Dublin City University
School of Biotechnology

PhD Thesis

An Investigation of Approaches and Analytical Tools to Disentangle Point and Diffuse Sources of Nitrate Contamination

by

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Cecilia Fenech	Date
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To my family.

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Abstract

An Investigation of Approaches and Analytical Tools to Disentangle Point and Diffuse Sources of Nitrate Contamination

Cecilia Fenech

Environmental forensics studies for nitrate source determination (NSD) have seen increased interest in recent years. However, the numerous approaches that have been suggested do not differentiate sewage and manure sources in surface waters. This differentiation is especially important as human-health risks are higher from human, than animal, faecal contamination. Therefore, human and veterinary pharmaceuticals were exploited as co-occurring sewage and manure markers. Through an understanding of pharmaceutical use, occurrence and fate, further source characterisation can also be achieved.

Pharmaceutical analysis in environmental waters has traditionally been carried out using SPE LC-MS/MS. A single method was developed and validated for six sewage and four manure markers at detection limits of up to 50 pg/L. Results from a one-year monitoring programme in Irish waters confirmed the selected suite's suitability for differentiating and characterising point and diffuse sources of sewage and manure. However, LC-MS/MS is costly, time-intensive and requires large sample volumes. Therefore, the novel application of NMR and immunoassays, was explored. The use of immunoassay techniques has shown great promise in this regard.

The potential for pharmaceutical entry into surface waters through alternative pathways than sewage and manure was also assessed. Data on medication use and disposal was collated following a survey of 1449 individuals. Results show that few participants dispose of unused pharmaceuticals in the sewer. Therefore, the potential for incorrect source attribution as a result of unused medication disposal is low, confirming the suitability of pharmaceuticals as chemical markers.

However, available data on NSD is highly fragmented and approach-dependent. Therefore, a decision-support tool that incorporates the considerations of interest was developed using IDEF0 modelling. This tool enables decision-makers to identify the most suitable NSD approach in a specific scenario. This tool was validated through interviews with key stakeholders, through which it was confirmed that there is, indeed, currently a need for such a decision tool.

List of Symbols and Abbreviations

% RSD	Percentage Relative Standard Deviation
ACN	Acetonitrile
ACT	Acetaminophen/Paracetamol
AIR	Atmospheric Nitrogen Isotopic Ratio
ANOVA	Analysis of Variance
BDL	Below Detection Limit
BOD	Biological Oxygen Demand
BQL	Below Quantification Limit
CAF	Caffeine
CBZ	Carbamazepine
CC$_{Ent}$P	Collision Cell Entrance Potential
CC$_{Ext}$P	Collision Cell Exit Potential
CE	Collision Energy
CHAID	Chi-squared Automatic Interaction Detection
COD	Chemical Oxygen Demand
COT	Cotinine
CSIA	Compound Specific Isotope Analysis
DAD	Diode Array Detector
DCU	Dublin City University
DP	Declustering Potential
DPH	Diphenhydramine
DTZ	Diltiazem
ELD	Environmental Liability Directive
ELISA	Enzyme-Linked Immunosorbent Assay
ENR	Enrofloxacin
EP	Entrance Potential
EPA	Environment Protection Agency (Ireland)
ESI	Electrospray Ionisation
ESKTN	Environmental Sustainability Knowledge Transfer Network
EtOAc	Ethyl Acetate
EU	European Union

FC	Faecal Coliforms
FIB	Faecal Indicator Bacteria
FIPS	Federal Information Processing Standards
FP	Focussing Potential
FS	Faecal Streptococci
GC	Gas Chromatography
GIS	Geographic Information System
GMS	General Medical Service
GP	General Practitioner/Family Doctor
HPLC	High Performance Liquid Chromatography
HSE	Health Service Executive
HWF	Hazardous Waste Facility
ICOM	Inputs Controls Outputs Mechanisms
IDEF0	Integration Definition Function modelling system
INAB	Irish National Accreditation Board
LC-MS/MS	Liquid Chromatography - Tandem Mass Spectrometry
LDM	Library Dependent Method
LIM	Library Independent Method
LIN	Lincomycin
LOD	Limit of Detection
LOQ	Limit of Quantification
LTI	Long-Term-Illness
MeOH	Methanol
MP	Mobile Phase
MS	Mass Spectometer
MST	Microbial Source Tracking
NI	Northern Ireland
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
NSD	Nitrate Source Determination
NVZ	Nitrate Vulnerable Zone
OSWTS	On-Site Wastewater Treatment System
OTC	Over-the-Counter
p.e.	Population Equivalents
PCR	Polymerase Chain Reaction
PFP	Pentafluorophenyl

RBD	River Basin District
RoI	Republic of Ireland
S.I.	Statutory Instrument
S.R.	Statutory Rules
SAQ	Self-Administered Questionnaire
SDM	Sulfadimethoxine
SI	Source Identifier
SMOW	Standard Mean Ocean Water
SNR	Signal:Noise Ratio
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring/Multiple Reaction Monitoring (MRM)
TSS	Total Suspended Solids
TYL	Tylosin
UK	United Kingdom
UKWIR	UK Water Industry Research Organisation
UWWTD	Urban Wastewater Treatment Directive
V-SMOW	Vienna Standard Mean Ocean Water
WFD	Water Framework Directive
WWTP	Wastewater Treatment Plant

Publications, Awards and Presentations

Peer-Reviewed Publications

C. Fenech, L. Rock, K. Nolan, J. Tobin, and A.J. Morrissey. The potential for a suite of isotope and chemical markers to differentiate sources of nitrate contamination: A review. *Water research*, 46(7):2023-2041, 2012.

C. Fenech, L. Rock, K. Nolan, J. Tobin, A. J. Morrissey. Attitudes towards the use and disposal of unused medications in two European countries. *Waste Management*, 33(2): 259-261, 2013.

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Non Peer-Reviewed Publications

C. Fenech, K. Nolan, L. Rock, A.J. Morrissey. Disposal of non-ingested pharmaceuticals within households: Is it a waste management concern? *Chartered Institute of Waste Management Magazine*, December 2012.

Honours and Awards

Postgraduate Prize at the International Society of Pharmaceutical Engineers (ISPE), Ireland Affiliate Event, Dublin (October 2011).

Research Mention: Research Trend. *Working with Water Magazine*, May 2012. (workingwithwater.filtsep.com/view/25453/ review-of-isotope-and-chemical-markers-to-differentiate-sources-of-nitrate-contamination/)

AVANTOR Presentation Award at the 8th Annual LC-MS/MS Workshop on Environmental Applications and Food Safety, Barcelona (July, 2012).

EPA Presentation Award at the Green Chemistry II in Ireland Conference, Dublin (July 2012).

Fixed Grant (Travel, Accommodation, and Living Expenses) from the ESF (European Science Foundation) to attend and present at the ESF Junior Summit. Water: Unite and Divide - Interdisciplinary approaches for a sustainable future, Italy (August 2012).

Presentation Award at the Sino-European Symposium on Environment and Health, Galway (August 2012).

Finalist in the Chemistry World Science Communication Competition, 2012 by the Royal Society of Chemistry (September 2012).

Student Bursary (Accommodation, Registration) for the Structure 2013 Conference at the University of Loughborough (February 2013).

Research Mention. Why throwing your unused meds in the bin is a bad idea, John Holden. *The Irish Times Health and Family Supplement*, pg. 5, 5^{th} March, 2013. (www.irishtimes. com/newspaper/health/2013/0305/1224330784738.html)

Research Mention: Video Interview. Environmental Science SelectScience Special Feature, 27^{th} March 2013. (www.selectscience.net)

Fixed Grant (Accommodation, Conference Fees) from the American Chemical Society (ACS) and the European Young Chemists Network (EYCN) to participate in the

Young Chemists Crossing Borders programme and attend the 246^{th} ACS National Meeting and Exposition in Indianapolis, USA (September 2013).

Oral Presentations

'Distinguishing sewage and manure derived nitrate' at the Dublin City University (DCU) School of Biotechnology Postgraduate Talks, Dublin, Ireland. 21 June 2011.

'An environmental forensics approach to differentiating sewage and manure nitrate inputs into surface waters' at the DCU, School of Biotechnology Research Day, Dublin, Ireland. 27 January 2012.

'Current attitudes to the use and disposal of medication' at the Institute of Global Health Inaugural Workshop, Dublin Ireland. 14 February 2012.

'Assessment of integrating stable isotope data and data on pharmaceuticals to disentangle sources of nitrate pollution' at the QUESTOR@DCU Annual Workshop, DCU, Dublin, Ireland. 28 February 2012.

'Using an environmental forensics approach to differentiate sewage and manure derived nitrate in Irish surface waters' at the 22nd Irish Environmental Researchers' Colloquium (ENVIRON 2012), Dublin, Ireland. 7-9 March 2012.

'Differentiation of nitrate sources: An environmental forensics approach' at the Danfoss Technology Centre, Nordborg, Denmark. 7 May 2012.

'An environmental forensics approach to identifying sewage and manure inputs to surface waters', at the Irish Mass Spectrometry Society AGM and Meeting, Dublin, Ireland. 9 May 2012.

'Micro pollutants as chemical markers for environmental forensics applications: Differentiating sewage and manure' at the 8th Annual LC-MS/MS Workshop on Environmental Applications and Food Safety, Barcelona. 2-4 July 2012.

'Pharmaceuticals in the environment: An environmental forensics application' at the Green Chemistry in Ireland Conference, Dublin, Ireland. 12 July 2012.

'Pharmaceuticals in the environment: Suitability for environmental forensics applications to identify nitrate sources' at the Sino-European Symposium on Environment and Health, Galway, Ireland. 21-24 August 2012.

'Environmental forensics: an Interdisciplinary Approach to Environmental Science' at the European Science Foundation Junior Summit on Interdisciplinary Research in Water, Stresa, Italy. 26-31 August 2012.

'Youth on the move: One person's experience', at the Youth on the Move at the Higher Options Fair, Dublin, Ireland. 21 September 2012.

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'Attitudes towards the use and disposal of unused medications in two European countries' at the 28th International Conference on Solid Waste Technology and Management, Philadelphia, USA. 10-13 March 2013.

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'Environmental forensics applications in identifying nitrate sources' at Sustainability Live, Birmingham, UK. 16-18 April 2013.

'Environmental forensics applications for sewage and manure differentiation: Is LC-MS/MS the only way?' at the Irish Mass Spectrometry Society AGM and Meeting, Dublin, Ireland. 1 May 2013.

Poster Presentations

'Assessment of the usefulness of integrating stable isotope data and data on pharmaceuticals to disentangle sources of nitrate pollution' at the DCU, School of Biotechnology Research Day, 27 January 2011.

'Distinguishing sewage and manure derived nitrate' at the Conference on Analytical Science, Ireland 2011 (6th CASi), Dublin, Ireland, 21-22 February 2011.

'Distinguishing sewage and manure derived nitrate' at the QUESTOR@DCU Annual Workshop, DCU, March 2011.

'Distinguishing sewage and manure derived nitrate' at the 21st Irish Environmental Researchers' Colloquium (ENVIRON 2011), Cork, Ireland, 6-8 April 2011.

'A method for the analysis of 10 chemical markers for the differentiation of sewage and manure nitrate in surface waters' at the 7th International Conference on Instrumental Methods of Analysis-Modern Trends and Applications (IMA 2011), Chania Crete, 18-22 September 2011.

'Pharmaceuticals as chemical markers for differentiating sewage and manure nitrate in surface waters' at the ISPE Ireland Affiliate Networking event, NIBRT facility, Dublin Ireland, 13 October 2011.

'An environmental forensics approach to differentiating sewage and manure nitrate inputs into surface waters', at the IWA World Congress on Water, Climate and Energy, Dublin, Ireland. 13-16 May 2012.

'Differentiating sewage and manure derived nitrate within surface waters' at the 17^{th} International Nitrogen Conference, Wexford, Ireland. 26-29 June 2012.

'An environmental forensics approach to differentiating sewage and manure nitrate inputs into surface waters' at the Sustainability @ DCU Meeting, Dublin, Ireland. 3 December 2012.

'Trace analysis for environmental forensics applications: The case of low level chronic contamination' at the Structure 2013 Conference, Loughborough, UK. 26-27 February 2013.

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Chapter 1

Scope of Work

1.1 Introduction

The nitrate ion (NO_3^-) occurs naturally as part of the nitrogen cycle. However, it is being detected at ever-increasing concentrations within water bodies due to increasing anthropogenic inputs, such as synthetic and natural fertilisers and leaking septic systems [1]. In addition, biogeochemical processes are known to modify nitrate concentrations such that different forms of nitrogen (e.g. NO_2, NH_3) can potentially be transformed into nitrate, resulting in further elevated concentrations [2].

The various point and diffuse (non-point) nitrate sources have caused nitrate to be considered a ubiquitous contaminant of natural water resources. Nitrate is considered to be a contaminant of concern because its presence within water bodies has been linked to various environmental and health effects. High nitrate concentrations in surface water[1] bodies can lead to eutrophication [3, 4] and cause biodiversity loss and ecosystem dysfunction [5]. High nitrate concentrations in drinking water have been linked to methemoglobinaemia in children (blue-baby disease) [3, 6] and cancer [2, 7]. However, the presence of a direct link is still an issue of debate [8, 9].

In order to achieve improved water resource management and preserve water quality, it is imperative that the sources of nitrate contamination can be identified [3, 10, 11]. Source identification allows for remediation actions to be targeted to the actual source. In this way, remediation efforts are more efficient, thus making them less costly and reducing public health and environmental concerns related to elevated nitrate concentrations. Furthermore, the identification of contamination sources allows for more effective application of the 'polluter pays principle', which is a cornerstone of the Environmental Liability Directive 2004/35/EC [12].

[1] Within this work the term surface waters is referring to fresh surface water bodies such as rivers and lakes.

Unfortunately, the sources of nitrate contamination may vary considerably, both between and within regions [13]. This is because the relationship between nitrate concentrations and the quantity of nitrate introduced from a specific source is complicated by a number of factors. These include the occurrence of multiple inputs, the presence of overlapping point and non-point sources, the coexistence of several biogeochemical processes that alter nitrate concentrations and the occurrence of considerable temporal variations dependent upon precipitation levels leading to inter-annual variations [3, 11, 13, 14].

To date, the differentiation of most nitrate sources has been achieved using nitrate stable isotope[2] compositions [3, 10]. However, they do not successfully differentiate sewage and manure sources. Nevertheless, such a differentiation is of particular importance as the risk to humans is considered higher from human faecal contamination (sewage) than from animal faecal contamination (manure). This increased risk is because viruses, which represent an important basis of illness resulting from faecal exposure, are highly host specific [16]. However, virological analyses of environmental waters are extremely complex and have significant limitations [17].

Therefore, this PhD study attempts to make a contribution to this effort by assessing the current state of knowledge and identifying gaps of knowledge in the field of nitrate source determination. In particular, efforts are focussed on the differentiation and characterisation of sewage and manure inputs to surface waters through the use of emerging contaminants, such as pharmaceuticals, as chemical markers for environmental forensics[3] applications.

The use of traditional and novel analytical techniques for the detection of chemical markers within surface waters was investigated, together with the potential for entry of the selected chemical markers through other sources than sewage and manure. Additionally, a decision tool was developed that brings together the current state of knowledge in the field of nitrate source determination and the differentiation requirements of such studies. Using this decision tool, decision-makers are able to identify the most suitable approach for achieving nitrate source determination for their specific scenario. Therefore, the effectiveness of environmental forensics studies for nitrate source determination is optimised.

[2] Stable isotopes, unlike radioactive isotopes, are nuclides which do not undergo radioactive decay [15].

[3] Environmental forensics is a multidisciplinary activity of recent formal development within the area of environmental measurement with the aim of identifying the sources, age and/or timing of contaminants into the environment [18–20].

1.2 Aim and Objectives

The main aim of this project is to investigate various approaches and analytical tools to disentangle point and diffuse sources of nitrate contamination. In order to reach this aim, the following objectives have been set:

- To review the current state of knowledge in the field of nitrate source determination and identify the key research gaps;
- To determine the most suitable approach for differentiating sewage and manure inputs into surface waters;
- To identify an appropriate suite of chemical markers for differentiating and characterising sewage and manure inputs into surface waters;
- To develop and validate a multi-residue chromatographic method for monitoring the suite of chemical markers within surface waters;
- To apply the validated chromatographic method to surface water monitoring sites within Ireland;
- To investigate the potential of alternative analytical techniques to traditional chromatographic methods to detect chemical markers for environmental forensics purposes;
- To obtain baseline data on the importance of disposal on the various routes of non-ingested pharmaceutical entry into the environment;
- To identify the current attitudes and awareness levels of the general public on the use, disposal and environmental effects of pharmaceuticals, and;
- To develop and evaluate a decision tool for the differentiation and characterisation of nitrate inputs into surface waters.

1.3 Thesis Organisation

This thesis is divided into eight chapters. In this first chapter, an overview of the research is given. Chapter 2, then, sets the context for the following chapters by providing an insight into the literature available on which this research builds. Current approaches to nitrate source determination are outlined and the potential for the use of chemical markers for the differentiation of point and diffuse sources of sewage and manure is critically examined.

Chapter 3 describes the methods adopted for investigating the different lines of research. Details of the sampling protocol adopted are included, together with

details of the method development and validation processes of the chromatographic and mass spectrometric techniques for the detection of the selected chemical markers. The use of alternative analytical techniques, namely nuclear magnetic resonance (NMR) and immunoassays for environmental forensics applications is also outlined. This is followed by a description of the approach undertaken for investigating current attitudes to the use and disposal of pharmaceuticals, which within this study are being used as chemical markers for the differentiation of sewage and manure.

A number of results and discussion chapters follow. Chapters 4 and 5 explore the use of chemical markers for the differentiation of sewage and manure inputs into surface waters. Chapter 4 discusses the use of standard chromatographic techniques for such an application, including details of the suite of chemical markers selected, method development and validation, and the application of the developed method to monitoring sites in Ireland. Chapter 5 discusses the potential of using alternative analytical techniques, namely NMR and immunoassays, as a way of reducing requirements for method development, sample processing and sample analysis. A particular focus is on the specific considerations for using such analytical techniques in environmental forensics studies.

The last results and discussion chapter, Chapter 6, investigates a lacuna in the current state of knowledge on the entry routes of pharmaceuticals into surface waters, namely that from incorrect disposal of medication. The outcomes of a questionnaire directed towards the general public on current medication use and disposal practices, and the disposal and environmental considerations of such practices are therefore discussed.

Chapter 7, then, discusses the development and evaluation of a decision support tool to assist the decision-maker in identifying the most suitable approach to achieve differentiation of nitrate inputs within surface water bodies. Details of the modelling system selected, the decision tool's development and its evaluation are given.

Finally, chapter 8 concludes the work by identifying the overarching conclusions and contributions of this work, as well as outlining suggestions for further studies.

Chapter 2

State of Knowledge: Nitrate Contamination and Nitrate Source Determination

As introduced in Chapter 1, this study aims to identify and contribute to research gaps within the field of nitrate source determination with a specific focus on sewage and manure differentiation. In order to achieve this, it is essential to first understand the context for this research and why such environmental forensics studies are becoming increasingly important.

Therefore, the legislative framework related to nitrate contamination within Europe and Ireland, and the current state of nitrate contamination is initially described in Section 2.1. Current approaches and limitations to differentiating the various sources of nitrate contamination are subsequently outlined in Section 2.2. This allows for a number of research gaps to be identified. To date, the use of nitrate stable isotope compositions has largely been adopted for nitrate source determination, whilst genetic markers have been adopted in an effort to achieve faecal source[1] tracking. Hence, a brief description of the current state of knowledge in these fields is given in Sections 2.3 and 2.4, respectively.

However, the use of nitrate stable isotope compositions has been shown to be unsuccessful in discriminating sewage and manure inputs [10]. Similarly, the use of genetic markers has been shown to have a number of limitations in this regard [21]. Therefore, this chapter focusses on identifying the potential of using chemical markers to achieve this differentiation (Section 2.5). Specifically, an in-depth

[1] Faecal contamination indicates the presence of constituents arising from human or animal waste matter. It is considered to be sewage when the source is human waste, whilst it is considered to be manure if the source is animal waste.

review of the considerations that need to be taken into account when using pharmaceuticals as chemical markers to differentiate sewage and manure sources of nitrate contamination is given.

Finally, in Section 2.6, the main conclusions arising from this chapter are given. The principal gaps in this field of research arising from the current state of knowledge, and which are tackled as part of this study, are also outlined.

2.1 Nitrate Contamination and Legislation

Currently, European legislation for the control of eutrophication and nutrient loading in water bodies is tackled in several pieces of legislation [22]. The Nitrates Directive, 91/676/EEC, is the most directly related legislation to nitrate pollution. It aims to protect water quality by preventing nitrates from agricultural sources (mainly diffuse sources) from polluting ground and surface waters [23]. To achieve these objectives, member states are required to identify waters that are affected or potentially affected by nitrate contamination[2], identify Nitrate Vulnerable Zones (NVZ)[3] and implement measures to limit nitrate contamination within NVZs [23].

The implementation of the Nitrates Directive, amongst other regulations, has led to decreased levels of nitrates in environmental waters since peak production of reactive nitrogen was reached in the 1980s [5]. Yet, even though nitrate concentrations have declined, nitrate levels have now stabilised at relatively high levels [5]. In fact, nitrate concentrations at a magnitude sufficient to promote eutrophication are still reported [26]. Thus, efforts for reducing nitrate contamination within water bodies are ongoing, especially as long time lags are required for the recovery of freshwater resources from nitrate contamination [5].

The Urban Wastewater Treatment Directive (UWWTD), 91/271/EEC, regulates effluents from a number of point sources, such as urban wastewater treatment plants (WWTP) and certain industrial sectors [27]. The directive requires that all agglomerations consisting of at least 2000 population equivalents[4] (p.e.) are equipped with collecting systems and that all wastewater discharged is subject to at least secondary treatment[5]. Where effluents are discharged into sensitive areas,

[2] Although no official standards have been set for nitrate concentrations in surface waters [24], the limit set within the Drinking Water Directive for drinking water quality (> 50 mg-NO_3 L^{-1}) [25] is used for this purpose.

[3] Areas draining zones identified to be affected or potentially affected by nitrate contamination.

[4] 1 population equivalent corresponds to the organic biodegradable load equivalent to a five-day biochemical oxygen demand (BOD_5) of 60 g of oxygen per day.

[5] Secondary treatment is an additional step of treatment following primary treatment, which involves the removal of residual organic matter and suspended solids. Primary treatment involves the removal of settleable organic and inorganic solids by sedimentation and scum by skimming.

more stringent treatment is required.

The Water Framework Directive (WFD), 2000/60/EC, provides the framework legislation in which the other directives related to water contamination operate [28]. Its aim is to achieve a good ecological and chemical status for all European waters by 2015. In order to achieve this status, member states are obliged to analyse the pressures and impacts on surface and subsurface water resources, set up monitoring networks, and develop river basin management plans. This directive has provided a framework for integrated water management by river basins, which requires the identification of point or diffuse sources of contamination [29]. Therefore, increased efforts related to source identification have become necessary.

The Environmental Liability Directive, 2004/35/EC (ELD), is also directly related to this study since its objectives include the application of the 'polluter pays principle' [12]. The deadline for transposition into law was April 2007. However, only four European Union (EU) member states (Italy, Lithuania, Latvia and Hungary) met this deadline. Seven countries (France, Finland, Slovenia, Luxembourg, Greece, Austria and the United Kingdom) received a European Court of Justice Judgement in 2008 and 2009, whilst the remaining countries had infringement procedures started against them but became compliant in the interim [30]. The delays in ELD transposition have resulted in only around 50 ELD cases having been initiated within Europe [30]. None had been initiated within Ireland by November 2012, which is from when the last data is available [31].

Nevertheless, the ELD has led to the area of environmental forensics being labelled a priority technology area by the Environmental Sustainability Knowledge Transfer Network (ESKTN) within the United Kingdom (UK) with an estimated market value of £10-15 million per annum by 2015 from a minimal current valuation [20]. This market value has largely been inferred from estimates related to remedial measure costs in the UK resulting from the implementation of the ELD and indicates the increased importance of such studies.

As with all European-wide directives, the various directives have been transposed into each country's national law. In the Republic of Ireland (RoI) and Northern Ireland (NI), these directives have been transposed into national law as specified in Table 2.1. Other measures related to nitrate contamination include the ongoing Common Agricultural Policy reforms. Through reforms initiated in 2003, subsidies have been decoupled from production levels and linked to the application of statutory minimum requirements that focus on increased sustainability in resource usage and the implementation of 'good farming practices' [32]. Amongst the requirements arising from the Common Agricultural Policy reforms there is that for additional

payments to be attained through the Rural Development Programmes, decreased fertiliser use is necessary [33]. Thus, this facilitates a reduction in the emission levels of nutrients, including nitrates, to water bodies.

Table 2.1: Matrix of relevant directive transpositions into Irish law (as amended, where applicable). S.I.: Statutory Instruments, S.R.: Statutory Rules.

	EU Directive	**RoI**	**NI**
Nitrates Directive	91/676/EEC	S.I. 610 of 2010	S.R. 2010 No. 411
Urban Wastewater Directive	91/271/EEC	S.I. 48 of 2010	S.R. 2007 No. 187
Water Framework Directive	2000/60/EC	S.I. 722 of 2003	S.R. 2003 No. 544
Groundwater Directive	2006/118/EC	S.I. 9 of 2010	S.R. 2009 No. 254
Environmental Liability Directive	2004/35/EC	S.I. 547 of 2008	S.R. 2009 No. 252

2.1.1 Situation in Ireland

Within Ireland, overall nitrate concentrations in rivers are generally lower than in most other European countries. In fact, according to the report on water quality in Ireland issued by the Environmental Protection Agency (EPA), only 0.3% of monitored sites achieved a poor status as a consequence of nitrate contamination during the period 2007-2009 [34]. This value represents a reduction in overall nitrate concentrations within Irish fresh surface waters over previous years.

These low nitrate concentrations have been attributed to the predominance of pasture[6], rather than tillage[7], as the main agricultural land-use [35]. The relevance of land-use characteristics can be seen in that average nitrate concentrations in rivers located in the southeast of the island are generally much higher than in the west, where the extent of pasture is higher (Figure 2.1) [24, 35].

In addition to land-use characteristics, a number of other factors have a bearing on nitrate concentrations within Irish waters. For example, the reduction in nitrate concentrations in 2007-2009, mentioned above, has been attributed to a combination of increased rainfall, reduced inorganic fertiliser use, improvements in organic fertiliser storage and the implementation of land spreading restrictions [34]. Nevertheless, there are nearly 140,000 farms within the RoI, which are a potential source of nitrogen to water bodies [36]. In fact, the quantities of nutrients, including nitrate, contained in animal manures are much larger than in sewage, largely due to cattle and sheep numbers greatly exceeding the human population in Ireland [35].

[6] Pasture refers to animal husbandry that is based on animals being allowed to graze on land.
[7] Tillage farming refers to the use of mechanical agitation for soil preparation and cultivation.

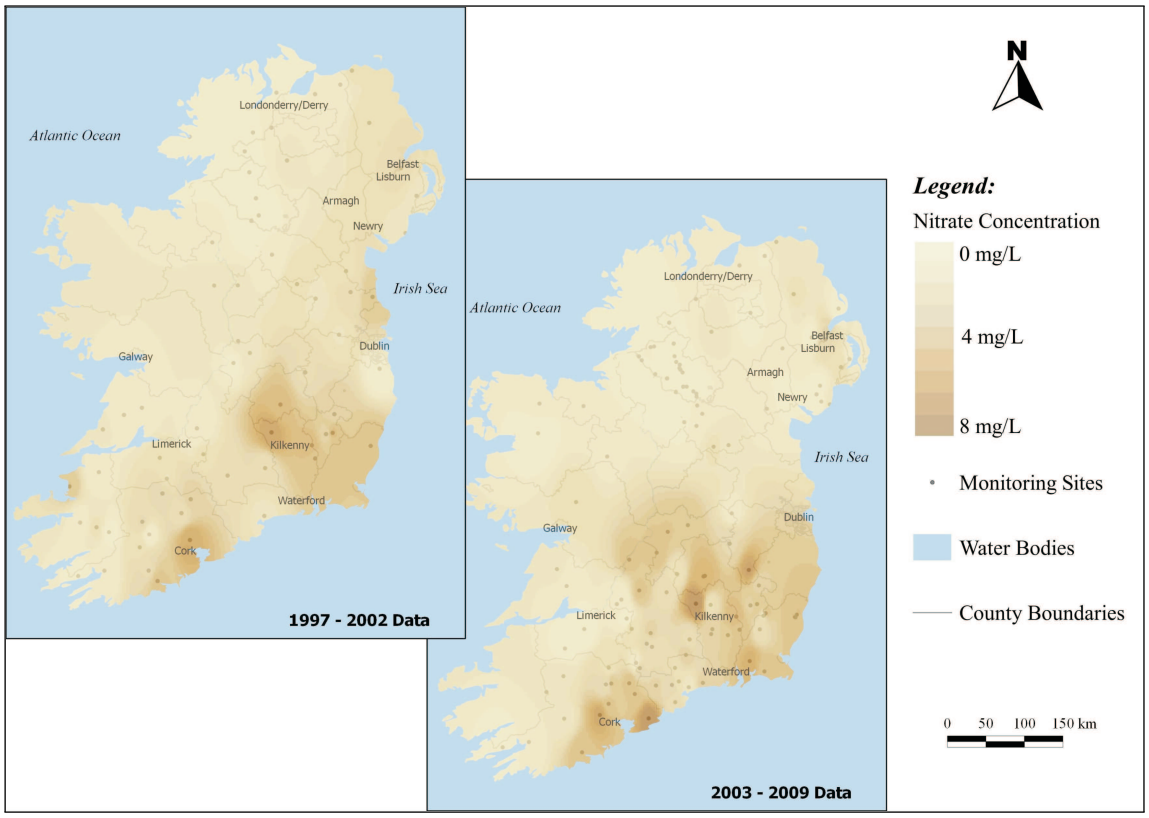


Figure 2.1: Annual average nitrate concentrations (mg-NO_3 L^{-1}) in lakes and rivers for data available for the years 1997-2002 (left) and the years 2003-2009. Mapping details are given in Appendix A.

In an effort to limit nitrate contamination, the whole island of Ireland (RoI and NI) has been designated an NVZ[8] [38]. This designation does not necessarily mean that the whole country is vulnerable to excessive nitrate levels but that the same level of protection is afforded to the whole territory [23]. The implemented action programmes in Ireland include measures related to farmyard manure management, storage requirements, closed periods for fertilisation (Table 2.2), distance rules to waters for fertilised sites, procedures governing land fertiliser application and provisions for nutrient management [38]. The closed periods for fertilisation and storage capacities for bovine livestock manure largely depend upon the location of the specific farm (Figure 2.2).

Yet, the RoI and NI have a derogation from certain elements of the Nitrates Directive. Within the RoI, this is by decision 2007/697/EC and its renewals[9] [41].

[8] Nine other EU countries: Austria, Denmark, Finland, Germany, Lithuania, Luxembourg, Malta, the Netherlands and Slovenia have also designated the whole territory as an NVZ [37].

[9] The latest renewal of the derogation is valid until the end of 2013. This deadline coincides with the next review of Ireland's Nitrates Action Programme, which defines the various actions being implemented to minimise nitrate contamination.

Table 2.2: Prohibited periods for the application of certain types of fertiliser to land (both dates inclusive) by zone [39, 40].

Zones	Chemical Fertiliser	Organic Fertilisers Excluding Farmyard Manure	Farmyard Manure
A	15 Sep to 12 Jan	15 Oct to 12 Jan	1 Nov to 12 Jan
B	15 Sep to 15 Jan	15 Oct to 15 Jan	1 Nov to 15 Jan
C	15 Sep to 31 Jan	15 Oct to 31 Jan	1 Nov to 31 Jan
D	15 Sep to 31 Jan	15 Oct to 31 Jan	31 Oct to 31 Jan

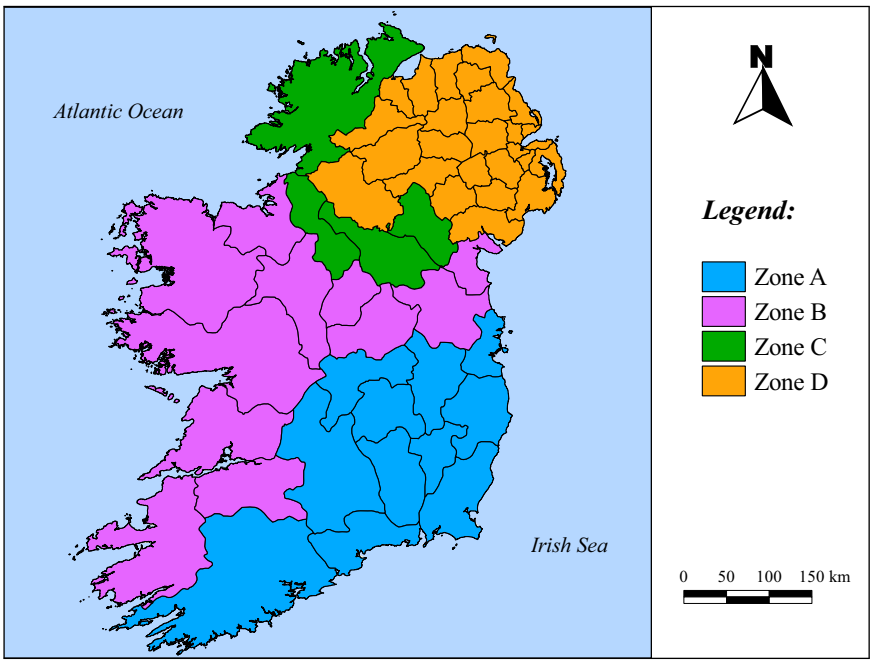


Figure 2.2: Zone delineation for the Irish nitrates action programme. Adapted from [39, 40].

As a result, farmers are allowed to operate at levels of manure land spreading up to 259 kg-N ha^{-1} yr^{-1}, if they adhere to the nitrates action programme, whilst a limit of 170 kg-N ha^{-1} yr^{-1} is allowed in the absence of a derogation [37, 42]. These derogations are considered to have contributed to Ireland representing some of the highest agricultural emissions of nitrogen to freshwater within Europe, with values exceeding 30 kg-N ha^{-1} yr^{-1} within certain regions (Figure 2.3) [5]. Such levels make the contribution of agricultural nitrogen loads to Irish waters an estimated 82% of all nitrogen sources in Ireland [39]. This proportion is at the top end of the contribution of agriculture to total nitrogen loads in Europe, which is estimated at 50-80% [22].

Apart from agricultural nitrate inputs, sewage sources of nitrate are another concern. Within the RoI, the treatment of urban wastewater has greatly increased

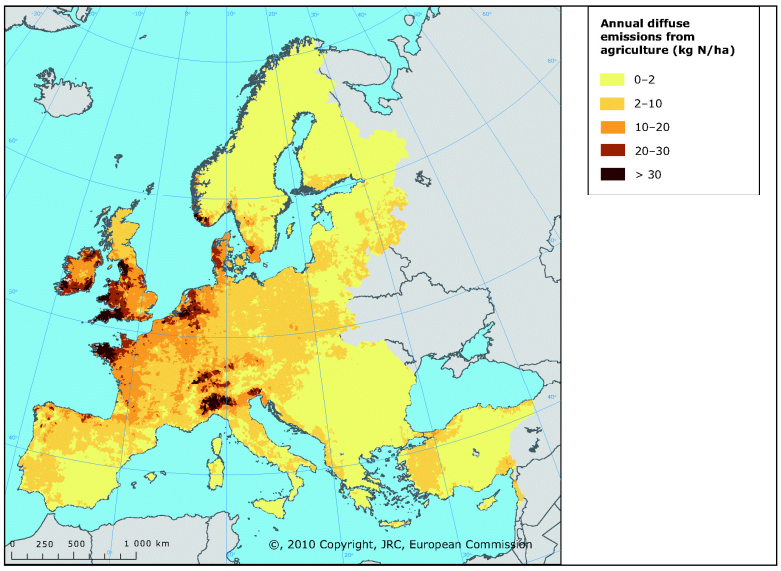


Figure 2.3: Annual diffuse agricultural emissions of nitrogen to freshwater (kg N ha^{-1} of total land area) in 2010 [5].

over the past decade (Figure 2.4) such that 94% of urban wastewaters now receive at least primary treatment. Nevertheless, urban wastewaters are still considered to be a significant threat to the quality of receiving waters in many areas [43]. By 2011, only 21% of WWTP effluents (by p.e.) were undergoing nutrient reduction in addition to secondary treatment. In particular, eight urban areas, including Dublin, Cork and Kilkenny, do not meet the UWWTD requirement for the provision of nutrient reduction, and 160 urban areas have secondary treatment that did not meet biological oxygen demand (BOD), chemical oxygen demand (COD) and total suspended solids (TSS) standards in 2011 [44, 45].

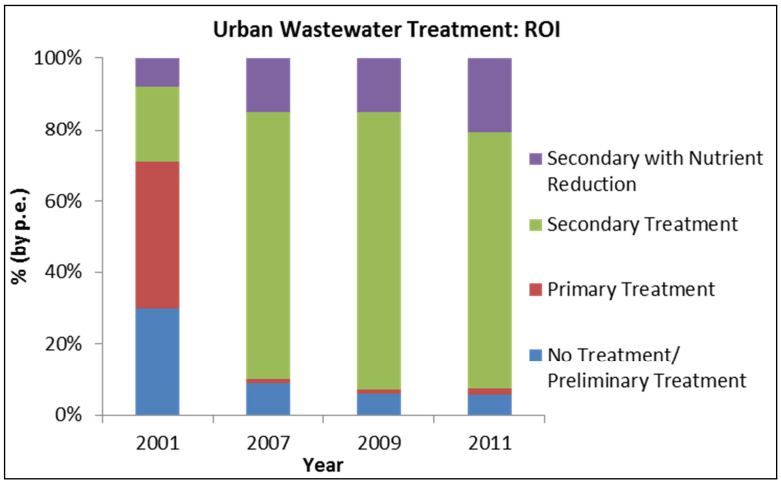


Figure 2.4: Trends in the degree of treatment applied to urban wastewater discharges in the RoI. Adapted from [43] and [44].

2.2 Differentiating Sources of Nitrate

Arising from the above, the differentiation of nitrate sources is of great importance due to legislative, health and environmental implications. Various approaches have been examined in an effort to distinguish between sources of nitrate contamination in water bodies. These have been applied with varying degrees of success. To date, no single technique has been identified to be suitable for differentiating all sources of nitrate contamination [3, 10]. Rather, it is expected that a suite of techniques and indicators will need to be utilised in conjunction with each other in order to achieve successful differentiation and characterisation of nitrate sources.

Currently, the use of nitrate stable isotope compositions represents the technique of choice in differentiating broad classes of nitrate contamination. However, it is not suitable for differentiating closely related sources of nitrate contamination, such as sewage and manure, because both sewage and manure nitrate undergo similar isotopic fractionation processes, which leads to overlapping isotopic compositions [10]. Yet, the differentiation of sewage and manure is of particular importance. The risk to humans arising from human faecal contamination (sewage) is considered to be higher than from animal faecal contamination (manure) since viruses, which represent an important basis of illness resulting from faecal exposure, are highly host specific [16]. Therefore, discriminators other than nitrate stable isotopes must be utilised in differentiating sewage and manure inputs.

2.2.1 Differentating Sewage and Manure

Faecal source tracking, where the aim is to detect and potentially differentiate sewage and manure inputs, has been attempted using a range of approaches. Unlike nitrate stable isotopes, such techniques do not measure nitrate itself. Rather, co-occurring markers of nitrate contamination are being monitored in order to gain an understanding of the various nitrate inputs.

Faecal indicator bacteria (FIB) represent the most commonly adopted faecal contamination markers. However, whilst they are useful for the detection of faecal contamination, it is currently not possible to distinguish between human (sewage) or animal (manure) sources on this basis. This is because the commonly used FIB, namely, *Escherichia coli* and enterococci, do not discriminate between human and animal faecal matter sources [29, 46–48].

Subsequently, in the 1960s, the ratio of faecal coliforms (FC) to faecal streptococci (FS) was put forward as a way to differentiate sewage and manure [49]. Samples having an FC-FS ratio greater than or equal to 4.0 were considered to be

impacted by human faeces, whilst ratios below 0.7 were considered to be associated with animal faeces. As a result of variable survival rates of the bacterial species and the differences in FC-FS ratios within different animals, the use of these ratios is no longer considered to be suitable [48].

For this reason, other tracers must be used to achieve this differentiation. The use of genetic markers for microbial source tracking (MST) through, for example, antibiotic resistance [50], biochemical fingerprinting [51], DNA fingerprinting [52] and bacteriophage occurrence [53] has also been investigated [16]. Yet, they can only function in the identification of the host from which the source of nitrate (or faecal) contamination is initiated. Therefore, by using such techniques, it would not be possible to provide source characterisation based on the entry pathway of contamination, e.g. between raw and treated sewage.

Another potential way to achieve the specific differentiation of sewage and manure inputs is through the use of chemical markers. Within this study, the potential of pharmaceuticals and related compounds, such as food additives and metabolites, as suitable chemical markers for differentiating and characterising sewage and manure inputs to surface waters is focussed upon.

In view of the above, this chapter continues with a discussion of the current state of knowledge in the use of nitrate stable isotope compositions for differentiating sources of nitrate contamination (Section 2.3). The use of other isotope tracers is also explored and their limitations for differentiating sewage and manure inputs outlined. This section is followed by an overview of the use of genetic markers for MST to differentiate sewage and manure with an emphasis on the challenges faced by this approach in differentiating and characterising sewage and manure inputs (Section 2.4). Then, the use of chemical markers as an alternative approach for differentiating sewage and manure inputs into surface waters is evaluated in Section 2.5.

2.3 Use of Stable Isotope Tracers

Stable isotopes are nuclides, which, unlike radioactive isotopes, do not decay [15]. These result in elements having one or more stable isotopes naturally occurring as nuclides of different mass due to differences in the number of neutrons they contain. A typical use of isotope tracers is in exploring a number of hydro-geochemical issues

such as the identification of contaminant sources within catchments [54]. This usage is possible for a variety of reasons, including that:

- Waters originating at different times and locations often have distinctive isotopic compositions;
- Environmental isotopes are not normally considered to react significantly with catchment materials, and;
- Changes in solute isotopic ratios generally occur in predictable and recognisable directions, which allows them to be reconstructed from the isotopic compositions [3, 54].

Isotope ratios are reported relative to a specific reference standard using delta (δ) units and the per mil (‰) notation. They are defined using the following equation where R represents the ratio of the heavy isotope to the light isotope (e.g. $^{15}N/^{14}N$) and R_{sample} and R_{std} are the ratios in the sample and reference standard, respectively:

$$\delta_{sample}(‰) = \frac{R_{sample} - R_{std}}{R_{std}} \times 1000$$

A positive δ_{sample} indicates enrichment in the heavy isotope, i.e. the isotopic ratio of the sample is higher than that of the standard. Conversely, a negative δ_{sample} indicates that the isotopic ratio of the sample is lower than that of the standard, i.e. depletion of the heavy isotope. For example, a $\delta^{15}N$ value of +30‰ means that the $^{15}N/^{14}N$ of the sample is 30 parts per thousand (3%) higher than the $^{15}N/^{14}N$ of the standard [54].

Isotopic fractionation is the underlying cause for the suitability of stable isotope compositions in source identification. Fractionation occurs because atomic masses and bond strengths are isotope dependent [55]. Hence, isotopes of the same element would have slightly different chemical and physical properties. These differences can result in mass-dependent isotope partitioning causing distinctive isotopic compositions based on the contaminant entry pathway [54].

Isotopic fractionation can occur through either reversible equilibrium reactions or irreversible non-equilibrium reactions [3]. Non-equilibrium effects, also referred to as kinetic isotope fractionation, result in products being depleted of the heavy isotope, while the substrate becomes increasingly enriched with the heavy isotope [56]. This fractionation has been attributed to an increased stability of molecules containing the heavy isotope because of higher dissociation energies than molecules with lighter isotopes [54, 56]. The extent of kinetic fractionation depends on the

reaction pathway, the reaction rate and the relative bond energies of the bonds being broken and formed by the reaction [54].

Equilibrium reactions tend to cause stable isotope fractionation to a smaller extent than non-equilibrium fractionations [15]. They are greatest for elements with low atomic weights, such as hydrogen, oxygen, nitrogen, silicon and sulfur since the difference in mass arising from a single neutron results in larger relative differences in the stable isotope forms [15].

Of note is that the isotopic composition of a particular water body can be influenced by isotopic fractionation during the transport and chemical transformation of the compounds and does not only reflect the composition of the original source or of mixed sources having different compositions [55, 57, 58]. Therefore, fractionation should ideally be minimal during transport from the source to nearby surface waters so that the transported products would inherit the source isotopic ratios [55]. Nevertheless, this is not always the case, such as when denitrification occurs during transport [59, 60].

Nitrate is a typical contaminant for which stable isotopic compositions have been used to achieve source differentiation [10]. This use is possible since most nitrogen sources are interrelated in the biochemical nitrogen cycle and measurable differences in the isotopic composition of nitrogen source materials persist as nitrogen-containing compounds are transported from the source [55]. Furthermore, both nitrogen and oxygen within nitrate have naturally occurring stable isotopes, which may be exploited in isotope tracer studies.

2.3.1 δ^{15}N of Nitrate Sources

There are two naturally occurring stable isotopes of nitrogen, ^{14}N and ^{15}N. The majority of nitrogen in the atmosphere is as ^{14}N (99.633%), with 0.3663% as ^{15}N [61]. Nitrogen isotope ratios ^{15}N:^{14}N are generally reported relative to N_2 in the reference standard gas AIR (Atmospheric nitrogen Isotopic Ratio), which is defined as 0‰ [62]. δ^{15}N compositions of most terrestrial materials fall between -10‰ and +25‰ (Figure 2.5) [3].

One of the first natural abundance tracer studies utilising nitrate nitrogen stable isotopes was carried out in 1971 [63]. It involved the use of δ^{15}N-NO_3 for the estimation of fertiliser contribution to nitrate in the Sangamon River (Illinois, USA). Since then, stable nitrogen (δ^{15}N) isotope data of nitrate have been widely used in the identification of sources and fates of nitrate in water bodies (Figure 2.6) [11, 64–73].

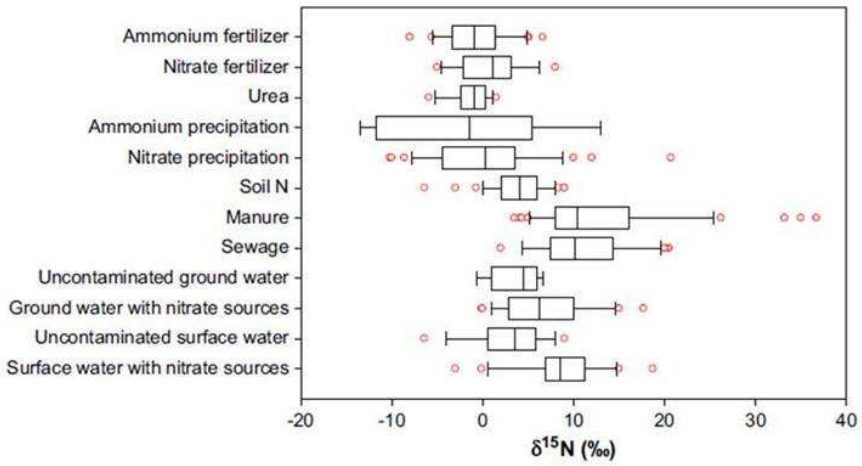


Figure 2.5: Box plots showing 25^{th}, 50^{th} and 75^{th} percentiles for δ^{15}N values of nitrate from various sources and sinks. The whiskers show the 10^{th} and 90^{th} percentiles, and the circles represent outliers [10].

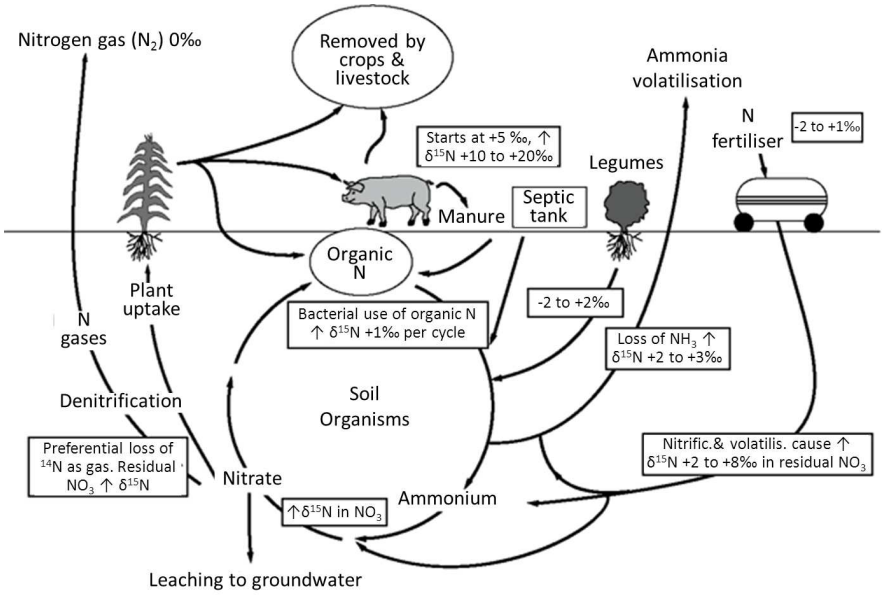


Figure 2.6: Effects of isotopic fractionation on different nitrogen cycle processes [74].

The use of nitrate isotopes to trace nitrate sources has been shown to provide significant information even where the nitrogen inputs from different sources can be estimated and the outputs into the surface water body can be measured. This is because the physical, chemical and biological processes that control the nitrogen cycle act unequally upon nitrogen from different sources [62]. Therefore, the different sources may contribute nitrogen disproportionally to their inputs within the catchment [62].

Nitrate recharged under septic systems and manure application typically has en-

riched δ^{15}N values as compared to other nitrate sources. Enrichment arises as values from animal wastes, including humans, are generally between +8‰ and +20‰ [68, 75–77]. This enrichment is mainly attributed to ammonia volatilisation during the storage and application of animal wastes, which causes enrichment of ^{15}N in animal waste products (manure and sewage) as it produces ^{15}N-enriched residual NH_4^+. Subsequently, this is converted into ^{15}N-enriched nitrate [3, 10, 69]. Furthermore, biologically mediated reactions, such as fixation, assimilation and denitrification, commonly undergo kinetic isotopic fractionation. As stated previously, this results in increased δ^{15}N values within the substrate and a decrease in the product's δ^{15}N [3, 78]. Therefore, animals are enriched in ^{15}N within their tissues and solid wastes as compared to their diet, whilst ^{14}N is largely eliminated in urine [3, 76].

Although variations in δ^{15}N values allow for the differentiation of a number of nitrate sources, many others cannot be distinguished on this basis. This difficulty in differentiating numerous nitrate sources, including sewage and manure, based solely on the δ^{15}N isotopic compositions has led to the application of a dual isotope approach.

2.3.2 The Dual Isotope Approach

The dual isotope approach involves the determination of both nitrogen and oxygen isotopic compositions. Oxygen (O) exists as three stable isotopes, namely ^{16}O (99.63%), ^{17}O (0.0375%) and ^{18}O (0.1995%) [79]. δ^{18}O values (^{18}O:^{16}O) are reported relative to the reference standard SMOW (Standard Mean Ocean Water) or V-SMOW (Vienna SMOW). Generally, ^{17}O values are not used due to their low natural occurrence, which leads to limited fractionation effects [80]. For a particular isotope to be useful as a tracer of contamination the rare isotope must be present at sufficient levels for detection [81]. Additionally, the relative mass difference of common to rare isotopes of the element should be large [81].

The dual isotope approach has three main benefits as compared to the use of δ^{15}N in isolation [3]. First of all, oxygen isotopic separation of some sources is greater than for nitrogen isotopes, with the spread of δ^{18}O being at around 70‰ whilst that for δ^{15}N is at 35‰. Therefore, better source resolution is possible through the use of both δ^{18}O and δ^{15}N values (Figure 2.7).

Secondly, some nitrate sources that are presently indistinguishable by using δ^{15}N values alone (e.g. fertiliser vs soil nitrate, or atmospheric vs soil nitrate) can be identified once δ^{18}O values are incorporated. Lastly, oxygen isotopic compositions of nitrate vary systematically with nitrogen isotopic compositions during denitrification.

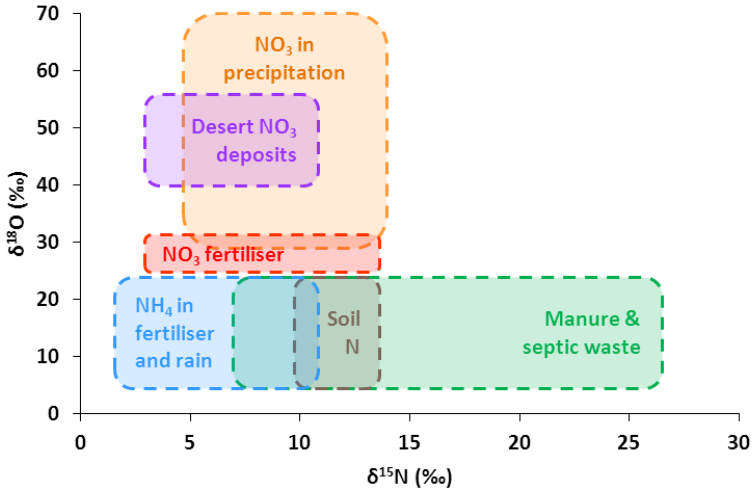


Figure 2.7: A general depiction of dual isotopic separation for the dominant sources of nitrate. Adapted from [3].

Thus, in systems where the dominant sources of nitrate are isotopically distinctive, source contributions can be determined despite significant denitrification [3].

In a similar manner to nitrogen stable isotope compositions, a number of factors have been found to alter the $\delta^{18}O$ isotopic values from the source to the sink and, thus, the obtained values are not simply an average of the various source values. For example, $\delta^{15}N$ values in groundwater nitrate beneath excessively fertilised cropland are somewhat higher, and $\delta^{18}O$ values are considerably lower than nitrate fertiliser values on their own [60, 68, 75, 76]. This has been attributed to factors such as the mixing of fertiliser nitrogen with other nitrogen reservoirs in soils with higher $\delta^{15}N$ values [70]. Furthermore, nitrate is usually not the main form of nitrogen fertiliser and reduced forms of nitrogen, such as urea and ammonia, acquire low $\delta^{18}O$ values from water when nitrified microbially to produce nitrate [70]. Therefore, it is not possible to transmit source data directly to ground or surface waters [70].

In general, typical $\delta^{18}O$ values of nitrate from nitrification (including values from microbial production of ammonium in fertiliser and precipitation, nitrate derived from soil nitrogen and nitrate derived from manure and sewage) are lower than those of nitrate from precipitation and nitrate in fertiliser [10]. $\delta^{18}O$ values of nitrate sources are largely dependent upon the origin of the nitrate oxygen atoms. For example, the three oxygen atoms in fertiliser nitrate derive from atmospheric oxygen resulting in the $\delta^{18}O$ of the nitrate being in a similar range, whilst two of the oxygen atoms in soil nitrate derive from water oxygen molecules; hence it reflects the $\delta^{18}O$ of water [82].

To date, the dual isotope approach has been applied successfully to numerous

scenarios, such as hydrologic studies into the transformation pathways of denitrification [59, 60, 83–86]. Following denitrification, nitrate concentrations decrease, whilst the residual nitrate becomes enriched in ^{15}N and ^{18}O in the ratio 2.1:1 for $\delta^{15}N$:$\delta^{18}O$ [59, 60]. Other applications of this approach have focussed on the identification and quantification of diffuse nitrate inputs into a water body [1, 65, 73, 84, 87–92] and studies on seasonal variations of dissolved nitrate [89, 93, 94].

Unfortunately, in the case of sewage and manure, data on $\delta^{18}O\text{-}NO_3^-$ do not contribute to source identification [10, 58]. Such a difficulty is likely due to the similarities between the isotopic fractionation pathways undergone by nitrate arising from animal and human waste. Therefore, other methods need to be found in order to carry out this differentiation.

2.3.3 Other Isotope Tracers

The differentiation of sewage and manure nitrate has been attempted using a number of other geochemical tracers. These include linking $\delta^{15}N$ and $\delta^{18}O$ values to land use types or physico-chemical properties of water, such as pH, conductivity, ammoniacal, sterol and nitrate nitrogen concentrations, chloride, and the levels of dissolved organic and inorganic carbon and nitrogen [69, 95–105].

Another approach has been to make use of isotope tracers that co-migrate with nitrate, such as boron and strontium [58]. Of these, the use of boron isotopes represents the most widely investigated approach, yet the available research into this area is still extremely limited [106–110]. Two stable isotopes of boron are known: ^{11}B at 80% natural abundance and ^{10}B at 20% natural abundance [10]. $\delta^{11}B$ values for sewage, manure and nitrogen fertilisers[10] have been determined [10].

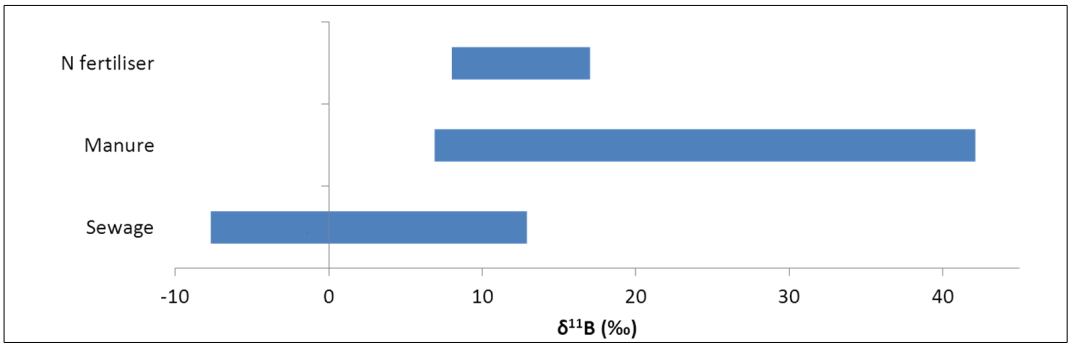


Figure 2.8: Range of boron stable isotope values for different sources of boron. Adapted from [10, 58].

[10] Nitrogen fertilisers contain boron as a minor/trace element [10].

However, as can be observed from Figure 2.8, there is still a considerable overlap between sewage and manure isotopic compositions. Furthermore, for successful application of boron isotopes to a particular site, all relevant inputs need to be well characterised [111]. Therefore, the approach of using boron stable isotope compositions, in isolation or in conjunction with other isotopes, is not easily transferred between watersheds.

In conclusion, the dual isotope approach and the use of other geochemical tracers have been identified to be, indeed, useful for the differentiation of numerous nitrate sources. However, these methods are not particularly suitable for the discrimination of sewage and manure inputs. Therefore, other approaches need to be considered for this purpose.

2.4 Use of Genetic Markers

The use of genetic markers has been exploited in a large number of studies related to microbial source tracking (MST). They involve the detection and quantification of specific genome[11] segments or their expression (Figure 2.9) [112]. Although they are most commonly applied to bacterial targets, they may also be applied to other targets, such as viruses and protozoans [112].

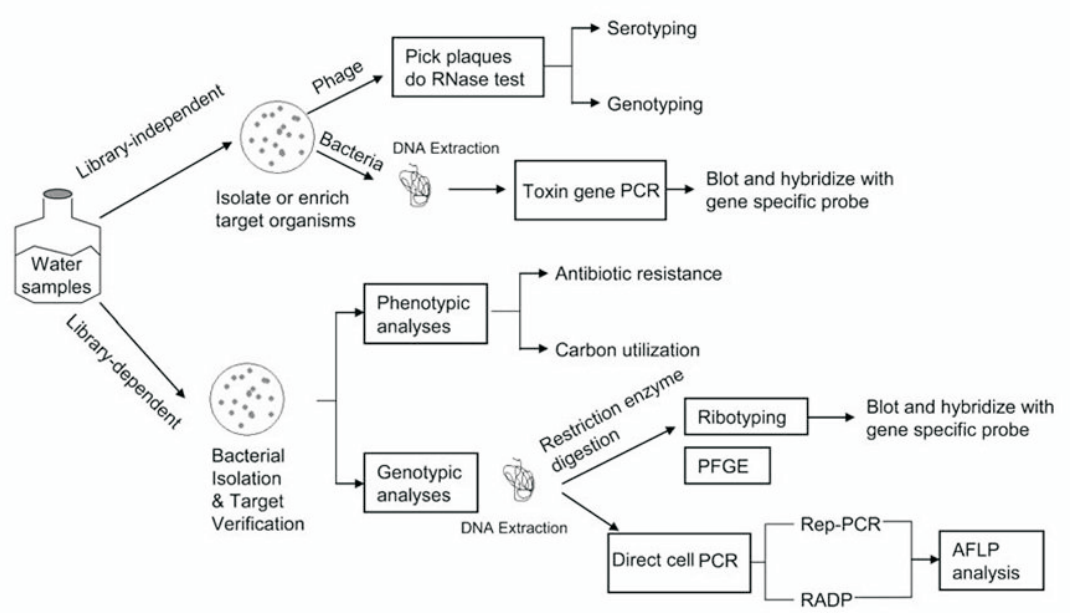


Figure 2.9: A summary of available microbial source tracking techniques [113].

[11] The genome consists of an organism's hereditary information, which is generally formed of DNA or RNA.

Most MST methods consist of a similar overall protocol:

1. Concentration of the organism of interest from the environmental water sample into a suitable volume;
2. Extraction of the genetic material from the target organisms;
3. Nucleic acid amplification, and;
4. Detection and/or quantitation of the amplified genomic sequence [112].

This field has been relatively well investigated and has been the subject of a number of recent reviews and books [16, 112, 114–116]. Therefore, only a short summary of the state of the art is given in this section, with the focus being on the considerations for using such an approach.

2.4.1 Types of MST Techniques

MST techniques are largely classified in two different manners. The first classifies MST techniques depending on the need or absence of a library[12] for identifying sources of genetic material. The second classifies MST techniques depending upon the need for the set-up of a culture in the MST method, or lack thereof. The details of the different techniques are given below.

2.4.1.1 LDMs and LIMs

Library-dependent methods (LDMs) involve matching parts of the organisms' genome to strains in a database. Phenotypic LDM analyses represent the first techniques developed for MST, where the outcome of the genome's expression is assessed, e.g. antibiotic resistance [113]. This development was followed by the introduction of genotypic analyses where the genetic material itself, and not its expression, is determined [113]. Genotypic LDMs include the use of fingerprints arising from the use of e.g. pulsed-field gel electrophoresis (PFGE) and direct cell polymerase chain reaction (PCR) techniques [48].

The set-up of suitable libraries has proven to be a particular challenge in the use of LDMs [48]. For this reason, LDMs are largely limited to relatively simple monitoring scenarios and restricted to regions from which the fingerprint databases (libraries) are primarily developed [117]. In addition, although the techniques themselves may be relatively simple and inexpensive, they become time-consuming, and

[12] A library is a database of characteristics (e.g. genetic fingerprints or antibiotic resistance profiles) of microorganisms from known sources/isolates [48].

consequently expensive, due to the large number of samples and isolates that need to be processed. The interpretation of results is also quite challenging due to the high frequency of cosmopolitan isolates [113].

The development of library-independent methods (LIMs), which are based on the analysis for unique genetic markers that are host-specific, followed. These include the detection of genetic material from phages[13] of specific strains of *Bacteriodes* species and enteroviruses [48]. LIMs have a number of advantages over LDMs, primarily that the need for a representative library to be built is eliminated [48].

Additionally, a large number of steps during sample processing and analysis may be automated (e.g. DNA extractions and PCR set up), multiple samples can be processed at a time and multiple assays can be performed against an individual DNA extract [113]. Nevertheless, although such methods eliminate the need for validating library size and representation up-front, they still require significant efforts in the validation of host-species targets during the development of the library-independent host-specific assays, potentially over a temporal and spatial scale to assess genetic drift [48, 115].

A critical limitation of most LIMs is that, commonly, only one gene is targeted within the host, which may have a low number of replicates within a cell [48]. Therefore, current efforts involve the development of MST assays involving the monitoring of a number of genetic targets [113].

2.4.1.2 Culture Based and Culture Independent Methods

Culture based and culture independent methods are classified on the basis of the enrichment technique adopted. Culture based MST techniques are centred around the growth of bacterial cultures and include tests for antibiotic resistance, DNA fingerprinting and bacteriophage methods [112]. The culturing procedure is laborious, and it is, generally, a challenge to achieve sufficient enrichment [112]. Due to the culturing requirements of such methods, they are limited to microbes that can be easily cultivated [16]. Additionally, such analyses are commonly semi-quantitative since it is difficult to achieve reproducible bacterial cultures, which are a critical requirement of quantitative analyses [16].

Culture independent methods eliminate the need for a culture to be set up. Generally, they make use of PCR techniques for enriching the relevant genetic markers, which makes them rapid and relatively cost-effective tools as compared to culture based methods and result in a high level of sensitivity and specificity [112]. A challenge in the application of culture independent methods has been to discriminate

[13] A phage is a virus that infects bacteria.

signals arising from viable[14] cells from those arising from non-viable cells, or naked DNA[15] [112, 113].

Culture independent methods have presented the additional difficulty of identifying the number of bacterial cells that are present within a specific environmental sample. Many bacteria are known to produce aggregates and may contain more than one copy of the genome in each cell, depending upon the organisms' physiological state [112]. Thus, depending upon the gene markers being targeted, different values may be obtained. Furthermore, in the application of PCR techniques to environmental waters a number of PCR inhibitors are known to be of particular concern. These include such components as humic acids, calcium ions and bile salts and act to increase the technique's complexity for use [118].

2.4.2 Considerations in Using Genetic Markers

In the application of genetic markers for MST, a number of considerations must be taken into account. A summary of the major considerations is given in Figure 2.10.

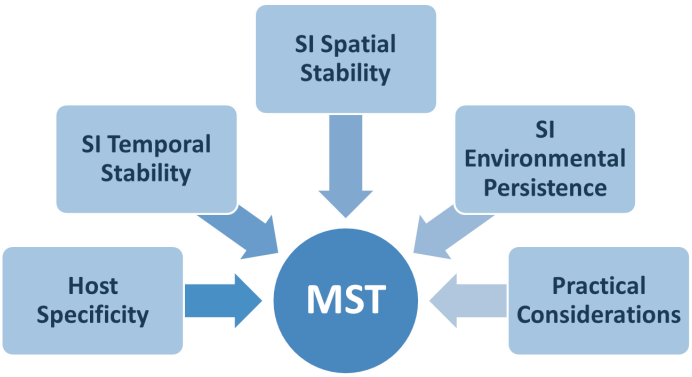


Figure 2.10: Considerations in the use of MST techniques. SI: Source Identifier. Adapted from [48, 119].

Host specificity is one of the major challenges in the development of genetic markers for MST. While host specificity of gene targets is commonly assumed a significant number of cosmopolitan strains are present, where the same strain occurs within different hosts [113, 116, 120]. A specific source identifier (SI) would display differential distribution within different organisms, such that it is found at a higher frequency or density within certain hosts [119]. However, it might still be present within other organisms, which can lead to incorrect source attribution.

[14] Viable cells are those that are still alive and capable of growth [113].
[15] DNA bound to particulate matter [113].

Furthermore, SIs are known to vary on temporal and spatial scales [119]. Differences in dietary regimes are amongst the major contributors to this variability as they cause inconsistencies in the occurrence and distributions of bacterial groups within the intestinal tract [113]. Hence, relevant SIs for a specific temporal period and geographical area might not be relevant in a different scenario [113, 116]. Yet, if the level of understanding on spatial variability is greatly increased, it could provide a means for discriminating between geographically separate populations of host animals [121]. However, it is believed that, even if this discrimination is possible, it is currently not practical due to the infancy of this aspect of the MST field [119].

The SI's environmental persistence is another consideration. This is because the species' clonal composition might differ between the environmental samples and the host populations [121]. Such a factor must be validated in order to ensure correct source attribution as it may result in over- or under- estimation of the contribution of a particular source [119]. Yet, even though studies have shown that variability does indeed exist, it is commonly assumed to be consistent between SIs [119].

Lastly, practical considerations, in particular related to the method's transferability and application, must be taken into account. These include factors such as the technique's availability and complexity, the cost of analysis and the level of expertise required for successful data interpretation [48].

In conclusion, the use of genetic markers for MST allows for highly specific information on the presence of faecal indicators such as bacteria, viruses and protozoans. However, a number of challenges have been identified, and whilst multiple techniques are currently available, many have not yet been fully tested and validated to the stage of application in field studies [16, 21, 112, 113]. In fact, a number of studies carried out by the US Geological Survey to assess available techniques concluded that none of the methods investigated were ready for field application [21, 113, 120, 122]. An additional consideration of using genetic markers is that they can only function in the identification of the host from which the source of nitrate (or faecal) contamination is initiated. Therefore, using such techniques, it would not be possible to provide source characterisation based on the entry pathway of contamination, e.g. between raw and treated sewage.

2.5 Use of Chemical Markers

A chemical marker is a chemical that is normally present together with a target analyte, which is nitrate in this case. Such markers are commonly used where it is

difficult for the target analyte to be measured. Alternatively, as in this case, they may be used where further information is required about the target analyte, such as the source of contamination, that is not possible to obtain if one solely analyses for the target analyte.

Nowadays, a wide range of chemicals exist in nature or are produced synthetically. Therefore, the selection of suitable chemicals to achieve differentiation is critical. Ideal chemical markers of faecal contamination (sewage and manure) meet a number of criteria. They are ubiquitous in the source and derive only from the particular source being investigated. At the same time, they are persistent and present at detectable concentrations in contaminated environmental samples but not in clean waters.

Suitable chemical markers of sewage and manure contamination to surface waters commonly have a number of specific physico-chemical characteristics. These include a high water solubility, low K_{OW}[16] and low volatility, which allow for their use as tracers for water-soluble components originating in sewage and manure [123].

Likely chemical markers of faecal contamination can fall into three main classes, namely:

- Chemicals that are produced by the body e.g. faecal sterols and fatty acids;
- Chemicals that are ingested and pass through the body e.g. pharmaceuticals, food additives and their metabolites, and;
- Chemicals that are associated with waste systems e.g. fragrances and detergents [46, 123].

Associated chemical markers, such as fragrances and detergents, are regarded to be the weakest chemical markers of faecal contamination of the three since they are not excreted by consumers and, thus, do not indicate a direct relationship [46]. Additionally, they do not have the capacity to identify manure contamination since very few, if any, of these chemicals are used in animal husbandry.

Chemical markers that are produced by the body are amongst the most commonly used chemical markers of faecal contamination, in particular faecal sterols such as phytosterols, cholesterol and coprostanol [124–126]. Their potential arises from the presence of distinct sterol distributions in different warm-blooded animals depending upon the identity of the animal, the food that has been ingested and the bacteria within their digestive tracts [105].

[16] The octanol-water partition coefficient. The lower the K_{OW}, the more polar is the compound and the higher the solubility.

However, faecal sterols are pervasive in nature. Although sterol distributions differ, a specific sterol may arise from a number of organisms and may be present within the natural background. In addition, sources of faecal sterols arising from ingested material can act to confound the profile of excreted sterols for a specific organism [127, 128]. Recent efforts have focussed on the incorporation of additional information, such as the use of sterol carbon compound specific isotope analysis (CSIA) to achieve an improved understanding of the sterols' origin [128]. Yet, this greatly increases the analytical requirements of such analyses, and the field of CSIA is, still, in its infancy.

Overall, the use of pharmaceuticals and related compounds, such as food additives and metabolites[17], has been considered to be more desirable than others. Therefore, in the following sections, a review of the current state of knowledge on the use of pharmaceuticals as co-occurring chemical markers of sewage and manure inputs to surface waters is given. The section begins by describing the factors of interest in using pharmaceuticals as chemical markers, namely their use within the community being studied (Section 2.5.1), their fate on being transported from the source to surface waters (Section 2.5.2) and their occurrence within surface waters (Section 2.5.3). This is followed by a discussion on the use of pharmaceuticals as sewage and manure markers (Section 2.5.4), and the specific considerations in the development of a suite of chemical markers for differentiating sewage and manure (Section 2.5.5).

2.5.1 Use of Pharmaceuticals in Human and Veterinary Treatment

The development of pharmaceuticals has greatly improved human and animal health [129]. Consequently, their consumption has reached substantial levels. In the EU, over 3000 different substances are used in human medicine [130], and a similar number of pharmaceuticals is used in veterinary medicine [131]. Yet, pharmaceuticals are not normally produced or used in high volumes. Consequently, expected environmental concentrations are low. Nevertheless, as a result of their pervasive use, pharmaceuticals have been inadvertently introduced into surface and ground waters [129].

The presence of pharmaceuticals in the environment was first noted more than 35 years ago [132]. However, the increased attention to their discharge, presence and potential adverse effects on ecosystems and human health is largely a recent effort

[17] These are subsequently collectively referred to as pharmaceuticals.

[133]. Accordingly, pharmaceuticals are nowadays considered emerging environmental micro contaminants. Most of the studies carried out to date have focussed on human pharmaceuticals. However, the recent intensification of animal production, in particular the proliferation of large-scale animal feeding operations during the last decade, has resulted in increased water quality concerns related to the production and disposal of animal waste generated by these operations [133, 134].

Of note is that a reduction of pharmaceutical entry into the environment through restricting or banning their use in view of ever-increasing environmental concerns is not perceived to be feasible due to their beneficial health effects and economic importance [130, 135]. On the contrary, pharmaceutical use is expected to grow as a result of the increasing numbers of pharmaceuticals being developed and the ageing population [130]. An increasing projected use makes pharmaceuticals well positioned for use as chemical markers of sewage and manure contamination.

2.5.2 Fate of Pharmaceuticals in Aquatic Environments

Pharmaceuticals enter the aquatic environment through a variety of routes (Figure 2.11). The route taken is largely dependent upon whether the pharmaceutical was intended for human or veterinary use. Thus, this difference could potentially be exploited to differentiate sewage and manure inputs to surface waters. However, first it is necessary to understand the fate of pharmaceuticals on their passage from their source to environmental waters before determining their applicability as chemical markers of sewage and manure inputs to surface waters.

2.5.2.1 Exposure

Excretion after normal pharmaceutical use is a major pathway for most pharmaceuticals arising from human and veterinary treatment [139]. The form in which the pharmaceutical is emitted following treatment depends upon the manner of administration and the pharmaceutical's physico-chemical characteristics. Generally, treatment of humans and animals is received topically, orally or as an injection [140, 141]. Topical treatments may be washed off unchanged prior to carrying out their intended action, whilst most other pharmaceuticals would be somewhat metabolised prior to being excreted.

The metabolisation pathways taken by pharmaceuticals are a determining factor of the form in which the pharmaceuticals are excreted. Most pharmaceuticals are metabolised to phase I or phase II metabolites (Figure 2.12) before being excreted [139]. The level of metabolisation is quite variable depending upon the specific

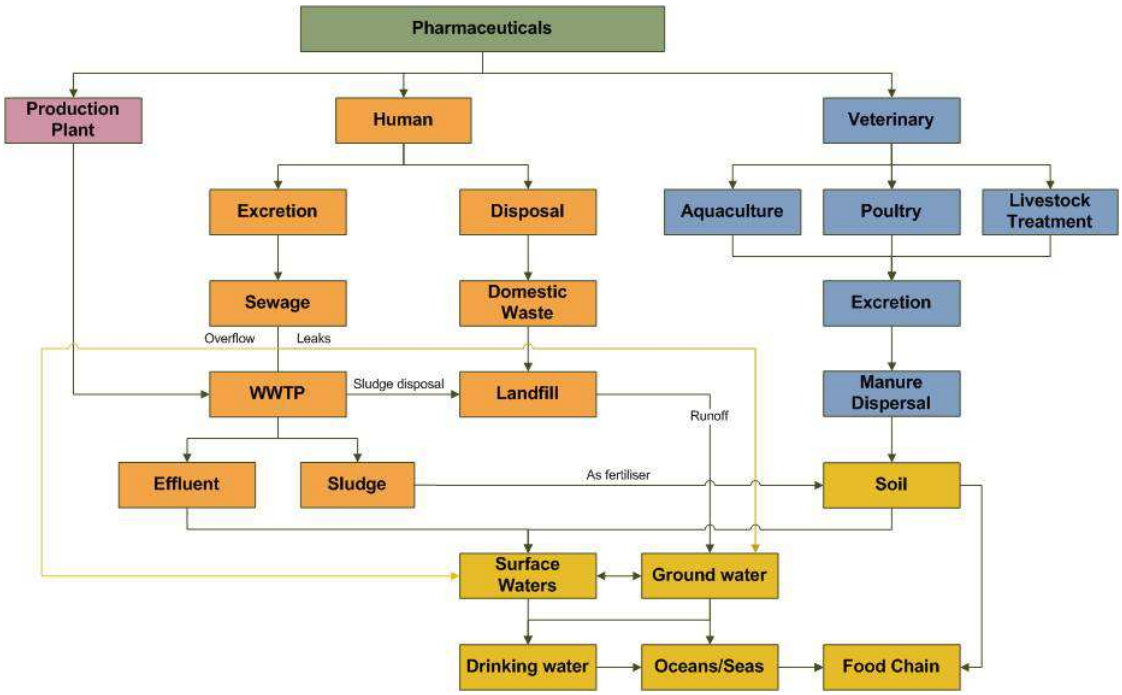


Figure 2.11: Scheme indicating the sources and pathways resulting in the presence of pharmaceuticals in the aquatic environment. Adapted from [136–138].

pharmaceutical and the organism being treated. Within humans, excretion levels of the unchanged parent compound have been estimated to range from less than 10% to more than 90% depending upon the pharmaceutical's identity [142, 143]. Thus, excretion may be as an unchanged parent compound, in the form of metabolites, or as conjugates of glucoronic and sulfuric acid [130, 144].

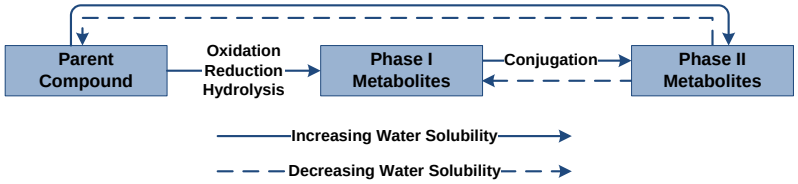


Figure 2.12: An overview of metabolic transformations of pharmaceuticals and the resulting changes in solubility and polarity. Adapted from [139, 145].

Both phase I and phase II reactions cause a change in the compound's physicochemical characteristics. Generally, metabolisation renders the metabolites more polar than the parent compounds [139]. Thus, pharmaceutical metabolites would have a greater likelihood of being detected within the environmental water phase than the parent compound as they become more water soluble. Hence, metabolites may be exploited as chemical markers of faecal contamination in instances where the degree of metabolisation is high since this would result in extremely low con-

centrations of the parent compound. For example, while clofibrate, etofibrate and fenofibrate are not generally detectable in WWTP effluents, their polar metabolites (clofibric acid and fenofibric acid) are more commonly detected [146, 147].

Disposal of unused and expired pharmaceuticals is another potential route of entry. Pharmaceuticals would enter the solid waste or sewage streams in an unmodified form having avoided metabolism in the body. As a result, their contribution to environmental contamination can be disproportionate. However, there is a current lack of knowledge on the significance of the disposal of unused medicines in relation to its extent, characteristics and environmental loading [148, 149].

2.5.2.2 Fate and Transport

The fate and transport of human and veterinary wastes largely depend upon the original source. On excretion, human waste streams are generally diverted towards a WWTP where the resultant sewage stream is treated before being released into discharging water as effluent [146]. As with metabolisation within the organisms being treated, the physico-chemical characteristics of specific pharmaceuticals are a determining factor in pharmaceutical removal within WWTPs [146, 150]. Within the EU, the percentage of the population connected to a WWTP varies between around 40% in South-East Europe and over 80% in Central, North and South Europe [151]. However, the number of inhabitants per WWTP and the operating technical standards at the WWTP are highly variable [146, 152], consequently resulting in highly variable capabilities for pharmaceutical removal [152, 153].

Notwithstanding differences in WWTP treatment levels and pharmaceutical physico-chemical characteristics, studies have suggested that as much as 80% of the total load of pharmaceuticals entering WWTPs is discharged in effluents unchanged [154]. For example, in a study of seven WWTPs in four European countries (Italy, France, Greece and Sweden) it was reported that, of the more than 25 pharmaceuticals monitored, all antibiotics, most β-blockers, gemfibrozil, ibuprofen, naproxen, diclofenac and carbamazepine were detected in almost every sample of WWTP effluent analysed [155].

Additionally, some metabolites are known to be converted back into the parent compound on passage through WWTPs. For example, metabolites of chloramphenicol, sulfadiazine, oestrogen and sulfamethazine are known to be converted into their parent compound during sewage treatment [130].

In addition to discharge waters, sludge disposal is another potential source of nitrate contamination and pharmaceutical entry to surface waters. Sludge and sludge components may be disposed of in three ways: deposition in a landfill or sludge

deposits, used as a land fertiliser or soil conditioner, or recycled for the productivity of sludge [156]. The wastewater composition (dependent upon the source of wastewater), the type of wastewater treatment (primary, secondary or tertiary[18]) and the treatment applied to the resulting sludge all affect the sludges' characteristics, such as its chemical composition, biological constituents and quantity produced [156]. Within the RoI, around 106,000 tonnes of sewage sludge were generated by WWTPs in 2009, of which 62% was reused in agriculture [43]. However, most pharmaceuticals are highly water soluble compounds [153] and, therefore, adsorption to WWTP sludges is limited [157, 158].

Generally, veterinary pharmaceuticals can enter the environment in a more direct manner than human drugs, particularly when used in aquaculture where they are released directly into the surface water [159]. Furthermore, unlike sewage, which is usually treated, animal excrement from intensively reared livestock is normally piled, composted or stored as a slurry in manure tanks, lagoons or pits without any deliberate treatment [147, 160], with manure from medicated animals being typically managed in the same way as manure from unmedicated animals [161]. Pharmaceuticals used for animals raised on pastures are excreted directly onto the grassland if applied orally or by injection. If the pharmaceutical has been applied topically, it can be washed off [160].

Animal manure tends to be more highly concentrated and has a higher BOD as compared to treated sewage sludge [147]. Under such conditions of high BOD, the existing pharmaceutical compounds in manure are even less likely to be degraded [147]. The field application of manure from treated animals may cause their release to soils during the slurry or manure application process and, subsequently, transported to surface or groundwater via surface runoff or leaching [160].

Of note is that livestock waste treatment plants have been developed. These are mainly based on anaerobic digestion [162]. Although they have not yet been adopted on a large scale, they present another source of veterinary pharmaceutical contamination [162, 163]. Such treatment plants function similarly to sewage WWTPs and, hence, the same considerations regarding pharmaceutical removal apply.

2.5.2.3 Persistence within the Environment

Regardless of the source of pharmaceuticals, the compound's persistence within the environment is a determining factor for its ultimate fate. The ultimate fate of pharmaceuticals may be classified into three principal routes:

[18] The use of advanced wastewater treatment techniques for the removal of specific wastewater constituents e.g. nutrients.

- Substance is ultimately mineralised;
- Substance is not readily degradable and part of the substance is retained within the environment unchanged, and;
- Substance is metabolised to a more hydrophilic form and persists in passing to the receiving waters [139].

Pharmaceuticals that are readily mineralised are largely unsuitable for use as chemical tracers as these would not be easily detected within the environment. In order for pharmaceuticals to be suitable as chemical tracers, their degradation must be limited or, alternatively, form relatively stable transformation products that could be detected within the environmental phase of interest.

Biodegradation, sorption and photodegradation are considered to be the major pathways for removal of pharmaceuticals from aquatic environments [135]. Thus, both abiotic and biotic mechanisms determine the fate of organic compounds in the aquatic environment. Generally, pharmaceuticals are developed in such a manner as to limit biodegradation and resist hydrolysis, thereby largely eliminating one of the major pathways for removal [139, 147, 159, 164].

In general, the efficiency of pharmaceutical removal from the aqueous phase is mainly influenced by the chemical marker's ability to interact with solid particles, which may be natural (soils, clay, sediments, microorganisms) or added to the medium (active carbon, coagulants). Compounds with low adsorption coefficients tend to remain in the aqueous phase, which favours their mobility through the WWTP or infiltration through the land mass to the receiving environment [144, 165]. Therefore, many pharmaceuticals, such as anti-inflammatories, that remain in the aqueous phase are suitable as chemical indicators of sewage or manure contamination, whilst others, such as musks, oestrogens, tetracyclines and quinolones, which are readily adsorbed to solid particles, are unsuitable for this application [144].

Photodegradation is another pathway that can play an important role in the fate and transport of pharmaceuticals to the environment. Many pharmaceuticals contain a number of aromatic rings, heteroatoms and other functional groups that play a role in direct and indirect photolysis and photochemical processes [135, 166]. This effectively removes the pharmaceutical from the aquatic environment, although it may result in the formation of persistent by-products that may, in turn, be used as chemical markers [167]. However, to date studies on the photodegradation by-products of pharmaceuticals are quite limited and, consequently, knowledge on the by-product suitability for use as chemical markers are not known.

2.5.3 Pharmaceutical Occurrence within Surface Waters

Understanding the occurrence of pharmaceuticals within surface waters is essential in determining their applicability as indicators of sewage or manure inputs. Their occurrence depends upon the use of the particular pharmaceutical in human or veterinary treatment (Section 2.5.1) and their fate within the aquatic environment (Section 2.5.2). In addition, the volume of the receiving water body is another aspect to be considered since this affects the degree of dilution [130].

Occurrence characteristics that are of importance in relation to the use of a suite of pharmaceuticals as chemical markers for differentiating sewage and manure inputs are the concentrations and detection frequencies at which they occur in water bodies. Pharmaceuticals are commonly detected at concentrations reaching several μg L^{-1} in surface waters downstream of WWTP discharges [129]. Some of the highest reported concentrations of pharmaceuticals within surface waters are given in Table 2.3.

Table 2.3: Concentrations of some of the pharmaceuticals detected at the highest concentrations within surface waters from an assessment of over 50 articles related to pharmaceuticals in surface waters (n: no. of samples, SW: surface water). The table includes the highest concentration reported in Irish waters for comparison.

Contaminant	**Matrix**	**Country**	**Max**	**Mean**	**Median**	**Ref.**
			(ng L^{-1})			
Lincomycin	SW: After treatment (n=4)	UK	21100			[160]
Acetaminophen	Streams	US	10000			[168]
Tramadol	SW (Downstream of WWTP)	Wales	5970	3522		[169]
Ibuprofen	SW (Downstream of WWTP) (n=5)	UK	5044	1105	826	[170]
Sulfamethazine	River Water (n=18)	China	4660		100	[171]
Oxytetracycline	SW: After treatment (n=4)	UK	4490			[160]
Diclofenac	SW (n = 3)	Pakistan	4400		1000	[172]
Sulfadiazine	SW: After treatment (n=4)	UK	4130			[160]
Salicylic Acid	River Water (n=43)	Germany	4100		25	[146]
Sarafloxacin	Streams/River near poultry farm (n=8)	US	4000			[173]
Tramadol	SW (Upstream of WWTP)	Wales	3468			[169]
Bezafibrate	River Water (n=43)	Germany	3100	956		[146]
Bisoprolol	River Water (n=43)	Germany	2900		350	[146]
Caffeine	SW (Downstream 1 of WWTP)	US	2600			[46]
Chlortetracycline	River Water (n=18)	China	2420		41	[171]
Ibuprofen	SW (n=18)	UK	2370		320	[174]
Oxytetracycline	River Water (n=18)	China	2200			[171]
Metoprolol	River Water (n=43)	Germany	2200		220	[146]
Caffeine	River Water (n=1)	Ireland	389	389	389	[175]

Two groups of pharmaceuticals are expected to be detected at the highest concentrations in environmental matrices. The first are those pharmaceuticals that are used by a large number of individuals, albeit for a short period of time, such as over-the-counter painkillers or regularly used food additives. These include acetaminophen (paracetamol), ibuprofen, diclofenac, salicylic acid (aspirin metabolite) and caffeine, as can be observed in Table 2.3. The second group consists of pharmaceuticals that are used by a small number of individuals consistently. These are mainly pharmaceuticals used in the treatment of long-term illnesses and include tramadol[19] and metoprolol[20]. Of particular importance are those pharmaceuticals that are used in both scenarios, such as aspirin and acetaminophen.

Detection frequencies are another important consideration. An analysis of occurrence data from more than 200 articles for the 50 most frequently studied pharmaceutical compounds in freshwater ecosystems reported mean detection frequencies of between 3% and 100% [176]. In several studies, numerous chemical markers have been detected within 100% of samples analysed [176]. These include acetaminophen [169], atenolol [169, 177, 178], naproxen [146, 169, 179], and lincomycin [169, 179]. These data indicate their ubiquitous presence within surface waters downstream of WWTPs or agricultural land and their suitability for use in such an application.

2.5.3.1 Occurrence within Irish Surface Waters

A small number of previous studies have explored the occurrence of pharmaceuticals within the Irish environment. Only one study has looked at pharmaceuticals within Irish surface waters, as part of a European Wide study by the European Commission Joint Research Centre [175]. Concentrations of up to 389 ng L^{-1} (caffeine) were reported [175].

Pharmaceuticals have also been detected in Irish WWTP effluents at concentrations of up to 4090 ng L^{-1} (metoprolol) [180]. Pharmaceutical residues have also been detected within digested sludge [181]. A source of particular concern within Irish waters is the effluent from pharmaceutical production plants due to their large presence in Ireland. In fact, in Ireland, the pharmaceutical industry generates over 50% of the country's exports and maintains operations, including manufacturing sites, belonging to 120 companies, including 13 of the top 15 pharmaceutical companies in the world [182]. A study on WWTP effluents from pharmaceutical manufacturing sites within Ireland has shown that removal of pharmaceuticals is commonly incomplete leading to the presence of active ingredients within effluents [183].

[19] A painkiller used in the treatment of a variety of diseases resulting in chronic pain such as rheumatoid arthritis and fibromyalgia.

[20] A beta-blocker used in the treatment of angina, hypertension and congestive heart failure.

Overall, the available studies on pharmaceutical contamination within the Irish environment are extremely limited. Mainly, the studies are related to human pharmaceuticals in the environment, and there is a dearth of information on the occurrence of veterinary pharmaceuticals, even though such inputs may be considerable in view of the importance of the agricultural industry in Ireland.

2.5.4 Pharmaceuticals as Sewage and Manure Markers

Pharmaceuticals have a number of factors that make them suitable as chemical markers of sewage and manure contamination. Their physico-chemical properties indicate that they are relatively water-soluble and non-volatile [153], and their natural background levels are low due to their synthetic nature [184]. Furthermore, they are commonly developed in a manner to increase their persistence in order to avoid the substance becoming inactive before having a curing effect [139, 147, 159, 164]. Through the careful selection of pharmaceuticals showing human or animal source specificity, it is expected that sewage and manure contamination can be differentiated and characterised.

Numerous compounds have been reported to be suitable chemical markers of sewage contamination. Caffeine is one of the most studied chemical tracers of sewage to date [e.g. 29, 47, 185–191]. Other pharmaceuticals that have been identified as being suitable indicators of sewage contamination are shown in Table 2.4. Of note is that most studies only investigated a small number of pharmaceuticals for their applicability as chemical markers for sewage contamination. Therefore, a compound may have not been suggested by a particular study because it was not investigated and not necessarily because it is not useful for such an application.

In relation to using pharmaceuticals as sewage markers, specific considerations related to their fate in WWTPs are essential. For example, an understanding of the level of biodegradability and overall removal rates of specific pharmaceuticals within WWTPs allows for the distinction between contamination of water with treated and raw sewage [123]. Therefore, it could provide a more comprehensive characterisation of the input, including information on the type of sewage (raw or treated) discharged into the water body in addition to sewage and manure differentiation [123].

Labile chemical markers[21] that are susceptible to removal in WWTPs include acetaminophen, ibuprofen, ketoprofen, naproxen, atenolol, gabapentin, caffeine and triclosan [46, 123, 191, 196, 197]. Their presence in water bodies should be a direct consequence of raw sewage inputs e.g. spillage of sewage, leaking sewage pipes and

[21] Labile chemical markers are those that are readily removed from the aqueous environment as a result of processes such as biodegradation, adsorption and photodegradation.

Table 2.4: Pharmaceuticals suggested (S) as being suitable as indicators of sewage contamination by different studies. 1: [123], 2: [46], 3: [192], 4: [184], 5: [193], 6: [47], 7: [194, 195], 8: [111], 9: [191].

Pharmaceutical	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Caffeine	S	S	S	S	S	S	S		S
Carbamazepine	S	S	S	S	S			S	S
Cotinine				S	S		S		
Codeine	S	S							
Gabapentin	S	S							
Ibuprofen	S		S						
Nicotine				S			S		
Acetaminophen	S			S				S	
Triclosan			S			S		S	
Atenolol	S							S	
Cimetidine	S								
Crotamiton			S						
Diclofenac	S							S	
Diltiazem	S								
Diphenhydramine		S							
Erythromycin-H_2O	S							S	
Ethyl citrate		S							
Galaxolide HHCB		S							
Ketoprofen			S					S	
Mefenamic Acid			S						
Metoprolol	S							S	
Naproxen			S					S	
Paraxanthine				S					
Propanolol	S							S	
Sulfamethoxazole				S				S	
Sulfasalazine	S								
Thymol			S						
Tonalide AHTN		S							
Tramadol	S								
Valsartan	S								

septic tanks, sewer overflows and illegal discharge from households since they would not be present within suitably treated effluents [123].

Other compounds are not successfully removed during wastewater treatment. These include carbamazepine, diclofenac, mefenamic acid, codeine, cotinine, diphenhydramine, tramadol and diltiazem [123, 164, 189, 191, 195, 197]. Consequently, such conservative markers[22] are good indicators of treated sewage. They would be detected in WWTP effluents, whilst the labile compounds would be absent or largely removed.

Of note is that most of the studies to date have focussed on identifying inputs of WWTPs into surface waters, whilst the specific distinction between sewage and

[22] Conservative chemical markers are those that are resistant to removal, by e.g. biodegradation and adsorption, and, therefore, persist within their environment for long periods of time.

manure inputs has not been investigated. In fact, a number of the markers suggested as indicators of sewage, e.g. ketoprofen, diclofenac and cimetidine, are also used in veterinary treatment [198–200] in addition to human treatment. Hence, they are unsuitable as chemical markers for differentiating sewage and manure.

No study is known where the suitability of veterinary pharmaceuticals as markers of manure contamination was specifically investigated. However, studies on human pharmaceuticals indicate that the occurrence of pharmaceuticals within the aquatic environment is directly related to their use within the community being studied, their metabolic degradation, and environmental degradation. Furthermore, a number of studies have determined lists of pharmaceuticals that are, for example, important in animal medicine [201], have a high potential to reach the environment and are commonly used [202] or of potential concern in relation to the aquatic environment [140]. Hence, these data may be utilised to determine the suite of chemical markers through understanding the veterinary pharmaceuticals that have the highest potential to act as chemical markers.

In relation to using veterinary pharmaceuticals as markers for manure, it is important to note that the use of antibiotic growth promoters was banned in Europe in 2006 through regulation No. 1831/2003 of the European Parliament and of the Council of 22^{nd} September 2003 on additives for use in animal nutrition [201]. This ban has led to a decline in the use of antibiotics such as erythromycin, virginiamycin, bacitracin, tylosin, oxytetracycline, sulfathiazole, lincomycin and apramycin [201]. Nevertheless, such pharmaceuticals are still found within European surface waters, indicating their high usage in livestock treatment in addition to their previous role in nutrition.

2.5.4.1 Advantages and Limitations

The use of chemical markers to track faecal contamination is considered to afford a number of advantages over biological methods, in particular due to their reduced sample preparation and analysis time requirements and the increased temporal and spatial stability of most chemicals [203]. Additionally, they are considered to show increased source-specificity as there are no issues related to environmental regrowth, which is a concern when carrying out microbial source tracking [204]. Furthermore, generally they are synthetic chemicals. Thus, no natural sources or analogs are known [203].

However, research into this area is quite limited as compared to MST techniques. The major challenges to using chemical markers for identifying the sources of faecal contamination include that dilution within environmental matrices may result in

the chemicals occurring at concentrations below the method detection limits [203]. Additionally, standard sample preparation protocols are unavailable for a large number of compounds [203]. These factors make the use of a suite of chemical markers as opposed to a single marker approach, the selection of the analytical suite and validation of the analytical protocol to be critical in such analyses.

2.5.5 Considerations for the Suite of Chemical Markers

The selection of a suitable suite of chemical markers is a critical factor of environmental forensics studies for the differentiation and characterisation of sewage and manure inputs to surface water bodies. Two critical considerations have been identified, namely marker specificity and detection frequency.

The selected chemical markers must be specific to human or veterinary treatment to allow for the differentiation of sewage and manure inputs. A number of pharmaceuticals are consumed by both humans and livestock [123]. Some examples of pharmaceuticals that are used for the treatment of humans, animals or both within Ireland and the UK are given in Table 2.5, indicating suitable indicators of human and animal sources.

Table 2.5: Classification of pharmaceuticals by intended user within Ireland and the UK [198–200, 205].

Use	Examples
Human	acetaminophen (paracetamol), caffeine, carbamazepine, codeine, diltiazem, diphenhydramine, ibuprofen, propranolol, meclofenamic acid, gabapentin
Veterinary	enrofloxacin, tylosin, sulfadimethoxine, lincomycin, doramectin, tilmicosin, ivermectin, diazinon, cypermethrin, cloxacillin, sulfadiazine
Human & Veterinary	cimetidine, ketoprofen, sulfamethoxazole, thymol, amoxicillin, ampicillin, erythromycin, neomycin, trimethoprim, oxytetracycline, tetracycline

Of note is that the list of approved pharmaceuticals for use and, consequently, their usage characteristics may vary between countries. These differences make it important to understand pharmaceutical use and application within a particular community prior to adopting a suite of pharmaceuticals as chemical markers for distinguishing sewage and manure inputs into surface waters.

Other considerations, such as environmental fates and transformations and specific uses (e.g. used to treat a specific group of animals or only used in hospital treatments), would allow for further characterisation of the nitrate source as being e.g. raw or treated sewage, or emanating from a particular type of manure. There-

fore, such an approach allows for further characterisation of sewage and manure inputs, in addition to their differentiation.

A high detection frequency within the environmental matrix being monitored is another crucial consideration when establishing a suitable suite of pharmaceuticals. The usefulness of a particular marker decreases if it is infrequently detected downstream of its particular source since this would require a much larger complement of markers within the suite for conclusive source differentiation.

The use of a smaller suite of chemical markers reduces method development complexity and analysis times. Furthermore, the occurrence of a particular chemical marker at higher concentrations within the environmental matrix of interest facilitates its detection, particularly when taking into account the complex matrices that are commonly investigated in relation to trace contaminants within surface waters.

Where actual data on pharmaceutical occurrence is absent, an indication could be achieved through an understanding of the persistence of the chemical markers (a persistent chemical marker is more likely to have a high detection frequency) and the usage levels within the area being investigated. Usage levels can be determined by an investigation of the prescription and sales levels of the different chemical markers within the community being studied. Studies on consumption patterns and volumes in the water body's catchment area are critical and, if they differ widely, can result in a suitable analytical suite within one geographical or temporal area being unsuitable in another setting [130, 206]. This is because pharmaceuticals that are detected most frequently represent those that are dispensed at the highest levels in that particular community [169].

Another important factor is that the suite of chemical markers must be periodically reviewed for potential changes in usage characteristics, which may have an impact on the detection frequency. For example, clofibric acid (the major metabolite of clofibrate and etofibrate), which was reported as one of the most common pharmaceutical residues in effluents from WWTPs and in natural waters in Germany in 1998 [146], was only detected in about half of the studied effluents five years later [155]. This change in detection frequency has been attributed to the drugs that are metabolised to clofibric acid being replaced with others, such as gemfibrozil and fenofibrate, within the studied communities [155].

2.6 Conclusion

The contamination of water bodies by nitrate has been linked to various environmental and health concerns, which has made nitrate source determination an area of growing importance as it allows for inputs to be identified. Thus, it results in more effective and, consequently, less costly remediation. Furthermore, it has legislative importance in relation to the EU WFD and ELD. This makes the development of methods for nitrate source identification and characterisation of interest.

As has been shown in Section 2.3, the use of nitrate stable isotope compositions has been successfully employed in discriminating most nitrate sources. However, these methods have been unsuccessful in differentiating sewage and manure due to similar isotopic fractionation processes undergone by sewage and manure nitrate. Nevertheless, the specific differentiation of sewage and manure is of particular importance due to related health risks. Therefore, alternative means to the use of nitrate isotopes to distinguish sewage and manure inputs must be identified.

A potential way to achieve such faecal source tracking is through the use of faecal indicator bacteria and genetic markers for microbial source tracking. The use of genetic markers is one of the approaches that has received significant interest in recent years. Yet, as outlined in Section 2.4, a number of challenges have been identified for the use of such an approach to achieve sewage and manure differentiation and characterisation. Through the use of pharmaceuticals as chemical markers, the specific differentiation of sewage and manure may be achieved, together with further source characterisation on the basis of the entry pathway (Section 2.5).

Two significant gaps in research were identified within this area. The first involves the development and application of a suite of chemical markers for the differentiation of sewage and manure. Therefore, a suite of chemical markers was identified and a multi-residue chromatographic method for monitoring the suite of chemical markers was developed (Chapter 4). In addition to the use of a standard chromatographic approach, alternative analytical tools were also explored with regards to their potential applicability for the detection of pharmaceuticals in surface waters for such an environmental forensics function (Chapter 5).

The second research gap is that, as described in Section 2.5.2.1, so far there is insufficient data on the use and disposal of medications within households. This lack of data is in contrast to the considerable body of research that focusses on pharmaceutical transport through WWTPs. Hence, in order to obtain a more comprehensive understanding of the use and disposal of medications within households and identify current attitudes of the general public to the use, disposal and environmental effects of medication, a questionnaire was devised and the gathered data

analysed (Chapter 6).

Once these two research gaps were tackled, an additional contribution was made to bring the current state of knowledge in the field of nitrate source determination and the differentiation requirements of various stakeholders together. Therefore, a decision tool was developed to facilitate the decision-making process in identifying the most suitable approach for achieving nitrate source determination. The development and evaluation of the decision tool are, thus, discussed in Chapter 7.

Chapter 3

Materials and Methods

This chapter outlines the research materials and methods adopted in this study in an effort to differentiate sewage and manure point and diffuse inputs into surface waters through the use of chemical markers. Details of the sampling protocol, including site selection and sample processing are described in Section 3.1. Then, the method followed in the selection of a suitable suite of chemical markers and the development of the relevant chromatographic and alternative analytical methods is outlined in Sections 3.2 and 3.3. Finally, the administration of a questionnaire for identifying current attitudes to the use and disposal of medications within households is described (Section 3.4).

3.1 Sampling Protocol

Surface water grab samples[1] were collected from three sites in Ireland in order to be able to test the analytical methods developed and discussed further on in this chapter. The three sites, Tullow, Baunreagh and Kilcruise, form part of the South Eastern river basin district (Figure 3.1). They were selected to represent different catchment types, namely upstream (Baunreagh and Kilcruise) and downstream (Tullow) of WWTPs, in order to be able to assess the suitability of the selected chemical markers to differentiate between them. The land-cover of each sub-basin is as described in Table 3.1. Samples were collected monthly over a twelve month period between October 2011 and September 2012 by T.E. Laboratories (Carlow, Ireland), who were project partners in this study.

[1] The use of grab sampling was used due to increased ease of sample collection protocols in this regards. Composite sampling would result in the collection of more representative samples. However, a number of issues are related to their use, particularly in relation to the cost of composite samples and sampling site security.

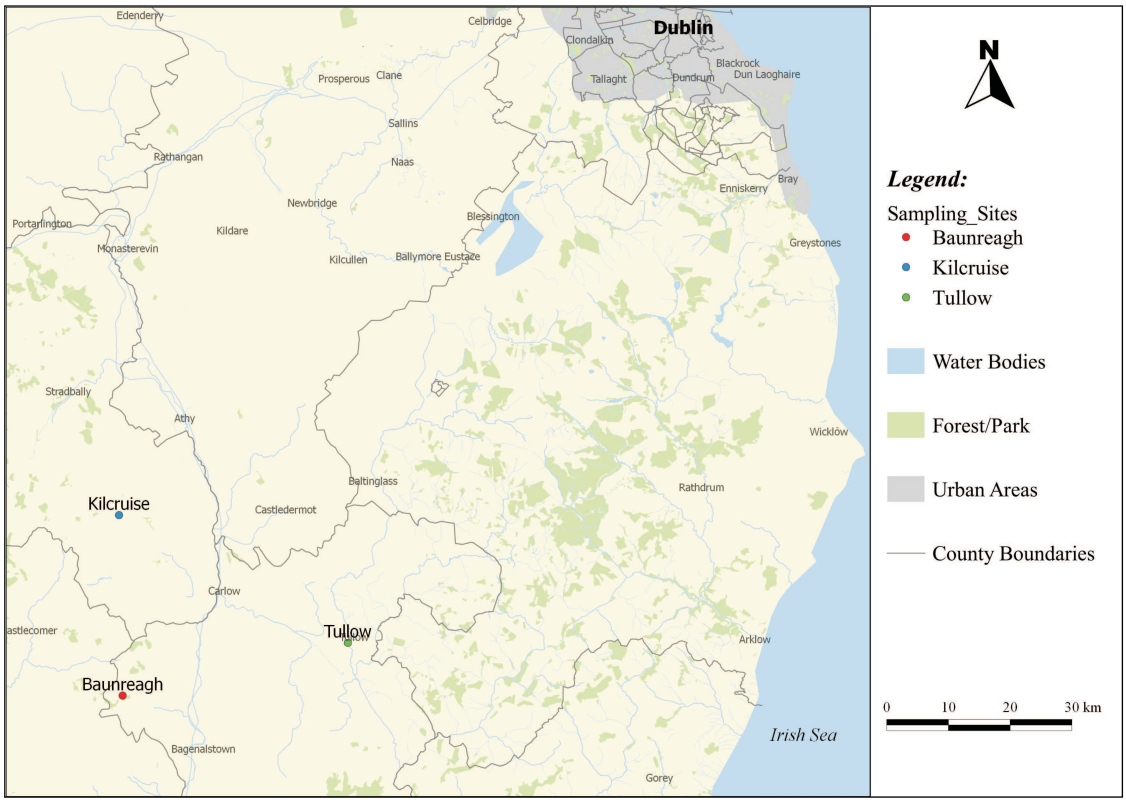


Figure 3.1: Location of sampling sites.

Table 3.1: Land cover within the Tullow (TL), Baunreagh (BR) and Kilcruise (KC) river sub-basins.

Corine Land-Cover Designation		% Cover		
Level 1	**Level 2**	**TL**	**BR**	**KC**
Artificial surfaces	Urban Fabric	4		
	Artificial non-agricultural vegetated areas	1		
Agricultural Areas	Arable Land	23		1
	Pastures	70	59	71
	Heterogeneous agricultural areas	2		21
Forest and seminatural areas	Forest		9	3
	Scrub and/or herbaceous vegetation associations		32	4

Samples were collected in two 5 L high density polyethylene sampling containers according to the method statement in Appendix B. One container was kept by T.E. Laboratories for nitrate analysis using an Irish National Accreditation Board (INAB) accredited ion chromatographic method. The other container was transported to Dublin City University (DCU) for further processing. A chain of custody form (Appendix C) accompanied the samples at all times during transit from site to the different labs.

3.1.1 Chemicals and Materials

Methanol (MeOH), dichlorodimethylsilane and toluene were purchased from Sigma-Aldrich. Whatman GF/C 1.2 µm filters were purchased from VWR Ireland.

3.1.2 Glassware Preparation

All glassware used for sample processing and chemical marker analysis was silanised by rinsing thoroughly with a 50% (v/v) solution of MeOH in water, followed by a 10% (v/v) solution of dichlorodimethylsilane in toluene, two toluene rinses and two MeOH rinses [180]. Silanisation was carried out in order to prevent pharmaceutical residues from adsorbing to the glassware.

3.1.3 Sample Processing

On arrival in the lab, the surface water samples were vacuum filtered through Whatman GF/C 1.2 µm filters and divided into five 1 L aliquots labelled A, B, C, D and E. Aliquots A-D were used for chromatographic analysis (Section 3.2). Aliquot E was used, as required, for analysis using NMR or immunoassays (Section 3.3) and the remainder maintained as a retention sample. The samples were all stored at 4℃ in the dark.

3.2 Chromatographic Techniques

3.2.1 Chemical Marker Selection

A suite of chemical markers composed of two separate groups, indicative of sewage and manure respectively, was initially determined.

3.2.1.1 Sewage Chemical Markers

In order to identify a suitable suite of sewage chemical markers, a literature search was initially completed. This search established the analytes that have been previously suggested to be suitable chemical markers of sewage contamination. Any pharmaceuticals in the list that were not approved for use in Ireland [205] and the UK [141] were excluded, as it would be unlikely to find them within Irish waters. Pharmaceuticals that have been approved for use in veterinary treatment [198–200] were also excluded from the list, because their presence would not be solely indicative of sewage inputs.

Further information was, then, collated on the remaining chemical markers in the list. This included their prescribing frequency, whether they are conservative or labile on passing through WWTPs, and details of their concentrations and detection frequencies within surface waters. This information allowed for the remaining chemical markers to be classified depending upon their characteristics. Subsequently, the list was reduced in number by limiting the presence of pharmaceuticals with overlapping characteristics. This led to the selection of the final suite of sewage chemical markers (acetaminophen, caffeine, carbamazepine, cotinine, diltiazem and diphenhydramine) that is believed to have the greatest ability to fully characterise the sewage input whilst, at the same time, being limited in size for ease of implementation within other laboratories. Further details on the selected suite of sewage chemical markers are given in Section 4.1.1.

3.2.1.2 Manure Chemical Markers

In determining the suite of manure chemical markers, a literature search returned no suggestions of manure chemical markers. Therefore, the initial list of potential analytes included veterinary pharmaceuticals that other researchers listed as being priority contaminants [160]; are highly likely to be transferred to surface waters [202]; or have been frequently detected in the environment [133, 160, 173]. The process from this point on parallelled the method used in the determination of the suite of sewage chemical markers, namely, the exclusion of compounds that are not approved for veterinary use in Ireland or the UK [198, 199] and compounds that are also approved for use in human treatment [141, 205]. Then, information on the remaining chemical markers in the list was collated from literature. This data included the target species and use scenario. Further details on the selected manure chemical markers (enrofloxacin, lincomycin, sulfadimethoxine and tylosin) are given in Section 4.1.2.

3.2.2 Method Development

3.2.2.1 Chemicals and Materials

Mobile phase solvents of high-performance liquid chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) quality were purchased from BDH Prolabo (VWR, Ireland) and Optima LC/MS (Fisher Scientific, Ireland). Mobile phase additives were purchased from Sigma-Aldrich. Prepared mobile phases were filtered (Pall nylon filters, 0.2 µm pore size) and sonicated prior to use.

High purity (>95%) chemical marker standards were purchased from Sigma-Aldrich (Table 3.2). Individual stock solutions were prepared by dissolving the required mass of powdered sample in methanol to a concentration of 1000 mg L^{-1}. Single[2] and mixed[3] standard solutions were prepared by diluting the stock solution in the starting mobile phase, as necessary. All standards were stored in silanised amber vials in the dark at 4°C.

Table 3.2: List of analytes with selected details and properties. Further parameters and analyte structures are given in Appendix D.

	Analyte	Symbol	CAS-No.	Formula	Mol. Mass
Human	Acetaminophen	ACT	103-90-2	$C_8H_9NO_2$	151.16
	Caffeine	CAF	58-08-2	$C_8H_{10}N_4O_2$	194.19
	Carbamazepine	CBZ	298-46-4	$C_{15}H_{12}N_2O$	236.27
	Cotinine	COT	486-56-6	$C_{10}H_{12}N_2O$	176.22
	Diltiazem	DTZ	2399-41-7	$C_{22}H_{26}N_2O_4S$	414.52
	Diphenhydramine	DPH	58-73-1	$C_{17}H_{21}NO$	255.35
Veterinary	Enrofloxacin	ENR	93106-60-6	$C_{19}H_{22}FN_3O_3$	359.4
	Lincomycin	LIN	406.537	$C_{18}H_{34}N_2O_6S$	406.54
	Sulfadimethoxine	SDM	122-11-2	$C_{12}H_{14}N_4O_4S$	310.33
	Tylosin	TYL	1409-61-0	$C_{46}H_{77}NO_{17}$	916.1

3.2.2.2 Sample Preparation

The pH of aliquots A-D (Section 3.1.3) was modified to pH 4±0.2 using sulfuric acid (Sigma-Aldrich). Then, sample A was kept as is, whilst samples B, C and D were spiked to 0.25 µg L^{-1}, 0.5 µg L^{-1} and 0.75 µg L^{-1}, respectively, using the 10 mg L^{-1} mixed standard solution containing all 10 analytes. The samples were all stored at 4°C in the dark.

3.2.2.3 HPLC Method Development

An Agilent 1200 HPLC system was used to achieve chromatographic separation of the analytes. Wavelength optimisation was carried out on a Beckman DU 520 UV/Visible Spectrophotometer. Then, the mobile phase composition for eluting each analyte was separately identified by injecting 10 mg L^{-1} single standard solutions into the HPLC according to the method outlined in Lacey et al. [207]. In summary, a chromatographic scan was carried out from 20% acetonitrile (ACN) to

[2] Solutions prepared by dilution of the stock standard solution and consisting of a known concentration of a single analyte.

[3] Solutions prepared by dilution of the stock standard solution and consisting of a known concentration of two or more analytes.

80% ACN in water with 0.1% ammonium acetate (v/v) at pH 6.1 on a Sunfire C18 3.5 µm, 2.1x150 mm column.

Subsequent investigations consisted of using a different stationary phase (Phenomenex Luna PFP 5 µm 2.0x150 mm) in an effort to improve selectivity and sample throughput. This was followed by chromatographic parameter optimisation, including mobile phase composition, mobile phase pH, mobile phase additives, injection volume and gradient profile, which was carried out for both stationary phases. Further details are given in Section 4.2.

3.2.2.4 SPE Method Development

To select the most appropriate Solid Phase Extraction (SPE) cartridge, a literature search was conducted to identify the SPE cartridge types used in similar applications. Three cartridges were selected for initial screening, namely Oasis HLB from Waters, Lichrolut EN from Merck, and Strata X from Phenomenex. Extraction was carried out in duplicate using the manufacturer's recommended methods (Table 3.3) for 6 mL, 500 mg cartridges on 1000 mL of ultra-pure water spiked to 1.25 µg L^{-1} of each analyte.

Table 3.3: Methods used for the selection of the most appropriate SPE cartridge.

	Oasis HLB	**Lichrolut EN**	**Strata X**
Condition	6 mL MeOH	6 mL MeOH	6 mL MeOH
Equilibrate	6 mL H_2O	6 mL H_2O	6 mL H_2O
Load	1000 mL Sample	1000 mL Sample	1000 mL Sample
Wash	6 mL 5% MeOH in H_2O	6 mL 5% MeOH in H_2O	6 mL 5% MeOH in H_2O
Dry	20 minutes	20 minutes	20 minutes
Elute	6 mL MeOH	6 mL MeOH:ACN 1:1	6 mL 1% CH_3COOH in MeOH

After elution, the samples were dried using a miVac sample concentrator (Genevac) and then re-suspended in 0.5 mL of the starting mobile phase. Pre-and post-extraction spiked samples were compared to determine the percentage recovery of each analyte. This allowed for the optimum cartridge for this application to be identified, namely the Oasis HLB cartridge. Use of this cartridge was then optimised by investigating the use of different eluents.

3.2.2.5 MS/MS Method Development

Two separate mass spectrometer (MS) methods were developed due to the use of two instruments. The tandem mass-spectrometer (MS/MS) method development

followed similar processes, as outlined below.

AB Sciex Instrument. The first MS system used was an AB Sciex API 2000™ Triple-Quad LC-MS/MS in positive mode, in combination with a Hewlett Packard 1100 Series HPLC. This instrument uses TurboIonSpray technology[4] as an ion source. The LC-MS/MS system was controlled by AB Sciex Analyst software version 1.4. Direct infusion of 1 mg L^{-1} single standards was carried out at a flow rate of 3 µL min^{-1} using a 1000 µL, 2.3 mm diameter glass syringe together with a Harvard syringe pump. The analyte precursor ions were identified through a full scan using the first quadrupole as the mass filter. Following this, the product ions were identified through an MS/MS scan[5]. This scan resulted in the identification of all products of the previously identified precursor ion. Using the 'Compound Optimisation' setting in the Analyst software, the acquisition parameters were further optimised and the settings saved.

A Selected Reaction Monitoring (SRM) method was then developed by the addition of the ten separate single analyte compound optimisation methods into one. SRM data are obtained by monitoring a single fragment ion of a specific precursor mass of the parent compound in tandem mass-spectrometry. This method provides greater confirmatory detail of the analyte than when looking at a single parent ion in isolation. It also results in peak resolution issues to be eliminated providing that the various analytes or matrix components do not undergo the same transition. Therefore, all analytes were injected in isolation into the combined method to ensure unique transitions had been selected.

Bruker Instrument. A Bruker Daltronics HCT ion trap MS with electrospray ionisation (ESI), in combination with an Agilent 1200 HPLC, operated in positive mode, was the second instrument used. The LC-MS/MS system was controlled by Bruker Compass HyStar version 3.2. Direct infusion of 1 mg L^{-1} single standards was carried out at a flow rate of 5 µL min^{-1} using a 500 µL glass syringe and a Cole-Parmer syringe pump.

The optimal trap drive voltage was initially identified, followed by the optimal conditions for the capillary, skimmer, capillary exit and octopole voltages and the lens conditions. Following the identification of the separate standard optimal acquisition conditions, an average value for the various acquisition parameters was

[4] TurboIonSpray is a form of electrospray ionisation in which ionisation occurs within the gas phase unlike in most other ionisation processes in mass spectrometry [208].

[5] Scan in which the first quadrupole is set to a fixed mass and the third quadrupole sweeps through the mass range.

determined for use throughout the analysis. Then, these parameters were utilised for mass spectrometric analysis carried out in SRM mode.

3.2.3 Method Validation

Three separate methods were validated. The first is the developed HPLC-Diode Array Detector (DAD) method using the Sunfire C18 column. This method was used for SPE cartridge selection and optimisation. The second and third methods were the two LC-MS/MS methods developed using the Luna PFP HPLC column i.e. using the developed AB Sciex and Bruker mass spectrometry parameters. The requirements for validation, as set for this analysis, are given in Table 3.4.

Table 3.4: Validation requirements for the developed method.

Parameter	Requirement
Precision	RSD <10% (6 injections)
Linearity	R^2 >0.99
LOQ	0.5 mg L^{-1} (as injected)
LOD	0.05 mg L^{-1} (as injected)

Linearity was based on the injection of at least 6 standards containing the 10 analytes over the concentration range of 10 mg L^{-1} to 0.001 mg L^{-1}, which is typical of the concentrations found after extraction. Instrumental precision was calculated from 6 repeated injections of a solution containing 1 mg L^{-1} of the 10 analytes. The method's limit of quantification (LOQ) and limit of detection (LOD) were determined to be the lowest concentration at which six repeated injections of the analyte within the mixture yielded a relative standard deviation of <15% and a signal:noise ratio of >3:1, respectively.

3.2.4 Application to Surface Water Samples

The validated multi-residue SPE LC-MS/MS method was applied to three river monitoring sites within Ireland on samples collected according to the methods specified in Section 3.1. Two separate LC-MS/MS methods were used. The AB Sciex instrument was used to analyse samples collected between October 2011 and March 2012, whilst the Bruker instrument was used for samples collected between April 2012 and September 2012. This change was necessary due to instrumental breakdown of the AB Sciex MS mid-way through the project.

3.2.4.1 Data Analysis

Once the results of chemical marker occurrence were collected, they were transferred to IBM SPSS Statistics version 19 for data analysis. Below Detection Limit (BDL) values were substituted by 0, whilst Below Quantification Limit (BQL) values were substituted by the mid-point value between the quantification limit and the detection limit. A significance level of $p < 0.05$ was used for all statistical tests. The statistical tests carried out on the data are as follows.

Levene's Test. Levene's test for homogeneity compares the variances within different groups and confirms whether the differences between the variances is 0 [209]. The hypotheses tested were:

H_o: The data sets have variances that are not statistically different.

H_1: The data sets have variances that are statistically different.

The null hypothesis was accepted if the p value was greater than 0.05.

In this assessment, the assessed independent factors were for differences by site and season[6].

ANOVA. Analysis of variance (ANOVA) analyses compare the means of independent data sets and confirm whether the means of these sets are statistically significant [209]. The hypotheses tested were:

H_o: The data sets have means that are not statistically different.

H_1: The data sets have means that are statistically different.

The null hypothesis was accepted if the p value was greater than 0.05.

Where the alternative hypothesis of ANOVA was accepted, the Tukey post-hoc test[7] was carried out on the data to identify homogeneous subsets within the data. The Tukey test was adopted since the datasets used were of equal size and the population variances were similar, as tested by the Levene's test of homogeneity [209]. The hypotheses tested were:

H_o: The mean of change between two factors is statistically insignificant.

H_1: The mean of change between two factors is statistically significant.

The null hypothesis was accepted if the p value was greater than 0.05, indicating homogeneity between the subsets.

[6] The seasons used were Spring (February, March, April), Summer (May, June, July), Autumn (August, September, October) and Winter (November, December, January), as per the Irish Metereological Calendar (Colm Faherty, Senior Meteorological Officer, Ireland. Personal Communication (e-mail) 29 August 2011).

[7] Post-hoc tests involve comparing the means of all combinations in pairs of groups in order to identify sub-groups (homogeneous subsets) within the dataset [209].

Kendall's τ Correlation Analysis Kendall's τ correlation analysis is a non-parametric correlation test used with data sets which are small and have a large number of tied ranks, as are present here [209]. It is used to measure the association between two continuous variable. A correlation close to +1 indicates a very strong positive correlation, while a correlation close to -1 indicates a very strong negative correlation. A correlation coefficient close to 0 indicates no relationship between the two variables [209]. The hypotheses tested were:

H_o: There is no correlation between the two variables.

H_1: There is significant correlation between the two variables.

The null hypothesis was accepted if the p value was greater than 0.05, indicating that no correlation occurs between the two variables.

3.3 Alternative Analytical Techniques

3.3.1 NMR Techniques

3.3.1.1 Chemical Marker Selection

In selecting a chemical marker for this pilot study investigating the suitability of using NMR as an analytical technique in environmental forensics studies, the characteristics of the analytes previously selected to form part of the chemical marker suite were established. In particular, the nuclei present within the various compounds and their activity within an NMR spectrophotometer were identified. From these, enrofloxacin was selected for the pilot study due to the presence of a ^{19}F nucleus within its structure.

3.3.1.2 Chemicals and Materials

NMR spectra were obtained using a Bruker Avance 400 and a Bruker Avance 500 NMR spectrophotometer using 5mm borosilicate tubes. These instruments result in a ^{19}F frequency of 376.3 MHz and 470.4 MHz, respectively. Deuterated solvents were purchased from Sigma-Aldrich (Ireland).

3.3.1.3 Method Development

Dissolution of enrofloxacin was assessed by dissolving the powdered standard in D_2O, acetonitrile, methanol, ethyl acetate, acetone, DMSO and alkaline D_2O. Alkaline D_2O was prepared by the addition of 2 mL of NaOD per litre of D_2O and selected as the most suitable solvent. The limit of detection was determined by modifying the

concentration of the analyte, the number of acquisitions and acquisition parameters. The obtained spectra were processed using Bruker Topspin 2.1 software.

3.3.2 Immunoassay Techniques

3.3.2.1 Chemical Marker Selection

In determining the chemical marker to use in this proof-of-concept study investigating the suitability of using immunoassay techniques for chemical marker detection, the commercial availability of antibodies for the 10 analytes within the analytical suite was initially investigated. From these, enrofloxacin was selected due to the commercial availability of an Enzyme-Linked Immunosorbent Assay (ELISA) kit for its detection.

3.3.2.2 Chemicals and Materials

An enrofloxacin ELISA kit was purchased from Randox (UK), which included the required 96-well microtitre plate, enrofloxacin standards (6 x 2 mL), enrofloxacin spiking material (2 mL), conjugate concentrate[8] (2 mL), conjugate diluent (20 mL), concentrated wash buffer[9] (32 mL), one shot substrate[10] (15 mL) and a stop solution[11] (15 mL). These were stored at +2 to +8°C.

Prior to use, the conjugate was diluted according to the conjugate dilution procedure specific for the purchased kit and the wash buffer prepared by diluting 6.25 mL of wash buffer concentrate with 250 mL of distilled water. Additional materials included Phenex Nylon 0.2 µm syringe filters, lint free tissue paper, microtitre plate sealers and an Infinite 200 Tecan plate reader.

3.3.2.3 Method Development

The intended use of the ELISA kit used is for the quantitative determination of enrofloxacin in prawn and fish tissue samples. Therefore, the suitability for use with surface water samples was assessed through the comparison of standard curves for enrofloxacin using the kit's enrofloxacin standards and spiked surface water samples.

Standard solutions at 9 ng mL^{-1}, 3 ng mL^{-1}, 1 ng mL^{-1}, 0.33 ng mL^{-1}, 0.11 ng mL^{-1}, 0.037 ng mL^{-1} and 0.012 ng mL^{-1} were prepared with distilled water and filtered (0.2 µm) surface water samples (Tullow: March 2012; Kilcruise: March

[8] Horseradish peroxidase enzyme (HRP).
[9] Trisp buffered saline solution.
[10] A chemiluminescent signal reagent that generates the light reaction.
[11] 0.2 M sulfuric acid solution.

2012). Then, the procedure provided by Randox Ltd. for extracted solid samples was followed. 50 µL of each standard solution and 75 µL of the diluted conjugate were pipetted into the appropriate wells of the microtitre plate. The standard solutions in distilled water were analysed in duplicate, whilst the surface water samples were analysed as singular analyses.

Then, the microtitre plate was covered with a plate sealer and incubated at 25°C in the dark. After 30 minutes, the plate was inverted, all the liquid tapped out, washed three times with the diluted wash buffer over a six minute period and the microtitre plate dried completely by tapping onto lint-free tissue paper. After washing, 125 µL of the one-shot substrate solution was pipetted into each well and then incubated for 20 minutes in the dark at 25°C. The colour reaction was stopped by adding 100 µL of stop solution per well, which resulted in a colour change from blue to yellow. Optical density at 450 nm using a 630 nm reference wavelength was measured within 10 minutes of stopping the colour solution using the plate reader.

3.4 Current Attitudes to the Use and Disposal of Medication

3.4.1 Data Collection

A 38-point web-based self-administered questionnaire (SAQ) was developed using the open-source[12] online questionnaire tool LimeSurvey 1.91+. LimeSurvey was deployed on a basic LAMP[13] server running Apache version 2.2.21, MySQL version 5.1.56 and PHP version 5.2.17. The questionnaire explored aspects of current medication use, disposal practices, disposal considerations, environmental considerations and demographics. The UK Data Archive Survey Question Bank [211] was utilised in order to achieve alignment of certain questions, in particular demographic data, with other large scale surveys. Participants responded to a selection of the 38 questions depending upon their response profile.

Ethical approval was sought and received from the DCU research ethics commission under the notification procedure for low risk social projects, with reference DCUREC/2011/104. Anonymity was ensured through the use of the 'Anonymize' facility within the Limesurvey software. The use of the 'Exit and Clear' button available on all pages of the questionnaire allowed the participants to cease their

[12] Licensed under General Public Licence v. 2 [210].

[13] LAMP refers to a combination of open-source software systems used to build a web server, through the utilisation of a Linux operating system, Apache HTTP server, MySQL as a database software and PHP scripting language.

participation in the questionnaire at any time. It also caused all data entered by them till that point to be deleted from the database.

At the start, the questionnaire was piloted by sending a draft version to 14 individuals. They were requested to review the draft version for technical issues, errors, clarity of instructions and design preferences and to give any other constructive suggestions. The comments gathered during this process were subsequently considered in the development of the final questionnaire version, which can be found in Appendix E.

The final version was activated for a month between the 16^{th} of January and the 15^{th} of February, 2012. Potential participants received a short introduction to the questionnaire and a URL address in the form of a hypertext link. The message invited the recipient to visit the web page and complete the questionnaire. Participants were recruited using a convenience sampling method linked to snowball sampling.

3.4.2 Data Analysis

The responses obtained from the various participants were automatically stored in an online database. Once data collection was finalised, the data were exported to IBM SPSS Statistics 19.0 for Windows. Data screening and cleaning was initially carried out to identify e.g. missing data. Missing data could be due to connection time out or closure of the web browser without going through the resume later procedure. Also, classification of responses to open questions was carried out where possible. Data mining and statistical analysis was carried out in order to assess relationships in the data. Significance levels were set at $p < 0.05$ for all statistical tests.

Pearson's chi-square test. Pearson's χ^2 test of independence is used when the independent and dependent variables are both categorical to compare the observed frequencies. Since the data gathered as part of this questionnaire were largely categorical, χ^2 analysis was used throughout. The hypotheses tested were:

H_o: Differences between observed and expected frequencies are not significant.

H_1: Differences between observed and expected frequencies are significant.

The null hypothesis was rejected if the χ^2 value was less than 0.05.
The χ^2 test makes 2 assumptions:

- each person, item or entity contributes to only one cell of the contingency table i.e. cannot be used on repeated measures (before-after) analyses, and;

- the sample should be large enough that expected frequencies are larger than 5.

Where a 2×2 contingency table[14] was set up Yate's correction was used, which compensates for the occurrence of Type I[15] errors, which are known to arise in such situations since the Pearson's χ^2 test tends to produce low significance values. Then, the χ^2 analysis was incorporated into a Chi-squared Automatic Interaction Detection (CHAID) model[16] using SPSS AnswerTree 3.1.

[14] A 2×2 contingency table is where two categorical variables with two categories each are used.
[15] Type I errors occur where the significance of an effect in a population is overestimated (false positive), as opposed to type II errors which result in false negatives.
[16] CHAID models use χ^2 statistics to identify optimal splits in data to build classification trees.

Chapter 4

Results & Discussion: Chromatographic Techniques for Chemical Marker Detection

Chromatography represents the routinely used analytical technique for determining pharmaceuticals within environmental waters. Within this study, such compounds are being proposed as chemical markers of sewage and manure. Therefore, this chapter focusses on the use of chromatographic techniques for chemical marker detection on the basis of the methods specified in Chapter 3.

The selection of a suite of chemical markers is described in Section 4.1. This section is followed by a discussion of the development and validation of a single multi-residue chromatographic method for the selected suite of chemical markers in Section 4.2. Then, the results from the application of the developed method to three monitoring sites within Ireland are reviewed in Section 4.3. This section includes a discussion on the characterisation of sewage and manure inputs at the three monitoring sites.

4.1 Analyte Selection

The suite of chemical markers selected for differentiating sewage and manure inputs into surface waters was identified through a desk study. Various considerations were taken into account, as described in Section 3.2.1. These include their frequency of use within Ireland, their reported occurrence in literature within surface waters and their fates within the environment. The analytes include pharmaceuticals used as antibiotics, analgesics, stimulants and anticonvulsants, food additives and metabolites.

4.1.1 Sewage Chemical Markers

Six chemical markers of sewage contamination were selected. The selected chemical markers represent some of the most highly prescribed pharmaceuticals within Ireland, as reported by the Health Services Executive in Ireland e.g. acetaminophen, carbamazepine and diltiazem [212]. This factor indicates their prevalent use, which would result in a higher potential for occurrence within wastewater streams. Furthermore, acetaminophen and diphenhydramine are over-the-counter medications; hence their frequency of use has the potential to be high. Meanwhile, caffeine and cotinine, being a food additive and a nicotine metabolite, respectively, are also expected to be commonly present within wastewater streams due to their widespread use. Characteristic details of the selected chemical markers are given in Table 4.1.

Table 4.1: Notes on the sewage chemical markers.

Analyte	**Function**	**Notes**
Acetaminophen/ Paracetamol (ACT)	Analgesic, Anti-pyretic	·Not conservative in WWTPs [46, 123, 191, 197]. ·Detected at 100% occurrence in UK WWTP receiving waters and absent in clean waters [123] ·Ranked 5^{th} in isolation and 33^{rd} when considering its combinations on the General Medical Service (GMS) prescribing frequency list in Ireland [212]
Caffeine (CAF)	CNS stimulant	·Not conservative in WWTPs [123, 184, 192] ·Detected at 13.9% occurrence in effluent from 3 Irish WWTPs [207]
Carbamazepine (CBZ)	Anti-convulsant	·Conservative in WWTPs [123, 184, 192, 197] ·Detected at 100% occurrence in UK WWTP receiving waters and absent in clean waters [123] ·Ranked 16^{th} on the prescribing frequency list for Long Term Illness [212] ·Detected at 88.9% occurrence in effluent from 3 Irish WWTPs [207]
Cotinine (COT)	CNS Stimulant	·A nicotine metabolite ·Not conservative within WWTPs [184]
Diltiazem (DTZ)	Anti-angina, Anti-hypertensive	·Conservative in WWTPs [169] ·Detected at 100% occurrence in UK WWTP receiving waters [123] ·Ranked 100^{th} on the Drugs Payment Scheme and 74^{th} on the Long Term Illness prescribing frequency list in Ireland [212]
Diphenhydramine (DPH)	Anti-histamine	·Conservative in WWTPs [213] ·Detected at 100% occurrence in WWTP receiving waters in the US [189]

The chemical markers were selected in such a way that, through their presence or absence, further source details could be elucidated. For example, caffeine and acetaminophen are highly labile within WWTPs [214]; hence, their presence is an indicator of raw sewage inputs rather than treated sewage. Other markers, such as carbamazepine and diltiazem, have been identified to be conservative on passing through a WWTP [214]. Therefore, if the conservative markers are present while the labile chemical markers are absent, this indicates the presence of treated sewage.

4.1.2 Manure Chemical Markers

Four chemical markers of manure contamination were chosen (Table 4.2). The chemical markers were selected to represent a wide range of sources and usage characteristics. For example, tylosin is mainly used in a pasture scenario, whilst lincomycin and enrofloxacin are used in both an intensive and pasture scenario. In addition, different chemical markers are used to treat different groups of animals. Thus, such data could be used to elucidate further information about the manure input.

Table 4.2: Notes on the manure chemical markers.

Analyte	Function	Notes
Enrofloxacin (ENR)	Fluoroquinolone antibiotic	·Used to treat most animals, including cattle, pigs, dogs, cats and poultry [199] ·Use scenario: Intensive, pasture
Lincomycin (LIN)	Lincosamide antibiotic	·Used to treat pigs, dogs, cats and poultry [199] ·Use scenario: Intensive, pasture ·Is a veterinary pharmaceutical classified to have a high risk of reaching surface water [140, 160]
Sulfadimethoxine (SDM)	Sulfonamide antibiotic	·Widely used to treat cattle, dogs, cats and birds [199, 215, 216] ·Is an important pharmaceutical in veterinary medicine [201]
Tylosin (TYL)	Macrolide antibiotic	·Used to treat cattle, pigs and poultry [199] ·Use scenario: Pasture ·Is highly used and has a high potential to reach the environment [202] ·Is a veterinary pharmaceutical classified to have a high risk of reaching surface water [140, 160]

4.2 Method Development and Validation

In this section, the results from the development and validation of a multiresidue SPE LC-MS/MS method, as described in Section 3.2, is discussed. The method re-

quirements for successful implementation of the developed method in such a study are initially described (Section 4.2.1). This section is followed by a review of the results obtained during the optimisation of the separate method components (SPE for sample concentration in Section 4.2.1.2; HPLC for separation in Section 4.2.1.3; and MS/MS for identification in Section 4.2.1.4). The separate methods are subsequently combined and validated (Section 4.2.2). An overview of the undertaken method development, optimisation and validation process is given in Figure 4.1.

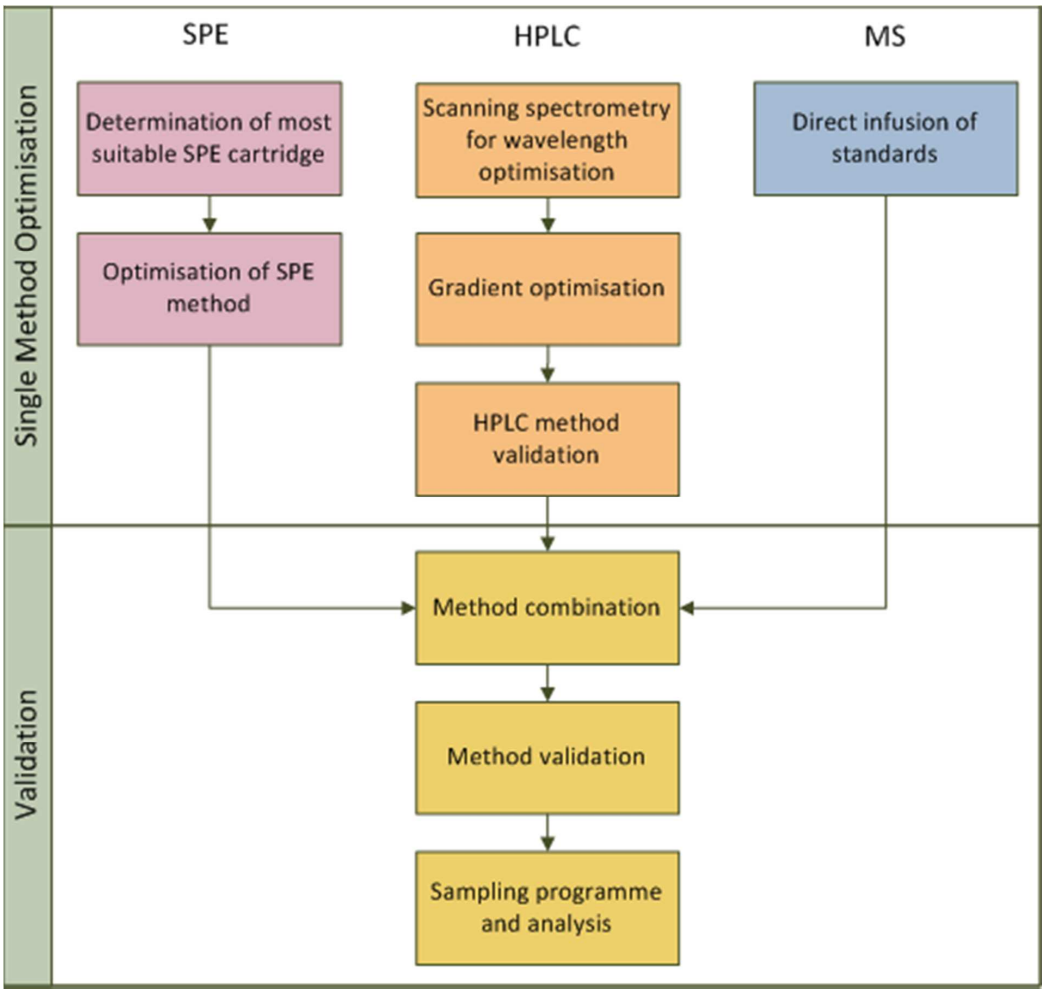


Figure 4.1: Overview of SPE LC-MS/MS method development, optimisation and validation. Adapted from [207].

4.2.1 Method Development

The developed analytical method had to meet a number of criteria for it to be suitable for the present application. The main criteria identified were that the developed method must:

- Consist of a single method for the simultaneous determination of the selected chemical markers. This would result in lower time requirements for system setup and equilibration and a higher sample throughput;
- Have a limit of detection (LOD) for the combined SPE LC-MS/MS of less than 50 ng L^{-1} since this corresponds to the concentration at which these chemical markers normally occur in surface waters;
- Be validated to allow for confidence in the results obtained, and;
- Be suitable for use with surface water samples.

4.2.1.1 Sample Preparation

The nature of surface water samples is that they contain a large number of compounds in addition to the analytes of interest. This makes sample preparation prior to analysis an important consideration. Sample filtration was initially carried out to remove particulates, which reduces problems in later steps, such as blockage of the SPE cartridges or HPLC column. Also, pH adjustment of the filtered samples to pH 4 was carried out in order to ensure a consistent pH and, thus, a consistent level of analyte ionisation. Therefore, improved reproducibility between samples is achieved.

As a final step during sample preparation, the samples were prepared for quantification using the standard addition method. Three standard additions were used for each sample, with 1 L aliquots of each sample being spiked to 0.25 μg L^{-1}, 0.5 μg L^{-1} and 0.75 μg L^{-1} prior to SPE, in addition to an unspiked aliquot. Although the use of the standard addition method is laborious and time-consuming, it allows for issues related to matrix effects to be eliminated [217].

4.2.1.2 SPE Method Development

Solid Phase Extraction (SPE) was selected as the technique of choice for sample clean up and concentration. SPE achieves the removal of interfering substances within the matrix being analysed, thus improving chromatographic separation and detection. In addition, through sample concentration, improved limits of detection and quantification may be attained, which is critical in view of the low concentrations at which the analytes of interest occur within surface waters.

A large number of SPE cartridges are available commercially. A literature search identified the Oasis HLB cartridge to be the cartridge of choice for most studies of a similar nature [e.g. 46, 218–220]. To verify this choice, three cartridges (Oasis HLB, Lichrolut EN and Strata X) were sourced from different suppliers and evaluated for

their recovery and reproducibility of the suite of chemical markers of interest to this study.

The selected cartridges contain polymeric reversed-phase sorbents and are suitable for applications where analytes with a range of polarities are to be extracted together. The obtained pre-and post-extraction spiked samples were analysed using the validated Sunfire HPLC method (Section 4.2.1.3). The percentage recoveries achieved by the different cartridges, on using the manufacturers' recommended methods, were compared in order to determine the optimum cartridge for this application (Table 4.3).

Table 4.3: Average (n=2) percentage recoveries for the three SPE cartridges. Values in red indicate the value closest to 100% recovery.

Analyte	Lichrolut EN	Strata X	Oasis HLB
ACT	63.95	20.24	59.12
CAF	98.93	78.49	100.5
CBZ	54.32	74.53	90.46
COT	82.55	84.54	94.46
DPH	3.01	67.79	52.64
DTZ	37.27	67.06	67.52
ENR	22.36	38.68	72.88
LIN	113.43	157.92	79.92
SDM	34.75	114.89	76.48
TYL	19.17	49.26	57.61

The Oasis HLB cartridge resulted in the values closest to 100% percentage recovery for most analytes of interest. Furthermore, there were no recoveries lower than 50% for any of the analytes. Conversely, the Lichrolut EN and Strata X cartridges had recovery values of less than 50% in 44% and 22% of the analytes, respectively. Moreover, the Oasis HLB cartridge resulted in the lowest standard deviations reported for more than half of the analytes. This outcome indicated that Oasis HLB is the cartridge that affords the most consistent results of the three investigated. Therefore, the obtained results confirmed the trend in literature regarding the suitability of this SPE cartridge for such an application.

The Oasis HLB sorbent is a macroporous copolymer of lipophilic divinylbenzene and hydrophilic N-vinylpyrrolidone (Figure 4.2). This composition renders the sorbent suitable for retaining compounds with a range of polarities, as is the case in this study, due to the range of polarities represented in the sorbent material itself.

Optimisation of the elution solvent used was carried out next. Specifically, the use of a 1:1 mixture of ethyl acetate (EtOAc) and acetone as an elution solvent, in addition to the use of methanol (MeOH), as suggested by the supplier, was in-

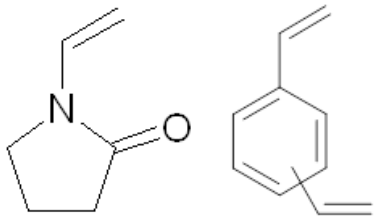


Figure 4.2: Structure of the Oasis HLB sorbent monomers: N-vinylpyrrolidone (left) and Divinylbenzene (right).

vestigated (Table 4.4). Minor differences could be noted between the two elution solvents studied, even though they have considerably different eluent strengths[1], at 0.95, 0.58 and 0.56 for MeOH, EtOAc and acetone, respectively [222]. In view of this, MeOH was selected as the eluent of choice since it was more readily available. The optimised SPE method is given in Table 4.5.

Table 4.4: Average (n=2) percentage recoveries for different eluents.

Analyte	**MeOH**	**EtOAc:Acetone** (1:1)
ACT	59.12	61.92
CAF	100.5	107.26
CBZ	90.46	89.26
COT	94.46	101.78
DTZ	67.52	77.79
DPH	52.64	69.92
ENR	72.88	74.19
LIN	79.92	78.65
SDM	76.48	57.46
TYL	57.61	45.88

Table 4.5: Finalised SPE method.

Cartridge	**Oasis HLB**
Condition	6 mL MeOH
Equilibrate	6 mL Water
Load	1000 mL Sample
Wash	6 mL 5% MeOH in H_2O
Cartridge Drying	20 minutes
Elute	6 mL MeOH
Sample Drying	MiVac Sample Concentrator
Sample Reconstitution	0.5 mL 1% ACN in H_2O (pH3, formic acid)

[1] Eluent strength is a measure of the solvent adsorption energy [221].

4.2.1.3 HPLC Method Development

Chromatographic separation of analytes is a precursor to analytical detection in many analytical techniques. Various chromatographic techniques are available of which HPLC and gas chromatography (GC) are amongst the most commonly used. HPLC was selected as the technique of choice in this research project, because unlike in GC, analyte derivatisation is not required. Therefore, sample preparation times are shorter, whilst still showing sufficient selectivity and sensitivity [223].

Wavelength Optimisation. The optimum wavelength for the simultaneous detection of the 10 analytes was determined by obtaining UV-Visible spectra of the 10 analytes at 10 mg L^{-1} (Figure 4.3). A number of analytes do not absorb at higher wavelengths. Hence, a wavelength of 206 nm was selected for initial method development. Additional detection was carried out using 300 nm as a secondary wavelength.

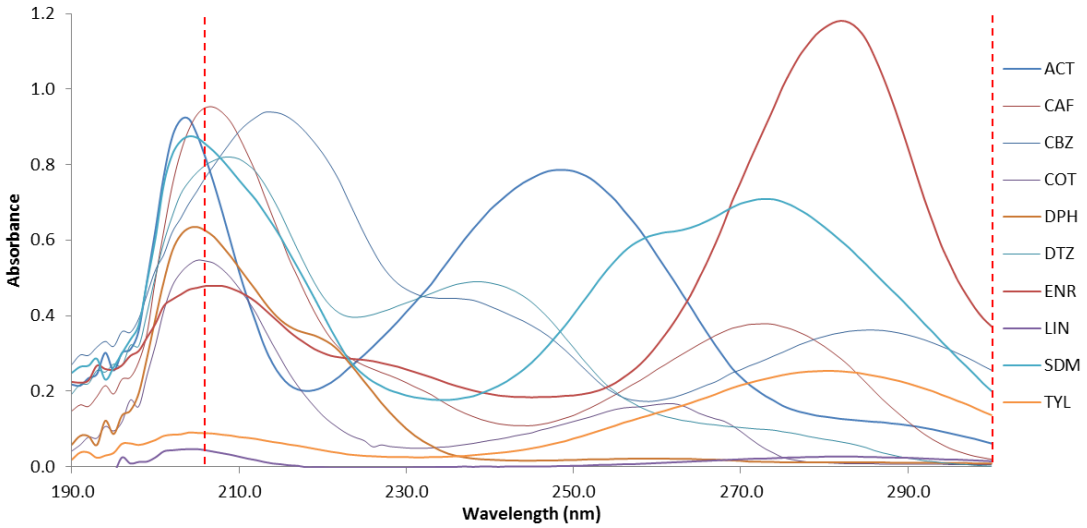
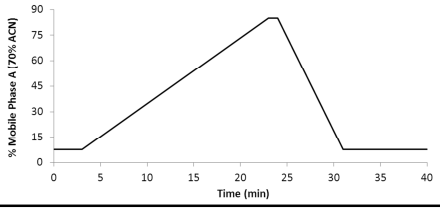


Figure 4.3: Scanning spectrometry between 190nm and 300nm for the 10 analytes. The wavelengths used (206 nm, 300 nm) are marked by a dotted line.

Gradient Optimisation. A previously developed gradient method for the chromatographic separation of 20 pharmaceuticals within wastewater treatment plant influent and effluent samples [180] was used as a starting point for method development. However, both the start and end mobile phase organic content had to be modified for optimal separation of the 10 analytes, due to differences in the analytical suite being monitored. In the final method (subsequently referred to as the Sunfire method), the two mobile phases (MP) used were 70:20 ACN:water with 0.1% ammonium acetate (MP A) and 5:95 ACN:water with 0.1% ammonium acetate (MP

B). The column was a Sunfire C18 3.5 µm, 2.1x150 mm column, the injection volume was 20 µL and the flow rate 0.3 mL min^{-1}. The optimised gradient is shown in Table 4.6 and the corresponding chromatogram in Figure 4.4. Then, this method was validated using an HPLC-DAD (Section 4.2.2.1) and used for SPE cartridge selection and eluent optimisation (Section 4.2.1.2).

Table 4.6: Optimised HPLC gradient for the Sunfire column.

Time (min)	**% MP A** (70% ACN)	**% MP B** (5% ACN)	**Gradient**
0	7.7	92.3	
3	7.7	92.3	
23	85.1	14.9	
24	85.1	14.9	
31	7.7	92.3	
40	7.7	92.3	

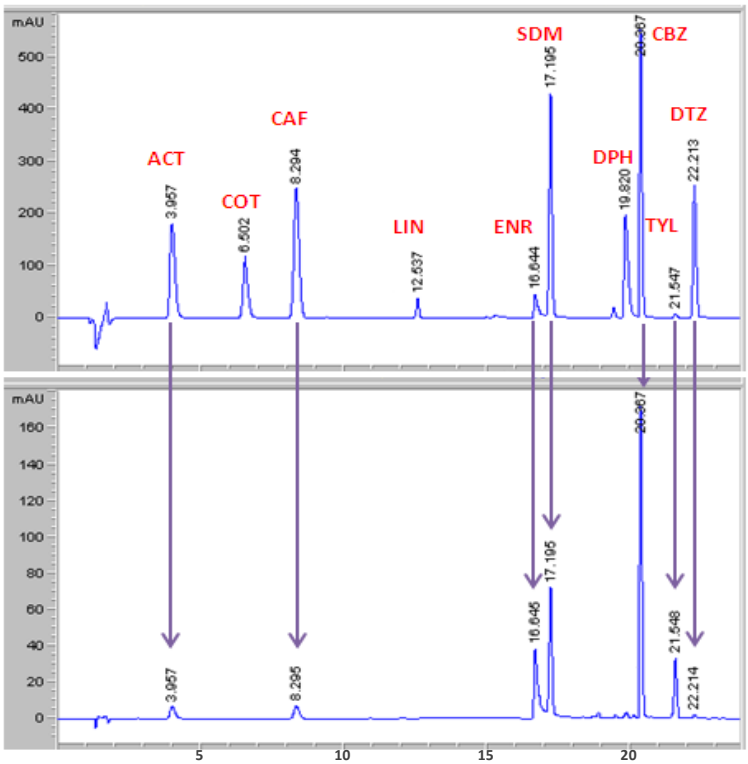


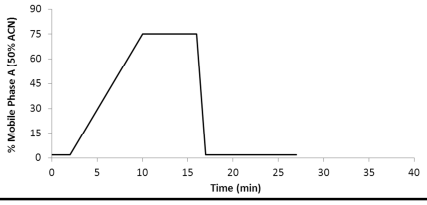
Figure 4.4: HPLC chromatogram for the 10 analytes at 15 mg L^{-1} with detection at 206nm (top) and 300nm (bottom) for the Sunfire C18 column.

A separate method was developed for the use of a Phenomenex Luna PFP (Pentafluorophenyl) 5 µm, 2.0x150 mm column (subsequently referred to as the Luna PFP method, Table 4.7, Figure 4.5). Phenyl stationary phases, such as within the Luna PFP column, show higher aromatic selectivity as compared to C18 columns,

which represent the most widely used columns in reverse-phase chromatographic analyses [224].

Therefore, the potential for using this stationary phase to achieve improved selectivity and sample throughput was investigated. This required further optimisation of the Sunfire method. The mobile phases used were 50:50 ACN:H_2O (MP A) and 100% H_2O (MP B). The pH was modified to pH 3.0 using formic acid. No buffer was added, as it was noted that it did not affect the resulting chromatogram or MS detection (Section 4.2.1.4). An injection volume of 20 µL and a flow rate of 0.3 mL min^{-1} were used.

Table 4.7: Optimised HPLC gradient for Luna PFP column.

Time (min)	**% MP A** (50% ACN)	**% MP B** (100% H_2O)	**Gradient**
0	2	98	
2	2	98	
10	75	25	
16	75	25	
17	2	98	
27	2	98	

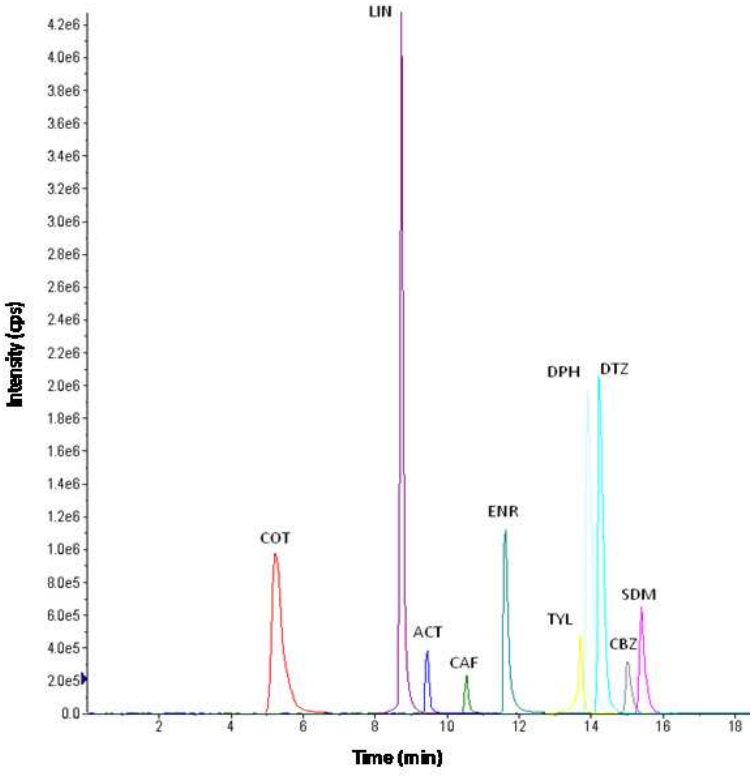


Figure 4.5: HPLC Chromatogram for the 10 analytes at 10 mg L^{-1} using the Luna PFP column and an MS detector.

Use of the Phenomenex Luna PFP column allowed for the run time to be decreased from 40 minutes to 27 minutes. This difference in run times corresponds to a 30% improvement in sample throughput. Therefore, further investigations related to LC-MS/MS hyphenation and validation were carried out using the Luna PFP method (Section 4.2.2.2).

4.2.1.4 MS/MS Method Development

MS was selected as the detector of choice since it provides more conclusive identification of the analyte being detected than UV detection, by providing structural information on the analytes [225]. Furthermore, through the use of tandem mass spectrometry, enhanced analyte selectivity can be achieved [223].

AB SCIEX Instrument. Direct infusion of single standards of the 10 analytes allowed for ideal mobile phase pH and buffer requirements to be identified. The use of a mobile phase at pH 3±0.2 using formic acid without buffer resulted in improved sensitivity for a number of analytes, in particular caffeine, as compared to the use of ammonium acetate or a pH of 6 or 4. Chromatographic separation was still successful when using mobile phases at pH 3 and in the absence of a buffer. Hence, these conditions were used for further development.

The mass spectrometer acquisition parameters for the optimal mobile phase identified were, then, determined. The acquisition method was built using the 2 most intense product peaks for each analyte. As a result, each analyte had 2 precursor/product pairs being investigated (Table 4.8). The second precursor-product pair acted as a confirmatory peak.

Bruker Instrument. Method development on the Bruker MS was carried out in a similar manner to that on the AB Sciex mass spectrometer. Direct infusion of single standards of the 10 analytes within the starting mobile phase (1% acetonitrile in water at pH 3 using formic acid) was initially carried out. This step allowed for the optimal mass spectrometer acquisition parameters for the different analytes to be identified (Table 4.9). The same major precursor and product ions as the AB Sciex instrument were used.

However, due to the operation of the Bruker instrument, only a single set of acquisition parameters can be utilised during a particular run, which is unlike the AB Sciex instrument. Therefore, a number of LC-MS/MS runs of a 1 mg L^{-1} mixed standard of the 10 analytes were carried out using various combinations of average optimised MS parameters. The trap drive was identified to be the parameter with

Table 4.8: Acquisition parameters for the 10 analytes for the Luna PFP method using the AB Sciex Instrument. DP: declustering potential, FP: focussing potential, EP: entrance potential, $CC_{Ent}P$: collision cell entrance potential, CE: collision energy, $CC_{Ext}P$: collision cell exit potential.

Analyte	**Precursor Ion** (Da)	**Product Ion** (Da)	**DP** (V)	**FP** (V)	**EP** (V)	**$CC_{Ent}P$** (V)	**CE** (eV)	**$CC_{Ext}P$** (V)
ACT 1	152.17	109.9	31	370	7	8	23	6
ACT 2	152.17	93.1	31	370	7	8	31	4
CAF 1	195.15	138.0	26	370	12	12	27	8
CAF 2	195.15	110.1	26	370	12	12	33	6
CBZ 1	237.20	194.2	26	370	12	10	27	10
CBZ 2	237.20	193.0	26	370	12	10	45	10
COT 1	177.13	80.1	26	370	9.5	10	35	4
COT 2	177.13	98.1	26	370	9.5	10	29	6
DTZ 1	415.15	178.1	16	340	9	20	37	8
DTZ 2	415.15	109.0	16	340	9	20	85	6
DPH 1	256.17	167.1	1	350	7	12	17	8
DPH 2	256.17	165.2	1	350	7	12	51	8
ENR 1	360.18	316.1	26	370	12	20	25	18
ENR 2	360.18	245.1	26	370	12	20	37	14
LIN 1	407.26	126.0	31	370	10.5	20	41	6
LIN 2	407.26	82.0	31	370	10.5	20	121	10
SDM 1	311.13	156.1	21	360	10	16	29	8
SDM 2	311.13	92.0	21	360	10	16	45	4
TYL 1	916.39	174.2	96	340	12	36	49	10
TYL 2	916.39	100.8	96	340	12	36	67	6

Table 4.9: Acquisition parameters for the 10 analytes for the Luna PFP method using the Bruker instrument.

Analyte	**Capillary** (V)	**Skimmer** (V)	**Cap Exit** (V)	**Oct 1 DC** (V)	**Oct 2 DC** (V)	**Trap Drive** (V)	**Oct RF** (V)	**Lens 1**	**Lens 2**
ACT	-3933	41	125	9	1	25	142	-6	-75
CAF	-3800	15	129	7	1	26	138	-2	-42
CBZ	-4200	83	150	7	2	29	204	-1	-37
COT	-4133	28	122	8	1	25	108	-5	-55
DPH	-4267	36	96	7	1	28	200	-3	-43
DTZ	-4067	26	163	7	2	38	300	0	-34
ENR	-4333	32	163	6	2	35	300	-2	-37
LIN	-4267	41	175	6	2	38	279	0	-31
SDM	-4400	38	154	7	2	34	300	0	-30
TYL	-4467	22	250	6	2	60	300	-2	-37

the greatest control on instrumental sensitivity. Specifically, the higher the parent ion mass, the higher the trap drive voltage is required to be.

Due to the wide range in target analyte molar masses, it was not possible to achieve optimal detection for all analytes, and a compromise had to be made between the number of target analytes and the method's sensitivity. Nine of the original target analytes (Parent ions: 152-415 Da) required an optimal trap drive voltage of 25-40 V, whilst the last (Tylosin, Parent ion: 916 Da) required an optimal trap drive voltage of 60 V. Therefore, it was decided to eliminate tylosin from the analytical suite. Then, the overall optimal parameters for the remaining nine analytes were identified to be as outlined in Table 4.10.

Table 4.10: Average optimised parameters for the Bruker mass spectrometer.

Capillary (V)	4200		
Skimmer (V)	30		
Cap Exit (V)	170	**Nebuliser (psi)**	20
Oct 1 DC (V)	7.5	**Dry gas (l min^{-1})**	8
Oct 2 DC (V)	1.5	**Dry temperature (℃)**	300
Trap Drive (V)	35		
Oct RF (V)	150		
Lens 1	-2		
Lens 2	-40		

4.2.2 Method Validation

4.2.2.1 HPLC Instrumental Validation

The Sunfire HPLC method was validated using spiked HPLC grade water with a DAD as a detector. The requirements for precision and linearity set for validation (Section 3.2.3) were all met. Linearity was determined using a six-point calibration curve at 0.5 mg L^{-1}, 2.5 mg L^{-1}, 5 mg L^{-1}, 7.5 mg L^{-1}, 10 mg L^{-1} and 15 mg L^{-1}. The LOQ was determined to be 0.5 mg L^{-1} for the 10 compounds, with six repeated injections at this concentration resulting in a percentage relative standard deviation (% RSD) of less than 5%. The LOD was determined to be 0.05 mg L^{-1} for the 10 compounds. Further details of method validation and system suitability are given in Appendix F.

4.2.2.2 LC-MS/MS Instrumental Validation

The Luna PFP method was validated using both the AB Sciex and Bruker instrumental setups. The requirements for precision and linearity set for validation (Section 3.2.3) were all met. Precision was assessed at a concentration of 1 mg L^{-1},

as injected[2]. Linearity was assessed by the injection of standards containing all the analytes at concentrations of 10 mg L^{-1}, 5 mg L^{-1}, 1 mg L^{-1}, 0.5 mg L^{-1}, 0.1 mg L^{-1}, 0.01 mg L^{-1} and 0.001 mg L^{-1}, as injected. The methods' LOQ and LOD were determined to be as presented in Table 4.11 and Figure 4.6. Further details of method validation and system suitability are given in Appendix F.

Table 4.11: LOQ and LOD values for the different analytes and mass spectrometers for concentrations as injected. The use of SPE results in a 2000 fold decrease in the specific limit of quantification or detection.

	AB Sciex MS		**Bruker MS**	
Analyte	LOQ (mg L^{-1})	LOD (mg L^{-1})	LOQ (mg L^{-1})	LOD (mg L^{-1})
ACT	0.01	0.0005	0.1	0.01
CAF	0.0005	0.0001	0.1	0.001
CBZ	0.001	0.0001	0.01	0.001
COT	0.01	0.0001	0.01	0.001
DTZ	0.0005	0.0001	0.01	0.001
DPH	0.0005	0.0001	0.001	0.0005
ENR	0.0005	0.0001	0.001	0.0005
LIN	0.0005	0.0001	0.001	0.0005
SDM	0.0005	0.0001	0.001	0.0005
TYL	0.033	0.010		

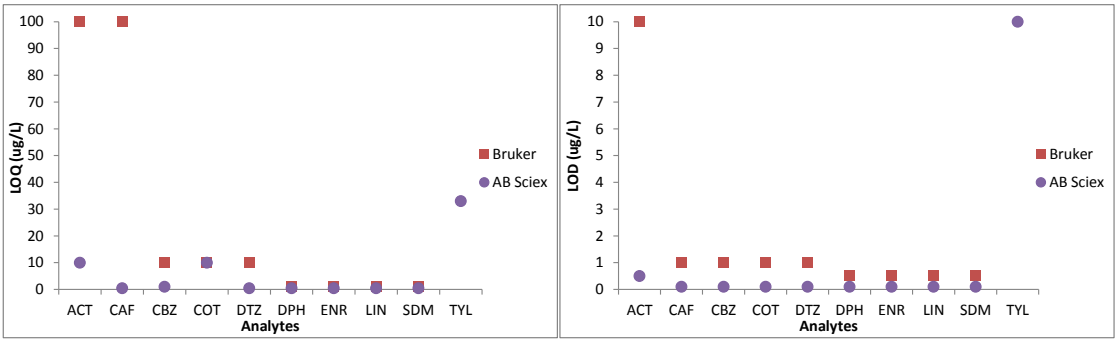


Figure 4.6: Differences in mass spectrometer instrumental sensitivities for the AB Sciex and Bruker instruments for the LOQ (left) and LOD (right) for concentrations as injected.

On comparing the LOQs and LODs on the two instruments, one can note that the overall sensitivity of the AB Sciex instrument is around 2 to 20 times higher than that of the Bruker instrument. This result is largely due to the compromise required in the Bruker mass spectrometer parameters, because the AB Sciex instrument operates by periodically tuning to each analyte's optimal acquisition parameters.

[2] 'As injected' refers to the concentration of the sample on injection into the instrument. When considering the use of SPE a lower actual concentration is effectively being measured.

Therefore, whilst each set of acquisition parameters is not continuously sampled, it is still possible to achieve improved sensitivities. The use of the compromise acquisition parameters for the Bruker instrument further explains why some of the analytes have considerably lower sensitivities on this instrument than the AB Sciex instrument. This difference in sensitivity is particularly true for the lower mass analytes, such as caffeine and acetaminophen, since the parameters were largely directly relevant to higher molar mass analytes.

Nevertheless, although the two instruments had different sensitivities, this was not deemed to be a major factor in the outcome of the present research: although caffeine and acetaminophen have a significantly higher LOQ in the Bruker MS as compared to the AB Sciex MS, they are present at the highest concentrations within surface waters as evidenced through literature and previous analyses carried out using the AB Sciex MS. The removal of tylosin as a manure marker from the analytical suite was considered to be of limited consequence upon the quality of analysis. It was largely a redundant marker for manure when considering the presence of the other manure markers also present within the analytical suite.

4.3 Application of Chromatographic Method to Surface Water Samples

In order to determine the suitability of the selected suite of chemical markers for characterising sewage and manure inputs to surface waters, surface water samples were collected from three monitoring sites in Ireland. These samples were analysed using the developed multi-residue MS methods. The results obtained during the one-year monitoring programme are presented in the next section followed by a discussion of their implications.

4.3.1 Results

Details of the detection frequencies, maximum and mean values of the suite of chemical markers at the three monitoring sites for samples collected between October 2011 and September 2012 are given in Table 4.12. A presence-absence chart for each sample is provided in Figure 4.7. It is evident from the obtained results that the three sites indeed have different characteristics. Of note is that samples collected between October 2011 and March 2012, and those collected between April 2012 and September 2012 were analysed using methods having different LOQs and LODs, as described in Section 4.2.2.2.

Table 4.12: Detection frequency (Freq., %), maximum concentrations (Max, ng L^{-1}) and mean concentrations (Mean, ng L^{-1}) for the 3 sites monitored (n = 12 for each site, except for TYL where n = 6).

		Tullow			**Baunreagh**			**Kilcruise**		
		Freq. (%)	**Mean** (ng L^{-1})	**Max**	**Freq.** (%)	**Mean** (ng L^{-1})	**Max**	**Freq.** (%)	**Mean** (ng L^{-1})	**Max**
Human	ACT	83.3	62.8	203.2	91.7	45.6	152.2	66.7	12.8	70.7
	CAF	91.7	108.6	303.9	83.3	43.4	161.0	58.3	59.2	246.2
	CBZ	75.0	11.6	30.3	25.0	2.4	15.0	58.3	5.4	34.5
	COT	75.0	22.1	93.9	83.3	28.9	92.2	41.7	6.4	24.7
	DPH	58.3	28.4	148.1	33.3	15.2	122.1	33.3	4.4	25.0
	DTZ	58.3	21.6	71.0	75.0	40.9	139.6	41.7	7.3	31.3
Veterinary	ENR	25.0	9.5	97.8	50.0	68.9	215.6	58.3	52.5	217.0
	LIN	16.7	2.6	29.8	100.0	11.5	35.9	91.7	29.1	173.9
	SDM	58.3	53.3	233.5	58.3	56.3	236.0	83.3	94.9	257.2
	TYL	33.3	3.0	17.7	66.7	10.7	29.1	66.7	6.3	21.2

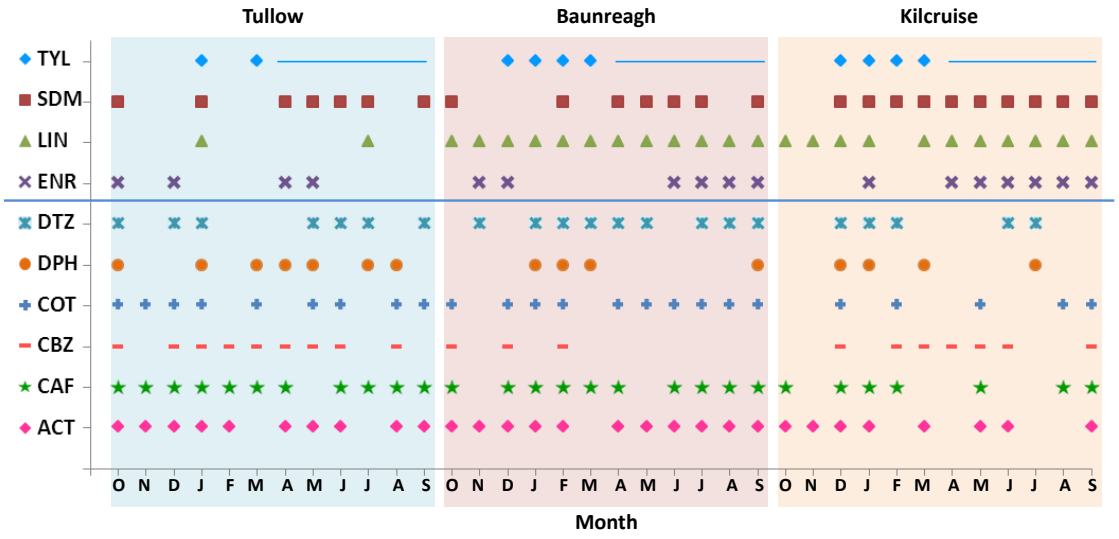


Figure 4.7: Presence-absence chart for data from the monitoring programme (October 2011 - September 2012). Chemical markers above the line are veterinary pharmaceuticals, whilst those below the line are human pharmaceuticals.

Results for the months October 2011 to March 2012 were obtained using the AB Sciex instrument, whilst results for the months April 2012 to September 2012 were obtained using the Bruker instrument. Therefore, there are differences in the analytical method's limits of detection and quantification for the two sample sets, and thus potentially differences in the detection frequencies. Additionally, data on tylosin are only available for the first six months i.e. October 2011 to March 2012.

Concentrations of nitrate were also determined for each sample collected, using ion chromatography by T.E. Laboratories. These results are given in Table 4.13.

Table 4.13: Nitrate concentrations (mg L^{-1} as N) for the collected samples.

Sampling Session		Nitrate as N (mg/L)		
		Tullow	**Baunreagh**	**Kilcruise**
2011	**Oct**	9	3	3
	Nov	8	3	3
	Dec	13	4	4
2012	**Jan**	13	3	3
	Feb	17	4	4
	Mar	15	2	3
	Apr	17	2	2
	May	11	3	3
	Jun	11	2	2
	Jul	14	3	3
	Aug	12	2	2
	Sep	15	3	3

4.3.1.1 Data Analysis

Data analysis was carried out in order to assess for significant variations by monitoring site and season of sample collection. Levene's test for homogeneity identified the presence of significantly different variances within the various chemical marker data sets. This is due to the nature of the data available, where a considerable number of values are equal. These represent those values that are below the detection limit (inserted as 0) or below the quantification limit (inserted as the mid-point value between the detection and quantification limit). Therefore, data transformation of the obtained chemical marker data sets was required. A global rank method of data transformation was used[3]. However, for tylosin, when using the season as a dependent variable, and for enrofloxacin, when using the site as a dependent variable,

[3] Rank transform tests have been widely studied for use in studies with null values [226, 227] as a method of using existing statistical analyses to compute non-parametric statistics [228]. Furthermore, it allows for multiple comparison analyses, such as Tukey's post-hoc test, to be carried out [228]. A limitation of such analyses is that they are only suitable for testing for main effects and not interactions in two-way layouts [229].

homogeneity was still not achieved. Therefore, they were excluded from further statistical analyses.

Two-way ANOVA analysis was performed on the transformed pharmaceutical concentration data sets and untransformed nitrate concentrations to assess for variances within the data set by season and site (Table 4.14). This established a number of significant factors. Four chemical markers (ACT, CBZ, DTZ, LIN) and nitrate were identified to be present at significantly different concentrations depending upon the sampling site, whilst two chemical markers were identified to be present at significantly different concentrations depending upon the season (ACT, SDM).

Table 4.14: Results for ANOVA analyses for differences by site and season. Values in red are significant terms ($p<0.05$).

	Site		Season	
Analyte	**F**	**Sig**	**F**	**Sig**
ACT	7.356	0.003	5.098	0.006
CAF	2.363	0.111	2.658	0.066
CBZ	3.650	0.038	0.498	0.686
COT	3.215	0.054	0.553	0.650
DPH	0.986	0.385	0.431	0.732
DTZ	4.089	0.027	1.642	0.200
ENR			1.722	0.184
LIN	11.670	0.000	0.589	0.627
SDM	1.957	0.159	5.587	0.004
TYL	0.679	0.524		
Nitrate	131.142	0.000	12.892	0.927

These significant differences were further explored using multiple comparison analysis, specifically the Tukey post-hoc test. The resulting homogeneous subsets are presented in Table 4.15.

Table 4.15: Homogeneous subsets using Tukey's post-hoc test for parameters found to be significant using ANOVA. The different homogeneous subsets are grouped and shaded in alternate colours ($p<0.05$).

Season		Site					
ACT	**SDM**	**ACT**	**CBZ**	**DTZ**	**LIN**	**Nitrate**	
Spring	Winter	KC	BR	KC	TL	BR	↓
Summer	Spring	BR	KC	TL	KC	KC	increasing
Autumn	Autumn	TL	TL	BR	BR	TL	concentration
Winter	Summer						

In order to assess for the presence of correlations between the various chemical markers and nitrate, Kendall's τ coefficient was also determined (Table 4.16). As can be observed from the table below, none of the chemical markers were significantly

correlated to the concentration of nitrate. This is likely related to the small size of the data set.

Table 4.16: Results for Kendall's τ correlation analysis for the various chemical markers and nitrate (n=36, except TYL: n = 18; $p<0.05$).

Analyte	Nitrate Kendall's τ Correlation Coefficient	Sig
ACT	0.171	0.091
CAF	-0.038	0.383
CBZ	0.134	0.156
COT	0.043	0.371
DPH	0.002	0.494
DTZ	0.070	0.304
ENR	-0.063	0.321
LIN	0.022	0.432
SDM	-0.035	0.393
TYL	0.065	0.369

4.3.2 Discussion

From the results obtained during the one-year monitoring programme, a number of observations could be made in relation to the potential for a suite of chemical markers to differentiate and characterise sewage and manure inputs into surface waters. The three monitoring sites selected (Section 3.1) are all located within the Irish south eastern river basin district but they form part of separate river catchments (Figure 4.8) and have differing site characteristics.

Baunreagh and Kilcruise represent sites upstream of any WWTPs. The Baunreagh sampling site is in the Nore river catchment and, more specifically, the Dinin-Coolcullen Upper river sub-basin, which is 11.5 km^2 in size. The Kilcruise sampling site forms part of the Barrow river catchment and is located within the Upper Douglas sub-basin, which is 16 km^2. Being upstream catchments, with no evident point sources of nitrate contamination, diffuse nitrate inputs are expected to predominate at these locations. On the other hand, the Tullow sampling site forms part of the Slaney river catchment. The river sub-basin within which the site is located is around 58 km^2 and is considered to be a downstream sub-catchment with the upstream portion of the river passing through agricultural and urbanised areas. Additionally, the sampling site is located a few metres downstream of the Tullow WWTP discharge point, which would be expected to elevate nitrate concentrations within receiving waters.

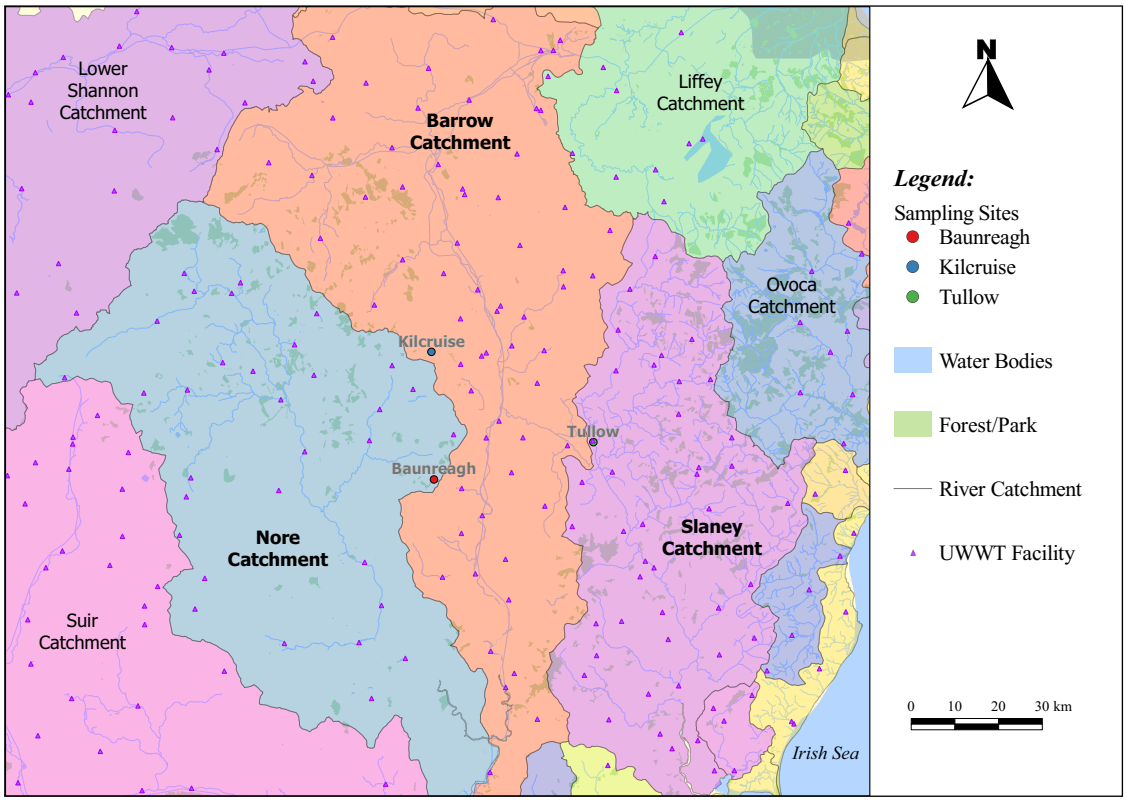


Figure 4.8: Map of the river catchment to which the sampling sites pertain together with the locations of the urban wastewater treatment facilities.

As expected, on the basis of the potential inputs of nitrate, dependent of river basin size and vicinity to point sources of nitrate contamination at the three sites, nitrate concentrations are significantly higher within samples collected from Tullow (Tables 4.13 and 4.15). This is true for all collected samples, with samples from Tullow being 3 to 9 times more contaminated by nitrate than samples from Baunreagh and Kilcruise. Meanwhile, nitrate concentrations at Baunreagh and Kilcruise were not observed to differ significantly from each other within the samples collected (Table 4.15).

Of note is that, although no significant differences in nitrate concentrations were observed for samples collected from Baunreagh and Kilcruise, the sources of nitrate and the proportion of the various sources might differ at the two sites. Through the use of data on the presence of the selected suite of sewage and manure chemical markers, further differentiation and characterisation of the sewage and manure inputs can be achieved. These observations and their implications are discussed in the following two sections.

4.3.2.1 Sewage Chemical Markers

Based on site characteristics, it is expected that samples from Tullow would be contaminated by sewage to the greatest extent. This expectation is largely in view of the Tullow WWTP discharging effluent just upstream of the sampling site and acting as a point source for such contamination. At Baunreagh and Kilcruise, no WWTPs discharge upstream of the sampling sites. Therefore, no evidence of sewage contamination was expected to be found.

An unanticipated finding was the detection of sewage chemical markers, including raw sewage markers such as acetaminophen and caffeine, within most samples (Figure 4.7). This finding indicates infiltration of raw sewage at the three sampling sites and is of particular concern due to health and environmental concerns associated with sewage infiltration, as discussed previously in Section 2.2. There are several possible explanations for these results. At Baunreagh and Kilcruise, two potential diffuse sources of sewage chemical markers were identified. These are, namely, sewage sludge used as a fertiliser for agricultural land and effluent from domestic on-site wastewater treatment systems[4] (OSWTS).

Of the two explanations, sewage infiltration arising from the application of sewage sludge from WWTPs as an organic fertiliser is not expected to be a major contributor of sewage chemical markers within the collected samples. This factor is because pharmaceuticals are largely water soluble compounds (Section 2.5.4). Therefore, their adsorption to WWTP sludges is expected to be extremely limited. In fact, studies on adsorption of caffeine to sewage sludges has been found to be low [158]. Similarly, studies on the sorption of acetaminophen to WWTP biosolids have concluded that, although removal rates of acetaminophen within WWTPs are high, the primary removal mechanism is through microbial degradation [157]. The importance of microbial degradation for acetaminophen removal could be an important factor in the seasonal variation observed for acetaminophen concentrations. During the colder months, acetaminophen concentrations were observed to be significantly higher, which corresponds to the period when microbial action would be expected to be lowest.

Since sorption to sludges is limited and it is these sludges that are being spread as organic fertiliser, they would be expected to contain very low concentrations of adsorbed pharmaceuticals. Consequently, it is unlikely that they would be detected in receiving waters. Therefore, it is believed that effluent from OSWTSs is the

[4] These are systems "involving physical, chemical, biological or thermal processes, or a combination ... utilised for the treatment or disposal of domestic wastewater, or the sludge derived from domestic wastewater" [230].

major contributor at these two sites. Although no WWTPs are present upstream of the sampling sites, a number of buildings are present within the corresponding river sub-basins (Figure 4.9). Each building would be expected to have a form of OSWTS since they are outside of any WWTP agglomeration catchment boundaries.

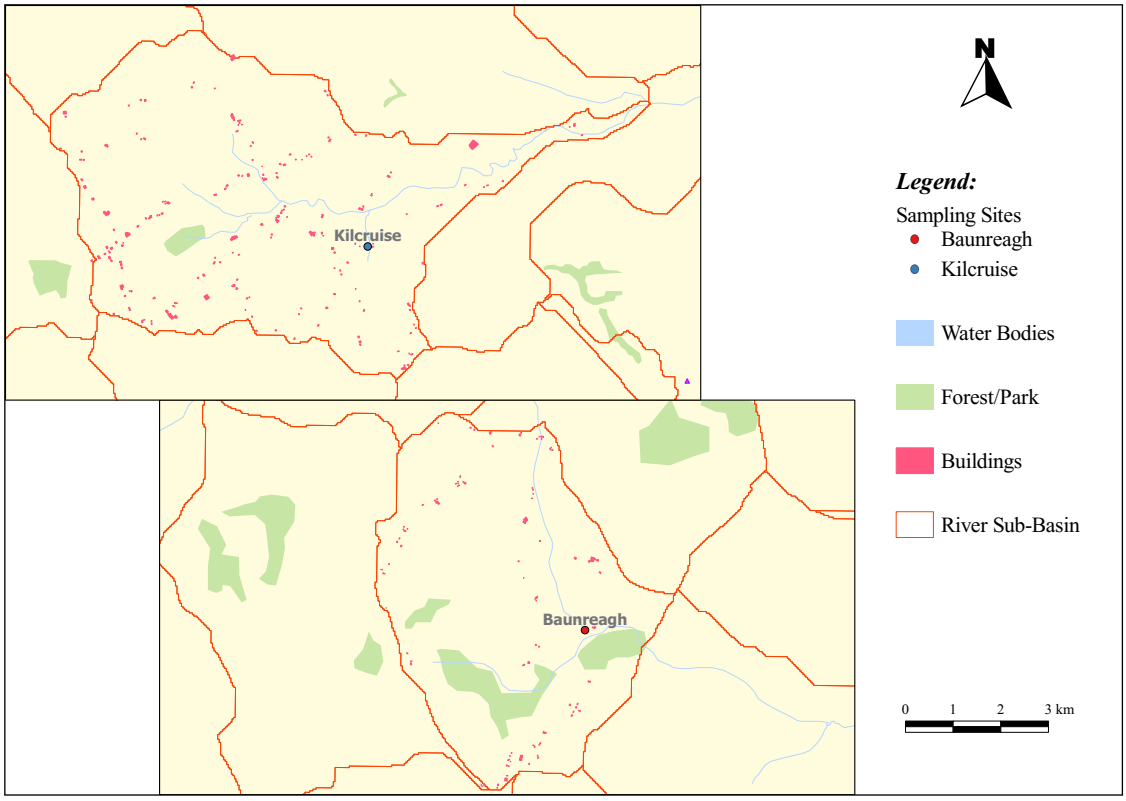


Figure 4.9: Map of the river sub-basins for Kilcruise and Baunreagh together with the locations of buildings in the area.

In Ireland, OSWTSs generally consist of a septic tank in which primary treatment occurs followed by a percolation system in the soil, which provides limited secondary and tertiary treatment (Figure 4.10) [231]. The operation of OSWTSs is of particular importance in Ireland where over one third of the population's wastewater is treated by such systems [232]. This factor, linked to the fact that bedrock formations in Ireland are commonly fissured or fractured [233], leads to a more direct transport pathway between the septic tank and the ground or surface water body. In fact, a new code of practice for OSWTSs in Ireland has recently been published [231]. In particular, it outlines improved OSWTSs designs to ensure environmental protection and a reduction in the range of acceptable subsoils receiving effluent depending upon attenuation potential [233].

Nevertheless, since most pharmaceuticals are highly water soluble (Section 2.2.1), they would be expected to be readily transported to the groundwater or surface

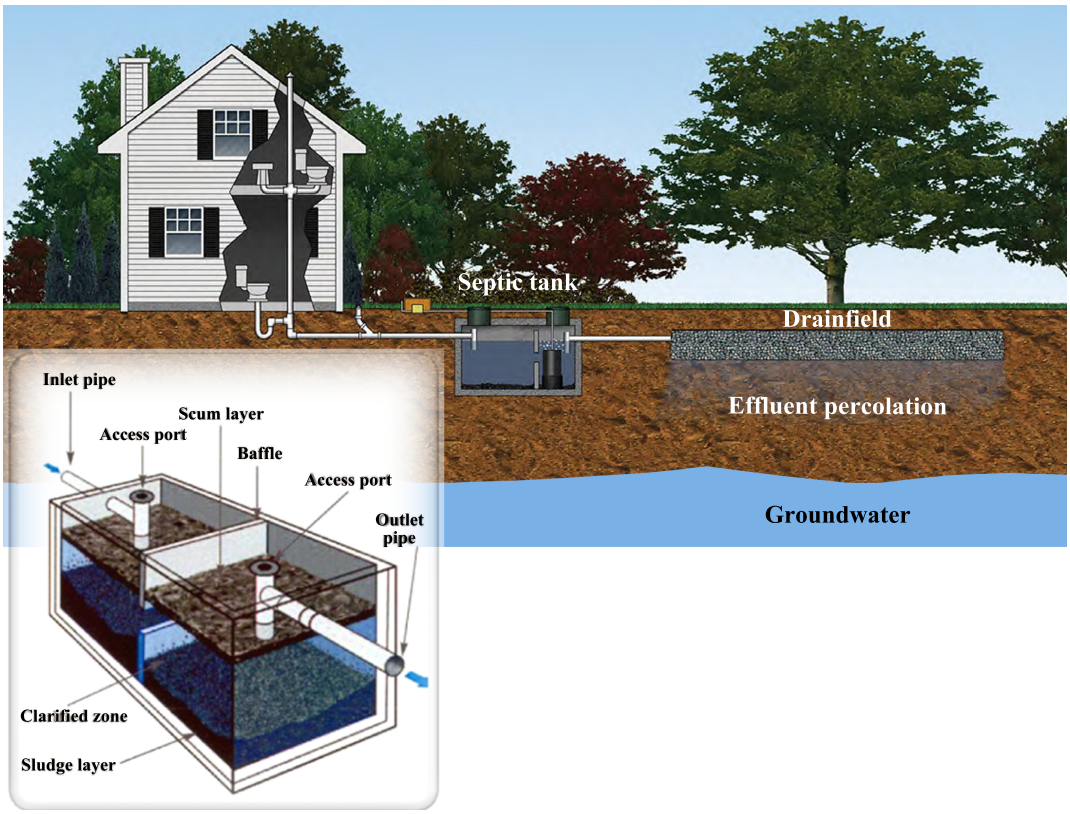


Figure 4.10: Schematic diagram of an on-site wastewater treatment system. Inset: Septic tank design detail. Adapted from [234, 235].

water body. This is especially so if the microbial communities within the soil percolation layers are not suited for biodegradation of the particular pharmaceutical or have alternative sources of nutrition, such as soil organic matter. Furthermore, within the Baunreagh and Kilcruise catchments, the predominant subsoil material is of limestone sand and gravel [236]. Such subsoil types can be accompanied by extensive fracturing and faulting, which would act to further enhance the limestone permeability [237], and consequently direct infiltration from upstream OSWTSs.

In contrast, the unexpected presence of raw sewage chemical markers within samples collected from the Tullow sampling site is attributed to a different source. At this site, infiltration from OSWTSs is expected to be largely diluted by effluent from the Tullow WWTP. This negligible effect is notwithstanding the fact that within the sub-basin there are dwellings lying outside of the Tullow WWTP agglomeration catchment boundary (Figure 4.11) and which would be expected to have OSWTSs. A similar dilution effect is expected to occur for any manure chemical markers from upstream flows.

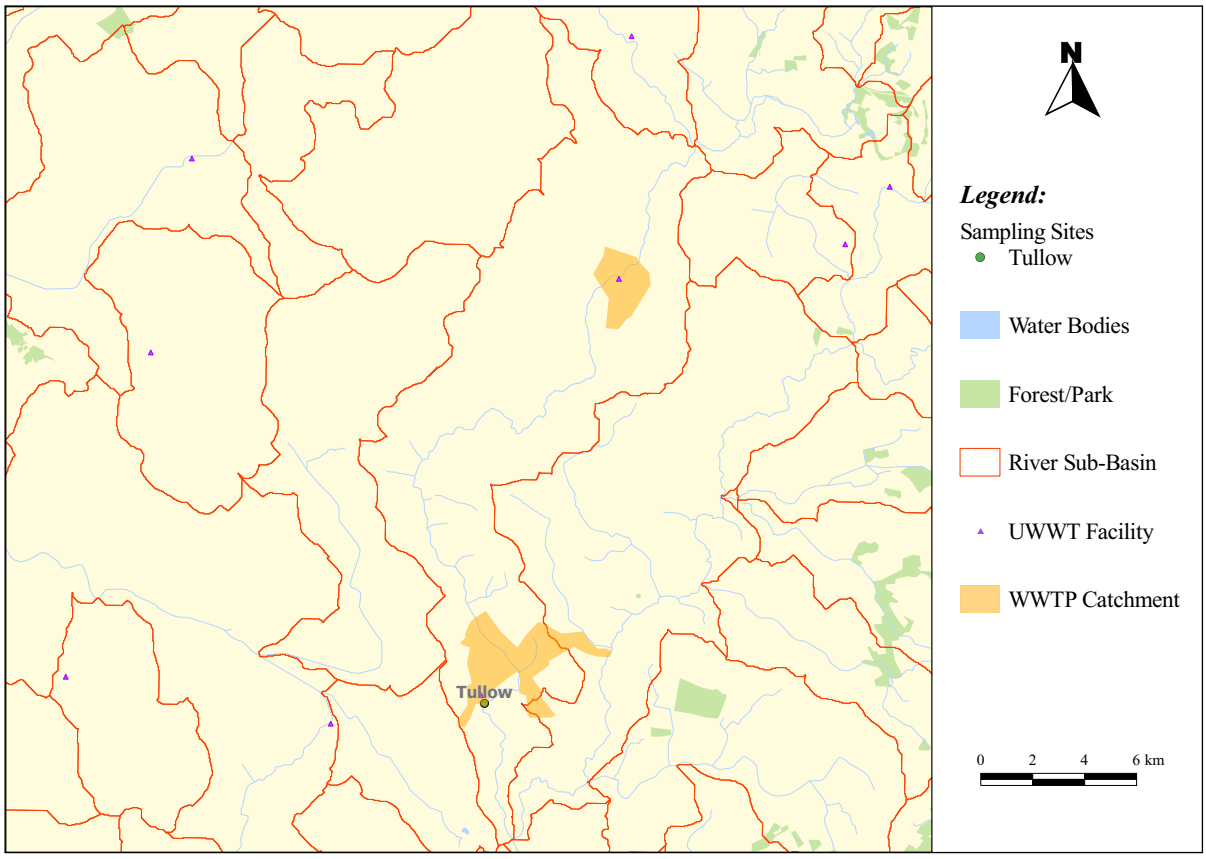


Figure 4.11: Map of the river sub-basins for Tullow together with the location of the WWTP catchment.

At Tullow, the potential for incompletely treated WWTP effluent was identified to be particularly high and is expected to be the major reason for raw sewage infiltration. The Tullow WWTP is a secondary treatment plant commissioned in 1989 and designed with a capacity of 4000 p.e. [238]. However, it is currently receiving a wastewater load of around 5000 p.e. [239] and is failing to meet requirements. In fact, during 2009, the Tullow WWTP was considered to have failed to reach UWWTD requirements due to the quality of samples collected [240], and there are plans for upgrading the current plant [238]. Therefore, the plant is currently functioning at over capacity indicating that limitations for treatment within the plant are indeed present, resulting in incomplete effluent treatment. Additionally, around 15km further upstream of the Tullow WWTP is another WWTP, at Rathvilly, which has also been identified to require significant upgrading [239].

Although all three monitoring sites were identified to be contaminated by sewage, the Tullow monitoring site is the site contaminated to the greatest degree. In fact, the detection frequency of sewage:manure chemical markers (normalised for the number of analytes in each group) is in the ratio of 3:1 within samples collected from

the Tullow monitoring site, indicating that sewage contamination is much higher than manure contamination at this site. Meanwhile, within samples collected from Baunreagh or Kilcruise, the detection of sewage:manure indicators is in the ratio of 1:1.

The sewage chemical marker carbamazepine was detected at significantly higher concentrations at Tullow as compared to the other sites (Table 4.15). Nevertheless, there is also considerable sewage infiltration at Baunreagh (Figure 4.12). Samples collected from this site fell within the subset containing the highest concentrations for both acetaminophen and diltiazem (Table 4.15). For acetaminophen, the homogeneous subset also contained Tullow, whilst for diltiazem it formed a unique homogeneous subset. Meanwhile, concentrations of sewage chemical markers at Kilcruise are the lowest of the three sites, which indicates the importance of carrying out environmental forensics studies in determining sources of nitrate contamination. A case in point is that, although both Baunreagh and Kilcruise were determined to have similar levels of nitrate contamination, sewage contamination was higher at Baunreagh.

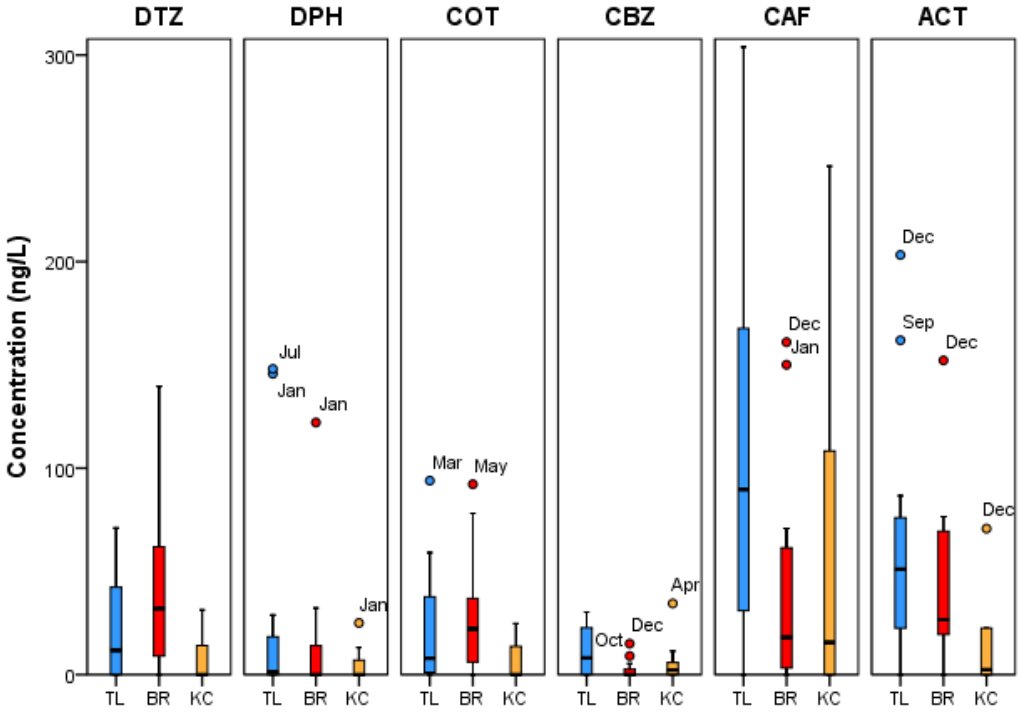


Figure 4.12: Box plots showing 25^{th}, 50^{th} and 75^{th} percentiles for sewage marker concentrations at the three monitoring sites. The whiskers show the 10^{th} and 90^{th} percentiles, and the circles represent outliers.

The findings described within the previous paragraphs relate specifically to current efforts within Ireland in the area of OSWTS. These are largely in relation to

the Water Services (Amendment) Act of 2012 [230] as a result of a European Court of Justice judgement made against Ireland in 2009 [241] due to Ireland's failure to fully transpose and implement European requirements for the disposal of domestic wastewaters for OSWTSs and ensuring the protection of human health and the environment. As a consequence of this judgement, a national inspection plan for OSWTSs will be initiated in the coming months [45].

Considering the extent of OSWTS usage in Ireland, it is clear that it is not possible for all systems to be monitored. The use of proxy inspections and routine inspections for identifying contamination risk have been suggested by the Irish Environmental Protection Agency (EPA) to the water services authorities (generally the local authorities) who are to implement the plan [242]. By monitoring chemical markers within water bodies, risk-based prioritisation for OSWTS inspection plan implementation could be carried out. Therefore, taking as an example Baunreagh and Kilcruise, it would allow for dwellings upstream of the monitoring site at Baunreagh to be prioritised over those upstream of the Kilcruise monitoring site.

4.3.2.2 Manure Chemical Markers

When considering manure chemical markers, from the results obtained it is clear that lincomycin is a particularly good indicator of manure contamination. Its frequency of detection is much higher at Baunreagh (100%) and Kilcruise (92%) as compared to Tullow (18%) (Figure 4.7). Additionally, lincomycin was observed to be present at significantly higher concentrations at Baunreagh and Kilcruise than Tullow (Table 4.15 and Figure 4.13). These observations indicate increased manure inputs at these sites as compared to Tullow.

Lincomycin is used to treat a wide range of animals and is used in both intensive and pasture scenarios (Table 4.2). Such activities, which act as diffuse sources of nitrate contamination, are expected to occur upstream of the sampling sites at Baunreagh and Kilcruise considering land use within the two sub-basins (Table 3.1). Land cover within the Baunreagh and Kilcruise sub-basins is exclusively agricultural, forest or semi-natural with no artificial cover such as developed and urbanised areas.

Although lincomycin represents the only manure chemical marker that showed significant differences by site, a number of other observations further corroborate these findings. Enrofloxacin and tylosin could be noted to be present at higher frequencies of detection and higher mean and maximum concentrations at Baunreagh and Kilcruise than Tullow (Figures 4.7 and 4.13), indicating a higher contribution of manure contamination at these sites.

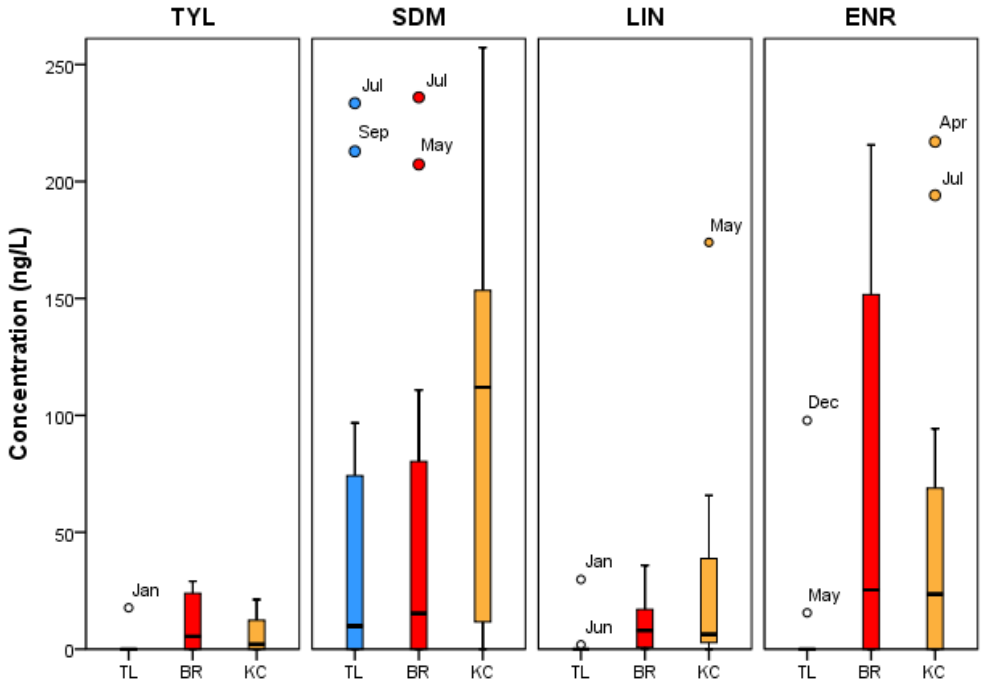


Figure 4.13: Box plots showing 25^{th}, 50^{th} and 75^{th} percentiles for manure marker concentrations at the three monitoring sites. The whiskers show the 10^{th} and 90^{th} percentiles, and the circles represent outliers.

Overall, sulfadimethoxine was identified to show the least potential as a chemical marker of manure contamination. Although its mean and maximum concentrations of detection are higher at Baunreagh and Kilcruise than Tullow, they are only marginally higher. In particular, maximum concentrations at Baunreagh and Kilcruise are less than 10% higher than those at Tullow. Additionally, its frequency of detection at Tullow and Baunreagh is identical, which could be explained by the fact that one of the main applications of sulfadimethoxine is in the treatment of cats and dogs [215], which are generally domestic animals. Although it is approved for use in the treatment of cattle, only comparatively low doses of sulfadimethoxine are required for effective treatment [216]. These factors would, therefore, contribute to such observations.

Nevertheless, the potential of sulfadimethoxine to be a suitable chemical marker of manure contamination is particularly low. It was detected in significantly different concentrations depending upon the sampling season (Table 4.15). Therefore, great attention should be paid to the sampling period if it were to be used as a chemical marker. If samples are to be collected in winter, its contribution is likely to be greatly underestimated, whilst if samples are collected in summer, they could be overestimated (Figure 4.14).

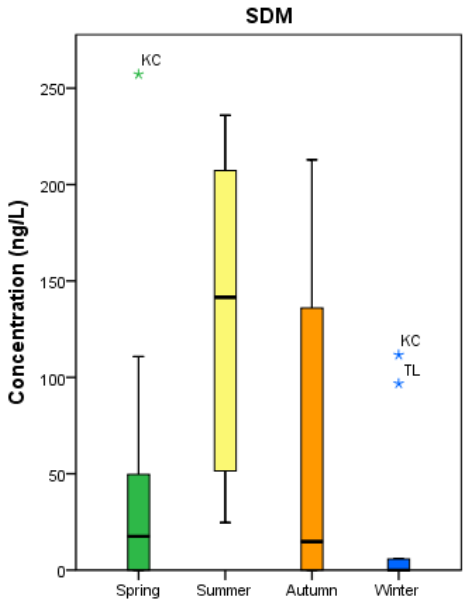


Figure 4.14: Box plots showing 25^{th}, 50^{th} and 75^{th} percentiles for sulfadimethoxine within the different seasons. The whiskers show the 10^{th} and 90^{th} percentiles, and the stars represent outliers.

4.4 Conclusion

Within this chapter, the potential for using pharmaceuticals as chemical markers of sewage and manure contamination was presented. 10 chemical markers were selected, six of which are sewage indicators whilst the remainder are indicators of manure. Through an understanding of their physico-chemical characteristics additional source characterisation could be achieved.

Two multi-residue mass spectrometric methods were developed and validated for the simultaneous analysis of the suite of chemical markers selected within surface waters. Chromatography was chosen as it represents the routinely used analytical technique for determining pharmaceuticals within environmental waters. Since method detection limits are in the pg L^{-1} to ng L^{-1} range, sites with low levels of contamination could also be studied and the sources of contamination identified.

The application of the validated methods to samples from three monitoring sites in Ireland allowed for sewage and/or manure point and diffuse sources of contamination to be characterised. Of particular mention is the identification of raw sewage infiltration within all three monitoring sites. At Baunreagh and Kilcruise, this has been attributed to effluent from OSWTSs. At Tullow, a different factor is expected to be the main contributor of such raw sewage, namely ineffective treatment of wastewaters reaching the Tullow WWTP. These findings are particularly relevant to current efforts within Ireland related to OSWTSs and the implementation of a

national inspection plan for such systems.

The obtained results also have important implications for the use of chemical markers as indicators of sewage and manure inputs. As previously described (Section 2.5.4), some studies for assessing sewage inputs into surface waters have been carried out. However, in instances where these have been applied, it is commonly the case that just one chemical marker is used [e.g. 186–188, 190, 243]. Yet, as shown by the obtained results, the use of a suite of chemical markers to achieve input characterisation is necessary because it is only through the use of a suite of chemical markers that the different sources of contamination, e.g. raw and treated sewage, can be identified. Furthermore, an increased confidence in results would be obtained as it could help limit the effects of changes in such factors as seasonal variations and prescribing frequencies.

The use of SPE LC-MS/MS is one of the most widely adopted analytical techniques for the determination of chemical markers within environmental samples. However, there are a number of issues related to its use. In particular are the time requirements related to method development and validation as well as sample processing. Furthermore, the utilised analytical instrumentation, and in particular mass spectrometers, require frequent and costly servicing in order to achieve optimal performance. Additionally, relatively large sample volumes are necessary, 4 litres in this case, which makes it a cumbersome method to carry out. Therefore, having shown the strength of using a suite of pharmaceuticals and related compounds as chemical markers to differentiate and characterise sewage and manure inputs into surface waters, the potential of using alternative analytical techniques to chromatography is described in the coming chapter (Chapter 5).

Chapter 5

Results & Discussion: Alternative Analytical Techniques

This chapter focusses on the use of alternative analytical techniques that might eliminate, or at least reduce, dependency on LC-MS/MS analysis in environmental forensics studies. To date, far too little attention has been paid to alternative means for chemical marker detection in environmental monitoring studies. Therefore, two proof-of-concept studies were performed for the application of alternative analytical techniques to LC-MS/MS, namely, NMR spectroscopy (Section 5.1) and immunoassays (Section 5.2). The techniques' potential suitability, as well as limitations, for achieving chemical marker detection in surface water samples is assessed. Particular focus is placed on achieving limits of detection relevant to pharmaceutical concentrations within surface waters.

5.1 NMR Spectroscopy

In this section, an overview of the pertinent characteristics of NMR as an analytical tool for environmental forensics applications is given (Section 5.1.1). This overview allows for a comparative analysis of NMR and chromatographic techniques, which have been discussed in Chapter 4. It is followed by a discussion of method development (Section 5.1.2) and an investigation of the technique's potential for use with surface water samples (Section 5.1.3). Finally, the outcomes of the present investigation and the implications for using NMR as an environmental forensics tool is discussed (Section 5.1.4).

5.1.1 Using NMR in Environmental Forensics

NMR spectroscopy is a technique that exploits differences in energy absorption by nuclei when they transition between nuclear spin states. During NMR analysis the sample is placed in an external magnetic field, which causes the magnetic nucleus to adopt an orientation of different energy [244]. Then, electromagnetic radiation is applied at a frequency that causes the nuclide to go from the lower energy level to the upper one [245]. The nuclide's identity and its chemical environment determine the frequency required [245], which allows the identification of molecular structures.

NMR spectroscopy is widely employed to determine organic structures and provide quantitative information at the molecular level. It has been used in a wide range of fields, including chemistry, food science, biology and medicine. Its applications include the analysis of complex mixtures, such as biological fluids, foods, drugs, cells and intact living systems [246]. However, so far, its application to environmental samples has been largely lacking.

5.1.1.1 Advantages of using NMR

The use of NMR as an analytical tool for environmental forensics applications is expected to afford a number of advantages over LC-MS/MS. First of all, method development requirements for NMR analysis are low, thereby reducing time constraints. As a result, the extensive time, and consequently cost, investments for method development, transfer and validation associated with LC-MS/MS techniques are eliminated. Furthermore, minimal sample preparation is generally required in NMR analysis [246].

Since NMR is a non-destructive technique, it also allows for the sample to be available for further analysis at a later stage [246, 247], unlike LC-MS/MS. This characteristic is of particular relevance to environmental forensics studies where NMR could be used for early screening, yet the sample could still be available for further analysis as necessary. NMR also provides rich structural and quantitative information on compounds and nuclei of interest [245, 246]. At the same time it is non-selective, thus eliminating the need for analyte-specific method development.

However, the non-selective nature of NMR analysis can be a limitation for environmental forensics applications where the NMR-active nucleus being monitored is ubiquitous in nature. When using ubiquitous nuclei, such as proton, carbon or phosphorus, signals arising from all sample components that contain the specific nucleus are aggregated into a single NMR spectrum. This aggregation leads to extremely complicated and overlapping spectra. As a result the spectra would be difficult to interpret, especially if an NMR expert is unavailable within the analytical labora-

tory or legal forum where decisions emanating from environmental forensics studies are taken.

In order to limit interference from sample components other than the analyte of interest, the ^{19}F nucleus was selected for the present application. Only around a dozen naturally occurring organic compounds that contain fluorine have been identified to date [248, 249]. This is because, although fluorine is the most abundant halogen in the earth's crust, most terrestrial fluorine is bound in an insoluble form hindering uptake by bio-organisms [249, 250].

On the other hand, over 30% of synthetic pharmaceuticals available on the market are fluorinated [251]. These include two of the top 15 most-prescribed pharmaceuticals in Ireland [252]. This development has largely occurred within the last two decades [253] as a result of fluorine's physico-chemical characteristics, such as its small size, strong electronegativity and the C-F bond's low polarisability [253, 254]. These characteristics lead to increased metabolic stability of pharmaceuticals due to a lower susceptibility of nearby moieties to cytochrome P450 enzymatic oxidation [253, 254]. Therefore, the synthesis and use of fluorinated pharmaceuticals is expected to rise in the coming years. This aspect increases the scope for using NMR spectroscopy in environmental forensics studies identifying anthropogenic inputs.

Additionally, the ^{19}F nucleus is particularly suited to NMR analysis. It is present at 100% natural abundance unlike for example ^{13}C at 1.108% natural abundance [245]. Thus, lower detection limits may be achieved. Moreover, the ^{19}F nucleus is highly sensitive to NMR [255, 256]. In a field of 9.39 T the ^{19}F NMR frequency is of 376.3 MHz as compared to 400 MHz for proton NMR [245]. Meanwhile, for example the frequency for ^{13}C is of just 100.6 MHz [245]. The ^{19}F nucleus' high natural abundance and relatively high sensitivity result in a reduced number of acquisitions being required as compared to other nuclides that may be used. Consequently, time requirements for analysis are lower.

A further advantage of using ^{19}F NMR is that fluorine chemical shifts occur over a wider range than for proton NMR [248, 256]. For example, aromatic fluorine shifts occur over a range of over 40 ppm (Figure 5.1), as opposed to just around 2 ppm for aromatic proton shifts [245]. This range arises from the fluorine nucleus being typically surrounded by a complement of nine electrons as opposed to a single electron in protons [256]. The nucleus' sensitivity to fluoroaromatic ring substituents and the solvent and solute concentrations cause further shifts [248]. This wider range of chemical shifts would allow for increased resolution as there would be a decreased scope for overlap.

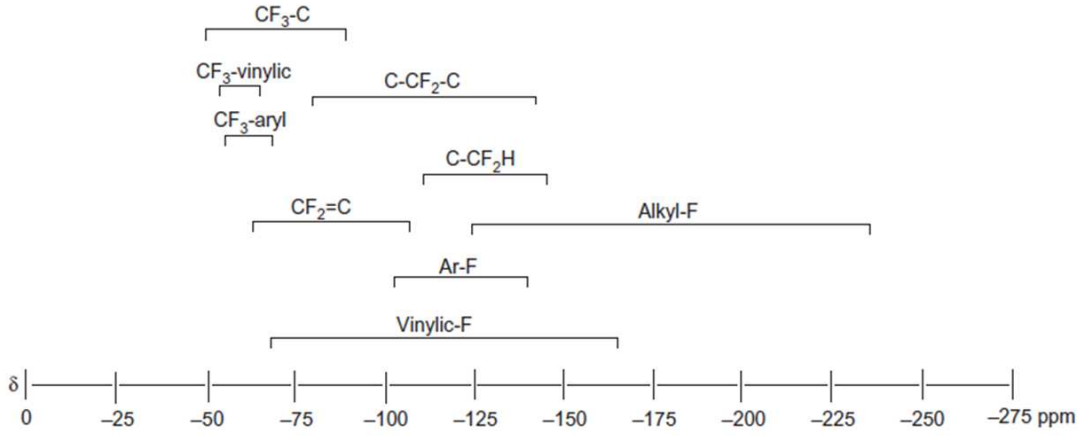


Figure 5.1: Overview of fluorine shifts relative to $CFCL_3$ [247].

5.1.1.2 Limitations of using NMR

Although the use of NMR has a number of advantages, its limitations must also be considered. The main perceived limitation is that the use of NMR in this capacity has yet to gain widespread acceptance. In addition, the use of NMR for the detection of chemical markers within surface waters is mainly limited to fluorinated compounds, unless a knowledgeable NMR user is available.

A second limitation is that within fluorinated pharmaceuticals suitable for such environmental forensics applications, only one fluorine nucleus is generally present within each molecule. Thus, the detection limit of the method is increased, unlike the case of protons, of which there is likely to be a higher number of equivalent nuclei in each molecule.

A final limitation to adopting NMR as an analytical tool in environmental forensics studies is the availability of NMR instruments within environmental laboratories. However, most academic chemistry departments would have an NMR instrument on their premises and the technology to obtain fluorine NMR spectra is practically identical to that required for obtaining proton NMR data [256]. Since the time required for analysis is not too extensive, access to an NMR should be achieved with ease. Furthermore, the cost for sub-contracting such analyses to academic institutions are minimal, due to the reduced sample preparation, time method development and instrument usage requirements.

5.1.2 Method Development

5.1.2.1 Analyte Selection

Arising from the factors outlined in Section 5.1.1, the fluorine nucleus was selected for investigation. As described previously, there exist a large number of fluorine containing pharmaceuticals. These include some of the most widely used pharmaceuticals such as:

- Atorvastatin (Lipitor): a statin, used for lowering blood cholesterol levels in humans. Within Ireland, it is one of the top four most prescribed medications within the general medical services, drugs payment and long term illness schemes [252];
- Fluoxetine (Prozac): an antidepressant, which is in the top 100 most prescribed medications in Ireland on the general medical services and drugs payment schemes;
- Ciprofloxacin (Ciprobay): a fluoroquinolone broad spectrum antibiotic used in the treatment of gram negative pathogens, and;
- Enrofloxacin: a fluoroquinolone antibiotic used in veterinary treatment.

Enrofloxacin (CAS: 93106-60-6) was selected as the fluorinated chemical marker for this scoping study on the use of NMR as an analytical tool in environmental forensics. It is a mono-fluorinated aromatic compound whose structure is given in Figure 5.2 and is a chemical marker of manure contamination within surface waters, as discussed in Chapter 4. The other analytes are expected to be suitable chemical markers for sewage for NMR analysis.

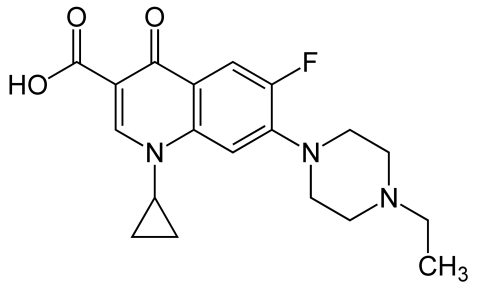


Figure 5.2: Structure of enrofloxacin.

5.1.2.2 Dissolution of Enrofloxacin

The solubility of enrofloxacin in different solvents was initially investigated. Solubility is an important consideration in such studies in order to determine the most

appropriate and cost effective solvent for use (Table 5.1). Irrespective of the solvent selected, costs should be much lower than purchasing LC-MS grade solvents, because in NMR analyses, solvents are used as diluents rather than as mobile phases and, therefore, much smaller volumes are required.

Table 5.1: Solubility of enrofloxacin in different solvents at a concentration of 4 mg ml^{-1} and room temperature.

Solvent	Result
D_2O	Suspension
Acetonitrile-d_3	Partially dissolved
Methanol-d_6	Dissolved completely
Ethyl Acetate-d_8	Partially dissolved
Acetone-d_6	Dissolved completely
DMSO-d_6	Dissolved completely
Alkaline D_2O	Dissolved completely

Alkaline D_2O was selected as the solvent of choice due to its dissolution capabilities and low cost. The use of deuterated solvents is necessary, since the NMR instrument requires an NMR-active nucleus within the solvent to lock onto. Acquired ^{19}F spectra of enrofloxacin show the presence of a signal peak at -124.7 ppm (Figure 5.3). This chemical shift corresponds to the presence of a fluorine substituent on an aromatic ring (Figure 5.1), as is expected for enrofloxacin.

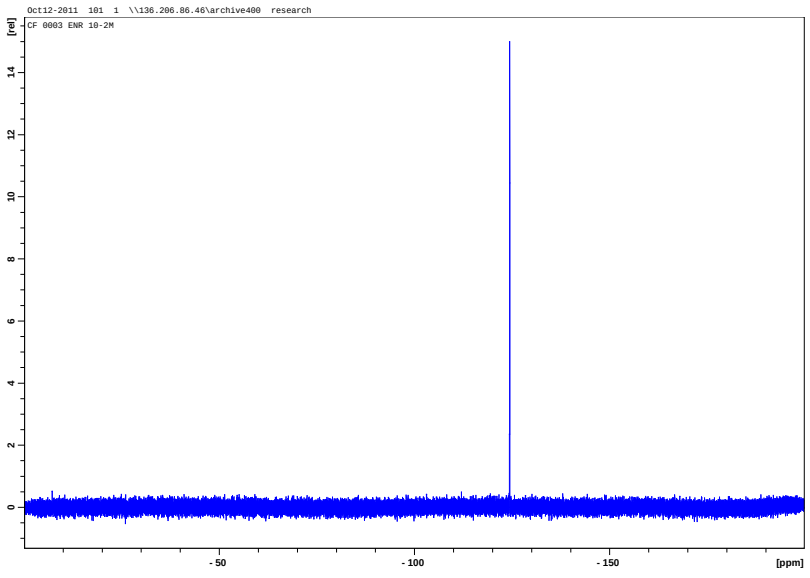


Figure 5.3: Acquired F NMR spectrum for enrofloxacin [Bruker Avance 400, 7000ppm 16 scans].

5.1.2.3 Acquisition Parameters

A number of acquisition parameters were assessed for their potential to achieve improved limits of detection and sample acquisition times. These were the:

- number of acquisitions;
- number of data points, and;
- proton coupling and decoupling.

Number of Acquisitions. The number of acquisitions was modified and its effect on the peak obtained assessed. As can be observed (Figure 5.4), an increase in the number of acquisitions corresponds to an increase in the signal:noise ratio (SNR). Therefore, by increasing the number of acquisitions, it would be possible to achieve improved detection limits.

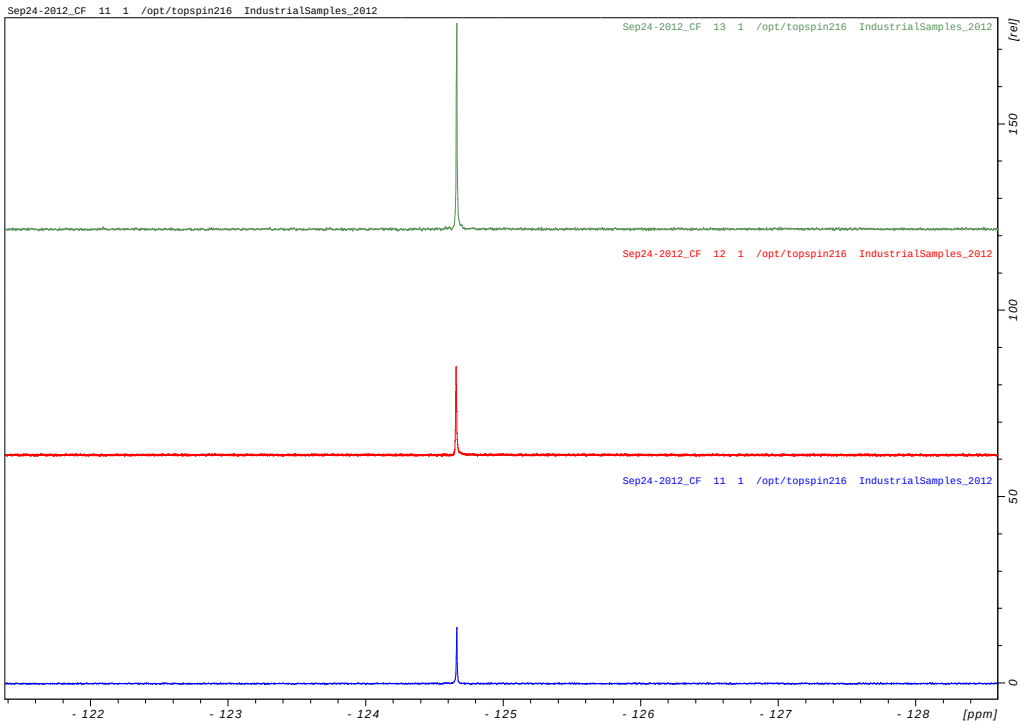


Figure 5.4: Change in peak height for a 0.002 M enrofloxacin solution at 64 scans (green), 32 scans (red) and 16 scans (blue).

However, the improved detection limit comes at the expense of increased acquisition times. For example, while a 16-scan acquisition can be obtained within 43 seconds, it takes nearly 2 minutes for 64 scans. The acquisition time is of low consequence at low scan numbers. Yet, going to ever higher acquisition scan numbers

can result in acquisitions taking hours, in particular since the improvement in SNR changes as a function of the inverse square law. As a rough estimate, for the SNR to be doubled, quadrupling of the number of acquisitions is required. In fact, the SNR is 99.8, 114.8 and 206.6 at 16, 32 and 64 acquisitions, respectively. Consequently, on going to lower concentrations, the number of acquisitions required might effectively require acquisition times that are not practical.

Number of Data Points. The number of data points was subsequently modified. The use of 262144, 16384 and 8192 data points[1] was assessed (Figure 5.5). The number of data points determines the NMR spectrum's quality as it controls the resolution of data acquisition. However, increasing the number of data points requires an increased duration for each acquisition. For example, an acquisition of 8192 data points takes 0.036 s, but it takes 1.153 s for 262144.

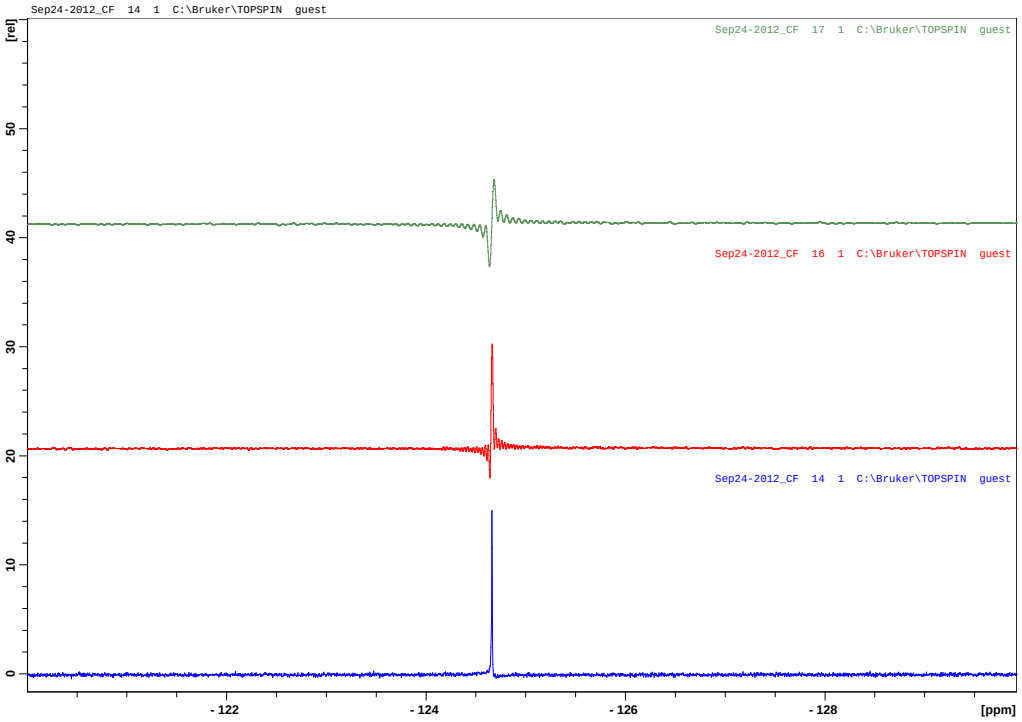


Figure 5.5: Change in the NMR spectrum acquired at a concentration of 0.002 M and 8192 (green), 16384 (red) and 262144 (blue) data points, respectively.

Although there is a decrease in the acquisition time required, one can note a decreased peak height on reducing the number of data points. There are minimal

[1] The data point resolution steps are such due to the digital nature of the instrument, which requires that the number of data points changes in bit increments.

differences in the SNR at 99.7, 122.1 and 111.3 for 262144, 16384 and 8192 data points, respectively. Nevertheless, a balance must be found between acquisition times and the resulting spectral quality.

Proton Coupling and Decoupling. In enrofloxacin, the fluorine nucleus is coupled to protons on the aromatic ring. Nuclear spin-spin coupling[2] arises as a result of two NMR-active nuclei interacting with each other [245]. Since enrofloxacin is a mono-fluorinated compound, heteronuclear coupling of the fluorine nucleus to proton nuclei is occurring. In order to assess the effect of proton coupling on the NMR spectra obtained, spectra were obtained under both proton coupled and proton decoupled conditions (Figure 5.6).

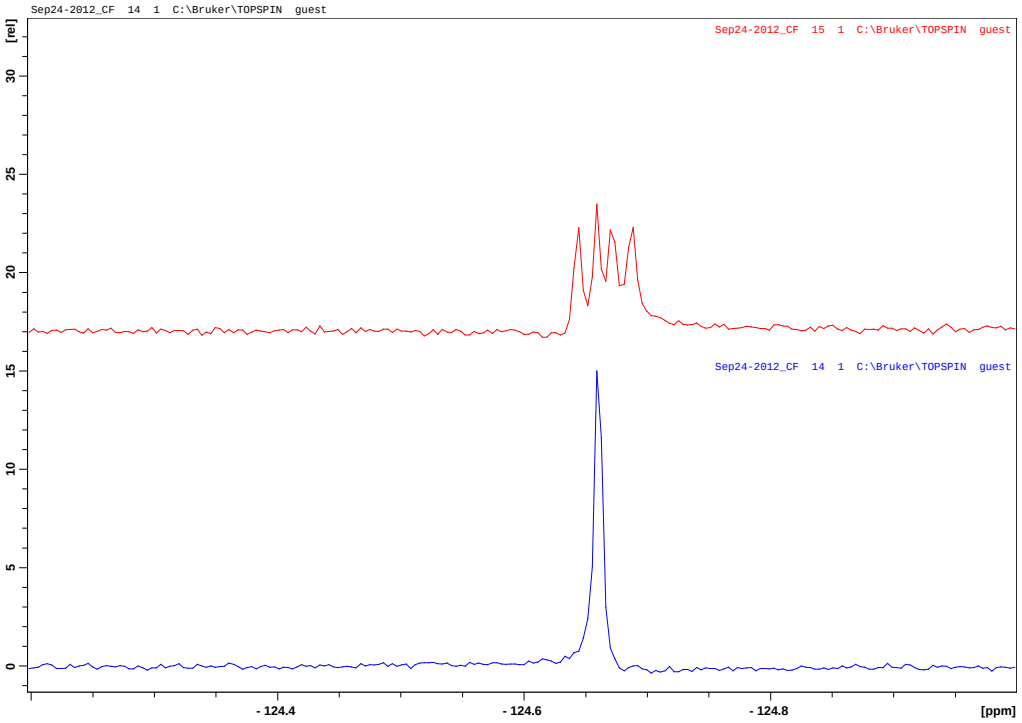


Figure 5.6: Change in the NMR spectrum acquired as a consequence of proton coupling (red) and proton decoupling (blue).

As can be observed from Figure 5.6, spin-spin coupling effectively results in a reduced NMR signal height, thus resulting in a stronger signal in the proton decoupled spectrum (bottom) as compared to the proton coupled spectrum (top). The enrofloxacin F-signal is split into a doublet of doublets. This splitting is as a result of J_3 coupling arising from n+1 coupling to the proton on the adjacent carbon,

[2] Spin-Spin coupling is also called J-coupling, scalar coupling or coupling [257].

which is followed by J_4 coupling caused by meta-coupling to the other proton on the aromatic ring. Therefore, proton decoupling was used in further analysis.

5.1.2.4 NMR Instrument Frequency

An alternative means of improving the limit of detection is to change the NMR instrument being used and going to a higher frequency instrument. In this section, the relative limits of detection of two NMR instruments operating at different frequencies is assessed. The first is a Bruker Avance 400 instrument, whilst the second is a Bruker Avance 500 instrument. The spectra were obtained under proton decoupled conditions and 262144 data points for both instruments. The number of acquisitions was, then, increased accordingly on decreasing the concentration of the enrofloxacin solution.

On the Bruker Avance 400 NMR spectrometer, the limit of detection was determined to be at 70 mg L^{-1}, with an acquisition time of around 30 minutes (Table 5.2, Figure 5.7). Detection of a 7 mg L^{-1} solution was not achieved after 8192 acquisition scans, which take around 4 hours to be acquired.

Using a Bruker Avance 500 NMR spectrometer, a limit of detection of 0.07 mg L^{-1} was not even obtained with a run time of around 14 hours (Table 5.3). A longer run time is likely to be considered to be excessively long for it to be feasibly carried out on a routine basis. Therefore, the limit of detection could be considered to be 7 mg L^{-1} with an acquisition time of 30 minutes. At this acquisition time, a limit of detection at an order of magnitude higher than that reached on the Bruker Avance 400 instrument is achieved.

Table 5.2: Limit of detection determination on a Bruker Avance 400 NMR.

Conc. (M)	**Conc.** (mg L^{-1})	**No. of Scans**	**Duration**	**Detected?**	**Figure 5.7**
0.02	7000	16	1 min	Yes	green
0.002	700	16	1 min	Yes	red
0.0002	70	16	1 min	No	
0.0002	70	128	5 min	No	
0.0002	70	1024	30 min	Yes	blue
0.00002	7	1024	30 min	No	
0.00002	7	8192	4 hr	No	

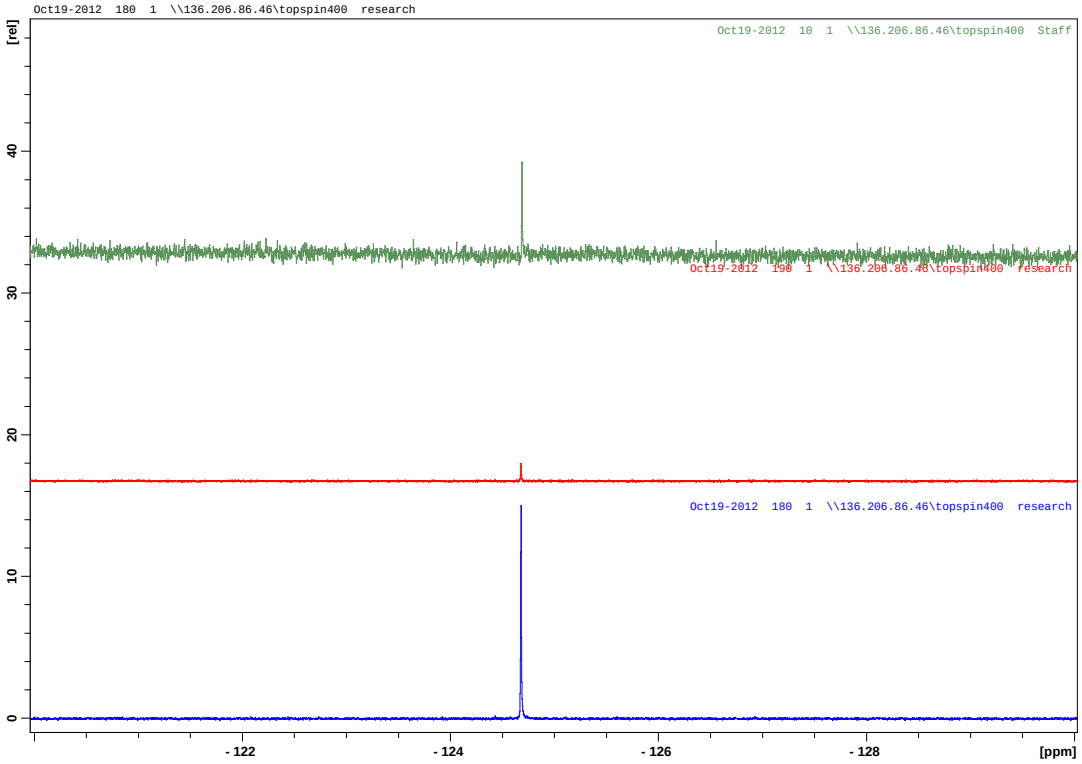


Figure 5.7: Acquired F NMR spectrum for different enrofloxacin concentrations and acquisition scans on a Bruker Avance 400 NMR as described in Table 5.2. From top to bottom, concentrations are at 7000 mg L^{-1} (green), 700 mg L^{-1} (red) and 70 mg L^{-1} (blue), respectively.

Table 5.3: Limit of detection determination on a Bruker Avance 500.

Conc. (M)	**Conc.** (mg L^{-1})	**No. of Scans**	**Duration**	**Detected?**	**Figure 5.9**
0.002	700	16	1 min	Yes	yellow
0.0002	70	32	1 min	No	
0.0002	70	128	5 min	Yes	purple
0.00002	7	1024	30 min	Yes	green
0.000002	0.7	1024	30 min	No	
0.000002	0.7	10400	6 hr	Yes	red
0.0000002	0.07	10400	6 hr	No	
0.0000002	0.07	48000	14 hr	No	blue

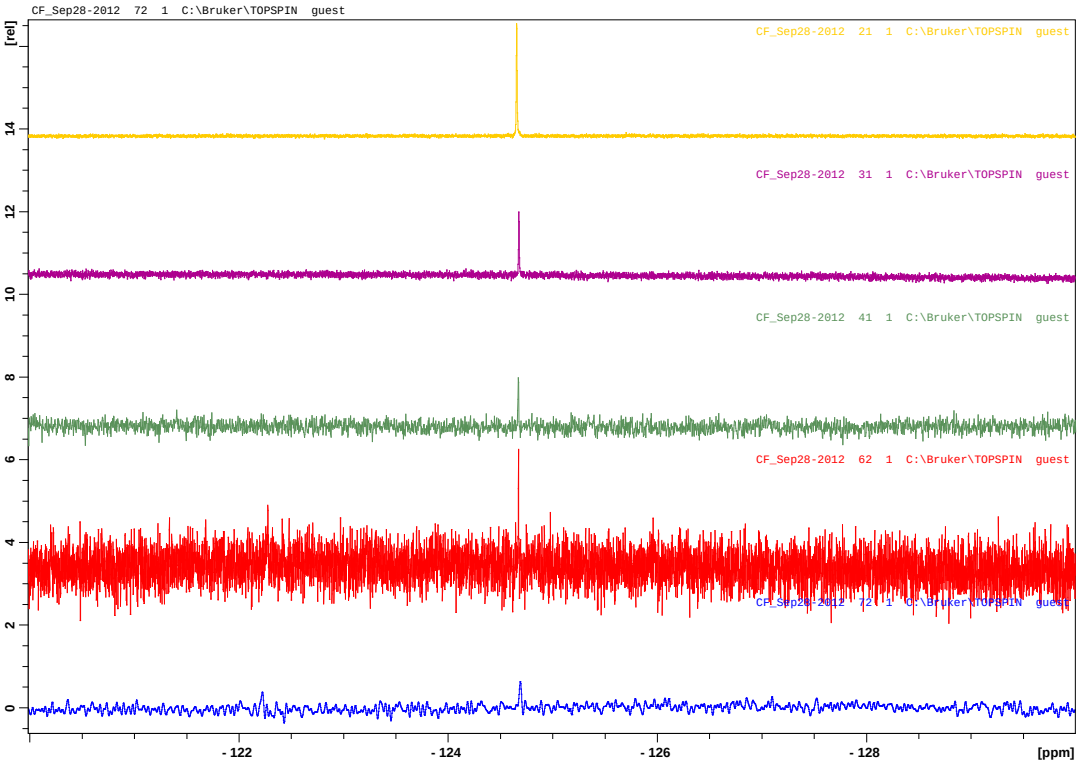


Figure 5.8: Acquired F NMR spectrum for different enrofloxacin concentrations and acquisition scans on a Bruker Avance 500 NMR as described in Table 5.3. From top to bottom, concentrations are at 700 mg L^{-1} (yellow), 70 mg L^{-1} (purple), 7 mg L^{-1} (green), 0.7 mg L^{-1} (red), 0.07 mg L^{-1} (blue), respectively.

5.1.3 Application of NMR Spectroscopy to Surface Water Samples

The limits of detection, achieved above on spiked alkaline D_2O samples, were then determined on spiked surface water samples. This work was carried out in order to identify whether there are any interferences from other fluorine nuclei that might be present within the sample matrix. As can be noted from Figure 5.9 no other interferences are evident.

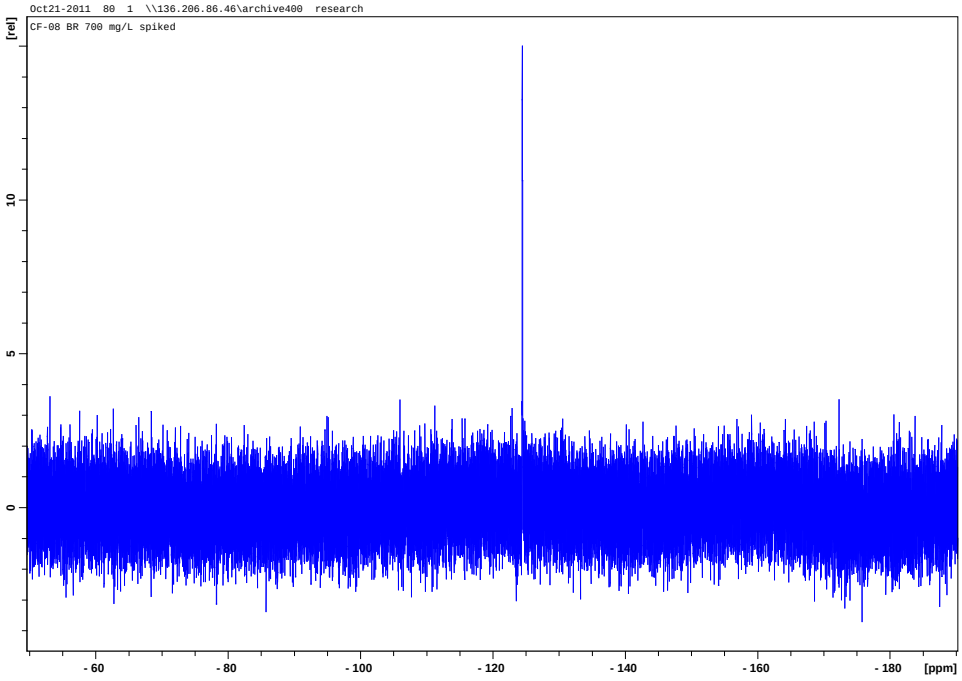


Figure 5.9: Acquired NMR spectrum for spiked surface water samples from Baunreagh at 700 mg L^{-1}.

During sample preparation, the main requirements are related to the introduction of D_2O within the sample, which is necessary to provide a nucleus for the NMR spectrometer to lock onto during acquisitions. At least 10% of D_2O is required within a sample for successful data acquisition.

Two sample preparation techniques were identified. The first involved the addition of 10% of D_2O to the filtered surface water sample, whilst the second involved the use of a sample concentrator (miVac sample concentrator, Genevac) to carry out solvent exchange. The former method effectively results in an increase in the limit of detection as a result of sample dilution. However, while the sample preparation time requirements are largely reduced by using NMR spectroscopy, the latter

involves considerable time requirements for sample concentration. Nevertheless, improved overall limits of detection would be possible, by increasing the volume of sample that is concentrated.

5.1.4 Considerations for using NMR

This study set out to determine the applicability of using NMR spectroscopy as an environmental forensics tool for identifying the presence of chemical markers within surface waters. It has shown that the use of NMR provides a number of benefits over the use of chromatographic techniques, because the technique is non-destructive, method development and validation requirements are practically non-existent, and costs related to chemical purchase are limited as compared to the LC-MS grade reagents required for chromatography. Additionally, sample preparation requirements for analysis are largely reduced, with filtered raw samples to which 10% D_2O has been added being suitable for analysis.

The main limitation in using NMR as an analytical technique is the associated high LOD. The lowest LOD determined for NMR was of 0.07 mg L^{-1} and an acquisition time of 15 hours on a Bruker Avance 500 instrument. However, an acquisition time of 30 minutes is considered to be more practical, which corresponds to a LOD of 7 mg L^{-1} on the same instrument. With LC-MS/MS, the LOD for enrofloxacin was four orders of magnitude lower (Table 4.11). When considering the further lowering of the LOD through the use of SPE in combination with chromatographic methods, a considerable difference in LODs could be noted between the two techniques.

Therefore, the results of this study indicate the suitability of using NMR for achieving contamination characterisation only in instances where considerable levels of contamination are expected at the current point in time. These are likely to be industrial settings, but unlikely to be useful for environmental monitoring of low-level contamination arising from, for example, manure dispersal. However, commercially available NMR instruments now reach 1000 MHz. Therefore, a theoretical LOD which is five orders of magnitude lower could be achieved, which corresponds to 70 ng L^{-1} with a 30 minute acquisition time. In addition, fluorinated surfactants, which may contain multiple equivalent fluorine nuclei, could be used as target analytes, rather than pharmaceuticals.

5.2 Immunoassay Techniques

Immunoassay techniques are the second alternative analytical technique assessed in this study. This section addresses the potential for using immunoassays in en-

vironmental forensics applications on the basis of the current state of knowledge (Section 5.2.1). This is followed by a discussion of the method development undertaken as part of this proof-of-concept study into the use of immunoassays in environmental forensics applications (Section 5.2.2). Finally, the considerations for using immunoassays in environmental forensics analyses are outlined on the basis of the outcome of this study (Section 5.2.3).

5.2.1 Using Immunoassays in Environmental Forensics

Immunoassays are bioanalytical techniques that depend upon the formation of an antibody-antigen complex. Antibodies are proteins that are naturally produced by animals as part of their immune system in order to recognise foreign substances (antigens) and facilitate their removal [258]. Within immunoassay analyses, antigens are typically the analytes of interest, which would bind to antibodies added to the test system to cause complex formation. Antibody-antigen complex formation is highly specific and it occurs through non-covalent interactions between defined portions of the antigen and the antibody [258]. This fundamental principle of antibody-antigen binding has been widely exploited in the development of immunoassay techniques.

Enzyme-Linked Immunosorbent Assays (ELISA) are amongst the most commonly adopted immunoassay techniques. They make use of enzymes conjugated to antibodies that upon the formation of the antibody-antigen complex and the subsequent addition of a chromogenic enzyme substrate form coloured reaction products [258]. Various ELISA configurations are known (Figure 5.10) including direct, indirect, and sandwich ELISAs [259].

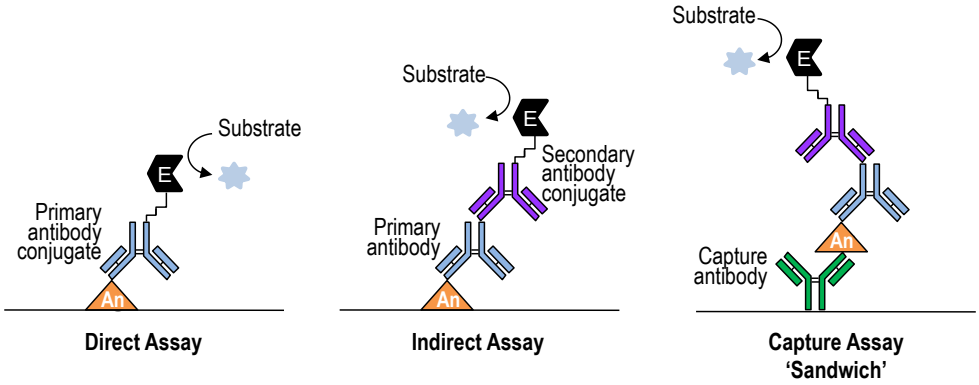


Figure 5.10: ELISA formats. Adapted from [259]. An = antigen, E = enzyme.

The various ELISA configurations may be operated within a competitive or non-competitive environment. Non-competitive ELISA is as depicted in Figure 5.10,

where a labelled primary or secondary antibody is conjugated to an enzyme. Therefore, increased sample concentrations cause increased antibody-antigen binding, resulting in a direct relationship between analyte concentrations and absorbance. In competitive ELISA (Figure 5.11), the antigen of interest 'competes' for antibody binding sites with a labelled 'competitor' that is conjugated to an enzyme [258]. Increased sample concentrations cause decreased antibody-competitor binding, which results in an inverse relationship between analyte concentration and absorbance.

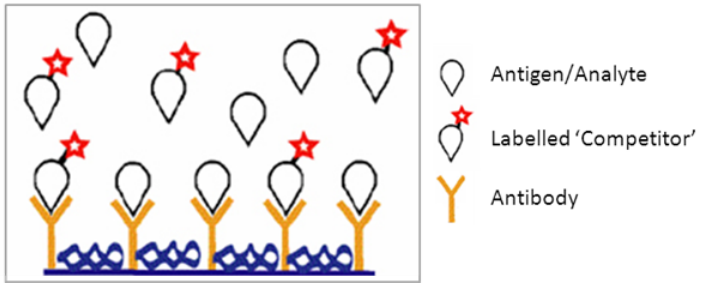


Figure 5.11: Schematic of competitive ELISA. Adapted from [260].

5.2.1.1 Advantages of Using Immunoassays

The use of antibodies as reagents within immunoassay techniques renders a number of advantages over other analytical techniques. Since the antibody-antigen complexes form through relatively weak interactions, which function over short distances, a close antibody-antigen fit is required for complex formation [258]. Therefore, complex formation confers a high degree of specificity to antibody-antigen binding and immunoassays have the capability of measuring the concentrations of antigens within complex matrices with limited or no pre-treatment, extraction, purification or concentration [261].

Furthermore, immunoassay analyses have the potential for high-throughput analysis [261] with the use of microtitre plates containing multiple 'wells' that function as small test tubes. The 96 well-plate format is the most commonly used but the use of larger microtitre plate formats and robotic handling can facilitate sample analysis even further. Combined with the various well-plate formats are multi-channel pipettes, which are used for the simultaneous addition of reagents to multiple wells, to greatly facilitate reagent and sample handling, and multi-channel spectrophotometers, which allow for entire plates to be read within a few seconds [259, 262].

These factors render great potential to the application of ELISA analyses to environmental analyses. However, their application has largely been focussed upon

clinical analyses and matrices such as bodily fluids [261]. Furthermore, despite the first studies on using ELISA to detect pharmaceuticals in surface waters showing up around 10 years ago for the detection on diclofenac [263], they have received limited further attention. This may be due to the limited availability of antibodies showing reactivity to chemical targets such as pharmaceuticals, as well as the skills set of environmental scientists.

5.2.1.2 Limitations of Using Immunoassays

As with all analytical techniques, the use of immunoassays has a number of limitations that need to be considered. One of the main limitations is the potential for cross-reactivity[3] or interference within immunoassay analyses (Figure 5.12). Therefore, factors that are used, such as the uniqueness of the epitope[4], are critical as they determine antibody-antigen selectivity.

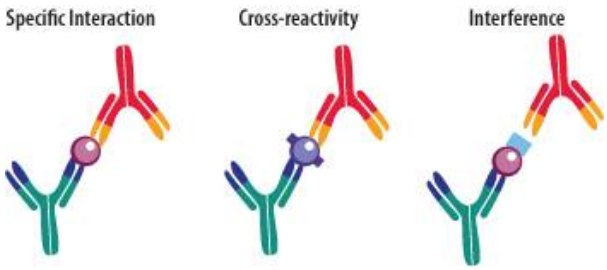


Figure 5.12: Potential for cross-reactivity and interference within immunoassay analyses [264].

Furthermore, the level of confirmatory detail on the presence of a particular analyte within a sample is reduced as compared to that obtained through mass spectrometric analyses. This lack of confirmatory detail is especially true when considering the potential variability in surface water matrices.

5.2.2 Method Development

5.2.2.1 Analyte Selection

The availability of the specific antibodies required is the critical factor in ELISA analyses. A number of antibodies for chemical markers forming part of the analytical suite discussed in Chapter 4 are commercially available. These include caffeine (Thermo-Scientific/Pierce Antibodies); cotinine, enrofloxacin, tylosin/tilmicosin and

[3] Ability of a particular antibody to form an interaction with two or more antigens possessing a common epitope [258].
[4] An epitope is a part of the antigen that is recognised by the antibody.

sulphadimethoxine (Randox Life Sciences); and acetaminophen (Beckmann Coulter). Similar methodological capabilities and characteristics, such as limits of detection, would be expected with all chemical markers. Therefore, one of the analytes was selected for use in this proof-of-concept study.

As described in Section 3.3.2.1, the enrofloxacin antibiotic was selected. This selection was largely based on the commercial availability of an ELISA kit for its detection and its suitability for use within the NMR analyses (Section 5.1.2.1). For the other analytes, although antibodies are commercially available, these are not available for purchase in the form of a kit, thus resulting in higher costs. This is because only the necessary volumes of reagents required are provided within a kit, thus reducing costs arising from minimum pack sizes. Meanwhile, the selection of enrofloxacin for the immunoassay studies would also allow for comparisons to the NMR analyses carried out to be made. The enrofloxacin ELISA kit used makes use of competitive ELISA in a capture assay (sandwich) format.

5.2.2.2 Sample Preparation

During method development, the starting point was the 'instructions for use' of the purchased ELISA kit. However, since the intended use of the kit is for tissue samples as a matrix, as opposed to surface waters, this method was modified for use in consultation with scientists at Randox Food Diagnostics (UK). The major difference was the elimination of tissue sample preparation since the analyte was already in a dissolved state. Instead, a filtration step, using 0.2 μm syringe filters, was added to reduce the potential interferents within the assay.

5.2.2.3 Limit of Detection

In determining the limit of detection, three sets of assays were carried out. The first involved the use of spiked distilled water. The other two sets of analyses made use of spiked surface water samples, one being from a site expected to be contaminated by sewage (Tullow: March 2012), whilst the other was from a site expected to be contaminated by manure (Kilcruise: March 2012).

The kit's limit of detection has been set by the manufacturer at 0.22 ng g^{-1} when considering solid samples, which corresponds to 110 ng L^{-1} as the extracted sample. However, due to differences in the matrix, it was expected to be possible to achieve even lower limits of detection, since there are reduced matrix interferents as compared to solid samples. Hence, linearity was determined between 12 ng L^{-1} and 9 μg L^{-1}. The three calibration curves obtained (Figure 5.13) had a coefficient of determination (R^2) of between 0.95 and 0.98, which show that the assay is linear

over this range. Furthermore, there is no significant shift in the calibration curves for the three samples, indicating limited matrix effects.

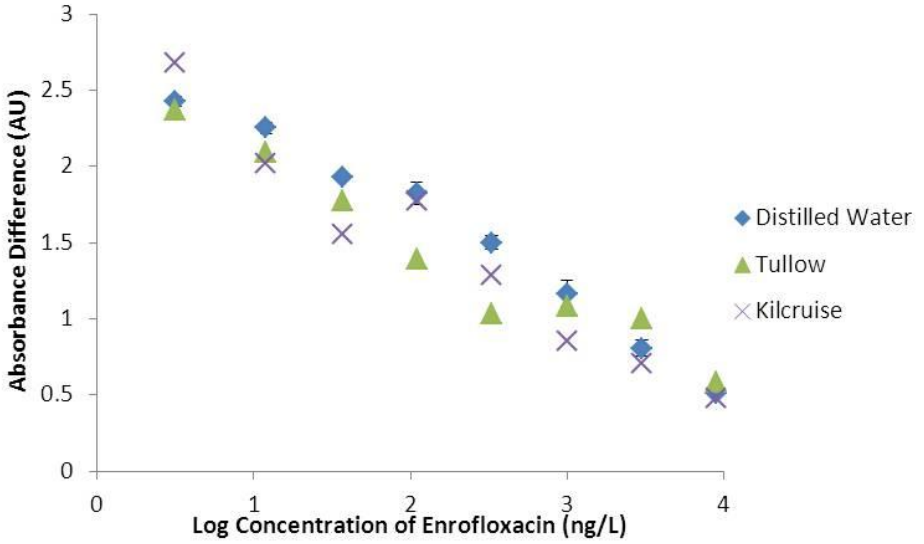


Figure 5.13: Calibration curves for spiked distilled water (n=2), Tullow and Kilcruise (n=1) samples between 12 ng L^{-1}1 and 9 μg L^{-1}.

Repeated analysis (n=3) at a concentration of 12 ng L^{-1} resulted in a % RSD of 19%. This is considered to be a relatively low level of variation in data in relation to the use of immunoassays with environmental samples where [265, 266]. Additionally, when comparing the level of absorbance at this lowest level on the calibration curve to unspiked samples/blanks (n=2), the differences between the two groups were identified to be statistically significantly different using univariate ANOVA ($p<.05$). This result confirms that the limit of detection was not yet reached.

5.2.3 Considerations for Using Immunoassays

This study has demonstrated the potential in using immunoassays for the determination of pharmaceuticals within surface waters at low concentrations. With minimal changes, commercially available kits could be easily adapted for use within surface water samples. The use of immunoassays allowed results to be obtained within a short time-frame. It only took around 1.5 hours for a 96 well-plate to be prepared and processed. When running analyses in duplicate, this effectively means that more than 40 samples can be analysed within each microtitre plate, even when taking into account system suitability samples. The potential for multiplex screening, where different antibodies are used within a single well allowing for multi-analyte analyses, would act to further reduce analysis times for such screening analyses.

However, for multiplex screening, there is increased cost and complexity related to the preparation of the microtitre plates and spectrophotometer specifications.

Nevertheless, these sample throughput levels represent a significant improvement over LC-MS/MS analyses, where sequential analysis is carried out and only one sample is analysed every 27 minutes, even when excluding SPE and system suitability samples. Additionally, sample preparation requirements are reduced to simply filtering surface water samples through a syringe-filter, when using immunoassays as an analytical tool.

The limits of detection obtained (12 ng L^{-1}) are also suitable for environmental forensics purposes. These could be further lowered through the incorporation of sample concentration through, for example, solid phase extraction, as this allows for even lower limits of detection. However, this would be at the expense of sample-throughput.

Nevertheless, a number of concerns must be addressed in relation to the technique's limitations, as outlined in Section 5.2.1.2. The major consideration is related to the confirmatory potential afforded by ELISA analysis as compared to LC-MS/MS. However, the potential of immunoassay techniques for high-throughput analyses makes them particularly suitable as screening analyses. Data obtained from such analyses would allow for reduced analysis by LC-MS/MS being required, since it is only where a high level of confirmatory detail is required that it is carried out. Therefore, the use of immunoassays can result in significant cost- and time-savings as compared to LC-MS/MS analysis.

5.3 Conclusion

The purpose of this chapter was to assess the current potential of alternative analytical techniques to traditional LC-MS/MS for determining the presence of chemical markers of sewage and manure within surface water samples. Two alternative analytical techniques were assessed, namely NMR spectroscopy and immunoassay techniques. Although both techniques have been widely used in a number of scientific fields, their potential for use in environmental forensics studies has been largely ignored. Therefore, the techniques' capabilities for such an application were investigated. Enrofloxacin was used as a model analyte due to its suitability to ^{19}F NMR spectroscopy and the commercial availability of an ELISA kit. Enrofloxacin is also one of the manure chemical markers that form part of the analytical suite of chemical markers discussed in Chapter 4.

Of the two techniques, the current potential for using NMR in environmental

forensics studies was determined to be more limited. This reduced potential is largely due to the technique's high limit of detection. Other factors reduce its potential applicability further. These include the fact that proton NMR would result in spectra that are too complicated when considering the complex matrix being analysed. Thus, only analytes having one of a small number of other NMR active nuclei, such as ^{19}F, would be useful.

On the other hand, immunoassay techniques were identified to show great potential for use in environmental forensics studies. Immunoassay techniques afford considerably higher sample throughputs to LC-MS/MS since they allow for concurrent sample preparation and analysis through the use of microtitre plates and multi-channel pipettes and spectrophotometers. In addition, limits of detection in the low ng L^{-1} were obtained, making them suitable for the analysis of chemical markers within surface water bodies with limited sample preparation and concentration. The development of multiplex screening plates for environmental forensics purposes, incorporating antibodies for a range of chemical markers within a single well, would make the use of immunoassays as an alternative analytical technique to LC-MS/MS even stronger.

Yet, it is unlikely that immunoassay techniques will completely eliminate the need for LC-MS/MS in environmental forensics studies due to the high level of confirmatory analysis conferred through LC-MS/MS analysis. Nevertheless, immunoassays have a role in reducing dependence on LC-MS/MS to achieve initial high-throughput screening. Then LC-MS/MS, would only be carried out as necessary, depending upon the results of immunoassay analysis and the confirmatory level required in the specific study.

Chapter 6

Results & Discussion: Current Attitudes to the Use and Disposal of Medication

Within this body of research, pharmaceuticals have been proposed as chemical markers of sewage and manure contamination. As discussed in Section 2.5.2.1, the disposal of unused medications has received scant attention to date. Previous studies in Ireland are generally small-scale projects as part of university course work, which are, then, only published in a small minority of cases [267]. This scenario is largely the rule within the European context, though some more studies are available for the US [268], Canadian [269] and New Zealander [270] contexts.

To establish current practices for unused medication disposal, a survey was undertaken to explore awareness levels of the consequences of different disposal practices and the reasons for current medication disposal practices. The resulting data aimed to achieve two main objectives. The first is that, through an understanding of current disposal practices, the importance of the various routes of unused pharmaceutical entry into the environment could be assessed. If disposal of non-ingested pharmaceuticals within sewage streams is significant, it brings up concerns related to overestimation of faecal contamination. This is because the entry of unused pharmaceuticals into the sewage stream could cause elevated pharmaceutical concentrations within surface waters and result in an overestimation of faecal inputs since they would be correlated to the pharmaceutical concentrations.

Another outcome of this study is that baseline data on the use and disposal of medications is collected. These data could be used to ensure that policy development in this area is matched to expectations and communication activities are tailored to meet current awareness levels. This is because this research identifies the pertinent

factors to target in an educational campaign and which disposal options would be most likely to receive wide acceptance by a community.

6.1 Data Collection

Data collection was carried out using a web-based self-administered questionnaire (SAQ). A questionnaire was selected as the survey instrument, because such an approach allows for data collection to be standardised and be administered and analysed with relative ease [271]. Also, the use of questionnaires allows for much larger numbers of a target population to be reached, as compared to for example face-to-face interviews [272]. Furthermore, the use of a web-based strategy for data collection allows for a larger, cross-cultural sample to be obtained, whilst keeping data collection costs at a minimum [273]. Finally, web-based strategies allow for more complex branching to be included, as opposed to paper-based SAQs where such branching is likely to introduce data collection errors and item non-response [274].

An SAQ, rather than face-to-face, approach was used because it has been found that this increases the participant's willingness to report sensitive information [274, 275]. Additionally, by allowing participants to decline filling in a response, an increased response accuracy has been shown to be achieved [273]. This is due to the fact that participants are not forced into making a response to all questions, which may result in arbitrary answering in order to allow for progress to the next screen.

LimeSurvey 1.91+ was used for deploying this SAQ, because the software allows for an unlimited number of questions and participants within the questionnaire, whilst most other available open source software do not provide this facility. LimeSurvey also allows for anonymous surveys to be devised, which ensures participant anonymity.

Nevertheless, the use of web-based questionnaires has a number of limitations particularly related to sampling, which need to be taken into account. Such a strategy omits individuals who do not have access to computers or who are not computer literate [272]. Also, having the questionnaire written in the English language limits responses to individuals with a good command of the language. This limitation is reflected in the participants' demographic characteristics, which must be considered during data analysis.

The convenience sampling strategy adopted, being a non-probability sampling strategy, is known to introduce sample bias within the participants. However, it is increasingly being adopted in such surveys where public attitudes are being in-

vestigated [271, 276–278]. This is because the strategy, although being rarely fully representative of the general population, has been shown to closely approximate data obtained through random sampling of a population in comparative studies [279]. Furthermore, such sampling strategies allow for exploratory research especially in areas, such as this, where little previous work is available [280]. In order to limit the level of bias in the data, the convenience sampling strategy was linked to a snowball strategy to increase the spread of data and access other sub-populations that would not have otherwise been reached.

6.1.1 Sample Characteristics

Following data collection and data cleaning, a useable sample of 1449 individuals remained. This sample size was considered satisfactory as it is more than the sample size needed for a significant χ^2 contingency table with an effect size[1] of 0.5 and a power[2] of 0.95 [281, 282]. Furthermore, the use of larger samples such as this allows for risks associated with skewness[3] and kurtosis[4] to be largely reduced [283].

The majority of the participants were females (67%) and less than 30 years old (57%). Education levels were roughly equally distributed between participants having achieved a post-graduate degree (39%), an undergraduate degree (32%) and a post-secondary level of education (26%). The remaining participants (3%) had achieved secondary or primary education, or had no formal qualifications. A wide variety of nationalities (52 nationalities) and countries of residence (32 countries) are represented within this questionnaire. Participants predominantly resided in Europe (98%) and in particular Malta (53%), Ireland (27%) and the UK (10%). These three countries represented the targeted populations due to the language of the questionnaire being English since English is an official language in the three countries. Therefore, participant nationalities and residences were re-classified as Ireland, Malta, the UK, Other European and Other.

Participant residencies ranged from big cities (16%) to a farm or home in the country (4%), with most living in a small city or town (49%). Such a cross-section of responses is similar to that obtained in other questionnaires carried out in recent years [284–286]. With respect to the Irish participants, these were from all four provinces. In a reflection of the Irish population, most participants came from Leinster followed by Connacht, Munster and Ulster [287].

[1] By convention, an effect size of 0.1 is small, 0.3 is medium and 0.5 is large. The larger the effect size, the greater is the magnitude of the difference required for statistical significance [209].
[2] Power refers to the probability of falsely accepting the null hypothesis [209].
[3] Measure of the symmetry of the frequency distribution [209].
[4] Measure of the degree to which scores cluster in the tails of a frequency distribution [209].

6.2 Data Analysis

In this section, the results from data analysis are discussed. As described previously, the main focus of this data analysis is to understand the relevant considerations for using pharmaceuticals as chemical markers of sewage and manure and to understand current attitudes towards medication use and disposal. Therefore, only the most pertinent results are discussed.

6.2.1 Domestic Use of Medication

In the first part of the questionnaire, the respondents' usage of medication over the previous six month period was assessed. 83% of participants reported that they had taken over-the-counter (OTC) medication, 52% that they had taken prescription medication and 23% were on long-term-illness (LTI) medication.

In the use of pharmaceuticals as chemical markers of sewage and manure, two groups are likely to be extremely useful as described in Chapter 2. The first are those pharmaceuticals that are used by a large number of individuals, albeit for a short period of time, namely OTC medications. The OTC medications most commonly listed as being used within the previous six months are pain killer medications such as acetaminophen, aspirin and ibuprofen. The second are those pharmaceuticals that are used by a small number of individuals consistently, such as those used in the treatment of LTIs. In this regards, bronchodilators (e.g. salbutamol/ventolin), hormone supplements (e.g. Levothyroxine as a thyroid steroid supplement or ethinylestradiol and drospirenone as hormonal contraceptives) and statins (e.g. atorvastatin/Lipitor for lowering blood cholesterol) were some of the most commonly listed medications.

Of the four human pharmaceuticals within the analytical suite (Chapter 4), only acetaminophen was amongst the most commonly listed medications and carbamazepine was only listed by name once. However, a number of participants only mentioned the general class of medication they had taken, e.g. anti-depressants or anti-histamines, without mentioning the specific active ingredient or brand. Most of the pharmaceuticals mentioned by name and the classes of medications listed, correspond to those listed by the Health Service Executive (HSE) to be amongst the most commonly prescribed in Ireland [252].

The obtained results show that gender is the most significant determinant for OTC ($\chi^2(1)=14.76$, $p<.001$) and prescription medication intake ($\chi^2(1)=9.24$, $p<.005$), with females taking significantly more OTC (86%) and prescription (55%) medications than males (78% and 47% respectively). Of interest are the differences in

medication intake by residence and nationality. Participants from Ireland and the UK use OTC medications to a larger extent than individuals in Malta and other countries (nationality: $\chi^2(4)=13.46$, $p<.01$; residence: $\chi^2(4)=12.79$, $p<.05$). This variability suggests reduced contact between UK and Irish residents and their General Practitioner (GP). Hence, a GP is particularly unlikely to be best suited to provide information on the correct disposal of medications in these countries. Rather, it would be suggested that the pharmacist is better suited. Also, a media campaign is likely to be more effective particularly when considering that a number of OTC medications can be bought in supermarkets, thereby cutting off the link with the pharmacist.

As is to be expected, the use of LTI medication increases significantly by age ($\chi^2(4)=49.05$, $p<.001$). The elderly are more likely to have been diagnosed with an LTI. This is of concern considering that the world population is ageing [288]. In addition, pharmaceutical development in the area of LTI management is ever increasing, such that some LTIs that were previously considered fatal can nowadays be managed. Therefore, the use of LTI medication is expected to rise in the coming years, further increasing the potential of LTI medication as chemical markers of sewage contamination.

6.2.2 Storage of Unused Medication

Medication storage is an important consideration in the disposal of unused medications. Ideally, all medication that has been purchased is taken until the course is finished. This scenario eliminates any concerns related to the storage and disposal of unused medications. However, this is often not the case and results in both health and environmental considerations. In this research, the environmental considerations related to medication disposal once they are no longer used are of specific interest, rather than the potential for ineffective treatment resulting from incomplete medication intake.

86% of participants reported that they keep medications other than those they would be currently taking within their home, which indicates that they either buy extra or do not finish their medications. This percentage compares well to studies from other regions, where between 62% and 98% of participants were identified to generate leftover pharmaceuticals [268–270]. Furthermore, whilst 74% of participants keep some basic medications such as pain killers and sore throat lozenges within their home, 16% keep prescription medications, such as antibiotics.

Medication intake is one of the significant determinants for the level of medication storage within households, with a higher level of medication intake corresponding to

increased medication storage. In fact, the major determinant of medication storage was the intake of OTC medication ($\chi^2(3)=60.90$, $p<.001$). Also, there was a significant association between the type of medication kept by participants and nationality ($\chi^2(4)=51.80$, $p<.001$), with the differences depicted in Figure 6.1. The differences between participants from European countries and other countries is of particular interest, indicating that current attitudes on medication storage in Europe are quite different from other countries. Participants from outside Europe were two to three times more likely to keep no pharmaceuticals within their household, other than those being taken at a particular point in time. However, further analysis would be required in this regard, particularly due to the small sample size for participants from non-European countries.

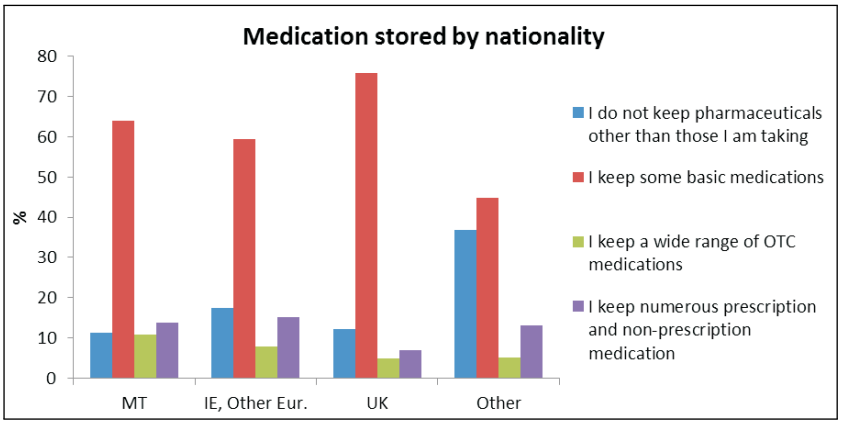


Figure 6.1: Medication stored by participants with different nationalities. Grouping of nationalities is by significant differences using a CHAID model, as described in Section 3.4.

6.2.3 Current Medication Disposal Practices

Disposal of unused medications within the sewerage system could result in an overestimation of risk arising from sewage streams. The relationship between faecal contamination and the chemical marker might no longer hold since the chemical marker is not detected as a function of faecal contamination but as a function of disposal within the sewer system. Therefore, it is relevant to the use of chemical markers as tracers of sewage contamination.

As discussed earlier, another practical advantage of gaining information on disposal practices is that it provides a significant amount of information that is relevant in understanding current medication disposal practices. This information allows for an understanding of current practices that need to be targeted in education campaigns, if these are to be implemented with maximum success. From Figure 6.2, it

is evident that disposal with the solid household waste is preferred for syrups, pills and veterinary pharmaceuticals.

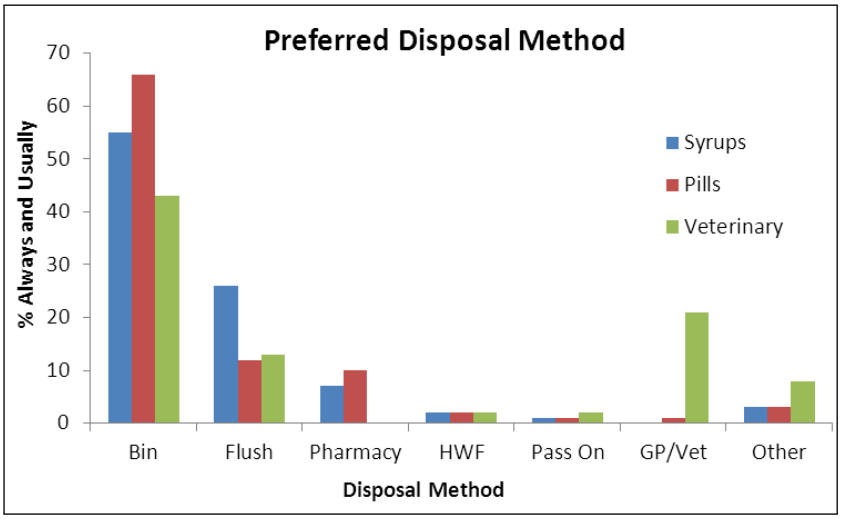


Figure 6.2: Graph showing % of participants always or usually selecting a particular disposal method for medicinal syrups, pills and veterinary medications. Taking to a pharmacy was not an option for the veterinary medications.

With respect to the disposal of veterinary medications, only 8% of participants dispose of them in a different manner to those intended for humans. However, of these, 21% take their unused/unwanted medication back to the vet rather than disposing them by placing in the bin or flushing down the toilet. This is a considerable increase compared to those who take human-intended medications to the GP, pharmacy or hazardous waste facilities (HWF). On analysing the other methods for disposal, what was extremely interesting was that three participants of different nationality (Ireland, Malta and Greece) stated that they feed their unwanted medications to their plants, with some going on to say that to date they have had positive results in this regards.

Disposal within the sewer stream (flush) was the preferred choice of disposal for 25% of syrups, 13% of veterinary medications and 12% of pills, making it the second most common means of disposal. Yet, it only represents a small proportion of pharmaceuticals used. Therefore, the scope for incorrect source attribution as a result of unused medication disposal is expected to be low. Furthermore, the majority of participants (50%) perceive that disposal of medication within the sewage stream is the worst method of pharmaceutical disposal (Figure 6.3). This perception makes it less likely that this disposal practice will increase considerably in the coming years.

A possible means of managed disposal is to return unused medication to a GP or pharmacy. Although not selected as the worst means of disposal in this survey,

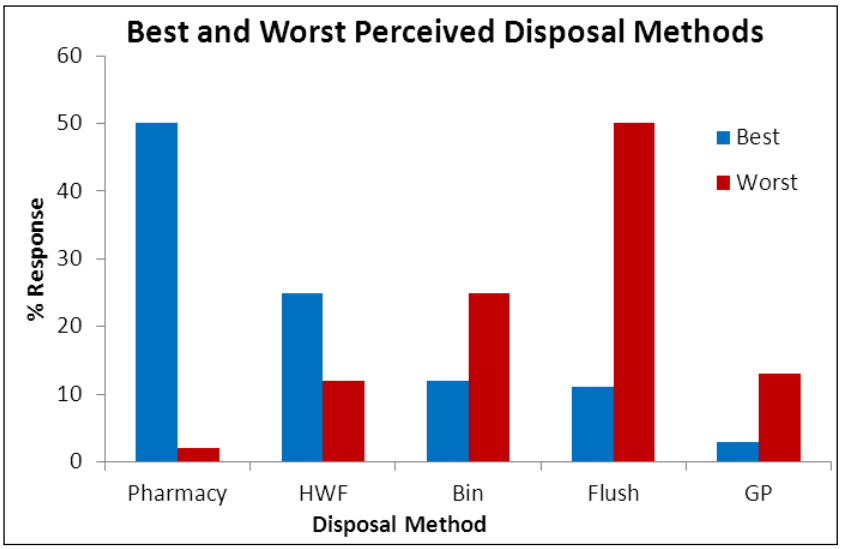


Figure 6.3: Graph showing the best and worst perceived disposal methods.

take-back to GPs was least often selected as the best means of disposal. A significantly larger proportion of people living in or from Malta considered this to be the worst option as compared to all other countries (nationality: $\chi^2(4)=16.43$, $p<.005$; residence: $\chi^2(4)=11.33$, $p<.05$). This difference is likely due to the health care structure in Malta, whereby the GP does not generally have a nurse or receptionist in the surgery. Therefore, returning unwanted pharmaceuticals to the GP is time consuming and potentially costly. On the other hand, although less than 10% of participants return their unused pharmaceuticals to the pharmacy, most perceive it to be the best option.

6.2.4 Medication Disposal Advice

Current avenues for receiving advice on unused medication disposal were also explored. When developing policies and programmes on the disposal of unused pharmaceuticals, an understanding of the level of public awareness is critical. This understanding may explain discrepancies between actual practices and perceived best practices by the general public, and allows for the need for educational campaigns and potential target audiences to be identified.

What is of concern is that only 14% of participants have ever been advised on the best way for medication disposal (Figure 6.4). This observation might explain the discrepancy between current practices and perceived best practice, as is evident from Figures 6.2 and 6.3, indicating that strategies for educational campaign implementation need to be rethought. In fact, a number of participants commented that they have never thought about this aspect of disposal, even though they might do all

their recycling and waste separation and consider themselves to be environmentally aware.

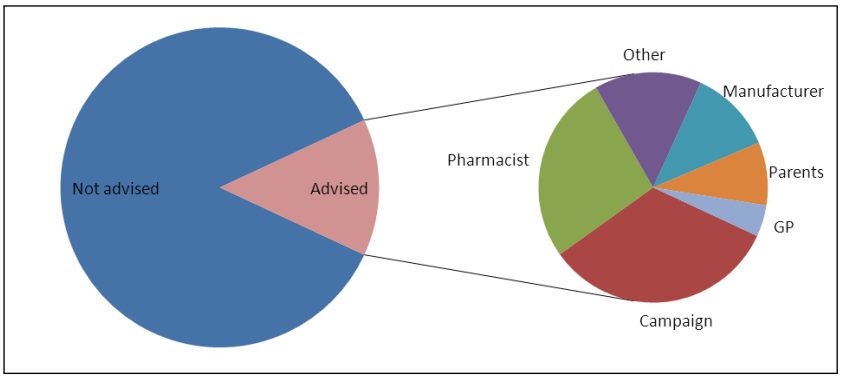


Figure 6.4: Graph showing whether participants have received advice on pharmaceutical disposal and the way advice has been obtained where this is the case.

The most common means for obtaining information on medication disposal was through an educational campaign and the pharmacist. Additionally, a considerable portion of participants stated that they obtained data from other means of information. Amongst the other sources mentioned most commonly are the line of work or study (65%), the media (18%), and nurses and hospital staff (15%). What is interesting to note is that the least common method of obtaining information is through the GP (6%). Reasons for this outcome could be that doctors are more focussed on patients finishing their medication courses than on medication disposal. Therefore, a take-back to GP system is likely to be unsuccessful.

Another consideration in developing targeted campaigns is an understanding of the demographic variations in awareness. Age ($\chi^2(4)=48.62$, $p<.001$), nationality ($\chi^2(4)=89.58$, $p<.001$), residence (χ^2 $(4)=63.72$, $p<.001$) and education level ($\chi^2(5)=49.00$, $p<.001$) were all significant determinants of whether advice has been received on unused medication disposal. For example in Malta, only 5% of participants under 45 years of age have received advice compared to 22% of participants over 46 years. In Ireland, the major determinant for whether participants have received information is the level of education, with 27% of participants with a postgraduate degree having received advice compared to 11% of those without a postgraduate degree. Therefore, an educational campaign in these countries should initially target these sectors of society that are not receiving advice.

In general, older participants and those with a higher level education were identified to have a statistically significantly higher probability of having received advice on pharmaceutical disposal. Participants from Malta were around three times less likely to have received advice on pharmaceutical disposal as compared to other coun-

tries, with only 7% of participants having received advice as compared to e.g. 21% of Irish participants. This outcome indicates a significant lack of awareness in Malta.

A factor that may contribute to the dearth of information reaching the public is that there is a lack of consensus on the optimal approach to dispose of unused medications [149]. The optimal disposal method largely depends upon the most critical consideration that is taken into account, which may be environmental, human health risk (including suicide prevention), costs or practicality. In relation to environmental considerations, studies that compare environmental emissions from different disposal options are only a recent development [149]. Based on current knowledge, a 100% solid waste disposal programme for pharmaceuticals has been identified to be the 'better option' as it results in significantly lower costs and emissions while increasing convenience and chance of compliance. Indications for higher compliance are also reflected in the present study since this is the preferred disposal method for most respondents. However, further in-depth studies are needed in order to understand the benefits and limitations of other methods of disposal [149].

6.2.5 Disposal Considerations

The factors participants took into account in their current practices for the disposal of medications were subsequently explored. This provided an understanding of what motivates individuals to act in a certain way and what are the considerations to be taken into account in setting up future educational campaigns. For individuals who already dispose their unused medications by returning to pharmacies, GPs or HWF, the main motivators were listed as being safety and environmental health (Figure 6.5).

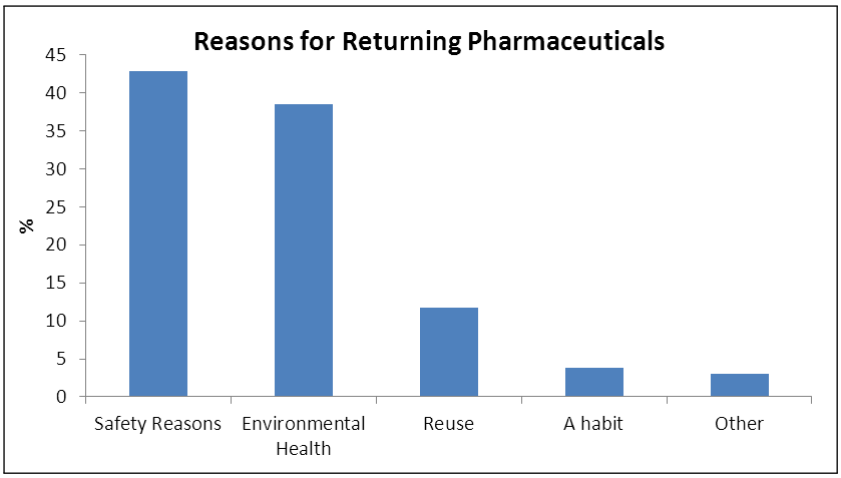


Figure 6.5: Reasons given for returning pharmaceuticals to pharmacies, GPs, HWFs or vets.

When considering individuals who do not dispose of unwanted/expired pharmaceuticals by taking back to pharmacies, GPs, HWFs or vets, the most common reason by far was that they were unaware of the possibility (Figure 6.6). The fact that the facilities are too far away was the least selected option (3%). Therefore, if a take-back to pharmacy system is implemented, the network currently in place is likely to be sufficient for the successful implementation of the programme. The most honest answer was probably "Because I am human...I guess laziness".

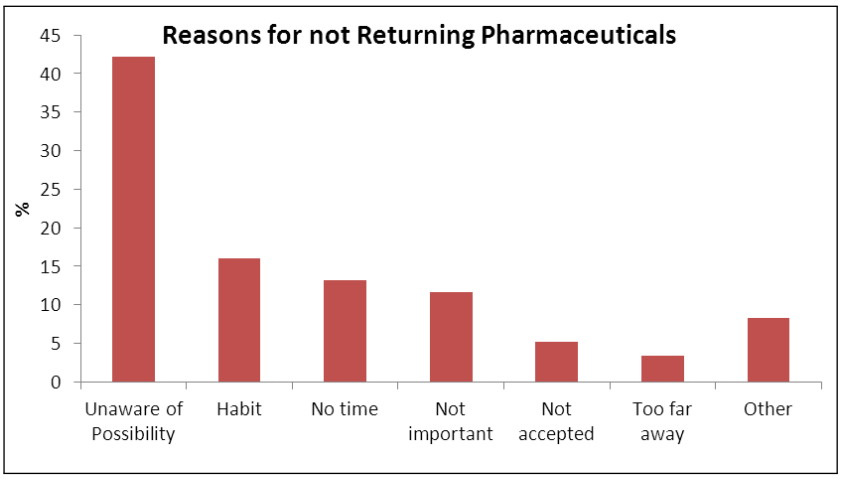


Figure 6.6: Reasons given for not returning pharmaceuticals to pharmacies, GPs, HWFs or vets.

A number of participants raised the issue that they are concerned that the pharmacist might try to re-sell returned pharmaceuticals. Thus, they effectively might be buying someone's old medication. In contrast, others commented that they would make more of an effort to return pharmaceuticals if they knew they would be donated. In fact, 12% of participants who do return pharmaceuticals do so for reuse (Figure 6.5). Therefore, if a take-back option for disposal is to be promoted, information on the end-point for the returned pharmaceuticals is an important consideration.

A number of additional considerations must be taken into account in implementing a take-back to pharmacy programme. These are mainly related to economies of scale and system feasibility. Informal discussions with individuals working in pharmaceutical retail in Ireland mentioned the fact that such a system comes with significant administrative efforts and costs for disposing of unwanted medications both at the pharmacy and distributor levels. Therefore, further studies on this topic among pharmacists are required to provide the necessary information on the best way to achieve a take-back system.

6.2.6 Environmental Awareness

Finally, the level of environmental awareness on the presence of pharmaceuticals within surface waters was assessed. It is necessary to determine how the general public perceives the effect of disposal practices on the environment, as this could influence compliance.

41% of participants were aware that pharmaceuticals have been detected within surface waters. A number of demographic parameters were found to be significant in this regard. Older participants ($\chi^2(4)=10.94$, $p<.005$) with a higher level of education ($\chi^2(5)=108.60$, $p<.001$) were significantly more likely to be aware of this fact. With respect to country of residence ($\chi^2(4)=92.66$, $p<.001$), participants from Malta had the lowest level of awareness (30%), followed by participants from the UK and other countries (47%), and finally Ireland (58%). Meanwhile, the gender of participants does not seem to have a bearing on the level of awareness.

Most participants perceive that the major source of pharmaceuticals in surface waters is unwanted medication flushed down the toilet/sink (Figure 6.7). Meanwhile, placing medications in rubbish bins was considered to contribute only a little to the presence of pharmaceuticals in surface waters. This result corresponds well to the best and worst perceived methods for medication disposal (Figure 6.3). Only 17% of participants considered the introduction of pharmaceuticals to surface waters in conjunction with sewage following medication ingestion to be the major source of pharmaceuticals to surface waters. This contrasts widely with the currently accepted major sources of pharmaceuticals to surface waters, i.e. as a result of excretion following normal use [137, 164].

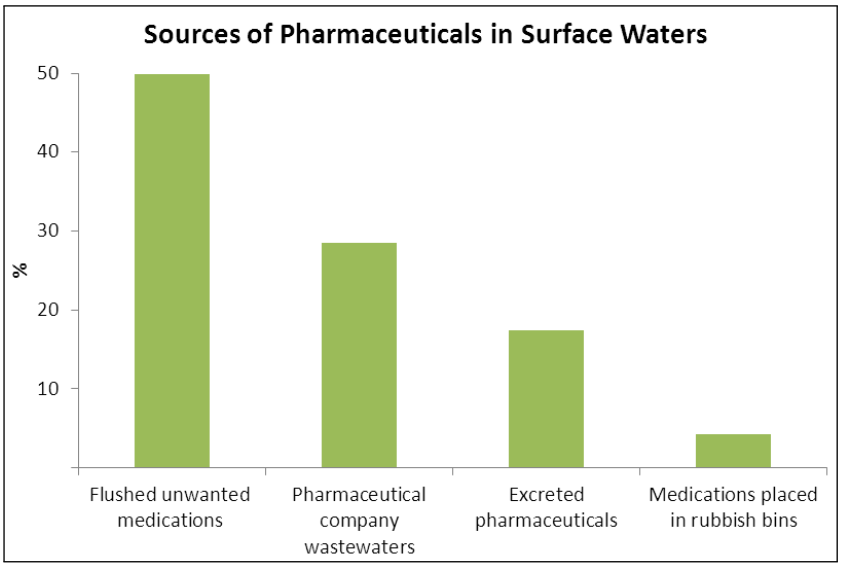


Figure 6.7: Perceived major sources of pharmaceuticals to surface waters.

Of interest is the perceived greater importance of pharmaceutical company wastewaters for participants from Ireland and Malta as compared to participants from the UK and other countries. Within Ireland and Malta, the pharmaceutical industry plays an important role, contributing to 50% of the country's exports in Ireland [182] and is the third highest export revenue source in Malta [289]. Therefore, participants from these countries are likely to be more aware of the potential risks arising from this industry. Meanwhile, such a perception is not as acute within other countries where the pharmaceutical industry does not play such a major role.

The major risks linked to the presence of pharmaceuticals in surface waters were perceived to be risk to aquatic life (Figure 6.8). The lowest risk was perceived to arise from the development of antibiotic resistant strains. The risks to human health and drinking water were similar, which might be because the major risk to human health from water bodies would be expected to arise from drinking water, leading to individuals considering them to offer similar levels of risk.

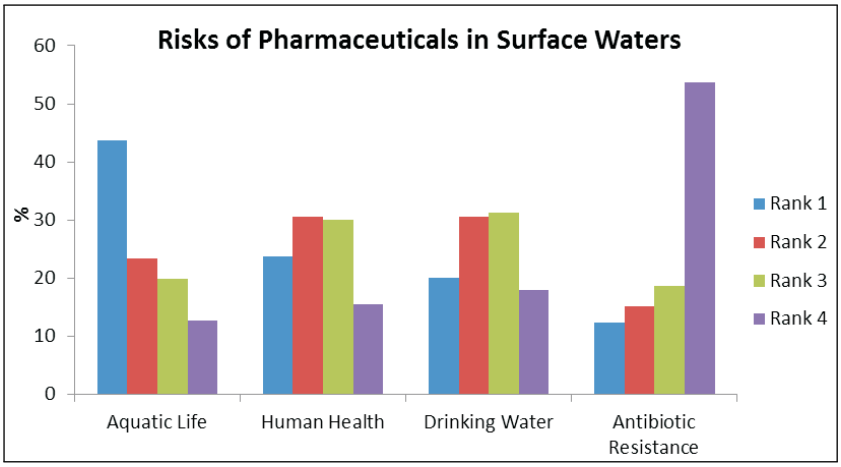


Figure 6.8: Ranking of perceived risks of the presence of pharmaceuticals in surface waters. Rank 1 indicates the highest perceived risk whilst rank 4 indicates the lowest perceived risk.

6.3 Conclusion

The presence of pharmaceuticals within surface waters is an area that has received considerable attention in recent years. However, the potential for environmental entry as a consequence of disposal of unused pharmaceuticals has been largely ignored. It is only in recent years that some research into this area is emerging. This route of entry needed to be investigated as part of this research in order to understand the extent of unused pharmaceutical disposal and particularly disposal into the sewer-

age system. At the same time, the considerations for current disposal practices and the level of awareness on the consequences of different disposal practices were evaluated. This evaluation allows for the identification of the main factors that need to be addressed should a successful disposal and educational campaign be developed.

With respect to the first aim of this study, it was determined that whilst 83% of participants store pharmaceuticals within their household, only around 17% would always or usually dispose of them by flushing in the sewage system. Since such an action would be expected to be carried out as a rare occurrence, it could be considered to provide limited scope for incorrect source attribution as a result of the disposal of non-ingested pharmaceuticals.

Considering the development of relevant policies and educational campaigns, it was identified that there is a significant lack of education in relation to the optimal way for disposal, with only 14% of participants having received any advice. This lack of awareness could be due to a lack of consensus on the best way for disposal and is notwithstanding the fact that within the EU, Directive 2004/27/EC specifies that member states are to "ensure that appropriate collection systems are in place for medicinal products that are unused or have expired" within article 127b [290].

Based on current levels of awareness, it is likely that a take-back to pharmacy system would receive the greatest acceptance. Twice as many participants consider this to be the optimal means for disposal as compared to the second-highest selected disposal method. Additionally, only 3% of participants stated that distance to a pharmacy is a reason for non-return of pharmaceuticals, indicating that the network currently in place is likely to be sufficient. It is the lack of awareness of the possibility to return pharmaceuticals to a pharmacy that was most commonly cited as a reason for non-return. Finally, due to the success of take-back systems, such as those implemented for batteries, it is most likely that such a system would receive greatest acceptance.

Chapter 7

Decision Tool for Nitrate Source Determination

To date, numerous approaches have been suggested for differentiating point and diffuse sources of nitrate contamination. These include the use of nitrate stable isotopes, microbiological analyses, genetic markers and chemical markers. The existing approaches each have their own strengths and limitations. As a result, the most appropriate approach to use largely depends upon the specific scenario and the context of the study. However, available data on nitrate source determination is highly fragmented and approach dependent, with very little or no interface between the different techniques. This makes it difficult for stakeholders to identify the most suitable approach to adopt in a specific scenario.

Aggregating the present knowledge into one unified system would make it easier for stakeholders to assess and implement the most appropriate approach. Therefore, a decision-support tool was developed using the Integration Definition Function (IDEF0) modelling system [291]. Its aim is to provide a generic framework that formalises the thought processes that need to be carried out in order to identify the most suitable approach to adopt for achieving nitrate source determination in a specific scenario. Hence, through the tool's application, the outcomes of such studies are standardised and more easily justified.

Through the inclusion of supplementary material, which brings together the current state of knowledge in the area of nitrate source determination (Chapter 2) and the differentiation requirements of key stakeholders, the selection of the most appropriate approach is further facilitated. In addition to the differentiation potential afforded by each approach, considerations such as cost, time, sample volumes and the state of the approach are taken into account. Hence, this tool optimises the effectiveness of environmental forensics studies for nitrate source determination by

assisting in the process of ensuring that the most suitable approach is applied within a specific scenario.

7.1 System Selection

A number of multi-criteria decision analysis[1] tools can be used in the development of decision tools. These are largely functional modelling methods, where activities, actions, processes and/or operations, collectively known as functions, are represented in a systematic manner. Examples include functional flow block diagrams, hierarchical input process output models and the integration definition function modelling system. The specific method utilised largely depends upon the decisions being made and the person making the decisions [293].

Within this study, the IDEF0 modelling system was adopted for the development of a decision tool for nitrate source determination. IDEF0 is a public domain modelling system that outlines the way a model is developed and depicted [291]. As a result, it allows for a consistent representation of the various functions[2] and functional relationships[3] that are necessary for the overall model aim to be achieved.

To date, IDEF0 has been used to model a number of systems, to ensure process consistency and rigour, whilst at the same time having logical data flows [294]. In particular, IDEF0 methodologies have been widely applied within the industrial and manufacturing sectors to gain an understanding of current systems as a way to initiate improvements [295]. However, it is increasingly being applied to project management scenarios [296] such as the development of hospital waste management programmes [294] and emergency management procedures [297]. By depicting and formalising the thought processes that need to be carried out, bottle-necks and/or deficiencies within the methodology can be more easily identified, and the outcomes from method application can be standardised and more easily justified.

The strengths of the IDEF0 modelling system include that it is generic, rigorous and precise, concise, conceptual and flexible [291]. In the context of this research, these strengths are of particular relevance. By being generic, conceptual and flexible yet at the same time rigorous and precise, IDEF0 allows for correct and usable models to be produced, which may be successfully applied to scenarios with varying purposes, scopes and complexities. The reasons for this is that IDEF0 focusses on

[1] Multi-criteria decision analysis is a means adopted in operations research where a number of criteria are taken into account during the decision-making process [292].

[2] Functions are activities, actions, processes and/or operations that need to be fulfilled [291].

[3] Functional relationships are the way the various functions interlink together as inputs, controls, outputs and mechanisms [291].

the identification of functional requirements as opposed to physical or organisational requirements. At the same time, the developed model is concise, thereby facilitating the modelled system's communication and validation.

7.1.1 IDEF0 Modelling System

An IDEF0 model consists of a hierarchical series of IDEF0 diagrams, which display increasing levels of detail representing a particular group of functions (Figure 7.1). The top-level context diagram, termed the A-0 diagram, outlines the method's overall functional relationship. Thus, it represents the model's most general description. The high-level function that is outlined in the A-0 context diagram is then decomposed into the main sub-functions, which can then be further sub-divided into more detailed lower-level diagrams until all processes are outlined. Hence, on moving from a parent-diagram[4] to its child-diagram/s[5] further detail is provided.

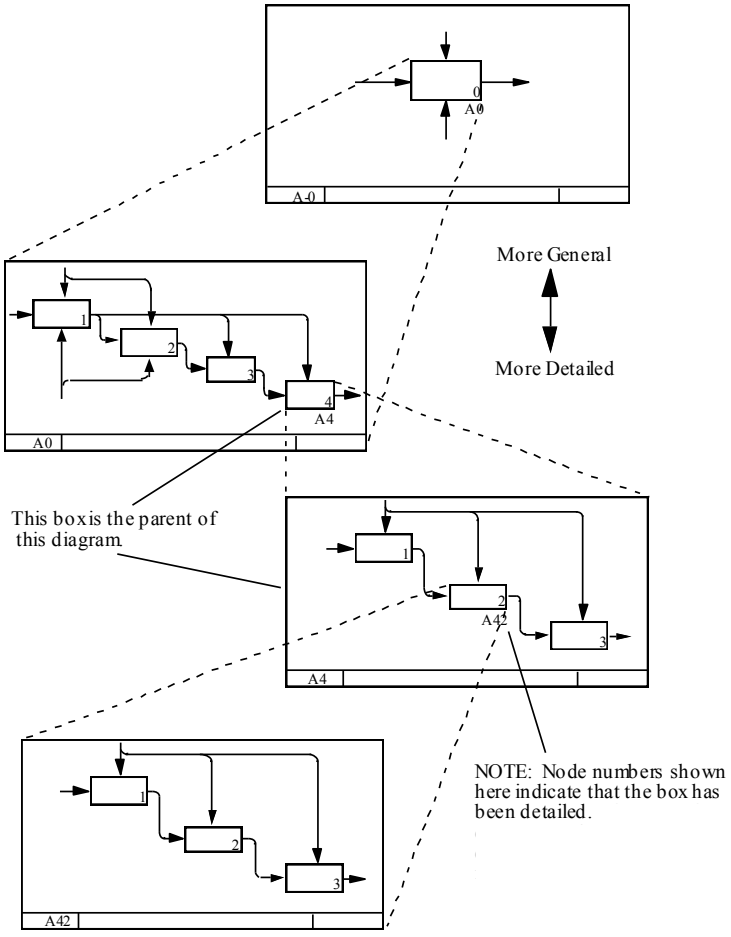


Figure 7.1: General structure of an IDEF0 model. Adapted from [291].

[4] A diagram in which the process outlined can be sub-divided into further sub-processes.
[5] A diagram that explains a parent-diagram in further detail.

Each diagram within the hierarchical decomposition consists of a series of boxes and arrows (Figure 7.2). Boxes depict the functions that need to be fulfilled and are identified by a verb or verb phrase describing what must be accomplished. Meanwhile, the arrows represent the functional relationships, which may be inputs (I), controls (C), outputs (O) and mechanisms (M). These are collectively known as ICOM arrows. The inputs represent data that is transformed into an output as a result of the function being carried out. The controls represent conditions that are required to produce the correct output, whilst mechanisms represent the means by which a function is carried out through the use of human or material resources [291]. Also, each box has a process ID, which is used to identify the particular process, and may have a node ID, which identifies the presence of further sub-divisions within a child-diagram.

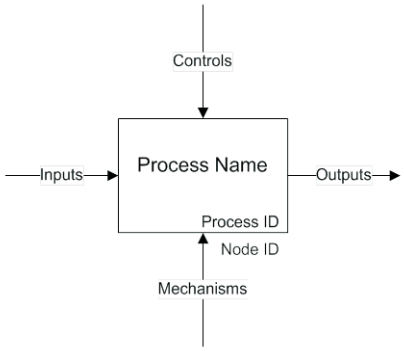


Figure 7.2: IDEF0 basic components.

7.2 Decision Tool Development

The decision tool was developed according to the methods specified by Federal Information Processing Standards (FIPS) publication 183 [291], which is maintained by the National Institute of Standards and Technology (NIST) and is the standard for IDEF0 modelling. The method consists of the initial identification of the model's context, viewpoint and purpose, which is also known as the model's orientation (Section 7.2.1). It is followed by the model's development, which includes the creation of the hierarchical series of diagrams (Section 7.2.2), and any supporting materials as necessary (Section 7.2.3). Of note is that, although the decision tool's development is here presented as a serial process, it largely occurs through an iterative process with additional refining as the tool's development proceeds.

7.2.1 Model Orientation

The decision tool's orientation is depicted by the top-level context diagram, which is termed the A-0 diagram (Figure 7.3). This diagram outlines the model's overall context and, consequently, the model's boundaries. Thus, the A-0 diagram allows the model's user to gain an overall understanding of the model's function. In addition to the model's viewpoint and purpose, the model's overall inputs, outputs, controls and mechanisms are included.

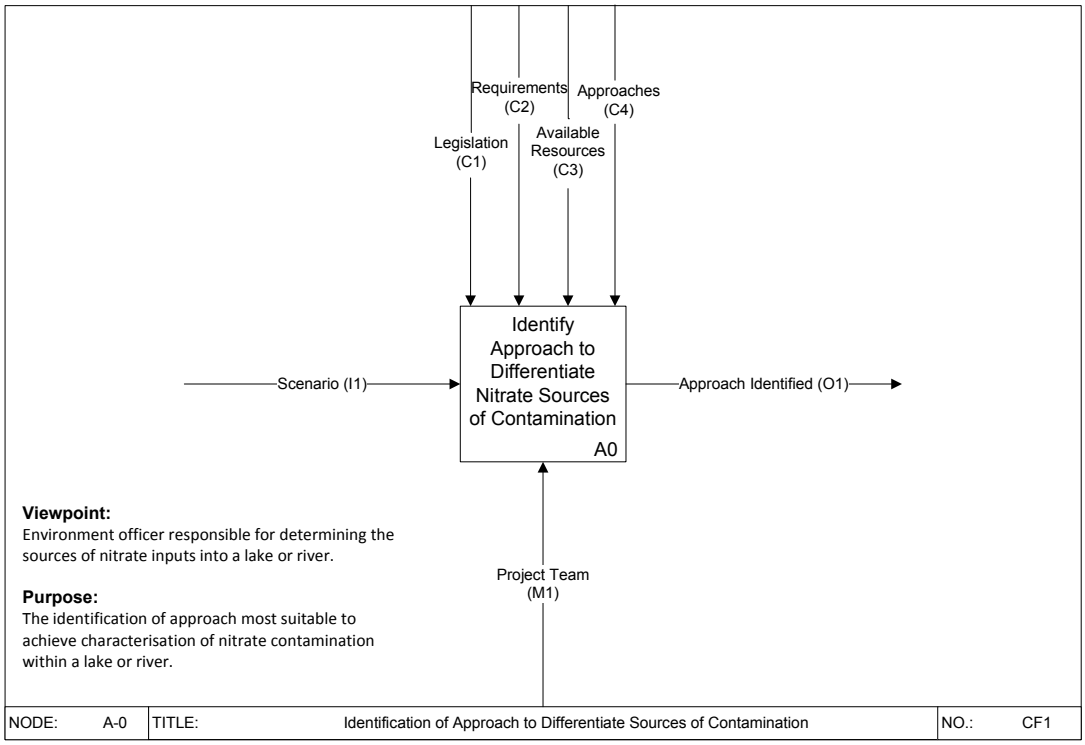


Figure 7.3: The model's top-level context diagram (A-0).

For the purpose of this decision tool, the selected viewpoint was that of an individual responsible for identifying the most suitable approach for achieving nitrate source differentiation within surface waters. This viewpoint may be held by people who may be approaching a need for differentiation from slightly different perspectives but who would, nevertheless, have the same functional requirements and purpose. The first are environmental protection officers within government entities, such as environment protection agencies, county councils and river basin management. For these stakeholders, the aim for applying the model may be to identify sources of nitrate contamination in order to achieve cost- and time-efficient remediation, and/or to apply the 'polluter pays principle' in situations where nitrate contamination has

occurred. This represents the major viewpoint used to build this model.

On the other hand, environmental officers within industries that produce nitrate containing effluents, e.g. WWTPs and farms, would also find this model's application useful. In such a setting, the scenario may be to show culpability, or lack thereof, with respect to their contribution to nitrate contamination. The data obtained through the application of the identified approach may be used to justify further investment into technologies to reduce nitrate concentrations in effluents and, consequently, nitrate inputs into surface waters. Additionally, it may be used as a defence in cases where the 'polluter pays principle' is being implemented against them.

Within the overall model, one input (I1), one output (O1), four controls (C1, C2, C3, C4) and one mechanism (M1) have been identified as depicted in the A-0 diagram (Figure 7.3). Since the approach to be taken (O1) largely depends on the scenario under study (I1), these have been identified as the model's only overall output and input, respectively. However, additional interim outputs do emerge throughout the course of the model's application. These are described in the coming sections as they arise.

A number of controls were identified to constrain the transformation of the scenario (the input) into the identified approach (the output). The relevant legislation under which this study is operating is the first control (C1). The legislative control depends on the particular scenario. It is likely to be the ELD (or equivalent outside of Europe) and additional legislation such as the UWWTD, the Nitrates Directive and relevant case law. The specific requirements (C2) of the entity carrying out the study are a second control mechanism. They determine the scope of the study and the extent to which the sources of nitrate contamination are differentiated and, therefore, the approach to be taken. These are largely related to the organisation's need leading to the study being carried out.

The third control mechanism is that of available resources (C3). This mechanism takes into account factors such as time and budgetary restrictions, as well as the level of expertise available. The level of expertise refers to both that available within the organisation carrying out the study as well as that available to the organisation from external sources e.g. consultants. The final control mechanism represents the characteristics of the various approaches (C4) that determine the outcome of the model's implementation.

The functions within this model are carried out by the project team that is identifying the most suitable approach to differentiate the sources of nitrate contamination under the guidance of the environmental officer. Therefore, the project

team represents the model's only mechanism (M1). The project team would be headed by the environmental officer from whose viewpoint this model is developed. However, this individual would require inputs from relevant individuals within and external to the organisation as necessary, such as financial officers, legal experts and scientific personnel.

7.2.2 Model Development

On the basis of the model's context given in the A-0 diagram, further functional decomposition was carried out, thereby representing the process of transforming the model's overall input (I1) into an output (O1) in greater detail. The top-most diagram (A0) is the only child-diagram of the A-0 top-level context diagram, where the model's global function defined within the A-0 diagram is sub-divided into the second tier of functionality. Each function within the A0 diagram is, then, further decomposed into a corresponding child-diagram. Within the model presented here, no additional child-diagrams arising from this third tier were necessary, because the model was considered to be sufficiently detailed at this stage.

The A0 Diagram. This diagram outlines the three functions that have been recognised to be necessary for identifying the most appropriate approach to determine the source of nitrate contamination (Figure 7.4). These are, namely:

1. Determine the context of the scenario in which this model is to operate (Function 1): Why is differentiation needed?
2. Determine the differentiation criteria of interest within the specific scenario (Function 2): What should differentiation achieve?
3. Determine the differentiation approach (Function 3): How is differentiation going to be achieved?

Each function in the A0 diagram is further sub-divided into a corresponding child-diagram (A1, Figure 7.5; A2, Figure 7.6; A3, Figure 7.7), as specified by the node ID. The child-diagrams have a number of sub-functions for each parent function outlined above. Sub-functions are labelled as *'parent-function' 'child-function'*. For example, Function 11 is the first child-function of parent-function 1, whilst Function 32 is the second child-function of parent-function 3. A number of interim outputs have also been identified. These have been labelled as *'function number'*.O*'output number'*. For example, 13.O1 means Function 13, Output 1, whilst 22.O2 means Function 22, Output 2.

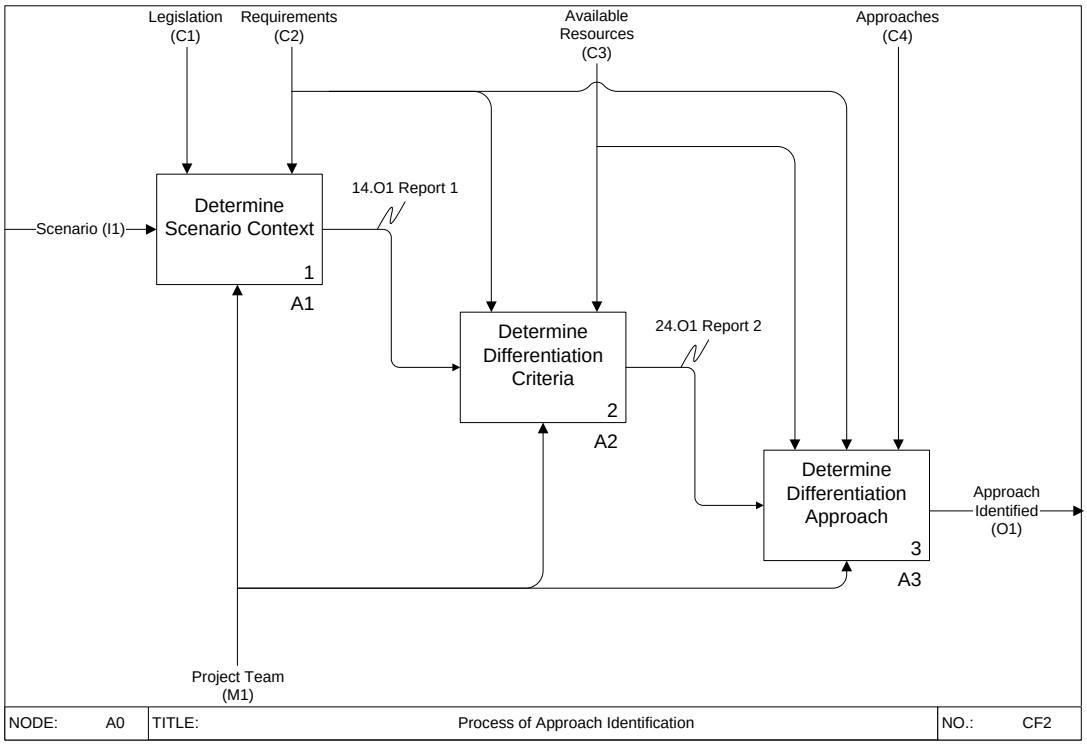


Figure 7.4: The top-most diagram A0.

The A1 Diagram. The first child-diagram (A1, Figure 7.5) delineates the functions and functional relationships required to successfully achieve Function 1 within the model i.e. determine the scenario context. It consists of four sub-functions. Initially, the scenario characteristics need to be identified (Function 11). The differentiation objectives (Function 12) and evaluation criteria (Function 13) can be determined concurrently with the determination of the scenario characteristics (Function 11), because Functions 12 and 13 are not under the control of any of the outputs from the previous functions.

Function 11 results in a number of outputs, namely an identification of the site characteristics (11.O1), the need for nitrate source determination (11.O2) and potential sources of nitrate within the site (11.O3). An important factor at this point is an understanding of potential temporal and spatial changes within the specific catchments being studied. Different sources of contamination might be more relevant at different periods of the year or locations. These are necessary for establishing the temporal and spatial resolution of sampling and, consequently, costs. The requirements of the study are an essential control (C2) as they limit the extent to which scenario characterisation is required and, consequently, carried out.

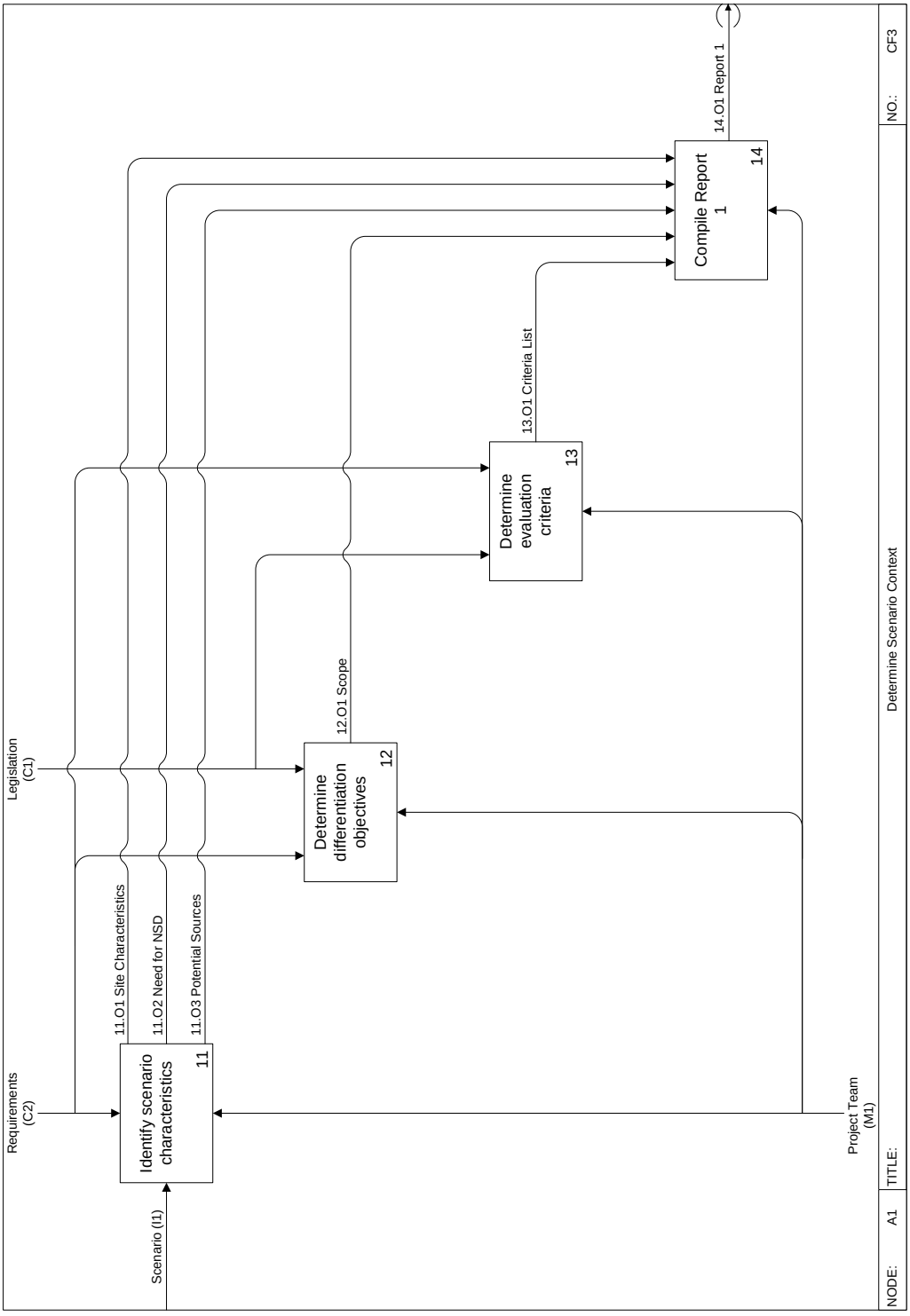


Figure 7.5: A1 child-diagram. NSD: Nitrate Source Determination.

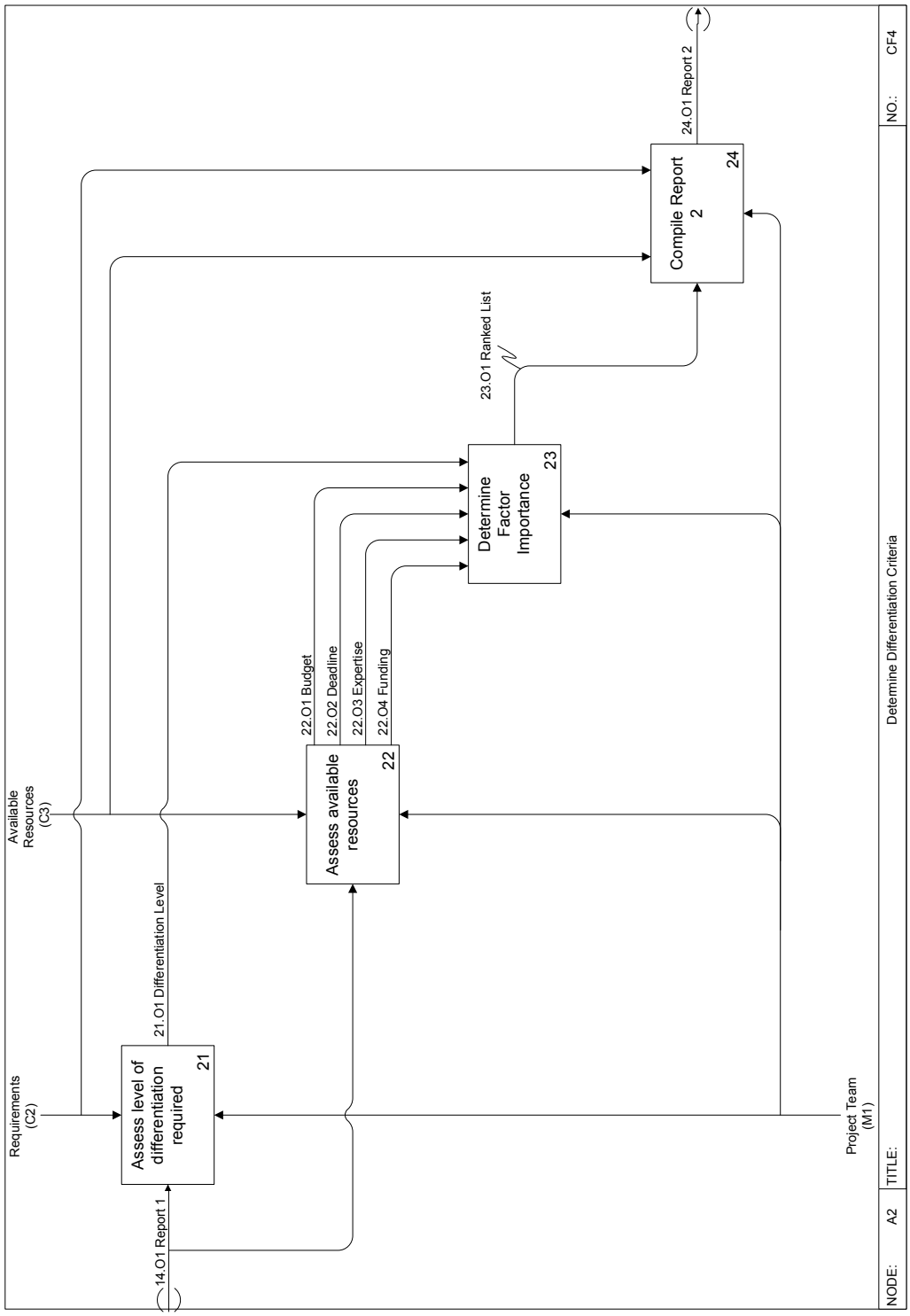


Figure 7.6: A2 child-diagram.

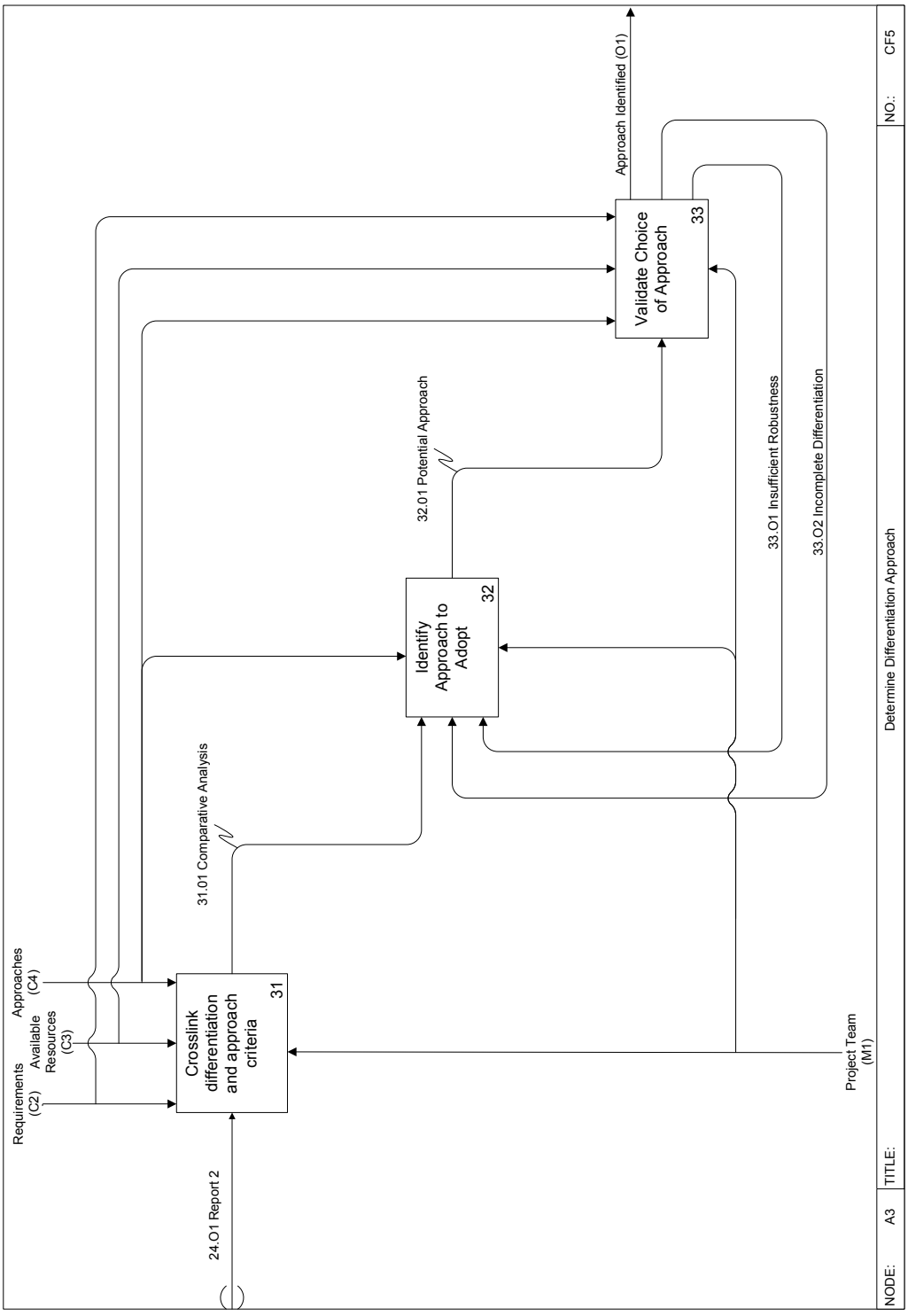


Figure 7.7: A3 child-diagram.

The next function, Function 12, involves the determination of the study's differentiation objectives resulting in a definition of the study's scope (12.01). These objectives are controlled by the study's requirements (C2) and legislation (C1) because, for example, the methods chosen for an initial scoping study differ from those that are used where the study is intended for court litigation. For instance, it may be relevant to look at an approach that differentiates between a wide range of nitrate sources within a scoping study. Meanwhile, in a court case, it may be more relevant to distinguish between the specific sources of interest to the case, potentially through the use of multiple approaches to increase support to the resulting position. An understanding of governing legislation and relevant case law (C1) would also be necessary in instances where litigation is within the study's outcome. As a result, the approaches taken during previous court cases and which have been successful or unsuccessful are identified.

The legislative and study requirements (C1 and C2) are also critical in determining the evaluation criteria to be utilised (Function 13). This understanding leads to the development of a study criteria list (13.01), which identifies the factors acting as operational limitations and that have decisive roles in determining the approach to be undertaken, e.g. cost, time, expertise and robustness.

Then, the data compiled through Functions 11, 12 and 13 are collated to form the first report (14.O1). This report outlines the context of the scenario being investigated, both on a technical and operational level, and is the only overall output of the A1 child-diagram. It is used to inform the second step of the process, as depicted by the corresponding child-diagram to Function 2, namely child-diagram A2 (Figure 7.6).

The A2 Diagram. Function 2 (Determine Differentiation Criteria) is further elaborated upon within the A2 child-diagram in order to define the process of determining the differentiation criteria in greater detail. It consists of four separate subfunctions. On the basis of the first report produced by the project team (14.O1), the level of differentiation required (Function 21) and the available resources (Function 22) may be assessed independently of each other.

In assessing the level of differentiation required (21.O1), a number of factors need to be considered. These include issues such as:

- Is the presence/absence of a particular source or source attribution required?
- Is differentiation between inorganic and organic sources of nitrate sufficient, or do the various inorganic or organic sources need to be further differentiated?

- In differentiating sources of organic contamination, is differentiation between the route of entry required (e.g. raw and treated sewage)?

The assessment of available resources for carrying out the study (Function 22), requires considerations such as budget availability (22.O1), time availability for the study's completion (22.O2), available in-house or independent expertise (22.O3) and available funding from external sources (22.O4). An understanding of available resources is critical when carrying out environmental forensics studies, because available resources frequently have a decisive role in determining the approach to be undertaken to achieve nitrate source determination.

Subsequently, the outputs of sub-functions 21 and 22 are utilised in the preparation of a ranked list (23.O1) conveying the relative importance of the various technical and operational factors of relevance to the study (Function 23). In particular, the ranked list allows for operational decisions related to the study being undertaken to be made, e.g. whether cost or the extent of differentiation are a critical component of the study and, thus, have decisive roles.

The data collected within Functions 21, 22 and 23 would, then, be used in the compilation of the second report (24.O1), which outlines the criteria for differentiation. Therefore, this report includes the main criteria to be considered in selecting the differentiation method to be adopted, as well as their ranking scheme according to the various budgetary, time and expertise availability constraints. This second report is, then, utilised to determine the most suitable differentiation approach (Function 3) as outlined in child-diagram A3 (Figure 7.7)

The A3 Diagram. In determining the most suitable approach, the various factors defined in Report 2 (24.O1) are cross-linked against the characteristics of the different approaches (C4) that may be adopted to achieve differentiation (Function 31). By cross-linking the differentiation and approach criteria (Function 31), it would be possible to identify the approach that best meets the differentiation criteria determined (Function 32, 32.O1).

The initially identified method is subsequently validated through an assessment of the approach taken (Function 33) in relation to the study's requirements (C2), the available resources (C3) and the approaches that may be adopted (C4). In case that increased robustness is required (33.O1) or the required differentiation is not complete (33.O2), this need is fed back to Function 32 and a different or additional approach is followed. When a satisfactory level of differentiation is obtained, the final approach, or set of approaches, to be adopted is identified (O1), which is the model's final output.

7.2.2.1 Model Summary

To facilitate the model's application by individuals who are not familiar with the IDEF0 methodology, a summary was developed to complement the IDEF0 model. The summary is wholly based on the developed IDEF0 model described, which ensures the model's robustness. Two complementary summaries were prepared, one in the form of a flow-chart (Figure 7.8) and another as an accompanying table of questions (Table 7.1).

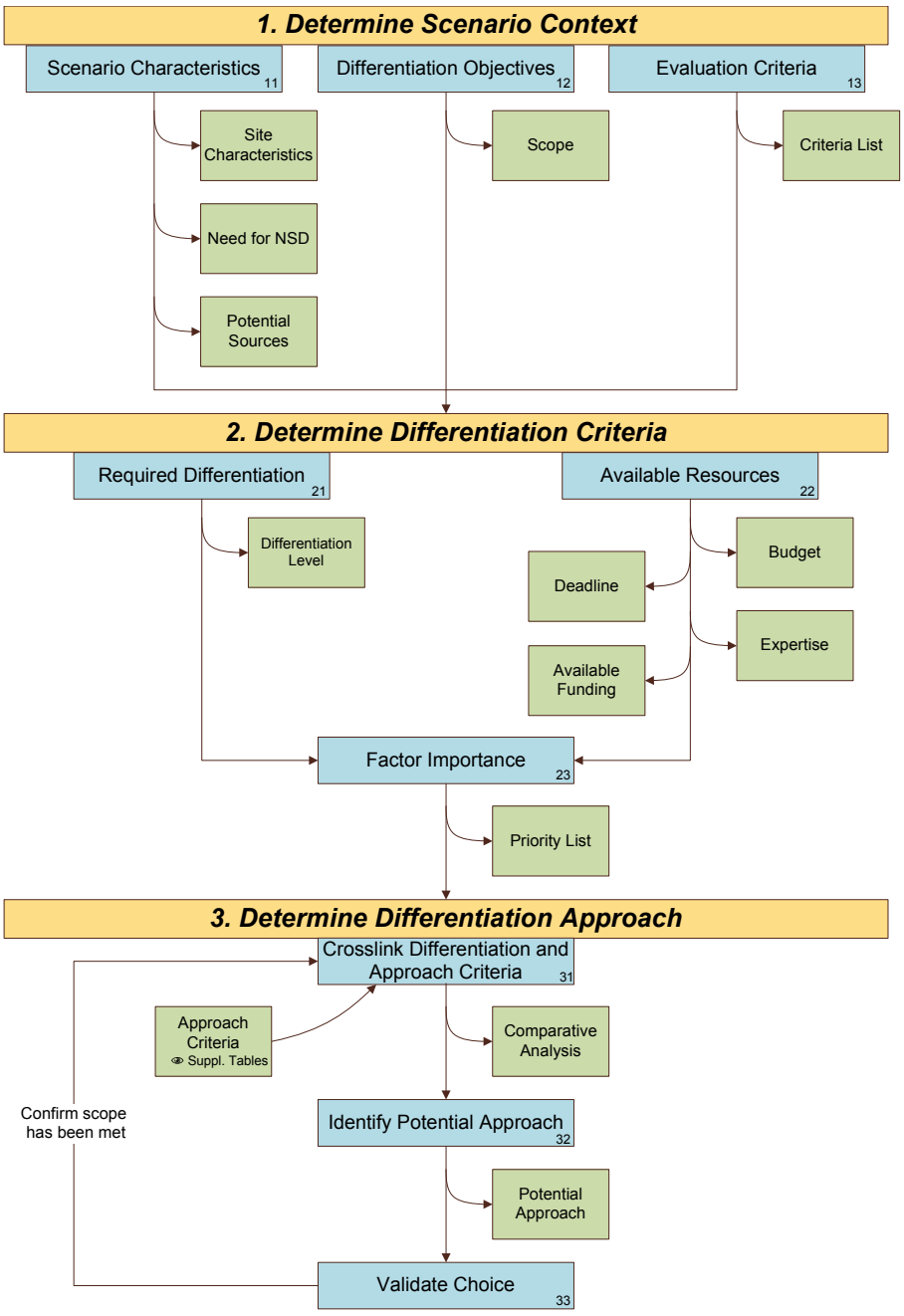


Figure 7.8: Decision tool summary flow chart.

Table 7.1: Model Summary.

A0 Identify Approach to Differentiate Nitrate Sources of Contamination	
A1 Determine Scenario Context	**Why is differentiation needed?**
11 Identify Scenario Characteristics	What are the site characteristics? e.g. What is the composition of the catchment? What is the expected temporal and spatial variability? Why is nitrate source determination required? What are the potential sources?
12 Determine Differentiation Objectives	What are the study's objectives? e.g. Is it a scoping study or is it for legal action?
13 Determine Evaluation Criteria	What are the factors of interest in this study? e.g. Operationally, do cost, time, expertise and/or robustness have decisive roles?
A2 Determine Differentiation Criteria	**What should differentiation achieve?**
21 Identify Level of Differentiation Required	What level of differentiation is required? e.g. Is presence-absence of a particular source or source attribution required? Do the various inorganic or organic sources need to be further differentiated? Is differentiation based on entry route (e.g. raw and treated sewage) required?
22 Identify Available Resources	What budgets are available for this study? What is the time-line for completing the study? What is the in-house available expertise? What expertise can be sub-contracted? What potential for external funding is there?
23 Determine Relative Importance of Factors	What are the most critical criteria of the study? What factors of functions 21 and 22 are must-haves or just good-to-haves?
A3 Determine Differentiation Approach	**How is differentiation going to be achieved?**
31 Crosslink Differentiation and Approach Criteria	How do the differentiation requirements relate to available approaches?
32 Identify Approach to Adopt	Which approach satisfies the most differentiation criteria?
33 Validate Choice of Approach	Does the selected approach satisfy the study requirements? Are there any differentiation requirements that have not been achieved?

7.2.3 Supporting Materials

As previously mentioned, a limitation of currently available data on nitrate source determination is that it is highly fragmented and approach dependent. This factor makes it very difficult for stakeholders to identify the most suitable approach for a specific scenario, unless resources are available for a comprehensive review of literature. Since IDEF0 models are largely conceptual, the developed model is not suitable for resolving issues related to the fragmented nature of the current state of knowledge. This role is fulfilled through the addition of supporting material to complement the decision framework illustrated within the IDEF0 model.

The availability of supporting material was identified to be particularly critical in relation to the model's fourth control (C4: Approaches), which plays a central role within the A3 child-diagram (Figure 7.7). Data related to controls C1, C2 and C3 (legislation, requirements and available resources) are largely scenario dependent. Therefore, they need to be identified by the organisation carrying out the analysis. However, the potential pool of approaches is universal.

Of note is that an advantage resulting from the nature of the IDEF0 modelling system, whereby it is conceptual and flexible, is that the decision tool developed would not require frequent updating. Rather, it is this supporting material that needs to be updated on the basis of new advances in the number and variability of approaches that are developing in this evolving field. This factor facilitates the application of the developed decision tool as it eliminates the need for an overhaul of the entire tool on a regular basis.

7.2.3.1 Approach Criteria

To date, four main approaches have been largely adopted for nitrate source determination (Chapter 2). These are, namely, nitrate isotopes, genetic markers, microbiological analyses and chemical markers. The four approaches have a number of applications, depending upon the factors under consideration. Some of the main considerations include the level of differentiation that they can achieve and the specific operational characteristics of the various approaches.

The first supporting material outlines the level of differentiation that the various approaches can achieve (Table 7.2). As can be observed from Table 7.2, whilst all four approaches may identify the presence of faecal contamination (manure and sewage), only nitrate isotopes can identify inorganic nitrogen fertiliser. Similarly, only chemical markers are able to differentiate between raw and treated sewage. Genetic markers are, then, generally host specific. Therefore, whilst they are unable

to differentiate between raw and treated sewage, they may differentiate between different sources of manure.

Table 7.2: The differentiation characteristics of the four main approaches outlined in literature. Consecutive sources of contamination with the same shade cannot be differentiated using that particular approach. For example, nitrate isotopes cannot differentiate between different sources of manure and sewage (both in group 1 for nitrate isotopes), but can differentiate between fertiliser nitrate (group 3) and soil nitrogen (group 4) and ammonium in fertiliser (group 6). Chemical markers, then, can differentiate between three classes (manure, raw and treated sewage) but cannot differentiate between different sources of manure.

Source of Contamination	Nitrate Isotopes	Genetic Markers	Micro-biological	Chemical Markers
Manure (organism *1*)	1	1	1	1
Manure (organism *2*)		2		
Manure (organism *x*)		3		
Raw Sewage		4		2
Treated Sewage				3
Nitrate in precipitation	2	NA	NA	NA
Inorganic N fertiliser	3			
Soil nitrogen	4			
Desert nitrate deposits	5			
Ammonium in fertiliser	6			

In addition to the differentiation potential of the various approaches, a number of other considerations are of importance in determining the most suitable approach for a particular scenario. These are largely operational parameters. The most pertinent are outlined in Table 7.3, and the corresponding characteristics of the four main approaches are given.

Of note is that, certain factors, such as cost and technique availability, may be very subjective depending upon the entity carrying out the study. This difference is mainly related to in-house expertise as compared to sub-contracted analyses. Similarly, the level of expertise required might not be considered as important in a particular scenario, as an 'expert' in a particular area requiring a high level of expertise may be employed within the entity itself. Furthermore, where sample volumes are given, these are based on the most commonly applied technique to date. Thus, for chemical markers, a sample volume of 'Litres' is given on the basis of SPE LC-MS/MS analysis even though through the use of, e.g. immunoassays (Chapter 5), sample volumes may be greatly reduced.

This supporting material would, therefore, be used in conjunction with the IDEF0 model developed to facilitate the selection of the most suitable approach

Table 7.3: Characteristics of the four main approaches. An increased number of '+' symbols indicates an increase in cost, time, level of expertise, state of approach, sample volumes and technique availability.

	Nitrate Isotopes	**Genetic Markers**	**Micro-biological**	**Chemical Markers**
Instrumentation	IRMS	Various	Incubator	Various
Multi-Source Determination	No*	Yes	No	Yes
Time Requirement	Days	Hours	Days	Hours
Sample Volume	Millilitres	Millilitres	Centilitres	Litres
Typical Cost	++	+++	+	++
Level of Expertise	++	+++	+	++
State of Approach	+++	+	+++	++
Technique Availability	+	++	+++	++

* Main source or average of the various sources determined.

to achieve nitrate source differentiation. Taking the example of a scenario where the presence of faecal contamination is to be identified, all four approaches would be suitable (Table 7.2). Thus, it is likely that the available resources and approach characteristics are the determining factors in identifying the most suitable approach to be adopted. In this case, it is most likely that microbiological analysis, involving the determination of faecal indicator bacteria (e.g. faecal coliforms) is used. This is because this method is relatively low cost, requires a low level of expertise for its application, is a well-defined approach and is a widely available (routine) technique. However, it has significant time constraints, in that these methods are culture-based. Therefore, sufficient time for culture growth is required before results can be obtained e.g. 24 hours for faecal coliforms, with limited opportunity for sample storage. On the other hand, if time is deemed a more critical differentiation criterion than e.g. cost, one of the other methods might be more suitable.

Of note is that, if the data obtained from the investigation is to be used in a law suit, it is likely that a multitude of methods need to be adopted in order to strengthen the case that is being put forward (33.O1). It might also be the case that more than one approach needs to be used in order to achieve the required differentiation (33.O2). These include situations where, for example, differentiation of sewage, manure and fertiliser nitrate is required where a multi-pronged approach would be necessary.

7.3 Model Evaluation

Model evaluation was carried out in a two-step process of model verification followed by model validation. Model evaluation is necessary to ascertain the usefulness of the

developed decision tool for identifying the most suitable approach to differentiate between sources of nitrate contamination in surface waters. Model verification and validation are discussed in Sections 7.3.1 and 7.3.2, respectively.

7.3.1 Model Verification

Verification involves ensuring that the model was correctly developed, thereby allowing for the required specifications to be achieved. The major considerations are that no gaps are present in the model and that consistency is ensured in the ICOM arrow depictions. This factor is particularly critical for those ICOM arrows linking to a parent box and, therefore, needing to be depicted on the corresponding child-diagram. Thus, four matrices were constructed. These were for the inputs (Table 7.4), controls (Table 7.5), outputs (Table 7.6) and mechanisms (Table 7.7).

The matrices were constructed as follows:

- The ICOM arrows linking to the A-0 diagram were inputted into separate columns within their relevant matrix (Input arrows in the input matrix etc.);
- The function/s to which each of the arrows links on the A0 diagram is noted in the first row, labelled A0;
- The specific function/s to which each of the arrows links within the child-diagram is noted within the corresponding child-diagram row;
- The presence of an entry in the first row is cross-checked against the presence of an entry/entries within the relevant child-diagram rows, and;
- Any identified discrepancies, where an arrow linking to a parent-diagram is not reflected on the relevant child-diagram or vice-versa, were corrected as required.

Within the developed model, there is only one parent-diagram (A0, Figure 7.4). Therefore, only one set of matrices was required in order to ensure consistency in the ICOM arrow depictions throughout the model.

	I1	**14.O1**	**24.O1**
A0	A1	A2	A3
A1	11		
A2		21	
A3			31

Table 7.4: Matrix for input arrows.

	C1	**C2**	**C3**	**C4**
A0	A1	A1, A2, A3	A2, A3	A3
A1	12, 13	11, 12, 13		
A2		21, 24	22, 24	
A3		31, 33	31, 33	31, 32, 33

Table 7.5: Matrix for control arrows.

	O1	**14.01**	**24.01**
A0	A3	A1	A2
A1		14	
A2			24
A3	33		

Table 7.6: Matrix for output arrows.

	M1
A0	A1, A2, A3
A1	11, 12, 13, 14
A2	21, 22, 23, 24
A3	31, 32, 33

Table 7.7: Matrix for mechanism arrows.

7.3.2 Model Validation

Model validation ensures that the developed decision tool carries out its intended function and that it meets the requirements of its users. A comprehensive case study validation, where a particular organisation follows the entire decision-making process, was not possible within the confines of this research project. Thus, a limited validation was performed by surveying a number of key stakeholders in order to explore the potential and limitations of the developed decision tool[6]. Individuals from three stakeholder categories within the water and environmental management field were targeted - regulators, operators and environmental laboratories. The stakeholders included:

- A river basin district (RBD) coordinator;

[6] Ethical approval was received under the DCU Research Ethics Committee notification procedure for low risk social projects with reference DCUREC/2012/169.

- Two county council officials (a scientist and an engineer) within the environmental department;
- The head of environmental enforcement at a major water and sewerage provider, and;
- The managing director of an environmental laboratory.

The survey strategy adopted was that of face-to-face semi-structured interviews. These are widely used in exploratory and explanatory research, such as that carried out here, since they allow for probing answers and clarifications during the survey process [298]. An interview pack consisting of the IDEF0 model, the supplementary material, the model's flow chart summary, the model's question-based summary in the form of a reporting tool and a consent form was used during interviews (Appendix G). These materials, with the exception of the consent form, represent those that will be used for decision tool dissemination. Thus, stakeholder attitudes to the decision tool, as it will be disseminated, could be obtained.

A mixture of one-to-one and group interviews was undertaken, depending upon the interviewees' availability and setting. All interviews were audio recorded following an initial short explanation of the purpose of the interview and the provision of ethical consent. Audio recording allowed for a full record of the conversation to be maintained whilst allowing for increased engagement with the discussion, as compared to extensive note-taking. In order to maintain interviewee anonymity, the individual's position will be used as an identifier.

Within the following sections, the outcomes of the various interviews are presented. These are grouped into a number of headings as follows:

1. The currently adopted approach in scenarios requiring nitrate source determination;
2. The potential for using the decision tool in their organisation;
3. The perceived benefits of the proposed decision tool, and;
4. The perceived limitations of the proposed decision tool.

7.3.2.1 Currently Adopted Approach

In order to establish the need for a decision tool for nitrate source determination, issues related to the currently adopted approach were explored. In this way, the stakeholders' views, including limitations and benefits, of the current decision-making process could be identified.

River Basin District Coordinator. To date, they have carried out limited studies on nitrate source determination. Most efforts have been related to risk identification for nitrate contamination within catchments. These include looking at septic tank densities, WWTP capacities and the intensity of agricultural activities (e.g. number of livestock heads). Also, they participate in projects carried out by the EPA and the Agricultural Catchments project carried out by Teagasc[7], related to RBD management.

In their studies, they have mainly used inorganic chemical markers such as phosphorus concentrations. However, they expressed their concern that this currently adopted approach only allows for most of the major point sources to be identified. Following source apportionment of point sources of contamination, the remaining nitrate portion is termed 'diffuse pollution'. This has led to agricultural organisations tending to disagree with the outcome and believing that the onus is incorrectly put on them.

County Council Environmental Officials. The county council officials stated that they have not specifically looked at sources of nitrate contamination within their catchment. This is because surface waters within their region have generally fallen well within the legislative requirements, which have been their main concern. Nevertheless, they have coordinated with the relevant RBD management team on some studies related to nitrate source determination.

Head of Environmental Enforcement. As yet, they have had no experience of environmental forensics studies for nitrate sources. To date, their interest has mainly focussed on WWTP effluent monitoring. Since these effluents are largely point sources, there has been no scope for such studies. However, they are aware of some studies on nitrate source determination carried out by the environmental regulator in the region as well as by the UK Water Industry Research organisation (UKWIR) in relation to RBD management.

Environmental Laboratory Managing Director. The interviewed managing director of an environmental laboratory stated that they currently do not carry out environmental forensics studies. Within their operation as an environmental laboratory, they only analyse clients' samples depending upon their clients' requests. Generally, this testing is related to current legislation compliance.

[7] The Irish agriculture and food development authority.

7.3.2.2 Potential for Using the Decision Tool

Once the developed decision tool was explained to the interviewees, the potential for its use within their organisation was explored. This allowed for their view of their organisation's need for such a decision tool within the short and long-term, to be explored. Such information is of interest to this study, as the usefulness of the developed decision tool is directly linked to it being applied in practice within the various organisations.

River Basin District Coordinator. The RBD coordinator sees great potential for using such a decision tool within their organisation. In fact, a recent gap analysis carried out as part of the RBD management plan has recognised the need to identify specific sources of nitrate contamination, rather than assigning risks for contamination, as an issue that needs to be tackled.

County Council Environmental Officials. The county council officials stated that they see limited scope for applying the entire decision tool in terms of nitrate source determination within their organisation. However, they are interested in its partial application in order to identify sources of faecal contamination within their region. This interest is particularly in the differentiation of raw and treated sewage in view of their responsibilities related to the operation of various WWTPs and the recent introduction of the Water Services (Amendment) Act of 2012 [230], which requires that an inspection plan for septic tanks be implemented. With the use of such a decision tool, they can identify the most suitable approach to adopt in identifying areas contaminated by raw sewage, indicating septic tank infiltration, and utilising the data gathered in the prioritisation process for septic tank inspections.

Head of Environmental Enforcement. Although they have not had any need for nitrate source determination to date, they are aware of a simulated catchment modelling tool being developed for their region to which they would be feeding in data. They see such a decision tool as an important supporting mechanism for this catchment modelling tool, to inform the data being entered and to challenge or support the data being fed in from other sources. They perceive this tool to be useful in gathering a greater evidence base for nitrate pollution sources, in particular diffuse pollution, which is largely ignored to date. Within their organisation, the results from the application of such a decision tool would be useful in identifying the most pertinent sources of nitrate contamination. This is particularly so because, to date, the focus has been on simply ever tightening effluent standards, which can

result in no benefit on the overall levels of river contamination but that are easier targets. In this way, efforts could be more targeted to the actual source.

Environmental Laboratory Managing Director. Within the current scope of the environmental laboratory, the managing director could see very limited potential for using the developed decision tool. This is because the role of their environmental laboratory is to test samples to client specifications. The driving force for their business is largely legislation. Under current legislation, there is no real need for such studies. Therefore, the necessary customer base is not there. However, if the environmental enforcement agency, or equivalent, were to include requirements for such studies into source determination such a tool would be useful in the long-term.

7.3.2.3 Benefits of the Decision Tool

An important factor in the use of such a decision tool is that it is accepted by its users and they find it easy to apply. Therefore, the perceived benefits of the proposed decision tool, on the basis of the decision tool explanation given, were assessed.

River Basin District Coordinator. Through the application of the proposed decision tool, the RBD coordinator identified that they can make more objective decisions for identifying sources of nitrate contamination. In particular, it would allow them to narrow the field of 'uncertainty' in identifying sources of nitrate contamination and, particularly, diffuse sources of contamination, which so far are largely considered as a single entity. To date, they have generally made use of approaches they are familiar with. However, these might not be as appropriate for their catchment or provide them with all the information they might be interested in.

Through the use of the decision tool in their study set-up, the thought process could be clarified and streamlined, allowing them to more suitably justify the conclusions from nitrate source determination studies. Furthermore, by standardising the thought process, a greater uniformity could be obtained, since, at the present moment, a different outcome is given depending on the report being read and the approach undertaken.

Furthermore, the decision tool's application could reduce costs by allowing them to identify which measures should be applied at specific points in their study in order to get the best value for money and identify the sources. For example, they would be able to utilise a wide-encompassing approach in the initial stages, as this allows them to identify broad classes of nitrate sources. Then, they would be able

to re-use the decision tool to identify another, potentially more costly, approach within the previously prioritised sites. At the moment, a major drive is to prioritise measures being implemented. Therefore, in the coordinator's opinion, such a decision tool is the right way to go to bring together the current state of knowledge and environmental requirements.

County Council Environmental Officials. The supporting tables to the decision tool were identified to be of particular benefit to the county council as they allow the user to compare the capabilities of different approaches at a glance. They allow for the initial application of a suite of analysis to identify risk areas and, then, additional analysis may be carried out depending on the level of detail required within the risk areas. To date, they would be required to do a literature review themselves and assess the different approaches' capabilities, which is something they do not have the resources to achieve.

Additionally, the results from applying the approach identified as the outcome of the proposed decision tool were identified to be of use to the county council, particularly in the area of faecal source determination. This is as a result of their responsibilities related to the septic system National Inspection Plan following the implementation of the Water Services (Amendment) Act of 2012 [230], which is to be initiated in mid-2013, and their role of WWTP operators in the area.

Head of Environmental Enforcement. A number of benefits of the application of the proposed decision tool were identified by the head of environmental enforcement at a major water and sewerage provider. This is particularly so as it allows for competing considerations to be identified and laid out. Within their organisation, cost and available in-house expertise are amongst their major considerations. Therefore, through the use of such a decision tool in identifying the most appropriate approach for differentiating sources of nitrate contamination, they can be more objective in identifying the approach to adopt rather than trying to make use of in-house expertise for each scenario.

Also, they identified that the tool is set up in a user-friendly manner for them to apply. This observation is because of the way that the decision tool flow-chart and the reporting tool take the user through the various steps required to be undertaken in a logical manner. Hence, it allows for decisions to be taken in a more objective manner since the same decision-making process is undertaken by different individuals within the organisation, and it allows for the decision-making process to be more objectively justified.

Environmental Laboratory Managing Director. Although the managing director of the environmental laboratory sees no immediate potential for the incorporation of the proposed decision tool within their operation, a number of potential benefits for other entities were identified. Of mention is their experience as an expert witness in court cases where there are several advantages offered by such a tool to enforcers. This is because the source of contamination and proving the cause and effect upon a polluted river is commonly the defence strategy adopted. Therefore, the application of results from such a tool could help legislators, councils and the environmental protection agencies be more effective.

7.3.2.4 Limitations of the Decision Tool

In addition to the benefits arising from the application of the proposed decision tool, it is essential to consider and address its perceived limitations. This understanding allows for the tool to be modified depending upon the requested changes and to understand where the impediments to its successful application are likely to lie.

River Basin District Coordinator. Although the tool is perceived to be of great benefit by the RBD coordinator, a potential limitation is that people applying the decision tool consider results from the application of the identified analytical approach to be the reality at all periods of time. This mindset would result in an over-simplification of environmental complexity, because, for example, different sources of contamination occur at different proportions at different times of the year due to different proportions of ground and surface waters. Therefore, a greater emphasis was placed on this factor in the model by specifying temporal and spatial variability as a consideration in the identification of site characteristics as part of Function 11 and Output 11.O1 (Table 7.1).

County Council Environmental Officials. Whilst they see such a decision tool to be of use to them, particularly in relation to their interaction with the RBD personnel, they identified that, since they also function as WWTP operators, they are interested in having decision tools for additional parameters, namely ammonia and phosphorus. This is in addition to their immediate interests related to the implementation of the Water Services (Amendment) Act of 2012.

Head of Environmental Enforcement. The head of environmental enforcement suggested an important addition to the proposed decision tool. Within their organisation, the availability of external funding for taking the outputs from the

decision tool to the next stage and carrying out the actual analysis is an important consideration when assessing the available resources in determining the most suitable approach. This is because, if there is funding for a particular type of research, they might tend to focus on that aspect of nitrate source determination to a greater degree. Therefore, an additional output into the IDEF0 model from Function 22, i.e. available funding, was incorporated into the final model presented (Figure 7.6) in comparison to that presented in the interview pack (Appendix G). The availability of decision tools for other parameters was also mentioned to be a potential improvement to the tool. Specifically, the availability of decision tools for ammonia and phosphorus were mentioned.

Environmental Laboratory Managing Director. With regards to the interviewee's organisation, specific legislation requiring their client base to implement the output from such a decision tool is the major current limitation. This legislation is necessary to make the decision tool a viable business prospect and build the necessary client base. Following the enactment of such legislation, it would, then, be necessary for the tool's acceptance by the legislators and regulators, which would take further time and effort.

7.3.2.5 Summary

To date, it seems that very little effort has been made in the field of nitrate source determination. Where it has been carried out, this has largely been in a superficial manner. These have included the identification of risks for nitrate inputs or the use of simple inorganic markers. This scenario is evidence of a significant mismatch between technical advances in the area and what is being used in the field.

In fact, it was mentioned that the availability of such a tool is of benefit to the stakeholders interviewed, as it allows for the current state of knowledge in this area to be distilled and effectively communicated to the individuals who need to use it. This outcome indicates that there needs to be increased communication of the potential approaches that may be adopted for nitrate source determination and the advantages and limitations of the same. The use of this decision tool in the identification of diffuse sources of nitrate contamination (including faecal contamination) seems to be particularly pertinent for the various organisations. Indeed, diffuse nitrate source determination was recognised by most interviewees as a major factor contributing to the tool's potential, as it seems to have been largely ignored to date.

The decision tool was perceived to provide a number of additional benefits by all the stakeholders interviewed, particularly as it allows for a streamlined and more

objective thought process leading to the identification of the most suitable approach for differentiating sources of nitrate contamination. Furthermore, it allows for standardised data and, thus, comparisons between studies to be made. The format of the decision tool was also mentioned to be user-friendly.

A number of limitations were also identified through the stakeholder interviews. Some were immediately fed back into the development of the modified decision tool presented here. However, others could not be directly tackled. These include the requirement of relevant legislation and the development of decision tools for alternative parameters, which may be of greater interest to their specific organisation. At the same time, the latter issue shows the tool's initial acceptance by the interviewees in that they see its benefit in the area of nitrate source determination and that they would like it to be extended.

7.4 Conclusion

Technical advances in the field of nitrate source determination have occurred steadily in recent years, particularly in relation to the use of isotopic, genetic and chemical markers. Yet, these techniques have largely failed to transition from academic studies into their application within the field. A reason for this lack of transition is believed to stem from the highly fragmented nature of knowledge in the area, which is approach dependent.

Therefore, this study was undertaken to develop a decision-support tool for nitrate source determination and evaluate its applicability through interviews with key stakeholders. The IDEF0 modelling system was used for the decision tool's development in order to ensure the tool's robustness. This IDEF0 model was then translated into a simplified flow-chart to facilitate the model's application since most stakeholders would not be familiar with IDEF0 techniques. In addition to this, supporting material and a reporting tool were developed in order to further facilitate the model's application.

Through interviews held with key stakeholders, it was identified that there is, indeed, currently a need for such a decision tool. The tool's flexibility allows it to be utilised for a range of purposes, depending upon the user's requirements. The use of the tool to identify the most suitable approach for diffuse nitrate source determination and faecal contamination were widely recognised. The potential for standardisation and objectivity in determining the most suitable approach in nitrate source determination was an additional benefit that was mentioned. This increasing potential is particularly in view of the ever increasing number of numerical models

being developed where data from different sources is plugged in, which is currently resulting in incompatible results.

These findings suggest a number of important implications for practice. They identify that there is a definite need for the development of such decision tools in the area of environmental forensics in order to act as a bridge between the current state of technical knowledge and practice. In fact, a number of stakeholders outlined their need for additional tools, depending upon their current requirements. One issue that was not addressed within this study was where would the responsibility for updating the supporting material forming part of the decision tool lie. Whilst the general framework is not expected to require significant updating, the supporting material needs to be reviewed on a regular basis following technical advances in the various fields.

Chapter 8

Conclusions and Further Work

In this study, a number of issues within the field of environmental forensics for nitrate source determination in surface waters were addressed. This work was carried out in view of the fact that the field of environmental measurement is shifting towards increased efforts related to environmental forensics, where the focus is on gaining an understanding of sources as opposed to just levels of contamination.

This shift towards environmental forensics studies is well outlined by the UK Environmental Sustainability Knowledge Transfer Network (ESKTN), which estimates the UK environmental forensics market to have a value of around £10-15 million per annum by 2015 from a minimal valuation in 2008 [20]. This value is largely attributed to the implementation of the Environmental Liability Directive (ELD), which requires sources of contamination to be identified with the view of applying the 'polluter pays principle' [12]. Additionally, such studies would allow for more effective remediation of contaminated sites since actions would be targeted to the actual source of contamination as opposed to adopting broader measures.

Therefore, the development of environmental forensics studies is expected to become an increasingly important field of research in the coming years. Moreover, although the ESKTN study focusses on the UK market, a similar increase in the environmental forensics' market value would be expected to be observed within other countries, particularly those within the European Union. Consequently, suitable techniques that allow for the sources of different contaminants to be identified will be increasingly necessary.

In this chapter, the main conclusions and research contributions arising from this work are outlined (Sections 8.1 and 8.2, respectively). These are followed by a number of research questions that emerged during the present study and might be addressed in the future (Section 8.3).

8.1 Overview

1. To date, the identification of nitrate sources has relied heavily on the use of nitrate stable isotopes. However, these methods are not appropriate for fully differentiating nitrate sources, particularly sewage and manure, due to the similar pathway nitrate takes in animals and humans. Yet, such a differentiation is essential, especially as human health risks arising from sewage contamination are higher than those arising from manure contamination. Additionally, this differentiation would help in the application of the 'polluter pays principle' and for remediation of contaminated sites to be more effective.
2. The use of chemical markers, namely pharmaceuticals and related compounds such as food additives, was identified to provide the greatest potential for differentiating sewage and manure inputs into surface waters. This potential is largely due to their specificity and physico-chemical characteristics, such as a high water solubility and persistence. Differentiation is possible because, by identifying the presence of pharmaceuticals that are only approved for human and veterinary usage, the source of faecal contamination may, then, be determined.
3. The appropriateness of using pharmaceuticals is further strengthened by the fact that additional faecal contamination source characterisation is possible. Pharmaceutical physico-chemical properties determine their fate within the environment. For example, since acetaminophen is largely labile within WWTPs, its presence within surface water samples is indicative of raw sewage.
4. The application of a validated SPE LC-MS/MS method to samples collected from three monitoring sites in Ireland over a one year period (October 2011 to September 2012) confirmed the suitability of using pharmaceuticals as chemical markers for differentiating and characterising point and diffuse inputs of sewage and manure into surface waters. Of mention is the identification of raw sewage infiltration at sites upstream of WWTPs, indicating the relevance of diffuse sewage inputs in what are considered 'clean' waters, from e.g. on-site wastewater treatment systems.
5. SPE LC-MS/MS was the primary method investigated for the detection of pharmaceuticals within surface waters due to its ubiquity and acceptance for similar studies. However, it is worth looking at alternative analytical techniques that may allow current dependence on costly and time intensive LC-MS/MS analysis to be reduced. Factors such as reducing sample sizes and requirements for method development, validation and transfer associated with LC-MS/MS were

considered in two proof-of-concept studies on the potential of using NMR spectroscopy and immunoassay techniques as alternative analytical techniques for detecting pharmaceuticals within surface waters.

a) NMR spectroscopy: Fluorinated pharmaceuticals were identified to provide the greatest potential as analytes when using NMR spectroscopy. However, even with extensive optimisation efforts, a relatively high detection limit in the mg L^{-1} range was obtained. Therefore, it was concluded that NMR spectroscopy is largely unsuitable for detecting pharmaceuticals within surface waters using currently available technology.

b) Immunoassay Techniques: The use of immunoassay techniques has shown great promise for detecting pharmaceuticals within surface waters. Using the target analyte, enrofloxacin, a limit of detection of 12 ng L^{-1} was achieved, with limited sample preparation (filtration), low sample volumes (< 10 mL), high sample throughput and negligible matrix effects during analysis. The major consideration is the lower confirmatory potential afforded by immunoassay analysis as compared to LC-MS/MS due to the possibility of cross-reactivity and interferences within immunoassay analyses. However, the potential of immunoassay techniques for high-throughput analyses makes them particularly suitable for initial screening analyses, which can result in significant cost- and time-savings as compared to LC-MS/MS analysis.

6. In this work, pharmaceuticals are being used as chemical markers of sewage and manure contamination. In order to ensure that source characterisation is properly achieved, it was appropriate to look at other possible sources of pharmaceutical entry to surface waters, namely disposal. Results from a survey carried out to assess current attitudes to the use and disposal of medication show that very few people dispose of unused pharmaceuticals in the sewer. Therefore, the potential for incorrect source attribution as a result of unused medication disposal is low, thus confirming the suitability of pharmaceuticals as chemical markers of sewage and manure. However, the results obtained clearly show that there needs to be increased awareness on the subject. In fact, only 14% of all participants stated that they have ever received advice on pharmaceutical disposal, with certain cohorts being particularly uninformed.

7. Due to the wide variety of nitrate contamination sources and potential approaches that may be adopted for achieving nitrate source determination, it was necessary to take a broader look at the field of nitrate source determination. Therefore, a decision tool was developed to facilitate the process of selecting the

most suitable approach to achieve nitrate source determination. Interviews with key stakeholders confirmed that there is, indeed, a need for such a decision tool. Additionally, a number of stakeholders outlined their need for additional decision tools that facilitate environmental forensics studies for other contaminants.

8.2 Research Contributions

This study has attempted to make a contribution to the field of environmental forensics for the differentiation and characterisation of point and diffuse sources of nitrate contamination, with a specific focus on sewage and manure. The main research contributions arising from this study are outlined in the following points.

Consolidation of Research on Nitrate Source Determination. Available research on nitrate source determination is highly fragmented and approach dependent. Therefore, a literature review of the current state of knowledge was carried out to determine the legal and technological context of nitrate source determination. This review allowed for a number of research gaps in the area of nitrate source determination to be identified, upon which this study builds.

A Suite of Chemical Markers for Sewage and Manure Differentiation and Characterisation. Six sewage and four manure chemical markers, considered to provide the greatest potential for achieving differentiation and characterisation of sewage and manure, were identified. The suite of chemical markers was selected to be as compact as possible to facilitate method transfer between laboratories while allowing the sewage or manure source to be characterised as fully as possible, e.g. through the differentiation of raw and treated sewage inputs.

Multi-residue Chromatographic Method for Chemical Marker Detection. A single SPE LC-MS/MS method for the simultaneous analysis of 10 chemical markers within surface waters was developed and validated. The development of a single method for such an application was considered to be essential as it would allow for the method to be more easily transferred between laboratories, improve sample throughput and reduce costs. Detection limits for the developed method lie between 50 pg L^{-1} and 5 ng L^{-1}, depending upon the specific analyte and the mass spectrometric instrument used.

Proof-of-Concept Studies for Alternative Analytical Techniques. The use of alternative analytical techniques to traditional chromatographic and mass spectro-

metric techniques for detecting pharmaceuticals within surface waters was explored through two proof-of-concept studies. NMR spectroscopy has shown limited potential for this purpose. However, the use of immunoassay techniques has shown great potential and has an emerging role within such analyses. Data obtained from such analyses would allow for a decreased dependence on costly and time-consuming LC-MS/MS analyses since it is only where a high level of confirmatory data is required that such LC-MS/MS analysis needs to be carried out.

Baseline Data on Current Attitudes to the Use and Disposal of Medication. Baseline data on current attitudes to the use and disposal of medication was collated through a survey of 1449 individuals. The data obtained can be used to ensure that policy development in this area is matched to expectations and for communication activities to be tailored to meet current awareness levels. Specifically, a need for increased awareness on appropriate means of unused pharmaceuticals was identified. Within the European Union, collection systems should be in place for unused medications [290]. However, only a small proportion of participants have ever received advice on unused medication disposal and, thus, an even smaller proportion would be aware of the existence of such collection systems.

A Decision Tool for Nitrate Source Determination. A decision tool was developed that facilitates the process of selecting the most suitable approach to achieve nitrate source determination. A model that defines the processes necessary to identify the most suitable approach was developed using the IDEF0 modelling system in order to ensure the tool's robustness. An easily understandable model summary, supporting material and a reporting tool were, then, incorporated in order to increase the tools' usability.

8.3 Further Work

This work has shown that the use of chemical markers can be successfully applied to differentiate and characterise point and diffuse sources of sewage and manure contamination. Nevertheless, there are a number of further research avenues that could build-up on the knowledge that has been acquired throughout this research.

Assessment of the Chemical Marker Suite's Applicability Within Other Countries. This study mainly focussed on the Irish context during the development of the analytical suite of chemical markers. Details on, for example, usage

levels and human and veterinary authorisations for use, were obtained for the Irish community. Although such characteristics are expected to show little variability between countries, particularly within the European Union, it would be of interest to carry out an assessment of these factors within other countries and communities.

A Comparative Assessment of the Different Approaches. Within this study the focus was on assessing the potential of using chemical markers to differentiate and characterise sewage and manure inputs of nitrate contamination. However, it would be of interest to carry out simultaneous analysis of samples using the various approaches for achieving nitrate source determination, i.e. isotopic, microbiological, genetic and chemical marker analysis. This would allow for a more extensive assessment of the capabilities of the different approaches in relation to each other.

Studies on Chemical Marker Passage through Septic-Tank Systems. In the present study, the passage of chemical markers through septic-tank systems and into surface waters was based on an understanding of the chemical markers' physicochemical characteristics and other studies on the presence of pharmaceuticals within sewage sludge. In view of the large numbers of septic tank systems within Ireland, there is the need for a comprehensive study into the passage of pharmaceuticals through septic tank systems. In particular, it would be important to gain an understanding of their attenuation within different systems, e.g. different soil types and climatic factors, and the correlation between pharmaceutical attenuation and effluent health risks.

Development of Immunoassay Techniques for Pharmaceutical Analysis. Immunoassay techniques have been shown to afford a great potential for the analysis of pharmaceuticals within surface waters. However, this was through a proof-of-concept study that was based on a single analyte. Further research should, therefore, concentrate on the development of additional immunoassays for other pharmaceuticals, their incorporation into a multiplexed system and a full assessment of the capabilities of immunoassay analyses for such a purpose.

Determination of the Most Suitable Means of Unused Pharmaceutical Disposal. In this study, it was largely assumed that the ideal way to dispose of unused pharmaceuticals is through their return to pharmacists. This assumption is based on current knowledge in the area. However, within this study, the difficulties faced by pharmacists in implementing such a system were only touched upon in a superficial manner. Therefore, it is of importance to carry out an assessment of

current limitations in implementing such a system. At the same time, it is recommended that further research is carried out into identifying the most suitable way of disposing of unused pharmaceuticals. Various considerations of interest are to be taken into account, such as health risks, environmental risks and operational factors.

Full Case-Study of the Developed Decision Tool. Within this study, a limited validation of the decision tool was carried out. What is now needed is a full case-study of the developed decision tool in order to achieve further information on the benefits and limitations of the developed decision tool and modify it accordingly. Based on the outcomes of the various stakeholder interviews carried out, it is suggested that such a case-study is carried out with the collaboration of a river-basin district coordinator. The reason for this suggestion is because they seem to have the greatest need and motivation to support the development process, as opposed to simply applying the final version of the tool.

Extension of Decision Tool Mechanisms for Environmental Forensics Studies. An interesting outcome of the interviews with the various stakeholders, on the developed decision tool was the need for decision tools for other contaminants, particularly phosphorus and ammonia. Therefore, further research leading to the development of such decision tools would be valuable.

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Appendices

Appendix A

Mapping Data

All maps within this thesis (unless otherwise referenced) were prepared using the free and open-source software Quantum Geographic Information System (GIS) 1.7.4 Wroclaw (QGIS) by the open source geospatial foundation project. Layers within the developed maps were sourced from a number of public domain sources.

Base map details were obtained from Natural Earth (www.naturalearthdata.com), public domain map vector and raster map datasets, at the large scale (1:10m) levels. These included the country boundaries (version 1.4.0), urban area identification (version 1.3.0), ocean polygons (version 1.3.0), lakes, rivers and reservoirs (version 1.4.0). Details of administrative (county) boundaries and locations of towns and cities were obtained from OpenStreetMap (www.openstreetmap.org) open data, which is licensed under the Creative Commons Attribution-Share Alike 2.0 licence (CC-BY-SA) and the DIVA-GIS project.

Where further data sets other than the developed base map have been used, specific details of the data sets utilised are outlined below. In addition, details of any data manipulation carried out are also specified, as applicable.

A.1 Figure 2.1

Aggregate annual mean nitrate concentrations in Ireland 1997-2002 and 2003-2009 (interpolation maps):

Data sets.

- Nitrate concentrations: European Environmental Agency Waterbase data sets for lakes and rivers version 11.

Interpolation.

- Interpolation was carried out using the Interpolation plugin in QGIS with an inverse distance weight model with a distance coefficient (p) of 4. All available annual data for the year groups selected were used for model development. Depiction was then achieved using a 17 class custom colour map with an equal interval classification mode.

A.2 Figure 4.8

Map of the river catchments to which the sampling sites pertain, together with the locations of the urban wastewater treatment facilities.

Data sets .

- River Catchment Boundaries: EPA ENVision GIS data for River Basins (Catchments), under the WFD.
- UWWT Facilities: EPA ENVision GIS data on EPA Licensed UWWT Plants and 2009 status, under the EPA licensed facilities.

A.3 Figures 4.9 and 4.11

Map of the river sub-basins for Kilcruise and Baunreagh, together with the locations buildings in the area, and, Map of the river sub-basins for Tullow, together with the locations buildings. in the area.

Data sets.

- River Sub-Basin Boundaries: EPA ENVision GIS data for River Water Body Sub-Basins - South Eastern RBD 28-04-2011, under the WFD.
- UWWT Facilities: EPA ENVision GIS data on EPA Licensed UWWT Plants and 2009 status, under the EPA licensed facilities.

Georeferencing and Digitisation.

- Buildings: Digitised from the QGIS OpenLayers Plugin satellite images.

- WWTP Catchment: Catchment boundaries for the different WWTPs were obtained from the relevant IPPC application submission to the EPA, as available on the EPA website. These were subsequently georeferenced using the QGIS Georeferencer GDAL version 3.1.9 and subsequently digitised.

Appendix B

Method Statement

Method statement developed by T.E. Laboratories for sample collection from the three sampling sites is presented in this appendix.

Method Statement

Contractor	**Name:** **T.E. Laboratories Ltd.**	**Address:** **Tullow Industrial Estate** **Tullow, Carlow**	**Tel:** **059-9152881** **E-mail:** **info@tellab.ie**
Project Name	School of Biotech *(Bawnree, Kilcruise, Slaney River Sampling)*		
Description of the Task/Activity	Task involves collection of water sample from River Slaney in Tullow, Co. Carlow, Bawnree in Old Leighlin, Carlow, and Kilcruise, Co. Laois to be taken once a month		
Site Address/Location:	▪ River Slaney,(downstream of Tullow WWTP) ▪ Bawnree, Old Leighlin, Carlow ▪ Kilcruise, Laois	**Start Date/Time:** **Finish Date/Time**	On going Until notice
Personnel Involved	**Name**	**Role/Trade**	
	Ray Tuazon	Service Engineer	
	Przemek Klimek	Service Engineer	
Site Supervisor:	N/A	**Tel:**	
Safety Officer	N/A	**Tel:**	
Key Plant & Tools (Attach Certification)	N/A		
Key Materials	Static pole sampler, sampling bottles, cooler box		
Other Essential Equipment:	(i.e. access platforms/winches/ladders, etc) n/a		
Specific Identified Residual Hazards: (or refer to the task specific risk assessment(s))	Risk Assessment on a separate sheet.		
Specific Staff Training	Safe Pass, Manual Handling		

Method Statement

Sequence of Operations: (include sketches if required)	1. The Service Engineer(s), prior to arrival on site will ensure that they have all the necessary PPE's, sampling equipment and bottles, required to safely complete the task. 2. A copy of the location map (if available) where the sample is to be collected will also be given to the Service Engineer. 3. Upon arrival on site, follow and observe all safety procedures, and proceed to the sampling point. 4. Take sample directly into the sampling containers whenever possible and safe to do so. Rinse the sampling bottles with river water to wash out any contaminants that might be present. Use a telescopic pole sampler if it is not possible to take samples directly into the containers. 5. While facing upstream, collect water sample using pole sampler and fill the sampling bottles. 6. Perform on site measurements required and ensure that all bottles are labeled correctly. 7. Upon returning to the vehicle, place all bottles in a cooler box in an upright position.
Temporary Supports and Props needed to facilitate the works:	(if none, state none) none
Method of Access and Egress to the work area:	(i.e. Ladders/MEWPS/Scaffold/Trestles/Step Ladder, etc) Sampling point at Bawnree can be accessed by standing on the bridge, using a pole sampler. Sampling point at Kilcruise can be accessed by walking down a field. Sample can be taken directly into the bottles. Sampling point at Slaney River can be accessed through Tullow WWTP, a pole sampler is required to take sample.
Fall Protection Measures: (Where work at height cannot be eliminated – consider both Personnel & Materials)	(i.e. Guard Rails/Toe Boards/Brick Guard/Safety Harnesses/Exclusion Zones, etc.) N/A

Hazardous Substances: (Attach MSDS if required)	 Very Toxic	 Harmful/ Irritant	 Corrosive	 Dangerous For the environment	 Oxidising	 Highly flammable	 Explosives
Applicable:	No	No	No	No	No	No	No

Storage Arrangements:	N/A
Details of Permits to Work:	N/A
SWL's:	(Detail any limits on the loadings applicable to temporary plant/equipment or fixed elements of the structure where the work is taking place) N/A

Method Statement

Required Personnel Protective Equipment:	 Safety Boots		 Safety Gloves				Other: 1. Hi-Viz 2. Coveralls 3. Life Vest

Emergency Procedures:	Personal safety of Service Engineers engaged in any field work (in transit, walking or hiking, and any field activities while on site) is of primary importance. Walking along river banks are slip-and-fall conditions and it is the Service Engineers responsibility to take \appropriate precautions against sustaining personal injury. If there is an indication that the activity could cause bodily harm, it is recommended that the work be postponed until all hazards have been dealt with. A mobile phone will be provided to every Service Engineer while conducting field work. First Aid Kit will also be provided.		
 **First Aid Facilities:**	**Name of On-Site First Aider:**	N/A	
	First Aid Box Location:	N/A	
	Location of Nearest Hospital:	N/A	
Welfare Requirements	N/A		
Services to be supplied by Others	N/A		
Other information & Comments	N/A		

All work will be undertaken by qualified competent persons with experience of the type of work described above, and in all cases in full accordance with safety procedures specified in the companies health and safety Policy.

Prepared by:

Position: Date:

Reviewed by: John Mc Grath

Position: Sales Director Date:

Method Statement

Items Attached:	Yes	No
Sketches	☐	☑
Certification of Plant etc.	☐	☑
Programme of Work	☐	☑
Risk Assessments	☑	☐

Method Statement Briefing Record

Briefing delivered by:

Position: Service

Date:

We (the undersigned) have read and understood the attached method statement and will comply with the specified requirements and control measures. If the work activity changes or deviates from that originally envisaged, we will seek further advice and request an amended method statement.

Name (Print)	Signature	Date
Ray Tuazon		
Przemek Klimek		

Appendix C

Chain of Custody Form

A chain of custody form developed by T.E. Laboratories accompanied the samples at all times. A blank copy of the used chain of custody form used is presented in this appendix.

T.E. LABORATORIES LTD

Trading As

TelLab

Tullow Industrial Estate Tullow Co Carlow
Tel: 059 9152881 Fax: 059 9152886 E-mail: info@tellab.ie

CHAIN OF CUSTODY

Company Contact: _____ Telephone: _____

Company Name: _____ Fax: _____

Company Address: _____ Customer

_____ Order number: _____

Sample Identification	Time & Date of Sample Taken	Sample Taken By	Analysis Required	Turnaround Time Working Days

Signed (Customer): …….. Date: ……..……..

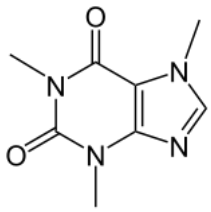
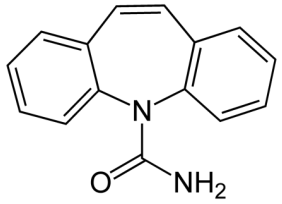
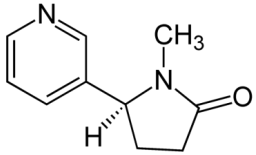
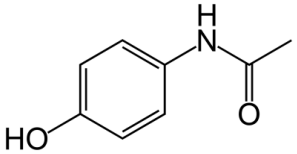
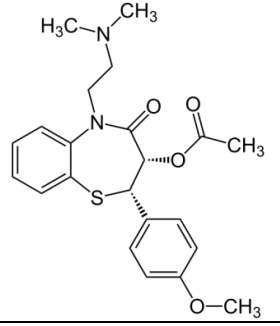
FOR LABORATORY USE ONLY:

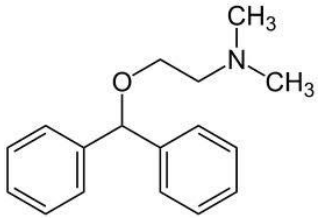
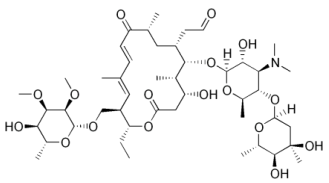
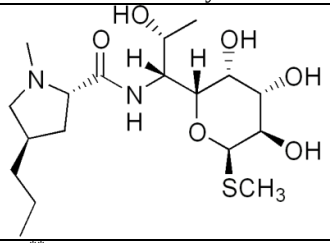
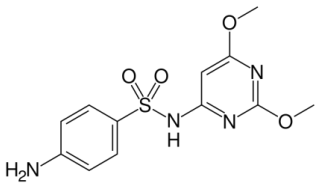
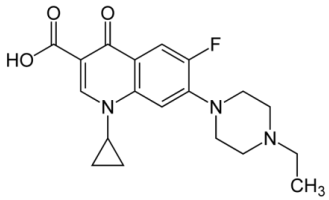
Time/Date samples Received	Received By	Sample condition	Lab Number	Due By Date

Appendix D

Analyte Details

This appendix contains the structures and further details on the 10 chemical markers selected to form part of the analytical suite as part of this study. Details of the analytes' use, pKa, log D, water solubility, marker type and structure are given.

	Caffeine	**Carbamazepine**	**Cotinine**	**Acetaminophen**	**Diltiazem**
Use	Stimulant	Antiepileptic	CNS Stimulant (Nicotine) metabolite	Analgesic	Antihypertensive/ Antiangina
pKa	10.4	13.9	8.8	9.38	8.06
Log D	-0.13	2.67	-0.30	0.48	1.86
Water Solub (25^oC)	21600 mg/L	18 mg/L	$1.0X10^6$ mg/L	14,000 mg/L	465 mg/L
Marker	Human, labile	Human, conservative	Human, labile	Human, labile	Human, conservative
					

	Diphenhydramine	**Tylosin**	**Lincomycin**	**Sulfadimethoxine**	**Enrofloxacin**
Use	Antihistamine	Macrolide Antibiotic	Lincosamide Antibiotic	Sulfonamide Antibiotic	Fluorochinolone Antibiotic
pKa	8.98*	7.73	7.6	6.91*	6.21**
Log D	0.13	1.49	-1.80	-0.31	0.02
Water Solub (25^oC)	3060 mg/L (at 37 °C)*	5 mg/L	927 mg/L	343 mg/L*	Slightly sol in water @ pH 7
Marker	Human, conservative	Veterinary	Veterinary	Veterinary	Veterinary
					

pKa and water solubility data from pubchem unless marked with *(drugbank) or **(Veterinary Substances DataBase)
Log D values are from the ChemSpider database as predicted using the ACD/Labs' ACD/PhysChem Suite at pH 5.5

Appendix E

General Public Questionnaire

Data on the current attitudes of the general public to the use and disposal of medication was achieved using an online questionnaire. A printable version of the research questionnaire is presented in this appendix.

My name is Cecilia Fenech and I am a PhD student at Dublin City University, Ireland. This project investigates the use and disposal practices of medications within households and your help is greatly appreciated. Data is being collected via this online survey on the 'Use and Disposal of Medications', which should take around 5 minutes. Your responses will provide a better understanding of the use and disposal of medications in households. It will also allow for the level of awareness on the environmental effects of different disposal practices to be assessed.

Please note, that the survey is completely anonymous and it will be impossible for me to link any answers back to you. You may also decide to withdraw from this research at any time by clicking on the 'Exit and Clear Survey' found at the bottom of each page. If you have any questions about this research, or would like to be informed of the results, you may contact me at cecilia.fenech2@mail.dcu.ie or +353 1700 5787, or my supervisor Dr Anne Morrissey (anne.morrissey@dcu.ie). If you wish to contact an independent person, please contact: The Secretary, DCU Research Ethics Committee, c/o Office of the Vice-President for Research, Dublin City University, Dublin 9, Ireland. Tel: +353 1700 8000.

I thank you in advance for your help. Your participation is greatly appreciated.

Cecilia Fenech

There are 38 questions in this survey

Introduction

1 [ICF 1]Informed Consent Questions: *

Please choose the appropriate response for each item:

	Yes	No
My participation is voluntary	○	○
I am aware that I may withdraw from the study at any point	○	○
I understand the requirements of the survey	○	○

2 [ICF3]

You replied no to one of the above questions and will therefore be unable to proceed with this survey. If you would like to clarify any issues and proceed with the survey, please contact me at cecilia.fenech2@mail.dcu.ie.

Thank you

Only answer this question if the following conditions are met:
°

------- Scenario 1 -------

Answer was 2'No' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

------- or Scenario 2 -------

Answer was 2'No' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point))

------- or Scenario 3 -------

Answer was 2'No' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

General Profile

3 [GP1]Age *

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose **only one** of the following:

- ○ <17
- ○ 18-30
- ○ 31-45
- ○ 46-60
- ○ 61+

4 [GP2]Gender

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **only one** of the following:

- ○ Female
- ○ Male

5 [GP3]Nationality

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please write your answer here:

6 [GP6]Where do you currently reside?

Please write your answer here:

7 [GP7]In which county do you currently reside?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point))

Please write your answer here:

8 [GP4]How would you classify the area you live in?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose **only one** of the following:

- ○ Big City
- ○ Suburbs or outskirts of a big city
- ○ Small city or town
- ○ Country village
- ○ A farm or home in the country

9 [GP5]What is the highest level of education you have achieved to date?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose **only one** of the following:

- ○ No Formal Qualifications
- ○ Primary Education
- ○ Secondary Education
- ○ Post Secondary Education
- ○ Undergraduate Degree (e.g. BA, BSc)
- ○ Postgraduate Degree (e.g. MSc, PhD)

Use of Medication

10 [UM1]Have you taken any over-the-counter medication (excluding herbal medication) in the past 6 months?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose **only one** of the following:

- ○ Yes
- ○ No

Over-the-Counter means medication that can be sold without a prescription e.g. mild pain killers.

11 [UM2]

If yes, how many times have you taken such medication? Optional: Please list which over the counter medications you have taken.

Only answer this question if the following conditions are met:
° Answer was Y'Yes' at question '10 [UM1]' (Have you taken any over-the-counter medication (excluding herbal medication) in the past 6 months?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose **only one** of the following:

- ○ 1-2
- ○ 3-5
- ○ 6-9
- ○ 10+

Make a comment on your choice here:

12 [UM3]

Have you been prescribed medication in the past 6 months?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose **only one** of the following:

- ○ Yes
- ○ No

13 [UM4]

If yes, how many times have you taken such medication? Optional: Please list which medication you have been prescribed.

Only answer this question if the following conditions are met:
° Answer was Y'Yes' at question '12 [UM3]' (Have you been prescribed medication in the past 6 months?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose **only one** of the following:

- ○ 1-2
- ○ 3-5
- ○ 6-9
- ○ 10+

Make a comment on your choice here:

14 [UM5]Over the past 6 months, have you been on any long-term medication e.g. for asthma, epilepsy, depression etc.?

Only answer this question if the following conditions are met:

° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **only one** of the following:

- ○ Yes
- ○ No

15 [UM6]

If yes, how many long-term medications have you been using? Optional: Please list the long-term medication you have been prescribed.

Only answer this question if the following conditions are met:
° Answer was Y'Yes' at question '14 [UM5]' (Over the past 6 months, have you been on any long-term medication e.g. for asthma, epilepsy, depression etc.?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **only one** of the following:

- ○ 1
- ○ 2
- ○ 3
- ○ 4-6
- ○ 7+

Make a comment on your choice here:

16 [UM7]Do you keep any medication (prescription, over-the-counter etc.) in your home, apart from any that you would be taking at that moment in time?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **only one** of the following:

- ○ Yes
- ○ No

17 [UM8]If yes, what type of medication do you keep?

Only answer this question if the following conditions are met:
° Answer was Y'Yes' at question '16 [UM7]' (Do you keep any medication (prescription, over-the-counter etc.) in your home, apart from any that you would be taking at that moment in time?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **only one** of the following:

- ○ I keep some basic medications e.g. pain killers, sore throat lozenges, burn cream
- ○ I keep a wide range of over-the-counter medications
- ○ I keep numerous prescription and non-prescription medications e.g. antibiotics

Medication Disposal Practices

18 [MD1]How do you dispose of medicinal syrups (e.g. cough syrup) after you have finished taking the course or they have expired? *

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose the appropriate response for each item:

	Always	Usually	Sometimes	Rarely	Never
Flush down the toilet/sink	○	○	○	○	○
Place in rubbish bin	○	○	○	○	○
Take to a pharmacy	○	○	○	○	○
Take to a GP	○	○	○	○	○
Take to a hazardous waste facility	○	○	○	○	○
Give to someone else	○	○	○	○	○
Other	○	○	○	○	○

Please select an option for each line. If you have never used any method of disposal (including 'Other') select 'Never' for that line.

19 [MD2]If you responded other, how do you dispose of medicinal syrups?

Only answer this question if the following conditions are met:
° Answer was 1'Usually' or 'Always' or 'Rarely' or 'Sometimes' at question '18 [MD1]' (How do you dispose of medicinal syrups (e.g. cough syrup) after you have finished taking the course or they have expired? (Other)) *and* Answer was 2'Usually' or 'Always' or 'Rarely' or 'Sometimes' at question '18 [MD1]' (How do you dispose of medicinal syrups (e.g. cough syrup) after you have finished taking the course or they have expired? (Other)) *and* Answer was 3'Usually' or 'Always' or 'Rarely' or 'Sometimes' at question '18 [MD1]' (How do you dispose of medicinal syrups (e.g. cough syrup) after you have finished taking the course or they have expired? (Other)) *and* Answer was 4'Usually' or 'Always' or 'Rarely' or 'Sometimes' at question '18 [MD1]' (How do you dispose of medicinal syrups (e.g. cough syrup) after you have finished taking the course or they have expired? (Other)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please write your answer here:

20 [MD3]How do you dispose of pills (prescription and non-prescription) after you have finished taking the course or they have expired? *

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose the appropriate response for each item:

	Always	Usually	Sometimes	Rarely	Never
Flush down the toilet/sink	○	○	○	○	○
Place in rubbish bin	○	○	○	○	○
Take to a pharmacy	○	○	○	○	○
Take to a GP	○	○	○	○	○
Take to a hazardous waste facility	○	○	○	○	○
Give to someone else	○	○	○	○	○
Other	○	○	○	○	○

Please select an option for each line. If you have never used any method of disposal (including 'Other') select 'Never' for that line.

21 [MD4]You replied other in the previous question. What other ways do you use to disposed of pills?

Only answer this question if the following conditions are met:
° Answer was 1'Always' or 'Usually' or 'Rarely' or 'Sometimes' at question '20 [MD3]' (How do you dispose of pills (prescription and non-prescription) after you have finished taking the course or they have expired? (Other)) *and* Answer was 2'Always' or 'Usually' or 'Rarely' or 'Sometimes' at question '20 [MD3]' (How do you dispose of pills (prescription and non-prescription) after you have finished taking the course or they have expired? (Other)) *and* Answer was 3'Always' or 'Usually' or 'Rarely' or 'Sometimes' at question '20 [MD3]' (How do you dispose of pills (prescription and non-prescription) after you have finished taking the course or they have expired? (Other)) *and* Answer was 4'Always' or 'Usually' or 'Rarely' or 'Sometimes' at question '20 [MD3]' (How do you dispose of pills (prescription and non-prescription) after you have finished taking the course or they have expired? (Other)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please write your answer here:

22 [MD5]

How should we dispose of unused medications, in your opinion?

Click on an item in the list on the left, starting with your highest ranking item (best disposal system), moving through to your lowest ranking item (worst disposal system).
*

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please number each box in order of preference from 1 to 5

- [ ] Flush down the toilet/sink
- [ ] Place in rubbish bin
- [ ] Take to a pharmacy
- [ ] Take to a GP
- [ ] Take to a hazardous waste facility

23 [MD6]Have you ever been advised as to the best way to dispose of medications?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point))

Please choose **only one** of the following:

- ○ Yes
- ○ No

24 [MD7]You replied yes in the previous question. How have you been advised?

Only answer this question if the following conditions are met:
° Answer was Y'Yes' at question '23 [MD6]' (Have you ever been advised as to the best way to dispose of medications?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **all** that apply:

- ☐ Pharmacist
- ☐ GP (family doctor)
- ☐ Educational campaign
- ☐ Parents
- ☐ Medicine Manufacturer
- ☐ Other

25 [MD8]In what other ways have you been advised about the best way to dispose of pharmaceuticals?

Only answer this question if the following conditions are met:
° Answer was Y at question '24 [MD7]' (You replied yes in the previous question. How have you been advised?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please write your answer here:

Veterinary Pharmaceuticals

26 [VP1]Do you have any pets/animals?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **only one** of the following:

- ○ Yes
- ○ No

27 [VP2]Have you given them any medication in the past year?

Only answer this question if the following conditions are met:
° Answer was Y'Yes' at question '26 [VP1]' (Do you have any pets/animals?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point))

Please choose **only one** of the following:

- ○ Yes
- ○ No

28 [VP3]What medications have you given your pets/animals?

Only answer this question if the following conditions are met:
° Answer was Y'Yes' at question '27 [VP2]' (Have you given them any medication in the past year?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point))

Please write your answer here:

29 [VP4]Do you dispose of unused or expired medications intended for your pets/animals in a different manner to those intended for humans?

Only answer this question if the following conditions are met:
° Answer was Y'Yes' at question '26 [VP1]' (Do you have any pets/animals?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose **only one** of the following:

- ○ Yes
- ○ No

30 [VP5]How do you dispose of unused or expired medication intended for your pets/animals? *

Only answer this question if the following conditions are met:
° Answer was Y'Yes' at question '29 [VP4]' (Do you dispose of unused or expired medications intended for your pets/animals in a different manner to those intended for humans?) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose the appropriate response for each item:

	Always	Usually	Sometimes	Rarely	Never
Flush down the toilet/sink	○	○	○	○	○
Place in the rubbish bin	○	○	○	○	○
Take to the vet	○	○	○	○	○
Take to a hazardous waste facility	○	○	○	○	○
Give to someone else	○	○	○	○	○
Other	○	○	○	○	○

If you have never used 'Other' methods of disposal select 'Never'.

31 [VP6]In what other ways do you dispose of your pets/animals medications?

Only answer this question if the following conditions are met:
° Answer was 1'Always' or 'Usually' or 'Sometimes' or 'Rarely' at question '30 [VP5]' (How do you dispose of unused or expired medication intended for your pets/animals? (Other)) *and* Answer was 2'Always' or 'Usually' or 'Sometimes' or 'Rarely' at question '30 [VP5]' (How do you dispose of unused or expired medication intended for your pets/animals? (Other)) *and* Answer was 3'Always' or 'Usually' or 'Sometimes' or 'Rarely' at question '30 [VP5]' (How do you dispose of unused or expired medication intended for your pets/animals? (Other)) *and* Answer was 4'Always' or 'Usually' or 'Sometimes' or 'Rarely' at question '30 [VP5]' (How do you dispose of unused or expired medication intended for your pets/animals? (Other)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I am aware that I may withdraw from the study at any point)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent

Questions: (I am aware that I may withdraw from the study at any point))

Please write your answer here:

Disposal Considerations

32 [DC1]You have said that you have returned medications to pharmacies/GPs/hazardous waste facilities/vets. Why is this?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **all** that apply:

- ☐ To safeguard the environment
- ☐ For safety reasons
- ☐ For reuse (in healthcare, developing countries etc)
- ☐ A habit
- ☐ Other:

33 [DC2]You have not always returned medications to pharmacies/GPs/hazardous waste facilities/vets. Why is this?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (I understand the requirements of the survey))

Please choose **all** that apply:

- ☐ Was not aware of this possibility
- ☐ Do not think it is important
- ☐ Do not have the time
- ☐ Pharmacy/GP/Hazardous waste facility/Vet is too far away
- ☐ Pharmacy/GP/Hazardous waste facility/Vet does not accept them
- ☐ Habit
- ☐ Other:

Environmental Considerations

34 [EC1]Are you aware that pharmaceuticals have been regularly found within surface waters (rivers, lakes, seas) worldwide?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **only one** of the following:

- ○ Yes
- ○ No

35 [EC2]Where do you think most pharmaceuticals in surface waters come from?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please choose **only one** of the following:

- ○ Unwanted medications flushed down the toilet/sink
- ○ From medications placed in rubbish bins
- ○ Pharmaceutical company waste waters
- ○ From sewage as a result of excreted pharmaceuticals from normal use

Make a comment on your choice here:

36 [EC3]

What do you consider to be the risks of the presence of pharmaceuticals within water bodies (e.g. rivers, lakes, seas)? *

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please number each box in order of preference from 1 to 4

- ☐ Risk to aquatic life
- ☐ Risk to human health
- ☐ Risk to drinking water quality
- ☐ Antibiotic resistance

37 [EC4]Do you think there are other risks associated to the presence of pharmaceuticals in water bodies? If yes, what are they?

Only answer this question if the following conditions are met:
° Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary)) *and* Answer was 1'Yes' or 'Yes' or 'Yes' at question '1 [ICF 1]' (Informed Consent Questions: (My participation is voluntary))

Please write your answer here:

Further Comments

38 [FC1]

Thank you for filling in this survey. Your help is greatly appreciated.

If you have any final comments, please leave them in the space provided before pressing submit.

Please write your answer here:

Thank you for taking some time to fill in this short survey. Your help is greatly appreciated. If you would like to be informed of the results or have any queries, please do not hesitate to contact me on cecilia.fenech2@mail.dcu.ie.

31.12.1969 – 16:00

Submit your survey.
Thank you for completing this survey.

Appendix F

Chromatographic Method Validation and System Suitability

In addition to the chromatographic method validation and system suitability results given in Chapter 4 further details are given on the results obtained within this appendix. Two separate chromatographic methods were used throughout this study, namely the Sunfire HPLC method and the Luna PFP LC-MS/MS method. Details of the precision, linearity, resolution, capacity factors, theoretical plates and the height equivalent theoretical plates (HETP) are given in Tables F.1 and F.2 for the HPLC and LC-MS/MS methods, respectively. For the LC-MS/MS methods developed a capacity factor was not determined since it is not possible to determine the retention time of the unretained species. The reason for this is that when carrying out SRM-based methods in tandem mass spectrometry the injection peak is not evident.

Table F.1: Details of method validation and system suitability for the HPLC Method.

Analyte	**Precision (%RSD)**	**Linearity**	**Resolution**		**Capacity Factor**	**Theoretical Plates**	**HETP (cm^{-1})**
ACT	0.31	0.9998			1.42	2266	0.006619
COT	0.30	0.9972	7.61		2.97	6088	0.002464
CAF	0.19	0.9998		5.73	4.06	12938	0.001159
LIN	2.74	0.9925	18.51		6.65	90534	0.000166
ENR	4.98	0.9941		16.43	9.15	39891	0.000376
SDM	0.88	0.9958	2.20		9.49	170305	0.000088
DPH	0.37	0.9921		12.60	11.09	100565	0.000149
CBZ	0.29	0.9993	4.66		11.74	160825	0.000093
TYL	2.88	0.9987		3.52	12.15	267421	0.000056
DTZ	2.38	0.9910	3.55		12.55	181893	0.000082

Table F.2: Details of method validation and system suitability for the LC-MS/MS Method.

Analyte	**Precision (%RSD)**		**Linearity**		**Resolution**		**Theoretical Plates**	**HETP (cm^{-1})**
	AB Sciex	Bruker	AB Sciex	Bruker				
COT	4.26	2.33	0.9995	0.9975			1510	0.009935
LIN	2.43	2.63	0.9999	0.9979	7.43		7186	0.002087
ACT	3.66	9.74	0.9969	0.9968		1.77	8433	0.001779
CAF	7.05	9.43	0.9953	0.9931	1.54		7132	0.002103
ENR	2.79	1.51	0.9990	0.9960		3.00	8176	0.001835
TYL	3.72		0.9999		4.30		17519	0.000856
DPH	3.30	3.33	0.9991	0.9923		0.49	6557	0.002288
DTZ	5.67	2.44	0.9994	0.9978	0.74		8667	0.001731
CBZ	7.92	3.95	0.9997	0.9983		1.18	13664	0.001098
SDM	2.77	2.808	0.9989	0.9977	0.65		12566	0.001194

Appendix G

Interview Pack

The interview pack used during the stakeholder interviews as part of the decision tool validation. This included the relevant plain language statement, consent form and interview aids.

Development of a Decision Tool for Identifying the Most Appropriate Approach to Differentiate Between Nitrate Sources

Identifying sources of nitrate contamination within water bodies is an area that has seen increasing interest in recent years. However, there are a considerable number of techniques that may be used in order to identify the source of nitrate, such as the use of isotopic, genetic and chemical markers. Each approach has its own set of benefits and limitations. For this reason, a decision tool has been developed that allows the user to identify the most suitable technique depending upon the potential sources of contamination, budgetary and time constraints and technique availability amongst others.

As part of the decision tool validation we invite you to participate in this short interview, which should take around 30 minutes to 1 hour. As a follow-up you may request the outcome of the interview for your comments and/or clarification and the final version of the decision tool for use within your organisation. The data collected through our conversation and subsequent communication will be used to improve the developed decision tool and will be utilised within the submitted thesis and any journal articles and or/presentations undertaken as part of this research.

Your participation in this study is voluntary and you may withdraw at any point. In order to protect confidentiality your name will not be mentioned in conjunction with this research (subject to legal limitations). Rather, your position as _____ will be used as an identifier.

Please complete the following (Circle Yes or No for each question)

I have read the Plain Language Statement and understood the information	*Yes/No*
I am aware that this conversation will be recorded	*Yes/No*
I am aware that my participation is voluntary and I may withdraw at any point	*Yes/No*

I have read and understood the information in this form. My questions and concerns have been answered by the researchers, and I have a copy of this consent form. Therefore, I consent to take part in this research project

Participants Signature: _____

Name in Block Capitals: _____

Witness: _____

Date: _____

If you have any concerns about this study and would like to contact an independent person, please contact: The Secretary, Dublin City University Research Ethics Committee, c/o Office of the Vice-President for Research, Dublin City University, Dublin 9. Tel 01-7008000

The Decision Tool

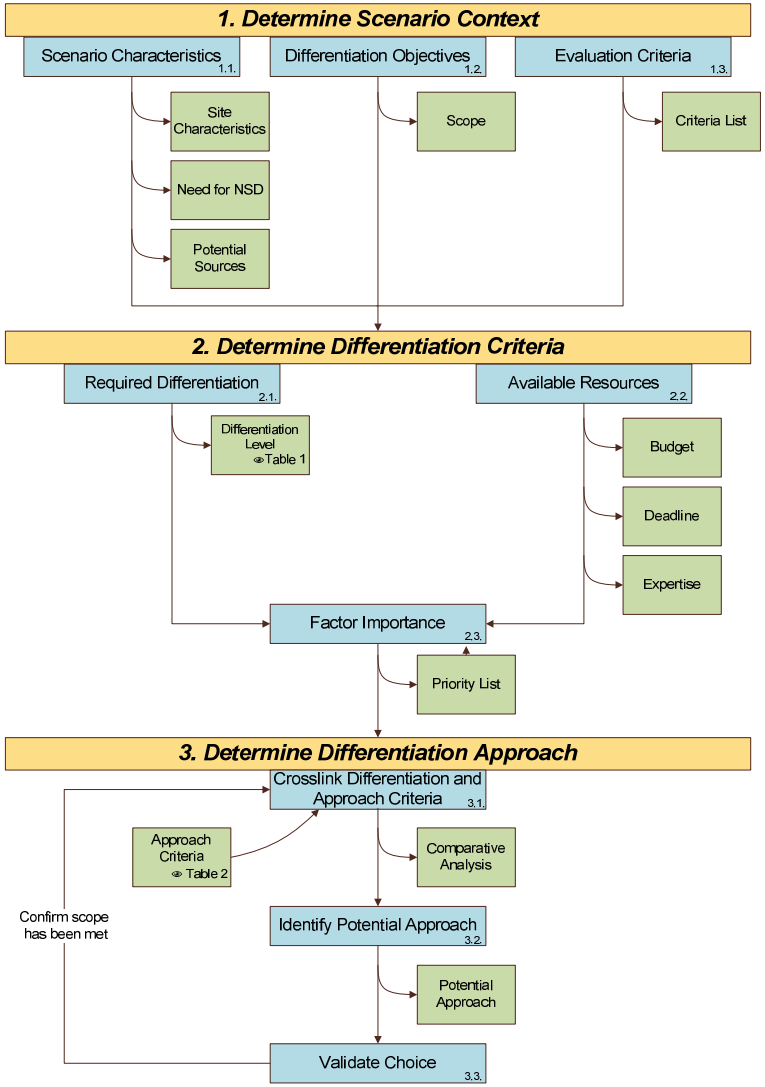


Table 1: Differentiation Potential: The differentiation characteristics of the four main approaches outlined in literature. Consecutive sources of contamination with the same shade cannot be differentiated using that particular approach. For example, nitrate isotopes cannot differentiate between different sources of manure and sewage (both in group 1 for nitrate isotopes), but it can differentiate between fertiliser nitrate (group 3) and soil nitrogen (group 4) or ammonium in fertiliser (group 6). Chemical markers, then, can differentiate between three classes (manure, raw and treated sewage) but cannot differentiate between different sources of manure.

Sources of Contamination	Approaches			
	Nitrate Isotopes	Genetic Markers	Micro-biological	Chemical Markers
Manure (organism 1)	1	1	1	1
Manure (organism 2)		2		
Manure (organism x)		3		
Raw sewage		4		2
Treated sewage				3
Nitrate in precipitation	2			
Nitrate in fertiliser	3			
Soil nitrogen	4	NA	NA	NA
Desert nitrate deposits	5			
Ammonium in Fertiliser	6			

Table 2: Approach Criteria: Characteristics of the four main approaches. An increased number of '+' symbols indicates an increase in cost, time, level of expertise, state of approach, sample volumes and technique availability

	Nitrate Isotopes	Genetic Markers	Micro-biological	Chemical Markers
Instrumentation	IRMS	Various	Incubator	Various
Time Requirement	Days	Hours	Days	Hours
Sample Volume	Millilitres	Millilitres	Centilitres	Litres
Multi-Source Determination	No	Yes	No	Yes
Typical Cost	++	+++	+	++
Level of Expertise	+++	+++	+	+++
State of Approach	+++	+	+++	++
Technique Availability	+	++	+++	++

Decision Tool Reporting

1. Determine Scenario Context: Why Is Differentiation Needed?

1.1. Identify scenario characteristics

1.1.1. What are the site characteristics? *e.g. Where is the site located?; What is the catchment composition?*

1.1.2. Why is nitrate source determination required?

1.1.3. What are the potential sources of nitrate at the site?

1.2. Determine differentiation objectives

1.2.1. What are the study's objectives? *e.g. Is it a scoping study, or is it for legal action?*

1.3. Determine evaluation criteria

1.3.1. What are the factors of interest in the study? *e.g. Will cost, time, expertise availability and/or robustness have a decisive factor?*

2. Determine Differentiation Criteria: What Should Differentiation Achieve?

2.1. Identify Level of Differentiation Required

2.1.1. What level of differentiation is required? *e.g. Do the various inorganic or organic sources need to be further differentiated?;Is differentiation of raw and treated sewage required or is the host to be identified? Table 1 identifies the potential levels of differentiation of the different approaches.*

2.2. Identify Available Resources

2.2.1. What budgets are available for this study?

2.2.2. What is the time-line for completing the study?

2.2.3. What is the expertise (in-house/external) available?

2.2.4. Are there any other potential resources of relevance to this study?

2.3. Determine Relative Importance of Factors

2.3.1. What are the most critical criteria and constraints of the study?

3. Determine Differentiation Approach: How is differentiation going to be achieved?

3.1 Cross-link Differentiation and Approach Criteria

3.1.1. How do the differentiation requirements relate to available approaches? *i.e. Through the use of associated tables 1 and 2, assess whether the required level of differentiation may be achieved (Table 1) using the available resources (Table 2).*

Nitrate Isotopes:

Genetic Markers:

Microbiological Markers:

Chemical Markers:

3.2. Identify Approach to Take

3.2.1. Which approach satisfies the most differentiation requirements?

3.3. Validate Choice of Approach

3.3.1. Does the selected approach satisfy the study requirements? *If a single approach is not suitable a combination of approaches may be required to achieve the required differentiation level or additional robustness.*