Determination of Pharmaceuticals in Irish Aquatic Ecosystems: An Evaluation of Occurrence and Risk.

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A thesis submitted for the award of PhD

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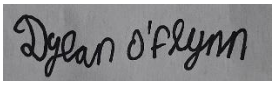
January 2024

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, and 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: 

ID No.: 15336691

Date: 03/01/2024

Acknowledgements:

I would like to acknowledge the funding of this study to both the IRC and the EPA under EMPIRE EVALUATE projects award number: GOIPG/2020/123 and EMPIRE. I wish to additionally extend my gratitude to Donata and Cara from the EPA for guidance and accommodating me during instrument downtime.

First, I would like to thank my supervisors, Prof. Fiona Regan and Blánaid White. Their dedication, guidance and support throughout this PhD has been invaluable, and I am deeply grateful for all the help they have provided me.

I would also like to thank all the technical staff and the Agilent support team, who have been instrumental in completing this project. Special thanks to Ambrose, Veronica, Vinny, Damien, Mary, Stephan, and John for their fantastic support, be it practical assistance or chats. You have helped me so much, and I am incredibly thankful.

To my lab mates who have become great friends: Mathavan, Imogen, Roberta, Chloe, Brian, Dan, Rachel, Shane, Steph, Krystina, Martin, Asmita, Helana, Jessica and Sean. You have all helped me beyond imagination, and I will cherish our nights out, get-togethers and dinner parties. I would particularly like to thank Mathavan, who has been like a brother to me throughout my PhD. You have helped me unimaginably from our sampling days, coffee breaks, late lab sessions and helped me become an overall better researcher. I cannot thank you enough. Imogen, I am so happy and thankful to have worked with you. You have been a fantastic source of both inspiration and experience. Looking back over our joint frustration over the LC-MS, nearly flooding your car during a sampling session and getting chased by cows, you have genuinely helped me make fantastic memories, and you have helped me keep positive and push forward.

To my friends outside of DCU, Jack, Richard, Rian, Ciaran, Connor, Paul, Marc, Michael, the two Seans, Cathal, Jake, Craig, Charlie, and Jeff. I am so thankful to have friends like you. From fishing, climbing, nights out, camping and trekking in the Kerry mountains, you have ensured that I did not burn out and that I take a break. You always remind me of maintaining a good work-life balance (when possible), and I am so grateful to have you in my life.

I would also like to express my gratitude to the Slaughter family, Fiona, Alan, Maria and Ciaran, for being so welcoming, kind, and supportive, as it has meant the world to me. From the beginning, you have treated me like one of your own, and I am genuinely thankful for that. To my best friend, partner and sampling buddy, Fiona, I cannot begin to thank you enough. Your love, eternally optimistic attitude and encouragement have been the foundation for completing this PhD, and I could not have done it without you. You have stuck by my side through thick and thin, and I will never forget it. From the big things like sampling in Donegal to the small things like reminding me to take breaks and to eat lunch, you never fail to support me. I cannot express how much you have helped me.

Finally, to my parents and sister, Muriel, Val and Gaëlle, I am so thankful and lucky to have such a fantastic family. You have always helped me so much and helped me cool down even when Microsoft Word crashes at 3 a.m. and deletes my whole document. The endless supply of love and cups of tea has helped me every step of the way. Your support means more to me than I can describe. I hope you know that I appreciate every second of your support and hope to do you proud.

Publications and Dissemination

Published papers:

Title: A review of pharmaceutical occurrence and pathways in the aquatic environment in the context of a changing climate and the COVID-19 pandemic

Authors: Dylan O'Flynn, Imogen Hands, Azeez Yusuf, Anne Parle-McDermott, Denise Harold, Thomas Mc Cloughlin, Linda Holland, Jenny Lawler, Blánaid White and Fiona Regan

Oral presentations:

Conference: International Conference on Sustainable Energy Water and Environment Nexus in Desert Climates, virtual conference, 22nd–25th November 2021

Title: Pharmaceuticals in surface waters: An overview of influencing sources and monitoring strategies.

Authors: Dylan O'Flynn, Imogen Hands, Azeez Yusuf, Anne Parle-McDermott, Denise Harold, Thomas Mc Cloughlin, Linda Holland, Jenny Lawler, Blánaid White and Fiona Regan

Conference: Society of Environmental Toxicology and Chemistry (SETAC) Europe, Dublin, Ireland, 30th April – 4th May 2023

Title: Study of the influence of seasonal conditions on the occurrence of pharmaceuticals in Irish surface waters

Authors: Dylan O'Flynn, Imogen Rose Hands, Anne Parle-McDermott, Denise Harold, Thomas Mc Cloughlin, Linda Holland, Stefania Scurtu, Jenny Lawler, Kristyna Mrstna, Blanaid White and Fiona Regan

Poster presentations:

Conference: Society of Environmental Toxicology and Chemistry (SETAC) Europe, Copenhagen, Denmark 15th -19th May 2022

Title: The presence of pharmaceuticals in our surface waters and the development of effective monitoring strategies

Authors: Dylan O'Flynn, Blánaid White, Imogen Hands, Anne Parle-McDermott, Denise Harold, Thomas Mc Cloughlin, Linda Holland, Jenny Lawler, Fiona Regan

Conference: Society of Environmental Toxicology and Chemistry (SETAC) Europe, Virtual Conference, 3rd - 7th May 2020

Title: Life cycle assessment of pharmaceuticals in Irish surface waters

Authors: Dylan O'Flynn, Blánaid White, Jenny Lawler, Fiona Regan

Conference: International conference on Risk Assessment of Pharmaceuticals in the Environment (ICRAPE), Barcelona, Spain, 28th -29th November 2019

Title: Lifecycle Assessment of Pharmaceuticals in Irish Surface Waters

Authors: Dylan O'Flynn, Jenny Lawler, Fiona Regan, Blánaid White

Conference: Symposium for European Freshwater Sciences (SEFS), Virtual Conference, 27th July 2021

Title: An HPLC-UV method for the determination of pharmaceuticals in surface water environments.

Authors: Dylan O'Flynn, Blánaid White, Imogen Hands, Jenny Lawler, Azeez Yusuf, Anne Parle McDermott, Denise Harold, Thomas Mc Cloughlin, Linda Holland, Fiona Regan

Conference: International Conference on Environmental Science & Technology (CEST), Virtual Conference, 1st - 2nd September 2021

Title: Occurrence of Emerging and Watch List pharmaceuticals present in Irish surface waters.

Authors: Dylan O'Flynn, Blánaid White, Jenny Lawler, Fiona Regan

Conference: ENVIRON, Virtual Conference, 20th – 22nd October 2020

Title: Occurrence, sources and fate of pharmaceuticals present in Irish surface waters.

Authors: Dylan O'Flynn, Blánaid White, Jenny Lawler, Fiona Regan

Conference: ENVIRON, Virtual Conference, 16-18th June 2021

Title: Method development for the determination of emerging pharmaceutical contaminants in surface water environments.

Authors: Dylan O'Flynn, Blánaid White, Imogen Hands, Jenny Lawler, Azeez Yusuf, Anne Parle McDermott, Denise Harold, Thomas Mc Cloughlin, Linda Holland, Fiona Regan

Conference: IPSW, Utrecht, Netherlands, 18th - 20th September 2022

Title: Method development for Chemcatcher passive sampling uptake studies for emerging pharmaceuticals

Authors: Dylan O'Flynn, Blánaid White, Stefania Scurtu, Jenny Lawler, Anne Parle-McDermott, Denise Harold, Thomas Mc Cloughlin, Linda Holland, Fiona Regan

Media Coverage:

TV interview: RTE 9 o'clock news, Date: 17/05/2023

Online article:

Irish examiner (https://www.irishexaminer.com/news/arid-41141032.html), Date: 17/05/2023

buzz.ie (https://www.buzz.ie/news/irish-news/water-pollution-drugs-rivers-hazardous-30013175), Date: 18/05/2023

RTE website (https://www.rte.ie/news/ireland/2023/0517/1384210-waste-water-treatment/), Date: 18/05/2023

Today FM (https://www.todayfm.com/podcasts/the-last-word-with-mattcooper/antidepressants-found-in-irish-rivers), Date: 18/05/2023

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Table of Contents

List of Abbreviations:

API	Active pharmaceutical ingredient
BCF	Bioaccumulation factor
E1	Estrone
E2	17 β -estradiol
EE2	17 α -ethinylestradiol
ERA	Environmental risk assessment
EU	European Union
WWTP	Wastewater treatment plant
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
OTC	Over the counter
MOA	Mode of action
IPCC	Intergovernmental panel on climate change
OECD	Organisation for Economic Co-operation and Development
WFD	Water Framework Directive
CECs	Chemicals/contaminants of emerging concern
EQS	environmental quality standards
CAS	Conventional activated sludge
RBMP	River basin management plan
AMR	Anti-microbial resistance
MeCN	Acetonitrile
MeOH	Methanol
HPLC	High Performance Liquid Chromatography
EMEA	European Medicines Evaluation Agency
UV	Ultraviolet
RQ	Risk Quotient
HRT	Hydraulic retention time
SRT	Solid retention time
CVD	Cardiovascular disease
PAC	Powdered activated carbon
GAC	Granular activated carbon
MRM	Multiple reaction monitoring
dMRM	Dynamic multiple reaction monitoring
CAV	Collision cell accelerator voltage
TIC	Total ion chromatogram
PuLE	Pulverised liquid extraction
SSRI	Selective serotonin reuptake inhibitors

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Thesis overview and author contributions

The fundamental research question guiding this thesis is: To what extent does the presence of pharmaceutical pollution pose a risk to river environments? This was accompanied by the selection and monitoring of pharmaceuticals to provide valuable insights into Irish water quality, which could help to benefit both the academic community and legislators. Each chapter of this thesis aims to address this core research question and is summarized below.

Chapter 1:

Chapter 1 provides a critical literature review examining the passage of pharmaceuticals from manufacturing to their impact on environmental endpoints. Additionally this work identifies 17 pharmaceuticals of interest, informing successive chapters on method development and environmental monitoring. Introduction was adapted from a published manuscript: O'Flynn, Dylan, Jenny Lawler, Azeez Yusuf, Anne Parle McDermott, Denise Harold, Thomas Mc Cloughlin, Linda Holland, Fiona Regan, and Blánaid White. 'A Review of Pharmaceutical Occurrence and Pathways in the Aquatic Environment in the Context of a Changing Climate and the COVID-19 Pandemic'. Analytical Methods 13, no. 5 (11 February 2021): 575–94. https://doi.org/10.1039/D0AY02098B. Research and writing of the literature review was completed by Dylan O'Flynn. Proofing of the manuscript was performed by Dr. Jenny Lawler, Dr. Azeez Yusuf, Dr. Anne Parle-McDermott, Dr. Denise Harold, Dr. Thomas Mc Cloughlin, Dr. Linda Holland, Prof. Blanaid White and Prof. Fiona Regan.

Chapter 2:

Chapter 2 shows the development, optimization and validation of analytical methods for the determination of emerging pharmaceuticals in complex river water matrices. The developed methods are used in Chapters 3, 4 and 5 for quantitative and qualitative analysis. Experimental work, research, analysis and writing of this chapter was completed by Dylan O'Flynn. Proofing of the document was performed by Prof. Blanaid White and Prof. Fiona Regan.

Chapter 3:

Chapter 3 details two years of pharmaceutical grab sampling monitoring data from four rivers (Nore, Liffey, Suir and Analee) in Ireland from September 2020 to March 2022, showing the occurrence of pharmaceutical mixtures in key Irish rivers and the potential linkage between health emergencies and environmental presence. Experimental work, research, analysis and writing of this chapter was completed by Dylan O'Flynn. Proofing of the document was performed by Prof. Blanaid White and Prof. Fiona Regan.

Chapter 4:

Chapter four presents the risk assessment through risk quotient analysis of pharmaceutical concentrations detected in Chapter 3, in addition to biological exposure studies. Risk assessment experimental work and biological uptake studies and writing of this chapter was completed by Dylan O'Flynn, with experimental work for chronic exposure studies performed by Kristina Mrstna and Stefania Scurtu. Proofing of the document was performed by Prof. Blanaid White and Prof. Fiona Regan.

Chapter 5:

Chapter 5 investigates the use of a combined monitoring strategy for passive and grab sampling for pharmaceuticals at three locations in the Liffey and five locations in the Donegal catchment. Experimental work, research, analysis and writing of this chapter was completed by Dylan O'Flynn. Proofing of the document was performed by Prof. Blanaid White and Prof. Fiona Regan.

Abstract

Determination of Pharmaceuticals in Irish Aquatic Ecosystems: An Evaluation of Occurrence and Risk.

Dylan O'Flynn

The frequent detection and occurrence of pharmaceuticals in surface waters pose a significant challenge to achieving good status for waterbodies under the Water Framework Directive. Active pharmaceutical ingredients (APIs) are increasingly recognised as contaminants of emerging concern (CECs) due to their biological activity and persistence in aquatic ecosystems.

Although previous studies have shown the presence of pharmaceuticals in Irish rivers, there is still the need for a greater focus on monitoring efforts to better understand the extent and potential environmental impact posed by APIs. Monitoring in the rivers Nore, Suir, Liffey, and Analee between 2020 and 2021 yielded crucial occurrence data that revealed the presence of 15 pharmaceuticals, with seven detected in all samples with concentration ranging from <LOD to 290.25 ng/L. Additionally, the observed increase in O-desmethyl venlafaxine concentrations following the easing of level 5 restrictions indicated that COVID-19 lockdown measures were a contributing factor to its presence in Irish rivers, aligning with studies from the UK and Italy. By integrating risk quotient analysis and leveraging effect-based biomonitoring tools, this study identified the antibiotic sulfamethoxazole and the antidepressant venlafaxine, along with its metabolite (O-desmethyl venlafaxine), as pharmaceuticals posing a high risk to Irish river ecosystems, in particular the Liffey at Lucan.

A thorough examination of passive and grab sampling passive techniques demonstrated the effectiveness of passive sampling in monitoring pharmaceuticals for point source identification. Source identification was achieved through the deployment of several passive samplers at strategic locations (upstream, discharge site and downstream), showing elevated concentrations of several pharmaceuticals at the discharge site. Moreover, this study demonstrated that employing passive samplers for pharmaceutical monitoring in remote locations can help to identify the presence of pharmaceuticals that could remain undetected through traditional grab sampling methods.

This project provided an in-depth investigation of pharmaceutical presence, occurrence and effect within Irish surface water environments, showing the importance of monitoring pharmaceuticals in surface waters and enhancing our understanding of water quality.

Chapter 1

Introduction

The contents of this chapter are adapted from a paper published in Analytical Methods 13, no. 5 (11 February 2021): 575–94. https://doi.org/10.1039/D0AY02098B.

1.1 Pharmaceuticals as an emerging contaminant

Surface water pollution is a topic of great concern as we are beginning to understand the true extent to which we influence our environment and the role that emerging contaminants may play in that context. The European Commission has defined contaminants of emerging concern (CECs) as "*substances that have /the potential to enter the environment and cause adverse ecological and human health effects, but are still largely unregulated and whose fate and potential effects are poorly understood.*".[1] CECs encompass a wide variety of chemical compounds, for example, micro/nanoparticles, pesticides, pharmaceuticals and personal care products, sweeteners, hormones and illicit drugs. A recent OECD workshop highlighted the need for a greater understanding of CECs. Indeed, of the 100,000 chemicals currently in use, only 1-5% of these chemicals have publicly available, albeit often poor quality, toxicity data.[2] Unlike many other environmental contaminants, pharmaceuticals are designed to have a high degree of stability and mediate their biological effect at low concentrations, which, as a consequence, leads to ideal conditions for an environmentally persistent and potentially damaging contaminant.[3,4] The increasing use of pharmaceuticals, coupled with the unknown ecological impact of pre-existing and novel active pharmaceutical ingredients (APIs), has led to increased concern.[5] Direct and indirect effects are exerted at or even below the measured environmental concentrations (MEC) in surface water.[6] Direct effects include the biochemical interaction with receptor molecules (e.g. hormone or enzyme receptors) and disruption of cellular processes, resulting in perturbations in gene expression, intracellular ion concentrations, cellular metabolism and the disruption of the endocrine system. [7] Indirect effects can include the proliferation of antimicrobial-resistance and the bio-accumulation of pharmaceuticals via trophic transfer from invertebrate larvae to predators, which consume

them. However, the risk posed by the transfer of pharmaceuticals through the food chain is not thoroughly understood.[8]

The intentional and unintentional release of APIs to the environment across a variety of point (illegal dumping, industrial wastewater and effluent from hospitals and domestic wastewater treatment plants (WWTPs)) and diffuse sources (runoff from agricultural farms and leaching from domestic septic tanks) leads to widespread contamination by both human and veterinary pharmaceuticals in surface waters across the European Union (EU).[3,9–12] The intentional release includes the purposeful disposal of pharmaceutical waste from manufacturing and improper disposal of unused or expired pharmaceuticals down the sink/toilet, ending up in WWTPs or disposal in a general waste bin, ending up in a landfill site or incineration. The presence of pharmaceuticals found within liquid waste streams of landfill can contain similar or even higher concentrations of medicine than is found in the influent from WWTPs.[13,14] Although lined and properly managed landfills should not affect the watercourse, landfill leachate treated in WWTPs is shown to be a potential source to the overall environmental load.[14–16] Un-intentional release of pharmaceutical medicines encompasses release through excretion and incomplete treatment of industrial effluent.[17,18] WWTPs with conventional activated sludge (CAS) treatment are one of the most common types of WWTPs for urban areas as they offer high removal of suspended solids, nutrients and organic matter at a low cost and ease of operation.[19,20] However, despite these apparent advantages, they are not typically tailored to remove pharmaceuticals or other CECs from wastewater (often as a result of associated prohibitively high costs), which accounts for the high variability of removal efficiencies between CAS WWTPs.[19] As a result, a primary source of pharmaceutical pollution in surface waters originates from effluent water discharged from WWTPs.[3] The continuous release of many APIs is reported to exceed the rate of degradation

in WWTPs and in the environment, which leads to a "pseudo-persistence" in surface waters.[3,4] Furthermore, the efficiency of single dwelling septic tanks has also been shown to significantly contribute to the overall pharmaceutical load, particularly in rural areas.[21]

The Water Framework Directive (WFD) 2000/60/EC is an integrative river basin management plan set up by the EU that commits all member states to achieving good water status.[22] In the case of surface waters, a part of achieving this good water status is determined by the concentration of priority substances in relation to the accepted environmental quality standard (EQS) limits. To determine if an EU-level EQS should be set, Union-wide monitoring data on suspected water pollutants are collected and assessed as part of the WFD Watch List (WL), and if hazards are recognised, they can be promoted to priority substances. As pharmaceuticals move from WL chemicals to designation as a priority substance, more chemicals will need to be monitored when determining a surface water's chemical status. In the 2018 second river basin management plan report, only 38% of EU surface waters were deemed to be of "good chemical status", while 46% did not meet requirements, and 16% were unknown.[23] The wide variety of APIs poses significant challenges for both environmental monitoring and toxicity testing. To address this knowledge gap and minimise environmental risk, existing environmentally relevant APIs must be identified and prioritised.[24] There are approximately 3000 APIs that are used to treat human or veterinary diseases and ailments within the EU.[25] As of 2019, a total of 381 different parent pharmaceutical compounds and 66 metabolites and transformation products have been found in European surface waters.[26] Although many studies have been conducted to determine the concentrations of APIs in surface water, the highest number of APIs are found in countries where monitoring is most frequently conducted, e.g. United Kingdom (UK), Germany, and Spain (Figure 1).[27] This may

suggest that countries with lower detections may have similar levels, but they are not sufficiently investigated.

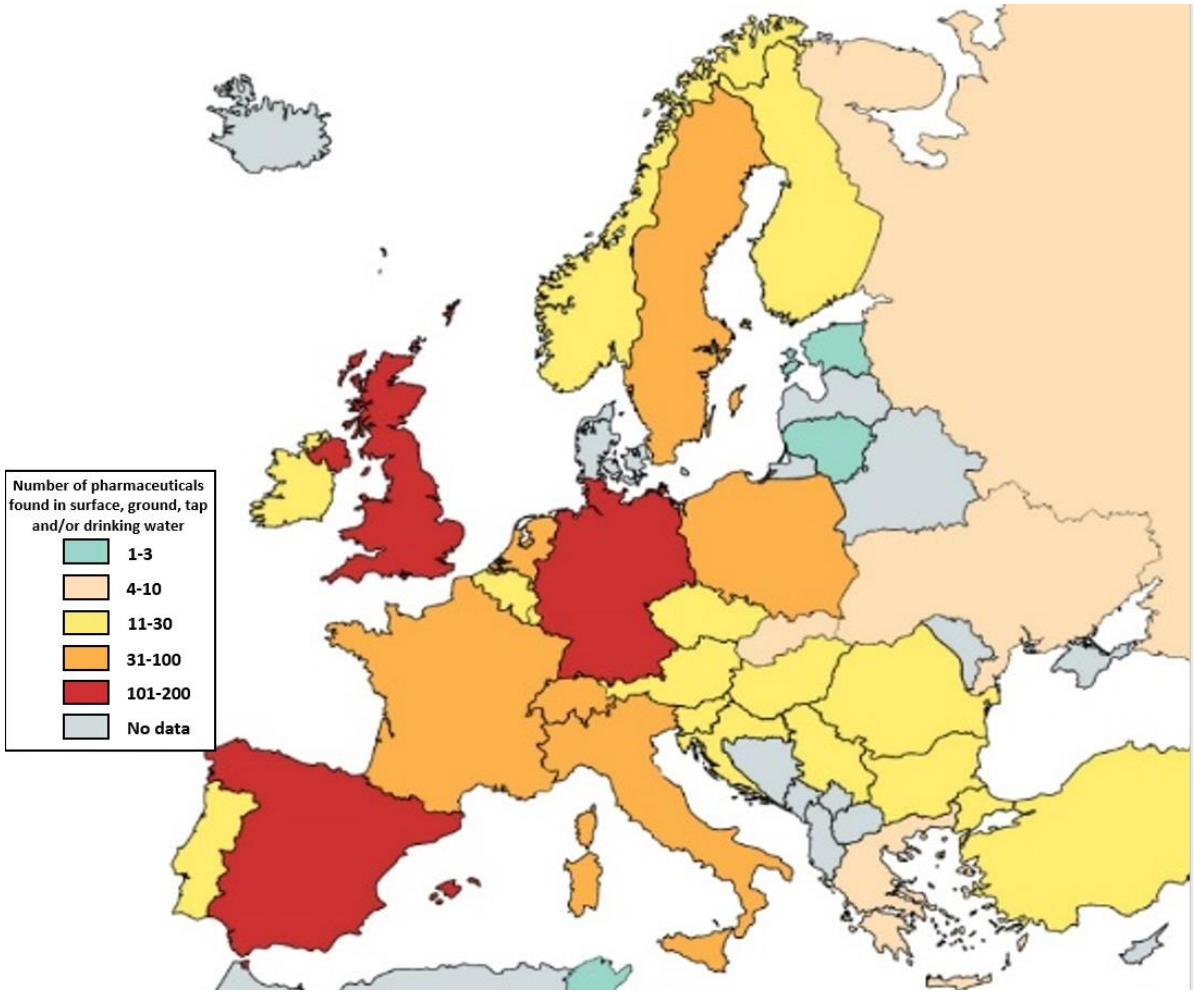


Figure 1. The number of pharmaceuticals found in tap water, drinking water surface and groundwater in the EU as of 2015. Reproduced from ref. [27] with permission from [John Wiley and Sons], copyright [2016].

1.2 Selection of Pharmaceuticals

For the purpose of this review, seventeen target APIs (Table 1) were selected as they were either highlighted as an antimicrobial resistance risk or represent a globally important selection of heavily prescribed and over the counter (OTC) pharmaceuticals. Many of these pharmaceuticals are known to be poorly removed from CAS WWTPs, are persistent in the aquatic environment and have all been included in numerous published prioritisation studies. The selected chemicals are on the WFD WL or are candidates for the updated WL and are

highlighted by the Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances (NORMAN) and Federal Environment Agency (Germany) as chemicals of emerging concern.[28–32] The inclusion of these pharmaceuticals into the WFD WL is necessary in order to accumulate monitoring data so that sufficient measures can be taken to address the potential risks posed by these pharmaceuticals. Although it is important to recognise the significance of metabolites in the evaluation of pharmaceutical pollution, their inclusion presents analytical challenges not least the lack of reference and internal standard compounds for identification and quantification. Additionally, their rate of formation, as well as a variety of formation pathways, can be influenced by a number of environmental factors, which further adds to their variability. Therefore, this study focuses on parent compounds, with the exception of the metabolite O-desmethyl venlafaxine, which is listed on the WFD WL.

Table 1: Description of the representative pharmaceuticals evaluated in this review.

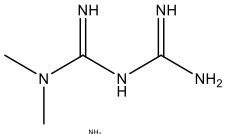
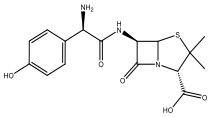
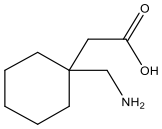
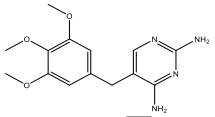
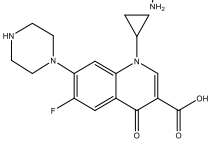
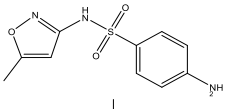
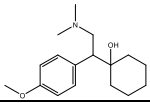
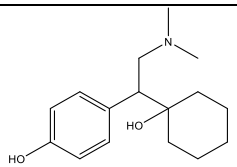
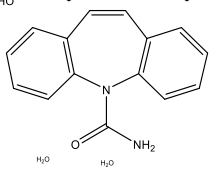
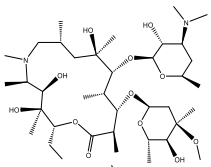
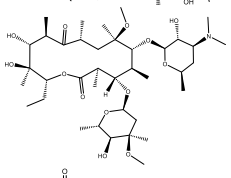
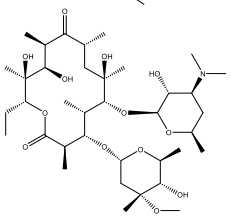
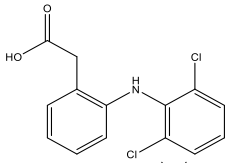
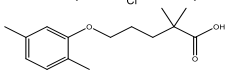
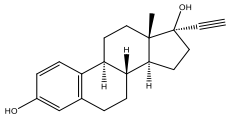
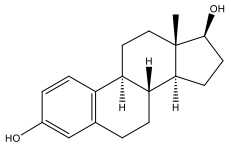
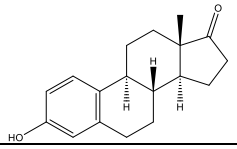
Pharmaceutical	CAS	Da	Structure	Drug class
Metformin	657-24-9	129.101440		Anti-diabetic
Amoxicillin	26787-78-0	365.104553		Antibiotic
Gabapentin	60142-96-3	171.125931		Anticonvulsant
Trimethoprim	738-70-5	290.137878		Antibiotic
Ciprofloxacin	85721-33-1	331.133209		Fluoroquinolone antibiotics
Sulfamethoxazole	723-46-6	253.052109		Sulphonamide antibiotic
Venlafaxine	93413-69-5	277.204193		Antidepressant

Table 1 (continued). Description of the representative pharmaceuticals evaluated in this review

Pharmaceutical	CAS	Da	Structure	Drug class
O-desmethyl- venlafaxine	93413-62-8	263.188538		Antidepressant
Carbamazepine	298-46-4	236.094955		Anticonvulsant
Azithromycin	117772-70-0	748.508545		Macrolide antibiotic
Clarithromycin	81103-11-9	747.476868		Macrolide antibiotic
Erythromycin	114-07-8	733.461243		Macrolide antibiotic
Diclofenac	15307-86-5	295.016693		Anti-inflammatory
Gemfibrozil	25812-30-0	250.156891		Lipid regulators
Ethinylestradiol	57-63-6	296.177643		Synthetic Hormone
Estradiol	50-28-2	272.177643		Hormone
Estrone	53-16-7	270.161987		Hormone

1.3 The lifecycle of pharmaceuticals.

A lifecycle assessment (LCA) is an inclusive tool, which gives the opportunity to measure all inputs, outputs and influencing factors from the creation to the disposal and the associated environmental effects as a result of a process (Figure 2).[33] An LCA additionally expands the discussion on the sources of pharmaceutical pollution by addressing a range of possible inputs, which enables smart decision-making for policymakers and stakeholders. Although a full LCA was not completed, for the context of this study, the pharmaceutical LCA is defined as the investigation of emerging pharmaceuticals with respect to manufacturing, use, waste treatment, occurrence and endpoints within river ecosystems.

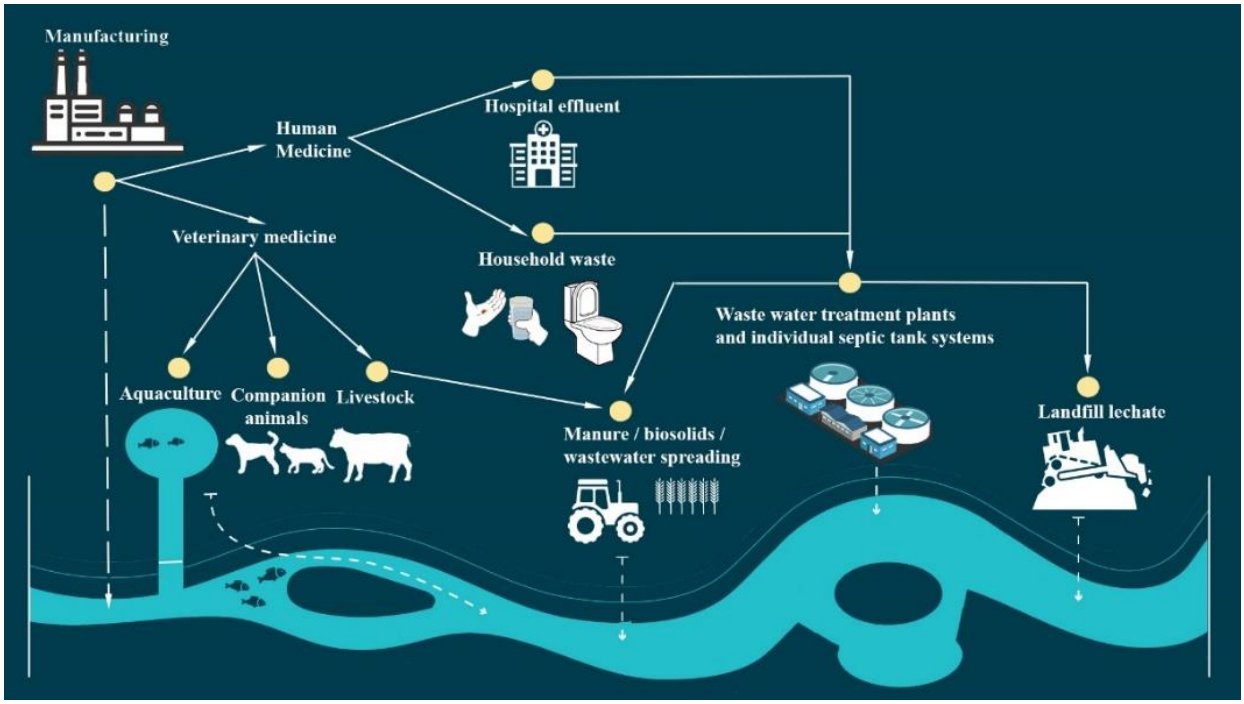


Figure 2. The lifecycle of pharmaceuticals from production to release into the aquatic environment. Created with BioRender.com

Conducting an LCA of pharmaceuticals not only helps track the pathway of pharmaceuticals into the environment, but it additionally helps meet the targets of the Sustainable Development Goals (SDGs). This chapter contributes to Sustainability Goals 3 (good health

and well-being), 6 (Clean Water and Sanitation), 12 (Responsible Consumption and Production) and 14 (Life Below Water) and the WHO One Health approach by highlighting influencing factors that lead to pharmaceutical pollution.[34,35]

1.3.1 Manufacturing and risk assessment

Industry and policymakers are gaining a better understanding of the environmental harm that trace levels of APIs may pose. This enhanced understanding triggers an urgency to innovate in green pharmacy, precision medicine and biological therapies to reduce potential environmental impacts.[3] Pharmaceutical companies are increasingly developing pharmaceutical products to be more environmentally friendly or "benign by design".[36] This is accomplished by reformulating pharmaceuticals to rapidly and totally mineralise upon reaching the environment or by changing how pharmaceuticals are administered (creams, tablets, patches, injection).[6,9,36,37] However, a "benign by design" API is not always feasible as many pharmaceuticals are "discovered" rather than designed. Additionally, changing an API or formulation strategy requires substantial resourcing and disrupts its stability, making the molecule ineffective.[38] Injections and ointments (creams, patches) generally have a low risk in terms of environmental contamination due to the uptake into the organism. However, ointments such as diclofenac and ciprofloxacin may still be released into wastewater streams in their parent form from showering, or they can be directly released into the environment from swimming or bathing in surface water.[39] Oral (e.g. tablets) and parenteral (injections/IV delivery) pharmaceuticals still account for the most substantial proportion of pharmaceuticals used and are utilised to administer all of the pharmaceuticals selected for this thesis. Orally administered pharmaceuticals pose a greater environmental risk as they have a higher

tendency to be excreted from the body as an active substance (parent compounds and metabolites) into wastewater streams.[13]

Market authorisation (MA) is a legislative requirement set out by the European Medical Agency (EMEA) for veterinary pharmaceuticals in 2005 and human pharmaceuticals in 2006.[40,41] If there is an unavoidable danger to the environment, risk mitigation measures can be implemented. However, it will not prevent the release of the pharmaceutical to market. Out of the 3000 APIs used in the EU, only about 500-600 have full Environmental Risk Assessments (ERAs).[42] These requirements do not apply to pharmaceuticals authorised prior to the implementation of the MA requirement, such as diclofenac and sulfamethoxazole. Therefore, this leads to a knowledge gap surrounding the environmental risks posed by legacy drugs.[13,43,44]

Quantitative structure-activity relationship models (QSAR) are predominantly used in drug discovery to establish a mathematical relationship between biological activity and physio-chemical parameters that represent properties such as lipophilicity, shape and electron distribution, significantly influencing drug discovery.[45] However, QSAR can also be used to identify potential risks for pharmaceuticals with minimal ecotoxicity data and to estimate the uptake of pharmaceuticals into invertebrates, algae, and fish in the form of toxicity predictions.[46] However, there are limitations with how representative these predictive models can be as they are based on single components and, therefore, do not consider the presence of pharmaceutical cocktails and their combined effect. Furthermore, in an environmental context, APIs have the capacity to be ionisable and have a broad range of polarities, with additional mechanisms influencing the uptake of pharmaceuticals, such as protein binding, ion trapping and carrier-mediated transport.[47]

ERAs of novel pharmaceuticals and medicinal products are listed as trade secrets and, therefore, remain confidential. This confidentiality further prevents individual pharmaceuticals from being cross-examined even if the pharmaceutical products share the same API.[13] This introduces a lack of transparency, which restricts the public, research bodies and water utilities from investigating potentially hazardous APIs, and it prevents the risk assessment of the true environmental load of a particular API entering the environment.[13,48]

An ERA consists of two phases (Figure 3): Phase I evaluates predicted environmental exposure and the potential to cause environmental harm. Phase I looks at the chemical properties, possible uses of a pharmaceutical, the route of environmental exposure, environmental concentrations and the pollution bioaccumulation toxicity (PBT) factor.[40,48] However, there is no specific guidance on how to implement a PBT screening into a risk-benefit analysis or a risk management procedure to make it eligible for MA.[13] Persistence of pharmaceuticals in the environment stems from a pharmaceutical's ability to resist degradation. Due to the persistence of pharmaceuticals, there is an increased risk of chronic and varied effects.[40] Bioaccumulation is the result of the uptake of pharmaceuticals into living tissue with limited or no excretion or degradation.[3] When the log K_{ow} of a pharmaceutical exceeds four, a bioaccumulation assay must be completed.[49] If a risk is identified, then risk management measures may be required, but compliance is voluntary.[13]

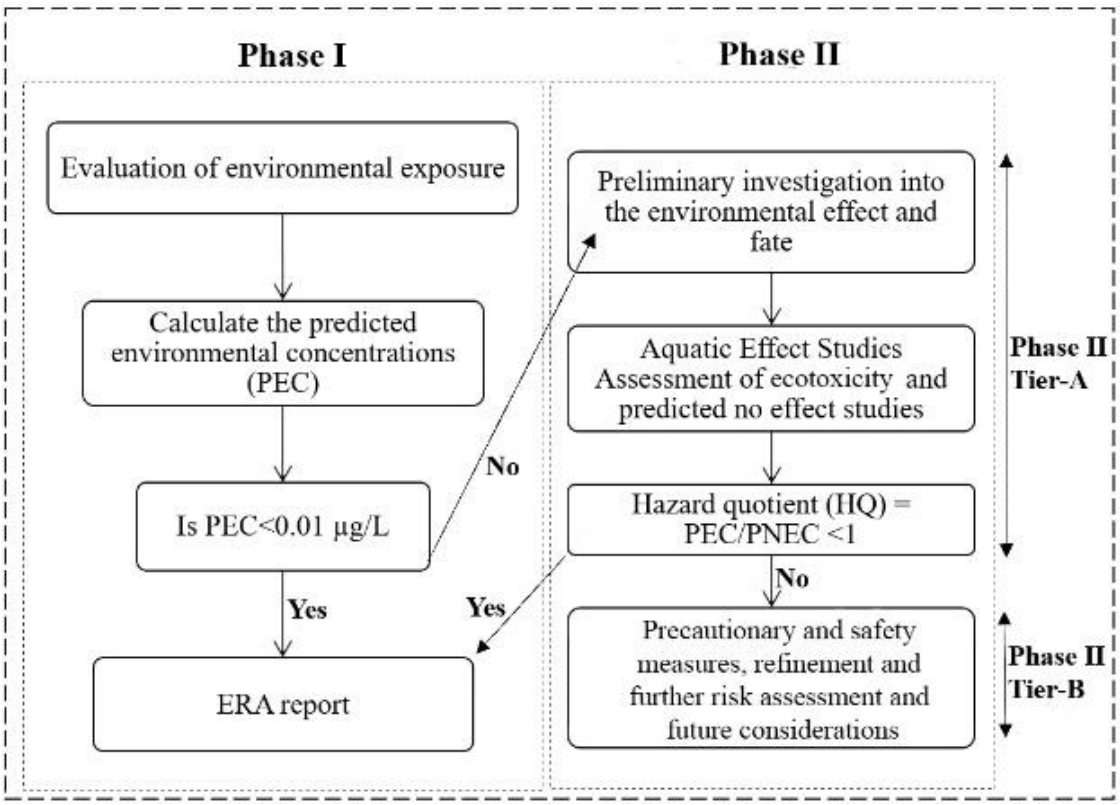


Figure 3. Depiction of the tiered approach of environmental risk assessment of the European Medicines Evaluation Agency (EMEA) during Market Authorisation. Reproduced from ref. [50] with permission from [Elsevier], copyright [2009].

In phase I, there is an assumption that any pharmaceutical that has limited use or low risk of being released into the environment does not need to undergo further testing, and an ERA report is synthesised.[40] Although the pharmaceutical product may be labelled as low risk, this does not take into account the multitude of pharmaceutical products that have the same API, therefore increasing its potential for environmental impact.[13]

Phase II assesses the potential environmental risks the pharmaceutical may pose. Phase II is split into Tier A and Tier B. Tier A screens for the initial risk prediction and provides a base aquatic toxicology and fate assessment by calculating the predicted no-effect concentration in surface water (PNEC).[40] The PNECs listed in the EU Water Framework Directive 2000/60/EC (WFD) are a step in the right direction towards setting water quality standards.[22] Although

chronic toxicity testing is preferred as it represents the pseudo-persistence caused by the continual release of pharmaceuticals into the environment, it imposes significant financial and experimental constraints. [51] A risk quotient (RQ) is generated to estimate the potential risk that a chemical may have in the environment. Pharmaceuticals which fail to pass Tier A (Figure 3.) with an RQ > 1 will undergo further testing in Tier B. Tier B will involve an extended analysis of consumption, metabolism and removal in WWTPs to better reflect the environmental concentrations, fate and effects. This information will help to ascertain if further precautionary and safety measures are needed to preserve and safeguard surface water quality. The multitude of potential sources of APIs and their mixtures and the use of RQ will be a conservative figure, as it does not represent the overall toxicity observed in the environment. A cocktail of pharmaceuticals can be more toxic to an organism in comparison to its individual constituents.[40,52]

1.4 Consumption of pharmaceuticals

Consumption of pharmaceuticals is one of the most significant contributors to an environmental load of API residues in water within OECD regions.[3,13,39] Human and veterinary pharmaceuticals are classified into two categories: Over-The-Counter (OTC) (e.g. diclofenac) and prescription (e.g. venlafaxine, azithromycin, ciprofloxacin, sulfamethoxazole, gemfibrozil).[3] The level of consumption of these pharmaceuticals is the largest influencing factor that will determine the final concentrations in the environment. However, in many European countries, there is limited publicly available data on the consumption of individual pharmaceuticals and in particular, OTC medicine.

Veterinary medicines, similarly to human medicine, aim to maintain animal health by treating and preventing infection, disease, parasites of livestock, companion animals

(sulfamethoxazole/trimethoprim, azithromycin, enrofloxacin which metabolises to ciprofloxacin) and aquaculture (sulphonamides/trimethoprim and enrofloxacin).[53–55] Although veterinary drugs are used in a lesser amount than human pharmaceuticals, they still play a significant role in the overall levels of pharmaceuticals seen in the environment.[13,39]

1.4.1 Factors impacting pharmaceutical consumption

Healthcare heavily relies on the use of pharmaceutical products. With an ever-developing global market and increasing population, its dependence is expected to increase. Emerging markets such as India, China, Brazil, Indonesia and Africa will see the most substantial increase in pharmaceutical consumption as more people have access to pharmaceuticals.[56] However, weak governance and limited resources in low-income and middle-income countries can lead to inappropriate use and an over-dependence on pharmaceuticals.[57] It is essential that legislation is implemented to address the potential risks posed by persistent and toxic pharmaceuticals without jeopardising their safety and effectiveness for either humans or animals.[6]

An older age profile of the population will additionally reflect a higher consumption rate of pharmaceuticals. A report conducted by The Irish Longitudinal Study on Ageing (TILDA) showed that in Ireland, 85% of the population over the age of 65 and 90% over the age of 75 reported taking pharmaceuticals regularly.[58] Furthermore, the same report showed that the consumption of multiple pharmaceuticals (polypharmacy) increases with an older population demographic (one in five over the age of 50 taking five or more pharmaceuticals). The rapidly growing (increase of 41.2 % from 2016 to 2051) and ageing population (an increase from 629,800 persons in 2016 to nearly 1.6 million in 2051) will lead to the increased use and

variety of pharmaceuticals being consumed, thus increasing the variety of pharmaceuticals entering surface water.[9,59]

Environmental pressures (such as natural disasters and the spread of disease created from a changing climate and an increase in tropospheric pollution) have been linked to a wide variety of chronic diseases, such as mental illness, respiratory disease and cardiovascular disease (CVD). With the increase of these chronic diseases, the prescription and consumption of pharmaceuticals increase in kind (Figure 4).[60]

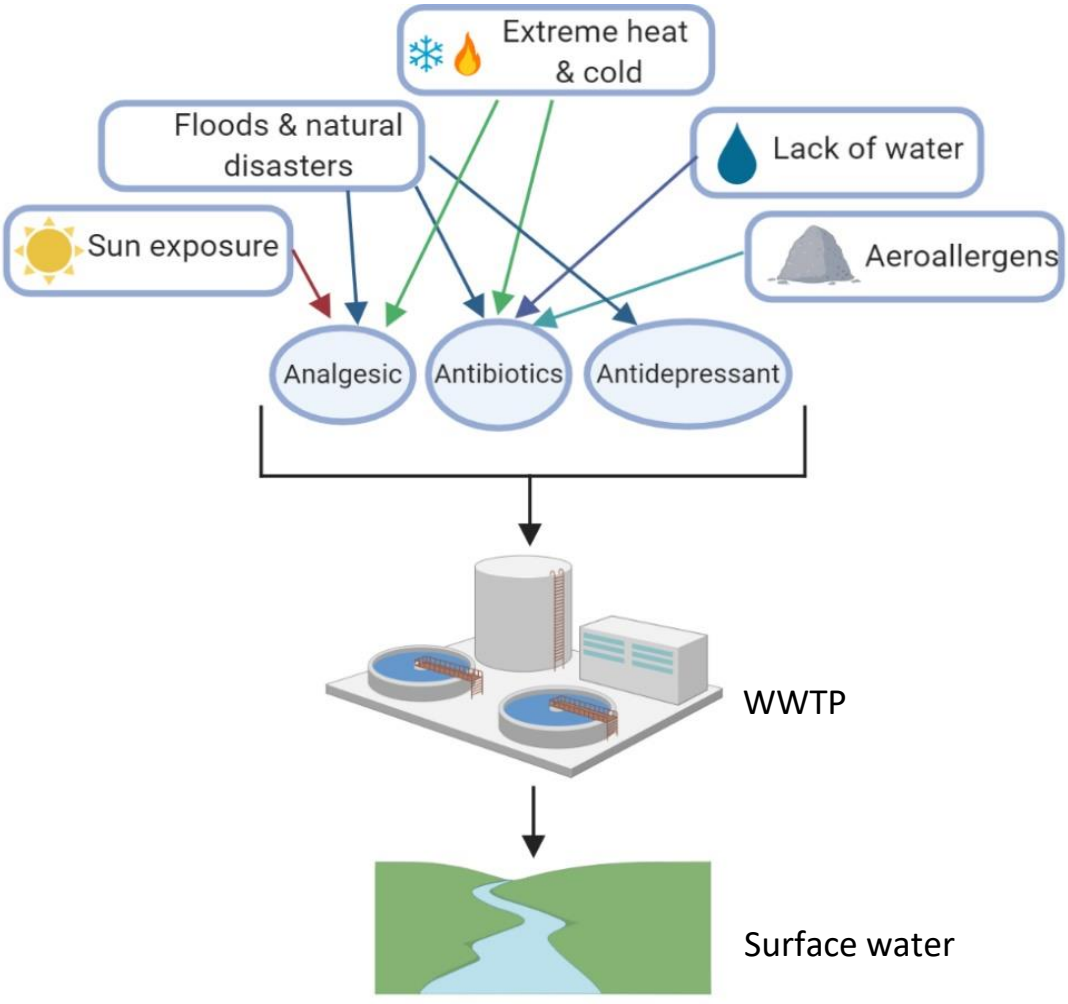


Figure 4. Linkage between environmental pressures and pharmaceutical usage. Created with BioRender.com

The consumption of antimicrobials (including antibiotics) has a marked seasonal trend of decreased usage during the summer and increased usage during the winter, which run in parallel with influenza cases, as well as typical increases in incidences of the common cold.[61,62] A 2017 study conducted by Bielen et al. showed a significant increase in the antibiotic azithromycin during winter and spring.[63] During viral outbreaks, medical health professionals widely prescribed antibiotics (e.g. macrolide antibiotics, quinolones), antiviral drugs and painkillers to treat the virus itself and any secondary symptoms such as pneumonia. This medical response to pandemics may lead to intermittent elevated usage of antibiotics, thus potentially increasing ecotoxicological hazards to surface water environment.[3,64,65]

During the 2009 A[H1N1]pdm09 pandemic, a study conducted by Singer et al. showed an increase in both the concentration and frequency of detection of antibiotics, including sulfamethoxazole, azithromycin and ciprofloxacin in surface waters during the pandemic in comparison to late and inter-pandemic phases.[66] As of yet, there has been a limited number of studies published investigating the effect of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) pandemic on an environmental load of pharmaceuticals present in surface water. However, a study conducted in the UK by Egli et al. in Rivers Hogsmill and Thames monitored the presence and trends of 390 contaminants of emerging concern throughout the SARS-CoV2 pandemic (2019 to 2021).[67] In this study, it was observed that during lockdowns, when movement restrictions were enforced, environmental concentrations of pharmaceuticals such as antidepressants decreased. However, these concentrations increased significantly as restrictions were lifted. While other pharmaceuticals, such as diclofenac, were found to increase significantly during lockdown periods. In addition to viral outbreaks, the obesity epidemic and increase in CVD caused by

the adoption of the "western lifestyle" has led to a global increase in the consumption of fibrate drugs such as Gemfibrozil.[68]

The lack of public knowledge surrounding the appropriate disposal of unused pharmaceuticals can lead to an increased risk of environmental exposure. Roughly 50%-90% of used pharmaceuticals dispensed in the EU are collected via take-back schemes in pharmacies, although some member states do not have take-back schemes in place.[69] Furthermore, stockpiling pharmaceuticals is a common practice in many countries, with a study conducted in Ireland by Vellingaa et al., 2014 showing that 88% out of 398 respondents reported keeping unused drugs.[17] Stockpiling pharmaceuticals for a later date can lead to the collection of expired pharmaceuticals.[70] This indicates a significant public knowledge gap surrounding the disposal of remaining unused medicines.

The degree at which a pharmaceutical is metabolised can significantly vary, with 30-90% of pharmaceuticals not being metabolised at all; this leads to the excretion of unchanged parent ions and pharmaceutical residues through faeces and urine, which end up in WWTPs and subsequently surface waters.[3,6,7,71] The presence of these metabolites in surface water can be transformed back to their parent compound through microorganisms.[72] Additionally, pharmaceutical breakdown and transformation during the transport of APIs through sewage pipes and during treatment in WWTPs can release pharmaceutical residues and lead to their presence in the environment.[7] For example, the metabolism of the commonly used veterinary pharmaceutical enrofloxacin has led to the detection of its metabolite ciprofloxacin in animal waste streams.[73] Furthermore, the metabolite O-desmethyl venlafaxine has been detected in surface waters at higher concentrations than its parent molecule, venlafaxine. A study conducted by López-Serna et al. (2012) in the Ebro River basin (Spain) found that

transformation products and metabolites were present in similar concentrations of their parent compound (representing 30-50 % of the total load of pharmaceuticals). [74] This raises serious concerns as there are a limited number of studies on the occurrence, fate and toxicity of transformation products and metabolites while being equally or more persistent and/or toxic than their parent compounds.[75,76]

Veterinary pharmaceuticals and their residues can enter the aquatic environment through direct and indirect pathways. Direct pathways include: 1. direct excretion from livestock and companion animals; 2. direct application into surface water from aquaculture; 3. direct release of wastewater from segregated aquaculture into surface water. Indirect pathways include: 1. leaching, drainage and runoff of urine and manure from agricultural land; 2. the release of incomplete treatment of wastewater from abattoirs, dairy industry and segregated aquaculture from WWTPs and topical treatments wash-off.[41,48,70,77–81]

As many pharmaceuticals are polar, non-volatile and relatively resistant to biodegradation, removal efficiencies for both primary and secondary treatment are not often sufficiently effective.[82] A list of the target pharmaceuticals included in this review and their removal efficiencies in activated sludge-type WWTPs can be found in Table 2. The removal efficiency of CAS WWTPs will significantly vary depending on the individual properties of the API (solid-water distribution coefficient (K_d) and ability to biodegrade), biomass concentration, hydraulic retention time (HRT), the solid retention time (SRT), redox conditions, pH and temperature.[19,20,70,83] Furthermore, the excretion of glucuronide metabolites after human metabolism will additionally end up in WWTPs. However, the cleavage of these glucuronide metabolites can convert back to their original form, increasing their overall concentration,

which leads to negative removal efficiency, as seen with diclofenac and venlafaxine (Table 2).[84]

K_d drives the sorption of APIs onto biomass, which is essential for the removal of pharmaceuticals in WWTPs. For this purpose, K_d is a ratio between the concentration of pharmaceuticals in the solids relative to the concentration of pharmaceuticals present in the aqueous phase. K_d is driven by hydrophobic interactions (K_{ow}) and electrostatic interactions (pK_a). K_{ow} determines the sorption of pharmaceuticals onto sludge over residing in the aqueous phase. A $\log K_{ow}$ below 2.5 results in low sorption potential (e.g. sulfamethoxazole, ciprofloxacin), a $\log K_{ow}$ between 2.5 and 4 signifies medium sorption potential (e.g. venlafaxine), and a $\log K_{ow}$ of greater than 4 shows a high adsorption potential (e.g. azithromycin, diclofenac and gemfibrozil).

Table 2: The removal efficiency of pharmaceuticals during the activated sludge treatment process.

Pharmaceutical	Log K_{ow} [85]	Excretion of parent molecule (%) [86–89]	Removal efficiency in activated sludge (%)	Reference
Azithromycin	4.02	12	7.6-79	90
Erythromycin	3.06	25	0-75	91,92
Clarithromycin	3.16	58	0-83, 8-73.8	91,93
Sulfamethoxazole	0.48	30	-138-99	84,94
Ciprofloxacin	0.28	70	0-96	91,93,95
Gemfibrozil	4.7	<2	0-75	91,94,96
Diclofenac	4.51	15	−143-80	93–96
Venlafaxine	3.2	5	7.7-56	84,97,98
17-α ethinylestradiol	3.67	40	44.1, 95	99,100
17-β estradiol	4.01	30	63.1, 99	99,100
Estrone	3.13	-	100, 99	99,100
Desvenlafaxine	2.72	-	40	98
Metformin	-2.6	100	78-99	89
Trimethoprim	0.65	80	40-70, 1.4-85	96,101
Amoxicillin	0.87	60	96, 88-100%	91,93
Carbamazepine	2.47	12	0, -44-7	92,95
Gabapentin	-1.10	100	87.6, 99.5	102,103

Sorption of pharmaceuticals can be improved by increasing concentrations of biomass, increasing the time that wastewater passes through the treatment process (hydraulic

retention time, HRT) and by lengthening the period in which the activated sludge solids or bacteria are maintained in the system (solids retention time - SRT). Increasing biomass will create a larger surface area for APIs to sorb. A longer HRT will increase the contact between the microorganisms and APIs present, thus enhancing treatment efficiency. A longer SRT will promote the sludge separation and the growth of slower-growing microorganisms that are more suited to removing particular pharmaceuticals such as ciprofloxacin and gemfibrozil.[102]

Both pH and temperature additionally play a significant role in the treatment of pharmaceuticals in WWTPs. The antibiotics azithromycin, sulfamethoxazole and ciprofloxacin have pH-dependent characteristics, affecting solubility, hydrophobicity, and hydrophilicity depending on the conditions present.[104] At a neutral pH, gemfibrozil is ionic, which causes it to have a low affinity to adsorb to sludge (a factor which accounts for its poor removal efficiencies in WWTPs).[53,105] The chemical modification of pharmaceuticals through biotransformation is highly dependent on pH and temperature during treatment, as microbes are extremely sensitive to environmental changes, thus affecting the removal efficiency.[106]

As with many other pharmaceuticals, ciprofloxacin, venlafaxine, and diclofenac are resistant to microbial degradation due to their stable chemical structure.[107–109] The use of tertiary treatment for wastewater treatment or for drinking water treatment provides the most robust removal of pharmaceuticals.[110] However, the increased efficiency of advanced treatment methods comes at a significant capital and operational cost that may be financially unsustainable for WWTPs, as it requires high-energy consumption for its relative efficiency and efficacy.[9] Some of the more common tertiary treatments include chlorination, advanced oxidation, UV-radiation and activated carbon.

Chlorination, as a tertiary treatment, involves the addition of free chlorine or chlorine dioxide. The chlorine acts as a strong oxidizing agent that can increase the removal/transformation of pharmaceuticals during wastewater treatment. A study conducted in 2011 by Li and Zhang showed that chlorination had shown an 11% increased removal of sulfamethoxazole and ciprofloxacin in comparison to just using a secondary treatment in the same WWTP.[20,111] Additionally, the use of ClO_2 has shown success in the treatment of gemfibrozil and diclofenac.[112]

Advanced oxidation involves the use of oxidising chemicals such as Fe (VI), ozone or hydrogen peroxide to break down pharmaceuticals. Ozonation is one of the most frequently used oxidation methods in post-treatment to remove pharmaceuticals in WWTPs. Pharmaceuticals eliminated through ozonation react directly with ozone or indirectly from the hydroxyl radicals that are formed during the degradation of ozone. Ozonation is commonly used in combination with H_2O_2 to further enhance the formation of hydroxyl radicals. Ozonation is effective across a wide range of pharmaceuticals with removal rates in excess of 90% for azithromycin, sulfamethoxazole, ciprofloxacin, gemfibrozil, diclofenac, venlafaxine.[113–116] However, further research is needed to determine the fate of the toxic by-products created through ozonation.[113]

Fe (VI) oxidation provides a broad spectrum of reaction rate constants during the treatment of pharmaceuticals. Additionally, utilizing Fe (VI) oxidation during wastewater treatment creates distinctive secondary reactive intermediates, which increase its selectivity.[117] Fe (VI) oxidation has previously been shown to have a high degree of removal of antibiotics such as azithromycin.[118] The use of UV radiation for the removal of pharmaceuticals can provide high removal rates. However, the combination of UV photolysis with H_2O_2 can drastically increase

the degradation rates in the same timeframe. A 2012 study conducted by De la Cruz et al. showed that the addition of UV photolysis with H_2O_2 within the same timeframe (10 minutes) had increased removal efficiencies from 48-69% for ciprofloxacin, 0-50% for azithromycin, 18-75% for gemfibrozil and 51-98% for sulfamethoxazole while removal efficiencies increased to 100% after 30 minutes of treatment with UV photolysis with H_2O_2. A further 2009 study by Kim et al. has shown that treatment of sulfamethoxazole and diclofenac with UV-H_2O_2 passing through WWTPs had removal efficiencies nearing 100%. However, in this study, the same parameters showed a low removal for azithromycin.[119] Other matrices such as surface water and ultrapure water have additionally been tested using UV-H_2O_2, showing >99-100% removal for sulfamethoxazole, ciprofloxacin, gemfibrozil and diclofenac.[113] Other combinations seen with UV photolysis, such as UV photolysis and titanium dioxide, provided a 96 and 100% degradation efficiency of the parent compound of diclofenac and venlafaxine, respectively.[120] Furthermore, a study by Batchu et al., 2014 recommended the addition of germicidal lamps with UV at 254 nm as an appropriate method to reduce the levels of sulphonamides passing through WWTPs.[121]

The use of granular activated carbon (GAC) and powdered activated carbon (PAC) can provide an effective alternative to improving removal efficiencies. The utilisation of GAC and PAC has been shown to remove sulfamethoxazole, diclofenac and gemfibrozil in excess of 80%. However, this is dependent on the presence of natural organic matter, which competes for binding sites.[122,123] PAC is a useful treatment strategy to employ because it can be seasonally added to the treatment process during periods of greater risk caused by low flow.[122] The sequestration of pharmaceuticals into the activated carbon does, however, pose a challenge. The solid contaminated GAC and PAC waste generated from this treatment process must be

incinerated to remove all traces of pharmaceuticals. If the solid waste is disposed of in landfills, the contaminated leachate will return back to the WWTP to be treated again.

1.5 Factors influencing environmental concentrations

Behaviour and environmental presence depend on geographical location, proximity to a WWTP, season, local administration practices (ease of disposal) and environmental factors (temperature, rainfall, sunlight hours and humidity).[3,13,107] The intergovernmental panel on climate change (IPCC) has presented various scenarios on how climate change will increase temperature and humidity (increasing degradation rates and reducing dilution) and increase precipitation (increasing the dilution rates in rivers).[3,124] Increase in precipitation may additionally lead to the mobilisation of pharmaceuticals into surface waters from surrounding soil and runoff from agricultural land.[3]

During winter months, there is an increased transport of pharmaceuticals into surface water as precipitation increases can cause storm water to bypass WWTPs directly into surface water. However, the expectation is that this is offset by the higher dilution rates within the receiving rivers. Furthermore, the reduction in thermal and photo-degradation may lead to pharmaceuticals persisting longer in the winter.[125] A study conducted by Lacey et al. 2012 showed during November to February, there was a significant decrease in pharmaceuticals detected. This was accounted for by the higher plant throughput, reduced ambient temperatures and higher dilution rates from increased rainfall.[82]

The decreased precipitation during summer, also exacerbated by climate change, may increase API concentrations in rivers due to decreased dilution. This is particularly relevant for countries which are expected to have dryer summers, causing more frequent low-flow events (Spain, France, Italy and Portugal).[107,126]

Cloud cover can have an influencing role in the degradation of pharmaceuticals in the environment. The photo-degradation of sulfamethoxazole gives a half-life between one to nine days in the environment.[121] However, it may persist much longer due to increased cloud cover (for example, Irish skies are entirely covered by clouds for over 50% of the year).[121,127]

1.6 Impact of pharmaceuticals on human health

Human exposure to pharmaceuticals comes from direct (actively taking medicine) and indirect (environmental exposure) pathways. The routes of human exposure to pharmaceuticals from environmental pathways are well understood, with the main routes coming from the consumption of contaminated food and drinking water. However, exposure to pharmaceuticals may additionally come from soils, dust and exposure to contaminated surface/coastal waters from swimming (Figure 5).[128] The incomplete removal of pharmaceuticals during drinking water treatment and their frequent detection in surface waters has resulted in a wide variety of APIs being found in drinking waters globally. Although there are many trace levels of pharmaceuticals being found in drinking water, the concentrations detected are unlikely to pose a considerable hazard to human health individually, even over a lifetime of exposure.[129] However, a study conducted by Bruce et al. investigated nineteen drinking water treatment plants in the United States of America and found trace levels of gemfibrozil, sulfamethoxazole and diclofenac in drinking water at levels suggesting that they may pose a low level of concern.[130,131] Preliminary screening using proxy indicators shows that the level of exposure is low. However, this form of screening does not fully represent the individual particularities of pharmaceutical exposure in drinking water. Thus, there is a knowledge gap surrounding the potential risk associated with chronic, low-level mixtures of pharmaceuticals to vulnerable/sensitive populations.[13]

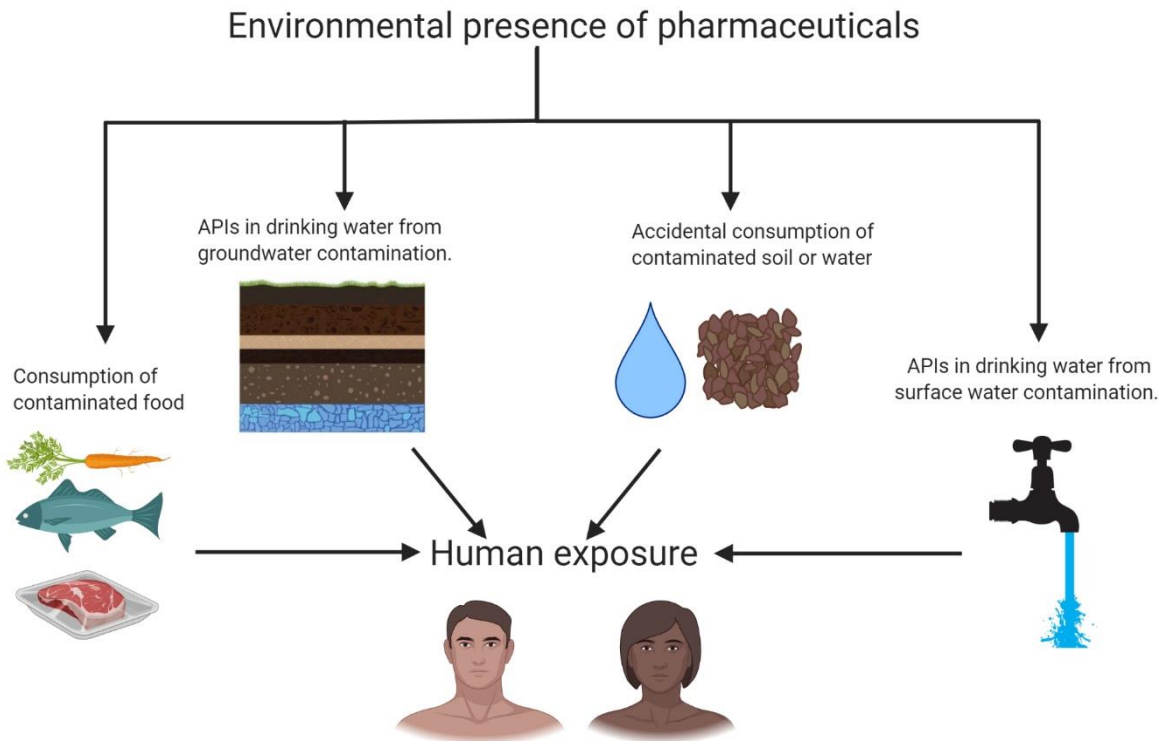


Figure 5. Routes of human exposure to pharmaceuticals.[13] Created with BioRender.com

An indirect consequence of pharmaceutical exposure for human health is associated with exposure to AMR organisms, as AMR poses a severe threat to both animal and human health.[132] The presence of the antibiotics such as sulfamethoxazole and ciprofloxacin in surface water and soil can lead to the development, maintenance and spread of AMR bacteria, fungi and biofilm in natural environments.[6,133–136] Furthermore, AMR bacteria present in fish from aquaculture have been shown to pass this resistance to humans.[137] However, this transfer is not currently fully known, and further investigation is needed.[138]

1.7 Impact of pharmaceuticals on the ecosystem

The increasing global awareness of the presence of pharmaceuticals in the environment has focused on the presence and transfer of APIs in different environmental compartments (biota, sediments and water).[4,13] However, even with the extensive research being conducted on the effects that pharmaceuticals have in aquatic environments, the impact of the long-term

presence of APIs on freshwater organisms is not fully understood.[42] When testing for aquatic toxicity, it is common practice to determine acute and chronic toxicity. Fish, invertebrates, algae, and aquatic plants are the four categories investigated when determining ecotoxicology endpoints.

Pharmaceutical exposure can disrupt physiological processes (e.g., cellular function and biochemical pathways) through toxicity, genotoxicity, and mutagenicity, ultimately affecting mortality.[139] The genetic damage associated with pharmaceutical exposure may potentially cause long-term damage to the populations of aquatic organisms.[139] Furthermore, the effect to the reproductive and endocrine system as a result of pharmaceutical exposure may lead to a population decline due to changes in hormonal balance, reproductive behaviours, growth, and egg and embryo development. For example, pharmaceuticals such as ciprofloxacin, venlafaxine, diclofenac and carbamazepine can potentially cause androgenic and/or estrogenic effects.[139]

Metformin, a medication used for treating type 2 diabetes, is one of the most frequently detected pharmaceuticals in aquatic environments as a result of its incomplete metabolism.[140] Metformin has been identified as an emerging contaminant due to its potential to influence the endocrine system, morphology, size, mobility, and reproductive capabilities of aquatic organisms.[140] For example, a study conducted by Godoy et al., which exposed metformin to *Daphnia similis,* observed that metformin had an effect on reproduction. However, the same study noted that at current environmental levels, metformin was not expected not pose a risk to aquatic organisms. [141]

The mechanisms by which carbamazepine, an antiepileptic pharmaceutical, may affect aquatic organisms is not fully understood. However, acute studies have shown that exposing

carbamazepine to zebrafish, *Daphnia rerio,* and *Hydra circumcincta* effected morphology.[142] Additionally, chronic exposure studies had also identified abnormal sperm production in *Daphnia rerio*, with effects observed on embryo development when in combination with acetaminophen.[142]

The presence of antibiotics in wastewater and surface waters has been shown to promote the selection of antibiotic-resistant genes, thus posing an environmental and human health risk.[143] To combat antimicrobial-resistant (AMR) bacteria, the EU have created the European One Health Action Plan and included antibiotics in the WFD to prioritise and improve our knowledge of the occurrence and persistence of antimicrobials in the environment.[3,32,144]

A 2020 study conducted by Li et al. investigated the potential toxicity of azithromycin to *Chlorella pyrenoidosa* (*C. pyrenoidosa*) and *Daphnia magna* (*D. magna*) via aqueous phase exposure and food phase exposure. In this study, azithromycin had inhibited digestive enzymes and was shown to cause oxidative stress within *D. magna*. This led to an alteration in feeding behaviour. Furthermore, azithromycin has also been shown to inhibit the growth and accumulation of crude fat, polysaccharides, and total protein content in *C. pyrenoidosa.*[145] Further studies by Fu et al. tested the toxicity of 13 antibiotics for their toxicity towards freshwater green algae. Out of the 13 antibiotics, azithromycin was shown to have the highest toxicity.[146]

Sulfamethoxazole's mode of action involves the inhibition of the folate biosynthetic pathway in bacteria. This mode of action is similar in many photosynthetic organisms, thus causing the inhibition of growth, as seen in *Lemna gibba.*[147,148] Sulfamethoxazole can be classified as highly toxic towards photosynthetic organisms, in particular aquatic plants, algae and cyanobacteria.[149] Furthermore, a 2020 study conducted by Liu et al. showed that the exposure

of sulfamethoxazole to healthy zebrafish larvae had led to an immune and inflammatory response that resulted in a delayed hatchment of embryos with shortened body length.[150] *D. magna* exposed to sulfamethoxazole at environmentally relevant concentrations has been linked with significant reproductive damage by inhibiting gene transcription associated with reproduction.[151]

Pharmaceuticals such as ciprofloxacin and diclofenac at environmentally relevant concentrations have been linked with oxidative stress, and neurotoxicity with diclofenac additionally linked to the onset of genotoxicity in the marine mussel Mytilus galloprovincialis.[152,153] Ciprofloxacin mode of action involves the disruption of DNA reparation and replication in bacteria. Ciprofloxacin has been identified as being very toxic, particularly to organisms such as *Pseudomonas putida*, *Microcystis aeruginosa*, *Synechococcus leopolensis* and *Cyclotella meneghiniana.*[154] A 2012 study conducted by Martins et al. used chronic toxicity assays to show that exposing *D. magna* to low concentrations reduced fecundity, the size of the neonates within the first brood and the number of broods per female. Furthermore, this study concluded that ciprofloxacin is a risk for the most sensitive aquatic ecosystems.[155]

Within the EU, diclofenac is one of the most frequently detected pharmaceuticals in the environment.[27] Diclofenac has been linked to the substantial reduction of Asian vulture populations on the Indian sub-continent as a result of consuming contaminated carcasses.[156,157] As a result, the EU has also shown increasing concern for the potential diclofenac might pose to other necrophagous birds.[48] Furthermore, the concentrations of Diclofenac in European surface waters are commonly greater than 100 ng/L, which is above the PNEC of 50 ng/L. In Germany, concentrations have reached levels of 2550 ng/L, which is

higher than the annual average annual environmental quality standard (AA EQS) threshold concentration (100 ng/L) for diclofenac.[5] Diclofenac has a high biological activity, which can potentially be toxic to non-target organisms.[158] The biotransformation of diclofenac into the reactive intermediate acyl-glucuronides, which can bond with intra and extracellular proteins, has ensuing toxicological results.[159] Diclofenac has been shown to cause oxidative stress and affect carbohydrate and fatty acid metabolism in *C. pyrenoidosa* at low concentrations.[160] It has been linked to reduced growth during the egg phase in Japanese medaka (*Oryzias latipes*) fish, causing a reduction in the ability to hatch and the time required to hatch.[161] Environmentally relevant concentrations of diclofenac have been shown to interfere with the biochemical functions of the rainbow trout (*Oncorhynchus mykiss*), resulting in tissue damage.[162]

The commonly used lipid regulator gemfibrozil is considered a good candidate for the WL as it is considered to be persistent and toxic with possible reproductive and carcinogenic effects on aquatic organisms.[32,163] A study conducted on the blood plasma of goldfish showed that gemfibrozil had over 14 days accumulated over 113 times the concentration that was measured in the surrounding water.[105] Gemfibrozil is reported to be an endocrine disruptor that has been shown to decrease testosterone levels in goldfish by 49%, and it has also been shown to increase oxidative stress in molluscs.[163,164] Gemfibrozil predominantly inhibits CYP2C9, whereas its metabolite Gemfibrozil 1-O-ß-glucuronide is a highly reactive electrophile which has a more significant effect on inhibiting CYP2C8 than its parent molecule.[165] These acyl-glucuronides have the potential to react with the nucleophilic centres in DNA and induce oxidative stress in organisms.[164,166,167] A study by Canesi et al. found that the exposure of gemfibrozil on the bivalve *Mytilus galloprovincialis* significantly affected its

immune and digestive gland function at concentrations commonly found in municipal effluents and surface waters.[168]

Venlafaxine and its metabolite O-desmethyl venlafaxine are prescription serotonin and norepinephrine reuptake inhibitor antidepressants. Venlafaxine is a prodrug, which means it is biologically inactive in its parent form. However, its metabolite, the biologically active drug desvenlafaxine, is classified as being both persistent and toxic in the environment.[73] There are a limited number of studies that look at the toxicological effects of venlafaxine in an environmental setting. However, a study conducted by Huang et al. showed that exposure to venlafaxine significantly increases hyperactivity in *Danio rerio* larvae by roughly 25%.[169] A study conducted by Painter et al. showed that low doses of venlafaxine decreased predator escape responses of *Pimephales promelas.* However, at higher doses, this behaviour was not observed.[170] This result is significant as more environmentally relevant lower doses of antidepressants that are found in surface waters showed more substantial behavioural effects than higher doses.[170] Grabicova et al. observed that Venlafaxine had the ability to accumulate in the brain and liver of rainbow trout (Oncorhynchus mykiss). [171] Venlafaxine was also observed to alter *D. magna* fecundity and behavioural response even at concentrations of ≤ 0.1 µg/L.[172,173] Furthermore, a study by Minguez et al. showed that venlafaxine had decreased the number of offspring in the F0 for *D. magna*. However, the F1 generation developed a drug tolerance limiting. Having successive generations with increased drug tolerance may not reduce the overall population of *D. magna* in an ecosystem. [174] However, as there has not been extensive research into the uptake of pharmaceuticals into *D. magna,* questions can be raised regarding the potential bioaccumulation risk for predators.[171]

1.8 The chemical cocktail

The risk of chronic exposure to an individual pharmaceutical is significant; however, a multi-component mixture of APIs and associated residues can activate multiple biological molecules within an organism.[175] A mixture of APIs in an organism can cause synergistic (the effect of the mixture of APIs is greater than the sum of its components), additive (the effect of the mixture is the sum of the effects from the specific APIs) or antagonistic effects (the mixture of APIs have a lessened effect than the effect of the single compound, e.g. enzyme induction).[175–177] Multiple permutations of chemicals must be tested as the mode of action (MOA), and effects can be unique to a specific chemical cocktail.[178]

Sulfamethoxazole and trimethoprim are commonly prescribed together as co-trimoxazole as they both inhibit different enzymes – trimethoprim affecting dihydrofolate reductase, while sulfamethoxazole targets dihydrofolate synthase, thus increasing their potency.[179] The presence of these antibiotics has provided evidence to suggest a synergistic interaction with primary producers (*S. leopoliensis*). However, further research is needed to confirm true synergism.[179]

A 2020 study by Drzymała et al. on *Aliivibrio fischeri*, *D. magna*, and *Lemna minor* suggested that the combination of sulfamethoxazole and diclofenac had a synergistic or partly additive effect with varying toxicity levels dependent on the test organisms.[180] Additionally, exposure to pharmaceuticals such as diclofenac and sulfamethoxazole has additionally been shown to cause interference in metabolic pathways. This has been linked with adverse health impacts concerning energy production.[151,181]

A study to evaluate the chronic toxicity of the presence of both sulfamethoxazole and ciprofloxacin on marine periphytic algae and bacteria showed the inhibition of the organism's

ability to metabolise carbon sources in a concentration-dependent manner. This change in metabolism indicated a change in the community's biodiversity and/or function.[182]

1.9 Effect Based Biomonitoring

Although there is a substantial amount of published literature on APIs in aquatic environments, there is an inherent difficulty in testing multiple pharmaceuticals and their residues. This has led to an insufficient understanding of the actual effects of pharmaceutical mixtures at environmentally relevant concentrations.[7] For this reason, supplementing Effect Based Methods (EBM) with chemical screening and impact modelling is proposed to help estimate the effect of a complex chemical mixture while keeping in line with the WFD.[183]

EBM is an integrative monitoring approach which combines exposure studies with effect-based trigger values to monitor water quality by determining the probability of adverse effects caused by CECs either individually or in a mixture.[183] EBM can be used to establish an early warning system that can be used to identify and prioritise water bodies that were previously deemed at low risk due to their location, to assess water quality, and to identify pollution hotspots. EBM considers three approaches, looking at ecological methods, biomarkers and bioassays.[184] Ecological methods involve observing the biological organisational levels, such as the population or community and measuring any changes within its structure. The changes in the structure of organisms with higher tolerance to pollutants indicate poorer water quality, as species without this tolerance cannot survive.[185] Biomarkers will measure the biological response/stress at the cellular or individual level in organisms collected from the test environment. The accumulation of several biomarkers can help to provide an early warning as it can detect the presence of harmful chemicals and other environmental pressures at an early stage.[184] Biomarkers are categorised into general and

specific biomarkers. General biomarkers are affected by multiple chemicals and environmental stressors, while specific biomarkers respond to particular substances.

Bioassays comprise of *in-vivo* (acute) and *in-vitro* (chronic) tests. *In-vivo* tests involve the exposure of a whole organism in a laboratory or field environment to determine various types of acute toxicity. Some standard *in-vivo* tests include fish embryo vitality, *D. magna.* immobilisation and algal growth studies. However, when conducting bioassays to investigate the individual or combined effects of pharmaceuticals, *D. magna.* Is typically the preferred choice as they are filter-feeders.[186] Exposing *D. magna.* To an individual or mixture of pharmaceuticals, can help to identify genetic responses, including the upregulation and downregulation of genes. Identifying genetic responses is critical when identifying the impacts of pharmaceutical residues in aquatic ecosystems. A 2023 study by He et al. conducted transcriptome analysis on *D. magna* that were exposed to carbamazepine.[187] This study observed that both F0 and F1 generations had altered genes related to reproduction and toxic metabolism, with the F1 generation showing reduced neonate production. Additionally, a study by O'Rourke et al. investigated pharmaceutical endpoints and patterns in biological responses in *D. magna*.[188] In this study, the individual exposure of *D. magna* to the pharmaceuticals metformin, gabapentin, amoxicillin, trimethoprim and β-estradiol was observed to cause the up regulation of endogenous metabolites, while the *D. magna* exposed to gemfibrozil, sulfamethoxazole and oestrone was observed to down-regulate endogenous metabolites.

In-vitro tests involve the assessment of a chemical's mode of action and measuring the chronic toxicological effects at a cellular/sub-cellular level (Figure 6).[189] *In-vitro* tests provide a significant advantage by limiting ethical complications seen with *in-vivo* tests while

facilitating high throughput analysis, thus reducing downtime during the screening of waterbodies. Furthermore, *In-vitro* studies can highlight both human and aquatic organisms sensitivity to chemical and ecological stressors through the expression of adaptive stress responses (e.g. oxidative stress). However, *in-vitro* tests are limited when addressing higher organisational levels.[189]

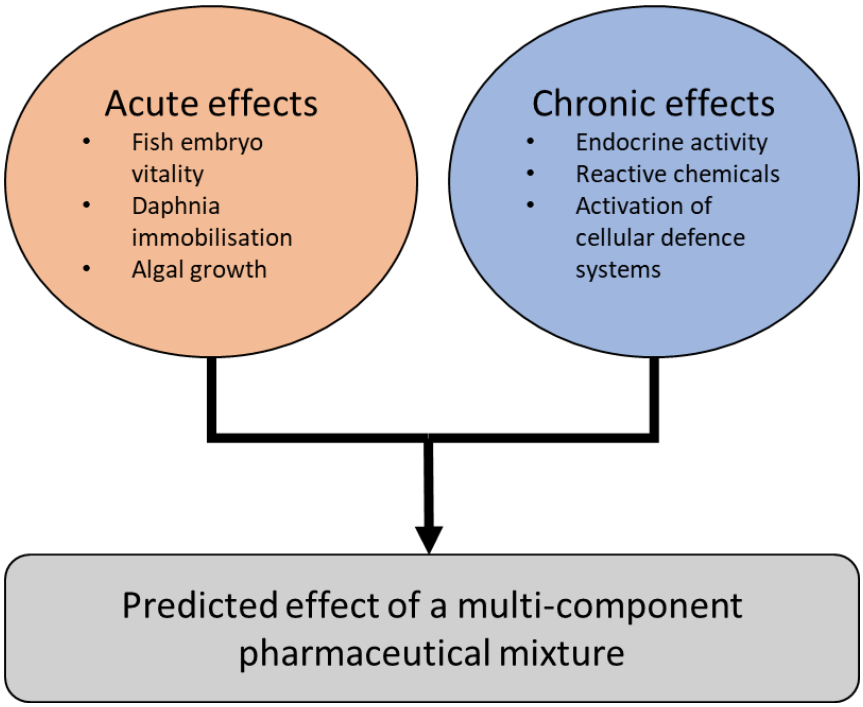


Figure 6. Recommended bioassays used for short term (orange) and long term (blue) effects. This modified work is licenced under a Creative Commons Attribution 4.0 License. It is attributed to Brack et al. 2019. [183]

1.10 Surface water sampling and analysis

Surface water monitoring is an essential practice to identify the presence of contaminants and maintaining regulatory compliance, ensuring environmental and public health. The creation of robust monitoring campaigns can help to identify potential sources and the extent of their impact while propagating the implementation of mitigation measures where necessary. Recent developments in chemical analysis have led to the discovery of a multitude

of trace-level hazardous chemicals and residues (metabolites, transformation products, conjugated products) in surface waters at µg/L to ng/L concentrations (Table 3).

Table 3:Examples of the concentration of pharmaceutical APIs in surface water – rivers/streams/lakes.

Pharmaceutical	Environmental concentrations min-max (ng/L)	Country	Reference
Metformin	13.2	Poland	[190]
Amoxicillin	1.16	Italy	[93]
Gabapentin	168.44-187.3	France	[191,192]
	595	Germany	[193]
Trimethoprim	0.1-61.6	France)	[194]
	5-43	Netherlands)	[195,196]
	20	Romania)	[197]
	0.1-110.4	Spain	[74,198–204]
Ciprofloxacin	15	Romania	[197]
	7.7	Italy	[93]
	17.78	Sweden	[205]
	1-93.3	Spain	[199–201,204]
	88.7	Portugal	[206]
Sulfamethoxazole	1.5-125.8	France	[191,194]
	140-469	Germany	[193,207]
	375	Luxembourg	[208]
	30	Romania	[197]
	0.51-149	Spain	[74,198–201]
	7.6	UK	[209]
Venlafaxine	18	Germany	[210]
	9-119	Finland	[211]
	16.17-17.1	France	[192]
	66.7-159	Portugal	[206]
	0.8-85.8	UK	[212]
	4-45	Spain	[201]
O-desmethyl venlafaxine	27.29-36.4	France	[191,192]
Carbamazepine	24.9-214	Portugal	[206,213]
	3.3-30.1	Ireland	[12]
	0.9- 163	Germany	[214]
Azithromycin	1-71.67	Spain	[74,198–201]
	11.1-29.6	Portugal	[206]
Clarithromycin	1.26-141	Spain	[74,198–202]
	4.6-149	Italy	[93,215]
	4.12	Sweden	[205]
	8.67-26.8	Portugal	[93]
Erythromycin	0.8-174.73	Spain	[198]
	25	Romania	[197]
	21.32	Sweden	[205]
	14	Poland	[190]

Table 3 (continued): Examples of the concentration of pharmaceutical APIs in surface water - rivers/streams/lakes.

Pharmaceutical	Environmental concentrations min-max (ng/L)	Country	Reference
Diclofenac	170-2550	Germany	207,214,216
	28.6-470	Poland	190,217
	830	Luxembourg	208
	22.8-841.5	Czech public	217,218
	0.5-330	Spain	198–200,202,204,219,220
	15-40	Romania	197
	1.16	Italy	93
	32-156	Denmark	221
	10-50	Netherlands	195
	46-700	Finland	211,222
	26.87-30.06	France	192
	38	Portugal	206
Gemfibrozil	6-30	Netherlands	195
	3	Poland	223
	50-78	Italy	224
	0.91-326	Spain	74,198–201,225
Ethinylestradiol	1.16	Hungary	226
	0.14	Spain	227
	20-117	Germany	228
Estradiol	0.449	Hungary	226
	14.1	Italy	229
Estrone	0.05-17	Spain	227
	13-18	Germany	228
	0.1-29.2	France	194

1.10.1 Grab sampling

Regulatory bodies in the European Union and the Irish government have mandated the regular monitoring of pharmaceuticals in water to benefit water quality.[230] The conventional monitoring method involves collecting grab samples, processing through solid phase extraction and quantification of contaminants using chromatographic analysis with mass spectrometry.[231] Grab samples are generally the preferred choice of sampling due to its simplicity, minimal preparation time and that it can be employed during known pollution events to get a snapshot of concentrations at a specific time.[232] However, it is not without its limitations. For continual monitoring, grab sampling is a time-consuming and costly process that can yield results lacking representativeness. This is particularly limiting in surface waters where pharmaceutical concentrations and legislated method detection limits are often in the range of low ng/L to pg/L.[231,233]

1.10.2 Passive sampling

Surface water passive sampling fundamentally relies on the diffusion of target analytes from the surrounding surface water into the receiving phase of the sampler. The diffusion occurs over a period of days to weeks and is driven by the chemical potentials between the sampler and the target analytes, such as electronic and hydrophobic interactions, until equilibrium is reached or sampling is discontinued.[234,235] Due to its capacity for longer deployments, passive sampling can provide a more in-depth understanding of contaminant concentrations that are known to substantially fluctuate.[236]

Passive sampling can be used qualitatively (where samplers are used for suspect screening) or quantitatively (separated into kinetic/integrative sampling and equilibrium sampling (Figure 7)). [237–240]

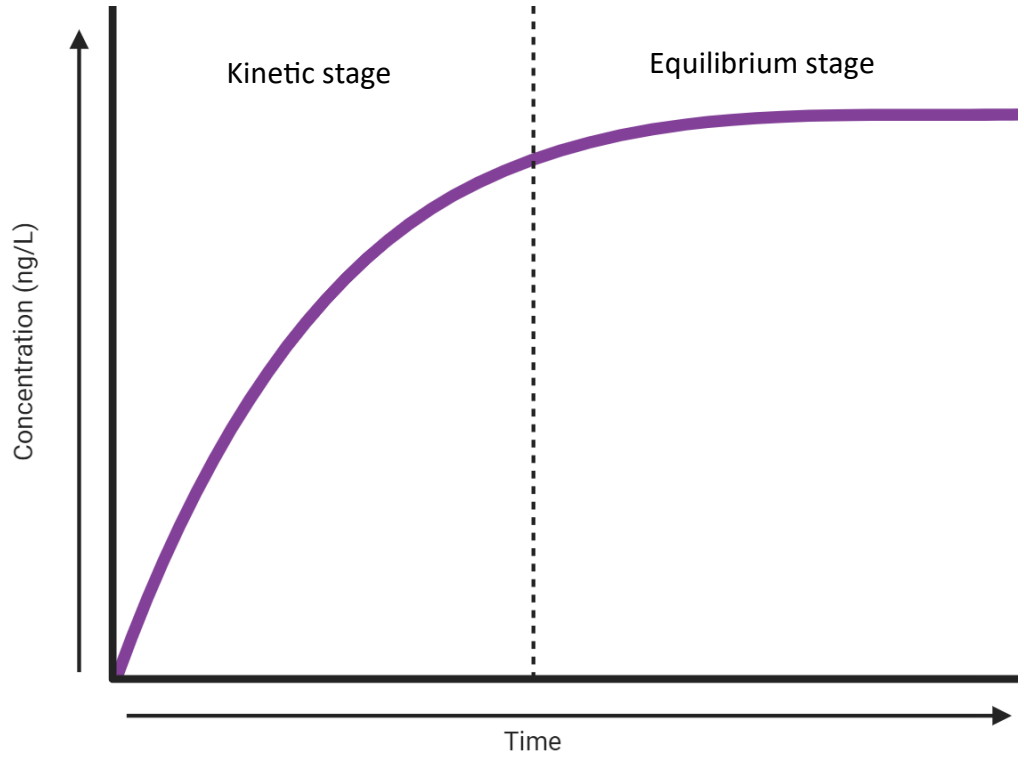


Figure 7. Passive sampling kinetic and equilibrium stages. Created with BioRender.com

The kinetic stage involves the unimpeded passage of target analytes into the sampler, and the mass of the target analyte diffused into the sampler is proportional to the time-weighted average (TWA) concentration in the surface water and estimated by compound-specific sampling rates (Equation 1).[241] The sampling rate (Rs) is generally calculated via laboratory or in situ calibration studies.[241] The determination of the TWA provides a more representative assessment of water quality.[235,241]

$$Concentration_{TWA} = \frac{mass\ of\ analyte_{sampler}}{Sampling\ upkake\ rate \times \text{deployment}\ time} \quad \textit{Equation 1}$$

As the receiving phase reaches equilibrium with the surrounding surface water, the concentrations of target analytes remain constant. At the equilibrium stage, the accumulation of pharmaceuticals in the receiving phase will be determined by the concentration within the surface water and the physiochemical properties of the pharmaceuticals.[234]

Surface water passive sampling of pharmaceuticals is predominantly accomplished using diffusion-limiting membranes with a sorbent (Chemcatcher, POCIS) or by means of analyte diffusion through the diffusive boundary layer into a binding layer (DGT).[242] Several studies have shown the effectiveness of passive sampling for pharmaceuticals (Table 4). [237,243,244]

A study by Rimayi et al. investigated the use of passive sampling with Chemcatcher HLB-L sorbents in combination with time-of-flight mass spectrometry for suspect screening. From this study, 152 medicines/drugs and metabolites were detected, including azithromycin, erythromycin, sulfamethoxazole, venlafaxine, carbamazepine, metformin and diclofenac. However, HLB-Ls was noted as having a limited capacity to extract anionic pharmaceuticals such as diclofenac.[237]

Table 4: Passive sampling devices previously used for the detection of pharmaceuticals in surface water/wastewater and associated sorbents used.

Passive sampling device	Sorbent	Pharmaceuticals	Reference
Chemcatcher	Horizon Atlantic™ HLB-L	Carbamazepine Diclofenac Erythromycin Venlafaxine Trimethoprim 17-α-Ethinylestradiol 17-β-Estradiol Amoxicillin Sulfamethoxazole Clarithromycin Azithromycin Metformin	236,245,246
Chemcatcher	EMPORE disk SDB-RPS	Carbamazepine Diclofenac Sulfamethoxazole Clarithromycin	222,247,248
Ceramic passive samplers	Sepra ZT Sepra SBD-L PoraPak Rxn RP	Carbamazepine Diclofenac Metformin Sulfamethoxazole Venlafaxine	231
POCIS	Oasis® HLB sorbent	Carbamazepine Diclofenac Erythromycin Venlafaxine Trimethoprim E1 E2 EE2 Sulfamethoxazole Erythromycin	236,249
DGT	Oasis® HLB binding gel	Carbamazepine Trimethoprim Sulfamethoxazole Diclofenac	250

Although both POCIS with HLB and Chemcatcher Horizon Atlantic HLB-L sorbents have both been successfully employed for pharmaceutical monitoring, the loose sorbent POCIS has a tendency to move during deployment, leading to variability in uptake rates and impacting robustness. [236,251,252] POCIS additionally faces challenges surrounding the loss of sorbent during deployment, which has been previously shown in the range of 11-51%.[236] However, Chemcatcher utilises a bound receiving phase, which can help

overcome this issue and improve reproducibility.[252] Additionally, Chemcatcher is reported to be advantageous over other passive sampling technologies, such as semipermeable membrane devices (SPMD), as they are less vulnerable to biofouling due to the overlaid membrane, reducing the effect on the sampling rate.[232,253]

1.10.3 Sample pre-treatment

Sample pre-treatment is a critical step to maximise recovery during solid phase extraction (SPE). The presence of suspended solids, colloids and microorganisms within surface water samples can reduce recoveries due to analyte sorption onto particulate matter and the clogging of sorbent within the SPE cartridges. [254] Na_2EDTA is a chelating agent used as a preservative to prevent the chemical change of pharmaceuticals within a sample. Na_2EDTA is shown to improve extraction efficiencies of some pharmaceuticals, e.g. antibiotics, as the Na_2EDTA forms complexes with metal ions, which would otherwise form a complex with the pharmaceuticals. [255–257] An additional preservation technique commonly employed is the acidification of a water sample. The acidification of water samples to pH 2 prior to storage has been shown to prevent microbial degradation and improve the stability of some pharmaceuticals.[88]

1.10.4 Solid phase extraction

SPE was developed as a cheaper and less solvent-intensive alternative to liquid-liquid extractions. It is a commonly employed extraction method for surface water analysis of pharmaceuticals. It provides sample clean-up and pre-concentration of target analytes while reducing matrix interferences and providing greater reproducibility.[256,258] Conventional SPE is most frequently performed through offline cartridges (>70%).

However, other forms of SPE, such as online SPE or disk SPE, have recently been developed but are less frequently used (9% and 3%, respectively).[256]

Conventional SPE is composed of five steps: conditioning, loading, washing, drying, and elution (Figure 8).[254] Conditioning involves wetting the activation of the sorbent with a solvent (e.g. MeOH) and removing this solvent (e.g. flushing with UPW). It is crucial to determine the optimum pH for SPE as it can enhance or decrease recovery efficiency. The acidification of SPE cartridges during conditioning can improve SPE efficiencies for acidic pharmaceuticals. However, depending on the pharmaceutical, it can also prevent the sorption of pharmaceuticals such as metformin to the SPE cartridges. [259]

Loading requires the addition of a selected sample. The sample will pass through the sorbent, and analytes with an affinity to the sorbent will be retained.

The washing step involves passing a weak solvent through the cartridge to reduce the number of sample interferences present in the cartridge. The drying step is essential for surface water extraction as it reduces the volume of water present in the cartridge and the eluting solvent. The presence of excess water in the elution solvent will otherwise impede the dry down of the eluent.[260] Elution utilises a strong solvent such as MeCN or MeOH to elute analytes of interest captured by the cartridge sorbent into a vial for further processing. Further processing often involves the evaporation of this eluent to dryness or near dryness to concentrate the sample to improve method detection limits.

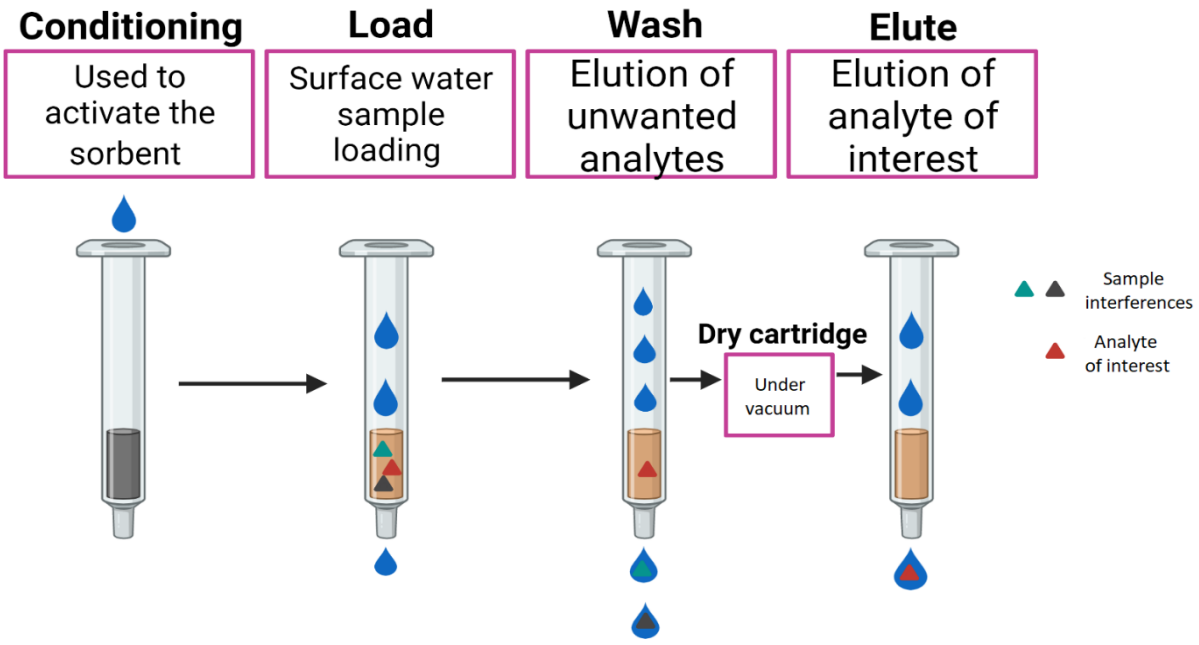


Figure 8. Diagram of the solid phase extraction process and purpose of each step from conditioning, loading sample washing and elution. Created with BioRender.com

OASIS HLB cartridges are the most frequently used sorbent within this category for their ability to target a wide variety of hydrophobic and lipophilic analytes.[256,261] OASIS HLB cartridges are made from a hydrophilic lipophilic balanced polymer (poly(N-vinylpyrrolidone-divinylbenzene)), composing of non-polar and polar regions, which allows for good wettability and interaction between the sorbent and surface water sample, with the ability to target acidic, basic and neutral pharmaceuticals.[256,261,262] This makes its application ideal for the pharmaceuticals selected for this project and has been successfully used in previous studies for the pharmaceuticals selected in this study (Table 5).[263]

Table 5: Reported SPE methods for LC-MS/MS determination of pharmaceuticals in surface water.

Analyte	Cartridge	Condition	Load Volume mL	Wash	Elute	Evaporation & Reconstitution	% Recovery	Ref
Trimethoprim Ciprofloxacin Sulfamethoxazole Azithromycin Erythromycin A Carbamazepine Diclofenac Gemfibrozil Estrone 17α-ethinylestradiol 17β-oestradiol	Oasis HLB	5 mL of MeOH and 5 mL of H_2O	200	H_2O	8 mL of MeOH	Evaporation to dryness under a gentle nitrogen stream, Reconstitution with 1 mL of H_2O /MeOH (75/25 v/v).	37.0-41.4 94.2-100.0 44.3-56.7 98.1-155.7 18.6-36.6 97.1-97.9 100.1-107.1 105.8-113.2 75.8-90.4 60.6-74.8 61.1-74.7	[255]
Azithromycin Erythromycin Trimethoprim Ciprofloxacin Carbamazepine	Oasis HLB	5 mL of MeOH and 5 mL of H_2O	200	-	6 mL of MeOH	Evaporation to dryness under a gentle nitrogen stream at 50 ℃, Reconstitution with 800 µL of methanol/0.1% (v/v) formic H2O (10/90, v/v) mixture.	87-109.9	[264]
Sulfamethoxazole, Diclofenac	Oasis HLB	6 mL of MeOH and 6 mL of H_2O	500	-	6 mL of MeOH → 3 mL of acetone: MeOH: ethyl acetate (2:2:1 v/v/v) → 3 mL of MeOH 0.1 % with ammonia.	Evaporation to 0.1 mL under a gentle nitrogen stream at 35 °C and Reconstitution by adjusting to 1 mL with MeOH	97.21 72.99	[265]
Sulfamethoxazole, Trimethoprim, Ciprofloxacin, Azithromycin Clarithromycin, Amoxicillin Diclofenac	Oasis HLB	3 mL of MeOH and 3 mL of UPW, and 3 mL of pH 3.0 H_2O	1000	3 mL of H_2O	6 mL of MeOH	Evaporation to near dryness under a gentle stream of nitrogen and Reconstitution with (methanol: 0.1% formic acid, 40:60, v/v)	77-116	[266]

Table 5 (continued): Reported SPE methods for LC-MS/MS determination of pharmaceuticals in surface water.

Analyte	Cartridge	Condition	Load Volume mL	Wash	Elute	Evaporation & Reconstitution	% Recovery	Ref
Metformin, Clarithromycin, Erythromycin, Sulfamethoxazole, Trimethoprim, Carbamazepine, Gabapentin	Oasis HLB and Supelco LC-18	4 mL of MeOH and 6 mL of H_2O	200	-	6 mL of 1% NH_4OH in MeOH.	Evaporation to 0.5 mL under a gentle nitrogen stream at 35 °C	75.33- 103.36	[267]

1.10.5 Chromatographic analysis

Liquid chromatography is most frequently used for surface water analysis in the reverse phase due to its versatility and reproducibility for quantitative and qualitative analysis.[254] Reverse phase liquid chromatography (RP-LC) involves the movement of molecules through an aqueous (water) and an organic mobile phase (e.g. acetonitrile (MeCN) or methanol (MeOH)). These molecules are separated by the affinity of the analytes' surface hydrophobicity with the solid stationary phase of a fixed column versus the affinity to the mobile phase. With RP-LC, as the polarity of a molecule decreases, the retention within the column increases, thus leading to a later elution time. Once the analyte has been eluted, it will enter a detector for analysis.[96,254]

Detectors such as ultraviolet (UV) or mass spectrometers (MS) are frequently applied for a wide range of quantitative or qualitative analyses. UV detectors measure the absorption of light at a selected wavelength or range of wavelengths. UV detection provides a cost-effective way to analyse samples of interest. However, this depends on the analytes having a chromophore to absorb the UV light, resulting in reduced selectivity for substances that have similar polarities and chromophores.[268] Mass spectrometers operate by ionising a molecule within a vacuum. This ionised molecule produces characteristic fragmentation ions specific to the molecule. The ratio of the mass to charge (*m/z*) of these parent ions and fragmentation ions are used to identify the analyte of interest, and their relative abundance detail the concentrations detected within a sample. Due to the wide variety of pharmaceutical classes being analysed and their low environmental concentration, creating a method which can analyse samples in the low ng/L concentrations for both the Limit of Detection (LOD) and the Limit of Quantification (LOQ) can be analytically and technically challenging. [232] Reaching low

LODs and LOQs can be challenging due to matrix interference/effect. The matrix effect is the result of the co-elution and chemical interaction of undesirable surface water matrix constituents with target analytes. This matrix effect can decrease the ionisation efficiency of target analytes, affecting the resulting LOD and LOQ.

Internal standards are often used to address this matrix interference. Internal standards should have a similar physiochemical makeup to the analyte of interest but not be in the test sample. Two internal standards frequently used in LC-MS analysis are Stable Isotopically Labelled Analogues (SILAs) and Structural Analogues (SAs). SILAs are an analyte that has an attached Deuterium, 13C or 15N. SILAs are generally preferred over SAs as SILAs are identical to the target analyte. If the SAs internal standard is not adequately structurally similar to the target analyte, the variations of the detector response (caused by ion enhancement/suppression) of an analytes/internal standard can jeopardize an analyte quantitation. However, the availability and costs of SILAs can limit their application; thus, SAs can be used in their stead.[269]

1.11 Conclusion

This chapter investigates 17 pharmaceuticals (azithromycin, erythromycin, clarithromycin, sulfamethoxazole, ciprofloxacin, gemfibrozil, diclofenac, venlafaxine, 17-α ethinylestradiol, 17-β estradiol, estrone, Desvenlafaxine, metformin, trimethoprim, amoxicillin, carbamazepine and gabapentin) to understand their passage from manufacturing to uptake into an organism. Dealing with a variety of disposal methods frequently creates more waste streams, making waste management a more complicated process. Waste treatment poses a unique challenge when considering pharmaceuticals, as higher treatment costs typically accompany targeted treatment processes with increased efficiency. This puts severe financial barriers on how we deal with pharmaceuticals in wastewater and increases the need to consider strategies to reduce APIs entering wastewater streams.

To identify which APIs pose an environmental hazard and therefore need to be prioritised, both novel and targeted monitoring strategies must be developed. However, in the absence of a robust monitoring strategy, the precautionary principle must be used to address the risk of pharmaceutical pollution, as the complexity of risk assessing a multi-component pharmaceutical mixture may underestimate the actual effects.

The persistence, ability to bioaccumulate and toxicity of pharmaceuticals pose a largely unknown threat to surface water environments. Antibiotics are of particular concern due to their ability to encourage the growth of AMR organisms in an environmental setting. Furthermore, environmental factors, obesity, population growth and an ageing population have led to the increased usage of many environmentally relevant pharmaceuticals, such as gemfibrozil, diclofenac and venlafaxine.

The increasing number of pharmaceutical mixtures, limited occurrence data and tremendous diversity of APIs pose a significant challenge to ecotoxicology.[131,189] To address this knowledge gap, a robust monitoring strategy that includes EBM with chemical analysis is advised. The importance of addressing each aspect of the lifecycle of pharmaceuticals which will mitigate release into surface waters has been recognised across multiple published articles. With the projected increased demand for pharmaceuticals due to climate change related impacts and COVID-19, source-directed and end-of-pipe measures must be implemented to educate the broader public on the responsible use and disposal of pharmaceuticals.

1.12 Aims and objectives

The aim of this thesis is to determine the occurrence and risk of pharmaceuticals pharmaceutical pollution in Irish rivers. Additionally, this thesis aims to serve as a proof of concept for integrating effect-based tools and passive sampling for monitoring pharmaceuticals in rivers.

The main objectives are to:

- Develop and optimise analytical methods for pharmaceuticals in environmental monitoring.
- Assess the occurrence of pharmaceutical cocktails in four key Irish rivers, highlighting trends over the course of a two-year sampling campaign.
- Highlight the utility of effect-based tools in determining the effects pharmaceuticals have on aquatic organisms.
- Investigate grab and passive sampling as a monitoring approach for the detection of pharmaceuticals in rivers.

Chapter 2 :

Method development for the detection of pharmaceuticals of emerging concern in environmental samples.

2.1 Introduction

Policy documents such as the Directive 2000/60/EC (2000) Water Framework Directive (WFD) were created to achieve "good ecological potential and good surface water chemical status". [22] A defining part of the WFD is the "watchlist". The watchlist is a policy document aiming to monitor chemicals suspected of causing a substantial risk to the aquatic environment and chemicals with inadequate environmental monitoring/ exposure modelling, for which insufficient data is available to consider their potential inclusion on the priority substance list. The substances are included as a result of the lack of information available (monitoring data) and their potential to pose a significant risk to the aquatic environment (Table 6).[270] However, contaminants of emerging concern (CECs) can be removed from the watchlist in three ways;

1. Under the WFD, the continuous monitoring watchlist substances duration cannot exceed four years (e.g. macrolide antibiotics and oestrogens),

2. Watchlist substances that pose a significant risk may be removed if they qualify for inclusion on the priority substances list. This would involve the designation of acceptable concentrations of these pollutants in the form of environmental quality standards (EQS),

3. Watchlist substances can be removed if a risk assessment can be performed without the addition of further monitoring data. [73,230,271–273]

Pharmaceuticals, particularly antibiotics such as macrolides, trimethoprim, sulfamethoxazole, amoxicillin and ciprofloxacin, have been included in past or current

WFD Watch List based on their toxicity to aquatic organisms and their potential to contribute to the presence of antimicrobial resistance in the environment.

Table 6: Presence/absence of pharmaceuticals on EU WFD watchlist chemicals with associated PNEC values [73,230,271–275]

Analyte of interest	Most recent PNEC (µg/L)	Candidate for 1st watchlist	Candidate for 2nd watchlist	Candidate for 3rd watchlist	Candidate for 4th watch list	Provisional Candidate for 5th watch list
17-alpha-Ethinylestradiol	0.000035	Y	Y	N	N	N
17-beta-Estradiol	0.0004	Y	Y	N	N	N
Estrone	0.0036	Y	Y	N	N	N
Diclofenac	0.05	Y	N	N	N	N
Erythromycin,	0.2	Y	Y	N	N	N
Clarithromycin	0.12	Y	Y	N	N	N
Azithromycin	0.019	Y	Y	N	N	N
Amoxicillin	0.078	N	Y	Y	N	N
Ciprofloxacin	0.089	N	Y	Y	N	N
Trimethoprim	0.05	N	N	Y	Y	Y
Sulfamethoxazole	0.1	N	N	Y	Y	Y
Venlafaxine	0.0061	N	N	Y	Y	Y
O-Desvenlafaxine	0.0061	N	N	Y	Y	Y
Gemfibrozil	0.8519-1.56	N	N	Y	N	Y
Gabapentin	10	N	N	N	N	Y
Metformin	10-160	N	N	N	Y	Y
Carbamazepine	0.5	N	N	N	N	N

Each of the three options outlined above requires data to inform decision making. As such, the lack of available data surrounding the presence of pharmaceuticals in Irish surface water environments poses challenges for maintaining good chemical surface water quality. To address this knowledge gap, it is imperative that highly sensitive analytical techniques and monitoring campaigns are developed and applied. With its application, vital information can be collected, such as wastewater treatment efficiency, identification of contamination sources and changes in pharmaceutical use patterns. This information will help to identify if mitigation measures are needed and ultimately help to protect aquatic ecosystems.

Given that pharmaceutical concentrations in surface waters are frequently detected within the lower ng/L range, it is essential to develop methods with low detection limits. To achieve this high degree of sensitivity on and offline, solid phase extraction (SPE) is often employed as a means for sample cleanup and concentration, while typically, Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) is the preferred analysis method for trace level analysis of polar pharmaceuticals. An example of analysis methods for analytes of interest can be seen in Table 7 below.

Table 7: LC-MS/MS analysis methods reported in literature for the determination of pharmaceuticals in surface water

Location	SPE extraction volume	Analyte	Column	Flow rate (mL/min)	Injection volume (µL)	LOQ (ng/L)	ref
EL lobregat River (Spain)	200 mL	Sulfamethoxazole Trimethoprim Azithromycin Erythromycin Clarithromycin Gemfibrozil Diclofenac Amoxicillin	Acquity BEH C_{18} column (100 × 2.1 mm, 1.7 µm)	0.4	10	1.1 1.1 1 0.2 0.2 4 3 115	[198]
Confidential (Ireland)	100 mL	E1 E2 EE2	InfinityLab Poroshell 120 EC-C_{18} (2.1 x 150 mm, 1.9 µm)	0.35	100	4.94 2.09 0.24	[276]
River Rakkolanjoki, Lake Haapajärvi (Finland)	400 mL	Diclofenac Venlafaxine Carbamazepine	Waters X-bridge analytical column C_{18} (2.1 × 50 mm, 3.5 µm)	0.3	30	10 1 1	[211]
Mijares river and Clot de la Mare de Déu river	50 mL	Diclofenac Carbamazepine Clarithromycin Erythromycin Sulfamethoxazole Trimethoprim Venlafaxine	CORTECS C_{18} analytical column (2.1 x 50 mm, 2.7 µm)	0.4	50	5 10 35 40 20 25 25	[277]

The choice of SPE sorbent material hinges on the analytes' specific chemistries, with analyte polarity playing a pivotal role in SPE cartridge selection. For pharmaceutical

analysis, Oasis HLB cartridges are often used due to their ability to target a wide variety of polar and nonpolar compounds while providing cleaner extracts in comparison to other Oasis SPE sorbents, e.g. MAX and WAX (

Table 8). [278,279] This chapter outlines the development of HPLC-UV and SPE-LC-MS/MS methods for the analysis of pharmaceuticals of emerging concern.

Table 8: Range of logK_{ow} values for pharmaceuticals selected for environmental analysis.

Pharmaceutical	Log K_{ow} [85]
Metformin	- 2.64
Amoxicillin	0.87
Gabapentin	-1.1
Trimethoprim	0.91
O-Desmethylvenlafaxine	2.72
Venlafaxine	3.2
Carbamazepine	2.45
Sulfamethoxazole	0.48
Azithromycin	4.02
Clarithromycin	3.16
Azithromycin	4.02
Ciprofloxacin	0.28
Gemfibrozil	4.7
Diclofenac	4.51
EE2	3.67
E2	4.01
E1	3.2

2.2 Aim and objectives

The aim of this chapter is to optimise and apply liquid chromatographic methods in conjunction with SPE for the determination of pharmaceuticals identified in Chapter 1 (Table 6). Furthermore, this study aims to validate the developed methods for the quantification of the selected pharmaceuticals in river water matrices.

The objectives of this chapter are to:

- Use information gathered in Chapter 1 to provide a foundation for HPLC-UV and LC-MS/MS work.
- Optimise LC-MS/MS method parameters for pharmaceutical analysis.
- Validate LC-MS/MS methods for selected pharmaceuticals in sampled river water matrices.

2.3 Experimental

2.3.1 Reagents and chemicals

HPLC and LC-MS (Honeywell CHROMASOLV™) grade acetonitrile, isopropanol, methanol, and sulphuric acid were purchased from Fisher Scientific (Dublin, Ireland). LC-MS grade dichloromethane, EDTA, dichloro dimethyl silane and Ammonium formate were purchased from Merck Life Sciences (Arklow, Ireland). Formic acid was acquired from TCI EUROPE. 0.45 µm pore size Supelco nylon filter membranes were supplied by Merck Life Sciences (Arklow, Ireland). Ultra-pure water (UPW) (18.2 MΩ cm) was obtained from a PURELAB® Ultra water purification system (Veola, Lab water, and High Wycombe, United Kingdom). Oasis hydrophilic-lipophilic balanced (HLB) 6 cc, 200 mg bed mass, 30 µm particle size and Oasis HLB cartridges (500 mg/6 mL) were acquired from Waters (Milford, Massachusetts, United States). Analytical reference standards of estrone (≥98 %), venlafaxine hydrochloride (≥99), sulfamethoxazole (≥98 %), ciprofloxacin (≥98 %), o-desmethyl venlafaxine and as well as surrogate standards, sulfamethoxazole-^{13}C6, gabapentin-d9, carbamazepine-^{13}C6 and were all obtained from Merck Life Sciences (Arklow, Ireland). Gemfibrozil (≥98 %), metformin, erythromycin (≥98 %), trimethoprim (≥98 %), β-estradiol (≥98 %), carbamazepine (≥98 %), 17α-ethylene estradiol (≥98 %), azithromycin, amoxicillin (≥96%), diclofenac sodium salt (≥98 %) were all obtained from Fisher Scientific (Dublin, Ireland). Clarithromycin was obtained from TCI (Belgium). Azithromycin-d3 was obtained from 2B Scientific Ltd (Oxfordshire, United Kingdom). Gabapentin (≥98 %) and Carbamazepine-D10 were obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Estrone-d4 was

obtained from Insight Biotechnology Limited (Wembley, United Kingdom). Diclofenac-d3 was obtained from Qmx Laboratories Limited (Essex, United Kingdom).

2.3.2 Silanization and glassware pre-treatment

Glassware was silanized by 1. Washing with MeOH: H_2O (50:50), 2. Rinsing with dichloromethane (3 times), 3. Washing with Dimethyldichlorosilanel: dichloromethane (10:90), 4. Rinsing with dichloromethane (3 times), 5. Rinsing with MeOH: UPW (50:50) (3 times), 6. Rinsing with UPW (3 times). Between each use, glassware was cleaned by rinsing glassware with MeOH (3 times), followed by a subsequent rinse with UPW (3 times).

2.3.3 Standard preparation

All standard stock solutions were prepared in silanized glass vials. 1000 mg/L individual pharmaceutical standard stock solutions were prepared from powder form and dissolved in 5 mL of MeOH, with the exception of amoxicillin, which was prepared in UPW. Furthermore, to improve the solubility of ciprofloxacin into MeOH, (>3 µL) formic acid was included to enhance solubility. The rapid degradation of amoxicillin and ciprofloxacin required standards to be prepared at most two days in advance to improve stability.[39] The 1000 mg/L Individual stock solutions were used to make a 1 mg/L pharmaceutical working standard mix in a 10 mL volumetric flask with 95:5 H_2O: ACN.

100 µg/mL individual internal standard stock solutions were prepared in MeOH from powder form, from which a 100 µg/L internal standard working standard mix was made in a 10 mL volumetric flask with 95:5 H_2O: ACN. Standard and internal stock solutions

were stored in a freezer at −20 °C, while working standards were stored in the dark at 4 ˚C before use.

2.3.4 Surface water collection

Surface water samples for HPLC-UV method validation were collected at Griffith Park, Co. Dublin (53°22'11.6"N 6°15'42.0"W). For method validation on the HPLC-UV analysis, 20 L of river water was collected in a Nalgene™ opaque HDPE carboy. Surface water samples for LC-MS/MS method development and sample analysis were collected from 5 locations (Nore, Liffey, Suir and Analee River) across 6 sampling time points from September 2020 to March 2022. Site information and locations are detailed in Chapter 3. x2 1 L samples were collected in 1L Nalgene™ Amber HDPE bottles. Surface water environmental conditions (temperature, pH, turbidity, conductivity and Dissolved Oxygen (DO)) were measured utilizing a YSI EXO3 Multiparameter Water Quality Sonde.

Prior to sample collection, sampling bottles were pre-rinsed in triplicate with MeOH, UPW and surface water at the sample site. During transportation, samples were stored in a cooler with ice. Collected samples were brought to a pH of 3 and subsequently stored at -20 ˚C until extraction to reduce biological activity and chemical reactions.

2.3.5 Sample pretreatment and Solid Phase Extraction

2.3.5.1 Sample pretreatment

Prior to extraction, samples were thawed in a refrigerator (4 °C) overnight, filtered through a Nalgene rapidflow™ 0.45 µm aPES membrane filter (Thermofisher) to remove particulate matter and spiked with 0.1M EDTA to a final conc. of 0.1% and internal standards. To ensure sample homogeneity for method validation for LC-MS/MS analysis,

a composite sample was prepared by placing 100 mL of grab sample from each location and sampling timepoint into a silanized amber Winchester glass (2.5 L). However, the samples for HPLC-UV analysis were already mixed due to their collection in a 25 L carboy at one location. Composite matrix-matched calibration standards were prepared in individual 1 L (HPLC-UV)/100 mL (LC-MS/MS) Nalgene bottles prior to Solid Phase Extraction (SPE). Calibration was performed using the matrix composite by adding the necessary concentrations of pharmaceutical standard mix prior to extraction. The preliminary HPLC-UV test calibration range was from 57 – 3000 µg/L (in final 1 mL extract), and for LC-MS/MS calibration range was 0 – 1000 or 2000 ng/L (in final 1 mL extract). Similarly, for LC-MS/MS analysis, the internal standard mix was added prior to SPE in the 100 mL matrix validation and river water samples to a constant concentration of 700 ng/L (in the final 1 mL reconstituted extract).

2.3.5.2 Solid phase extraction

SPE for HPLC-UV and LC-MS/MS analysis was adapted from previous work by Rapp Wright et al., where the sample load volume was changed (1 L for HPLC-UV and 100 mL for LC-MS/MS).[276] The SPE method was carried out on a vacuum manifold system (Supelco, Bellefonte, PA, USA). Oasis HLB cartridges (200 mg, 6 mL barrel, 30 µm, Hertfordshire, UK) were conditioned with 4 mL of MeOH followed by 4 mL of UPW. After conditioning, samples were loaded (1 L for HPLC-UV and 100 mL for LC-MS/MS) at a rate where discrete drops of liquid eluted from the cartridge were visible. The cartridges were washed using 4 mL of UPW and vacuum-dried for 20 min. SPE cartridges containing the extracted sample were kept at -20°C until the time of elution and analysis or eluted immediately following the drying step.

A 5 mL solution of ACN (0.1% formic acid) was used as the eluent, and extracts were placed into a 10 mL silanized amber glass vials. Extracts were carefully evaporated until complete dryness using a gentle stream of nitrogen at room temperature. The resulting residue was reconstituted to 1 mL with 95:5 UPW/acetonitrile (0.1% formic acid), vortexed for 1 min, sonicated for 10 min and vortexed again for 1 min to ensure thorough mixing (Figure 9).

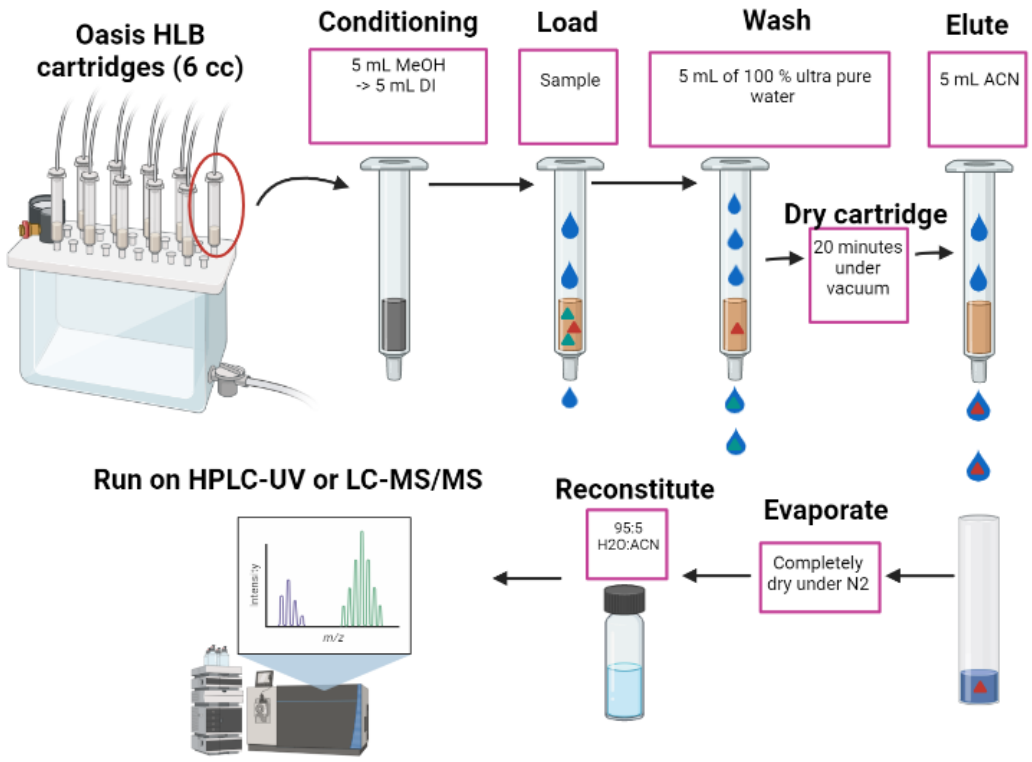


Figure 9. Representation of Solid-Phase Extraction (SPE) Workflow for Surface Water Sample Analysis by HPLC-UV and LC-MS/MS. The process involves conditioning, loading the water sample (1 L HPLC-UV, 100 mL LC-MS/MS), washing, elution and reconstitution of the extracts for subsequent analysis. Created with BioRender.com

2.3.6 Liquid Chromatography

The initial mobile phase and SPE optimisation was carried out using HPLC-UV. To improve sensitivity, reduce sample load volume and to include more pharmaceuticals, LC-MS/MS was later employed and optimised for sample analysis.

2.3.6.1 HPLC-UV instrumental conditions

HPLC-UV analysis was carried out using a Shimadzu prominence HPLC-UV instrument equipped with a SIL-20AC XR autosampler, DGU-20A5R degassing unit, LC-20AD XR binary pump and an SPD-20A UV detector. Chromatographic separation was carried out with an InfinityLab Poroshell 120 EC-C18 column 2.1 × 250 mm, 4 µm particle size equipped with a Poroshell 120 UHPLC EC-C18 2.1 mm 4 µm guard column (Agilent Technologies, Cheadle, UK) at room temperature.

The mobile phase used was 0.1% formic acid in ultrapure water (mobile phase A) and 0.1% formic acid in HPLC grade Acetonitrile (mobile phase B) at a flow rate of 0.4 mL/min in a gradient separation. Starting conditions were 5% B, from time 0 - 5 min %B increased to 10%, from time 5 - 14 min B increased to 40% B, from time 14 - 30 min B increased to 70%, from time 30.01-37 min B stayed at 100% B, %B was then returned to 5% for a re-equilibration time of 11 min. An injection volume of 30 µL was selected. HPLC-UV analysis was conducted at 275 nm and included 11 pharmaceuticals (Amoxicillin, trimethoprim, ciprofloxacin, diclofenac, venlafaxine, sulfamethoxazole, gemfibrozil, carbamazepine, E1, E2, EE2).

2.3.6.2 LC-MS/MS instrumental conditions

LC-MS/MS analysis was completed with an Agilent HPLC instrument equipped with a 1290 Infinity II LC multi-sampler, temperature-regulated sample tray, and binary pump with a temperature-regulated column compartment. Detection was performed by a 6470A triple quadrupole mass spectrometer equipped with electrospray ionisation (Agilent Technologies, Cheadle, UK), which used helium as collision gas and N_2 as a nebulising and desolvation gas. Data was collected using MassHunter Data Acquisition software.

Chromatographic separation and method optimisation was initially achieved using an InfinityLab Poroshell 120 EC-C18, 2.1 x 150 mm, 1.9 µm LC column with Column ID equipped with an InfinityLab Poroshell 120 EC-C18, 2.1 mm, 1.9 µm UHPLC guard column. Field samples analysis and final method validation was conducted with a Zorbax eclipse plus C18 2.1 x 50 mm 1.8 µm LC column equipped with a Zorbax eclipse plus C18, 2.1 x 5 mm, 1.8 µm UHPLC guard column at 30 ˚C.

Analysis of pharmaceuticals by LC-MS/MS was separated into two methods (method 1 and method 2) which were run in dMRM. With dMRM, the delta retention times for each pharmaceutical were targeted to enhance dwell times within the time window without compromising sensitivity. dMRM cycle times were set to 500 ms with dwell times set at 20 to 50 ms.

Delta electron multiplier voltage (EMV) of 200 V (positive mode) for method 1 and 200 V (negative mode) for method 2. A cell accelerator voltage (CAV) was set to 4 V for both methods. Both methods had set MS1 and MS2 resolution to wide.

To optimise the analyte and method conditions for each pharmaceutical, 10 µL of a 100 ppb standard mix of individual analytes were injected and passed through the column into the MS/MS (per manufacturer recommendation). Source conditions for method 1 was optimized using source optimiser software (Agilent Technologies, Cheadle, UK), source conditions for method 2 were selected based on previous work conducted by Rapp Wright et al.[276] The fragmentor voltage, collision energy and a minimum of 2 transition ions (qualifier and quantifier) of each analyte were determined using the Agilent Optimizer software (Agilent Technologies, Cheadle, UK).

2.3.6.2.1 Method 1

Method 1 was run in a positive mode, which included 13 pharmaceuticals (azithromycin, clarithromycin, diclofenac, erythromycin, amoxicillin, ciprofloxacin, venlafaxine, o-desmethyl venlafaxine, sulfamethoxazole, trimethoprim, carbamazepine, gabapentin and metformin). The mobile phase consisted of 5 mM ammonium formate and 0.1% formic acid in H_2O (mobile phase A), and 0.1% formic acid in ACN (mobile phase B) at a flow rate of 0.4 mL/min. The following gradient was used for separation; starting conditions were 5% B, from time 0-9 min %B increased to 40%, from time 9-15 min B increased to 100%, from time 15-17 min %B was maintained at 100%, %B was then returned to 5% from 17-17.5 min and re-equilibration time was 4 min. An injection volume of 30 µL was selected as a result of no observable sample overloading and previous optimisation work conducted within Dublin City University by Hands et al.[232]

2.3.6.2.2 Method 2

Method 2 was run in a negative mode, which included 4 pharmaceuticals (E1, E2, EE2 and gemfibrozil) and was adapted from previous work conducted by Rapp Wright et al.

[276] The mobile phase consisted of 1 mM ammonium fluoride in H_2O (mobile phase A) and acetonitrile (mobile phase B) with a flow rate of 0.4 mL/min with initial starting conditions set at 5%. The following gradient was used for separation: starting conditions were 70% B, from time 0-2.2 min %B was maintained at 70%, from time 2.2-2.7 min B increased to 100%, from time 2.7-3.7 min %B was maintained at 100%, from time 3.7-4 min % B was then returned to 70% and stayed at 70% from time 4-5 min. Re-equilibration time was 4 min. An injection volume of 100 µL was selected as a result of no observable sample overloading and previous optimisation work conducted within Dublin City University by Rapp Wright et al.[276]

2.3.7 Method Performance and validation

The validation of the HPLC-UV and LC-MS/MS methods followed guidelines set out by the International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use guidelines.[280] During the initial stages of HPLC-UV method validation, a composite sample from one location was made, and for LC-MS/MS method validation, a composite of the four sample sites from 6 sampling time points (n = 24) was made. This sample was used in several studies to determine the method linearity, recovery, the limit of detection (LOD) and limit of detection quantification (LOQ) and the associated matrix effects from these samples. Data analysis for method development was completed using Microsoft® Office Excel (WA, USA).

The quantification of pharmaceuticals was carried out utilising a minimum of five calibration points from matrix-matched composite samples (LC-MS/MS) or extracted deionised water (HPLC-UV). The peak areas were used for validation calculations on the HPLC-UV, and on the LC-MS/MS, peak area ratios were used for the pharmaceuticals

with their associated SILA internal standards. Linearity was calculated using a linear/logarithmic linear regression model. For LC-MS/MS analysis, responses are calculated using their peak area, divided by the internal standard to account for instrumental or matrix interferences.

In the calibration process, the existence of pharmaceuticals within the composite matrix was addressed with a matrix blank. This blank was prepared by spiking the composite matrix (n = 3) with only the internal standard mix to a concentration of 700 ng/L in the 1 ml reconstitute. The average peak area ratio associated with these contaminants was subtracted from the peak area ratio at each calibration point to account for any pre-existing pharmaceuticals. This approach effectively adjusted the calibration values to accurately reflect the presence of pharmaceuticals in the composite matrix.

To further mitigate contamination and carryover risks, a three-stage wash (1. 90:10 H_2O:Isopropanol, 2. 50:50 MeOH:ACN, 3. 95:5 H_2O:ACN) was introduced to clean the injection needle (inside and outside) and needle seat between each injection. Furthermore, two solvent blank injection runs were carried out (100 µL injection volume) between sample runs. The first injection containing 100% ACN, and the second injection run consisting of the initial mobile phase (95:5 H_2O:ACN).

SPE recovery was determined by comparing the peak area ratios of pharmaceutical concentrations from an extracted composite sample (100 mL) that was spiked prior to solid phase extraction (n = 3) against a composite sample (100 mL) that was spiked post-extraction (n = 3). Both samples were spiked to 700 ng/L in the final 1 mL reconstitute by adding the required concentrations of the pharmaceutical standard mix and internal

standard mix. Method precision was calculated by calculating the Relative Standard Deviation (%RSD) across a triplicate spiked sample.

LOD and LOQ were calculated by dividing the standard deviation of the intercept from the calibration line by its slope and multiplying by 3.3 (LOD) or 10 (LOQ) (Equation 2 and Equation 3).[281]

$$LOD = 3.3 \times \frac{standard\ deviation\ of\ the\ intercept}{slope} \quad \textit{Equation 2}$$

$$LOQ = 10 \times \frac{standard\ deviation\ of\ the\ intercept}{slope} \quad \textit{Equation 3}$$

The matrix effects ratio (recommended value = 1) was additionally calculated to determine the level of ion suppression (matrix effect ratio less than one)/enhancement (matrix effect ratio greater than one) that matrix interference can have on a sample (Equation 4). This was calculated by extracting triplicate UPW water and spiked matrix prior to extraction.

$$Matrix\ effects\ ratio = \frac{Peak\ area\ ratio\ of\ analyte\ in\ spiked\ matrix}{Peak\ area\ ratio\ of\ analyte\ in\ spiked\ UPW} \quad \textit{Equation 4}$$

Triplicate matrix blanks were extracted to deduct the concentrations of pharmaceuticals already present in the sample. The % recovery of pharmaceuticals was calculated using matrix match samples with spiked samples 500 µg/L for HPLC-UV and 700 ng/L for LC-MS/MS. Matrix effects were calculated through triplicate spiked DI and spiked matrix-matched samples.

2.3.8 Chromatography

Several factors, such as retention drift, selectivity, retention factor, and peak shape, were assessed to ensure the quality of the chromatographic method used. Symmetry

(S) was calculated using Agilent MassHunter software. The retention factor (k) was calculated in Equation 5 where k = retention factor, tr = retention time of pharmaceutical, and t_0 = void time of the column. The mobile phase, column temperature, and stationary phase influence the retention factor and it is recommended that k > 1.

$$k = \frac{t_r - t_0}{t_0}$$ *Equation 5*

2.4 Results and discussion

The optimisation of methods was conducted to establish a robust and reproducible method for the analytical determination of pharmaceutical analysis in surface water analysis. To achieve this, the optimisation of solid-phase extraction, mobile phase composition, and instrumental parameters was assessed.

2.4.1 SPE-HPLC-UV Evaluation

2.4.1.1 SPE Evaluation

To determine recovery in the absence of matrix interference, 1 L DI water samples were spiked to 500 µg/L. To increase method sensitivity, 1 L was used. This significantly increased the extraction time to 2/3 days. The prolonged extraction time was incompatible with analytes that have a higher susceptibility to degradation, such as amoxicillin and ciprofloxacin.[232] To limit the degradation of analytes overnight, the water samples were kept in the dark at 4 ˚C along with the SPE cartridges, and during extraction, the water samples were placed in a water cooler with ice.

Two sorbent sizes (200 mg and 500 mg) were trialled to optimise SPE using the same extraction method outlined in section 2.3.5.2. In addition to testing for sorbent sizes, both extractions were tested to determine the effects of a filter vs an unfiltered sample. Although these steps were taken to control external environmental factors, the extraction time and degradation of pharmaceuticals was reflected in poor recoveries and reproducibility seen during extractions, as seen in Table 9.

Table 9: Percentage recovery of pharmaceuticals from the extraction of 1 L H_2O with two types of OASIS HLB cartridges (200 mg and 500 mg sorbent packing (n=3).

Pharmaceutical	200 mg Filtered Recovery %	% RSD	200 mg Un-filtered Recovery %	% RSD	500 mg Filtered Recovery %	% RSD	500 mg Un-filtered Recovery %	% RSD
Amoxicillin	26.89	47.89	13.83	48.59	52.35	26.77	38.70	40.36
Trimethoprim	71.78	30.47	75.01	34.00	27.55	26.25	18.85	56.26
Ciprofloxacin	9.85	17.23	8.02	14.88	31.49	18.33	26.49	83.84
Venlafaxine	48.49	7.67	48.21	12.27	84.62	29.12	82.91	20.26
Sulfamethoxazole	71.87	9.02	59.88	50.97	66.22	30.44	29.47	33.48
Carbamazepine	76.40	7.55	77.85	6.84	94.75	5.96	80.40	13.32
EE2	26.03	48.02	24.14	55.99	18.99	20.94	19.48	21.68
E2	24.09	43.68	34.65	66.22	28.63	47.75	32.41	60.68
E1	59.21	13.70	60.93	34.81	57.94	21.78	51.18	78.89
Diclofenac	71.49	148.98	20.36	45.25	48.48	1.35	25.64	67.66
Gemfibrozil	77.80	4.33	95.22	47.50	52.35	26.77	62.08	57.21

In addition to challenges faced with recovery efficiencies using deionised water, the significant matrix interference observed after the extraction of 1 L of surface water created challenges with reproducibility and did not allow for the differentiation between the analytes of interest in comparison to extracted H_2O samples (Figure 10). To progress a method for pharmaceutical analysis in surface waters, future method development was performed using LC-MS/MS.

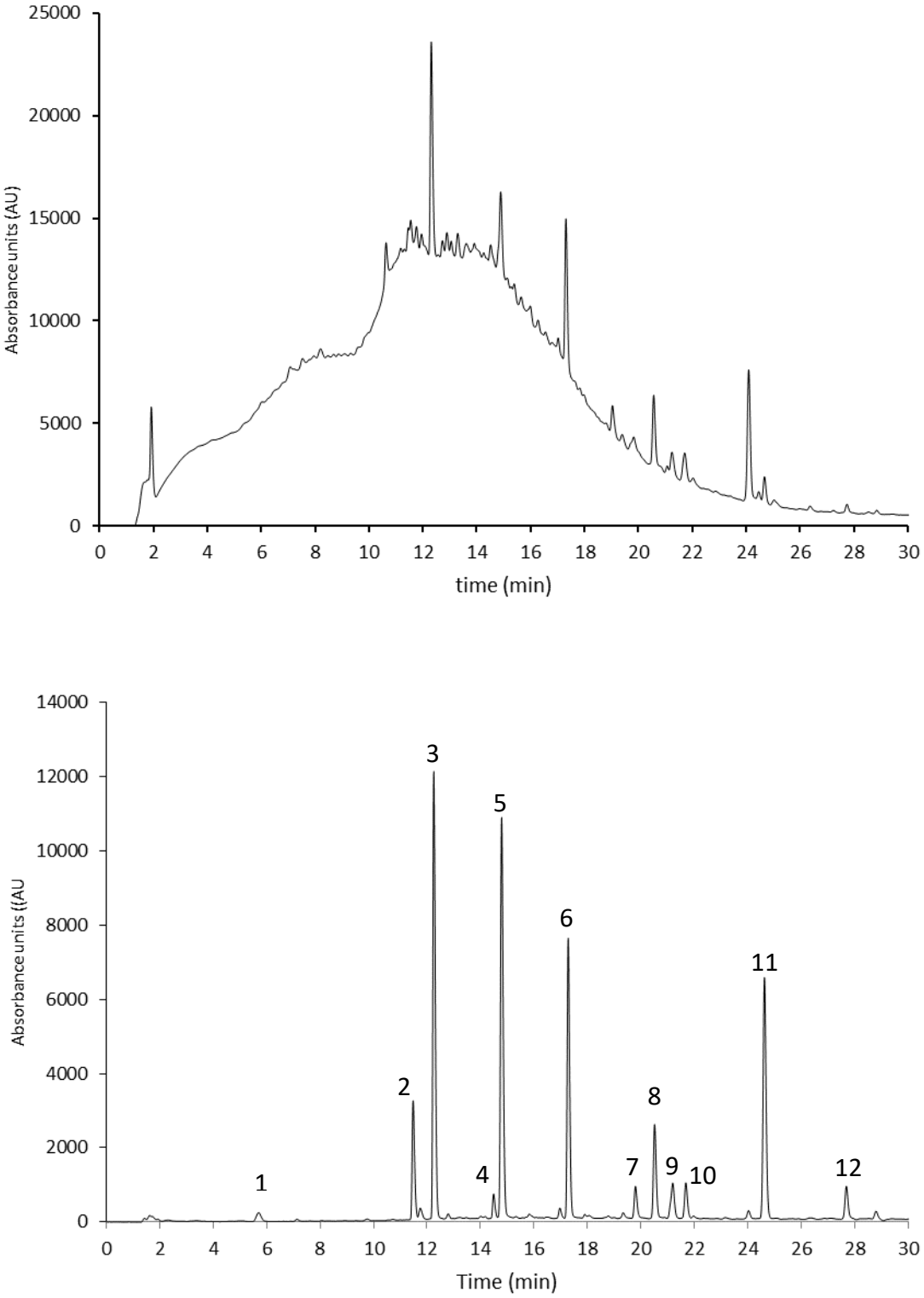


Figure 10. Chromatograms demonstrating the comparison between SPE of surface water samples (top) and DI spiked (bottom) with 500 µg/L of pharmaceutical mix injection volume of 30 µL. HPLC-UV Starting conditions: 5 % B, 0 - 5min % B increased to 10%, 5 - 14 min B increased to 40 % B, 14-30 min B increased to 70%, from time 30.01-37 min B stayed at 100% B, %B was then returned to 5% for a re-equilibration time of 11 min, UV detector conditions: 275 nm. 1. Amoxicillin, 2. Trimethoprim, 3. Ciprofloxacin, 4. Venlafaxine, 5. Sulfamethoxazole, 6. Carbamazepine, 7. EE2, 8. Ketoprofin (trialled I.S.), 9. E2, 10. E1, 11. Diclofenac, 12. Gemfibrozil

2.4.1.2 HPLC-UV method development and optimisation

HPLC-UV was selected as the initial instrument for method development with the analysis of eleven pharmaceuticals (amoxicillin, trimethoprim, ciprofloxacin, venlafaxine, sulfamethoxazole, carbamazepine, E1, E2, EE2, diclofenac and gemfibrozil). Pharmaceuticals such as macrolide antibiotics were included in LC-MS/MS analysis, which was excluded from HPLC-UV analysis due to analytical challenges and the lack of a suitable chromophore that was detectable by HPLC-UV analysis.[276,282]

2.4.1.2.1 Mobile phase optimisation

Reverse phase HPLC is one of the most commonly used HPLC methods to separate acidic pharmaceuticals.[283] The pH of a mobile phase plays a significant role in the pharmaceuticals of interest being kept in an ionised state. Maintaining pharmaceuticals in an ionised state suppresses the presence of dissociation groups, increasing retention of the analytes within the column.[283] For weakly acidic pharmaceuticals (e.g. venlafaxine and oestrogens), the pH of the mobile phase is independent of the retention time. However, increasing the pH of the mobile phase acidic pharmaceuticals (e.g. diclofenac and gemfibrozil) can lead to their dissociation and decrease their retention within the reverse phase C-18 HPLC column (Figure 11). This has additionally been previously documented by Stafiej et al.[283]

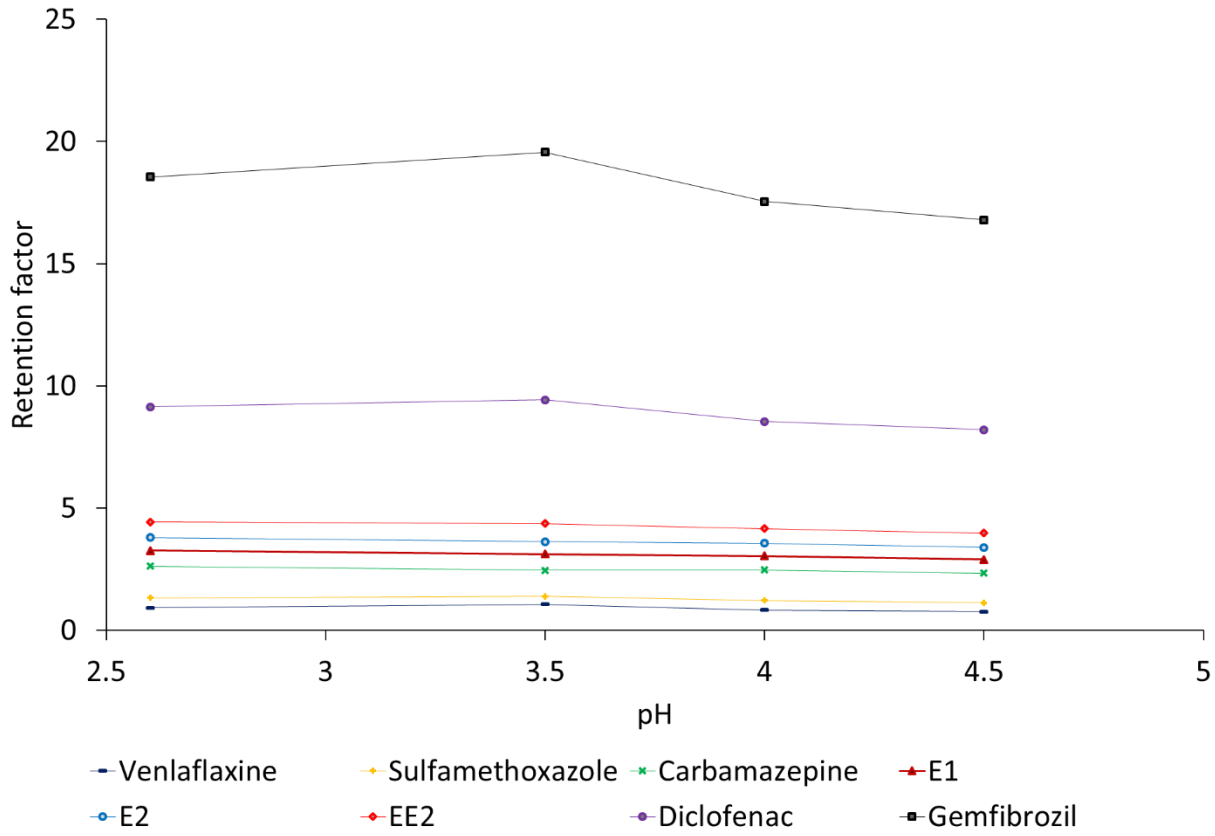


Figure 11. Relationship between the mobile phase pH and the retention factor of selected pharmaceuticals using a 55:45 (H_2O:MeCN) isocratic gradient.

To ensure the robustness of the retention times within this method, the pH of the mobile phase was adjusted to be 2 pH units above the acidic pKa of pharmaceuticals analysed to ensure they were sufficiently ionised during analysis. [284] Acidification of the mobile phase was accomplished using 0.1% formic acid in both the aqueous and organic mobile phases. Furthermore, using 0.1% formic acid for pharmaceuticals has been widely documented and is within the safe operational level for the separation column. [285–287]

Figure 12 shows the influence of % organic (acetonitrile) on the retention factor of the pharmaceuticals of interest. As the % organic increases, the retention factor decreases due to the reduction of hydrophobic interactions between the pharmaceuticals and the C-18 stationary phase within the column.

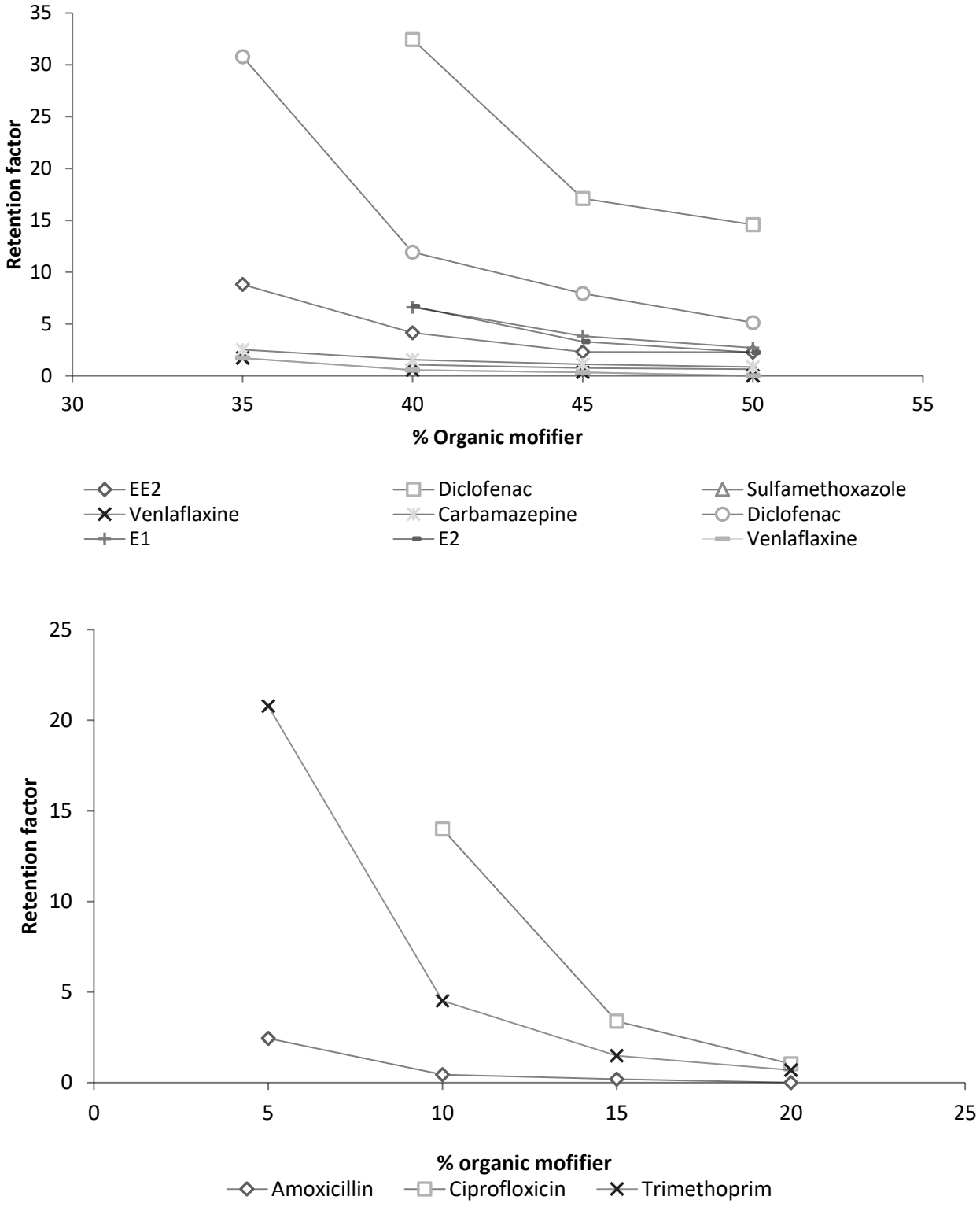


Figure 12. Relationship between % organic (MeCN) in the mobile phase at a constant pH on the retention factor of tested pharmaceutical, graphs are separated to show the more hydrophobic (top) and more hydrophilic pharmaceuticals (bottom).

To achieve sufficient retention of all 11 analytes, a gradient was chosen over an isocratic run, as there was no % organic modifier composition, which allowed for the successful elution of all pharmaceuticals (Figure 13).

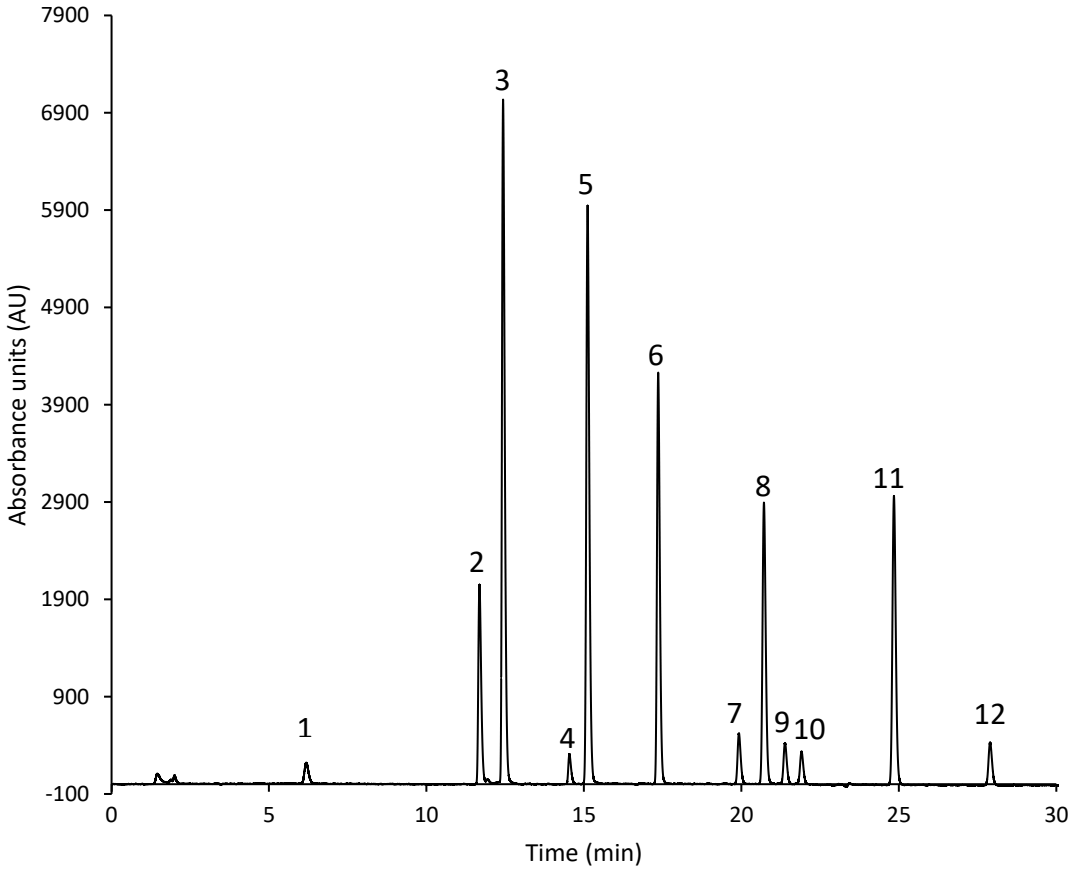


Figure 13. Blank subtracted chromatogram of pharmaceutical separation in a 500 µg/L standard mix injection volume 30 µL. Starting conditions: 5% B, 0 – 5 min % B increased to 10%, 5 - 14 min B increased to 40 % B, 14-30 min B increased to 70%, from time 30.01-37 min B stayed at 100% B, %B was then returned to 5% for a re-equilibration time of 11 min. 1. Amoxicillin, 2. Trimethoprim, 3. Ciprofloxacin, 4. Venlafaxine, 5. Sulfamethoxazole, 6. Carbamazepine, 7. EE2, 8. Ketoprofin, 9. E2, 10. E1, 11. Diclofenac, 12. Gemfibrozil

2.4.1.2.2 Preliminary validation of HPLC-UV method

To determine the appropriate concentrations for surface water analysis, a preliminary assessment of method linearity, LOD and LOQ was evaluated by analysing 12 concentrations (57-3000 µg/L) of a pharmaceutical mix.

LOD and LOQ were determined based on the RSD of the response (y-intercept) and the slope of the calibration curve. An assessment of instrument sensitivity using standard injections (Table 10) was carried out. As pharmaceuticals are frequently detected in surface waters in ng/mL-ng/L concentrations, this developed HPLC-UV method required the SPE of a 1 L sample (x 1000 concentration factor) to successfully detect pharmaceuticals at environmentally relevant concentrations, as represented in Table 10. However, this is under the assumption of 100% recovery during SPE and no matrix interference.[42]

Table 10: LOD and LOQ determined by HPLC-UV analysis of standard injections from 57-3000 µg/L of a pharmaceutical standard mix.

ANALYTES	LOD µg/L	LOQ µg/L	R^2	Theoretical LOD ng / mL (1 L SPE concentration)	Theoretical LOQ ng / mL (1 L SPE concentration)
Amoxicillin	139.94	424.07	0.9994	0.14	0.42
Trimethoprim	233.71	708.21	0.998	0.234	0.71
Ciprofloxacin	482.59	1462.4	0.9915	0.48	1.46
Venlafaxine	262.69	796.02	0.9977	0.26	0.80
Sulfamethoxazole	240.54	728.91	0.9979	0.24	0.73
Carbamazepine	236.55	716.81	0.9979	0.23	0.72
EE2	253.52	768.26	0.9976	0.25	0.77
E2	292.8	887.27	0.9974	0.29	0.89
E1	230.3	697.87	0.9974	0.23	0.70
Diclofenac	403.64	1.22	0.9941	0.40	1.22
Gemfibrozil	240.54	728.91	0.9919	0.24	0.73

2.4.2 SPE-LC-MS/MS method development

2.4.2.1 Solid phase extraction

Oasis HLB SPE cartridges were chosen due to their documented success in efficiently extracting each of the pharmaceuticals selected for this study.[288–291] Further optimisation steps include spiking water samples with 0.1 M EDTA to make a concentration of 0.1% EDTA. EDTA complexes with the metal ions within surface water samples, freeing up the pharmaceuticals that would otherwise be complex with the metal ions. [255–257]

2.4.2.2 LC-MS/MS optimisation

Pharmaceuticals assessed in this study were individually prepared for direct infusion in positive and negative modes to select a minimum of two transitions that provided the greatest signal. The highest transition was selected as the qualifier and the second highest to the qualifier ion.

Initially, an MRM method was utilised during method optimisation to identify pharmaceuticals and potential isomers of the analytes of interest. Once retention times of these 17 analytes were recorded, a Dynamic Multiple Reaction Monitoring (dMRM) method was created to enhance dwell times and to reduce the potential of false identification caused by similarly weighted compounds found in environmental samples.[276]

Two chromatographic methods were developed to achieve a lower LOD and LOQ of target analytes. Method 1 was run in positive mode and analysed 13 pharmaceuticals (metformin, amoxicillin, trimethoprim, ciprofloxacin venlafaxine, o-desvenlafaxine,

carbamazepine, sulfamethoxazole, diclofenac, gabapentin, clarithromycin, azithromycin, erythromycin). Method 2 was run in negative mode, targeting 17-alpha-ethinylestradiol, 17-beta-estradiol, estrone and gemfibrozil.

2.4.2.3 Analyte optimisation

Agilent Masshunter Optimizer software was used to individually select the optimal MRM parameters of the MS for each analyte. The software parameters were set to have a low mass cut-off of 40 m/z, fragmentor voltage of 0—180 V, and collision energy range of 0-50 V. During each optimisation run, 10 µL of a 100 ppb analytical standard was injected into the LC-MS/MS. This software optimised fragmentor voltage and collision energy in positive and negative modes. In addition, this software assessed both [M+H] + and [M-H] – for each pharmaceutical to identify the product and two precursor ions with the greatest intensity/signal. The fragmentation of an analyte, as seen with sulfamethoxazole in Figure 14 involves the dissociation of an analyte during its ionised state into unique product ions. Further MRM transitions can be found in Figure 15. These fragmentations and their associated retention time are used for identification and quantification.

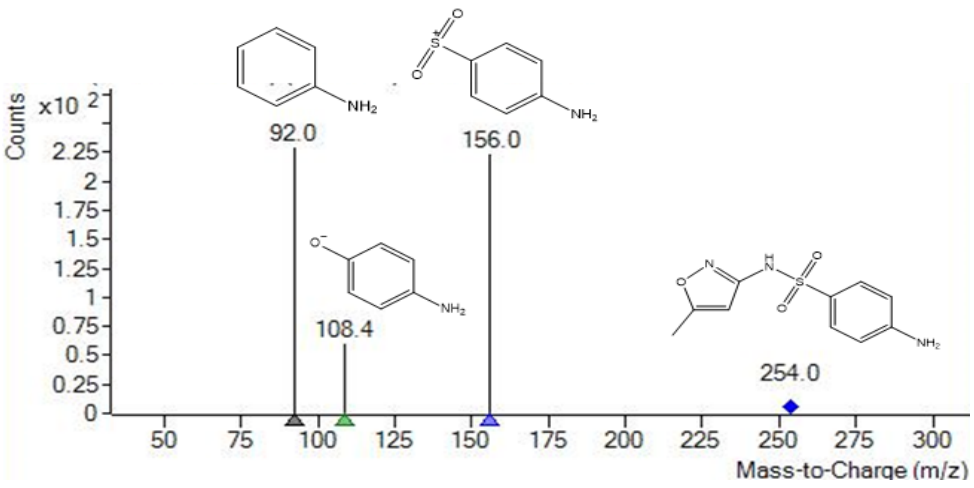


Figure 14. Mass spectrum of sulfamethoxazole analytical of a 75 ng/L standard onto the MS in dMRM mode (positive).

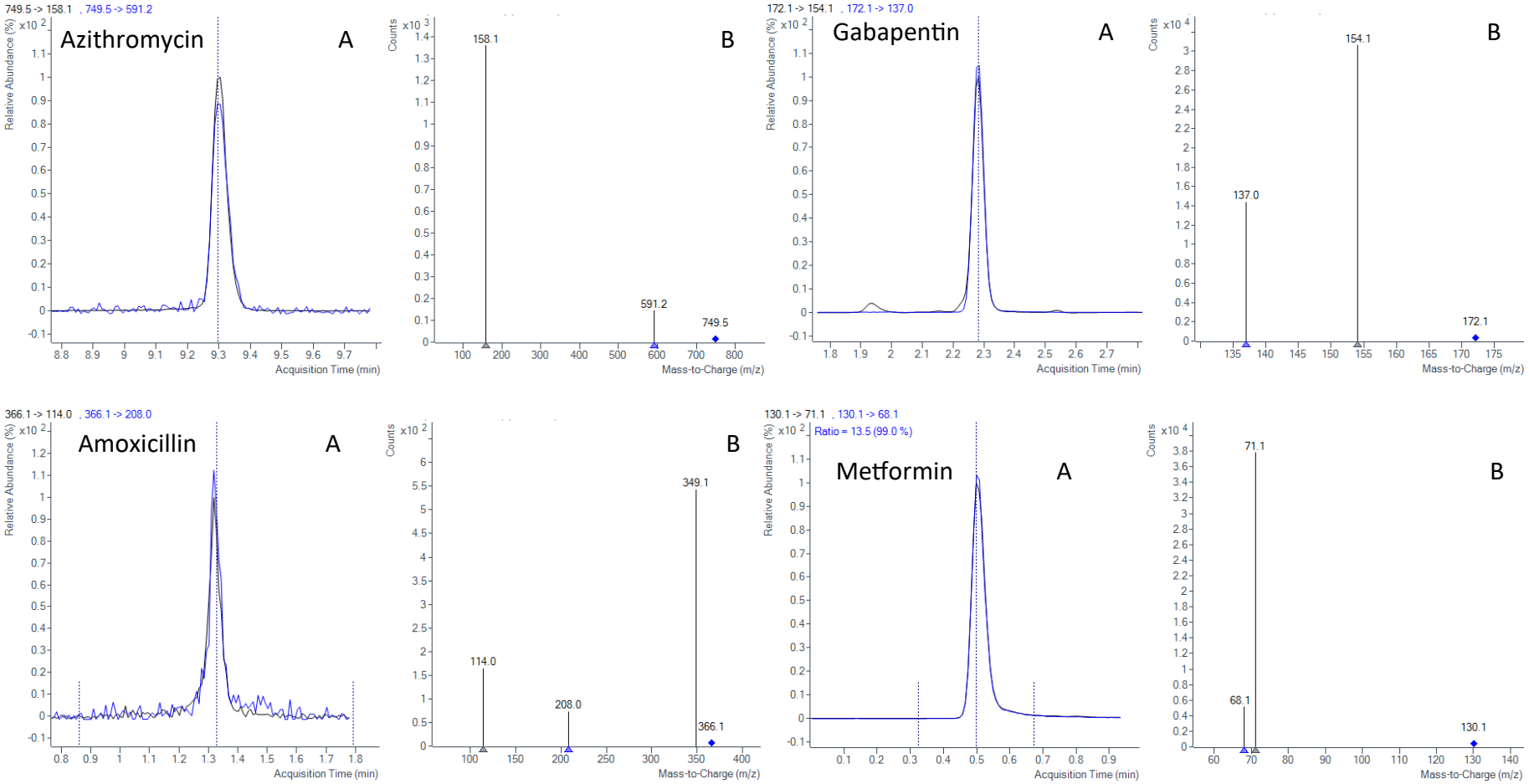


Figure 15. MRM of pharmaceuticals in a composite matrix spiked with 700 ng/L pharmaceutical mix with the overlay of quantitation and qualification MRM (A) and their associated mass spectrum (B).

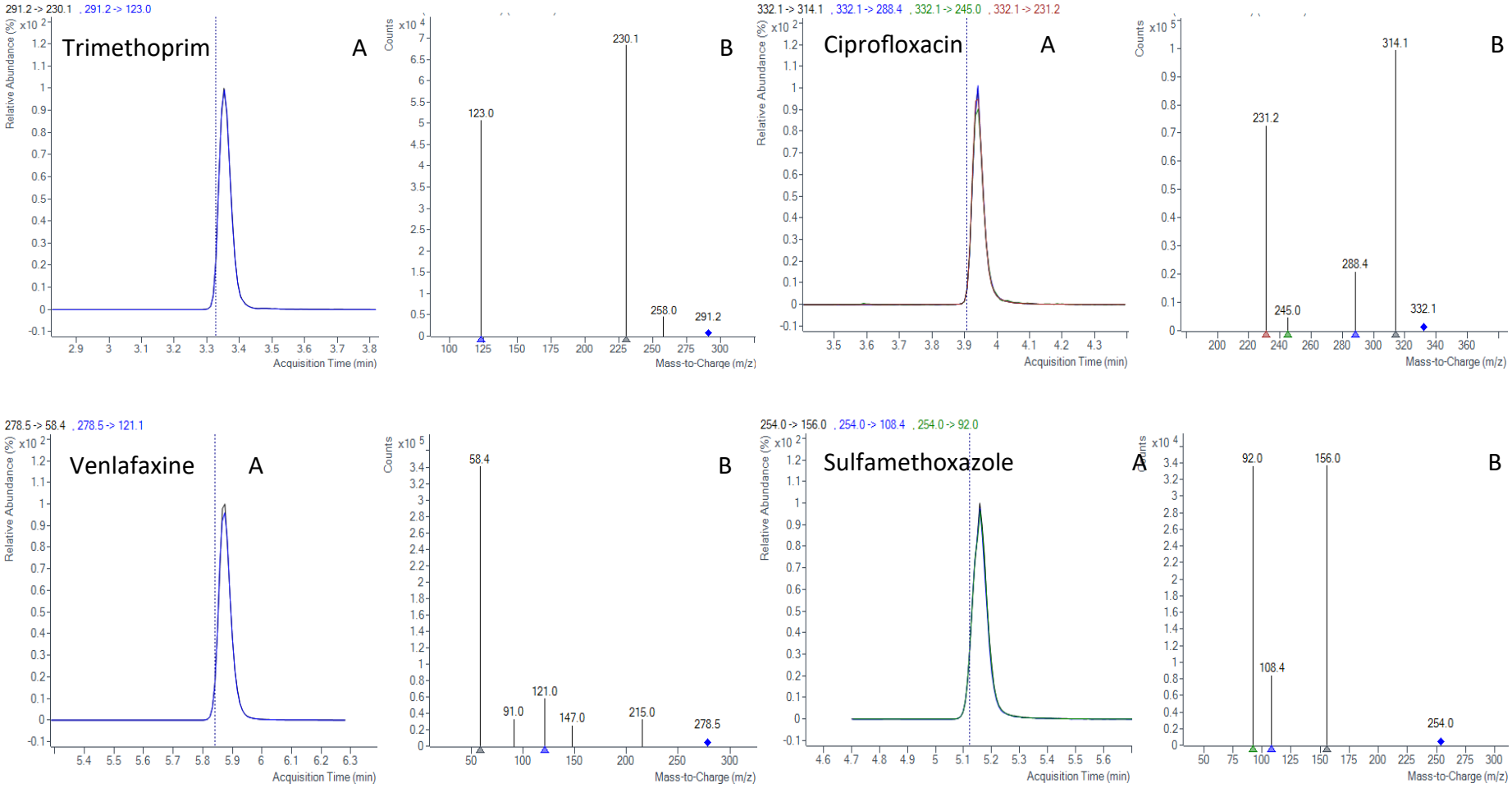


Figure 15 (continued). MRM of pharmaceuticals in a composite matrix spiked with 700 ng/L pharmaceutical mix with the overlay of quantitation and qualification MRM (A) and their associated mass spectrum (B).

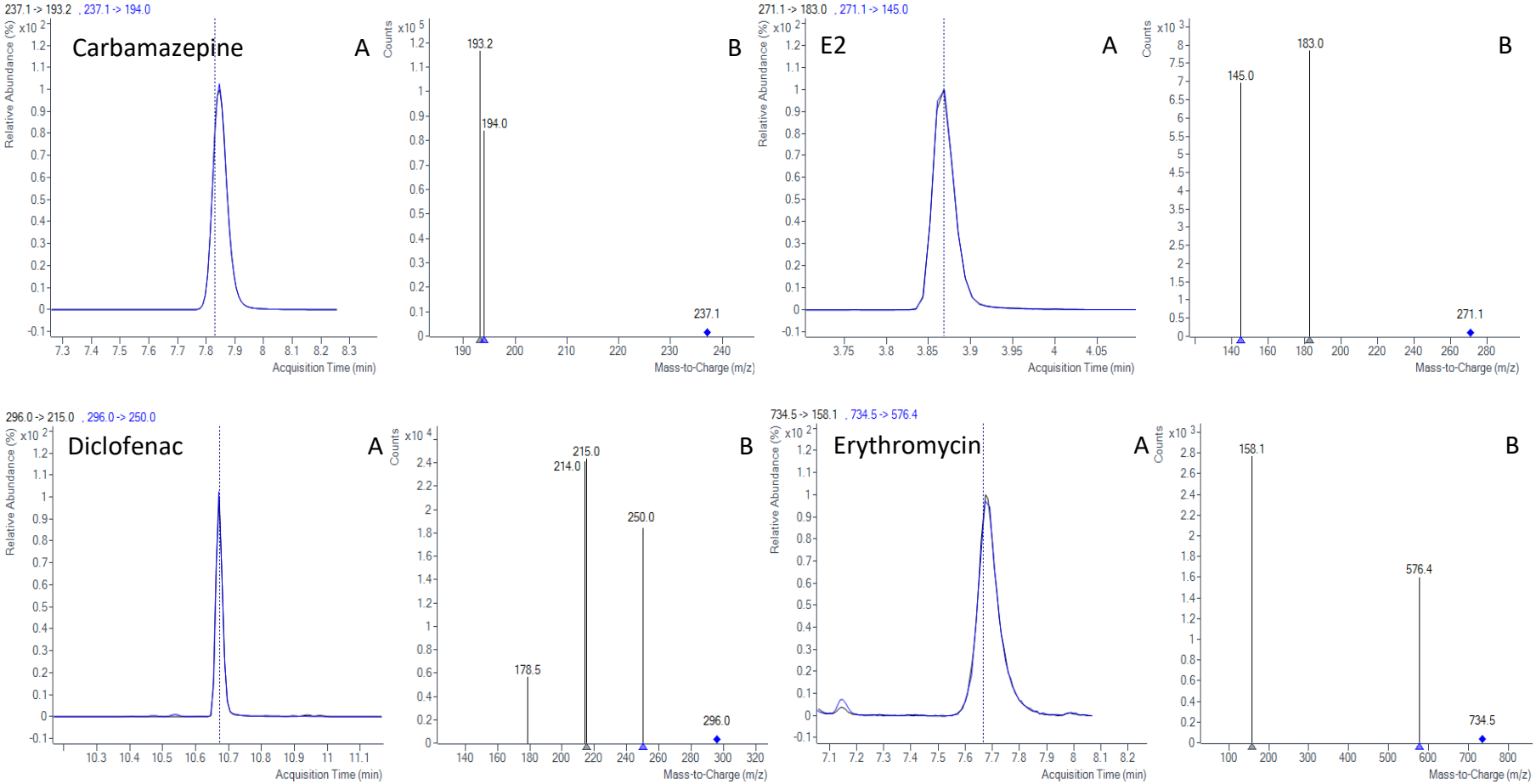


Figure 15 (continued). MRM of pharmaceuticals in a composite matrix spiked with 700 ng/L pharmaceutical mix with the overlay of quantitation and qualification MRM (A) and their associated mass spectrum (B).

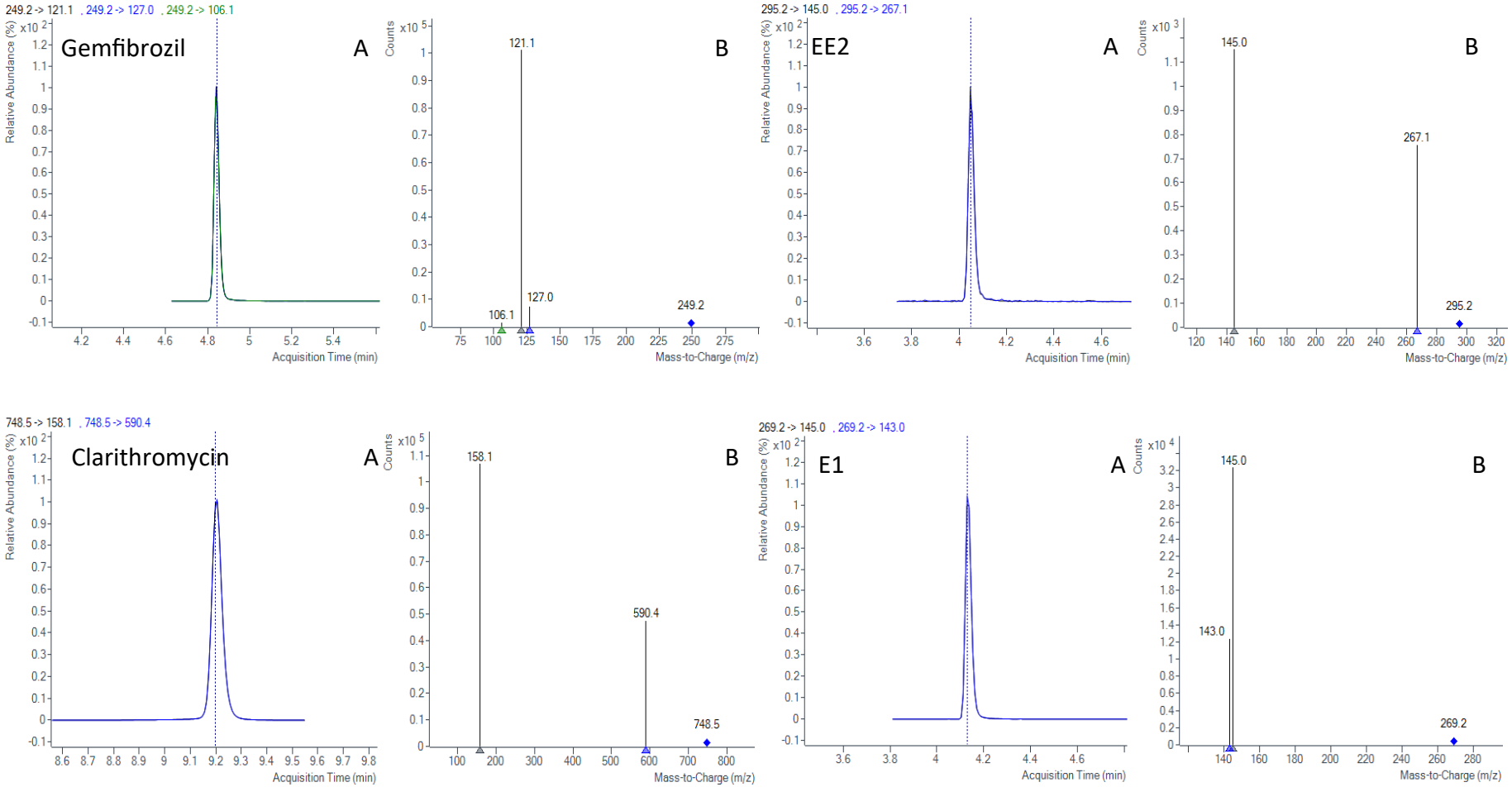


Figure 15 (continued). MRM of pharmaceuticals in a composite matrix spiked with 700 ng/L pharmaceutical mix with the overlay of quantitation and qualification MRM (A) and their associated mass spectrum (B).

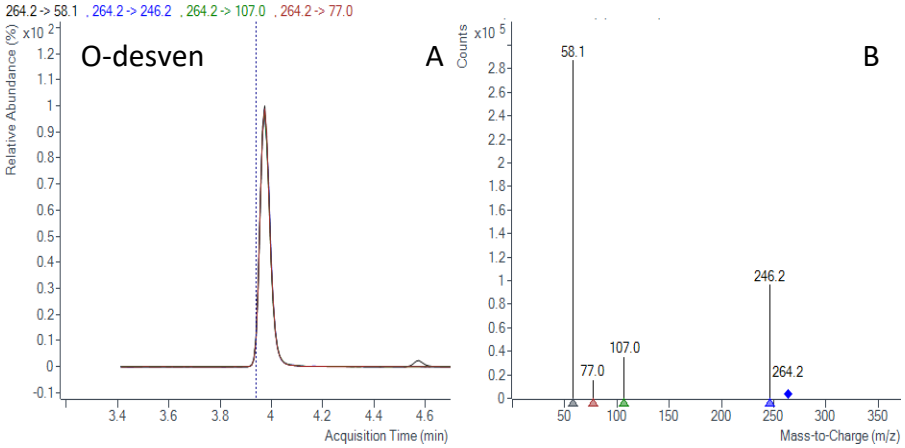


Figure 15 (continued). MRM of pharmaceuticals in a composite matrix spiked with 700 ng/L pharmaceutical mix with the overlay of quantitation and qualification MRM (A) and their associated mass spectrum (B).

Further optimisation of the MS involved altering the resolution of each MS quadrupole (MS1 and MS2), the electron multiplier voltage (EMV), cell accelerator voltage (CAV) and dwell times (Table 11).

Table 11: Final optimisation parameters for retention time, quantifier, and qualifier ions with their associated collision energy (CE) and fragmentor voltage for pharmaceuticals of interest.

Pharmaceutical	t_r (min)	Quantification (CE)	Identification (CE)	Fragmentor voltage (V)
Azithromycin	9.202	749.51 > 158.1 (45)	749.51 > 591.2 (33)	215
Clarithromycin	9.207	748.48 > 158.1 (33)	748.48 > 590.4 (17)	165
Diclofenac	10.672	296.03 > 215.0 (16)	296.03 > 250 (12)	125
Erythromycin	7.675	734.46 > 158.1 (29)	734.46 > 576.4 (17)	145
Amoxicillin	1.077	366.1 > 349.1(5)	366.1 > 208.0 (17) 366.1 > 114 (21)	80
Ciprofloxacin	3.941	332.1 > 314.1 (24)	332.13 > 231.2 (41)	150
Venlafaxine	5.875	278.5 > 58.4 (18)	278.5 > 121.1 (29)	72
o-desmethylvenlafaxine	3.974	264.2 > 58.1 (20)	264.2 > 246.2 (12)	115
Sulfamethoxazole	5.158	254 > 156.0 (16)	254 > 92.04 (28)	75
Carbamazepine	7.847	237.1 > 193.2 (36)	237.1 > 194 (40)	140
Gabapentin	2.283	172.1 > 154.1 (12)	172.1 > 137 (16)	100
Metformin	0.499	130.1 > 71.1 (24)	130.1 > 68.1 (40)	100
Trimethoprim	3.353	291.2 > 230.1(28)	921.2 > 123 (24)	10
17-β-estradiol	4.045	271.1 > 183 (49)	271.1 > 145 (45)	155
17-α-ethinylestradiol	4.24	295.2 > 145.0 (29)	295.2 > 267.1 (45)	185
Estrone	4.324	269.2 > 145 (45)	269.2 > 143 (60)	165
Gemfibrozil	5.119	249.2 > 121.1 (20)	249.2 > 127 (8) 249.2 > 106.1 (43)	100

To optimise MS resolution, three resolution settings (unit (0.7 u), wide (1.2 u) and widest (2.5u)) were tested on MS1 and MS2 for a total of 9 combinations. The appropriate resolution setting was based on maximum signal enhancement without compromising mass accuracy during analysis. Signal enhancement increased from unit to widest. However, to increase accuracy, MS1 and MS2 were set to wide.

Dwell times were selected to retain >1.2 cycles/s or <800 ms/cycle during each analyte elution window, with a delta EMV (+) of 200 for method 1 and a delta EMV (-) of 200 for method 2. Optimisation of CAV was tested by running a standard pharmaceutical mix (100 ppb) using a CAV of 2, 4 and 7 V. This optimisation step had not shown a significant

change between tests; therefore, a CAV of 4 V was included in both methods. The source optimiser software analysed a range of conditions and generated a report for the following parameters: gas temperature, gas flow, nebuliser, sheath gas temperature, sheathe gas flow, capillary voltage and nozzle voltage. For temperature-based conditions, duplicate runs were carried out, and the second run was used for comparison. The Total Ion Chromatogram (TIC) of each parameter were compared, as seen in Figure 16, and the setting with the greatest sensitivity (CAV = 3000) was selected (Table 12).

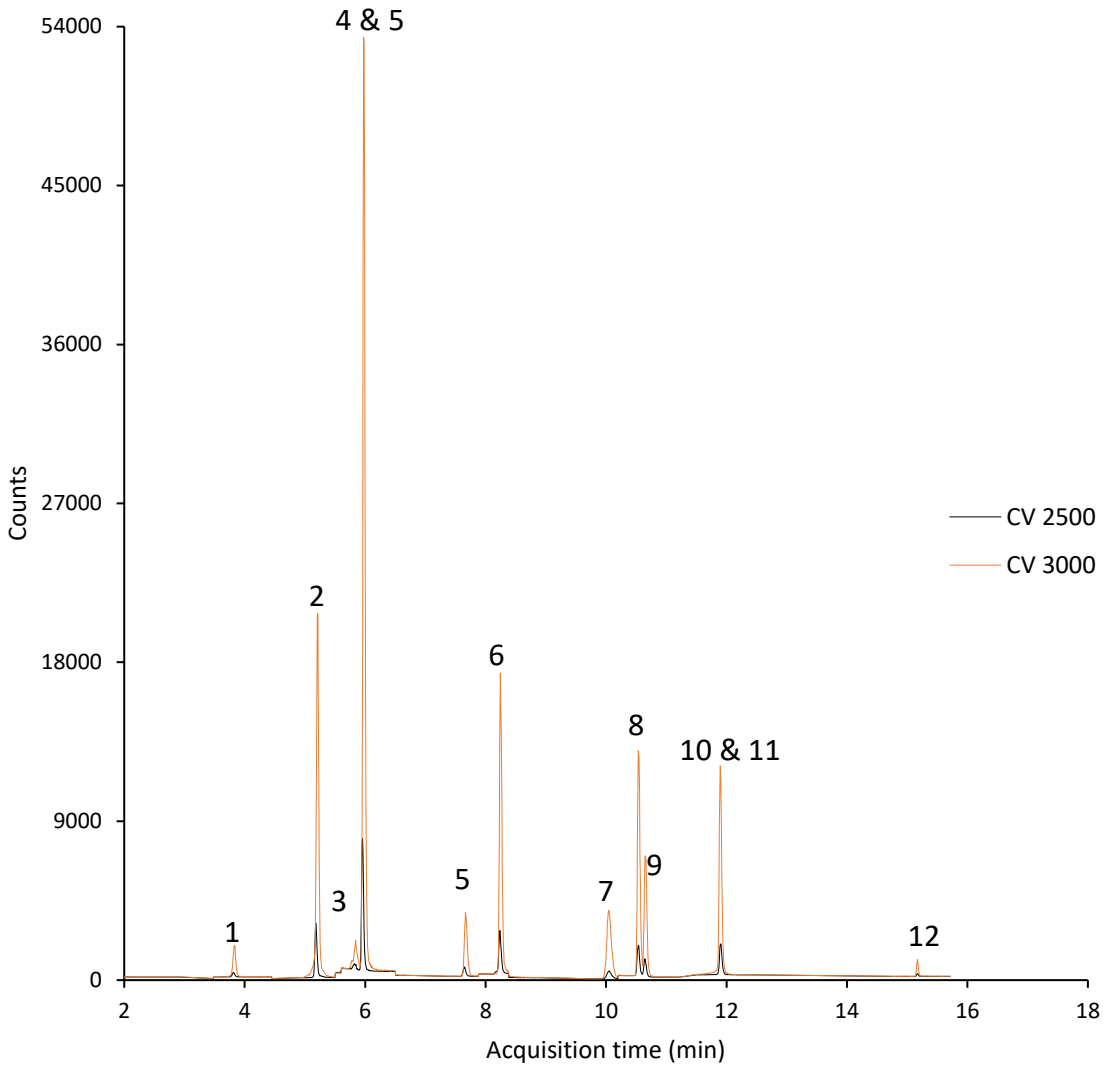


Figure 16. TIC example of capillary voltage source optimisation (Orange - 3000, Black - 2500), injection volume 40 µL of a 100 µg/L standard mix. A capillary voltage of 3000 was selected as it provided the greatest overall sensitivity for the following pharmaceuticals: 1. Gabapentin & Gabapentin-d4, 2. Trimethoprim, 3. Ciprofloxacin, 4.O-desmethylvenlafaxine & O-desmethylvenlafaxine-d5, 5. Sulfamethoxazole & Sulfamethoxazole-13C6, 6. Venlafaxine, 7. Erythromycin, 8. Carbamazepine, 9. Carbamazepine-d10 10. Azithromycin, 11. Clarithromycin, 12. Diclofenac & Diclofenac-d4.

Table 12: Source conditions for LC-MS methods one and two.

Source condition	Method 1		Method 2	
Gas Temperature	230 ℃		340 ℃	
Gas Flow	8 L / min		8 L / min	
Nebuliser	40 psi		40 psi	
Sheath Gas Temperature	400 ℃		350 ℃	
Sheathe Gas Flow	11 L/min		12 L/min	
Capillary voltage	Positive	Negative	Positive	Negative
	3000 V	3000 V	3000 V	3000 V
Nozzle Voltage	2000 V	2000 V	500 V	1500 V

A second method was necessary to accommodate the poor ionisation of hormones (E1, E2, EE2) and gemfibrozil. Hormones have been previously reported as being difficult to detect and quantify due to poor method sensitivity, ionisation efficiency and matrix interference. [292–295] The presence of a phenolic hydroxyl group in these three oestrogens can be ionised using negative-mode ESI; however, in positive-mode ESI, oestrogens' weak gas phase protonation involves the formation of adducts rather than $[M+H]^+$. [295] It is suggested that ammonium fluoride enhances the sensitivity in negative mode ESI due to the fluoride ions' strong basicity in the gas phase, which aids in forming $[M+F]^-$ and $[M+FHF]^-$ clusters.[276,296] Furthermore, the use of ammonium fluoride has been shown to provide higher analyte responses over other mobile phase additives such as ammonium hydroxide.[297]

2.4.2.4 Mobile phase optimisation

The selection of an appropriate mobile phase, pH and mobile phase additives is an important step as it can affect the ionisation and retention of analytes. pH adjustment of a mobile phase is necessary to enhance the degree of ionisation of each pharmaceutical and to optimise retention time within a column.[283] Many pharmaceuticals selected within this study have a weak acidic character and, therefore,

have a retention independent of the pH of the mobile phase. However, the inclusion of relatively strong acidic pharmaceuticals such as amoxicillin, gabapentin, diclofenac, gemfibrozil, sulfamethoxazole and ciprofloxacin requires the pH of the mobile phases to be lower than their acidic pKa. The pH was adjusted using 0.1% formic acid and ammonium formate as a mobile phase modifier due to their LC-MS/MS compatibility and frequent use in literature.[291]

Two frequently used LC-MS/MS solvents, MeOH and ACN, were evaluated using several concentrations of ammonium formate as a mobile phase additive (Table 13). [276,291,298–300] Ammonium formate and its conjugate acid, formic acid, was used as a buffer to resist changes in mobile phase pH as changes in pH can affect the ionisation and retention of analytes.[301]

Table 13: Seven mobile phase variations selected in mobile phase optimisation study.

Mobile phase mix	Mobile phase A (H_2O)	Mobile Phase B
1	0.1% formic acid with 2 mM ammonium formate	Methanol with 0.1% formic acid and 2 mM ammonium formate
2	0.1% formic acid with 5 mM ammonium formate	Methanol with 0.1% formic acid and 5 mM ammonium formate
3	0.1% formic acid with 10 mM ammonium formate	Methanol with 0.1% formic and acid 10 mM ammonium formate
4	0.1% formic acid	Acetonitrile with 0.1% formic acid
5	0.1% formic acid with 2 mM ammonium formate	Acetonitrile with 0.1% formic acid
6	0.1% formic acid with 5 mM ammonium formate	Acetonitrile with 0.1% formic acid
7	0.1% formic acid with 10 mM ammonium formate	Acetonitrile with 0.1% formic acid

MEOH was initially evaluated as the organic modifier for the mobile phase (see Table 13, mixtures 1-3). The highest response was observed from 0.1% formic acid with 5 mM ammonium formate in H_2O and 0.1% formic acid + 5 mM ammonium formate in MeOH (Figure 17).

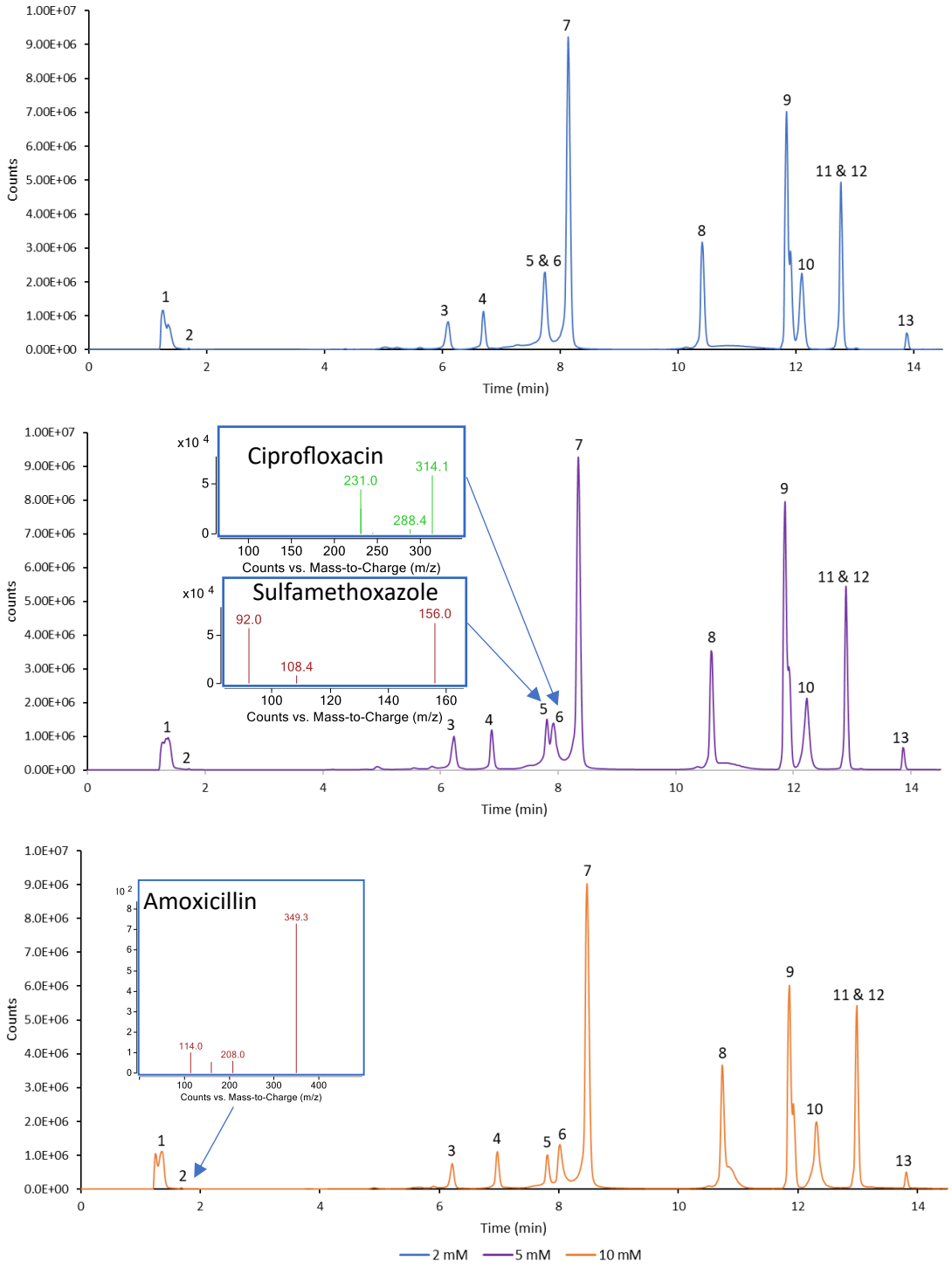


Figure 17. TIC overlay of standard injections with analyte confirmation by MRM for 0, 2, 5 and 10 mM ammonium formate in water + 0.1% formic acid with 0, 2, 5 and 10 mM ammonium formate in MeOH + 0.1% formic acid. Pharmaceuticals; 1. Metformin, 2. Amoxicillin, 3. Gabapentin & internal standard, 4. Trimethoprim, 5. Sulfamethoxazole & internal standard, 6. Ciprofloxacin, 7. O-desmethylvenlafaxine & internal standard, 8. Venlafaxine, 9. Carbamazepine & internal standard, 10. Erythromycin, 11. Azithromycin & internal standard, 12. Clarithromycin, 13. Diclofenac & internal standard.

As higher back pressures were associated with MeOH, mobile phases 4-7 in Table 13 were assessed. Due to the insolubility of ammonium formate in acetonitrile, it was not included in mobile phase B. The highest instrumental response was observed using 0.1% formic acid with 5 mM ammonium formate in UPW and 0.1% formic acid in CAN. Minor variations in retention times and peak shape were observed with lower buffer concentrations when performing the mobile phase optimisation. The effect of ammonium formate on peak shape was best observed on metformin, where the peak symmetry (S) for mobile phases 4-7 were S = 4.73, 3.61, 1.16, and 2.03, respectively (Figure 18). These variations are a result of a reduced buffering capacity, and therefore, slight variations in pH can influence an analytes ionisation and retention within the column.[302] There was a negligible difference in resolution between 10 mM ammonium formate and 5 mM ammonium formate. However, improved peak symmetry was observed with the addition of 10 mM ammonium formate over 5 mM ammonium formate for O-desmethyl venlafaxine (S = 1.27, 1.46), sulfamethoxazole (S = 1.05,1.47), erythromycin (S = 0.95,1.33), azithromycin (S = 1.17, 2.23), clarithromycin (S = 1.16, 2.13) and diclofenac (S = 0.89,1.51). However, the addition of 10 mM ammonium formate led to a rapid build-up of mobile phase buffer on the source, potentially jeopardising sensitivity for further injections.

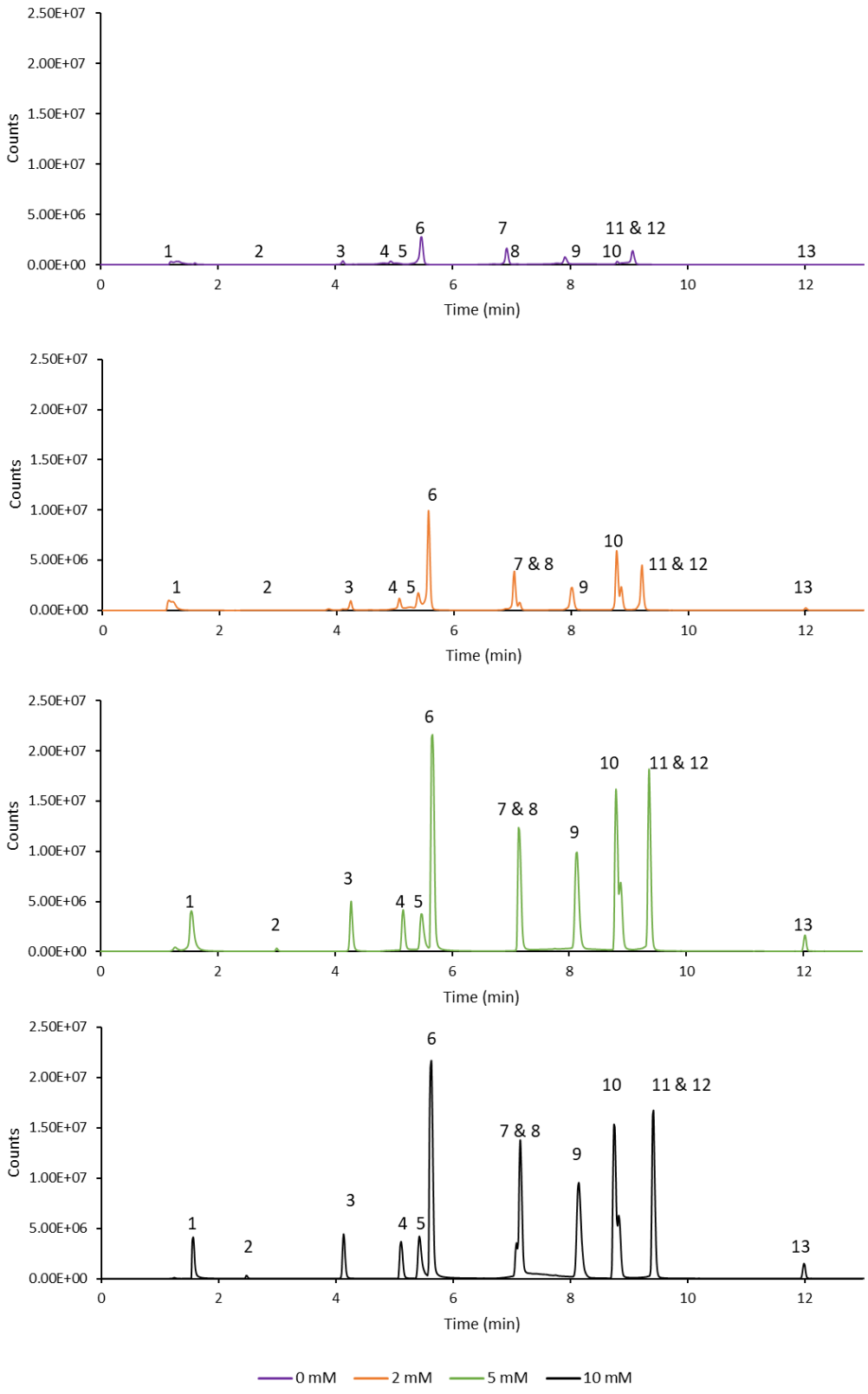


Figure 18. TIC overlay of 100 ppb standard injections for 0, 2, 5 and 10 mM ammonium formate in water + 0.1% formic acid with ACN + 0.1% formic acid. Pharmaceuticals; 1. Metformin, 2. Amoxicillin 3. Gabapentin & internal standard, 4. Trimethoprim, 5. Ciprofloxacin, 6. O-Desmethylvenlafaxine & internal standard, 7. Sulfamethoxazole & internal standard, 8. Venlafaxine, 9. Erythromycin, 10. Carbamazepine & internal standard, 11. Azithromycin & internal standard, 12. Clarithromycin, 13. Diclofenac & internal standard

Comparing the two methods that used different organic mobile phases (mobile phase mix 2 and 6), The highest instrumental response was observed using 0.1% formic acid with 5 mM ammonium formate in H_2O and 0.1% formic acid in acetonitrile. Furthermore, due to backpressure, the use of acetonitrile facilitated an increased flow rate of 0.4 mL/min over a flow rate of 0.3 mL/min, which was used with MeOH. This increased flow rate reduced the method's overall runtime. Additionally, the peak shape of some pharmaceuticals (e.g. metformin, ciprofloxacin, venlafaxine, sulfamethoxazole) was greatly improved using acetonitrile over MeOH (Figure 19).

Furthermore, it is adventitious to use acetonitrile over MeOH as MeOH can potentially interfere with the analysis of β-lactam pharmaceuticals (amoxicillin) due to degradation.[303] Thus, this mobile phase 6 was selected for method 1.

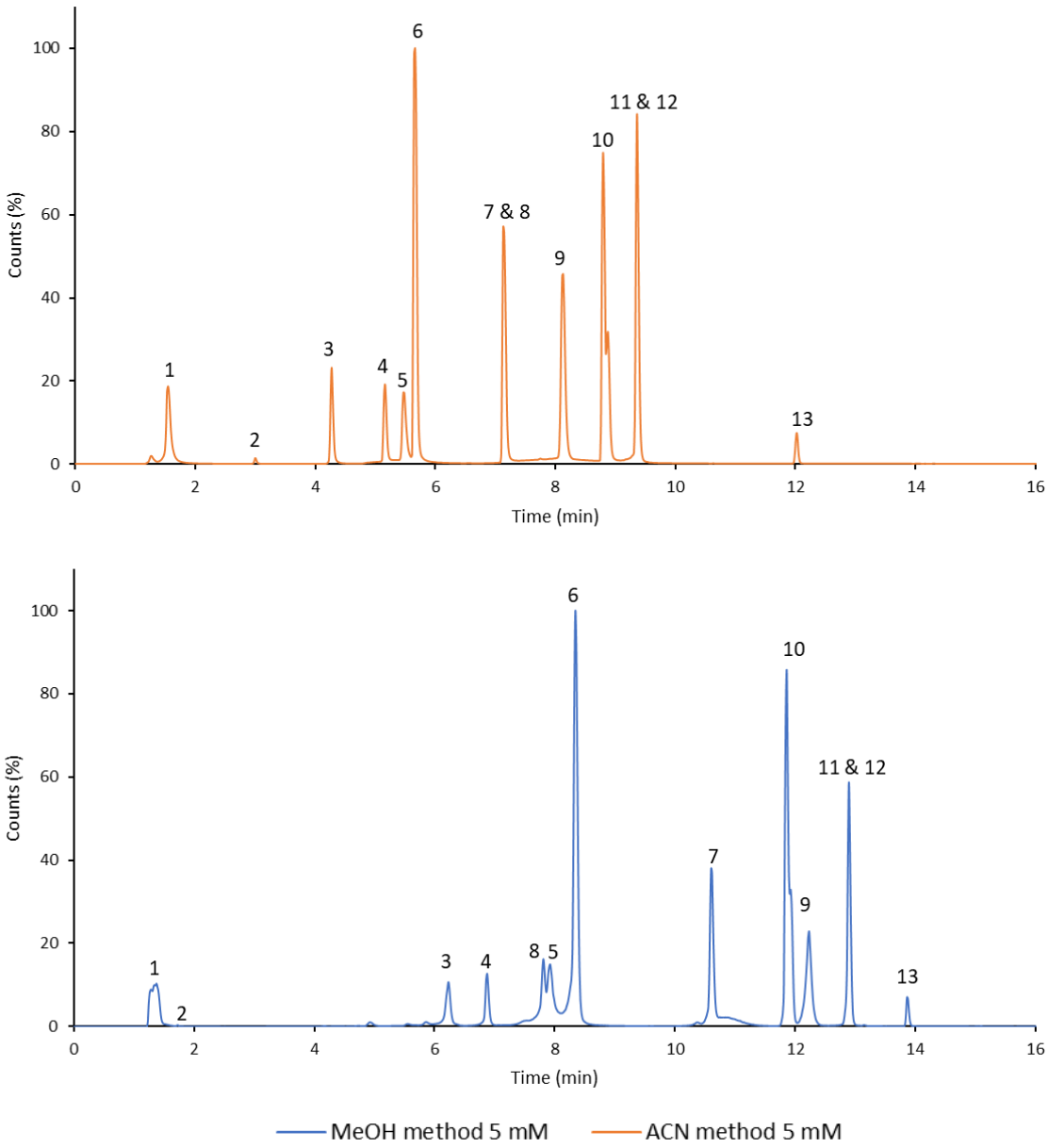


Figure 19. TIC overlay of standard injections for 5 mM ammonium formate in water and MeOH + 0.1% formic acid vs 5 mM ammonium formate in water + 0.1% formic acid and ACN + 0.1% formic acid. Pharmaceuticals; 1. Metformin, 2. Amoxicillin 3. Gabapentin & internal standard, 4. Trimethoprim, 5. Ciprofloxacin, 6. O-Desmethylvenlafaxine & internal standard, 7. Venlafaxine, 8. Sulfamethoxazole & internal standard, 9. Erythromycin, 10. Carbamazepine & internal standard, 11. Azithromycin & internal standard, 12. Clarithromycin, 13. Diclofenac & internal standard.

2.4.2.5 Chromatography and column selection

The mobile phase optimisation work completed on the HPLC-UV demonstrated that a gradient analysis was preferential to isocratic elution to reduce the presence of co-eluting compounds and to improve the separation of pharmaceuticals.

A flow rate of 0.4 mL/min was selected as it provided acceptable backpressure and reduced method analysis time. Lower back pressure is beneficial as it reduces wear on the HPLC system components and column. Furthermore, this flow rate gave a sufficient amount of time for the tested pharmaceuticals to interact with the stationary phase without compromising the quality of the chromatography.

Figure 20 and Figure 21 show the optimised separation conducted on Infinity Lab Poroshell 120 EC-C18 column with its corresponding column guard (Agilent Technologies, Cheadle, UK) for method 1 and method 2, respectively. The retention factor, selectivity and symmetry were calculated for all pharmaceuticals (Table 14).

Pharmaceutical separation/selectivity of most pharmaceuticals was within the acceptable range where $\alpha > 1.1$. However, some analytes co-eluted irrespective of the gradient applied, such as ciprofloxacin and O-desmethyl venlafaxine, azithromycin and clarithromycin. For this reason, the MRM was utilised to differentiate between these compounds. Analysis of peak symmetry was conducted using Agilent MassHunter software. Peak symmetry of analytes showed peak tailing in a few of the pharmaceuticals tested. Evidence of fronting and tailing can be seen in injected standards (Table 14). This could be a result of secondary interactions between analytes and acidic silanols in the column stationary phase.

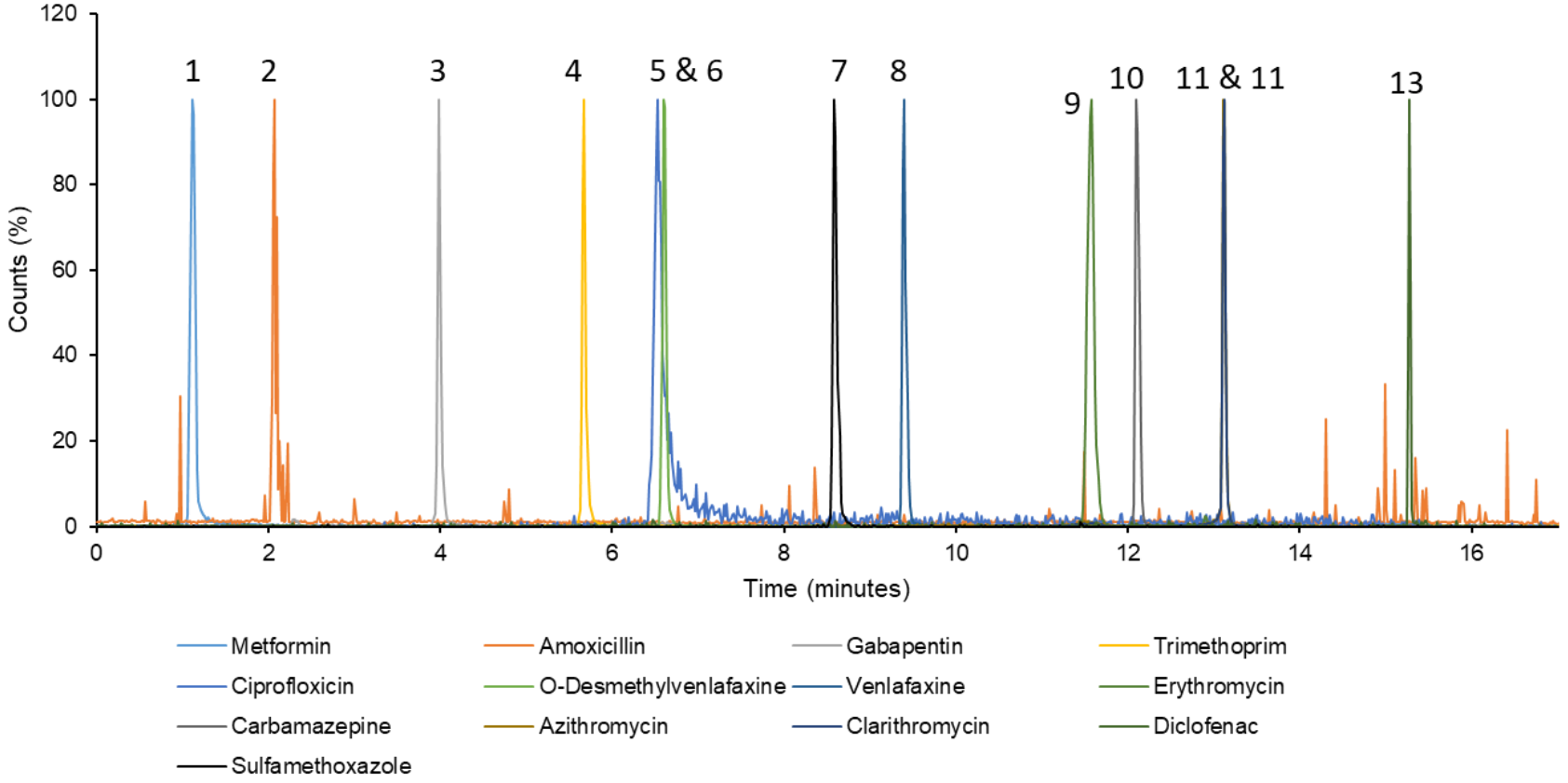


Figure 20. Peak-to-peak adjusted MRM overlay of the separation of pharmaceuticals selected for Method 1, using a Poroshell 120-EC C18 column, 0.4 mL/min flow rate, gradient elution of 5 mM ammonium formate with 0.1% formic acid in ultrapure water (A) and 0.1% formic acid in acetonitrile (B) at a concentration of 100 ppb. Pharmaceuticals; 1. Metformin, 2. Amoxicillin 3. Gabapentin, 4. Trimethoprim, 5. Sulfamethoxazole, 6. Ciprofloxacin, 7. O-Desmethylvenlafaxine, 8. Venlafaxine, 9. Carbamazepine, 10. Erythromycin, 11. Azithromycin, 12. Clarithromycin, 13. Diclofenac.

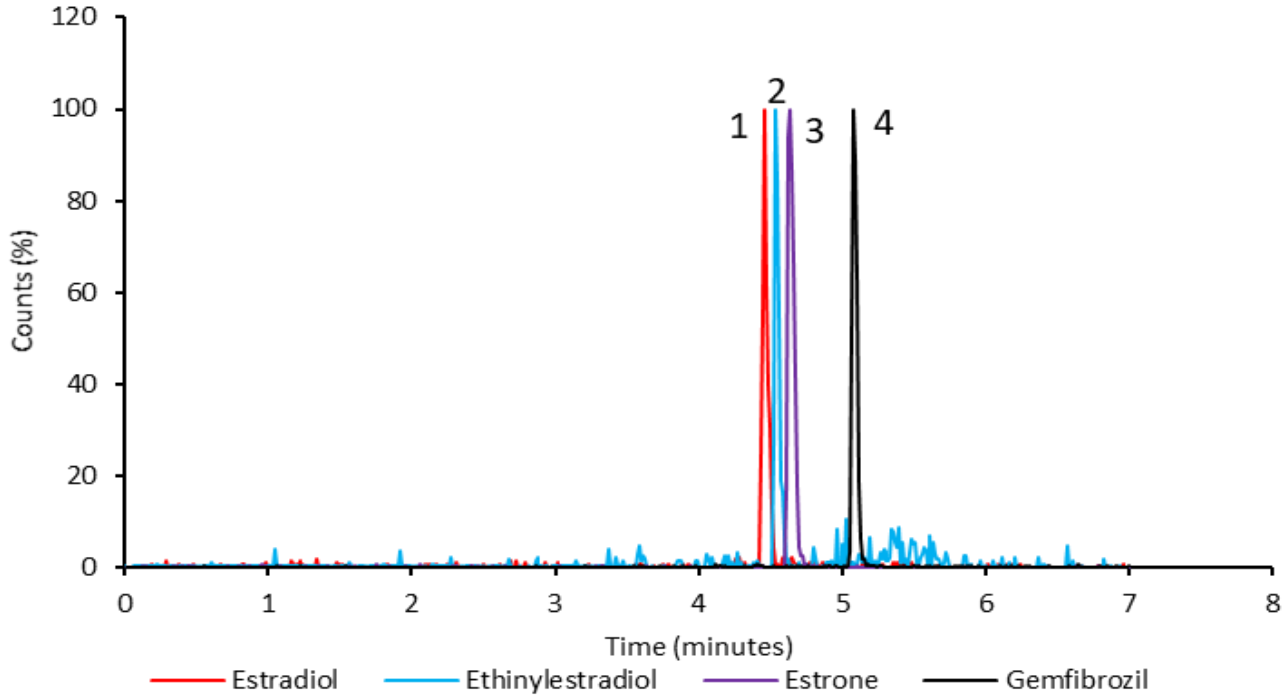


Figure 21. Peak-to-peak adjusted MRM overlay of the separation of pharmaceuticals selected for Method 2, using a Poroshell 120-EC C18 column, 0.4 mL/min flow rate, gradient elution of 1 mM ammonium formate in ultrapure water (A) and acetonitrile (B) at a concentration of 100 ppb. Pharmaceuticals; 1. E2, 2.EE2, 3. E1 and 4. gemfibrozil.

Table 14: Chromatographic parameters for analytes and internal standards in LC-MS/MS methods 1 and 2 using a Poroshell 120-EC C18 column.

Pharmaceutical	Rt (min)	Capacity Factor K	Symmetry
Method 1			
Metformin	1.112	0.112	1.63
Amoxicillin	2.069	1.069	0.72
Gabapentin	3.984	2.984	1.47
Gabapentin-d4	3.951	2.951	1.85
Trimethoprim	5.672	4.672	1.16
Ciprofloxacin	6.525	5.525	2.02
O-desmethyl venlafaxine	6.594	5.594	2.08
O-desmethyl venlafaxine-d6	6.593	5.593	1.21
Sulfamethoxazole	8.596	7.596	1.88
Sulfamethoxazole-13C6	8.596	7.596	1.85
Venlafaxine	9.400	8.400	1.12
Erythromycin	11.557	10.557	1.53
Carbamazepine	12.100	11.100	1.96
Carbamazepine-d10	12.002	11.002	1.01
Azithromycin	13.105	12.105	2.07
Clarithromycin	13.122	12.122	0.93
Diclofenac	15.273	14.273	1.17
Diclofenac-d4	15.256	14.256	1.8
Method 2			
Ethinylestradiol (EE2)	4.535	3.535	2.84
Estradiol (E2)	4.456	3.456	1.67
Estradiol-d2	4.636	3.636	1.64
Estrone (E1)	4.640	3.640	1.8
Estrone-d4	4.647	3.647	2.86
Gemfibrozil	5.090	4.090	1.98

As a result of a symmetry > 2 for three pharmaceuticals, the method developed was adapted to an alternate column (Zorbax eclipse plus C18, 2.1 x 50 mm with its associated guard column). Although Poroshell 120-EC and Zorbax eclipse plus columns are designed with similar stationary phases, the Zorbax eclipse plus column proved to be more effective in reducing analysis time (Figure 22 and Figure 23) while enhancing peak shape/symmetry for tested pharmaceuticals; therefore, it was selected for sample analysis Table 15.

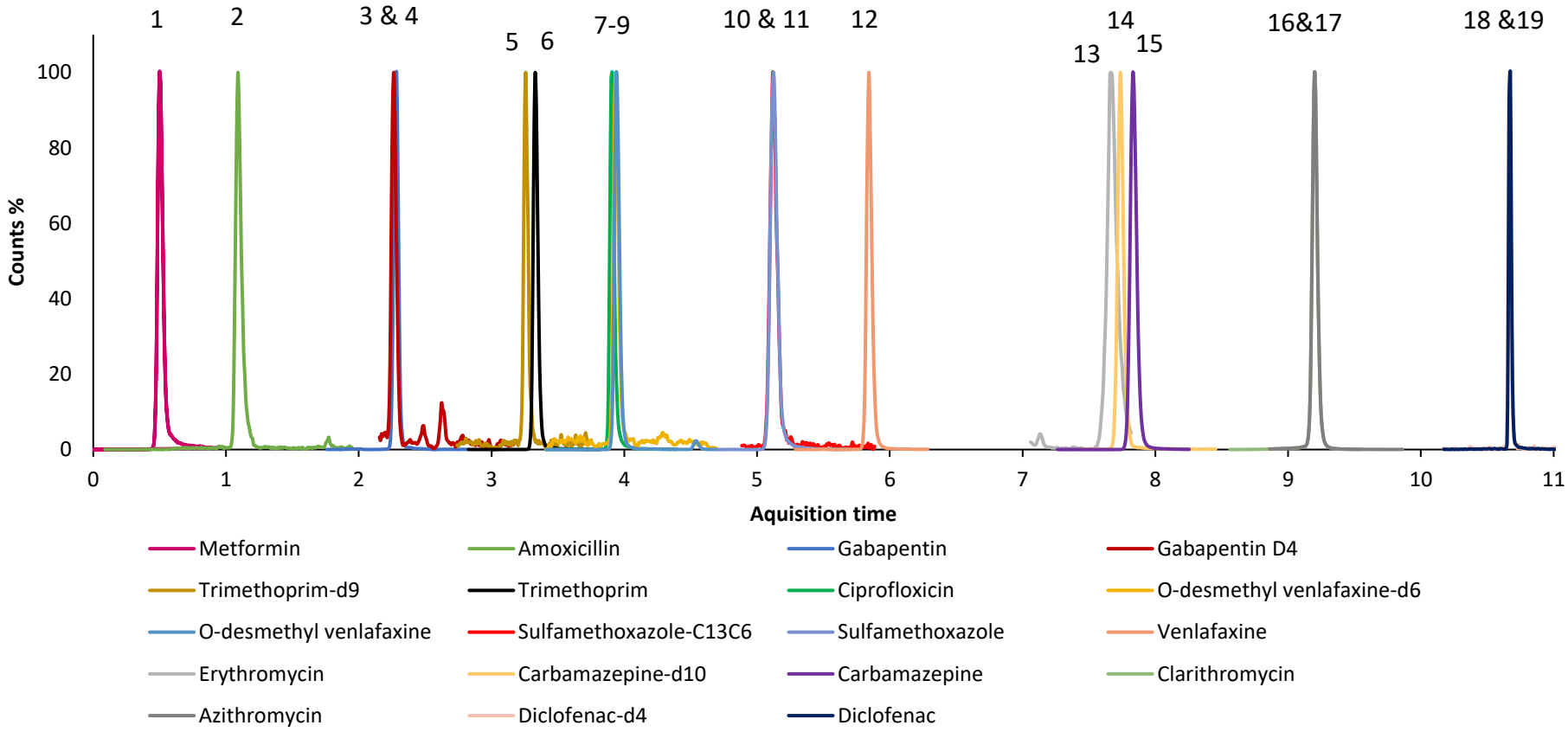


Figure 22. Peak-to-peak adjusted MRM separation of a 700 ng/L matrix spiked with pharmaceuticals and internal standards ran with Method 1, using a Zorbax eclipse plus C18 2.1 x 50 mm 1.8 µm LC column equipped with a Zorbax eclipse plus C18, 2.1 x 5 mm guard column, 0.4 mL/min flow rate, gradient elution of 5 mM ammonium formate with 0.1% formic acid in ultrapure water (A) and 0.1% formic acid in acetonitrile. Pharmaceuticals; 1. Metformin, 2. Amoxicillin, 3 & 4. Gabapentin & Gabapentin-d4, 5. Trimethoprim-d5, 6. Trimethoprim, 7-9. Ciprofloxacin, O-desmethyl venlafaxine and O-desmethyl venlafaxine-d6, 10 & 11. Sulfamethoxazole and Sulfamethoxazole-13C6, 12. Venlafaxine, 13. Erythromycin, 14. Carbamazepine-d10, 15. Carbamazepine, 16 &17. Clarithromycin & Azithromycin, 18 & 19. Diclofenac & Diclofenac-d4

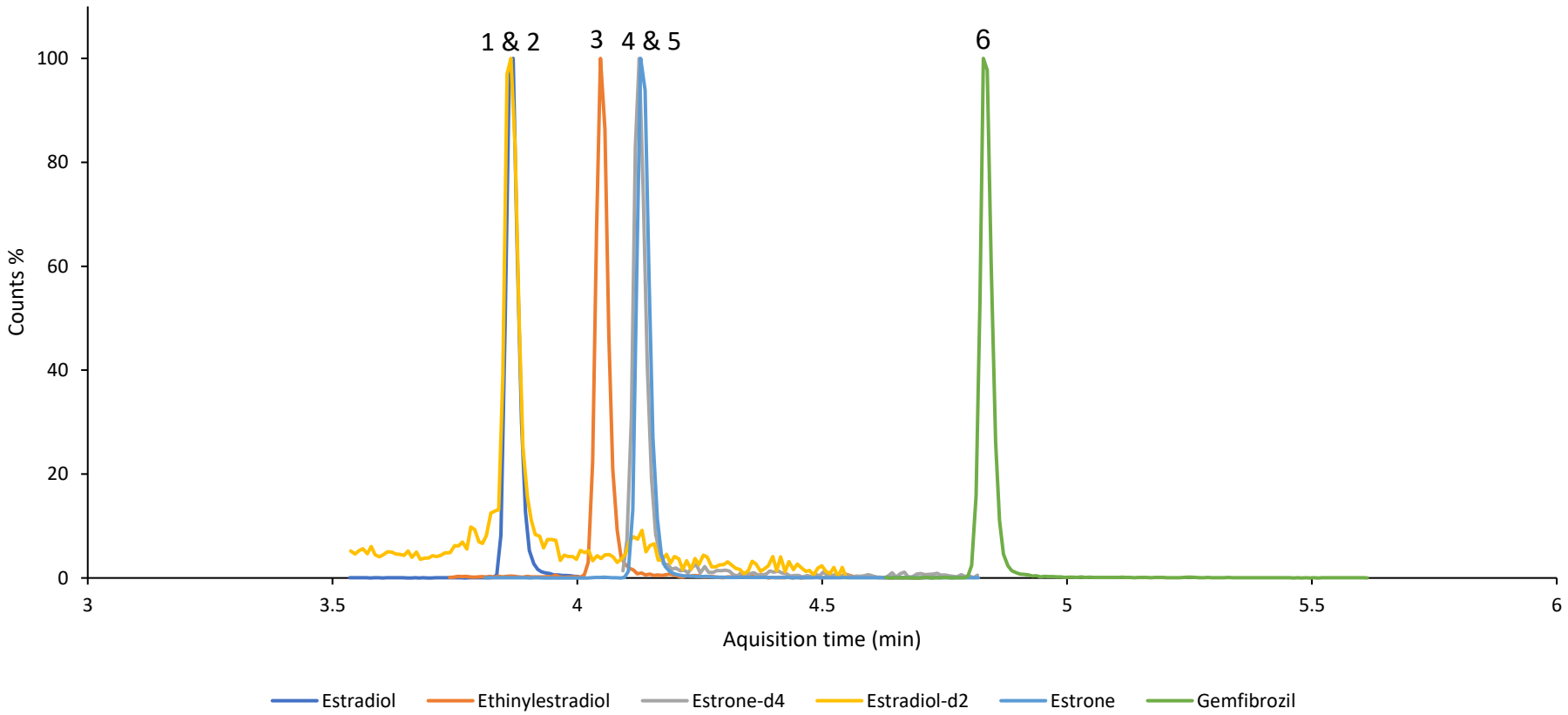


Figure 23. Peak-to-peak adjusted MRM separation of a 700 ng/L matrix spiked with pharmaceuticals and internal standards ran with Method 2, using a Zorbax eclipse plus C18 2.1 x 50 mm 1.8 µm LC column equipped with a Zorbax eclipse plus C18, 2.1 x 5 mm guard column, 0.4 mL/min flow rate, gradient elution of 5 mM ammonium formate with 0.1% formic acid in ultrapure water (A) and 0.1% formic acid in acetonitrile. Pharmaceuticals; 1 & 2. E2 & E2-d2, 3. EE2, 4 & 5. E1 & E1-d4, 6. Gemfibrozil.

Table 15: Chromatographic parameters for analytes ran with Zorbax eclipse plus C18 column in extracted matrix spiked with 700 ng/L of pharmaceutical mix and internal standard using the updated LC-MS/MS methods 1 and 2.

Pharmaceutical	Rt (min)	Capacity Factor k	Symmetry
Method 1			
Metformin	0.482	0.21	0.95
Amoxicillin	1.102	1.76	1.35
Gabapentin	2.299	4.75	0.79
Gabapentin-d4	2.654	5.64	0.96
Trimethoprim	3.361	7.40	1.68
Trimethoprim-d9	3.290	7.23	1.34
Ciprofloxacin	3.949	8.87	1.21
O-desmethyl venlafaxine	3.828	8.57	1.29
O-desmethyl venlafaxine-d6	3.971	8.93	1.58
Sulfamethoxazole	5.122	11.81	1.14
Sulfamethoxazole-13C6	5.156	11.89	1.22
Venlafaxine	5.883	13.71	1.34
Erythromycin	7.700	18.25	1.22
Carbamazepine	7.856	18.64	1.20
Carbamazepine-d10	7.760	18.40	1.16
Azithromycin	9.211	22.03	1.37
Clarithromycin	9.215	22.04	1.15
Diclofenac	10.672	25.68	1.13
Diclofenac-d4	10.669	25.67	2.05
Method 2			
Ethinylestradiol (EE2)	3.868	8.67	1.57
Estradiol (E2)	4.047	9.12	1.24
Estradiol-d4	3.866	8.67	1.4
Estrone (E1)	4.129	9.32	1.11
Estrone-d4	4.125	9.31	1.39
Gemfibrozil	4.843	11.11	1.20

2.4.2.6 Method performance

Utilising LC-MS/MS for pharmaceutical analysis provided clear advantages over HPLC-UV as it helped include pharmaceuticals that did not contain chromophores, reduced necessary sample volumes as a result of increased sensitivity and reduced analysis time, reduced matrix interference and increased method sensitivity.

Method calibration and validation experiments were executed in accordance with the method outlined in 2.4.2. The method developed was adapted to configured with the use of a separate column (Zorbax eclipse plus C18 2.1 x 50 mm 1.8 µm LC column

equipped with a Zorbax eclipse plus C18, 2.1 x 5 mm) as it reduced overall method run time.

Seventeen pharmaceuticals were extracted from a pharmaceutical composite in the range of 0 ng/L to 2000 ng/L. With the exception of EE2, which was not detected, and metformin and amoxicillin, analyte recoveries were between 80-131% with linearity R^2>0.96, which were deemed acceptable due to the complexity of the sample matrix tested (Table 16).

Surface water samples were acidified as a preservation step, and this may have led to the low recovery rates observed with metformin.[259] In acidic environments, metformin has a low distribution coefficient due to its doubly charged cationic form, rendering the molecule highly polar and, therefore, reducing retention on Oasis HLB cartridges.[259] Therefore, maintaining a basic pH during extraction is recommended to increase metformin's hydrophobicity and improve recoveries.[259] Previous studies have additionally shown that recovery rates for metformin can often be below 1% due to its high polarity.[304] However, with the proposed method validation via matrix matching, the loss in analyte recovery will account for this reduced recovery rate.

Method LOQs shown in Table 16 were below the recommended maximum LOQ by the European Union Joint Research Centre (JRC), with the exception of oestrogens, which are often noted for their analytical challenges in meeting the required detection limits.[232,305] These challenges are a result of their rapid degradation and low environmental concentrations. Furthermore, as quantification limits are commonly set in accordance with predicted no-effect concentrations (PNEC), the low LOQ-PNEC criterion set for oestrogens by the Water Framework Directive (WFD) (PNEC: 17-alpha-

Ethinylestradiol = 0.035 ng/L, 17-beta-Estradiol = 0.4 ng/L, Estrone = 3.6 ng/L) has highlighted the need for a global effort in improving method sensitivity for these free and conjugated oestrogens.[73] While other analytical methods, like gas chromatography and radioimmunoassay, have been employed for oestrogen analysis, SPE–LC–MS/MS is currently the most favourable analytical technique in use.[305]

SPE–LC–MS/MS optimisation work to improve method sensitivity was previously conducted by Hands et al. and Rapp-Wright et al., where parameters such as increased sample volume. However, employing larger extraction volumes provided no significant improvements in the LOD while presenting other analytical challenges, including increased matrix effect, increased preparation time and a higher risk for degradation.[232,276] Therefore, the extraction volume of 100 mL was selected.

Currently, the mechanisms of the underlying matrix effects is not fully understood. However, it has been attributed to the presence of co-eluting constituents, which are competing for droplet surface and available charges and in Electro Spray Ionisation (ESI), leading to matrix-induced suppression or enhancement of target analytes. [306–308] The matrix enhancement observed with Gemfibrozil can be associated to its higher $\log_{kow}$ as it would have a greater affinity for organic particulates in the matrix rather than in the aqueous phase.

Table 16: Calibration and validation results of pharmaceuticals from a composite sample made from the Nore/Liffey/Suir/Analee surface water grab samples. Matrix-matched calibration curves are included in Figure A 1 found in the appendix.

Pharmaceutical	Internal standard used (at 700 ng/L)	LOD (ng/L)	LOQ (ng/L)	R^2 (n ≥ 5)	Recovery% ± %RSD (n = 3 at 700 ng/L)	Matrix effects ratio	% Matrix effect ± %RSD (n = 3 at 700 ng/L)
Metformin	O-desmethyl venlafaxine-d5	1.74	5.26	0.982	19.38 ± 13.38	0.92	92.4 ± 13.37
Amoxicillin	Trimethoprim-d9	6.51	19.72	0.982	52.15 ± 38.57	6.72	671.56 ± 38.57
Gabapentin	O-desmethyl venlafaxine-d5	2.98	9.03	0.982	101.2 ± 3.57	0.96	96.16 ± 35.7
Trimethoprim	Trimethoprim-d9	1.37	4.14	0.994	127.96 ± 3.26	2.41	240.9 ± 32.6
Ciprofloxacin	Trimethoprim-d9	0.77	2.32	0.989	112.11 ± 0.59	1.07	106.56 ± 5.9
Sulfamethoxazole	Sulfamethoxazole-13C6	0.91	2.76	0.997	129.61 ± 2.39	0.9	89.77 ± 23.9
O-Desmethyl Venlafaxine	O-desmethyl venlafaxine-d5	0.82	2.48	0.997	99.03 ± 1.05	3.24	323.56 ± 10.5
Venlafaxine	O-desmethyl venlafaxine-d5	1.58	4.78	0.99	98.4 ± 0.94	1.26	126.35 ± 9.4
Carbamazepine	Carbamazepine-d10	1.63	4.94	0.989	112.43 ± 9.47	1.08	108.41 ± 94.7
Erythromycin	Carbamazepine-d10	6.21	18.82	0.973	89.16 ± 8.85	0.98	98.41 ± 8.41
Clarithromycin	Carbamazepine-d10	2.51	7.61	0.984	116.6 ± 4.5	1.95	195.27 ± 4.5
Azithromycin	Carbamazepine-d10	2.80	8.50	0.980	118.07 ± 3.91	1.84	184.02 ± 3.91
Diclofenac	Carbamazepine-d10	1.51	4.59	0.986	96.79 ± 2.85	1.51	151.3 ± 2.85
Gemfibrozil	Estrone-d4	1.63	4.93	0.984	80.36 ± 6.5	14.46	1445.81 ± 6.5
E1	Estrone-d4	2.64	8.01	0.968	130.94 ± 1.6	1.22	122 ± 1.6
E2	Estrone-d4	4.44	13.45	0.99	120.43 ± 4.58	0.74	74.05 ± 4.58

Additional sampling was conducted in three locations on the River Liffey (upstream, at the WWTP outfall and downstream) and five locations in Donegal; further details on site location can be found in Chapter 5 (section 5.2.2). Table 17 shows the results of the method calibration and validation experiments, which were executed in Dublin City University using the same method parameters as above at alternate sampling sites with a different LC-MS/MS instruments (however, the same model). Pharmaceutical concentrations were calculated through the creation of matrix-matched calibration curves, which can be found in Figure A 1 in the appendix. The results from Table 17 showed that the method developed here has good reproducibility and robustness.

Furthermore, as the method was applied to a separate instrument (although the same model of instrument), a slight improvement in the method sensitivity was observed when comparing LODs and LOQs from Table 16 and Table 17. This improvement in sensitivity could be a result of the instrument itself and/or the composition of the matrix tested. Most notably, metformin was observed to encounter significant matrix-induced ion suppression in comparison to Table 16, where minimal suppression was seen. Furthermore, the antibiotic amoxicillin experienced matrix enhancement in both sampling campaigns. However, matrix enhancement in the river Liffey/Donegal sampling campaign was much greater (2737.69%) than observed in Table 16. Amoxicillin has been previously reported to be particularly affected by matrix interference, with previous studies showing over 4000% matrix enhancement.[232] Matrix interference is a commonly reported analytical challenge during HPLC-UV and LC-MS/MS analysis of surface water.[309–312] While SPE can reduce matrix interference, it may not be eliminated entirely for all target analytes, as the interfering compounds can also become concentrated with the pharmaceuticals, giving rise to a notable matrix effect.[313]

As field measurements were assessed in both sampling campaigns it was observed that the site locations for the Liffey/Donegal sampling campaign had a lower conductivity (30.3 – 428.7 µS/cm) and turbidity (0.58 – 8.56 FNU) than the Liffey/Nore/Suir/Analee sampling campaign whose conductivity were 149.6 – 629.8 µS/cm and 0.61 – 17.22 FNU respectively. The lower turbidity and conductivity levels in the Liffey/Donegal sampling campaign in comparison to the river Liffey, Nore, Suir and Analee sampling campaign could explain the reduction in % matrix effect of gemfibrozil, trimethoprim and O-desmethyl venlafaxine (Table 16 and Table 17). The higher conductivity levels in the Liffey/Nore/Suir/Analee sampling campaign indicate the presence of more ions in a sample than in the Liffey/Donegal sampling campaign. These ions can play a contributing role to matrix interference as they can affect the ionization process and potentially causing ion suppression or enhancement during mass spectrometric analysis. Furthermore, high turbidity levels may play a contributing role to matrix enhancement or suppression as a result of analytes of interest sorbing onto suspended particles in a matrix. [232,314]

Table 17: Calibration and validation results of pharmaceuticals from a composite sample made from the Liffey/ Donegal surface water grab samples. Matrix-matched calibration curves are included in Figure A 1.4 found in the appendix.

Pharmaceutical	Internal standard used (at 700 ng/L)	LOD (ng/L)	LOQ (ng/L)	R^2	%Recovery ± %RSD (n =3 at 700 ng/L)	Matrix effects ratio	% Matrix effect ± % RSD (n =3 at 700 ng/L)
Metformin	O-desmethyl venlafaxine-d5	2.71	8.22	0.9681	0.47 ± 14.37	0.06	6.12 ± 14.37
Amoxicillin	O-desmethyl venlafaxine-d5	3.09	9.37	0.9786	58.99 ± 13.99	27.38	2737.69 ± 13.99
Gabapentin	O-desmethyl venlafaxine-d5	1.61	4.89	0.9883	24.37 ± 11.54	7.41	740.75 ± 11.54
Trimethoprim	O-desmethyl venlafaxine-d5	1.06	3.22	0.9899	96.74 ± 3.77	0.83	83.12 ± 3.77
Ciprofloxacin	O-desmethyl venlafaxine-d5	1.26	3.83	0.9835	55.98 ± 9.66	1.07	106.53 ± 9.66
Sulfamethoxazole	Sulfamethoxazole-13C6	0.39	1.18	0.9986	100.44 ± 1.91	1.02	101.57 ± 1.91
O-Desmethyl Venlafaxine	O-desmethyl venlafaxine-d5	0.35	1.05	0.9989	123.14 ± 1.79	1.04	103.58 ± 1.79
Venlafaxine	O-desmethyl venlafaxine-d5	1.08	3.27	0.9896	124.94 ± 2	1.36	135.88 ± 2
Carbamazepine	Carbamazepine-d10	0.49	1.48	0.9979	95.39 ± 2.74	1.05	104.61 ± 2.74
Erythromycin	Carbamazepine-d10	1.20	3.63	0.9951	74.42 ± 12.46	1.26	125.98 ± 12.46
Clarithromycin	Carbamazepine-d10	0.89	2.69	0.9918	83.02 ± 5.81	1.11	110.95 ± 5.81
Azithromycin	Carbamazepine-d10	0.83	2.51	0.9928	82.39 ± 7.16	1.14	113.95 ± 7.16
Diclofenac	Carbamazepine-d10	1.93	5.84	0.9793	48.39 ± 11.06	1.26	126.06 ± 11.06
Gemfibrozil	Estrone-d4	1.23	3.72	0.9941	94.96 ± 6.1	1.38	138.1 ± 6.1
E1	Estrone-d4	0.40	1.20	0.9985	105.72 ± 4.25	0.91	91.1 ± 4.25
E2	Estrone-d4	2.24	6.80	0.9821	87.93 ± 7.06	0.85	85.4 ± 7.06
EE2	Estrone-d4	0.89	2.71	0.9948	100.28 ± 2.98	1.40	139.57 ± 2.98

In Figure 24, two calibration curves are shown. The calibration curve created from reference standards achieved a higher slope than with a matrix-matched calibration curve. A lower slope in matrix-matched calibration standards can attributed to analyte loss during sample extraction and/or the presence of interfering compounds within a matrix affecting analyte response. This highlights the influence of matrix on method sensitivity and the importance of considering sample matrix during method development.

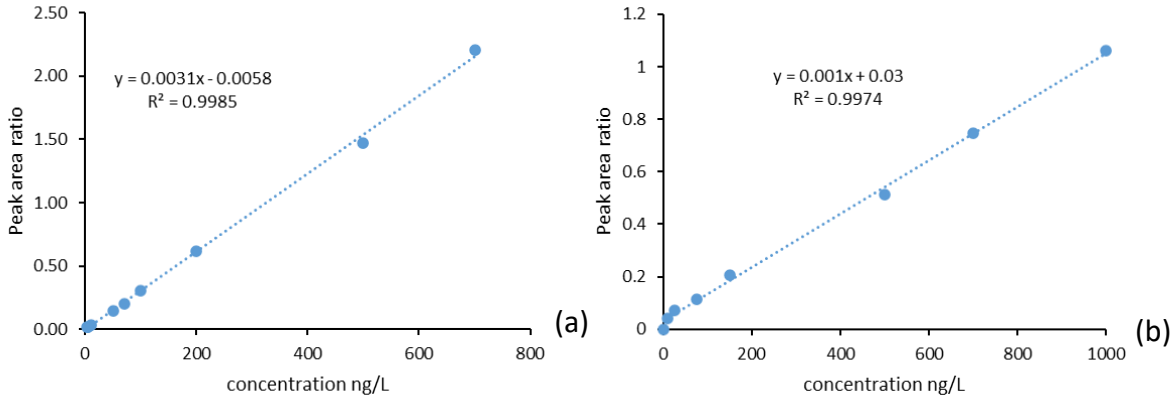
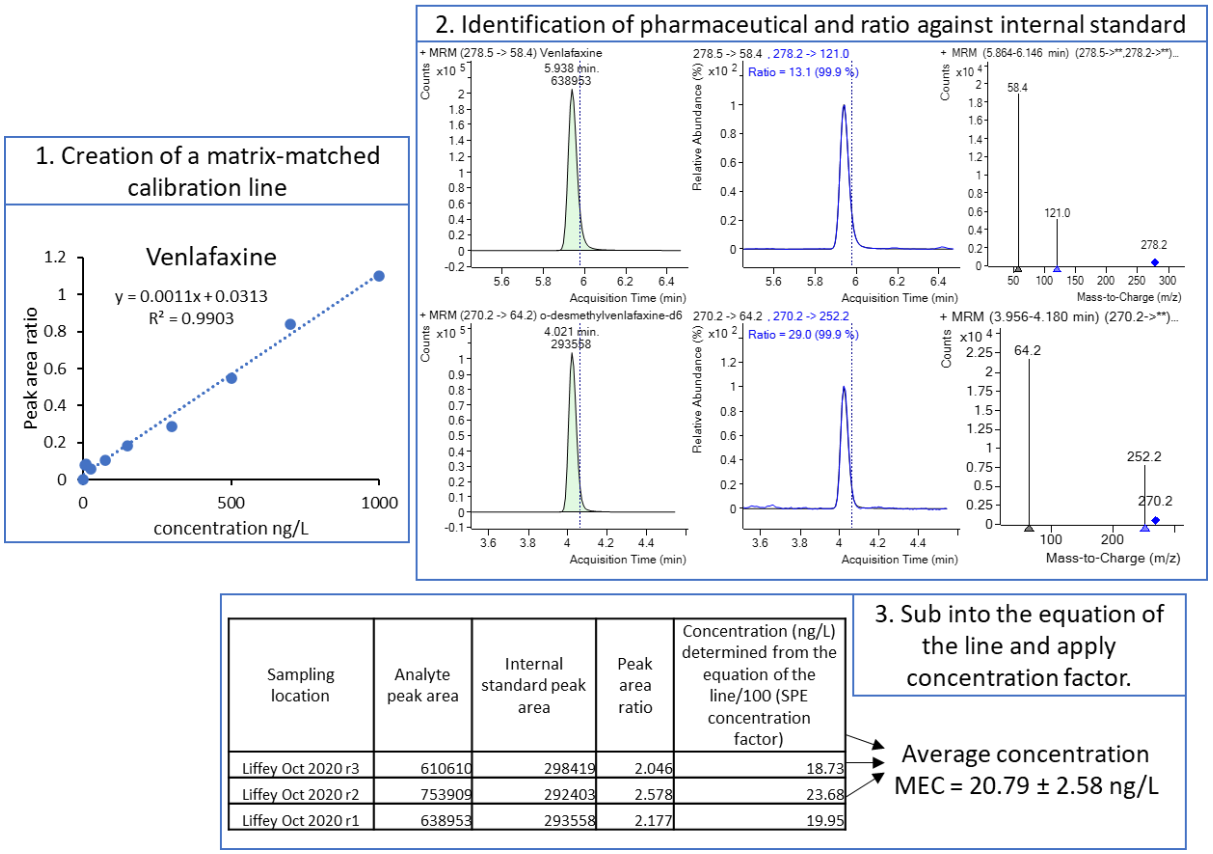


Figure 24: Calibration curve of the pharmaceutical sulfamethoxazole acquired from direct injection of reference standards (a) and matrix-matched calibration (b).

Following the identification of the qualifier and quantifier along with the associated ion ratio and retention time, the concentrations of the detected pharmaceuticals were determined through peak area ratios of detected pharmaceuticals and associated internal standards. This involved applying the equation of the line and dividing by the concentration factor (100). An example of the quantification of pharmaceutical concentrations can be seen in Figure 25.



Sampling location	Analyte peak area	Internal standard peak area	Peak area ratio	Concentration (ng/L) determined from the equation of the line/100 (SPE concentration factor)
Liffey Oct 2020 r3	610610	298419	2.046	18.73
Liffey Oct 2020 r2	753909	292403	2.578	23.68
Liffey Oct 2020 r1	638953	293558	2.177	19.95

Figure 25: Example workflow used to determine and quantify pharmaceuticals in river water samples.

2.4.3 Contamination

Contamination, whether from sample carryover, system components, or environmental factors, can compromise the accuracy and reliability of analytical results. The presence of contaminants poses a significant challenge as surface water sampling often involves the analysis of trace-level contaminants that require low detection levels.

One common source of contamination is sample carryover from a previous injection persisting in the LC-MS/MS system.[315] This can be observed in blue in Figure 26, where two peaks of venlafaxine was observed along with the presence of several other pharmaceuticals.

Appropriate sample cleanup, such as SPE, can minimise the presence of problematic contaminants entering the system. Furthermore, the inclusion of solvent-only samples (e.g. starting mobile phase and ACN) between sample runs can help identify contamination as a result of sample carryover. Additional steps include using high-purity solvents and the frequent flushing and cleaning of components prone to contamination, such as the column, injector, capillaries, spray chamber, and ion capillary.

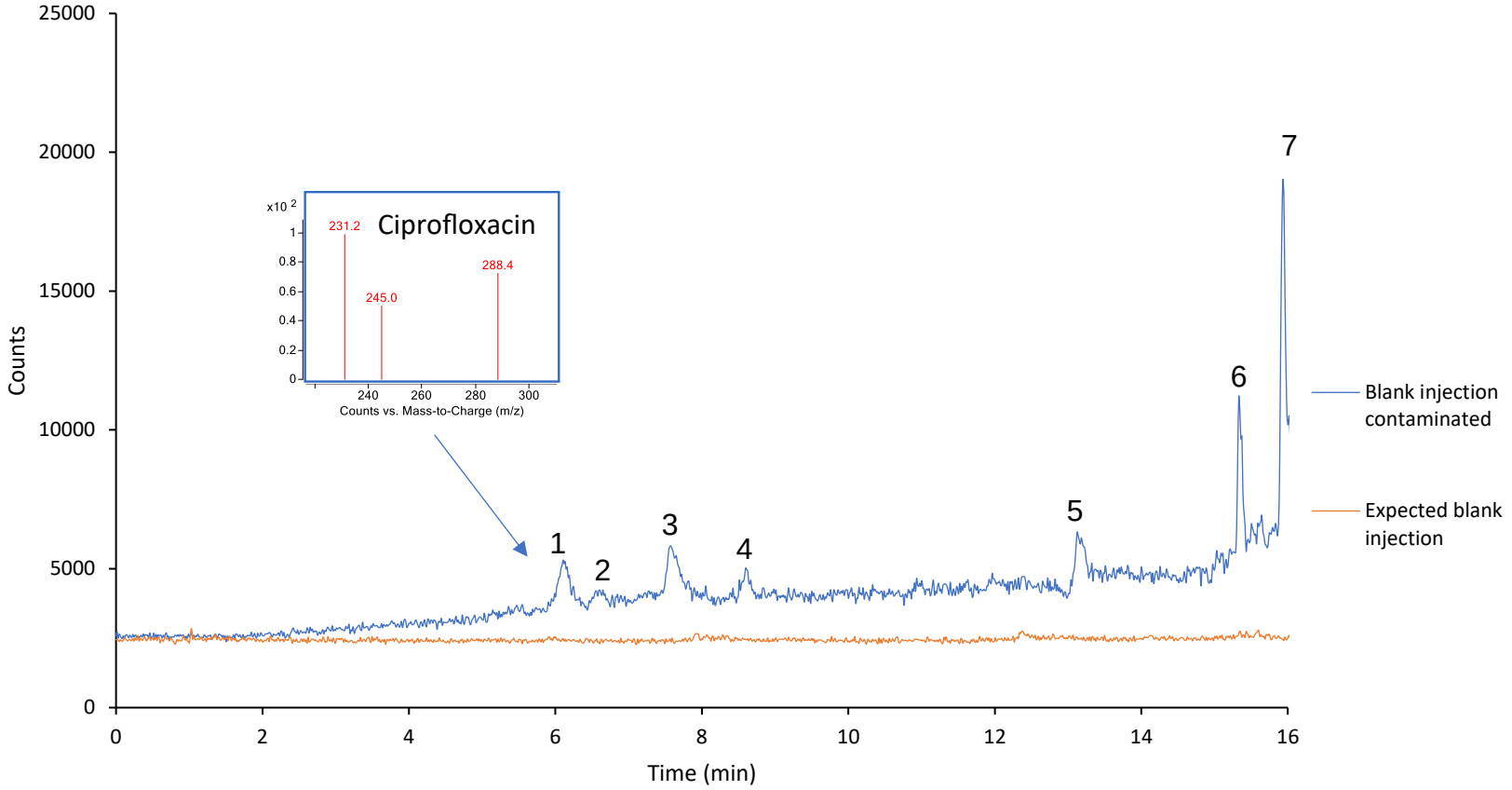


Figure 26: TIC example of blank injections from clean blank injection (orange) and contaminated (blue) confirmed by MRM. Ciprofloxacin (1), O-Desmethylvenlafaxine (2), sulfamethoxazole-13C6 (3), venlafaxine (4), sulfamethoxazole (5), O-desmethyl venlafaxine (6), venlafaxine (7).

2.5 Conclusion

The objective of this chapter was to develop and validate an analytical method to monitor pharmaceuticals at environmentally relevant concentrations in surface water environments. Initially, a SPE-HPLC-UV method was developed to analyse eleven pharmaceuticals with 1L sample extraction volume to reach environmentally relevant concentrations. However, complications arising from the extraction of large volume SPE and the substantial matrix interference associated with this large sample volume prevented further sample analysis. Therefore, a SPE-LC-MS/MS method was developed for further analysis.

The utilization of LC-MS/MS for pharmaceutical analysis has demonstrated significant advantages over HPLC-UV. This analytical approach has not only expanded the scope of detectable pharmaceuticals by enabling the analysis of compounds without chromophores but has also allowed for the reduction of required sample volumes and analysis time, minimized matrix interference, and enhanced method sensitivity.

The LC-MS/MS method developed incorporated 17 pharmaceuticals which have been highlighted as a potential risk to aquatic organisms. Furthermore, the achieved linearity was acceptable for all pharmaceuticals tested with an $R^2 > 0.96$ and analyte recoveries fell within an acceptable range, considering the complex sample matrix. The successful analysis of different sampling locations and instruments has indicated the method's reproducibility and robustness across varying matrices.

It is worth considering that some challenges, such as meeting low detection limits, are especially pertinent during oestrogen analysis due to their instability and low environmental concentrations. Furthermore, the substantial matrix interference observed is a frequently

reported issue in surface water analysis. These challenges highlight the importance of considering environmental factors in water analysis.

The work presented in this chapter demonstrates the development and assessment of analytical methods to monitor pharmaceuticals in surface waters. The application of the developed method in collected surface water samples has helped to provide valuable insight into pharmaceutical contamination in Irish rivers to inform future monitoring campaigns.

Chapter 3 :

Determination of pharmaceuticals in surface water samples

3.1 Introduction

The use of pharmaceuticals for the treatment of livestock, companion animals and human diseases/ailments has led to the contamination of tap, ground and surface waters. With many of these pharmaceuticals having the potential to cause harmful effects on aquatic organisms and ecosystems, this contamination has led to concerns within the European Union and the publication of a plethora of associated scientific research studies, which has been increasing annually (Figure 27).[316,317]

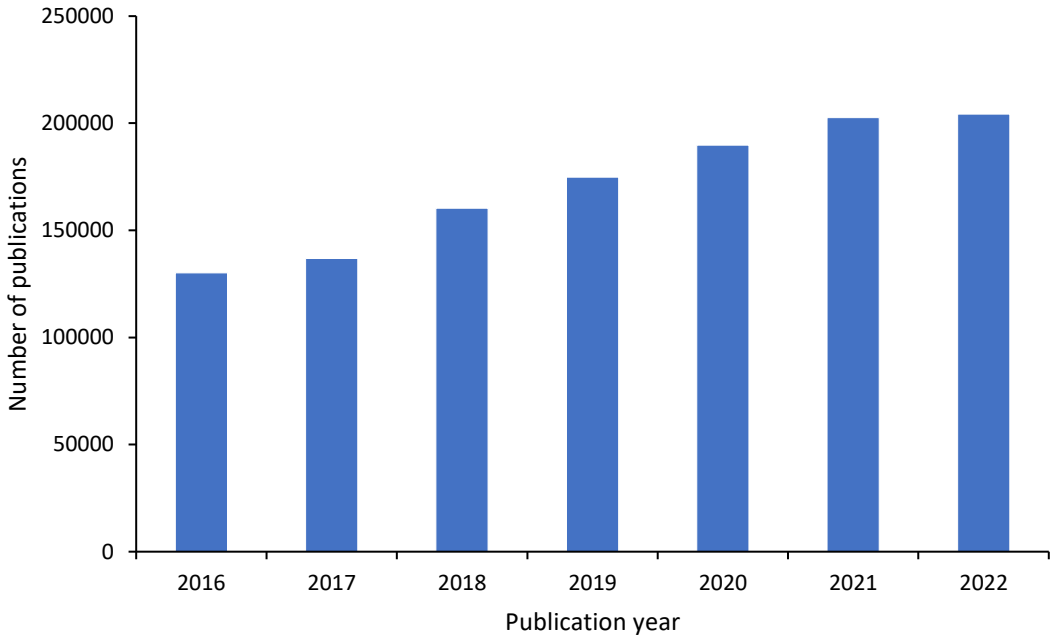


Figure 27: Published literature accessible from Web Of Science using keywords "pharmaceutical" or "drugs" or "medicine" or "API" and "surface water" or "river water" or "freshwater" from 2016-2022.

Pharmaceuticals prescribed for human consumption can enter into surface water environments in their parent or metabolite form via discharge from wastewater treatment plants (WWTP), improper disposal and runoff from agricultural regions.[42]

Although the pharmaceuticals selected in this thesis are frequently detected in European rivers (see Chapter 1, Table 3), there is limited data available surrounding their occurrence in

Irish rivers as studies predominantly focus on influent, effluent and marine surface waters rather than river water and do not include many of the pharmaceuticals from past or present Water Framework Directive (WFD) watchlists. However, given the appropriateness of the studies from a geographical perspective, these surface water studies were analysed to obtain an initial representation of categories of pharmaceuticals detected in Irish surface waters.

Quinn et al. examined Galway and Dublin Bay effluent and seawater for carbamazepine, diclofenac, trimethoprim and gemfibrozil.[318] Concentrations in WWTP effluent ranged from 0.2-3.9 µg/L for carbamazepine, 0.2-2.8 µg/L for diclofenac, 0.1-1.6 µg/L for trimethoprim and, <0.04-1.7 µg/L for gemfibrozil. While seawater concentrations ranged from 0.004-2 µg/L for carbamazepine, <0.02-0.7 µg/L for diclofenac, <0.006-0.9 µg/L for trimethoprim and <0.04-1 µg/L for gemfibrozil.

A 2012 study by Lacey et al. investigated carbamazepine, diclofenac, gemfibrozil, and trimethoprim investigated influent and effluent at three WWTP locations (Ringsend, Leixlip and Swords)[82] with concentrations ranging from <LOQ-6.5 µg/L for carbamazepine, <LOD-2.95 µg/L for diclofenac, <LOD-0.15 for gemfibrozil and <LOD-15.7 µg/L for trimethoprim.

Rapp Wright et al. 2021, investigated the presence of amoxicillin, ciprofloxicin, E1, E2, EE2, erythromycin, carbamazepine, and venlafaxine in surface waters at undisclosed rural and urban areas in Ireland from 2018-2019.[276] In this study, amoxicillin and erythromycin were predominantly <LOD with some detections <LOQ at both sites, ciprofloxicin was <LOD at the rural site and <LOD-5 ng/L at the urban site, E1, E2 and EE2 were <LOD-LOQ at both sampling locations, trimethoprim ranged between <LOD-LOQ and was only measured at the urban sampling site. Venlafaxine levels were <LOD at the rural sampling site but ranged between <LOD-32 ng/L at the urban sampling site.

Hence, the pharmaceuticals shown in Table 18 were selected to expand upon previous work and to improve understanding of the presence of pharmaceuticals recognised as potentially hazardous to aquatic ecosystems.

Table 18: Pharmaceuticals selected, their chemical structures and prioritisation.[73,230,271–275,320]

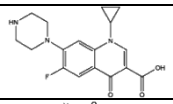
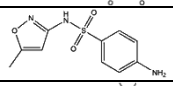
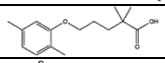
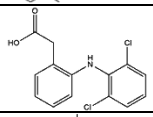
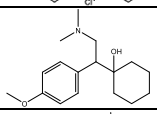
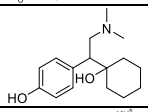
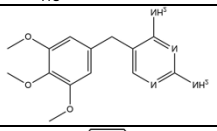
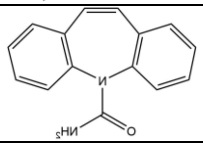
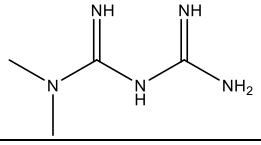
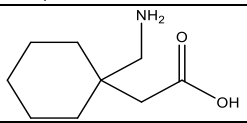
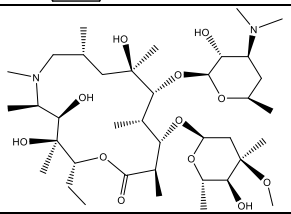
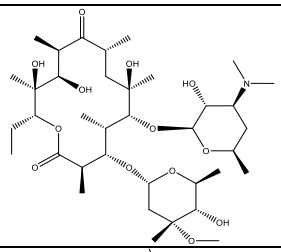
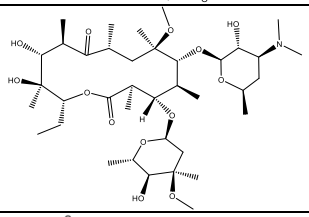
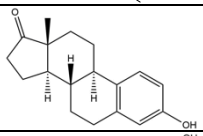
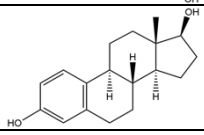
Pharmaceutical	Structure	Prioritisation
Ciprofloxacin (Fluoroquinolone antibiotics)		Inclusion in the 2nd & 3rd Watchlist
Sulfamethoxazole (Sulphonamide antibiotic)		Inclusion in the 3rd & 4th Watchlist
Gemfibrozil (Lipid regulators)		Provisional Candidate for 5th Watchlist
Diclofenac (Anti-inflammatory)		Priority substance
Venlafaxine (Antidepressant)		Inclusion in the 3nd & 4th Watchlist, Provisional Candidate for 5th watch list
O-Desmethylvenlafaxine (Antidepressant metabolite)		Inclusion in the 3nd & 4th Watchlist, Provisional Candidate for 5th Watchlist
Trimethoprim (Antibiotic)		Inclusion in the 3nd & 4th Watchlist, Provisional Candidate for 5th Watchlist
Carbamazepine (Anticonvulsant)		Priority substance
Metformin (Biguanide)		Inclusion in the 4th and Provisional Candidate for 5th Watchlist
Gabapentin (Anticonvulsant)		Provisional Candidate for 5th Watchlist
Azithromycin (Macrolide antibiotic)		Priority substance

Table 18 (continued): Pharmaceuticals selected, their chemical structures and prioritisation.[73,230,271–275,320]

Pharmaceutical	Structure	Prioritisation
Erythromycin (Macrolide antibiotic)		Priority substance
Clarithromycin (Macrolide antibiotic)		Priority substance
E1 (Steroid hormone)		Priority substance
E2 (Steroid hormone)		Priority substance

The implementation of monitoring strategies can help improve our understanding of the effect that human activities have on the environment and identify areas where mitigation strategies would be beneficial to protect the environment. Monitoring surface waters can serve as a valuable indicator for public health; for example, monitoring illicit drugs could identify regions where misuse is occurring, thus informing authorities of the need for increasing spending on substance abuse programs and policing. The determination of community use/abuse and health trends of pharmaceuticals through the analysis of wastewater and surface water has been conducted in several European countries, including Ireland, Italy, Switzerland, Spain and Belgium.[321] Monitoring for the presence, concentration, and types of pharmaceuticals in surface waters may provide valuable insight into the level of medication within a community that is serviced by a WWTP. This information could provide critical insight into disease patterns and health conditions.

Elevated levels of specific pharmaceuticals may help identify public health issues, and authorities could use this information to recognise challenges a community may face and implement strategies to address these issues.[322] For example, the presence of antidepressants or antibiotics can help to identify overprescription in a community, highlight the need for education surrounding the appropriate disposal of these medications or indicate a public health issue in that area.[323]

The influence of environmental factors such as pandemics (e.g. H1N1 virus) and natural disasters have been linked with mental illness and respiratory and cardiovascular diseases, resulting in increased consumption and prescription of pharmaceuticals.[60] To date, no Irish study has investigated the effect of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) pandemic on surface water concentrations of pharmaceuticals. However, there is a risk that, as with previous pandemics, the increased consumption of pharmaceuticals may lead to elevated environmental levels.[324]

Further research and monitoring campaigns are needed to better inform on the presence and associated risks posed by APIs in Irish surface waters to inform policymakers and governmental officials to create prevention and mitigation strategies such as improving WWTPs to include additional treatment technologies, increasing funding to public awareness and pharmacy takeback schemes.

3.2 Aims and objectives

The aim of this chapter is to determine the occurrence and frequency of pharmaceuticals in key Irish rivers and to show how environmental factors such as public health crises can influence their environmental presence.

Objectives of this research

- Investigate potential sources of pharmaceutical pollution at sampling sites.
- Using the methods developed in Chapter 2, generate a dataset for pharmaceutical occurrence from field samples collected in the Nore, Liffey, Suir and Analee rivers.
- Assess temporal trends in pharmaceutical occurrence and in the context of the COVID-19 pandemic.

3.3 Experimental

3.3.1 Materials and methods

All materials and methods used are detailed in Chapter 2, section 2.3, with validation parameters detailed in Table 16 in Section 2.4.2.6 of this thesis. Additionally, the results presented were calculated as described in section 2.4.2.6 of this thesis.

3.3.2 Sampling and study area

Surface water grab samples were collected at 4 sample sites in four catchments across Ireland (Figure 28), with samples collected in September 2020, October 2020, March 2021, May 2021, September 2021 and March 2022. The sampling sites were selected as they were used for previous and existing EU WFD Watch list monitoring sites (2013-2021), which were advised by the 2013 EQS directive (2013/39/EU). River samples were collected at locations that are influenced by urban, rural and agricultural inputs with a range of water quality (Q-value) health statuses. The Q-value system used by the EPA can help to reflect the presence of environmental pollutants by assessing macroinvertebrate abundance, diversity and identified sensitivity. The Q-values derived from this assessment fall within a nine-point scale from a Q-value of 1, indicating poor quality, to a Q-value of 5, indicating high water quality.[325] Environmental parameters of the water were additionally taken (e.g. pH, conductivity, temperature, turbidity and dissolved oxygen). A summary of the sampling sites and potential pressures is detailed below.

Figure 28: Map of Ireland with grab sampling locations in the Rivers Analee, Liffey, Nore and Suir for pharmaceutical detection from September 202 to March 2022.

The water quality of the River Nore sampling sites increased from moderate to good from 2016-2018, assessed by fish status. However, it was downgraded to moderate (q value of 3-4) in 2022.[326] Concurrently, chemical conditions dropped from high to good. Upstream of the Nore sampling site, there are several wastewater treatment plants (WWTPs) which drain directly into the river: Thomaston (7500 PE, tertiary treatment with added phosphorus removal); Bennetsbridge (475 PE, primary treatment); Kilkenny (77000 PE, tertiary phosphate removal); Ballyraggart (1920 PE, secondary treatment); Castletown (500 PE, secondary treatment) and Borris-in-Ossory (1626 PE, secondary treatment) with possible domestic misconnections.[326] Additionally, as the Nore passes through rural areas, the large volume of septic tanks used may contribute to the overall levels of pharmaceuticals present as it is in

the locality of well-drained soils. Finally, the Nore River has a protected status and a history of good water quality without any identified significant pressures.[327,328]

Upstream of the River Annalee sampling site, the water quality is considered to be of good water quality status (q value of 4) as of 2022. However, the Cavan River, which is classified as poor (q value of 3), enters the Analee at the sampling site.[326] The Cavan River faces considerable challenges due to the impact of urban and agricultural pressures. WWTPs influencing the sampling site include upstream at Butlersbridge, which is just upstream of the sampling point (<500 PE, Primary treatment); Cavan (30000 PE, tertiary phosphate removal); Ballyhaise (905 PE, tertiary phosphate removal); Shercock (1000 PE, tertiary phosphate removal) and is identified to be connected with a large volume of septic tanks.[326] However, the soil in the locality is poorly drained.

The River Suir sampling site water quality declined from good to moderate from 2016 to 2018, assessed by invertebrate status and has been recorded as moderate (Q value of 3-4) in 2022.[326] Significant pressures recorded in the River Suir include agriculture and urban runoff. Upstream of sampling sites, there are several small and medium-sized wastewater treatment facilities: Clonmel (80000 PE tertiary phosphate removal), Ardfinnan (1100 PE, tertiary phosphate removal), Cahir (5000 PE, tertiary phosphate removal), Holycross (600 PE, tertiary phosphate removal), Thurles (15000 PE, tertiary phosphate removal) and Templemore (6000 PE, tertiary phosphate removal) with possible domestic misconnections and a large number of large volume septic tanks in well-drained soils.[326] Although the Suir is categorised as not at risk, future projections indicate that the river may transition into an at-risk river.[232]

The River Liffey sampling site water quality improved from moderate to good from 2016-2018 but returned to moderate in 2019-2022.[326] Urban runoff and urban wastewater were

identified as significant pressures in the River Liffey. For example, raw sewage was released in the Rye River, which is a tributary to the Liffey near the sampling point, between September 2003 and June 2022, with the latter release killing over 500 brown trout.[329–331] The River Liffey sampling site is downstream of several wastewater treatment plants in the Liffey catchment, including Golden Falls (2000 PE, tertiary treatment with phosphate removal, Osberstown (130000 PE, tertiary treatment with phosphate removal) and Leixlip (150000 PE, tertiary treatment equipped with added nitrogen and phosphate removal) with possible domestic misconnections.[326] The WWTP located at Leixlip additionally has been reported to have had several instances of uncontrolled release of wastewater with 6 in 2019 and 8 in both 2020 and 2021.[332] Furthermore, large pharmaceutical facilities are connected to WWTPs at Leixlip and Osberstown. Additionally, the River Liffey contains tributaries which flow through moderately intensive agricultural and urban areas that could add to the total pollution load due to runoff. The sampling site at the River Liffey was selected based on its likelihood of being at a higher risk of pharmaceutical contamination due to urban pressures and WWTPs.

3.3.3 Environmental conditions

During the collection of these samples, conductivity, temperature, turbidity, pH and dissolved oxygen (DO) were recorded (Table 19). Conductivity at sampling locations ranged from 149.6 to 629.8 µS/cm, with the River Annalee having the lowest conductivity out of all sites. However, the conductivity levels recorded are characteristic of surface water systems. Although Dissolved Oxygen (DO) fluctuations were observed potentially as a result of temperature or anthropogenic factors, the DO for all sampling sites remained above 8 mg/L, indicating that the tested rivers are in good status to support aquatic organisms such as brown trout.[333] Surface water pH across all sites were predominantly alkaline and remained relatively stable across all sites. However, In September 2020, the pH of the Analee slightly

decreased, which may have been a result of anthropogenic sources such as agricultural runoff or wastewater discharge. However, without further assessment, no conclusive determination could be made.

Turbidity, which is caused by the presence of suspended particles, can pose a significant challenge to water quality monitoring as it can enhance matrix interference during sample analysis, thereby affecting the precision of measurements. For example, as shown in Chapter 2, section 2.4.6.2, significant matrix enhancement was observed for amoxicillin (671 %), O-Desmethyl Venlafaxine (323 %), and gemfibrozil (1445.81 %). Although matrix interference cannot be removed, it is an important environmental parameter to measure during environmental monitoring. [232]

For the River Nore, turbidity values range from 0.32 FNU in September 2021 to a relatively high value of 21.75 FNU in October 2020, which was associated to increased rainfall. In the River Liffey, turbidity values exhibit less variation, with the lowest value at 0.83 FNU in September 2021 and the highest at 4.65 FNU in March 2021. The River Suir shows a similar pattern, with turbidity values ranging from 0.61 FNU in September 2020 to 17.22 FNU in March 2022, with a spike in March 2022 as a result of rainfall. Finally, the River Annalee displays turbidity values ranging from 0.84 FNU in September 2021 to 4.48 FNU in October 2020. While there is some fluctuation, these values indicate moderate water clarity throughout the monitoring period.

Table 19: Environmental conditions during the River Nore, Liffey, Suir and Analee grab sampling campaign.

	Sample	Turbidity (FNU)	Temperature (°C)	DO (mg L^{-1})	pH	Conductivity (µS cm^{-1})
River Nore	**Sep-20**	1.31	15.573	8.77	7.18	475.1
	Oct-20	21.75	11.111	8.9	7.9	243.7
	Mar-21	1.75	8.464	10.65	8.23	396.5
	May-21	1.09	11.34	10.39	8.43	431.3
	Sep-21	0.32	15.727	9.11	8.42	534.6
	Mar-22	12.29	7.91	10.46	8.48	330.8
River Liffey	**Sep-20**	2.33	16.05	8.88	7.42	393.5
	Oct-20	3.12	11.68	9.29	8.03	412.4
	Mar-21	4.65	7.60	11.09	8.22	349.9
	May-21	2.99	13.94	10.62	8.42	629.8
	Sep-21	0.83	16.75	12.19	8.41	486.9
	Mar-22	3.56	6.79	10.90	8.42	455.2
River Suir	**Sep-20**	0.61	15.77	9.55	7.5	467.5
	Oct-20	1.64	11.18	9.70	8.13	399.3
	Mar-21	1.24	8.32	10.36	8.07	404.8
	May-21	1.62	12.36	10.86	8.36	466.3
	Sep-21	3.01	15.28	10.90	8.45	448.4
	Mar-22	17.22	7.51	10.41	8.42	371.7
River Annalee	**Sep-20**	2.7	15.97	7.12	6.2	247.9
	Oct-20	4.48	11.60	8.38	7.55	246.8
	Mar-21	3.24	7.25	9.88	7.42	193.6
	May-21	2.73	13.40	10.71	8.71	314.8
	Sep-21	0.84	16.08	8.04	8.02	282.9
	Mar-22	4.18	5.61	10.93	8.16	149.6

3.4 Results and discussion

Despite the increased global focus on pharmaceutical occurrence in river waters, limited research is being conducted in Ireland. This knowledge gap has hindered efforts to understand the scale and impact of pharmaceutical pollution and usage from a public health perspective. To address this knowledge gap, a set of 24 river water samples composed of 6 sampling time points in 4 locations were collected from September 2020 to March 2022. Method sensitivity employed in this study was below the maximum level of detection (LOD) for all pharmaceuticals, with the exception of Estrone (E1) and estradiol (E2) (Table 20). The concentrations of pharmaceuticals detected are detailed in Table 21 to Table 24. From the samples analysed, all 15 pharmaceuticals were detected above LOD, and 14 were detected above LOQ at least once in the 24 samples analysed.

Table 20: Method LOD/LOQ and Watchlist Maximum acceptable method detection or quantification limit.[230,270,272,275]

Pharmaceutical	LOD ng/L	LOQ ng/L	Maximum LOQ Policy requirement (ng/L)
Metformin	1.74	5.26	No guidance
Gabapentin	2.98	9.03	No guidance
Trimethoprim	1.37	4.14	100
Ciprofloxacin	0.77	2.32	89
Sulfamethoxazole	0.91	2.76	100
O-Desmethyl Venlafaxine	0.82	2.48	6
Venlafaxine	1.58	4.78	6
Carbamazepine	1.63	4.94	No guidance
Erythromycin	6.21	18.82	19
Clarithromycin	2.51	7.61	19
Azithromycin	2.80	8.50	19
Diclofenac	1.51	4.59	10
Gemfibrozil	1.63	4.93	No guidance
E1	2.64	8.01	0.035
E2	4.44	13.45	0.4

Table 21: Concentrations of pharmaceuticals detected in the River Nore surface water samples from September 2020 to March 2022 (n=3).

	Nore					
Pharmaceutical	Mar-22 (ng/L ± SD)	Sep-21 (ng/L ± SD)**	May-21 (ng/L ± SD)	Mar-21 (ng/L ± SD)	Oct-20 (ng/L ± SD)	Sep-20 (ng/L ± SD)
Metformin	7.06 ± 0.09	<LOQ	<LOQ	10.61 ± 0.76	15.07 ± 0.25	5.28 ± 0.2
Gabapentin	10.64 ± 0.58	31.59 ± 0.83	22.81 ± 1.13	<LOQ	10.54 ± 0.09	14.43 ± 1.39
Trimethoprim	4.28 ± 0.08	<LOQ	<LOD	<LOD	9.35 ± 0.3	<LOQ
Ciprofloxacin	8.16 ± 0.02	6.09 ± 4.11	2.99 ± 0.14	15.12 ± 0.6	32.21 ± 0.89	17.02 ± 1.44
Venlafaxine	4.82 ± 0.32	18.26 ± 1.4	7.86 ± 0.64	<LOQ	8.31 ± 0.67	10.9 ± 0.38
O-desvenlafaxine	6.69 ± 0.32	32.76 ± 0.76	10.19 ± 0.17	4.63 ± 0.31	8.27 ± 0.2	15.33 ± 0.45
Sulfamethoxazole	6.28 ± 0.11	13.07 ± 1.58	15.55 ± 0.42	7.93 ± 0.76	29.54 ± 0.95	39.51 ± 0.28
Carbamazepine	<LOQ	12.53 ± 0.53	<LOQ	<LOD	<LOQ	4.95 ± 0.12
Diclofenac	7.69 ± 0.38	14.79 ± 0.61	5.39 ± 0.07	5.38 ± 0.17	5.34 ± 0.23	4.6 ± 0.1
Erythromycin	<LOQ	288.32 ± 201.53 *	<LOD	<LOD	<LOQ	<LOQ
E1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
E2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Gemfibrozil	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Azithromycin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Clarithromycin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

* Inconclusive due to high standard deviation and therefore not included in reported concentrations.

** Quant and qual ion for pharmaceutical identification at selected sampling time points are detailed in Figure B 2.1, available in the appendix.

Table 22: Concentrations of pharmaceuticals detected in the River Liffey surface water samples from September 2020 to March 2022 (n=3).

	Liffey					
Pharmaceutical	Mar-22 (ng/L ± SD)	Sep-21 (ng/L ± SD)	May-21 (ng/L ± SD)	Mar-21 (ng/L ± SD)**	Oct-20 (ng/L ± SD)	Sep-20 (ng/L ± SD)
Metformin	<LOQ	<LOQ	6.55 ± 0.38	6.46 ± 0.33	<LOQ	5.48 ± 0.24
Gabapentin	75.87 ± 5.2	50.74 ± 1.8	119.29 ± 4.16	<LOQ	54.4 ± 1.74	45.76 ± 2.33
Trimethoprim	26.72 ± 1.74	13.86 ± 0.23	8.52 ± 0.21	4.62 ± 0.47	9.33 ± 0.25	8.01 ± 0.7
Ciprofloxacin	4.33 ± 0.39	19.29 ± 1.68	4.31 ± 0.23	6.09 ± 0.37	3.2 ± 0.2	11.25 ± 0.41
Venlafaxine	37.27 ± 1.25	64.45 ± 4.9	56.85 ± 0.7	16.13 ± 0.78	20.79 ± 2.58	19.91 ± 0.23
O-desvenlafaxine	58.57 ± 2.02	90.88 ± 5.74	85.55 ± 1.76	20.6 ± 1.22	27.58 ± 0.26	28.04 ± 0.34
Sulfamethoxazole	290.25 ± 2.61	204.78 ± 26.54	102.62 ± 5.23	69.32 ± 1.33	171.05 ± 1.6	151.28 ± 3.9
Carbamazepine	15.94 ± 0.21	26.44 ± 3.14	22.61 ± 0.75	8.48 ± 0.22	7.88 ± 0.1	8.2 ± 0.1
Diclofenac	<LOQ	6.76 ± 0.65	6.87 ± 0.08	92.34 ± 2.02	5.83 ± 0.21	6.39 ± 0.58
Erythromycin	<LOD	<LOQ	<LOD	41.39 ± 5.3	<LOD	<LOQ
E1	<LOD	<LOD	<LOQ	18.61 ± 0.6	<LOD	<LOD
E2	<LOQ	<LOD	<LOD	<LOQ	<LOD	<LOD
Gemfibrozil	<LOD	<LOD	85.68 ± 0.88	283.63 ± 4.92	11.24 ± 0.06	5.47 ± 0.01
Azithromycin	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOD
Clarithromycin	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOD

** Quant and qual ion for pharmaceutical identification at selected sampling time points are detailed in Figure , available in the appendix.

Table 23: Concentrations of pharmaceuticals detected in the River Suir surface water samples from September 2020 to March 2022 (n=3).

	Suir					
Pharmaceutical	Mar-22 (ng/L ± SD)	Sep-21 (ng/L ± SD)	May-21 (ng/L ± SD)	Mar-21 (ng/L ± SD)	Oct-20 (ng/L ± SD)**	Sep-20 (ng/L ± SD)
Metformin	<LOQ	5.66 ± 0.27	6.34 ± 0.16	<LOQ	7.15 ± 0.24	16.54 ± 0.41
Gabapentin	54.31 ± 4.33	<LOQ	30.59 ± 0.53	<LOQ	14.46 ± 0.95	16.77 ± 0.51
Trimethoprim	<LOQ	7.38 ± 0.35	7.89 ± 0.38	<LOQ	7.42 ± 0.17	<LOQ
Ciprofloxacin	9.56 ± 0.19	9.87 ± 0.25	4.55 ± 0.08	10.54 ± 0.84	10.08 ± 0.73	13.53 ± 0.75
Venlafaxine	12.81 ± 0.55	8.02 ± 0.35	7.26 ± 0.63	<LOQ	7.72 ± 0.19	5.74 ± 0.55
O-desvenlafaxine	13.65 ± 0.24	12.35 ± 0.53	10.79 ± 0.28	3.54 ± 0.07	10.94 ± 0.34	8.22 ± 0.24
Sulfamethoxazole	24.71 ± 1.67	126.99 ± 8.37	8.79 ± 0.33	20.28 ± 1.55	199.21 ± 8.42	8.41 ± 2.85
Carbamazepine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Diclofenac	<LOQ	9.25 ± 0.52	10.64 ± 0.3	7.27 ± 0.18	6.83 ± 0.08	18.96 ± 4.53
Erythromycin	<LOQ	<LOD	<LOD	<LOQ	26.17 ± 4.74	<LOD
E1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
E2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Gemfibrozil	<LOD	<LOD	<LOD	4.99 ± 0.46	<LOD	<LOD
Azithromycin	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD
Clarithromycin	<LOD	<LOQ	<LOD	<LOQ	<LOD	<LOD

** Quant and qual ion for pharmaceutical identification at selected sampling time points are detailed in Figure , available in the appendix.

Table 24: Concentrations of pharmaceuticals detected in the River Analee surface water samples from September 2020 to March 2022 (n=3).

	Analee					
Pharmaceutical	Mar-22 (ng/L ± SD)	Sep-21 (ng/L ± SD)	May-21 (ng/L ± SD)	Mar-21 (ng/L ± SD)**	Oct-20 (ng/L ± SD)	Sep-20 (ng/L ± SD)
Metformin	<LOQ	<LOQ	<LOQ	8.01 ± 0.11	25.11 ± 0.38	13.15 ± 0.08
Gabapentin	<LOQ	16.69 ± 0.25	68.25 ± 3.35	19.03 ± 0.15	27.12 ± 0.22	23.29 ± 0.53
Trimethoprim	<LOD	<LOQ	<LOQ	9.27 ± 0.38	14.54 ± 0.2	<LOQ
Ciprofloxacin	7.26 ± 0.38	2.84 ± 0.11	<LOQ	24.44 ± 1.15	8.16 ± 0.62	<LOQ
Venlafaxine	<LOQ	12.05 ± 0.34	14.3 ± 0.91	10.25 ± 0.79	11.61 ± 0.05	<LOQ
O-desvenlafaxine	<LOQ	13.16 ± 0.47	19.03 ± 0.29	7.65 ± 0.35	17.45 ± 0.08	5.45 ± 0.62
Sulfamethoxazole	14.11 ± 1.69	11.04 ± 0.34	91.22 ± 9.54	233.84 ± 11.19	67.71 ± 0.93	5.22 ± 0.67
Carbamazepine	5.49 ± 0.15	6.06 ± 0.27	10.95 ± 0.06	6.55 ± 0.27	6.2 ± 0.08	<LOQ
Diclofenac	<LOQ	8.66 ± 0.13	8.85 ± 0.16	23.99 ± 0.31	5.64 ± 0.15	<LOQ
Erythromycin	<LOQ	<LOD	<LOQ	22.21 ± 1.47	<LOD	<LOD
E1	<LOD	<LOQ	<LOD	<LOQ	<LOD	<LOD
E2	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD
Gemfibrozil	<LOQ	124.41 ± 1.83	<LOD	129.13 ± 1.38	<LOQ	<LOQ
Azithromycin	<LOD	<LOD	<LOD	<LOD	14.86 ± 0.29	<LOD
Clarithromycin	<LOD	<LOD	<LOQ	<LOD	14.92 ± 0.61	<LOD

** Quant and qual ion for pharmaceutical identification at selected sampling time points are detailed in Figure , available in the appendix.

The identification and quantification of pharmaceuticals in sampled rivers through LC-MS/MS involved the detection of quantifier and qualifier ions at their correct ratios and retention time, as seen for venlafaxine and sulfamethoxazole in a spiked matrix vs surface water samples (Figure 29 and Figure 30).

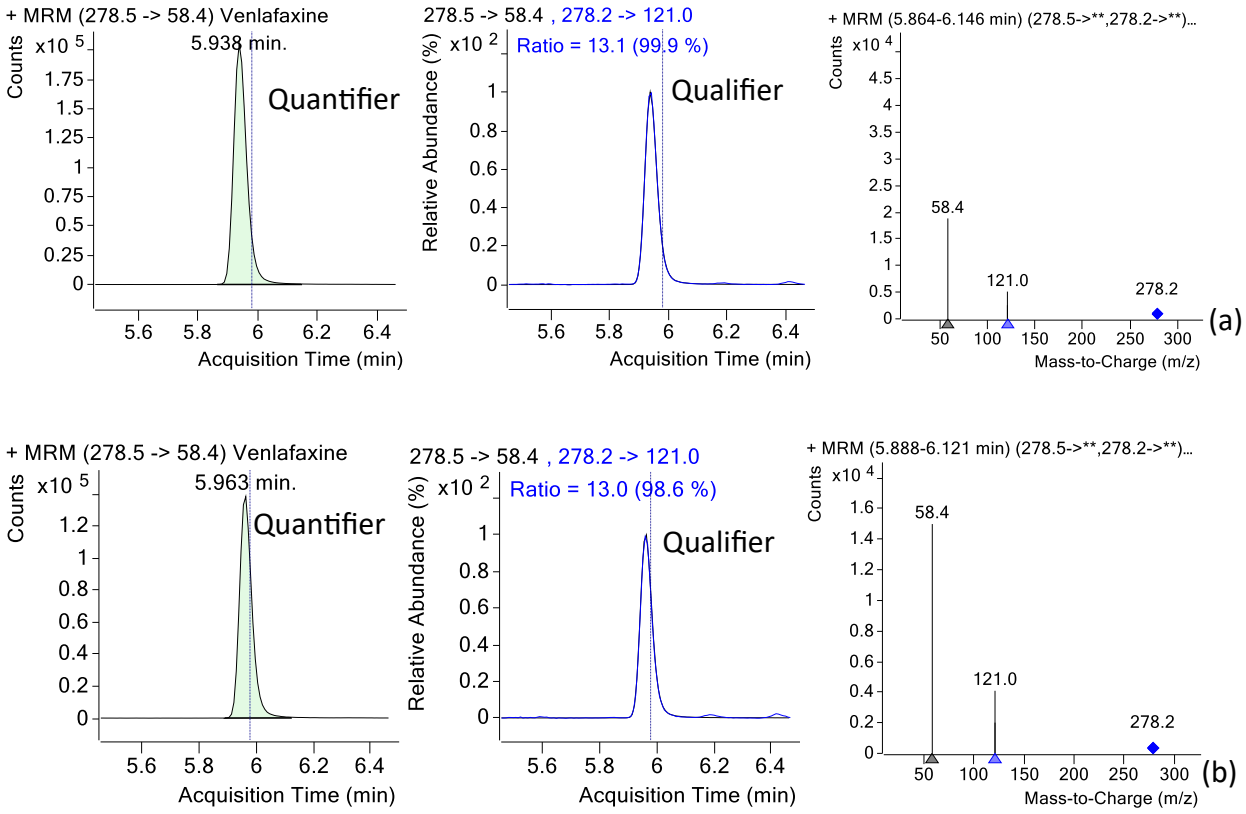


Figure 29. Extracted MRM for venlafaxine quantifier (278.5 → 58.4) and qualifier ion (278.5 → 121.0) in (a) the river Liffey October 2020 and (b) matrix composite spiked with 75 ng/L of venlafaxine reference standard.

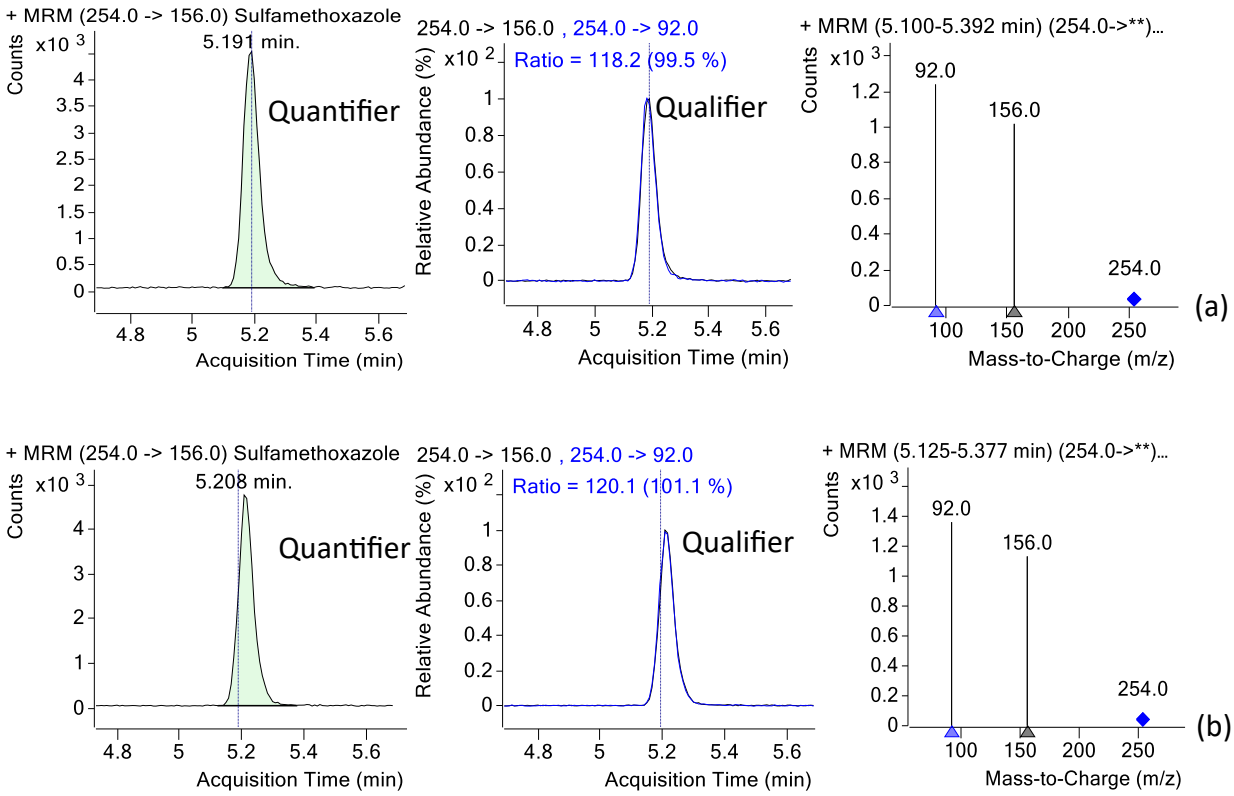


Figure 30: Extracted MRM for Sulfamethoxazole quantifier (254.0→156.0) and qualifier ion (254.0→92.0) in (a) the river Analee October 2020 and (b) matrix composite spiked with 75 ng/L of sulfamethoxazole reference standard.

3.4.1 Trends in pharmaceutical detection and frequency

Across all sampling sites, 15 of the 16 pharmaceuticals were detected above the LOD, with 14 quantifiable (Figure 31). The most frequently detected pharmaceutical was sulfamethoxazole, which was found above LOQ in all 24 samples taken. Other pharmaceuticals frequently detected above LOQ include O-desvenlafaxine (23 samples), ciprofloxacin (22 samples), diclofenac and venlafaxine (20 samples), metformin (16 samples); trimethoprim and carbamazepine (13 samples). The least frequently detected pharmaceuticals include E1 (4 samples), erythromycin, clarithromycin and azithromycin (1 sample), and E2 was detected but not quantifiable. The high detection frequency is a cause for concern as it indicates a chronic toxicological effect for aquatic organisms.

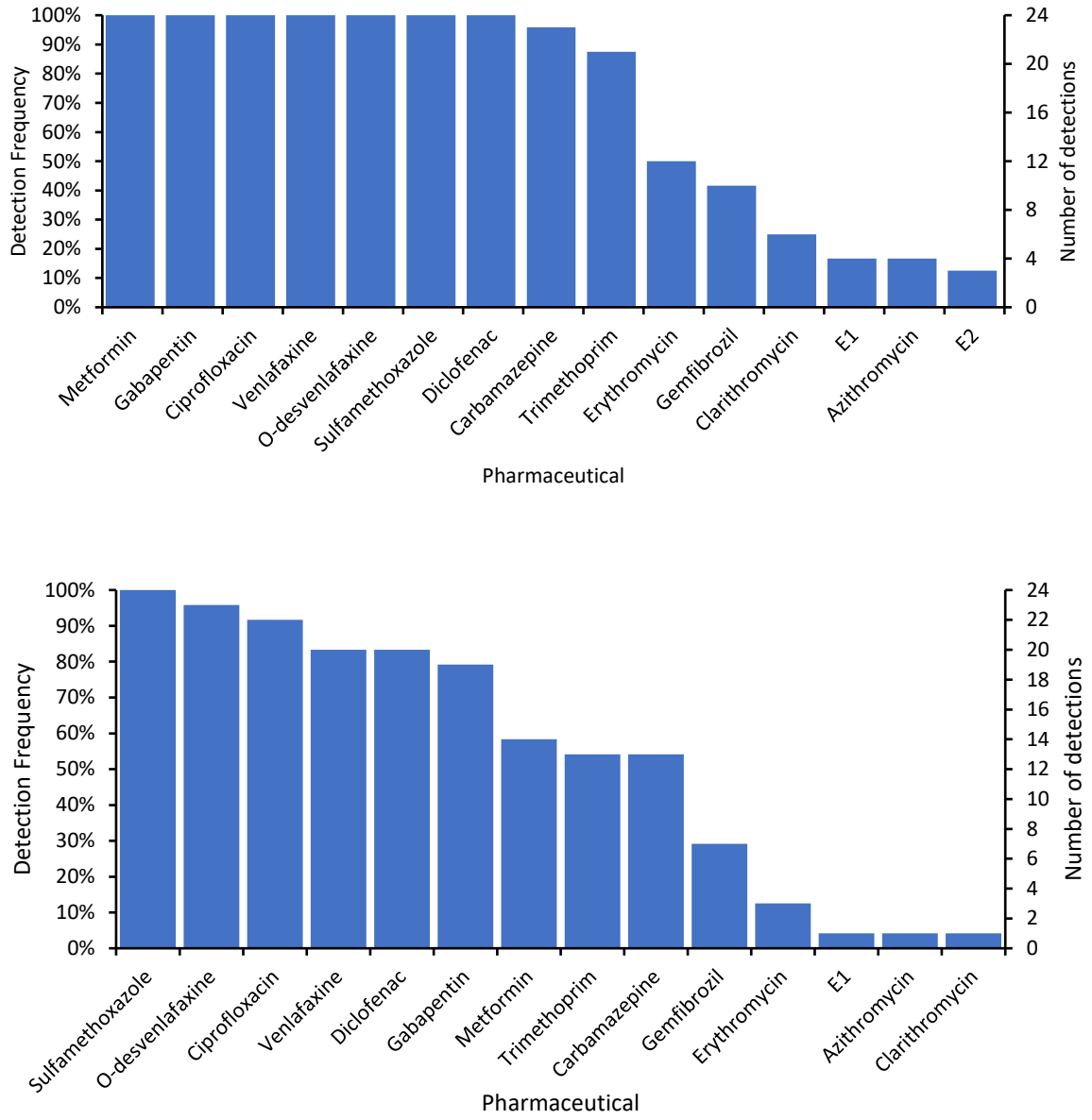


Figure 31. Frequency of pharmaceutical detections in all sites over 6 month sampling period (n=24), detections >LOD (Top), detections >LOQ (Bottom) from 2020 to 2022.

Samples from the River Liffey were collected downstream of the Leixlip WWTP. The effluent from this WWTP has been previously documented to contain several pharmaceuticals investigated in this study, including carbamazepine (up to 0.70 µg/L), diclofenac (up to 0.73 µg/L), gemfibrozil (up to 0.15 µg/L) and trimethoprim (up to 0.57 µg/L).[82] As WWTPs are not typically equipped with the treatment of pharmaceuticals, the dilution effect from treatment

and rivers plays a pivotal role in diminishing risk to aquatic organisms.[276,334] With this, an insufficient dilution effect was also observed in the River Liffey surface waters as it had the highest occurrence and concentration of pharmaceuticals detected in all sample sites (Figure 32**Error! Reference source not found.**).

Pharmaceuticals were detected in concentrations ranging from below LOD to 290.25 ng/L (Table 21 to Table 24). Among the tested pharmaceuticals, the antibiotic sulfamethoxazole was the most frequently found pharmaceutical and had the highest maximum concentration of 290.25 ng/L in the River Liffey and the second highest mean concentration of 79.70 ng/L. The maximum measured environmental concentrations (MEC) levels of sulfamethoxazole were comparable to MECs observed in Europe, where concentrations generally range between <1 ng/L to 286 ng/L with a maximum of 4072 ng/L.[335] For example, previous studies have found sulfamethoxazole concentrations in Germany at 469 ng/L and Spain at 600 ng/L, respectively.[42,271] It is important to mention that the frequent prescription, poor removal rates and sulfamethoxazole's classification as persistent has led to its detection worldwide and is often found in EU rivers at levels comparable to or higher than what has been reported here.

Trimethoprim is frequently prescribed with sulfamethoxazole under the name co-trimoxazole in a 1:5 ratio. This likely explains the frequent presence of both antibiotics in samples analysed in this study, where both pharmaceuticals were detected at an average 1:8 ratio. The maximum and mean of the antibiotic ciprofloxacin were 32.21 ng/L and 10.50 ng/L, respectively, which is consistent with concentrations found in other EU countries, such as Sweden and Portugal, for the compound which was included in the first WFD watchlist.[205,206] The appearance of ciprofloxacin in the tested samples may have resulted from the use of oral

or topical treatments for humans or from the breakdown of enrofloxacin, an antibiotic used in livestock.[73] While macrolide antibiotics such as erythromycin, clarithromycin, and azithromycin were detected, their levels were generally below the detection or quantification limits.

Antidepressants are reported to be increasing in consumption and use in Ireland from 2012 to 2017, and there was a 28% increase in prescriptions and an 18% increase in patients that were prescribed some form of antidepressant by general practitioners.[336] The antidepressant venlafaxine and its metabolite O-desmethyl venlafaxine are the most common Serotonin–Norepinephrine Reuptake Inhibitors (SSRIs) prescribed in Ireland and saw a 48% increase in prescriptions from 2007 to 2017.[337] In this study, venlafaxine and its metabolite O-desmethyl venlafaxine were among the most commonly found pharmaceuticals across all samples, with a frequency (< LOQ) of 83 and 96%, respectively. Venlafaxine maximum and mean are 64.45ng/L and 17.77ng/L, respectively, and O-desmethyl venlafaxine maximum and mean are 90.88ng/L and 22.23ng/L, Respectively. Furthermore, a review by Zhou et al. identified the mean and maximum concentration of venlafaxine in 33 European countries to be 131 ng/L and 575 ng/L, respectively.[26] The pattern of venlafaxine and O-desmethyl venlafaxine is often observed and supported by literature that O-desmethyl venlafaxine is found over its parent molecule in surface water environments.[338]

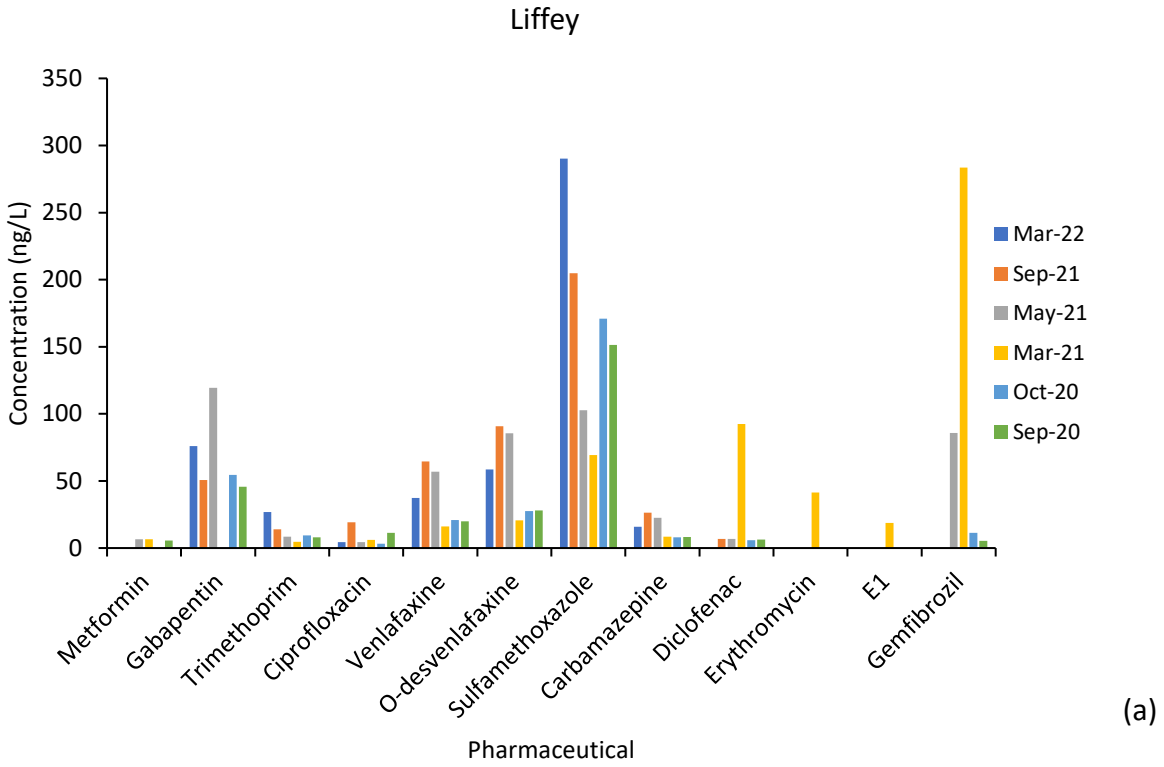
Gabapentin and carbamazepine, used as antiepileptics and anticonvulsants, were found in 79% and 54% of the samples, respectively. Concentrations of gabapentin were generally higher than that of carbamazepine, with a mean concentration of 30.63 ng/L and 10.94 ng/L, respectively. This may be attributed to the greater metabolization of carbamazepine over gabapentin.[339] Mean concentrations of gabapentin were lower than the European mean (437

ng/L). However, they are in line with individual European countries where concentrations were detected at 58 – 75 ng/L (Cardiff, UK), 42 – 64 ng/L (Poznań, Lechicka Street, Poland), 17.7 -126 ng/L (Belgrade, Serbia).[338,340]

While no prescription data is available for Ireland, a study by Ferencik et al. suggested that gabapentin is more commonly used than carbamazepine.[339] The presence of these compounds in surface water samples can be attributed to their poor removal rates during wastewater treatment processes and their resistance to degradation.[339,341]

Diclofenac was detected at a max and mean concentration of 92.34 ng/L and 13.07 ng/L, respectively. These concentrations are in agreement with other reported levels in EU countries such as France (26.87-30.06 ng/L), Poland (28.6-470 ng/L), Denmark (32-156 ng/L) and Spain (0.5-330 ng/L).[42]

Although a matrix-matched calibration was performed, low SPE recoveries were associated with metformin (~19%) as a result of the acidification of samples to prevent against microbial degradation.[88,259] However, from samples analysed, metformin maximum and mean concentrations were determined as 25.11 ng/L and 19.89 ng/L, respectively. Notably, these values are relatively lower than those found in several European countries, where the levels are often reported above 100 ng/L to µg/L concentrations.[338] However, the average concentrations detected in rivers located in Tallinn, Estonia (21.8-29.6 ng/L), Madeira Portugal (20.4 ng/L), and Wales River Dee (27.2 ng/L) are consistent with this report's findings.[338]



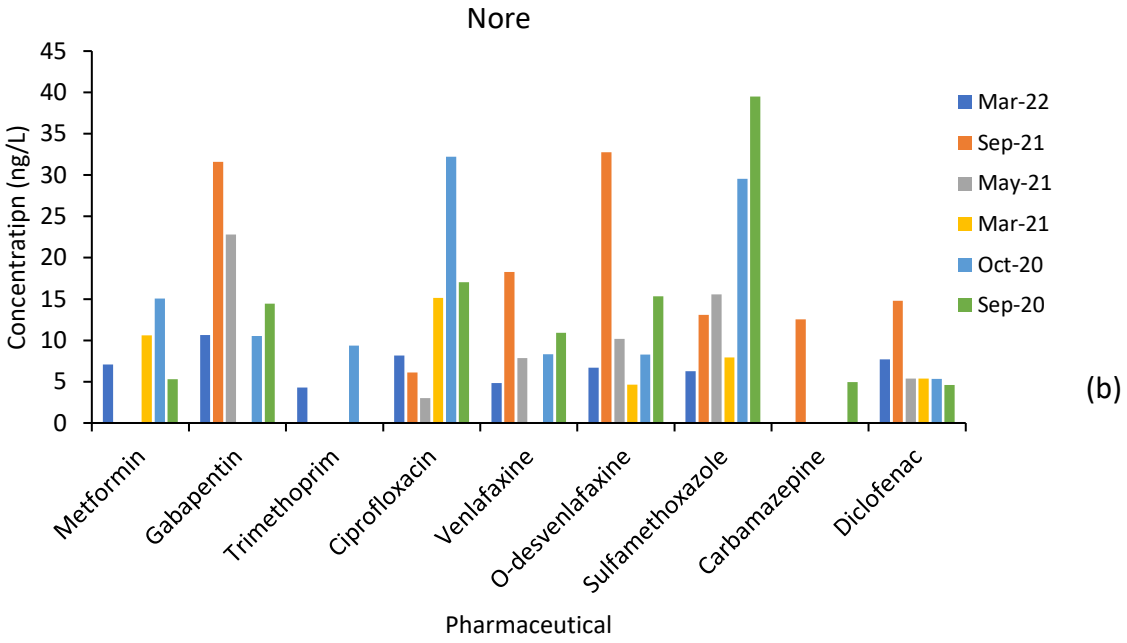
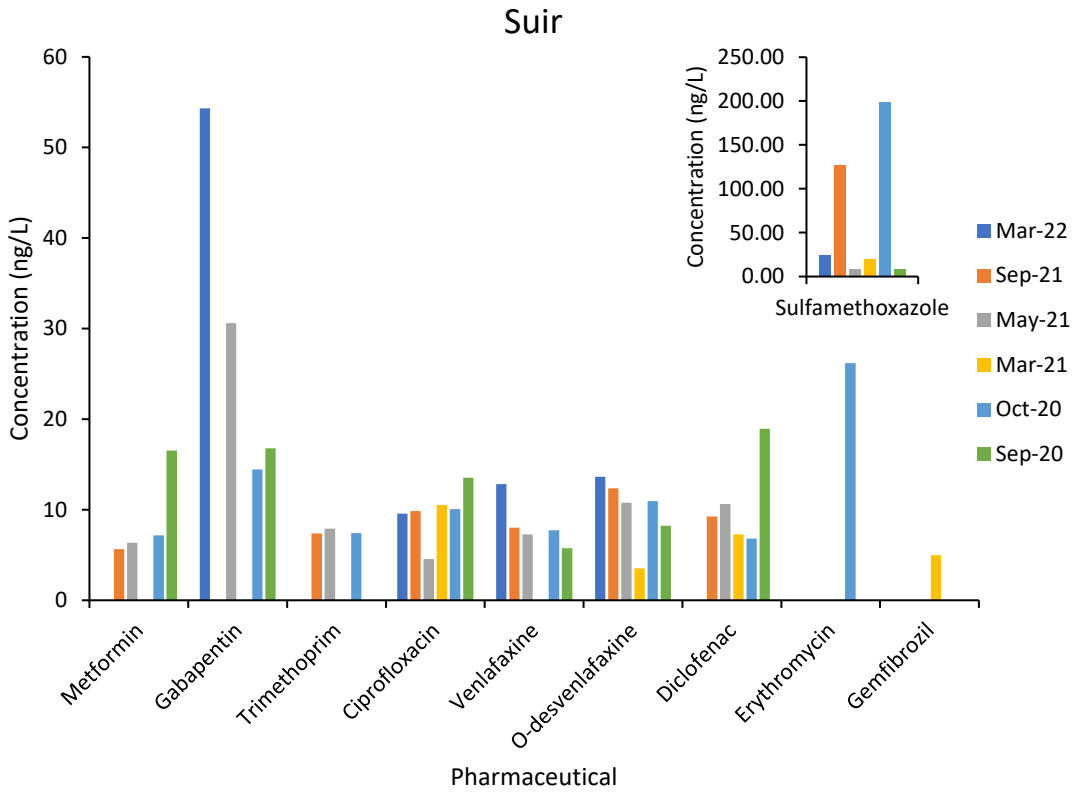
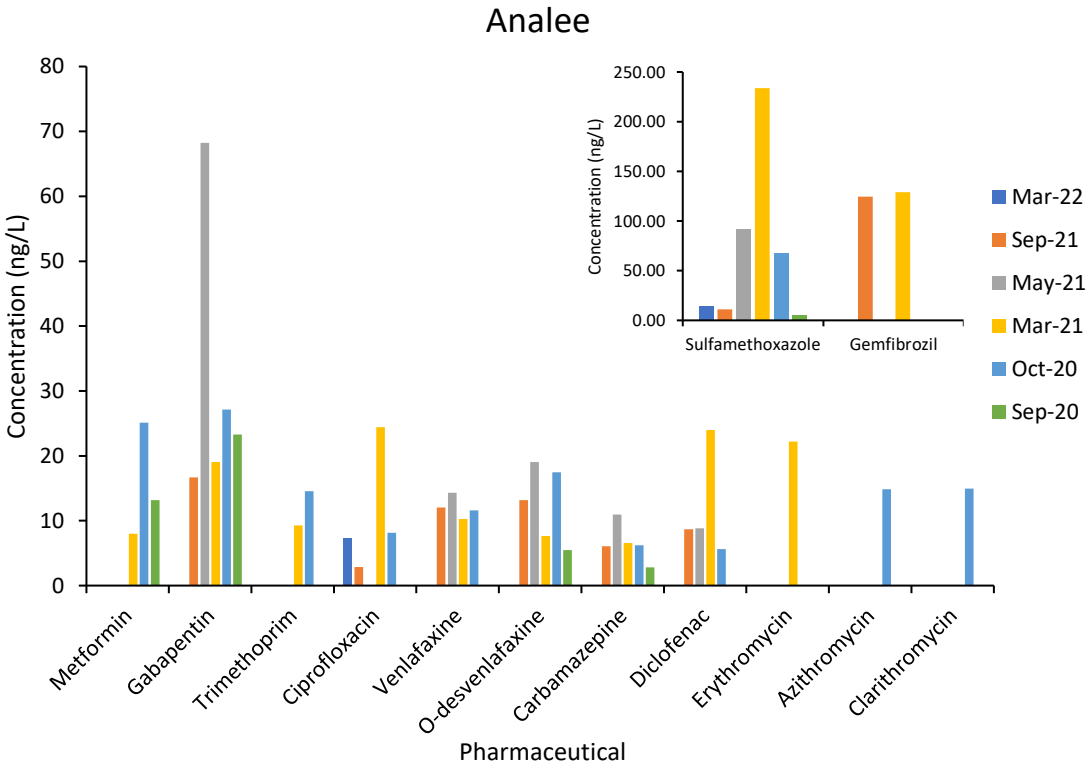


Figure 32. Pharmaceutical concentrations detected in the Nore (a), Liffey (b), Suir (c), and Analee (d) during six sampling campaigns from 2020 to 2022 (light blue Mar-22, orange Sept-21, grey May-21, yellow Mar-21, blue Oct-20, green Sept-20). Inserts are included in Figures (c) and (d) for scaling purposes.



(c)



(d)

Figure 32 (continued). Pharmaceutical concentrations detected in the Nore (a), Liffey (b), Suir (c), and Analee (d) during six sampling campaigns from 2020 to 2022 (light blue Mar-22, orange Sept-21, grey May-21, yellow Mar-21, blue Oct-20, green Sept-20). Inserts are included in Figures (c) and (d) for scaling purposes.

3.4.2 Temporal assessment of pharmaceuticals detected.

The COVID-19 pandemic has created an unprecedented environment where severe restrictions have been implemented in many countries, including Ireland. Initial sampling began in September 2020 when the Irish government announced the "living with COVID" plan, which included five levels designated increasing restrictions with each level.[342] Ireland was initially placed under level 2 restrictions in September, which was later revised to level 3 for selected counties depending on infection rates. This meant that people were only allowed to move within their county of residence. At this first sampling timepoint across all sample sites, 30 quantifiable pharmaceutical detections were found, equating to a quantifiable detection rate of 50%. During October 2020, the second sampling timepoint, level 5 restrictions were adopted, which introduced a 5 km travel restriction from a place of residence. During this period, the number of pharmaceutical detections increased in the River Suir and Analee from 7 and 5 to 9 and 11 quantifiable detections, respectively.

The total number of quantifiable detections from September 2020 to September 2022 remained between 30-37 detections, with a decrease to 22 detections in March 2022, by which time all Covid-19 restrictions had been lifted. The number of detections in the River Nore and the River Suir decreased from March 2021 to March 2022, as shown in Figure 33. The River Liffey is situated in an area with many commuter towns, which may have resulted in increased household wastewater being treated by the wastewater services during this period, as most people were restricted from travelling to their place of work. Restrictions were eased on the 12th of April 2021, and the number of pharmaceutical detections decreased in May 2021, which continued to March 22, as shown in Figure 33. Furthermore, in contrast to rural sampling locations, the levels of pharmaceutical detections remain relatively similar.

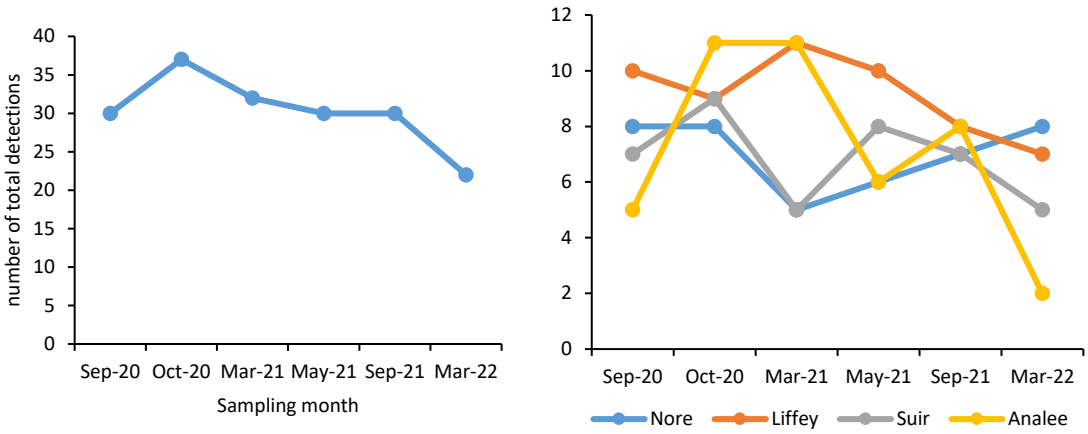


Figure 33. Temporal Trends in combined pharmaceutical detections (<LOQ) across all rivers (Left) and The Rivers Nore, Suir, Analee, and Liffey (right) from 2020 to 2022.

Variations in antidepressant concentrations (O-desmethyl venlafaxine) could also be seen during lockdown to post-lockdown phases. The impact of COVID-19 on mental health, resulting in an increased diagnosis of depression, anxiety and post-traumatic stress disorder, has been previously reported.[343] The effect of COVID-19 on antidepressant use could potentially be reflected by the increase of antidepressant concentrations in the River Liffey and Nore from May 2021 to March 2022, coinciding with the easing of COVID-19 restrictions, which began in April 2021 (Figure 34). It is possible that the increase in surface water concentrations was due to higher consumption of antidepressants, which may, in turn, be a result of the impact restrictions had on mental health. However, additional factors that may have led to increased antidepressant concentrations could have resulted from greater access to healthcare professionals who could provide prescriptions. Although there is limited Irish data on the influence of COVID-19 on antidepressant use, a study by Antonazzo et al., 2022 in Italy showed similar trends with regard to the reduction of the prevalence and incidence of antidepressant use during COVID-19 lockdowns, which were followed by a sharp increase post-lockdown.[343] It has previously been suggested that the reduced concentrations

observed during lockdown could be a cause for concern as it may allude to the increased probability of adverse events such as self-harm [343]. Research into the effect lockdowns had on antidepressant use in Ireland is limited and requires greater investigation as it could aid in outlining future plans to sufficiently address mental health during periods of extenuating circumstances (i.e. natural disasters, pandemics). Furthermore, monitoring for pharmaceuticals in river waters may help address sustainable development goal 3, good health and well-being, by contributing to targets 3.5 (prevent and treat substance abuse) and 3.D (improve early warning systems for global health risks).[34,35]

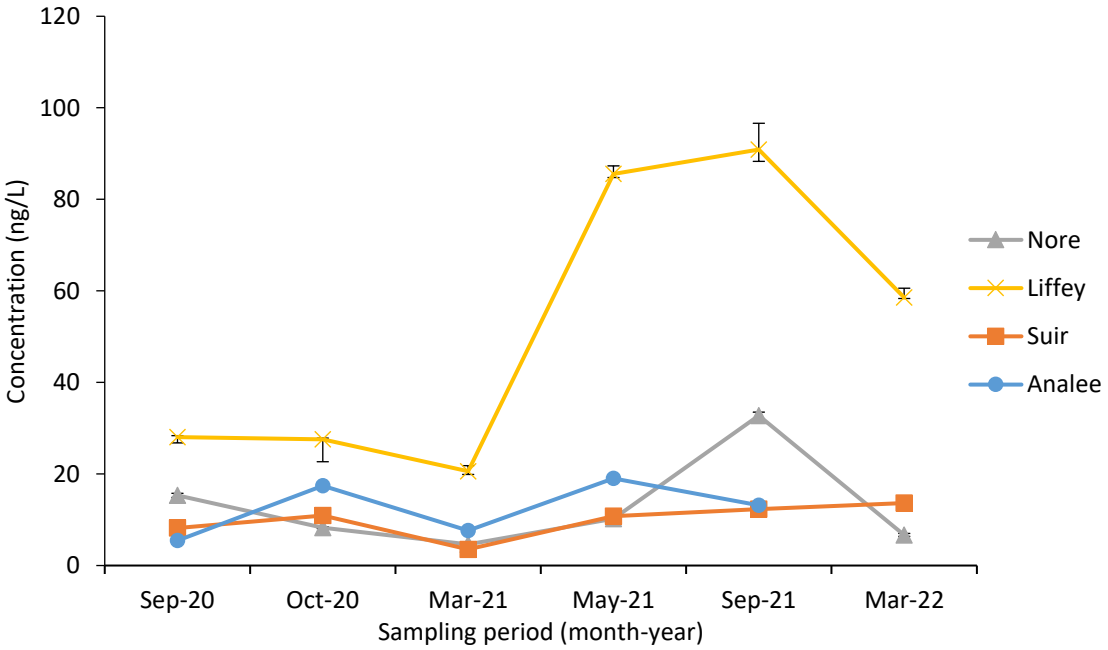


Figure 34. Temporal concentration fluctuation of O-Desmethylvenlafaxine across The Rivers Nore, Liffey, Suir and Analee form the sampling period 2020-2022.

The accumulated antibiotic concentrations in the Nore spiked in September and October 2020 but remained relatively stable across all other sampling time points. The river Liffey generally had the highest overall antibiotic load, with the exception of a spike in the River Suir in

October 2020 and the River Analee in March 2021 (Figure 35). The frequent detection of antibiotics such as sulfamethoxazole, ciprofloxacin and trimethoprim indicate potential seasonal fluctuations, likely influenced by community consumption, river dynamics and agricultural practices. Furthermore, a study by Ohoro et al. suggested that a neutral pH and an increase in conductivity and turbidity in surface waters can be associated with elevated pharmaceutical concentrations.[344] Additionally, turbidity can play an influencing role in the degradation of photosensitive compounds (e.g. sulfamethoxazole) due to the reduction of sunlight penetration.[345,346] However, the scope for drawing conclusions regarding seasonal variation is constrained due to the number of data points available (6 per site in total), with a maximum of 3 observed in any given calendar year.

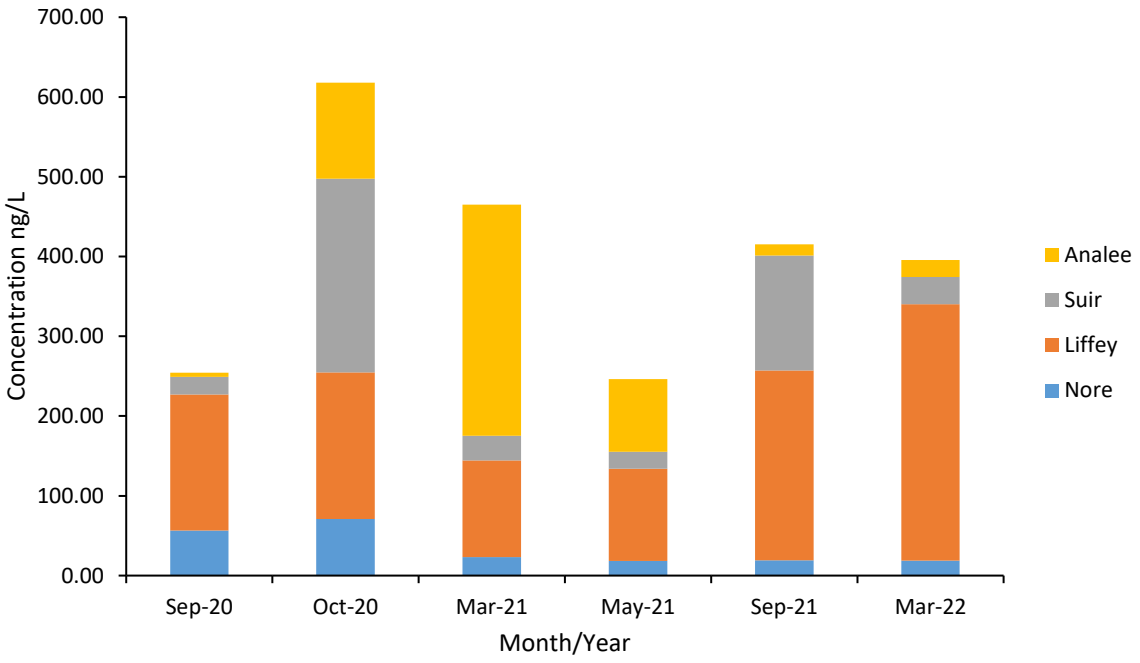


Figure 35. Temporal variation in combined concentration of the antibiotics sulfamethoxazole, ciprofloxacin, trimethoprim, azithromycin, clarithromycin, erythromycin across The Rivers Nore, Liffey, Suir and Analee from 2020 to 2022.

Gaining an understanding of these trends can be instrumental in formulating targeted monitoring strategies during periods when higher antibiotic/pharmaceutical levels are

anticipated while providing valuable insights into the potential threat of antibiotic resistance as a result of their presence. It can also pinpoint seasons and locations of heightened concern so that local wastewater treatment processes can be adapted to include and select the appropriate tertiary/quaternary treatment technologies or modification of biomass concentrations during treatment in accordance with the type and scale of pollution and the financial investment required. For example, increasing the hydraulic retention time increases pharmaceutical interaction with biomass and solid retention time, improving sludge separation and growth of microbes better equipped to remove pharmaceuticals, namely ciprofloxacin and gemfibrozil.[42] Furthermore, the utilisation of tertiary treatment, such as ozonation and activated carbon treatment, has been shown to be effective in enhancing removal rates (<90%) for sulfamethoxazole and venlafaxine, which were the most frequently detected pharmaceuticals in this study.[347–350]

The risk associated with the presence of these antibiotics comes from long-term exposure in surface water ecosystems. Their persistence/pseudo-persistence at sub-inhibitory concentrations may lead to the formation of antibiotic-resistant genes in aquatic ecosystems by horizontal drug-resistance gene transfer and pose a hazard for antibiotic treatment of human microbial communities.[42,351] In addition to microbial resistance, the implications for other microorganisms, such as algae, cannot be overlooked. Algae are an essential food source for the larva of molluscs and fish, and they play an important role in nutrient cycling. The frequent presence of antibiotics that are toxic to algae, such as sulfamethoxazole, is a cause for concern as it may affect not only the algae themselves but also higher trophic levels.[352] To assess the toxicological risks, multitrophic exposure studies could be employed at detected concentrations, the use of risk quotients (RQ) where the measured environmental concentrations (MEC) of pharmaceuticals are ratioed against the predicted no-effect values

(PNEC) have been shown as effective strategies to predict risk within a river system or where monitoring data is not available utilising Quantitative structural activity relationship modelling can provide an adequate predictive framework for assessing the potential interactions between chemical structures and biological endpoints.[232,341,353–355]

3.5 Conclusions

With the analytical methods developed and the monitoring of four sampling locations across six sampling time points, this study has provided data into the detection of 15 pharmaceuticals in Irish surface waters. The frequent detection of pharmaceuticals and their mixtures, including antibiotics, antidepressants, and anti-inflammatory drugs, raises concerns regarding the long-term ecological threat to Irish rivers.

The potential impact of COVID lockdown restrictions may have led to short-term spikes in pharmaceutical detections and, as a result, environmental risk. The data demonstrates the antibiotic burden in our surface water and the potential risk in relation to antibiotic resistance. However, further research is needed to fully understand the impact and associated environmental and human health risks.

Aligned with the United Nations' SDG 6, "*ensure availability and sustainable management of water and sanitation for all*", this study emphasises the need to minimize pharmaceutical occurrences to protect and restore surface water ecosystems. The chapter additionally aids in identifying critical locations and periods that may help empower local decision-makers to customize wastewater treatment processes.

Chapter 4 :

Assessing Pharmaceuticals' Risk and Ecotoxicological Effects: A Multifaceted Perspective on Surface Water Pollution

4.1 Introduction

The presence of pharmaceuticals in surface waters has been documented globally, with concentrations generally ranging from ng/L to µg/L concentrations.[42,338] This is a cause for concern as the presence has been shown to cause an array of harmful effects to aquatic organisms, such as toxicity and mortality, behavioural changes, reproductive and endocrine disruption, genotoxicity, immune and respiration disruption.[139] Furthermore, aquatic organisms exposed to pharmaceuticals may disrupt the immune system, decreasing species' resilience to diseases, infections and environmental stressors.[356]

A study conducted by Fong and Hoy showed that the pharmaceutical venlafaxine resulted in significant foot detachment in the freshwater snail *Leptoxis carinata* at concentrations as low as 313 pg/L.[357] A study by Ribeiro et al. observed that the exposure of zebrafish larvae to venlafaxine resulted in an increase in malformations and changes in behaviour. Additionally, in the same study, the exposure of *Daphnia* to venlafaxine over the course of 21 days resulted in a significant decrease in fecundity.[173] A study by Rafiq et al. on the marine mussel Mytilus galloprovincialis additionally showed that venlafaxine's metabolite, O-desmethyl venlafaxine, had caused a reduction in egg fertilization.[358]

Omotola et al. had shown that the exposure of *Daphnia magna (D. magna)* to antibiotic sulfamethoxazole had resulted in toxicity with a potential for mutagenicity.[359] Additionally, sulfamethoxazole has been shown to affect oxidative stress response, energy metabolism, and immune response in marine vertebrates and invertebrets.[360]

Yisa et al. had found that antibiotics Amoxicillin and ciprofloxacin could induce mortality and oxidative stress in *D. magna* as well as having the potential to change population dynamics within an ecosystem.[361] Niemuth et al. had shown that metformin exposure resulted in endocrine disruption in flat head minnows at environmentally relevant concentrations.[362] Nkoom et al. found that exposing *D. magna* to diclofenac can result in oxidative damage, reduce feeding rates and stimulate behavioural changes.[363]

The presence of a pharmaceutical cocktail may also result in synergistic effects (the effect of the mixture of APIs is greater than the sum of its components), additive (the effect of the mixture is the sum of the effects from the specific APIs) or antagonistic effects (the mixture of APIs have a lessened effect than the effect of the single compound, e.g. enzyme induction).[189] A 2023 study conducted by Duchet et al. investigated the combined effect of environmental stressors and community exposure to a mixture of 15 pharmaceuticals, which included venlafaxine, carbamazepine sulfamethoxazole, trimethoprim and clarithromycin, at concentrations found in surface waters.[364] In this study, it was found that the combination of pharmaceutical exposure with warming stressors (associated with summer months) resulted in the delayed or accelerated emergence of top insect predators. However, in the winter experiment, the effects were much weaker.

4.1.1 Environmental risk assessment

QSAR is a mathematical model that uses the molecular structure or physio-chemical properties (log_{kow} and logP) of Active Pharmaceutical Ingredients (APIs) to determine the biological and environmental fate. QSAR modelling is an invaluable first line of investigation to identify problematic compounds as toxicity testing comes at a great

expense, and it can assess legacy drugs legally exempted from environmental risk assessments, as mentioned in Chapter 1.

The risk assessment measure implemented by the European Union was the formation of Environmental Risk Assessments (ERAs). ERAs follow a systematic approach, with an initial screening phase (phase I), which addresses environmental exposure by predicting an APIs' ability to bioaccumulate and persist in the environment.[365] As a part of an ERA, the pharmaceutical risk to the environment is calculated by the Risk Quotient (RQ), which is the ratio of their predicted environmental concentration (PEC) and predicted no-effect concentration (PNEC). However, as the PEC does not account for compounding exposure as a result of multiple pharmaceuticals with the same Active Pharmaceutical Ingredients, in this chapter Measured Environmental Concentrations (MEC) is used instead of PEC to provide a greater outlook on APIs' impact on an aquatic ecosystem and the specific locations where surface water is monitored (Equation 6).

$$RQ = \frac{PEC}{PNEC} \rightarrow \frac{MEC}{PNEC}$$ *Equation 6*

PNECs are derived from acute and chronic toxicity tests from trophic levels (algae, Daphnia and fish); the lowest PNEC from these three trophic levels is selected, and an assessment factor (AF) is applied to provide a buffer of margin of safety. [366]

In order to comprehensively assess the risk posed by pharmaceuticals in surface water environments, a multifaceted approach needs to be developed (Figure 36). Initially, surface water samples must be analysed to ascertain the concentrations/types of pharmaceuticals present and the frequency of their occurrence. Subsequently, with this information, applying effect-based methods through exposure to invertebrates such as

D. magna will help to determine a pharmaceutical's capacity to enter into the river's food web and its impact.

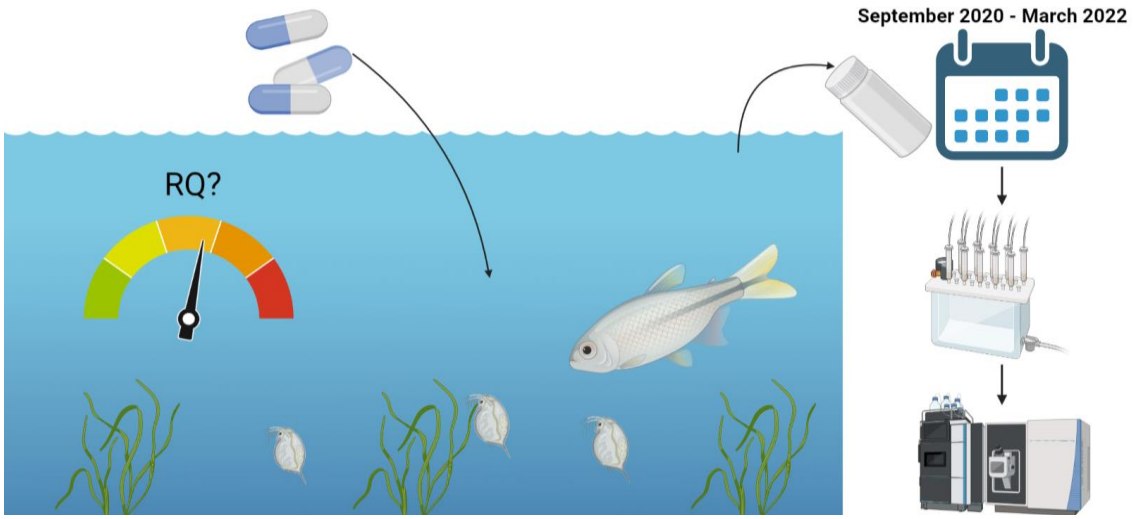


Figure 36. Summary of the assessment of risk in surface waters. Created with BioRender.com

Due to multiple uptake mechanisms and the presence of a mixture of pharmaceuticals in surface waters, a comprehensive assessment is required to understand the risks posed by APIs to aquatic organisms entirely. Current research into the bioaccumulation and effects of APIs in aquatic organisms has been predominantly focused on vertebrates; therefore, a knowledge gap persists regarding invertebrates. However, there is an increasing focus on addressing this issue.

4.2 Aims and objectives

This study aims to provide a comprehensive risk assessment by assigning a risk quotient value for pharmaceuticals identified in Chapter 3.

The objectives are to:

- Determine the Risk Quotient for selected pharmaceuticals across all sampling time points and locations;
- Identify pharmaceuticals of most concern;
- Highlight rivers that are most at risk of pharmaceutical pollution;
- Apply effect-based methods to determine their potential effect on aquatic invertebrates.

4.3 Experimental

4.3.1 Chemicals, reagents and instrumental conditions

All materials and methods used for sample collection, preparation and analysis are detailed in Chapter 2 (section 2.3).

4.3.2 Sampling, study area and river water extraction

Site location, environmental conditions and methodology are detailed in Chapters 2 (section 2.3) and 3 (section 3.2.1) of this thesis.

4.3.3 Daphnia culture and storage

D. magna were obtained through a parthenogenetic (asexual) reproduction initiated from a single mother hatched from the ephippium, according to Microbiotests Inc. (Belgium).[24] A maximum of 60 *D. magna* were maintained in an aerated 2.5 L tank (containing 2 L) to prevent overcrowding and were fed every 72 h with 600 µL of a 0.1 g/100 mL spirulina suspension (Figure 37). The medium used consisted of 16 mg of $NaHCO_3$, 100 mg of $CaSO_4 \cdot 2H_2O$, 20 mg of $MgSO_4$ and 3 mg of KCl per litre of deionised water, which was changed every 48 hours. Lighting consisted of a 16:8 light-to-dark cycle of cool white light between 1000-1500 lux.

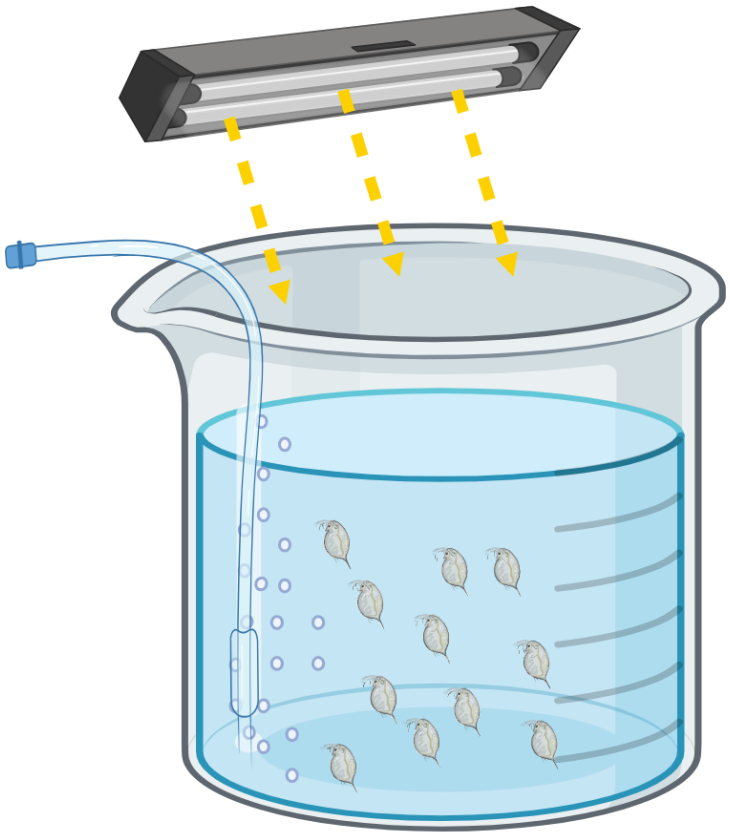


Figure 37. D. magna culturing apparatus. The tank was aerated and maintained at 18 ± 1°C, pH of 8 ± 1 unit and dissolved oxygen content of > 3 mg/L. Created with BioRender.com

4.3.4 Daphnia exposure and extraction protocol

4.3.4.1 Uptake study

Few studies report on the determination or quantification of pharmaceuticals in biological matrices due to variability and the difficulty of analysis.[368] *D. magna* exposure studies were adapted from work conducted by JDing et al. and from OECD Guideline for the Testing of Chemicals, Section 2 - Test No. 202: Daphnia sp. Reproduction Acute Immobilisation Test.[353,369] 30 adult (21-day old) *D. magna* were placed in 100 mL glass vials containing spring water to assess PNEC, PNEC x10 and PNEC x100 (n=3). At t = 48, *D. magna* were removed, rinsed with spring water, and depurated for 24 h. *D. magna* were collected, dried with filter paper, frozen in liquid nitrogen, and subsequently stored in a - 80 ℃ freezer.

Prior to SPE, 30 *D. magna* was placed in a 2 mL Eppendorf tube, and 1.5 mL of ACN was added along with glass beads. The samples were then homogenised at 300 rpm for 180 seconds using a Bead Bug microtube homogeniser. The use of pulverised liquid extraction (PuLE) has been previously reported as a rapid and more environmentally friendly extraction method.[368] Subsequently, the Eppendorf tube was sonicated for 20 min and centrifuged at 4000 RFC for 5 min. 1 mL of ACN extract was pipetted into 100 mL of deionised water, 1 mL of ACN was added to replenish the Eppendorf tube, and the process was repeated. The final step involved the removal of the remaining 1.5 mL of ACN in the Eppendorf tube (Total volume = 3.5 mL ACN into 100 mL of DI water) (Figure 38). Spiking for method validation was performed by spiking with known concentrations of target analytes and internal standards (700 ng/L) directly into the Eppendorf tube containing 30 *D. magna*. The 100 mL of DI water containing the 3.5 mL of extracts underwent SPE in accordance with methods developed in Chapter 2, with the exception of extracts being reconstituted in 250 µL of 95:5 H_2O: ACN.

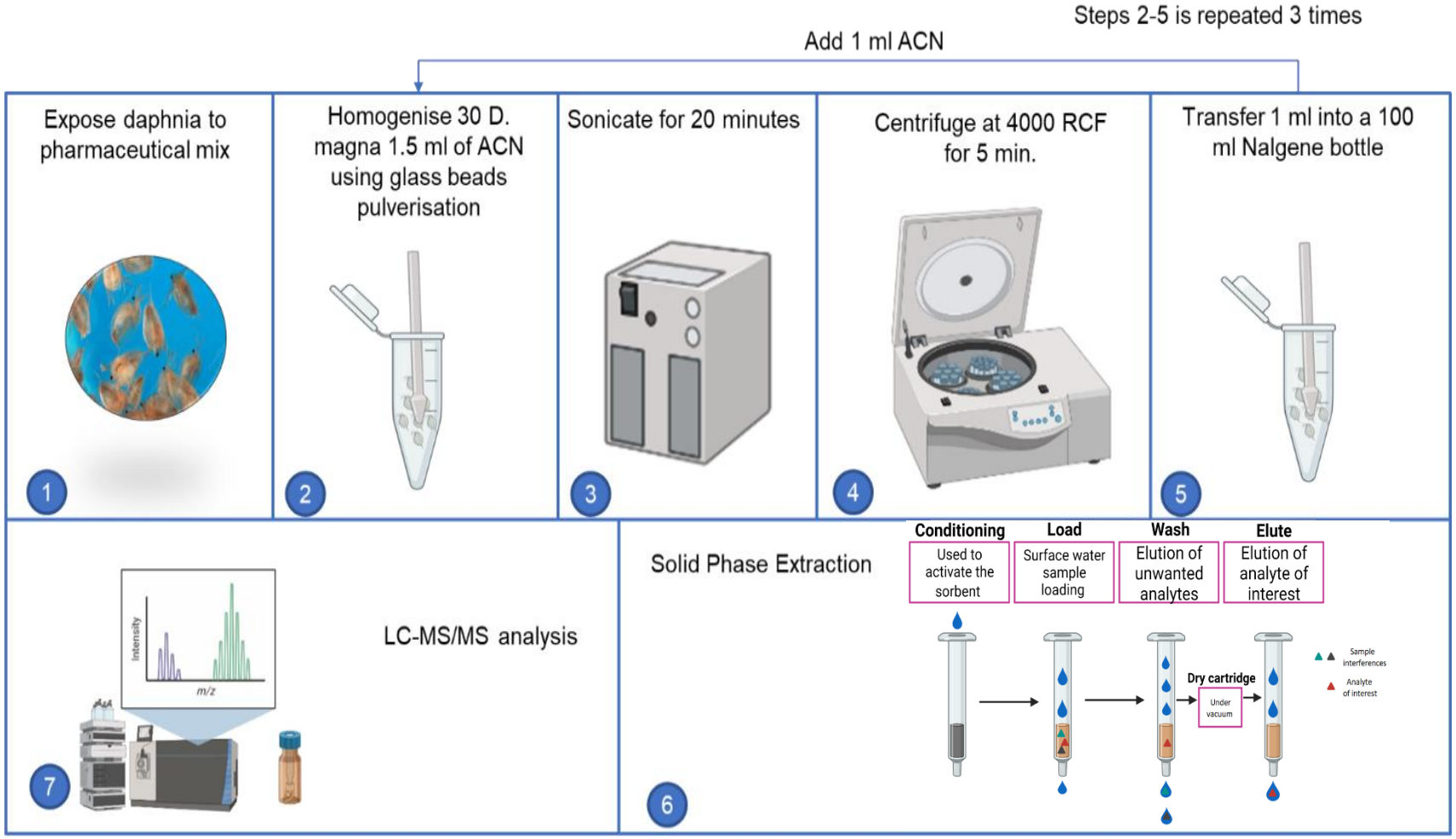


Figure 38. Extraction protocol for D. magna adapted from Miller et al., and J. Ding et al. [47,353,368] Created with BioRender.com.

4.3.4.2 Chronic effect exposure study

Adapting Test No. 211: *Daphnia magna* Reproduction Test Protocol, [370] venlafaxine was assessed for its potential to affect the reproductive output, heart rate, morphology and transcriptome of *D. magna*. The workflow for morphology and transcriptome can be seen in Figure 39.

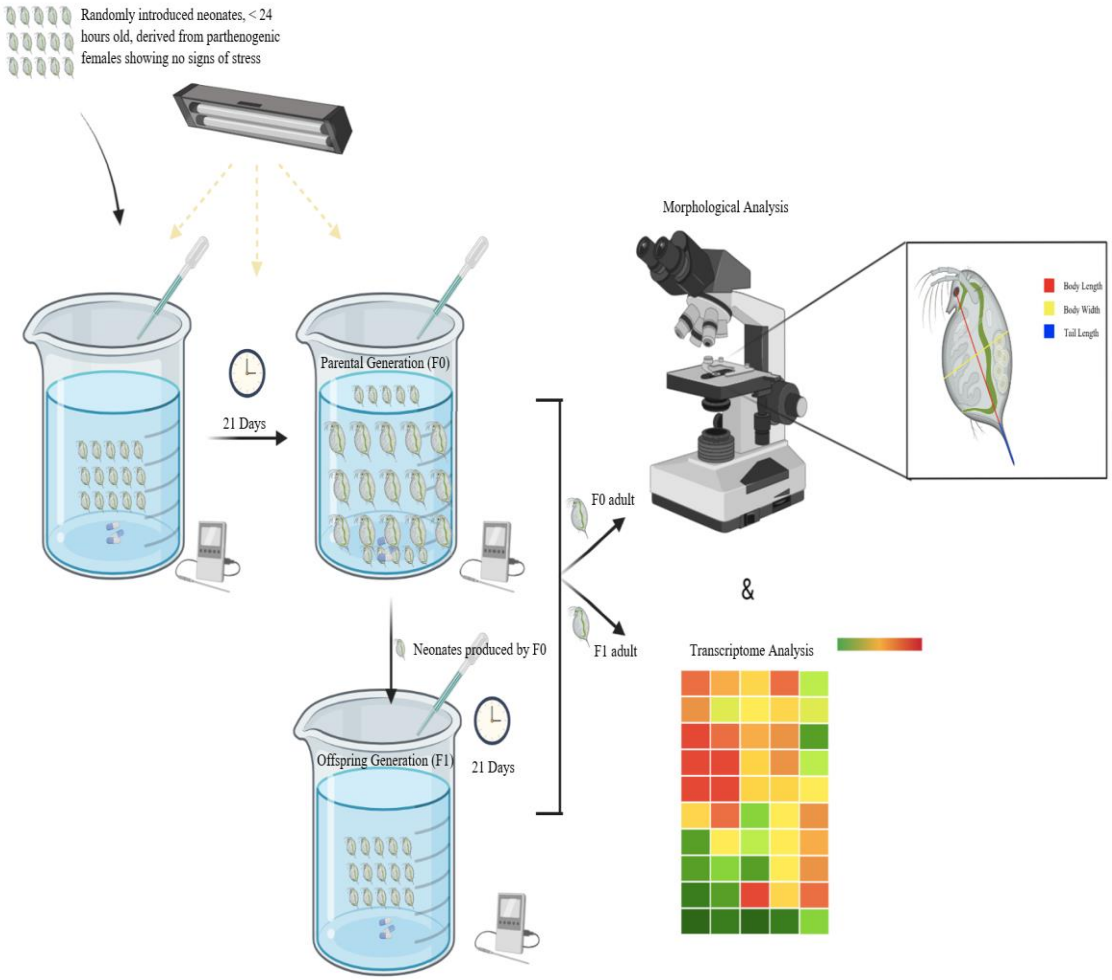


Figure 39. Workflow of chronic exposure study methodology for morphological and transcriptome analysis. The heat map is given as an example, not as an accurate representation of transcriptome analysis results. Created with BioRender.com

Heart rate was assessed by exposing 21-day-old *D. magna* to venlafaxine at three concentrations: PNEC (6.1 ng/L), PNEC x10 (61 ng/L) and PNEC x100 (610 ng/L) and the heart rate after 48 h was recorded using a microscope and timer.

Reproductive output was assessed by exposing 30 (n=4) female *D. magna* neonates (> 24 hours old) to venlafaxine at PNEC (6.1 ng/L) over 21 days. Parental (F0) and offspring (F1) generations were separated and reared until reaching 21 days old. At the end of the 21-day test, the total number of living offspring produced was individually counted and assessed. Furthermore, the number of surviving parent animals and the time it took to produce the first brood was counted.

Transcriptome analysis consisted of collecting the F0 and F1 generations from the reproduction study and was kept in RNAlater® solution (Merck Millipore Ltd., Ireland) at -4 ℃ until extraction. RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Ireland) and isolated using the RNeasy® Micro Kit (50) (Qiagen, Ireland) per the manufacturer's instructions. RNA was purified using the RNase-Free DNase Set (50) (Qiagen, Ireland) to remove genomic DNA. Total RNA was quantified using the NanoDropTM One/Onec Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Ireland), with all samples containing ≥ 400 ng of total RNA as per Novogene Co., Ltd. sample requirements. Transcriptome sequencing by mRNA-Seq and subsequent bioinformatics analysis were obtained commercially at Novogene Co., Ltd. (Cambridge Sequencing Centre, UK), screening relevant genes and pathways potentially altered by exposure to the pharmaceutical.

4.3.5 Risk assessment

QSAR modelling was carried out utilising two programs, Ecological Structure Activity Relationships (ECOSAR) and Toxicity Estimation Software Tool (TEST). A risk quotient (RQ) was generated to determine the potential ecological risk posed by each Measured environmental concentration (MEC) of a pharmaceutical detected. The RQ value was generated using Equation 7 [371], where the PNEC were selected from previous WFD "watchlist" documents.[73,230,271–275] Risk Quotients (RQ) are categorised into four groups: high risk (RQ > 1), medium risk (1 < RQ < 0.1), low risk (0.1 < RQ < 0.01), and negligible risk (RQ < 0.01), to give a numerical value to the risk of the environmental impact of the pharmaceutical concentrations present in the rivers in relation to their PNEC value.

$$RQ = \frac{MEC\ (ng/L)}{PNEC\ (ng/L)} \quad \textit{Equation 7}$$

The PNECs used for risk quotient analysis calculations were selected from acute and chronic toxicity tests from trophic levels (algae, Daphnia and fish) published in WFD watchlist documents or selected from literature. The selected PNEC values are summarised in Table 25.

Table 25: PNEC values used for risk quotient analysis calculations of pharmaceuticals of interest.

Analyte of interest	PNEC (µg/L)	Ref
Metformin	5	[274]
Gabapentin	100	[51]
Trimethoprim	0.5	[372]
Ciprofloxacin	0.089	[73]
Venlafaxine	0.0061	[372]
O-Desmethyl venlafaxine	0.0061	[372]
Sulfamethoxazole	0.1	[372]
Carbamazepine	0.5	[372]
Diclofenac	0.05	[195]
Erythromycin	0.2	[73]
17-beta-Estradiol	0.0004	[73]
Estrone	0.0036	[73]
Gemfibrozil	0.8519	[372]
Azithromycin	0.019	[73]
Clarithromycin	0.12	[73]

Bio-concentration Factor (BCF) used to determine the uptake of pharmaceuticals from the aqueous phase was calculated using Equation 8.[353] Equation 8 solely references the concentration of pharmaceuticals after the 24 h depuration phase where $C_{organism}$ = Pharmaceutical concentration in *D. magna* (µg/kg) and C_W =pharmaceutical concentration in spiked water.

$$BCF = \frac{C_{Organism}}{C_W} \quad \textit{Equation 8}$$

4.4 Results and discussion

This discussion explores the experimental approaches to increase our understanding of pharmaceutical risk assessment. These methods involve risk quotient determination through river water monitoring, evaluating pharmaceutical uptake into *D. magna*, and analysing changes in genetic expression, morphology and heart rate resulting from pharmaceutical exposure. Findings aim to provide a holistic assessment of pharmaceutical risks and potential environmental effects.

4.4.1 Risk assessment

4.4.1.1 Qsar modeling

NORMAN Substance Database and QSAR modelling programs, such as ECOSAR and TEST, were initially used to determine the LC_{50} for the pharmaceuticals of interest (Table 26).[373–375] Limitations of QSAR models are the quality of data used for toxicity assessments and their accuracy compared to real-world toxicity testing.[376] For this reason, PNEC values, which were experimentally determined and reported in watchlist reports, were selected for assessment over values determined through QSAR.[73,230,271–275]

Table 26: Calculated LC_{50} 48 h of D. manga using QSAR modelling (TEST and ECOSAR) and in comparison to toxicity predictions from NORMAN network.[375]

Pharmaceutical	Predicted toxicity		
	NORMAN LC50 mg/L	T.E.S.T. LC50 mg/L	ECOSAR LC50 mg/L
Amoxicillin	603.62	23.81	350.82 (Aliphatic Amines) 689.21 (Phenols) 7333.54 (Amides) 15.35 (Phenol Amines)
Trimethoprim	140.97	15.16	6.38 (Anilines (Unhindered) 226.37 (Anilines (Hindered) 4.53 (Anilines (amino-meta))
Erythromycin	38.37	160.53	8.62 (Aliphatic Amines) 101.86 (Esters) 11.34 (Ketone Alcohols)
Clarithromycin	0.0297	83.68	3.31(Aliphatic Amines) 37.61 (Esters) 4.20 (Ketone Alcohols)
Azithromycin	244.59	94.06	3.02 (Aliphatic Amines) 34.25(Esters)
Ciprofloxacin	51.51	2.84	1240.43 (Aliphatic Amines) 140379.81(Vinyl/Allyl/Propargyl Ketones)
Sulfamethoxazole	99.20	N/A	6.43 (Anilines (amino-meta)) 1319.42 (Amides)
Diclofenac	42.64	3.50	25.75 (Neutral Organics)
Venlafaxine	23.10	8.18	1.06 (Aliphatic Amines)
Estrone	3.96	3.60	3.16 (Phenols)
17-α-ethylene estradiol	5.08	N/A *	1.60 (Vinyl/Allyl/Propargyl Alcohols-Hindered Phenols)
β-estradiol	3.52	3.15	8.93 (Phenols)
Metformin	49.19	412.82	1.78 (Aliphatic Amines)
Gabapentin	118.37	36.27	1927.73 (Aliphatic Amines)
Gemfibrozil	14.05	5.13	4338.24 (Neutral Organics)
Carbamazepine	26.59	3.56	4.93 (Substituted Ureas)

*The consensus prediction for this chemical is considered unreliable since only one prediction can be made

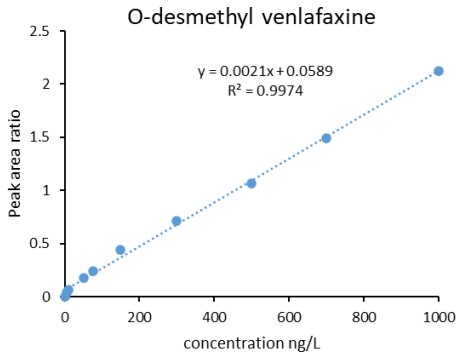
4.4.1.2 Risk Quotient analysis from field samples

With the detection of pharmaceuticals in the Nore, Liffey, Suir and Analee rivers (Chapter 3), a risk quotient analysis was conducted and is detailed in Table 27 to

Table 30. Within these tables, the substance-specific risks are linked to the associated risk quotient for each site and pharmaceutical compound. An example of how risk quotients were calculated can be seen in Figure 40.

Sampling location	Analyte peak area	Internal standard peak area	Peak area ratio	Concentration (ng/L) using the equation of the line / 100 (concentration factor)
Liffey May 2021 r3	4354527	243052	17.916	86.43
Liffey May 2021 r2	4711077	272110	17.313	83.52
Liffey May 2021 r1	4719948	262679	17.969	86.69

Average concentration
MEC = 85.55 ng/L



$$RQ = \frac{MEC}{PNEC}$$

$$RQ = \frac{85.55\ ng/L\ (MEC)}{6.1\ ng/L\ (PNEC)} = 9.32$$

Figure 40. Sample Risk Quotient analysis calculation using Measured Environmental Concentrations (MEC) and Predicted No Effect Concentrations (PNEC) reported in Table 25.

It can be seen that for each sampled river, there are substances that frequently present the highest level of risk. For instance, in the R. Liffey, venlafaxine, its metabolite O-desmethyl venlafaxine, and sulfamethoxazole pose a consistent risk. Notably, these substances likely originate from WWTPs (i.e., are present in WWTP influent but not effectively removed during treatment processes). However, further studies and extensive monitoring are required to confirm this.

Applying this form of risk assessment provides a straightforward mapping of pharmaceutical occurrence, and the inclusion of RQ facilitates the identification of locations, months and pharmaceuticals that are most critically in need of continual monitoring.

Table 27: R. Liffey risk quotient (RQ) values for pharmaceuticals that present negligible (blue), low (green), moderate (yellow) or high (red) risk based on calculated risk quotient (RQ).

Pharmaceutical	Mar-22	Sep-21	May-21	Mar-21	Oct-20	Sep-20
Metformin	<LOQ	<LOQ	0.0013	0.0013	<LOQ	0.0011
Gabapentin	0.00076	0.00051	0.00119	<LOQ	0.00054	0.00046
Trimethoprim	0.053	0.028	0.017	0.009	0.019	0.016
Ciprofloxacin	0.049	0.217	0.048	0.068	0.036	0.126
Venlafaxine	6.11	10.57	9.32	2.64	3.41	3.26
O-Desmethyl venlafaxine	9.60	14.90	14.02	3.38	4.52	4.60
Sulfamethoxazole	2.90	2.05	1.03	0.69	1.71	1.51
Carbamazepine	0.032	0.053	0.045	0.017	0.016	0.016
Diclofenac	<LOQ	0.14	0.14	1.85	0.12	0.13
Gemfibrozil	<LOD	<LOD	0.10	0.33	0.013	0.0064
E1	<LOD	<LOD	<LOQ	46.53	<LOD	<LOD
Erythromycin	<LOD	<LOQ	<LOD	0.21	<LOD	<LOQ

Table 28: R. Suir risk quotient (RQ) values for pharmaceuticals that present negligible (blue), low (green), moderate (yellow) or high (red) risk based on calculated risk quotient (RQ).

Pharmaceutical	Mar-22	Sep-21	May-21	Mar-21	Oct-20	Sep-20
Metformin	<LOQ	0.0011	0.0013	<LOQ	0.0014	0.0033
Gabapentin	0.00054	<LOQ	0.00031	<LOQ	0.00014	0.00017
Trimethoprim	<LOQ	0.015	0.016	<LOQ	0.015	<LOQ
Ciprofloxacin	0.11	0.11	0.051	0.12	0.11	0.15
Venlafaxine	2.10	1.32	1.19	<LOQ	1.27	0.94
O-Desmethyl venlafaxine	2.24	2.02	1.77	0.58	1.79	1.35
Sulfamethoxazole	0.25	1.27	0.088	0.20	1.99	0.084
Diclofenac	<LOQ	0.18	0.21	0.15	0.14	0.38
Gemfibrozil	<LOD	<LOD	<LOD	0.0059	<LOD	<LOD
Erythromycin	<LOD	<LOQ	<LOD	<LOD	0.13	<LOD

Table 29: R. Nore risk quotient (RQ) values for pharmaceuticals that present negligible (blue), low (green), moderate (yellow) or high (red) risk based on calculated risk quotient (RQ).

Pharmaceutical	Mar-22	Sep-21	May-21	Mar-21	Oct-20	Sep-20
Metformin	0.0014	<LOQ	<LOQ	0.0021	0.0030	0.0011
Gabapentin	0.00011	0.00032	0.00023	<LOQ	0.00011	0.00014
Trimethoprim	0.0086	<LOQ	<LOD	<LOD	0.0187	<LOQ
Ciprofloxacin	0.09	0.07	0.03	0.17	0.36	0.19
Venlafaxine	0.79	2.99	1.29	<LOQ	1.36	1.79
O-Desmethyl venlafaxine	1.10	5.37	1.67	0.76	1.36	2.51
Sulfamethoxazole	0.06	0.13	0.16	0.08	0.30	0.40
Carbamazepine	<LOQ	0.03	<LOQ	<LOD	<LOQ	0.010
Diclofenac	0.15	0.30	0.11	0.11	0.11	0.092

Table 30: R. Analee risk quotient (RQ) values for pharmaceuticals that present negligible (blue), low (green), moderate (yellow) or high (red) risk based on calculated risk quotient (RQ).

Pharmaceutical	Mar-22	Sep-21	May-21	Mar-21	Oct-20	Sep-20
Metformin	<LOQ	<LOQ	<LOQ	0.0016	0.0050	0.0026
Gemfibrozil	<LOQ	0.15	<LOD	0.15	<LOQ	<LOQ
Gabapentin	<LOQ	0.00017	0.00068	0.00019	0.00027	0.0002
Trimethoprim	<LOD	<LOQ	<LOQ	0.019	0.029	<LOQ
Ciprofloxacin	0.082	0.032	<LOQ	0.27	0.092	<LOQ
Venlafaxine	<LOQ	1.98	2.34	1.68	1.90	<LOQ
O-Desmethyl venlafaxine	<LOQ	2.16	3.12	1.25	2.86	0.89
Sulfamethoxazole	0.14	0.11	0.91	2.34	0.68	0.0522
Carbamazepine	<LOQ	0.012	0.022	0.013	0.012	0.0056
Diclofenac	<LOQ	0.17	0.18	0.48	0.11	<LOQ
Azithromycin	<LOD	<LOD	<LOD	<LOD	0.78	<LOD
Clarithromycin	<LOD	<LOD	<LOQ	<LOD	0.12	<LOD
Erythromycin	<LOQ	<LOD	<LOQ	0.11	<LOD	<LOD

Figure 41 summarises pharmaceutical risk classifications in the rivers Liffey, Nore, Suir, and Analee, combining the sampling period (September 2020-March 2022). The River Liffey has the highest number of high-risk detections (19), suggesting a greater potential for environmental impact compared to the other rivers. This may result from the connected WWTPs, which service a larger population equivalent.

However, the risk profile for the River Nore, Suir and Analee shows higher levels of moderate risk detections, which are sub-PNEC levels (RQ <1), which may pose a

potential risk for long-term/chronic exposure. Furthermore, as tested, pharmaceuticals have been detected in a cocktail, which could enhance risk due to the possibility of combined interactions/effects.[377] There is a cause for concern as these pharmaceuticals can ultimately impact ecosystem resilience, which may enhance an aquatic organism's vulnerability to the presence of other stressors such as pesticides and PAHs.[378]

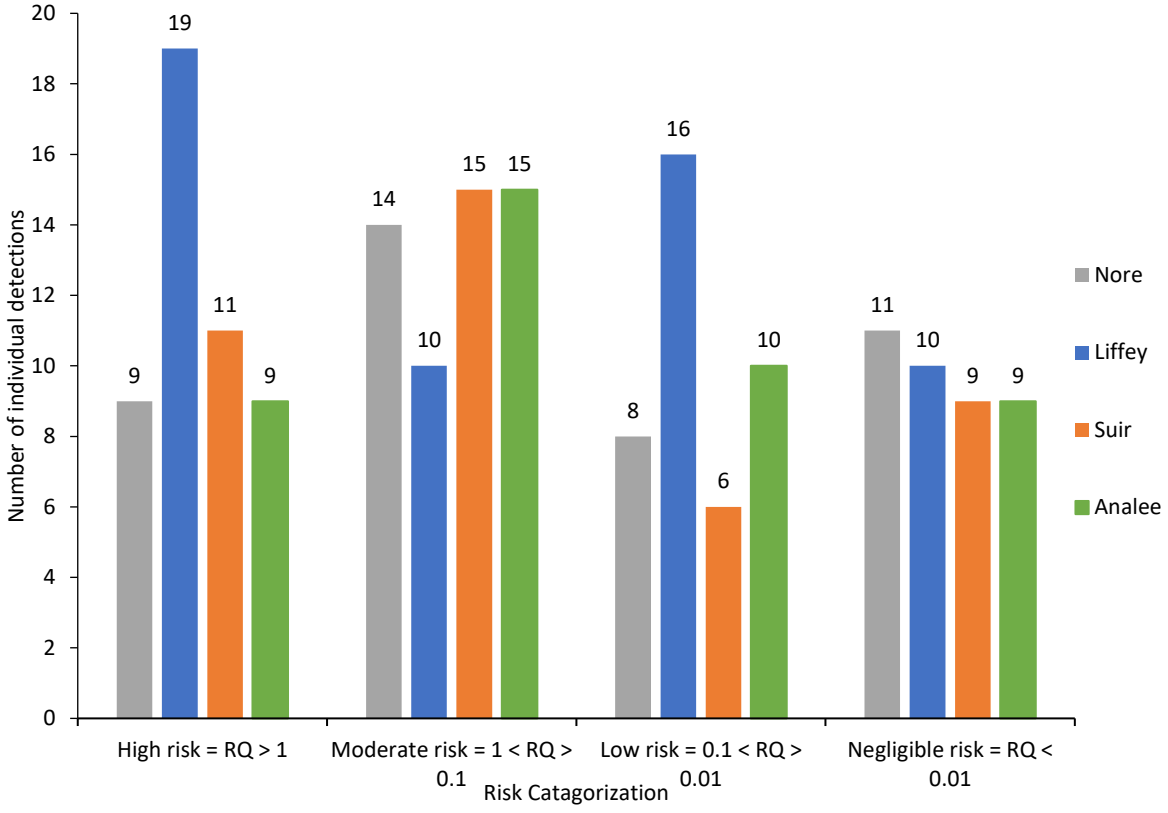


Figure 41. Risk categorisation of the Nore, Liffey, Suir and Analee for individual pharmaceutical detections over six sampling periods (>LOQ).

Venlafaxine and its metabolite O-desmethyl venlafaxine were found to have the highest mean risk of all pharmaceuticals tested across all sites (Figure 42). Due to the frequency of detection at above PNEC levels (RQ > 1), venlafaxine and its metabolite O-desmethyl venlafaxine are shown to be a persistent/pseudo-persistent high-risk pharmaceutical and toxic in aquatic ecosystems. RQ values observed in this study align with European

RQ values such as in Belgium (Brussels, Antwerp), Finland (Helsinki), the UK (London), and Germany (Berlin, Frankfurt).[338]

Venlafaxine and its metabolite O-desmethyl venlafaxine posed a high potential risk to the disruption to sensitive organisms in the form of population dynamics, behaviour, biodiversity, reproduction and development, leading to alterations to the composition of aquatic communities and overall ecosystem health. A study by Huang et al. observed that venlafaxine and its metabolite have been shown to increase hyperactivity in zebrafish.[169] Meanwhile, studies conducted by Painter et al. and Thompson and Vijayan et al. observed reduced growth performance and escape response in flat head minnow at environmentally relevant concentrations.[170,379]

Sulfamethoxazole was additionally found at mean RQ >1 in the River Liffey. Sulfamethoxazole is frequently found in European surface waters, posing a risk and having been shown to affect a wide range of photosynthetic aquatic plants, algae, and cyanobacteria.[42,149] The sustained high RQ values for these pharmaceuticals may induce chronic toxicity in the form of an ecosystem.

As pharmaceutical prioritization is typically evaluated under both normal and worst-case scenarios.[26] The maximum pharmaceutical concentration was selected for RQ analysis (Figure 43). The risk for several pharmaceuticals increased from moderate to high, such as sulfamethoxazole in the Suir and Analee, diclofenac in the Liffey, and estrone in the Liffey. Although the higher RQ values for this worst-case scenario were the exception rather than the norm, the values still fall within RQ values calculated from maximum concentrations detected in the European rivers. For example, using the PNEC values in Table 25 and maximum concentrations reported by Wilkinson et al., for

venlafaxine, Denmark (Odense), Luxembourg City, Switzerland (Basel), Scotland (Glasgow) had RQ values of 9.92, 58.85, 2.38, 61.31 respectively, while RQ values for sulfamethoxazole in Germany (Tubingen), Hungry (Budapest), Bulgaria (Sofia) were 9.22, 1.07, 0.776 respectively. [338]

Furthermore, calculated max RQ values were lower than reported by Zhou et al., who examined the maximum RQ values in 33 European countries surface waters.[26] In this study, the maximum RQ values for sulfamethoxazole, gemfibrozil, erythromycin, venlafaxine, carbamazepine, and diclofenac were 4.97, 4.99, 42.50, 94.26, 1156.10, 18,740.00 respectively.[26]

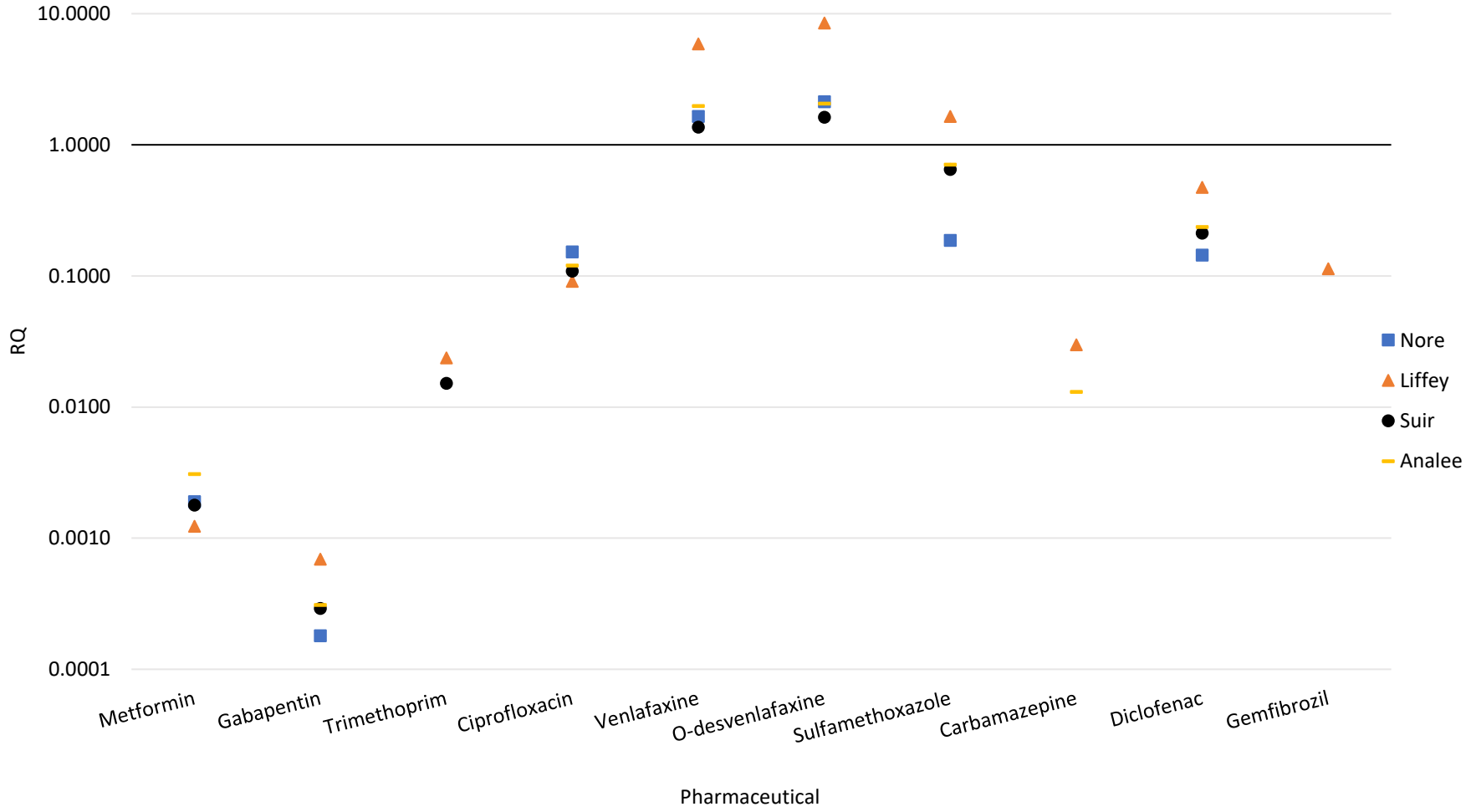


Figure 42. Average RQ value with single detections removed (> LOQ detections in ≥50% of samples) for pharmaceuticals detected between September 2029 to March 2022 (>LOQ). High risk (RQ > 1), medium risk (1 < RQ < 0.1), low risk (0.1 < RQ < 0.01), and negligible risk (RQ < 0.01).

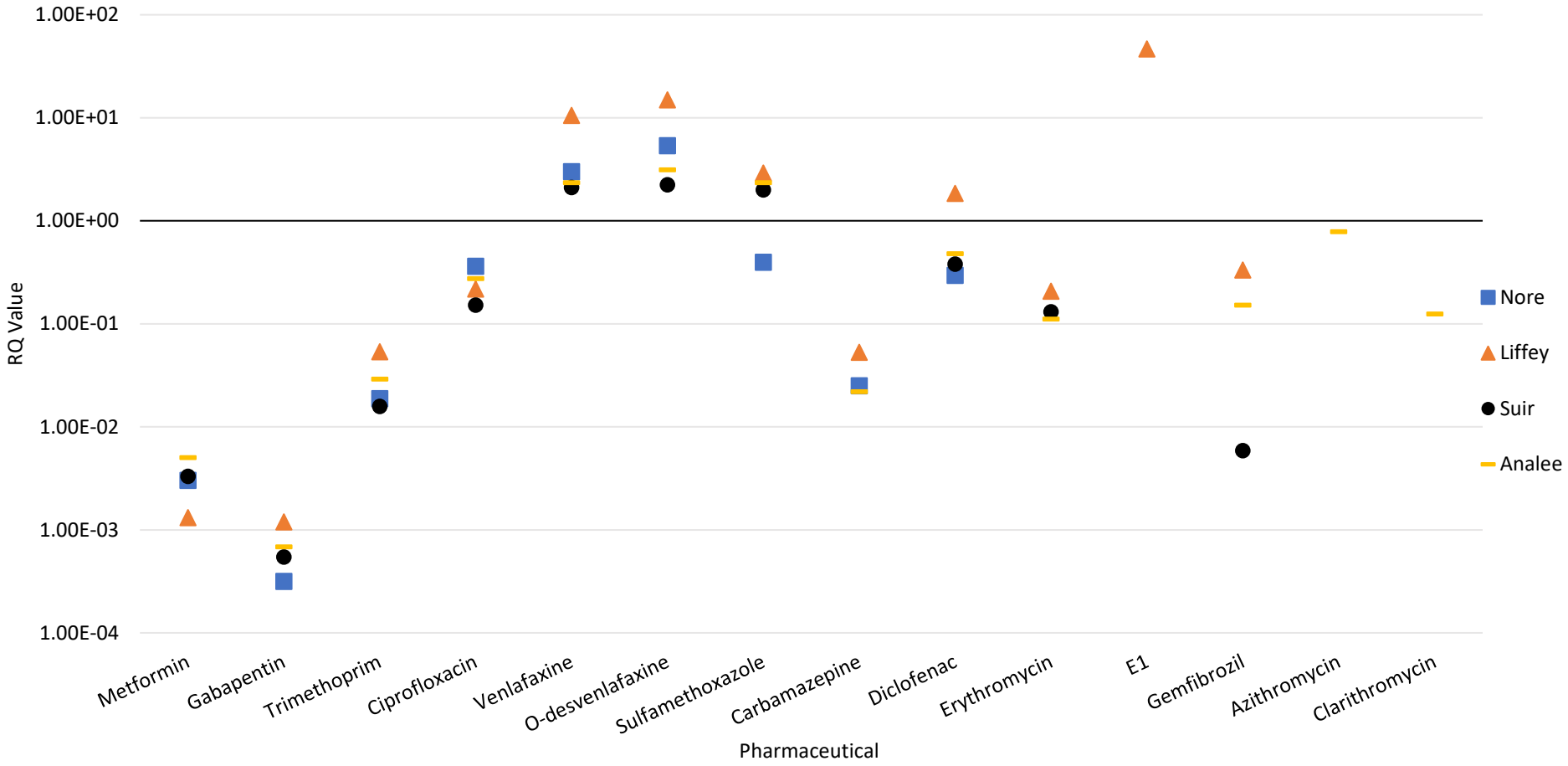


Figure 43. RQ value for the highest concentration of pharmaceuticals detected between September 2029 to March 2022 (LOQ). High risk (RQ > 1), medium risk (1 < RQ < 0.1), low risk (0.1 < RQ < 0.01), and negligible risk (RQ < 0.01).

The inclusion of a novel form of risk assessment by Zhou and Li et al., which was later used by Figuière et al., optimised RQ analysis through the development of a Risk Quotient Frequency (RQ_f).[380,381] RQ_f allows for the differentiation between pharmaceuticals frequently detected exceeding an RQ of 1 (e.g. O-desmethyl venlafaxine) and pharmaceuticals that only have one exceedance (e.g. diclofenac). RQ_f was calculated using Equation 9, where RQ_f values indicate risk in four categories: high risk ($RQ_f \geq 1$), moderate risk ($1 > RQf \geq 0.1$), limited risk ($0.1 > RQf \geq 0.01$) and negligible (risk $0.01 > RQf > 0$).[381]

$$RQ_f = \frac{MEC}{PNEC} \times \frac{Number\ of\ samples\ where\ MEC>PNEC}{Total\ number\ of\ samples\ (24)} \quad \textit{Equation 9}$$

In Table 31, a summary of the compounds considered to have the highest potential to pose an environmental risk ($RQ_f > 0$) is provided. Venlafaxine and O-desmethyl venlafaxine are suspected to be the most concerning to aquatic ecosystems in Irish freshwaters, with a high environmental risk potential. (RQ_f = 2.04 and 2.98, respectively). Whereas sulfamethoxazole posed a moderate risk (RQ_f = 0.27) and diclofenac was found to pose a limited/negligible effect risk (RQ_f =0.011). Figuière et al. investigated the RQ mean and RQf of several Swedish rivers. This study found that venlafaxine had an RQ of 3.6 with an RQ_f value of 1.4, O-desmethyl venlafaxine had an RQ of 0.84 with an RQ_f of 0.14, while diclofenac had an RQ of 0.61 with an RQ_f value of 0.1.[381] However, challenges arise when comparing RQ and RQ_f in the literature as PNEC values are being continually being updated and studies often use PNEC values from various sources rather.

Given the prevalence of these pharmaceuticals identified with high or moderate risks, it indicates insufficient removal of pharmaceuticals in WWTPs. Furthermore, due to the risks identified, it is imperative to maintain a continuous monitoring campaign to catch any

potential increase in their concentrations within surface waters and to determine the long-term effect these pharmaceuticals may have on aquatic ecosystems.

Table 31: List of compounds for which optimised risk quotient (RQf) was > 0 and detections comprising a minimum of 80% of the tested samples. These compounds are suspected to be the most concerning to aquatic ecosystems and should be prioritised for further work.

Substance	CAS	PNEC ng/L	LOQ ng/L	LOQ/PNEC	Number of samples Negligible risk RQ<0.01 (excluding <LOQ)	Number of samples Low Risk 0.01-0.1 (excluding <LOQ)	Number of samples Moderate risk 0.1-1 (excluding <LOQ)	Number of samples High Risk RQ>1 (excluding <LOQ)	Mean RQ (24 samples including non-detects)	RQ_f
Sulfamethoxazole	723-46-6	100	2.76	0.028	0	5	11	8	0.80	0.27
Venlafaxine	93413-69-5	6.1	4.78	0.41	0	0	2	18	2.72	2.04
O-Desmethyl venlafaxine	93413-62-8	6.1	2.48	0.78	0	0	3	20	3.58	2.98
Diclofenac	15307-86-5	50	4.59	0.091	0	1	18	1	0.27	0.011

4.4.2 Biological assessment of pharmaceuticals

The presence of moderate to high-risk pharmaceuticals in the selected rivers raises concerns. However, the direct link of exposure to effects could inaccurately represent the actual risk as the uptake of these pharmaceuticals into aquatic organisms is dependent on a variety of processes such as adsorption, distribution, metabolism, and excretion, which in effect can result in a range of toxicological consequences.[353,382] *D. magna* play a vital role in surface water food webs. Research by Ding et al. has shown that *D. magna* can accumulate pharmaceuticals and act as a pathway for the transfer of pharmaceuticals to higher organisational levels.[353] However, there is still a significant knowledge gap surrounding the uptake and effects of pharmaceuticals on aquatic invertebrates. For this reason, the following study aimed to measure the internal concentrations of test organisms at environmentally relevant concentrations and assess their associated morphological and genetic effect.

4.4.2.1 Biological uptake study method performance

A linearity of $R^2 > 0.98$ ($n \geq 5$) was achieved in triplicate with standard injections for 7 pharmaceutical API's. Limits of quantitation and quantification (LOD and LOQ) and recovery are shown in Table 32. Four APIs achieved a recovery of >70 %. With the current method employed, venlafaxine, sulfamethoxazole, metformin and trimethoprim were deemed acceptable for quantification.

Table 32: Method LOD and LOQ of target analytes and analytical recovery from spiked D. magna matrix and extracted with SPE as per section 4.3.4.1.

Pharmaceutical	% recovery (n=3 at 700 ng/L)	RSD %	LOD ng/L	LOQ ng/L
Venlafaxine	78.08	0.87	0.26	0.79
Sulfamethoxazole	71.96	3.67	0.19	0.56
Metformin	106.99	6.69	0.28	0.84
Carbamazepine	34.92	3.97	0.48	1.44
Erythromycin	54.23	106.78	0.30	0.92
Trimethoprim	102.32	4.57	0.16	0.48
Clarithromycin	33.26	63.43	0.10	0.31

4.4.2.2 Pharmaceutical concentrations detected in *D. magna*

The method employed allowed for the detection and quantification of pharmaceuticals in lab-grown *D. magna* with individual (Table 33) and mixture scenarios (Table 34). The pharmaceuticals venlafaxine, sulfamethoxazole, and metformin were exposed to *D. magna* at environmentally relevant concentrations (PNEC x10). Both Table 33 and Table 34 show that *D. magna* were unable to entirely remove all the tested pharmaceuticals during the 24 h depuration period. This observation aligns with observations of other pharmaceuticals, such as roxithromycin and propranolol.[353]

Table 33 shows the respective concentrations of pharmaceuticals found from individual exposure experiments. From the concentrations observed, all tested pharmaceuticals showed a low level of bioaccumulation, with venlafaxine having the highest propensity to accumulate in *D. magna* as expected due to its higher Log K_{ow} in comparison to metformin or sulfamethoxazole. An example of how the Bio-Concentration Factor (BCF) was calculated can be seen in Figure 44, where BCF represents the accumulation of pharmaceuticals in *D. magna* with respect to the concentrations in surrounding water. BCF was calculated by the determination of pharmaceutical concentrations (µg/kg) in 30 daphnia accounting for

recovery, concentration factor and weight and dividing it by the exposure concentration (PNEC x10).

$$\text{8.11E-03 µg/kg} \longrightarrow \text{BCF} = \frac{\text{8.11E-03 µg/kg}}{\text{0.061 µg/L (PNEC x 10)}} = 0.13$$

8.11E-06 ng/mg

$$\frac{\text{3.24 ng/L/100mg * volume reconstituted (0.00025L)}}{\text{100 mg}}$$

Average concentration = 3.24 ng/L/100mg

Peak area ratio - controls	Calculated concentration in ng/L adjusted for recovery (78.08%)	Calculated concentration/400 (concentration factor)	Weight of each 30 daphnia replicate (mg)	Uptake ng/L/100mg
32.718	1213.940	3.035	71.00	4.274
15.159	566.876	1.417	64.70	2.190
21.135	787.091	1.968	60.20	3.269

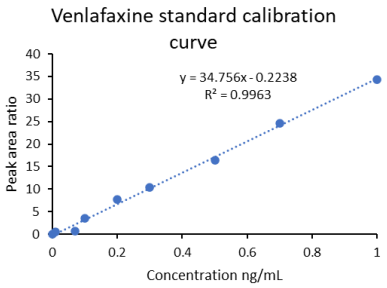


Figure 44. Example of Bioconcentration factor calculation (BCF) for venlafaxine.

The low BCFs observed in this study suggest that the tested pharmaceuticals were unlikely to bioconcentrate in *D.magna*. However, as these estimated values were derived under controlled laboratory conditions and the BCF was calculated assuming equilibrium between the pharmaceutical and *D. magna*, real-world scenarios may introduce variability as a result of diet, exposure concentration and duration of exposure. [383,384]

To the best of the authors' knowledge, no prior research has investigated the BCF of these pharmaceuticals on *D. magna*. However, studies on the Mussel (*Mytilus galloprovincialis*) by Serra-Compte et al. reported a BCF of 213-528 and Gomez et al. noted a BCF of 265.[385,386]

Table 33: Concentration of pharmaceuticals detected in individual exposure experiments of D. magna (dry weight) at x10 PNEC; Venlafaxine 61 ng/L; Metformin 5000 ng/L and Sulfamethoxazole 1000 ng/L.

Sample	Venlafaxine ng/L /100 mg *D. magna*	Metformin ng/L /100 mg *D. magna*	Sulfamethoxazole ng/L /100 mg *D. magna*
PNEC x 10	3.24 ± 1.042	13.2 ± 11.57	0.90 ± 0.80
Logkow [85]	3.2	-2.64	0.89
µg/kg	0.0081	0.0033	0.0023
Experimental BCF	0.13	0.00066	0.0023

Table 34 shows a subsequent test on the determination of pharmaceuticals in *D. magna* from a mixture, showing the capability of *D. magna* to uptake multiple pharmaceuticals simultaneously. This simultaneous uptake raises questions regarding the ecological and toxicological effect on surface water organisms, as exposure to pharmaceutical mixtures can potentially have synergistic or antagonistic effects.[42] Although this test illustrates the capacity of *D. magna* to uptake multiple pharmaceuticals, the variability in test samples precludes the derivation of definitive conclusions. However, a study by Miller et al., who investigated the presence of pharmaceuticals in wild *Gammarus pulex*, observed the presence of six pharmaceuticals (carbamazepine, trimethoprim diazepam, nimesulide, and warfarin) at low ng/g concentrations.[368]

Table 34: D. magna exposure to a pharmaceutical mixture.

Pharmaceutical at PNEC x10 Mix	ng/L/(30 *D. magna*)
Venlafaxine	1.16 ± 0.44
Metformin	3.65 ± 0.77
Erythromycin	3.39 ± 2.05
Trimethoprim	3.69 ± 1.12
Sulfamethoxazole	0.59 ± 0.63
Carbamazepine	4.36 ± 2.10
Clarithromycin	14.26 ± 5.28

4.4.2.3 Assessment of low-level pharmaceutical effects on invertebrate growth

PNECs are predicted no-effect concentrations, and therefore by implication, no adverse effects should be observed on exposure to these concentrations. However, the validity of this assumption should be evaluated as an integral component of assessing the ecological and physiological consequences of chemical contaminants in aquatic ecosystems. To achieve this, an informed assessment conducted by Stefania Scurtu and Kristyna Mrstna (researchers in

DCU Water Institute) was carried out on the effect of PNEC on *D. magna* morphology, reproduction, heart rate, and transcriptome in response to exposure.

4.4.2.3.1 Morphological analysis

Exposing *D. magna* to venlafaxine at PNEC (6.1 ng/L) concentrations resulted in a significant decrease in the mean body length, width and tail length in the parent (F0) generation. Furthermore, these reductions in morphological markers were observed in the subsequent F1 generational offspring, as shown in Figure 45 to Figure 47; however, there was no statistical difference between the F0 and F1 generation.

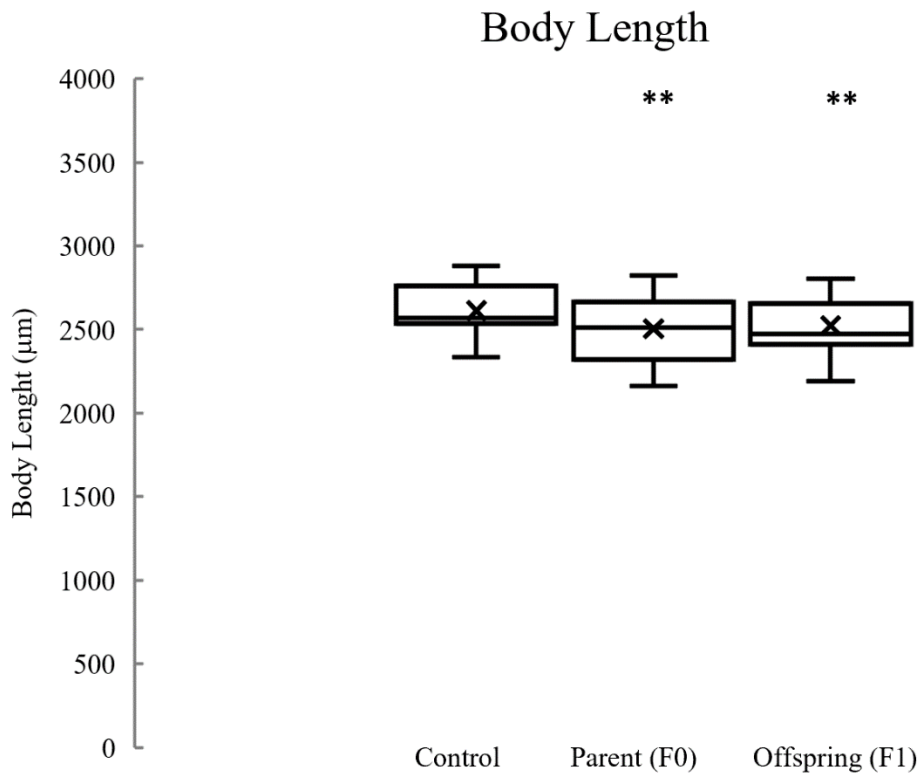


Figure 45. Box plots depicting Body length measurements in 21-day-old D. magna in the control (not exposed), parental (F0) and offspring (F1) generation (exposed to venlafaxine at of PNEC concentration (6.1 ng/L)), where n= 42. Crosses denote mean values, while the central horizontal line denotes the median. The higher and lower lines represent the maxima and minima, respectively. Limits show the third and first quartiles, respectively. Asterisks denote statistical significance compared to the respective control: p ≤ 0.05 (*), p ≤ 0.01(**), p ≤ 0.001 (***).

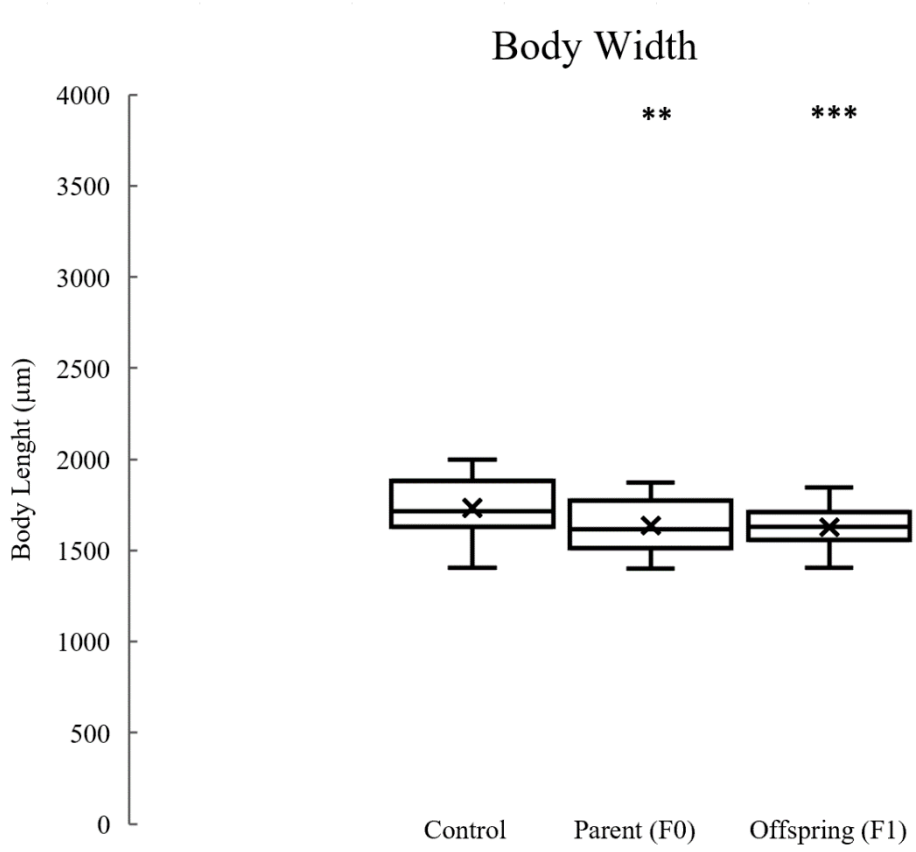


Figure 46. Box plots depicting Body width measurements in 21-day-old D. magna in the control (not exposed), parental (F0) and offspring (F1) generation (exposed to venlafaxine at of PNEC concentration (6.1 ng/L)), where n= 42. Crosses denote mean values, while the central horizontal line denotes the median. The higher and lower lines represent the maxima and minima, respectively. Limits show the third and first quartiles, respectively. Asterisks denote statistical significance compared to the respective control: p ≤ 0.05 (*), p ≤ 0.01(**), p ≤ 0.001 (***).

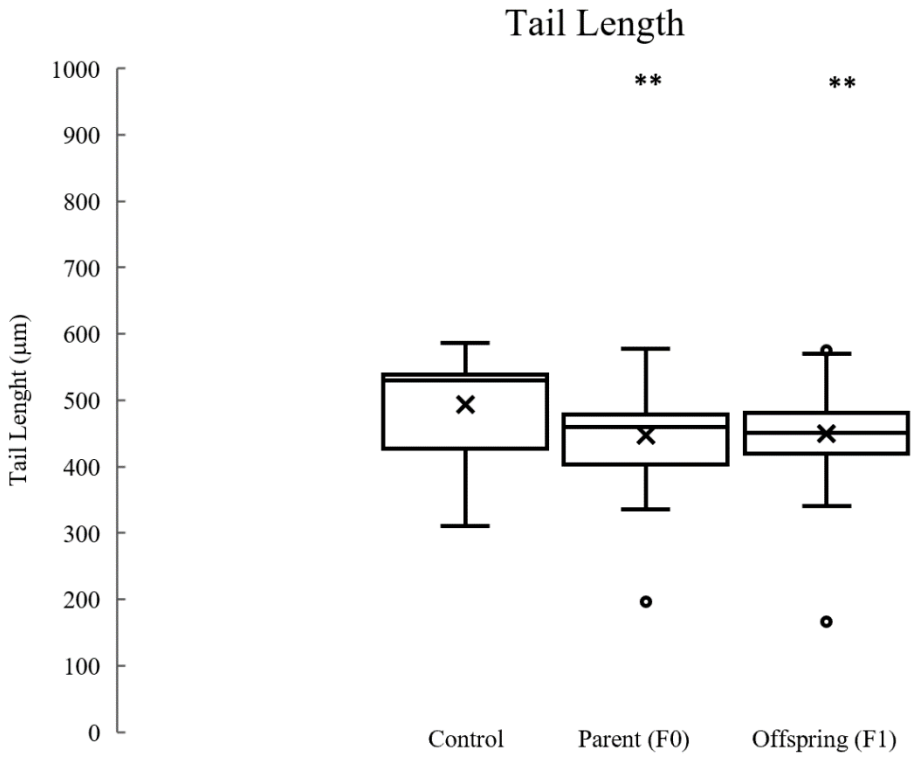


Figure 47. Box plots depicting tail length measurements in 21-day-old D. magna in the control (not exposed), parental (F0) and offspring (F1) generation (exposed to venlafaxine at of PNEC concentration (6.1 ng/L)), where n= 42. Crosses denote mean values, while the central horizontal line denotes the median. The higher and lower lines represent the maxima and minima, respectively. Limits show the third and first quartiles, respectively. Asterisks denote statistical significance compared to the respective control: p ≤ 0.05 (*), p ≤ 0.01(**), p ≤ 0.001 (***).

4.4.2.3.2 Reproduction effects

Figure 48 shows that on exposure of the test organisms to venlafaxine at PNEC concentrations (6.1ng/L), the brood size and number are impacted significantly. Neonates were first produced on Day 11 for the control and the venlafaxine-exposed animals. A significant difference in average accumulated offspring number between the control and exposed *D. magna* was observed on Days 19 and 21.

These experiments are carried out at ng/L concentrations and demonstrate the potential for concentrations that are considered to have no effect can have an effect on the reproduction of aquatic invertebrates. The *D. magna* females exposed to venlafaxine at a PNEC

concentration produced, on average, ~29% less offspring than the control on Day 19. This is a significantly lower value with a p-value of 0.0002 provided by Welch's unpaired T-Test. Day 19 control had a value of 51.88 ± 16.54, while venlafaxine had a value of 31.56 ± 7.899. Median values occurred at 48 and 34, respectively. Day 21 showed a significant difference between control and venlafaxine-exposed animals with a p-value of <0.0001 provided by Welch's unpaired T-Test. A ~51% decrease in accumulated offspring number was observed with values of 73.69 ± 8.26, control, and 37.8 ± 8.523, venlafaxine exposed. Median values were 74 and 38, respectively.

The drop in *D. magna* fecundity when exposed to 100 µg/L of venlafaxine has been previously reported by Minguez et al., who observed a drop of 44% over a 21-day period, with drug tolerance observed in successive broods.[174]

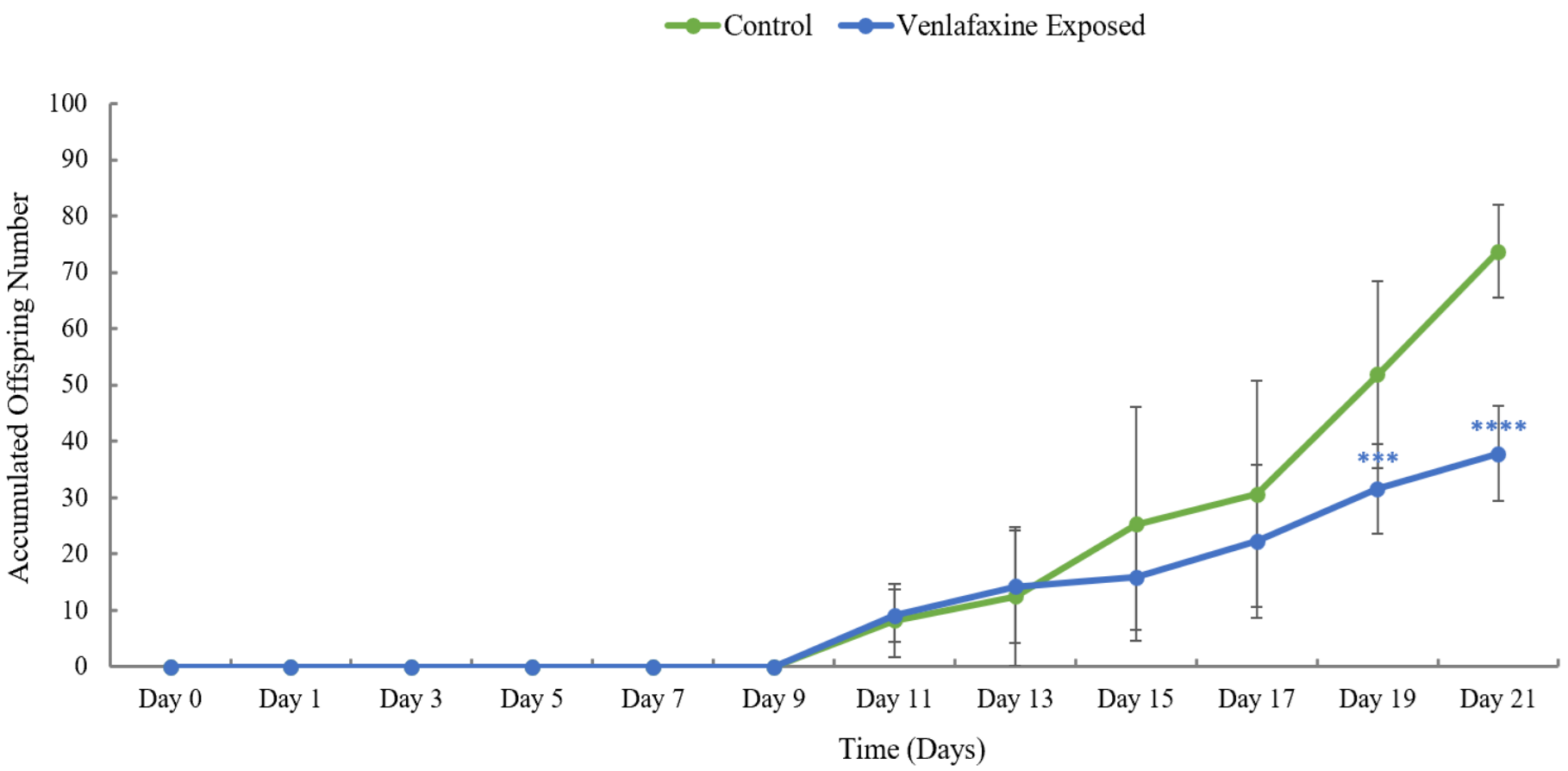


Figure 48. Graph depicting mean accumulated offspring number per female of control and D. magna exposed to venlafaxine at PNEC (6.1 ng/L) level over 21 days, where n= 16. Values as mean ± standard deviation. Asterisks denote statistical significance compared to the control: p ≤ 0.05 (*), p ≤ 0.01(**), p ≤ 0.001 (***), p ≤ 0.0001 (****).

4.4.2.3.3 Heart rate study

There are various physiological endpoints in ecotoxicological studies, such as feeding activity, thoracic limb movement, heart rate, cardiac area, respiratory activity, compound eye, mandible movements and post-abdominal claw contractions.[387] Each compound was tested at three concentrations after 48 h exposure. The average heart rate is different from publication to publication. According to Corotto et al., the average heart rate is 354 beats minute^{-1} (range: 91–521 beats minute^{-1}).[388] This parameter is easily affected by temperature and slows with decreasing temperature. However, the variation in heart rate cannot be attributed to variation in *D. magna* size. [388]

Figure 49 shows the average heart rates of *D. magna* exposed to venlafaxine at PNEC to PNEC x100 concentrations. *D. magna* exposed to PNEC x10 of venlafaxine (61 ng/L) was observed to increase heart rate by ~13.3% with a value of 462.6 ± 31.97 average beats per minute (p-value: 0.0075). In comparison, the PNEC x100 heart rate increased by ~32.9% with a value of 500.5 ± 39.86 average beats per minute (p-value: <0.0001). The respective control averaged 376.4 ± 44.27 beats per minute. Significant differences were also observed between PNEC and PNEC x100 (p-value: < 0.0001), as well as PNEC x10 and PNEC x100 (p-value: < 0.0036). However, in contrast to these findings, a study conducted by Oliveira et al. reported a reduction in the heart rate of *Danio rerio* embryos following acute venlafaxine exposure, potentially eluding to species-specific effects.[389]

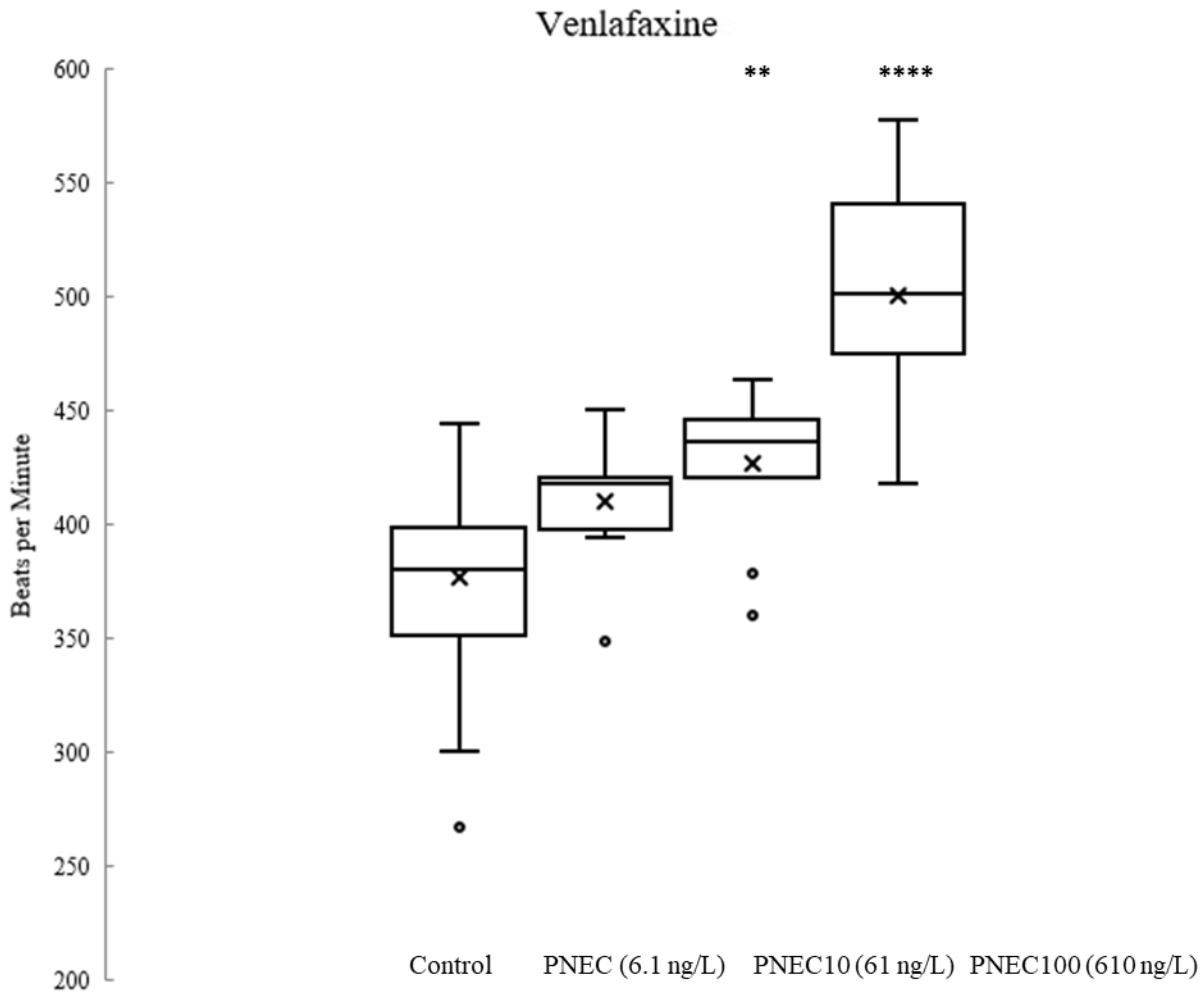


Figure 49. Box plots depicting average beats per minute (mean ± sd) of control and venlafaxine-exposed D. magna adults for 48 hours, where n= 20. Venlafaxine concentrations included PNEC, PNEC10 and PNEC 100. The D'Agostino & Pearson normality test classed the data as nonparametric, and therefore, it was analysed using the Kruskal-Wallis test, followed by Dunn's multiple comparisons tests. Asterisks denote statistical significance compared to the control: p ≤ 0.05 (*), p ≤ 0.01(**), p ≤ 0.001 (***), p ≤ 0.0001 (****).

Figure 50 shows the average heart rates of *D. magna* exposed to sulfamethoxazole at PNEC to PNEC x100 concentrations. Heart rate upon PNEC exposure decreased by ~15.1% with a value of 423.9 ± 17.19 average beats per minute (p-value: 0.0004). In comparison, PNEC x10 led to a decrease of ~ 17.8% with a value of 410.3 ± 30.99 average beats per minute (p-value: <0.0001). PNEC x100 reduced heart rate by 21.7%, averaging 390.7 ± 24.96 beats per minute compared to the control (p-value: <0.0001). The respective control averaged 499.1 ± 20.35 beats per minute. A significant difference was also observed between PNEC and PNEC x100

(p-value:< 0.0181). This finding is in contrast to the results of Zhang et al., who observed a decreased heart rate in *D.magna* at PNEC x100 concentrations. However, this study observed a reduction in *D.magna* heart rate at higher concentrations.[151] As all three tested concentrations of venlafaxine and sulfamethoxazole were observed to affect D. magna heart rate, this raises concerns about the validity of relying on PNEC values as an indicator of safety, as sublethal effects still warrant assessment when examining environmental risk.

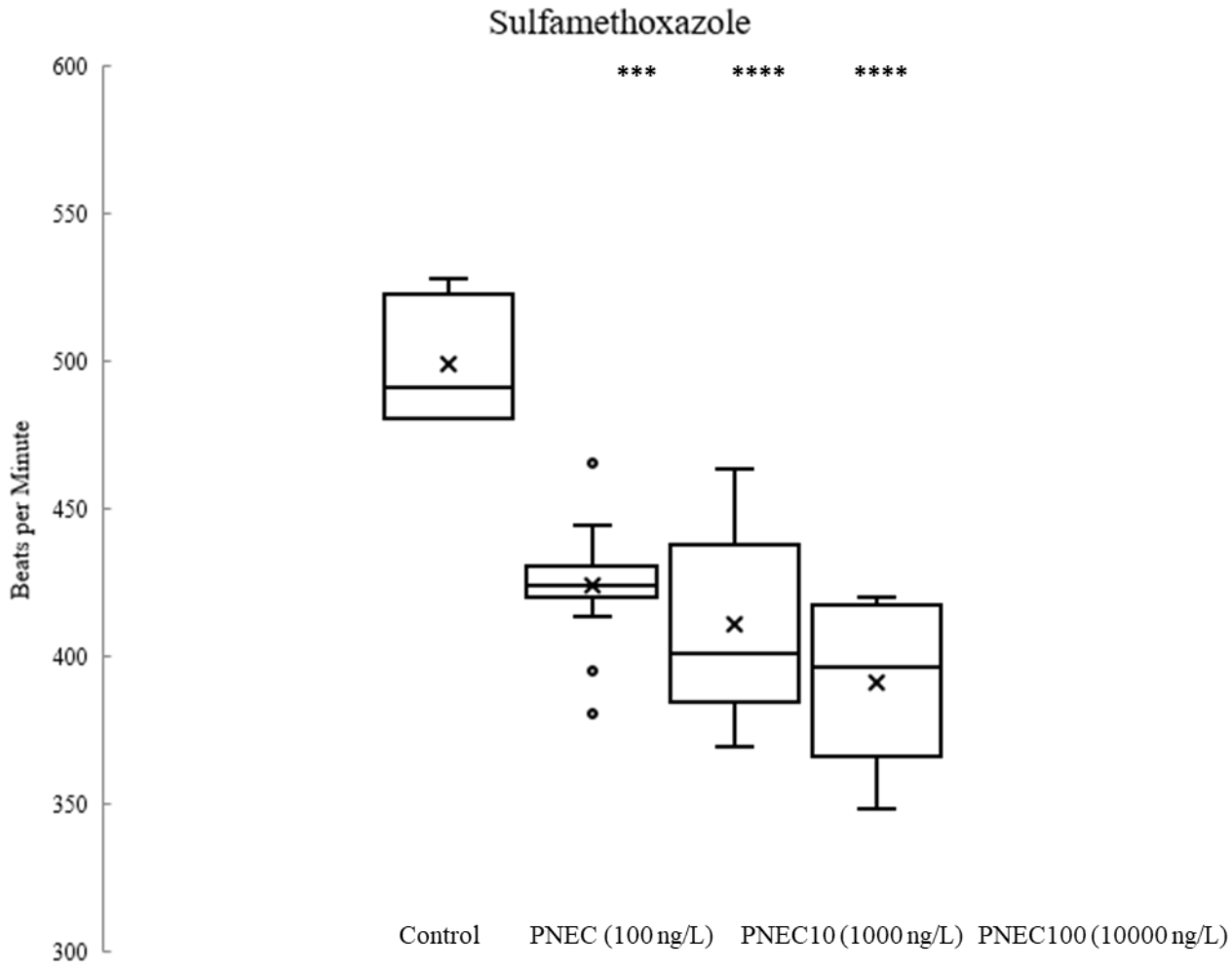


Figure 50. Box plots depicting mean heart rate (beats per minute) of control and sulfamethoxazole-exposed D. magna adults for 48 hours, where n= 20. Sulfamethoxazole concentrations included PNEC, PNEC x10 and PNEC x100. The D'Agostino & Pearson normality test classed the data as nonparametric, and therefore, it was analysed using the Kruskal-Wallis test, followed by Dunn's multiple comparisons tests. Asterisks denote statistical significance compared to the control: p ≤ 0.05 (*), p ≤ 0.01(**), p ≤ 0.001 (***), p ≤ 0.0001 (****).

4.4.2.3.4 Transcriptome analysis

Table 35 summarises the difference in expressed genes of the Parent (F0) and Offspring (F1) generations relative to the control of the *D. magna* following exposure to venlafaxine. The data shows the upregulation of oxidoreductase in both the parent (F0) and offspring generations (F1), with Iron binding in the F0 generation. As these categories have been previously associated with antioxidant responses, this may indicate that under PNEC concentrations, *D. magna* is experiencing oxidative stress.[390]

The Offspring (F1) generation appears to be experiencing a higher level of oxidative stress, with the upregulation of genes responsible for oxidation-reduction processes. The oxidative stress response from aquatic organisms is not unique to *D. magna*. A study by Ziegler et al. additionally identified biomarkers linked to oxidative stress in larvae of brown trout.[391] Furthermore, Ribeiro et al.'s study suggested that the observed disruption in zebrafish embryo development, resulting from exposure to venlafaxine, may be attributed to the disruption of enzymatic and non-enzymatic defence mechanisms induced by oxidative stress.[173]

Figure 51 summarises the counts of differentially expressed genes (DEG) of F0 and F1 generations who were exposed to PNEC concentrations of venlafaxine. In the F1 generation, 19 genes were downregulated, with 103 genes being upregulated, and in the F0 generation, six genes were downregulated, with 159 being upregulated. Both generations appear to upregulate bio-transforming and detoxifying genes in response to pharmaceuticals.

Table 35: List of significant GO enrichment analysis terms depicting gene properties categorised as cellular component (CC), molecular function (MF), and biological process (BP) following chronic exposure to 6.1 ng/L venlafaxine hydrochloride of parent (F0) and offspring (F1) D. magna generations. 4 biological replicates containing a pool of 30, 21-day-old D. magna, adult animals were sampled (n=4) (p< 0.05) (padj) < 0.05 and Log2 (FoldChange) > 0.

GOID	Category	Description	p-Value	Gene Count		GOID	Category	Description	p-Value	Gene Count
Parent						Offspring				
Upregulated						Upregulated				
GO:0006030	BP	chitin metabolic process	1.45E-14	13		GO:0006508	BP	Proteolysis	0.015769	6
GO:0006040	BP	amino sugar metabolic process	1.45E-14	13		GO:0006629	BP	lipid metabolic process	0.040451	3
GO:1901071	BP	glucosamine-containing compound metabolic process	1.45E-14	13		GO:0008610	BP	lipid biosynthetic process	0.041568	2
GO:0006022	BP	aminoglycan metabolic process	2.01E-14	13		GO:0055114	BP	oxidation-reduction process	0.043902	5
GO:0017144	BP	drug metabolic process	1.22E-12	13		GO:0016705	MF	oxidoreductase activity	0.010415	3
GO:1901135	BP	carbohydrate derivative metabolic process	1.16E-09	14		GO:0070011	MF	peptidase activity	0.014458	6
GO:0005576	CC	extracellular region	2.47E-09	13		GO:0008233	MF	peptidase activity	0.015418	6
GO:0008061	MF	chitin binding	1.80E-13	13		GO:0008146	MF	sulfotransferase activity	0.022188	2
GO:0042302	MF	structural constituent of cuticle	2.98E-10	12		GO:0008237	MF	metallopeptidase activity	0.022864	3

Table 36 (continued): List of significant GO enrichment analysis terms depicting gene properties categorised as cellular component (CC), molecular function (MF), and biological process (BP) following chronic exposure to 6.1 ng/L venlafaxine hydrochloride of parent (F0) and offspring (F1) D. magna generations. 4 biological replicates containing a pool of 30, 21-day-old D. magna, adult animals were sampled (n=4) (p< 0.05) (padj) < 0.05 and Log2 (FoldChange) > 0.

GOID	Category	Description	p-Value	Gene Count		GOID	Category	Description	p-Value	Gene Count
Parent						Offspring				
Upregulated						Upregulated				
GO:0005198	MF	structural molecule activity	6.65E-07	12		GO:0016782	MF	transferase activity	0.023243	2
GO:0016705	MF	oxidoreductase activity	0.000687	5		GO:0004222	MF	metalloendopeptidase activity	0.025412	2
GO:0005506	MF	iron ion binding	0.005021	4		GO:0020037	MF	heme binding	0.028891	3
GO:0020037	MF	heme binding	0.02052	4		GO:0046906	MF	tetrapyrrole binding	0.032194	3
GO:0046906	MF	tetrapyrrole binding	0.023557	4		GO:0016491	MF	oxidoreductase activity	0.041345	5
GO:0016747	MF	transferase activity	0.033766	2		GO:0005198	MF	structural molecule activity	0.043599	4
GO:0046914	MF	transition metal ion binding	0.045445	6						
Downregulated						Downregulated				
GO:0042302	MF	structural constituent of cuticle	0.001327	2		GO:0005326	MF	neurotransmitter transporter activity	0.000895	2
GO:0005198	MF	structural molecule activity	0.005122	2		GO:0005328	MF	neurotransmitter: sodium symporter activity	0.000895	2
GO:0004867	MF	serine-type endopeptidase inhibitor activity	0.011952	1		GO:0015294	MF	solute: cation symporter activity	0.000895	2

Table 36 (continued): List of significant GO enrichment analysis terms depicting gene properties categorised as cellular component (CC), molecular function (MF), and biological process (BP) following chronic exposure to 6.1 ng/L venlafaxine hydrochloride of parent (F0) and offspring (F1) D. magna generations. 4 biological replicates containing a pool of 30, 21-day-old D. magna, adult animals were sampled (n=4) (p< 0.05) (padj) < 0.05 and Log2 (FoldChange) > 0.

GOID	Category	Description	p-Value	Gene Count		GOID	Category	Description	p-Value	Gene Count
Parent						Offspring				
Downregulated						Downregulated				
GO:0061135	MF	endopeptidase regulator activity	0.018431	1		GO:0015293	MF	symporter activity	0.000975	2
GO:0030414	MF	peptidase inhibitor activity	0.01897	1		GO:0015291	MF	secondary active transmembrane transporter activity	0.001737	2
GO:0061134	MF	peptidase regulator activity	0.01897	1		GO:0015081	MF	sodium ion transmembrane transporter activity	0.002446	2
GO:0004857	MF	enzyme inhibitor activity	0.020584	1		GO:0046873	MF	metal ion transmembrane transporter activity	0.005072	2
GO:0030234	MF	enzyme regulator activity	0.03557	1		GO:0022804	MF	active transmembrane transporter activity	0.006013	2
						GO:0015077	MF	monovalent inorganic cation transmembrane transporter activity	0.006613	2
						GO:0022890	MF	inorganic cation transmembrane transporter activity	0.010758	2
						GO:0008324	MF	cation transmembrane transporter activity	0.013733	2

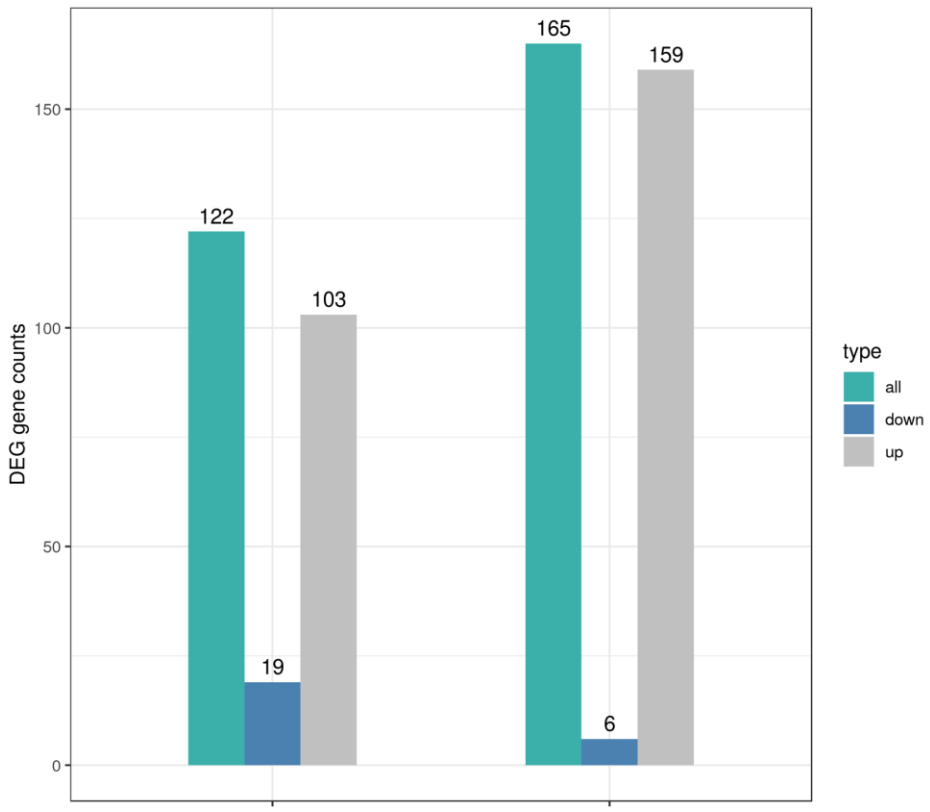


Figure 51. Chart of differentially expressed genes in Parent (F0) and Offspring (F1) generations exposed to venlafaxine relative to control, where up = upregulated genes, down = downregulated genes, all = total genes (n=4) (padj) < 0.05 and Log2 (FoldChange) > 0.

4.5 Conclusions

The aim of this study was to address the lack of substantial environmental risk assessment data available for pharmaceuticals in Irish surface waters. Conducting a risk assessment showed that the presence of pharmaceuticals and their mixtures found in the River Nore, Liffey, Suir, and Analee above or near PNEC is a cause for concern. In particular, the River Liffey was observed to have the highest frequency of moderate and high-risk detections, which warrants further investigation into possible mitigation strategies. Venlafaxine, its metabolite O-desmethyl venlafaxine and sulfamethoxazole are the most frequently detected pharmaceuticals across all sites and were identified as pharmaceuticals of most concern, with levels posing a moderate/high risk to the aquatic environment.

While PNECs serve as important guidance for setting environmental quality standards and are subject to regular updates, it is important to note that effects may occur at concentrations below the PNEC values, as observed in this study. This study provides a valuable insight into the application of effect-based methods, illustrating that the presence of venlafaxine and sulfamethoxazole at PNEC levels can still affect aquatic invertebrates. For example, sulfamethoxazole was observed to decrease mean heart rate, while venlafaxine exhibited alterations in gene expression, elevated heart rate, and morphological changes. This study shows the necessity of including effect-based methods during toxicity assessment, even if the predicted environmental concentrations are at or below PNECs.

Chapter 5 :

Evaluation of alternate sampling tools in environmental monitoring for pharmaceuticals

5.1 Introduction

Although many pharmaceuticals are selected for monitoring, there are challenges associated with developing highly sensitive methods that can reach legislated detection limits.[392] Passive sampling has been highlighted as a promising approach to address these challenges. As passive sampling functions as an infinite sink for the continuous accumulation of pharmaceuticals over time, it can improve method detection limits for ultra-trace level contaminants, overcoming limitations associated with traditional grab sampling methods providing a more representative picture of pharmaceuticals present in surface water environments.[232]

A review of 43 articles by Nitti et al. highlighted that the three most commonly used passive samples employed for pharmaceutical and personal care products are diffusive gradient in thin-film (DGT), polar organic chemical integrative sampler (POCIS) and Chemcatcher®.[242]

Yu et al. employed the use of DGTs for the detection of the pharmaceuticals gemfibrozil, carbamazepine and clarithromycin (1.9, 1.9-3.9 and 5.7-16.1 ng/L, respectively) in the Yangtze River, China.[393] Furthermore, Ren et al. showed that Time Weighted Average (TWA) concentrations of the antibiotics sulfamethoxazole, trimethoprim and ciprofloxacin from DGT samplers were comparable to the concententratins obtained from grab sampling.[394] However, a study by Buzier et al. highlighted that DGT samplers demonstrated poorer sensitivity than other passive samplers, such as POCIS. [395]

An Irish study by Jones et al. demonstrated the effectiveness of POCIS and its value as a screening tool for monitoring oestrogens (E1, E2 and EE2) and diclofenac. [243] In this study, passive sampling was conducted at the same sampling locations selected in this chapter (Osberstown and Lucan). The calculated TWA concentrations of E1 (0.29-0.42 ng/L), E2 (<0.6-

0.6 ng/L) and EE2 (<0.6 ng/L) and the mass per device of Diclofenac (0.93-17.48 ng/device) were shown to be comparable to concentrations found in European studies. Despite its proven effectiveness, POCIS has drawbacks, such as sorbent movement and potential loss during deployment.[236] This emphasizes the advantage of using a passive sampler with a bound receiving phase, as seen in Chemcatcher®.

Chemcatcher® has consistently demonstrated its efficacy as a passive sampling tool in multiple studies conducted by Rimayi et al., Gravell et al., Petrie et al., and Römerscheid et al. These studies have successfully employed Chemcatcher passive samplers to monitor a broad spectrum of pharmaceuticals, including metformin, amoxicillin, trimethoprim, venlafaxine, sulfamethoxazole, diclofenac, erythromycin, azithromycin, clarithromycin, carbamazepine, as well as estrogenic compounds such as E1, E2, and EE2.[236,237,245,246]

Even though the application of passive sampling for water quality monitoring is promising, grab sampling is still the predominant method. This lack of adoption can be addressed by increasing efforts to showcase the benefits of passive sampling over traditional grab sampling and building evidence of its utility in field studies.

5.2 Aims and objectives

This research chapter aims to investigate both traditional grab sampling and passive sampling methodologies to highlight their respective strengths and limitations in monitoring pharmaceuticals in surface waters while contributing occurrence data to benefit the scientific community.

The objectives of this study are to;

- Perform lab uptake calibration studies comparing the outcomes with findings from existing published literature.
- Undertake field sampling campaigns in catchments with a high or low likelihood of pharmaceutical contamination.
- Investigate the advantages and limitations of passive sampling in contrast to conventional grab sampling techniques.

5.3 Materials and Methods

5.3.1 Materials and reagents

All materials used are detailed in Chapter 2, section 2.3 of this thesis. Additional materials include PTFE Chemcatcher® housings (Atlantic design, 52 mm), Horizon Atlantic hydrophilic-lipophilic balanced (HLB-L, 52 mm) receiving phase, and polyethersulfone (PES) membranes were obtained from T.E Laboratories (Co. Carlow, Ireland).

5.3.2 Carousel manufacture and sampler preparation

To enable effective deployment of the PS disks, a carousel was designed and fabricated in the DCU School of Engineering using PTFE (Figure 52). The carousel, glass tank and Chemcatcher PTFE housings were pre-cleaned by soaking overnight in 2% Decon 90 solution and rinsed three times with MeOH, with housings being additionally placed in an ultrasonic bath for 10 min and dried.

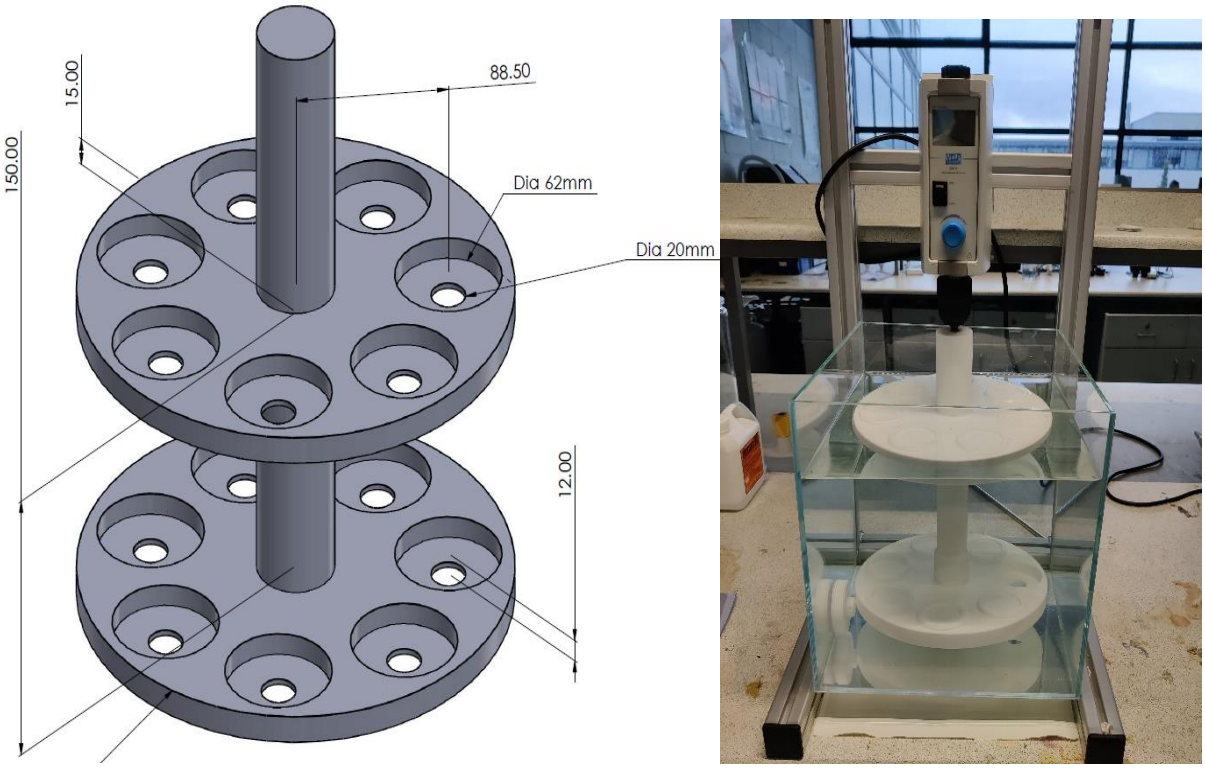


Figure 52. PTFE Passive sampling carousel and dimensions.

HLB-L sorbents were prepared by soaking in LCMS-grade methanol overnight. Sorbents were then preconditioned with 50 mL of MeOH and 100 mL of ultra-pure water under a gentle vacuum before use. PES membranes were prepared by soaking in methanol for 30 min, followed by soaking in UPW for 30 min.

Samplers were assembled by placing the PES membrane onto the smooth side of the receiving phase water (preventing air bubbles between the two) and screwing the retaining lid onto the housing (Figure 53), maintaining a watertight seal. Samplers were stored in water before exposure to prevent the sorbent from drying.

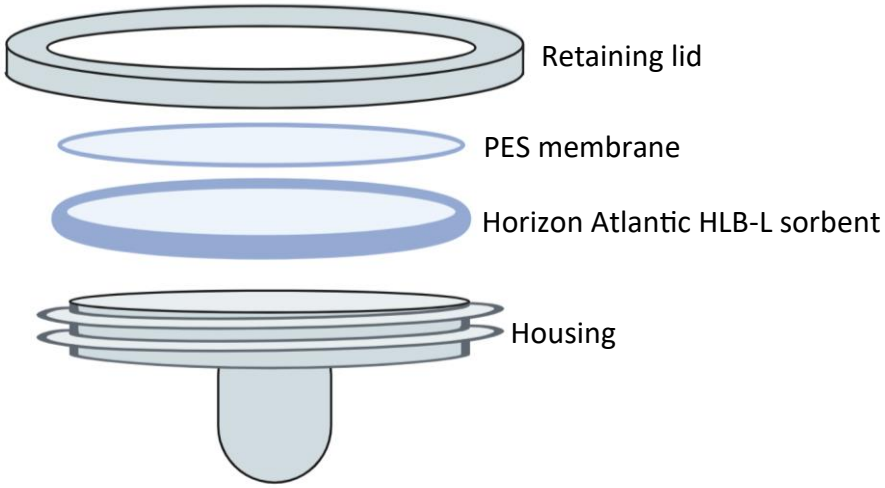


Figure 53. Assembly and constituents of a Chemcatcher passive sampler. Created with BioRender.com

5.3.3 Uptake study

Twenty litres of UPW were spiked with a 1 ug/L pharmaceutical mix and placed in a glass tank containing 14 samplers secured to the carousel. An additional sampler was placed next to the tank and opened during sampler removal from the carousel to account for air contamination. For the 14-day deployment, a carousel was rotated in the tank at 50 rpm to simulate natural river flow, and tank water was renewed daily, with pH and temperature monitored before and after renewal.

Each day (24 h), 2 mL water samples were collected before and after renewal along with a sampler. A dummy precleaned sampler housing was placed in its stead to keep hydrodynamic conditions. Collected samplers were vacuum-dried for 1 h and stored at -20 °C until extraction.

Using Equation 10, the sampling rate (R_s) of individual pharmaceuticals was calculated, where the known average spike concentration (C_w), the mass of analyte sorbed on the receiving phase (m_s) and the duration of the linear uptake phase.[245]

$$R_s = \frac{m_s}{C_W * t} \quad \textit{Equation 10}$$

5.3.4 Passive and grab sample extraction

Grab samples were extracted as per Chapter 2, section 2.3.5 of this thesis.

The extraction protocol for passive samplers was adapted from Petrie et al.[246] HLB-L receiving phases were initially brought to room temperature before extraction. The receiving phase disks were then placed into a Büchner funnel and eluted under gravity with methanol (40 mL) spiked with internal standards (to 700 ng/L when reconstituted in 1 mL) into a pre-washed glass vial (60 mL). Extracts were evaporated under nitrogen using a Biotage Turbovap II ® (Uppsala, Sweden) to dryness and reconstituted to 1 mL with 5:95 ACN: H_2O for analysis.

5.3.5 Sample site Location

Two deployment campaigns were completed, one in Kildare consisting of three sites, and the second sampling campaign in Donegal consisted of five sampling locations, which were selected to build upon earlier investigations conducted by the DCU Water Institute.[232]

5.3.5.1 Liffey sampling campaign

The River Liffey sampling campaign was conducted upstream, at the discharge location and downstream of Osberstown WWTP (Figure 54). It is important to note that these sampling sites were distinct from the Leixlip WWTP sampling site selected in Chapter 3, Section 3.3.2. These sampling sites were selected to capture the effect of large wastewater treatment facilities where "upstream" represents the background pharmaceutical concentration within the river prior to mixing with effluent from the WWTP. Pharmaceutical concentrations found at the upstream sampling site could be influenced by factors like local pollution sources, domestic misconnections, septic tanks and other WWTPs located upstream, such as Golden Falls WWTP (2000 P.E., tertiary treatment with phosphate removal). The sampling site upstream of the WWTP has been recorded as having high water quality (Q value 4-5).

The sampling site at the discharge location for Osberstown WWTP (130000 PE, tertiary treatment with phosphate removal) was selected to investigate the potential of pharmaceutical pollution due to the incomplete removal of pharmaceuticals or the formation of transformation products. The sampling site downstream was selected to understand the role of dilution and persistence of pharmaceuticals downstream of the sampling site. The water quality at the WWTP and downstream sampling sites is documented as not at risk, with good water quality (Q value 4).

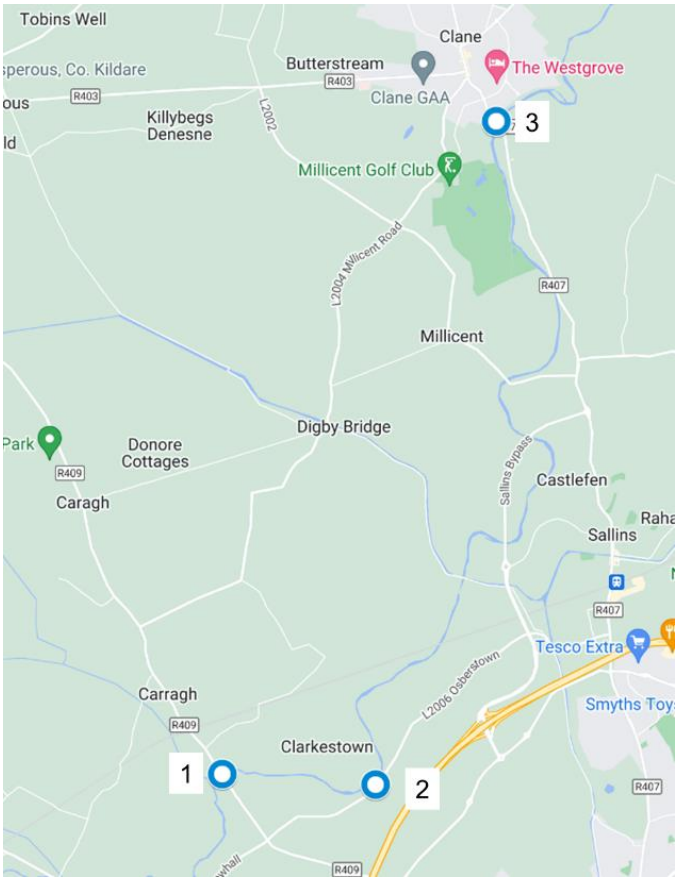


Figure 54. Sampling locations for deployed passive samplers (left) Liffey catchment: (1) upstream Osberstown WWTP, (2) Osberstown WWTP discharge site, (3) Downstream Osberstown WWTP.

5.3.5.2 Donegal sampling campaign

The Donegal sampling campaign consisted of five sampling locations: Big Burn, Glen Upstream, Glen Downstream, Owenveagh and Clogher (Figure 55). Big Burn, Glen Upstream and Glen Downstream, situated along the Glen River, are classified as a water framework directive (WFD) at risk location with poor water quality in Big Burn (Q value 3) and moderate water quality at both Glen sites (Q value 3-4).[326] Agriculture is the predominant pressure for these sampling locations.[326] However, all sites are located outside of Urban Waste Water Treatment (UWWT) Agglomeration Boundaries (towns/cities which are serviced by wastewater treatment plants with a PE <500), indicating that residences in the vicinity are likely served by septic tanks, which are known as potential sources of pharmaceutical

contamination. [326,396–399] The threat posed by improperly sealed septic tanks to private drinking wells has been highlighted in Donegal and across Ireland, with the presence of E. coli from human faecal matter being a significant concern. [400,401]

The sampling site at River Owenveagh was selected as it is categorised as being not at risk with a high water quality status (Q value 5). Although there are some settlements which are also outside UWWT Agglomeration Boundaries, this site was selected as a clean site with minimal likelihood of pharmaceutical contamination. The Clogher sampling site has a good water quality status (q value 4). However, it has been identified as a river which faces agricultural pressures with residences outside UWWT Agglomeration Boundaries.[326]

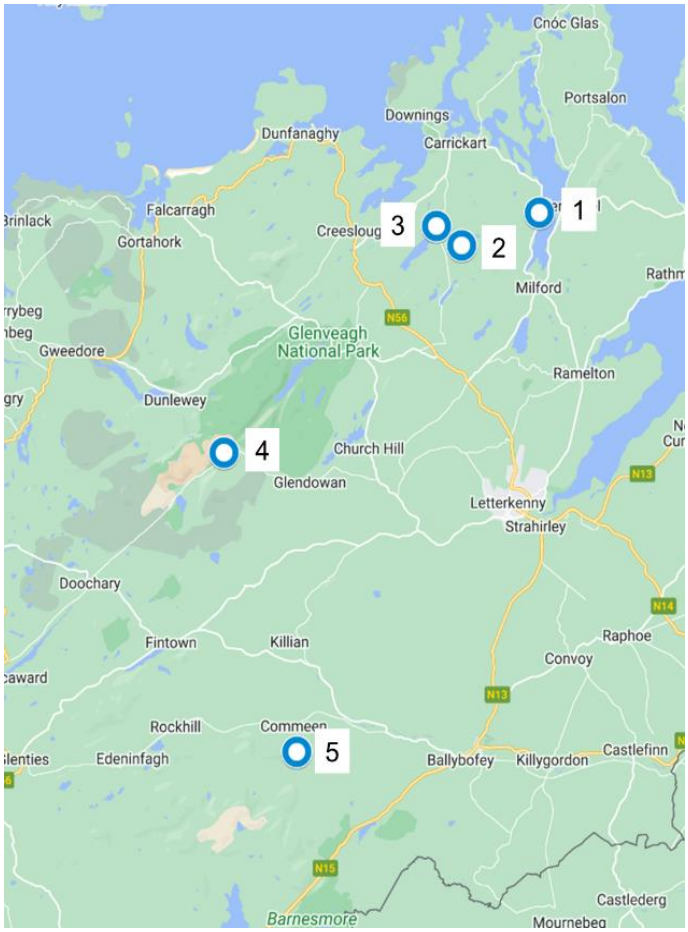


Figure 55. Sampling locations for deployed passive samplers in the Donegal Catchment: (1) Big Burn, (2) Glen Upstream, (3) Glen Downstream, (4) Owenveagh, (5) Clogher.

5.4 Results and Discussion

The evaluation of passive samplers as an additional monitoring strategy was carried out by assessing literature sampling rates, in-lab uptake rate calibration studies, collection of grab samples and the deployment of passive samplers in the River Liffey and Donegal catchment.

5.4.1 Passive sampling uptake study

Water temperature (17.0 ± 0.56 °C), pH (7.48 ± 0.31) and concentration of pharmaceuticals (which had been spiked at 1000 ng/L) remained relatively stable. Over the course of the 14 days, tank water was measured at the start and end of each day (n = 28) with a standard deviation of ± 27.9% for diclofenac, ± 17% for O-desmethyl venlafaxine and ± 9% for venlafaxine over the course of the 14-day study. Furthermore, paired sample t-tests showed no daily significant difference between spiked tank water at the start and end of each sampling day, and concentrations of blank samplers were below LOD. Figure 56 to Figure 58 show the linear uptake of the mass of each pharmaceutical accumulated on the passive sampling receiving phase. O-Desmethyl venlafaxine and venlafaxine remained linear for a period of 8/9 days, and diclofenac had not reached equilibrium by the end of the 14 days. Environmental conditions and analyte physiochemical properties play a significant role in the uptake of target analytes.[402] For example, higher temperature has been associated with increased diffusion coefficients increasing uptake, while flow rate has been shown to affect uptake based on an analyte's molecular weight.[402]

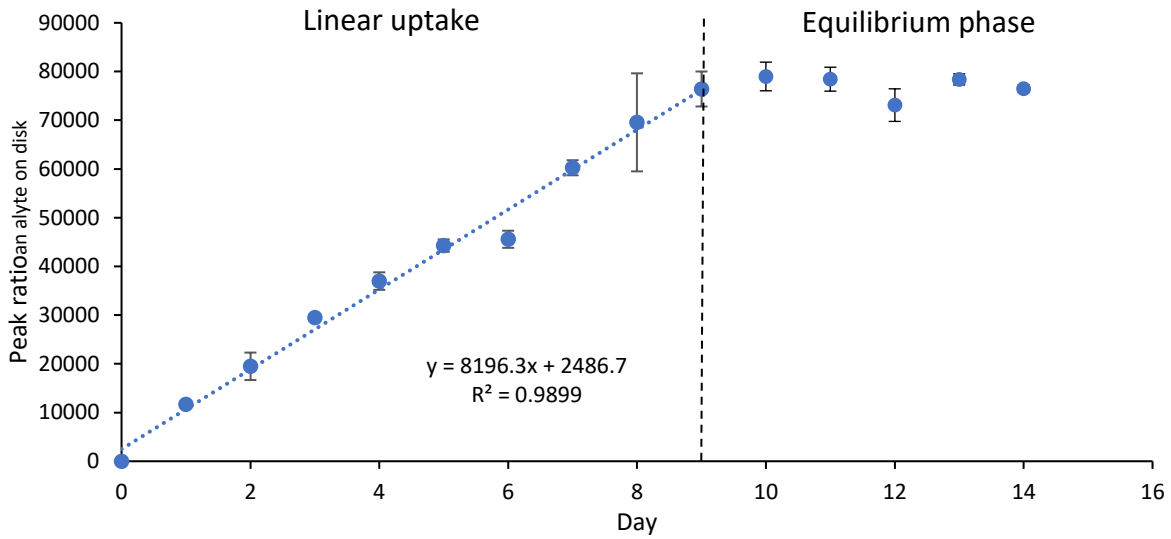


Figure 56. Linear uptake and equilibrium phase of the pharmaceutical O-desmethyl venlafaxine in Chemcatcher HLB-L passive sampling disks during a 14-day exposure study at 1000 ng/L. The line fitted a linear regression curve (R^2 = 0.9899, slope = 0.8196.3).

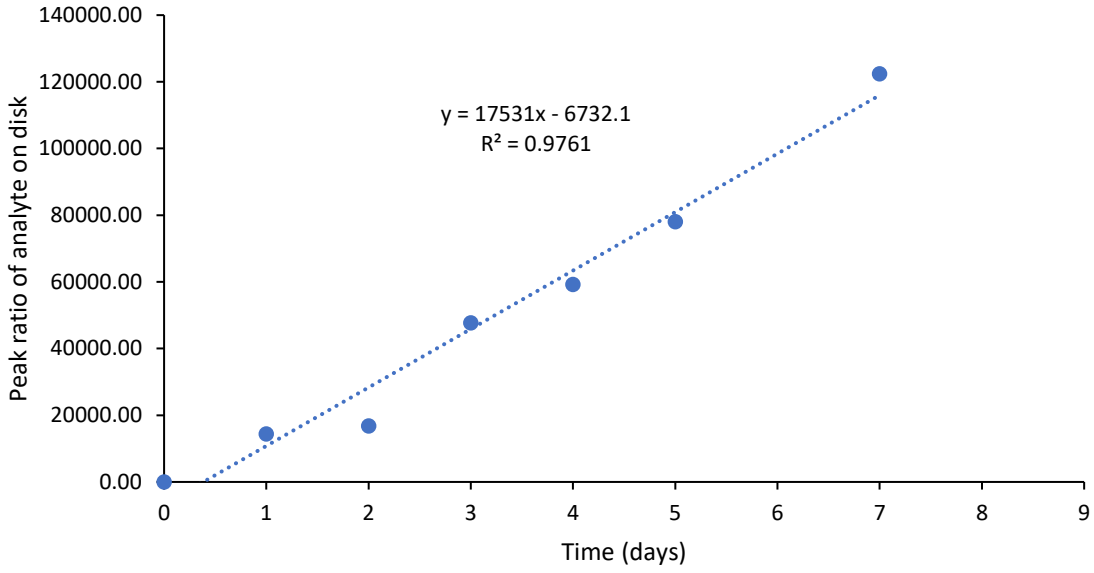


Figure 57. Linear uptake phase of the pharmaceutical venlafaxine in Chemcatcher HLB-L passive sampling disks during a 14-day exposure study at 1000 ng/L. The line fitted a linear regression curve (R^2 = 0.9761, slope = 17531).

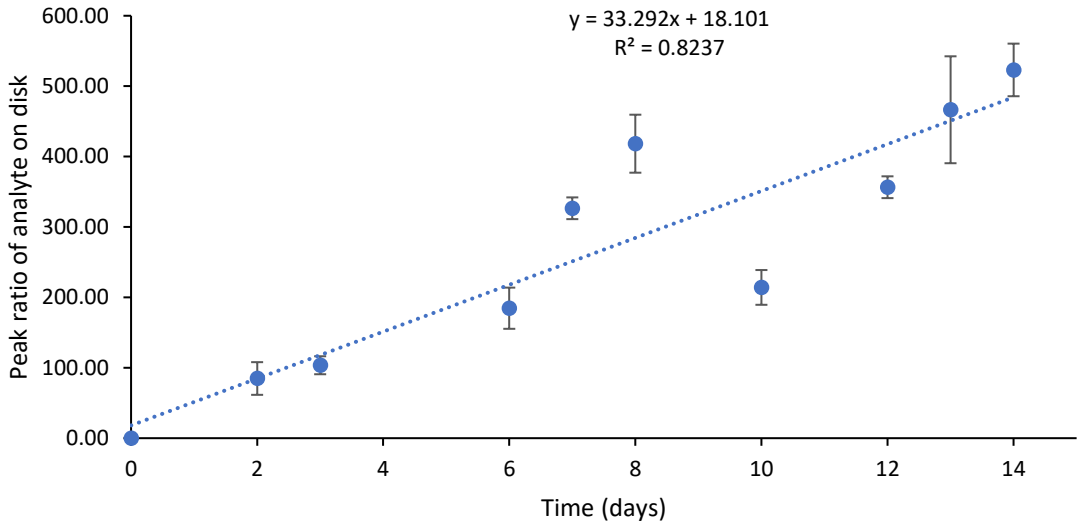


Figure 58. Linear uptake phase of the pharmaceutical diclofenac in Chemcatcher HLB-L passive sampling disks during a 14-day exposure study at 1000 ng/L. The line fitted a linear regression curve (R^2 = 0.8237, slope = 33.292).

Table 36 shows the sampling rates of 3 pharmaceuticals determined from this studies 14 day lab-based calibration studies and 11 pharmaceuticals from published literature on Chemcatcher HLB sorbents in surface water.[245,246] As a result of lab-based calibration studies being conducted in spiked UPW while the selected literature sampling rates were conducted in surface water and effluent, variations in sampling rates were observed. The experimental design and test environmental conditions (matrix, temperature, pH and flow rate) have been previously noted to cause a high degree of variability.[245,246] Due to the increased sampling rates observed with lab-based uptake studies, literature values were used during sample analysis to determine time-weighted average concentrations. However, future work should investigate the in-situ calibration of these pharmaceuticals as a limited number of field calibration studies are conducted due to its financial costs and labour-intensive requirements.[246]

Table 36: Sampling rates used from literature and lab-based studies. [245,246]

Pharmaceutical	Literature uptake rate Rs (L/day)	Uptake study Rs (L/day)
Amoxicillin	0.022	-
Trimethoprim	0.04	-
Sulfamethoxazole	0.023	-
Venlafaxine	0.065	0.404
Carbamazepine	0.052	-
Clarithromycin	0.024	-
Azithromycin	0.024	-
Diclofenac	0.038	0.020
E1	0.071	-
E2	0.04	-
EE2	0.031	-
O-desmethyl venlafaxine		0.117

A lag phase was identified during the uptake study for the antibiotics trimethoprim, ciprofloxacin, erythromycin, clarithromycin and azithromycin (Figure 59). The lag phase has been previously observed and attributed to the equilibration period of the PES membrane placed on top of the receiving phase, and the duration of this lag phase is compound-specific.[247] Therefore, the interpretation of sampling uptake rates was inconclusive due to the short sampling window (14 days).

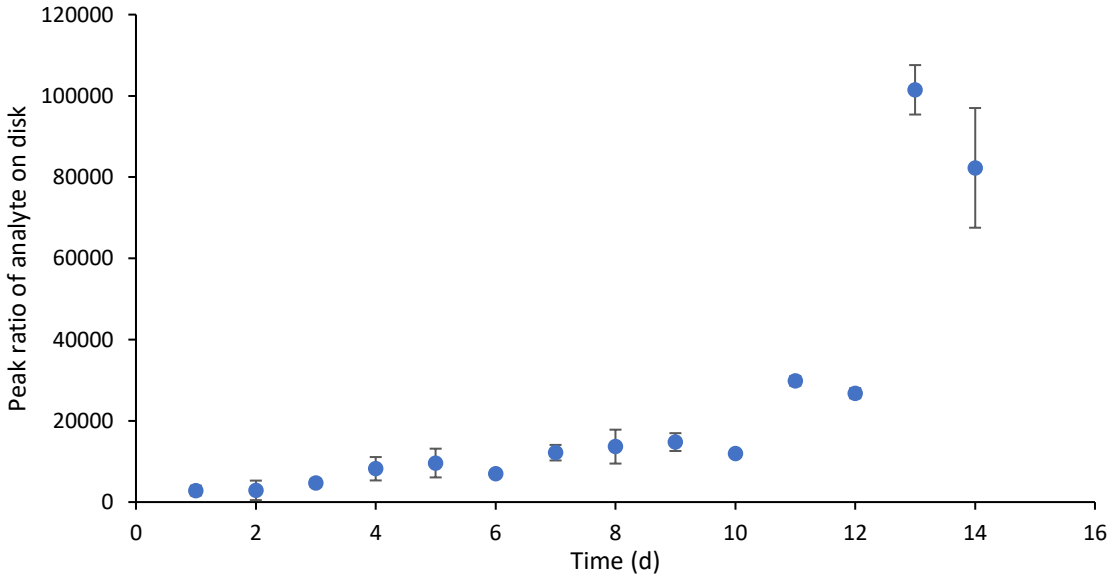


Figure 59. Uptake of Trimethoprim in Chemcatcher HLB-L passive sampling disks during a 14-day exposure study at 1000 ng/L.

5.4.2 Site location and Field Measurements

Due to the risk of theft/damage from continual measurement, physical/chemical conditions (pH temperature, turbidity, dissolved oxygen and conductivity) were only monitored during deployment and collection. Environmental conditions remained relatively stable except for the sampling sites at Glen A and B and the national park, which had increased turbidity in July due to heavy rainfall. Although conditions were monitored at the time of deployment and collection, the ability to draw conclusions in terms of temporal trend monitoring was not possible as not enough data points were collected. However, the environmental conditions are summarised in Table 37. Nonetheless, it is important to assess these physical/chemical conditions as flowrate temperature and turbidity fluctuations can impact the uptake mechanism of passive samplers, which could affect the resulting calculated pharmaceutical concentrations.[403]

Table 37: Environmental conditions at the point of deployment and retrieval.

	Sample	pH	Temperature (°C)	Turbidity (FNU)	Conductivity (µS/cm)	D.O. (mg/L)
Liffey Upstream WWTP	June	8.16	15.077	0.8	369.2	8.53
	July	8.17	15.781	0.84	375.2	7.89
Liffey Discharge Point (Osberstown WWTP)	June	8.36	15.201	0.67	376	8.35
	July	8.11	15.949	0.6	384.8	8.3
Liffey Downstream WWTP	June	8.17	15.868	0.58	421.2	8.81
	July	8	16.129	0.69	428.7	7.74
National Park	July	7.96	14.414	6.25	77.3	9.22
	August	6.66	13.155	0.67	32.8	9.26
Cranford (Big Burn)	July	7.11	14.354	1.81	164.7	8.79
	August	7.66	13.138	2.14	187.9	8.96
Glenadowan (Clogher)	July	6.14	14.931	1.02	48.2	9.02
	August	6.11	14.057	0.69	30.3	9.35
Glen A	July	7.49	14.084	4.62	89.9	9
	August	7.12	12.7	1.1	84.8	9.07
Glen B	July	7.08	14.586	8.56	81.5	8.56
	August	7.45	12.896	1.53	93.8	9.18

5.4.3 Field sample analysis

5.4.3.1 Grab samples

5.4.3.1.1 Liffey sampling site

Three of the eleven pharmaceuticals investigated during the passive sampling uptake study were detected during the Liffey sampling campaign (Table 38). The concentrations of EE2 in June and O-desmethyl venlafaxine in June and July at the WWTP sampling site indicate that they are introduced into the river in these concentrations via wastewater discharges, as these compounds were not detected upstream. As expected, the concentrations decreased back to <LOD downstream due to the natural dilution.

Diclofenac was detected (<LOQ) at the upstream sampling site. However, it was not detected at the WWTP and downstream sampling sites, indicating WWTP removal and/or dilution at and below the discharge location, reducing its environmental presence.

The concentrations observed of O-desmethyl venlafaxine at the Osberstown WWTP discharge site and those detected in the Leixlip WWTP in Chapter 3 highlight the need to include this pharmaceutical metabolite in future monitoring campaigns.

Table 38. Pharmaceuticals concentrations quantified from June-July grab sampling campaign in the River Liffey (n=3 per site).

	Liffey					
	Upstream (ng/L± %RSD)		WWTP discharge site (ng/L± %RSD)		Downstream (ng/L± %RSD)	
Pharmaceutical	Jun-21	Jul-21	Jun-21	Jul-21	Jun-21	Jul-21
Amoxicillin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Trimethoprim	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Venlafaxine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
O-desmethyl venlafaxine	<LOD	<LOD	2.53 ± 2.6	2.6 ± 5.23	<LOD	<LOD
Sulfamethoxazole	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Carbamazepine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Clarithromycin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Azithromycin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Diclofenac	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD
E1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
E2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
EE2	<LOD	<LOD	7.07 ± 8.73	<LOD	<LOD	<LOD

5.4.3.1.2 Donegal sampling sites

Concerning the Donegal sampling campaign, as these sample sites were in rural river locations, the concentrations of pharmaceutical compounds generally remained below LOQ when detected (Table 39). However, the detection (<LOQ) of EE2 and diclofenac in August at Glenadowan and diclofenac in August at Glen A could be attributed to potential runoff from nearby agricultural lands and improperly sealed septic tanks. The low levels of diclofenac and EE2 highlight the potential for pharmaceuticals in aquatic environments, even in remote settings. However, further investigation is needed to understand the pathways and sources of these compounds in the sampled rivers. The detection of pharmaceuticals in remote areas with low anthropogenic influence has been previously observed in a study by Royano et al., who identified the presence of carbamazepine, clarithromycin, erythromycin, O-desmethyl venlafaxine, sulfamethoxazole, trimethoprim and venlafaxine in the Tagus River Basin.[404] Moreover, the detection of pharmaceuticals (e.g. diclofenac, venlafaxine clarithromycin) and

synthetic hormones in surface waters in Antarctica shows that pharmaceuticals can be present even in the most remote and pristine environments.[405]

Table 39. Pharmaceuticals concentrations quantified through grab sampling campaign in the Donegal (n=3 per site).

	Glen A (ng/L ± %RSD)		Glen B (ng/L ± %RSD)		Billies pit (ng/L ± %RSD)		Glenadowan (ng/L± %RSD)		Big burn (ng/L± %RSD)	
Pharmaceutical	Jul-21	Aug-21	Jul-21	Aug-21	Jul-21	Aug-21	Jul-21	Aug-21	Jul-21	Aug-21
Amoxicillin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Trimethoprim	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Venlafaxine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Sulfamethoxazole	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Carbamazepine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Clarithromycin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Azithromycin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Diclofenac	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD
E1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
E2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
EE2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD

5.4.3.2 Detection of pharmaceuticals through passive sampling

Although Chemcatcher HLB-L passive sampling has been previously used to investigate pharmaceuticals in other countries, e.g. U.K. and South Africa, [237,285] this study applied passive sampling to selected monitoring locations, thereby contributing valuable insights into Irish river water quality and practical application of passive sampling.

Time weighted averages (C_{TWA}) were calculated as shown in Figure 60. Where m_s is the mass in ng on disk. m_s was calculated by 1. Determining the analyte peak ratio by dividing the analyte peak area from the internal standard, 2. Using the equation of the line from a matrix-matched composite sample from Donegal and Liffey sampling sites, the average concentration of the 1 mL extract from each deployed sampler was determined and represented as ng/L, 3. The average concentration was divided by 1000 to get mass in ng in the 1 mL extract. Sampling rates (R_s), which represent litres of water sampled per day, were obtained from literature values shown in Table 36 and time (t) was the duration of the sampling period (14 days).

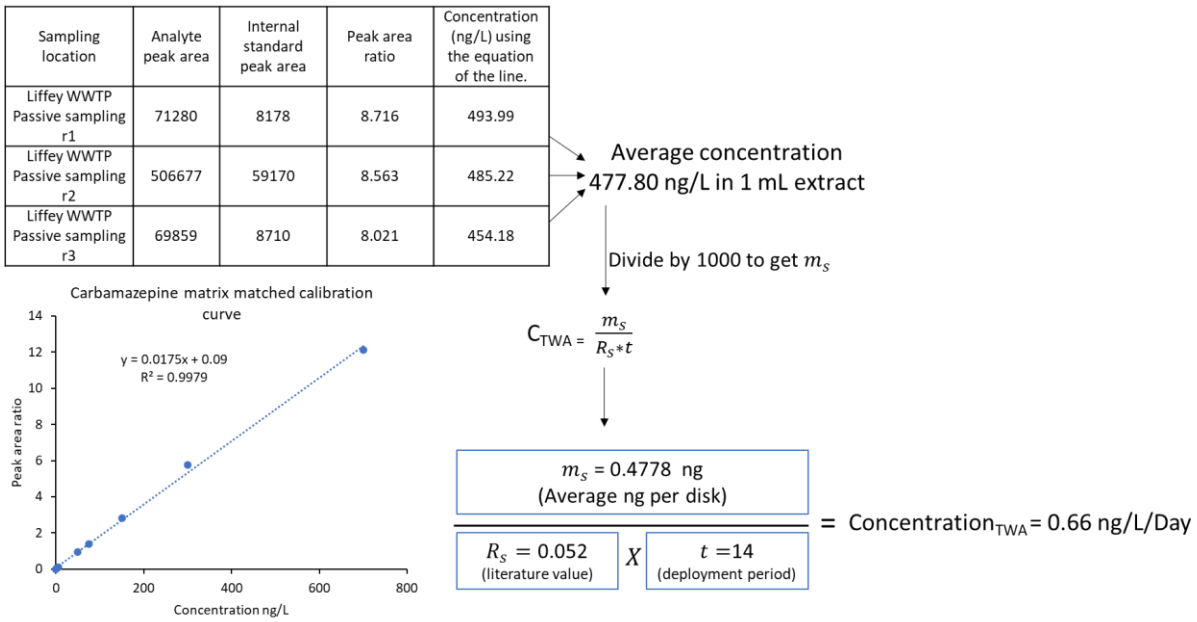


Figure 60. Workflow for the determination of time weighted average concentrations from monitored river water.

5.4.3.2.1 Liffey sampling campaign

This passive sampling work detected a higher number and concentration of pharmaceuticals relative to traditional spot/grab sampling at the same sample sites. The time-weighted average results from passive sampling can be seen in Table 41. This can be attributed to passive samplers sampling larger volumes of water, which allows for the detection of contaminants at lower concentrations than would be commonly found through grab sampling.[406] However, the calculated TWA concentrations depend on the sampling rate, which estimates the volume of sampled water, and if there are differences in environmental conditions, there is the possibility of over or underestimating contaminant concentrations.

The WWTP sampling site showed the highest concentrations and number of pharmaceutical detections from all sampling sites. Pharmaceuticals such as trimethoprim, diclofenac, clarithromycin, azithromycin, carbamazepine, venlafaxine, E1 and EE2 were previously undetected at the WWTP sampling site through grab sampling. Whereas through grab sampling, only O-desmethyl venlafaxine and EE2 were detected at the WWTP.

The concentrations for both grab and passive sampling at the WWTP discharge location were lower than what was recorded in Chapter 3. Factors which may have led to the variation in concentrations include;

- As of 2021, the Osberstown WWTP serviced a lower Population Equivalent (PE) than the Leixlip WWTP with a maximum PE of 130,000 PE and weekly PE of 100425 (weekly) in comparison to the Leixlip WWTP with a maximum PE of 150,000 and weekly PE of 138675 (weekly).[332] The higher population that the WWTP serviced could lead to an increase in pharmaceutical input into the river.

- Lower consumption of pharmaceuticals such as antibiotics can contribute to reduced environmental presence, as explained in Chapter 1. Where the lower consumption of antibiotics during summer periods.[407–409]
- Increased efficiency of WWTPs during summer months. The higher temperatures associated with summer months have been recorded to play a significant role in the degradation of pharmaceuticals. A study by Vieno et al. compared the elimination of pharmaceuticals during the winter and summer months and observed an average reduction of 25% compared to the summer, resulting in higher pharmaceutical concentrations in effluent water. [410] Whereas a study by Castiglioni et al. observed that removal rates can be temperature-dependent, with increased microbial activity. For instance, the antibiotics amoxicillin and sulfamethoxazole exhibited lower removal rates in winter, 75% and 17 %, respectively, than in summer, 100% and 71%, respectively. [411]
- Furthermore, as the passive sampling campaign was conducted in summer (July and August), increased sunlight and higher river temperatures were observed than during the monitoring campaign in Chapter 3. As discussed in Chapter 3, these environmental factors play a role in increasing the biodegradation and photodegradation degradation of pharmaceuticals. Notably, lower levels of turbidity and conductivity were recorded during the summer sampling campaign compared to those at the Leixlip WWTP sampling location, which can increase pharmaceuticals' biodegradation and photodegradation degradation.[345,346] Furthermore, as discussed in Chapter 2, section 2.4.2.6. lower conductivity levels can also be an indicator of the presence of less contaminants.

The ability of passive sampling to capture intermittent or low-level releases offers a greater insight into surface water pharmaceutical concentrations, particularly at critical locations like WWTPs. This information is invaluable for assessing the potential environmental impact and identifying potential sources of pharmaceutical contamination.

Table 40: Time-weighted averages of pharmaceuticals assessed on Chemcatcher HLB-L receiving phase disks deployed over 14 days in the June – July Liffey sampling campaign (n=3 per site). TWAs were calculated using literature uptake rates shown in Table 36.

Pharmaceutical	Upstream (ng/L ± %RSD)	WWTP discharge site (ng/L ± %RSD)	Downstream (ng/L ± %RSD)
Amoxicillin	<LOD	<LOD	<LOD
Trimethoprim	0.18 ± 27.5	0.81 ± 8.28	0.37 ± 28.57
O-Desmethyl venlafaxine*	0.099 ± 20.01	2.12 ± 5.49	0.11 ± 22.62
Venlafaxine	0.038 ± 18.52	0.83 ± 25.36	0.057 ± 31.03
Sulfamethoxazole	<LOD	<LOD	<LOD
Carbamazepine	0.061 ± 10.11	0.66 ± 4.38	0.078 ± 11.44
Azithromycin	0.059 ± 35.37	1.88 ± 18.52	0.20 ± 19.27
Clarithromycin	0.097 ± 17.61	1.62 ± 17.29	0.21 ± 15.69
Diclofenac	0.12 ± 10.63	1.28 ± 15.2	0.13 ± 23.22
E1	0.0016 ± 58.24	0.0050 ± 19.54	0.0084 ± 29.81
E2	<LOD	<LOD	<LOD
EE2	1.03 ± 136.42	0.28 ± 39.12	0.38 ± 38.62

*mass (ng) on sampler, not TWA

5.4.3.2.2 Donegal sampling campaign

Most pharmaceuticals assessed were below LOD during the Donegal passive sampling campaign, which was expected due to its rural locality. However, low levels of the pharmaceuticals trimethoprim and EE2 were detected. The high standard deviations observed between the triplicate deployed disks may originate from variations in analyte uptake rates as a result of hydrodynamic conditions, biofouling and sediment obstructing the passive sampler disk.[412,413]

The presence of trimethoprim in the Donegal sampling sites could indicate that livestock emissions could be a potential contributing source. Trimethoprim is a common antibiotic that is generally administered orally to calves, pigs, horses, and poultry.[414] Furthermore, the

tonnage of antibiotics used for livestock (trimethoprim and macrolide antibiotics) in Ireland has been increasing [415], but the usage data for specific categories of livestock are generally not available or are confidential.[415] Therefore, conclusions could not be made. Furthermore, the presence of human pharmaceuticals such as EE2, venlafaxine, and O-Desmethyl venlafaxine at the Donegal sampling sites signals that the source is of human origin. As previously mentioned, the sampling sites are outside the UWWT agglomeration; therefore, the presence of these pharmaceuticals could be a result of septic tanks.

Table 41: Time-weighted averages of pharmaceuticals analysed from Chemcatcher HLB-L receiving phase disks deployed over 14 days in the July - August Donegal sampling campaign (n=3 per site). TWAs were calculated using literature uptake rates shown in Table 36.

Pharmaceutical	Glen A (ng/L ± %RSD)	Glen B (ng/L ± %RSD)	Billies pit (ng/L ± %RSD)	Big burn (ng/L ± %RSD)	Glenadowan (ng/L ± %RSD)
Amoxicillin	<LOD	<LOD	<LOD	<LOD	<LOD
Trimethoprim	0.044 ± 28.08	0.019 ± 19.12	0.016 ± 4.89	0.030 ± 28.91	0.060 ± 11.89
O-Desmethyl venlafaxine*	0.0037 ± 32.78	0.0037 ± 18.84	0.0025 ± 6.18	0.0042 ± 27.52	0.0036 ± 21.95
Venlafaxine	<LOQ	<LOQ	<LOD	0.0064 ± 0	0.0037 ± 103
Sulfamethoxazole	<LOD	<LOD	<LOD	<LOD	<LOD
Carbamazepine	<LOD	<LOD	<LOD	<LOD	<LOD
Azithromycin	<LOD	<LOD	<LOD	<LOD	<LOD
Clarithromycin	0.027 ± 15.28	<LOQ	<LOQ	0.022 ± 14.29	0.024 ± 24.03
Diclofenac	0.015 ± 71.02	0.047 ± 35.18	0.036 ± 1.11	0.052 ± 27.35	0.030 ± 126.82
E1	0.0042 ± 9.29	0.0052 ± 64.33	0.0035 ± 18.63	0.0014 ± 7.61	0.0014 ± 1.6
E2	<LOD	<LOD	<LOD	<LOD	<LOD
EE2	0.013 ± 66.07	0.055 ± 69.32	0.023 ± 82.34	0.0095 ± 34.28	0.169 ± 0.47

*mass (ng) on sampler, not TWA

5.5 Evaluation of monitoring techniques

Passive sampling analysis improved the overall picture of pharmaceutical contamination compared to grab sampling, specifically in terms of the number of compounds detected. Using literature-based sampling rates to calculate TWA values may not fully account for environmental conditions (matrix temperature, pH, and salinity) at the selected sample sites, potentially influencing the accuracy of reported concentrations. However, the semi-quantitative data can offer valuable information for future monitoring strategies. Incorporating passive sampling into non-target monitoring campaigns can help identify pharmaceuticals otherwise missed by grab sampling.[232] A 2016 study by Petrie et al. investigated using Chemcatcher containing an Atlantic HLB disk for 57 compounds in wastewater effluent via LC-MS/MS.[246] This study found that several micropollutants, including pharmaceuticals, were detected in passive sampling, which was not detected through traditional grab sample monitoring.

Chemcatcher passive samplers measure the presence of micropollutants in the freely dissolved fraction of surface water. Thus, detected pharmaceutical concentrations better reflect the bioavailability and potential risks these contaminants pose within an aquatic ecosystem.[233] Furthermore, biomonitoring often faces many challenges, including ethical issues, cost, reproducibility of results, and upkeep of test organisms. Using passive samples as a proxy for traditional monitoring could help conserve resources by identifying specific areas of concern. [233] Additionally, Passive samplers can capture pharmaceuticals with short residence times, provide critical information on the TWA of pharmaceutical pollution, and capture pollution events that would otherwise be missed during grab samples (Figure 61).[234]

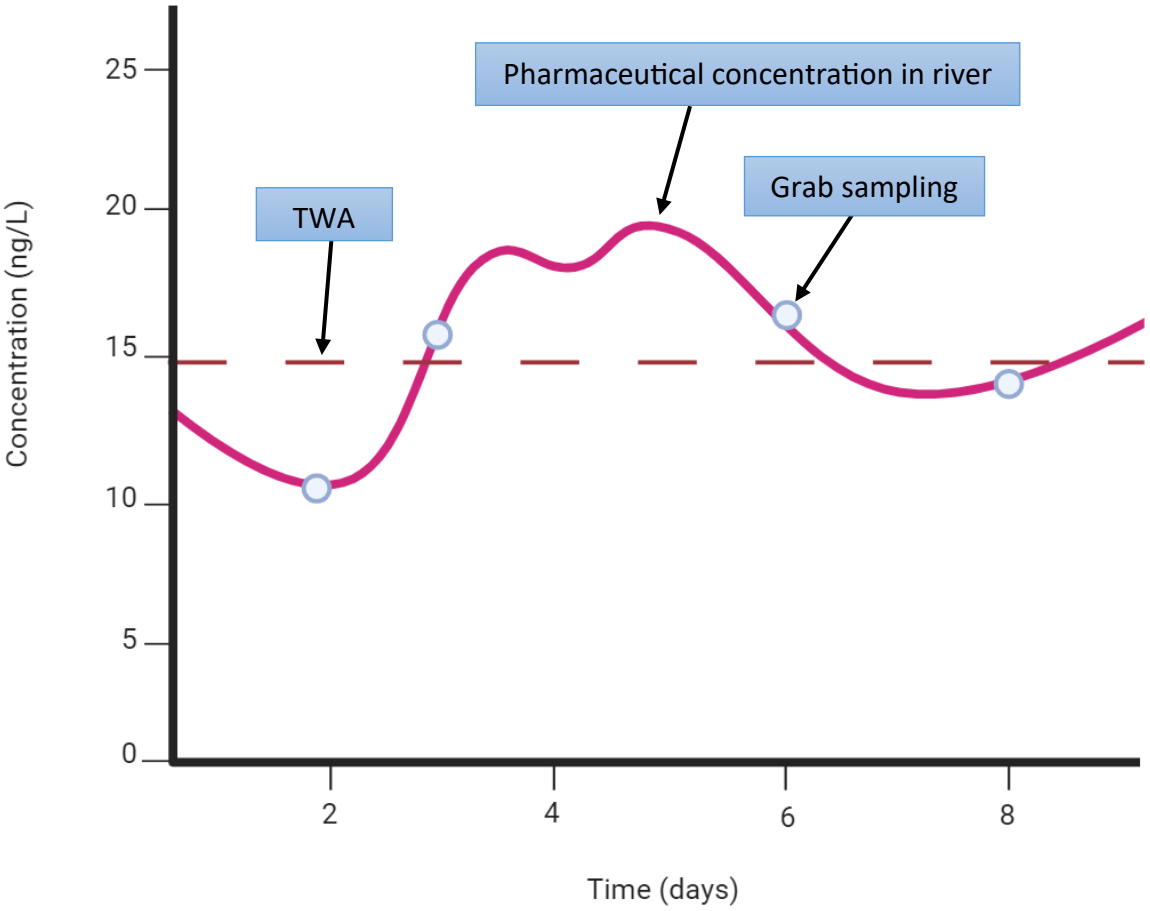


Figure 61. Example of the comparison between the actual pharmaceutical concentration in surface water (purple line) and that detected by TWA from passive sampling vs. continuous grab sampling. Created with BioRender.com

However, adopting passive sampling for pharmaceutical detection is not without challenges and critical considerations. Samplers must also remain untampered with by members of the public, which can be challenging to ensure. Therefore, deployment sites must be investigated for suitability prior to deployment. The deployment cage relies upon its weight and a chain to an external fixture to secure it. This poses labour challenges during the transport of the cage to the sampling site and the site suitability if no external fixture is available. Other challenges faced with passive sampling include potential biofouling and variation of uptake between replicate deployed samplers, which can lead to high standard deviations.

Passive samplers are typically deployed for a duration of two weeks. During this period, environmental factors such as flow rate and temperature can lead to variations in the sampling rate. In contrast, to grab sampling, prolonged deployment periods (>5 days) can affect the uptake rates with biofouling and the build-up of organic matter on the diffusive membrane, thereby affecting accurate quantification.[232,416] Additionally, with the nature of passive sampling providing an average concentration over a period of time, it is incapable of providing the identification and resolution of daily concentration fluctuations.[246] However, as passive samplers sample larger volumes of water, typically between 0.05 and 0.35 L day^{-1}, this can considerably increase detection capabilities for ultra-trace level pharmaceutical contamination.[234] This was observed during both the Liffey and Donegal sampling campaigns, where trace-level pharmaceuticals, which would have otherwise gone unreported, were detected. Furthermore, monitoring both the Liffey and Donegal catchments through passive sampling proved to be cost and resource-effective, as continual monitoring through grab sampling would have incurred labour and financial constraints. This cost and labour benefit has also been documented in previous studies on passive sampling.[417–419]

5.6 Conclusions

This chapter details the application and feasibility of passive sampling as an additional monitoring technique to traditional grab sampling. Lab-based uptake studies were completed to determine the sampling rates for pharmaceuticals, and surface water sampling was conducted in the River Liffey and Donegal catchment to capture a variety of potential sources.

The findings from this research serve as a proof of concept for adopting a diverse approach to surface water monitoring. This study highlights that relying solely on grab sampling as a monitoring strategy may result in the under or over-reporting of pharmaceutical concentrations in surface waters, leading to an inaccurate representation of water quality.

Pharmaceuticals were detected in both sampling campaigns through grab or passive sampling. The highest concentrations were observed at the WWTP discharge location on the River Liffey for both passive and grab sampling.

In the River Liffey, through passive sampling, the pharmaceuticals trimethoprim, diclofenac, clarithromycin, azithromycin, carbamazepine, venlafaxine, E1 and EE2 were detected. However, only O-desmethyl venlafaxine and EE2 were detected at the WWTP discharge site.

Furthermore, the detection of trimethoprim, O-desmethyl venlafaxine, clarithromycin, diclofenac, E1 and EE2 in the Donegal passive sampling campaign, while only diclofenac was detected (<LOQ) using grab sampling highlights the utility of passive sampling in remote locations where the anthropogenic input of pharmaceuticals is expected to be low.

Consequently, this study shows that employing both monitoring strategies could yield a more comprehensive and holistic water quality assessment. However, due to the challenges associated with passive sampling, traditional grab sampling still has an essential role in water quality monitoring.

Chapter 6 :

Conclusions and future work

6.1 Conclusions:

The aim of this thesis was to assess pharmaceuticals as emerging environmental contaminants. This was achieved through several key components, including gathering occurrence data from a two-year study, risk assessment methods, utilising effect-based tools, and evaluating monitoring strategies. From this research, the following contributions were made:

An investigation of emerging pharmaceuticals from manufacturing to their endpoints revealed that the primary source of pharmaceutical pollution in rivers is attributed to wastewater treatment plants (WWTPs). WWTPs are typically not designed to remove these compounds from WWTP influent, and the subsequent discharge of treated wastewater, which has accumulated pharmaceutical concentrations, exposes aquatic organisms to largely unknown consequences. Furthermore, with the use of over 3000 active pharmaceutical ingredients and the limitations regarding their legitimacy for an Environmental Risk Assessment, it is vital that significant advancements are needed in quantifying these contaminants. Unlike many countries, such as the United Kingdom and Germany, where extensive monitoring campaigns are in place, research in Ireland has only begun to investigate this form of pollution. However, even with established monitoring campaigns and evidence of pharmaceutical pollution, it is imperative that monitoring should result in proactive measures to address and mitigate pollution rather than simply accumulating data.

The analytical methods for assessing the presence of pharmaceuticals in complex surface water and biological matrices were developed on HPLC-UV and LC-MS/MS. However, LC-MS/MS analysis afforded greater sensitivity and reproducibility and reduced sample extraction and analysis time. The developed LC-MS/MS method achieved a high degree of

sensitivity with LODs and LOQs in the ng/L concentrations. Although matrix-matched calibration standards, internal standards and solid phase exaction was used during sample analysis to minimize the impact of matrix effects, it could not be removed completely. As a result, there is the potential to either over or underestimate actual environmental concentrations. The methodology developed in this study contributes significantly to monitoring efforts in the European Union and the broader scientific community by providing a robust framework for monitoring emerging pharmaceuticals. Moreover, it is a critical step in addressing the knowledge gap concerning concentration levels in Irish surface waters.

This research has yielded several key findings that significantly contribute to understanding pharmaceutical contamination in Irish surface waters. Firstly, the study detected the widespread presence of pharmaceutical cocktails in the analysed Irish rivers. Secondly, this research identified the high-risk pharmaceuticals venlafaxine and sulfamethoxazole, which exceeded established risk thresholds. Lastly, the study identified the value of grab and Chemcatcher passive sampling for pharmaceutical monitoring. These findings collectively enhance our knowledge of pharmaceutical contamination in Irish aquatic environments.

The assessment of individual risks of pharmaceuticals detected in the River Liffey, Nore, Suir and Analee showed two pharmaceuticals that had a high-risk categorisation for venlafaxine and sulfamethoxazole, with venlafaxine's primary metabolite O-desmethyl venlafaxine being observed more frequently than its parent compound and also occurring at high risk (RQ > 1), which is consistent with findings commonly reported in the literature.[420–422]

Assessing the presence of the pharmaceuticals detected in this study in conjunction with published wastewater treatment removal rates shows that current technologies employed are insufficient and improvements need to be made regarding prescription practices,

pharmaceutical waste disposal and the development of pharmaceuticals which are benign by design. Prescription levels can heavily influence the presence of human pharmaceuticals in surface waters. The influence of health emergencies such as pandemics may create periods where the prescription/consumption of pharmaceuticals may fluctuate. This may lead to periods of heightened risk for aquatic organisms, therefore demanding the implementation of robust monitoring strategies and the implementation of mitigation measures, supporting Sustainable Development Goal 6. The analysis of antidepressant concentrations in river waters during the COVID-19 pandemic underscored that there is a link between human health and water quality. For this reason, it is imperative to promote good well-being (Sustainable Development Goal 3) and practice responsible consumption (Sustainable Development Goal 12) so that good water quality can be achieved (Sustainable Development Goal 6) while protecting life below water (Sustainable Development Goal 14).

With the detection of pharmaceuticals in Irish surface waters, grab sampling proves to be a vital part of monitoring campaigns. However, it was observed that solely relying on grab sampling can lead to the underreporting of pharmaceutical concentrations, with the possibility that pollution events could be missed. This work shows the necessity of including passive sampling in future monitoring campaigns. However, its inclusion does not come without challenges, such as upfront cost, damage, outside interference and accurate determination of sampling rates. A combined passive and grab sampling approach should be taken to get a holistic outlook on the presence of pharmaceuticals in aquatic ecosystems.

6.2 Recommendations for future work:

The importance of developing robust analytical testing is required to determine micropollutants in surface water. However, matrix interference during surface water analysis

can pose substantial challenges to the accurate analysis and quantification of pharmaceuticals. Further research into identifying pharmaceuticals particularly influenced by matrix interference could help introduce additional sample pre-treatment measures, thereby increasing the reliability of results. Furthermore, evaluating the role of environmental factors and potential sources of the matrix interfering compounds would be valuable to suggest mitigation strategies while highlighting this challenging yet frequently observed phenomenon.

The presence of pharmaceuticals in a cocktail raises concerns regarding their potential combined effects, as this area of research has had limited attention to date. A cornerstone of the European Green Deal is the Zero Pollution Action Plan, which aims to deliver zero pollution for air, water and soil.[423] To implement this strategy, scientific bodies must better understand and mitigate the environmental risks posed by these pharmaceutical mixtures in aquatic ecosystems.[424] To address this, future research should explore the effects of these low-level mixtures and whether their combination has additive, synergistic, or antagonistic effects on aquatic life. Furthermore, chronic ecotoxicological work and risk assessments should be expanded to gain insight into identifying vulnerable organisms and sub-lethal effects such as behaviour and reproduction. The investigation of pharmaceutical mixtures should be expanded to include their interaction with other environmental stressors to predict disruptions to ecosystem dynamics, such as the food web and aquatic species composition.

The monitoring campaigns and analytical methods developed in this research provide the foundation for further research into the link between pharmaceutical usage and their environmental presence, particularly in a health crisis such as a pandemic. Implementing epidemiological studies and environmental monitoring could provide valuable information

regarding public health data. Taking this form of holistic monitoring can help to inform policies and mitigation strategies so that proactive measures and mitigation strategies can be taken in times of crisis.

The adoption of passive samplers for quantitative water quality monitoring is hampered by the lack of reliable data regarding the sampling rates and the challenges faced with calibration studies. Further work into developing and implementing calibration studies would greatly benefit its adoption in future monitoring campaigns. However, passive sampling is still a valuable tool for qualitative assessments. The significant deficit of available data for the presence and concentration of pharmaceuticals in Irish aquatic environments poses a severe risk to our understanding of river water quality. Future research that integrates passive sampling with non-targeted screening methods has the potential to increase our understanding of the presence of pharmaceuticals that would otherwise remain undetected through traditional grab sampling.

This research has advanced our understanding of pharmaceutical contaminants in Irish environmental waters by providing insights into the prevalence and ecological risks posed to surface water ecosystems. Recognising that the health of people, animals, and ecosystems are interconnected, measures must be taken to safeguard aquatic ecosystems, particularly in a rapidly changing climate. The findings from this study contribute to Irish and European policy decisions while contributing to water quality assessment and mitigation strategies.

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Appendix

Appendix A: Matrix matched calibration curves for targeted pharmaceuticals.

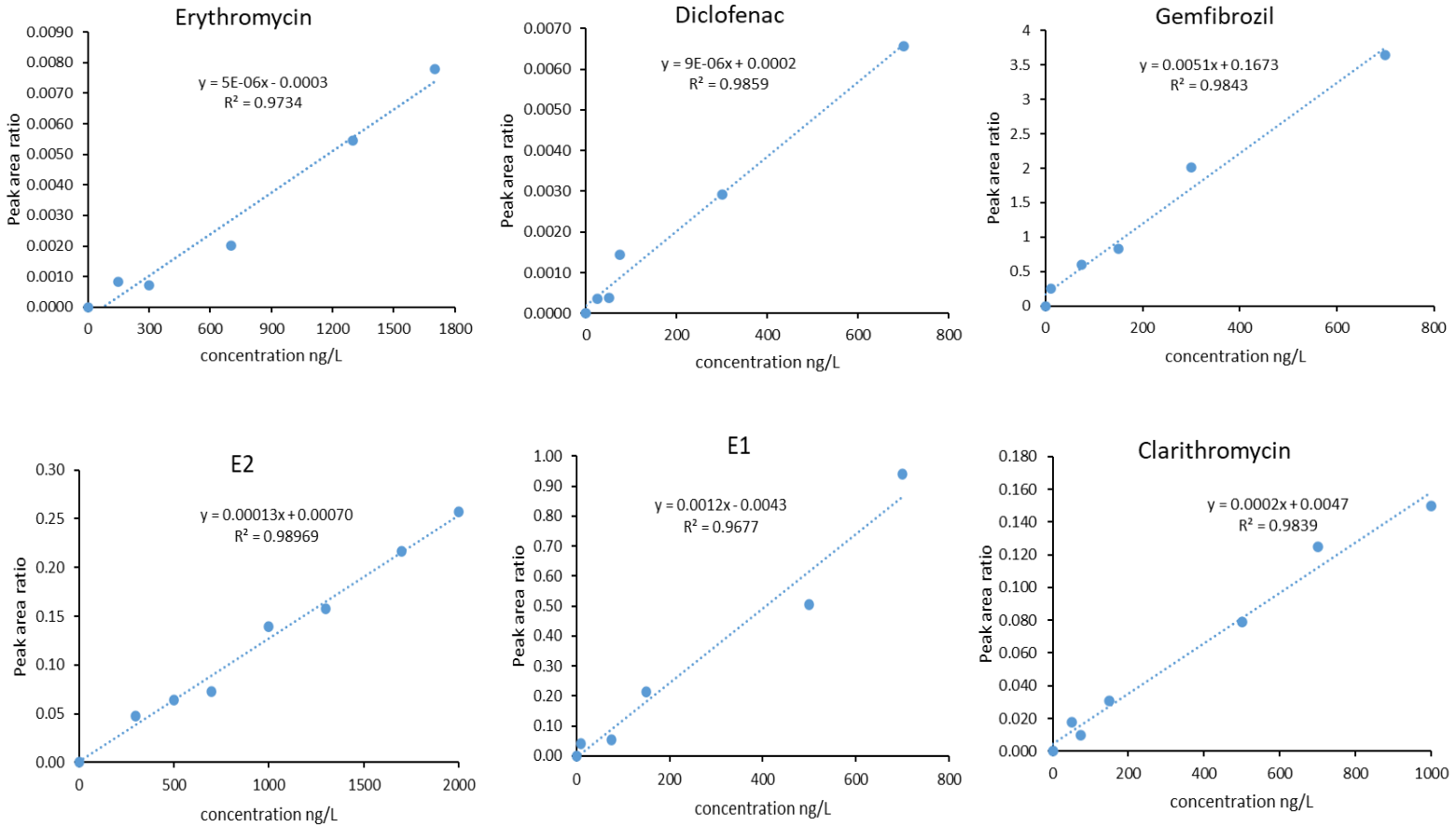

Figure A 1.1 Matrix matched calibration curves used in LC-MS/MS method validation in Chapter 2, Section 2.4.2.6 and to determine Measured Environmental Concentrations in Chapter 3.

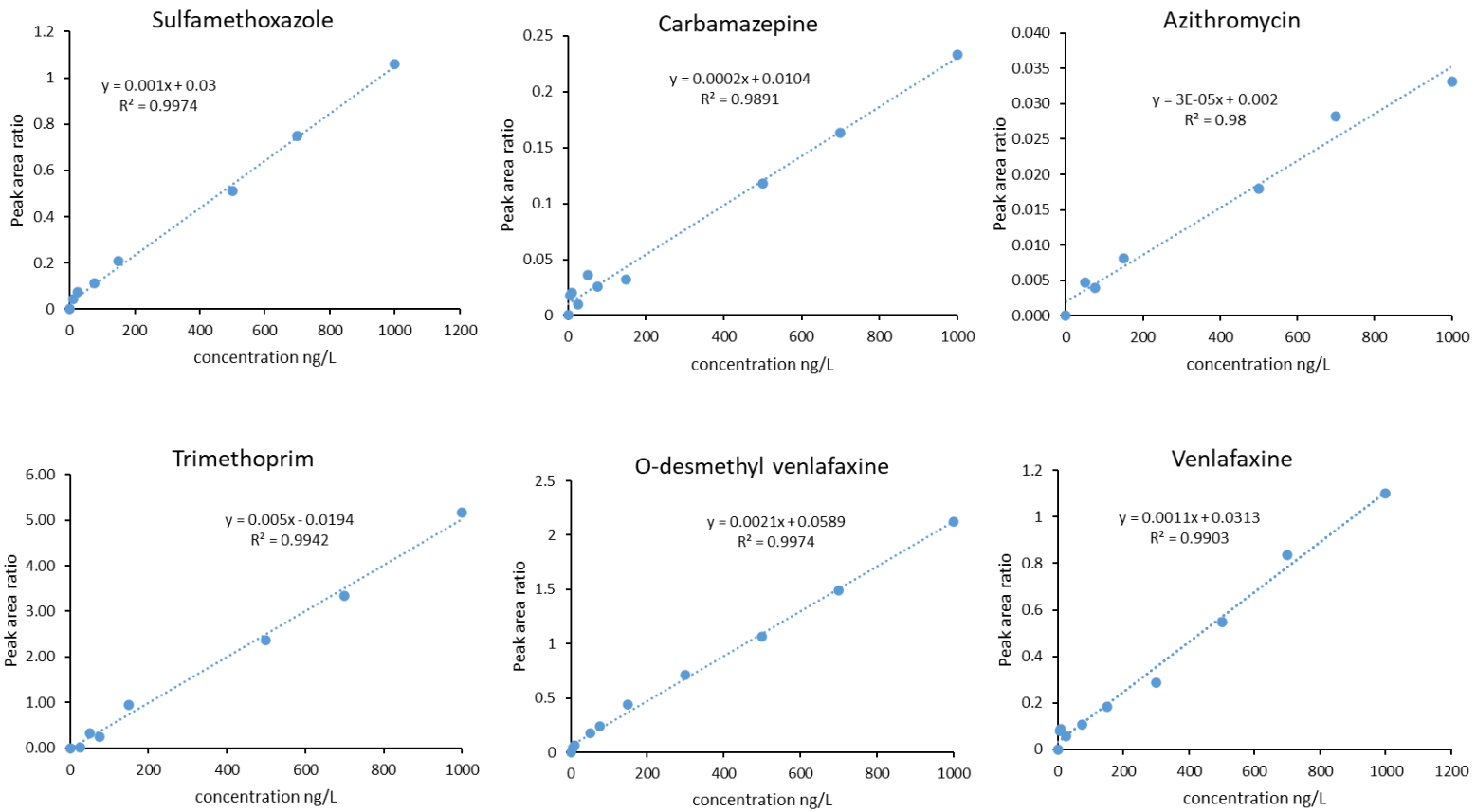

Figure A 2.1 (continued) Matrix matched calibration curves used in LC-MS/MS method validation in Chapter 2, Section 2.4.2.6 and to determine Measured Environmental Concentrations in Chapter 3.

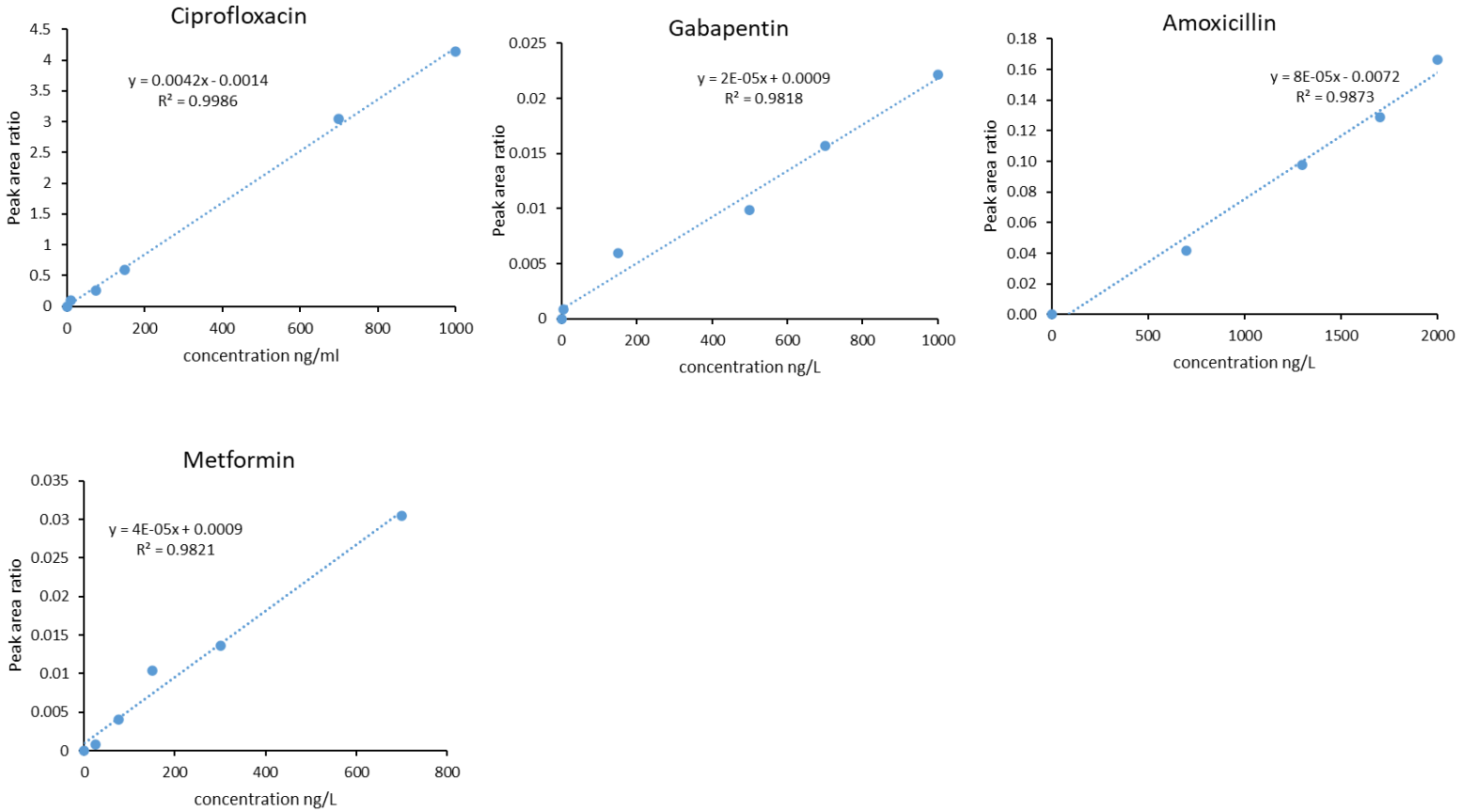

Figure A 3.1 (continued) Matrix matched calibration curves used in LC-MS/MS method validation in Chapter 2, Section 2.4.2.6 and to determine Measured Environmental Concentrations in Chapter 3.

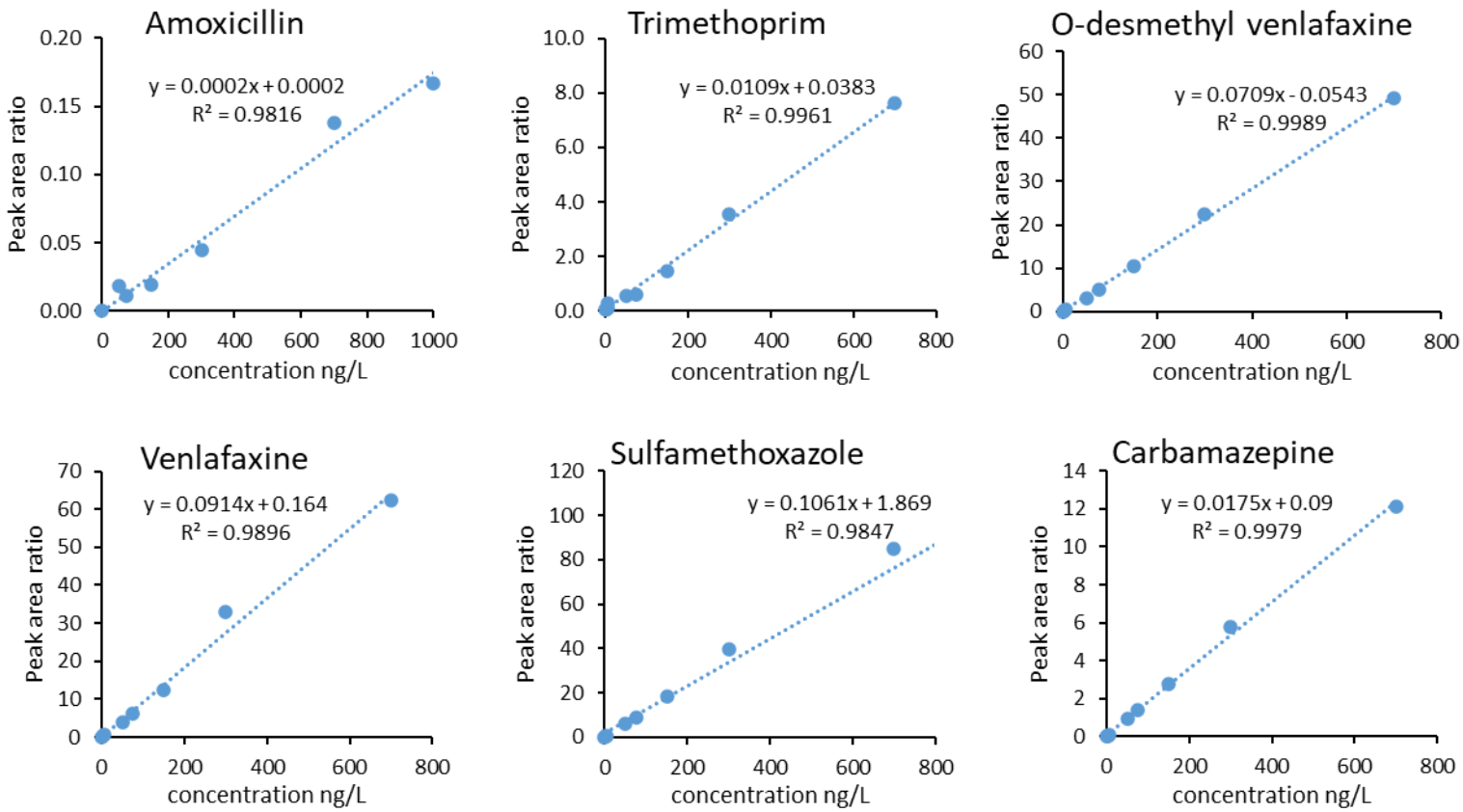

Figure A 1.4 Matrix matched calibration curves used in LC-MS/MS method validation in Chapter 2, Section 2.4.2.6 *and to determine Measured Environmental Concentrations in Chapter 5.*

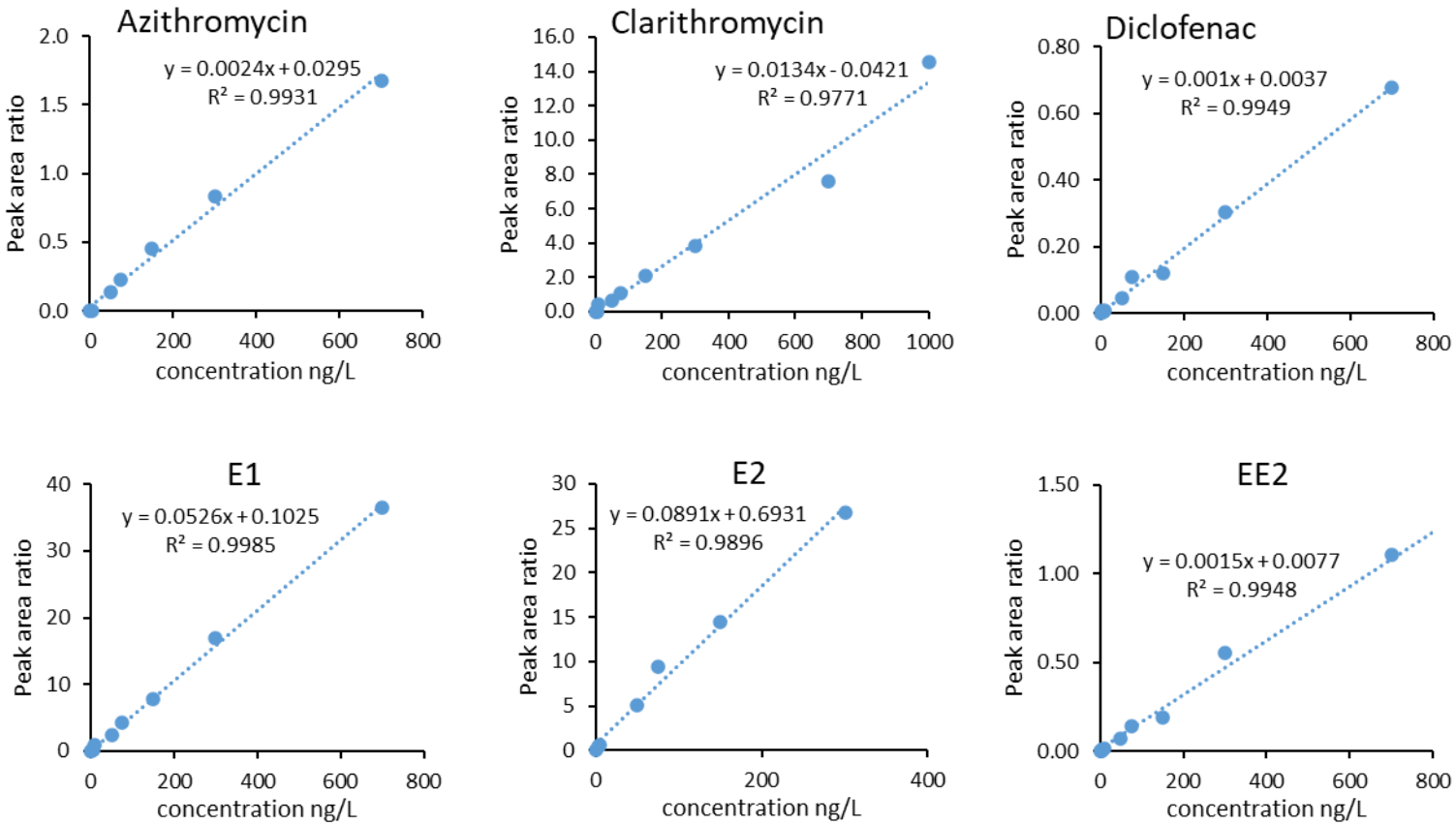

Figure A 1.2 (continued) Matrix matched calibration curves used in LC-MS/MS method validation in Chapter 2, Section 2.4.2.6 and to determine Measured Environmental Concentrations in Chapter 5.

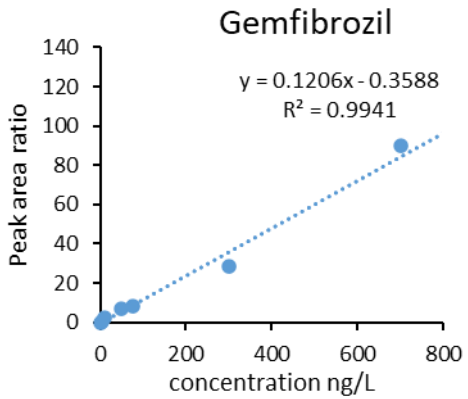

Figure A 1.2 (continued) Matrix matched calibration curves used in LC-MS/MS method validation in Chapter 2, Section 2.4.2.6 and to determine Measured Environmental Concentrations in Chapter 5.

Appendix B: Representative quantifier and qualifier ions for detected pharmaceuticals.

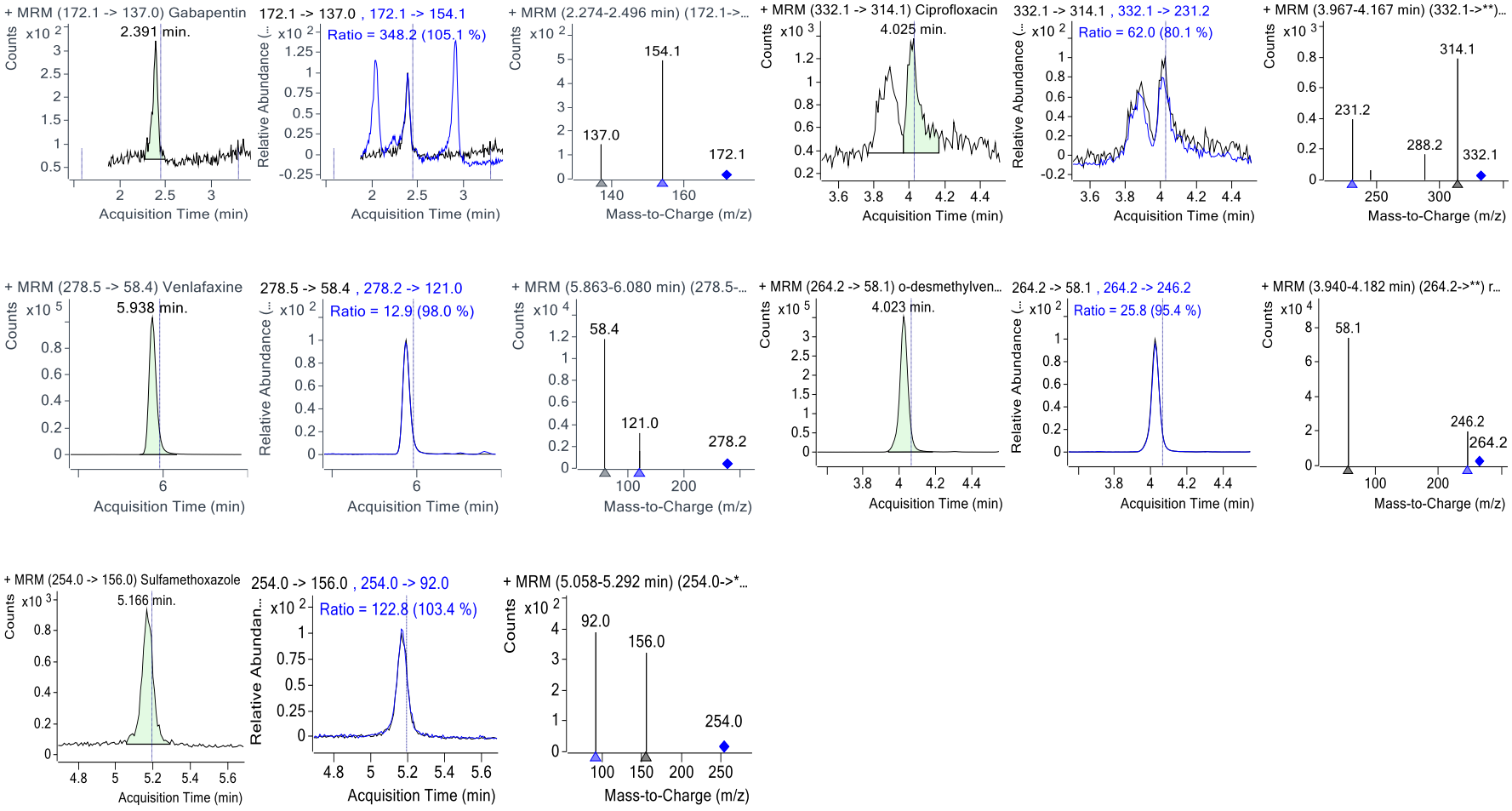

Figure B 2.1 Quantifier and qualifier ions for quantifiable pharmaceuticals detected in September 2021 in the river Nore.

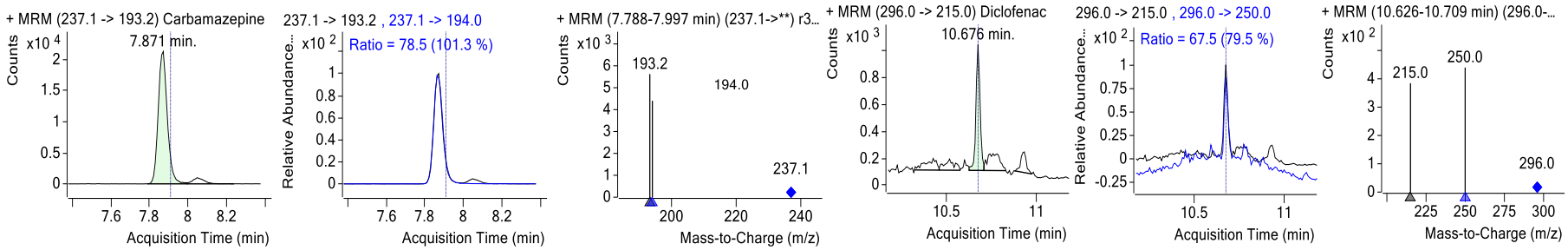

Figure B 2.1 (continued) Quantifier and qualifier ions for quantifiable pharmaceuticals detected in September 2021 in the river Nore.

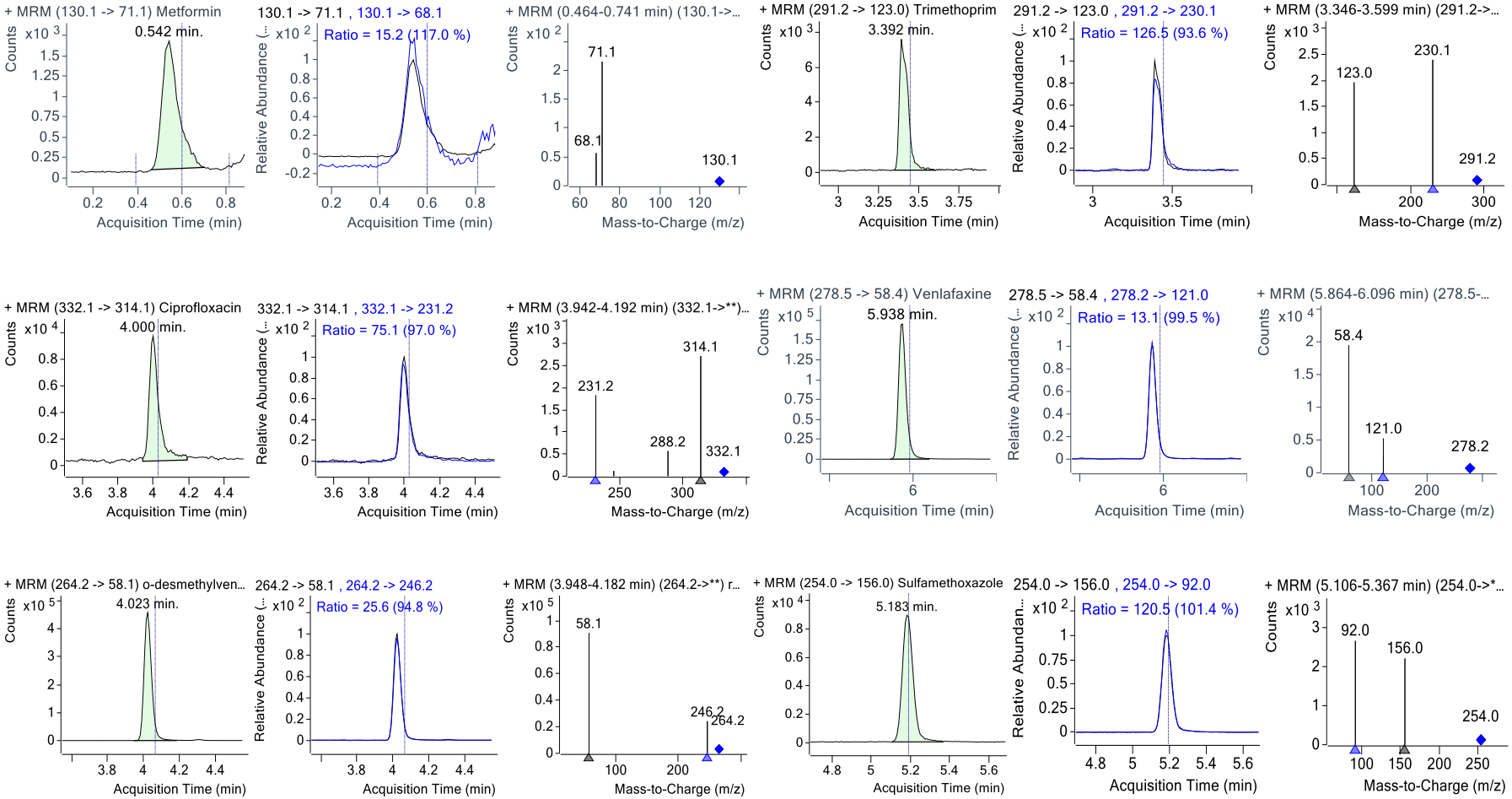

Figure B 2.2 Quantifier and qualifier ions for quantifiable pharmaceuticals detected in March 2021 in the river Liffey.

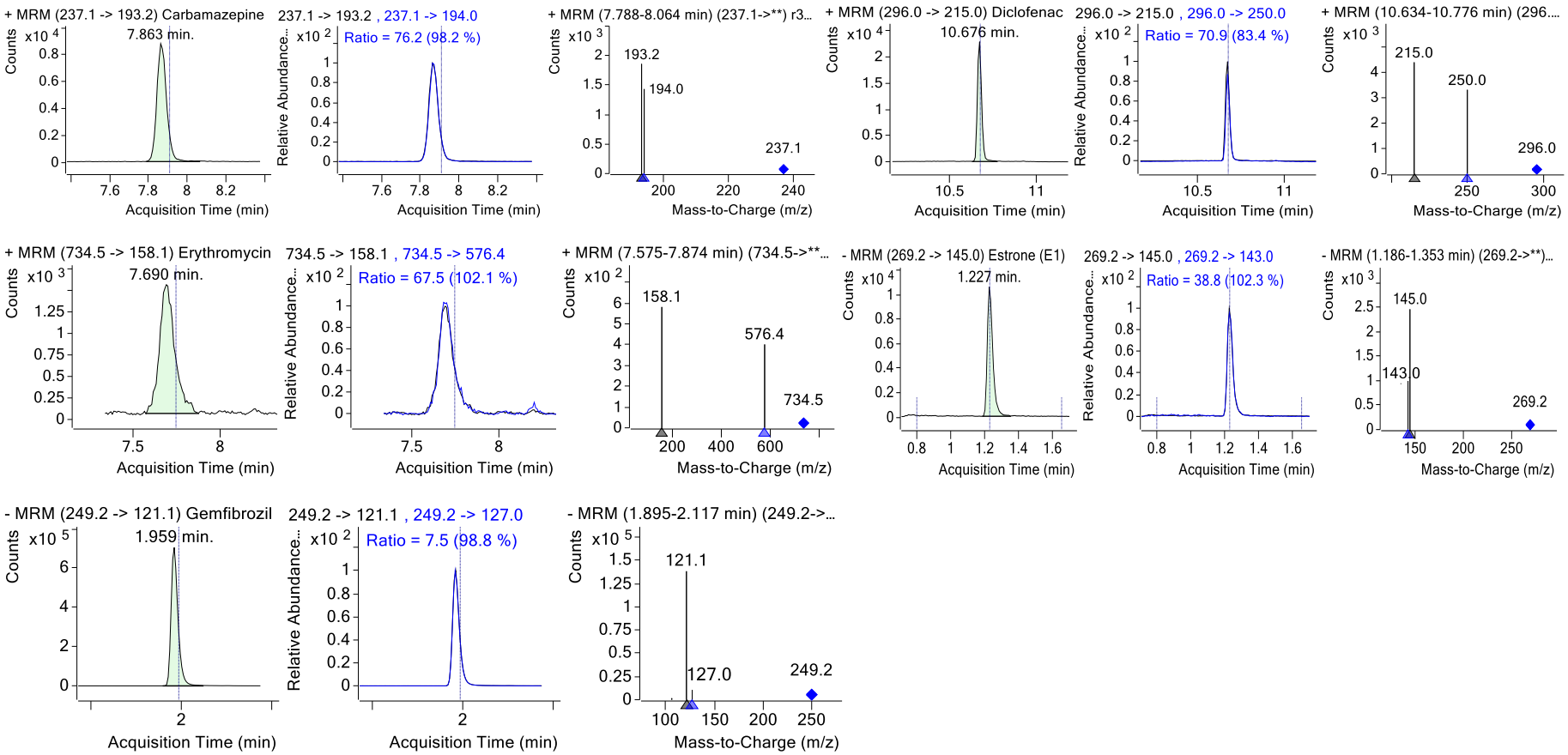

Figure B 2.2 (continued) Quantifier and qualifier ions for quantifiable pharmaceuticals detected in March 2021 in the river Liffey.

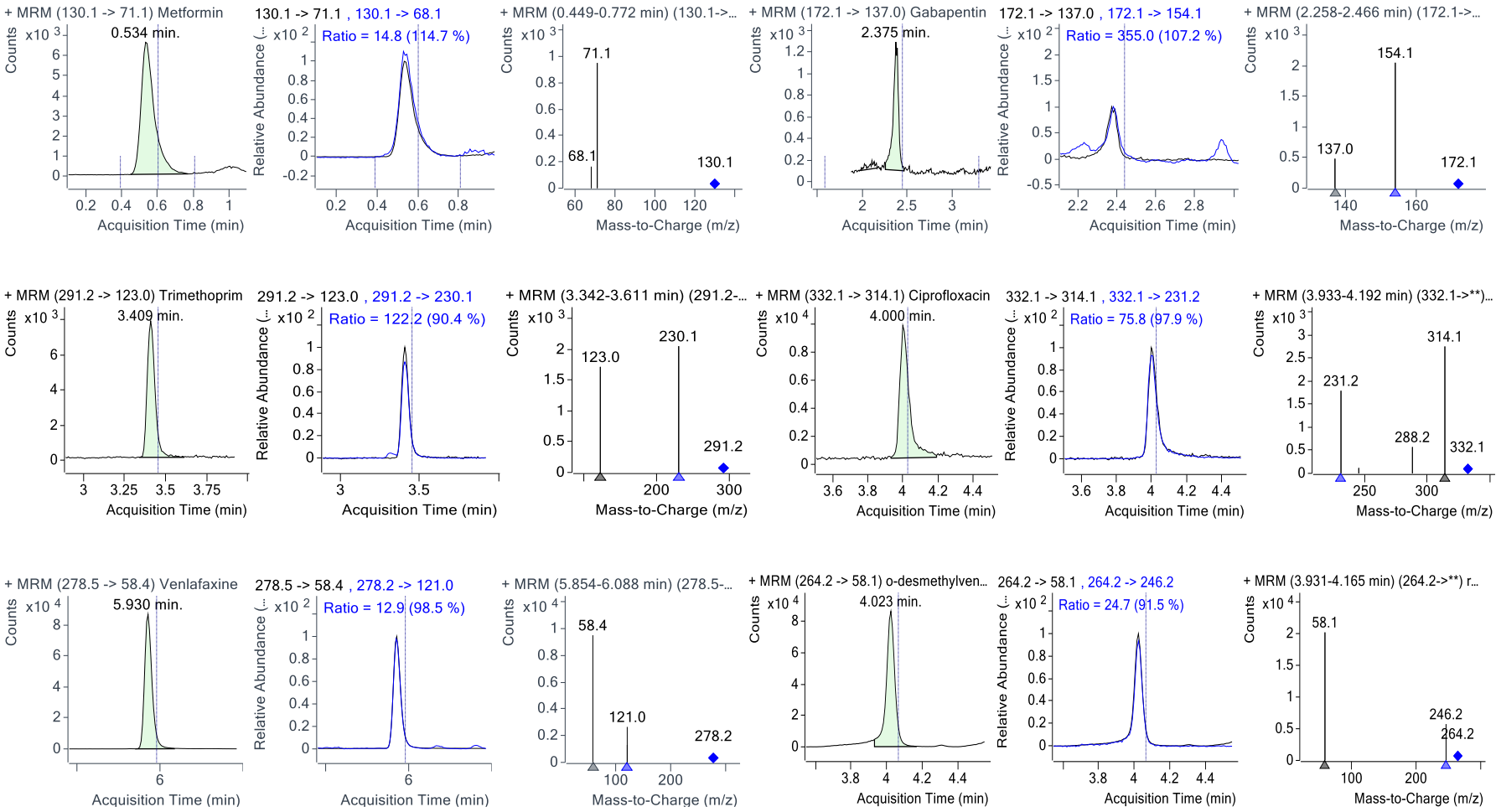

Figure B 2.3 Quantifier and qualifier ions for pharmaceuticals detected in October 2020 in the river Suir.

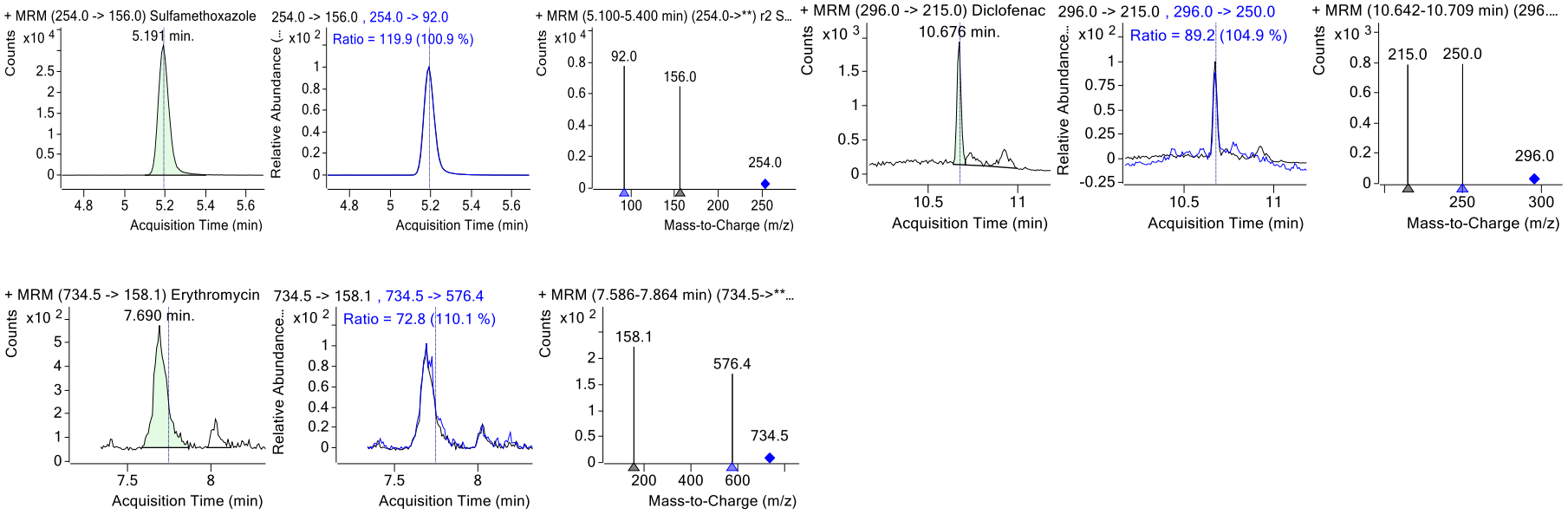

Figure B 2.3 (continued): Quantifier and qualifier ions for pharmaceuticals detected in October 2020 in the river Suir.

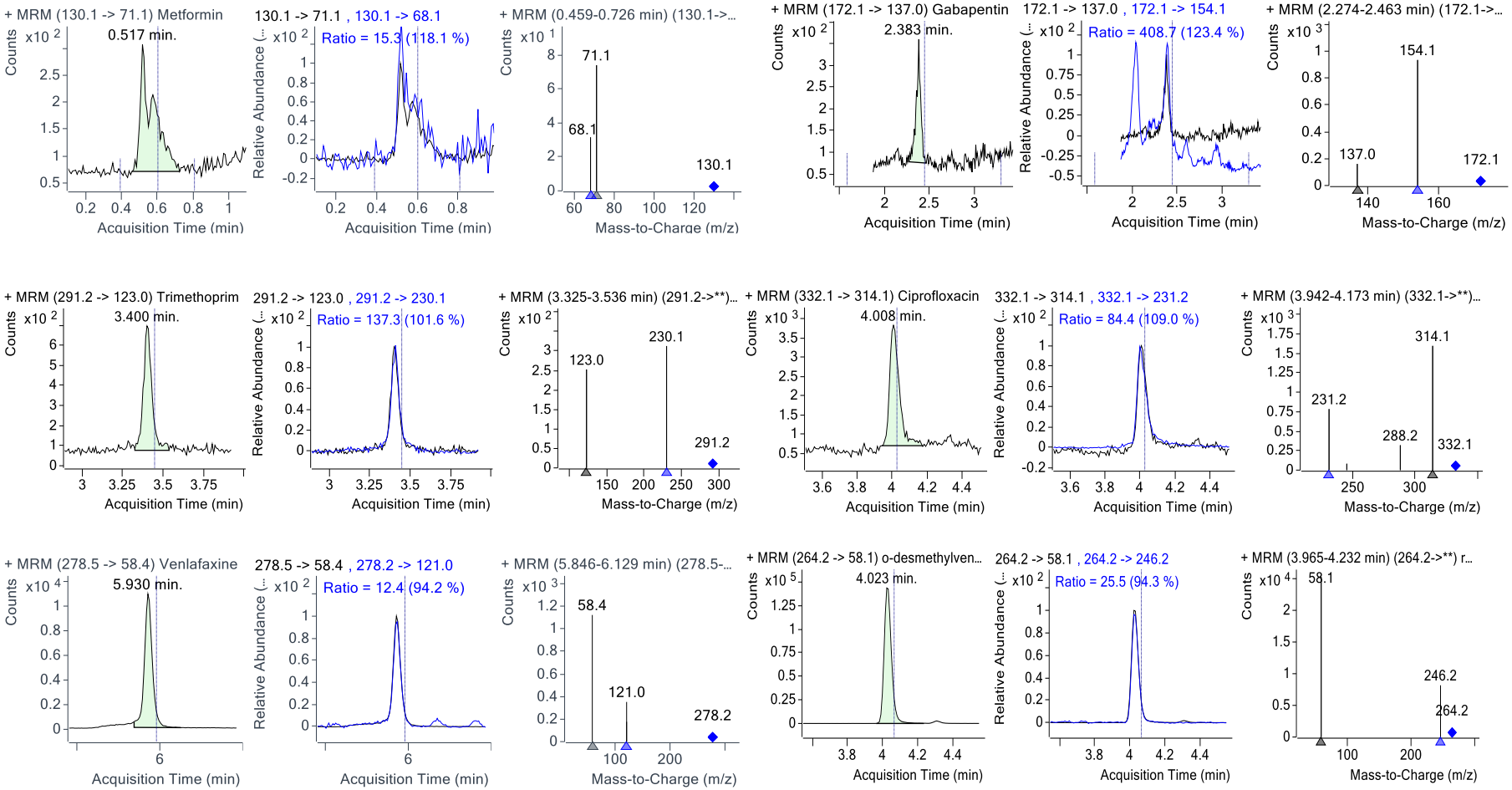

Figure B 2.4 Quantifier and qualifier ions for quantifiable pharmaceuticals detected in March 2021 in the river Analee.

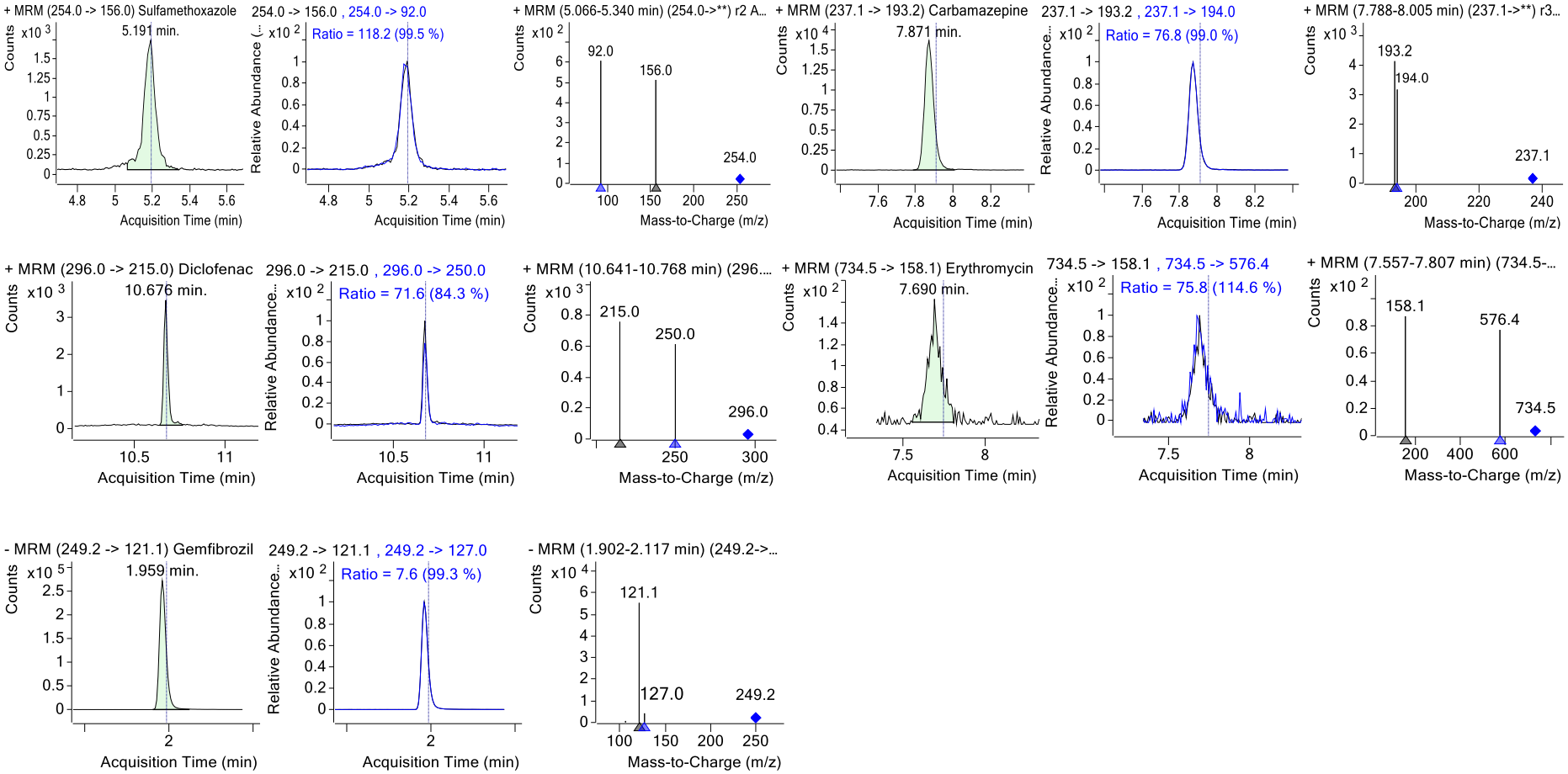

Figure B 2.4 (continued) Quantifier and qualifier ions for quantifiable pharmaceuticals detected in March 2021 in the river Analee.