 50, 60	49
H	43, 49, 41, 43, 59, 51	48
I	57, 58, 52, 51, 57, 47	54

the caudal vein, was placed in a vial for later biomarker analysis. The liver (average weight of 0.5 g), kidney (average weight of 0.8 g) and fillet (average weight of 12 g ×2) of fish were collected at each sampling time point, dissected and placed in labeled vials or plastic bags. All samples were transported back to the laboratory on dry ice and frozen at −80 °C. Aqueous samples were stored at 4 °C and extracted by SPE within 24 h of collection.

3.2.3 Food preparation and feeding

Blue mussels (*Mytilus* spp.) were sourced from a Class A bivalve mollusc production area, under EC Regulation 854/2004, off the west coast of Ireland and used as feed for the fish in the mussel control tanks. Fish in the mussel exposure tanks were fed blue mussels collected from one of the most highly impacted sites off the coast of Ireland, representative of a worst-case effluent exposure site. Pharmaceutical residues were previously detected in caged mussels exposed to the contaminated marine surface waters at this site and were expected to be present in the wild mussels residing at this site for a longer period of time. The animals chosen for this study were of the same size class (4-6 cm) and were collected at the end of August, before the spawning period in September. After collection, mussels were transported back to the laboratory in a cooler box, wiped free of debris, de-shelled, chopped into small pieces, weighed into feed bags for each day of exposure and stored at −80 °C. Bagged mussel feed was removed from the freezer, cut into small frozen pellets and fed to the corresponding tank. All tanks were fed daily at 2 % the total fish weight in the tank. From t_1 to t_{14}, each tank contained approximately 300 g (6×50 g) of fish and received 6 g of feed. After sampling at t_{14}, each tank received 3 g of feed until t_{28}. For the control tanks, fish were fed commercial fish feed in the form of pellets at the same quantities fed to the fish in the mussel control and exposure tanks. Fish faeces were removed approximately 6 h after feeding by siphoning from the tank bottom.

3.2.4 Sample pre-treatment, extraction and clean-up

3.2.4.1 Glassware preparation and silanisation

All glassware, including the exposure tanks, was pre-cleaned and silanised using

the method described in Section *2.2.3.1*.

3.2.4.2 Carbon filtered municipal water

The optimised analytical method applied to ultra-pure water samples (Section *2.2.3.2*) was also applied to the carbon filtered municipal water samples collected from each tank. Briefly, samples (500 mL) were filtered, adjusted to pH 4 and extracted onto Strata-X SPE cartridges (6 mL, 200 mg, Phenomenex, Cheshire, UK). Samples were eluted with ethyl acetate:acetone (50:50), evaporated and reconstituted in 250 µL starting mobile phase before LC-MS/MS analysis.

3.2.4.3 Marine mussels

For marine mussels, the analytical method optimised described in Section *2.2.3.3* was applied. Prior to sample extraction, mussels were frozen at −80 °C, freeze-dried, homogenised and sieved to 125 µm. PLE was carried out on 1 g of freeze-dried mussel tissue. Sample extracts were dried down to <10 mL and diluted to 200 mL with ultra-pure water before undergoing SPE. Samples were reconstituted in 250 µL of mobile phase A and analysed by LC-MS/MS.

3.2.4.4 Fish liver

The analytical method for marine mussels was also applied to fish liver tissues with some minor adjustments. Prior to sample extraction, fish livers were frozen at –80 °C, freeze-dried, pooled according to tank and sampling time, homogenised and sieved to 125 µm. PLE was carried out using the same conditions as in Section *2.2.3.3* but with different sample measurements added to the extraction cell. The activated neutral Al_2O_3 (20 g) (Sigma Aldrich, Steinheim, Germany) was placed in the cell followed by a mixture of freeze-dried fish liver sample (0.4 g) mixed with 10 g of ottawa sand (20-30 mesh, Fisher Scientific, Cheshire, UK). The remaining dead volume of the cell was filled with sand. PLE was carried out as before and sample extracts were dried down to <10 mL and diluted to 200 mL with ultra-pure water. Samples underwent SPE and sample reconstitution to 200 µL before LC-MS/MS analysis.

3.2.4.5 Fish fillet

After freeze-drying, the fish fillet was too tough to be ground to a fine powder and could not be processed any further. The fish skin was not removed from the fillet before freeze-drying and may have been the reason the fillet did not freeze-dry sufficiently.

3.2.5 Instrumental conditions

The Agilent 1200 LC system (Agilent Technologies, Palo Alto, CA, USA) consisted of a binary solvent manager, autosampler, UV detector and Waters Sunfire C_{18} column (3.5 µm, 150 mm×2.1 mm, Waters Corp., MA, USA) and guard column (3.5 µm, 2.1 mm×10 mm, Waters Corp., MA, USA). Operating conditions were as previously described in Section *2.2.4*. A Bruker Daltonics Esquire HCT Ion Trap mass spectrometer equipped with an API-ESI source was used for pharmaceutical identification and quantification. Nitrogen was used as a nebulising and desolvation gas (on site tap) and helium (99.999 %) (Air Products Plc, Crewe, UK) was used as a collision gas. Ionisation and mass spectrometric conditions (see Table 2.2) were optimised by direct infusion using a syringe pump (Cole-Parmer 74900 Series) to deliver 300 $\mu L.h^{-1}$ of analyte solution. The nebuliser pressure and drying gas flow were increased slightly after optimisation to account for the higher flow rates set for sample analysis. Mass spectrometric analysis was carried out in SRM mode for selected transitions (see Table 2.3).

3.2.6 Method performance

Method performance in ultra-pure water and marine mussels was previously carried out and discussed in Section *2.2.5*. Method performance data for fish liver was obtained by replicating the optimised method for marine mussels, as described in Section *2.2.3.3*, with a change in the sample mass added to the PLE extraction cell. Calibration curves (n=3) were constructed in fish liver tissues (0.4 g), sourced from rainbow trout bought from a local fishmonger, to assess method performance. Fish liver was spiked with the selected pharmaceuticals at concentrations from 5 to 5000 $ng.g^{-1}$. LOD (S/N ratio=3:1) and LOQ (S/N ratio=10:1) were calculated using triplicate injections of three low-level spiked samples. Precision, reproducibility

and recovery studies were carried out by spiking sample matrices (n=6) pre-extraction to pharmaceutical concentrations of 0.5 µg.g^{-1} DW. Recovery was calculated by comparing spiked extracts to final extracts of the unspiked matrix (n=3) reconstituted in 200 µL of mobile phase A containing the expected 100 % recovery concentration. Ion suppression and enhancement was also investigated by comparing sample extracts spiked post-extraction to a 1 mg.L^{-1} analyte mixture prepared in mobile phase A. None of the target analytes were detected in solvents, reagents, ultra-pure water or fish liver tissue used for the method performance studies. All sample and standard measurements were carried out in triplicate unless otherwise stated.

3.3 Results and Discussion

3.3.1 Fish liver sample preparation

Prior to PLE, the freeze-dried biotic tissue was ground with a pestle and mortar and sieved to a particle size of approximately 125 µm. Most of the sample was ground to a fine powder with very few larger remains in the sieve (<5 %). PLE was based on the method previously optimised for marine mussels with slight modifications. Extraction solvents were tested using 1:1 methanol/water, 1:1 acetonitrile/water, 3:1 acetonitrile/water and 3:1 acetonitrile/water with 1 % formic acid). A mixture of 3:1 acetonitrile/water again provided the best recovery. The optimal temperature (60 °C), static time (5 min), number of cycles (3) and flush volume (100 %) remained the same as those for the PLE method for marine mussels. Next, the addition of various quantities of Al_2O_3 (10, 15 and 20 g) added to the extraction cell and the quantities of ottawa sand (2, 4 and 10 g) mixed with the sample were evaluated. Higher quantities of Al_2O_3 (20 g) and ottawa sand (10 g) achieved slightly higher recoveries and S/N ratios. The sample mass itself was tested by analysing the sieved freeze-dried biotic tissue (0.1, 0.2 and 0.4 g) spiked at 500 ng.g^{-1}. Due to the small size of fish liver samples, the higher sample mass of 0.4 g was made up of three fish liver tissues combined. Higher analyte recoveries were achieved with 0.4 g of sample in comparison to 0.1 g. The PLE extract was further cleaned up using Strata-X SPE cartridges. The analytical method applied to marine

mussels was used for the extraction of pharmaceuticals from fish liver tissues. Eluting solvents were re-investigated here as a means of increasing percentage recovery of analytes from a more complex matrix. Both methanol and 50:50 (v/v) ethyl acetate/acetone exhibited similar results with ethyl acetate/acetone achieving slightly higher recoveries on average.

3.3.2 Instrumental and analytical method performance

Method performance results for individual pharmaceuticals in ultra-pure water, marine mussel tissue and fish liver are shown in Table 3.2. Instrumental precision measured ≤1.3 % for retention time of all analytes in ultra-pure water, marine mussel tissues and fish liver tissues. As results for ultra-pure water and marine mussels have been previously discussed in Section *2.3.3*, this section will focus on the results of the method performance when applied to fish liver samples. Fish liver samples (0.4 g, n=11 in triplicate) were spiked in the extraction cell, after mixing with sand, with a standard mixture resulting in final concentrations of 5-5000 $ng.g^{-1}$ of each pharmaceutical (solvent volume added <250 µL). Linearity was achieved for analytes in negative mode from 10 to 2500 $ng.g^{-1}$ ($R^2 \geq 0.992$) and in positive mode from 5 to 1000 $ng.g^{-1}$ ($R^2 \geq 0.988$). LOQs ≤53 $ng.g^{-1}$ were achieved for analytes in fish liver. Method recoveries were in the range of 41 to 97 % with precision varying ≤18 % for n=6 replicates of a 500 $ng.g^{-1}$ standard mix. Signal suppression ranged from 32 to 68 % for analytes in fish liver tissue with the signal for acidic compounds exhibiting the greatest suppression.

In order to minimise the matrix effect on the quantification of ions, calibration curves were prepared in each sample matrix and run before and after each series of samples. For quality control of the method, an injection of starting mobile phase was run between each sample with no carry over observed. EICs of pharmaceuticals spiked in fish liver at 250 $ng.g^{-1}$ are shown in Figure 3.3.

Table 3.2: Method performance data for pharmaceuticals in ultra-pure water (500 mL), marine mussel tissue (1 g) and fish liver (0.4 g)

Compound			Ultra-pure water			Marine mussels			Fish liver		
	t_r * (min)	SRM transitions	LOQ ($ng.L^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6	LOQ ($ng.g^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6	LOQ ($ng.g^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6
Trimethoprim	5.9	291→<u>123</u> (+) 291→230 (+)	2	0.993	84±3	4	0.985	91±9	4	0.990	97±18
Diclofenac	8.1	294→<u>250</u> (−) 294→236 (−)	5	0.997	98±8	29	0.990	83±8	53	0.992	52±8
Carbamazepine	8.2	237→<u>194</u> (+) 237→192 (+)	2	0.991	93±9	6	0.987	100±5	8	0.988	54±5
Mefenamic acid	9.0	240→<u>196</u> (−) 240→223 (−)	6	0.999	96±10	23	0.990	104±12	44	0.996	42±6
Gemfibrozil	11.5	249→<u>121</u> (−) 249→127 (−)	8	0.999	79±7	18	0.993	100±20	49	0.999	41±6

*Average time recorded for each pharmaceutical in marine mussel tissues

[a] Three data points carried out in triplicate

[b] Eight data points carried out in triplicate

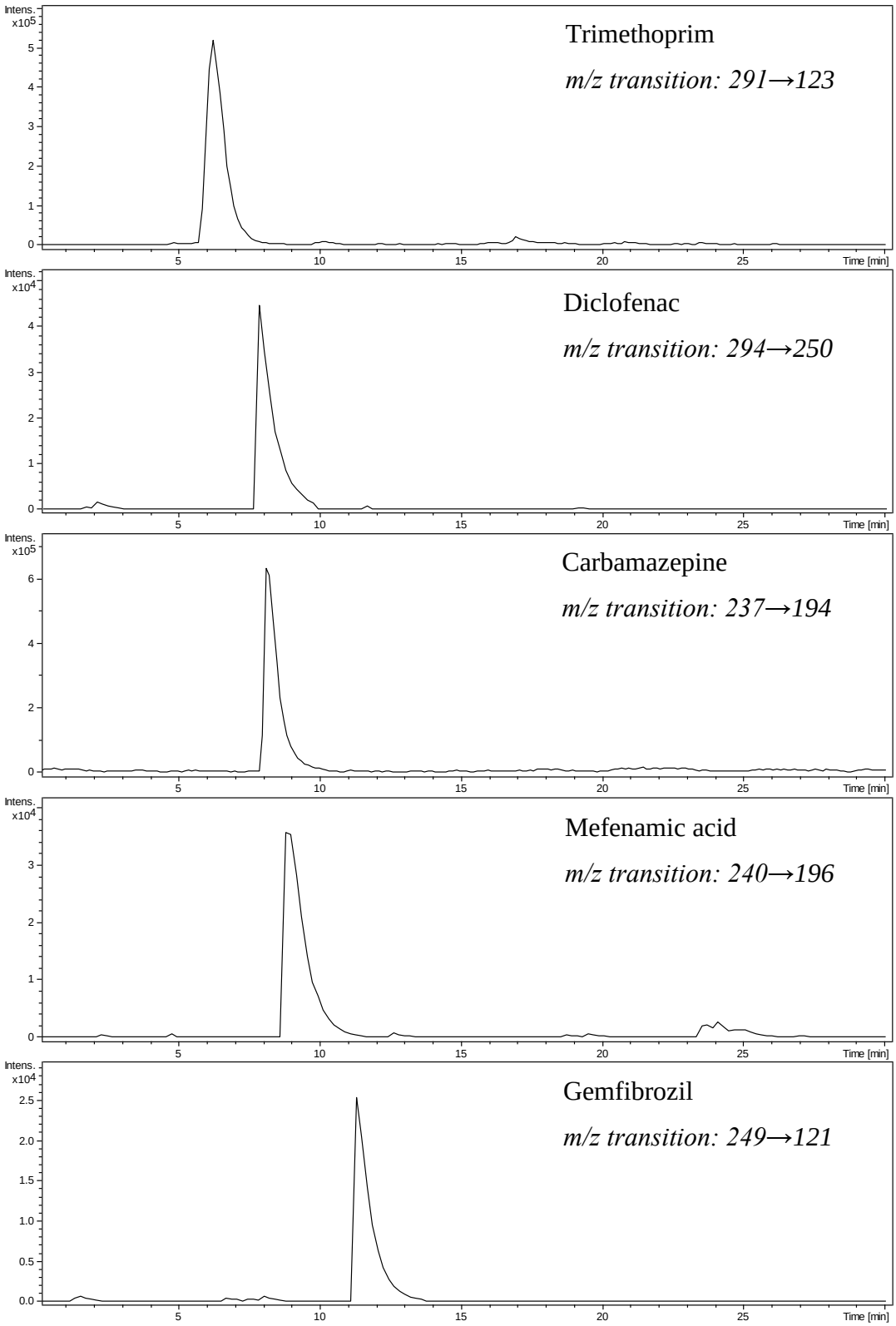


Figure 3.3: EICs of selected pharmaceuticals (250 ng.g^{-1} DW) spiked in fish liver

3.3.3 Application to marine mussels sampled from a wastewater effluent exposure site

The method previously developed for the analysis of pharmaceuticals in marine mussel tissues was applied to the identification of pharmaceuticals in both the marine mussels collected from the pristine site off the west coast of Ireland and the effluent exposed marine mussels collected from the highly contaminated site off the east coast of Ireland. As expected, no pharmaceuticals were detected in the marine mussels collected from the pristine site and used as feed in the mussel control tanks. Similar to the results obtained for pharmaceutical residues in the caged mussels (Table 2.7), residues of trimethoprim were quantified in the tissues of the wild effluent exposed mussels at 6.68 $ng.g^{-1}$ DW. An EIC of trimethoprim residues detected in the wild marine mussel tissues is shown in Figure 3.4. None of the other selected pharmaceuticals were detected in the wild marine mussels.

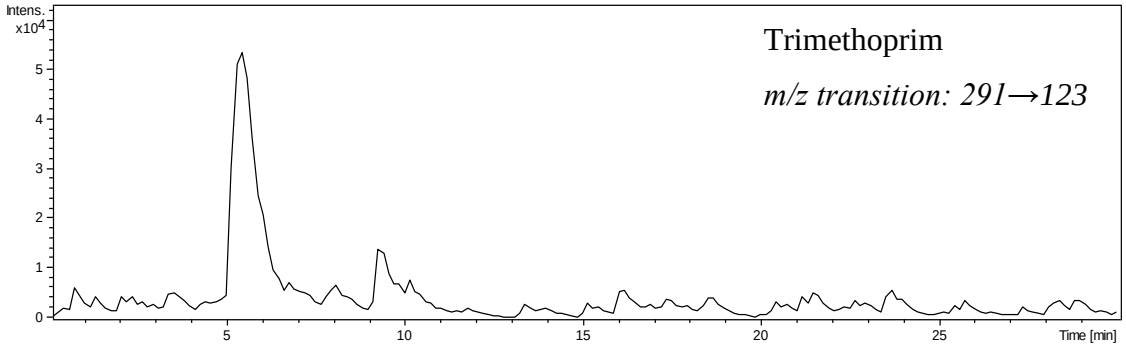


Figure 3.4: EIC of trimethoprim in wild marine mussel tissue sampled from a wastewater effluent exposure site

Although trimethoprim was the only pharmaceutical quantified in the effluent exposed mussel tissue, this does not rule out the presence of the other selected pharmaceuticals in the contaminated mussel tissue considering the ubiquitous presence of the same pharmaceutical residues in surrounding marine waters at concentrations up

to 1.41 $\mu g.g^{-1}$ (see Tables 2.6 (a), (b) & (c)). If there is a potential for pharmaceuticals to bioaccumulate and even biomagnify through the food chain, pharmaceuticals too low to be quantified in marine mussels using this analytical method may be detected or even quantified in the tissues of organisms at higher trophic levels, feeding on the exposed marine mussel tissues over time.

3.3.4 Application to fish tissues collected from juvenile rainbow trout feeding on contaminated marine mussel tissue for 28 days

The developed methods were applied to the identification of pharmaceutical residues in fish liver following a 28-day *in vivo* exposure. Fish and carbon filtered municipal water were sampled from each of the nine tanks at t_{14} and t_{28} in an attempt to evaluate the potential for selected pharmaceuticals to bioaccumulate in specific tissues of rainbow trout feeding on contaminated mussel tissue. Fish liver and fillet were collected from each fish sample however, pharmaceutical analysis was only carried out on the fish liver due to problems with the freeze-drying of the fillet. Due to the small sample size after freeze-drying, fish livers (n=3) sampled from fish collected from the same tank at the same time were combined prior to PLE and analysed as one sample via triplicate injections on the LC-MS/MS. One sample of carbon filtered water was also collected per tank at each sampling time and analysed via triplicate injections on the LC-MS/MS. Water pH, temperature, dissolved oxygen (DO) content, total organic carbon (TOC) and hardness were measured throughout the 28-day *in vivo* experiment on the days marked in Table 3.3. Average results for each parameter are shown in Table 3.4.

From the results in Section *3.3.3*, it can be accepted that juvenile rainbow trout were exposed to environmentally relevant concentrations of trimethoprim through a diet of naturally contaminated marine mussels. However, following analysis of liver tissues from the exposed juvenile rainbow trout, no pharmaceutical residues were detected in the fish liver sampled at t_0, t_{14} or t_{28}. There were also no pharmaceutical residues detected in the liver tissues of the rainbow trout fed uncontaminated mussel tissue or commercial fish feed.

Table 3.3: Days on which water measurements, such as temperature (°C), pH, DO (%), TOC ($mg.L^{-1}$) and hardness ($CaCO_3$ $mg.L^{-1}$), were collected

Parameter	Time (days)																												
	t_0	t_1	t_2	t_3	t_4	t_5	t_6	t_7	t_8	t_9	t_{10}	t_{11}	t_{12}	t_{13}	t_{14}	t_{15}	t_{16}	t_{17}	t_{18}	t_{19}	t_{20}	t_{21}	t_{22}	t_{23}	t_{24}	t_{25}	t_{26}	t_{27}	t_{28}
Temperature (°C)	√	√	√	√			√	√	√	√	√			√	√	√	√	√			√	√	√	√	√			√	√
pH	√	√		√			√		√		√			√		√		√			√		√		√			√	√
DO (%)	√		√	√			√	√			√			√		√		√					√		√			√	√
TOC ($mg.L^{-1}$)		√													√														√
Hardness ($CaCO_3$ $mg.L^{-1}$)	√														√														√

Table 3.4: Average measurements of temperature (°C), pH, DO (%), TOC ($mg.L^{-1}$) and hardness ($CaCO_3$ $mg.L^{-1}$) collected from each tank during the 28 d *in vivo* exposure

Parameter	Tanks								
	Control			*Mussel Control*			*Mussel exposure*		
	A	B	C	D	E	F	G	H	I
Temperature (°C)	14.7±1.7	14.7±1.7	14.7±1.4	14.8±1.6	14.7±1.5	14.7±1.5	14.8±1.6	14.8±1.7	14.5±.5
pH	7.1±0.5	7.2±0.5	7.2±0.4	7.1±0.5	7.2±0.5	7.3±0.3	7.3±0.5	7.3±0.5	7.2±0.3
DO (%)	92±6	91±6	89±6	89±9	96±4	95±6	92±6	93±4	95±3
TOC ($mg.L^{-1}$)	1.3±0.2			1.4±0.2			1.2±0.2		
Hardness ($CaCO_3$ $mg.L^{-1}$)	149.5±6.3			148.9±7			148.5±6.4		

The experimental design of this study was similar to that carried out by Haukas *et al.* (2009) in that it was based on an organisms ability to graze on lower trophic species and the feed administered was collected from an impacted site in the natural aquatic environment assumed to contain environmentally relevant concentrations of the contaminants. The test compound HBCD has a logK_{ow} value higher than the selected pharmaceuticals, at approximately 5.6, therefore, it has a higher affinity for solid matrices and is most likely removed by adsorption to sludge during wastewater treatment (Barron *et al.*, 2009). However, when this compound is released with industrial wastewaters, directly into the aquatic environment, its potential for bioaccumulation and trophic level transfer in aquatic organisms was found to be high (Haukas *et al.*, 2009). Jaffe (1991) proposed that dietary exposure of pharmaceuticals in the environment and biomagnification could be relevant for compounds with logK_{ow}>6. Considering most pharmaceuticals are polar non-volatile compounds with low logK_{ow} values, biomagnification of pharmaceuticals in aquatic ecosystems is not likely, however, low bioaccumulation and trophic level transfer of pharmaceuticals may still occur within food webs.

Low-level residues of trimethoprim were quantified in the mussel tissue feed but not in the fish liver, implying the antibiotic was either readily metabolised in the fish tissues or eliminated at a higher rate than the uptake rate. The latter explanation seems more feasible based on the results from previous exposure studies which highlight the long residence times of trimethoprim in fish species after exposure and its low biotransformation in the renal tubule and liver in rats and humans (Chair *et al.*, 1996; Liu *et al.*, 2012). Unlike the bioaccumulation study of trimethoprim carried out by Chair *et al.* (1996), bioaccumulation of trimethoprim in fish via trophic level transfer was not observed in this experiment. However, this is most likely due to the environmentally relevant and much lower concentrations exposed to the fish which may have been too low to measure in the fish liver. Although trimethoprim was not detected in the exposed fish tissues, it cannot be concluded that bioaccumulation did not occur, as trimethoprim residues can only be detected as low as the detection limits set for this analytical method i.e. <1 ng.L^{-1}.

Carbamazepine was not detected in the contaminated mussel feed, previously shown to be exposed to marine surface waters contaminated with this compound at concentrations up to 1.41 µg.L^{-1}. However, trace residues of carbamazepine have been previously detected in mussel tissue collected from the same exposure site but, at a different time of year (see Tables 2.6 (a), (b) & (c)).

Studies have shown carbamazepine to have a low propensity to bioconcentrate in fish (Huggett *et al.*, 2004; Ramirez *et al.*, 2007; Zhou *et al.*, 2008) but, when exposed to green algae then fed to the crustacean, *T. platyurus*, high bioaccumulation of the compound was reported via dietary exposure (Vernouillet *et al.*, 2010). This trophic level study also reported very low bioaccumulation of carbamazepine in the cnidarian, *H. attenuata*, following exposure to the compound via the food chain (Vernouillet *et al.*, 2010). Biochemical biomarkers such as Phase I (cytochrome P450-3A4) activity and oxidative stress were monitored during this exposure to provide an insight into the biotransformation capacities of each species. In comparison to unexposed control species, Phase I activity was inhibited in the exposed algae and crustacean but, was induced by 433 % in exposed cnidarians. This implies low biotransformation of carbamazepine within all species except the *H. attenuata* spp. which explains the low bioaccumulation measurements made for carbamazepine in the cnidarians. Previous exposure studies support the induction of biotransformation enzymes in the cniadarian, *H. attenuata*, in the presence of carbamazepine (Quinn *et al.*, 2004). The metabolism of pharmaceuticals in aquatic organisms has not been previously studied in great detail, but in the case of fish, both Phase I and II metabolism have been found to occur (Lahti *et al.*, 2011). Major metabolites of three acidic compounds (diclofenac, ibuprofen and naproxen) were detected in the bile of exposed rainbow trout at concentrations greater than the parent compounds, suggesting efficient biotransformation of the studied pharmaceuticals in fish. As highlighted by these previous studies, aquatic species at lower trophic levels may not possess a metabolic system as efficient or complex as their predators, increasing their susceptibility to pharmaceutical bioaccumulation.

3.4 Conclusions

In this study, PLE, SPE and LC-MS/MS methods were optimised and validated for the identification and quantification of five pharmaceuticals in fish liver tissues. Linearities for this method were $\geq$0.988 and LOQs $\leq$53 ng.g^{-1} were achieved. This analytical method and the methods previously optimised for pharmaceutical analysis in marine mussels and ultra-pure water were applied to samples collected from a 28 d *in vivo* exposure investigating the uptake of pharmaceutical residues in

juvenile rainbow trout via dietary intake of wild marine mussels. Despite the presence of trimethoprim residues in the tissues of the *Mytilus* spp., no pharmaceutical residues were detected in the fish liver tissues or the carbon filtered municipal water supplied to the fish tanks, after 28 days of feeding. It cannot be concluded that trimethoprim was not bioaccumulated in the fish tissues as residues may have been too low to be detected by the analytical method. The monitoring of pharmaceutical metabolites is suggested for future studies assessing the risk of pharmaceutical exposure to aquatic species via dietary intake.

References

Barron, L., Havel, J., Purcell, M., Szpak, M., Kelleher, B., Paull, B. (2009) Predicting sorption of pharmaceuticals and personal care products onto soil and digested sludge using artificial neural networks. *Analyst* **134**, 663-670.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A. (2009) Pharmaceuticals and endocrine disrupting compounds in US drinking water. *Environmental Science & Technology* **43**, 597-603.

Chair, M., Nelis, H.J., Leger, P., Sorgeloos, P., DeLeenheer, A.P. (1996) Accumulation of trimethoprim, sulfamethoxazole, and N-acetylsulfamethoxazole in fish and shrimp fed medicated *Artemia franciscana*. *Antimicrobial Agents and Chemotherapy* **40**, 1649-1652.

Daughton, C.G., Brooks, B.W. (2011) *Active pharmaceutical ingredients and aquatic organisms*. In: Beyer, W.N. and Meader, J.P. (eds.), *Environmental Contaminants in Biota: Interpreting Tissue Concentrations*. 2nd edn. Taylor and Francis, Boca Raton, 286-347.

EFSA (2013) *Report for 2011 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products*. European Food Safety Authority.http://www.efsa.europa.eu/en/supporting/doc/363e .pdf, Accessed September 2013.

Focazio, M.J., Kolpin, D.W., Barnes, K.K., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Barber, L.B., Thurman, M.E. (2008) A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States - II) Untreated drinking water sources. *Science of the Total Environment* **402**, 201-216.

Gobas, F., Morrison, H. (2000) *Bioconcentration and biomagnification in the aquatic environment*. In: Mackay, D. and Boethling, B. (eds.), *Handbook of Property*

Estimation Methods for Chemicals: Environmental and Health Sciences. Lewis Publishers, Boca Raton, 189-231.

Haukas, M., Mariussen, E., Ruus, A., Tollefsen, K.E. (2009) Accumulation and disposition of hexabromocyclododecane (HBCD) in juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* **95**, 144-151.

Haukas, M., Ruus, A., Hylland, K., Berge, J.A., Mariussen, E. (2010) Bioavailability of hexabromocyclododecane to the polychaete *Hediste diversicolor*: exposure through sediment and food from a contaminated fjord. *Environmental Toxicology and Chemistry* **29**, 1709-1715.

Heberer, T., Fuhrmann, B., Schmidt-Baumler, K., Tsipi, D., Koutsouba, V., Hiskia, A. (2000) Occurrence of pharmaceutical residues in sewage, river, ground-, and drinking water in Greece and Germany. *Abstracts of Papers of the American Chemical Society* **219**, U623-U623.

Huerta, B., Jakimska, A., Gros, M., Rodriguez-Mozaz, S., Barceló, D. (2013) Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A* **1288**, 63-72.

Huggett, D.B., Ericson, J.F., Cook, J.C., Williams, R.T. (2004) *Plasma concentrations of human pharmaceuticals as predictors of pharmacological responses in fish*. In: Kummerer, K. (ed.) *Pharmaceuticals in the Environment - Sources, Fate, Effects and Risks*. Springer, Berlin, 373-386.

Jaffe, R. (1991) Fate of hydrophobic organic pollutants in the aquatic environment - a review. *Environmental Pollution* **69**, 237-257.

Konwick, B.J., Garrison, A.W., Avants, J.K., Fisk, A.T. (2006) Bioaccumulation and biotransformation of chiral triazole fungicides in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* **80**, 372-381.

Lacey, C., McMahon, G., Bones, J., Barron, L., Morrissey, A., Tobin, J.M. (2008) An LC-MS method for the determination of pharmaceutical compounds in wastewater treatment plant influent and effluent samples. *Talanta* **75**, 1089-1097.

Lahti, M., Brozinski, J.M., Jylha, A., Kronberg, L., Oikari, A. (2011) Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout. *Environmental Toxicology and Chemistry* **30**, 1403-1411.

Liu, Z.Y., Wu, Y., Sun, Z.L., Wan, L.R. (2012) Characterization of *in vitro* metabolites of trimethoprim and diaveridine in pig liver microsomes by liquid chromatography combined with hybrid ion trap/time-of-flight mass spectrometry. *Biomedical Chromatography* **26**, 1101-1108.

Nie, X.-P., Chen, J.-F., Wang, X., Zhou, X.-Z., Lu, J.-Y., Yang, Y.-F. (2008) Bioaccumulation of ciprofloxacin in allogynogenetic crucian carp and its toxic effects (in Chinese). *Acta Ecologica Sinica* **28**, 246.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., Mahmood, S., Ali, A., Khan, A.A. (2004) Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **427**, 630-633.

OECD 9789264070462 (2012) *Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure*. Organisation for Economic Co-Operation and Development.

Quinn, B., Gagne, F., Blaise, C. (2004) Oxidative metabolism activity in *Hydra attenuata* exposed to carbamazepine. *Fresenius Environmental Bulletin* **13**, 783-788.

Ramirez, A.J., Mottaleb, M.A., Brooks, B.W., Chambliss, C.K. (2007) Analysis of pharmaceuticals in fish using liquid chromatography-tandem mass spectrometry. *Analytical Chemistry* **79**, 3155-3163.

Stan, H.J., Heberer, T. (1997) Pharmaceuticals in the aquatic environment. *Analusis* **25**, M20-M23.

Tittlemier, S.A., Van de Riet, J., Burns, G., Potter, R., Murphy, C., Rourke, W., Pearce, H., Dufresne, G. (2007) Analysis of veterinary drug residues in fish and shrimp composites collected during the Canadian Total Diet Study, 1993-2004. *Food Additives and Contaminants* **24**, 14-20.

Vernouillet, G., Eullaffroy, P., Lajeunesse, A., Blaise, C., Gagne, F., Juneau, P. (2010) Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. *Chemosphere* **80**, 1062-1068.

Zhou, S.N., Oakes, K.D., Servos, M.R., Pawliszyn, J. (2008) Application of solid-phase microextraction for *in vivo* laboratory and field sampling of pharmaceuticals in fish. *Environmental Science & Technology* **42**, 6073-6079.

4.0 The Determination of Pharmaceutical Residues in Cooked and Uncooked Marine Bivalves using PLE and SPE with LC-MS/MS

Published as:

McEneff, G., Barron, L., Kelleher, B., Paull, B., and Quinn, B. (2013) The determination of pharmaceutical residues in cooked and uncooked marine bivalves using pressurised liquid extraction, solid phase extraction and liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* **405**, 9509-9521.

Abstract

The effect of cooking (by steaming) on pharmaceutical residues present in marine mussels (*Mytilus* spp.) was investigated to determine any potential difference in human exposure risk. Selected pharmaceuticals included two non-steroidal anti-inflammatory drugs (diclofenac and mefenamic acid), an antibiotic (trimethoprim), an antiepileptic (carbamazepine) and a lipid regulator (gemfibrozil). A preliminary *in vivo* exposure experiment was set up in the laboratory in which mussels were exposed either directly by injection (10 ng) or daily through spiked artificial seawater (ASW) over 96 h. The solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, optimised for marine surface water in Chapter 2.0, was applied to ASW samples. Limits of quantification (LOQs) $\leq$46 ng.L^{-1} were achieved for extracted cooking water and ASW, $\leq$64 µg.L^{-1} for ASW in exposure tanks, and $\leq$29 ng.g^{-1} for mussel tissue. Method linearities were achieved for pharmaceuticals in each matrix with correlation coefficients of $R^2 \geq 0.977$. A selection of exposed mussels was cooked (via steaming) and analysed using the optimised method to observe any effect on detectable concentrations of parent pharmaceuticals present. An overall increase in pharmaceutical residues in the exposed mussel tissue and cooking water were observed after cooking.

Aims and Objectives

- Develop and validate analytical methods for the quantitation of selected pharmaceuticals in artificial seawater (ASW) using solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). These pharmaceuticals include an antiepileptic (carbamazepine), two non-steroidal anti-inflammatory drugs (NSAIDs) (diclofenac and mefenamic acid), a lipid regulator (gemfibrozil) and an antibiotic (trimethoprim).
- Set up a preliminary experiment exposing marine mussels *in vivo* to the pharmaceutical mix via direct injection or water exposure for a 96-h period.
- Apply previously developed methods for pharmaceutical extraction and analysis to ASW, cooking water and marine mussels samples collected from this preliminary exposure.
- Following the steaming of 96-h exposed mussels, examine the net changes of pharmaceutical residues in the mussel tissue after cooking.
- Compare the effect of cooking on mussels exposed to elevated pharmaceutical concentrations to those exposed to effluent impacted marine surface waters.

4.1 Introduction

Studies have shown that chemicals present in the aquatic environment have the ability to be absorbed by humans. The Fourth National US Report on Human Exposure to Environmental Chemicals published the exposure levels of 250 chemicals, such as heavy metals, polychlorinated biphenyls (PCBs), chlorinated pesticides, phytoestrogens, and triclosan, in blood and urine samples from over 6000 participants (US Department of Health and Human Services, 2009). Pharmaceuticals are designed to have a biological effect at low levels and exposure of humans to these compounds through the environment may act as a serious health risk either through direct effect or indirectly through potential antimicrobial resistant species. It is therefore of interest to determine possible routes of exposure that may pose as a threat to human health.

The presence of pharmaceuticals in drinking water has been previously reported at low $ng.g^{-1}$ concentrations in countries such as the U.S. and Germany (Benotti *et al.*, 2009; Focazio *et al.*, 2008; Heberer *et al.*, 2002). However, levels of pharmaceutical residues found in drinking water are usually much lower than those detected in foodstuffs as most pharmaceutical residues in drinking water are sourced from environmental contamination alone. Food safety appears to be more of a concern for regulatory authorities in the E.U. following the reports of contaminated foodstuffs in the news, such as the presence of the hormone medroxyprogesterone acetate in pig feed in Belgium and Holland and antibiotics in shrimp imported from Asia (Holmstrom *et al.*, 2003; Van Leengoed *et al.*, 2002). Yearly residue monitoring programmes are carried out by the EU on various foodstuffs such as meat, meat products and aquaculture in a bid to improve the quality of consumed food (EFSA, 2013).

Due to their widespread use in aquaculture and livestock, veterinary drug residues, in particular antibiotics, are among the most commonly reported chemical contaminants in foodstuffs in the EU (Kleter *et al.*, 2009). Most veterinary drug residues occur in food as a result of the direct administration of pharmaceuticals to farmed animals and seafood in an effort to reduce disease outbreak and promote growth efficiency (Turnipseed and Andersen, 2008). Besides exposure to dispensed veterinary drugs, seafood in particular is also exposed to contaminants present in the aquatic environment which may potentially accumulate in their tissues.

Most raw seafood is thermally treated by cooking before consumption but, little information is available regarding the potential effects of these treatments on pharmaceutical residue content. At present, studies on the effect of thermal treatments have only been applied to veterinary antibiotic residues, mainly in fish and crustacean species. Kitts *et al.* (1992) carried out one of the first thermal treatment studies on OTC residues in salmon with reductions of approximately 30 % measured in fried salmon fillet. The effect of cooking on the residues of two quinolones, oxolinic acid (OA) and flumequine, was also investigated in salmon and reported residue persistence during baking and boiling and leakage from contaminated tissue to uncontaminated tissues (Steffenak *et al.*, 1994). Uno *et al.* (2002; 2006a; 2006b; 2010) investigated the persistence of the residues of OA and OTC in shrimp and prawns, reporting residue elimination to be incomplete with reductions measuring between 20-60 % in the muscle and shell. The reduction of the antibiotics, ormetoprim, sulfadimethoxine and OTC, after cooking were also reported at approximately 50 % from raw catfish fillet (Huang *et al.*, 1997; Xu *et al.*, 1996). The transfer of biologically active compounds to humans is of major concern and may occur through mechanisms as simple as the consumption of seafood products containing pharmaceutical residues.

Marine blue mussels are natural filter feeders, filtering approximately 1.8 $L.h^{-1}$ (Clausen and Riisgard, 1996). Dissolved or suspended contaminants present in the water column may result in uptake within the mussel and potential exposure to humans via dietary exposure. Pharmaceutical residues were detected in caged and wild marine mussel tissues in Chapters 2 and 3 and have also been detected in mussels used in monitoring studies in Belgium, China and the U.S. (Bringolf *et al.*, 2010; Klosterhaus *et al.*, 2013; Li *et al.*, 2012; Wille *et al.*, 2011).

A preliminary experiment was set up whereby marine mussels were exposed *in vivo* to a pharmaceutical mixture (carbamazepine, diclofenac, gemfibrozil, mefenamic acid and trimethoprim) via direct injection or water exposure. This study was carried out to investigate if cooking will decrease pharmaceutical residues to negligible levels, in the event that pharmaceuticals are present in the raw marine mussels. No previous studies exist on the effect of thermal treatment on pharmaceutical residues, other than veterinary antibiotics, in biological tissues.

4.2 Experimental

4.2.1 Reagents and chemicals

LC-MS grade acetonitrile and water, and analytical grade acetone, acetonitrile, ethyl acetate and methanol, were purchased from Fisher Scientific (Cheshire, UK). Dichloromethane, dichlorodimethylsilane, dimethyl sulfoxide, ammonium hydroxide solution, acetic acid and sulphuric acid were purchased from Aldrich (Gillingham, UK). Analytical grade carbamazepine (≥98 %), diclofenac sodium salt (≥98 %), gemfibrozil (≥99 %) and mefenamic acid (≥99 %) were obtained from Sigma-Aldrich (Steinheim, Germany) and trimethoprim (≥98 %) was ordered from Fluka (Buch, Switzerland). Ultra-pure water was obtained from a Millipore Milli-Q water purification system (Bedford, MA, USA).

A stock solution of the analyte mix, including carbamazepine, diclofenac, gemfibrozil, mefenamic acid and trimethoprim, was prepared in dimethyl sulfoxide (DMSO) at a concentration of 10,000 $mg.L^{-1}$. Working mixed standard solutions were prepared by appropriate dilution of the stock solutions in DMSO. Stock 1,000 $mg.L^{-1}$ standards were also prepared for each pharmaceutical in methanol for method performance and quantification purposes. Working mixed standards were prepared weekly in either methanol or, where required, in 80:20 v/v 13 mM ammonium acetate in water/acetonitrile. All solutions were stored in a freezer at −20 °C and in the dark for optimum stability.

4.2.2 Sampling and experimental design

Blue mussels (*Mytilus* spp.) were collected in the west of Ireland (Lettermullen, Co. Galway), from a Class A bivalve mollusc production area, designated by the Sea-Fisheries Protection Authority of Ireland under EC Regulation 854/2004. The animals chosen for this study were of the same size class (4-6 cm) and were between summer spawning periods. The mussels were transported back to the laboratory in a cooler box, wiped free of debris and seaweed and acclimatised over 3.5 days in a large tank of ASW. ASW consisted of Peacock Seamix (NaCl 65.5 %, $MgSO_4$ 8.25 %, $MgCl_2$ 6 %, $CaCl_2$ 3 %, KCl 1.6 %, insolubles 0.05 %, H_20 15.6 %) dissolved in dechlorinated tap water to a salinity of 33 $g.kg^{-1}$ at 13 °C (±1 °C).

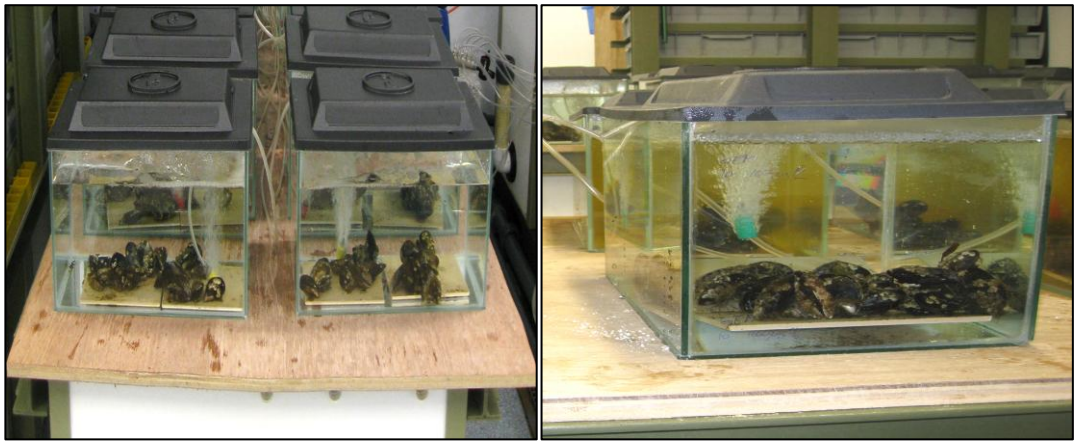


Figure 4.1 (a) & (b): Images of the *in vivo* experimental set-up exposing pharmaceuticals to *Mytilus* spp. via direct injection and water uptake

Preliminary experiments were set up in four silanised glass tanks (15 L total volume) containing ASW under semi-static conditions (24-h ASW renewal).

Mussels were exposed to 1 $mg.L^{-1}$ of a pharmaceutical mixture via direct injection into the haemolymph of the mussel or via water uptake. Each tank was supplied with an air stone to maintain dissolved oxygen concentrations and covered with a black plastic cover, shown in Figure 4.1 (a) & (b). For the direct injection exposure, 10 ng of a 1 $mg.L^{-1}$ pharmaceutical mixture in DMSO was injected into the adductor muscle of the mussel following the protocol described by Schmidt *et al*. (2011). For the water uptake exposure, mussels were exposed to 1 $mg.L^{-1}$ of each selected pharmaceutical with water replenishment and fresh chemical addition occurring daily. To fortify 10 L ASW at this concentration, 1 mL of the 10,000 $mg.L^{-1}$ stock solution was added directly into the exposure tank on Day 1. The volume of ASW and stock solution added to the tank for Days 2, 3 and 4 were reduced to 6,666 mL and 0.66 mL, respectively, to compensate for the reduction in mussels sampled after 24 hours of exposure (t_{24h}) and maintain the same volume of ASW per mussel throughout the experiment. The mixture was stirred immediately with a glass rod for 1 min to hasten equilibration of pharmaceuticals throughout the tank. Forty mussels were attached to two tiles and placed at the bottom of each tank after spiking and the pH was recorded (7.95±0.02). Solvent control tanks were prepared in parallel with each exposure and DMSO concentrations corresponding to those in the exposure tanks were used. Fresh ASW was supplied to all tanks daily

and spiked as necessary. Mussels were not fed during acclimatisation and exposure. Water temperature and salinity were measured (WTW Cond 1970i meter with a TetraCon 325 probe) at the mussel collection site and for each exposure tank daily. The experiment was carried out over 96 h and conducted under 10 h of light per day. ASW (500 mL) was sampled from the acclimatisation tank before the exposure commenced and from each tank (1.5 mL) at t_{1h}, t_{24h} and t_{96h} of the exposure. The concentration of compounds in water over a 19-h period was monitored during Day 2 of the water exposure experiment. ASW (1.5 mL) was collected in silanised amber vials at t_{2min}, t_{1h}, t_{3h}, t_{7h} and t_{19h} and frozen immediately at −20 °C until analysis. At t_{2min}, t_{24h} and t_{96h}, mussels were sampled from each tank, de-shelled and the visceral mass was dissected and frozen at −80 ^{o}C. For statistical analysis, 10 mussels were sampled from each tank at t_{2min} and again at t_{24h} and 20 mussels were sampled at t_{96h}.

The frozen visceral mass collected at t_{96h} was halved and allowed to defrost at room temperature. Following a basic cooking recipe, mussels were steamed in approximately 50 mL of ultra-pure water in a silanised glass pot, as shown in Figure 4.2, at 100 °C for 15 minutes, removed and allowed to cool (Mussel Industry Council of North America, 2009). After cooling, cooked mussels were frozen again

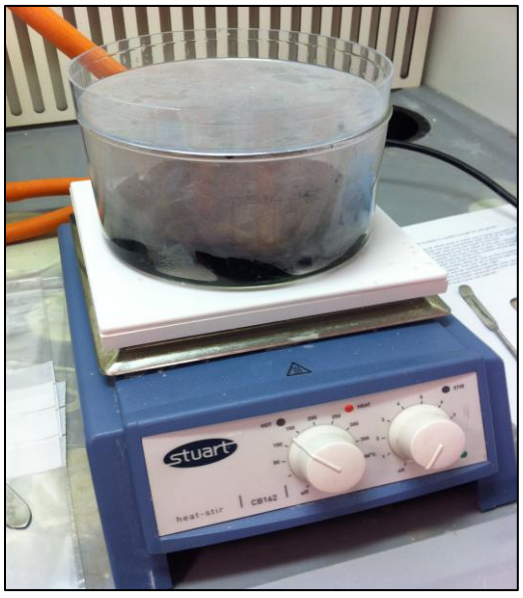


Figure 4.2: The steaming of water-exposed mussels collected at t_{96}

at –80 °C until analysis. The remaining cooking water was diluted up to 250 mL before undergoing SPE and LC-MS/MS analysis. From the experiment described in the previous chapter, mussel samples collected from EXP1 in October 2011 were also cooked, following the same procedure, to investigate the effect of cooking on pharmaceutical residues present in the mussel tissues at environmentally relevant concentrations.

4.2.3 Sample pre-treatment, extraction and clean-up

4.2.3.1 Glassware preparation and silanisation

All glassware, including the exposure tanks, was pre-cleaned and silanised using the method described in Section *2.2.3.1*.

4.2.3.2 Artificial sea water

The optimised analytical method applied to effluent and marine surface water samples (Section *2.2.3.2*) was also applied to artificial seawater samples. Briefly, samples were filtered, adjusted to pH 4 and extracted onto Strata-X SPE cartridges (6 mL, 200 mg, Phenomenex, Cheshire, UK). Samples were eluted with ethyl acetate:acetone (50:50), evaporated and reconstituted in 250 µL starting mobile phase before LC-MS/MS analysis.

4.2.3.3 Marine mussels

For marine mussels, the analytical method optimised and discussed in Section *2.2.3.3* was applied to all cooked and uncooked mussel samples collected. Prior to sample extraction, both cooked and uncooked mussels were frozen at −80 °C, freeze-dried, homogenised and sieved to 125 µm. PLE was carried out on 1 g of freeze-dried mussel tissue. Sample extracts were dried down to <10 mL and diluted to 200 mL with ultra-pure water before undergoing SPE and sample reconstitution prior to LC-MS/MS analysis.

4.2.4 Instrumental conditions

The Agilent 1200 LC system (Agilent Technologies, Palo Alto, CA, USA) consisted of a binary solvent manager, autosampler, UV detector and Waters Sunfire C_{18} column (3.5 µm, 2.1 mm×150 mm, Waters Corp., MA, USA) and guard column (3.5 µm, 2.1 mm×10 mm, Waters Corp., MA, USA). Operating conditions were as previously described in Section *2.2.4*. A Bruker Daltonics Esquire HCT Ion Trap mass spectrometer equipped with an API-ESI source was used for pharmaceutical identification and quantification. Nitrogen was used as a nebulising and desolvation gas (on site tap) and helium (99.999 %) (Air Products Plc, Crewe, UK) was used as a collision gas. Ionisation and mass spectrometric conditions (see Table 2.2) were optimised by direct infusion using a syringe pump (Cole-Parmer 74900 Series) to deliver 300 µL.h^{-1} of analyte solution. The nebuliser pressure and drying gas flow were increased slightly after optimisation to account for the higher flow rates set for sample analysis. Mass spectrometric analysis was carried out in SRM mode for selected transitions (see Table 2.3).

4.2.5 Method performance

Calibration curves (n=3) were constructed in ASW (500 mL and 1.5mL) to assess method performance. Method performance in marine mussels was previously discussed in Section *2.2.5*. ASW (500 mL, n=12 in duplicate) was spiked with the selected pharmaceuticals at concentrations from 5 to 5000 ng.L^{-1}. For ASW (1.5 mL, n=12 in triplicate) collected from each tank during the exposure, ASW was spiked at concentrations measuring from 5-5000 µg.L^{-1}. LOD (S/N ratio=3:1) and LOQ (S/N ratio=10:1) were calculated using triplicate injections of three low-level spiked samples. Precision, reproducibility and recovery studies were carried out by spiking sample matrices (n=6) pre-extraction to pharmaceutical concentrations of 0.5 µg.L^{-1}. Recovery was calculated by comparing spiked extracts to final extracts of the unspiked matrix (n=3) reconstituted in 250 µL of mobile phase A containing the expected 100 % recovery concentration. Ion suppression and enhancement was also investigated by comparing sample extracts spiked post-extraction to a 1 mg.L^{-1} analyte mixture prepared in mobile phase A. None of the target analytes were detected in solvents, reagents, ultra-pure water, ASW or marine mussel tissue (sampled from Lettermullan, Co. Galway) used for method performance studies.

All sample and standard measurements were carried out in triplicate unless otherwise stated.

4.3 Results and Discussion

4.3.1 Instrumental and analytical method performance

Upon investigation of instrumental precision, retention times varied ≤1.3 % for all analytes in each matrix. Despite high levels of salt in the ASW matrix, correlation coefficients of greater than 0.990 (n≥8) were achieved for all analytes over the ranges of 10 to 2500 $ng.L^{-1}$ in negative ESI-MS/MS mode and 5 to 1000 $ng.L^{-1}$ in positive ESI-MS/MS mode. All analytes were quantifiable at concentrations as low as 2 to 46 $ng.L^{-1}$ in ASW. Method performance in ASW correlates to data for marine surface water in Chapter 2.0 and to that reported by Wille *et al.* (2010) for the analysis of 13 pharmaceuticals in natural sea water. The % recovery of each analyte in ASW using the combined SPE and LC-MS/MS method was 45 to 87 % for n=6 replicates of a 1 $\mu g.L^{-1}$ standard mix (RSD≤12 %) (Table 4.1). An investigation into the matrix effects of ASW was carried out by comparison of extracts spiked post-extraction (1 $\mu g.L^{-1}$) to a standard in mobile phase A. Signal suppression of <29 % was observed for all analytes in ASW, with gemfibrozil subject to the most suppression. For ASW (1.5 mL) collected from each tank during the exposure, instrumental correlation coefficients of R^2≥0.977 and LOQs ≤64 $\mu g.L^{-1}$ were achieved for concentrations measuring from 5 to 2500 $\mu g.L^{-1}$. Ion suppression ranged from 7 to 26 % for all pharmaceuticals except gemfibrozil, which underwent a signal enhancement of 7 %. Recoveries of between 79-98 % were achieved for analytes in ultra-pure water (cooking water) with LOQs measuring up to 8 $ng.L^{-1}$. Method performance in marine mussels has been previously discussed in Section *2.3.3*. All method performance results for individual pharmaceuticals in ultra-pure water, ASW (1.5 mL), extracted ASW (500 mL) and marine mussel tissue are shown in Table 4.1.

Table 4.1: Method performance data for pharmaceuticals in ultra-pure water, extracted ASW (500 mL), ASW from exposure tanks (1.5 mL) and marine mussels

Compound		Ultra-pure water			Artificial sea water (500 mL)		
	t_r* (min) n=12	LOQ ($ng.L^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6	LOQ ($ng.L^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6
Carbamazepine	8.2	2	0.991	93±9	4	0.994	87±4
Diclofenac	8.1	5	0.997	98±8	46	0.992	63±7
Gemfibrozil	11.5	8	0.999	79±7	16	0.996	45±12
Mefenamic acid	9.0	6	0.999	96±10	24	0.989	49±3
Trimethoprim	5.9	2	0.993	84±3	2	0.992	70±4
Compound		Artifical sea water (1.5 mL)			Marine mussels (DW)		
		LOQ ($\mu g.L^{-1}$) n=9[a]	R^2 n≥21[c]	Ion suppression (%) n=3	LOQ ($ng.g^{-1}$) n=9[a]	R^2 n≥24[b]	Recovery (%) n=6
Carbamazepine		7	0.991	13±5	6	0.987	100±5
Diclofenac		16	0.998	26±7	29	0.990	83±8
Gemfibrozil		64	0.999	−7±15	18	0.993	100±20
Mefenamic acid		37	0.997	7±4	23	0.990	104±12
Trimethoprim		2	0.977	9±3	4	0.985	91±9

*Average time recorded for each pharmaceutical in marine mussel tissues

[a] Three data points carried out in triplicate

[b] Eight data points carried out in triplicate

[c] Seven data points carried out in triplicate

In order to minimise the matrix effect on the quantification of ions, calibration curves were prepared in each sample matrix and ran before and after each series of samples. For quality control of the method, an injection of starting mobile phase was run between each sample with no carry over observed.

4.3.2 Application to marine mussels exposed *in vivo* via direct injection or water exposure

The developed methods were applied to the identification of pharmaceutical residues in marine mussels and ASW collected during a preliminary 96 h *in vivo* exposure. Ten mussels and 1.5 mL aliquots of ASW were sampled from each of the four tanks at t_{2min}, t_{24h} and t_{96h} in an attempt to evaluate the potential of selected pharmaceuticals to bioconcentrate within the tissues of mussels over time. Five 1.5 mL aliquots of ASW were also collected from the water exposure tank over 19 h to investigate any reduction in pharmaceutical concentration over time. Due to small sample volumes, marine mussel samples were analysed in duplicate and ASW samples were determined via triplicate injections on the LC-MS/MS.

This preliminary exposure experiment was carried out to ascertain any possibility for pharmaceutical uptake by mussels through direct injection and water exposure. For the direct injection exposure, pharmaceutical concentrations of ≈10 ng (using a 1 $mg.L^{-1}$ standard in DMSO) were injected into the adductor muscle of the mussel, as previously described by Schmidt *et al.* (2011). No pharmaceuticals were detected in the injected mussel tissue or ASW. This may be due to the natural detoxification abilities of mussels, but is more likely to be related to the method detection limits given the concentration administered. Therefore, larger pharmaceutical concentrations than normally observed in environmental waters (Fatta-Kassinos *et al.*, 2011; Wille *et al.*, 2010) were exposed to the mussels in the water tank to ensure analytical method measurement reliability. Environmental exposure to pharmaceutical concentrations >1 $mg.L^{-1}$ are generally considered rare, but have been previously reported in effluent from an Indian WWTP serving several drug manufacturers (Larsson *et al.*, 2007). Upon fortification with pharmaceuticals, most analyte concentrations did not reach equilibrium in the ASW until 3 h after spiking. That said, after this initial stabilising period, concentrations were significantly less than the spiking level and continued to decline over time,

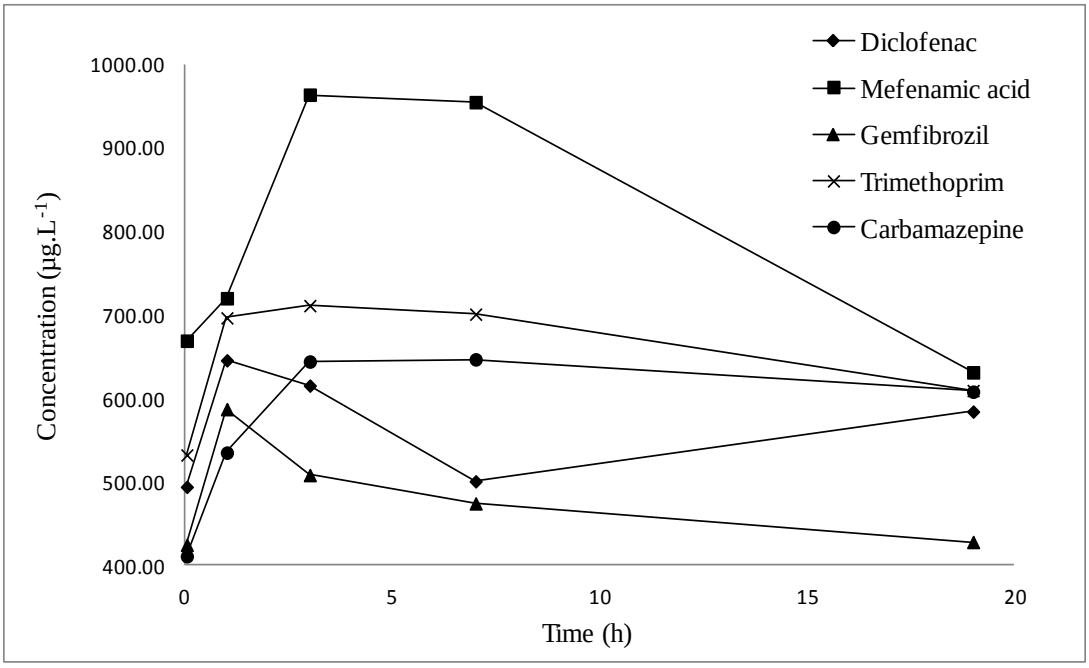


Figure 4.3: Pharmaceutical concentrations in ASW (n=1) from water exposure tank spiked at approximately 1 mg.L^{-1} and measured over 19 h

as shown in Figure 4.3. Therefore, and as tanks were silanised before use, reduced pharmaceutical concentrations are most likely due to a combination of uptake within the mussels, photolysis, microbial transformation and sequestration via sorption to the mussel shell, although this is difficult to determine definitively.

As can be deduced from Table 4.2, all pharmaceuticals were detected in the mussel tissues sampled from the water exposure tank from as soon as 2 min post-exposure. Mussels were originally placed on tiles in the acclimation tank and directly transferred to the exposure tank with minimum handling, where they were observed to have their siphons extended almost immediately, indicating feeding activity. EICs of the most concentrated occurrence of selected pharmaceuticals in the water-exposed marine mussel samples are shown in Figure 4.4. No pharmaceutical residues were detected in the mussels and ASW collected from the acclimation tank and solvent control tanks.

Table 4.2: Concentrations (μg.g^{-1} DW) of carbamazepine (CBZ), diclofenac (DCF), gemfibrozil (GEM), mefenamic acid (MFA) and trimethoprim (TRM) detected in water-exposed mussel tissue over 96 h

Time	Pharmaceutical concentrations detected in mussel tissue (μg.g^{-1} DW)				
	CBZ	*DCF*	*GEM*	*MFA*	*TRM*
t_{2min}	4.3±0.1	7.2±1.0	2.4±0.5	9.4±1.0	2.6±0.1
t_{24h}	12.8±1.1	13.5±2.4	1.0 ±0.0	11.7±1.0	18.8±0.9
t_{96h}	12.1±0.4	1.3±0.2	0.4±0.0	1.6±0.5	21.7±1.4

It is generally assumed that organic compounds with logK_{ow} ≥3 have the potential to bioconcentrate within biotic tissues (Huerta *et al.*, 2012). However, bioconcentration depends on numerous factors relating to the compound i.e. physicochemical nature and bioavailability, the aquatic organism i.e. rates of metabolism and uptake and depuration kinetics (Van Der Oost *et al.*, 2003), and its residing waters i.e. quality and pH (Bremle *et al.*, 1995; Nakamura *et al.*, 2008; Rendal *et al.*, 2011). Due to the ionisable nature of most pharmaceutical compounds, logD_{ow} is a more reliable quantity in this case as it takes into account the pH of the ASW. Trimethoprim is mostly ionised under experimental conditions but, with the second highest logD_{ow} value (0.63) after carbamazepine, it was found to be the most bioconcentrated pharmaceutical detected in the mussel tissue at the end of the 96 h exposure. On the contrary, gemfibrozil has only a slightly lower logD_{ow} value of 0.36 and was the least concentrated pharmaceutical measured in the mussel tissue at t_{96h}. Similarly, diclofenac and mefenamic acid also display positive logD_{ow} values and were poorly bioconcentrated in the mussel tissue. Low bioavailability due to limited solubility or adsorption to shell surfaces for less polar compounds may be one reason for this observed trend in results. There is also the possibility that the pharmaceuticals were detoxified and metabolised within the mussel and therefore immeasurable using a SRM-based method. A glucuronidated metabolite cleaving enzyme, β-glucuronidase, has been previously extracted from marine molluscs, such as the large periwinkle (*Littorina littorea*) and the limpet (*Patella vulgata*), suggesting the presence of Phase II enzymatic activity

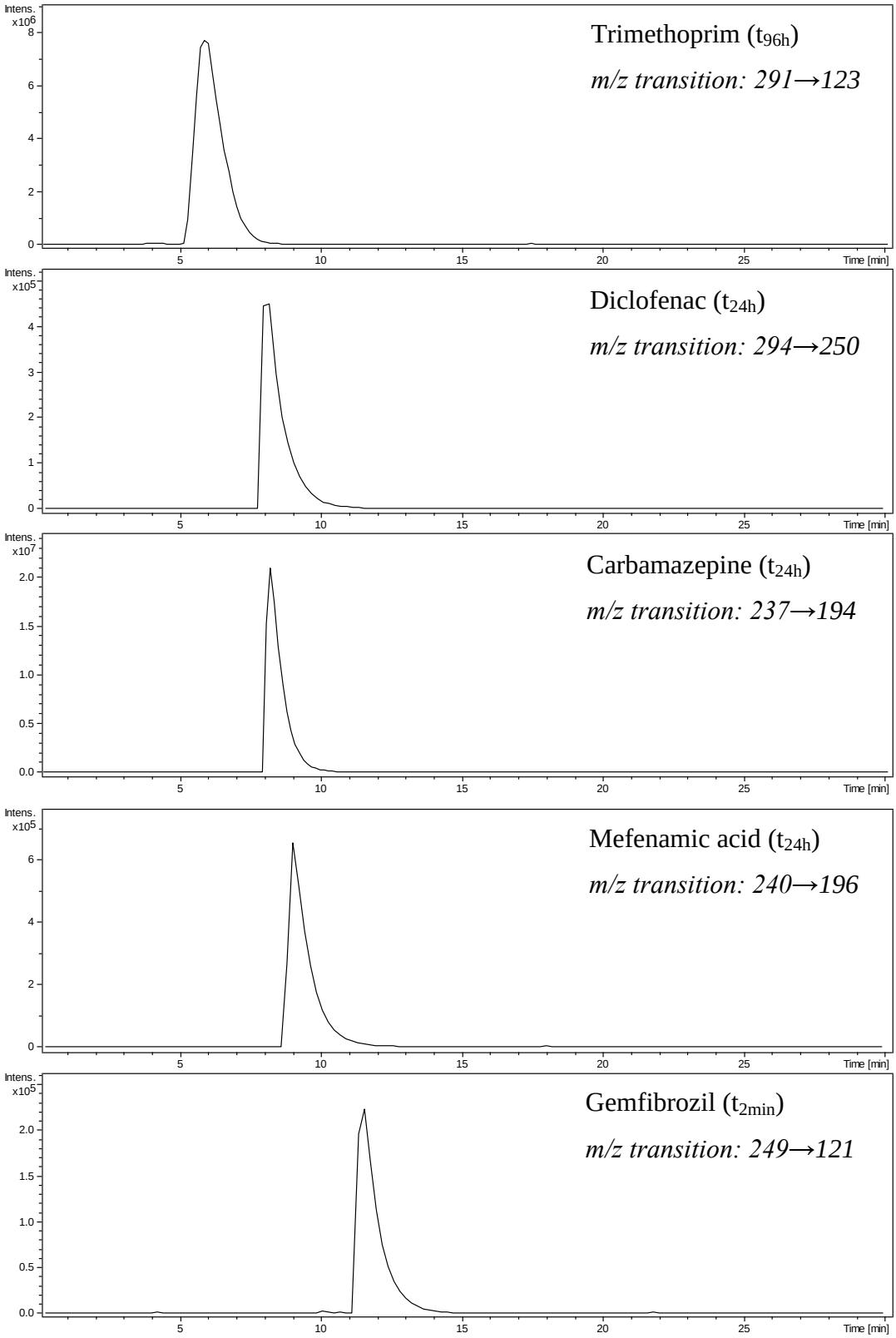


Figure 4.4: EICs of the most concentrated occurrence of selected pharmaceuticals in the water-exposed marine mussel samples

in invertebrates (Dodgson *et al.*, 1953). Exposure to two carboxylic acid containing drugs (diclofenac and gemfibrozil) was found to significantly increase expression of the Phase II detoxification enzyme GST in marine and freshwater mussels at environmentally relevant concentrations (Quinn *et al.*, 2011; Schmidt *et al.*, 2011). Acyl glucuronides are reactive electrophilic metabolites, responsible for the elimination of carboxylic acid containing drugs (Horng *et al.*, 2013). From the selected pharmaceuticals in this study, diclofenac, gemfibrozil and mefenamic acid contain carboxylic acid moieties and are primarily released in their acyl glucuronated form in the bile of humans, rats and fish (Grillo *et al.*, 2012; Kallio *et al.*, 2010; Lahti *et al.*, 2011; Mano *et al.*, 2007). If mussels share similar metabolic pathways to fish, this may explain the lower concentrations of the three acidic pharmaceuticals detected in the mussel tissue sampled at t_{96h}.

An overall reduction in mussel residues was observed for all pharmaceuticals except trimethoprim after 96 h. A reported study carried out by Le Bris and Pouliquen (2004) highlighted the ability of blue mussels to bioaccumulate two antibiotics, OTC and OA, over an eight hour *in vivo* exposure. Reductions in concentrations during exposure were observed between six to eight hours for OTC in the viscera and gills and between two to four hours for OA in the gills. The mussels may have undergone a detoxification process whereby the rate of elimination is greater than the rate of uptake, resulting in an overall loss in contaminant concentration within the mussel. As mentioned previously, metabolism within the mussel must also be considered. An in depth metabolic pathway study in the mussel model is required to account for any definite losses through biotransformation.

4.3.3 Effect of cooking on pharmaceutical residues in marine mussel tissue

As clearly observed in Figure 4.5, steaming of the water-exposed mussels resulted in an overall increase of pharmaceutical concentrations in the mussel tissue. Diclofenac, gemfibrozil and mefenamic acid residues in the mussel tissue increased by more than a factor of 20 and in the case of mefenamic acid, concentrations increased from 1.6 $\mu g.g^{-1}$ in the raw mussel to 89.6 $\mu g.g^{-1}$ after cooking (Table 4.3). The basic compound, trimethoprim, was the only pharmaceutical to undergo a reduction of 23 %, similar to results for other antibiotics, such as OTC and OA,

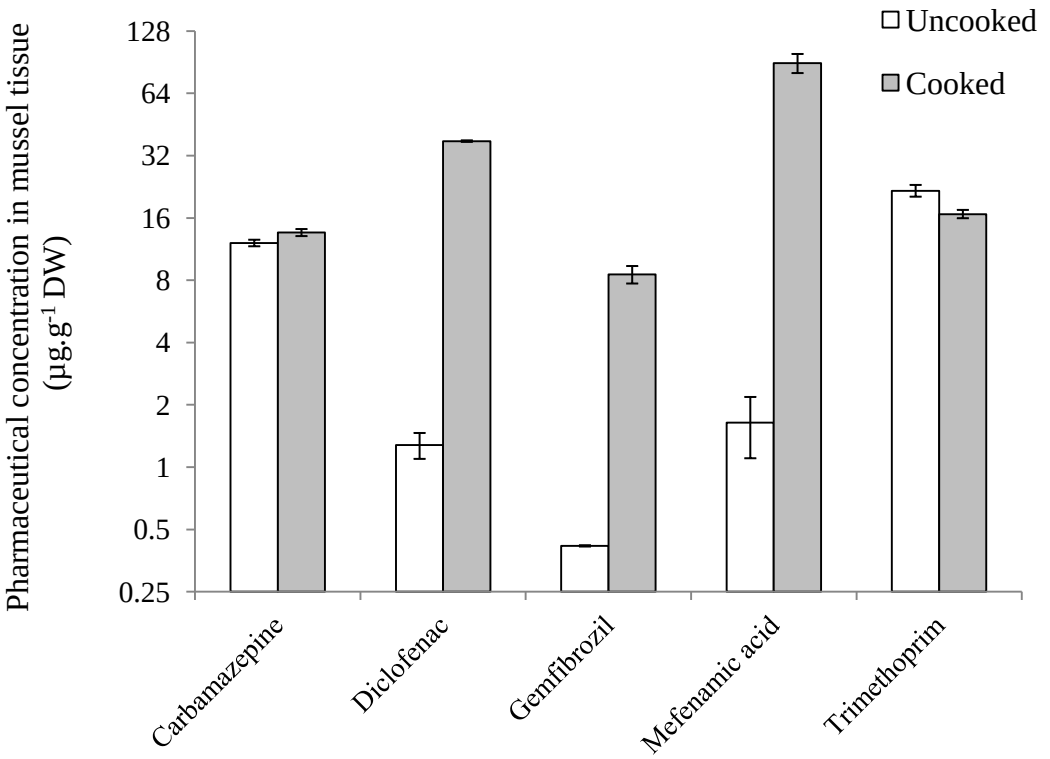


Figure 4.5: Pharmaceutical concentrations (µg.g^{-1} DW) detected in mussel tissue extracts before (n=2) and after cooking (n=2). Water-exposed mussels sampled at t_{96h} were steamed and compared to uncooked mussels also sampled at t_{96h}.

which were reduced in shrimp muscle after baking (Uno *et al.*, 2006a; Uno *et al.*, 2006b). Carbamazepine, a neutral compound, underwent the least change in concentration, with an increase of 12 % after cooking. Previous work in our laboratory has shown that temperatures of 450 ^{o}C in activated sludge drying processes perform only moderate transformation of this pharmaceutical residue in particular and remarkable stability in soils was also demonstrated during a two week leaching experiment (Barron *et al.*, 2008; Barron *et al.*, 2010). Studies exposing carbamazepine to aquatic species such as, *H. attenuata*, have reported the induction of biotransformation enzymes within the organism (Quinn *et al.*, 2004; Vernouillet *et al.*, 2010). Interestingly, significant increases in concentrations after cooking were only observed for acidic compounds.

As shown in Table 4.3, the steaming of the mussels exposed *in vivo* resulted in the detection of all selected pharmaceuticals in the cooking water, indicating

leakage or extraction from the mussel tissue when heated. Carbamazepine and trimethoprim had the highest concentrations detected in the cooking water at 5.2 $\mu g.g^{-1}$ and 4.4 $\mu g.g^{-1}$ WW, respectively. Considering their higher logD_{ow} values, this is not entirely unsurprising as water displays similar properties to organic solvents when heated under pressure to subcritical point (100-374 °C) and has been previously used as an extraction solvent for various environmental contaminants such as PAHs, PCBs and pesticides (Smith, 2002). Therefore, the presence of less polar pharmaceutical residues in the cooking water might be expected.

Table 4.3: Comparison of concentrations of carbamazepine (CBZ), diclofenac (DCF), gemfibrozil (GEM), mefenamic acid (MFA) and trimethoprim (TRM) detected in raw mussel tissue ($\mu g.g^{-1}$ DW) (n=2), steamed mussel tissue ($\mu g.g^{-1}$ DW) (n=2) and cooking water ($\mu g.g^{-1}$ WW) (n=1) after steaming

	Pharmaceutical concentrations ($\mu g.g^{-1}$)				
	CBZ	*DCF*	*GEM*	*MFA*	*TRM*
Uncooked mussels	12.1±0.4	1.3±0.2	0.4±0.0	1.6±0.5	21.7±1.4
Cooked mussels	13.6±0.5	37.6±0.3	8.5±0.8	89.6±9.5	16.7±0.8
Cooking water	5.2	0.3	0.1	0.9	4.4

Significant increases in the production of GST in mussels exposed to low concentrations of acidic pharmaceuticals (Quinn *et al.*, 2011; Schmidt *et al.*, 2011) suggests a strong possibility of metabolic activity within the mussels. As previously mentioned, acidic compounds were found to undergo biotransformation to their glucuronated metabolites in fish bile (Lahti *et al.*, 2011). Although not an acidic compound, carbamazepine has been found to undergo primary amine glucuronidation in human tissues (Staines *et al.*, 2004) and trimethoprim metabolites were subsequently conjugated with glucuronides in metabolic studies carried out in pigs (Mengelers *et al.*, 1997). Glucuronides are generally stable and can undergo deconjugation experimentally by harsh enzyme or chemical digestions but, in the case of acyl glucuronides in particular, these display instability (via cleavage) at mild temperatures and hydrolysis at pH>5 (Horng *et al.*, 2013;

Skonberg *et al.*, 2008; Trontelj, 2012). The observed increases in concentration of diclofenac, gemfibrozil and mefenamic acid in the cooked mussels may be as a result of the reconversion of acyl glucuronides to the parent compound after such thermal treatment. However, this would need to be confirmed in further studies as these metabolites were not available to analytical grade standards. The increase in concentration of carbamazepine in the mussel tissue after cooking was very low in comparison to the more acidic pharmaceuticals, however, this may be due to the higher stability of the formed amide linkage in n-glucuronides in comparison to the more reactive ester linkage in acyl glucuronides (Nassar *et al.*, 2004). The reconversion of glucuronised parent compounds has been observed previously for estrogens following activated sludge treatment in WWTPs (Ternes *et al.*, 1999). The presence of glucuronidase activity during secondary treatment is considered a rational explanation for the increase in concentration of numerous pharmaceuticals, including carbamazepine, in the discharged effluent of WWTPs (Jelic *et al.*, 2012).

Previous studies investigating the effect of cooking on pharmaceuticals have only determined veterinary antibiotics, such as OTC, OA and flumequine, directly exposed to farmed crustaceans and fish via medicated feed, with reported reductions, if any, less than 60 % after cooking (Huang *et al.*, 1997; Kitts *et al.*, 1992; Steffenak *et al.*, 1994; Uno, 2002; Uno *et al.*, 2006a; Uno *et al.*, 2006b; Uno *et al.*, 2010; Xu *et al.*, 1996). No previous studies on the effect of cooking on human pharmaceuticals in aquatic biota exist. Other environmental contaminants, such as metals, hexachlorobenzene (HCB) and polycyclic aromatic carbons (PAH), have been measured in fish, specifically hake, and mussel tissues at increased levels after undergoing several cooking processes (Kalogeropoulos *et al.*, 2012; Perello *et al.*, 2008; Perello *et al.*, 2009).

As mentioned previously, pharmaceutical residues in the natural aquatic environment are most often detected at concentrations in the high $ng.L^{-1}$ to low $\mu g.L^{-1}$ range. Marine mussel samples collected in October 2011 from the effluent exposure site, EXP1, described in Chapter 2, were found to contain residues of trimethoprim at 9.22 $ng.g^{-1}$ (Table 2.6 (b)). These environmentally contaminated mussel samples were cooked (n=2) and both mussel tissue extracts and cooking water (n=1) were analysed via triplicate injection on the LC-MS/MS. No pharmaceutical residues were detected in either sample after steaming. Trimethoprim residues in mussels exposed to elevated pharmaceutical

concentrations were not significantly reduced after steaming but, in comparison, trimethoprim residues detected in naturally exposed marine mussels were so low, they were completely eliminated after steaming.

4.4 Conclusions

In this study, PLE, SPE and LC-MS/MS methods were optimised and validated for the identification and quantification of five pharmaceuticals popularly prescribed in Ireland, in ultra-pure water, ASW and *Mytilus* spp. tissues. Linearities in each matrix were ≥0.977 and LOQs ≤46 $ng.L^{-1}$ were achieved for extracted cooking water and ASW, ≤64 $\mu g.L^{-1}$ for unprocessed ASW, and ≤29 $ng.g^{-1}$ in *Mytilus* spp. tissues. Application of these analytical methods to samples collected from a preliminary 96 h *in vivo* exposure, investigating the uptake of elevated pharmaceutical concentrations in mussel tissues, revealed the bioconcentration of selected pharmaceuticals in water-exposed mussel tissue only, at up to 21.7 $\mu g.g^{-1}$ DW. Domestic cooking by steaming resulted in an overall increase in pharmaceutical residues in the contaminated mussel tissue and cooking water. Mussels exposed to effluent impacted marine surface waters were found to contain low-level residues of the antibiotic trimethoprim but, these residues were eliminated after steaming. The potential risk of pharmaceutical exposure to humans through the food chain is low when pharmaceuticals are present in foods at environmentally relevant concentrations. However, this work has highlighted the possibility for pharmaceutical residues in food, particularly acidic compounds, to increase in concentration after thermal treatment and potentially pose a risk to human health via ingestion of farmed fish or animals regularly administered medication at high doses.

References

Barron, L., Tobin, J., Paull, B. (2008) Multi-residue determination of pharmaceuticals in sludge and sludge enriched soils using pressurized liquid extraction, solid phase extraction and liquid chromatography with tandem mass spectrometry. *Journal of Environmental Monitoring* **10**, 353-361.

Barron, L., Nesterenko, E., Hart, K., Power, E., Quinn, B., Kelleher, B., Paull, B. (2010) Holistic visualisation of the multimodal transport and fate of twelve pharmaceuticals in biosolid enriched topsoils. *Analytical and Bioanalytical Chemistry* **397**, 287-296.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A. (2009) Pharmaceuticals and endocrine disrupting compounds in US drinking water. *Environmental Science & Technology* **43**, 597-603.

Bremle, G., Okla, L., Larsson, P. (1995) Uptake of PCBS in fish in a contaminated river system - bioconcentration factors measured in the field. *Environmental Science & Technology* **29**, 2010-2015.

Bringolf, R.B., Heltsley, R.M., Newton, T.J., Eads, C.B., Fraley, S.J., Shea, D., Cope, W.G. (2010) Environmental occurence and reproductive effects of the pharmaceutical fluoxetine in native freshwater mussels. *Environmental Toxicology and Chemistry* **29**, 1311-1318.

Clausen, I., Riisgard, H.U. (1996) Growth, filtration and respiration in the mussel *Mytilus edulis*: no evidence for physiological regulation of the filter-pump to nutritional needs. *Marine Ecology Progress Series* **141**, 37-45.

Dodgson, K.S., Lewis, J.I.M., Spencer, B. (1953) Studies on sulphatases. 3. The arylsulphatase and beta-glucuronidase of marine molluscs. *Biochemical Journal* **55**, 253-259.

EFSA (2013) *Report for 2011 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products*. European Food Safety Authority. http://www.efsa.europa.eu/en/supporting/doc/363e .pdf, Accessed September 2013.

Fatta-Kassinos, D., Meric, S., Nikolaou, A. (2011) Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. *Analytical and Bioanalytical Chemistry* **399**, 251-275.

Focazio, M.J., Kolpin, D.W., Barnes, K.K., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Barber, L.B., Thurman, M.E. (2008) A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States - II) Untreated drinking water sources. *Science of the Total Environment* **402**, 201-216.

Grillo, M.P., Lohr, M.T., Wait, J.C.M. (2012) Metabolic activation of mefenamic acid leading to mefenamyl-S-acyl-glutathione adduct formation *in vitro* and *in vivo* in rat. *Drug Metabolism and Disposition* **40**, 1515-1526.

Heberer, T., Reddersen, K., Mechlinski, A. (2002) From municipal sewage to drinking water: fate and removal of pharmaceutical residues in the aquatic environment in urban areas. *Water Science and Technology* **46**, 81-88.

Holmstrom, K., Graslund, S., Wahlstrom, A., Poungshompoo, S., Bengtsson, B.-E., Kautsky, N. (2003) Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International Journal of Food Science & Technology* **38**, 255-266.

Horng, H., Spahn-Langguth, H., Benet, L.Z. (2013) *Mechanistic role of acyl glucuronides*. In: Kaplowitz, N. and DeLeve, L.D. (eds.), *Drug-Induced Liver Disease*. 3rd edn. Elsevier Science, San Diego, 35-70.

Huang, T.S., Du, W.X., Marshall, M.R., Wei, C.I. (1997) Determination of oxytetracycline in raw and cooked channel catfish by capillary electrophoresis. *Journal of Agricultural and Food Chemistry* **45**, 2602-2605.

Huerta, B., Rodríguez-Mozaz, S., Barceló, D. (2012) Pharmaceuticals in biota in the aquatic environment: analytical methods and environmental implications. *Analytical and Bioanalytical Chemistry*

Jelic, A., Gros, M., Petrovic, M., Ginebreda, A., Barceló D. (2012) *Occurrence and elimination of pharmaceuticals during conventional wastewater treatment*. In: Guasch, H., *et al.* (eds.), *Emerging and Priority Pollutants in Rivers*. Springer, Berlin, 1-24.

Kallio, J.M., Lahti, M., Oikari, A., Kronberg, L. (2010) Metabolites of the aquatic pollutant diclofenac in fish bile. *Environmental Science & Technology* **44**, 7213-7219.

Kalogeropoulos, N., Karavoltsos, S., Sakellari, A., Avramidou, S., Dassenakis, M., Scoullos, M. (2012) Heavy metals in raw, fried and grilled Mediterranean finfish and shellfish. *Food and Chemical Toxicology* **50**, 3702-3708.

Kitts, D.D., Yu, C.W.Y., Aoyama, R.G., Burt, H.M., McErlane, K.M. (1992) Oxytetracycline degradation in thermally processed farmed salmon. *Journal of Agricultural and Food Chemistry* **40**, 1977-1981.

Kleter, G.A., Prandini, A., Filippi, L., Marvin, H.J.P. (2009) Identification of potentially emerging food safety issues by analysis of reports published by the European Community's Rapid Alert System for Food and Feed (RASFF) during a four-year period. *Food and Chemical Toxicology* **47**, 932-950.

Klosterhaus, S.L., Grace, R., Hamilton, M.C., Yee, D. (2013) Method validation and reconnaissance of pharmaceuticals, personal care products, and alkylphenols in

surface waters, sediments, and mussels in an urban estuary. *Environment International* **54**, 92-99.

Lahti, M., Brozinski, J.M., Jylha, A., Kronberg, L., Oikari, A. (2011) Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout. *Environmental Toxicology and Chemistry* **30**, 1403-1411.

Larsson, D.G.J., de Pedro, C., Paxeus, N. (2007) Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *Journal of Hazardous Materials* **148**, 751-755.

Le Bris, H., Pouliquen, H. (2004) Experimental study on the bioaccumulation of oxytetracycline and oxolinic acid by the blue mussel (*Mytilus edulis*). An evaluation of its ability to bio-monitor antibiotics in the marine environment. *Marine Pollution Bulletin* **48**, 434-440.

Li, W.H., Shi, Y.L., Gao, L.H., Liu, J.M., Cai, Y.Q. (2012) Investigation of antibiotics in mollusks from coastal waters in the Bohai Sea of China. *Environmental Pollution* **162**, 56-62.

Mano, Y., Usui, T., Kamimura, H. (2007) The UDP-glucuronosyltransferase 2B7 isozyme is responsible for gemfibrozil glucuronidation in the human liver. *Drug Metabolism and Disposition* **35**, 2040-2044.

Mengelers, M.J.B., Kleter, G.A., Hoogenboom, L.A.P., Kuiper, H.A., VanMiert, A. (1997) The biotransformation of sulfadimethoxine, sulfadimidine, sulfamethoxazole, trimethoprim and aditoprim by primary cultures of pig hepatocytes. *Journal of Veterinary Pharmacology and Therapeutics* **20**, 24-32.

Mussel Industry Council of North America (2009) *How to cook mussels*. Mussel Industry Council of North America. http://www.discovermussels.com/how-cook-mussels, Accessed March 2013.

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T., Hirai, N., Tatarazako, N. (2008) The effects of pH on fluoxetine in Japanese medaka (*Oryzias latipes*): acute toxicity in fish larvae and bioaccumulation in juvenile fish. *Chemosphere* **70**, 865-873.

Nassar, A.-E.F., Kamel, A.M., Clarimont, C. (2004) Improving the decision-making process in the structural modification of drug candidates: enhancing metabolic stability. *Drug Discovery Today* **9**, 1020-1028.

Perello, G., Marti-Cid, R., Llobet, J.M., Domingo, J.L. (2008) Effects of various cooking processes on the concentrations of arsenic, cadmium, mercury, and lead in foods. *Journal of Agricultural and Food Chemistry* **56**, 11262-11269.

Perello, G., Marti-Cid, R., Castell, V., Llobet, J.M., Domingo, J.L. (2009) Concentrations of polybrominated diphenyl ethers, hexachlorobenzene and polycyclic aromatic

hydrocarbons in various foodstuffs before and after cooking. *Food and Chemical Toxicology* **47**, 709-715.

Quinn, B., Gagne, F., Blaise, C. (2004) Oxidative metabolism activity in *Hydra attenuata* exposed to carbamazepine. *Fresenius Environmental Bulletin* **13**, 783-788.

Quinn, B., Schmidt, W., O'Rourke, K., Hernan, R. (2011) Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (*Dreissena polymorpha*) and their comparison with standardised toxicity tests. *Chemosphere* **84**, 657-663.

Rendal, C., Kusk, K.O., Trapp, S. (2011) The effect of pH on the uptake and toxicity of the bivalent weak base chloroquine tested on *Salix Viminalis* and *Daphnia Magna*. *Environmental Toxicology and Chemistry* **30**, 354-359.

Schmidt, W., O'Rourke, K., Hernan, R., Quinn, B. (2011) Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus* spp.) and their comparison with standardized toxicity tests. *Marine Pollution Bulletin* **62**, 1389-1395.

Skonberg, C., Olsen, J., Madsen, K.G., Hansen, S.H., Grillo, M.P. (2008) Metabolic activation of carboxylic acids. *Expert Opinion on Drug Metabolism and Toxicology* **4**, 425-438.

Smith, R.M. (2002) Extractions with superheated water. *Journal of Chromatography A* **975**, 31-46.

Staines, A.G., Coughtrie, M.W.H., Burchell, B. (2004) N-glucuronidation of carbamazepine in human tissues is mediated by UGT2B7. *Journal of Pharmacology and Experimental Therapeutics* **311**, 1131-1137.

Steffenak, I., Hormazabal, V., Yndestad, M. (1994) Effect of cooking on residues of the quinolones oxoinic acid and flumequine in fish. *Acta Veterinaria Scandinavica* **35**, 299-301.

Ternes, T.A., Kreckel, P., Mueller, J. (1999) Behaviour and occurrence of estrogens in municipal sewage treatment plants - II. Aerobic batch experiments with activated sludge. *Science of the Total Environment* **225**, 91-99.

Trontelj, J. *Tandem mass spectrometry - applications and principles*. InTech, (2012) http://www.intechopen.com/books/tandem-mass-spectrometry-applications-and-principles/quantification-of-glucuronide-metabolites-in-biological-matrices-by-lc-ms-ms, Accessed May 2013.

Turnipseed, S.B., Andersen, W.C. (2008) *Veterinary drug residues*. In: Picó, Y. (ed.) *Wilson & Wilson's Comprehensive Analytical Chemistry: Food Contaminants and Residue Analysis*. Elsevier, Amsterdam, 307-338.

Uno, K. (2002) Oxytetracycline and oxolinic acid residues in kuruma prawn (*Penaeus japonicus*) and the effect of cooking procedures on the residues. *Journal of the Food Hygienic Society of Japan* **43**, 62-67.

Uno, K., Aoki, T., Kleechaya, W., Ruangpan, L., Tanasomwang, V. (2006a) Pharmacokinetics of oxolinic acid in black tiger shrimp, *Penaeus monodon Fabricius*, and the effect of cooking on residues. *Aquaculture Research* **37**, 826-833.

Uno, K., Aoki, T., Kleechaya, W., Tanasomwang, V., Ruangpan, L. (2006b) Pharmacokinetics of oxytetracycline in black tiger shrimp, *Penaeus monodon*, and the effect of cooking on the residues. *Aquaculture* **254**, 24-31.

Uno, K., Chaweepack, T., Ruangpan, L. (2010) Pharmacokinetics and bioavailability of oxytetracycline in vannamei shrimp (*Penaeus vannamei*) and the effect of processing on the residues in muscle and shell. *Aquaculture International* **18**, 1003-1015.

US Department of Health and Human Services (2009) *Fourth National Report on Human Exposure to Environmental Chemicals*. US Department of Health and Human Services. http://www.cdc.gov/exposurereport/pdf/FourthReport.pdf, Accessed Sep 2013.

Van Der Oost, R., Beyer, J., Vermeulen, N.P.E. (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* **13**, 57-149.

Van Leengoed, L., Kluivers, M., Herbes, R., Langendijk, P., Stephany, R., van den Berg, M., Seinen, W., Grinwis, G., van der Lugt, J., Meulders, F., Geudeke, J., Verheijden, J. (2002) The weakest link: medroxyprogesterone acetate in pig feed. *Tijdschrift voor Diergeneeskunde* **127**, 516-519.

Vernouillet, G., Eullaffroy, P., Lajeunesse, A., Blaise, C., Gagne, F., Juneau, P. (2010) Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. *Chemosphere* **80**, 1062-1068.

Wille, K., Noppe, H., Verheyden, K., Vanden Bussche, J., De Wulf, E., Van Caeter, P., Janssen, C.R., De Brabander, H.F., Vanhaecke, L. (2010) Validation and application of an LC-MS/MS method for the simultaneous quantification of 13 pharmaceuticals in seawater. *Analytical and Bioanalytical Chemistry* **397**, 1797-1808.

Wille, K., Kiebooms, J.A.L., Claessens, M., Rappe, K., Vanden Bussche, J., Noppe, H., Van Praet, N., De Wulf, E., Van Caeter, P., Janssen C.R., De Brabander H.F., Vanhaecke L. (2011) Development of analytical strategies using U-HPLC-MS/MS and LC-ToF-MS for the quantification of micropollutants in marine organisms. *Analytical and Bioanalytical Chemistry* **400**, 1459-1472.

Xu, D.H., Grizzle, J.M., Rogers, W.A., Santerre, C.R. (1996) Effect of cooking on residues of ormetoprim and sulfadimethoxine in the muscle of channel catfish. *Food Research International* **29**, 339-344.

5.0 Final Conclusions and Future Work

5.1 Summary

Pharmaceuticals have been previously detected in the aquatic environment, particularly in freshwater environments, and have been reported to exert specific biological effects on aquatic organisms exposed *in vivo*. However, knowledge on the occurrence, fate and effects of pharmaceuticals in the marine environment is still lacking and their potential to bioaccumulate and biomagnify in aquatic organisms and potentially pose a threat to human health is relatively unknown. Therefore, the main goal of this thesis was to investigate the prevalence of selected pharmaceuticals in the Irish marine environment with the optimisation of novel analytical methods for their determination in various environmental matrices, such as wastewater effluent, marine surface waters, bivalves and fish. Additionally, the potential for trophic level transfer of pharmaceuticals through the food chain and human exposure via ingestion of contaminated seafood was also addressed. The main outputs of this thesis are as follows:

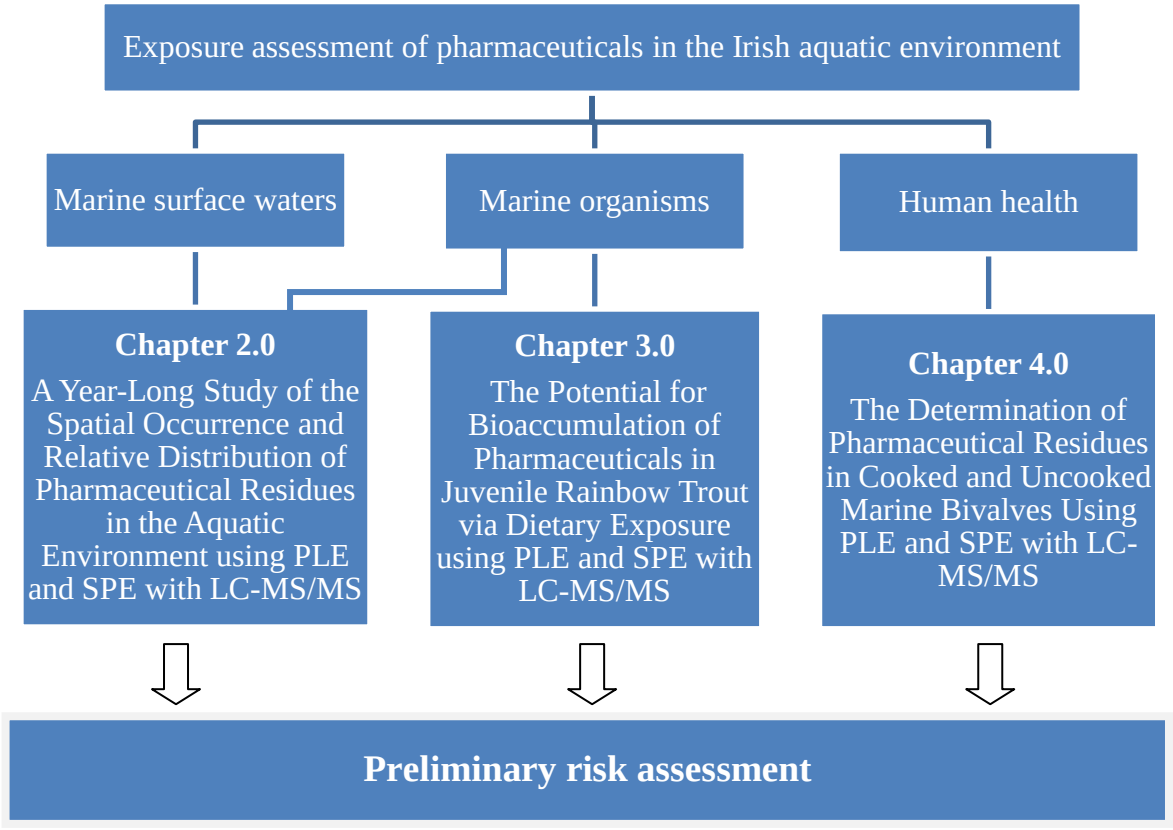


Figure 5.1 Schematic overview of the main outputs of this thesis

The research carried out investigates the occurrence of pharmaceuticals in the Irish aquatic environment and their potential to bioaccumulate and pose a risk to human health via dietary intake of contaminated seafood. The current literature on the presence of pharmaceuticals in the aquatic environment focuses on residues detected in wastewater and freshwater and to date the occurrence and fate of pharmaceuticals in the natural marine environment is still unknown. For this reason, **Chapter 1.0** reviews the presence of pharmaceuticals in the marine environment with a focus on previous monitoring studies conducted in marine surface water and marine biota and recent trends in analytical techniques.

For the first time, a year-long monitoring study was carried out to determine the spatial occurrence of five targeted pharmaceuticals in the Irish marine environment (**Chapter 2.0**). Analytical techniques such as PLE, SPE and LC-MS/MS were combined, optimised and applied to wastewater effluent, marine surface water and *Mytilus* spp. samples collected from two impacted sites and a control site along the Irish coastline. The presence of all five targeted pharmaceuticals was confirmed at concentrations in the low $\mu g.L^{-1}$ range in effluent and in the high $ng.L^{-1}$ range in exposed marine surface water. Three of the five detected pharmaceuticals in marine surface waters were also found to occur in exposed *Mytilus* spp., with residues of trimethoprim measuring at concentrations up to 9.22 $ng.g^{-1}$ DW. This study has confirmed the uptake of pharmaceuticals in marine bivalves at measurable quantities. The presence of very low antibiotic residues in the aquatic environment has been shown to cause antimicrobial resistance in natural bacteria which could be of concern for potential consumers (Kummerer, 2009). Thus, for the remaining work (Chapters 3 & 4), the possibility of pharmaceutical uptake in exposed biota was assessed and the potential for exposure to humans via dietary intake was also investigated.

Pharmaceutical bioaccumulation in juvenile rainbow trout via trophic level transfer was investigated in **Chapter 3.0**. A 28-day *in vivo* bioaccumulation experiment was carried out in a flow-through system in which the exposed fish were fed effluent exposed marine mussels. The PLE, SPE and LC-MS/MS methods previously developed were applied to the water and biotic samples. The pharmaceutical extraction method applied to marine mussels was further optimised for fish liver samples. Trimethoprim was the only pharmaceutical from the five selected to be quantified in the *Mytilus* spp. at concentrations of 6.68 $ng.g^{-1}$ DW.

After 28 days of feeding and despite the presence of trimethoprim residues in the tissues of the *Mytilus* spp., no pharmaceutical residues were detected in the fish liver tissues or the carbon filtered municipal water supplied to the fish tanks. The presence of pharmaceuticals in other fish tissues were not assessed as the fillet could not be processed and other organs were used for biological analysis. The potential for pharmaceuticals, in particular trimethoprim, to biomagnify in the liver via trophic level transfer is not likely considering the LOD and LOQ for trimethoprim in mussel tissue and fish liver tissues were of equal value. However, bioaccumulation of pharmaceutical residues via dietary exposure may have occurred and gone undetected if pharmaceutical concentrations present in the mussel tissue and fish liver were less than the LOD value. Considering the possibility of pharmaceutical bioaccumulation in fish via trophic level transfer, the next step was to investigate the potential risk of pharmaceutical exposure to humans.

The effect of cooking on the concentrations of five pharmaceutical residues in exposed mussel tissue was investigated in an attempt to assess the potential risk of exposure to humans via ingestion of contaminated seafood (**Chapter 4.0**). A preliminary *in vivo* experiment was carried out exposing marine mussels to high pharmaceutical concentrations via direct injection or water uptake over 96 h. After cooking (via steaming) a selection of the water-exposed mussels, an overall increase in pharmaceutical residues in the contaminated mussel tissue and cooking water was observed. In contrast, mussels exposed to effluent impacted marine surface waters were found to contain low-level residues of the antibiotic trimethoprim but, these residues were eliminated after steaming. It is for this reason why mussels were also exposed to pharmaceutical concentrations approximately a thousand times higher than those in the environment as the effects of cooking on pharmaceutical residues could be observed at a higher scale and changes in concentrations, which may have gone unnoticed at environmentally relevant concentrations close to the method LOD values, could be reported. The potential risk of pharmaceutical exposure to humans through the food chain is low but may exist if sourcing seafood from surface waters receiving highly contaminated effluent such as that reported in India by Larsson *et al.* (2007).

5.2 Final Conclusions

5.2.1 Distribution of pharmaceuticals in the Irish aquatic environment

It is clear from the results generated during this project that pharmaceutical residues are present in quite substantial quantities in the Irish aquatic environment. It has been shown that a number of commonly used pharmaceuticals persist beyond the wastewater treatment process and enter receiving surface waters. In general, research into the occurrence and fate of pharmaceuticals in the marine environment is limited in comparison to the number of studies carried out in various freshwater environments. This was previously addressed in Chapter 1.0 and a summary of the studies carried out to date in marine surface waters was provided in Section *1.7.1*. Typical concentrations in marine surface waters were quantified in the very low to mid $ng.L^{-1}$ concentration range with slightly higher pharmaceutical concentrations measured in estuarine water samples (Thomas and Hilton, 2004; Wille *et al.*, 2010). In comparison to the results from other monitoring studies, pharmaceutical residues measured slightly higher in Irish marine surface waters, with the highest residues detected at 1.41 $\mu g.L^{-1}$ for carbamazepine and all other selected compounds quantified in the mid to high $ng.L^{-1}$ concentration range.

When comparing marine surface waters from the east and west coasts of Ireland, pharmaceutical concentrations measured in both locations were directly related to the concentration of pharmaceuticals in the wastewater effluent released into each exposure site. Much higher concentrations of carbamazepine were detected in marine surface waters from the east coast (EXP1) whereas higher concentations of trimethoprim and gemfibrozil were detected in marine surface waters from the west coast (EXP2). Unlike WWTP1, effluent from WWTP2 was discharged through a diffuser pipe in the bay and impacted waters were predicted to contain much lower pharmaceutical residues than EXP1 due to the greater extent of dilution. However, high residues of the antibiotic, trimethoprim, in particular, at EXP2 may be due to the treatment of effluent from four major hospitals in a volume of wastewater almost ten times smaller than that treated by WWTP1. It has been previously highlighted that in some respects effluent from major hospitals differs in composition to general urban effluent and the need for specific monitoring and control may need to be considered particularly for antimicrobial residues (Cormican *et al.*, 2012).

An *in situ* study carried out the previous year in the same estuary but at 700 m from the effluent outfall pipe, revealed pharmaceutical residues of carbamazepine and trimethoprim in marine suface waters at concentrations up to 30 $ng.L^{-1}$. Taking these results into account, a decrease in the concentrations of pharmaceutical residues in marine surface waters could be observed with increasing distance from the coast. The reason for the decrease in pharmaceutical residues is believed to be as a result of both dilution and degradation processes hindering their transport further out of the estuary.

5.2.2 Uptake of pharmaceuticals in non-target aquatic biota

This research has highlighted the ability of marine mussels to bioaccumulate low-level pharmaceutical concentrations present in their surrounding aquatic environment. The uptake of the antibiotic, trimethoprim, was reported in wild and caged *Mytilus* spp. at concentrations of 6.68 $ng.g^{-1}$ and 9.22 $ng.g^{-1}$, respectively, after exposure to one of the most contaminated marine surface water sites on the Irish coast, EXP1. Carbamazepine and mefenamic acid were also detected in caged marine mussel tissues from this site and from another heavily impacted marine site (EXP2) but residues were too low to be quantified. Previous pharmaceutical fate studies carried out in soils and sludges support some of the findings from this study, in particular, the higher concentration ability of antimicrobial agents, such as triclosan and triclocarban, in soils and sludges in comparison to pharmaceuticals from other classes (Barron *et al.*, 2008).

Selected pharmaceuticals were continuously detected in surrounding marine surface waters at average concentrations in the mid to high $ng.g^{-1}$ range, except for gemfibrozil which was detected sporadically. Previous chronic exposure studies (see Table 1.3) have tested environmentally relevant concentrations of pharmaceuticals on the *Mytilus* spp. and other aquatic organisms with potentially lethal effects observed, such as increased oxidative stress, lesions on vital organs and reduced enzyme function in cells (Contardo-Jara *et al.*, 2011; Cuklev *et al.*, 2011; Lajeunesse *et al.*, 2011; Mehinto *et al.*, 2010; Quinn *et al.*, 2004; Quinn *et al.*, 2011; Schmidt *et al.*, 2011; Schmidt *et al.*, 2013; Schwaiger *et al.*, 2004). Due to the ability of these compounds to readily metabolise, there is a need to combine both chemical and biological analysis particularly for the assessment of

pharmaceutical uptake in naturally exposed aquatic organisms, as seen in this case where low uptake does not necessarily indicate low exposure or risk.

In Chapter 4.0, marine mussels were exposed to artificial seawater spiked daily with elevated pharmaceutical concentrations of 1 $mg.L^{-1}$ over a 96-h period. Acidic compounds in the mussel tissue decreased drastically in concentration over the 96 hrs in contrast to carbamazepine and trimethoprim, respectively neutral and basic compounds, which gradually increased in concentration over time. The possibility of metabolic activity within the mussel was suggested following the overall reduction in concentrations of acidic compounds which have been previously shown to undergo efficient glucuronisation in fish bile (Lahti *et al.*, 2011). This possibility was further supported when higher pharmaceutical residues, of acidic compounds in particular, were detected in the mussels after thermal treatment, most likely due to the cleaving of glucuronised metabolites back to the parent compound. No correlations between the $\log D_{ow}$ values and the extent of uptake of pharmaceuticals in biota could be observed. Bioaccumulation does not seem to be exclusively driven by the hydrophobicity of the compound at a certain pH which makes it difficult to predict the partitioning of pharmaceuticals into different aqueous, solid and biotic phases of the aquatic environment. For this reason and their ability to extensively undergo biotransformation within biological tissues, information regarding their presence in both the aquatic phase and biotic tissue is required, which is provided within this thesis.

5.2.3 The use of sample extraction and mass spectrometry in the analysis of pharmaceuticals in environmental matrices

Chemical analysis in this area still proves to be very challenging, primarily due to the complexity of the environmental sample matrices involved. In particular, the co-extraction of the sample matrix with selected analytes, adversely affecting method sensitivity and detection. Ion suppression further affects the reliable quantification of pharmaceuticals. Deuterated standards can be used to address this problem, but this can add considerable cost to the analysis, coupled with their extremely limited availability. Standard addition and matrix-matched calibration are other commonly used quantification methods that prove more cost effective however, may be difficult to perform if there are very few samples available to

analyse or if it is difficult to obtain blank matrix-matched samples. The use of new separation technologies such as UHPLC can somewhat tackle this problem by providing better resolution from interfering compounds although this may not be the most cost effective option.

The distinct diversity across the range of sample matrices investigated proved more problematic for some than others. A solution is to develop several separate matrix specific extraction methods coupled to LC-MS/MS analysis. For optimal performance, each extraction method must be tailored for compounds with similar physicochemical properties. However, such an approach is timely and costly and is therefore, not a feasible solution. Chemical analysis, in particular, LC-MS/MS, remains the technique of choice for trace analysis of pharmaceuticals in environmental matrices, however, matrix effects and laborious sample preparation processes continue to limit its applicability to all samples.

5.2.4 Risk assessment

The requirement for a detailed risk assessment of pharmaceuticals is a priority to ensure there are no major risks to the environment and human health. The risks for the aquatic environment, associated with the measured water concentrations of the targeted pharmaceuticals within this thesis, cannot be fully assessed as there are currently no regulatory guidelines established for widely used and widespread occurring pharmaceuticals. Only recently, diclofenac has been added to a new 'watch list' included in the EU Water Framework Directive and may be added to the priority list at a later date following further monitoring and toxicity studies (EU Directive 2013/39/EU). As demonstrated in this thesis, there are numerous other pharmaceutical residues present at substantial concentrations in marine surface waters which are also known to cause chronic effects in aquatic organisms at low doses (Quinn *et al.*, 2004; Quinn *et al.*, 2011; Schmidt *et al.*, 2011; Schmidt *et al.*, 2013).

As mentioned previously, the EMEA is the regulatory body responsible for carrying out environmental risk assessments on pharmaceuticals prior to their licensing. If PECs are >0.01 $\mu g.L^{-1}$, risk is then determined by the ratio of PEC/PNEC, where PNEC values are based on results from acute toxicity studies. In order to perform a more comprehensive risk assessment, available information on

the chronic toxic effects of the selected pharmaceuticals i.e. lowest observed effect concentration (LOEC), were used instead of PNEC values and any uncertainty regarding single-species analysis was accounted for using the necessary assessment factor (European Chemicals Bureau, 2003). Based on previous chronic toxicity studies, the pharmaceutical monitoring data produced for carbamazepine and diclofenac within this thesis highlights a potential risk for chronic effects in aquatic species residing in the selected Irish coastal zones (Triebskorn *et al*., 2007).

The measurement of pharmaceuticals in aquatic species that are important in terms of human consumption, such as mussels, is very useful in estimating the human exposure and dietary intake of these pharmaceuticals. It was observed that the European maximum residue limit (MRL) for trimethoprim in all food producing species (50 $ng.g^{-1}$) was not exceeded in marine mussels collected from one of the most highly contaminated sites in Ireland, deeming it safe for human consumption (EU Regulation 37/2010). After cooking the wild contaminated mussels, trimethoprim was not detected and appeared to be eliminated in the mussel tissue. The effectiveness of thermal treatment was then assessed at higher pharmaceutical concentrations and interestingly, an overall increase in pharmaceutical residues in contaminated mussel tissue and cooking water was reported after thermal treatment. In the case of trimethoprim, this was the only pharmaceutical to be reduced after cooking, hence, marine mussels with MRL values acceptable before cooking should still be acceptable for human consumption after cooking. However, all of the other selected pharmaceuticals showed an increase in concentration in the marine mussels after cooking, with exceptionally high increases measured for acidic pharmaceuticals in particular. Although diclofenac and several other acidic pharmaceuticals are not monitored for in seafood, they are controlled in other foodstuffs in the EU. The research carried out in Chapter 4.0 highlighted the possibility for pharmaceutical residues in approved foodstuffs to exceed the assigned MRL values following thermal treatment of the food, potentially posing a risk to human health via dietary ingestion.

In Ireland, the lowest oral dosages of the selected pharmaceuticals for humans on the market are approximately 80 mg and higher, except for diclofenac which is available in 25 mg tablets (IPHA, 2013). Considering medicines are usually taken every several hours and at various doses, the concentration of pharmaceutical residues in the environment may be considered negligible in

comparison. On the other hand, the lowest oral doses available for these pharmaceuticals are still very potent and should not be confused with LOEC values of pharmaceuticals in the human body, for which data is not publicly available.

The scope of the work presented in this thesis has provided knowledge of the occurrence and distribution of pharmaceuticals in natural marine ecosystems in Ireland and the potential risk of pharmaceutical exposure to human health. It is suggested there is a very low risk of pharmaceutical exposure to humans via dietary ingestion of marine mussels, however, there may be potential for pharmaceutical exposure to humans via dietary intake of other exposed aquatic species. The information gathered using the developed analytical techniques has enhanced Ireland's capacity towards the integrated monitoring of contaminants in the marine environment and these findings will contribute to future pharmaceutical fate studies and evaluations of the human risks posed by these emerging environmental pollutants.

5.3 Future Work

The following areas have been identified as possibly requiring further research and may be of relevance to those undertaking similar studies:

- Following method performance testing, the measured ion suppression values for pharmaceuticals in complex matrices, such as wastewater effluent, marine surface water and biological tissues, were very high even after undergoing at least one extraction step. There is a need for more selective extraction techniques using efficient SPE sorbents to remove unwanted matrix components and allow for the accurate quantification of compounds present in the environment at sub $ng.g^{-1}$ concentration levels. Although MIP is a very selective extraction technique, this is only suitable for target analyte screening of samples as only one compound or a class of structurally related compounds can be extracted at any one time, hindering the development of multi-class methods of analysis (Mahony *et al.*, 2005).

- ‘Prevention is better than cure’ is the message from the Irish Environmental Protection Agency when it comes to water quality. Besides encouraging doctors against the unnecessary prescribing of pharmaceuticals and providing a greater awareness of correct ways of disposing unused or out of date pharmaceuticals, there is no alternative to reduce the presence of pharmaceuticals in sewerage systems. Removal technologies for pharmaceuticals should be employed in the wastewater treatment plant to prevent the release of potentially harmful pharmaceuticals into the environment however, emission limits first need to be set before the most cost effective treatments can be implemented. Previous studies have reported that a combination of advanced treatment technologies, such as UV/H_2O_2 and O_3/H_2O_2, have proven successful in the efficient removal of pharmaceuticals from wastewater (Ternes, 2004). Although these technologies are costly to implement, they eliminate the need for water remediation and greatly reduce the human impact and potential biological effects on the natural aquatic environment.

- The toxicity of a mixture of pharmaceuticals with similar modes of action has been previously found to be substantially higher than the toxicity of individual pharmaceuticals at the same concentrations (Cleuvers, 2003). As pharmaceuticals do not occur isolated in the natural environment, further study is required to assess the ecological relevance and ecotoxicological potential of prevalent pharmaceutical mixtures in the aquatic environment.

- The inclusion of metabolites in analytical methods should be addressed, especially for the likes of prodrugs, which release the active compound after undergoing metabolism (Rautio *et al.*, 2008). This will require availability of pharmacokinetic data and reference standards from pharmaceutical manufacturers. A current limitation in the area is the lack of reference standards, especially of pharmaceutical metabolites, and even if available, they are very expensive. Other products formed via environmental processes, such as photo transformation, should also be examined, thereby pharmaceuticals and their relevant product compounds can be included in analytical methods for a more complete environmental risk assessment.

- Based on the conclusions of this thesis, the possibility for antibiotics, such as trimethoprim, to bioaccumulate in aquatic species and potentially through the food chain was highlighted. The input of resistant bacteria into the environment has been previously shown to affect the health of aquatic organisms and may potentially pose a threat to human health (Kummerer, 2009). A more in depth study of the fate of persistent antibiotics in the aquatic environment should be carried out and their abilities to bioaccumulate in other aquatic species further assessed.

- The risk of biological effects occurring in aquatic organisms was assessed using previous chronic toxicity data, however, LOEC values were used due to the lack of PNEC data available. Chronic toxicity studies need to be carried out on a wider range of aquatic species and should include exposure to pharmaceutical concentrations lower than 1 $ug.L^{-1}$ in order to produce PNEC data and allow for the accurate environmental risk assessment of pharmaceuticals. More of a focus should be certainly placed on the combination of chemical and biological analysis of aquatic biota to fully assess the exposure and uptake of pharmaceuticals and the effects exerted on the aquatic organism. As mentioned before, low measurements of pharmaceutical residues in the exposed organism does not necessarily indicate low exposure, especially in the case of highly metabolised compounds such as acidic pharmaceuticals.

- Following the results of this thesis, it may be necessary to further investigate animal food products, containing pharmaceutical residues at concentrations close to the defined MRL values, after thermal treatment. This investigation should particularly target carboxyl containing compounds which are highly susceptible to glucuronidation.

References

Barron, L., Tobin, J., Paull, B. (2008) Multi-residue determination of pharmaceuticals in sludge and sludge enriched soils using pressurized liquid extraction, solid phase extraction and liquid chromatography with tandem mass spectrometry. *Journal of Environmental Monitoring* **10**, 353-361.

Cleuvers, M. (2003) Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicology Letters* **142**, 185-194.

Contardo-Jara, V., Lorenz, C., Pflugmacher, S., Nutzmann, G., Kloas, W., Wiegand, C. (2011) Exposure to human pharmaceuticals carbamazepine, ibuprofen and bezafibrate causes molecular effects in *Dreissena polymorpha*. *Aquatic Toxicology* **105**, 428-437.

Cormican, M., Cummins, E., Morris, D., Prendergast, M., O'Donovan, D., O'Flaherty, V. (2012) *Enhancing human health through improved water quality*. Irish Environmental Protection Agency.

Cuklev, F., Kristiansson, E., Fick, J., Asker, N., Forlin, L., Larsson, D.G.J. (2011) Diclofenac in fish: blood plasma levels similar to human therapeutic levels affect global hepatic gene expression. *Environmental Toxicology and Chemistry* **30**, 2126-2134.

EU Regulation 37/2010 (2009) *European Commission Regulation no. 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.* Official Journal of the European communities, OJL 15

EU Directive 2013/39/EU (2013) *European Union water framework directive 2013/39/EU*. Official Journal of the European Communities, OJL226.

European Chemicals Bureau (2003) *Technical guidance document on risk assessment in support of Commission Directive 93/67/EEC, Commission Regulation (EC) No. 1488/94 and Directive 98/8/EC. Part II: Environmental risk assessment.* Institute for Health and Consumer Protection.

IPHA (2013) Medicines information online, *Irish Pharmaceutical Healthcare Association*, http://www.medicines.ie, Accessed Nov 2013.

Kummerer, K. (2009) Antibiotics in the aquatic environment - a review - Part II. *Chemosphere* **75**, 435-441.

Lahti, M., Brozinski, J.M., Jylha, A., Kronberg, L., Oikari, A. (2011) Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout. *Environmental Toxicology and Chemistry* **30**, 1403-1411.

Lajeunesse, A., Gagnon, C., Gagne, F., Louis, S., Cejka, P., Sauve, S. (2011) Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment - Evidence of biological effects. *Chemosphere* **83**, 564-571.

Larsson, D.G.J., de Pedro, C., Paxeus, N. (2007) Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *Journal of Hazardous Materials* **148**, 751-755.

Mahony, J.O., Nolan, K., Smyth, M.R., Mizaikoff, B. (2005) Molecularly imprinted polymers—potential and challenges in analytical chemistry. *Analytica Chimica Acta* **534**, 31-39.

Mehinto, A.C., Hill, E.M., Tyler, C.R. (2010) Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). *Environmental Science & Technology* **44**, 2176-2182.

Quinn, B., Gagne, F., Blaise, C. (2004) Oxidative metabolism activity in *Hydra attenuata* exposed to carbamazepine. *Fresenius Environmental Bulletin* **13**, 783-788.

Quinn, B., Schmidt, W., O'Rourke, K., Hernan, R. (2011) Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (*Dreissena polymorpha*) and their comparison with standardised toxicity tests. *Chemosphere* **84**, 657-663.

Rautio, J., Kumpulainen, H., Heimbach, T., Oliyai, R., Oh, D., Jarvinen, T., Savolainen, J. (2008) Prodrugs: design and clinical applications. *Nature Reviews Drug Discovery* **7**, 255-270.

Schmidt, W., O'Rourke, K., Hernan, R., Quinn, B. (2011) Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus spp.*) and their comparison with standardized toxicity tests. *Marine Pollution Bulletin* **62**, 1389-1395.

Schmidt, W., Rainville, L.-C., McEneff, G., Sheehan, D., Quinn, B. (2013) A proteomic evaluation of the effects of the pharmaceuticals diclofenac and gemfibrozil on marine mussels (*Mytilus spp.*): evidence for chronic sublethal effects on stress-response proteins. *Drug Testing and Analysis*, DOI: 10.1002/dta.1463.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R.D. (2004) Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part 1: histopathological alterations and bioaccumulation in rainbow trout. *Aquatic Toxicology* **68**, 141-150.

Ternes, T. (2004) *Assessment of technologies for the removal of pharmaceuticals and personal care products in sewage and drinking water facilities to improve the*

indirect potable water reuse. Poseidon EU Project Report. http://www.eu-poseidon.com, Accessed Sep 2013.

Thomas, K.V., Hilton, M.J. (2004) The occurrence of selected human pharmaceutical compounds in UK estuaries. *Marine Pollution Bulletin* **49**, 436-444.

Triebskorn, R., Casper, H., Scheil, V., Schwaiger, J. (2007) Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). *Analytical and Bioanalytical Chemistry* **387**, 1405-1416.

Wille, K., Noppe, H., Verheyden, K., Vanden Bussche, J., De Wulf, E., Van Caeter, P., Janssen, C.R., De Brabander, H.F., Vanhaecke, L. (2010) Validation and application of an LC-MS/MS method for the simultaneous quantification of 13 pharmaceuticals in seawater. *Analytical and Bioanalytical Chemistry* **397**, 1797-1808.

Appendix

Table A.1: Pharmaceutical residues detected in wastewater effluent (µg.L^{-1}), marine surface water (MSW) (µg.L^{-1}) and mussels (ng.g^{-1} DW) sampled from WWTP1 and EXP1 from May to August 2011. Salinity and water temperature of MSW taken on site at 1 m below surface. Data for BOD, COD, suspended solids and flow rate of effluent provided with weather data from WWTP1 and Met Eireann

EXP1	**May 2011**			**Jun 2011**			**Jul 2011**			**Aug 2011**		
	Effluent	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*
Trimethoprim	0.07±0.11	0.20±0.06	<4*	1.19±0.16	0.56±0.06	<4*	0.49±0.13	0.24±0.08	<4*	0.65±0.07	0.57±0.02	<4*
Diclofenac	0.75±0.06	0.11±0.11	n.d.	1.22±0.30	0.46±0.05	n.d.	0.84±0.20	n.d.	n.d.	1.52±0.15	0.06±0.05[a]	n.d.
Carbamazepine	0.94±0.13	0.18±0.18	n.d.	0.86±0.07	0.31±0.06	n.d.	1.91±0.08	0.51±0.03	<6*	2.37±0.14	0.64±0.09	<6*
Mefenamic acid	1.25±0.11	n.d.	n.d.	0.54±0.10	0.08±0.08	n.d.	0.73±0.12	n.d.	n.d.	0.42±0.18	0.14±0.01[a]	<23*
Gemfibrozil	0.57±0.15	n.d.	n.d.	0.45±0.14	0.29±0.04	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Salinity (g.kg^{-1})		36			29							
Water temp. (°C)		6			9							
BOD (mg.L^{-1})	10			13			5			15		
COD (mg.L^{-1})	59			76			50			63		
SS (mg.L^{-1})	16			15			11			9		
pH	7.6	8.0		7.7	7.8		7.4	8.0		7.2	8.0	
Flow rate (m^3.day^{-1})	343908			310622			339884			318524		
Rainfall (mm)		0.0			0.0			0.3			2.0	
Temp. range (°C)		10-16			10-19			13-20			13-21	

n.d. not detected

[a] Quantification carried out using a three point standard addition calibration

*Pharmaceutical residues detected at concentrations <LOQ

Table A.2: Pharmaceutical residues detected in wastewater effluent (µg.L^{-1}), marine surface water (MSW) (µg.L^{-1}) and mussels (ng.g^{-1} DW) sampled from WWTP1 and EXP1 from September to December 2011. Salinity and water temperature taken on site at 1 m below surface. Data for BOD, COD, suspended solids and flow rate of effluent provided with weather data from WWTP1 and Met Eireann

EXP1	**Sep 2011**			**Oct 2011**			**Nov 2011**			**Dec 2011**		
	Effluent	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*
Trimethoprim	0.59±0.19	0.35±0.08	<4*	0.85±0.05	0.16±0.07	**9.22**±1.54	0.34±0.05	0.29±0.10	**7.28**±1.70	1.08±0.21	0.35±0.04	<4*
Diclofenac	0.51±0.04	0.30±0.01	n.d.	0.88±0.13	0.39±0.03	n.d.	1.22±0.16	0.46±0.12	n.d.	0.31±0.07	0.26±0.02	n.d.
Carbamazepine	2.33±0.19	1.30±0.20	<6*	2.49±0.13	1.41±0.15	n.d.	3.16±0.32	0.97±0.19	n.d.	1.86±0.16	0.88±0.13	<6*
Mefenamic acid	0.49±0.04	0.13±0.11[a]	<23*	0.46±0.04	0.31±0.11	<23*	1.07±0.22	0.11±0.11	n.d.	0.42±0.11	0.27±0.05	n.d.
Gemfibrozil	0.10±0.09	n.d.	n.d.	0.16±0.07	n.d.	n.d.	0.31±0.05	n.d.	n.d.	0.09±0.13	0.11±0.09	n.d.
Salinity (g.kg^{-1})											38	
Water temp. (°C)											13	
BOD (mg.L^{-1})	9			14			22			10		
COD (mg.L^{-1})	55			44			61			54		
SS (mg.L^{-1})	10			14			28			12		
pH	7.6	7.7		7.5	8.1		7.4	8.1		7.6	8.1	
Flow rate (m^3.day^{-1})	325659			362697			395197			467155		
Rainfall (mm)		0.0			0.0			0.3			1.0	
Temp. range (°C)		5-18			8-13			4-11			1-10	

n.d. not detected

[a] Quantification carried out using a three point standard addition calibration

*Pharmaceutical residues detected at concentrations <LOQ

Table A.3: Pharmaceutical residues detected in wastewater effluent (µg.L^{-1}), marine surface water (MSW) (µg.L^{-1}) and mussels (ng.g^{-1} DW) sampled from WWTP1 and EXP1 from January to April 2012. Salinity and water temperature taken on site at 1 m below surface. Data for BOD, COD, suspended solids and flow rate of effluent provided with weather data from WWTP1 and Met Eireann

EXP1	**Jan 2012**			**Feb 2012**			**Mar 2012**			**Apr 2012**		
	Effluent	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*
Trimethoprim	1.20±0.10	0.21±0.02	<4*	0.65±0.07	0.07±0.03	<4*	0.60±0.16	0.52±0.06	<4*	0.06±0.11	0.21±0.05	<4*
Diclofenac	0.60±0.17	0.25±0.08	n.d.	0.99±0.15	0.37±0.12	n.d.	1.40±0.17	0.38±0.03	n.d.	1.69±0.20	0.42±0.13	n.d.
Carbamazepine	0.62±0.09	0.52±0.11	<6*	0.51±0.10	0.58±0.12	<6*	0.76±0.16	0.47±0.05	<6*	0.99±0.05	0.36±0.09	<6*
Mefenamic acid	1.50±0.19	0.37±0.05	<23*	0.80±0.05	0.32±0.09	<23*	1.52±0.26	0.48±0.09	n.d.	1.55±0.31	0.41±0.07	<23*
Gemfibrozil	0.36±0.04	0.24±0.10	n.d.	0.65±0.15	0.37±0.10	n.d.	0.43±0.11	0.14±0.15	n.d.	0.53±0.07	0.40±0.07	n.d.
Salinity (g.kg^{-1})		42			16			24			23	
Water temp. (°C)		10			12			13			15	
BOD (mg.L^{-1})	11			16			11			13		
COD (mg.L^{-1})	54			45			54			63		
SS (mg.L^{-1})	15			22			16			13		
pH	7.3	7.5		7.5	8.1		7.5	7.8		7.6	7.7	
Flow rate (m^3.day^{-1})	409324			474047			339308			344219		
Rainfall (mm)		1.0			0.0			0.0			0.0	
Temp. range (°C)		2-10			-6-3			5-13			2-17	

n.d. not detected

[a] Quantification carried out using a three point standard addition calibration

*Pharmaceutical residues detected at concentrations <LOQ

Table A.4: Pharmaceutical residues detected in wastewater effluent (µg.L^{-1}), marine surface water (MSW) (µg.L^{-1}) and mussels (ng.g^{-1} DW) sampled from WWTP2 and EXP2 from May to August 2011. Salinity and water temperature of MSW taken on site at 1 m below surface. Data for BOD, COD, suspended solids and flow rate of effluent provided with weather data from WWTP2 and Met Eireann

EXP1	**May 2011**			**Jun 2011**			**Jul 2011**			**Aug 2011**	
	Effluent	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*	*Effluent*	*MSW*	*Mussels*	*Effluent*	*MSW*
Trimethoprim	0.95±0.25	0.76±0.10	<4*	1.17±0.26	0.80±0.14	<4*	0.50±0.08	0.74±0.11	<4*	1.03±0.14	0.87±0.14
Diclofenac	2.63±0.19	0.22±0.19	n.d.	1.61±0.04	0.55±0.05	n.d.	0.45±0.16	n.d.	n.d.	1.01±0.14	0.24±0.01[a]
Carbamazepine	0.57±0.16	0.08±0.04	n.d.	0.40±0.19	0.05±0.12	n.d.	0.27±0.06	0.16±0.10	n.d.	1.71±0.21	0.40±0.10
Mefenamic acid	2.42±0.11	n.d.	<23*	2.80±0.15	0.61±0.14	<23*	0.52±0.07	0.27±0.09[a]	<23*	0.53±0.11	0.21±0.15[a]
Gemfibrozil	0.84±0.20	n.d.	n.d.	1.48±0.25	0.49±0.16	n.d.	1.57±0.24	0.64±0.14[a]	n.d.	0.57±0.21	n.d.
Salinity (g.kg^{-1})		30			28						
Water temp. (°C)		10			12						
CBOD (mg.L^{-1})	2			3			2			<4	
COD (mg.L^{-1})	29			28			28			27	
SS (mg.L^{-1})	<5			7			<5			<5	
pH	7.60	7.97		7.23	7.95		7.29	8.21		7.12	8.20
Flow rate (m^3.day^{-1})	40103			46306			47145			38223	
Rainfall (mm)		4.0			0.1			0.8		2.0	
Temp. range (°C)		9-14			6-12			9-15		11-17	

n.d. not detected

[a] Quantification carried out using a three point standard addition calibration

*Pharmaceutical residues detected at concentrations <LOQ