INVESTIGATING VETERINARY ANTIPARASITIC DRUGS AS EMERGING CONTAMINANTS IN IRISH GROUNDWATER

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DECLARATION

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SUMMARY

Due to increased intensification of the food production system, veterinary drugs have become a critical component in animal husbandry in Ireland and more broadly within the European Union. The administration of such substances can potentially lead to their occurrence in the environment, primarily as a result of the direct excretion on land, or excretion in faeces and urine which is subsequently land spread in manure or slurry. This work specifically focuses on two groups of antiparasitic agents commonly used in Irish agriculture, the anthelmintics and the anticoccidials, covering a total of 66 antiparasitic drugs. Anthelmintic drugs are used to control helminthic parasites (nematodes, cestodes and trematodes) that infect animals, particularly those exposed through pasture-based production systems, such as cattle and sheep. Anticoccidials are used to control coccidiosis and other protozoan infections in food producing animals, with primary prophylactic use in poultry production. Very limited information is available on the occurrence of anthelmintics and anticoccidials in the environment, particularly in groundwater, which has resulted in them being considered potential emerging organic contaminants of concern. Information on their environmental transformation products is even more scant. This dearth of information has been attributed (in part) to a lack of suitable analytical methodologies. The overall aim of this research was to investigate the occurrence of these antiparasitic drugs in Irish groundwaters, to help broaden the overall knowledge and understanding of the fate of these contaminants in the environment. This was achieved through the development and application of more sensitive and comprehensive analytical methods, as presented in this thesis.

A multi-residue solid phase extraction (SPE) method was developed for the extraction of 40 anthelmintic compounds in surface water and groundwater, with determination using ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). This method, which includes 27 parent drugs and 13 transformation products, was validated and applied in a spatial occurrence study comprising a total of 106 sites, including 88 groundwater and 18 surface waters. The groundwater sites were selected to be representative of different karstic and fractured bedrock aquifers. During spring of 2017, 17 out of 40 anthelmintics were detected, with one or more anthelmintic residues found at 22% of sites. Detected anthelmintic concentrations were of the order of 1- 41 ng L^{-1}. A temporal study

carried out over 13 months highlighted the importance of anthelmintic usage patterns and meteorological events in controlling the occurrence of anthelmintics in groundwaters that are most sensitive to contamination. This work not only presents the most comprehensive method currently available (to the best of the author's knowledge) for detecting anthelmintics in surface and groundwaters, it also reports on some of the first occurrences of these contaminants in Irish groundwater.

A second SPE method was also developed for the determination of 26 anticoccidial compounds by UHPLC-MS/MS and allows for the simultaneous analysis of both the ionophore and synthetic anticoccidials, including several analytically problematic polar compounds which previously required separate analysis. This method was applied as part of a comprehensive spatial occurrence study during autumn 2018, in which water samples from sites representative of different source and pathway factors were analysed for anticoccidial drugs. Up to seven different compounds were detected at 24% of sites, at concentrations ranging from 1 to 386 ng L^{-1}. The anticoccidials detected were in line with expected usage, with statistical analysis indicating that poultry activity was a significant driver of anticoccidial occurrences. This work presents the most comprehensive and sensitive method for the determination of anticoccidial drugs in groundwater amongst current literature, and reports the first groundwater detections of several anticoccidials, not only in Ireland, but also in Europe and perhaps globally.

This thesis is dedicated to my Nanny

Mrs. K. Whelan

Pain Keeps You Alive

(The Devil, *Pers. Comm. 2020)*

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ABBREVIATIONS

Amm. Form.	Ammonium formate
ASE	Accelerated solvent extraction
BH	boreholes
C_{18}	Octadecyl bonded silica
C_8	Octyl bonded silica
$CaCO_3$	Calcium carbonate
CE	Collision Energy
CH_3COOH	Acetic acid
cv	Coefficient of variance
CV	Collision voltage
CXP	Collision cell exit potential
DAD	Diode array detector
DAFM	Department of Agriculture, Food and Marine
DELG	Department of the Environment and Local Government
DI-H_2O	De-ionised water
DMSO	Dimethyl sulphoxide
DP	De-clustering potentials
DSPE	Dispersive solid phase extraction
DVB	Divinylbenzene
EC	European Commission
EEC	European Economic Community
EFSA	European Food Safety Authority
EG	Ethylene Glycol
EIC	Extracted Ion Chromatography
EMA	European Medicines Agency
EOC	Emerging organic contaminant
EP	Entrance Potential
EPA	Environment Protection Agency
EQS	Environmental Quality Standard
ESI	Electrospray ionisation
EtOAc	Ethyl Acetate
EU	European Union
FDA	Food and Drug Administration
FIA	Flow injection analysis
GC	Gas chromatography
GC-µECD	Gas chromatography coupled to micro electron capture detector
GC-ECD	Gas chromatography coupled to electron capture detector
GC-MS	Gas chromatography-mass spectroscopy
GC-MS/MS	Gas chromatography coupled to tandem mass spectrometry
GSI	Geological Survey of Ireland

GW	Groundwater
GWSs	Group water Schemes
H_2O	Water
HCl	Hydrochloric Acid
HCOOH	Formic acid
HILIC	Hydrophilic Interaction Liquid Chromatography
HLB	Hydrophilic-lipophilic balance
HPLC	High Performance Liquid Chromatography
IPA	Isopropanol (propan-2-ol)
IS	Internal Standard
K_a	Acid dissociation constant
K_{oc}	Organic carbon-water partition coefficient
K_{ow}	Octanol-water partition coefficient
LC	Liquid chromatography
LC-MS	Liquid chromatography - mass spectrometry
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass to charge ratio
MAE	Microwave assisted extraction
ME	Matrix effect
MeCN	Acetonitrile
MeOH	Methanol
MISPE	Molecular imprinted polymer solid phase extraction
ML	Macro-cyclic lactone
mLLE	Micro liquid-liquid extraction
MLOD	Method limit of detection
MLOQ	Method limit of quantification
MPs	Monitoring points
MRM	Multiple reaction monitoring
MTBE	Methyl tert-butyl ether
N_2	Nitrogen gas
NaOH	Sodium hydroxide
NFGWS	National Federation of Group Water Schemes
NH_4OH	Ammonium hydroxide
NSAI	National Standards Authority of Ireland
OC	Organic carbon
P	Parent compound
PDAD	Photo-diode array detector
PFPP	Pentafluorophenylpropyl

pH	Potential of Hydrogen
pKa	Negative log of acid dissociation constant
PLE	Pressurized liquid extraction
POCIS	Polar organic chemical integrative sampler
PPCP	Pharmaceutical and personal care product
PTFE	Polytetrafluoroethylene
PV	Parametric value
QQQ	Triple quadrupole
QTRAP	Quadrupole linear ion trap
R^2	Regression coefficient values
rpm	Revolutions per minute
RRT	Relative retention time
RSD	Relative standard deviation
RSD_r	Relative standard deviation under repeatability conditions
RSD_{wR}	Relative standard deviation under reproducibility conditions
RSM	Response surface methodology
S/N	Signal to noise ratio
SBSE	Stir-bar sorptive extraction
SFE	Supercritical fluid extraction
SMD	Soil moisture deficit
SP	Spring
SPE	Solid phase extraction
SPMD	Semi-permeable membrane device
SPME	Solid phase micro extraction
SRM	Selected reaction monitoring
S_w	Water solubility
TD	Thermal desorption
TP	Transformation product
t_R	Retention Time
TV	Threshold value
UAE	Ultrasonic assisted extraction
UHPLC-MS/MS	Ultra - high pressure liquid chromatography- tandem mass
Ultra-H_2O/UPW	Ultra-pure water
WFD	Water framework directive
WL_r	Within-lab repeatability
WL_R	Within-lab reproducibility
WS	Working Standard
ZOC	Zone of contribution

Unit	Description	Unit	Description
%	percentage	ng	nanogram
°C	degrees Celsius	ng L^{-1}	nanogram per litre
µg kg^{-1}	microgram per kilogram	ng mL^{-1}	nanogram per millilitre
µg mL^{-1}	microgram per millilitre	pg L^{-1}	picogram per litre
µL	microliter	ppb	parts per billion
µm	micrometer	ppm	parts per million
g	gram	ppq	parts per quadrillion
kg	kilogram	ppt	parts per trillion
L	litre	psi	pound per square inch
M	molarity (moles per litre)	rpm	revolutions per minute
mg	milligram	s	seconds
mg kg^{-1}	milligram per kilogram	V	voltage
mg mL^{-1}	milligrams per millilitre	v	volume
min	minute	*v/v*	volume per volume
mL	millilitre	*w/v*	weight per volume
mL min^{-1}	millilitres per minute		
mM	millimolar		
mm	millimetre		
mol	moles		

THESIS STRUCTURE

This thesis is presented across 6 chapters as summarised below. The four main chapters of this thesis (chapter 2, 3, 4 and 5) consist of three published papers (2, 3 and 5) and one draft paper that is intended for submission for peer review. Chapters produced from work already published have been constructed in a fashion as to remain consistent with the structure and order of content in the published paper. As a result, it should be noted that the order and flow of content in chapter 2 differs to the other chapters, as a result of the un-conventional structure of the *Molecules* MDPI journal publications. In line with the guidelines in the Geology Department Requirements and Procedures for Research Students Document (2020), a lay abstract (in addition to the traditional abstract) has been provided for each of the four publication-based chapters.

Chapter 1 provides a detailed overview of the project background and the rationale for the research, with a comprehensive literature review presented thereafter. This is followed by the specific project aims and objectives. The literature review focused particularly on three groups of antiparasitic drugs commonly used in Ireland, the anthelmintics, the anticoccidials and the pyrethroid insecticides. This review sought to inform on the current state of research on these groups of veterinary drugs as emerging contaminants in groundwater, with an overall goal of selecting and prioritising these groups for the main investigation. The literature review was also heavily focused on the different aspects of chemical analysis since the need for suitably sensitive analytical methodologies was crucial for fulfilling the project objectives in terms of assessing the environmental occurrence.

Chapter 2 describes the analytical method development work carried out for the anthelmintic drugs. The main goal of this work was to develop, optimise and validate a comprehensive extraction and detection method to allow for the analysis of a more extensive suite of anthelmintic drugs in water samples, which literature currently lacks. This work sought to include anthelmintic compounds from across all anthelmintic classes, with an emphasis also on the incorporation of their transformation products. This work has been published in the Journal *Molecules*.

Chapter 3 describes the analytical method development work carried out for the anticoccidials. The main goal of this was also to develop, optimise and validate an analytical method to allow the extraction and determination of a larger number of anticoccidial

compounds from water samples. This work also focused on developing a new chromatographic separation method to allow the simultaneous determination of both ionophore and synthetic anticoccidials, which includes several problematic polar compounds, that otherwise require separate analysis. This work has been published in *Journal of Chromatography A.*

Chapter 4 describes the application of the analytical method developed for the anthelmintic drugs in chapter 2, in order to investigate the spatio-temporal occurrence of the 40 different drug residues in groundwater within karstic and fractured bedrock aquifers. A temporal occurrence study was carried out at catchment level to assess the potential variation in anthelmintic occurrence in sensitive waters, due to seasonal events such as usage patterns and meteorological events. This work is presented as a draft manuscript intended to be submitted for peer review by the journal *Science of the Total Environment.*

Chapter 5 presents a comprehensive spatial occurrence study of 26 anticoccidial compounds in groundwater. This study involved the application of the anticoccidial analytical method developed in chapter 3, to a large sampling network consisting of 109 sampling sites, selected to be representative of different source and pathway pressures. This study sought to investigate the frequency of occurrence of the different anticoccidial compounds and to examine any potential drivers of these occurrences. This work has been published in *Science of the Total Environment.*

Chapter 6 summarises the findings of this research and provides some insights into potential future work in this area that would further advance on the findings in this thesis.

CHAPTER 1 – INTRODUCTION AND LITERATURE REVIEW OF VETERINARY ANTIPARASITIC DRUGS AS EMERGING CONTAMINANTS IN GROUNDWATER

1.1 Project Background and Rationale

Emerging organic contaminants (EOCs) are becoming a growing international concern with respect to their occurrence in groundwater bodies (Lapworth et al., 2012; Stuart et al., 2012; Meffe and de Bustamante, 2014; Postigo and Barcelo, 2015). EOC is a term used to describe both newly developed compounds and newly discovered compounds present in the environment (Lapworth et al., 2012). Some examples of the most common EOCs include pesticides, veterinary drug products and pharmaceutical and personal care products (PPCPs), often with respect to both the parent compound and any metabolites and transformation products (TPs). Sources in the rural environment include agricultural usage of pesticides, veterinary usage of pharmaceuticals (including antibiotics and antiparasitic drugs), bovine endocrine disrupting compounds and domestic usage of pharmaceuticals and personal care products.

The administration and application of such pharmacologically active chemicals can potentially lead to the occurrence of such compounds in groundwater bodies. Potential entry routes to groundwater include both point source contamination (e.g. farmyard wastes, leachates from agricultural land and from animal holding, feeding and waste storage areas and septic tank effluent discharge) and diffuse source contamination (e.g. spreading of fertilisers and pesticides) (Boxall, 2018). Entry of veterinary drugs to groundwater from livestock wastewater impoundments has been documented in the United States (U.S.) (Bartelt-Hunt et al., 2011), and oestrogens from cattle manure, which are potential endocrine disruptors, have been detected in different environmental compartments (Schuh et al., 2011; Adeel et al., 2017; Popova and Morra, 2017). Occurrences of synthetic organics associated with domestic wastewater have also been recorded (Barnes et al., 2008; Arrubla Vélez et al., 2016; Tran et al., 2018). If such compounds reach aquifers and hence groundwater supplies, they may not be effectively removed by drinking water treatment (Stuart et al., 2012; Charuaud et al., 2019a).

In addition to these potential entry routes, there are several factors which can affect the movement of contaminants into groundwater, including the physicochemical properties of the contaminants themselves, and geological factors such as overlying soil and underlying aquifer properties. The nature of Irish bedrock aquifers (with fracture permeability and no significant primary permeability, and the widespread occurrence of karstification in Irish

limestone aquifers with unique features such as swallow holes) may facilitate transport (and in some cases direct entry) of such contaminants through the unsaturated and saturated zones, without sufficient attenuation. As a result, such karstic and fractured aquifers are of most interest due to increased vulnerability.

The production of livestock for meat and the cultivation of crops is an essential feature of the food production system within Ireland and more broadly within the European Union. Due to an ever-increasing global population as well as a huge focus on land development, inevitably there is a demand for more food while utilising less space. In order to improve the efficiency of production of foods (particularly of animal origin), more intensive food producing practices have been introduced. As a result, veterinary agrochemicals have now become a critical component in animal husbandry and crop cultivation respectively, which has led to such substances being primary EOCs of concern. Antiparasitic drugs are of interest for this project given the predominance of grassland based agricultural systems in Ireland. Such systems are heavily reliant on antiparasitic drugs, as animals are more exposure to parasites from pastures (Bloemhoff et al., 2014). Furthermore, intensively reared species, such as poultry, are also heavily reliant on the prophylactic usage of antiparasitics to control infection outbreak amongst large flocks confined to indoor housing.

Given the high demands on the performance of Irish agriculture in terms of the food production system, in addition to the imminent pressures attributed to Irish Government policies including Food Harvest 2020 (DAFM, 2010) and more recent FoodWise 2025 (DAFM, 2015), heavy usage of veterinary drugs is set to continue, if not increase. As a result, loss of veterinary products such as antiparasitic agents to groundwater is not only a matter of international scientific interest, but also a matter of public concern. Such usage is likely to be exacerbated as a result of potential impacts of climate change, with unpredictable weather patterns likely to alter farming practices which could increase the need for veterinary drugs (Phelan et al., 2015). At present, there is only limited information available on the quantities of veterinary drugs used in Ireland, with no information available for the antiparasitic drugs. This is due to the fact that, up until recently, Ireland availed of an exemption in Directive 2006/130/EC (European Communities, 2006) which required veterinary medicinal products intended for use in food producing animal, to be subject to a veterinary prescription. This exemption meant that antiparasitics could be sold without prescription, which resulted in difficulties in collating and tracking information on

antiparasitic usage. However, according to a recent report (ACVM, 2019) by a task force of the Health Product Regulatory Authority's (HPRA) Advisory Committee for Veterinary Medicines (ACVM), due to evidence of widespread resistance issues and the environmental safety concerns of extensive use, it has been determined that Ireland no longer qualify for derogation from veterinary prescription under the new EU regulation 2019/6 (European Parliament, 2019). Therefore, from 2022 onwards, all antiparasitic drugs sold in Ireland will require a valid prescription and as a result, it is anticipated that more reliable data on usage quantities will become more readily available in the coming years, through the development of a National Secure Veterinary Prescription System (Bolton, *Pers. Comm., 2021)*.

Internationally, the majority of research on veterinary drug occurrence in the environment has mainly focused on surface and waste waters, with little research concerning these EOCs in groundwater. There is limited information available on the occurrence and associated concentrations of these antiparasitic drugs in the environment with information on the occurrence of metabolites and TPs even more scarce. As part of a prioritisation exercise in the United Kingdom (UK), 56 different veterinary drugs were classified as being "high priority" in terms of risk to the environment, based on their potential to reach the environment in large amounts and their hazard to aquatic and terrestrial organisms, with many of the drugs being antiparasitics (Boxall et al., 2003a). The same working group also noted the lack of suitably sensitive analytical methodology, specifically for TPs, as one of the main contributors to inadequate environmental risk assessment (Boxall et al., 2002; Boxall et al., 2003b). This issue has been echoed in many critical reviews by experts in the fields, even up to the present date (Hansen et al., 2009a; Horvat et al., 2012; Snow et al., 2016; ACVM, 2019). Very little research has been undertaken in Ireland to date on other emerging organic groundwater contaminants besides pesticides, particularly herbicides (McManus et al., 2013; McManus et al., 2014a; McManus et al., 2014b); some research has been undertaken on endocrine disrupting chemicals arising from on-site domestic wastewater treatment systems (Gill et al., 2009; Súlleabháin et al., 2009) but no research has been undertaken on entry of veterinary products to Irish groundwaters.

Overall, this project aimed to build on and complement this work, to fill the void in current research which lacks any insight into the occurrences of emerging veterinary antiparasitic contaminants in Irish groundwater, thus representing an important advancement in the knowledge and understanding of Irish groundwater quality. It is anticipated that this work

will also contribute to international research by providing more comprehensive analytical methods for determination of both parent drugs, and more importantly TPs. These methods will allow us to obtain a better understanding of the occurrence and fate (i.e. mobility) of TPs in the environment, which lacks understanding at present. More specific aims and objectives of this work are as set out in Section 1.9.

1.2 An Introduction to Groundwater

Of all the available freshwater on earth that is not frozen, 97% is accounted for as groundwater, which constitutes the largest reservoir of fresh water in the world (European Commission, 2008b). Approximately 75% of European Union (EU) residents depend on groundwater as a source of drinking water (European Commission, 2019). In Ireland approx. 26% of the public and private drinking supply is provided by groundwater sources (EPA, 2008; EPA, 2013a), with some areas even more reliant e.g. Roscommon with up to 75% of the drinking water sourced from groundwater (EPA, 2007). In addition to its use as a drinking water source, groundwater is extensively used for industrial and agricultural purposes. Not only is groundwater important as a natural reservoir, it also plays an essential role contributing to and maintaining surface water flow. In Ireland groundwater contributes 5–90% to the overall surface water flow depending on the aquifer productivity (EPA Ireland, 2010; EPA, 2015b). As a result, groundwater quality issues can be reflected in the surface water bodies, further magnifying the importance and need for groundwater protection and risk assessment.

In terms of groundwater protection, risk assessment is the most important step in water resource management to ensure the protection of human health and the environment. All groundwater protection schemes and regulations in Ireland (and the EU), are underpinned by the Source-Pathway-Receptor (SPR) model approach to risk assessment (Daly, 2009; EPA, 2013b). This model seeks to identify potential source(s) of contamination, the potential receptor(s) that are at risk of such contamination, and the transfer pathway(s) between the source and receptor. Figure 1-1 below depicts the SPR model for rural groundwater. Sources of groundwater contamination in rural environments include both point source contamination (localised and discrete) and diffuse source contamination (over a large area).

Point sources include farmyard wastes from animal holding, feeding and waste storage areas and septic tank effluent discharge, while diffuse sources include animal grazing and application of fertilisers and pesticides to land (Ritter et al., 2002). Receptors include existing and future groundwater resources both in terms of drinking water supply (e.g. springs and abstraction wells) (Daly, 2004), and groundwater dependent ecosystems which may be groundwater-fed rivers, lakes and wetlands or ecosystems within the aquifer (Knight and Penk, 2010).

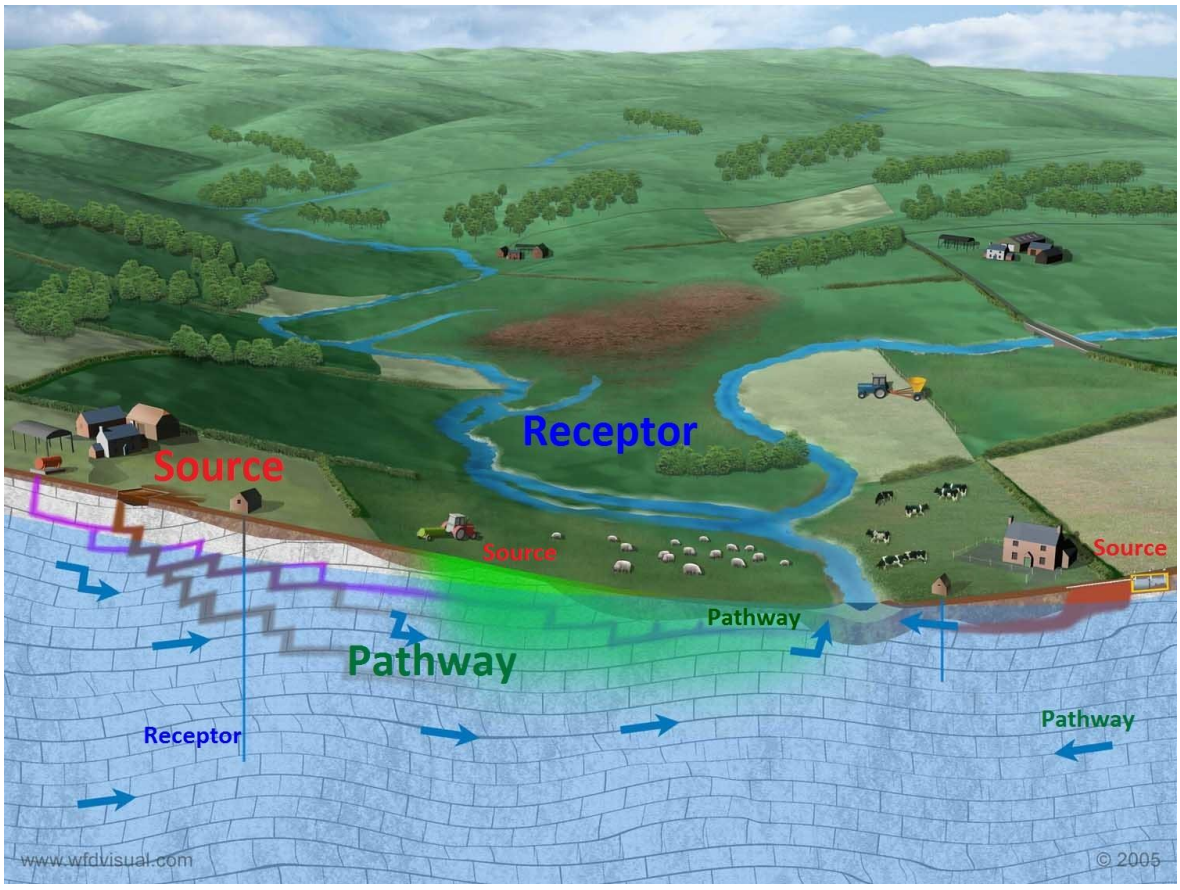

Figure 1-1 Sources, pathways and receptors in a rural agricultural environment (WFD visual, 2005)

We must also consider the main hydrological pathways by which water moves above and below the ground given water is the main vehicle for transporting contaminants. Water arriving at the ground surface can take 3 main pathways: it can flow at or near the surface (overland flow), it can enter the top layers of soil and move laterally (interflow) or it can percolate through the overlying soils to form groundwater (Mistear et al., 2009; O'Brien et

al., 2013). Soil permeability, as determined by soil texture, is the main factor influencing which hydrological pathway the water may take (Swartz et al., 2003). Depending on the pathway, attenuation of contaminants may occur to varying degrees (EPA, 2013b; McManus et al., 2014b).

There are considered to be two main ways in which water percolates through the overlying soils to the bedrock aquifers. **Matrix flow** is the slow even movement of water through the primary openings of the matrix material (soil or rock), while **preferential flow** is the fast heterogeneous movement of water through soils or rocks along certain pathways such as macropores, worm holes, root holes, cracks and fractures, bypassing the bulk media through which it flows (Kramers et al., 2009). Artificial pathways such as drainage ditches and pipes can also provide transport to groundwater (EPA, 2013b).

Ultimately it is such hydrological pathways between the land surface and groundwater and the extent to which they attenuate contaminants, that determine the groundwater vulnerability. Vulnerability is defined by the Geological Survey of Ireland (GSI) as the intrinsic geological and hydrogeological characteristics that determine the ease with which groundwater may be contaminated by human activities (DELG/EPA/GSI, 1999). It is the combination of subsoil permeability and thickness that determines the vulnerability category of extreme (E), high (H), moderate (M) or low (L) (Swartz et al., 2003). The nature of the aquifers themselves is particularly important given this project focuses specifically on Irish karst and fractured bedrock aquifers. In Ireland, bedrock aquifers have secondary permeability only, with flow via fissures and fractures (Swartz et al., 2003; EPA Ireland, 2010). In addition karst regions possess solutionally widened fractures and openings, and unique feature such as swallow holes and sinking streams, which allow for point recharge to groundwater with minimal attenuation (Daly, 2000; Coxon, 2014) (Figure 1-2).

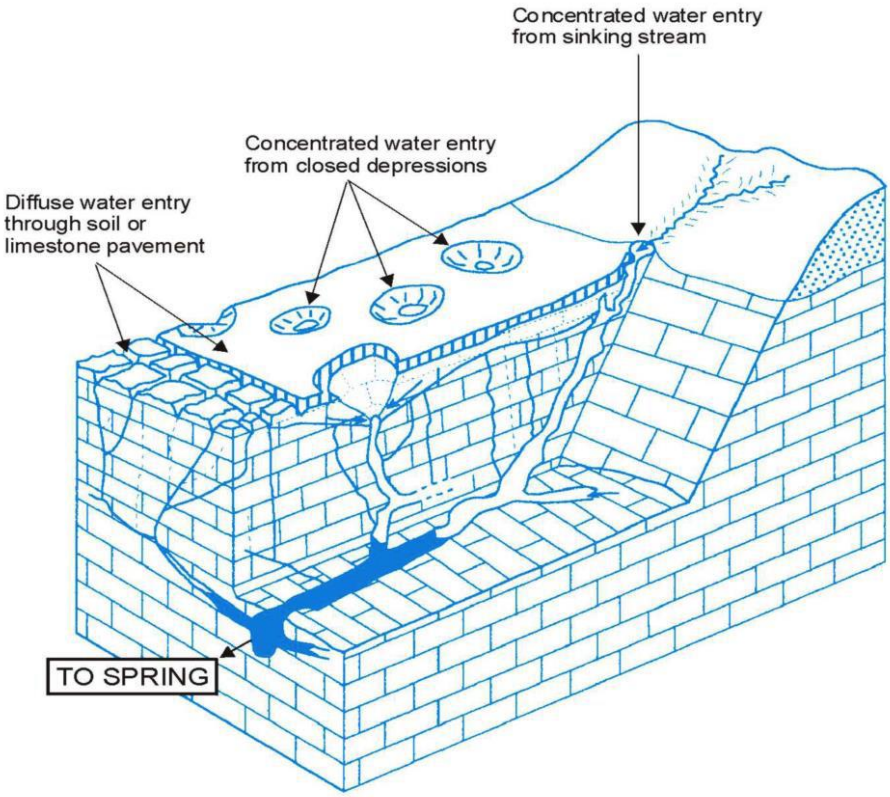

Figure 1-2 Possible contaminant entry routes to karst groundwater from (Groundwater, 2005; Coxon, 2014)

The majority of groundwater issues in rural environments are generally directly related to agricultural practices which pose risk of contamination from nutrients (nitrates, phosphates), and pesticides (Böhlke, 2002; Panno and Kelly, 2004; Levison and Novakowski, 2008; Coxon, 2011). A number of reviews, both nationally and internationally, have been published examining pressures, pathways and/or effects of nitrate contamination (Di and Cameron, 2002; Fenton et al., 2009; Premrov et al., 2012; Tedd et al., 2014) and pesticide contamination (Tiktak et al., 2004; Worrall and Kolpin, 2004; Chilton et al., 2005; McManus, 2012; McManus et al., 2014b) in rural groundwaters. Septic tank systems and the spreading of slurry and farmyard wastes can also pose risk of microbial contamination such as *E. coli* and *Cryptosporidium* (Ball, 1997; Gill et al., 2007; Chique et al., 2020), which are of high importance when it comes to public health and drinking water (EPA, 2015a).

1.3 What are Emerging Organic Contaminants (EOCs)?

Emerging Organic Contaminants (EOCs), often known as synthetic organic compounds, are becoming more and more of a growing international concern with respect to their occurrence in, and contamination of, groundwater bodies (Jurado et al., 2012; Lapworth et al., 2012; Stuart et al., 2012; Meffe and de Bustamante, 2014; Postigo and Barcelo, 2015). The definition of an EOC is quite broad given it includes any chemical that has not been included in national or international monitoring programmes and is not currently included in existing environmental quality regulations, but is continually introduced into the environment by anthropogenic activities (Norman Network, 2012). To be classified as an EOC, a compound does not necessarily have to be new, rather it is a chemical whose environmental fate and toxicology have yet to be established, thus has the potential to be harmful in the environment (Horvat et al., 2012; Lapworth et al., 2012; Čelić et al., 2017). EOCs include pharmaceuticals, agrochemicals (veterinary agents and crop protection agents), endocrine disruptors, pesticides, drugs of abuse, lifestyle products, industrial compounds and personal care products. Not only do they include parent molecules, but also any metabolites or transformation products. Figure 1-3 below gives a broad overview of some of the main groups of EOCs and the sub-groups into which they can be divided. As previously highlighted, this research will focus on one strand of the EOCs, the veterinary pharmaceuticals, and in particular, this literature review will consider three different groups of antiparasitic agents, the anthelmintics, anticoccidials and pyrethroid insecticides, as highlighted in red in Figure 1-3.

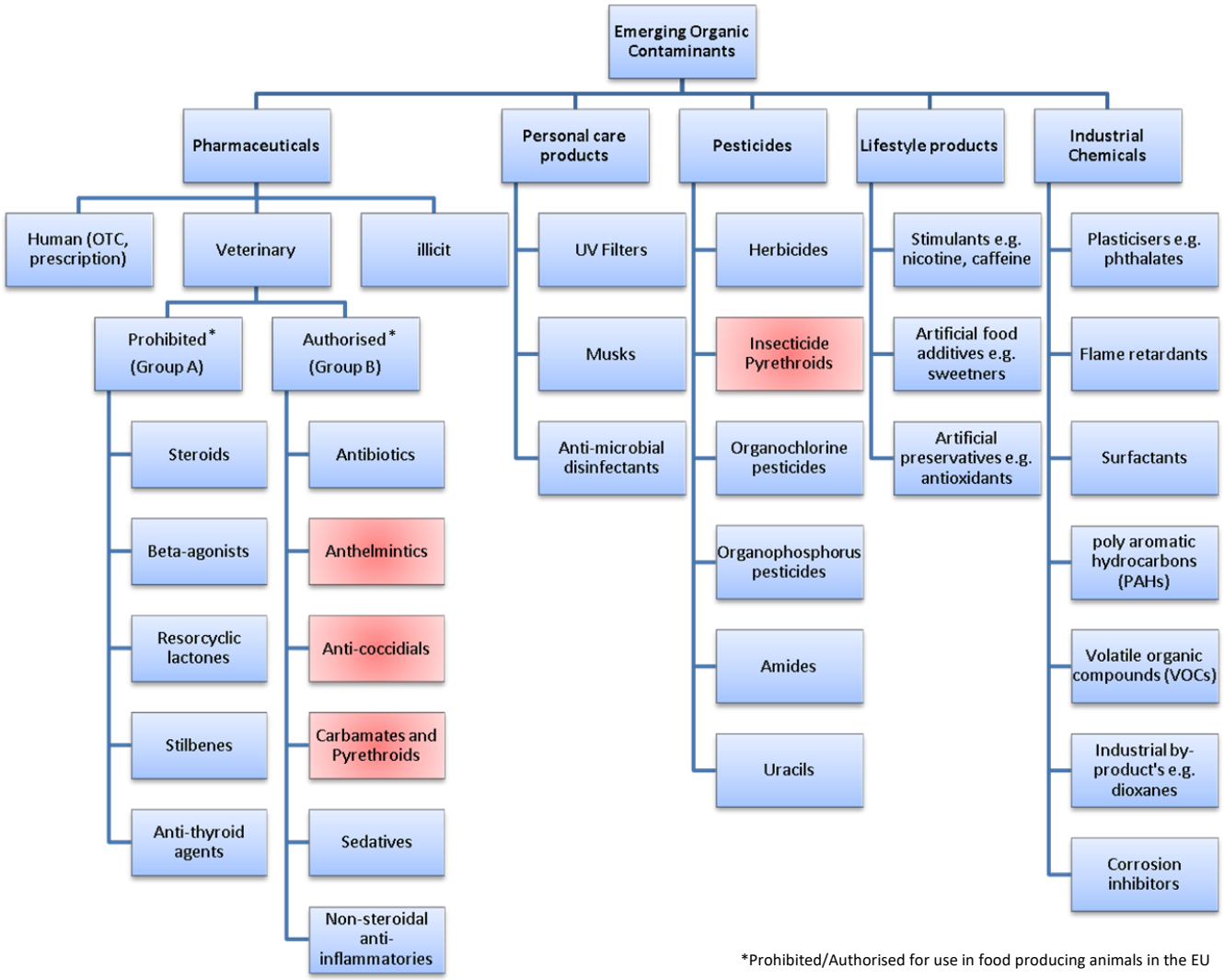

Figure 1-3 Schematic of the main groups of substances that are classified as emerging organic contaminants, with the antiparasitic drugs of interest indicated in red

1.4 Overview of Antiparasitic Agents

1.4.1 Antiparasitics and their use in agriculture

Antiparasitic agents cover a broad range of compounds that are used as veterinary drugs, feed additives and insecticides. Appendix A1 provides a list of names and the structures of commonly used antiparasitic drugs belonging to the anthelminthic, anticoccidial and pyrethroid groups. Many of these agents have applications in the areas of animal production and crop protection. The anthelmintic veterinary drugs are used in food producing animals to control internal parasites (Botsoglou, 2001). They are normally applied as a herd treatment (as opposed to individual animals) for the prevention of infection by a range of helminthic parasites, including roundworms, tapeworms and flukes (Danaher et al., 2006; Danaher et al., 2007; Tuck et al., 2016). The macrocyclic lactone (ML) type anthelmintic drugs can be applied to treat both internal and external parasitic infections, and are frequently referred to as the endectocides (Kahn and Line, 2010). Insecticides cover a broad range of substances used in agriculture. These include the pyrethrins and their synthetic pyrethroid derivatives (Feo et al., 2010b). The pyrethroids are one of the most widely used insecticides due to their higher potency for treating external parasites on livestock, crops and domestic applications (Beyond Pesticides, 2003; Albaseer et al., 2010). Anticoccidials are used to control coccidiosis and other protozoan infections in food producing animals (NFRD, 2011; Moloney et al., 2012). They are most widely used in broiler chicken production but are also used to a much lesser extent in other food producing animals such as pigs, calves and lambs (NFRD, 2011). There are two classes of anticoccidials, namely, the ionophore and the synthetic anticoccidial compounds (Clarke et al., 2014).

Antiparasitic drugs can enter into the environment through a number of different routes as summarised by Boxall (2010) and in Figure 1-4. The most important routes of entry into the environment from an Irish perspective are mostly due to direct application as plant protection agents, the excretion of substances in urine and faeces of livestock animals and direct application of slurries. Notably, Boxall also highlights the importance of wash-off of topical treatments and spillage during application as other important entry points (Boxall, 2010; Boxall, 2018).

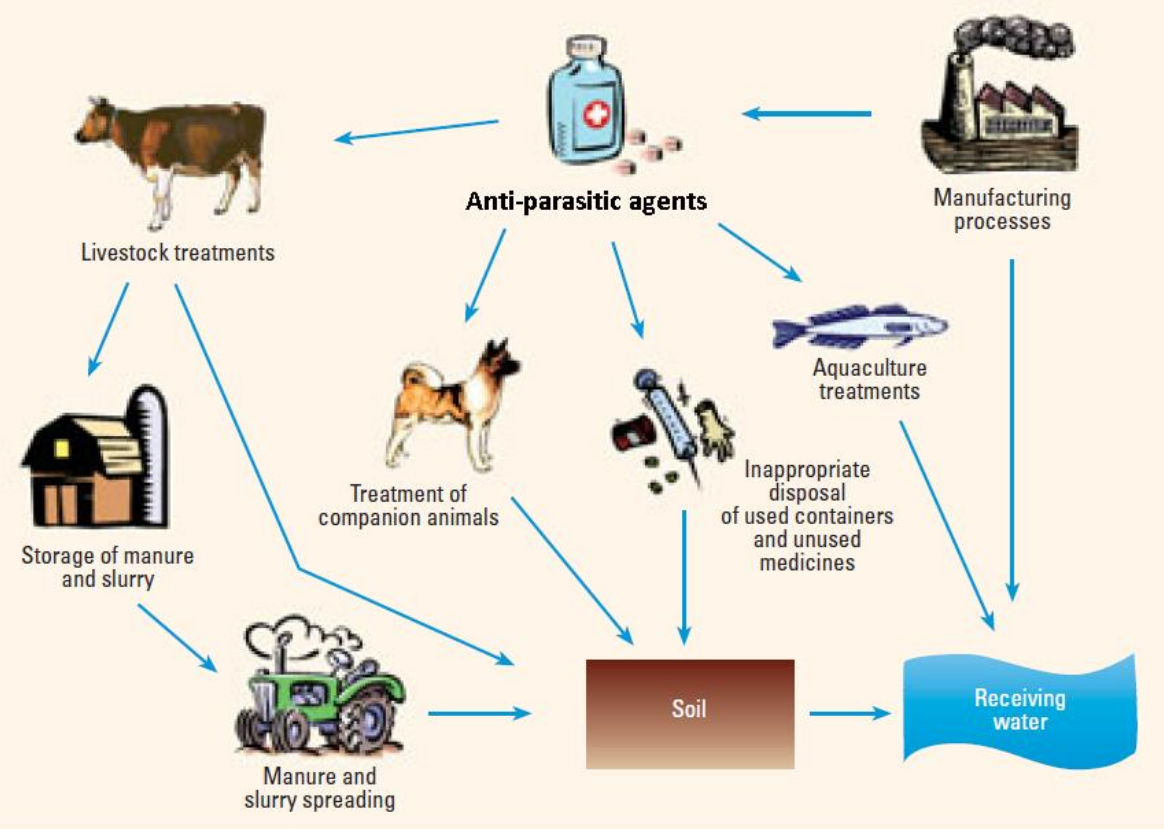

Figure 1-4 Schematic of the main entry routes of antiparasitic drugs into the environment from Boxall et al. (2003b)

1.4.2 Metabolites and transformation products of antiparasitics in the environment

Knowledge of the excretion, metabolism and environmental transformation of antiparasitic drugs is important to help identify the most appropriate target analytes for monitoring purposes. Once administered, a drug or substance is absorbed into the bloodstream of the animal, where it gets distributed around the body to the site of action. Any drug not absorbed is generally excreted in faeces. Once absorbed into the blood stream there is the potential for the drug to be metabolised, the degree of which will depend on the type of substance, the species treated, and the age and condition of the treated animal (Boxall et al., 2003b). If the compound is not metabolised, it will be excreted unchanged as the parent molecule. Consequently, urine and/ or faeces from a treated animal may contain a mixture of the parent compound and metabolites. The exact excretion path and profile depends on the drug physicochemical properties, route of administration and animal species (Boxall et al., 2003b). Available excretion data for antiparasitic drugs are limited and often difficult to interpret or compare due to differences with commercial products (Wardhaugh, 2005).

The fate of the MLs has been well researched; it has been shown that >90% of the administered dose is excreted in faeces as unchanged parent form regardless of the route of administration (Campbell, 1985; Hally et al., 1989; M.S. Maynard, 1989; Danaher et al., 2006; Beynon, 2012a; Horvat et al., 2012). In contrast, benzimidazoles, levamisole and tetrahydropyrimidines (Figure A1-1) are mainly excreted in urine (McKellar, 1997). It has been shown that 94% of the dose of netobimin was excreted in urine following parental administration to sheep and cattle (Danaher et al., 2007).Morantel (MOR) tartrate is excreted in faeces with up to 60% in the un-metabolised form (Horvat et al., 2012).

In terms of anticoccidials, the literature shows very little or no information on the metabolic behaviour and profile in animals. Of the limited information that is available, several anticoccidials (e.g. lasalocid (LAS) and diclazuril (DICLAZ)) can be excreted in sizeable amounts (up to 95% of administered dose) as un-metabolised active substances (EFSA, 2004; Hansen et al., 2009a). A European Food Safety Authority (EFSA) report indicated that ionophore type anticoccidials, such as lasalocid, are absorbed to a considerable extent in poultry, however metabolites account for no more than 10% of the total excreta (EFSA, 2004). There is limited information on the excretion of the pyrethroid insecticides; similar to the MLs, >96% of the pour-on dose of deltamethrin administered to cattle was eliminated in faeces (Wardhaugh, 2005), with the same authors also indicating that other pyrethroids including cypermethrin (CYPER), cyhalothrin (CYHALO) and cyfluthrin (CYFLU) are also excreted mainly in faeces.

The analysis of antiparasitic drugs in the environment is further complicated because of the need to not only monitor metabolites formed following the administration to animals, but also transformation products (TPs), which can result from the breakdown of parent drug in the environment, or the further breakdown of excreted metabolites. In some cases the TPs are more toxic than the parent drug (Boxall et al., 2002; Boxall et al., 2003a; Danaher et al., 2012). Furthermore, environmental marker residues are not well defined in legislation in contrast to those listed under food safety legislation. Horvat et al. (2012) comprehensively reviewed anthelmintic residues and their transformation in the environment. The MLs have been researched extensively leading to identification of a range of TPs including hydrolysis and photo-degradation products (Danaher et al., 2012). The benzimidazole drugs are extensively metabolised in animals and as many as three major metabolites have to be monitored in food in the EU (Danaher et al., 2007). Due to the sulphide linkages within their

structure, albendazole (ABZ), fenbendazole (FBZ) and triclabendazole (TCB) are susceptible to sulphide oxidation to their respective sulphoxide (SO) metabolites: ABZ-SO (aka ricobendazole), FBZ-SO (oxfendazole, OXF) and TCB-SO (Horvat et al., 2012). These sulphoxide metabolites can undergo further transformation to their respective sulphone (SO_2) metabolites (Danaher et al., 2007). Notably the sulphoxide metabolites, particularly for ABZ, have the highest activity, thus anthelmintic efficacy is attributed to these primary metabolites. Flubendazole (FLU) and mebendazole (MBZ) contain carbonyl groups which are susceptible to reduction forming hydroxyl groups. All of the above BZs (ABZ, FBZ, FLU and MBZ) possess carbamate groups which can be hydrolysed to form their counter-part amino-metabolites. The insecticides are normally monitored in food and environmental samples as the parent chemistry. However, it has been reported that the pyrethroid insecticides can undergo degradation to more toxic carboxylic acid derivatives and phenoxybenzoic acid TPs (ATSDR, 2003; Wielogórska et al., 2015; Cycon and Piotrowska-Seget, 2016; Li et al., 2016).

1.4.3 Dynamics influencing the fate and mobility of antiparasitic drugs in the environment

Once released into the environment, contaminants will be transported and distributed between the major environmental compartments (e.g. soil, water). Agrochemicals reaching soil may partition to the soil particles, leach to groundwater (via hydrological pathways discussed in section 1.2) and/or be degraded (Arias-Estévez et al., 2008). The extent of transport and distribution of agrochemical is often referred to as the mobility of the compound and is mainly influenced by the physicochemical properties of the contaminant and the environmental characteristics (including geological conditions) (Ritter et al., 2002). Persistence of a contaminant relates to the residence time of the contaminant in a given compartment, subject to degradation or removal processes (McManus, 2012).

The main physicochemical properties of the contaminants that influences their environmental fate include:

- The octanol–water partition coefficient (K_{ow}) (usually expressed as log K_{ow}) can be defined as the ratio of the compound's concentration in a known volume of n-octanol (non-polar) to its concentration in a known volume of water (polar), once equilibrium has been reached. Log K_{ow} is often used as a relative indicator of the tendency of an organic compound to adsorb to soil and Log K_{ow} values are generally inversely

related to aqueous solubility. If a compound has a log K_{ow} value of less than 0, it is said to be hydrophilic, while values greater than 0 indicate it is hydrophobic.

- The soil organic carbon-water partitioning coefficient (K_{oc}) (often expressed as log K_{oc}), is the ratio of the mass of a chemical that is adsorbed in the soil per unit mass of organic carbon in the soil per the equilibrium chemical concentration in solution. LogK_{oc} values are useful in predicting the mobility of organic soil contaminants, where higher logK_{oc} values indicate less mobility, while lower logK_{oc} values indicate higher mobility.
- The acid dissociation constant (K_a) (usually expressed as pK_a) is a measure of how likely a chemical is to ionise in solution; pK_a values therefore provide important information regarding solubility and ion exchange properties of residues.
- The water solubility (S_w), often called aqueous solubility, refers to the maximum amount of a substance that can be fully dissolved per unit volume of water at a given temperature. The European Pharmacopoeia defines different solubility ranges as summarised in Table A1-1, Appendix 1B.

Overall, the higher the S_w and the lower the log K_{ow} and log K_{oc} values, the more chance the contaminant has of reaching groundwater (McManus, 2012). Physicochemical data for antiparasitic drugs are limited to a few sources (Oudou and Hansen, 2002; ATSDR, 2003; Danaher et al., 2007; Krogh et al., 2008a; Ochiai et al., 2008; Hansen et al., 2009b; Albaseer et al., 2010; Feo et al., 2010b; Horvat et al., 2012; Santaladchaiyakit and Srijaranai, 2012; Bak et al., 2013a; Bak et al., 2013b; Zrncic et al., 2014) and are summarised in various tables throughout this thesis (Table 4-1 for the anthelmintics, Table 5-1 for the anticoccidials and Table A1-2 for the pyrethroids).

Most anthelmintic drugs are practically insoluble or very slightly soluble in water, with S_w values ranging from 1.5×10^{-5} mg L^{-1} (Closantel (CLOS)) to 407.2 mg L^{-1} (OXF) (see Table 4-1 in Chapter 4). A few anthelmintics (PIP, LEV and MOR) are soluble or highly soluble and more likely to be transported in water. The octanol water coefficient (K_{ow}) of an organic molecule is a measure of the hydrophobicity and potential for persistence in the environment, particularly those with logKow values >4 (CVMP, 2016). Many ML and flukicide anthelmintics (TCBs, bithionol (BITH), CLOS, niclosamide (NICLOS) and rafoxanide (RAFOX)) have logK_{ow} values of >4, indicating they may persist in the environment. Such an example is the ML moxidectin, which has been determined to meet the criteria for

classification as a persistent bioaccumulative toxic substance (PBTs) (CVMP, 2016). Conclusive decisions on the PBT status of other parasiticides could not be made during the same assessment due to a lack of necessary data. The potential persistence of these compounds is also reflected in terms of the relatively high $logK_{oc}$ values, which implies that organic carbon seems to be very important for the binding of anthelmintics in soil. This is particularly the case for the MLs and the flukicides given their higher $logK_{oc}$ values, indicating relatively low mobility in soil.

There is scant data published in literature on the physicochemical properties of the anticoccidials, with most related to the ionophores (Table 5-1). S_w values range from low mg L^{-1} to a few hundred mg L^{-1}. Diclazuril and narasin would appear to be the most hydrophobic anticoccidials with $logK_{ow}$ values of >4. Based on their physicochemical properties (Table 5-1), it is expected that ionophore compounds will be more associated with soil and sediment once in the environment. The insecticides are all highly hydrophobic and are practically insoluble in water, given their low S_w values (Table A1-2, Appendix 1A). Log K_{ow} values for the selected insecticides listed are all >4 (range 4.0–8.35) indicating they are likely to persist in the environment with low mobility (Feo et al., 2010b). This is further supported by their relatively high $logK_{oc}$ values, the majority of which are ≥5 (Hladik and Kuivila, 2009).

In addition to the intrinsic properties of the contaminants themselves, environmental factors such as land use, soil type and properties, aquifer (geological) properties and climate can affect the movement of contaminants to groundwater (Worrall and Kolpin, 2004; Essaid et al., 2015). Soil and Quaternary deposit (subsoil) properties such as texture, structure, permeability and thickness can influence the transport pathway (thus groundwater vulnerability) taken by contaminants through the unsaturated zone to groundwater. On taking such pathways discussed in Section 1.2, contaminants are subject to a number of complex physical, chemical and biological transformation processes that can provide attenuation (Arias-Estévez et al., 2008). The most important of these for agrochemicals are adsorption (to organic matter, organic carbon (OC) and/or clay minerals), ion exchange and chemical and biological degradation (Estevez et al., 2012).

Considering the physicochemical properties and based on relatively high hydrophobicity of the compounds, sorption is likely to be the most important attenuation process, besides

degradation, for the majority of the antiparasitic drugs included in this work. Soil and subsoil properties, namely organic carbon content, clay content and soil pH influence sorption of contaminants to soil; as a result, contaminants can be adsorbed to varying extents in different soils and subsoils. Particularly Quaternary deposits consisting of clayey tills will provide more attenuation due to longer travel times and naturally higher organic carbon (OC) and clay mineral content, compared to sand or gravelly subsoils with higher permeability (lower travel times) and less clay content (Swartz et al., 2003).

While agrochemicals that are strongly adsorbed to soil or surface particulate matter can remain at or near the site of application, there is also the potential for transportation to groundwater via preferential flow pathways in which the contaminant is transported in the sorbed-phase attached to colloidal particles (Foster and Chilton, 1991) . This introduces fast direct entry of contaminants to groundwater, bypassing many of the attenuation process. Notably, current risk assessment models for veterinary medicinal products do not consider this potential route of exposure (Boxall et al., 2002). This phenomenon combined with fractured and fissure flow aquifers and karst aquifers mentioned previously, pose vulnerability to groundwater contamination. Particularly karst groundwater systems are most vulnerable due to fast-flowing water in karst conduits with transport times ranging from only hours to days over long distances (Einsiedl et al., 2010).

1.5 Groundwater Legislation and Policy

1.5.1 Legislation pertaining to groundwater

The Water Framework Directive (WFD) 2000/60/EC (European Parliament, 2000) is the most important legislation to date for the protection and management of groundwater in Ireland and the European Union. This directive manages both surface and ground water as a continuum (as opposed to separate entities) in an integrated approach which now recognises the underground component of the hydrological cycle. The WFD's objectives for groundwater focus on quantitative and chemical status, whereas the objectives for surface waters concern ecological and chemical status (Daly, 2009). While the quantitative status objectives of the WFD are clear, the chemical-status criteria are more complex and were not fully resolved at the time the WFD was adopted. As a result, a new Groundwater Directive (GWD) was developed and implemented in 2006 (2006/118/EC) (European Parliament, 2006), which completely superseded the original directive 80/68/EC (EEC, 1979). This

GWD highlights criteria for the assessment of good groundwater chemical status and for the identification and reversal of significant and sustained upward trends. In addition, the GWD requires member states to identify threshold values for pollutants, and to adopt measures to prevent or limit inputs of pollutants into groundwater. In Ireland, the Irish Groundwater Regulations (Government of Ireland, 2010) implement strengthened measures for the protection of groundwater in line with the requirements of the Water Framework Directive (2000/60/EC) and the Groundwater Directive (2006/118/EC).

In addition to groundwater legislation there is also legislation relating to drinking water. The EU Drinking Water Directive 98/83/EC (European Commission, 1998) concerns the quality of water intended for human consumption and was established to protect human health from the adverse effects of any contamination of water by ensuring that it is wholesome and clean. In Ireland, these pieces of legislation are given formal effect in Irish law in the form of the Irish Drinking Water Regulations 2014 (Government of Ireland, 2014a) since amended in 2017 (Government of Ireland, 2017a). These Regulations establish strict quality standards for water used for human consumption and set out the maximum and guideline values for various different physical, bacteriological and chemical contaminants.

1.5.2 Applicability to EOCs

Currently there is no specific legislation relating to EOCs in groundwater. This is primarily due to there being very little information known about the fate and toxicity of EOCs. However, there are pieces of legislation relating to groundwater pesticides, which are commonly transposed for application to other pesticide like contaminants, such as some of the antiparasitic veterinary drugs included in this work. Such pieces of legislation include the aforementioned EU Drinking Water Directive (implemented in Ireland by the Drinking Water Regulations 2017) and the Groundwater Directive 2006/118/EC. Drinking water standards specified under both of these pieces of legislation state that individual pesticide concentrations must adhere to the parametric value (PV) of 0.1 µg L^{-1}, while total pesticides (sum of all individual pesticides found) must adhere to the PV of 0.5 µg L^{-1}. Furthermore, the Drinking water Regulations 2017 (Government of Ireland, 2017a) also specify performance characteristics for pesticides whereby the method of analysis used must, at a minimum, be capable of measuring concentrations equal to the parametric value with a trueness, precision and limit of detection (LOD) of 25%. Ultimately this indicates that the

analytical method must be capable of detecting levels of individual pesticide compounds of the order of equal to or less than 0.025 µg L^{-1}.

Notably under these pieces of legislations the term pesticides is defined to include all of the following:

- organic insecticides,
- organic herbicides,
- organic fungicides,
- organic nematocides,
- organic acaricides,
- organic algicides,
- organic rodenticides,
- organic slimicides and
- relevant metabolites, degradation and reaction products.

As a result, the scope of this definition allows such PVs and performance characteristics to be directly applied to the insecticide compounds in this review, in addition to several of the anthelmintic drugs which are used to treat nematodes. In addition to the PVs, under the Groundwater Regulations 2010 (Government of Ireland, 2010), the Environmental Protection Agency (EPA) of Ireland have the responsibility for establishing and maintaining a list of Threshold Values (TVs) for pollutants that are causing risk to groundwater bodies. For pesticides, including the pyrethroid cypermethrin, such TVs have been set as 0.075 µg L^{-1} and 0.375 µg L^{-1} for individual and total pesticides, respectively. Any exceedance of TVs at a representative monitoring point triggers further investigation to confirm whether the criteria for poor groundwater chemical status are being met.

In more recent years there has been some movement on legislation for EOC's, however this mainly pertains to surface waters. Good chemical status in surface waters implies compliance with community Environmental Quality Standards (EQS) as defined by Annex IX and X of the WFD 2000/60/EC (Jones et al., 2015). Specifically, Annex X of the WFD has identified a list of priority and priority hazardous substances, and EQS's have subsequently been established for 33 substances in a WFD daughter Directive, the EQS Directive 2008/105/EC (European Commission, 2008a). Recently an expanded list of proposed emerging compounds was introduced in the form of Directive 2013/39/EU

(European Parliament, 2013). This list includes a number of industrial chemicals, endocrine disrupting compounds and pesticides, in which the pyrethroid cypermethrin is specified (Jones et al., 2015). Maximum allowed concentration (MAC) EQS values set for cypermethrin range from 0.06 to 0.6 ng L^{-1} in different surface waters. Technical specifications listed under Directive 2009/90/EC (European Commission, 2009) define minimum performance criteria for methods of analysis whereby each member state must ensure that the minimum performance criteria for limits of quantification are equal to or below a value of 30 % of the relevant EQS standard. This poses an immense analytical challenge for emerging priority pollutants such as cypermethrin, which would require LOQs of $\leq$ 0.02 or 0.2 ng L^{-1} (depending on the type of water body). Directive 2013/39/EU also provided for the establishment of a "watch list" for Union-wide monitoring of so-called contaminants of emerging concern (CECs) (Sousa et al., 2018), which are substances deemed as having the potential to pose significant risk to the aquatic environment. A list of 17 CECs, including several veterinary steroid hormones and antibiotics, comprised the first watchlist, published in 2015 under Decision 2015/495/EU (European Commission, 2015). This watchlist was updated in 2018, as set out on Commission Implementing Decision 2018/840 (European Commission, 2018), by the removal of five substances and addition of three new substances. The watchlist was most recently updated in August 2020 under Decision 2020/1161 (European Commission, 2020) which now includes 19 antibiotic type CECs. There are currently no EQS values set for such CECs.

1.6 Previous Occurrence studies and Levels in Environmental Water Samples

The majority of research on EOC occurrence in the environment has focused on surface and wastewater systems, with very little research concerning EOCs in groundwater (Table A1-3, Appendix 1C). Of these studies in surface waters, while some research focuses on the occurrence of a wide array of EOCs (Murray et al., 2010; Pal et al., 2010; Gonzalez et al., 2012; Riva et al., 2019; Köck-Schulmeyer et al., 2021) (e.g. in freshwater bodies or wastewaters), the majority of research focuses more specifically on the occurrence and/ or fate of specific groups of EOC's such as pharmaceuticals (Mompelat et al., 2009; Rivera-Jaimes et al., 2018; Casado et al., 2019) or endocrine disrupting compounds (Liu et al., 2009; Silva et al., 2012).

In terms of groundwaters, Stuart et al. (2012) reviewed the risk of EOCs in UK groundwater, while Meffe and de Bustamante (2014) published the first comprehensive review of EOC occurrence in both surface and groundwater in Italy. Lapworth et al. (2012) summarised the sources, fate and occurrence of EOCs in the groundwater of Europe, while Loos et al. (2010) carried out a pan-European (including Ireland) survey on the occurrence of polar organic contaminants. Extending the work of Lapworth et al., Jurado et al. (2012) carried out a review of the source, occurrence and fate of EOCs in Spanish groundwaters. More recently Lapworth et al. (2015) investigated the occurrence of 42 contaminants including industrial compounds, pesticides and personal care products in chalk aquifers across the UK and France, while Dodgen et al. (2017) investigated the occurrence of PPCPs and hormones in karstic aquifers in the USA. Kivits et al. (2018) investigated the presence and fate of veterinary antibiotics in age-dated groundwater, in areas with intensive livestock farming in the Netherlands.

There is limited information available on the occurrence and associated levels of antiparasitic agents in the environment with information on the occurrence of metabolites and TPs even more scarce (Horvat et al., 2012). A comprehensive review has been undertaken on such occurrences for this report as presented in Appendix 1C Tables A1-4, A1-5, and A1-6, and as summarised in the text below.

1.6.1 Previous anthelmintic occurrences

The majority of occurrences of anthelmintic compounds in the environment (Table A1-4, Appendix 1C) relate to detections in wastewaters from treatment plants (Van De Steene and Lambert, 2008; Bartelt-Hunt et al., 2009; Babic et al., 2010; Sim et al., 2013), surface waters (Alvarez et al., 2005; Bartelt-Hunt et al., 2009; Sim et al., 2013; Zrncic et al., 2014; Wagil et al., 2015b) or manure leachate (Raich-Montiu et al., 2008; Weiss et al., 2008). Based on the literature search on this occasion, no data was found on occurrences of anthelmintic compounds in groundwaters, with the exception of thiabendazole (TBZ) detected in a single farm well in Norway, detected in <1% of total samples analysed (Haarstad and Ludvigsen, 2007). However, detections in surface waters and waste treatment effluent gives an indication of possible amounts of anthelmintics being released into the environment.

The benzimidazole, flubendazole (FLU), was most commonly detected in the environment. FLU was detected in both influent and effluent waters of a pharmaceutical waste water treatment plants (WWTP) at levels up to 89 µg L^{-1} (Van De Steene and Lambert, 2008; Van De Steene et al., 2010), with the later study also detecting FLU at low levels (< 20.2 ng L^{-1}) in fifteen of sixteen river water samples in the later study by these authors (Van De Steene et al., 2010). These levels in river water are similar to those reported by Wagil et al. (2015b) who reported the occurrence of FLU in river water samples in Poland at levels of 5.4–39.3 ng L^{-1}, with detection at 4 out of 8 sites sampled. As part of the same study, fenbendazole (FBZ) was also detected in the same (4 of 8) river samples at levels in the range of 7.1–87.5 ng L^{-1}. Polar organic chemical integrative samplers (POCIS) were used in two different studies which allowed the detection of another benzimidazole, thiabendazole (TBZ) (Alvarez et al., 2005; Bartelt-Hunt et al., 2009).

The most comprehensive surveys on anthelmintic occurrence were those by Sim et al. (2013) and Zrncic et al. (2014). Sim et al. (2013) included 8 anthelmintics in an investigation of influent and effluent wastewaters including sewage treatment plants (STPs) and livestock wastewater treatment plants (LWTPs). Of more importance, levels of FLU in the range of 2–1170 ng L^{-1} were found in 35 out of the 38 samples (>90%) collected from receiving river waters. The highest of these detections were found downstream relative to the waste treatment site, which indicates the potential influence of effluents on the receiving waters. In terms of the LWTPs, FBZ and its metabolites OXF, FBZ-SO_2, FBZ-NH_2 and FBZ-OH were found at relatively high levels in effluent (15–1490 ng L^{-1}), however, the corresponding levels of FBZ and metabolites in receiving river waters were found to be in the range of 0–63 ng L^{-1}. Zrncic et al. (2014) analysed 11 samples from the Llorbregat River in Spain for 10 different anthelmintics. The authors reported the detection of eight anthelmintics, including albendazole (ABZ), FBZ, FLU, levamisole (LEV), triclabendazole (TCB) and moxidectin (MOXI), typically at concentrations ranging from 1–5 ng L^{-1}, except for LEV which was detected at concentrations up to 39 ng L^{-1}.

1.6.2 Previous anticoccidial occurrences

The majority of reported occurrences of anticoccidials in the environment (Table A1-5, Appendix 1C) are in surface waters and runoff (Cha et al., 2005; Hao et al., 2006; Kim and Carlson, 2006; Song et al., 2007; Thompson et al., 2009; Iglesias et al., 2012; Sun et al.,

2013; Bak and Björklund, 2014) or solid agricultural samples (including soil, sediment, manure/litter) (Kim and Carlson, 2006; Olsen et al., 2012; Bak et al., 2013b; Herrero et al., 2013; Sun et al., 2013; Bak and Björklund, 2014), with very few studies on occurrences in groundwater (Watanabe et al., 2008; Bartelt-Hunt et al., 2011). Notably, the ionophores (Cha et al., 2005; Hao et al., 2006; Kim and Carlson, 2006; Watanabe et al., 2008; Thompson et al., 2009; Bartelt-Hunt et al., 2011; Herrero et al., 2012; Bak et al., 2013b; Sun et al., 2013; Bak and Björklund, 2014) are by far the most studied anticoccidials compared to chemical (synthetic) anticoccidials (Song et al., 2007; Iglesias et al., 2012; Olsen et al., 2012). Monensin (MON), salinomycin (SAL) and narasin (NAR) were the most commonly detected ionophores in environmental samples.

On examining the occurrence of antibiotics in shallow groundwater, Bartelt-Hunt et al. (2011) reported levels of the anticoccidial MON up to 2350 ng L^{-1} in monitoring wells at two different concentrated animal feeding operations (CAFO) in the USA. One of the two sites had considerably lower levels (20–60 ng L^{-1}), while much higher levels were detected in livestock waste lagoons (12900 ng L^{-1}). The only other occurrence of anticoccidials in groundwater found as part of this review was reported by Watanabe et al. (2008), who detected MON in four of sixteen groundwater samples at concentration from 40 to 390 ng L^{-1}. Other occurrences of MON at relatively high concentrations were reported for surface waters, with levels of up to 843 ng L^{-1} detected in 85 of 237 river water samples (Thompson et al., 2009). The ionophores NAR and SAL were also detected in this study, but at much lower frequencies, with detected concentrations <20 ng $L^{-`1}$. NAR and SAL were also detected in surface waters as part of several different studies, with levels generally in the range of 2–20 ng L^{-1} (Kim and Carlson, 2006; Thompson et al., 2009; Iglesias et al., 2012) and certainly not exceeding 60 ng L^{-1} (Cha et al., 2005). Of notable interest, Sun et al. (2013) detected high level of MON, SAL and NAR (levels of up to 2389, 9022 and 358 ng L^{-1} respectively) in run-off from agricultural land to which poultry litter had been applied, indicating a potential transport route for these relatively immobile compounds. Other than the ionophores discussed, there are very few reported occurrences of chemical coccidiostats (synthetic anticoccidials) in environmental samples; of notable interest, amprolium (AMP) was detected in 9 out of 11 surface run-off samples at levels up to 288 ng L^{-1} (Song et al., 2007).

1.6.3 Previous pyrethroid occurrences

The majority of reported studies for insecticides (Table A1-6, Appendix 1C) relate to detections of insecticides in solid environmental samples such as soil and sediment (Yasin et al., 1996; Weston et al., 2004; You et al., 2004; Rissato et al., 2005; Woudneh and Oros, 2006), with few detections in environmental waters (Xue et al., 2005; Feo et al., 2010a). There has also been very few reported occurrence of insecticides in groundwaters (Kumari et al., 2008), although a few studies have looked at the potential of leaching of individual pyrethroids to groundwater (Dousse et al., 2014). Kumari et al. (2008) reported the occurrence of three pyrethroids in soil and groundwater samples. Of twelve groundwater samples taken at depths of 20–60 m (below ground level.), six were found to contain levels of cypermethrin (22–90 ng L^{-1}), while four contained levels of deltamethrin (17–61 ng L^{-1}). In a study assessing the occurrence of 10 pyrethroids in surface and groundwaters from paddy fields in Spain, Aznar et al. (2016) report the detection of up to nine pyrethroids in surface waters at concentrations ranging from 14 to 1450 ng L^{-1}, while up to eight pyrethroids were detected in groundwaters al levels ranging 6 to 833 ng L^{-1}. The detected compounds in both surface water and groundwater included resmethrin (RESM), bifenthrin (BIFE), fenpropathrin (FENP), cyfluthrin (CYFL), cyhalothrin (CYHALO), cypermethrin (CYPER) and esfenvalerate (ESFEN).

The majority of other detections of pyrethroids in water all relate to surface waters. Feo et al. (2010a) developed a method for determination of 14 pyrethroids in water and subsequently applied it for the analysis of 16 “real life” samples from the Ebro River delta (Tarragona, Spain). The results state that CPYER was found in 14 out of 16 samples, with levels ranging from 4.93–30.5 ng L^{-1}. As part of study to screen for 31 endocrine disrupting compounds in surface waters and sediment,. Xue et al. (2005) reports the detection of CPYER at levels of 1.89 and 8.87 ng L^{-1} in surface waters and soil pore water respectively. Fenvalerate (FENV) and deltamethrin (DELT) were also detected as part of this study at concentrations up to 26.3 ng L^{-1} 54.2 ng L^{-1}, respectively.

Since carrying out the initial literature review, Tang et al. (2018) have produced a more comprehensive review of pyrethroid occurrences worldwide, with further detailed information reported for pyrethroid occurrences in different matrices including soil, water (surface waters and waste waters) and sediments. This review provides example of the rapid

and drastic increase in the number of publications produced concerning the occurrence of pyrethroids, particularly in the last decade.

1.7 Sampling Techniques

A sample should be representative of the original matrix of interest, sufficient for the required analysis and maintained free from contamination during collection, transport and storage. Procedures should be selected to minimise changes in water chemistry. Incorrect sampling technique can be detrimental to the integrity of the sample rendering the analytical result invalid (Górecki and Namiesnik, 2002). In Ireland there are standard protocols and procedures relating to sampling of water (e.g. ISO 5667 parts 3, 5 and 11 (NSAI, 2006; NSAI, 2009; NSAI, 2018)) as provided by the National Standards Authority of Ireland in conjunction with the EPA (EPA, 2016; NSAI, 2016b). The two main modes of sampling for environmental water sample ("once-off" sampling including grab and pump sampling techniques, and the more modern passive sampling technique) are briefly discussed below with emphasis on groundwater.

1.7.1 Once-off sampling

Once-off sampling, often called "grab" or "spot" sampling, involves the physical collection of a single sample of a water body at one given moment in time and subsequent storage in an appropriate container until analysis. Typical once-off sampling techniques include traditional grab sampling with an appropriate container or pump sampling (whether pumped to a tap or via a portable peristaltic pump). The aim of groundwater sampling is to recover a representative sample of the groundwater in the formation adjacent to screened or open portion of a well/borehole (ITRC, 2007). If there is no obvious point of sampling such as a tapped public supply, groundwater wells/boreholes can be sampled using manual devices such as bailers (Figure 1-5) and/or pumps. Peristaltic pumps are commonly used for low flow sampling of narrow diameter piezometers or even large diameter where they allow sampling with minimal disturbance of the water column. Generally, it is recommended to purge a well/borehole to remove stagnant water, thus ensuring the water being sampled is most representative of the formation. Usually at least three well volumes are removed prior

to sampling, to ensure the sample representative of the active groundwater (WYG Environmental, 2008; In-Situ, 2016).

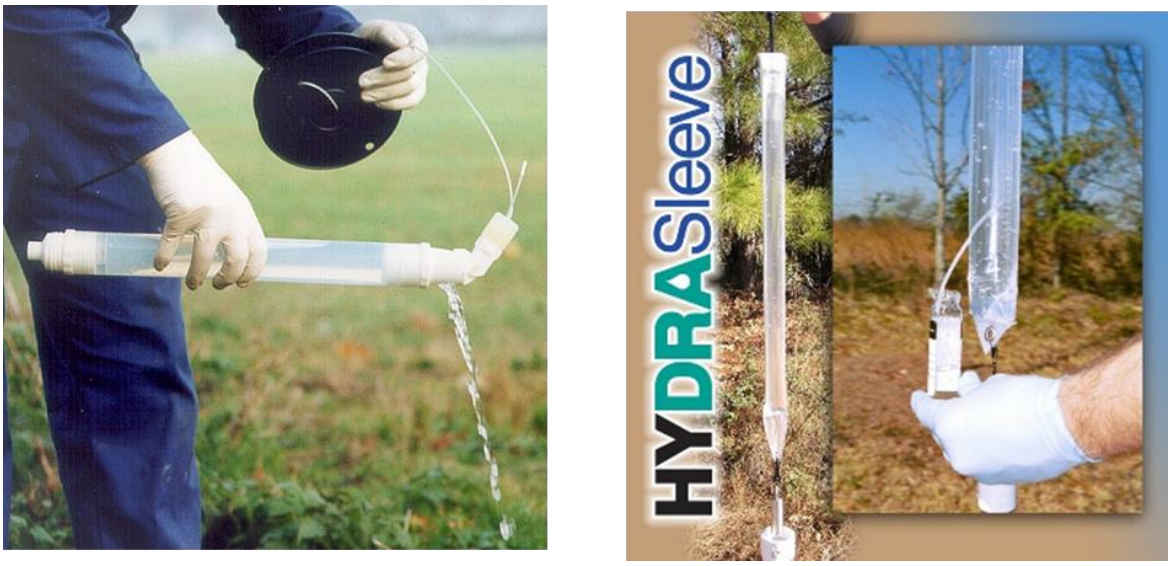

Figure 1-5 (a) example of a traditional bailer sampler and (b) example of a HydraSleeve sampler

Currently grab sampling is the only sampling method that meets the requirement set out by the WFD (2000/60/EC) (European Parliament, 2000) and the EQS Directive 2008/105/EC (European Commission, 2008a), given it allows for the collection and subsequent determination of "whole water" concentrations. Regardless of this, there still remains to be a number of disadvantages associated with grab or spot sampling that may need to be addressed in the future (Vrana et al., 2005; Mills et al., 2007; Jones et al., 2015). The main disadvantage being that grab sampling only provides a snapshot of the water quality at that given moment in time. It does not provide any means of capturing episodic pollution events or temporal variation, nor does it allow any long-term exposure assessment. In order to provide a sufficiently representative sample to account for any temporal variation and pick-up any transient pollution events, a large number of grab samples would be required, resulting in extremely high costs due to the amount of work force regularly required on site, as well as the increased number of analyses that comes with the added samples (Vrana et al., 2005).

1.7.2 Passive sampling

As a result of the above issues associated with once-off sampling, alternative approaches have been highly sought after. Passive sampling is one such technique proving more promising as a tool for monitoring priority contaminants in environmental matrices such as water, soil, sediment and biota (Górecki and Namiesnik, 2002; David A. Alvarez et al., 2004; Vrana et al., 2005; Seethapathy et al., 2008). Passive sampling is defined as any sampling technique based on free flow of analyte molecules from the sampled medium to a collecting medium, as a result of difference in chemical potential between the two media (Górecki and Namiesnik, 2002).

Since its first application to water in the mid-late 1980s (Figure 1-6), there has been over twenty-six different passive samplers developed for application to environmental waters (Vrana et al., 2005; Seethapathy et al., 2008). Common amongst these are semi-permeable membrane devices (SPMD), polar organic chemical integrative sampler (POCIS) and Chemcatcher® samplers (Vrana et al., 2005; Albaseer et al., 2010; Jones et al., 2015), which have been applied for the monitoring of various chemical groups such as pharmaceuticals (steroids, antibiotics), personal care products and pesticides and herbicides (David A. Alvarez et al., 2004; Alvarez et al., 2005; Esteve-Turrillas et al., 2006; Mills et al., 2007). However, choice of passive sampler is mainly governed by the hydrophobicity of the individual analytes (Vrana et al., 2005; Seethapathy et al., 2008; Jones et al., 2015). Only a few studies have shown the applicability of passive samplers to the antiparasitics in this study. These include thiabendazole (anthelmintic) (Alvarez et al., 2005; Bartelt-Hunt et al., 2009) and 12 insecticides (Moschet et al., 2014).

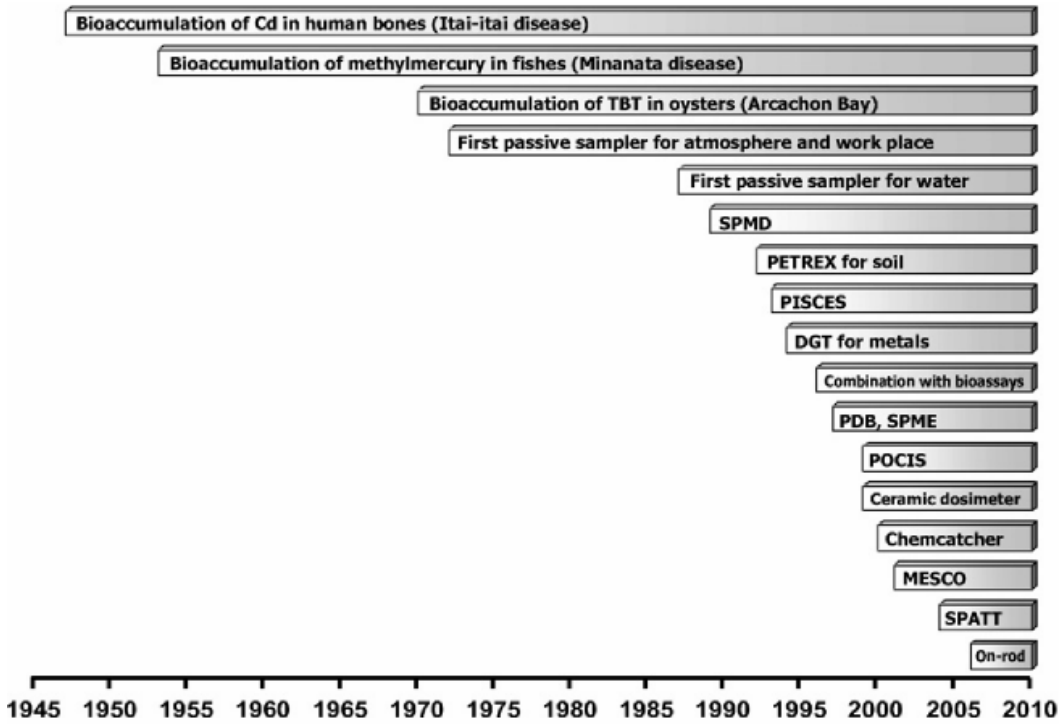

Figure 1-6 Milestones in the development of passive techniques for different media (from Kot-Wasik et al. (2007))

The application of passive samplers to groundwaters is not as well reported amongst literature. The main difficulty with deployment of passive samplers to groundwater include the requirement of a consistent flow of water through the passive sampling device and the physical size/shape restrictions for deployment of a passive sampler down a borehole (piezometer). While a number of passive samplers have been applied to groundwater monitoring, practically all of these are only suitable for inorganic, volatile organic compounds (VOC) or polycyclic aromatic hydrocarbon (PAH) analysis (ITRC, 2006; ITRC, 2007). While passive sampling allows for monitoring of water bodies for longer periods of time and provide time-weighted average concentrations of trace pollutants, the downfall is that they are only capable of measuring freely dissolved molecules (Vrana et al., 2005), which does not meet the requirements to measure "whole water" concentrations under the WFD (2000/60/EC) (European Parliament, 2000) and Directive 2013/39/EC (European Parliament, 2013). This means that the sampling and analytical technique must be capable of including and measuring both the dissolved fraction of analyte as well as any suspended matter fractions, and it is for this reason that traditional grab sampling is the only recognised sampling technique under the aforementioned legislative documents.

1.8 Chemical Analysis

1.8.1 Sample storage considerations

Sample storage, handling and preservation methods for environmental samples are a very important part of any analytical procedure. Naturally these factors should always be considered as part of sampling protocols and it is just as important to control these factors in order to maintain sample integrity during analytical determinations (Albaseer et al., 2011). Often it is the case that extraction and analysis of environmental samples cannot be performed on-site, instead samples are collected in a suitable container, transported to the laboratory and then stored until such a time that the analysis can be performed. As a result, there are several considerations that need to be taken into account in order to ensure that the quality and chemistry of the samples remain the same during the transport and subsequent storage. Such factors include sampling container type and composition, matrix composition and storage conditions such as temperature (Lee et al., 2002; Albaseer et al., 2011). These factors may also need to be considered when it comes to the extraction and analysis of the sample.

The main issues posed by transport and storage in containers is the possibility of loss of analyte, mainly by degradation or adsorption, leading to low recoveries and a result which is under-estimated in terms of the true concentration of the water body sampled. Adsorption of analytes to solids, whether it be to the solid surface of a sample container or suspended solids within the sample matrix, is one of the main contributors to loss of the analytes that are highly hydrophobic (high $\log K_{ow}$) and this is particularly the case with the compounds being studied as part of this project. This phenomenon is particularly well reported amongst literature for pyrethroid compounds with a several studies demonstrating strong adsorption to container walls (Sánchez-Brunete et al., 1998; Oudou and Hansen, 2002). In particular Zhou et al. (1995) examined the partitioning of pyrethroids between the dissolved and particulate phase and reported that up to 60% of the compounds were adsorbed on glass walls. The extent of adsorption can depend on a number of factors including the volume to contact ratio, the concentration of analyte in the sample, the organic matter and suspended solid content of the sample and the sample container material (e.g. plastic, glass) (Albaseer et al., 2011). It is evident however that this effect is not widely considered as part of analytical method development for many of the reported methods for the anthelmintics and anticoccidials (Section 1.8.2), or if it has been considered, it is not widely reported.

Based on review of literature there are some common trends in procedures with regards to sample containers and subsequent storage that have been adopted in order to minimise any compromise of the quality of the sample. These include:

- Use of glass containers where possible and to avoid plastics (specifically for trace analysis). A number of studies have shown that analytes such as pyrethroids and anthelmintics have a lower sorption affinity for glass opposed to plastics (Wheelock et al., 2005; Hladik et al., 2009).
- Use of amber glass to attenuate incident light and reduce the possibility of photodegradation.
- Vigorous agitation prior to extraction has shown to significantly improve the recovery of analytes. This is believed to be due to the re-suspension of some analytes that may be adsorbed to suspended material which has settled to the bottom of the container. Similarly, agitation helps desorb analytes from container walls.

While there is a consensus on these aspects of sample storage, there is less so regarding sample filtration prior to storage. In-fact, most reported methodologies (Section 1.8.2) incorporate a filtration step for water samples prior to sample preparation. With consideration to legislative requirements specified in different drinking water and groundwater standards (Section 1.5), samples should not be filtered in order to allow the measurement of "whole water" concentrations. Filtration of sample prior to storage and subsequent analysis, could result in the potential loss of analyte that may be associated with suspended solids in the sample, which in turn could lead to misleadingly lower concentrations detected. As a result, any methodologies adopted as part of this project will focus on measurement of unfiltered samples in order to comply with legislation.

1.8.2 Extraction, purification and pre-concentration of antiparasitic drugs in environmental water samples

Sample preparation is a fundamental component of any chemical analysis and is essential in achieving high sensitivity in order to detect low concentrations. Effective sample preparation is necessary in attaining robustness and repeatability of sample results. In simple terms, sample preparation involves the extraction of the analytes of interest, in this case antiparasitic residues, from the matrix of interest (water) followed by subsequent purification of the extracts prior to determination, generally by an instrumental technique. The main purpose of the purification step is to remove (or at least reduce) any matrix component that

can undesirably affect the detection capabilities of the detection system. Such phenomenon of alteration of the detected signal(interference) for an analyte due to matrix components is referred to as a matrix effect, with the component causing the effect know as an interferent (which causes the interference) (Nic et al., 2006). Interferences may be exogenous (components which are introduced from external sources into the matrix) or endogenous (components which naturally occur in the matrix itself) (Chambers et al., 2007; Ismaiel et al., 2010) to the sample, with the overall matrix effects presenting as enhancement (increase) or suppression (decrease) of an analyte signal. While a number of steps can be taken to minimise matrix effects, the modification of sample preparation procedures remains to be the only method that has the potential to definitively remove interfering components (Hall et al., 2012), thus highlighting the importance of effectual sample preparation procedures.

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A number of aspects make the analysis of antiparasitic compounds in environmental matrices a challenging task. These include the broad range of substances with different physicochemical properties to be analysed, the complexity of the matrices involved, and the high sensitivity required to be capable of detecting the low levels associated with environmental samples. The requirement to measure sub parts per billion (ppb) and even parts per trillion (ppt) concentrations results in sample pre-concentration being necessary, however this results in many matrix components being concentrated also. As a result, sample preparation is inevitable, with sample extraction and purification considered the most crucial steps in achieving the required sensitivity for environmental water samples.

There are several different sample preparation techniques that can be applied for the extraction of emerging contaminants, such as these antiparasitic drugs, from environmental water samples. Such techniques include the traditional liquid-liquid extraction (LLE), supported liquid extraction (SLE), solid phase extraction (SPE), solid phase micro-extraction (SPME) and stir bar sorptive extraction (SBSE). While the more modern SPME and SBSE techniques are favoured in theory due to their “greener” approach, SPE is still considered the most effective and widely accepted sample preparation technique for extracting trace contaminants from water samples. This is reflective by the fact that the recent EU legislation establishing a watch list for a number of contaminants of emerging concern, specifies SPE as the preferred technique (European Commission, 2015). The main advantage of SPE is that it allows for both sample extraction and clean-up to be performed at the same time. It

also has the potential to achieve a much higher enrichment factor of the analytes of interest from the water sample (Horvat et al., 2012).

SPE works on the principle of the binding affinity of analytes dissolved or suspended in a solution to a solid stationary phase (sorbent) packed within an SPE cartridge (Figure 1-7). Liquid samples are passed through the SPE cartridge in which analytes with a strong affinity for the stationary phase (SPE sorbent) are retained on the SPE sorbent thus isolating the analytes from the bulk of the matrix. The popularity and efficacy of SPE is mainly due to the fact that there are various different sorbent chemistries available which can be chosen to selectively retain the analytes of interest based on their properties. In addition, not only does SPE allow for the analytes to be isolated from the matrix, it also allows for potential interfering components to be removed from the SPE cartridge by selectively choosing appropriate wash solvents. Once purified, by using a solvent that the analytes have more affinity for compared to the stationary phase, the analytes can be eluted from the SPE to obtain an overall purified extract.

The following sections detail the most common approaches used for the extraction and purification of each of the three groups of antiparasitic drugs (anthelmintics, anticoccidials and pyrethroid) under investigation as part of this study. While this review provides a broad overview of techniques that have previously been applied, for the aforementioned reasons outlined above, the primary focus of this work will be on analyte extraction using SPE.

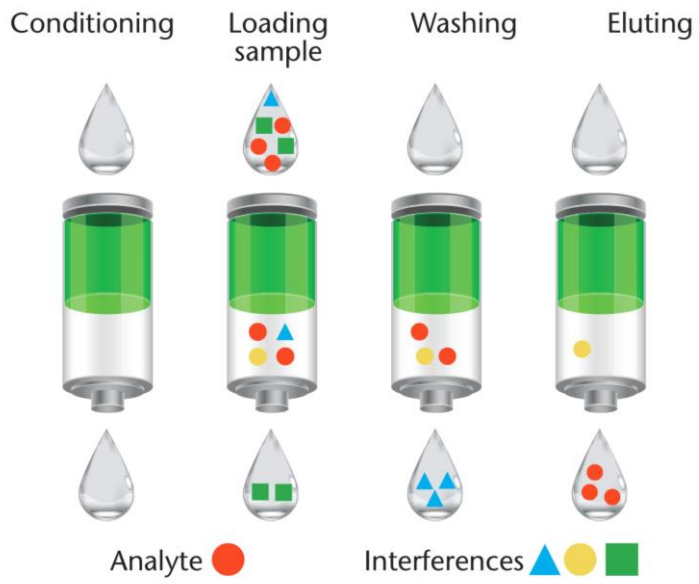


Figure 1-7 Solid Phase Extraction (SPE)

1.8.2.1 Anthelmintic extraction from water samples

A comprehensive overview of the extraction and purification techniques that have been applied for the determination of anthelmintics in environmental water samples are as summarised in Table A1-7, Appendix 1D. Of these methods, the majority are considered multi-residue methods incorporating more than one anthelmintic, however in most cases these methods are limited to <10 anthelmintic compounds (Krogh et al., 2008a; Cacho et al., 2009; Islam et al., 2013; Zrncic et al., 2014). Amongst publications, various different SPE sorbents have been assessed for the extraction of anthelmintics from water including modified silica (C_8 and C_{18}), polymeric (HLB, Strata-X, EN, ENV+) and polymeric with cation-exchange capacity (MC, MCX) (Krogh et al., 2008a; Islam et al., 2013; Zrncic et al., 2014). Of these sorbents assessed, Oasis polymeric hydrophilic-lipophilic balanced (HLB) (Krogh et al., 2008a; Islam et al., 2013; Zrncic et al., 2014) seems to be most commonly used, however, cartridge size and sample size vary from one method to another ranging from 100 mL sample extracted with a 60 mg, 3 mL HLB (Zrncic et al., 2014) to 1000 mL samples extracted with 500 mg, 6 mL HLB cartridges (Islam et al., 2013). In general the HLB cartridge is activated and pre-conditioned with aliquots of pure methanol or methanol-water mixtures and pure water (Islam et al., 2013; Zrncic et al., 2014).

Krogh et al. (2008a) presented a method for the extraction and determination of 7 avermectins from surface water by SPE. Having assessed sorbent type, sample pH and elution conditions, the best extraction was achieved whereby samples (500 mL) were pH adjusted (pH 7) and extracted using a HLB (200 mg, 6 mL) cartridge which was previously activated and conditioned with n-heptane (6 mL), acetone (2 mL), MeOH (6 mL) and finally groundwater (at pH 7). The cartridges were washed with MeOH (5 mL), dried under flow of nitrogen (1 h) and subsequently eluted with MeOH (2×2 mL) and then acetone (2×2 mL). The recoveries of the 7 analytes were relatively low with values in the range of 38–67%, while sensitivity in terms of limits of quantification (LOQ) was in the range of 2.4–13.6 ng L^{-1}. Zrncic et al. (2014) proposed a similar method for the determination of 10 anthelmintics from river water. Samples (100 mL) pH adjusted to pH 7, were extracted using HLB (60 mg, 3 mL) cartridges previously conditioned with MeOH and water. The cartridges were eluted with MeOH (3 mL), with recoveries of the analytes in the range of 76.5–102.8%, except for LEV (42.8%) and MOXI (56.6%). Instrument detection limits were estimated to be in the range of 0.02–0.33 ng L^{-1}. Sim et al. (2013) achieved similar results for nine anthelmintics in river, sea and waste waters using auto-SPE with HLB disks. Recoveries ranged from 50–

120% with LOQs of 0.1–0.4 ng L^{-1}. Other sorbents have also been used to successfully extract anthelmintics from water, however, the majority only incorporate 1–2 anthelmintics as part of multi-class determinations of various pharmaceuticals (Van De Steene and Lambert, 2008; Weiss et al., 2008; Babic et al., 2010; Wagil et al., 2015b).

Other than the more conventional forms of SPE, molecular imprinted polymer SPE (MISPE) is a relatively new technique which could be promising in the future. The principle of MISPE uses molecular imprinted polymers (MIPS) which are synthetic polymers with highly specific recognition ability for target molecules (He et al., 2007). Cacho et al. (2009) developed an off-line MISPE method for the extraction of 7 benzimidazole compounds from river, tap and well water using an off-line procedure. The authors report method recoveries of 99–106%, 95–104% and 90–105 % for tap, river and well water samples respectively. Method detection limits ranged from 2–11 ng L^{-1}. The same authors also proposed an on-line pre-concentration procedure based on the use of MIP as selective stationary phase in HPLC, however the achieved sensitivities were poorer (30–90 ng L^{-1}).

1.8.2.2 Anticoccidial extraction from water samples

There are several methods published that detail the analysis of anticoccidials in environmental water samples as summarised in Table A1-8, Appendix 1D. Notably, amongst published methods, the majority are for the multi-residue (single class) determination of ionophores only (Cha et al., 2005; Kim and Carlson, 2006; Herrero et al., 2012; Bak et al., 2013a; Sun et al., 2013) or multi-class methods incorporating a limited number of ionophore compounds (Song et al., 2007; Zhang and Zhou, 2007; Martinez-Villalba et al., 2009; Thompson et al., 2009; Iglesias et al., 2012), but very few methods include synthetic anticoccidials (chemical coccidiostats) alone (Olsen et al., 2012) or synthetic anticoccidials and ionophores together. In the case where some methods do include both synthetic and ionophores anticoccidials together, these generally only include two to three of the synthetic compounds (Martinez-Villalba et al., 2009; Iglesias et al., 2012).

As with the anthelmintics, the majority, if not all, extraction methods for the anticoccidials (Table A1-8 Appendix 1D) involve reversed-phase SPE using sorbents such as C_{18} (Martinez-Villalba et al., 2009; Olsen et al., 2012), HLB (Cha et al., 2005; Kim and Carlson, 2006; Song et al., 2007; Zhang and Zhou, 2007; Watanabe et al., 2008; Bak et al., 2013a;

Sun et al., 2013) and Strata-X (Iglesias et al., 2012). Again HLB SPE is most common, with cartridge size varying from 60 mg (Cha et al., 2005; Kim and Carlson, 2006; Watanabe et al., 2008) to 200 mg (Song et al., 2007; Zhang and Zhou, 2007; Bak et al., 2013a) or 500 mg (Sun et al., 2013). Elution solvent is generally pure methanol or methanol acidified with formic acid (Song et al., 2007; Martinez-Villalba et al., 2009). Based on a comprehensive review of extraction procedures, Hansen et al. (2009a) report the optimal sample pH for extraction of ionophores to be in the range of pH 7–9, which ensures the ionophores remain unprotonated.

The best performing method was that proposed by Herrero et al. (2012) for the determination of five ionophores from river water and sewage treatment plant (STP) influent/effluent using HLB (150 mg) SPE cartridges. Samples (1000 mL river water, 500 mL effluent or 250 mL influent) were loaded, washed, dried and then eluted with MeOH (10 mL). Very good recoveries of 85–97, 86–97 and 87–100% and LODs of 0.5–1 ng L^{-1}, 2–10 ng L^{-1} and 1–5 ng L^{-1} were obtained for river water, STP influent and STP effluent respectively. Martinez-Villalba et al. (2009) proposed a method for the determination of eight anticoccidials (including two chemical anticoccidials) using C_{18} SPE. The reported SPE steps are similar to those used with HLB by other authors, except both samples and elution solvents were acidified with 0.1% formic acid. Recoveries of all analytes were in the range of 85–100% except for robenidine (60%), while LODs were in the range of 11–71ng L^{-1}.

In addition to the SPE methods available, Thompson et al. (2009) have proposed an alternative extraction approach based on large volume injection online enrichment for the direct analysis of ionophores (n=4) and avermectins in surface water. This approach is a lot simpler and uses smaller sample volumes compared to SPE. The method involves samples (50 mL), pH adjusted to pH 7± 0.5 and mixed with MeOH (2 × 25 mL aliquots), resulting in an overall 1:1 sample: MeOH mixture. Analysis was performed by large volume injection (3 mL loop) liquid chromatography tandem mass spectrometry with positive mode electrospray ionisation (LC-ESI (+)-MS/MS). Recoveries are reported to be in the range of 96.7–114.2% with detection limits in the range of 1 to 2 ng L^{-1}.

1.8.2.3 Pyrethroid extraction from water samples

Table A1-9 of Appendix 1D summarises extraction and purification techniques that have been applied for the extraction of pyrethroid insecticides from environmental water samples. These include liquid-liquid extraction (Fernández-Gutiérrez et al., 1998; Mekebri et al., 2008), SPE (Van Der Hoff et al., 1996; Xue et al., 2005; Gil-Garcia et al., 2006) and more modern techniques such as SPME (Casas et al., 2006; Van Hoeck et al., 2007; Vazquez et al., 2008; Li et al., 2009) and SBSE (Serodio and Nogueira, 2005; Ochiai et al., 2008). Reverse phase SPE using C_{18} (Van Der Hoff et al., 1996; Gil-Garcia et al., 2006) or Oasis HLB (Xue et al., 2005) sorbent is most commonly used for extraction of insecticides from water. Gil-Garcia et al. (2006) provide a method for the extraction of seven pyrethroids from groundwater and seawater samples. Samples (800 mL) modified with MeOH (200 mL) were extracted using C_{18} (360 mg, 3 mL) SPE cartridges pre-conditioned with MeOH, hexane, methanol and water. Extracts were eluted with hexane (7 mL), evaporated to dryness and reconstituted in MeCN: water (70:30, *v/v*). Recoveries were reported to be in the range of 71.8–110% and 80–115.6%, with sensitivity (LODs) of 0.2–0.5 ng L^{-1} and 0.3–0.7 ng L^{-1} for groundwater and seawater samples respectively. In contrast, Xue et al. (2005) reports a multi-class method for the determination of 31 endocrine-disrupting pesticides, including three insecticides. Reservoir water (1000 mL) was extracted by Oasis HLB SPE with elution using MeOH: MeCN (50:50, *v/v*), with dried extracts reconstituted to 500 µl. Recoveries were between 70–89% while the authors claim LODs for all compounds in the range of 0.5–15 pg L^{-1}.

In more recent times, more comprehensive and rapid procedures have been reported. Aznar et al. (2016) report a simple ultrasonic assisted liquid-liquid extraction procedure for 10 pyrethroids whereby sample (20 mL) is shaken and sonicated with ethyl acetate: hexane (90:10, *v/v*) followed by evaporation and analysis by GC-MS, with LODs ranging from 0.2–4.8 ng L^{-1}. Such an approach could be benefitted by the vibrational shaker technique adopted by de Oliveira et al. (2019) who extracted 22 antiparasitic residues from fish. Feo et al. (2010a) proposed a method for the extraction of 14 pyrethroids using ultrasound-assisted emulsification-extraction (UAEE) of a water-immiscible solvent in an aqueous medium. The extraction solvent, chloroform (1 mL), was added to the water sample (20 mL) and the mixture was sonicated, centrifuged (3500 rpm, 5 min) and the organic layer was subsequently dried to dryness and reconstituted with ethyl acetate for analysis by gas chromatography-negative ion chemical ionisation mass spectrometry (GC-NCI-MS). Of the

total 12 analytes, 8 had recoveries >93% in tap water and 10 analytes had recoveries >87% in river water. Notably, 8 out of 12 analytes reported had LODs of <0.5 ng L^{-1}.

Both SPME and SBSE provide a more modern alternative to SPE or SLE, with both techniques considered simple and solventless. Above all, both have the potential for significantly enhancing sensitivity given the extracted fraction (extracted directly onto a fibre or stir bar) can be quantitatively introduced into the GC system by thermal desorption (Feo et al., 2010b). As with SPE, selectivity and sensitivity depend on the choice of stationary phase; however, unlike SPE, due to the limited number of phases available for SPME and SBSE, multi-class residue extraction is difficult. Other disadvantages include the lower sample throughput for both, and longer extraction time required for SBSE can sometimes compromise analyte recoveries (Albaseer et al., 2010).

1.8.3 Instrumental detection

Determination of veterinary drug residues such as the antiparasitics typically involves some sort of chromatographic separation coupled to a sensitive instrumental detection system. Traditional analytical methods developed for veterinary and related residues in complex matrices were only capable of analysing individual or a small number of analytes within a single class of drugs, with the majority focused on biological matrices. These methods generally involved quite complex and exhaustive purification procedures such as LLE, which requires large sample sizes and large volumes of extraction solvents, in order to make extracts suitable for determination methods such as high performance liquid chromatography (HPLC) with ultra violet (UV) detection or gas chromatography (GC) (Schnitzerling and Nolan, 1985; Vuik, 1991; Dowling et al., 2005; Danaher et al., 2006). The pre-requisite for some form of derivatisation adds to the non-specific nature of such approaches (Balizs and Hewitt, 2003).

In recent years, advances in detection systems, namely the coupling of chromatography to the mass spectrometer and the subsequent use of tandem mass spectrometry (MS/MS), has made it possible to potentially analyse hundreds of analytes in one method (Kaufmann et al., 2008; Stolker et al., 2008; Ortelli et al., 2009). Such capabilities are owed to the increased sensitivity and selectivity of the mass spectrometer, which has been attributed to the successive mass filtrations and the ability of MS/MS to perform selected and multiple reaction monitoring (SRM and MRM) experiments. As a result, techniques such as liquid

chromatography tandem mass spectrometry (LC-MS/MS) are currently considered the most powerful technique for the quantitative determination of a large number of veterinary residues in complex matrices (Le Bizec et al., 2009; Čelić et al., 2017). While such technologies have been advantageously applied for the determination of veterinary drugs in biological and food matrices (Geis-Asteggiante et al., 2012) , there have been limited advancement on their application for the multi-residue and multi-class determination of veterinary drugs in environmental waters, with the majority of methods including much fewer analytes (<40) and are generally limited to no more than 10 analytes from any one given class (Xue et al., 2005; Van De Steene and Lambert, 2008; Sim et al., 2013).

A comprehensive overview of the analytical detection methods that are currently available and that have been previously applied for the determination of anthelmintics, anticoccidials and pyrethroids in environmental waters, is as summarised in Table A1-10, Table A1-11 and Table A1-12 (Appendix 1E), respectively. LC-MS/MS has been widely applied for the determination of both anthelmintics and anticoccidials in environmental samples (Table A1-10 and A1-11, Appendix 1E). For both groups, LC separation is generally achieved on a C_{18} column with acidified (formic acid) or buffered (ammonium acetate or formate) water, methanol and/or acetonitrile as mobile phase in a gradient elution. To improve the ionization of analytes and the sensitivity of MS detection, the mobile phase is usually modified with volatile additives such as formic acid, acetic acid or ammonia (Horvat et al., 2012). Electrospray ionisation (ESI) is the most common ionisation technique for these residues. Methods for environmental samples for both groups of compounds are not very extensive and only include a limited number of analytes from the same class (<12). The most comprehensive detection method that has been applied to environmental waters for the anthelmintics was that developed by Zrncic et al. (2014) who detected 10 benzimidazoles using a UHPLC-MS/MS Qtrap (quadrupole linear ion trap) system. Analytes were chromatographically separated on a BEH C_{18} column using a gradient elution with 10 mM ammonium aqueous phase, and an acetonitrile organic phase. Reported instrumental detection limits were of the order of sub picograms (pg), which translates to estimated method detection limits ranging from 0.02 to 0.33 ng L^{-1} when coupled with SPE extraction. For the anticoccidials, Martinez-Villalba et al. (2009) also proposed a detection method using LC-MS/MS whereby 8 anticoccidials (5 ionophores and 3 synthetic) were separated using a Hypersil Gold C_{18} chromatographic column, eluted with acidified aqueous and organic (both 0.1% formic) gradient. Method detection limits ranged from 11 to 71 ng L^{-1}

In contrast, gas chromatography (GC) separation prior to ECD (electron capture detector) or MS detection is the preferred determining system for the insecticides in environmental samples (Corcellas et al., 2013; Aznar et al., 2016) (Table A1-12, Appendix 1E). However, LC-MS/MS techniques are being increasingly applied for insecticide determination in environmental matrices. The advantage of LC over GC is due to less extensive clean-up required by LC (Feo et al., 2010b), attributed to the high selectivity and sensitivity of LC-MS/MS. Gil-Garcia et al. (2006) separated seven insecticides on a C_{18} column by gradient elution with MeCN and an ammonium acetate (50 mM): MeCN 95:5 (*v/v*) solution, with LODs ranging from 0.2 to 0.6 ng L^{-1}. More recently, one of the most extensive LC-MS/MS detection methods applied to environmental water and sediment samples was presented by Ccanccapa-Cartagena et al. (2017) which allows for the separation and simultaneous determination of 17 synthetic and natural pyrethroids using a triple quadrupole. The 17 compounds were separated on a Luna C_{18} column suing a 10 mM ammonium formate aqueous phase, and a 10 mM methanol organic phase, with all compounds eluted within 25 minutes. This method reports limits of detection ranging from 0.12 to 0.62 ng L^{-1} for water samples.

Besides these detection methods that have been applied to environmental matrices, in recent times, there has been a significant amount of work carried out on more extensive instrumental detection methods for the anthelmintics, anticoccidials and pyrethroids in various matrices of food of animal origin (e.g. poultry eggs, muscle, milk and liver). A number of critical and comprehensive reviews summarise these methods: Clarke et al. (2014) carried out a comprehensive overview of anticoccidial analysis in meat and other food products, Tuck et al. (2016) provided an updated overview of detection methods for the anticoccidials, in addition to the anthelmintics, in animal-derived foods, while Tuck et al. (2018) provided a review of methodology for the detection of pyrethroids from foods of animal origin. As part of these reviews, several of the comprehensive and extensive detection methods were identified as outlined below.

Building on the original work carried out by Kinsella et al. (2009), Whelan et al. (2010) developed a UHPLC-MS/MS detection method capable of quantifying 38 anthelmintic drugs residues in milk, including anthelmintic compounds from all structural classes, in one analytical injection with a 13 minute duration. The method employed the use of a QqQ (triple quadrupole) detection system equipped with an ESI source and allowed for rapid polarity

switching which allowed the simultaneous detection of positive and negative analyte ions. For the anticoccidials, Moloney et al. (2012) developed a UHPLC-MS/MS detection method for residues of 20 anticoccidials (including 7 ionophores and 13 synthetic anticoccidials) in eggs and poultry muscle, with the method shown to be fit for purpose for the quantitative analysis of 13 compounds, and for screening of the remainder. This method was improved upon by Clarke et al. (2013) who further extended the scope of analysis to bovine milk and poultry muscle. Validation of this method with a new clean-up approach showed fitness for purpose for all 20 analytes except the toltrazurils, which were insufficiently fragmented and did not meet identification criteria. While this method is considered one of the most comprehensive, there is still some scope for improvement. Both the Clarke et al. (2014) and Tuck et al. (2016) reviews highlighted the complexity of anticoccidial analysis due to the broad range physicochemical properties of the compounds, and identified a number of particularly problematic compounds such as amprolium, cyromazine and clopidol, which are highly polar and typically require separate analysis (due to the need for as separate chromatographic separation) which is costly and time consuming. In terms of the pyrethroids, at the time of this initial review, an in-house detection method (at Teagasc, the host laboratory) existed for the determination of 19 insecticide compounds in animal fat. This has since been revised and published by Moloney et al. (2017). Chromatographic separation was carried out on an Acquity C_8 BEH column, using a binary gradient separation comprising of mobile phase A, 5 mM ammonium formate in water: MeOH (80:20, *v/v*) and mobile phase B, 5 mM ammonium formate in MeOH. The insecticides were detected using ESI in positive polarity mode. This work was built upon by de Oliveira et al. (2019) who included three additional insecticides and shortened the analysis time from 21 min to just 7 min.

Overall, based on the above, it is evident that there are many extensive detection methods available, the most of these being for biological matrices. As a result, efforts of this research will focus on adapting and optimising such comprehensive methods currently developed for biological matrices, to allow application to environmental water samples. A significant challenge will be in achieving the required sensitivities for detecting the relevant environmental concentrations, and for this reason, extensive work will be carried out on developing and optimising SPE extraction protocols to achieve clean extracts for instrumental analysis.

1.9 Summary, Aims and Objectives

With consideration of this literature review, it is evident that there are two main gaps in knowledge of these antiparasitic drugs in environmental waters, particularly groundwater, as summarised as follows:

1. There is a lack of comprehensive information on the environmental occurrence of antiparasitic drugs and their associated environmental water concentrations, while information on transformation (breakdown) products is even more scant. This in turn has led to a lack of understanding of the environmental fate of these contaminants, which hinders the adequate assessment of their environmental risk.
2. There is a lack of suitably sensitive multi-class analytical methods for the extraction and instrumental determination of these contaminants in groundwater and surface waters. The lack of such comprehensive methods has contributed to the overall lack of detailed information on the environmental occurrence and fate of antiparasitic drugs.

While these gaps in knowledge for all three groups of antiparasitics still remain, it is evident that the void in information is much bigger for the anthelmintics and anticoccidials, with analytical methodologies for water samples for both of these groups being less developed compared to the pyrethroids. Furthermore, in recent times, more and more studies are reporting new information on the occurrence of pyrethroids in different environmental waters, and this is as a result of the availability and continued development of suitable analytical methods, which are currently lacking for the anthelmintics and anticoccidials. For this reason, the anthelmintics and anticoccidials were prioritised for the work carried out in this thesis.

Considering the above, the overarching aim of this project was to investigate the occurrence of two groups of veterinary antiparasitic drugs as emerging contaminants in Irish groundwaters, the anthelmintics and the anticoccidials, which represent two of the most widely used groups of veterinary drugs in Irish agriculture. Furthermore, the project was specifically focused on examining the occurrence of these contaminants in Irish karst and fractured bedrock aquifers, due to the prevalence of these aquifers throughout Ireland and the inherent vulnerability to contamination that can be associated with such hydrogeological settings.

Some of the main project objectives are as listed below:

1. To develop and validate comprehensive, and suitably sensitive, analytical methodologies for the extraction and quantification of these antiparasitic veterinary agents in environmental waters. Specifically, the work will seek to:
    a. Develop a comprehensive solid phase extraction (SPE) method to allow for the extraction of a broad range of anthelmintic residues (from all structural classes), including their known transformation products, from unfiltered water samples, with subsequent determination using an optimised UHPLC-MS/MS detection method.
    b. Develop a comprehensive SPE-UHPLC-MS/MS method to allow for the extraction, simultaneous chromatographic separation and detection of both the ionophore anticoccidials and synthetic anticoccidials (coccidiostats), from unfiltered water samples.

2. To apply said methodologies to investigate the frequency of occurrence of the different compounds (and TPs where applicable) and its relationship to the chemical characteristics of the compounds. Where applicable, the work will seek to establish whether TPs are more prevalent in groundwater than the parent drug.

3. To investigate the spatial occurrence of anthelmintics and anticoccidials in groundwater, specifically in relation to source factors. In particular, the work will seek to test the hypothesis that these contaminants are more likely to occur in groundwater in regions where high usage is expected, e.g. areas with intensive cattle and sheep production or areas with intensive poultry activity.

4. To investigate any physical pathway factors associated with the occurrence of anthelmintics and anticoccidials. In particular, the work will assess whether these compounds are more prevalent in karst aquifers due to the intrinsic vulnerability whereby contaminants can be carried overland and enter groundwater via karst features such as sinking streams.

5. To investigate any temporal variations in the occurrence of antiparasitic drugs in sensitive groundwaters. The work will seek to establish whether any temporal variations

in antiparasitic agrochemical occurrence in groundwater are influenced by timing of agricultural practices, such as when animals are housed versus on pasture. Furthermore, it will establish whether such variations are also influenced by meteorological conditions and timing of groundwater recharge.

1.10 Authorship and Contributions

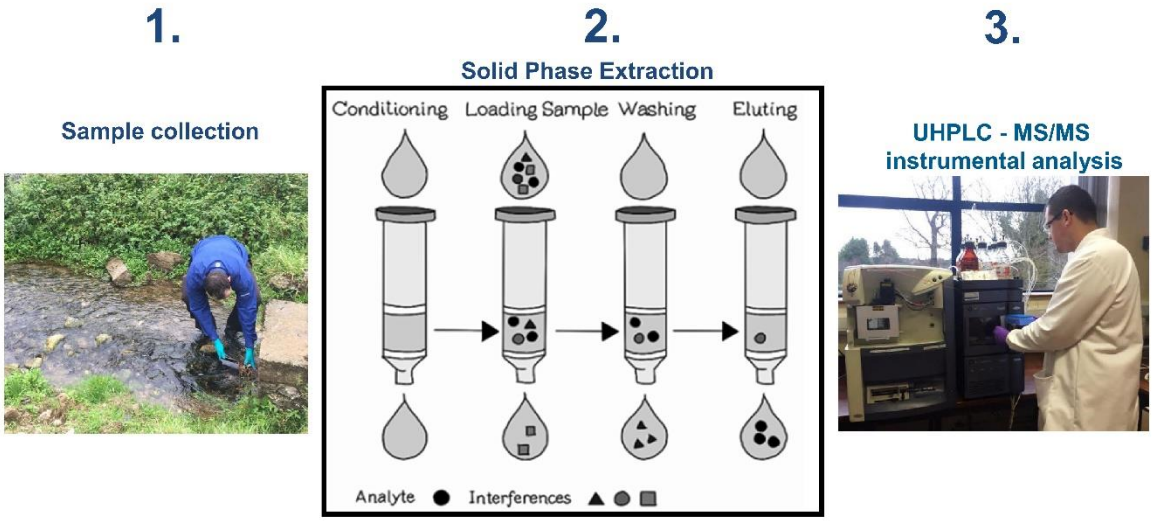
I am the lead author for all work currently published, submitted for peer review, or intended to be submitted for peer review publication, emanating from the research presented in this thesis. As the lead author of these manuscripts, I was responsible for experimental design, sample preparation and analysis, data acquisition, processing and interpretation and the overall writing of the original draft (including the creation of all figures and tables) and any subsequent revised drafts. I am also the noted corresponding author for all related manuscripts.

For all publication-based chapters (Chapters 2,3, 4 and 5), a list of the appropriate authors is provided at the beginning of each chapter. For the three chapters that have already been published (Chapter 2, Chapter 3 and Chapter 5), authorship contributions are provided at the end of each chapter, in the format of the CRediT author statement, and as submitted to the respective journal. Same contributions are also described for Chapter 4, as intended to be submitted for peer review.

For all paper-based chapters, the publication status and details are provided at the beginning of each chapter clearly identifying if the chapter has been (a) published, (b) submitted for peer review or (c) in preparation for submission for per review.

CHAPTER 2 – DEVELOPMENT AND OPTIMISATION OF A MULTIRESIDUE METHOD FOR THE DETERMINATION OF 40 ANTHELMINTIC COMPOUNDS IN ENVIRONMENTAL WATER SAMPLES BY SOLID PHASE EXTRACTION (SPE) WITH LC-MS/MS DETECTION

Graphical Abstract



Lay Abstract

This chapter reports on the work carried out to develop a new analytical method for the extraction and subsequent detection of a broad range of anthelmintic drugs in water samples using solid phase extraction (SPE) with ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) detection. SPE is a technique that involves the use of a chemical sorbent which acts like a "filter", and allows for the simultaneous removal, purification and concentration of organic contaminants from a water sample. UHPLC-MS/MS is a technique involving sophisticated instrumentation that firstly separates complex mixtures of drugs in a solution based on their physicochemical properties and subsequently detect them based on their mass, at very low levels. In this work a new method was developed that is capable of detecting 40 different anthelmintic drugs including 27 parent drugs and 13 transformation products in un-filtered surface water and groundwater samples at very low parts per trillion levels. The method was stringently tested and validated to ensure that it was fit for purpose before applying it as part of a pilot programme whereby 72 different surface water (rivers and lakes) and groundwater (wells and springs) samples were collected and analysed for the 40 anthelmintic drugs. Overall, this newly developed method is now the most comprehensive available for determining anthelmintic drugs in water samples given that it allows for the detection of larger number of drugs compared to other methods.

DEVELOPMENT AND OPTIMISATION OF A MULTI-RESIDUE METHOD FOR THE DETERMINATION OF 40 ANTHELMINTIC COMPOUNDS IN ENVIRONMENTAL WATER SAMPLES BY SOLID PHASE EXTRACTION (SPE) WITH LC-MS/MS DETECTION

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Abstract

A comprehensive multi-residue method was developed and validated for the determination of 40 anthelmintic compounds, including 13 transformation products, in surface and groundwater samples at sub nanogram per litre (ng L^{-1}) levels. Anthelmintic residues were extracted from unfiltered water samples using polymeric divinylbenzene solid phase extraction (SPE) cartridges and eluted with methanol: acetone (50:50, *v/v*). Purified extracts were concentrated, filtered and injected for UHPLC-MS/MS determination. The method recovery (at a concentration representative of realistic expected environmental water levels based on literature review) ranged from 83–113%. The method was validated, at three concentration levels, in accordance to Commission Decision 2002/657/EC and SANTE/11813/2017 guidelines. Trueness and precision, under within-laboratory reproducibility conditions, ranged from 88–114% and 1.1–19.4%, respectively. The applicability of the method was assessed in a pilot study whereby 72 different surface and groundwater samples were collected and analysed for the determination of these 40 compounds for the first time in Ireland. This is the most comprehensive method available for the investigation of the occurrence of both anthelmintic parent compounds and their transformation products in raw, unfiltered environmental waters.

Keywords: Veterinary drugs; anthelmintics; emerging organic contaminants; transformation products; environmental water; solid phase extraction; UHPLC-MS/MS

2.1 Introduction

Due to increased pressures on the food production system, veterinary antiparasitic agents, such as anthelmintic drugs, have become a critical component of animal husbandry in many countries, including Ireland. Anthelmintic drugs are widely used to control helminthic parasites that infect animals, particularly those exposed through pasture-based production systems. Anthelmintics are primarily used to treat and prevent the following parasitic worms in ruminants: nematodes, cestodes and trematodes, which are more commonly known as roundworms, tapeworms and liver flukes, respectively (Tuck et al., 2016). The anthelmintic family can be divided into a number of groups or classes, primarily based on their chemical structure, and their mode of action against the parasite (Kahn and Line, 2010). The main classes of anthelmintics include: the benzimidazoles, macrocyclic lactones (avermectins and milbemycins), salicylanilides and substituted phenols, tetrahydropyrimidines, imidazothiazoles, organophosphates and amino-acetonitrile derivatives. The compounds included in this study, as grouped into their respective anthelmintic class, are listed in Table 2-2, with their structures shown in Supplementary Information SI-2.1 Figure S2-1.

Anthelmintics can be administered orally (drench or bolus), as an injectable preparation or topically (pour-on). Once administered, the drug can undergo a series of transformations within the animal, eventually being excreted as the parent drug and/or metabolites in urine or faeces (Wardhaugh, 2005; Danaher et al., 2007; Beynon, 2012b), the exact excretion profile of which is equally dependent on the route of administration and the drug's physicochemical properties (Boxall et al., 2003b). As a result, the excretion data available for antiparasitic agents is limited and often difficult to interpret or compare (Wardhaugh, 2005). However, of the information available, it has been shown that >90% of the administered dose of avermectins can be excreted in faeces as the unchanged parent (Danaher et al., 2006; Liebig et al., 2010; Beynon, 2012a), while in contrast, the benzimidazoles, levamisole and tetrahydropyrimidines are mainly excreted in urine as parent and /or metabolites (McKellar, 1997). As a result, it is evident that the administration of such 'agrochemicals' can potentially lead to their persistence in the environment, posing a risk to water quality, which has led to anthelmintics being considered as emerging organic contaminants (EOCs) of potential concern (Horvat et al., 2012; Snow et al., 2016; Charuaud et al., 2019b)

The most important point of entry for anthelmintics into the environment is due to the direct excretion onto pastures and/ or by direct application of slurries to land (Boxall, 2010; Kim et al., 2010). Boxall et al. (2002) emphasised the importance of wash-off of topical treatments and spillage during application as other important routes to enter the environment. Once in the environment, the fate and transport of anthelmintic drugs is further complicated due to their breakdown into transformation products (TPs), which can be more toxic than the parent drug (Boxall et al., 2002; Boxall et al., 2003a). The ecotoxicity of anthelmintic drugs in the environment is not well established; however, some of these drugs have been found to be toxic to different organisms in the environment, as summarised in a recent review by Horvat et al. (2012). For example, the avermectins, as a group, have been found to have effects on the reproduction, biological function and survival of non-target terrestrial and aquatic organisms. For instance, ivermectin is acutely toxic to crustaceans, with an LC_{50} of low ng L^{-1} levels (Liebig et al., 2010). O'Hea et al. (2010) also highlighted the impact of ivermectin in the environment on dung beetle populations.

While there are well defined legislative requirements pertaining to veterinary residues of anthelmintic drugs in food of animal origin, there is no specific legislation relating to emerging organic contaminants in environmental waters. Environmental marker residues are not well defined in legislation in contrast to those listed under food safety legislation. This is due to the very nature of EOCs, since often there is very little information known about the fate and toxicity of such substances. There are some individual pieces of legislation relating to pesticides in environmental waters in the European Union (EU), such as the drinking water Directive 98/83/EC (European Commission, 1998) and groundwater Directive 2006/118/EC (European Parliament, 2006). Under such legislation, the term ‘nematocides’ is included under the definition of pesticide. As a result, the individual and total pesticide limits (100 ng L^{-1} and 500 ng L^{-1} respectively) specified, are applicable to some anthelmintic drugs.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is currently considered the most powerful technique for the quantitative determination of a large number of veterinary residues in complex matrices (Le Bizec et al., 2009). Advances in detection systems have allowed for the development of multiclass methods for determining pharmaceuticals and veterinary drugs in environmental samples. Detection methods for environmental samples are not as well established (generally <40 analytes) (Van De Steene and Lambert, 2008; Sim

et al., 2013), compared to those for biological matrices (hundreds of analytes) (Kaufmann et al., 2008; Geis-Asteggiante et al., 2012). Methods for the LC-MS/MS detection of anthelmintic in environmental water samples are not very extensive and only include a limited number of analytes from the one class (generally <12 residues) (Krogh et al., 2008a; Van De Steene and Lambert, 2008; Islam et al., 2013; Zrncic et al., 2014). Methodologies incorporating transformation products are scarce, with most methods covering just parent drugs and not metabolites/environmental TPs (only four or fewer metabolites/TPs included in any one method, all of which relate to fenbendazole (Santaladchaiyakit and Srijaranai, 2012; Sim et al., 2013; Zrncic et al., 2014)). More extensive and sensitive methods have been developed for biological food matrices, which allow simultaneous detection of many more anthelmintic residues (Peters et al., 2009; Whelan et al., 2010)

Regardless of the instrumental technique, due to the wide range of physicochemical properties of analytes and the complexity of environmental matrices, sample preparation steps are inevitable in order to achieve the required sensitivity. A number of different extraction and purification techniques have been applied for the determination of anthelmintic drugs, with solid phase extraction (SPE) being the most commonly used technique when it comes to environmental water samples. Of the available extraction methods specific to water matrices, the majority are considered multiresidue methods incorporating more than one anthelmintic; however, in most cases, these methods are limited to <10 anthelmintic compounds (Krogh et al., 2008a; Islam et al., 2013; Zrncic et al., 2014), or incorporate only 1–2 anthelmintics, as part of multiclass determinations of various pharmaceuticals (Van De Steene and Lambert, 2008; Babic et al., 2010). The most comprehensive method, to our knowledge, was that developed by Zrncic et al. (2014) who proposed a method for the multiresidue determination of ten anthelmintics from differing structural classes (the majority being from the benzimidazole class), from surface river water using SPE (HLB cartridge; 60 mg, 3 mL). Krogh et al. (2008a) presented a method for the extraction and determination of seven avermectins from surface water (500 mL), also using polymeric SPE; however, recoveries reported were relatively low (38–67%).

In a prioritisation exercise on veterinary medicines in the environment in the United Kingdom (UK), Boxall et al. (2003a) identified 56 different drugs, including eight anthelmintics, which they considered to be of high priority with regards to having a potential impact on the environment. The same working group also noted the lack of suitably sensitive

analytical methodologies, specifically for TPs, as one of the main contributors to inadequate environmental risk assessment (Boxall et al., 2002; Boxall et al., 2003b). Even though there are some methods available for a limited number of anthelmintics, information on the occurrence and associated levels in the environment is lacking. Information on the occurrence of anthelmintic metabolites and transformation products is even more scarce (Horvat et al., 2012), which further hinders sufficient environmental risk assessment. Some studies even go as far as questioning whether current legislation and environmental risk assessments of both human and veterinary products are sufficiently protective (Sebestyén et al., 2018). In order to better inform on the environmental fate and occurrence of anthelmintics in environmental waters, more comprehensive analytical methods capable of detecting many more anthelmintics and their TPs, at environmentally relevant detection levels, are required. The objective of this study therefore was to develop, optimise and validate a more comprehensive method for the multiresidue determination of a wide range of commonly used anthelmintics and their transformation products, incorporating clean-up by SPE. This method was then applied to a wide range of surface and groundwater samples from across Ireland, to help better understand the environmental fate and occurrence of anthelmintics.

2.2 Results and Discussion

2.2.1 Method Development: sample preparation—solid phase extraction

2.2.1.1 Assessment of SPE sorbents

Amongst the literature, polymeric hydrophilic-lipophilic type sorbents are most commonly used for the SPE of anthelmintic drugs from water, due to their all-purpose, strong hydrophilic reversed phase application for the extraction of pharmaceuticals (Krogh et al., 2008a; Islam et al., 2013; Sim et al., 2013; Zrncic et al., 2014). As a result, method development and optimisation tasks focused on the use of such sorbents, with initial experiments focusing on the use of Bond Elut ENV reversed phase polymeric cartridges, which have large particle size for high volume, fast flow-through application. Investigation of elution solvent composition ((methanol (MeOH)/ acetonitrile (MeCN)) and volume (0–15 mL) indicated optimum conditions with a MeOH: MeCN (50:50, *v/v*), 10 mL, elution (data not shown). However, no further optimisation of this particular sorbent was carried

out, due to inconsistencies with the SPE sorbent packing after vacuum drying, which produced large deviations in recoveries between replicates (RSD >30%). Using the same elution conditions optimised for the Bond Elut ENV cartridge above, three additional SPE cartridges (Bond Elut PLEXA, Oasis HLB, and UCT HL-DVB) were assessed for extraction, with the results as depicted in Figure 2-1(a). Oasis HLB performed best in terms of recoveries and precision, with 31 of 40 compounds extracted within the satisfactory recovery range and RSD values between 0.8–9.9%. UCT HL-DVB also achieved satisfactory recoveries for 31 of the 40 anthelmintics however for a few analytes the precision (%RSD) was larger (0.4–24.9%). Recovery of CLOS and RAFOX (<40 and <20% respectively) from all four sorbents were much lower than the minimum targeted recovery of 70%. Both these analytes have high octanol-water coefficients (Kow) (Supplementary File SI-2.1 Table S2-1), which implies that they are highly hydrophobic, thus the low recoveries were proposed to be due to inefficient elution from the sorbents, or adsorption of these analytes on the sample container wall. The UCT HL-DVB was selected for further assessment due to its faster sample load times compared to HLB and PLEXA, which both required much higher vacuum, increasing the load time by 60 min.

Further to this, sorbent mass (200 mg vs. 500 mg) and elution volume (10, 15 and 20 mL), were assessed for the HL-DVB cartridge, with the 200 mg cartridge combined with a 10 mL elution volume providing the best result (Supplementary Figure S2-2). CAM, TBZ and TBZ-OH all showed no extraction (all < 0.5% extraction) with the 500 mg sorbent mass; therefore, 200 mg was selected. This is most likely due to too much retention on the larger bed mass. The 10 mL elution volume was selected given there was no noticeable difference observed on increasing the volumes, in addition to the fact that larger volumes were restricted by evaporation capabilities (max. 15 mL tube in TurboVap LV). The selected elution volume was similar to those reported amongst other published methods (Krogh et al., 2008a; Van De Steene and Lambert, 2008; Babic et al., 2010)

2.2.1.2 Elution solvent composition and wash solvent assessment

Optimisation of elution solvent composition for the UCT HL-DVB 200 mg cartridge was performed given that increases in the eluent volumes (Section 2.2.1.1) did not improve recoveries. Seven different elution solvent compositions, (A)–(G), were assessed, with the mean recoveries and RSDs (n = 3) obtained for each composition presented in Figure 2-1(b).

These compositions were selected based on commonly used elution solvents for these compounds in the literature (Babic et al., 2010; Islam et al., 2013; Sim et al., 2013). The best results were determined to be with elution with composition (D) which produced recoveries in the range of 19–123% and precision of 0.5–18.8%. Eluent (A) produced similar recoveries (14–136%); however, (D) was preferred as it produced more precise results across replicates (improved RSD for ABA, CLOS, COUMA DORA, EMA, EPRINO, IVER and MOXI), with increased sensitivity also observed (higher analyte responses). This increased sensitivity was proposed to be due to less polar interferences being eluted by the more hydrophobic acetone solvent incorporated in Eluent (D) (compared to MeCN in (A)). There was still no significant improvement for CLOS and RAFOX indicating the lower recoveries may not be due to insufficient elution from the sorbent.

The effect of a mild wash solvent (90:10 (*v/v*) H_2O: MeOH) was assessed in order to remove undesirable matrix co-extractives from the SPE, prior to analyte elution. The use of no wash solvent was compared to the use of 15 mL or 25 mL wash aliquots (used to rinse the sample container, before being added to the SPE). The best results were achieved with use of the 15 mL wash solution (recoveries of 37–127%), with improved recoveries observed for all analytes, except ABA, IVER and COUMA, which had slightly decreased recoveries compared to no wash step (Figure 2-1(c)). While the avermectins showed improved recoveries when the larger wash aliquot (25 mL) was incorporated (recoveries increased by up to 33%), lower recoveries and poorer precision were demonstrated for a number of other analytes (e.g., CAM, TBZ and TBZ-OH). With both wash volumes, the recovery of CLOS and RAFOX increased by at least 10%, most likely due to more efficient extraction of analytes that may have remained adsorbed to the glass surface of the sample container.

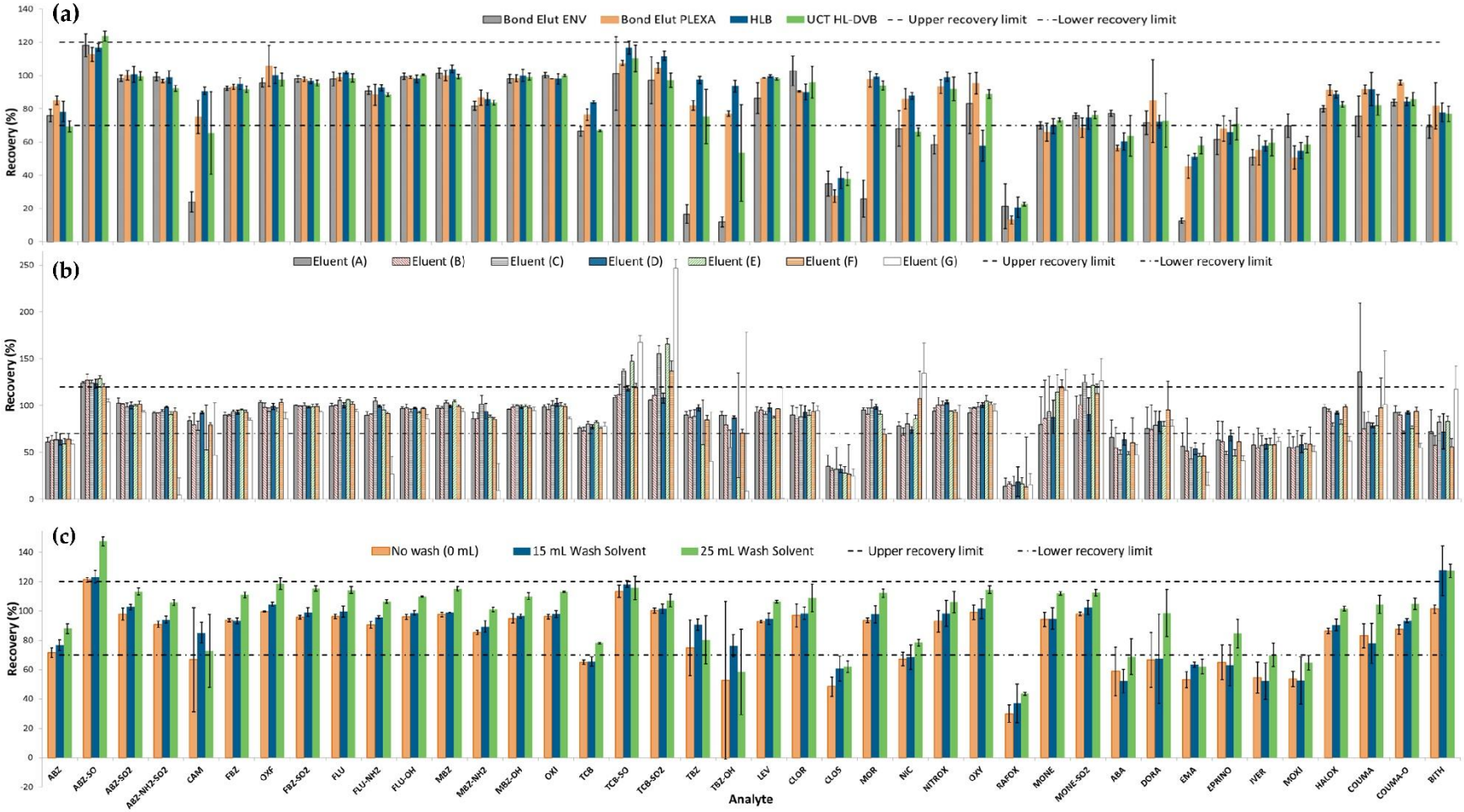


Figure 2-1 Mean recoveries (%) and precision (%RSD, shown by error bars)(n = 3) for assessment of: (a) four different SPE Cartridges (Bond Elut ENV, Bond Elut Plexa, Oasis HLB and UCT HL-DVB) eluted with 50/50 MeOH/MeCN (v/v) (10 mL) (b) seven different eluent compositions: (A) = 50/50 MeOH/MeCN (10 mL), (B) = 50/50 MeOH/MeCN (5 mL) + Acetone (5 mL), (C) = 50/50 MeOH/MeCN (5 mL) + MTBE (5 mL) (D) = 50/50 MeOH/Acetone (10 mL), (E) = 50/50 MeCN/MTBE (10 mL), (F) = 100% Acetone (10 mL) and (G) = 100% MTBE (10 mL) using the HL-DVB cartridge (200 mg, 6 mL) and (c) three different volumes (0, 15 and 25 mL) of water: methanol (90:10, v/v) wash solution using the same HL-DVB cartridge.

2.2.1.3 Sample modification (organic modifier and pH)

Sample modification was assessed to further investigate low recoveries of some analytes due to potential inefficient extraction of analyte from the sample, and its container. Thompson et al. (2009) report that the addition of MeOH to samples was necessary to prevent partial adsorption of analytes (which included avermectins) to container surfaces; thus, the use of a methanol modifier was assessed in this study. Krogh et al. (2008a) report that sample pH did not have a drastic effect on the extraction of seven avermectins using HLB SPE; however, Zrncic et al. (2014) indicated that pH can affect the recovery of anthelmintics from other structural classes. As a result, the effect of sample pH modification was also assessed.

The best overall conditions from the response surface methodology (RSM) optimiser, for 17 selected compounds (see section 2.3.6.1 below), were predicted to be extraction with 20% MeOH modifier at sample pH 7 (Supplementary Figure S2-3(a)). There was no notable change in predicted recoveries using 20 to 25% MeOH modifier; however, on further increasing the modifier (to 30%), the recoveries of a number of analytes greatly reduced (e.g., ABZ-SO, FBZ, TBZ-OH, LEV, CLOR and NITROX). In contrast, as the modifier is increased, the predicted recovery of CAM and a number of avermectins (EMA, EPRINO and MOXI) all increased, which is consistent with the necessity of organic modifier, as reported by Thompson et al. (2009). For sample pH, the optimum was predicted to be pH 7, with predicted recoveries of the majority of analytes consistent across the pH range of 6–8. At low pH (towards pH 4), recoveries were improved for a number of analytes, mainly belonging to the benzimidazole class (e.g., ABZ-SO, FBZ). This is due to the drugs becoming more ionised and more solubilised at lower pH, as a result of their first dissociation constant (pKa) being between 2.5–5.5 (Supplementary File SI-2.1 Table S2-1). The avermectins are neutral compounds, except EMA which is a salt and favours increased retention as the pH increases from 4 to 7, where the benzoate form will be prominent (pKa 4.7) and the epi methyl-amino ion will be almost 50% ionised (pKa 7.7) At basic pH, for a number of compounds, the recoveries predicted are lower compared to those at neutral and acidic pH, with the exception of CAM, FLU-NH_2, LEV and MBZ-NH_2, which all have basic functional groups and therefore will be less ionised and retained better at higher pH. These results are similar to that observed by Zrncic et al. (2014) who assessed the effect of pH on the extraction of 10 anthelmintics from river water. At pH 4.0 these authors report the recovery of all analytes to be >60% with the exception of LEV (<20%) and MOXI (approx.

40%); however, on further increasing pH from acidic to neutral (pH 7.0), recoveries for the majority of the analytes further increased or remained the same. At basic pH, the authors report that the recovery of most analytes significantly decreased; however, the recovery of LEV was at its highest (>55%), as was predicted for LEV by the RSM optimiser graph in this work. In this current work, the overall response surface methodology predicted extraction pH range of 6–8 is consistent with the findings of Zrncic et al. (final pH 7 selected) and with other methods reported amongst literature (Krogh et al., 2008a; Van De Steene and Lambert, 2008; Thompson et al., 2009). The RSM graphs for the remaining 23 anthelmintics (Supplementary Figure S2-3(b)) showed that the predicted optimum modifier (20%) and pH conditions (pH 7) also gave satisfactory predicted recoveries.

The predicted results for sample modifier (%) were verified by application to fortified groundwater samples (n = 3) in which the average recoveries of analytes in samples using the optimal conditions (20% modifier, pH 7) were compared to the average recoveries in fortified samples without modifier addition (0% modifier, pH 7) (results depicted in Supplementary Figure S2-4). Ten of the 40 compounds showed an increase in recovery with the addition of the 20% modifier, while three compounds had a notable decrease in recovery. Notably, for the first time, acceptable recoveries for CLOR and RAFOX were verified with the incorporation of the modifier (recoveries of 91 and 75% with modifier compared to 50 and 28% with no modifier). Levamisole (LEV) showed the greatest decrease in recovery with the addition of the modifier (reduced from 89 to 70%), which indicates that the MeOH modifier causes breakthrough of LEV while loading; however, this recovery was still acceptable.

2.2.2 Method validation

The method was validated at three concentration levels according to an amalgamation of criteria as specified in Table 2-1 (see Table 2-3 for validated concentration levels).

2.2.2.1 Identification

For each compound, one precursor and two daughter ions (one quantifier and one qualifier) were monitored, giving a total of four identification points, satisfying the confirmation criteria. Daughter ions were identified as part of the initial tuning of analytes on the MS detection system, with quantifier and qualifier ions generally selected as the two most intense

(abundant) ions. Careful consideration was given to ensure the ions chosen were suitably selective (i.e., not produced as a result of a common neutral losses e.g., loss of water (−18 amu) (Berendsen et al., 2013). The quantifier ion was assigned as the most abundant *m/z* ion of the two daughters. For the majority, the 2002/657 ion ratio criterion (≤20%) was adhered to, with the exception of a few analytes on a few occasions, where the SANTE criterion (≤30%) was necessary.

2.2.2.2 Specificity and linearity

The specificity of the method was investigated through monitoring for interferences in UHPLC-MS/MS traces from analytes or internal standards. Transitions for ABZ-SO_2 (*m/z* 298.1→ 266.2) and MBZ-OH (*m/z* 298.25→266.15) were prone to isobaric interference but were sufficiently separated in the UHPLC-MS/MS traces (3.44 vs. 4.09 min., respectively (Figure 2-2)). The absence of cross-talk interference was confirmed by injecting analytes and internal standards separately. The selectivity of the method was evaluated by application to 30 different groundwater and surface water samples, which were confirmed to be free of interferences, according to the 2002/657 criterion; however, in some instances, the SANTE criterion (≤30%) was more appropriate.

Linearity was assessed by visual inspection of these calibration curves (constructed with a linear fit and $1/x^2$ weighting), residual plots and coefficient of determination (R^2) values. For all analytes, R^2 values were >0.99, except for TCB-SO and TCB-SO_2 (0.97 and 0.89) (Table 2-2). Whelan et al. (2013) proposed the use of trifluoroacetic acid (TFA) as a mobile phase additive which allowed better ionisation of these two analytes by promoting the formation of the protonated pseudo-molecular ions in ESI positive (+ve) mode. This approach was beyond the scope of this work; therefore, these two analytes are only suitable for screening purposes (non-confirmatory) in this method.

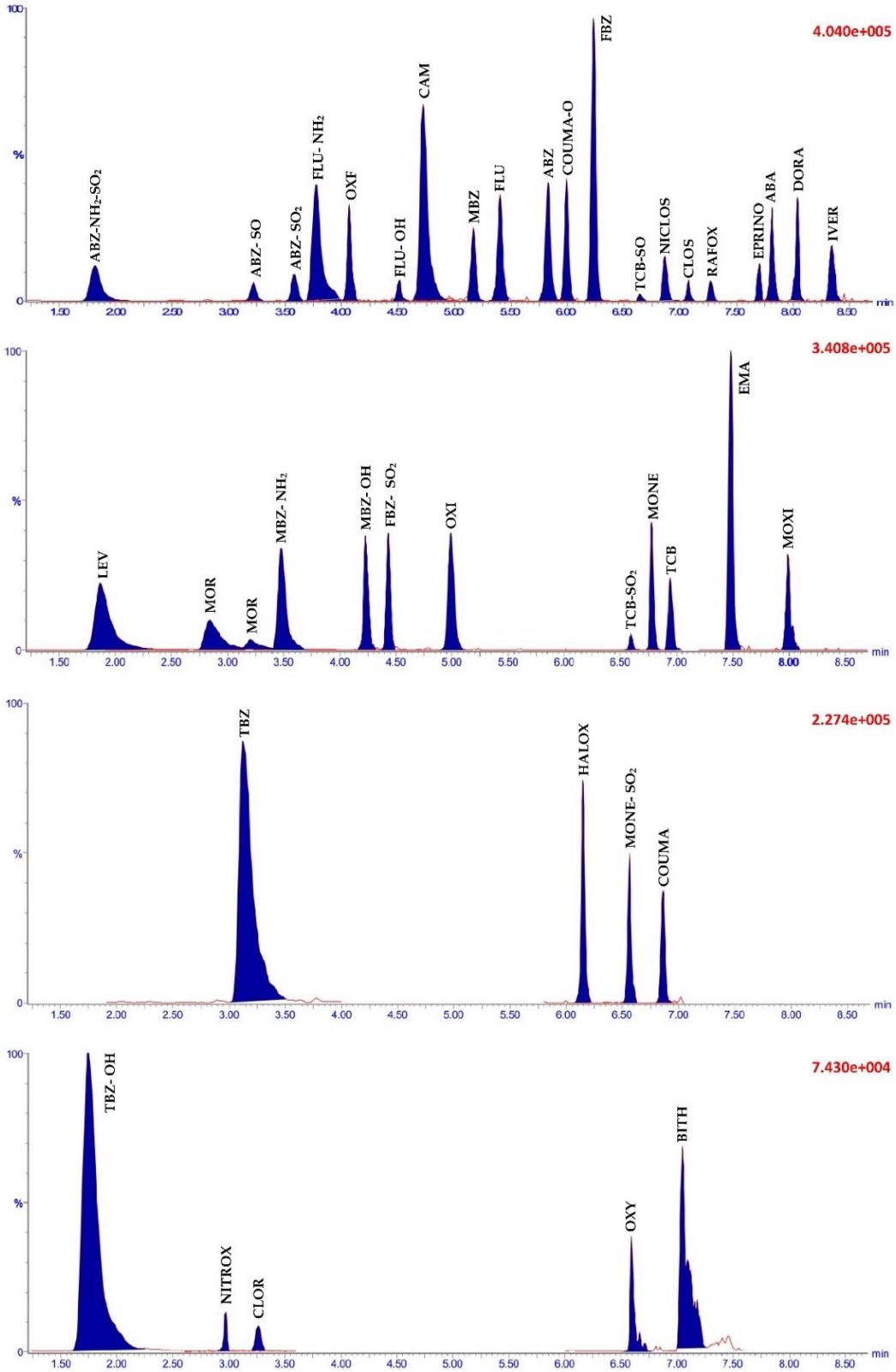


Figure 2-2 Overlay of LC-MS/MS chromatograms for the 40 anthelmintic residues in a blank water sample fortified at concentrations equivalent to the LOQ (see Table 2-3) for each analyte

2.2.2.3 Trueness and precision

Trueness and precision data under within-lab repeatability (WL_r) and within-lab reproducibility (WL_R) conditions are summarised in Table 2-3. Under WL_r conditions the trueness for all analytes was satisfactory and met the set criteria, with overall trueness in the range of 83–113%. WL_r precision (RSD_r) for all analytes across the three validation levels was in the range of 0.8–13.2%, with the exception of NITROX which had an RSD_r of 19.5% at the lowest validation concentration, which still met the acceptance criteria. The majority of analytes had RSD_r values ≤5%. Under reproducibility conditions (WL_R), trueness ranged from 88–114%, with all analytes meeting the acceptance criteria. Precision for all analytes under WL_R conditions (RSD_{wR}) were all under 12.4%, again with the exception of NITROX, which had an RSD of 19.4% at the lowest validated level. Overall, this method has been shown to be very accurate and precise for the 38 confirmatory analytes.

2.2.2.4 Recovery, limits of detection and quantification

The recovery of analytes (Table 2-3) at the higher concentration (200/400 ng L^{-1}) ranged from 71 to 114%, all within the acceptable criteria (70–120%), except for NITROX (56%) and MOXI (59%). The precision for all analytes was <8.7% RSD. At the lower concentration (20/40 ng L^{-1}) the overall analyte recoveries ranged from 83–113%, while RSDs ranged from 1.3–11.6%. Notably, the recoveries of NITROX and MOXI were satisfactory at the lower concentration (105 and 95%, respectively). This method performs better (in terms of recovery) when compared to other methods available. Krogh et al. (2008a) reported a recovery range of 38–67% for ABA, DORA, EMA, EPRINO, IVER and MOXI, using HLB SPE; however, individual recoveries for each analyte could not be clarified throughout the paper. Notably, Krogh et al. used a 4 mL MeOH wash step prior to drying and elution, which may have resulted in removal of analyte at the wash stage. In the method by Zrncic et al. (2014), using HLB SPE of water samples at pH 7, recovery ranged between 76.5 and 105.5% for ABZ, FBZ, FLU, MBX, OXI and TCB. Low recoveries of 42.8 and 56.6% were reported for LEV and MOXI respectively. The recovery of LEV reported in this current paper is much higher than that achieved by Zrncic et al., while the recovery of MOXI in this current work performs similarly, or better, depending on analyte concentration (much improved recovery at lower concentration in this work).

The LOQ for the majority of analytes corresponded to the lowest calibrant level of the calibration curve, with an overlaid LC-MS/MS chromatogram for all 40 analytes, fortified in blank water samples at the LOQ, shown in Figure 2-2. The LOQs ranged from 0.5–10 ng L^{-1}, with the exception of EPRINO and CLOR, which had LOQs of 20 and 40 ng L^{-1}, respectively. The LOQs for all compounds were lower than 25 ng L^{-1} detection capability required by the EU Drinking Water Directive (European Commission, 1998), and given that the method's LODs are inherently lower than the LOQs, this method more than meets this performance criterion. The exception to this is CLOR, which has an LOQ of 40 ng L^{-1}; however, the LOD was determined to be acceptable (10 ng L^{-1}). The performance of this method in terms of sensitivity, performs similar to or better (depending on the analyte) than other methods available.

Table 2-1 Validation criteria adhered to, with corresponding legislative guideline.

Parameter	Performance Criteria	Guideline [a]
Identification		
Points	Minimum 3	2002/657
Relative retention (RRT)	≤2.5%	2002/657
Ion ratio tolerance (ΔR)	20-50%	2002/657
	30%	SANTE
Selectivity	Interferences: ≤ 10% lowest calibrant	2002/657
	Interferences: ≤ 30% lowest calibrant	SANTE
Linearity	Regression coefficient $R^2 \geq 0.98$	2002/657
	Residuals ± 20%	SANTE
Trueness (WL_R and WL_r)	70–120%	SANTE
Precision (RSD_{wR} and RSD_r)	≤ 20%	SANTE
Recovery	70–120%	SANTE

[a] 2002/657 = European Commission Decision 2002/657/EC (European Commission, 2002), SANTE = SANTE/11813/2017 (European Commission, 2017)

Table 2-2 Calibration range, mean linearity (of $n = 5$ runs) and results of matrix effects (ME) ($n = 30$) for each of the 40 anthelmintic compounds.

Analyte	Abbreviation	P/ TP	Labelled IS Used	Calibration Range (ng L^{-1})	Linearity R^2	Mean ME (%) (n=30)	ME RANGE (%)		RSD No IS (%)	RSD with IS (%)
							Min	Max		
Benzimidazoles										
Albendazole	ABZ	P	ABZ-d_3	1–1000	0.997	27.1	8.2	47.3	9.3	3.0
Albendazole sulphoxide	ABZ-SO	TP	ABZ-SO-d_3	1–1000	0.994	93.4	13.8	212	31.6	7.1
Albendazole sulphone	ABZ-SO_2	TP	ABZ-SO_2-d_3	1–1000	0.996	60.8	29	120	18.2	6.5
Albendazole-amino-sulphone	ABZ-NH_2-SO_2	TP	ABZ-NH_2-SO_2-d_3	0.5–1000	0.998	16.9	-1.4	28.0	6.9	4.0
Cambendazole	CAM	P	FBZ-d_3	0.5–1000	0.997	9.7	-5.1	24.2	6.6	7.2
Fenbendazole	FBZ	P	FBZ-d_3	0.5–1000	0.995	23.1	1.0	44.9	9.3	2.3
Oxfendazole	OXF	TP	FBZ-SO-d_3	1–1000	0.993	42.0	11.6	106.2	18.5	6.4
Fenbendazole sulphone	FBZ-SO_2	TP	FBZ-SO_2-d_3	1–1000	0.998	47.5	8.1	165.7	25.5	3.3
Flubendazole	FLU	P	FLU-d_3	1–1000	0.996	33.3	7.4	108.2	14.1	3.7
Amino-flubendazole	FLU-NH_2	TP	TCB-NH_2 (pos)	1–1000	0.995	11.5	-3.7	29.8	8.0	8.8
Hydroxy-flubendazole	FLU-OH	TP	MBZ-OH-d_3	1–1000	0.997	3.7	-12.9	27.4	12.1	7.6
Mebendazole	MBZ	P	MBZ-d_3	1–1000	0.994	45.0	11.4	104.2	18.1	3.6
Amino-mebendazole	MBZ-NH_2	TP	TCB-NH_2 (pos)	1–1000	0.995	15.1	0	36.3	7.2	8.5
Hydroxy-mebendazole	MBZ-OH	TP	MBZ-OH-d_3	1–1000	0.998	27.4	3.8	64.2	13.1	4.6
Oxibendazole	OXI	P	OXI-d_7	0.5–1000	0.994	9.3	-2.5	21.6	5.8	4.5
Triclabendazole	TCB	P	TCB-d_3	0.5–1000	0.997	3.6	-14.2	27.4	8.0	3.3
Triclabendazole-sulphoxide	TCB-SO	TP	TCB-NH_2(neg)	4–20	0.967	-3.0	-45	47.8	25.2	24.7
Triclabendazole-sulphone	TCB-SO_2	TP	TCB-NH_2(neg)	4–20	0.891	5.2	-25.4	57.8	18.2	19.8
Thiabendazole	TBZ	P	TBZ-$^{13}C_6$	0.5–1000	0.999	9.1	-6.8	26.6	6.7	2.7
5-Hydroxy-Thiabendazole	TBZ-OH	TP	ABZ-NH_2-SO_2-d_3	0.5–200	0.991	-6.4	-23.8	12.7	9.6	7.2
Macrocyclic lactones (Avermectins & Milbemycins)										
Abamectin	ABA	P	SEL	10–2000	0.996	20.4	-4.1	45.7	9.7	7.5
Doramectin	DORA	P	SEL	1–1000	0.993	77.8	13.8	130.9	16.0	15.2
Emamectin	EMA	P	SEL	0.5–200	0.996	24.8	3.4	37.8	7.7	8.2
Eprinomectin	EPRINO	P	SEL	20–2000	0.997	6.8	-17.9	25.7	9.9	8.3
Ivermectin	IVER	P	SEL	10–2000	0.996	5.2	-22.5	27.2	9.6	7.9
Moxidectin	MOXI	P	SEL	10–2000	0.996	34.9	-9.1	76.3	16.3	13.7

Table 2-2 *continued*

Analyte	Abbreviation	P/ TP	Labelled IS Used	Calibration Range (ng L^{-1})	Linearity R^2	Mean ME (%) (n=30)	ME RANGE (%)		RSD No IS (%)	RSD with IS (%)
							Min	Max		
Salicylanilides and substituted phenols										
Bithionol	BITH	P	RAFOX-$^{13}C_6$	5–1000	0.995	32.0	-1.4	50	10.6	5.4
Closantel	CLOS	P	CLOS-$^{13}C_6$	2–1000	0.997	-3.9	-12.1	5.2	5.4	2.8
Niclosamide	NICLOS	P	SAL	1–200	0.991	13.0	-5	33.3	8.3	5.1
Nitroxynil	NITROX	P	NITROX-$^{13}C_6$	10–1000	0.993	28.6	-5.7	73.2	14.9	14.2
Oxyclozanide	OXY	P	OXY-$^{13}C_6$	5–1000	0.996	42.8	18.2	70.9	9.5	10.4
Rafoxanide	RAFOX	P	RAFOX-$^{13}C_6$	2–1000	0.994	23.0	2	41.2	10.5	3.4
Tetrahydropyrimidines										
Morantel	MOR	P	TBZ-$^{13}C_6$	1–1000	0.997	13.3	-2.5	34.1	7.4	1.6
Imidazothiazoles										
Levamisole	LEV	P	LEVA-d_5	0.5–1000	0.999	12.4	-2.5	33.7	7.4	2.0
Organophosphates										
Coumaphos	COUMA	P	ABZ-d_3	5–200	0.986	47.0	10.1	87.7	12.9	8.4
Coumaphos-Oxon	COUMA-O	P	FBZ-d_3	1–1000	0.992	16.2	3.9	31.9	6.0	7.6
Haloxon	HALOX	P	ABZ-d_3	5–500	0.989	25.5	-73.8	55	12.0	7.2
Amino-acetonitrile derivatives										
Monepantel	MONE	P	CLOS-$^{13}C_6$	5–400	0.991	16.7	-6.1	31.8	7.2	8.5
Monepantel-sulphone	MONE-SO_2	TP	CLOS-$^{13}C_6$	1–400	0.993	14.0	-4.4	28.8	7.0	7.4
Miscellaneous										
Clorsulon	CLOR	P	SAL	40–2000	0.991	-15.1	-48.8	9.2	18.7	15.6

P = Parent compound, TP = Transformation product, IS = Internal standard, R^2 = regression coefficient, ME = Matrix effects where positive values indicate ion enhancement, while negative values indicate ion suppression. Matrix effect study was carried out at a concentration of 100 ng L^{-1} for all analytes except CLOR, BITH and MOR, which were at 200 ng L^{-1} RSD = relative standard deviation

Table 2-3 Validation trueness and precision (RSD) under repeatability conditions (WLr) (n = 6) and reproducibility conditions (WLR) (n = 18) at three concentration levels for 40 anthelmintics with respective method recovery, LOD and LOQ values (ng L^{-1}).

Analyte	Validated Levels L1, L2, L3 (ng L^{-1})	WL_r Trueness (RSD_r) (%)[a]			WL_R Trueness (RSD_{WR}) (%)[b]			LOD [c] (ngL^{-1})	LOQ [d] (ngL^{-1})	Recovery % (RSD%, n = 3) at	
		L1	L2	L3	L1	L2	L3			20/40 ng L^{-1}	200/400 ng L^{-1}
Benzimidazoles											
ABZ	5, 50, 200	100 (5.6)	100 (3.0)	97 (1.5)	102 (3.6)	100 (3.2)	98 (2.8)	0.125	1.0	94 (4.7)	94 (0.5)
ABZ-SO	5, 50, 200	113(10.8)	97)7.3)	101 (4.7)	107 (13.5)	99 (9.9)	99 (5.2)	0.2	1.0	95 (1.3)	114 (5.5)
ABZ-SO_2	5, 50, 200	95 (7.3)	96 (4.5)	99 (2.5)	105 (6.4)	99 (3.2)	99 (3.2)	0.165	1.0	92 (2.1)	105 (5.6)
ABZ-NH_2-SO_2	5, 50, 200	103 (3.1)	101 (1.4)	101 (1.1)	101 (3.7)	99 (2.3)	100 (3.9)	0.165	0.5	93 (4.0)	91 (7.6)
CAM	5, 50, 200	103 (4.0)	96 (1.4)	97 (1.1)	102 (4.3)	101 (3.9)	100 (3.1)	0.165	0.5	94 (3.2)	92 (6.0)
FBZ	5, 50, 200	103 (4.5)	97 (2.0)	100 (1.3)	105 (6.7)	100 (3.8)	99 (2.3)	0.1	0.5	89 (4.6)	109 (1.9)
OXF	5, 50, 200	87 (11.4)	100 (5.4)	101 (3.3)	101 (15.1)	98 (6.3)	98 (6.4)	0.25	1.0	94 (6.5)	103 (4.8)
FBZ-SO_2	5, 50, 200	99 (2.7)	96 (1.6)	97 (0.8)	101 (5.1)	99 (3.0)	99 (1.7)	0.20	1.0	97 (3.2)	102 (5.5)
FLU	5, 50, 200	107 (7.2)	95 (5.5)	95 (2.1)	102 (7.1)	97 (4.3)	100(3.3)	0.1	1.0	97 (4.9)	97 (2.5)
FLU-NH_2	5, 50, 200	107 (3.6)	104 (3.4)	97 (2.4)	105 (4.8)	103 (2.9)	98 (3.4)	0.05	1.0	94 (5.1)	102 (1.8)
FLU-OH	5, 50, 200	97 (6.8)	109 (4.4)	103 (2.3)	99 (5.6)	102 (4.3)	101 (3.1)	0.3	1.0	95 (4.3)	99 (3.7)
MBZ	5, 50, 200	105 (5.3)	99 (3.6)	97 (2.0)	102 (6.1)	97 (3.9)	98 (2.6)	0.125	1.0	97 (4.0)	102 (0.9)
MBZ-NH_2	5, 50, 200	104 (3.4)	104 (3.1)	96 (3.8)	105 (4.8)	104 (3.5)	100 (4.1)	0.3	1.0	92 (2.0)	101 (2.4)
MBZ-OH	5, 50, 200	102 (2.6)	107 (1.0)	100 (1.0)	103 (4.3)	101 (4.2)	99 (2.5)	0.2	1.0	96 (3.6)	104 (5.2)
OXI	5, 50, 200	102 (2.7)	99 (2.7)	97 (1.0)	106 (5.2)	101 (3.3)	98 (3.2)	0.125	0.5	103 (3.3)	98 (2.4)
TCB	5, 50, 200	96 (6.9)	105 (4.5)	102 (3.5)	100 (7.6)	102 (3.5)	100 (3.4)	0.125	0.5	91 (2.0)	100 (4.0)
TCB-SO	6, 14, 20	-	-	-	-	-	-	1.0	4.0	80 (4.8)	92 (6.6)
TCB-SO_2	6, 14, 20	-	-	-	-	-	-	1.0	4.0	97 (7.5)	103 (4.8)
TBZ	5, 50, 200	102 (3.8)	99 (1.0)	98 (0.6)	103 (3.2)	99 (2.4)	100 (2.0)	0.1	0.5	99 (3.1)	98 (3.2)
TBZ-OH	5, 50, 150	110 (1.5)	101 (1.2)	93 (0.7)	109 (3.3)	100 (2.1)	92 (4.1)	0.1	0.5	104 (2.0)	80 (5.1)
Macrocyclic lactones (Avermectins & Milbemycins)											
ABA	40,150,500	104 (5.4)	99 (5.0)	98 (7.3)	98 (8.5)	100 (5.6)	99 (3.2)	1.0	10.0	110 (9.0)	90 (6.0)
DORA	20, 80, 200	103 (4.7)	97 (5.3)	103 (4.3)	98 (7.9)	97 (7.3)	99 (4.5)	0.5	10.0	105 (6.8)	87 (1.5)
EMA	5, 50, 150	107 (4.5)	96 (9.6)	104 (8.7)	108 (5.6)	104 (6.5)	102 (5.5)	0.05	0.5	102 (5.0)	87 (4.5)
EPRINO	40, 150, 500	96 (3.4)	99 (4.9)	104 (2.6)	100 (8.9)	101 (3.1)	102 (2.4)	5	20.0	109 (0.8)	91 (5.6)

Table 2-3 *continued*

Analyte	Validated Levels L1, L2, L3 (ng L^{-1})	WL_r Trueness (RSD_r) (%)[a]			WL_R Trueness (RSD_{WR}) (%)[b]			LOD [c] (ngL^{-1})	LOQ [d] (ngL^{-1})	Recovery % (RSD%, *n* = 3) at	
		L1	L2	L3	L1	L2	L3			20/40 (ng L^{-1})	200/400 (ng L^{-1})
Macrocyclic lactones (Avermectins & Milbemycins)											
IVER	40, 150, 500	104(4.1)	100(2.7)	107(5.4)	98(7.5)	100(2.9)	103(4.6)	2.5	10.0	113(10.9)	72(8.7)
MOXI	40, 150, 500	96(6.4)	92(8.7)	91(6.5)	101(7.8)	100(8.0)	98(6.5)	2.0	10.0	95(10.8)	59(5.0)
Salicylanilides and substituted phenols											
BITH	20, 80, 200	112(5.6)	112(4.7)	104(2.7)	114(7.2)	106(4.8)	101(3.8)	1.0	5.0	98(10.8)	84(3.7)
CLOS	5, 50, 200	105(4.8)	104(2.0)	101(1.0)	105(7.1)	101(3.7)	99(3.2)	0.5	2.0	103(3.6)	76(3.5)
NICLOS	5, 50, 150	107(10.3)	106(3.7)	96(2.0)	114(9.5)	105(7.2)	96(6.9)	0.125	1.0	94(7.0)	100(5.4)
NITROX	20, 80, 200	107(19.5)	107(13.2)	91(4.6)	96(19.4)	104(12.4)	96(8.7)	2.5	10.0	105(4.6)	56(4.7)
OXY	20, 80, 200	113(6.7)	108(7.4)	101(2.4)	109(9.6)	103(8.6)	101(4.1)	1.5	5.0	93(7.7)	104(5.6)
RAFOX	5, 50, 200	105(8.7)	101(3.0)	99(1.8)	102(10.3)	102(4.3)	101(2.5)	0.3	2.0	97(5.8)	86(4.8)
Tetrahydropyrimidines											
MOR	5, 50, 200	101(1.8)	98(1.4)	95(1.8)	100(2.3)	97(1.9)	98(2.8)	0.3	1.0	100(4.0)	100(2.5)
Imidazothiazoles											
LEV	5, 50, 200	102(1.5)	100(1.4)	100(0.7)	102(2.1)	100(1.1)	101(1.7)	0.125	0.5	89(5.7)	96(1.9)
Organophosphates											
COUMA	10, 50, 150	83(9.3)	93(2,9)	104(3.8)	88(8.3)	95(5.8)	106(4.7)	1.0	5.0	84(6.0)	99(3.6)
COUMA-O	5, 50, 200	95(3.7)	89(3.9)	98(1.6)	96(6.6)	92(3.4)	99(3.2)	0.25	1.0	93(5.6)	102(2.5)
HALOX	20, 80, 200	94(11.7)	94(3.6)	100(2.0)	90(11.8)	94(5.3)	102(3.1)	1.0	5.0	83(0.8)	99(0.6)
Amino-acetonitrile derivatives											
MONE	10, 50, 150	103(5.1)	96(4,3)	93(3.2)	104(12.1)	97(6.0)	94(5.2)	0.5	5.0	90(6.9)	96(3.0)
MONE-SO2	5, 50, 150	94(8.1)	91(6.2)	93(3.2)	98(8.9)	94(4.6)	98(5.3)	0.2	1.0	92(2.6)	102(1.7)
Miscellaneous											
CLOR	80, 300, 800	95(12.8)	97(5.8)	95(4.9)	96(14.9)	95(10.0)	94(8.4)	10	40.0	101(11.6)	110(3.6)

[a] WLr = Within-laboratory repeatability while RSD_r = Relative standard deviation under repeatability conditions, [b] WLR= Within-laboratory reproducibility, while RSD_{wR} = Relative standard deviation under reproducibility conditions [c] LOD = Limit of Detection based on S/N = 5, [d] LOQ = Limit of Quantitation based on S/N = 10, L1, L2 and L3, refer to each of the three levels at which the validation was performed

2.2.3 Matrix effects

In this study, matrix effects were calculated as follows: ME (%) = (B − A/A × 100), where A is the response of analyte in neat solution, and B is the response in post-extraction spiked samples. Using this approach, negative (−) ME values indicated suppression (decrease in analyte response due matrix components), while positive (+) values indicated enhancement (increase in analyte response). All anthelmintic compounds experienced ion enhancement due to matrix, with the exception of CLOR, CLOS, TBZ-OH and TCB-SO, which all showed ion suppression on average (Table 2-2). The mean matrix effects (n = 30) ranged from −15.1% for CLOR (analyte suppression) up to +93.4% for ABZ-SO (enhancement). The range of ME for each individual analyte across the entire 30 samples is shown in Table 2-2. The most suppression in any one sample (of total 30) was 74% (ME −74%) for HALOX, while the highest enhancement in any one sample was observed for ABZ-SO (+212%). In order to account for this observed enhancement or suppression due to ME, isotopically labelled internal standards (IS) were employed (IS as specified in Table 2-2). When the internal standards were incorporated into the method, the overall precision (RSD%) was improved for a number of analytes, particularly ABZ-SO with the RSD reduced from 32% to 7%. In cases where the IS did not drastically improve the precision (e.g., DORA and EMA), the exact deuterated form of the compound was not used as the IS, either due to unavailability or cost, in which case the addition of IS was used only to account for losses of analyte during extraction. Overall, the combination of the use of matrix matched calibration curves and internal standards (IS) compensated for any ME effects, thus satisfying validation criteria.

2.2.4 Applicability

The method presented above has been applied for the determination of the 40 anthelmintic compounds as part of an initial pilot sampling programme, whereby 72 environmental water samples were collected from different locations across Ireland during Autumn 2016 (September–October 2016). Overall, as part of this pilot study, 52 groundwaters (from boreholes, wells and springs) and 20 surface waters (from streams, rivers and lakes) were collected from 43 different sampling locations and analysed for the 40 anthelmintic compounds. Anthelmintic compounds were detected in 8 out the 72 samples (11%) with concentrations of the order of 1.0 ng L^{-1} to 30 ng L^{-1}. Of the eight samples with detections,

four were groundwater samples which contained up to three different anthelmintic compounds (detection in 7.7% of groundwater samples analysed), while the other four were surface waters with up to five different anthelmintics present (detections in 20% of surface waters analysed). The method has also been applied in more comprehensive spatial and temporal studies, which are currently in preparation.

2.3 Materials and Methods

2.3.1 Chemicals, standards and consumables

Ultrapure water (UPW) (18.2 MΩcm) was generated in house using a Millipore water purification system (Cork, Ireland). Romil "SpS" (super purity solvent) grade methanol (MeOH) 215, acetonitrile (MeCN) 200 far UV and propan-2-ol (IPA) were sourced from Romil Ltd. (Cambridge, UK). Acetone puriss was purchased from Honeywell Research Chemicals (Honeywell Riedel-de Haen; Seelze, Germany). Dimethyl sulfoxide (DMSO), 99.5% d-MeOH, ammonium formate puriss p.a. (puriss pro analysis), formic acid (HCOOH) 98–100%, methyl tert-Butyl ether (MTBE) (Fluka for GC) and sodium meta-bisulphite (>97%) were sourced from Sigma-Aldrich (Dublin, Ireland). Glacial Acetic acid (CH3COOH) (100%) and ammonia solution (25% *w/v*) were obtained from Merck (Darmstadt, Germany). Concentrated hydrochloric acid (HCl) (36%) was sourced from BDH Chemicals Ltd. (Poole, UK).

Neat analytical standards of abamectin (ABA), albendazole (ABZ), bithionol (BITH), clorsulon (CLOR), closantel (CLOS), coumaphos (COUMA), doramectin (DORA), Emamectin benzoate (EMA), eprinomectin (EPRINO), fenbendazole (FBZ), haloxon (HALOX), ivermectin (IVER), levamisole hydrochloride (LEV), morantel-tartrate-hydrate (MOR), moxidectin (MOXI) niclosamide (NICLOS), nitroxynil (NITROX), oxfendazole (OXF), oxyclozanide (OXY), rafoxanide (RAFOX), thiabendazole (TBZ), triclabendazole (TCB) and salicylanilide (SAL) were purchased from Sigma-Aldrich Ireland (Dublin, Ireland). Albendazole-sulphoxide (ABZ-SO), albendazole-sulphone (ABZ-SO_2), albendazole-amino-sulphone hydrochloride (ABZ-NH_2-SO_2), cambendazole (CAM), fenbendazole-sulphone (FBZ-SO_2), 5-hydroxy-thiabendazole (5-OH-TBZ), triclabendazole sulphoxide (TCB-SO), triclabendazole sulphone (TCB-SO_2) and amino-triclabendazole (TCB-NH_2) were purchased from Witega (Berlin, Germany). Coumaphos-oxon (COUM-O)

was purchased from Greyhound Chromatography and Allied Chemicals, (Merseyside, UK). Flubendazole (FLU), amino-flubendazole (FLU-NH_2), hydroxy-flubendazole (FLU-OH), mebendazole (MBZ), amino-mebendazole (MBZ-NH_2) and hydroxy-mebendazole (MBZ-OH) were obtained from Janssen Animal Health (Beerse, Belgium). Oxibendazole (OXI) was purchased from QMX Laboratories (Essex, UK), while selamectin (SEL) was acquired from Pfizer (Kent, UK). Monepantel (MONE) and monepantel-sulphone (MONE-SO_2) were purchased from Novartis Pharmaceuticals (Dublin, Ireland). All deuterated or isotopically labelled internal standards (specified for each compound in Table 2-2) were purchased from Witega (Berlin, Germany), except for flubendazole-d_3 (FLU-d_3) which was purchased from Sigma-Aldrich Ireland (Dublin, Ireland).

Duran style (GL45) glass amber bottles (1000 mL) were purchased from Scientific and Chemical Supplies Ltd. (Cork, Ireland). Analytical grade glass wool (silanised and unsilanised) were purchased from Sigma-Aldrich (Dublin, Ireland). Polypropylene tubes (15 mL, conical) were obtained from Sarstedt Ltd. (Wexford, Ireland). Isolute 150 mL frittless SPE reservoirs were purchased from Biotage (Uppsala, Sweden). Reservoirs were connected to the SPE cartridge using adapter caps for 1–6 mL cartridges, provided by Agilent Technologies Ltd. (Cork, Ireland). Captiva Econo PTFE 0.2 µm filters were also purchased from Agilent Technologies Ltd., as were the glass inserts (400 µL) used in the Waters HPLC vials (Waters; Dublin, Ireland) The different SPE sorbents evaluated as part of method development included: Bond Elut ENV (200 mg, 6 mL) and Bond Elut PLEXA (200 mg, 6 mL) from Agilent technologies Ltd. (Cork, Ireland), Oasis HLB (200 mg, 6 mL) from Waters (Dublin, Ireland) and UCT Enviro Clean HL DVB (200 mg, 6 mL) and UCT Enviro Clean HL DVB (500 mg, 6 mL) from United Chemical Technologies Ireland Ltd.

2.3.2 Preparation of standard solutions

Individual primary stock solutions were prepared from certified standard material at a concentration of 4 mg mL^{-1} in MeCN for EPRINO, in MeOH for BITH, CLOR, CLOS, MOR, NITROX and OXY and in DMSO for ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, FBZ-SO_2, MONE, MONE-SO_2 and OXF. Stock solutions at a concentration of 2 mg mL^{-1} were prepared in MeCN for ABA, DORA, EMA, IVER, MOXI and SEL, in MeOH for CAM, COUMA, COUMA-O, HALOX, LEV, NITROX, RAFOX, TBZ, TCB, TCB-SO, TCB-SO_2 and TCB-NH_2 and in DMSO for FLU, FLU-OH, FLU-NH_2, MBZ, MNZ-OH,

MBZ-NH_2, OXI and TBZ-OH. Ten mixed intermediate solutions were prepared in MeOH as follows: WS-A containing 50 µg mL^{-1} CLOR, WS-B containing 50 µg mL^{-1} of ABA, EPRINO, IVER and MOXI, WS-C containing 25 µg mL^{-1} DORA and NITROX, WS-D containing 25 µg mL^{-1} of EMA, MONE-SO_2, NICLOS and TBZ-OH, WS-E containing 25 µg mL^{-1} of CLOS, FLU-OH, FLU-NH_2 and RAFOX, WS-F containing 25 µg mL^{-1} of ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, CAM, COUMA-O, FBZ, FBZ-SO_2, FLU, LEV, MBZ, MBZ-OH, MBZ-NH_2, MOR, OXF, OXI, TBZ and TCB, WS-G containing 10µg mL^{-1} of TCB-SO, WS-H containing 10 µg mL^{-1} of TCB-SO_2, WS-I containing 25 µg mL^{-1} of BITH, HALOX and OXY, and WS-J containing 25 µg mL^{-1} of MONE and COUMA.

A set of seven mixed working calibration solutions (Calibrants 1–7) with concentration ranges of 100–2500 ng mL^{-1}, 50–2500 ng mL^{-1}, 25–1250 ng mL^{-1}, 2.5–500 ng mL^{-1}, 5–1250 ng mL^{-1}, 2.5–1250 ng mL^{-1}, 12.5–1250 ng mL^{-1} and 12.5–500 ng mL^{-1} were prepared in MeOH by dilution of the respective intermediate mixed working solution; WS-A to WS-F and WS-I to WS-J. For TCB-SO and TCB-SO_2, an intermediate calibration solution (INT-A) at a concentration of 20 ng mL^{-1} was prepared by dilution of WS-G and WS-H. Primary stock of all deuterated and labelled internal standards, in addition to SAL, were prepared at a concentration of 1 mg mL^{-1}. These single stocks were subsequently used to prepare an intermediate IS solution in deuterated MeOH containing 200 µg mL^{-1} SEL and TCB-NH_2, 40 µg mL−1 LEVA-d_5, TBZ-$^{13}C^6$ and SAL, and 20 µg mL^{-1} of all other deuterated/labelled internal standards. This intermediate IS solution was diluted 1 in 10 to give a 20/4/2 µg mL^{-1} working IS solution. All working solutions were stored at -18 °C or, below in glass amber vials, with equilibration to room temperature before use.

2.3.3 Sample collection, control samples and Quality Control (QC)

Water samples were collected (in 2.5 L amber bottles) by one of three techniques depending on the source: (a) traditional grab sampling direct into the sampling container; (b) grab sampling via a bailer device, or (c) by pump (peristaltic or submersible). The sampling container was rinsed three times with the source water prior to collection. Samples were transported to the laboratory under chilled conditions, in individual, sealed, polypropylene bags and stored at 4 ºC until analysis (within 7 days after collection, as determined by matrix stability studies (SI-2.2)).

Samples found to be free of analyte, or to contain analyte levels of <30% of the lowest calibration point (in accordance with SANTE (European Commission, 2017)), were deemed to be suitable as negative control samples for method development, matrix matched calibration and validation experiments. Negative control samples were also used to produce QC Trip (Field) blanks. A QC trip blank (500 mL negative control aliquot) was transported to and from field sites while sampling. In the field, at each sampling location, the trip blank was exposed (open capped) in the vicinity of the sampling point, for the duration of sampling, and accompanied samples back to the laboratory in the same cooler container and under the same conditions. This trip blank was subsequently analysed along with samples, to demonstrate there was no contamination of samples during collection and transport. Fortified QC samples were not used during this study as some sampling was carried out by external organisations, and fortified samples were not feasible in such cases.

For internal (within batch) QC, a system suitability check to monitor analyte response and retention was injected prior to each instrumental run, to ensure the instrument was performing as expected. Negative control samples (n = 2) were included to confirm no cross contamination during the extraction process. Post-extraction spiked recovery samples were included to ensure the performance of the method for each analyte. Solvent blank injections were incorporated following calibration samples, prior to injection of unknown samples, to demonstrate no carryover of analytes. Retention checks involved re-injection of a matrix calibrant several times throughout the analytical run to check for accuracy. A minimum of four retention check injections were used to ensure no drift in retention during the analytical run, and to ensure no variation in detector response.

2.3.4 Matrix matched calibration

Matrix matched calibration curves were prepared by fortification of negative control samples as described in Table 2-4. An additional lower and upper calibration point was produced for some analytes. A minimum of seven points was used to construct a calibration curve, with the individual calibration range for each analyte as shown in Table 2-2. For TCB-SO and TCB-SO_2, a calibration curve was prepared by spiking of respective calibration samples above with 50, 100, 150, 250, 350, 450, 500, 550 and 625 µL of INT-A to give the concentrations described in Table 2-4 (analyte group G and H). All calibrants, quality control samples and samples were fortified with internal standard (25 µL) corresponding to a sample

concentration 1000 ng L^{-1} SEL and TCB-NH_2, 200 ng L^{-1} LEVA-$_{d5}$, TBZ- $^{13}C_6$ and SAL, and 100 ng L^{-1} of all other deuterated/labelled internal standards.

Table 2-4 Preparation of matrix matched calibration, with corresponding sample concentrations

Spiking Vol. (µL)	Calibration Level	Concentration Ranges (ng L^{-1}) for Analyte Group [a]:									
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
100	0.5 × L1	20	10	5	0.5	1	0.5	2	2	2.5	2.5
200	L1	40	20	10	1	2	1	4	4	5	5
200	L2	80	40	20	5	5	5	6	6	20	10
200	L3	200	100	40	20	20	20	10	10	40	20
200	L4	300	150	80	50	50	50	14	14	80	50
200	L5	400	200	100	100	100	100	18	18	100	100
200	L6	800	500	200	150	200	200	20	20	200	150
200	L7	1000	1000	500	200	500	500	22	22	500	200
400	L8 (2 × L7)	2000	2000	1000	400	1000	1000	25	25	1000	400

[a] Analytes within each concentration range group are as described in Section 2.3.2.

2.3.5 UHPLC-MS/MS Determination

All analytes were chromatographically separated using an in-house method as previously described by Whelan et al. (2010). Here analytes were separated on a stainless steel HSS T3 (100mm × 2.1mm, 1.8 µm particle size) column on a Waters Acquity UHPLC system, with a binary gradient. Anthelmintic residues were detected by a Waters Quattro Premier XE triple quad mass spectrometer (Milford MA, USA) with an electrospray ionisation (ESI) interface, coupled to the LC. All analysis was performed using rapid polar switching using a modified version of the acquisition described by Whelan et al. (2010). Dwell times, collision energies (CE) and collision voltages (CV) were further optimised from the original method, with the modified conditions shown in supplementary information file SI-2.1 Table S2-2.

2.3.6 Sample preparation-solid phase extraction

2.3.6.1 Development and optimisation

The main experiments carried out for SPE optimisation are summarised in Figure 2-3. All experiments were performed by fortification (n = 3) of negative control water samples, giving a concentration of 200 ng L^{-1} for analytes except BITH, CLOR, MOR and OXY, which were fortified at 400 ng L^{-1}. All SPE cartridges were conditioned and equilibrated according to the final procedure as described below. For experiments 1 to 4, the SPE cartridges were washed with ultrapure water, with experiments proceeding experiment 5 incorporating the selected optimum wash solution. In experiment 6, a simple central composite design response surface methodology (RSM) experiment was employed to optimise sample pre-treatment steps. The experimental design was carried out using MiniTab® 17 Statistical Software version 17.1.0 (MiniTab Inc., PA, USA). This experiment investigated two independent factors: (a) MeOH modifier added to samples (0–40%) and (b) sample extraction pH (pH 4–10). A quadratic model was selected to generate 13 experimental combinations (Supplementary Table S2-3), including five central combinations to assess error within the model. In the first stage, data was acquired and evaluated for all 40 analytes, with 17 analytes further evaluated at Stage 2 using an RSM optimiser graph, to optimise these two sample modification factors. These 17 analytes were selected to include different anthelmintic compounds representative of the different structural classes, in addition to the analytes which demonstrated poor recoveries in previous experiments (e.g., CLOS and RAFOX). Predicted results were verified by the optimiser graphs for the remaining 23 of the total 40 analytes (Supplementary Figure S2-3 (b)).

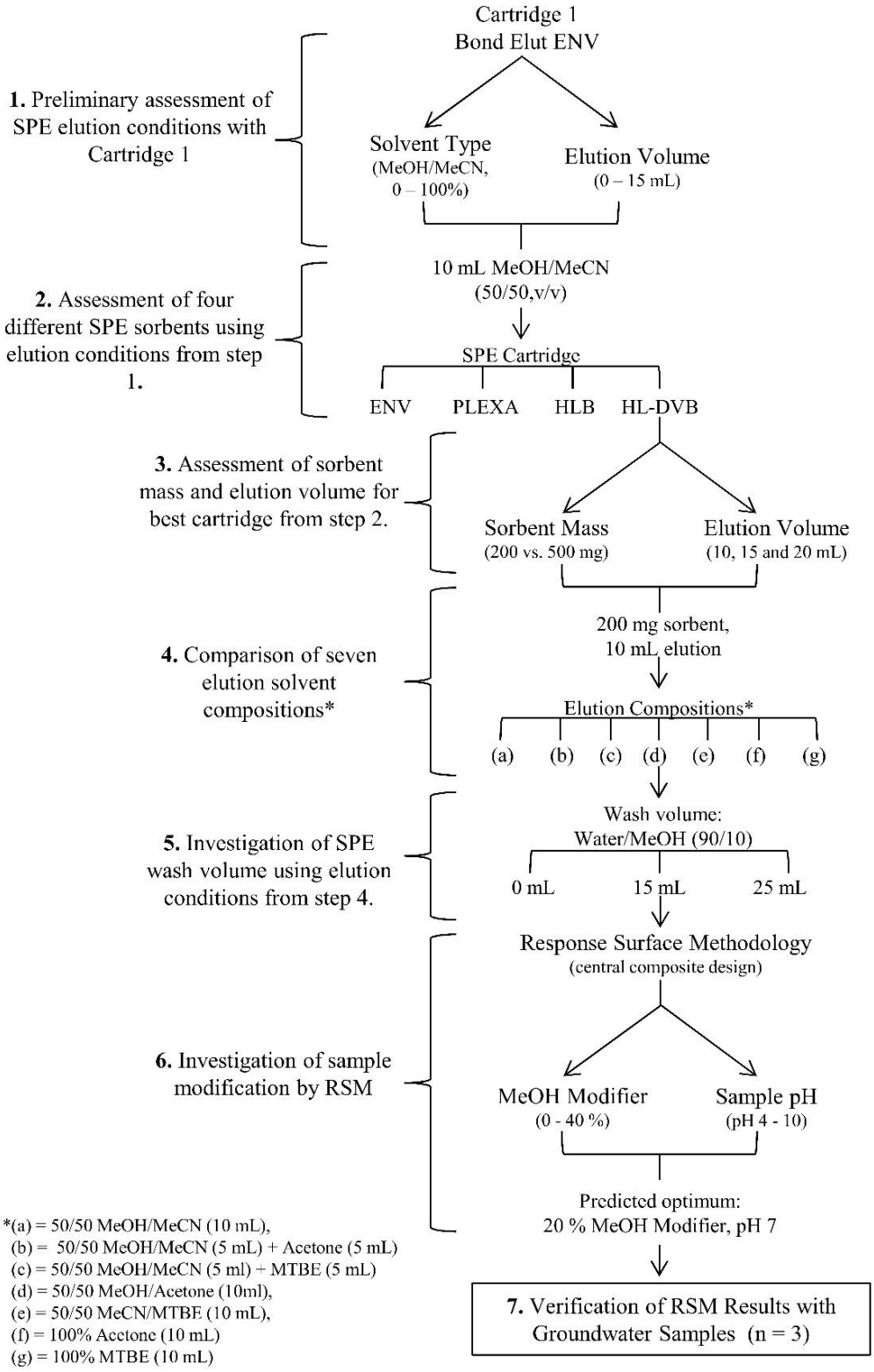


Figure 2-3 Summary of the main experiments carried out for the method development

2.3.6.2 final Method

Water samples (500 ± 0.1 g corresponding to 500 ± 0.1 mL), pre-shaken, were weighed directly into glass amber bottles (1000 mL) and equilibrated to room temperature. Matrix calibrants and samples were fortified with the working calibrant and IS solutions as described in Section 2.3.4. These were then shaken (60 s), modified with MeOH (100 mL), and shaken again (1 min.). Samples were subsequently adjusted to pH 7 ± 0.05 with HCl (0.1M) or NH_4OH (0.1M). The sample-modifier mixtures (600 mL) were purified on UCT Enviro Clean HL DVB (200 mg, 6 mL) SPE cartridges packed with glass wool (2.5 ± 0.2 g). Prior to loading, SPE cartridges were conditioned with MeOH: Acetone (50: 50, *v/v*) (5 mL) and MeOH (5 mL) and equilibrated with ultrapure water, pH 7 (5 mL). Samples were loaded under vacuum through large volume reservoirs (150 mL) on top of the SPE cartridge, at a rate of 6 mL min^{-1}. Once loaded, samples bottles were rinsed with H_2O: MeOH (90:10, *v/v*) (10 mL) and added to the SPE. The SPE cartridge was then washed with a further 5 mL aliquot of H_2O: MeOH (90:10, *v/v*). Cartridges were dried under vacuum (30 min.) and eluted with MeOH: Acetone (50:50, *v/v*) (10 mL) into 15 mL polypropylene tubes. DMSO (500 µL) was added to each sample as a keeper solvent and vortexed (30 s). Samples were evaporated under nitrogen using a TurboVap LV (50 °C, 15–20 psi, 60–90 min) until 500 µL DMSO remained. Extracts were sonicated (2 min.) and vortexed (30 s) prior to filtration through 0.22 µm syringe filters into glass HPLC vials for instrumental determination.

2.3.7 Method validation procedure

There are currently no legislative guidelines available for validation of veterinary residues in water matrix; therefore, a method validation approach was implemented based on a combination of criteria set out in SANTE/11813/2017 guidelines (European Commission, 2017) relating to pesticides in food and European Legislation 2002/657/EC (European Commission, 2002), pertaining to veterinary residues in food. As part of this validation the following performance parameters were examined: identification, selectivity, sensitivity/linearity, trueness, within-laboratory repeatability (WL_r or RSD_r) and within-laboratory reproducibility (WL_R or RSD_{wR}). Further to this, method recovery, limits of detection (LOD) and limits of quantification (LOQ) were also assessed as part of method validation. Validation was performed at concentration levels equivalent to a low, medium and high concentration across the calibration curve, to be consistent with the method sensitivities for the different analytes (described in Table 2-2 and 2-3).

The selectivity of the method was assessed by individually injecting standards and internal standards to check for isobaric interferences by monitoring all transitions. In addition, blank groundwater samples (n = 30, all from different sources) were analysed along with reagent blanks (both spiked with IS and non-spiked with IS) to determine any matrix interferences co-eluting with analytes. To assess linearity, matrix matched calibration curves, with at least seven points, were prepared by fortification of negative controls over a range of concentrations as described above (Table 2-3).

Trueness and Precision were both assessed in terms of within lab repeatability conditions (WL_r) and within lab reproducibility (WL_R) conditions, using fortified negative control samples, given that no certified reference material is available for these analytes in water. The WL_r study involved a negative control sample fortified at each of three validation levels in replicates of n = 6. For WL_R a similar experiment was carried out at the same three concentration levels, with a total of n = 18 replicates analysed over 5 different days (3 days with n = 4 replicates and 2 day with n = 3 replicates). In this case, negative control samples from different sources were used on each of the different days, with different negative controls also used for each of the three validation levels.

The dependence of recovery on analyte concentration was assessed whereby blank water samples were fortified pre- and post-extraction (n = 3) at two different concentrations; 20 ng L^{-1} and 200 ng L^{-1} for all analytes, except BITH, CLOR, MOR and OXY, which were at concentrations of 40 and 400 ng L^{-1}. Recovery was determined by comparison of analyte response in the pre-extraction spiked samples (spiked at the beginning, immediately prior to extraction) to that in the samples spiked post-extraction (spiked at the end, immediately prior to instrumental determination). Use of such approach allowed the effects of matrix on analyte response to be considered in calculating the recovery

LODs and LOQs were determined by fortification of blank samples at concentrations equivalent to the lowest calibrant level. The chromatographs of each analyte, on five different occasions, were visually inspected and the LOD and LOQ were given as the estimated analyte concentration that achieved a signal to noise (S/N) of 3 and 10, respectively, with consideration given to both quantifier and qualifier ions. The LOQ was assessed as the lowest spiking level which satisfied the method performance criteria set out by SANTE for trueness and precision, in combination with the minimum S/N.

2.3.8 Matrix effects

Matrix effects (ME) were assessed using the post-extraction spiking method adapted from Matuszewski et al. (2003) with matrix effects calculated as follows: ME (%) = (B − A/A × 100), where (A) is the response of analyte in neat solvent, and (B) is the response of analyte in matrix extract, spiked post-extraction. Negative control samples (n = 30), from different groundwater and surface water sources in Ireland (spring, boreholes, streams and lakes), were extracted and spiked post-extraction corresponding to a concentration of 100 ng L^{-1} for all analytes, except BITH, CLOR, MOR and OXY at 200 ng L^{-1}. These post-extraction spiked samples were compared to solvent standards to quantify the ion enhancement or suppression due to matrix. Using this approach, negative (−) ME values indicated suppression (decrease in analyte response due to endogenous and/or exogenous matrix components), while positive (+) values indicated enhancement (increase in analyte response due to matrix components).

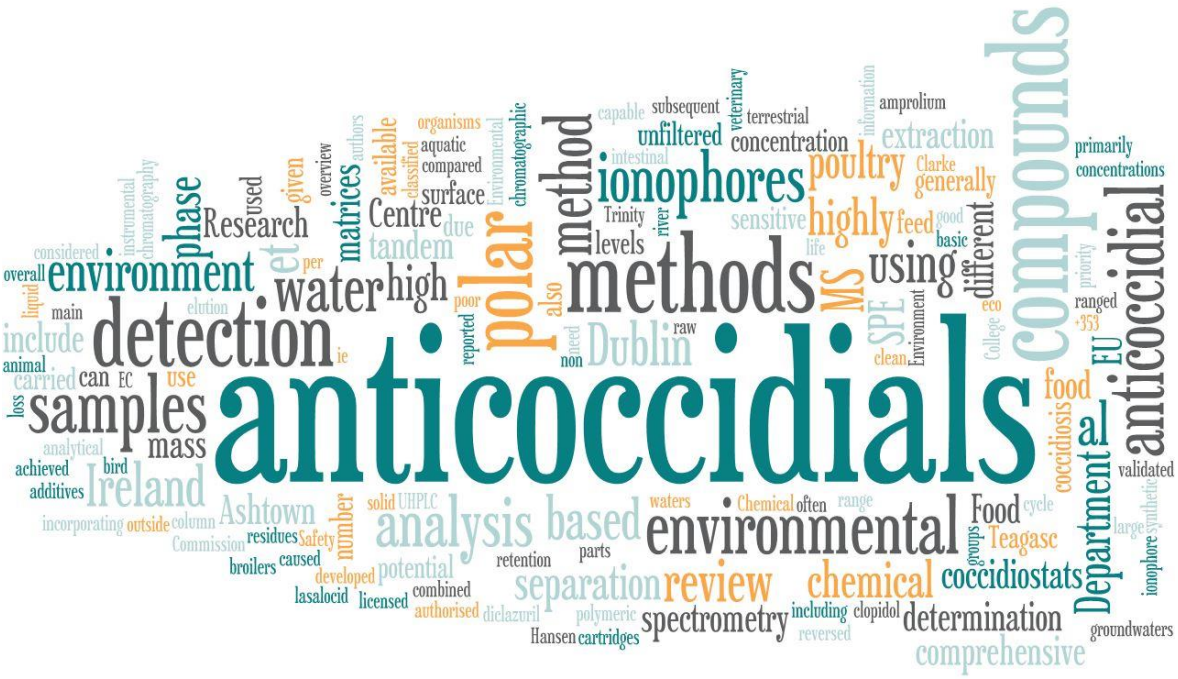
2.4 Conclusions

A comprehensive and sensitive analytical method, based on SPE followed by LC–MS/MS detection, has been developed for the quantitative confirmatory analysis of 38 anthelmintic compounds in raw, unfiltered, environmental water samples and screening analysis for a further two anthelmintic residues. The method has been extensively validated over a broad range of concentration levels, in-line with expected concentration in the environment, based on review of currently available literature. This method is advantageous compared to existing analytical methods for environmental samples because it allows for analysis of a wider range of anthelmintic residues (40), from different structural classes. Of these 40 compounds, 13 of them are metabolites/transformation products, for which currently available methods are lacking. This provides a more comprehensive application to improve understanding of the environmental occurrence of anthelmintics. The method development work carried out showed the impact of sample modification prior to extraction, which in this case aided desorption of some analytes from the sample container. The matrix effect study demonstrated the importance of assessing ion enhancement/suppression due to matrix, as part of method development and validation stages. The results of this study highlighted the significance of incorporating deuterated internal standards into the analytical methodology, which was shown to improve the overall accuracy and precision for the majority of analytes.

This work incorporates deuterated or surrogate internal standards for all 40 compounds. The overall method presented was validated according to appropriate guidelines and deemed to be fit for the purpose intended

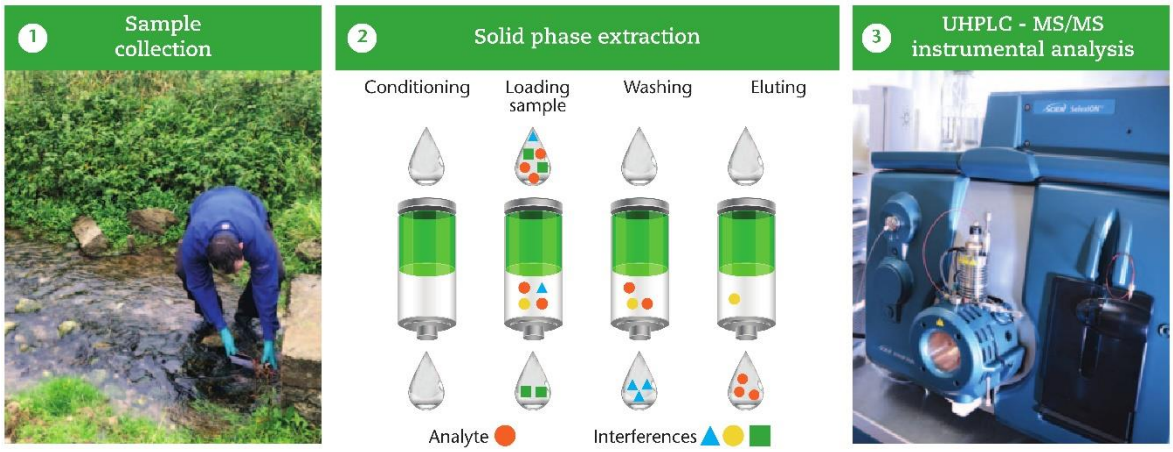
2.5 Author contributions (CRediT)

Conceptualization, D.M., C.C., K.G.R., M.D., L.G. and P.-E.M.; methodology, D.M. and M.D.; validation, D.M.; investigation, D.M.; resources, M.D. and P.-E.M.; writing—original draft preparation, D.M.; writing—review and editing, M.D., C.C., K.G.R., L.G. and P.-E.M.; visualization, D.M., C.C. and M.D.; supervision, M.D., C.C. and K.G.R.; project administration, D.M. and C.C.; funding acquisition, C.C., K.G.R., L.G. and M.D.



CHAPTER 3 – A NEW SENSITIVE METHOD FOR THE SIMULTANEOUS CHROMATOGRAPHIC SEPARATION AND TANDEM MASS SPECTROMETRY DETECTION OF ANTICOCCIDIALS, INCLUDING HIGHLY POLAR COMPOUNDS, IN ENVIRONMENTAL WATERS

Graphical Abstract



Lay Abstract

This work reports on the development of a new analytical method for the determination of a range of anticoccidial compounds, including both the ionophores and synthetic anticoccidials, in unfiltered environmental water samples using solid phase extraction (SPE) with ultra-high performance liquid chromatography detection. This work focused on assessing different SPE sorbents (chemical filters) and elution conditions (to remove the contaminants from the sorbent) for extracting anticoccidial compounds with a wide range of physicochemical properties. The work also focused on developing a new chromatographic separation to allow for the simultaneous analysis of these anticoccidials, including a number of problematic compounds which previously required a separate analysis. The overall developed method allows for the simultaneous determination of 26 anticoccidials, including 6 ionophore feed additives and 20 synthetic anticoccidials, in surface water and groundwater at very low levels, with detection capabilities of the order of parts-per-quadrillion (picogram per litre) to parts-per-trillion (nanogram per litre). The new chromatographic separation allows for the determination of all compounds in the same analysis, with an overall instrumental analysis time of 15 minutes per sample. Validation of the method showed it was fit for purpose, with high accuracy and precision.

A NEW SENSITIVE METHOD FOR THE SIMULTANEOUS CHROMATOGRAPHIC SEPARATION AND TANDEM MASS SPECTROMETRY DETECTION OF ANTICOCCIDIALS, INCLUDING HIGHLY POLAR COMPOUNDS, IN ENVIRONMENTAL WATERS

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Abstract

A sensitive and selective method was developed and validated for the determination of 26 anticoccidial compounds (six ionophores and twenty chemical coccidiostats) in surface and groundwater samples at parts-per-quadrillion (pg L^{-1}) to parts-per-trillion (ng L^{-1}) levels by ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). A range of different analytical columns and mobile phase compositions were evaluated to enhance selectivity and retention of a number of highly polar and basic anticoccidials along with other non-polar coccidiostats. A combined separation, including these problematic polar compounds, was achieved on a phenyl-hexyl column, by binary gradient elution with water/acetonitrile using ammonium formate and formic acid as additives. The anticoccidial residues were extracted from raw, unfiltered, water samples (250 mL) using polymeric divinylbenzene solid phase extraction (SPE) cartridges, with subsequent elution (methanol:acetonitrile:ethyl acetate, 40:40:20, *v/v*) and concentration prior to determination. The method recovery (at a concentration representative of realistic expected environmental water concentrations based on literature review) ranged from 81–105%. The method was successfully validated for 26 anticoccidials, at four concentration levels, in accordance to Commission Decision 2002/657/EC and SANTE/11813/2017 guidelines. Trueness and precision, under within-laboratory reproducibility conditions, ranged from 88–111% and 0.9–10.3% respectively.

Keywords: Chemical coccidiostats; Ionophores; Environmental water; SPE; UHPLC-MS/MS.

3.1 Introduction

Anticoccidials, interchangeably referred to as coccidiostats, are used to control coccidiosis and other protozoan infections in food producing animals (NFRD, 2011; Moloney et al., 2012). Coccidiosis is a parasitic intestinal disease caused by protozoa of the genus *Eimeria*. Anticoccidials can be classified into two main groups: the ionophores which are naturally occurring polyether antibiotic type compounds, and the synthetic/chemical anticoccidials (Clarke et al., 2014). Chemical anticoccidials are generally used at much lower concentrations compared to the ionophores, given that they have higher efficacy toward the parasites (Hansen et al., 2009a).

Poultry have a high susceptibility to coccidiosis, which causes intestinal lesions and diarrhoea in the animal, resulting in poor weight gain and poor feed conversion. Due to the high number of birds housed at any one time, outbreak of infection poses huge economical loss. Very often, the damage to the bird occurs before it becomes symptomatic and hence, if infected, it is often difficult for the bird to recover, given their very short life cycle (approx. 42 weeks) (O'Keefe, 2003). As a result, it is more financially viable to administer anticoccidials prophylactically as opposed to therapeutically, with broilers treated for a large portion of their life-cycle.

In the European Union (EU), there are 11 anticoccidials licensed as feed additives under Regulations 1831/2003/EC (European Parliament, 2003), for use on intensively reared species, primarily poultry (broilers, turkeys, and layers), where the substance is administered in feed. These include the ionophores salinomycin, narasin, monensin, lasalocid, maduramicin and semduramicin, and the chemical anticoccidials robenidine, decoquinate, halofuginone, nicarbazin and diclazuril. In addition, some anticoccidials are authorised in the EU as veterinary medicines as listed under Commission Regulation No 37/2010 (European Commission, 2010), which are used to a lesser extent in poultry, cattle, swine, sheep and rabbits. There are also a number of anticoccidials authorised for use outside of the EU, which include aklomide, arprinocid, clopidol, diaveridine, ethopabate, nequinate and roxarsone (Hansen et al., 2009a; Moloney et al., 2012).

Of the information available, it has been reported that up to 95% of some anticoccidials can be excreted as the unmetabolised active parent drug e.g. diclazuril (85–95%) (Broekaert et al., 2011) and lasalocid (74–77%) (EFSA, 2004). This, combined with the prophylactic use, provides for a potentially persistent source of anticoccidials that can enter the environment, primarily via the spreading of poultry manure and slurry (Boxall, 2010). Once in the environment, these compounds have the potential to: sorb and concentrate in soil, be washed to surface waters by overland flow, or be leached to groundwaters, depending on their mobility and fate, on which information is generally lacking. The main concern with anticoccidials in the environment relates to resistance issues caused by long term exposure to low levels, and potential eco-toxicological effects on aquatic and terrestrial organisms, given the antimicrobial potency of anticoccidials (Hernández et al., 2007; Hansen et al., 2009a; Hansen et al., 2009b). In a prioritisation exercise in the UK, Boxall et al. (2003a) classified 56 different veterinary drugs to be of "high priority" in terms of risk to the environment, based on (a) their potential to reach the environment in large amounts and (b) their hazard to aquatic and terrestrial organisms (based on available eco-toxicity data). Twelve different anticoccidial compounds were included in this high priority group.

There has been a significant amount of work carried out on instrumental detection methods for anticoccidials, with the majority, and most extensive, of these methods relating to matrices of food of animal origin (e.g. poultry eggs, muscle, milk and liver) (Shao et al., 2009; Moloney et al., 2012; Clarke et al., 2013). Clarke et al. (2014) carried out a comprehensive overview of anticoccidial analysis in meat and other food products, providing a good overview of their history and advancements in their analysis and detection techniques. Based on this review, and published methods, liquid chromatography tandem mass spectrometry (LC-MS/MS) is currently considered the most powerful technique for determining anticoccidial residues in complex matrices. Instrumental detection is usually carried out using a reversed phase separation, with detection by tandem mass spectrometry using rapid polar switching electrospray ionisation (ESI). Notably, the Clarke et al. review highlights the complexity of analysis due to the broad range of physicochemical properties of anticoccidial compounds (e.g. highly polar amprolium and clopidol in contrast with some non-polar ionophores), with the authors emphasising the need to improve anticoccidial analysis to include these polar compounds. Since this review, some attempts have been made to incorporate highly polar compounds such as amprolium; however retention and peak shape still remained an issue based on the chromatograms presented (Barreto et al., 2017).

In regard to environmental matrices, none of these comprehensive detection methods developed for food applications have been adapted and applied for environmental samples, with most methods for environmental samples incorporating no more than 12 anticoccidial compounds, and very few methods incorporating both groups of anticoccidials (Martinez-Villalba et al., 2009; Iglesias et al., 2012). Amongst the methods available for environmental water samples, extraction and clean-up is generally performed by solid phase extraction (SPE), typically using reversed phase polymeric sorbents (Cha et al., 2005; Kim and Carlson, 2006; Song et al., 2007; Zhang and Zhou, 2007; Watanabe et al., 2008; Bak et al., 2013a; Sun et al., 2013), eluted with methanol for subsequent evaporation and detection. The best overall method is considered to be that proposed by Herrero et al. (2012) for the determination of five ionophores from river water and sewage treatment plant influent/effluent using Oasis HLB SPE cartridges with good recoveries and sensitivity achieved for river water.

In a comprehensive review assessing analytical strategies for analysis in the environment, Hansen et al. (2009a) decided to report solely on ionophore compounds due to the scarcity of methods for the analysis of chemical anticoccidials in environmental samples. In concluding, the authors expressed an urgent need for development of robust, sensitive methods capable of monitoring both classes of anticoccidials in environmental matrices. Taking all of the above into consideration, the overall aim of this study was to firstly develop a more comprehensive chromatographic separation and detection method for the quantitative confirmatory determination of a larger suite of both ionophore and synthetic/chemical anticoccidials, particularly the highly polar and/or basic compounds, which to date have required separation on alternative column chemistries. This detection method would also include anticoccidials licensed outside the EU, to allow for a broader application in different geographical regions. The second focus of this study was to develop and optimise a sample clean-up procedure based on SPE, capable of extracting these anticoccidials from unfiltered raw samples, for particular application to surface and groundwaters. This extraction procedure would be more advantageous compared to previously reported methods as the analysis of unfiltered samples would avoid the loss of contaminants on filtering, which most methods to date have failed to consider, as was also highlighted by the Hansen et al. review.

3.2 Experimental

3.2.1 Chemicals, standards and consumables

Ultra-pure water (UPW) (18.2 MΩcm) was generated in house using a Millipore water purification system (Cork, Ireland). The following super purity grade solvents ("SpS") were purchased from Romil Ltd. (Cambridge, UK): acetonitrile (MeCN), ethyl acetate (EtOAc), methanol (MeOH) and propan-2-ol (IPA). Dimethyl sulfoxide (DMSO), ethylene glycol (EG), 99.5% deuterated MeOH (MeOH-$_d$), ammonium formate puriss p.a. (puriss pro analysis) and formic acid (HCOOH) (98-100%) were sourced from Sigma-Aldrich (Dublin, Ireland). Acetone puriss and ammonium acetate puriss p.a. (Fluka) (>98%) were purchased from Honeywell Research Chemicals (Honeywell Riedel-de Haen; Seelze, Germany). Acetic acid (CH_3COOH) (100%) and ammonia solution (25% w/v) were obtained from Merck (Darmstadt, Germany). The ammonia solution was used to prepare 0.1 and 0.5M ammonium hydroxide (NH_4OH) solutions for sample pH adjustment. Concentrated hydrochloric acid (HCl) (36%) was sourced from BDH Chemicals Ltd. (Poole, UK) and used to prepare a 0.1M HCl solution for pH adjustment.

Neat analytical standards of aklomide (AKLO), amprolium hydrochloride (AMP), clopidol (CLOP), cyromazine (CYROM), decoquinate (DECO), diaveridine (DIAV), diclazuril (DICLAZ), diminazene aceturate (DIMIN), dinitolmide (DINITOL), 4',4''-dinitrocarbanilide (DNC), ethopabate (ETHO), imidocarb dipropionate (IMIDO), maduramicin ammonium (MAD), monensin sodium salt hydrate (MON), nafamostat mesylate (NAFAM), narasin (NAR), nitromide (NITRO), pentamidine (PENT), piperazine (PIP), robenidine hydrochloride (ROB), roxarsone (ROX), salinomycin monosodium salt hydrate (SAL) and toltrazuril (TOL) were purchased from Sigma-Aldrich Ireland (Dublin, Ireland). Arprinocid (ARPRIN), 3-Amino-2-methyl-5-nitrobenzamide (3-ANOT), buquinolate (BUQUIN), halofuginone hydrobromide (HALO-HBr), isometamidium chloride hydrochloride (ISOMET), nequinate (NEQUIN), toltrazuril sulphoxide (TOL-SO) and toltrazuril sulphone (TOL-SO_2) were purchased from Witega (Berlin, Germany), as were the isotopically labelled internal standards: decoquinate-d_5 (DECO-d_5), dinitrocarbanilide-d_8 (DNC-d_8), ethopabate-d_5 (ETHO-d_5), halofuginone hydrobromide-$^{13}C_6$ (HALO-HBr-$^{13}C_6$), imidocarb-d_8 2HCl hydrate (IMIDO-d_8) and robenidine hydrochloride-d_8 (ROB-d_8). The deuterated Cyromazine internal standard cyromazine-d_4 (CYROM-d_4) was purchased from C/D/N Isotopes Inc. (Quebec, Canada). Semduramicin sodium (SEMD) was obtained

from the Community Reference Laboratory (CRL) (Berlin, Germany), while lasalocid A sodium (LAS) (Dr Ehrenstorfer GmbH, Augsburg, Germany) was sourced through LGC Standards (Middlesex, UK).

Glass amber bottles (1000 mL and 500 mL), were purchased from Sci Chem Scientific and Chemical Supplies Ltd. (Cork, Ireland). Glass wool (both silanised and unsilanised) was purchased Lennox Laboratory Supplies (Dublin, Ireland). Polypropylene tubes (15 mL, conical) were obtained from Sarstedt Ltd (Wexford, Ireland). Large volume SPE reservoirs (150 mL) were purchased from Biotage (Uppsala, Sweden) and connected on top of the SPE cartridge using 1–6 mL adapters purchased from UCT Ireland Ltd, (Wexford, Ireland). Final extracts were filtered through Captiva Econo PTFE 0.2 µm filters from Agilent Technologies Ltd. (Cork, Ireland).

A number of different SPE cartridges were assessed as part of the initial method development steps for sample preparation and clean-up including: Isolute ENV+ (200 mg, 6 mL) and Isolute ENV+/C_{18} dual layered (400 mg, 6 mL) purchased from Biotage (Upsala, Sweden), STRATA-X (200 mg, 6 mL) (Phenomenex, Cheshire, UK), UCT Enviro-Clean HL-DVB (200 mg, 6 mL) from United Chemical Technologies Ireland Ltd. (Wexford, Ireland), and Oasis HLB (200 mg, 6 mL) and Oasis MCX (500 mg, 6mL) from Waters (Dublin, Ireland). The analytical UHPLC column chemistries assessed for the chromatographic separation included: Luna Omega Polar C_{18} (50 × 2.1 mm, 1.6 µm) (Phenomenex, Cheshire, UK), Selectra PFPP (100 × 2.1 mm, 3.0 µm) (UCT, Wexford, Ireland), Triart C_{18} (100 × 2.0 mm, 1.9 µm) (YMC, Kyoto, Japan) and Zorbax Eclipse Plus Phenyl-Hexyl Rapid Resolution HD (100 × 3.0 mm, 1.8 µm) (Agilent, Cork, Ireland).

3.2.2 Preparation of standard solutions

Individual primary stock solutions were prepared by dissolving the appropriate weight of certified standard material in suitable solvents, selected based on solubility. CLOP (0.5 mg mL^{-1}), DIAV, HALO, NICARB, NITRO (all 2 mg mL^{-1}), DICLAZ and ROB (both 4 mg mL^{-1}) were prepared in DMSO. NEQUIN, BUQUIN and DECO (0.1, 1 and 2 mg mL^{-1} respectively) were prepared in 10% (*v/v*) formic acid in MeCN (quinolone solvent). ETHO (2 mg mL^{-1}) was prepared in MeCN, while all remaining analytical standards were prepared in MeOH at a concentration of 2 mg mL^{-1}, except CYROM, MAD, NAR and ROX which

were prepared at 4 mg mL^{-1}. All deuterated or labelled internal standards were prepared at a concentration of 1 mg mL^{-1}, in the same solvent as their corresponding analyte, from which a mixed intermediate solution was prepared for all internal standards, except DECO-$_{d5}$ which remained separate. Internal standards requiring MeOH, were prepared in MeOH-d.

Six mixed intermediate solutions were prepared at a concentration of 25 µg L^{-1}, each containing different analytes as specified in Table 3-1 (Std. Group A–F). In addition, 1 µg mL^{-1} intermediates were prepared for groups A-D. All intermediates were prepared in MeCN, except group C intermediates, which were prepared in quinolone solvent. This solvent was incorporated based on the work carried out by Moloney et al. (2012), who reported the necessity of the added formic acid to keep the group C analytes in solution. A set of eight mixed working calibration solutions (Calibrants 1–8) were prepared in MeCN by dilution of the respective intermediate mixed working solution (A, B and D-F), as described in Supplementary File SI-3.1 Table S3-1. A second series of calibrants for group C compounds were prepared in quinolone solvent. All working solutions were stored at –18 °C or below in glass amber vials with equilibration to room temperature before use.

3.2.3 UHPLC-MS/MS determination

Instrumental determination was performed using an Agilent 1290 Infinity™ II UHPLC system (equipped with an 8 tray multi-sampler and dual needle injector), coupled to an AB Sciex 6500+ quadrupole linear ion trap (QTRAP) mass spectrometer with IonDrive™ technology including a Turbo V source, an IonDrive QJet Guide and an IonDrive High Energy Detector+ (HED). The mass spectrometer was controlled using Analyst® software provided by Sciex (Version 1.7.0.). An Analyst® Device Driver (ADD) application (Version 1.3) provided by AB Sciex, was necessary to interface and control the Agilent LC. Data was processed and reviewed using MultiQuant™ (version 3.0.3) provided by AB Sciex.

3.2.3.1 UHPLC conditions

All analytes were chromatographically separated on an Agilent Zorbax Eclipse Plus Phenyl-Hexyl Rapid Resolution HD threaded analytical column (100 × 3.0 mm, 1.8 µm particle size) fitted with an in-line filter (0.2 µm pore size). A binary gradient elution was performed using 2mM ammonium formate + 0.01% formic acid in water (mobile phase A) and 0.1% formic acid in MeCN (mobile phase B), at a flow rate of 0.6 mL min^{-1}. The gradient starting

condition was 99.9% mobile phase A, with the profile as follows: 0.0–2.0 min (99.9% A), 2.0–4.0 min (70% A), 4.0–8.0 min (30% A), 8.0–11.0 min (30% A), 11.0–13.0 min (0.1% A), 13.0–14.5 min (0.1% A), 14.50–14.6 min (99.9% A) and 14.6–16.5 min (99.9% A). An integrated divert valve was incorporated to divert the LC flow to waste for the first and last 2 min of the gradient. Extracts were injected in pure DMSO, using a 2.5 µL injection volume. The autosampler needle was rinsed after each injection with a H_2O:MeOH:IPA (40:40:20, *v/v*) solution, while a H_2O:IPA (90:10, *v/v*) solution was used for seal wash. The column temperature was maintained at 40 ± 1 °C while the auto-sampler was maintained at 20 °C to prevent solidification of the DMSO extracts.

3.2.3.2 MS/MS conditions

Anticoccidial residue detection was performed using an electrospray ionisation interface with rapid polar switching i.e. in both ESI positive (+) and negative (-) mode. Data was gathered using multiple reactions monitoring (MRM) mode with the acquisition segmented to produce MRM windows around each analyte retention time with a span of 60 or 90 s, dependent on the peak width. Transitions were selected and adapted from the in-house methods described by Moloney et al. (2012), with some additional compounds included. Compound specific parameters were tuned using a teed infusion of individual compounds (100 or 500 ng mL^{-1}), using a Hamilton syringe (10 µL min^{-1}), into the MS source with mobile phase (A:B, 50:50 *v/v*, 0.6 mL min^{-1}). Generic source conditions were used to allow sufficient desolvation and ionisation in the source (± 4500V, 450^{o}C, curtain gas pressure 20 psi and GS1 and GS2 both at 40 psi). The transitions followed for each analyte are as summarised in Table 3-1. The MS/MS source conditions were then optimised for the least sensitive analytes using flow injection analysis (FIA) and the final optimised conditions are summarised as follows: ion spray voltage (+)4500V/(-)4500V; source temperature 550^{o}C; collision gas nitrogen (N_2); CAD gas High; entrance potential (EP) 10 volts; curtain gas pressure 40 psi; ion source gas 1 (GS1) pressure 60 psi; ion source gas 2 (GS2) pressure 60 psi and Q1/Q3 unit resolution. Collision energies (CE) and de-clustering potentials (DP) were optimised for each fragment, and are also summarised in Table 3-1.

Table 3-1 UHPLC–MS/MS conditions for anticoccidial residues and respective internal standards

Analyte	Abbreviation	Std. Group	t_R (min)	Pre-ion (*m/z*)	Product Ions[a] (*m/z*)	[M]	DP (V)	CE (V)	CXP (V)	ESI Polarity	IS
Cyromazine-d_4*	CYROM-d_4	IS	2.53	171.1	86.0	$[M+H]^+$	60	27	10	+	None
Cyromazine*	CYROM	D	2.56	166.9	**84.8**/124.9	$[M+H]^+$	30	25	12	+	Cyromazine-d_4
Roxarsone*	ROX	F	2.90	263.8	**217.8**/90.9	$[M-H]^-$	26	29	20	-	None
Amprolium*	AMP	A	3.61	242.8	**149.9**/94.0	$[M+H]^+$	60	17	14	+	None
Imidocarb-d_8	IMIDO-d_8	IS	4.00	357.1	191.9	$[M+H]^+$	26	39	22	+	None
Imidocarb	IMIDO	E	4.03	349.0	**187.9**/162.0	$[M+H]^+$	120	33	14	+	Imidocarb-d_8
Nafamostat*	NAFAM	E	4.16	348.1	**162.0**/186.9	$[M+H]^+$	120	23	8	+	None
Clopidol	CLOP	B	4.20	191.9	**100.9**/86.9	$[M+H]^+$	131	39	10	+	None
ANOT*	ANOT	E	4.35	196.0	**106.9**/153.0	$[M+H]^+$	26	23	12	+	None
Diaveridine	DIAV	B	4.37	261.1	**122.9**/244.9	$[M+H]^+$	1	29	14	+	None
Pentamidine*	PENT	F	4.56	341.1	**324.1**/120.0	$[M+H]^+$	121	43	12	+	None
Aklomide*	AKLO	F	5.11	201.0	**137.9**/154.8	$[M+H]^+$	36	37	16	+	None
Isometamidium*	ISOMET	F	5.13	461.1	**313.0**/298.0	$[M+H]^+$	36	29	16	+	None
Halo-HBr-$^{13}C_6$	HALO-$^{13}C_6$	IS	5.26	419.9	138.0	$[M+H]^+$	60	25	16	+	None
Halofuginone-HBr	HALO	A	5.27	414.3	**120.1**/100.1	$[M+H]^+$	61	27	14	+	Halo-HBr-$^{13}C_6$
Arprinocid	ARPRIN	B	5.30	278.0	**142.9**/106.9	$[M+H]^+$	20	79	12	+	None
Nitromide*	NITRO	F	5.48	209.9	**166.9**/62.9	$[M-H]^-$	-20	-20	-17	-	None
Dinitolmide*	DINITOL	E	5.52	223.9	**181.0**/77.0	$[M-H]^-$	-15	-14	-21	-	None
Ethopabate- d_5	ETHO-d_5	IS	5.57	243.1	211.0	$[M+H]^+$	35	15	12	+	None
Ethopabate	ETHO	A	5.61	238.0	**135.9**/206.0	$[M+H]^+$	35	35	14	+	Ethopabate- d_5
Robenidine-d_8	ROB-d_8	IS	6.88	342.0	342.0	$[M+H]^+$	100	63	12	+	None
Robenidine	ROB	A	6.92	334.0	**154.9**/137.9	$[M+H]^+$	100	27	18	+	Robenidine-d_8
Toltrazuril-SO	TOL-SO	F	7.19	440.0	440.0	$[M-H]^-$	-15	-6	-11	-	None
Buquinolone*	BUQUIN	C	7.40	362.0	**316.0**/203.9	$[M+H]^+$	20	47	22	+	None
Nequinate*	NEQUIN	C	7.50	366.0	**200.9**/144.9	$[M+H]^+$	20	61	16	+	None
DNC-D_8	DNC-d_8	IS	7.66	308.9	140.8	$[M-H]^-$	-30	-16	-15	-	None

Table 3-1 *continued*

Analyte	Abbreviation	Std. Group	t_R (min)	Pre-ion (*m/z*)	Product Ions[a] (*m/z*)	[M]	DP (V)	CE (V)	CXP (V)	ESI Polarity	IS
4'4''-dinitrocarbanilide**	NICARB**	D	7.69	300.9	106.8	$[M-H]^-$	-35	-52	-13	-	$DNC-D_8$
Toltrazuril-SO_2	TOL-SO_2	F	7.86	455.9	455.9	$[M-H]^-$	-50	-12	-23	-	None
Diclazuril	DICLAZ	A	8.32	404.8	**333.7**	$[M-H]^-$	-10	-28	-35	-	None
				406.8	335.7	$[M-H]^-$	-10	-28	-25	-	None
Toltrazuril	TOL	D	8.38	423.9	**423.9**	$[M-H]^-$	-20	-10	-5	-	None
Deco-d_5	DECO-d_5	IS	8.73	423.1	377.1	$[M+H]^+$	130	33	20	+	None
Decoquinate	DECO	C	8.75	418.1	**372.1**/203.9	$[M+H]^+$	130	55	22	+	None
Semduramicin	SEMD	A	9.43	890.4	**629.3**/727.2	$[M-Na+NH_4]^+$	80	37	4	+	None
Lasalocid	LAS	A	10.03	613.2	**377.1**/595.1	$[M+Na]^+$	130	53	20	+	None
Monensin	MON	A	10.33	693.0	**675.2**/461.1	$[M+H]^+$	80	55	36	+	None
Salinomycin	SAL	F	10.83	773.1	**431.1**/531.1	$[M+H]^+$	120	69	22	+	None
Maduramicin	MAD	D	11.33	934.4	**629.4**/647.4	$[M+H]^+$	60	41	20	+	None
Narasin	NAR	A	11.75	787.3	**431.0**/531.1	$[M+Na]^+$	91	71	22	+	None

t_R= Retention time, M Wt = molecular weight, Pre-ion = precursor ion, *m/z* = mass to charge ratio, [M] = molecular ion, DP = declustering potential, CE= collision energy, CXP = collision cell exit potential, ESI polarity mode; (+) = positive mode and (-) = negative mode IS= internal standard [a] Quantification Ion (bold) / Qualifier Ion
* denotes additional compounds included in this method, that were not included in the Moloney et al. (2012) method,
** Nicarbazin (NICARB) detected as 4'4''-dinitrocarbanilide

3.2.4 Sample collection, control samples and Quality Control

Samples were collected in the same manner as previously described by the authors in discussing the analysis of anthelmintic drug residues (Mooney et al., 2019). Samples were stored in the dark at 4 °C until analysis, which was always carried out within 7 days of collection, as determined by matrix stability studies (SI-3.2). Control samples were also produced as described in the previous paper (Mooney et al., 2019), with the exception of the negative control and QC samples, which in this instance consisted of a 250 mL negative control aliquot contained in a 500 mL glass amber bottle. Similarly, internal QC checks consisted of system suitability checks, negative control samples, solvent blank injections and retention checks.

3.2.5 Procedural matrix calibration

Matrix calibration curves were prepared by fortification of negative control water samples (250 mL) with 100 µL of both sets of calibrant standards (Calibrant 1–8) as described in Appendix 3A Table A3-1. An additional lower calibration point was produced for some analytes by fortification with 100 µL of a solution consisting of Calibrant 1 diluted 1 in 5, while an additional higher calibration point was produced by fortification with 200 µL of calibrant 8 (i.e. 2 × Cal 8). For each analyte, a minimum of 8 points were used to construct a calibration curve, with the individual calibration range for each analyte shown in Table 3-2. All calibrants, quality control samples and samples were fortified with the working mixed internal standard solution (100 µL) and DECO-d_5 (100 µL), corresponding to sample concentration of 100 ng L^{-1} for CYROM-d_4, DECO-d_5, ETHO-d_5, HALO-HBr $^{13}C_6$ and ROB-d_8 and 500 ng L^{-1} for DNC-d_8 and IMIDO-d_8.

Table 3-2 Retention time, calibration range, mean linearity (of n = 5 runs) and results of matrix effects (ME) at two concentrations (n = 25) for each of the 26 anticoccidials

Analyte	t_R ±SD min	Calibration Range (ng L^{-1})	Linearity R^2	Mean ME (%) (n-25)		ME RANGE (%)				RSD (n=25) (%)	
						[Low]		[High]			
				[Low]	[High]	Min	Max	Min	Max	No IS	With IS
Aklomide	5.11 ± 0.01	20.0–250	0.9966	8.6	1.2	-5.9	19.9	-13.3	13.0	6.8	-
Amprolium	3.61 ± 0.03	0.5–250	0.9995	3.3	2.5	-0.3	10.2	-3.9	8.7	2.8	-
ANOT	4.35 ± 0.01	10.0–150	0.9982	0.7	-2.9	-7.8	10.4	-21.9	11.8	7.2	-
Arprinocid	5.30 ± 0.00	0.5–150	0.9996	8.9	2.1	2.6	15.5	-4.2	7.4	2.9	-
Buquinolone	7.40 ± 0.00	0.5–150	0.9993	7.1	-0.2	0.1	12.0	-4.8	3.7	2.1	-
Clopidol	4.20 ± 0.00	0.5–150	0.9996	7.3	2.6	-1.2	13.8	-3.3	7.2	2.7	-
Cyromazine	2.56 ± 0.01	1.0–250	0.9997	14.1	-1.3	0.0	21.0	-5.8	3.7	2.6	0.9
Decoquinate	8.75 ± 0.01	0.5–150	0.9977	10.1	-1.7	1.2	17.3	-5.9	2.5	2.1	1.0
Diaveridine	4.37 ± 0.00	0.5–150	0.9995	7.0	2.6	-1.0	12.1	-1.8	8.6	2.5	-
Diclazuril	8.32 ± 0.00	0.1–250	0.9989	-5.9	1.1	-20.1	6.6	-5.3	11.9	3.9	-
Dinitolmide	5.52 ± 0.01	10.0–150	0.9989	-7.2	-12.4	-22.0	3.4	-19.3	0.1	7.0	-
Ethopabate	5.61 ± 0.00	0.1–250	0.9985	6.4	-1.7	-0.7	14.8	-7.2	1.8	2.3	1.8
Halofuginone	5.27 ± 0.00	0.1–250	0.9993	14.9	1.0	-2.1	24.0	-3.9	6.8	2.8	2.5
Imidocarb	4.03 ± 0.02	-	-	4.9	-2.5	-0.9	10.8	-7.8	2.7	2.6	1.4
Isometamidium	5.13 ± 0.08	-	-	-6.8	2.5	-14.1	1.8	-2.8	8.8	3.0	-
Lasalocid*	10.03 ± 0.00	0.1–250	0.9953	-5.0	-1.1	-14.1	1.3	-5.8	4.3	3.2	-
Maduramicin*	11.33 ± 0.01	1.0–250	0.9981	1.1	5.0	-16.2	20.9	-7.8	18.4	5.7	-
Monensin*	10.33 ± 0.01	0.1 - 250	0.9987	-4.8	3.8	-13.5	3.7	-3.5	11.5	3.8	-
Nafamostat	4.16 ± 0.02	-	-	9.2	-2.2	0.7	17.3	-9.0	2.4	2.9	-
Narasin*	11.75 ± 0.00	0.1–250	0.9985	-5.8	2.1	-16.2	3.1	-2.0	8.7	2.7	-
Nequinate	7.50 ± 0.00	0.5–150	0.9991	8.9	0.6	-0.4	14.7	-3.6	3.3	1.8	-

Table 3-2 *continued*

Analyte	t_R ±SD min	Calibration Range (ng L^{-1})	Linearity R^2	Mean ME (%) (n-25)		ME RANGE (%)				RSD (n=25) (%)	
						[Low]		[High]			
				[Low]	[High]	Min	Max	Min	Max	No IS	With IS
Nicarbazin	7.69 ± 0.00	1.0–250	0.9988	11.1	0.6	-1.1	20.3	-1.7	2.6	1.3	1.0
Nitromide	5.48 ± 0.00	20.0–250	0.9990	-5.3	-3.2	-16.7	7.2	-9.0	0.7	2.5	-
Pentamidine	4.56 ± 0.02	-	-	9.6	0.1	-3.8	19.7	-6.6	6.8	3.3	-
Robenidine	6.92 ± 0.02	0.1–250	0.9973	13.0	0.0	1.1	19.9	-3.4	4.0	2.1	1.0
Salinomycin*	10.83 ± 0.00	0.1–250	0.9985	-7.3	-1.8	-18.7	0.1	-7.2	5.4	3.6	-
Semduramicin*	9.43 ± 0.00	1.0–250	0.9970	-4.0	4.2	-16.9	17.3	-6.1	13.0	5.2	-
Toltrazuril	8.38 ± 0.00	20.0–250	0.9992	6.8	-3.7	-1.9	13.0	-10.3	2.0	3.0	-
Toltrazuril sulphone	7.86 ± 0.00	20.0–250	0.9988	-1.2	3.3	-14.3	11.0	-6.4	16.8	4.6	-
Toltrazuril sulphoxide	7.19 ± 0.00	20.0–250	0.9986	0.7	-0.4	-3.7	5.7	-8.9	11.2	3.5	-

* denotes ionophore compounds, t_R = retention time, SD = standard deviation, R^2 = coefficient of determination, ME = matrix effect, RSD = relative standard deviation, IS = internal standard, [Low] = concentration equivalent to calibrant 2 (2.5 ng L^{-1} for groups A, B and C and 7.5, 20 and 25 ng L^{-1} for D, E and F) [High] = concentration equivalent to calibrant 7 (125 ng L^{-1} for standard groups B, C and E and 200 ng L^{-1} for groups A, D, and F.

3.2.6 Sample preparation - Final SPE method

Water samples were weighed (250 ± 0.1 g corresponding to 250 ± 0.1 mL) directly into glass amber bottles (500 mL) and equilibrated to room temperature. Extracted matrix calibrants were fortified with the working calibrant solutions, with internal standard added to all calibrants, controls and test samples, as described above (Section 3.2.5). All samples were shaken (60 s), modified with MeOH (7.5 mL), and shaken again (60 s). Samples were subsequently adjusted to pH 8.5 ± 0.05 with NH_4OH (0.5M and/or 0.1M). The sample-modifier mixtures (257.5mL) were extracted using UCT Enviro Clean HL DVB (200 mg, 6 mL) SPE cartridges packed with glass wool (2.5 ± 0.2 g). Prior to loading, SPE cartridges were conditioned with MeOH:MeCN (50:50, *v/v*) (5 mL), MeOH (5 mL) and equilibrated with UPW, pH 8.5 (5 mL). Samples were loaded under vacuum at a rate of 5–6 mL min^{-1}. Once loaded, samples bottles were rinsed with H_2O: MeOH (95:5, *v/v*) (10 mL) and added to the SPE cartridge. The SPE cartridges were then washed with a further aliquot of H_2O: MeOH (95:5, *v/v*) (5 mL). Cartridges were dried under vacuum (30 mins) and eluted with MeOH:MeCN:EtOAc (40:40:20, *v/v*) (3 × 4 mL) into 15 mL polypropylene tubes. DMSO (500 µL) was added to each sample as a keeper solvent and then vortexed (30 s). Samples were concentrated under nitrogen using a TurboVap LV (50°C, 15–20 psi, 60–90 min). Extracts (in 500 µL DMSO) were sonicated (5 min) and vortexed (60 s) prior to filtration through 0.22 µm syringe filters into glass HPLC vials (Waters; Dublin, Ireland) containing 300 µL glass inserts, for instrumental determination.

3.2.7 Method validation procedure

There are no definitive legislative validation guidelines available pertaining to the performance of analytical methods for the determination of veterinary pharmaceuticals in environmental water samples. As a result the developed method was validated using a similar approach to that previously described by the authors of this work (Mooney et al., 2019), using an amalgamation of validation criteria from SANTE/11813/2017 (guidelines for pesticides in food) (European Commission, 2017) and European Legislation 2002/657/EC (guidelines for veterinary residues in food) (European Commission, 2002). Validation was performed at four concentration levels (Table 3-3) across the calibration curve, and around a target level (TL) of 100 ng L^{-1} (set based on pesticide legislation in drinking water (European Commission, 1998) and groundwater (European Parliament,

2006)), to be consistent with the method sensitivities for the different analytes. Identification, specificity, selectivity, matrix effects, limits of detection and limits of quantification were all assessed as per the Mooney et al. (2019) approach. Linearity was assessed by examining calibration curves produced with a minimum of 8 points using a $1/x^2$ fit, on five different occasions. Trueness and Precision as relative standard deviation (RSD) were both assessed under within lab repeatability (WL_r) and within lab reproducibility (WL_R) conditions, using fortified negative control samples. The WL_r study involved fortification at each of four validation levels in replicates of $n = 8$. For WL_R, a total of $n = 29$ replicates at each validated concentration level, were analysed over 5 different days ($n = 6$ on each day, except one day with $n = 5$). Matrix effects (ME) were assessed similarly using the post extraction spiking method as described by Matuszewski et al. (2003), using 25 negative control samples from different sources. ME were assessed at two concentration levels, equivalent to calibrant L2 and L7 for each analyte. The criteria adhered to for each parameter are specified in Supplementary Information File SI-3.1 Table S3-2.

Table 3-3 Validation trueness and precision (RSD) under repeatability conditions (WLr) (n = 8) and reproducibility conditions (WLR) (n = 29) at four concentration levels for the 26 anticoccidial compounds, with respective limit of detection (LOD) and limit of quantification (LOQ) values (ng L^{-1})

Analyte	Validated levels L1, L2, L3, L4 (ng L^{-1})	WL_r Trueness (RSD_r) (%)[a]				WL_R Trueness (RSD_{wR}) (%)[b]				LOD [c] (ng L^{-1})	LOQ [d] (ng L^{-1})
		L1	L2	L3	L4	L1	L2	L3	L4		
Aklomide	25, 75, 150, 200	102 (6)	110 (5.9)	98 (8.2)	93 (1.5)	97 (9.1)	102 (7.6)	97 (7.7)	96 (6.3)	5	20
Amprolium	2.5, 50, 150, 200	102 (4.5)	109 (3.5)	109 (3.5)	106 (3.6)	105 (8.6)	104 (5.4)	102 (6.1)	99 (8.8)	0.1	0.5
ANOT	20, 50, 100, 125	105 (6.5)	101 (3.4)	98 (4.1)	92 (5.2)	100 (7.0)	99 (3.3)	94 (8.1)	91 (7.4)	2.5	10
Arprinocid	2.5, 50, 100, 125	101 (3.4)	99 (1.9)	94 (3.6)	91 (1.9)	104 (9.6)	98 (5.6)	95 (6.5)	93 (7.4)	0.1	0.5
Buquinolone	2.5, 50, 100, 125	101 (1.3)	90 (1.8)	86 (3.7)	88 (2.6)	101 (6.8)	91 (4.3)	88 (5.1)	88 (8.0)	0.1	0.5
Clopidol	2.5, 50, 100, 125	104 (2.8)	100 (1.6)	96 (2.4)	93 (1.8)	103 (8.9)	98 (5.3)	94 (4.3)	91 (4.9)	0.1	0.5
Cyromazine	7.5, 50, 150, 200	100 (1.2)	103 (0.8)	101 (0.5)	100 (1)	101 (2.9)	101 (1.4)	100 (0.9)	99 (1.0)	0.1	1
Decoquinate	2.5, 50, 100, 125	113 (1)	105 (1.4)	97 (1.5)	93 (0.9)	111 (3.3)	103 (3.2)	97 (3.0)	95 (4.3)	0.1	0.5
Diaveridine	2.5, 50, 100, 125	100 (2.1)	96 (1.5)	93 (3.9)	92 (2.8)	103 (9.3)	95 (5.1)	91 (5.7)	89 (7.2)	0.15	0.5
Diclazuril	2.5, 50, 150, 200	100 (3.8)	102 (3.7)	97 (1.6)	98 (4.7)	105 (7.5)	104 (5.5)	100 (5.6)	99 (5.8)	0.02	0.1
Dinitolmide	20, 50, 100, 125	103 (3.1)	105 (2.1)	102 (2.4)	102 (2.4)	102 (4.4)	102 (4.9)	99 (8.0)	99 (8.6)	2	10
Ethopabate	2.5, 50, 150, 200	106 (1.9)	106 (2.3)	100 (1.5)	96 (1.8)	110 (9.1)	105 (2.0)	100 (1.9)	97 (2.3)	0.02	0.1
Halofuginone	2.5, 50, 150, 200	96 (3)	104 (2.1)	103 (1.9)	102 (2.1)	104 (8.1)	102 (2.7)	102 (2.6)	101 (2.2)	0.05	0.1
Lasalocid	2.5, 50, 150, 200	112 (1.2)	109 (1.3)	94 (2.6)	88 (1)	110 (3.6)	107 (3.8)	94 (4.7)	88 (4.8)	0.01	0.1
Maduramicin	7.5, 50, 150, 200	106 (3.7)	101 (5.4)	94 (8)	95 (5.9)	102 (6.6)	106 (7.1)	97 (8.1)	95 (9.4)	0.5	1
Monensin	2.5, 50, 150, 200	102 (2.4)	108 (3.6)	104 (3.3)	102 (3.3)	103 (5.6)	106 (7.0)	100 (7.5)	101 (10.3)	0.005	0.1
Narasin	2.5, 50, 150, 200	100 (3)	100 (3.3)	95 (3.7)	91 (2.3)	101 (4.7)	101 (5.2)	93 (3.9)	91 (4.5)	0.005	0.1

Table 3-3 *continued*

Analyte	Validated levels L1, L2, L3, L4 (ng L^{-1})	WL_r Trueness (RSD_r) (%)[a]				WL_R Trueness (RSD_{wR}) (%)[b]				LOD [c] (ng L^{-1})	LOQ [d] (ng L^{-1})
		L1	L2	L3	L4	L1	L2	L3	L4		
Nequinate	2.5, 50, 100, 125	96 (1.2)	87 (1.8)	86 (3.4)	87 (2.5)	101 (3.9)	90 (3.8)	88 (5.5)	89 (7.6)	0.1	0.5
Nicarbazin	7.5, 50, 150, 200	104 (0.7)	104 (1.8)	102 (1.5)	99 (1.9)	103 (1.7)	105 (1.5)	101 (1.5)	100 (1.5)	0.1	1
Nitromide	25, 75, 150, 200	103 (2.3)	101 (1.5)	101 (1.9)	99 (2.3)	98 (7.5)	100 (3.9)	99 (4.9)	98 (4.5)	5	20
Robenidine	2.5, 50, 150, 200	107 (1.5)	104 (0.9)	103 (1.3)	104 (0.6)	107 (2.9)	101 (1.9)	101 (1.4)	101 (1.6)	0.03	0.1
Salinomycin	2.5, 50, 150, 200	98 (3.4)	100 (3.1)	96 (4.6)	93 (2.4)	100 (5.6)	99 (5.6)	93 (5.8)	91 (6.4)	0.02	0.1
Semduramicin	7.5, 50, 150, 200	104 (4.4)	106 (7.7)	93 (4.7)	90 (4.2)	100 (5.6)	99 (6.2)	93 (9.6)	91 (8.5)	0.25	1
Toltrazuril	25, 75, 150, 200	102 (2.1)	101 (1.3)	102 (1.7)	99 (2)	99 (4.7)	99 (2.6)	100 (2.8)	99 (3.1)	4	20
Toltrazuril sulphone	25, 75, 150, 200	97 (2.3)	100 (3)	100 (3.1)	102 (2.4)	98 (3.1)	99 (3.2)	99 (3.1)	99 (3.0)	10	20
Toltrazuril sulphoxide	25, 75, 150, 200	97 (2.1)	98 (1.9)	99 (2.2)	98 (1.5)	99 (5.4)	100 (2.1)	99 (3.5)	98 (4.0)	4	20

[a] WL_r =Within-laboratory repeatability while RSD_r = Relative standard deviation under repeatability conditions,

[b] WL_R=Within-laboratory reproducibility, while RSD_{wR} = Relative standard deviation under reproducibility conditions

[c] LOD = Limit of Detection based on S/*N* = 5,

[d] LOQ = Limit of Quantitation based on S/N = 10., L1, L2, L3 and L4 refer to each of the four levels at which the validation was performed, equivalent to calibration points 2, 4, 6 and 7 respectively.

3.3 Results and Discussion

3.3.1 Method development:

3.3.1.1 UHPLC-MS/MS

Precursor and product ions were assessed by teed infusion of individual analytes along with mobile phase into the MS, with detection using generic source parameters that were further optimised by flow injection analysis (FIA) once transitions were selected (final conditions as in Section 3.2.3.2). This approach was used as mobile phase was necessary to assist with the formation of particular adducts. The product ion transitions obtained and selected (Table 3-1) were in agreement with the in-house method developed by Moloney et al. (2012) and consistent with those reported amongst literature, as summarised by the Clarke et al. (2014) review. NICARB was detected as its active component dinitrocarbanilide (DNC). Semduramicin-sodium was detected and fragmented using the 890 *m/z* precursor, which is produced by loss of the free sodium and subsequent formation of an ammonium adduct (895.1 - 23 + 18 *m/z*). Fragments (833.2 *m/z* and 851.1 *m/z*) were also obtained for the protonated semduramicin sodium molecular ion (896 *m/z*), however intensities were not very reproducible. TOL, TOL-SO and TOL-SO_2 proved difficult to fragment in either ESI (+) or (-), as experienced and discussed by previous authors. No product ions were achieved for TOL-SO and TOL-SO_2, however some product ions were obtained for TOL, as follows: *m/z* 371, *m/z* 367, *m/z* 99 and *m/z* 42. The authors were unable to verify any of these transitions given that no other method has been published with similar product ions, except for *m/z* 42, which may be unspecific and prone to background interference for such a small fragment ion.

A number of additional compounds, not included in the Moloney et al. (2012) paper, were incorporated as highlighted in Table 3-1. Tuning experiments, for the majority of these compounds, showed protonated $[M + H]^+$ molecular ions, with the exception of NITRO and DINITOL (dinitolmide also called zoalene) which formed deprotonated $[M–H]^-$ ions. The products formed from these additional compounds were in agreement with those included in the Clarke et al. (2014) review, or other literature (Wei et al., 2011; Chang et al., 2019; Rusko et al., 2019), with the exception of AKLO and ANOT. Clarke et al. noted that AKLO does not easily fragment, thus is monitored using the deprotonated $[M–H]^-$ *m/z* 199 only, and therefore is unsuitable for confirmatory analysis. In this current work, AKLO was monitored

using the protonated molecular ion *m/z* 201, with product ions observed at *m/z* 183, 155, 138 and 110, with the *m/z* 138 and 155 ions selected as quantifier and qualifier ions respectively. The quantifier ion monitored in this experiment for ANOT was consistent with other reported literature, however a *m/z* 133 qualifier fragment monitored by Wu et al. (2011) was not observed in this current work. Instead, a *m/z* 153 ion was monitored as a qualifier, likely formed by cleavage of the amide group.

Four different UHPLC column chemistries were assessed, namely PFPP, Triart C_{18}, Omega polar C_{18} and phenyl-hexyl. Initial work indicated that both the PFPP and phenyl hexyl columns showed good retention of most compounds, including the problematic highly polar compounds, which are not well retained by reversed phase chromatography chemistries and are normally analysed by HILIC phases. Further assessment of the PFPP indicated problems with drifting and inconsistent retention times for a number of compounds including CLOP, IMIDO, ISOMET and NAFAM. It is proposed that this issue is likely due to the capability of the PFPP stationary phase to operate in both reversed phase and HILIC mode, where the very polar basic compounds are retained initially by reverse phase interactions; however as the percentage of organic phase increases, the retention mechanism switches to HILIC mode. Efforts to address this issue resulted in a significantly increased run time, and as a result PFPP was omitted from further consideration, with phenyl-hexyl selected for final consideration. The particular phenyl-hexyl phase used contains a special high purity ZORBAX support that is designed to reduce or eliminate strong adsorption of basic and highly polar compounds.

A number of authors have reported improved retention and peak shape for a number of anticoccidials by incorporation of formic acid (HCOOH) into mobile phases (Moloney et al., 2012; Chang et al., 2019), therefore the effect of varying concentrations of HCOOH (0.01–1%, *v/v*), in both A and B mobile phases, was assessed using the phenyl hexyl column. Acetic acid was also assessed as a commonly used alternative additive. Chang et al. (2019) also reported the use of ammonium formate to further improve peak shape, and hence, varying concentrations (1–10 mM) of ammonium formate in mobile phase A were also assessed. Optimal results for 31 different anticoccidial compounds were achieved when using a binary gradient separation on the phenyl hexyl column using a 0.01% HCOOH and 2 mM ammonium formate aqueous phase (mobile phase A) and a 0.1% HCOOH in MeCN organic phase (mobile phase B). Higher concentrations (0.1%) of HCOOH in mobile phase

A, had a negative effect on peak shape and intensity for some analytes, as did the use of the acetic acid additive. In addition, a number of different injection solvents, including DMSO, EG and H_2O:MeCN (80:20, *v/v*) were assessed, with DMSO achieving better sensitivity and peak shape for a number of compounds, including AMP and CYROM.

The gradient profile was optimised in order to reach optimal chromatographic separation, with the overall conditions as previously described in Section 3.2.3.1. All 31 anticoccidials and the six internal standards were successfully eluted within the first 12 minutes of the gradient, as demonstrated by the extracted ion chromatograms (EIC) in Figure 3-1. After elution of the compounds, the gradient was held at 99.9% B for 1.5 min to remove any less polar co-extractives from the column. During this period the LC continued to flow directed into the MS source, with the organic solvent anticipated to provide some cleaning of the ion source probe and spray plate. Subsequently, the gradient was returned to the starting point (99.9% A), with a minimum 2 min hold determined to be necessary for column re-equilibration. On injecting a solvent standard on a number of different occasions (5 different runs), the gradient was found to be robust and reproducible, with the variation in retention times for all analytes ≤0.02 min (Table 3-2), except for AMP (≤0.03 min) and ISOMET (≤0.08 min). All analytes satisfied the SANTE criterion (± 0.1 min)(European Commission, 2017). Retention was also verified by injection on columns with different product batch numbers, with no adjustment necessary to retention windows.

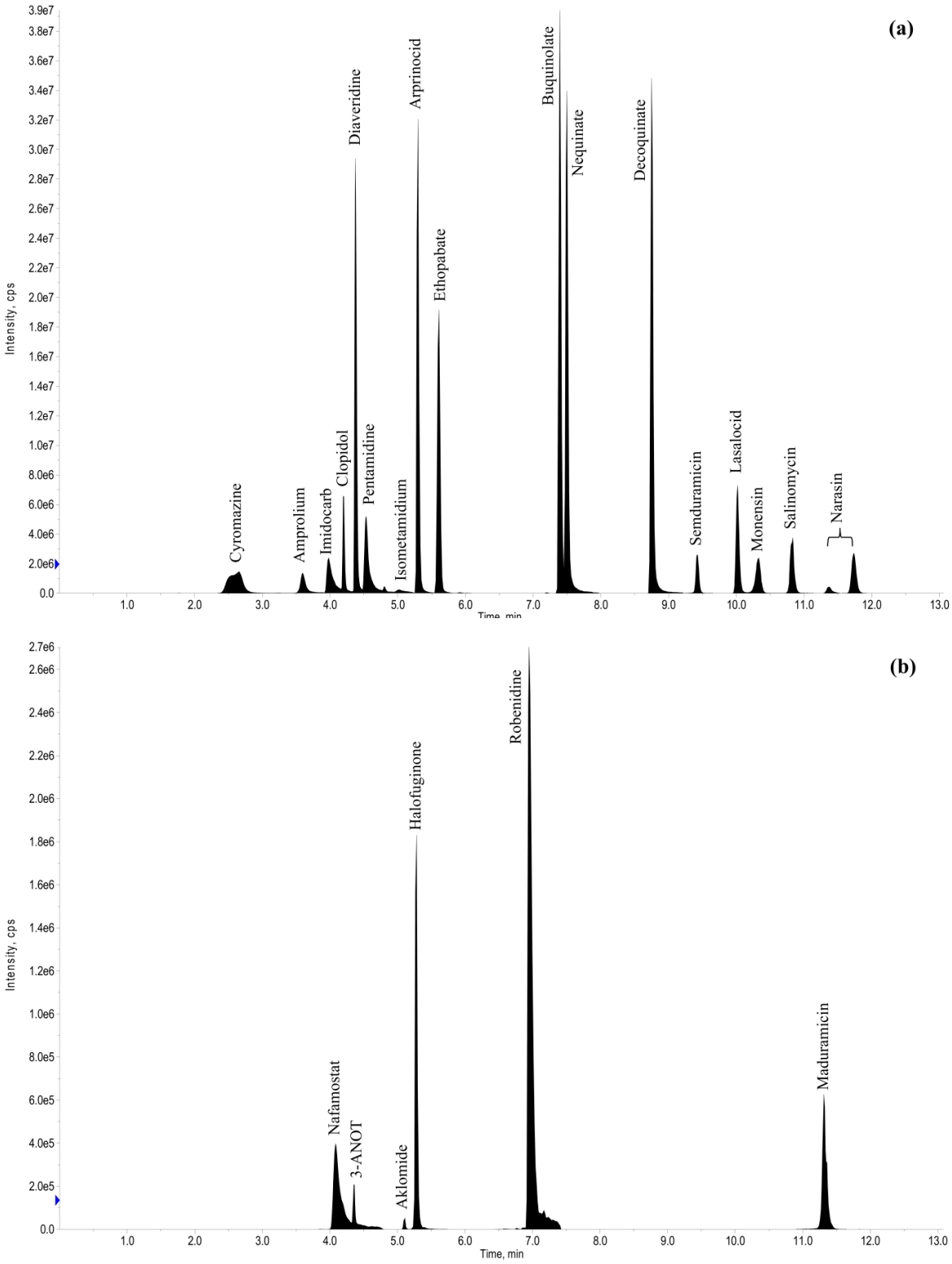


Figure 3-1 Overlay of LC-MS/MS extracted ion chromatograms (EIC) for all 31 anticoccidial analytes (positive mode (a-b) and negative mode (c)) at concentrations equivalent to calibrant level L2 (2.5/7.5/20/25 ng L^{-1}) (Table A3-1), and the seven internal standards (d), in a fortified blank water sample.

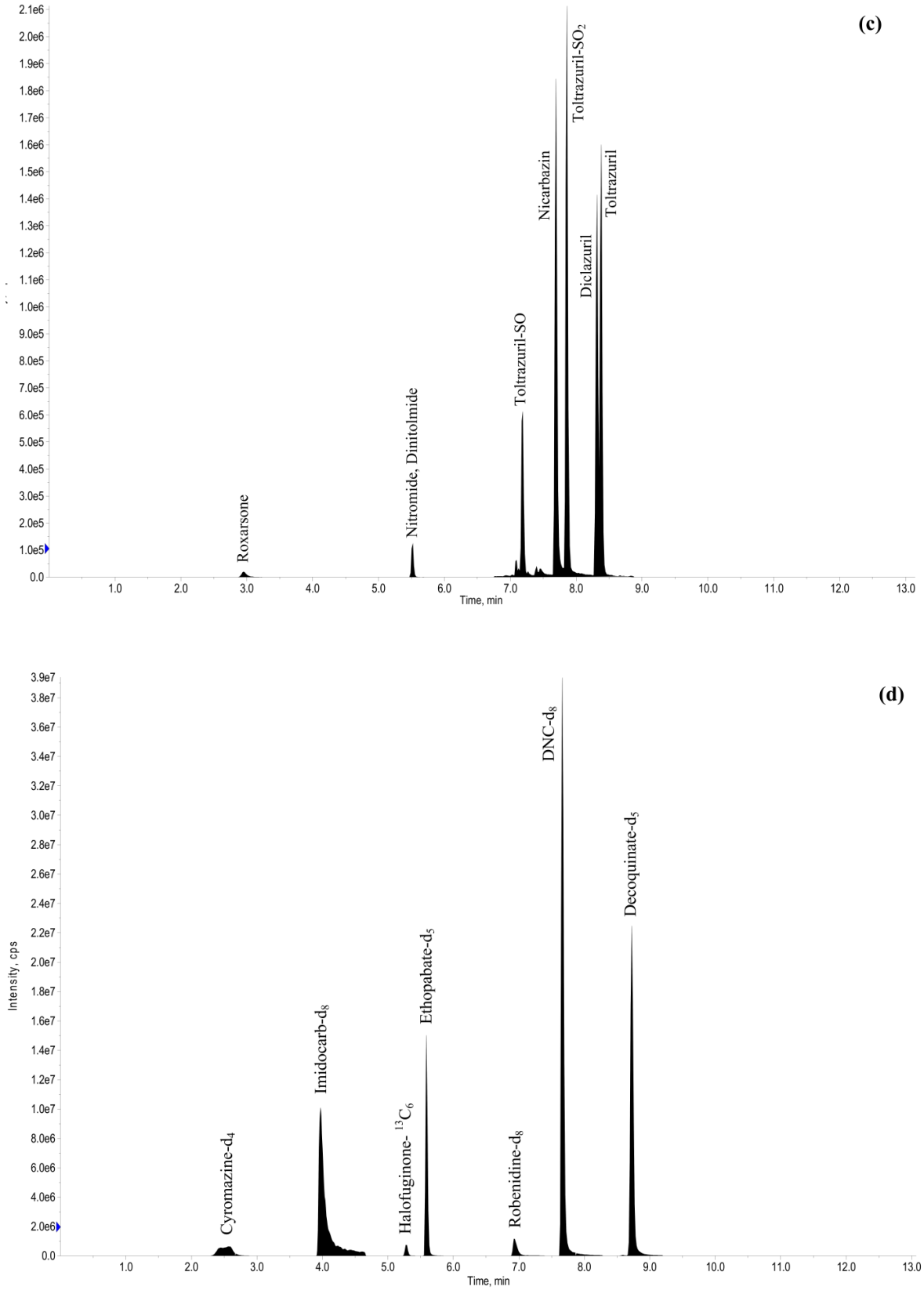


Figure 3-1 *Continued*

3.3.1.2 Sample preparation

The development and optimisation of a SPE procedure for anticoccidials was carried out using an approach similar to that described for anthelmintic residues (Mooney et al., 2019), with the main steps depicted in Figure 3-2. Six different polymeric sorbents (described in Section 3.2.1) were assessed as part of this work for the extraction of anticoccidials from water, given that they are the most commonly used amongst literature (Cha et al., 2005; Kim and Carlson, 2006; Song et al., 2007; Zhang and Zhou, 2007; Watanabe et al., 2008; Bak et al., 2013a; Sun et al., 2013). These included five different reversed phase sorbents and one mixed mode phase used for the extraction of basic compounds with cationic functional groups. The HLB, ENV+ and HL-DVB cartridges all performed similarly, with satisfactory recovery (>70%) for the majority of analytes, with the exception of CLOP, DIAV, HALO, IMIDO, ISOMET, NAFAM and PENT, which gave lower recovery (<50%), while AMP and CYROM demonstrated recoveries of 50 and 60% respectively. The dual layered ENV+/C_{18} cartridge also showed similar results, although the loading rate was much slower and a higher vacuum required. Recoveries of a number of the poorly recovered basic compounds were improved using the MCX cartridge, however this was at the expense of less basic and neutral compounds such as the toltrazurils (TOL, TOL-SO and TOL-SO_2), NICARB, DICLAZ and the quinolones (BUQUIN, DECO AND NEQUIN) which were not retained on this sorbent phase. Overall, the HL-DVB cartridge was selected for further assessment, given that better reproducibility (all <15% RSD) and more consistent flow rates were achieved compared to the other cartridges.

Further spiking experiments were carried out to assess the recovery of analytes on the HL-DVB cartridge. On spiking directly onto the cartridge (as opposed to loading in water), all analytes achieved satisfactory recoveries (69–116%) except HALO (22%), indicating that recovery losses occurred prior to, or during, the loading of samples onto the SPE cartridge. To further improve recovery, six different elution solvent compositions (described in Figure 3-2) were assessed, with the elution volume restricted to 12 mL due to tube size and evaporation time in the TurboVap LV evaporator. MeOH, MeCN and MeOH:MeCN (50:50, *v/v*) gave the best overall recovery results, but the MeOH:MeCN mixture provided better precision. Results indicated that EtOAc did not improve the recovery of analytes, however it provided enhanced sensitivity for analytes including the toltrazurils and two ionophores, namely, NAR and SAL. This was attributed to the more hydrophobic EtOAc extracting

fewer polar interferences. Additional elution compositions incorporating EtOAc were assessed, with the overall optimal elution solvent determined to be MeOH:MeCN:EtOAc (40:40:20, *v/v*). On assessing elution volumes, the 12 mL volume was maintained given there was no significant increase in recovery with the larger volumes. Following optimisation of the elution conditions, further experiments were carried out to identify the possible cause of lower recoveries for some analytes, namely AMP, CYROM, HALO, IMIDO, ISOMET, NAFAM and PENT. Breakthrough experiments (two stacked cartridges eluted and analysed separately) showed minimal breakthrough of analytes. Analysis of the sample bottle rinsate (rinsed with elution solvent) indicated that there was minimal adsorption of analyte to the bottle given that no more than 5% of any analyte was detected in rinsate.

The Water Framework Directive (European Parliament, 2000) and the Environmental Quality Standards Directive (European Commission, 2008a) require the measurement of "whole water" concentrations of pollutants (including both dissolved fractions and suspended solid fractions). Filtration of water samples prior to analysis may consequently remove any contaminants sorbed to suspended solids in the sample, therefore does not allow for the measurement of whole water concentrations. Glass wool was incorporated into the SPE procedure to allow for the analysis of the water without filtration, with the glass wool eluted simultaneously with the SPE cartridge. The glass wool was also necessary to prevent blocking of the SPE cartridge by the unfiltered samples. In order to investigate the effect of the glass wool on recovery, experiments were carried out using ultrapure water in which analytes were extracted with and without glass wool, assessing both silanised and unsilanised glass wool. Results indicated that IMIDO, ISOMET, NAFAM and PENT were strongly retained to active sites on the unsilanised glass wool, with subsequent elution failing to remove these analytes from the glass wool. However, use of the unsilanised glass wool proved beneficial for a number of analytes (AMP, ARPRIN, CLOP and CYROM) with up to a 70% improvement in recovery compared the use of silanised or no glass wool, indicating that the recovery of these compounds was primarily due to adsorption to the unsilanised glass wool, as opposed to retention on the sorbent. Overall, better recoveries were achieved for a greater number of analytes using the unsilanised glass wool.

Sample modification experiments assessed the use of organic modifier (MeOH, 0–30%) and pH adjustment (pH 2–10) to address the retention of analytes to the unsilanised glass wool. Addition of >10% MeOH modifier demonstrated a notable decrease in recovery for a few

compounds (e.g. AMP and CYROM), with recoveries dropping below 20%, while 15 other analytes showed a slight improvement in recovery with higher modifier, particularly the ionophores. Further experiments looked at refinement of the modifier, with the addition of 3% MeOH modifier selected as the optimum, despite no improvement in recovery of IMIDO, ISOMET, NAFAM and PENT. These findings are somewhat consistent with those reported by Song et al. (2007) who reported the use of approx. 9% MeOH. A pH range between 8.5 and 10 produced the best overall results, with improved recovery demonstrated for a number of compounds, namely the ionophores, AMP, CYROM and HALO. A pH of 8.5 was selected for the final method, given that there was evidence of precipitation of some compounds when the pH was adjusted to 10. The improved recovery of AMP and CYROM is proposed to be due to reduced adsorption of analyte on the glass wool, and more retention on the reversed phase sorbent as the analytes are fully unionised at the higher pH. This selected pH is also consistent with the findings of the Hansen et al. (2009a) review, which suggested a range of pH 7–9, as reported amongst literature, to be sufficient for extraction of the ionophore anticoccidials. At pH values greater than their pK_a (reported as 4–8 (Hansen et al., 2009a; Bak et al., 2013a; Bak et al., 2013b)), the ionophores remain un-protonated and form neutral highly lipophilic complexes with cations, allowing for better retention on the reverse phase SPE.

The overall optimised conditions for the final method (as described in Section 3.2.6) were assessed at two levels (one low and one high), with concentrations of each analyte equivalent to calibrant L2 and calibrant L7, respectively. The overall recovery results are as presented in Figure 3-3. The SPE procedure was unsuitable for the extraction of four compounds (IMIDO, ISOMET, NAFAM and PENT), due to what is proposed to be the lack of retention on the cartridge, or the retention and insufficient elution of analyte from the glass wool. For the other analytes, at the lower concentration, the recoveries ranged from 81–105%, with precision ranging from 0.9–8.8% RSD. At higher concentrations, recovery of some analytes was slightly lower, however the minimum criteria were satisfied (recovery of 70–120%) with recoveries ranging from 77–105% and precision between 0.8–5.8 %.

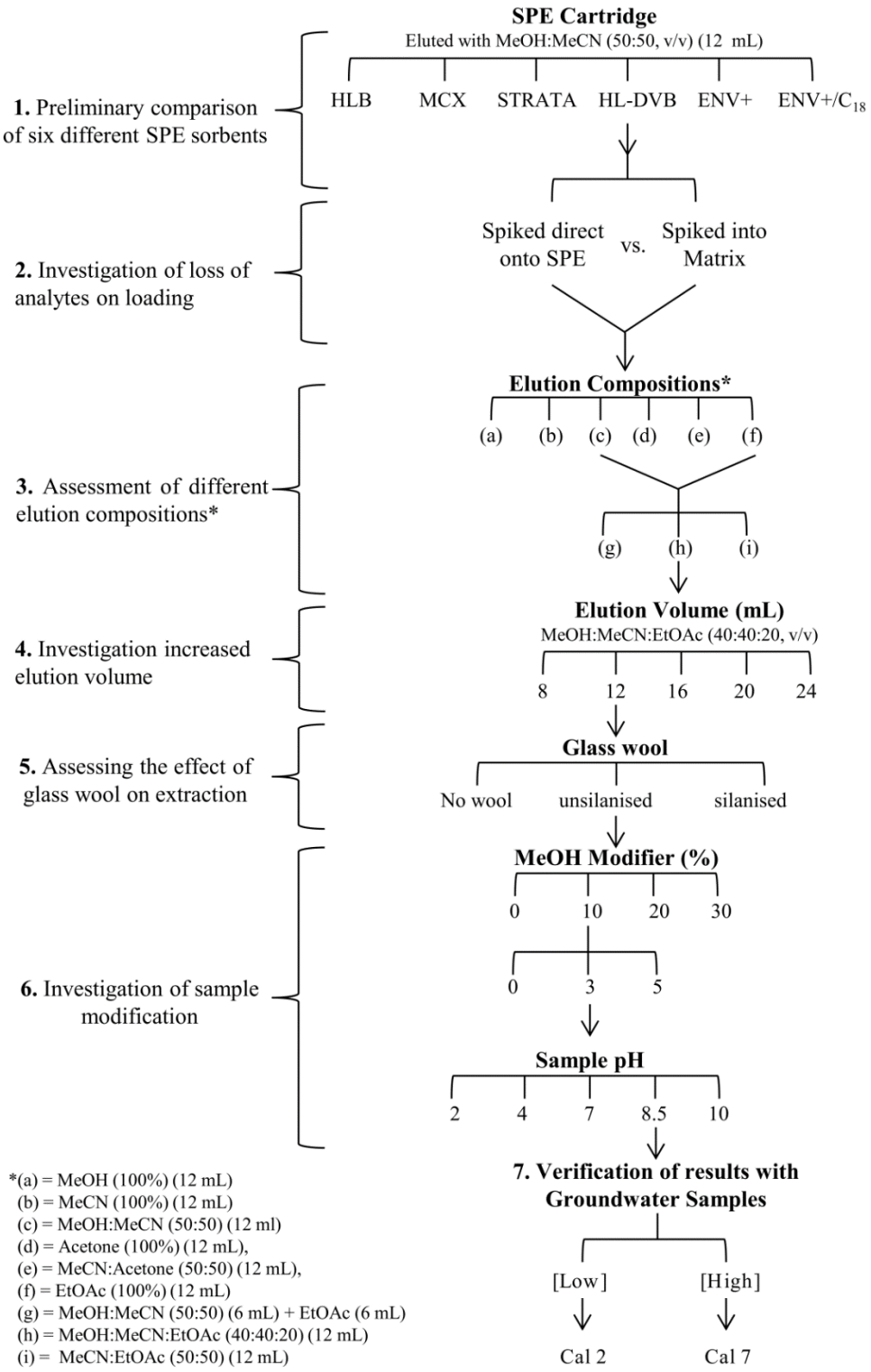


Figure 3-2 Summary of the main steps carried out as part of the development and optimisation of the SPE procedure for extraction of anticoccidial residues from water.

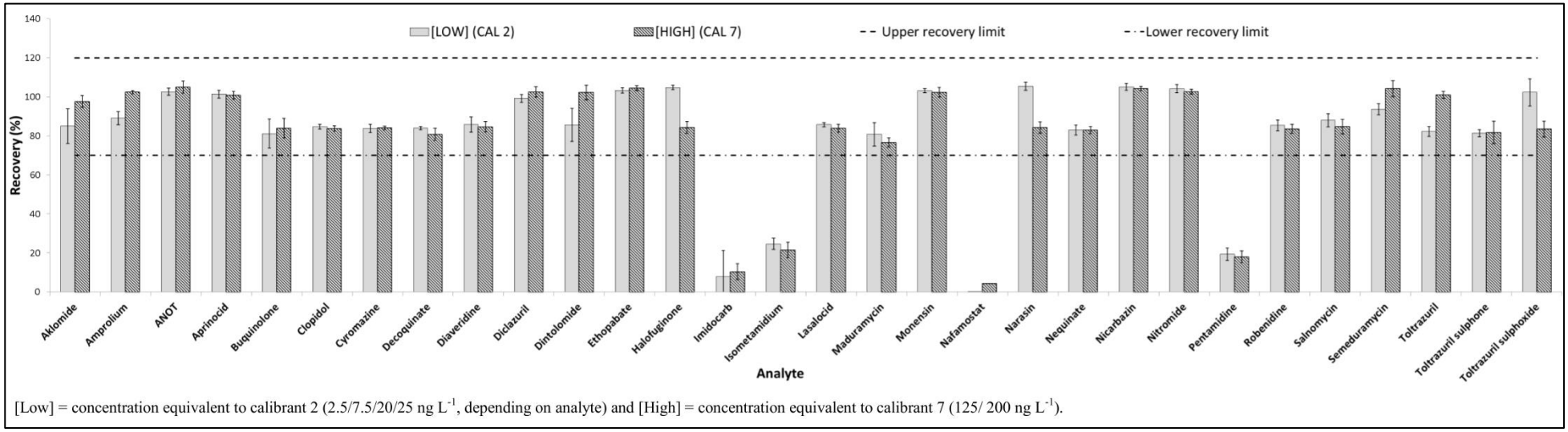


Figure 3-3 Overall mean recoveries and precision (% RSD shown by error bars) (n = 3) for all anticoccidial compounds, at two concentrations using the final optimised conditions: 250 mL environmental water samples, modified with MeOH (7.5 mL) and pH adjusted to pH 8.5, extracted using UCT-HL-DVB (200 mg, 6 mL) SPE cartridges, washed with MeOH:H_2O (95:5, *v/v*) and eluted with MeOH:MeCN:EtOAc (40:40:20, *v/v*) (3 × 4 mL).

3.3.2 Method validation

3.3.2.1 Identification

For each compound, one precursor and two daughter ions were monitored giving a total of four identification points, satisfying the confirmation criteria set out in 2002/657/EC. In some cases (e.g. DICLAZ), five points were achieved by monitoring two different precursor ions. TOL, TOL-SO and TOL-SO_2 failed to meet confirmatory criteria due to insufficient identification points, as a result of the poor fragmentation, commonly reported amongst literature. However, these three analytes were still incorporated for screening purposes. The 2002/657 criterion for relative retention time (RRT, ≤2.5% deviation) was adhered to and satisfied for all analytes. For ion ratio (R, relative intensities), the SANTE criterion of 30% (ΔR) was applied, given the value specified in 2002/657/EC varied from 20–50% (ΔR) depending on the magnitude of the value. In this work, the ion ratio criteria of < 20% deviation were for the majority of analytes.

3.3.2.2 Specificity, selectivity, linearity, limits of detection (LOD) and limits of quantification (LOQ)

No cross-talk or isobaric interferences were observed on injecting analytes and internal standards. The selectivity of the method was initially evaluated through application to 30 different groundwater and surface water samples collected from different sources. No major matrix interference peaks were observed at the same retention time of the analytes.

Linearity was assessed by visual inspection of calibration curves and by verification of residuals and coefficient of determination (R^2) values. Acceptable linearity was set as $R^2 \geq 0.98$ (2002/657) and residual deviations of no greater than ± 20% from the calibration plot. The majority of curves were produced with using a linear fit and $1/x^2$ weighting, however a number of analytes (ARPRIN, CLOP, DIAV, BUQUIN and NEQUIN) required a quadratic fit, attributed to the detector approaching saturation at the higher concentrations. For almost all analytes, mean R^2 values (n = 5 runs) were >0.99 (Table 3-2) meeting the validation criterion. The one exception was ROX, with insufficient linearity achieved through all validation runs, thus this analyte was omitted from the method.

The LOQ was determined as the lowest spiking level which satisfied the method performance criteria set out by SANTE for trueness and precision, in combination with the minimum signal to noise (S/N) (Supplementary File SI-3.1 Table S3-2). The LOQ for the majority of analytes corresponds to the lowest calibrant level of the calibration curve, ranging from 0.1–20 ng L^{-1} as summarised in Table 3-3. Adhering to minimum performance capabilities specified for pesticides under Council Directive 98/83/EC (European Commission, 1998) and assuming similar applicability to anticoccidials, the method LODs were required to be ≤ 25 ng L^{-1} (calculated as ≤ 25% of the specified parametric value for pesticides of 0.1 µg L^{-1}). LODs, as summarised in Table 3-3, ranged from 0.005 to 5 ng L^{-1} (ppt; parts-per-trillion), thus all analytes satisfied the minimum performance capability criterion. In terms of sensitivity, this developed method performs similar to, or better than other methods (see Section 3.4), with detection capabilities as low as part-per-quadrillion (ppq; pg L^{-1}) levels.

3.3.2.3 Matrix effects (ME)

Traditionally, ME are calculated using the formula first described by Buhrman et al. (1996):

$$ME\ (\%) = 100 - (B/A) \times 100 \quad \text{(Eq. 1)}$$

where A is the response of analyte in neat solvent and B is the response of analyte at the same concentration, in post spiked matrix extracted samples. However, this approach can be counter-intuitive, given that a resulting negative ME value represents ion enhancement (increase in response), while a positive value indicates ion suppression (decrease in response). In an attempt to avoid such confusion, Matuszewski et al. (2003) used an adapted approach whereby they measured ME as "absolute ME" calculated as (B/A × 100), in which a resulting ME value >100% indicated ion enhancement, while values <100% indicated suppression.

In this paper, a similar approach to Matuszewski et al. was used, where matrix effects were assessed at two levels, one low (Cal L2) and one high (Cal L7), and calculated as follows:

$$ME\ (\%) = (B - A)/A \times 100 \quad \text{(Eq. 2) (Hall et al., 2012)}$$

Using this approach, negative (-) ME values indicated suppression (decrease in analyte response due to endogenous and/or exogenous matrix components), while positive (+) values indicated enhancement (increase in analyte response due to matrix components). The mean matrix effects (n = 25) of analytes at the higher concentrations (equivalent to Cal L7, validation L4) ranged from –12% for DINITOL (analyte suppression) up to +5% for MAD (enhancement), satisfying the SANTE criteria (ME ± 20%). The range of ME for each individual analyte across the entire 25 samples is shown in Table 3-2. The most suppression in any one sample was 22% (ME −22%) for ANOT (as demonstrated in Figure 3-4(b)), while the highest enhancement in any one sample was observed for MAD (+18%) (Figure 3-4(a)). Very good precision was demonstrated between the 25 different samples, with RSD values for each analyte ranging between 1.3 and 7.2%. At lower concentrations (equivalent to Cal L2, validation L1) the effect of matrix was slightly more prominent, with the mean ME ranging from −22% (suppression) to +24% (enhancement). Precision at the lower concentration, however, was still satisfactory, with RSD for all analytes <9.9%. Isotopically labelled internal standards were incorporated into the method for six anticoccidials, with the precision further improved for these six analytes when the IS was employed for quantification. Suitable internal standards were not available for the majority of analytes and as a result matrix calibration was employed to address any potential matrix effects, further satisfying validation criteria.

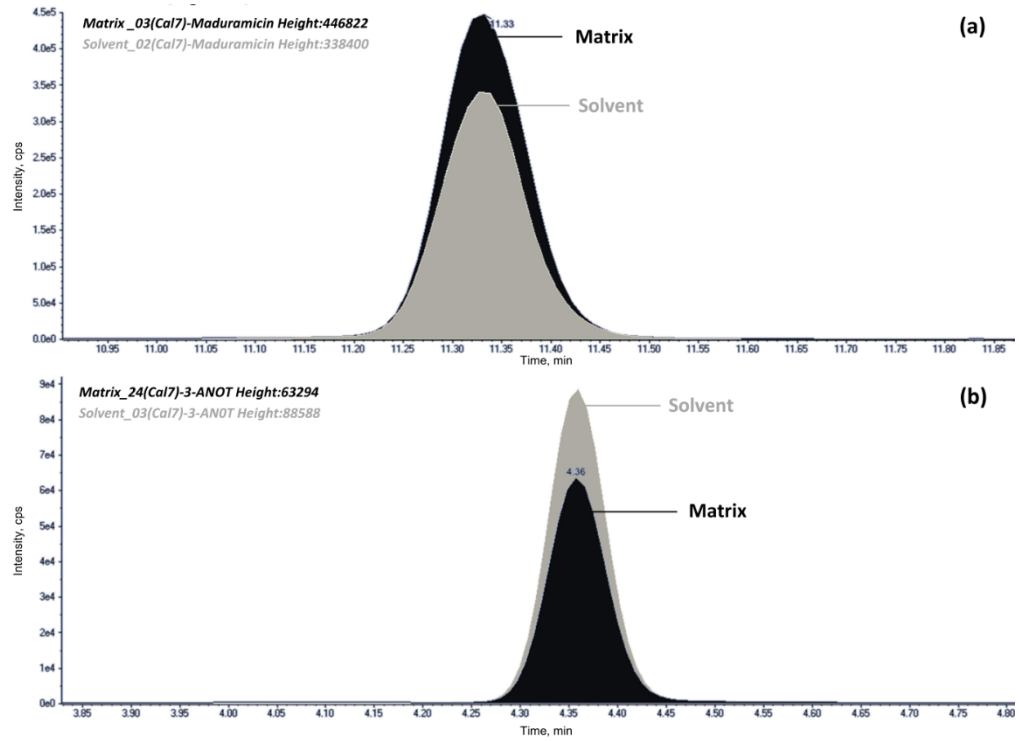


Figure 3-4 Extracted ion chromatogram (EIC) overlay of quantifier ions for a spiked solvent standard (grey) and spiked matrix sample (black), spiked at concentration equivalent to calibrant level L7 (125/200 ng L^{-1}), demonstrating (a) response enhancement for maduramicin and (b) suppression for 3-ANOT, due to matrix effects

3.3.2.4 Trueness and Precision

Trueness criteria were set as 70–120% based on SANTE guidelines, while precision (in terms of RSD) was set as ≤20% as the 2002/657/EC guidelines were not appropriate. Trueness and precision data for WL_r and WL_R conditions are summarised in Table 3-3. Under WL_r conditions, the trueness across the four validation levels ranged from 86–114%, with all analytes meeting the set criteria. The trueness for all analytes under WL_r conditions at the lowest validation level was >95%, demonstrating very high accuracy even at ppq (pg L^{-1}) to ppt (ng L^{-1}) levels. WL_r precision (RSD_r) for all analytes across the four validation levels was in the range of 0.5–8.2%. For a number of analytes such as NEQUIN and BUQUIN, the WL_r trueness decreased with increasing concentration, however it was were still acceptable. Under reproducibility conditions (WL_R), trueness ranged from 88–111%, with all analytes meeting the acceptance criteria. Precision for the majority of analytes under reproducibility conditions (RSD_{wR}) was <5%, with the overall range between 0.9–10.3%. Overall, this method has been shown to be very accurate and precise for the 23 confirmatory analytes and three screening analytes (TOL, TOL-SO and TOL-SO_2).

3.3.3 Applicability

The method presented above has been applied for the determination of the 26 anticoccidial compounds as part of a spatial sampling programme, whereby >100 groundwater samples were collected from sites throughout the Republic of Ireland during November/December 2018. Seven different anticoccidial compounds, consisting of four ionophores (lasalocid, monensin, narasin and salinomycin) and three chemical coccidiostats (amprolium, diclazuril and nicarbazin), were detected during the sampling campaign. The concentration ranges of each anticoccidial detected are as shown in Table 3-4. Further information and details of this spatial occurrence study are currently in preparation for publication.

Table 3-4 Summary of the seven anticoccidial compounds, and respective concentration ranges, detected during a spatial sampling campaign throughout the Republic of Ireland in 2018

Anticoccidial Compound	**Detected Concentration Range (ng L^{-1}) ***
Ionophores	
Lasalocid	≥LOQ–56
Monensin	≥LOQ–386
Narasin	≥LOQ–47
Salinomycin	≥LOQ–19
Chemical coccidiostats	
Amprolium	≥LOQ–50
Diclzauril	≥LOQ–66
Nicarbazin	≥LOQ–135

*See Table 3-3 for LOQ of each analyte

3.4 Comparison with other existing methods for environmental water samples

As highlighted in the introduction, based on literature review, there are very few methods available for the determination of anticoccidial residues in water samples, with the majority of methods reported being for the separate analysis of ionophores (Cha et al., 2005; Kim and Carlson, 2006; Herrero et al., 2012; Bak et al., 2013a; Sun et al., 2013) or a limited number of chemical anticoccidials (Olsen et al., 2012). The method proposed by. Herrero et al. (2012) extracted five ionophores (LAS, MAD, MON, NAR, SAL) from river water using HLB (150 mg) SPE cartridges, with good recoveries reported, ranging from 89–97%. An

LOQ of 1 ng L^{-1} was reported for all analytes except MAD (5 ng L^{-1}), with LODs ranging from 0.5–1 ng L^{-1}. Martinez-Villalba et al. (2009) proposed a method for the determination of eight anticoccidials (including the three chemical anticoccidials DICLAZ, NICARB and ROB) using C_{18} SPE. Recoveries of all analytes were in the range of 85–100% except for ROB (60%), while LODs were in the range of 11–71 ng L^{-1}. The method developed as part of this study is capable of determining 26 anticoccidial compounds, including six ionophores and 20 chemical anticoccidials. This new method performs better for all of the analytes reported by Herrera et al., with LOQs of 0.1 ng L^{-1} for LAS, MON, NAR and SAL and 1 ng L^{-1} for MAD. Similarly, detection capabilities of this developed method are much improved compared to the results reported by Martinez-Villalba et al. (2009). In particular, this work reports higher recovery of ROB, with lower reported detection limits (at least 50 times lower) for the three chemical anticoccidials reported by Martinez-Villalba et al. (2009). This new method allows for detection limits down to the part-per-quadrillion (pg L^{-1}) level, depending on the analyte.

3.5 Conclusions

A comprehensive LC-MS/MS detection method has been developed which allows for the simultaneous separation and detection of 31 anticoccidial drugs in one single injection. A sample extraction procedure based on SPE has been developed and optimised for the extraction of these anticoccidial residues from raw, unfiltered, environmental water samples at ppq to ppt levels. This extraction procedure was suitable for extraction of 26 anticoccidials, with four compounds not retained by the SPE due to their high hydrophilicity. The method has been extensively validated for these 26 analytes, over a broad range of concentration levels, in-line with expected environmental levels, based on review of currently available literature. The developed detection method is advantageous compared to other reported methods as it allows the simultaneous detection of highly polar, basic compounds such as amprolium and cyromazine, along with other analytes such as the ionophores, on the same analytical column. In addition, the combination of the developed SPE procedure with this detection method allows for the determination of a broader range of both ionophore and chemical anticoccidial residues (26), compared to currently available methods which incorporate <10 anticoccidials. Overall, the method has been deemed fit for purpose for the confirmatory analysis of 23 anticoccidials, and screening of an additional

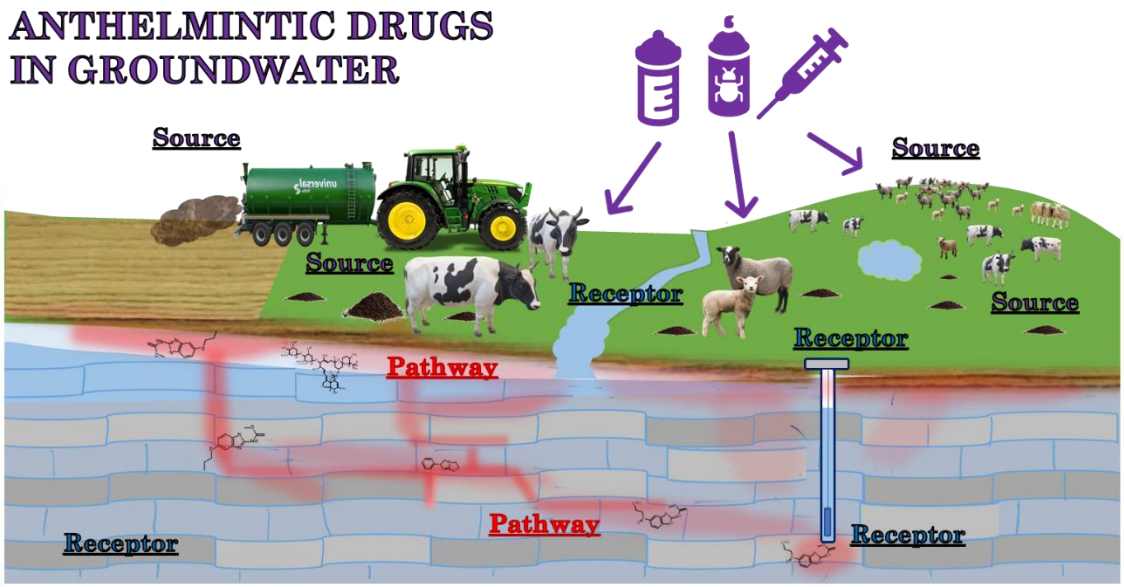
three compounds (TOL, TOL-SO and TOL-SO_2), according to appropriate validation guidelines.

3.6 CRediT author statement

Damien Mooney: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Visualization, Project Administration. **Catherine Coxon:** Conceptualization, Writing – Review and Editing, Visualization, Supervision, Project Administration, Funding Acquisition. **Karl Richards:** Conceptualization, Resources, Writing – Review and Editing, Supervision, Funding Acquisition. **Laurence Gill:** Conceptualization, Writing – Review and Editing, Funding Acquisition. **Per-Erik Mellander:** Conceptualization, Resources, Writing – Review and Editing. **Martin Danaher:** Conceptualization, Methodology, Resources, Writing – Review and Editing, Visualization, Supervision, Funding Acquisition.

CHAPTER 4 – AN ANALYSIS OF THE SPATIO-TEMPORAL OCCURRENCE OF ANTHELMINTIC DRUG RESIDUES IN GROUNDWATER

Graphical Abstract



Lay Abstract

Anthelmintics are a group of veterinary antiparasitic drugs use for treating parasitic worms known as helminths, in intensively reared animals such as cattle and sheep in Ireland. When administered to the animals, these drugs may enter the environment mainly by the direct excretion in urine or faeces onto the soil, or as a result of the spreading of potentially contaminated slurry and manure onto land. Once in the environment these drugs have the potential to be transported throughout the environment and can potentially end up in the groundwater. The aim of the work presented in this chapter was to carry out a study to determine whether anthelmintic drugs are present in groundwater in Ireland, and if so, to investigate any source factors (where the drugs came from) and pathway factors (how it got into the groundwater) that are related to the occurrence. A comprehensive study was carried out whereby 106 sites (88 groundwater and 18 surface waters) were sampled and analysed for 40 anthelmintic drugs. Of the total 40 anthelmintics tested for, 17 were found in 22% of the sites sampled. The most detected compounds (albendazole and its degradation products) belong to a class of anthelmintics commonly used in cattle and sheep production as a broad spectrum treatment. The overall anthelmintic occurrences were found to be statistically related to sheep density and tillage land. A temporal study was also carried out to investigate any seasonal variation in detections, with the results indicating that anthelmintics are likely to occur at the highest frequency and concentrations following periods of increase usage and/or heavy rainfall, such as February/March and August/September.

AN ANALYSIS OF THE SPATIO-TEMPORAL OCCURRENCE OF ANTHELMINTIC VETERINARY DRUG RESIDUES IN GROUNDWATER

D. Mooney, K. G. Richards, M. Danaher, J. Grant, L. Gill, P-E. Mellander, and C.E. Coxon

Draft prepared for submission to Science of the Total Environment for peer review

Abstract

Anthelmintics are antiparasitic drugs used to control helminthic parasites such as nematodes and trematodes in animals, particularly those exposed through pasture-based production systems. Even though anthelmintics have been shown to be excreted into the environment in relatively high amounts, as unmetabolized drug or transformation products (TPs), there is still only limited information available on their environmental occurrence, particularly in groundwater, which has resulted in them being considered as potential emerging contaminants of concern. A comprehensive study was carried out to investigate the occurrence of 40 anthelmintic residues (including 13 TPs) in groundwaters (and associated surface waters) throughout the Republic of Ireland. The study focused on investigating the occurrence of these contaminants in karst and fractured bedrock aquifers, with a total of 106 (88 groundwaters and 18 surface waters) sampled during spring 2017. Seventeen anthelmintic compounds consisting of eight parent drugs and nine TPs were detected at 22% of sites at concentrations up to 41 ng L^{-1}. Albendazole and its TPs were most frequently detected, found at 8% of groundwater sites and 28% of surface water sites. Multivariate statistical analysis identified several source and pathway factors (including tillage land use, sheep density and monitoring point type) as being significantly related to the occurrence of anthelmintics in groundwater, however there was an evident localised effect which requires further investigation. An investigation of temporal variations in occurrence over a 13 month period indicated a higher frequency and concentration of anthelmintics during February/March and later during August/September 2018, which coincided with periods of increased usage and intensive meteorological events. This work presents the first detections of these contaminants in Irish groundwater and it contributes to broadening our

understanding of anthelmintics in the environment. It also provides insight to seasonal trends in occurrence, which is critical for assessing potential future effects and implications of climate change.

Keywords: emerging contaminants; antiparasitic drugs; transformation products; karst; surface water; groundwater

4.1 Introduction

Pasture based animal production systems play an important role for sustainable farming in many temperate regions throughout Europe, including Ireland, with grass grazing providing a low cost, on-farm feed source for industries such as milk production (Hennessy et al., 2020). However, such systems in temperate climates are not without their disadvantages in terms of animal health, with pasture grazed animals (primarily cattle and sheep) highly susceptible to infection by helminthic parasites, which can be an economic burden on the food production system. According to Animal Health Ireland (2016), gastrointestinal nematodes (GIN) (roundworms), and trematodes (flukes) are the most economically detrimental groups of helminths infecting cattle and sheep livestock in Ireland. Typical issues associated with infection of livestock with roundworms or flukes includes poor appetite and feed intake resulting in poor digestion and absorption of nutrients, ultimately causing negative impacts on growth rate, fertility and production yield (e.g. milk or wool quality) (Miller et al., 2012; Animal Health Ireland, 2013). Consequently, anthelmintic agents have become a critical component in animal husbandry and pasture-based production systems (Patten et al., 2011; Bloemhoff et al., 2014).

There are several classes of anthelmintic drugs that can be used to treat these helminthic parasites, each class differing by their mode of action, with some classes more effective toward particular species of helminths than others. The 40 anthelmintic residues (including 27 parent drugs and 13 transformation products) included in this study, subdivided into the main anthelmintic classes, are listed in Table 4-1, with a broad classification of the primary usage of each drug also provided. In cattle and sheep production, the most important of these classes are the benzimidazole group (BZs), macrocyclic lactone group (MLs) and the imidazothiazole levamisole (LEV), given their broad-spectrum efficacy toward many helminths during most of their lifecycle (i.e. early immature, immature and adult parasites). Flukicides, a collective of drugs from across the different classes, used specifically to target liver flukes in intensive livestock production, are also of importance.

Although the classes of drugs used are similar, anthelmintic drug usage patterns are complex and can differ not only between production systems, but also from farm to farm. Recommended best practices are based around management systems which adopt the approach of dosing according to the onset and severity of infection, as informed by regular

testing for faecal egg counts. However, despite best efforts, traditional chemoprophylactic approaches involving a regular dosing programme, regardless of the infection status of the animals, are still prevalent on many farms resulting in the unnecessary over-use of anthelmintic drugs (Patten et al., 2011). Consequently, resistance of certain helminths to anthelmintics such as the BZs, has become an emerging issue, particularly in sheep, with reported treatment failure attributed to resistance (Keegan et al., 2017). This in turn adds burden on treatment regimes, often with higher doses required to achieve the required efficacy. Regardless of dosing regimen, common amongst both cattle and sheep rearing systems is the importance and prioritisation of treatment for young animals (i.e. calves and lambs) (Bloemhoff et al., 2014), given their susceptibility to infection due to the lack of previous exposure and immunity to the parasite. As a result, anthelmintic usage and application patterns are largely centred around protecting calves and lambs during periods of highest parasite burden, which are largely driven by climate and ground conditions. A summary of the main seasonal events of cattle and sheep production (i.e. housing, turn-out to pastures and grazing), around which anthelmintic dosing occurs in Ireland, is provided in Figure 4-1, with an example of potential anthelmintic dosing events also depicted (Bloemhoff et al., 2014) (Keane, *Pers. Comm*, 2020).

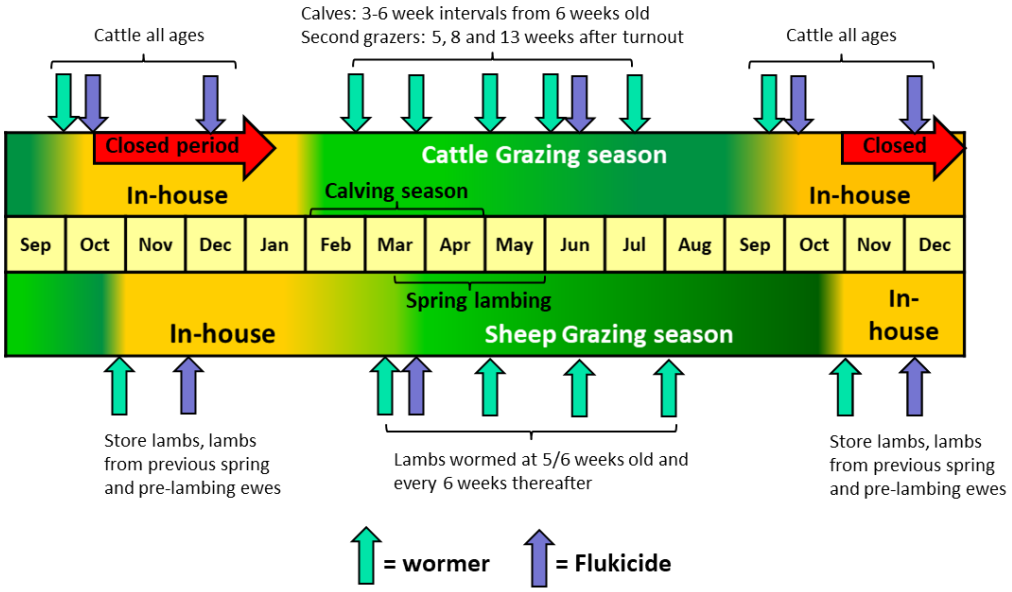


Figure 4-1 Overview of the main seasonal events of cattle and sheep production in Ireland, around which anthelmintic dosing occurs, with some potential dosing events for wormers and flukicides shown

Boxall (2010) provides a comprehensive overview of the factors controlling both the entry and movement of veterinary drugs such as the anthelmintics in the environment. Once administered, typically as an oral drench, topical pour-on, or parenteral injection, the drugs can undergo transformation by metabolism in the animal, the extent of which depends on both the drug and route of administration. For some (e.g. ivermectin (IVER) and abamectin (ABA)), extensive metabolism does not normally occur with a large proportion (often >90%) of parent drug shown to be excreted in urine or faeces (Liebig et al., 2010; Beynon, 2012a; Horvat et al., 2012). For others (e.g. fenbendazole (FBZ), morantel (MOR) and Closantel (CLOS)), more extensive metabolism has been reported (Wardhaugh, 2005). In some instances when the drugs are metabolized, the excreted metabolite(s) are often more active and toxic than the parent drug themselves (Danaher et al., 2007). Entry of anthelmintic residues into the environment is therefore primarily as a result of the direct excretion of the parent drugs and/or TPs onto pastures during grazing, or as a result of the land-spreading of manure and slurry containing anthelmintic residues, typically accumulated in large amounts during the housing period (Boxall et al., 2004). The latter of these exposure routes is likely to be of most importance; several studies have highlighted the potential for anthelmintic drugs to persist in the manure due to minimal or slow degradation during storage (Floate et al., 2005; Kreuzig et al., 2007). Application of stored manure following the housing period can therefore provide a concentrated source of anthelmintic residues in the environment with initial land-spreading. Once in the environment, anthelmintics (both parent and excreted metabolites) can further undergo degradation by processes such as oxidation, hydrolysis and photolysis (Horvat et al., 2012). As a result, breakdown products present in the environment (as a result of both excretion and environmental degradation) are collectively referred to as transformation products (TPs).

Once in the environment the anthelmintics have the potential to be transported between different environmental compartments, including soil, surface water and groundwater. The extent to which anthelmintics are mobile in the environment is controlled by a combination of factors such as the sorption behaviour of the substances (dictated by their physicochemical properties) and environmental factors such as soil characteristics, climate conditions and hydrogeology (e.g. aquifer type and properties) (Boxall, 2018). Based on their physicochemical properties (Table 4-1), with low water solubility and high sorption coefficients, the anthelmintics are likely to be found more associated with soil and sediment than water. Reflecting this, the majority of analytical methodologies and previous occurrence

studies have focused on soil or sediment (Moreno-Gonzalez et al., 2015), with only few on manure leachate (Raich-Montiu et al., 2008) and surface waters (Bartelt-Hunt et al., 2009; Sim et al., 2013).

To date, there is an apparent lack of research focusing specifically on groundwater occurrence, which may be as a result of the expected immobility of anthelmintics in the environment due to their affinity for soil. However, several studies have demonstrated the transport of anthelmintics via particle associated surface transport (Kreuzig et al., 2007) and preferential macropore flow (Weiss et al., 2008). Such pathways are of particular importance in hydrogeological settings dominated by secondary permeability, with flow primarily via fissures and fractures (Swartz et al., 2003; EPA Ireland, 2010). Furthermore, karstic aquifers possess solutionally widened fractures and openings, and unique features such as swallow holes and sinking streams, which allow for rapid point recharge to groundwater with minimal attenuation (Coxon, 2011). Groundwater bodies within such settings are therefore potentially vulnerable to contamination by anthelmintics. Groundwater is not only important as a natural drinking water reservoir, with up to 75% of European Union habitants relying on groundwater for their drinking water supply (European Commission, 2019), it also plays a vital role in supporting and maintaining many groundwater dependent ecosystems, on which anthelmintic drugs can have a non-targeted effect.

While some anthelmintics have been shown to exhibit toxicological properties such as teratogenicity, embryotoxicity, neurotoxicity, goitrogenicity and mutagenicity (Kinsella et al., 2009), the expected levels within environmental waters are not likely to be of major concern for human health (ACVM, 2019), certainly lower risk than those expected in foods of animal origin, for which safe maximum residue limits have been set. However, the main concern over the environmental occurrence of anthelmintics is due to their ecotoxicological effects on non-target organisms (Floate et al., 2005). The ecotoxicological effects of some classes of anthelmintics, such as the macrocyclic lactones, have been well documented, with negative effects observed for dung beetle populations (O'Hea et al., 2010; Jacobs and Scholtz, 2015) and different aquatic organisms (Sanderson et al., 2007; Liebig et al., 2010). Information on other classes is lacking (Wagil et al., 2015a), with ecotoxicological information on TPs even more scant, as highlighted by Horvat et al. (2012).

Early work by the Boxall research group highlighted gaps in current research regarding the environmental fate and ecotoxicity of veterinary medicines, with several anthelmintic drugs deemed of potential high priority in terms of risk to the environment (Boxall et al., 2003a; Boxall et al., 2004). On assessing more recent reviews on this topic, it is still evident that these gaps have yet to be filled (Horvat et al., 2012; Kaczala and Blum, 2016; Snow et al., 2016). A European Medicines Agency (EMA) reflection paper (CVMP, 2016) noted that a lack of information prevented a conclusive decision on 20 substances identified as potentially persistent bioaccumulative toxic substances (PBTs), the majority of which were parasiticides used in food-producing species. The anthelmintic moxidectin was deemed suitable for classification as a PBT. More recently, as part of the concluding remarks of a report reviewing the method of supply of anthelmintic veterinary medicinal products carried out by the Health Product Regulatory Authority (HPRA), it was also noted that there is a deficit in the monitoring of antiparasitic drugs in the environment (ACVM, 2019).

Finally, given the reliance of grass grazed production systems on anthelmintic drugs to control helminthic infections (whose growth are influenced primarily by rainfall and temperature), the ongoing threat of climate change is another important driver toward the need to adequately investigate and assess the environmental occurrence and risk of anthelmintic drugs in the environment. This is primarily because of the uncertainty around the future impacts of climate change in temperate regions, including altered rainfall patterns resulting in wetter milder winters, prolonged warmer summers, and more sporadic extreme weather events (van Dijk et al., 2010; Bloschl et al., 2019), all of which are likely to result in changes in farming patterns and practices, and thus parasite management approaches (Morgan and Wall, 2009). Longer warmer summers are likely to result in longer grazing seasons, as predicted by Phelan et al. (2015), providing an increased exposure of animals to helminths, which in turn increases the need for, and frequency of, anthelmintic drug treatments. Alternatively, harsher, wetter, winters will increase and shift the period of highest parasite burden, and may require longer housing periods for animals, which again further complicates and puts strain on parasite control strategies (Morgan and Wall, 2009). Evidently, the effects of climate change are only set to exacerbate anthelmintic usage, therefore more adequate assessment is of most importance.

Considering the above, the main aim of this study was to apply a newly developed comprehensive analytical procedure (Mooney et al., 2019) to investigate the spatial occurrence of anthelmintic drugs in environmental waters, particularly groundwaters, throughout Ireland. This study particularly focused on groundwater within karstic and fractured bedrock aquifers, given their prevalence throughout Ireland, and their potential to accommodate inadequately attenuated transport of contaminants to groundwater, through flow paths that are likely to be of most importance for the anthelmintics. The main objective of this work was to establish the frequency of occurrence of the drugs, and the relevant groundwater concentrations. This is necessary to broaden our understanding of, and provide additional information on, the fate and occurrence of these drugs in groundwater, which is currently lacking. This work also aimed to assess any potential temporal variations in anthelmintic drug occurrences due to seasonal factors such as drug usage patterns and meteorological events. This temporal study, although not fully representative of the different hydrogeological settings in Ireland, was carried out on two karstic areas representing the hydrogeological settings which are likely to be most sensitive to temporal variations, due to the potential for rapid transport of contaminants to groundwater. The results of this temporal study will provide an insight into any seasonal controls on anthelmintic occurrence, the understanding of which is critical for assessing potential future variations in occurrence caused by the effects of climate change.

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Table 4-1 Chemical and physicochemical data of the 40 anthelmintic compounds (grouped by class), investigated in the spatial-temporal occurrence study, with corresponding analytical method performance parameter and typical usage in livestock

Class/ Analyte	**Abbreviation**	**CAS number**	**Main Usage**	**LOD**	**LOQ**[a]	**Physicochemical properties**[b]			
				(ng L^{-1})		**S_w (mg L^{-1})**	**logK_{ow}**	**pK_a**	**log K_{oc}**
Benzimidazoles (BZs)									
Albendazole	ABZ	54965-21-8	Wormer + Flukicide (Adult)	0.125	1.0	10, 46.39	2.2-3.07	2.94 , 10.26	3.37 - 9.93, 13.1
Albendazole sulphoxide	ABZ-SO	54029-12-8	TP of parent	0.2	1.0	62	0.83 - 1.2	-	3.5, 9.8, 7.8
Albendazole sulfone	ABZ-SO_2	75184-71-3	TP of parent	0.165	1.0	-	0.9-1.01	-	-
Albendazole-amino-sulfone	ABZ-NH2SO_2	80983-34-2	TP of parent	0.165	0.5	-	0.69-0.75	-	-
Cambendazole	CAM	26097-80-3	Wormer	0.165	0.5				
Fenbendazole	FBZ	43210-67-9	Wormer	0.1	0.5	0.01-0.04, 6.38	1.95-4.01	3.37	5.12, 12.72
Oxfendazole	OXF	53716-50-0	Wormer (also TP of FBZ)	0.25	1.0	407.2	1.88-2.13	-	4.13, 11.79
Fenbendazole sulphone	FBZ-SO_2	54029-20-8	TP of parent	0.20	1.0	-	2.13-3.30	-	-
Flubendazole	FLU	31430-15-6	Wormer	0.1	1.0	194.3	1.98-2.91	3.05	3.6, 9.9
Amino-flubendazole	FLU-NH_2	82050-13-3	TP of parent	0.05	1.0				
Hydroxy-flubendazole	FLU-OH	82050-12-2	TP of parent	0.3	1.0				
Mebendazole	MBZ	31431-39-7	Wormer	0.125	1.0	10, 50.05	2.44-2.71	3.00	3.5, 9.2
Amino-mebendazole	MBZ-NH_2	52329-60-9	TP of parent	0.3	1.0	-	2.22-2.61	-	9.8
Hydroxy-mebendazole	MBZ-OH	60254-95-7	TP of parent	0.2	1.0	-	1.84-2.27	-	5.5
Oxibendazole	OXI	20559-55-1	Wormer	0.125	0.5	-	1.86-2.63	-	4.6, 9.6
Triclabendazole	TCB	68786-66-3	Flukicide (All stages)	0.125	0.5	-	4.90- 6.66	-	2.5, 10.5, 4.6
Triclabendazole-sulphoxide	TCB-SO	100648-13-3	TP of parent	1.0	4.0		3.39-3.66	-	-
Triclabendazole-sulphone	TCB-SO_2	106791-37-1	TP of parent	1.0	4.0	-	3.58-5.14	-	-
Thiabendazole	TBZ	148-79-8	Wormer	0.1	0.5	335.2	1.6-2.5, 5.3-6.2	2.69	2.5 -5.22, 12.83
Hydroxy-Thiabendazole	TBZ-OH	948-71-0	TP of parent	0.1	0.5	30		-	4.5
Macrocyclic lactones (MLs) (Avermectins & Milbemycin's)									
Abamectin	ABA	71751-41-2	Wormer + Ectoparasiticide	1.0	10.0	3.5×10^{-4}	4.0	3.72–4.48	-
Doramectin	DORA	117704-25-3	Wormer + Ectoparasiticide	0.5	10.0	-	4.1	3.88-4.94	-
Emamectin	EMA	155569-91-8	Wormer + Ectoparasiticide	0.05	0.5	-	5.0	4.39-5.86	4.2 -7.7

Table 4-1 *continued*

Class/ Analyte	**Abbreviation**	**CAS number**	**Main Usage**	**LOD (ng L^{-1})**	**LOQ[a] (ng L^{-1})**	**Physicochemical properties[b]** S_w (mg L^{-1})	**logK_{ow}**	**pK_a**	**log K_{oc}**
Eprinomectin	EPRINO	123997-26-2	Wormer + Ectoparasiticide	5	20.0	-	5.40	3.51-3.96	-
Ivermectin	IVER	70288-86-7	Wormer + Ectoparasiticide	2.5	10.0	4	3.22	3.60-4.41	4.2-4.9
Moxidectin	MOXI	113507-06-5	Wormer + Ectoparasiticide	2.0	10.0	4	4.77, 5.67	2.8-4.63, 12.6	-
Imidazothiazoles (collectively referred to as levamisole's (LVs))									
Levamisole	LEV	16595-80-5	Wormer (some adult only)	0.125	0.5	1116	2.87	1.88	4.83, 10.50
Amino-acetonitrile derivatives (AADs)									
Monepantel	MONE	851976-50-6	Wormer (roundworm only)	0.5	5.0	0.08	4.2-4.7	-	3.84-3.94
Monepantel-sulphone	MONE-SO_2	851976-52-8	TP of parent	0.2	1.0				
Tetrahydropyrimidines									
Morantel	MOR	26155-31-7	Wormer	1.0	5.0	1.5×10^5	3.69	2.9	
Salicylanilides and substituted phenols									
Bithionol	BITH	97-18-7	Wormer + Flukicide	1.0	5.0	0.2	5.91	4.67	4.83, 10.50
Closantel	CLOS	57808-65-8	Flukicide (immature/adult)	0.5	2.0	1.5×10^{-5}	8.11	5.72	4.26
Niclosamide	NICLOS	50-65-7	Wormer	0.125	1.0	10	4.56	3.58	-
Nitroxynil	NITROX	1689-89-0	Flukicide (immature/adult)	2.5	10.0		2.04		2.49
Oxyclozanide	OXY	2277-92-1	Flukicide (Adult only)	1.5	5.0	0.26	3.53	5.1, 10.8	3.42
Rafoxanide	RAFOX	22662-39-1	Flukicide (immature/adult)	0.3	2.0	4.6×10^{-5}	8.14	5.40	-
Organophosphates									
Coumaphos	COUMA	56-72-4	Wormer + Ectoparasiticide	1.0	5.0	-	-	-	-
Coumaphos-Oxon	COUMA-O	321-54-0	TP of parent	0.25	1.0	-	-	-	-
Haloxon	HALOX	321-55-1	Wormer	1.0	5.0	-	-	-	-
Miscellaneous									
Clorsulon	CLOR	60200-06-8	Flukicide (Adult only)	10	40.0		0.08	-	2.75

[a] LOD = Limit of detection, LOQ = Limit of quantification, data taken from Mooney et al. (2019)

[b] data primarily adapted and extracted from extracted from (O'Neill, 2001; Danaher et al., 2007; Krogh et al., 2008a; Krogh et al., 2008b; Horvat et al., 2012; Santaladchaiyakit and Srijaranai, 2012; Popova et al., 2013; van der Velde-Koerts, 2014; Zrncic et al., 2014) where S_w = water solubility, logK_{ow} = logarithm of octanol-water partition coefficient, pK_a = dissociation constant and logK_{oc} = logarithm of soil organic carbon-water partitioning coefficient

4.2 Materials and Methods

4.2.1 Sampling sites

4.2.1.1 Spatial site selection

This study was carried out as part of a project which contributed to a larger research challenge focused on securing and protecting groundwater within karst and fractured bedrock aquifers. In Ireland, karst aquifers are subdivided into two categories depending on the extent of conduit and diffuse flow within the system (DELG/EPA/GSI, 1999). As a result, groundwater sampling sites were mainly selected to investigate the relevance of the following three flow regimes to anthelmintic occurrence, with a representative number of sites selected broadly across these three regimes: (a) karst aquifers dominated by conduit flow (Rkc), (b) karst aquifers dominated by fracture diffuse flow (Rkd) and (c) non-karst bedrock aquifers with fracture flow. Samples of several associated surface waters were also taken, as explained below. Besides this main selection criterion, other factors that were taken into account were pre-existing research on sites (notably sites from the Teagasc Agricultural Catchments Programme (ACP) and from joint Geological Survey Ireland (GSI) and National Federation of Group Water Schemes (NFGWS) work), stakeholder interest and site ownership/ access considerations.

All groundwater sites were characterised based on the different land-uses (source factors) and hydrogeological properties (pathway factors) within their zone of contribution (ZOC), with the predominant class of each property recorded and input for statistical analysis (as described in Section 4.2.3). ZOCs for most of the sampling sites have been previously delineated using hydrogeological mapping and/or numerical modelling incorporating (but not limited to) data on topography, groundwater tracing experiments, recharge, extraction and previously determined boundaries, as described by Kelly (2010). ZOC boundary shapefiles for all EPA groundwater monitoring sites were downloaded from publicly available datasets (EPA Ireland, 2018), while ZOCs for private group water schemes (GWSs) were provided confidentially by the NFGWS in coordination with the GSI. ZOCs for several research observation piezometer boreholes were estimated in coordination with Teagasc ACP researchers.

Overall, 106 sites comprising 88 groundwaters (which included 41 boreholes (BHs) and 47 springs (SPs)) and 18 surface waters (SW) (which included 11 streams/rivers, 2 lakes, 3 swallow holes (SHs) and 2 drainage ditches), were sampled across the Republic of Ireland during March and April 2017 (Figure 4-2). This sampling period was selected to coincide with a period of active groundwater recharge and a period following the return of animals to pasture and the recommencement of manure spreading (Figure 4-1). A large proportion (42%) of sites were sampled in coordination with the Environmental Protection Agency of Ireland (EPA) in tandem with the national groundwater quality monitoring programme for the E.U. Water Framework Directive (WFD) (EPA Ireland, 2019). The remaining sites comprised private and/or semi-private group water schemes (GWSs) (28%) sampled in coordination with the NFGWS or observation/research sites (31%) sampled in coordination with Teagasc ACP. In total, 66% of groundwater sites were used for public drinking water supplies (including springs and BHs), with the remainder used for agricultural or observational purposes. Overall, 35% of groundwater sites selected fell within karstic conduit flow aquifers, with 24% and 41% of sites falling under karstic diffuse flow aquifers and non-karst fractured bedrock aquifers, respectively.

While this sampling network represents a variety of land use and hydrogeology, it is not necessarily representative of all groundwaters in Ireland, given the focus of the study was on karst and fractured bedrock aquifers. Some areas were also sampled at catchment level, resulting in several small clusters of sampling sites. The surface waters included are not intended to be representative of surface water quality in Ireland, rather these surface waters were sampled to complement some groundwater sampling in areas of karst groundwater-surface water interactions and in ACP catchments.

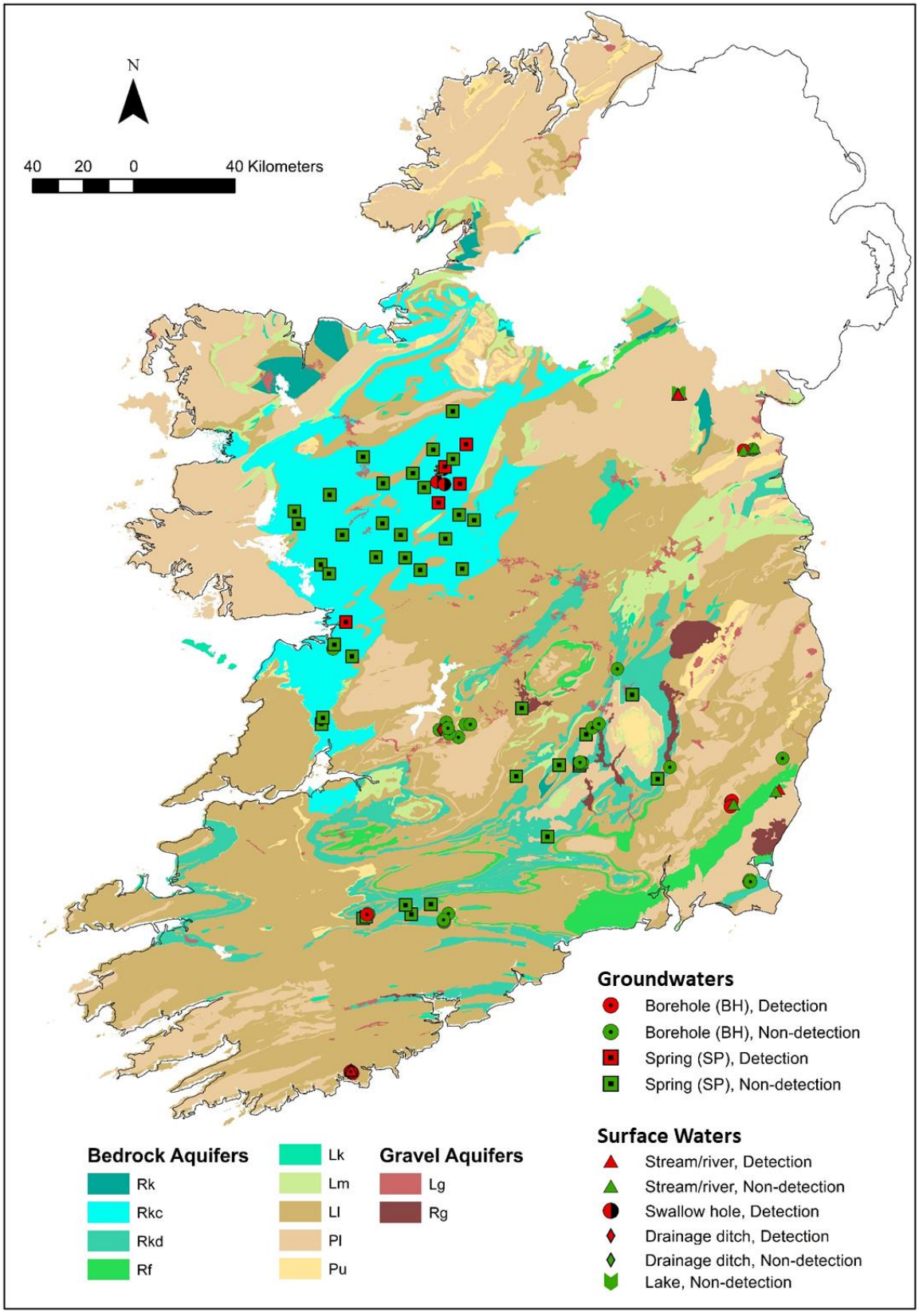


Figure 4-2 The spatial distribution of the 106 sampling points, classified based on sampling point type and detection (red) vs. non-detection (green), overlaid onto the GSI National Bedrock Aquifer Map (GSI, 2015b & 2015c)

4.2.1.2 Temporal study site selection

Following the spatial investigation in March/April, a temporal study was undertaken starting in November 2017. Temporal sampling was focused on karst groundwater with rapid flow and short travel times, carried out at catchment level in two regions of Ireland and comprised 11 sampling sites, which included four karst springs and three swallow holes (sinking streams) in County Roscommon, and four karst springs in County Clare (Figure 4-3). These sites were selected in consultation with the Irish hydrogeological community and based on pre-existing knowledge of, and research at, the study sites. Consideration was also given to such sites which had detections of anthelmintics during the spatial study. Based on previous dye tracing experiments, the three sinking streams sampled were individually linked to three of the springs sampled. Sampling of these sinking streams were therefore carried out to complement the sampling of the spring associated with the sink. Table S4-1 (of supplementary information file SI-4.1) summarises each site in terms of the predominant land-use and hydrogeological properties within their ZOC (characterised using the approach described in 4.2.1). All 11 sites are associated with conduit-flow dominated karst aquifers (Rkc) which provides the potential for rapid underground travel, with short residence times (Drew, 2008). Groundwater velocities across both Roscommon and Clare sites typically range from 19 to 224 m h^{-1}, with travel times ranging from a few hours to a few days e.g. positive dye tracing experiments to Clare Site A showed groundwater velocities of 163–224 m h^{-1}, resulting in a travel time of 6-8 hours, while similar studies in Roscommon showed a velocity of >157 m h^{-1} from a swallow hole (Roscommon site F) linked to the spring at Roscommon site E, with an overall travel time of <14.5 hours (GSI, 2014).

Historical daily meteorological data were obtained for the entire sampling period (and preceding months) from The Irish Meteorological Service (Met Éireann, 2020), for the synoptic weather stations nearest to each sampling location (Mount Dillon station for Roscommon and Shannon Airport station for Clare sites). This dataset included daily rainfall and soil moisture deficits (SMD) (calculated for well drained soils, according to the Schulte model (Schulte et al., 2005)), which were used to determine the overall daily effective rainfall (ER). ER was used as a proxy for groundwater recharge given it is a measure of the amount of incident rainfall that has the potential to percolate through the soil matrix and contribute to recharge. Rainfall data were also examined to account for the potential for contaminant transport to a karst spring via shallow surface pathways, and/or rapid transport

via solutionally widened conduits and fractures, which is plausible given the nature of the conduit flow karstic aquifers underlying each site.

Overall, each of the 11 sites were sampled on a monthly basis for 13 months, from November 2017 to November 2018, with each sample analysed for the 40 anthelmintic drugs. Due to logistical issues with site access, there is a gap in sampling for all seven Roscommon sites and one Clare site for December 2017. In addition, the summer of 2018 was atypical in terms of rainfall, with drought conditions throughout the country recorded for several months. July had just 42.2 mm of rainfall compared to the 30-year mean of 73.1 mm, with soil moisture deficits in the range of 40–90 mm for Clare and Roscommon with no effective rainfall occurring (Met Éireann, 2020). As a result, there are some gaps in sampling for the three sinking streams for the month of July and extending into August in some instances, because of insufficient water for sampling.

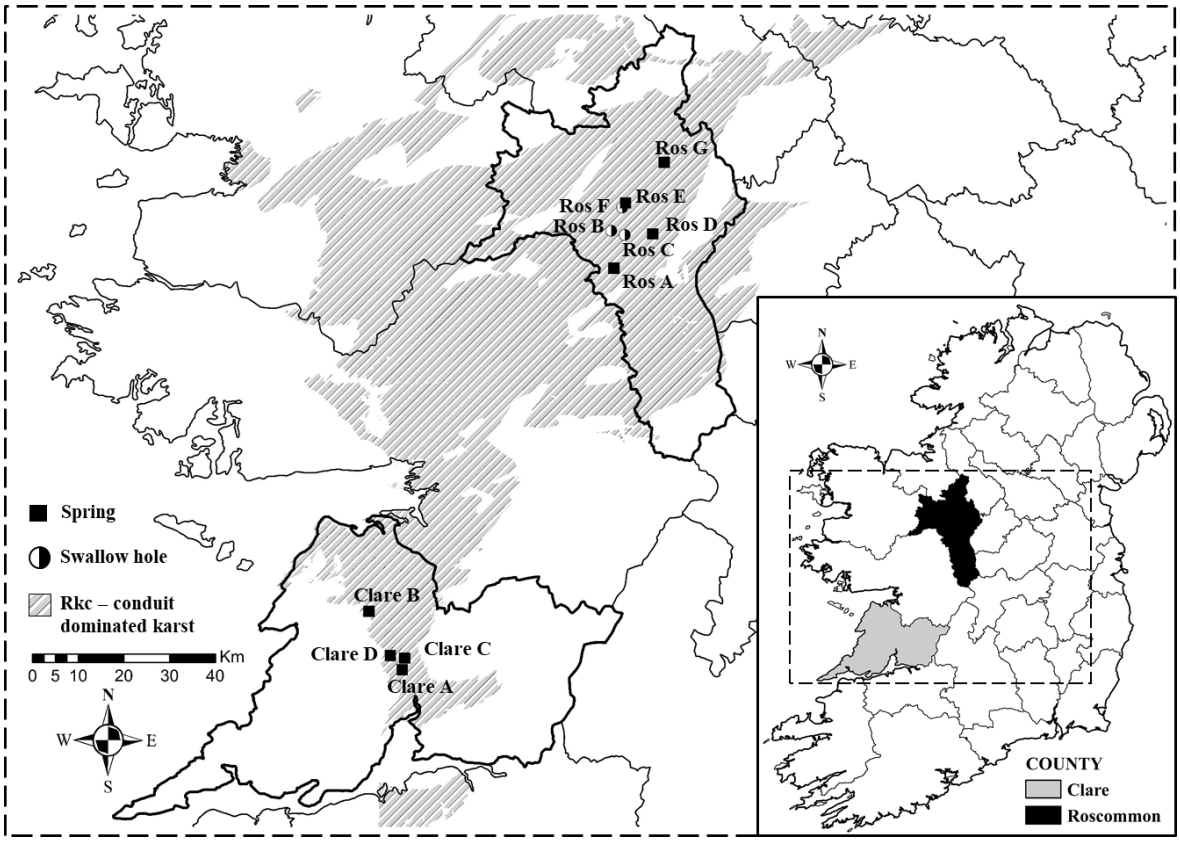


Figure 4-3 Geographical location of the 11 karstic sites sampling in Clare and Roscommon as part of the temporal occurrence study

4.2.1.3 Sample collection

Surface and groundwater samples were collected as once-off grab samples in accordance with relevant ISO EN 5667 standards (NSAI, 2006; NSAI, 2009; NSAI, 2016a; NSAI, 2018) and represented the water quality at that given moment in time. Surface water samples were collected directly into the sampling bottle, from a minimum depth of 0.5m from the surface where possible. Groundwater samples were sampled from either a raw water sampling tap at pre-existing distribution pump houses, or directly from the supply (as for several springs). Observation wells were sampled with a discrete depth sampler (closed bailer). In all cases, sample containers were rinsed with the source water prior to sample collection. Samples were received at the laboratory in a chilled condition and stored at <4°C until analysis which was normally carried out within 3 days of collection, and no longer than 7 days. Quality control field blanks were prepared and incorporated into sample collection where appropriate, as described by Mooney et al. (2019).

4.2.2 Anthelmintic analysis

The extraction and instrumental detection of the anthelmintics was carried out according to the method described by Mooney et al. (2019). Modified samples (500 mL sample + 100 mL methanol, adjusted to pH 7) were extracted using a highly linked divinylbenzene (HL-DVB) solid phase extraction cartridge and analysed for 40 anthelmintic compounds (consisting of 27 parent drugs and 13 transformation products) using ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) detection. This comprehensive method allows the analysis of unfiltered raw water sample, thus the measurement of "whole water" concentrations (i.e. analyte fractions in solution and fractions adsorbed to suspended solids). Details of this analytical procedure, including the chemicals, standards, equipment and instrumental parameters, are as described by Mooney et al. (2019). The method was extensively validated and is fit for purpose for the quantitative confirmatory analysis of all anthelmintic residues, besides triclabendazole sulphoxide (TCB-SO) and triclabendazole sulphone (TB-SO_2), which are included for screening only. Table 4-1 summarises the main method detection and quantification limits for all 40 compounds.

4.2.3 Site characterisation and dataset preparation

4.2.3.1 Land-use properties (source factors)

Two different sources of data were used for classification of land-use within the ZOC of the sampling sites, both offering a different level of detail. The first dataset used was the publicly available Corine (Co-ORdinated INformation on the Environment) Land Cover (CLC) datasets, which consists of geo-spatial information on natural and built environments across Ireland (Lyndon and Smith, 2014). Data were obtained (EPA Ireland, 2012) for 44 different land-use classes, and for this study, these were amalgamated into four classes (arable, non-arable pasture, forest and other), which allowed discrimination of the two main agricultural activities in Ireland.

To allow for a more accurate characterisation of agricultural activity with the ZOC of each site, higher resolution data on land-use were obtained from different Department of Agriculture, Food and Marine (DAFM) datasets, namely the Land-Parcel Information System (LPIS), Animal Identification and Movement (AIM) system and the agricultural sheep census (data obtained under license to the Teagasc ACP through a formal data sharing agreement). LPIS is a database maintained by DAFM that is used to identify the location and shape of agricultural land units, known as parcels. The land-use recorded on the LPIS is the crop description (from a pre-defined category) provided by farmers/landowners annually. Information was obtained for all parcels that fell within (fully or partially) the ZOC of each site, with information on the 95 original crop descriptions recorded for each parcel. For the purpose of statistical analysis, these 95 crop descriptions were amalgamated into four broad groups (grass, tillage, farmyard and other) as described in supplementary information file SI-4.1 Table S4-2.

The AIM system is a database which contains various levels of details on the identification and movement (herd activity) of cattle, sheep, goats, pigs and horses through a tag number system. Integration of the AIM data with LPIS allows the number of animals to be determined for each land parcel for a calendar year. Given the primary source of anthelmintics in Ireland is due to administration to cattle and sheep, data were obtained for these species only, with data broken down depending on sex or age. Using these data, the number of livestock units (LU) were calculated by application of different LU coefficients

as described in Annex I of Regulation 1200/2009/EC (European Parliament, 2009). LU values for individual parcels were then adjusted to account for the percentage of the parcel that fell within the ZOC of each site, with the overall total LU within the ZOC calculated by summing all adjusted values for the given ZOC. Cattle and sheep stocking density was determined by expressing the number of animals per unit area (hectare, ha), accounting for the entire area of each ZOC. Finally, the animal stocking data were also used to calculate the nitrogen load due to animal excretion, expressed as nitrogen per hectare (N ha^{-1}). Nitrogen load was calculated for each parcel by applying the different excretion rates for each animal, as described in Table 6 of the GAP (Good Agricultural Practice) Regulations (Government of Ireland, 2017b), and then related to the agricultural area within each ZOC.

4.2.3.2 Physical and hydrogeological properties (pathway factors)

For statistical analysis, groundwater sampling points were classified as either springs or BHs, with BHs further subclassified as abstraction boreholes or monitoring BHs. BHs pumped daily and used as drinking water supplies or for agricultural purposes, were classified as abstraction BHs, while BHs bailed or pumped for short periods less frequently (e.g. weekly or monthly) and used for research and observational purposes, were classified as monitoring BHs. The hydrogeological (pathway) properties used for sites characterisation are summarised in Supplementary file SI-4.1 Table S4-3, and included: (a) bedrock geology presented as 27 hydrostratigraphic units (GSI, 2016a) amalgamated into six lithological groups as described by Tedd et al. (2017), (b) GSI aquifer category comprising 11 aquifer classes (see supplementary file SI-4.1 for a description of the 11 classes) (GSI, 2015b; GSI, 2015c), (c) Water Framework Directive (WFD) flow regime (karstic, productive fractured and poorly productive fractured) as described by the Working Group on Groundwater (2001), (d) flow regime further categorised into three classes (conduit-dominated karstic, diffuse-dominated karstic and fractured) as previously described for site selection in Section 4.2.1.1 (e) groundwater vulnerability consisting of five vulnerability classes (X-Extreme, E-Extreme, H-High, M-Moderate and L-Low) (GSI, 2015d), (f) Irish Forests Soils (IFS) (Bulfin et al., 2002; Teagasc-EPA-GSI, 2006) consisting of 25 soil classes simplified and dichotomised based on the four main principal components (mineral/peat, acidic/basic, well drained/poorly drained, shallow/deep) used in the classification system described by Fealy et al. (2009), (g) Quaternary sediments, presented as 53 sediment classes (GSI, 2016b) amalgamated into seven geneses and (h) subsoil permeability, extracted from the GSI

Subsoil Permeability dataset (GSI, 2015a), and presented as four permeability classes (high, moderate, low and depth to bedrock less than 3m (DTB<3m) where permeability was not determined).

Data on the relative percentage of each property class was extracted using the ARCGIS intersect tabulate tool, and the class occupying the largest percentage of the ZOC was assigned as the predominant class, as summarised in Supplementary file SI-4.2 for all groundwater sites.

4.2.4 Statistical analysis of relationships with site characteristics

The non-random selection of the data sets for analysis introduces a risk of bias in the outcomes but the authors believe that there is sufficient value in the data to warrant an analysis. The interpretation should be used to guide future research rather than as the basis for definitive conclusions.

The association between detections and the recorded characteristics (land-use and hydrogeological properties) of the groundwater sites was analysed using SAS 9.4 (SAS Institute Inc, 2014). Any samples with a detection greater than the limit of detection (LOD) were recorded as a detect, with results <LOD recorded as a non-detect. The Logistic procedure was used to fit regression models with detection/non-detection as a binary response. Marginal tests were conducted for each characteristic and a variable selection procedure was used to build a conditional model. The former established the usefulness of each variable on its own as a predictor of detections while the latter checked for any combination of the characteristics that was useful for such prediction. The main focus in evaluating the results was on the interpretation of odds ratios and their 95% Wald confidence intervals. Characteristics with continuous variables were also analysed using marginal tests with detection/non-detection as the regressors, with the means of sites with detections compared to those recorded as non-detection. Significance was assigned as p values ≤ 0.05, with values slightly higher than 0.05 noted as being of interest. Due to the prevalence of quasi-complete separation in the regressions, a Firth penalty was used to achieve convergence. The overall analysis was complete for detections defined for several different groups of compounds as follows; (a) all anthelmintics, (b) BZs, (c) non-BZs, (d) parent compounds and (e) TPs.

4.3 Results

4.3.1 Anthelmintic spatial occurrence summary

The geographical spread of the 106 sampling sites, classified by sampling point type (surface water vs. groundwater) and detection vs. non-detection, is shown in Figure 4-2. One or more anthelmintic drugs were detected at 20% of the total sites (23 of 106 sites) at levels greater than the LOQ, while a further 2% of sites had detections at levels less than the LOQ but greater than the LOD (i.e. present but not quantified). In total, 17 out of the 40 different compounds were detected throughout the campaign, at concentrations ranging from 0.9 to 40.6 ng L^{-1} (Appendix 4A, Table A4-1). Thirteen of the seventeen detected compound belonged to the BZ class of anthelmintics.

The number of detections of each of the 17 anthelmintics at SW sites compared to GW sites is depicted in Figure 4-4, with eight different anthelmintic compounds detected across 39% of SW sites at levels >LOQ (1.0–40.6 ng L^{-1}) and up to five compounds detected at any one site. Seventeen compounds were detected across 16% of GW sites at levels >LOQ (0.9–22.2 ng L^{-1}), with a further 2% of GW sites with detections <LOQ but >LOD (Table 4-2). Up to six different anthelmintics were detected at any one GW site (Appendix A Table A1). The concentration range of each compound detected in SW and GW is also summarised in Table 4-2. Detection of an anthelmintic was shown to be 2.9 times more likely (p=0.057) at SW sites than at GW sites.

ABZ and its sulphoxide (SO), sulphone (SO_2) and amino-sulphone (NH_2-SO_2) transformation products (TPs) were the most detected compounds, each detected in 3.8%, 6.6%, 3.8% and 7.5% of total sites, respectively. ABZ and/or its TPs were detected at 8% of GW and 28% of SW sites. ABZ was also the compound detected at the highest concentration during the campaign, detected at 40.6 ng L^{-1} in a field drain, with all three transformation products also detected in this same sample (Site 089, see Appendix 4A Table A4-1). Converting the concentration of each of these TPs back to the parent equivalent, the combined concentration of ABZ in this sample was 73 ng L^{-1}. The ABZ-SO_2 TP was the compound detected at the highest concentration in GW (22.2 ng L^{-1}) (Site 079). The next most detected compounds in SW were TCB (16.7 % of SW) and OXYCLOZ (11.1% of SW), while OXYCLOZ was the second most detected in GW (4.5%), followed by FBZ, OXF, LEV and CLOS, all of which were detected at 3.4% of GW sites. Notably, TPs were

more predominant in groundwater compared to surface waters, with twenty-two detections of eight different TPs in eight groundwater samples, compared to seven detections of three different TPs in five surface water samples.

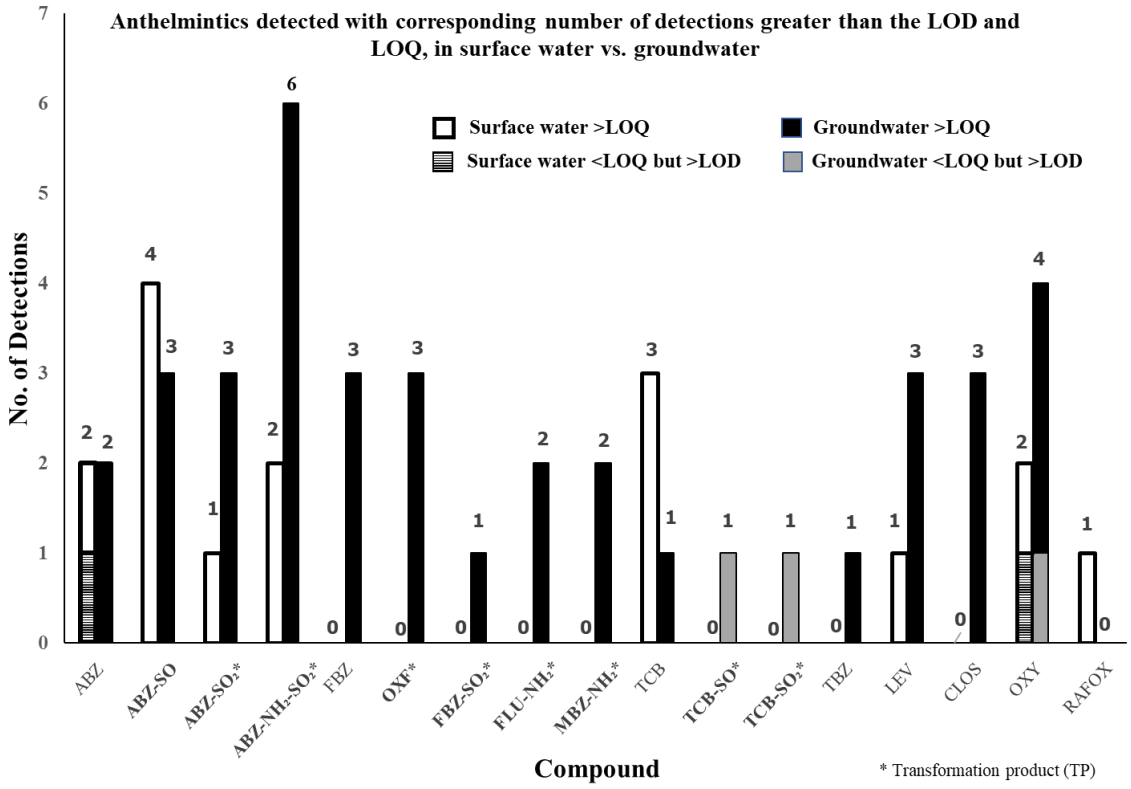


Figure 4-4 Summary of the different anthelmintic compounds detected in surface water (SW) versus groundwater (GW), with the respective number of detections of these compounds at levels greater than the limit of quantification (LOQ) or at levels less than the LOQ but greater than the limit of detection (LOD)

Table 4-2 Summary statistics for the anthelmintic compounds detected above the limit of detection (LOD) and limit of quantification (LOQ), at surface water (SW) and groundwater (GW) sites

Analyte	P /TP	Surface Water							Groundwater						
		% Samples		% of Total	Concentration (ng L^{-1})				% Samples		% of Total	Concentration (ng L^{-1})			
		>LOD	>LOQ	Detections	Min	Max	Mean	Median	>LOD	>LOQ	Detections	Min	Max	Mean	Median
Benzimidazoles															
Albendazole	P	11.1	5.6	29	0.7	40.6	20.7	20.7	2.3	2.3	13	1.2	4.9	3.1	3.1
- Sulphoxide	TP	22.2	22.2	57	1	21.3	6.3	1.4	3.4	3.4	19	3.2	8.5	5.6	5.2
- sulphone	TP	5.6	5.6	14	5.6	5.6	5.6	5.6	3.4	3.4	19	4.5	22.2	11.3	7.3
amino sulphone	TP	11.1	11.1	29	1.5	6.8	4.2	4.2	6.8	6.8	38	1.5	5.2	3.4	3.5
Fenbendazole	P	0.0	0.0	0.0	-	-	-	-	3.4	3.4	19	0.9	2.3	1.5	1.2
- Oxfendazole	TP	0.0	0.0	0.0	-	-	-	-	3.4	3.4	19	1.0	2.7	2.1	2.7
- sulphone	TP	0.0	0.0	0.0	-	-	-	-	1.1	1.1	6	0.9	0.9	0.9	0.9
Amino-flubendazole	TP	0.0	0.0	0.0	-	-	-	-	2.3	2.3	13	1.3	2.0	1.7	1.7
Amino-mebendazole	TP	0.0	0.0	0.0	-	-	-	-	2.3	2.3	13	1.4	2.2	1.8	1.8
Triclabendazole	P	16.7	16.7	43	1.3	4.7	2.5	1.4	1.1	1.1	6	1.5	1.5	1.5	1.5
- sulphoxide	TP	0.0	0.0	0.0	-	-	-	-	1.1	0.0	6		>LOD but <LOQ		
- sulphone	TP	0.0	0.0	0.0	-	-	-	-	1.1	0.0	6		>LOD but <LOQ		
Thiabendazole	P	0.0	0.0	0.0	-	-	-	-	1.1	1.1	6	1.1	1.1	1.1	1.1
Non-benzimidazoles															
Levamisole	P	5.6	5.6	14	1.2	1.2	1.2	1.2	3.4	3.4	19	1.3	1.4	1.3	1.3
Closantel	P	0.0	0.0	0.0	-	-	-	-	3.4	3.4	19	1.9	3.3	2.6	2.6
Oxyclozanide	P	11.1	5.6	29	2.6	9.4	6	6	4.5	1.1	25	3.2	5.0	4.0	3.9
Rafoxanide	P	5.6	5.6	14	2.2	2.2	2.2	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
All Anthelmintics	-	38.9	38.9	-	0.7	40.6	6.4	1.9	18.2	15.9	-	0.9	22.2	3.4	3.8

P = Parent compound, TP = Transformation Product, LOQ = Limit of Quantification, see Table 4-1 for LOQ values for individual analytes, >LOD but <LOQ indicates compound was detected but not quantifiable

4.3.2 Groundwater occurrence and factors controlling spatial distribution

4.3.2.1 Occurrence and zone of contribution (ZOC) land-use characteristics

Analysing categorical and continuous variables with detection/non-detection as a binary response showed that the detections of (a) all anthelmintics, (b) BZs, (c) non-BZs, (d) parent drugs and (e) TPs, were significantly associated (p<0.05) with one or more of the land-use properties used for site characterisation. The land-use characteristics shown to be associated with each detect grouping are summarised in Table 4-3, along with a description of odds ratios and their interpretation. Similar results were observed for the analysis of LPIS land-use and for the analysis of the Corine dataset, and this is believed to be due to aspects of the Corine data being derived from LPIS. To avoid duplication, results are therefore only presented for LPIS land-use in Table 4-3.

Detections of all anthelmintics, collectively, were related to sites with higher proportions of agricultural land in their ZOCs, in addition to higher sheep density (specifically ewe density and other (non-breeding) sheep density). This same trend was observed for detection defined for parent compounds only and non-BZ compounds. Ram density also produced results with p<0.05, however interpretation of these results was not practical due to the outcome being dependent on a limited number of sites only, producing a questionable model fit. Occurrence of BZ compounds was statistically related to higher proportions of agricultural land, a higher proportion of tillage crop and a lower proportion of grass crop, within the ZOCs. A similar trend was observed for TPs, with detections associated with a higher proportion of tillage and a lower proportion of grassland in the ZOC. However, the overall proportion of agricultural land within the ZOC was not statistically related to TP occurrence, neither were the LPIS land-use or Corine land cover classes. All other land-use characteristics not listed in Table 4-3 were not statistically related to anthelmintic occurrence, with a complete summary of p-values shown in Supplementary file SI-4.1 Table S4-5.

For several characteristics, analysis of the continuous variables using detect/non-detect as the regressors indicated a significant difference between the mean of sites that had a detection compared to the mean of sites with a non-detection. The mean percentage of grassland in the ZOC of sites with a BZ detection (52.4%) was significantly different from the mean of sites with no detection of BZs (74.9%) (p = 0.0356). The mean percentage of tillage within the ZOCs of sites with a BZ detection (46.5%) was also significantly different

from the mean of sites with no BZ detections (9.6%) (p = 0.0102). Like BZs, the mean percentage of tillage within the ZOC of sites with detections of TPs (38.1%) was also significantly different from the mean percentage of tillage at sites with no detection (10.8%) (p = 0.0345). The mean density of ewes at sites with a detection of non-BZ compounds (1.44 ewes per Ha) was significantly different to the mean density of ewes at sites with non-detection of non-BZ compounds (0.25 ewes per ha) (p = 0.008). Similarly, the mean density of "other sheep" at sites with a non-BZ detections (0.2 ewes per ha) was significantly different from the mean of sites with non-detection (0.11 ewes per ha) (p = 0.03).

4.3.2.2 Occurrence and ZOC hydrogeological characteristics

Analysis of the different groupings of anthelmintic detections indicated that occurrence of one or more groups was significantly related to several pathway factors including sampling point type, aquifer category, bedrock group, Quaternary sediments and IFS soil types. Detections of all anthelmintics collectively, were shown to be more likely in (a) monitoring BHs compared to abstraction BHs, or springs, (b) non-calcareous bedrock compared to pure limestone bedrock, and (c) poorly drained mineral soils (minPD), as opposed to deep well drained soils (minDW) (Table 4-4). While statistically significant, it must be noted that this relationship with bedrock is biased as a result of several detections in a cluster of sampling points within the same catchment.

Assessing parent compounds only, detections were shown to be more likely in non-calcareous bedrock as opposed to pure limestone, however the same issue remains with these results, due to the localised cluster of detections in one catchment. While not fully significant in terms of p-value, IFS soil type odds ratios analysis indicated a higher likelihood of a detection of a parent compound in poorly drained soils, as opposed to deep well drained soils. Non-BZ compounds (i.e. CLOS, LEV, OXYCLOZ and RAFOX, all of which are parent compounds) show similar relationships with bedrock and IFS soil type. Aquifer class was also shown to be significant, with detections more associated with poorly productive Pl aquifers. This relationship is also questionable, due to the same localised cluster of detections.

TPs on the other-hand were shown to be statistically associated with locally important (Ll) aquifers, with a detection 7.7 times more likely in (Ll) aquifers compared to regionally important conduit dominated (Rkc) aquifers, and 1.021 times more likely, for every one percent increase in the percent of Ll aquifer within the ZOC. TPs were more associated with impure limestone bedrock than pure limestone bedrock, and with Quaternary sediments comprised of tills derived from Namurian sandstone and shales (TNSSs) as opposed to tills derived from limestones (TLs). Similar associations (to TPs) for aquifer category, bedrock group and Quaternary sediments were shown for BZ compounds, which is not unexpected given that all detected TP compounds also belong to the BZ class. However, additional pathway factors were shown to be related to BZ occurrences: detections were more likely in monitoring BHs than in springs and more likely in shallower soils than deeper soils (based on IFS soil data).

Table 4-3 Summary of **land-use (source) characteristics** showing a significant relationship with the occurrence of a) all anthelmintics, (b) benzimidazoles, (c) non-benzimidazoles, (d) parent drugs and (e) transformation products (TPs), with corresponding *p*-values, confidence intervals and odds ratio likelihood interpretations

Land-use Characteristic	Detection defined as:	Odds ratio	95% confidence intervals	*p*-value	Odds ratio interpretation
LPIS land-use	Benzimidazoles	9.00	1.66–50.00	0.0114	**detection 9** times **more likely** at sites with tillage predominant in the ZOC compared to grass
% agriculture in the zone of contribution (ZOC)	Any anthelmintics	1.09	1.01–1.16	0.0093	**detection 1.1** times **more likely** than not, for every unit increase in % of agriculture in the ZOC
	Non-benzimidazole	1.12	1.01–1.25	0.0143	**detection 1.1** times **more likely** than not, for every unit increase in % of agriculture in the ZOC
	Parent drugs	1.17	1.05 –1.30	0.0004	**detection 1.2** times **more likely** than not, for every unit increase in % of agriculture in the ZOC
% grassland in ZOC	Benzimidazoles	1.03	1.01–1.06	0.0119	**detection 1.03** times **less likely** than not, for every unit increase in % of grassland in the ZOC
% tillage in ZOC	Benzimidazoles	1.03	1.01–1.05	0.0061	**detection 1.03** times **more likely** than not, for every unit increase in % of tillage in the ZOC
	TPs	1.02	1.001–1.04	0.0479	**detection 1.02** times **more likely** than not, for every unit increase in % of tillage in the ZOC
density of ewes	Any anthelmintics	2.64	1.16–5.99	0.0036	**detection 2.6** times **more likely** than not, for every unit increase in ewe density in the ZOC
	Non-benzimidazole	4.26	1.49–12.16	0.0001	**detection 4.26** times **more likely** than not, for every unit increase in ewe density in the ZOC
	Parent drugs	3.08	1.275 –7.47	0.0012	**detection 3.08** times **more likely** than not, for every unit increase in ewe density in the ZOC
density of other sheep	Any anthelmintics	8.02	1.39–48.11	0.0128	**detection 8.02** times **more likely** than not, for every unit increase in “other sheep” density in the ZOC
	Non-benzimidazole	29.24	3.39–251.97	0.0004	**detection 29.2** times **more likely** than not, for every unit increase in “other sheep” density in the ZOC
	Parent drugs	12.12	1.84 –80.08	0.0042	**detection 12.1** times **more likely** than not, for every unit increase in “other sheep” density in the ZOC

Table 4-4 Summary of physical hydrogeological (pathway) characteristics showing a significant relationship with the occurrence of (a) all anthelmintics, (b) benzimidazoles, (c) non-benzimidazoles, (d) parent drugs and (e) transformation products (TPs), with corresponding *p*-values, confidence intervals and odds ratio likelihood interpretations

Site Characteristic	Detection defined as:	Odds ratio	95% confidence intervals	*p*-value	Odds ratio interpretation
MP type	Any anthelmintics	4.66	0.0928–23.39	0.0419	**detection 4.7** times **more likely** at sites classified as monitoring BHs compared to sites classified as abstraction BHs
		4.02	1.20–13.51	"	**detection 4.0** times **more likely** at sites classified as monitoring BHs compared to sites classified as springs
	Benzimidazoles	6.07	1.20–30.72	0.0681	**detection 6.1** times **more likely** at sites classified as monitoring BHs compared to sites classified as springs
	Parent drugs	4.00	1.12–14.27	0.0368	**detection 4.0** times **more likely** at sites classified as monitoring BHs compared to sites classified as springs
Aquifer Class	Benzimidazoles	7.70	1.10–54.04	0.0640	**detection 7.7** times **more likely** at sites that have ZOCs predominantly underlain by Ll aquifers as opposed to Rkc
		1.017	1.001–1.034	0.0378	**detection 1.017** times **more likely** than not, for every unit increase in the % of the ZOC underlain by Ll aquifer
	Non-benzimidazoles	1.019	1.003–1.036	0.0239	**detection 1.019** times **more likely** than not, for every unit increase in the % of the ZOC underlain by Pl aquifer
	TPs	7.71	1.10–54.00	0.0346	**detection 7.7** times **more likely** at sites that have ZOCs predominantly underlain by Ll aquifers as opposed to Rkc
		1.021	1.003–1.038	0.0181	**detection 1.021** times **more likely** than not, for every unit increase in the % of the ZOC underlain by Ll aquifer
Bedrock (amalgamated)	Any anthelmintics	6.58	1.55–37.78	0.0524	**detection 6.6** times **more likely** at sites which have ZOCs dominated by non-calcareous sedimentary bedrock compared to pure limestone bedrock
	Benzimidazoles	9.91	1.28–75.76	0.0399	**detection 9.9** times **more likely** at sites with ZOCs dominated by impure limestone bedrock compared to pure limestone bedrock
		6.64	1.12–39.36	"	**detection 6.6** times **more likely** at sites with ZOCs dominated by metamorphic bedrock compared to pure limestone bedrock
Bedrock (amalgamated	Non-benzimidazole	6.99	1.47–33.33	0.0868*	**detection 7.0** times **more likely** at sites which have ZOCs dominated by non-calcareous sedimentary bedrock compared to pure limestone bedrock

Table 4-4 *continued*

Site Characteristic	Detection defined as:	Odds ratio	95% confidence intervals	*p*-value	Odds ratio interpretation
Continued...	Parent drugs	7.94	1.80 –34.48	0.049	**detection 7.9** times **more likely** at sites which have ZOCs dominated by non-calcareous sedimentary bedrock compared to pure limestone bedrock
	TPs	9.9	1.28 –76.7	0.0281	**detection 9.9** times **more likely** at sites with ZOCs dominated by impure limestone bedrock compared to pure limestone bedrock
Quaternary Sediment	Benzimidazoles	13.89	1.67–111.11	0.0536	**detection 13.9** times **more likely** at sites which have ZOCs dominated by TNSSs Quaternary sediments opposed to TLs Quaternary sediments
	TPs	13.89	1.67–111.11	0.0536	**detection 13.9** times **more likely** at sites which have ZOCs dominated by TNSSs Quaternary sediments opposed to TLs Quaternary sediments
Quaternary Genesis	Benzimidazoles	51.65	1.16–>999	0.0026	**detection 51.7** times **more likely** at sites which have ZOCs dominated by rock at surface compared to tills
Irish Forestry Soils (IFS)	Any anthelmintics	6.58	1.59–27.03	0.0172	**detection 6.6** times **more likely** at sites which have ZOCs dominated by minPD IFS soils opposed to minDW
	Benzimidazoles	6.71	1.02–43.48	0.0794*	**detection 6.7** times **more likely** at sites which have ZOCs dominated by minSW IFS soils opposed to minDW
	Non-benzimidazole	5.65	1.15–27.77	0.0843*	**detection 5.7** times **more likely** at sites which have ZOCs dominated by minPD IFS soils opposed to minDW
	Parent drugs	5.18	1.21–22.22	0.0806*	**detection 5.2** times **more likely** at sites which have ZOCs dominated by minPD IFS soils opposed to minDW

*p>0.05 however observation notes as interesting due to satisfactory odds ratio confidence intervals
LIPS = Land Parcel Information System, TPs = Transformation Products, BH = Borehole, ZOC = Zone of Contribution, Ll = Locally Important Aquifer - Bedrock which is Moderately Productive only in Local Zones, Rkc = Rkc = Regionally Important Aquifer-Karstified (conduit flow), Pl = Poor Aquifer - Bedrock which is Generally Unproductive except for Local Zones, TLs = Tills derived from limestones, TNSSs = Tills derived from Namurian shale and sandstone, minPD= poorly drained mineral soils, minDW = Deep well drained mineral soils, minSW= Shallow well drained mineral soils.

4.3.3 Analysis of anthelmintic temporal variation at catchment level

There were 11 anthelmintic compounds detected across the 11 sites at some point throughout the temporal study. These compounds included ABZ and its three TPs (SO, SO_2 and NH_2SO_2), FBZ and its TP (OXF), TCB, LEV, CLOS, OXY and IVER. The anthelmintics detected at each of the 11 sites across the 13 months, with their respective concentrations, are shown in Tables S4-7 and S4-8 (Supplementary File SI-4.1). The frequency of detection of anthelmintic compounds, in addition to the average number of anthelmintic detections per site (DPS), is shown in Figure 4-5 (a), while Figure 4-5 (b) depicts the mean concentration of anthelmintics detected at both Clare and Roscommon sites, on each sampling occasion. Considering these data, there is a trend evident at both locations, with two spikes in anthelmintic detections, firstly during the months of February and March, followed by a gradual decline, and a second spike during the month of August. These spikes occur not only for the frequency of detection and mean DPS, but also the mean concentration of anthelmintics. During February and March, an average of 1.6 anthelmintic compounds were detected at 86% of Roscommon sites, with an average of 1.5 compounds detected at 75% of Clare sites. The mean concentration detected in February was 10.3 ng L^{-1} at Roscommon sites and 14.6 ng L^{-1} at Clare sites, which further increased to 23.7 and 18.9 ng L^{-1} in March, respectively. In August, the spike in detection is most evident in terms of the mean concentration, with the frequency and mean DPS slightly lower in comparison to the Feb/March period.

Comparison of anthelmintic detections across all 11 sites using statistical analysis indicated a significant association with month (p=0.0021). Detections of anthelmintic compounds were 26.6 times more likely in February compared to January (95% intervals: 2.66–266.5), 26.3 times more likely in March compared to January (95% intervals: 2.65-250), 10.1 times more likely in August compared to January (95% intervals: 1.14–90.1) and 11.6 times more likely in September compared to January (95% intervals: 1.34–100). Furthermore, detections were 9.9 and 11.4 times more likely in both February and March compared to July (95% intervals: 1.18–82.63) and November respectively (95% intervals: 1.96–66.18). These initial observed trends appear to coincide with periods of expected anthelmintics application as part of seasonal events for the different production systems (Figure 4-1) e.g. animal turn-out to pastures and spread of manure around February/March, as well as summer dosing of spring lambs and calves. These trends are discussed in more detail in Section 4.4.3.1.

In order to further assess and explain these trends, daily rainfall and the calculated daily ER were examined for the period during and prior to each sampling event. Figure 4-6 presents the concentration of total anthelmintics detected at each of the seven Roscommon sites, with the data overlaid by the daily rainfall (black bars) and daily effective rainfall (blue bars) (bottom chart). Figure 4-6 also shows the breakdown of these concentrations for parent compounds compared to transformation products (top chart). Similar data are presented for the Clare sites in Figure 4-7. It is evident from these graphs that the spikes in anthelmintics detections and concentrations in Feb/March, and August, also coincide with period of increased rainfall and/or ER, during the days immediately prior to the sampling date. The importance of rainfall is more evident for Clare sites, which show no apparent ER occurring prior to the spike in detections in August.

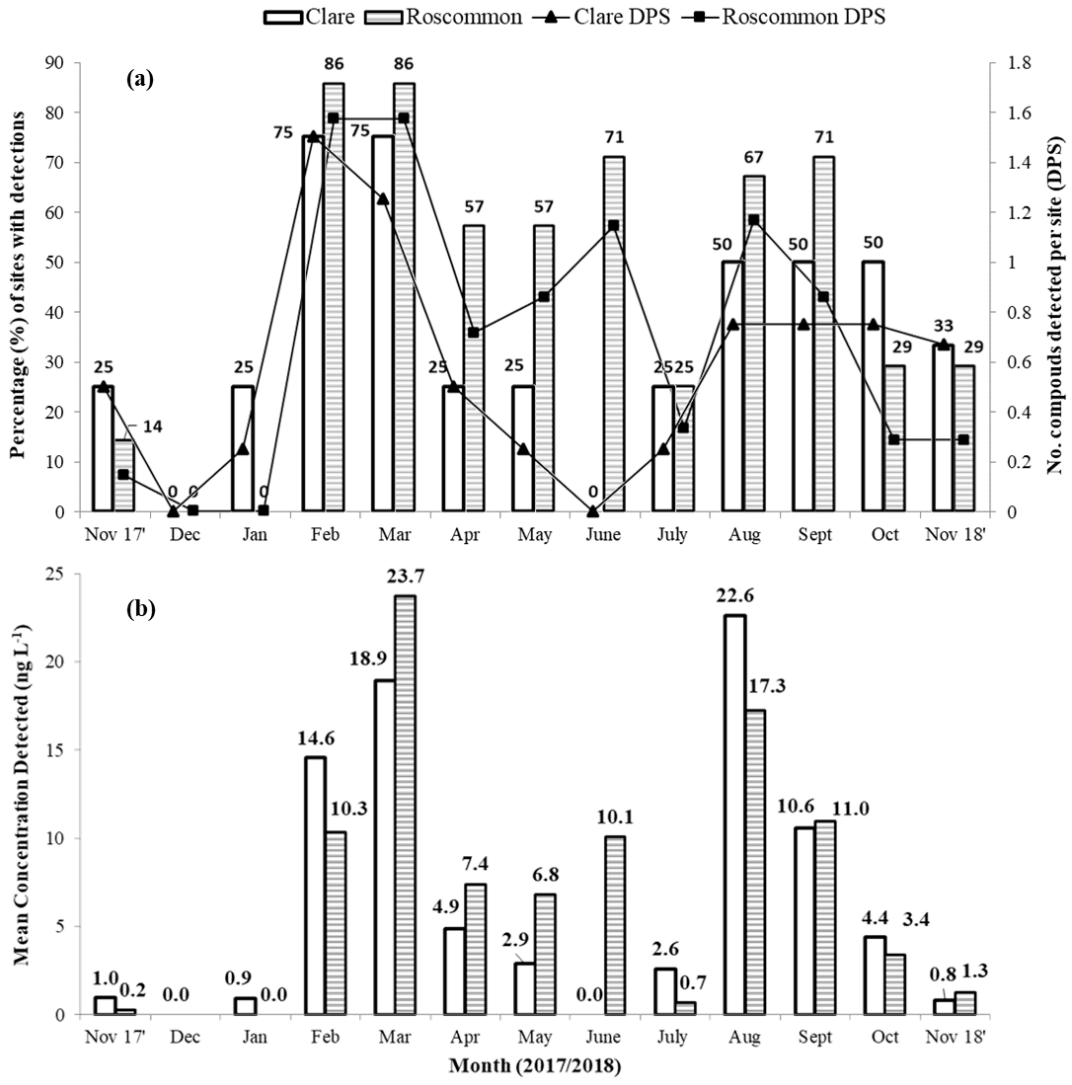


Figure 4-5 An overview of (a) the percentage frequency and mean number (per site) of anthelmintics detections and (b) the mean anthelmintic concentration detected, for Roscommon and Clare locations, across the thirteen-month temporal occurrence study.

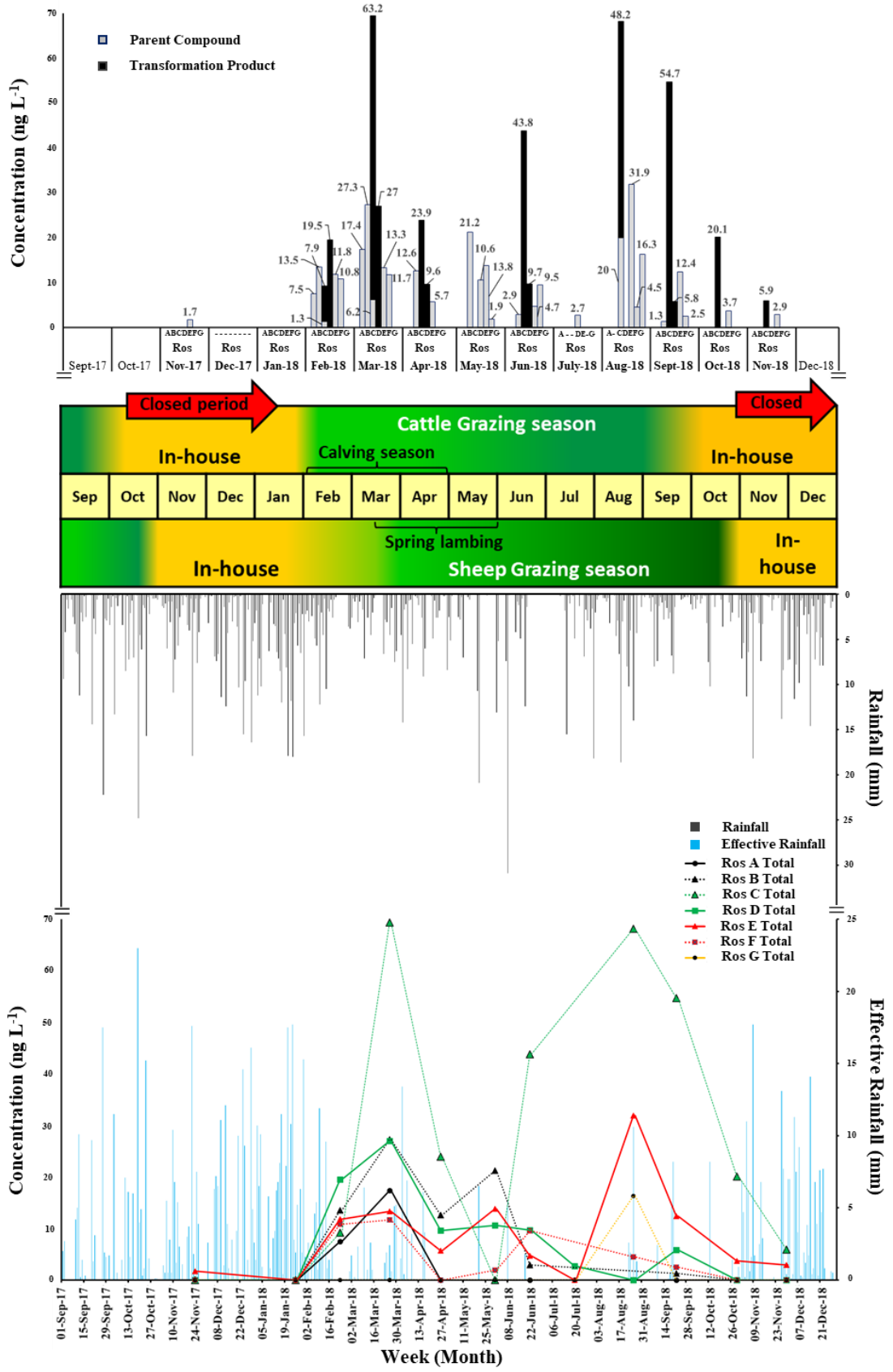


Figure 4-6 Total anthelmintic concentrations detected at each of the seven Roscommon sites (A-G) across the 12 sampling events (Nov 2017-2018), overlaid by daily effective rainfall (ER) (primary vertical axis) and rainfall (secondary vertical axis), with respective concentrations of parent (grey bar) and transformation products (black bar) shown

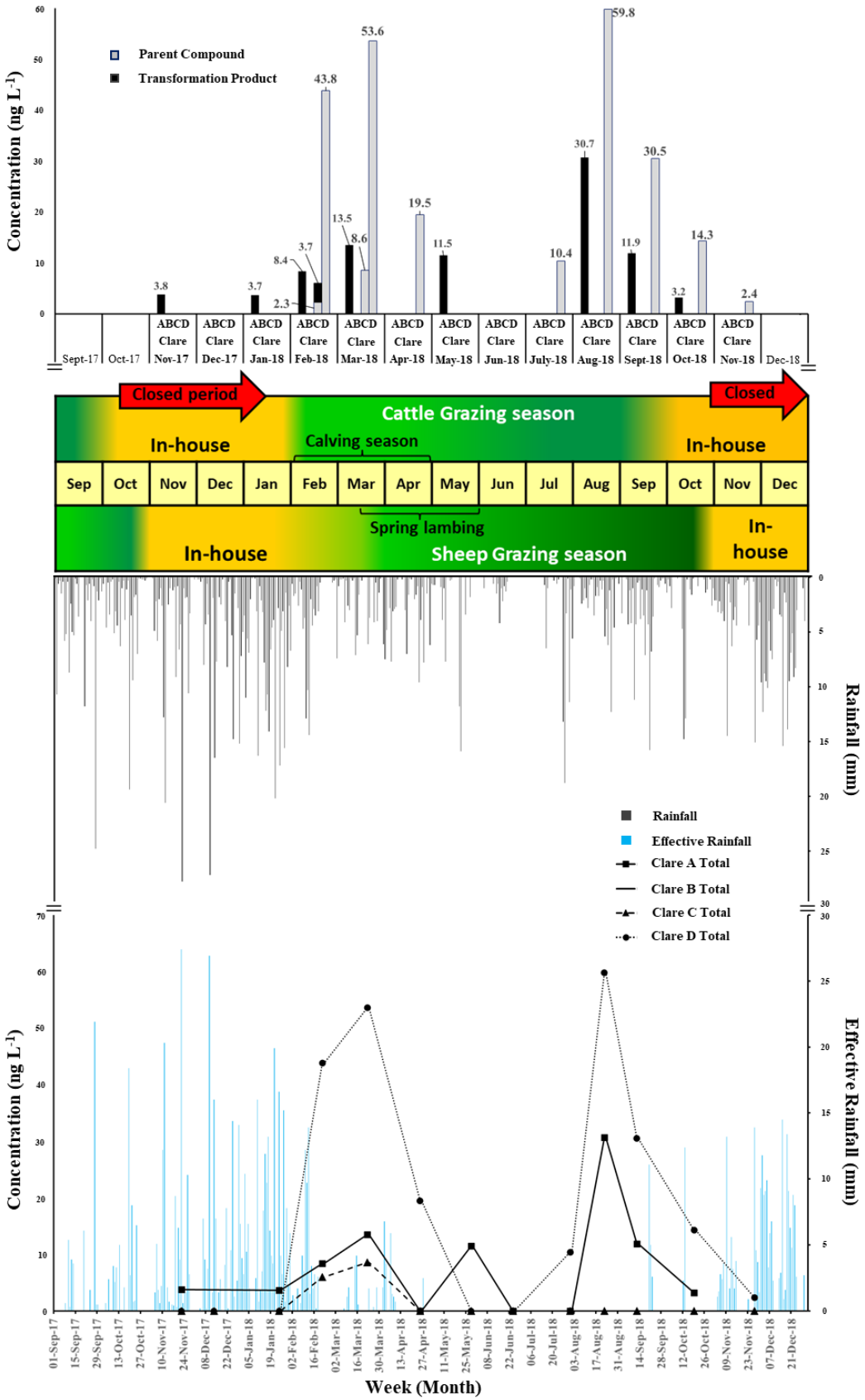


Figure 4-7 Total anthelmintic concentrations detected at each of the four Clare sites (A-D) across the 13 sampling events (Nov 2017-2018), overlaid by daily effective rainfall (ER) (primary vertical axis) and rainfall (secondary vertical axis), with respective concentrations of parent (grey bar) and transformation products (black bar) at each site also shown

4.4 Discussion

4.4.1 Anthelmintic occurrence

4.4.1.1 Timing of application

The timing of the spatial investigation (March/April 2017) coincided with a period which falls during, or just after, two of the main seasonal dosing occasions: animal housing and turnout to pasture (Figure 4-1). A general understanding of these usage patterns is therefore important in interpreting the anthelmintic occurrences discussed below.

Housing represents a period of indoor confinement of the animals following the transition from pasture, typically during winter months (e.g. Sept/October onwards for cattle (Bloemhoff et al., 2014)), or at defined stages of the production calendar (e.g. drying-off of dairy cows, finisher stage of beef or during lambing season for ewes). Anthelmintic dosing during housing is important to prevent the spread of worms and flukes acquired by the animal on pastures, prior to housing. The housing period typically occurs during what is known as the "closed-period" (typically November to mid-late January) whereby spreading of manure, slurry and chemical fertilisers is prohibited under the GAP (Good Agricultural Practice) Regulations (Government of Ireland, 2017b). As highlighted in the introduction, many anthelmintic drugs are excreted in high amounts, in faeces and/or urine. During housing, this potentially contaminated animal waste is stored for land-spreading once the closed-period has ended. Therefore, whilst anthelmintic drugs administered at housing are not likely to be at immediate risk of entry into the environment, there is the potential of entry from mid-January onwards, as a result of the re-commencement of land-spreading. This entry route is complex depending on the management practices on different farms, such as the amounts and form in which the waste is stored (i.e. as slurry versus solid farmyard manure) and timing of land application, which is also dictated by climate. According to a National Farm Survey Report (Buckley et al., 2020), at national scale, for the year 2017, 81% of all farmyard waste produced during the related housing period was stored as slurry, 46% of which was spread during the period of January to April, thus contributing a potential source of anthelmintics.

The turn-out stage represents the period where housed animals are returned to pastures. Depending on climate and ground conditions, turn-out of cattle can occur as early as the beginning of February (Bloemhoff et al., 2014), with anthelmintic treatment focusing on

dosing of young calves who are being exposed to pastures, thus the potential of worms for the first time. Turn-out of sheep generally coincides with the lambing period, which can occur anytime from mid-March through to May (Patten et al., 2011). Initial treatment for cattle at turn-out focuses on treating roundworms in calves, normally within the first three weeks of turn-out, with a typical dosing regimen every 3-6 weeks thereafter throughout the summer grazing, up until the end of July (Figure 4-1). For sheep production, new-born lambs are particularly susceptible to infection by roundworms on pastures from the age of 5 weeks onwards, with the highest risk of infection by *Nematodirus* spp. worms during April and May (Animal Health Ireland, 2020a). Lambs are therefore normally treated with wormers from the age of 5 or 6 weeks, with further doses every 6 weeks.

Considering the above, it is certainly plausible that the detections during this study in March and April relate to an increased source of anthelmintic due to landspreading of slurry and dosing of animal on pastures at periods of high parasite burden. However, it must be noted that this association with recent usage is not definitive given the potential of time lags for the transport of contaminant to water bodies, depending on environmental conditions and the properties of the hydrogeological setting into which they are entering.

4.4.1.2 Compounds detected

The compounds detected, and their frequency of detection, are consistent with the overall usage of the anthelmintics in Ireland. The BZs were the most detected class of anthelmintics, with 14 of 17 compounds detected belonging to this class of anthelmintic. The prevalence of BZ detections is not surprising given they are one of the most commonly used classes of anthelmintics, both in cattle and sheep production, due to their broad-spectrum efficacy toward nematodes at all stages of their lifecycle (i.e. immature and mature worms). In a three year study investigating anthelmintic resistance in sheep farms throughout Ireland, Keegan et al. (2017) reported that BZ drugs were the most popular anthelmintic class used by farmers, with usage in 42% of the cases. BZs are commonly administered at housing as part of a combination product, typically containing a BZ and a flukicide. ABZ is the most common BZ administered at housing, owing to its dual efficacy toward both nematodes and mature liver flukes. A survey of 312 pasture based dairy farms in Ireland found ABZ to be the most commonly used BZ drug for treating heifers and calves on pasture based dairy farms, with 30% of farms reported to be using ABZ in the 2009 survey, with the usage further

increasing to almost 58% in a follow-up survey in 2011 (Bloemhoff et al., 2014). Such usage could therefore account for the higher frequency of detection of ABZ, with ABZ and/or its TPs occurring in 52% sites with detections recorded in this study. TCB and its TPs (SO and SO_2) were the second most detected BZs, the frequency of which (22% of sites with a detection) was much lower than the ABZ compounds. TCB is the only drug effective toward early-immature flukes; however due to reported widespread resistance issues, it is only used for treating acute infections of liver fluke (Animal Health Ireland, 2013; Animal Health Ireland, 2020b), and this is likely to account for lower frequency of detection. All other BZs that were detected are authorised for used in both cattle and sheep; however, their usage trends are less predictable given that anthelmintic usage typically depends on factors such as availability, cost and the farmer's preference and past experience (Patten et al., 2011) e.g. besides ABZ, FBZ and OXF are sometimes favoured for treating nematodes in lactating cows, due to the short milk withholding times, which could account for the detection for both of these compounds at 19% of sites with detections.

In order to further assess these trends in BZ detections, it is also important to consider other factors, such as the environmental mobility, that may influence the environmental occurrence. A comprehensive review of such factors has previously been carried out by Horvat et al. (2012), who provide a critical overview of the current knowledge on the fate and the ecotoxicology of anthelmintics and their TPs. Considering their physiochemical properties (Table 4-1), with poor water solubility, high octanol-water coefficients and high organic-carbon water coefficients, on application to soil, it is expected that BZs will be relatively immobile and more associated with soil and sediment compartments than aqueous phase. Such effect is demonstrated by Kreuzig et al. (2007) who investigated the fate of FLU and FBZ in manure and manure amended soil. Both BZs demonstrated slow degradation in manure, and on application to soil, both were extractable from the near surface soil. Notably, this study also demonstrated the degradation of FBZ to its metabolites FBZ-SO (OXF) and FBZ-SO_2 in the manure amended soils, with OXF increasing to 45% within 14 days, after which is was further converted to the SO_2 derivative, which amounted to 12% of the amount applied. This is consistent with the findings of our study, with the FBZ parent and OXF TP detected more frequently than the FBZ-SO_2 (Table 4-2).

As part of similar adsorption studies Mutavdzic Pavlovic et al. (2018) indicated the medium to strong sorption of ABZ on sediment and soil. The authors note that the sorption of ABZ

is particularly dependent on pH, which dictates the overall ionic form of the drug. Considering the pKa of ABZ, and other BZs, and the typical pH of soils in Ireland (typically between 5.5 and 6.5 (Teagasc, 2018)), there is the potential for the BZs to be ionized to a certain extent that may increase their mobility, however, Mutavdzic Pavlovic et al. (2018) suggest that the neutral form of ABZ is predominant between both pKa's, so this form may control the mobility across the typical soil pH range. This strong sorption could account for the lower frequency of ABZ parent in groundwaters compared to its SO, SO_2, and NH_2SO_2 TPs, all of which are more polar and likely to have greater water solubility; the increased solubility of ABZ-SO (the main animal metabolite) compared to ABZ (Horvat et al., 2012) and the increased polarity of the amino-sulphone could enhance the transfer of these TPs to surface and groundwater.

Regardless of the apparent immobility, various studies have indicated the potential for transport of veterinary drugs including the anthelmintics, via preferential pathways, or as a result of surface runoff (discussed further in Section 4.4.2.2). Such a transport route is likely to have a further influence on the overall fate of the BZs. As highlighted by Horvat et al. (2012), a number of the BZs including ABZ, FBZ, FLU, MBZ and TCB, have the potential to undergo extensive transformation (by oxidation, reduction or hydrolysis) not just as a result of metabolism within the animal, but also while in the environment, to produce various sulphoxide, sulphone, amino and hydroxy derivatives. These authors note that photolysis is the main degradation pathway for most BZs, particularly ABZ and MBZ, with the amino derivative being the common product, which are likely to be slightly more water soluble. Such degradation pathways could also account for the overall prevalence of TPs found in this study; however, if transport is facilitated by underground pathways, the contaminants will be less exposed and persist for longer.

Beside the BZ compounds, all of the other compounds that were detected are commonly used as flukicides (CLOS, OXYCLOZ and RAFOX), except for LEV, which belongs to its own class entirely. Fluke infection of cattle, particularly spring born calves, normally occurs later into the autumn period with the onset of wetter conditions which favour the hatching and growth of fluke on pastures. Therefore, spring calves are typically dosed with a flukicide, such as CLOS and OXYCLOZ, initially at turn-out (if not during housing) and again during late summer and early autumn. Sheep are much more susceptible to liver fluke than cattle during this period since they graze closer to the ground, with treatments involving flukicides

such as CLOS or RAFOX used for spring dosing to treat chronic and subacute fluke infection. Such usage can therefore account for the occurrence of these compounds. The environmental fate and degradation pathways of these compounds are not widely reported, however CLOS and RAFOX are highly hydrophobic and may also be subject to similar transport mechanisms as the BZs. LEV is a drug used as a wormer, and is often used in combination with TCB, with products containing LEV often favoured later in the season, if BZ efficacy fails. Liver fluke burden on pastures is typically particularly high during the autumn months, due to the onset of wetter conditions, therefore treatments at or near housing are particularly important. While LEV has been shown to have poor mobility in the environment due to strong sorption to soil (Ma et al., 2019), its high solubility and relatively low $LogK_{ow}$ indicates it is susceptible to preferential and conduit flow pathways in which the bulk soil matrix is bypassed.

Finally, it is also important to note the lack of detections of the anthelmintic ML class (endectocides) as part of this study, which is somewhat surprising given this class of drugs is used just as heavily as the BZs. In terms of usage patterns, IVER and EPRINO are the most heavily used MLs. The former is typically used at housing and can often be favoured due to its efficacy as both a wormer, but also as an ectoparasiticides for external parasites such as ticks and lice (Animal Health Ireland, 2013). The lack of groundwater occurrence of IVER may be explained by its lack of mobility in the environment. Krogh et al. (2008b) suggest that ivermectin binds to soil by formation of complexes with immobile, inorganic soil matter. Prasse et al. (2009) also highlight the importance of photodegradation of IVER in water, with DT_{50} (time to degrade to 50% of initial concentration) reported from 6–39 h. It must be noted however, given the typical concentrations of BZs detected (majority <10 ng L^{-1}), the lack of detection of the MLs relative to the BZs may be of a consequence of the lower sensitivity of the analytical method for MLs, with method limits for the BZ 10 times lower for the MLs.

4.4.2 Relationship of anthelmintic groundwater occurrence with site characteristics

4.4.2.1 Source factors

Statistical analysis indicated that occurrence of BZs and their TPs was more likely at sites with ZOCs dominated by tillage land compared to grassland, while the occurrence of non-BZ compounds was significantly related to the density of sheep (detections associated with

higher densities of ewes and "other sheep"). Interpretation of the relationship of detections with tillage is difficult given that the sites dominated by tillage land represented only a small sample size (n=7) compared to those dominated by grassland (n=81), with the overall statistical relationship based on BZ detections at just 3 of 7 tillage dominated sites, two of which were in the same surface catchment. Therefore, while significant, this relationship cannot be regarded as conclusive, given the evident bias by the small sample size.

The higher likelihood of BZ detections in tillage vs. grassland may be initially explained as a result of the method of application of animal waste to both systems. A national study by Hennessy et al. (2011) reports that the majority manure applied to tillage was solid manure incorporated by ploughing (compared to liquid manure on grassland by spray plate), which may potentially facilitate the direct entry of the contaminants to lower depths below the surface, where they may be less attenuated due to the lower soil organic carbon and microbial activity (Alletto et al., 2010). It is also noted that the incorporation of the manure by ploughing was not carried out until more than 48 h after the initial manure application. This could explain the association with TPs, with parent drug subject to photodegradation while exposed on the soils surface, the process of which may be retarded following ploughing, since the compounds will be less exposed to light. However, this explanation is complicated by several other factors. In Ireland, up to 67% of farms that are designated as tillage also having some form of livestock enterprise, sizeable enough to account for up to one third of annual outputs (Buckley *Pers. Comm., 2020)*. As a result, assumptions around landspreading as a source cannot be made with any certainty, with the livestock enterprises also potentially contributing to the occurrences. Although there was negative association found between detections and the percentage of grassland within the ZOC, there was still an appreciable proportion of grassland within the ZOCs of sites that had a detection (mean of 52%) compared to sites with non-detections (mean of 75%). The transport of BZs through the soil matrix has been demonstrated by Weiss et al. (2008) who report loss rates of up to 16% as a result of preferential flow through macropores, of which permanent pastures have a multitude. It is therefore reasonable to suggest that the relationship observed with tillage may actually be caused by a combination of both tillage farming and grassland, with a much more focused and detailed study required to properly disentangle this relationship.

The association of the non-BZ detections with sheep density can be accounted for by a combination of both source and pathway factors (discussed in 4.2.2 below). Two frequently

detected non-BZ compounds at the sites with higher sheep density were OXYCLOZ and CLOS, detected at 66% of sites that had non-BZ detection. The association of these compounds with sheep density is not surprising given both compounds are used as flukicides for treating liver flukes, to which sheep are particularly susceptible, as previously discussed. Geography may also have an effect, with most of the detections of these flukicides occurring in the west of Ireland. This may be due to the expected higher fluke burden in western regions of Ireland, as a result of wetter climate and poorer ground conditions, which is known to favour fluke growth (Parr and Gray, 2000). Bloemhoff et al. (2014) reported a higher usage of flukicides on dairy farms in western regions and attributed this to the higher fluke burden, as well as the higher proportion of mixed cattle and sheep farms in the western counties. It is also notable that these regions are typically the areas of Ireland where the majority of sheep production occurs (DAFM, 2017). Overall, it is evident that the relationship of non-BZ detections with sheep intensity, is likely localised to such regions and may be dependent on environmental conditions.

4.4.2.2 Pathway factors

A number of pathway factors (including sampling point type, aquifer type, bedrock group, Quaternary sediment and IFS soil type) were shown to be statistically related to the occurrence of anthelmintic compounds (of one or more classes of these). However, meaningful interpretation of the relationship with a number of these hydrogeological factors (namely aquifer class, bedrock group and Quaternary sediment) is complicated as a result of clusters of sites at catchment level and/or a relatively low number of detections from a small sample size, causing a bias in the statistical analysis. It must therefore be stressed that these observations should only be considered in an exploratory manner and may be used for informing future studies.

The occurrence of anthelmintics (collectively) was shown to be more associated with monitoring BHs than abstraction BHs, and this may be reflective of the larger ZOCs associated with abstraction BHs, compared to the monitoring BHs (Median ZOC area of monitoring BHs was 0.09 km^2 compared to 0.50 km^2 for ZOCs of abstraction BHs). Monitoring BHs, having smaller ZOCs, are likely to be more sensitive to contamination by nearby localised activities and sources of anthelmintics. For abstraction BHs, a localised area of contamination within a larger ZOC is more likely to undergo dilution due to the larger

volume of aquifer from which the water is drawn, with longer distances and travel times to the BH providing more opportunity for attenuation of the contaminants.

Detections defined for all anthelmintics (collectively), were shown to be more likely in poorly drained soils (minPD) compared to deep well drained soils (minDW). This relationship appears to be driven more by the non-BZ and parent drug groups of detections, which showed similar relationships with poorly drained soils. There is also evidence that this association with poorly drained soil is confounded by the relationship with sheep density. Sheep are commonly grazed on poorly drained soils, which typically have wetter ground conditions that are more favourable for growth of parasites such as the flukes on pastures, thus increasing the parasite burden and need for anthelmintic drugs such as the flukicides. With an assumed source of the contaminants, transport to the groundwater below the poorly drained soils is likely to be facilitated by two potential pathways, either as a result of preferential flow through the soil via macropores (which has previously been indicated to be an potential pathway for anthelmintics (Weiss et al., 2008)), or via surface or near surface pathways (particularly with heavy rainfall) (Stamm et al., 2002; Kreuzig et al., 2007) to exposed features such as karstic sinking streams, which can then provide further rapid groundwater transport via conduits. The latter of these seems a potentially plausible explanation, with 3 of the 5 minPD sites with detections being karstic springs which are known to be fed by conduit flow with rapid travel times. The influence of surface pathways in poorly drained soils is also reflected by the higher frequency of anthelmintic detections in the surface waters: almost half of the detection in surface water streams occurred in catchments dominated by minPD soils.

The relationships of anthelmintic detections with different aquifer classes (Pl and Ll) and bedrock groups (non-calcareous and metamorphic) were also shown to be statistically significant despite the small number of sites involved, however as a consequence of such small sample numbers, these relationships are likely being influenced more by the localised characteristics of these sites. An example of this is the association of BZ detections with the Ll aquifer class. Of the five sites with BZ detections that had ZOCs dominated by Ll aquifers, two sites (site 032 and 076) had an appreciable proportion of karstic aquifer within the ZOC (43% Rkd aquifer for site 076 and 42% Rkc aquifer for site 032), with these karstic aquifers supplying the borehole (076) and spring (032). In addition, three of the five sampling points were classified as monitoring BHs, which have been shown to have a higher likelihood of

anthelmintic detections, as discussed previously. It is therefore believed that the occurrence of anthelmintics at these sites are more likely related to a combination of such localised factors, as opposed to the dominant aquifer class in the ZOC.

Further evidence of the importance of combined localised factors is evident when considering the groundwater vulnerability dataset analysis. Although the relationship of anthelmintic detections with groundwater vulnerability was not statistically significant (Table S4-6), there is some evidence of a weak relationship with extreme (E) vulnerability. Whereas 34% of the 88 groundwater sites had ZOCs dominated by E vulnerability, 56% of the 16 groundwater sites with detections were dominated by E vulnerability. Further investigation of the relationship between detections and the percentage of the ZOC with E vulnerability showed only a weak relationship ($p = 0.13$), but it is possible that more targeted studies with fewer confounding factors could determine a clearer relationship with groundwater vulnerability.

4.4.3 Interpretation of temporal trends

Two of the most important factors likely to be influencing the temporal variation in occurrence of these drugs at the conduit dominated karstic sites are (a) the usage patterns and timing of application of the drugs (source factor), and (b) meteorological events, primarily rainfall events controlling recharge (pathway factor). As previously highlighted, the routes of entry to the environment are also important and therefore must be consider alongside the usage pattern, when discussing the temporal trends below.

4.4.3.1 Temporal trends in relation to usage patterns

Considering the same usage patterns discussed for the spatial study (Section 4.4.1.1 and also as summarised in Figure 4-1), and assessing the temporal trends observed as part of this study, it is evident that the spikes in detections during Feb/March and August/September of 2018, coincide with an increased source of the drugs entering the environment as a result of periods of heavier usage. The increased detections in Feb/March 2018 occurred during or following a period where cattle and sheep are turned-out from housing and dosed for worms and flukicides (in some instances), providing the potential of direct excretion of the drugs onto pasture while grazing. Spreading of slurry and manure collected during the housing period at the end of 2017 and into 2018, also provides a potential source of anthelmintics.

According to the 2018 Teagasc National Farm Survey on bovine manure management (Buckley et al., 2020), on a national scale, 42% of bovine slurry gathered during housing was land-spread between January (mid-late) and April of 2018, with a further 41% of slurry spread through the summer months of May to July. Both study catchments are dominated by grassland, onto which 97% of bovine slurry is spread (based on national average (Hennessy et al., 2011)), so it is evident that the first spreading of slurry on re-commencement after the closed period is also likely contributing a potentially significant source of anthelmintics. This combined with the usage at turn-out provides reasonable explanation for the initial spike in detections in February/March. Further application (of the 41% of stored slurry) through the summer months, in addition to the regular dosing of spring lambs and calves in pastures (dosed with wormers through to the Autumn), is likely contributing to the spike in detections in August. The lower frequency of detections throughout the months preceding August, is interpreted as a result of meteorological factors, which are discussed further in Section 4.4.3.2.

In terms of the specific anthelmintics detected throughout the temporal study, there are several trends that are likely explained by usage patterns. As with the spatial study, ABZ and its TPs were commonly detected at several sites throughout the temporal study. When administered at housing to treat nematodes and liver flukes, ABZ will be excreted primarily in urine (Wardhaugh, 2005) , after which it will be stored, and later land spread in the form of slurry. It may also be used as a wormer for treating animals at turn-out, and furthermore for spring lambs and calves through the summer months. This could account for the initial early Feb/March detections of the ABZ parent drug, with the increasing concentrations of the ABZ TPs in the months thereafter likely due to a combination of both degradation and a potential increased source from animal excreta. The main sulphoxide metabolite that is excreted by animals (Junquera, 2015) can undergo further transformations to the amino-sulphone derivative, which is also the main environmental photodegradation product of ABZ (Horvat et al., 2012). The occurrence of OXYCLOZ is also probably attributable to land spreading of the drug administered during housing, given that it is only effective for treating mature liver fluke (Bloemhoff et al., 2014). LEV was typically only detected from the month of May onward, and this can be explained by the fact that LEV is used for treating *Teladorsagia* spp. and *Trichostrongylus* spp. roundworm, which pose the highest risk of infection from June to August. It is also notable that IVER was detected on a number of sampling occasions at one site (Clare D), with the highest concentrations detected during

February and March 2018. IVER was not previously detected as part of the spatial study in March/April 2017.

4.4.3.2 Temporal trends in relation to meteorological factors

Having established plausible sources of anthelmintics in the environment, assessing the meteorological conditions prior to the sampling events can provide further explanation for the trend in detections. Since the sites sampled as part of this temporal study have the potential for rapid transport of contaminants from sinking streams to springs in as little as 8 hours, meteorological events during the hours and days just prior to the sampling events are likely to have the most influence on the transport of the contaminants. It is important to note however that this is not always the case, with the potential of longer lag times (from months to years) in other hydrogeological settings such as low permeability soils and less transmissive aquifers (Fenton et al., 2011), so recent meteorological events may be of less importance in such situations.

For the Roscommon sites, there is a clear trend of significant rainfall events occurring during the periods just prior to the February and March spike (Figure 4-6), with most of the incident rainfall was recorded as effective rainfall, thus contributing to recharge. The mean daily rainfall and effective rainfall for the week prior to the sampling event was 2.8 and 2.3 mm per day, with as much as 12 mm of effective rainfall occurring on a given day during this period, which is likely sufficient to provide for the transport of the veterinary drugs to groundwater. It is also notable that month of January 2018 was particularly wet, with an average monthly rainfall of 177 mm (compared to a 30-year average of approx. 92 mm). Consequently, although permitted by legislation, there is the potential that manure spreading was not carried out immediately after the end of the closed period, rather later into February, providing a potential increased source of the drug residues closer to the sampling event, with contaminants carried to groundwater with any subsequent rainfall.

The summer of 2018 was atypical, with drought conditions present across most of the country during July and into August. For Roscommon, there were very few ER events occurring from April through July, with accumulated soil moisture deficits and higher evapotranspiration rates consuming any incident rainfall. Although there was a potential regular source of drugs throughout these summer months, these dry conditions may account

for the decrease in frequency and concentration of the detected anthelmintics, due to the contaminants remaining on the surface or in the soil matrix. The spike in detections in August can be explained by the evident ER events just prior to the sampling. The increase in concentrations is potentially as a result of the flushing of a concentrate of the contaminants from the soil, with this significant ER event.

Similar ER trends were observed for Clare sites (Figure 4-7), to those described for Roscommon, with one main difference; the detections at Clare sites in August do not appear to coincide with any ER event, with large SMDs and no ER occurring from mid-April through to early September. These sites provide evidence of the importance of surface pathways in such karst hydrogeological settings. The meteorology data show up to 20 mm of rainfall occurring in Clare during the period prior to the sampling event. Although not contributing to ER due to the accumulated SMD, it is plausible that this high incidence of rainfall resulted in rapid run-off and/or direct infiltration to karstic aquifers.

4.4.3.3 Surface-groundwater connections

Interesting trends were observed for two of the Roscommon karst springs (C and E), with the same compounds detected on a number of sampling events, both at the spring and at a sinking stream known to be connected (based on previous dye tracing (GSI, 2014)). At Roscommon site C (sinking stream), the parent compound ABZ, was detected initially during February and March, with an increasing concentration of TPs (particularly the amino sulphone TP) detected for several of the months thereafter (data provided in Table S4-7). Site observations indicated evidence of cattle grazing and sheep farming in the immediate vicinity of the sinking stream, thus providing a likely source of the contaminants. At the associated spring (Roscommon D), the same ABZ TPs were detected during a number of sampling events (February, March, April, June and Sept), albeit at lower concentrations, which provides evidence of a potential poorly attenuated surface to groundwater pathway for anthelmintics. The lower concentrations combined with the lack of detection of parent drug and the prevalence of the amino-sulphone at the spring (compared to the sinking stream), does however indicates some degree of attenuation and/or degradation during transport. Another example is provided by Roscommon sites E and F. OXYCLOZ was detected during February and March, both at Roscommon spring E and at the sinking stream (site F). In this case the concentrations detected at the sinking stream (10.8 and 11.7 ng L^{-1}

in Feb and March) and the spring (11.8 and 13.3 ng L^{-1}) were quite comparable. While there is very little information reported on the fate of OXCLOZ in the environment, its relatively high $LogK_{ow}$ and $LogK_{oc}$ values (Table 4-1) indicate that it is likely to persist in the environment within the soil compartments. The occurrences at Roscommon spring E further indicates the potential for conduit fed systems to accommodate the unattenuated transport of some anthelmintics, which would otherwise persist and remain in the soils. While the suggested transport in both examples is not definitive, given that the groundwater arriving at a karstic spring is supplied by a network of conduits and fractures rather than a single sinking stream, it does clearly demonstrate the vulnerability of these systems and the need for further site-specific investigations

4.4.4 Overall comparison to previous occurrence studies

There are only a limited number of studies available reporting on the occurrence of anthelmintics in environmental waters, the majority of which related to surface waters (Bartelt-Hunt et al., 2009; Sim et al., 2013; Wagil et al., 2015b) or agricultural leachate/ seepage water (Raich-Montiu et al., 2008; Weiss et al., 2008). To the best of our knowledge, there have been no reported occurrences of the majority of anthelmintics in groundwaters, except for thiabendazole, detected in a single farm well in Norway (Haarstad and Ludvigsen, 2007). Of the data available, the BZs seems to be the most commonly detected anthelmintic in surface waters. Van De Steene et al. (2010) detected FLU in 15 of 16 surface waters at concentrations ranging from 0.3–20.2 ng L^{-1}, with the majority of samples having concentrations <5 ng L^{-1}. Wagil et al. (2015b) reported the detection of FLU and FBZ in 4 of 8 surface waters in Poland at concentrations ranging from 5.4–87.5 ng L^{-1}. Prior to this current study, the most comprehensive investigation of anthelmintics in environmental waters was carried out by Zrncic et al. (2014) who analysed 11 samples from the Llorbregat River in Spain for 10 different anthelmintics. The authors reported the detection of eight anthelmintics, including ABZ, FBZ, FLU, LEV, TCB and MOXI, typically at concentrations ranging from 1–5 ng L^{-1}, except for LEV which was detected at concentrations up to 39 ng L^{-1}. The overall findings of the current work are consistent with these previously reported environmental occurrence, both in terms of the compound detected, and the concentrations detected as part of the spatial study(1–41 ng L^{-1}) and the temporal study (1.3–47.5 ng L^{-1}). One noticeable difference, however, is the prevalence of TP detections in the current study, which most other studies lack, primarily due to the failure to include such TPs in their

analytical methodology. Detection of additional compounds in the current work, such as the flukicides OXYCLOZ and CLOS, may be more reflective of usage patterns, with the wetter, damper climate in Ireland having a higher fluke burden, thus the need for flukicides, compared to compared to Mediterranean countries such as Spain .

While some of the anthelmintic compounds are covered as "nematocides" under the pesticide definition specified in the EU Drinking Water Directive (European Commission, 1998) and Groundwater Directive (European Parliament, 2006), there is currently no regulatory monitoring of these contaminants in groundwater or drinking water. Furthermore, there are no environmental quality standards (EQS) set for these contaminants in environmental waters. Considering the concentrations of anthelmintics detected in the current study, and accounting for the TP concentrations, although there were no breaches of the pesticide parametric value of 100 ng L^{-1} (0.1 µg L^{-1}), there were a number of occasions where the concentrations of anthelmintics (e.g. albendazoles) met or approached the pesticide threshold value of 75 ng L^{-1} (Government of Ireland, 2014a), indicating that such contaminants may require additional investigation. Such investigations should also prioritise the assessment of the environmental impacts of anthelmintics. While the majority of the benzimidazoles concentrations detected are not likely to pose an acute environmental risk given the reported ecotoxicological data have shown half maximum effective concentrations (EC_{50}) of the order of low µg L^{-1} for aquatic organisms such as *Daphnia magna*, there is a concern for the levels of the ML IVER, detected at concentrations up to 47.5 ng L^{-1} as part of the temporal study. Previous studies have shown that IVER is highly toxic to a number of aquatic organisms, including the *Neomysis integer*, *Gammarus* sp. and *Daphnia magna,* with reported lethal concentration (50%) (LC_{50}) values ranging from 25–70 ng L^{-1} (Hally et al., 1989; Horvat et al., 2012). Even higher acute toxicity to *Daphnia magna* has been reported by Garric et al. (2007) with even lower LC_{50} values of 5.7 ng L^{-1}. The same authors report extremely high chronic toxicity to IVER at levels of 0.3–1 pg L^{-1}.

4.5 Conclusion

This study, the first of its kind in Ireland, reports on the most comprehensive investigation of an extensive suite anthelmintic drugs (including 27 parent drugs and 13 transformation products) in groundwaters and a limited number of surface waters throughout Ireland. Up to sixteen different anthelmintic residues were detected across 18% of the groundwater sites, with up to 8 compounds detected in 39% of the surface waters sampled. The overall detected concentrations of anthelmintics ranged from 1–41 ng L^{-1}. Of the 17 compounds detected, 13 belonged to the benzimidazole class of anthelmintics which are commonly used in Ireland. Of these, albendazole and its three transformation products (albendazole-sulphoxide, albendazole-sulphone and albendazole-amino-sulphone) were the most commonly detected, found at 52% (12 of 23) of the sites with detections, and at 11% of the total (106) sites, with occurrence in both groundwater and surface waters. Fenbendazole and its two metabolites were also frequently detected, in this case only occurring in groundwater.

Sheep density and the proportion of tillage land within the zone of contribution (ZOC) of sampling sites were shown to be statistically significant source factors associated with anthelmintic occurrences, with detections more likely at sites with higher sheep density and with higher proportions of tillage land use in their ZOC. Several pathway factors were statistically related to occurrences, however, many of these were confounded by small clusters of sites within the same catchment. Sampling point type and soil type were shown to be related to anthelmintic occurrences, with detections more likely in monitoring boreholes compared to abstraction boreholes and in sites with ZOCs dominated by poorly drained soils. However, it was evident that the occurrence of anthelmintics in groundwater was not solely related to any one source or pathway factor at national scale, rather a combination of multiple factors on a more localised level, which varied on a site by site basis. The findings of the temporal investigation indicated that the periods of highest risk of anthelmintics occurrence was February/March following return of animals to pastures, pre-spring dosing and landspreading of manure, and again later during August/September, which was interpreted to be as a result of the transition from dry summer conditions to wetter in autumn. The outcome of this temporal investigation also highlighted the potential importance of unattenuated surface to groundwater pathways, such as sinking streams, for the transport of anthelmintics to groundwater.

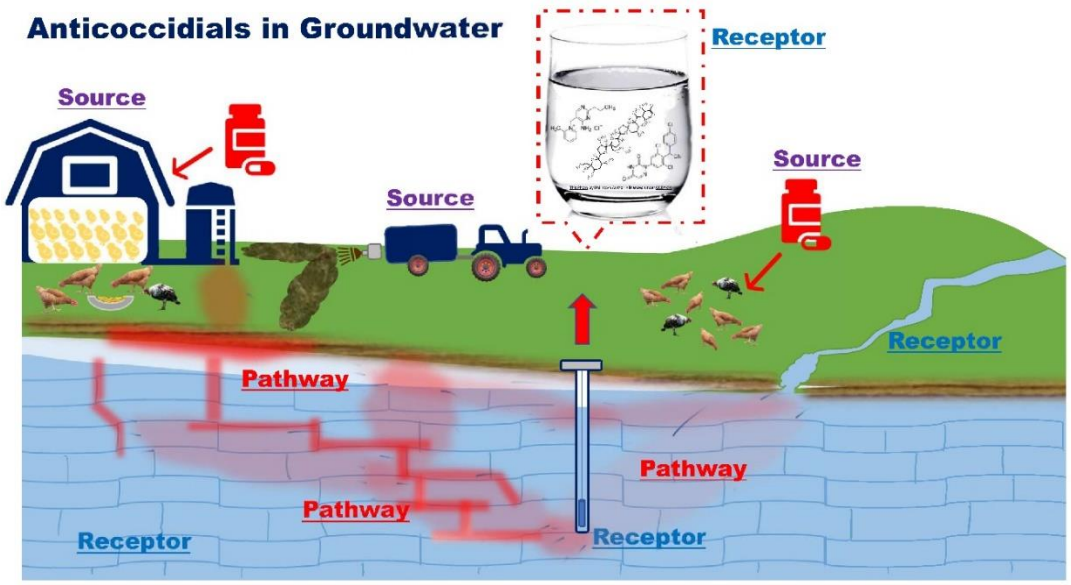
Overall, while the results of this study may not be suitable for use as definitive predictors of anthelmintic occurrence, they are suitable for application as a broad tool for identifying potential sites for targeted anthelmintic monitoring. The results suggest that groundwater monitoring points with small ZOCs, with a high density of sheep within the ZOC, and in areas with poorly drained soils and a higher parasite burden due to wetter climate, would be potential candidates for anthelmintic monitoring. Furthermore, monitoring of the benzimidazole anthelmintics, may be more worthwhile at sites with a mixture of tillage and grassland agriculture within the ZOC.

4.6 CRediT author statement

Damien Mooney: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Visualization, Project Administration. **Karl Richards:** Conceptualization, Resources, Writing – Review and Editing, Supervision, Funding Acquisition. **Martin Danaher:** Conceptualization, Methodology, Resources, Writing – Review and Editing, Visualization, Supervision, Funding Acquisition. **Jim Grant:** Software, Formal Analysis, Writing – Review and Editing. **Laurence Gill:** Conceptualization, Writing – Review and Editing, Funding Acquisition. **Per-Erik Mellander:** Conceptualization, Resources, Writing – Review and Editing. **Catherine Coxon:** Conceptualization, Writing – Review and Editing, Visualization, Supervision, Project Administration, Funding Acquisition.

CHAPTER 5 – AN INVESTIGATION OF ANTICOCCIDIAL VETERINARY DRUGS AS EMERGING ORGANIC CONTAMINANTS IN GROUNDWATER

Graphical Abstract



Lay Abstract

Anticoccidials are a group of antiparasitic agents used as feed additives or veterinary medicines for treating coccidiosis, which is an intestinal disease caused by parasites. In Ireland and more broadly in the European Union, the primary use of anticoccidials is in poultry productions as a prophylactic treatment, which means every bird is treated regardless of whether they are infected or not. As a result, there is a potential continuous source of anticoccidials that may enter the environment by landspreading of contaminated poultry manure and litter. There is very little information known about the occurrence of anticoccidials in the environment, particularly groundwater. The aim of this work was to use a newly developed analytical method to investigate whether these drugs are getting into our groundwater, and if so, at what levels they are present. This work also aimed to assess where the contaminants came from, and how they got to the groundwater. This was done by carrying out a comprehensive occurrence study involving 109 groundwater sites that were selected to be representative of different source and pathway pressures. Up to 7 different anticoccidials were found at 24% of the sites sampled, with the anticoccidials that were most often detected, being consistent with the reported usage patterns. The overall results indicated that poultry activity (poultry farms and manure spreading) was the most significant driver of anticoccidial occurrence. This work reports some of the first detections of anticoccidials in groundwater Ireland and in Europe.

AN INVESTIGATION OF ANTICOCCIDIAL VETERINARY DRUGS AS EMERGING ORGANIC CONTAMINANTS IN GROUNDWATER

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Abstract

Intensification of the food production system to meet increased global demand for food has led to veterinary pharmaceuticals becoming a critical component in animal husbandry. Anticoccidials are a group of veterinary products used to control coccidiosis in food-producing animals, with primary prophylactic use in poultry production. Excretion in manure and subsequent land-spreading provides a potential pathway to groundwater. Information on the fate and occurrence of these compounds in groundwater is scant, therefore these substances are potential emerging organic contaminants of concern. A study was carried out to investigate the occurrence of anticoccidial compounds in groundwater throughout the Republic of Ireland. Twenty-six anticoccidials (6 ionophores and 20 synthetic anticoccidials) were analysed at 109 sites (63 boreholes and 46 springs) during November and December 2018. Sites were categorised and selected based on the following source and pathway factors: (a) the presence/absence of poultry activity (b) predominant aquifer category and (c) predominant groundwater vulnerability, within the zone of contribution (ZOC) for each site. Seven anticoccidials, including four ionophores (lasalocid, monensin, narasin and salinomycin) and three synthetic anticoccidials (amprolium, diclazuril and nicarbazin), were detected at 24% of sites at concentrations ranging from 1 to 386 ng L^{-1}. Monensin and amprolium were the two most frequently detected compounds, detected at 15% and 7% of sites, respectively. Multivariate statistical analysis has shown that source factors are the most significant drivers of the occurrence of anticoccidials, with no definitive relationships between occurrence and pathway factors. The study found that the detection of anticoccidial compounds is 6.5 times more likely when poultry activity is present within the

ZOC of a sampling point, compared to the absence of poultry activity. This work presents the first detections of these contaminants in Irish groundwater and it contributes to broadening our understanding of the environmental occurrence and fate of anticoccidial veterinary products.

Keywords: Emerging contaminants; Ionophores; Coccidiostats; Feed additives; Poultry production; Groundwater

5.1 Introduction

According to the Groundwater Directive 2006/118/EC (European Parliament, 2006), groundwater is the largest body of fresh water within the European Union (EU), with up to 75% of EU residents depending on groundwater as a source of drinking water. In Ireland, approx. 26% of the public and private drinking water supply is provided by groundwater sources, with more localised regions relying on groundwater for up to 75% of their needs (EPA Ireland, 2010). There are approx.172,000 households (equating to 17% of the national population) that obtain their drinking water supply from private groundwater sources and springs (CSO, 2017), which do not fall under the regulations implementing the EU Directive on water for human consumption (98/83/EC) in Ireland (Government of Ireland, 2014a). Most of these private water supplies are more susceptible to contamination given that the onus for monitoring and maintenance is with the individual owners (EPA Ireland, 2010). Poorly sited and/or constructed supplies are likely to have an increased risk of contamination (Misstear et al., 2017; Gill et al., 2018). Groundwater also plays an essential role in contributing to and maintaining surface water flow, and as a result, groundwater quality issues are often reflected in surface water bodies and wetlands. This further magnifies the importance and need for groundwater protection and risk assessment, both for the benefit of drinking water consumers, but also for a wide range of groundwater dependent ecosystems.

An Environmental Protection Agency (EPA) of Ireland groundwater pressure risk assessment report indicated that nutrient pressures from agricultural activities (including livestock farming, arable activities and intensive enterprises) and usage of dangerous substances such as agrochemicals, are the most widespread, and nationally significant, anthropogenic pressure on groundwater in Ireland (Clabby et al., 2008). In recent years, synthetic organic compounds, often known as emerging organic contaminants (EOCs), are becoming more of a growing international concern regarding their occurrence in, and contamination of, groundwater bodies (Lapworth et al., 2012; Postigo and Barcelo, 2015). Veterinary pharmaceuticals have become a critical component in Irish agriculture because of the ever-increasing demands on the production systems to provide more foods, particularly of animal origin. The administration of such veterinary products can potentially lead to their occurrence in groundwater once excreted by the animal. As a result, all veterinary pharmaceuticals are potentially emerging groundwater contaminants of concern,

depending on their fate and eco-toxicological behaviour in the environment (Lapworth et al., 2012).

In recent analytical method development work by this research group, 26 anticoccidials (6 ionophores and 20 synthetic anticoccidials (often referred to as chemical coccidiostats)) were selected as a group of veterinary products/feed additives of interest, due to their potential to be emerging groundwater contaminants of concern (Mooney et al., 2020). These compounds were selected due to the current lack of information on their environmental occurrence and fate, with more emphasis and priority given to the 11 compounds currently licensed as feed additive or veterinary medicines in the EU (Table 5-1). To broaden the scope, and to enhance the applicability of the methodology, some additional compounds that are authorised outside of the EU (e.g. in the USA) were also included in the original method development work. All 26 compounds (and their abbreviations) investigated in this study are listed in Table 5-1, with compounds grouped as ionophores or synthetic anticoccidials.

While there are 11 licensed anticoccidial feed additives in the EU, the usage (and therefore potential environmental source) of anticoccidials differs from one country to another; for example, in Denmark only four of the six licensed ionophores are used as feed additives (Bak and Björklund, 2014). In Ireland, the majority of licensed anticoccidials are used exclusively in poultry species, with the primary source in the environment likely to be a consequence of their prophylactic use in intensive poultry production. A very limited number of anticoccidials (e.g. diclazuril and toltrazuril) are also used in Ireland as therapeutics in other production systems such as cattle and sheep, however to a much lesser extent than as feed additives.

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Table 5-1 Chemical and physicochemical data of the 26 anticoccidial compounds (grouped by class), investigated in the spatial occurrence study, with corresponding analytical method performance parameter

Analyte	Abbreviation	Formula	CAS number	Calibration[a] Range (ng L^{-1})	LOD[a] (ng L^{-1})	LOQ[a] (ng L^{-1})	Physicochemical properties[c]			
							S_w (mg L^{-1})	$logK_{ow}$	pK_a	log K_{oc}
Ionophores				-	-	-				
Lasalocid	LAS	$C_{34}H_{53}NaO_8$	25999-20-6	0.1–250	0.01	0.1	1060	2.3	5.66	2.9–4.2
Maduramicin	MAD	$C_{47}H_{83}NO_{17}$	84878-61-5	1.0–250	0.5	1	-	-	-	2–2.4
Monensin	MON	$C_{36}H_{61}NaO_{11}$	22373-78-0	0.1–250	0.005	0.1	8.8	3.8 - 4.4	4.5, 6.6	1.9–3.8
Narasin	NAR	$C_{43}H_{72}O_{11}$	55134-13-9	0.1–250	0.005	0.1	102–681	4.85	7.9	2.9–3.6
Salinomycin	SAL	$C_{42}H_{70}NaO_{11}$	53003-10-4	0.1–250	0.02	0.1	623–1371	5.12	6.4	1.9–3.2
Semduramicin	SEMD	$C4_5H_{76}O_{16}$	113378-31-7	1.0–250	0.25	1	163–1240	2.6	5.4	1.4–3.3
Synthetic Anticoccidials				-	-	-				
Aklomide	AKLO	$C_7H_5ClN_2O_3$	3011-89-0	20.0–250	5	20	-	-	-	-
Amprolium	AMO	$C_{14}H_{19}N_4$ Cl	125-25-2	0.5–250	0.1	0.5	540×10^3	-2.5	4.65	3–3.7
ANOT	ANOT	$C_8H_9N_3O_3$	3572-44-9	10.0–150	2.5	10	-	-	-	-
Arprinocid	ARPRIN	$C_{12}H_9ClFN_5$	55779-18-5	0.5–150	0.1	0.5	-	-	-	-
Buquinolate	BUQUIN	$C_{20}H_{27}NO_5$	5486-03-3	0.5–150	0.1	0.5	-	-	-	-
Clopidol	CLOP	$C_7H_7Cl_2NO$	2971-90-6	0.5–150	0.1	0.5	-	-	-	-
Cyromazine	CYROM	$C_6H_{10}N_6$	66215-27-8	1.0–250	0.1	1	$8–13 \times 10^3$	0.069	5.22	-
Decoquinate	DECO	$C_{24}H_{35}NO_5$	18507-89-6	0.5–150	0.1	0.5	0.06	5.2–5.5	-	>5.6
Diaveridine	DIAV	$C_{13}H_{16}N_4O_2$	5355-16-8	0.5–150	0.15	0.5				-

Table 5-1 *continued*

Analyte	Abbreviation	Formula	CAS number	Calibration[a] Range (ng L^{-1})	LOD[a] (ng L^{-1})	LOQ[a] (ng L^{-1})	Physicochemical properties[c] S_w (mg L^{-1})	$logK_{ow}$	pK_a	$log\ K_{oc}$
Diclazuril	DICLAZ	$C_{17}H_9Cl_3N_4O_2$	101831-37-2	0.1–250	0.02	0.1	1.44–2.6	3.6	5.89	3.7–4.0
Dinitolmide	DINITOL	$C_8H_7N_3O_5$	148-01-6	10.0–150	2	10	-	-	-	-
Ethopabate	ETHO	$C_{12}H_{15}NO_4$	59-06-3	0.1–250	0.02	0.1	-	-	-	-
Halofuginone	HALO	$C_{16}H_{17}BrClN_3O_3$	55837-20-2	0.1–250	0.05	0.1	3000	1 - 2.5	-	-
Nequinate	NEQUIN	$C_{22}H_{23}NO_4$	13997-19-8	0.5–150	0.1	0.5	-	-	-	
Nicarbazin (DNC)[b]	NICARB	$C_{13}H_{10}N_4O_5 \cdot$	330-95-0	1.0–250	0.1	1	<0.02	>3.6	12.44	4.2–5.1
Nicarbazin (HDP)		$C_6H_8N_2O$					$>60 \times 10^3$	-0.94	3.75	1.5–2.2
Nitromide	NITRO	$C_7H_5N_3O_5$	121-81-3	20.0–250	5	20	-	-	-	-
Robenidine	ROB	$C_{15}H_{13}Cl_2N_5$	25875-51-8	0.1–250	0.03	0.1	118	3.3	3.4	5.6
Toltrazuril	TOL	$C_{18}H_{14}F_3N_3O_4S$	69004-03-1	20.0–250	4	20	-	-	-	-
Toltrazuril sulphone	TOL-SO_2	$C_{18}H_{14}F_3N_3O_6S$	69004-04-2	20.0–250	10	20	1	2.49	7.15	2.8
Toltrazuril sulphoxide	TOL-SO	$C_{18}H_{14}F_3N_3O_5S$	69004-15-5	20.0–250	4	20	-	-	-	-

[a] Calibration range, LOD and LOQ data taken from (Mooney et al., 2020)

[b] Nicarbazin detected as DNC

[c] physicochemical data extracted from the EFSA Journal (EFSA, 2020)

LOD = Limit of detection, LOQ = Limit of quantification, S_w = water solubility, $logK_{ow}$ = logarithm of octanol-water partition coefficient, pK_a = dissociation constant and $logK_{oc}$ = logarithm of soil organic carbon-water partitioning coefficient

The introduction of anticoccidials into the environment is potentially from direct excretion of faeces and/or urine on land, spreading of manure and slurry collected from the production/housing units, or because of point source contamination at or near the production facilities (Boxall, 2010; Alonso et al., 2019). There is a lack of information on anticoccidials and the factors that can influence their entry to the environment; there is a shortage of data in international literature on the metabolism and excretion of anticoccidials following administration, while information on the attenuation and degradation processed for the contaminants, once in the environment, is scant. Of the limited information that is available, several anticoccidials (e.g. lasalocid (LAS) and diclazuril (DICLAZ)) can be excreted in sizeable amounts (up to 95% of administered dose) as un-metabolized active substances (EFSA, 2004; Hansen et al., 2009a)

Movement of these contaminants to groundwater can depend on different environmental and hydrogeological factors such as land use, soil properties, geological and hydrogeological properties and climate (Essaid et al., 2015). Meteorological conditions, particularly the timing of effective rainfall, are an important factor which can produce temporal variability in contaminant transport (Harman et al., 2011). The intrinsic physicochemical properties of the individual compounds themselves are also vital (Table 5-1). While in transport, contaminants are subject to several complex physical, chemical and biological transformation processes that can provide attenuation, depending on the pathway taken (Arias-Estévez et al., 2008). Based on the physicochemical properties of the anticoccidials (mostly highly hydrophobic, with high organic carbon sorption coefficients), the most important of these environmental factors are soil and Quaternary deposit properties (such as pH, texture, structure, organic content, permeability and thickness), with adsorption to soil likely to be a significant attenuation process as these contaminants move through the unsaturated zone to groundwater. The pH of both soil and water has been shown to play a critical role in the transport of anticoccidials, particularly the ionophores, with different chemical speciation occurring at varying pH (Hansen et al., 2009a; Alonso et al., 2019). Given the potential for the strong adsorption of anticoccidials to soils and sediment, there is the potential for transport of the contaminants to groundwater via colloidal transport (Foster and Chilton, 1991) through preferential flow pathways, therefore increasing the vulnerability of groundwater to these contaminants. In a study in Brazil, Yopasá-Arenas and Fostier (2018) produced vulnerability maps for a qualitative approach to risk assessment of the exposure of Brazilian soils and groundwater to anticoccidials (monensin (MON) and

salinomycin (SAL)) and antimicrobial growth promoters, which indicated that groundwater was more vulnerable than soil.

There is also a dearth of information on the occurrence of anticoccidials in environmental waters. Mooney et al. (2020), attributed this to the lack of suitably sensitive and comprehensive analytical methods. Most published environmental detections of anticoccidials relate to the ionophores (Watanabe et al., 2008; Bartelt-Hunt et al., 2011; Sun et al., 2013; Bak and Björklund, 2014), with very few relating to synthetic anticoccidials (Song et al., 2007; Iglesias et al., 2012). Some studies have reported on anticoccidial occurrence in surface waters and agricultural runoff (including agricultural land drains) (Kim and Carlson, 2006; Song et al., 2010; Iglesias et al., 2012; Sun et al., 2013; Bak and Björklund, 2014; Alonso et al., 2019) or in solid agricultural samples (including soil, sediment, manure/litter) (Biswas et al., 2012; Sun et al., 2013), but very few have reported occurrence in groundwater bodies (Watanabe et al., 2008; Bartelt-Hunt et al., 2011). MON, SAL and NAR (narasin) are by far the most commonly detected ionophores in environmental water samples, with detected concentrations in groundwater and surface waters between 2 and 390 ng L^{-1}. However, some studies have reported concentrations of the order of thousands of nanogram per litre (i.e. microgram per litre) e.g. 2350 ng L^{-1} of MON detected in groundwater (Bartelt-Hunt et al., 2011) and 9022 ng L^{-1} of SAL in run-off (Sun et al., 2013).

The key concerns over the occurrence of anticoccidials in groundwater primarily relate to consumption in drinking water, and environmental effects on non-target organisms. Boxall et al. (2003a) included ten anticoccidial compounds (including MON, SAL, LAS, NICARB (nicarbazin) and DICLAZ) on a list of 56 veterinary medicines which are high priority in the environment, based on usage, amounts likely to enter the environment, and their environmental toxicity. Capleton et al. (2006) carried out a similar study but looked at the risk of indirect exposure and toxicity to humans, with several anticoccidials (MON, SAL and toltrazuril (TOL)) also classified as high priority veterinary medicines in need of detailed risk assessments. Both studies cite the lack of complete information on usage as a limitation to the study and risk assessment. The primary concern over human consumption is the long term exposure to low levels, leading to potential chronic toxicity (Biswas et al., 2012; Roila et al., 2019). Some acute effects of anticoccidials, such as the ionophores, have been observed in humans, with muscle cell necrosis occurring as a result of the increased

intracellular concentrations of sodium and calcium, which is transported across membranes in ionophore complexes (Dorne et al., 2013). Previously documented clinical symptoms include skeletal and cardiac muscle loss and/or weakness, with some cases resulting in organ failure leading to death (Caldeira et al., 2001). However, all such instances have occurred in cases of unintended ingestion of the substances, with exposure to concentrations much higher than those expected in environmental waters. Recently concerns have been raised that the use of anticoccidials in feed could cause the development of bacteria with antimicrobial resistance in both humans and animals (VKM, 2015).

The aim of this work was to investigate the occurrence of a wide range of anticoccidial compounds in Irish groundwaters, with a focus on karstic and fractured bedrock aquifers, which dominate the hydrogeology of Ireland. This work specifically aimed to determine the frequency of occurrence of the different anticoccidial compounds, and to investigate the drivers behind detections (such as land-use and hydrogeological characteristics) to help advance our understanding of their fate.

5.2 Materials and Methods

5.2.1 Sampling sites

5.2.1.1 Sampling pool

Sampling points (referred to as monitoring points, MPs) were selected from a pool of over 320 pre-existing, groundwater sampling sites from across the Republic of Ireland. This extensive list of potential MPs incorporated >280 MPs included as part of the national groundwater quality monitoring programme for the E.U. Water Framework Directive (WFD), implemented in Ireland by the EPA (EPA Ireland, 2019). The remaining sites comprised private and/or semi-private group water schemes (GWSs), sampled in coordination with the National Federation of Group Water Schemes (NFGWS). The EPA groundwater monitoring network mainly comprises public and private drinking water abstraction points, represented by production boreholes (BH) or springs (SP) with large abstraction and discharge rates. GWSs vary in size and capacity, with individual schemes providing for a few houses, up to hundreds of houses. The EPA national groundwater

monitoring programme was designed to represent the variation in hydrogeology and pressures across the various groundwater bodies (Craig et al., 2006).

5.2.1.2 Zone of contribution (ZOC)

A zone of contribution (ZOC) is described as the catchment area that contributes water to a borehole or spring (Kelly, 2010; Misstear et al., 2017). Delineation of such a ZOC provides the area boundary, within which, the anthropogenic activities and geological and hydrogeological properties can influence the water quality at the associated MP. ZOC's for all the aforementioned potential sampling sites have previously been delineated using the approach summarised by Kelly (2010). For this study, ZOC data were obtained in the form of shapefiles (EPA Ireland, 2018), which were manipulated using ArcGIS 9.3 (Geological Information System) for site classification.

5.2.1.3 Selection process

Sites were classified based on land use and physical hydrogeological properties, selected to account for different source and pathway factors. The three key site characteristics selected were: (a) aquifer category/flow regime, (b) groundwater vulnerability (both pathway factors) and (c) poultry activity (source factor). Aquifer category was determined using the Geological Survey of Ireland classification system, with aquifer classes divided into three main groups based on their resource potential (Regionally Important, Locally Important or Poor Aquifers), and further subdivided based on the openings through which groundwater flows (DELG/EPA/GSI, 1999) (Table 5-2). These 11 GSI aquifer classes were also amalgamated into four WFD flow regime categories (Working Group on Groundwater, 2001). Groundwater vulnerability, defined as the intrinsic geological and hydrogeological characteristics that determine the ease with which groundwater may be contaminated by human activities (DELG/EPA/GSI, 1999), was classified into four major categories: Extreme (subdivided into X and E), High (H), Moderate (M) and Low (L), based primarily on the subsoil permeability and thickness (depth to bedrock). Poultry source factors were taken into consideration when selecting sites by using data on poultry farms and poultry manure spreading within the ZOC of each MP, from a limited dataset provided by the Department of Agriculture, Fisheries and Marine (DAFM) (unpublished data, see Supplementary File SI-5.1). Poultry activity was classified by the presence or absence of

poultry activity within the ZOC, with poultry activity defined as poultry farms and/or manure spreading.

Data for each of these site characteristics were extracted in ARCGIS using the tabulate intersection tool, which computes the intersection between two feature classes and cross-tabulates the area of the intersecting features (ESRI, 2016). This tool output the percentage area of the ZOC accounted for by the different classes within each property layer (except for poultry farms, presented as no. of farms within the ZOC). Each MP was characterised based on the most predominant class of each physical site characteristic within the ZOC. Predominance, in most cases, was assigned to the class which accounted for >50% of the ZOC area.

5.2.1.4 Final sampling sites

For the overall sampling campaign, 109 MPs, comprising 63 BHs and 46 springs, were selected from across 25 of the 26 counties in the Republic of Ireland. Samples were collected during November and December 2018, during a period with active groundwater recharge conditions. An approximate 70/30 ratio was adopted between MPs with poultry activity present vs. absent, with 68% classified as having poultry activity present. Table 5-3 summarises the spread of the 109 MPs, selected across the different bedrock aquifer categories (GSI), and the three bedrock aquifer flow regime classes. Overall, 50% of the total MPs selected fell within the karstic flow regime, with 17% and 33% of sites falling under the productive fractured and poorly productive categories, respectively. This spread achieves a representative sample of each of the three bedrock aquifer flow regimes, with a focus on productive aquifers, in broad agreement with the proportions of groundwater bodies in Ireland within each regime (Daly, 2009). Table 5-3 also shows the spread of the number of MPs across the five different groundwater vulnerability classes.

Table 5-2 Summary of some of the main land use and physical hydrogeological site properties, used to characterise sampling MPs for site selection and statistical analysis, with the corresponding national dataset source

Property[b]	**MP Type**	**Corine Land Cover**	**Bedrock Geology**	**Aquifer Category***	**WFD Flow Regime***	**Groundwater vulnerability***	**Irish Forestry Soils (IFS)**	**SIS Irish Soils**	**Quaternary Sediments**	**Subsoil Permeability**
Data source	EPA	Corine Land Cover 2012 Digital Map	Hydrostratigraphic Rock Units Group Map 1:100,000 (Digital) GSI	Groundwater Bedrock Aquifers Map 1:100,000 & Gravel Aquifers 1:50,000(Digital)	Amalgamated from GSI Aquifer Categories	Groundwater Vulnerability Map1:40,000 (Digital) GSI	IFS National Soil Map 1:50,000 (Digital) from the EPA	The Irish Soils Information System National Map 1:250,000 (Digital) from the EPA	Quaternary Sediments Map 1:50,000 (Digital) GSI	Groundwater Subsoil Permeability Map 1:40,000 (Digital) GSI
Ref	(EPA, 2011)	(EPA Ireland, 2012)	(GSI, 2016a) (Tedd et al., 2017)	(GSI, 2015b) (GSI, 2015c)	(Working Group on Groundwater, 2001)	(GSI, 2015d)	(Teagasc-EPA-GSI, 2006)	(Teagasc-EPA, 2014)	(GSI, 2016b)	(GSI, 2015a)
Classes	• **Borehole** • **Spring**	• **Corine (level 3):** o See Supplementary file SI-5.1 • **Corine (amalgamated)** o Arable o non arable (pasture) o forest o other	27 rock units amalgamated into six lithological groups • **Sand and gravel** • **Impure limestone** • **Pure limestone** • **Non-calcareous sedimentary** • **Igneous** • **Metamorphic**	11 classes as follows: • **Regionally Important** o Rk o Rkc o Rkd o Rf o Rg • **Locally Important** o Lm o Lk o Ll o Lg • **Poor Aquifer** o Pl o Pu	• **Karstic** o Rk, Rkc, Rkd & Lk • **Productive fractured** o Rf & Lm • **Poorly productive** o Ll, Pl and Pu • **Intergranular** o (Rg & Lg)	• **X**- Extreme (exposed) • **E** – Extreme • **H**- High • **M**-Moderate • **L**-Low	**Type I:** Acid vs. Base **Type II:** Mineral vs Peat **Type III:** Deep vs. Shallow **Type IV:** Wet vs. Dry	• Soil Association - 61 • **Drainage** o Excessive o Well o Moderate o Imperfect o Poor • **Texture**	**(Genesis)** • Alluvium • Irish Sea Tills • Karstified rock • Peat • Sand and Gravels • Tills • Bedrock at surface	• **High** • **Moderate** • **Low** • **DTB<3m *****

Rk = Regionally Important Aquifer-Karstified , Rkc = Regionally Important Aquifer-Karstified (conduit flow), Rkd = Regionally Important Aquifer-Karstified (diffuse flow), Rf = Regionally Important Aquifer – Fissured bedrock, Lm = Locally Important Aquifer – Bedrock which is Generally Moderately Productive, Lk = Locally Important- Karstified, Ll = Locally Important Aquifer - Bedrock which is Moderately Productive only in Local Zones, Pl = Poor Aquifer - Bedrock which is Generally Unproductive except for Local Zones and Pu = Poor Aquifer - Bedrock which is Generally Unproductive Rg = Regionally Important Gravel Aquifers, Lg = Locally Important gravel aquifer

[a] Indicates property was used in the overall selection of the final sampling sites [b] see supplementary file SI 5.1 for a detailed description of each property

[c] Subsoil permeability could not be ranked for areas with less than 3 meters depth to bedrock, and were therefore assigned as DTB<3m

Table 5-3 The relative proportion of the 109 sampling sites, subdivided into poultry present vs. absent, spread across nine GSI bedrock aquifer classifications, three WFD bedrock aquifer flow regime categories and the groundwater vulnerability categories

Poultry Activity [a]	No. Sites	Aquifer Category (GSI Classification) [b]									WFD Flow Regime [c]		Groundwater Vulnerability [d]				
		Rk	Rkc	Rkd	Lk	Rf	Lm	Ll	Pl	Pu	Karstic	Productive Fractured	Poorly Productive	Extreme (X)[d]	Extreme (E)	High (H)	Moderate (M)	Low (L)
Present [a]	74	1	22	18	1	3	6	18	4	1	41	10	23	7	13	29	13	12
Absent [a]	35	0	7	6	0	7	2	8	4	1	13	9	13	9	7	10	5	4
Total	109	1	29	24	1	10	8	26	8	2	54	19	36	16	20	39	18	16
% of total sites		1	27	22	1	9	7	24	7	2	50	17	33	15	18	36	17	15

[a] Presence/Absence of poultry activity according to DAFM dataset only, with a "poultry activity" being a poultry farm and/ or poultry manure spreading activity.
[b] Rkc = Regionally Important Aquifer-Karstified (dominated by conduit flow), Rkd = Regionally Important Aquifer-Karstified (dominated by diffuse flow), Ll = Locally Important Aquifer - Bedrock which is Moderately Productive only in Local Zones, Pl = Poor Aquifer - Bedrock which is Generally Unproductive except for Local Zones and Pu = Poor Aquifer - Bedrock which is Generally Unproductive
[c] Groundwater flow regime classified by grouping GSI aquifer categories, where karstic = Rk, Rkc, Rkd and Lk, Productive Fractured = Rf and Lm while Poorly Productive = Ll, Pl and Pu

[d] Extreme-X = rock at or near surface or karst

5.2.2 Sample collection, storage and chemical analysis

Raw, unfiltered, groundwater samples were taken in accordance with ISO EN 5667 standards (NSAI, 2009; NSAI, 2018) and EPA protocols. Samples were collected in pre-cleaned amber borosilicate glass bottles, with a 1 L sample enough to allow for initial analysis and further repeat analysis in duplicate. Depending on the MP, sampling was carried out directly from the source, or via pre-existing distribution pump houses (where the source was not accessible or enclosed). As a result, sampling was carried out by one of three techniques: (a) directly into the sampling container (e.g. from a tap or shallow spring); (b) using a discrete depth sampler, in this case a closed bailer device or (c) by pump using Teflon tubing. BHs, lacking a raw water sampling tap, with standing water levels >2 m below ground level were sampled by pump (WaSP Five Stage 12 V Submersible Pump (In-Situ, Europe, Worcestershire, UK)) and those with <2 m were sampled by bailer (100 mL or 250 mL). Prior to collection, the sampling bottle was rinsed (a minimum of three times) with the source water. For sites included as part of the EPA monitoring network (98 sites, as listed in Supplementary File SI-5.2), groundwater physicochemical "field" parameters (listed in Supplementary File SI-5.1 Table S5-5) were measured and the sample was taken after four consecutive, stable readings.

Once collected, samples were transported to the laboratory under chilled conditions (<4 °C), arriving at the laboratory no later than 24 h after collection and remained in storage at 4 °C until analysis, within 7 days of collection (SI-3.2). Analysis of groundwater samples was carried out according to the method previously developed and validated by Mooney et al. (2020), for the determination of 26 anticoccidial compounds (as listed in Table 5-1) in environmental waters. An organic modifier (7.5 mL methanol) was added to samples (250 mL) to assist with desorption of residual analytes potentially sorbed on the sample container, with the modified samples subsequently pH adjusted (pH 8.5) and extracted using Enviro Clean HL-DVB solid phase extraction (SPE) cartridges (200 mg, 6 mL), packed with glass wool. The SPE cartridges were eluted (MeOH:MeCN:EtOAc, 40:40:20, v/v, 12 mL) and evaporated (0.5 mL) for final instrumental analysis by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). All analytes were chromatographically separated using a Zorbax Eclipse Plus Phenyl-Hexyl Rapid Resolution HD analytical column on an Agilent 1290 Infinity™ II UHPLC system and detected using an AB Sciex 6500+ quadrupole linear ion trap (QTRAP) mass spectrometer. This method

was deemed fit for purpose for the confirmatory analysis of all analytes except toltrazuril and its two transformation products toltrazuril sulphoxide and toltrazuril sulphone, which were included in this study for screening only. All samples were initially analysed singly, with any samples with detections further repeated in duplicate, with the result reported as the mean concentration of the triplicate analyses.

Negative control samples were used to produce QC Trip (Field) blanks, also as described by Mooney et al. (Mooney et al., 2019; Mooney et al., 2020). QC trip blanks were transported to the sampling sites, exposed to the environment while sampling, and transported back to the laboratory alongside normal samples for analysis, to demonstrate a lack of cross contamination in the field and during transportation. Fortified QC field samples were not feasible, given sampling was carried out by varying personnel from different organisations. There were no measurable detections of any target compounds found in field blanks during this campaign.

The 98 EPA samples underwent additional analysis at the EPA laboratories for several water quality parameters including the major ions, nutrients, metals and faecal microorganisms using standard methods. A full list of these parameters is provided in Supplementary File SI-5.1 Table S5-5, which also highlights the method of analysis and relevant detection capabilities. Results of these analyses were provided by the EPA (unpublished data) and used to investigate any association with detection of the anticoccidial contaminants that could be used as a surrogate indicator for potential anticoccidial contamination of groundwater.

5.2.3 Statistical analysis

While the datasets used below for statistical analysis cannot be claimed to be free of bias because of the non-random sampling, the authors contend that the sample selection corresponds to an expert sample and therefore the statistics calculated from the data contains useful information on the processes that were being examined. The scope of any statistical inference should be viewed as exploratory in the sense that it could form a basis for designing future studies, rather than claiming firm associations from these data.

5.2.3.1 Additional dataset preparation for statistical analysis

Several other physical site properties were also used for statistical analysis, to investigate any association between anticoccidial detections, and these site characteristics. These properties and the classes into which they were subdivided, are listed in Table 5-2, and are described in more detail in Supplementary File SI-5.1. The predominant class of each of these properties was determined using the same process described in Section 5.2.1.3, with the predominant class within the ZOC of each MP recorded (Supplementary File SI-5.2) and input for statistical analysis. In order to provide enough observations for a more meaningful statistical analysis, and to allow a more stable, accurate logistic regression analysis, several datasets with many levels, were amalgamated into fewer categories/classes (preferably <10 classes), as also described in supplementary file SI-5.1. Assignment of the predominant class for a number of site characteristics was problematic, therefore statistical analysis was also carried out on the relative percentage data, of each class within the ZOC.

5.2.3.2 Site physical characteristics

Testing for association between detection of anticoccidials and physical site characteristics was carried using SAS 9.4 (SAS Institute Inc, 2014). Since the overall prevalence of detections above the LOQ was very low, resulting in biased estimation of means (Helsel, 2011), a positive result in any test for presence of the contaminants was coded as a detection and the resulting classification of each sample as Detect/Non-detect was analysed as a binary response. The Logistic procedure in SAS was used to fit a regression to quantify the relationship between the binary response and the explanatory variables (Supplementary File SI-5.2). Initially a variable selection procedure was used to identify variables associated with detection of contaminants and then the marginal effect of each explanatory variable was tested. Relationships were quantified using odds ratios and their 95% Wald confidence limits. Sampling MP type was included as a factor in the modelling to correct for any possible confounding of effects. Where the regression failed to converge, Fisher's Exact Test with Monte-Carlo simulation was used to test for independence of the explanatory variable and detection.

Several definitions of detection were analysed as follows; (a) any detection across the full set of 26 contaminants, (b) detection of an ionophore compound and (c) detection of a synthetic anticoccidial. In addition, MON was also analysed individually, given it is the most

commonly reported anticoccidial detected in groundwater. All analyses were repeated for a subset of the data (74 sampling points) where only those observations with poultry "present" within the ZOC of the sampling point, were included. This approach was deemed reasonable given MPs with confirmed contaminant sources were more informative in distinguishing between MPs that have a source of and detection of anticoccidials, compared to MPs that had a source, but non-detection, of anticoccidials.

5.2.3.3 Water quality parameters

A similar approach was used to test for relationships with anticoccidial occurrence and water quality parameters, but on a reduced number of MPs (n = 98), given such data was only available for the EPA MPs. The analysis was problematic because of varying degrees of censoring of the quality parameters at the limit of quantitation (LOQ). Water quality parameters with no censoring, and those with one to three values missing or censored, were straightforwardly modelled using logistic regression with detection/non-detection as the response. Measurements with very high levels of censoring (>70%) are very subject to bias in estimating mean values and so these were recoded as present/not-present and tabulated for testing against contaminant detections. Characteristics with intermediate levels of censoring were recoded and tested in the same way but were further explored. For these, substitution of zero and LOQ values was used to examine the maximum and minimum potential outcome differences and a nonlinear model was fitted that incorporated the calculation of the statistical likelihood of censored data (Long, 1997). Dealing with the censoring in the explanatory variable was also problematic and rather than using a censored characteristic as an explanatory variable to model detections, detection and non-detection were treated as a grouping variable and the means for those two groups were compared. This allowed methods for censored responses to be used.

5.3 Results

5.3.1 Groundwater Occurrence – summary of anticoccidials detected

Of the 109 MPs, 24% (26 sites) had a detection of at least one anticoccidial compound, with 7 out of the 26 different compounds detected throughout the campaign, at concentrations ranging from 1 to 386 ng L^{-1}. Up to three different compounds were detected at any given MP. Table 5-4 outlines some summary statistics for the seven compounds detected, while

Table 5-5 summarises the 26 sites that had anticoccidial detections, and the associated concentrations. Ionophore compounds were detected at 19.3% of sites, while synthetic anticoccidials were detected at 11% (Table 5-4). The ionophore MON was the most frequently detected anticoccidial, detected at 14.7% of the total sites (16 of 109). MON was detected at the highest concentration with one sample containing up to 386 ng L^{-1}, however the overall median concentration amongst the 26 sites was much lower (17.5 ng L^{-1}). The next most often detected compound was the synthetic anticoccidial amprolium (AMP), detected at 7.3% of sites at concentrations up to 49.8 ng L^{-1}, with an overall median concentration of 11.9 ng L^{-1}. The ionophore LAS was detected at 5.5% of sites, and at the lowest concentration throughout the study (1.9 ng L^{-1}), however the overall median concentration (14.7 ng L^{-1}) was similar to other ionophores. SAL had the overall lowest median concentration (9.8 ng L^{-1}) detected across just 4% of the total sites

Table 5-4 Summary statistics for the seven anticoccidial compounds detected above the limit of quantification (LOQ)

Analyte	No. samples > LOQ [a]	% Samples >LOQ [a]	% of Total Detections	Concentration (ng L^{-1})			
				Min	Max	Mean	Median
Ionophores [b]	21	19.3	81	-	-	-	-
Lasalocid (LAS)	6	5.5	23.1	1.9	55.7	19.5	14.7
Monensin (MON)	16	14.7	61.5	4.5	385.7	47.1	17.5
Narasin (NAR)	4	3.7	15.4	6.5	46.7	19.1	11.6
Salinomycin (SAL)	4	3.7	15.4	6.5	18.6	11.2	9.8
Synthetic Anticoccidials [b]	12	11	42	-	-	-	-
Amprolium (AMP)	8	7.3	30.8	2.8	49.8	14.5	11.9
Diclazuril (DICLAZ)	5	4.6	19.2	3.7	65.6	21.8	14.7
Nicarbazin (NICARB	2	1.8	7.7	29.5	134.9	82.2	82.2

[a] LOQ = Limit of Quantification, see Table 5-1 for LOQ values for individual analytes
[b] Data presented for ionophores collectively and synthetic anticoccidials collectively, indicates the number of sites (and respective percentages) which had detection of one or more ionophores/ one or more synthetic anticoccidials at concentrations greater than the LOQ.

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Table 5-5 Summary of the anticoccidial compounds detected, and corresponding concentrations, at each of the 26 sites that had a detection recorded

MP No.	MP Type	Region	Aquifer Class [a]	Flow Regime [a]	Groundwater Vulnerability[a]	Poultry Activity	Analyte, Mean Concentration (ng L^{-1}) (n = 3 replicates)						
							LAS	MON	SAL	NAR	AMP	DICLAZ	NICARB
16	BH	Border	Rf	Productive Fractured	H	Present	5.2	n.d.	6.5	n.d.	n.d.	3.7	n.d.
19	BH	Border	Rkd	Karstic	L	Present	n.d.	39.2	n.d.	n.d.	n.d.	n.d.	n.d.
38	BH	Border	Rk	Karstic	M	Present	24.2	n.d.	n.d.	n.d.	13.7	14.7	n.d.
90	BH	Border	Rkc	Karstic	M	Present	n.d.	n.d.	n.d.	n.d.	n.d.	16.0	134.9
108	BH	Border	Ll	Poorly Productive	L	Present	n.d.	112	n.d.	15.1	10.8	n.d.	n.d.
109	BH	Border	Ll	Poorly Productive	M	Present	n.d.	24.0	n.d.	n.d.	n.d.	n.d.	n.d.
13	BH	Mid-East	Rkd	Karstic	H	Present	n.d.	14.7	n.d.	6.5	n.d.	n.d.	n.d.
8	BH	Midland	Rf	Productive Fractured	H	Absent	n.d.	385.7	n.d.	46.7	n.d.	n.d.	n.d.
50	BH	Midland	Ll	Poorly Productive	H	Present	n.d.	11.7	n.d.	n.d.	n.d.	n.d.	n.d.
59	Spring	Mid-West	Ll	Poorly Productive	E	Present	n.d.	n.d.	n.d.	n.d.	49.8	n.d.	n.d.
14	BH	South-East	Pl	Poorly Productive	E	Present	n.d.	5.6	n.d.	n.d.	n.d.	n.d.	n.d.
72	BH	South-East	Rkd	Karstic	M	Present	n.d.	12.8	n.d.	8.1	n.d.	n.d.	n.d.
85	BH	South-East	Rkd	Karstic	E	Present	n.d.	20.3	n.d.	n.d.	n.d.	n.d.	n.d.
103	BH	South-East	Rf	Productive Fractured	X	Absent	n.d.	n.d.	n.d.	n.d.	5.1	9.3	n.d.
107	BH	South-East	Rkd	Karstic	H	Present	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	29.5
79	BH	South-West	Ll	Productive Fractured	E	Present	55.7	4.5	n.d.	n.d.	n.d.	n.d.	n.d.
96	Spring	South-West	Ll	Poorly Productive	H	Present	n.d.	9.0	n.d.	n.d.	n.d.	n.d.	n.d.
97	Spring	South-West	Ll	Poorly Productive	H	Present	25.5	n.d.	n.d.	n.d.	14.3	n.d.	n.d.
9	BH	West	Pl	Poorly Productive	E	Present	n.d.	44.8	18.6	n.d.	6.2	n.d.	n.d.
23	Spring	West	Rkc	Karstic	L	Present	n.d.	22.7	7.9	n.d.	2.8	n.d.	n.d.
32	BH	West	Ll	Poorly Productive	L	Present	n.d.	11.5	n.d.	n.d.	n.d.	n.d.	n.d.
45	Spring	West	Rkc	Karstic	E	Present	n.d.	27.4	11.8	n.d.	n.d.	n.d.	n.d.
58	Spring	West	Rkc	Karstic	M	Present	1.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
66	Spring	West	Rkc	Karstic	E	Present	n.d.	n.d.	n.d.	n.d.	12.9	n.d.	n.d.
70	Spring	West	Rkc	Karstic	M	Present	4.4	n.d.	n.d.	n.d.	n.d.	65.6	n.d.
71	Spring	West	Ll	Poorly Productive	H	Present	n.d.	7.1	n.d.	n.d.	n.d.	n.d.	n.d.

*Predominant class within the zone of contribution, MP = monitoring point, BH = borehole, SP = spring, Border region = counties Cavan, Donegal, Leitrim, Louth and Monaghan

5.3.2 Analysis of factors controlling spatial distribution

5.3.2.1 Occurrence and ZOC characteristics

The geographical spread of the 109 MPs classified by MP type and detect/non-detect is shown in Figure 5-1 below. All sites with detections, except for two, were classified as having poultry activity present within their ZOC. BHs accounted for 65% of the sites with detections and anticoccidials were recorded at 27% of BHs. Fig. 5-2 gives an overview of the distribution of detections across the different classes of aquifer category, WFD flow regime, and groundwater vulnerability. These summary statistics do not indicate any clear relationship between detections and aquifer category, flow regime or groundwater vulnerability, with detections spread relatively even across the different classes of each of these three site characteristics. Statistical analysis further confirmed this observation, with no significant relationship shown (Supplementary Table S5-6 of file SI-5.1).

Detections of (a) all anticoccidials, (b) ionophore compounds and (c) synthetic anticoccidial compounds were observed to be significantly ($p < 0.05$) related, or almost significantly related ($p<0.06$), to several site characteristics, namely poultry activity, poultry farm density, poultry manure spreading and IFS (Type I) acidic/basic soils (Table 5-6). For relationships that were significant ($p < 0.05$) the interpretation of the odds ratio likelihood outputs and the associated 95% confidence intervals are presented (Table 5-6). All other characteristics were not statistically related to anticoccidial occurrence, with a full summary of *p*-values for all characteristics, for each of the detection definitions provided in Supplementary File SI-5.1 Table S5-6. The Fisher Exact test returned a significant result for SIS soil association (p = 0.0335) but interpreting the detail of the detected association is not practical because of the large number of classes explaining the detection and the resulting sparseness of the tabulation, including many zeros.

Focusing on MPs classified as “poultry present” produced similar results to the above for the different detect definitions, however, IFS Type I class (Acidic vs. basic soils) was significantly related to detections of synthetic anticoccidials (p = 0.0268), ionophores (0.0392), and anticoccidials collectively (p = 0.0183). In all cases, detections were more likely in acidic soils (synthetic anticoccidials 7.7 times more likely, an ionophore 3.3 times more likely and anticoccidial 3.6 times more likely). These effects were confirmed by analysis of the percentage of each IFS Type 1 class, which indicated that a detection of any

anticoccidial was 1.016 times more likely for every percentage increase of IFS acidic soil within the MP ZOC (p = 0.0212, 95% confidence interval is 1.002–1.030). A similar relationship with the percentage of acidic soils was observed for ionophores only (p = 0.0566) and synthetic anticoccidials only (p = 0.0559)

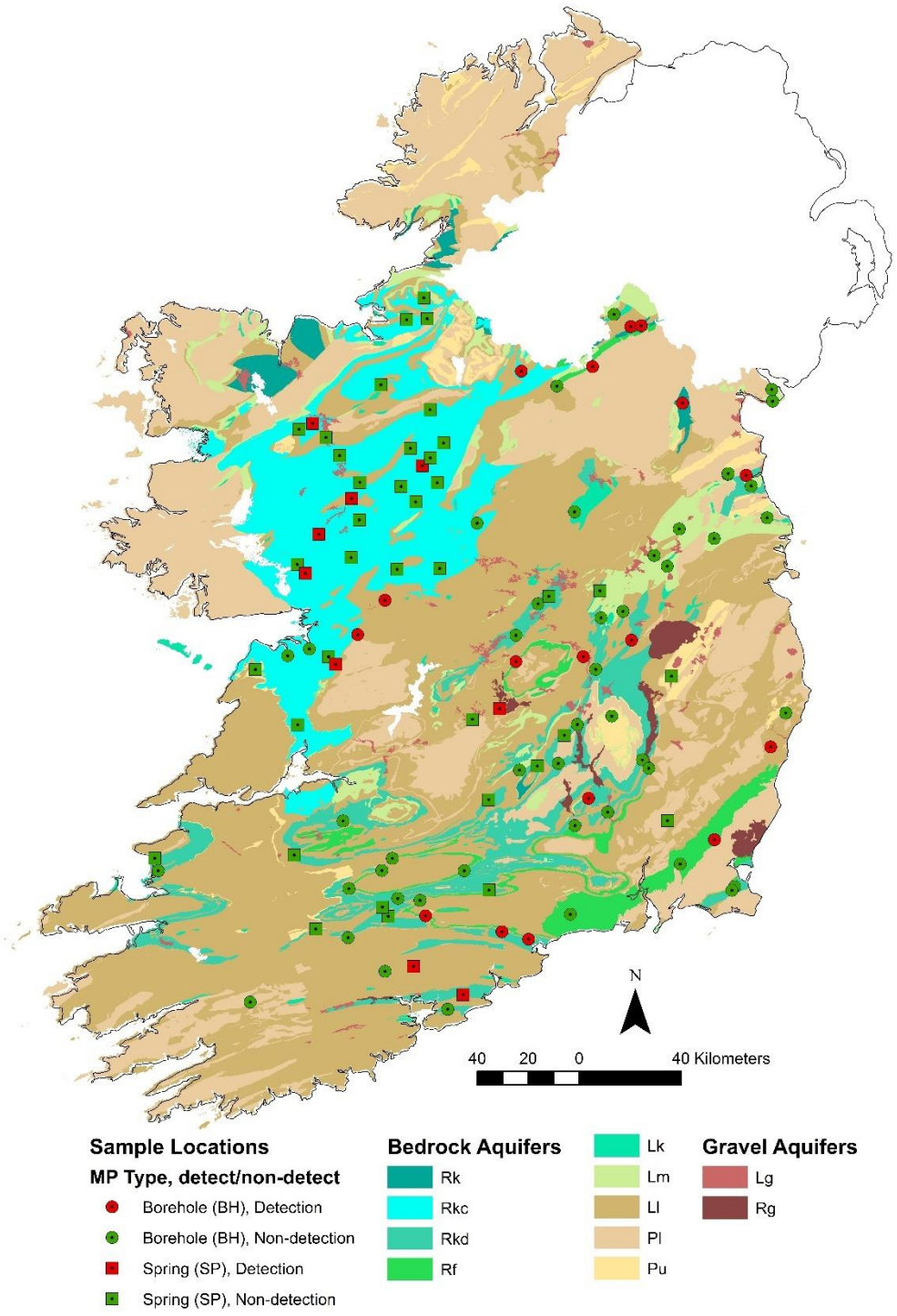

Figure 5-1 The spatial distribution of the 109 sampling monitoring points, classified as boreholes (BH) vs. springs (SP) and detection (red) vs. non-detection (green), overlaid onto the GSI national bedrock aquifer Map (GSI, 2015c; GSI, 2015b)

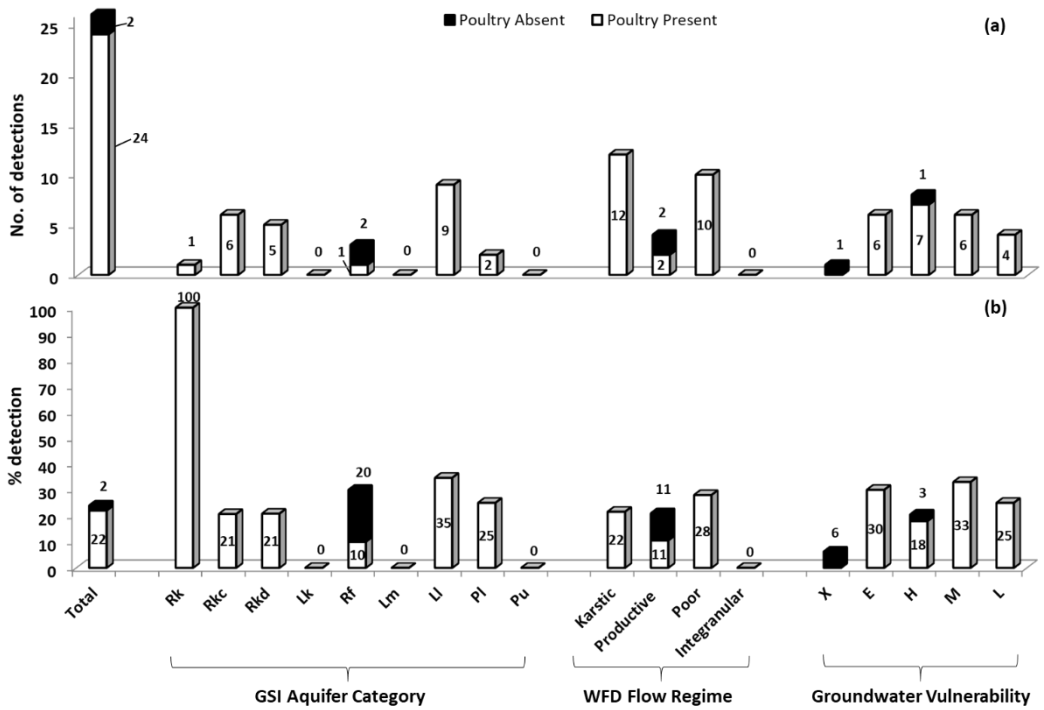

Figure 5-2 (a) Summary of the number of sites within each of the GSI aquifer category, WFD flow regime and groundwater vulnerability classes, that had detections and **(b)** the percentage (%) of sites within each category that had detections, broken down into sites with poultry activity present vs. absent.

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Table 5-6 Summary of site characteristics showing a significant relationship with the occurrence of anticoccidials, defined as all anticoccidials, ionophores and synthetic anticoccidials, with corresponding *p*-values, confidence intervals and odds ratio likelihood interpretations

Site Characteristic	Detection defined as:	Odds ratio	95% intervals	*p*-value	Odds ratio interpretation
Poultry activity	All anticoccidials	6.5	1.62–26.32	0.0083	6.5 times more likely to have "any anticoccidial" detection with poultry activity present in the ZOC, compared to when poultry activity is absent
	Ionophores	8.6	1.53–50.00	0.0148	8.6 times more likely to have an ionophore detection with poultry activity present in the ZOC, compared to when poultry activity is absent
	Synthetic anticoccidial	–	–	0.1150	Not significant
Poultry farm density	All anticoccidials	4.6	2.04–10.25	0.0002	4.6 times more likely to have "any anticoccidial" detection than not, for every unit increase in poultry farm density within the ZOC
	Ionophores	4.9	2.14–11.39	0.0002	4.9 times more likely to have an ionophore detection than not, for every unit increase in poultry farm density within the ZOC
	Synthetic anticoccidial	–	–	0.3555	Not significant
Poultry manure	All anticoccidials	9.3	2.62–32.26	0.0005	9.3 times more likely to have "any anticoccidial" detection with poultry manure spreading in the ZOC, compared to no poultry manure spreading
	Ionophores	7.3	2.01–26.31	0.0025	7.3 times more likely to have an ionophore detection with poultry manure spreading in the ZOC, compared to no poultry manure spreading
	Synthetic anticoccidial	6.1	1.66–22.73	0.0065	6.1 times more likely to have a synthetic anticoccidial detection with poultry manure spreading in the ZOC, compared to no poultry manure spreading
Irish Forestry Soils (IFS) acidic/basic	All anticoccidials	4.0	0.978–6.589	0.0555	4.0 times more likely to have a synthetic anticoccidial detection in acidic soils, compared to basic soils *marginal significance*
	Ionophores	–	–	0.1327	Not significant
	Synthetic anticoccidial	7.2	1.24–42.11	0.0282	7.2 times more likely to have a synthetic anticoccidial detection in acidic soils, compared to basic soils

5.3.2.2 Occurrence and water quality parameters

A number of water quality parameters, as discussed below, were shown to be associated with the detection of (a) all anticoccidials, (b) ionophore anticoccidials and (c) monensin, with all other water quality parameters showing no association (Supplementary File SI-5.1, Table S5-7). There were no relationships identified between any water quality parameters and synthetic anticoccidials. Groundwater pH and ammonium were both significantly related to the detection of ionophore compounds, while groundwater pH was also shown to be significant for detections MON alone. Results showed that for every unit increase in groundwater pH, detection of an ionophore was 3.03 times less likely compared to a non-detection (p = 0.027, 95% confidence intervals: 1.14–8.13), while a detection of MON was 4.33 times less likely with every unit increase in pH (p = 0.0066). Detection of an ionophore compound was 3.9 times more likely at a site that had ammonium detected compared to a site that did not have ammonium detected (p = 0.0266, 95% confidence intervals: 1.17–12.92). MON occurrence was shown to be less likely when zinc is present in the groundwater (p = 0.0514). When accounting for confounding effects of MP type, faecal coliforms showed evidence of an effect on the detection of anticoccidial compounds (p = 0.066). The median faecal coliform count at sites where any anticoccidial compounds were detected was 4.7 times higher than the median faecal coliform count at the sites where no anticoccidial compounds were detected.

Again, focusing on MPs classified as “poultry present”, there was a significant association with anticoccidial detections and groundwater pH, field conductivity and calcium concentration. For every unit increase in field pH, detection of any anticoccidial was 0.15 times more likely (thus 6.7 times less likely), compared to a non-detection (p = 0.0183, 95% intervals of 0.031–0.725). For every unit increase in conductivity, detection of any anticoccidial was 1.003 times less likely, compared to a non-detection (p = 0.0454, intervals 1.0001–1.006). For every unit increase in calcium concentration, a detection of an anticoccidial was 1.016 times less likely compared to a non-detection (p = 0.0196, 95% confidence intervals: 1.003–1.030).

Ionophore compounds were related to groundwater pH (p = 0.0059), ammonium (p = 0.0258), calcium (p = 0.0334) and uranium (p = 0.0517). For every unit increase in field pH, detection of an ionophore was 12.8 times less likely, compared to a non-detection (95%

intervals of 2.09–76.92), while an ionophore detection was 4.7 times more likely at MPs that had ammonium detected vs. MPs that had no ammonium. For every unit increase in calcium concentration, a detection of an ionophore was 1.014 times less likely compared to a non-detection (95% confidence intervals: 1.001–1.030). The effect observed for uranium suggested that an ionophore detection was more likely at MPs with no uranium, compared to MPs with uranium. MON was statistically related to groundwater pH (p = 0.0022), calcium (p = 0.0228), uranium (p = 0.0129) and zinc (p = 0.0456). As with the ionophores collectively, a detection of MON was shown to be less likely with every unit increase in field pH (23.6 times less likely for every unit increase in pH). Detection of MON was 6.67 times less likely (95% intervals: 1.49–29.41) at MPs where uranium was detected, compared to MPs where uranium was not detected, and 4.81 times less likely at sites that had zinc recorded at the MPs, compared to sites that had no zinc.

5.4 Discussion

5.4.1 Anticoccidial compounds detected

5.4.1.1 Ionophores

The top four most frequently detected ionophores in groundwater were MON, LAS, NAR and SAL. The trend in the compounds detected, and their frequency of detection, may be attributed to the overall usage of the compounds in Ireland, on the assumption that the primary source of these drugs in the Irish environment is as a result of the use in poultry production. These compounds make up four of the eleven anticoccidial compounds licensed in the EU for use as zootechnical feed additives in intensively reared species, under Regulations 1831/2003/EC (European Parliament, 2003). All four are licensed as feed additives intended for the control of systematic coccidiosis in different types of poultry, namely chickens reared for laying and/or chickens for fattening and/or turkeys, depending on the compound. LAS and MON are also licensed as a veterinary medicine according to Directive 2019/6/EC (European Parliament, 2019), and as listed under Commission Regulation No. 37/2010 (European Commission, 2010). However, such use of ionophores as veterinary medicines is not common in Ireland. A review of anticoccidial residues in poultry in Ireland reported that 100% of the producers used both MON and NAR, while 66% and 25% of producers reported the use of SAL and LAS respectively, for treatment of

coccidiosis at their facilities (O'Keefe, 2003). The compounds detected are in relative agreement with such usage patterns, but there are other factors, as summarised below, that may influence this trend in the occurrence of ionophores.

Based on their physicochemical properties (Table 5-1), it is expected that ionophore compounds will be more associated with soil and sediment once in the environment, however the extent of association of soil/sediment versus water is both pH and compound dependent (discussed in Section 5.4.2). Hansen et al. (2009a) give a comprehensive overview of the occurrence of ionophores in the environment, with various studies reporting the detection of ionophores in manure and manure amended soil e.g. Furtula et al. (2009) reported the detection of three ionophores (MON, NAR and SAL) and one synthetic anticoccidial (NICARB) in poultry litter, at concentrations of the order of mg kg^{-1}. While the degradation half-lives of ionophores in manure have been shown to be of the order of 4–17 days (Dolliver and Gupta, 2008; Hansen et al., 2009a), some studies have shown longer stability in stored manure for over three years (Biswas et al., 2012; Doydora et al., 2015). On application to the environment, these ionophores have the potential to persist, with MON, NAR and SAL stable under photolytic conditions, while LAS was shown to be unstable from photolysis (Bohn et al., 2013). This could explain the lower frequency of detection of LAS compared to MON, due to photodegradation prior to entering groundwater. In the same study, MON, NAR and SAL were prone to hydrolysis at pH 4 (half-lives of 0.6–13.3 days), but relatively stable under more realistic neutral and alkaline conditions. The lack of photolytic or hydrolytic degradation suggests the potential for these contaminants to persist and reach aquifers.

Sassman and Lee (2007) carried out sorption and desorption studies of MON and LAS in eight different soils and found LAS to be more associated with soil matrix, with MON shown to be more hydrophilic and associated with both aqueous and suspended solid phases. MON was also found at higher concentrations in agricultural run-of compared to sediment, a further indication of the potential mobility of MON (Davis et al., 2006). These findings are consistent with that reported by Furtula et al. (2009), with lower levels of MON associated with poultry litter, suggesting loss of MON to the solution phase during storage. Hussain and Prasher (2011) studied the sorption of MON, NAR and SAL in two wetlands and found that NAR exhibited the highest hydrophobicity, with MON being the least hydrophobic in

both soils. The higher hydrophobicity of NAR and SAL could account for the lower frequency of detection of these compounds in this current study, compared to MON.
Overall, the reported detections of ionophores, particularly MON, in run off from agricultural land (Sun et al., 2013), in surface waters (Cha et al., 2005; Kim and Carlson, 2006), and groundwaters (Watanabe et al., 2008; Bartelt-Hunt et al., 2011), indicates the relative mobility of these ionophores in the environment. All the above factors, combined with the usage discussed, and a sampling period of active groundwater recharge, provides reasonable explanation for the occurrence of these compounds.

5.4.1.2 Synthetic Anticoccidials

The most frequently detected synthetic anticoccidial in groundwater was AMP, followed by DICLAZ and NICARB. The detection and frequency of AMP in groundwater is surprising, given it is no longer authorised for used as a feed additive since its withdrawal in 2001 by Commission Regulation No. 2205/2001 (European Commission, 2001). However, it is licensed as a veterinary medicine and listed in Regulation 37/2010, for use in poultry species, but is therefore assumed to be present in lower amounts than the compounds used as feed additives. All other detected synthetic anticoccidials are licensed as feed additives, for treatment of coccidiosis in poultry. According to the previously mentioned O'Keefe (2003) review, 100% of producers reported the use of NICARB, as part of a combination product also containing NAR, while 51% were reported to have used DICLAZ. The review also reported that 70% of the producers used robenidine (ROB) as part of treatment, however it was not detected in groundwater as part of this study. This may be attributed to the relative instability of ROB, which has been shown to be prone to photolysis and hydrolysis at low pH, both with degradation half-lives of approx. 4 days (Hansen et al., 2009b).

AMP is relatively mobile in the environment due to its hydrophilicity, as indicated by its water solubility (>500,000 mg L^{-1}) and a log K_{ow} of -2.5 (Table 5-1). On assessment by EFSA, biodegradation studies carried out in five different soils indicated that AMP can persist in the environment, with degradation half-lives ranging from 60 to 417 days in the different soils (EFSA, 2018a). Further data on other degradation pathways in the environment, such as photolysis and hydrolysis, are scant for AMP. The apparent mobility and persistence of AMP, combined with its hydrophilicity, suggests the capability for AMP

to be transported to groundwater, albeit at relatively low concentrations, as shown by leaching studies summarised in the EFSA assessment. Song et al. (2007; 2010) report AMP to be the most frequently detected of four pharmaceuticals, also including MON, in surface water runoff from agricultural land. This study also detected AMP in different soil samples. Song et al. (2010) highlight the potential for association of the strong cationic AMP with dissolved organic matter, such as humic acids in solution, under realistic field conditions. This further suggests enhanced mobility of amprolium to groundwater, particularly in high and extreme vulnerability areas, with little soil protection, or the potential for unattenuated bypass flow. The detection of AMP in a range of hydrogeological settings (including karstic aquifers) (Supplementary File SI-5.2), combined with its high mobility, refutes the potential for the occurrences of AMP to be as a result of persistence and longer lag times. This suggests that its occurrence is potentially as a result of more recent use as a veterinary medicine, as opposed to its historical use (prior to its withdrawal in 2001) as a feed additive.

DICLAZ is licensed both as a feed additive for treating coccidiosis in poultry (primarily) and as a veterinary medicine for therapeutic use in ruminants. It exists in two forms in the environment; at low pH it remains in a neutral form and exhibits very low water solubility, high sorption and high persistence in soil, while at high pH (>7) the anionic form has much higher solubility and much lower sorption and persistence (EFSA, 2018b). Degradation studies in soil have indicated that DICLAZ is stable at low pH with a degradation half-life of >2000 days and a degradation half-life of 70–97 days in higher pH soils. A photolytic degradation half-life for DICLAZ was reported to be between 10 and 308 days (Hansen et al., 2009a). Given a pK_a of 5.9 for DICLAZ (Table 5- 1) and considering that the typical pH of soils in Ireland is generally > 5.5 (Teagasc, 2018), it is unlikely for DICLAZ to be present in its fully neutral form, and therefore as the soil pH approaches and goes above 5.9, it will convert to its more mobile anionic form, allowing potential transport to groundwater. This is reflected in the common occurrence of this synthetic anticoccidial in the groundwater samples.

NICARB is an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP), which on administration, splits into the two components which are excreted separately as DNC and HDP (EFSA, 2010; EFSA, 2017). While HDP is considered to have moderate environmental mobility, due to its water solubility and low soil sorption (Table 5-1), aerobic stability studies in different soils have shown degradation half-

lives between 3 and 7 days. In contrast, DNC has very poor water solubility, binds strongly to soil and has been shown to persist in different soils with half-lives ranging from 193 to 257 days under aerobic conditions. NICARB was detected in the form of DNC in the analytical method used for the groundwater analysis (Mooney et al., 2020), because of the relative instability of HDP. Potential transport of DNC to groundwater may be facilitated by colloidal transport, or sorption of the contaminants onto suspended material, which may reach the aquifer via preferential pathways, bypassing the soil matrix. Both MPs which had detections of NICARB had hydrogeological properties which might facilitate such transport i.e. one site was underlain by a regionally important karst aquifer dominated by conduit flow (solutionally widened openings) with evidence of surface-groundwater interactions, while the second MP was underlain predominantly by a diffuse flow dominated karst aquifer (Rkd) and a regionally important fractured aquifer (Rf), with groundwater within the ZOC predominantly classified as having Extreme X and E vulnerability. It must be noted however that these observations are based on a very limited number of detections, and while the results suggest the possibility of these factors being important for the occurrence of NICARB, it is difficult to make any strong conclusion.

5.4.1.3 Comparison to previous studies

Various ionophores and synthetic anticoccidials have been detected in surface waters and agricultural run-off (Song et al., 2010; Sun et al., 2013; Bak and Björklund, 2014), but the ionophore MON has been the only anticoccidial detected in groundwater based on literature review at the time of this study. Bartelt-Hunt et al. (2011) reported the occurrence of MON in groundwater underlying two different concentrated animal feeding operations housing cattle in the United States of America (USA). Although MON is not used in cattle in Ireland, it is authorised and used heavily as a growth promoter in cattle in the USA. Detected concentrations in groundwater at one site studied by Bartelt-Hunt et al. ranged from 180 to 2350 ng L^{-1}, with monitoring wells downgradient of the facilities more susceptible to contamination. The second site had relatively lower MON concentrations detected (20–68 ng L^{-1}). Watanabe et al. (2008) also reported the detection of MON in shallow groundwater underlying two different dairy facilities in the USA, with concentrations ranging from 40 to 390 ng L^{-1}. The concentrations of MON detected in this current study (4.5–386 ng L^{-1}) are in relative agreement with both studies, apart from the previously mentioned Bartelt-Hunt et

al. study which had a higher range at one site. The ionophores LAS, NAR, and SAL, in addition to the synthetic anticoccidials AMP, DICLAZ, and NICARB were also detected in this study, the first reported detections of these anticoccidials in groundwater to the best of our knowledge in Ireland, and broadly within the EU. Concentrations detected ranged from 1.9 to 139.9 ng L^{-1}. There are currently no legislative limits applicable to anticoccidial compounds in groundwater or drinking water, however on application of the pesticides parametric value specified under the EU Directive on the quality of water intended for human consumption (European Commission, 1998), there were three sites with levels in exceedance of the 100 ng L^{-1} individual pesticide limit. Further research is therefore needed to establish acceptable concentration ranges for these products to protect human and aquatic health.

5.4.2 Relationship of anticoccidial occurrence with site characteristics

5.4.2.1 Source factors

The occurrence of ionophore anticoccidials was significantly related to both the presence of poultry farms and poultry manure spreading within the ZOC, while the occurrence of synthetic anticoccidials was only significantly related to poultry manure spreading. This trend may be as a result of the different application and amounts used of the two groups of anticoccidials. Synthetic anticoccidials are generally used at much lower concentrations, given their better efficacy towards the parasites (Hansen et al., 2009a). Ionophores, however, are more widely used in larger amounts, because of their broad spectrum activity and slow development of resistance (Chapman et al., 2010; Chapman, 2014). There is evidence that several of the ionophores are lost from manure in solution (Section 5.4.1) therefore there is the potential for leaching losses to groundwater, while most of the detected synthetic anticoccidials are strongly sorbed to manure, with the proposed pathways to groundwater likely via preferential flow pathways.

All but two MPs (MP008 and MP103) (Table 5-5) that had detections of anticoccidial compounds, were shown to have a source of poultry activity present within their ZOC. The absence of poultry at these sites is not definitive and may be due to the limitations of the data used for determining poultry activity and poultry manure spreading data. The poultry manure spreading data were based on self-declaration by the individual farmers, with access to more detailed data on poultry manure transport and usage through EPA being restricted due to the

General Data Protection Regulation (European Parliament, 2016). The detections may therefore be due to undisclosed land-spreading of poultry manure on vulnerable soils within the ZOCs of the supplies.

5.4.2.2 Pathway factors

Pathway factors, with the exception of IFS Type I soils discussed below, were not significantly related to the occurrence of anticoccidials, however the authors believe this may be due to the scale of the investigation, which employed the predominant pathway classes within the ZOC, determined from national datasets. There is some evidence of the influence of more localised pathway factors, examples of which are discussed later in this section. There was an association ($P < 0.06$) of detections with IFS Type I classification (acidic/basic soils), showing a higher likelihood of detections in groundwaters overlain by acidic soils, particularly when analysing just the MPs classified as having poultry activity present. Sorption/desorption studies for the ionophore compounds have reported log K_{oc} to be inversely proportional to soil pH, which indicates less adsorption (log K_{oc} decreases) to soil as pH increases (Davis et al., 2006; Sassman and Lee, 2007; Hussain and Prasher, 2011). This is likely due to the formation of anionic molecules as the soil pH approaches and increases above the pK_a of the compound, resulting in repulsion from soil surfaces. Ionophores will primarily be anionic in neutral and alkaline environments and therefore have the potential to migrate to groundwater. An interesting trend has been observed for MON whereby the log K_{oc} increased (instead of further decrease) at higher pH (> 7 as reported by Sassman and Lee (2007) and > 8.5 by Hussain and Prasher (2011)). This effect was attributed to the complexation of MON with cationic metals and this could account for the lack of association expected in this study, between anticoccidial detections and basic soils. In higher pH soils, ionophores such as MON form lipophilic neutral complexes with metal cations such as calcium and sodium, which can consequently bind to soil, thus increasing the sorption behaviour, and reducing the mobility to groundwater.

Given the potential primary source of anticoccidials in poultry manure or litter, it is important to consider how the application can alter the behaviour of the contaminants in the receiving soil. Poultry/broiler litter is typically alkaline, with a reported pH of 8–9 (Nicholson et al., 1996; Doydora et al., 2015). Given the alkaline nature, there is the potential for a localised pH increase in soils receiving such manure/litter, e.g. Doydora et al. (2017)

reported an overall increase of one pH unit in soil with long term broiler litter amendment, compared to unamended soils. There is also the potential for an accumulative effect if more than one type of manure is spread on the land. Whalen et al. (2000) demonstrated the effect of cattle manure application, with amended soils shown to have an increased pH, with the increase lasting for at least 8 weeks. The increase in soil pH from manure application has been attributed mainly to the presence of $CaCO_3$ (calcium carbonate) in the manure, particularly poultry litter, however, there is also the potential of an increased pH because of the release of hydroxide (OH-) as a by-product of the ammonification of urea/uric acid in the manure.

A localised increase in the pH of an acidic soil of one unit would likely be enough to alter the sorption behaviour of compounds such as the ionophores, given their physicochemical properties (Table 5-1). In the Doydora et al. (2017) study, 46% less MON was sorbed in poultry litter amended fields, compared to unamended fields. As part of the national soil sampling carried out by Teagasc, (the Agricultural and Food Development Authority of Ireland), 55% of soils sampled (based on 45,157 samples) had a pH between 5.5 and 6.5, with just 9% having a pH of <5.5 and 36% having a pH >6.5 (Teagasc, 2018). At this typical pH range, several anticoccidials will be sufficiently ionised and less sorbed to the soil, therefore they have the potential to be transported to groundwater, thus reasonably explaining their occurrence in this study. Any potential localised increase in pH on the application of manure is likely to amplify such effect, with increased mobility of compounds such as DICLAZ and the ionophores, because of less sorption to soil at the higher pH. Application of manure onto soils that are already alkaline could consequently increase the pH even further and exacerbate the complexation effects of the ionophore mentioned previously.

There was no significant relationship between anticoccidial detections and the predominant groundwater vulnerability, which is somewhat surprising given it was hypothesized that the occurrence of such contaminants would be associated with areas of higher groundwater vulnerability, because of the inherently higher susceptibility of higher vulnerability groundwaters to contamination (Daly, 2004). MPs with anticoccidial detections were relatively evenly spread across the different groundwater vulnerability classes (Figure 5-2 and Table 5-5). Notably, the percentage of detections at MPs classified as low (L) (25%) and moderate (M) (33%) was a lot higher in comparison to extreme (X) (6%) vulnerability MPs

(Figure 5-2). Further analysis carried out on the actual percentage of each vulnerability within the ZOC (as opposed to the predominant vulnerability class) still showed no significant relationship. This suggests that the overall predominant groundwater vulnerability within the MP ZOC does not adequately reflect the potential for anticoccidial contamination at an MP, with the occurrence of these contaminants more likely accounted for on a site-specific basis, due to more localised factors.

The effect of localised factors can be illustrated with examples of two of the four MPs with anticoccidial detections that were classified as having predominantly low groundwater vulnerability. MP023 (with 67% M and L vulnerability) nevertheless has a sizeable proportion of the ZOC classified as X, E or H vulnerability (33%), and is a karst spring fed by a regionally important karst aquifer dominated by conduit flow (Rkc). This MP has several karstic solution features within its ZOC, including several sinking streams, which allow for rapid and unattenuated direct entry of contaminants to groundwater (Karst Working Group, 2000; Coxon, 2014). MP019 (BH with 99% of ZOC being L vulnerability) has a history of elevated phosphorus (P) levels in the water supply and a site report for this BH indicated the potential for in-flow of surface runoff due to the wellhead construction (specifically a lack of protective caps), with evidence of surface ingress directly into the well. The detections of anticoccidials at the MP is therefore likely to be via the same localised pathway contributing to the high P in the BH and could potentially result from inadequate wellhead protection.

5.4.3 Anticoccidial occurrence and water quality parameters

The relationship shown between the presence of ammonium in groundwater and detections of the ionophore compounds, is interesting for several reasons. Ammonium can be formed as a result of the mineralisation of organic nitrogen in poultry manure, for example, uric acid/urea can undergo ammonification to ammonium. Poultry litter also generally contains relatively high amounts of ammonia (NH_3) and on application to acidic soils, ammonia is converted to ammonium (NH_4^+) which will bind to negatively charged sites such as clay, in the soil (Wlazlo et al., 2016). In this regard, any ammonium that is found in groundwater is likely to have reached there by preferential flow pathways to groundwater, similar to phosphorus, which is not leached easily. Leaching studies have indicated the potential for

ammonium to be leached via macropores (Silva et al., 2000). The overlying soils and subsoils also act as a natural protective layer for attenuating microbial contaminants such as faecal coliforms, with agriculture (farmyard run-off, grazing animals and land-spreading of manure and slurry) being one of the main sources of such contaminants in water (EPA, 2015b). The relationship of anticoccidial detections with faecal coliforms in this study is a further indication of the importance of localised groundwater vulnerability and the potential influence of preferential secondary flow pathways on the transport of anticoccidials to groundwater. Further work would need to be carried out, possibly at the field scale, using lysimeter and leaching studies to confirm these relationships.

A significant relationship was shown between anticoccidial occurrence (particularly the ionophores) and lower groundwater pH, however, it is difficult to draw conclusion given that the overall mean groundwater pH at sites with detections was 6.8 ± 0.6 (range from 5.5 to 7.4), with 90% of these sites having a pH > 6. Any inferences made are therefore limited to this relatively narrow pH range. At a pH > 6, an appreciable fraction of most of the ionophores will be ionised and in solution phase given their pK_a's of 4.5–6.6. This groundwater pH relationship does however agree with the relationship found between detections and IFS acidic soils. The statistical analysis also showed that detections of any anticoccidial, and the ionophores as a subgroup, were more likely in groundwaters with lower calcium and lower conductivity, with both of these trends also linked to lower pH conditions. The relationship between anticoccidial occurrence and the absence of zinc and uranium, we believe, are not causal relationships, rather are a by-product of their relationship with pH, and are more likely to be linked to the geology. However, the lack of relationship with geology, namely the bedrock units, does not strengthen this, and further investigations would need to be carried out to further explore these potential relationships.

5.4.4 Future considerations and applicability

Summarising the outputs of this work, the results suggest that anticoccidial drug occurrence may be more associated with MPs that have a known source of poultry and where there is evidence of contamination from poultry manure in the form of ammonium and/or faecal coliform detections, with MPs with lower groundwater pH of more interest. While these observations were shown to be statistically significant, there are a number of limitations that

should be considered as part of any future application. Due to the relatively low number of detections, some of the inferences made cannot be considered as conclusive, rather should only be considered as evidence of an effect that merits further investigation. In this regard, the outputs of this work are suitable for application as a broad indicator tool only (as opposed to a predictor), for selection of potential sites for future monitoring of anticoccidial occurrence. Future work, as highlighted previously in the discussion, is required to further investigate the relationship with groundwater ammonium.

Regarding the association of anticoccidial detections with poultry activity, it should be noted that this effect is influenced by the fact that poultry production is the primary source of anticoccidials in Ireland. This observation therefore does not imply that poultry is the only driver of anticoccidial occurrence in the environment, and on future application of the approach adopted in this work, consideration should be given to other sources of anticoccidials, which can vary from one country to another (e.g. the USA where anticoccidials are more heavily used in cattle). Finally, the authors also note that this study was carried out as part of one sampling season only during November and December 2018, which coincided with a period of active groundwater recharge. As a result, the study does not assess the potential influence of meteorological effects, such as the timing of effective rainfall, on the overall occurrence of anticoccidials in groundwater. A comprehensive temporal occurrence study was beyond the scope of this work, however this is something that needs to be considered in future work, not only to assess the potential temporal variation of occurrence as a result of meteorological conditions, but also to assess variations due to timing of manure application and lag times through the unsaturated zone.

5.5 Conclusion

This study, we believe, is the first of its kind to assess the occurrence of anticoccidials in groundwater, given the comprehensive suite of anticoccidials investigated, which included six ionophore and twenty synthetic anticoccidials. Up to seven different anticoccidial compounds were detected across 24% of groundwater monitoring points, at concentrations ranging from 1.9 to 386 ng L^{-1}. On average, 1.7 compounds were detected at each of the 26 monitoring points with anticoccidial detections, with five monitoring points having up to

three compounds detected together. Monensin, a commonly used ionophore feed additive in poultry production, was the most frequently occurring compound, detected in 15% of all samples, while amprolium, a veterinary medicine exclusively used in poultry, was the second most frequently detected, detected in 7% of all samples.

Poultry activity was statistically shown to be a significant driver of the occurrence of anticoccidial compounds in Irish groundwater, with the occurrence of an anticoccidial 6.5 times more likely at supplies which had a source of poultry (poultry farm and/or poultry manure spreading), compared to an absence of poultry activity. Statistical analysis did not identify any clear relationships with physical site properties, however, the occurrence of any anticoccidial compounds was found to be more associated with monitoring points which contained a higher proportion of acidic soils within their zone of contribution (ZOC). Assessment of the MPs with detections and their hydrogeological properties, on a site by site basis, indicated that occurrence was likely explained by more localised factors. Several water quality parameters (groundwater pH, calcium, conductivity, ammonium, and faecal coliforms) were shown to be significantly related to the occurrence of anticoccidial compounds, or one of their subgroups (ionophores or synthetic anticoccidials). Due to the limitations of the data involved, these inferences are not definitive predictors, with further work required to confirm the relationships.

This work not only reports on the first detections of anticoccidials in an Irish groundwater context, it also reports, to the best of our knowledge, some of the first reported occurrences of lasalocid, narasin, salinomycin, amprolium, diclazuril and nicarbazin, in groundwater in Europe. Such detections indicate that these contaminants may require greater consideration in groundwater quality monitoring programmes, given that their use is anticipated to continue, if not increase, as a result of agricultural intensification. This work contributes additional information on the overall environmental groundwater occurrence of anticoccidials, thus helping to advance our understanding of their fate. The results and outputs of this work may also provide a broad preliminary tool for the identification of potential sites for regulatory monitoring of anticoccidials.

5.6 CRediT author statement

Damien Mooney: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Visualization, Project Administration. **Karl Richards:** Conceptualization, Resources, Writing – Review and Editing, Supervision, Funding Acquisition. **Martin Danaher:** Conceptualization, Methodology, Resources, Writing – Review and Editing, Visualization, Supervision, Funding Acquisition. **Jim Grant:** Software, Formal Analysis, Writing – Review and Editing. **Laurence Gill:** Conceptualization, Writing – Review and Editing, Funding Acquisition. **Per-Erik Mellander:** Conceptualization, Resources, Writing – Review and Editing. **Catherine Coxon:** Conceptualization, Writing – Review and Editing, Visualization, Supervision, Project Administration, Funding Acquisition.

CHAPTER 6 – CONCLUSIONS AND FUTURE WORK

6.1 Conclusion

The outputs of this thesis provide some of the first insights into the occurrence of anthelmintic and anticoccidial antiparasitic veterinary agents in groundwater throughout Ireland. To the best of the author's knowledge, it also represents the most significant body of research carried out on anthelmintic and anticoccidial occurrence in groundwater in Europe and perhaps globally. This was achieved by the development of two new comprehensive analytical methodologies for detecting and quantifying anthelmintics and anticoccidial in surface water and groundwater samples, which were subsequently applied as part of comprehensive occurrence studies which were designed to be reflective of the different source and pathway pressures for each respective group of contaminants. With consideration of the project objectives specified in Chapter 1, the main findings and outputs of this work are summarised below, as divided into these two main aspects of the project i.e. developing analytical methodology and investigating groundwater occurrence.

Analytical Methodology

Two new comprehensive analytical methodologies based on solid phase extraction (SPE) with UHPLC-MS/MS detection were developed and validated for the determination of two groups of antiparasitic agents in water samples as follows:

- In Chapter 2, a comprehensive multiresidue method based on SPE with UHPLC-MS/MS detection was developed and validated for the determination of 40 anthelmintic compounds, including 13 transformation products (TPs), in unfiltered surface and groundwater samples (Mooney et al., 2019). This anthelmintic method is the most comprehensive method now available for application to unfiltered environmental water, for the determination of a broad range of compounds, from all anthelmintic structural classes, at sub part per trillion (ppt) levels (ng L^{-1}).

- In Chapter 3, a sensitive and selective SPE UHPLC/MS/MS method was developed and validated for the determination of 26 anticoccidial compounds (six ionophores and twenty synthetic anticoccidials) in surface and groundwater samples at parts per quadrillion (pg L^{-1}) to parts per trillion levels (Mooney et al., 2020). This anticoccidial method is the first reported method that allows the simultaneous analysis of all

ionophores in addition to a large suite of synthetic coccidiostats, including traditionally problematic polar compounds, in one analytical run. It is also considered the most comprehensive method now available for application to environmental waters that covers all of the anticoccidials licensed for used within the EU, in addition to several licensed outside of the EU, this broadening its applicability.

Groundwater Occurrence

Application of the new methodologies developed in Chapter 2 and 3 allowed for comprehensive occurrence studies to be carried out, the results of which show that anthelmintic (Chapter 4) and anticoccidial (Chapter 5) antiparasitic drugs are occurring in Irish groundwaters. Both studies report on the first occurrences of each respective group of antiparasitic agents in groundwater in Ireland, and in some cases globally. The main conclusions of these occurrence studies are discussed below, with consideration of the remaining objectives outlined in Chapter 1.

- Frequency of occurrence
    - In Chapter 4, as part of the occurrence study, 17 different anthelmintic drugs were detected at 22% of the total sites sampled, with occurrences in 18% of groundwater samples and 39% of the associated surface waters. The overall detected concentrations ranged from 1 to 47.5 ng L^{-1} (parts per trillion). The benzimidazoles, one of the most used broad spectrum classes of anthelmintics, were detected most frequently, with benzimidazole TPs more prevalent than their respective parent compound, in both surface and groundwaters. Albendazole, the most heavily used benzimidazole due to its dual efficacy toward worms and flukes, and its transformation products, were the most commonly occurring anthelmintics.
    - In Chapter 5, seven different anticoccidials consisting of four ionophores and three synthetic anticoccidials, were detected at 24% of groundwater sites sampled, at concentrations ranging from 1.9 to 386 ng L^{-1}. The ionophore monensin was the most frequently occurring anticoccidial, detected at 15% of the total sites sampled. Monensin was also detected at the highest concentration. The higher frequency of monensin compared to other ionophores such as lasalocid or narasin, is reflective of the higher apparent mobility of monensin due to less

sorption to manure and soil, based on its physicochemical properties. The second most detected anticoccidial was the synthetic anticoccidial amprolium, detected at 7% of sites. The higher occurrence rate of amprolium compared to other synthetic anticoccidials was attributed to the relatively higher mobility of amprolium in the environment, owed to its high water solubility and low degradation rate, thus persistence, in the environment.

Overall considering both studies, the frequency of detections in groundwater is of a similar magnitude for both groups of compounds, however it is difficult to make any direct comparisons given both studies were independent from one another, with different sites sampled for each study due to the different source pressures for the respective contaminant groups (i.e. cattle and sheep for anthelmintics, and poultry production for the anticoccidials). In addition, both studies were carried out on different sampling occasions, further complicating any comparisons. It is noted however that one site had both anthelmintic and anticoccidial detections recorded.

- Spatial occurrence in relation to source pressures

  Both occurrence studies were designed to account for the different source pressures for the respective groups of contaminants. In each case, source factors were shown to have a significant relationship with the spatial occurrence as follows:

  - In Chapter 4, the anthelmintic detections were shown to be statistically related to sheep density and the proportion of tillage land within the zones of contribution (ZOC) of the sampling points. The anthelmintic parent compounds were shown to be associated with sites with a higher sheep density within the ZOC, with the compounds detected at such sites, consistent with those used heavily as flukicides to treat flukes, to which sheep are most susceptible. Benzimidazole anthelmintics and their TPs, were shown to be associated with sites with a higher percentage of tillage land within their ZOC. Although significant, this result was confounded at a more localised level with evidence of influence of grassland agriculture, with most tillage farms in Ireland also having some degree of livestock enterprise.
  - In Chapter 5, poultry activity was shown to be the most significant driver of anticoccidial occurrences, with detections found to be statistically related to both the presence of poultry farms and poultry manure/litter spreading within the ZOC of the monitoring points. Anticoccidial occurrence was more likely at monitoring points with a higher density of poultry farms/housing units with the ZOC, and

more likely at sites that had manure spreading in the ZOC, compared to those that did not.

- Pathway factors

  As part of each occurrence study the different physical pathway factors within the ZOC of the sampling points were analysed for relationships with contaminant occurrence, with several statistical relationships identified:

  - For the anthelmintics (Chapter 4), monitoring point type and soil type were found to be the physical characteristics most associated with detections, with occurrence more likely in monitoring boreholes with smaller ZOCs compared to abstraction boreholes, and more likely at sites with ZOCs dominated by poorly drained soils. Such relationships suggested the potential importance of surface or preferential pathways for the transport of anthelmintics.
  - For the anticoccidials (Chapter 5), the main physical characteristic associated with occurrence was soil pH, with detections more associated with sites with a higher proportion of acidic soils within their ZOC, compared to basic soils. A further statistical relationship with anticoccidial occurrence and the presence of ammonium in groundwater provided insight to potential transport pathways for anticoccidials to groundwater, with preferential pathways via macropores, the most important for ammonium, which may therefore be indicative of transport routes for the anticoccidials.

  While these aforementioned relationships were significant, for both studies there was evidence of an effect of more localised factors influencing the relationships, and therefore it is concluded that the assessment of the predominant site characteristics within the ZOC of monitoring points using national scale datasets, does not adequately reflect the potential for anthelmintic or anticoccidial occurrence. There was no association found between anthelmintic or anticoccidial occurrences and aquifer type or groundwater vulnerability and this was also attributed to be as a consequence of such national scale investigations, with the occurrence of these contaminants more likely accounted for on a site-specific basis, due to more localised factors.

- Temporal variations

  A temporal occurrence study was carried out for the anthelmintics only (Chapter 4). The results showed that both anthelmintic drug usage and recent meteorological events were

important factors influencing the temporal occurrence of anthelmintics at eight springs in two karst regions. The periods of highest frequency and concentration of anthelmintic occurrence were during February/March following landspreading of manure gathered during housing and the return of animals to pastures, and again later during August/September, which were interpreted to be as a result of the transition from dry summer conditions to wetter in autumn. While these trends were observed in conduit flow karstic aquifers, the findings can be broadly applied to other hydrogeological settings which are sensitive to recent meteorological events and vulnerable to groundwater contamination. While the karstic aquifers were not shown to be significantly related to anthelmintic occurrence as part of the spatial study, this temporal study demonstrates the vulnerability of such systems to anthelmintic contamination, with the outputs of the temporal study highlighting the potential importance of unattenuated surface to groundwater pathways, such as sinking streams, for the transport of anthelmintics to groundwater.

Overall, it is very evident that both groups of antiparasitic agents are occurring in Irish groundwater, which indicate that these contaminants may require greater consideration in groundwater quality monitoring programmes, given that their use is anticipated to continue, if not increase, as a result of agricultural intensification and/or climate change (as in the case for the anthelmintics). The results of this work can be used as a broad tool for selecting suitable sites that may be of most interest for the monitoring of anthelmintics and anticoccidials in groundwater. This work provides additional information on the overall groundwater occurrence of anthelmintics and anticoccidials, thus contributes toward filling the gaps in knowledge, which is crucial for advancing our understanding of the environmental fate and risk of these contaminants. The availability of the newly developed comprehensive methods will allow more comprehensive occurrence studies to be carried out in future, thus further advancing our understanding of the environmental fate of these contaminants.

6.2 Future Considerations

There are several aspects of this work that could be built upon, to further advance on the findings reported in this thesis. Examples of such are provided below in the context of (a) analytical methodology, (b) national (local) level investigations and (c) international considerations.

(a) Analytical methodology

- While both newly developed analytical methods were more than capable of detecting environmentally relevant concentrations and performed better than other available methods, there is still a need for continual efforts to achieve lower detection limits. This is important to be able to satisfy the analytical performance requirements of any potential legislative limits, or environmental quality standards, that may be set in the future. In terms of the work reported in this study, it was noted that the lack of detections of the macrocyclic lactone anthelmintics (spatial occurrence study in Chapter 4) was surprising given their usage in relatively high amounts. A potential explanation of this was the lower sensitivity of the detection method for these compounds, with limits of detection approximately 10 times higher than the benzimidazoles. Future analytical development work should therefore focus on increasing the method sensitivity for the macrocyclic lactones (i.e. achieving lower limits of detection). This could be achieved by transfer of the detection method to more sensitive instrumentation, such as the QTRAP mass spectrometer used for the anticoccidial analysis (which was not available in the laboratory at the time of the anthelmintic analytical work). This would allow for lower limits of detection to be achieved, while at the same time, the use of more sensitive instrumentation could allow for reduction the sample volume required for analysis, which in turn reduces the sample extraction time and could potentially increase the throughput of the method.

- The developed SPE methods involved the extraction of unfiltered water samples to comply with legislative requirements to measure "whole" water concentrations. Such an approach allowed for the determination of both the fraction of analytes associated with suspended sediment including colloidal material in the sample and the dissolved fractions in solution phase. However, as part of the occurrence studies it was proposed that the occurrence of some antiparasitic drugs in groundwater, may be as a result of sediment associated transport via surface-runoff or preferential flow pathways. It would

therefore be of benefit for future studies to analyse both a filtered and unfiltered aliquots of raw water samples to determine the extent to which the detected drugs are associated with the solution phase or suspended sediment phase of the water sample. This could provide further evidence of particle/sediment bound transport pathways for these contaminants.

- Although passive sampling has its disadvantages, the most important being its ability to measure only the dissolved "free-water" fraction of contaminants, it could be useful as a complementary tool to supplement the results obtained from grab-sampling. As previously described, a grab sample only represents the water quality at any one moment in time and therefore has a high risk of missing both sporadic and episodic pollution events. The use of passive samplers, which can be deployed for longer periods of time, would allow for such events to be picked-up, and can provide a time-weighted average concentration of pollutants. This would be of particular benefit in hydrogeological settings which are sensitive and very responsive to episodic events such as intense rainfall. As evident from the investigation of temporal variations in anthelmintic occurrences (Chapter 4), anthelmintic compounds were detected at two sinking streams and at the associated karstic springs to which the streams had previously been traced. This highlighted the potential importance of surface to groundwater transport in such settings. The use of passive samplers would allow such relationships to be investigated more thoroughly.

(b) National investigations

- At a national level, there is an evident need to carry out additional studies at a catchment or field scale to further investigate and disentangle several relationships found for the occurrences of anthelmintics and anticoccidials, as listed below:
    - The association of anthelmintic occurrence with tillage land-use was hindered by the presence of an appreciable proportion of grassland agriculture within the ZOC. As a result, it was difficult to conclusively attribute the anthelmintic occurrence at these sites to be solely as a result of the tillage activity. Future investigations could involve a study at farm level to quantify the different anthelmintic loading on the tillage land, compared to the grass land, while field scale leaching studies could investigate the effect, if any, of the incorporation of

contaminated manure applied to tillage land by ploughing, compared to spray application to grassland.

- The occurrence of anticoccidials was shown to be statistically related to several water quality parameters, such as the presence of groundwater ammonium and the presence of faecal coliforms, which inferred the potential of contaminant transport via preferential flow pathways. Further work would need to be carried out, possibly at the field scale, using lysimeter and leaching studies to confirm these relationships.

- There were several limitations to the data used for characterising the poultry activity within the ZOCs of sites sampled as part of the occurrence study for the anticoccidials. Due to GDPR (General Data Protection Regulation), the data provided by the Department of Agriculture only accounted for poultry premises included on the poultry premises register, and the form in which the information was provided did not allow for the relative scale of the poultry premises to be determined. The information provided on poultry manure spreading was gathered based on self-declaration by the individual farmers. As a result, there was the potential for the influence of unregistered poultry premises, or undeclared poultry manure spreading, on the occurrence of anticoccidials, that was not accounted for in the study. Further efforts should be made as part of future studies to ensure a more complete data-set, to provide for a more accurate assessment of the source pressures for anticoccidials.

- Due to time constraints, a temporal occurrence study for the anticoccidial compounds was not feasible as part of this project, however, this is something that needs to be considered in future investigations. Although there is not expected to be a significant variation in the amounts of the anticoccidial drugs used in poultry production due to the almost constant prophylactic use, future investigations are required to assess not only the potential temporal variation of occurrence as a result of meteorological conditions, but also to assess variations due to timing of poultry litter/manure application and lag times through the unsaturated zone.

(c) International context

- Application of the new, and more comprehensive, analytical methodologies to groundwaters and surface waters throughout Europe, and further afield, will contribute a better understanding of the occurrence and associated environmental concentration of a comprehensive suite of antiparasitic agents. On future application of the approach adopted in this work, consideration should however be given to other sources of contaminants not considered in this study, given that they can vary from one country to another. For example, the primary source of anticoccidials in Ireland and more broadly within the EU is in poultry production, however, in the USA, anticoccidials are also licenced and heavily used as growth promoters in cattle.

- Considering the evidence of groundwater occurrence of both the anthelmintics and anticoccidials presented in this thesis, there is a clear and evident need for more research and monitoring of both groups of contaminants in groundwater not only in Ireland, but more broadly within the EU. There are currently no legislative limits applicable to anticoccidial compounds in groundwater or drinking water in the EU, however, on application of the pesticides parametric value specified under the EU Drinking Water Directive (European Commission, 1998) and the Groundwater Directive (European Parliament, 2006), there were three sites with levels (up to 386 ng L^{-1}) in exceedance of the 100 ng L^{-1} individual pesticide parametric value set. This provides evidence of the need to consider such contaminants as a risk to groundwater, and to either extend the scope of the pesticide definition to include these, or to implement new guidelines to establish acceptable concentration ranges for these products to protect human and aquatic health. For the anthelmintics, given their use to treat nematodes, several of these compounds fall under the pesticide definition specified in legislation (Section 1.5). Although there were no exceedances of the pesticide parametric value for the anthelmintics, on a number of occasion (and accounting for the transformation products), the concentrations of albendazole matched or approached the threshold value (TV) of 75 ng L^{-1} (0.075 µg L^{-1}) set for pesticides in Ireland. It was noted however that the anthelmintic ivermectin was detected on several occasions at concentrations in excess of the 50% lethal concentration (LC_{50}) reported amongst literature for different aquatic organisms. The lack of monitoring, or capability to monitor (up until now) the anthelmintics which are occurring at levels close to the TVs and in excess of such LC_{50}

values further highlights the need for more consideration of these contaminants in groundwater quality standards.

Overall, the work presented in this thesis reports the most significant advancement in recent times toward filling the current gaps in knowledge of the environmental occurrence and fate of these contaminants in groundwater. However, there is still an evident need for much more research to compliment and further build on these efforts.

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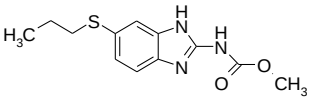
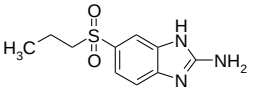
APPENDICES

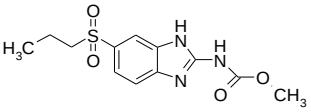
Appendix 1A - Groups of antiparasitic agents and their structures



Albendazole
ABZ



Albendazole amino sulphone *ABZ-NH₂-SO₂*



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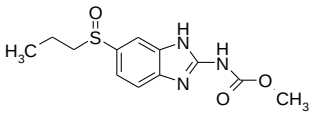

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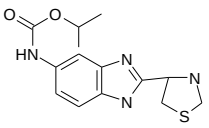
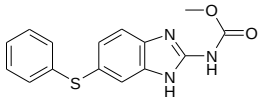
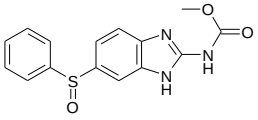
ABZ-SO



Cambendazole
CAM



Fenbendazole
FBZ



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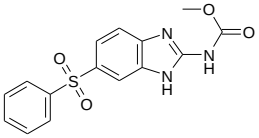
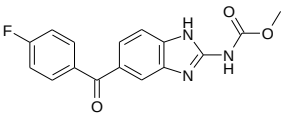
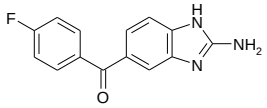
OXF



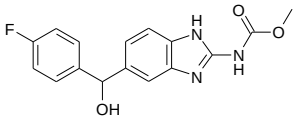
Fenbendazole sulphone
FBZ-SO₂



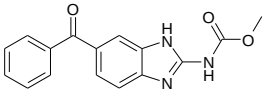
Flubendazole
FLU



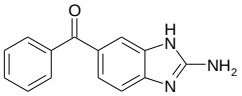
Amino-Flubendazole
FLU-NH₂



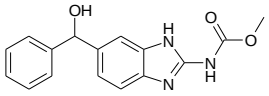
Hydroxy-Flubendazole
FLU-OH



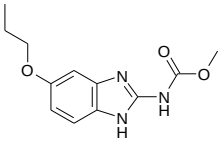
Mebendazole
MBZ



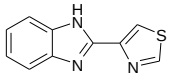
Amino-Mebendazole
MBZ-NH₂



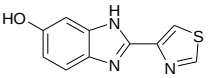
Hydroxy-Mebendazole
MBZ-OH



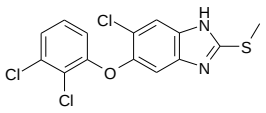
Oxibendazole
OXI



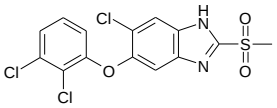
Thiabendazole
TBZ



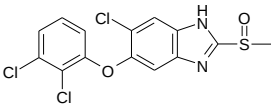
5-Hydroxy Thiabendazole
TBZ-OH



Triclabendazole
TCB

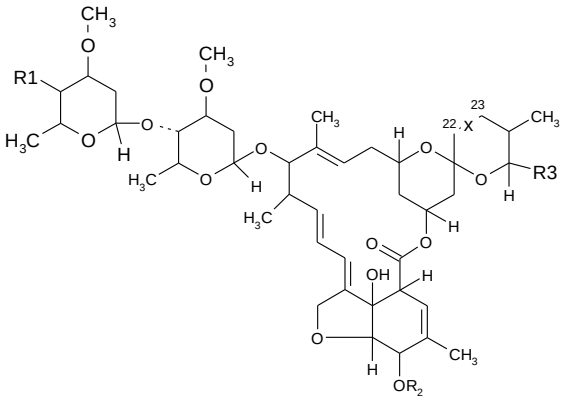


Triclabendazole sulphone
TCB-SO₂

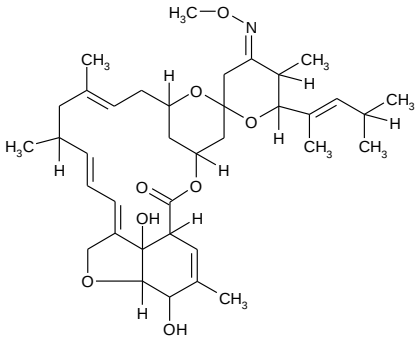


Triclabendazole sulphoxide
TCB-SO

Figure A1-1 (a) structures of benzimidazole anthelmintics

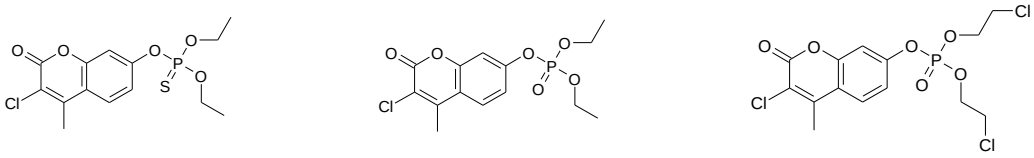


Avermectin	R1	R2	R3	C_{22}-x-CC_{23}
Abamectin B1a (ABA)	OH	H	$CHCH_3CH_2CH_3$	-CH=CH-
Doramectin (*DORA*)	OH	H	C_6H_{11}	-CH=CH-
Emamectin Benzoate B1a (*EMA*)	$C_6H_5COOHCH_3NH$	H	$CHCH_3CH_2CH_3$	-CH=CH-
Eprinomectin B1a (*EPRINO*)	$NHCOCH_3$	H	$CHCH_3CH_2CH_3$	-CH=CH-
Ivermectin B1a (*IVER*)	OH	H	$CHCH_3CH_2CH_3$	-CH_2-CH-



Moxidectin
MOXI

Figure A1-1 (b) structures of macrocyclic lactone anthelmintics

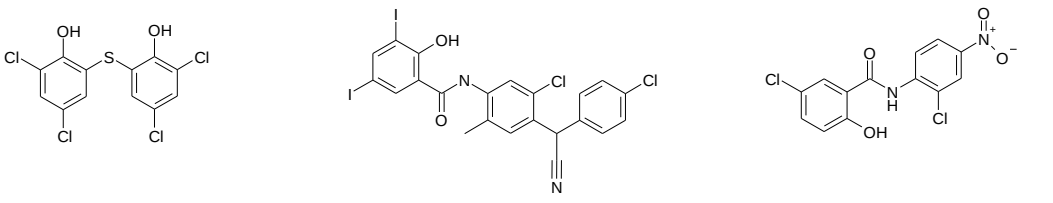


Coumaphos
COUMA

Coumaphos-oxon
COUMA-O

Haloxon
HALOX

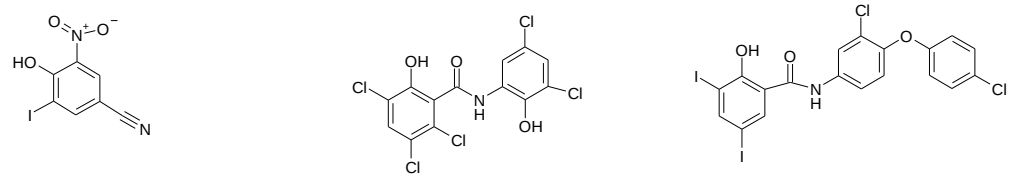
Figure A1-1 (c) structures of organophosphate anthelmintics



Bithionol
BITH

Closantel
CLOS

Niclosamide
NICLOS

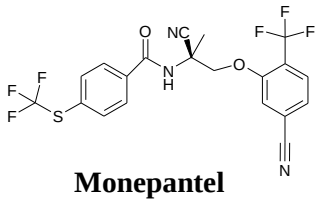


Nitroxynil
NITROX

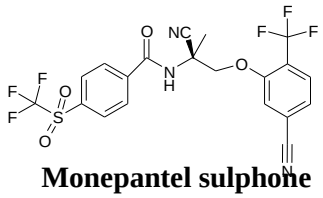
Oxyclozanide
OXY

Rafoxanide
RAFOX

Figure A1-1 (d) structures of salicylanilide and substituted phenol anthelmintics

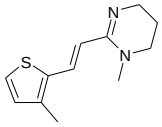


Monepantel
MONE

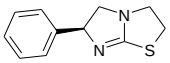


Monepantel sulphone
MONE-SO_2

Figure A1-1 (e) structures of amino-acetonitrile derivative anthelmintics

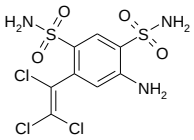


Morantel
MOR



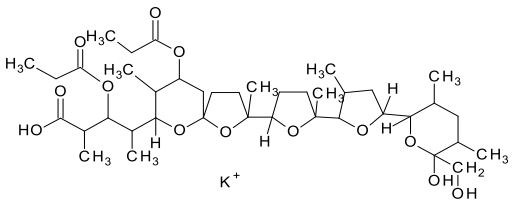
Levamisole
LEV

Figure A1-1 (f) structures of tetrahydropyrimidines (MOR) and imidazothiazole (LEV) anthelmintics

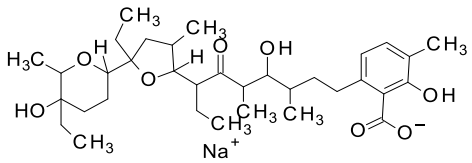


Clorsulon
CLOR

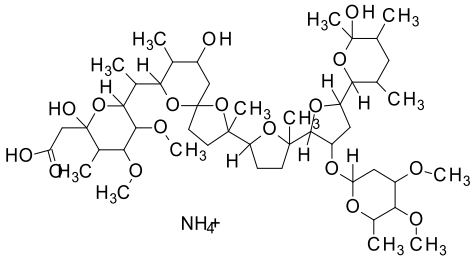
Figure A1-1 (g) structure of one miscellaneous anthelmintic (CLOR)



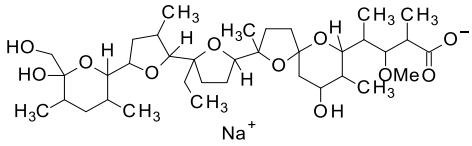
Laidlomycin
LAID



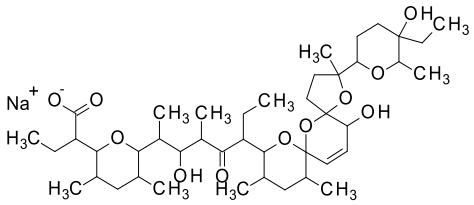
Lasalocid
LAS



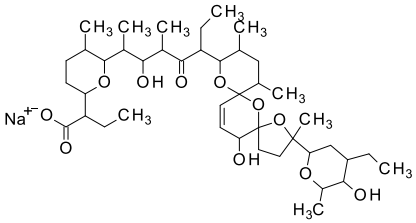
Maduramycin
MAD



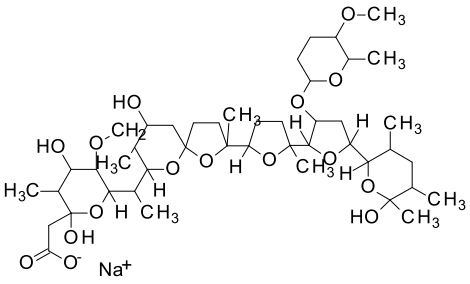
Monensin
MON



Narasin
NAR

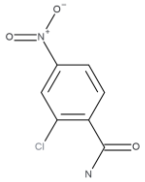


Salinomycin
SAL

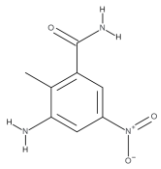


Semduramicin
SEMED

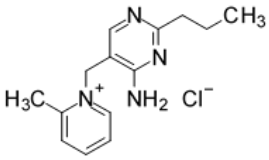
Figure A1-2 (a) Structures of ionophore anticoccidials



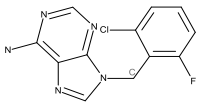
Aklomide
AKLO



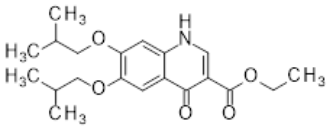
3-Amino-2-methyl-5-Nitrobenzamide (*3-ANOT*)



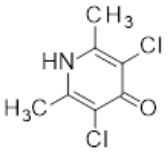
Amprolium
AMP



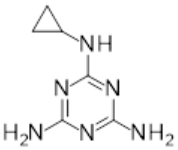
Aprinocid
APRIN



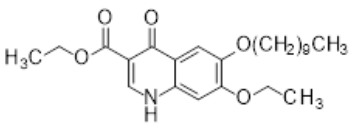
Buquinolate
BUQ



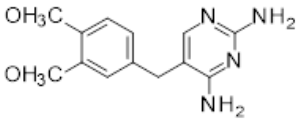
Clopidol
CLOP



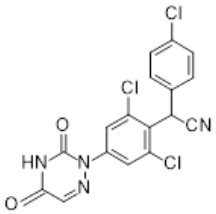
Cyromazine
CYRO



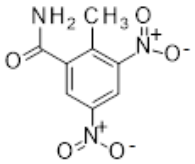
Decoquinate
DECOQ



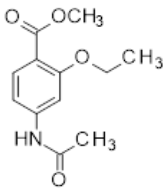
Diaveridine
DIAV



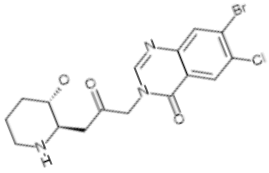
Diclazuril
DICLAZ



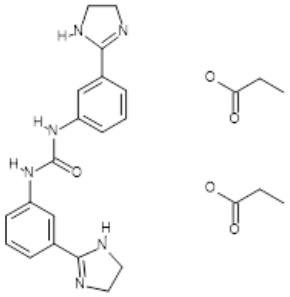
Dintolmide
DINITOL



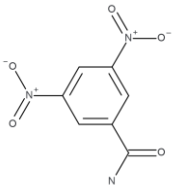
Ethopabate
ETHO



Halofuginone
HALO

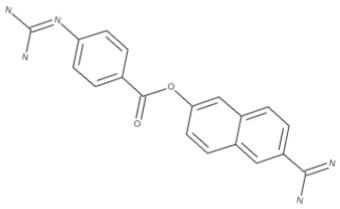


Imidocarb dipropionate
IMIDO

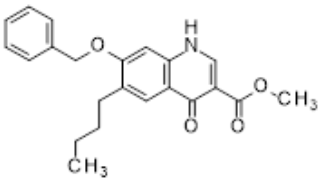


Nitromide
NITRO

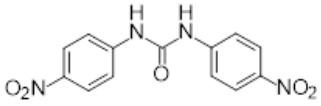
Figure A1-2 (b) Structures of synthetic anticoccidial compounds



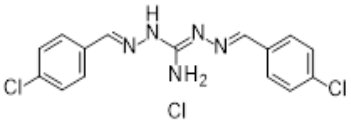
Nafamostat
NAFAM



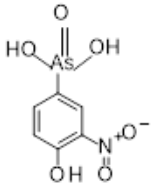
Nequinate
NEQ



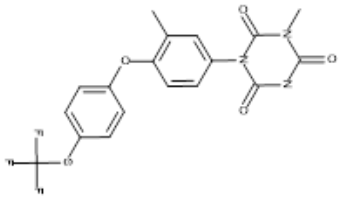
4,4-Dinitrocarbanilide
NICARB



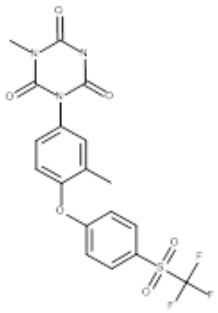
Robenidine
ROB



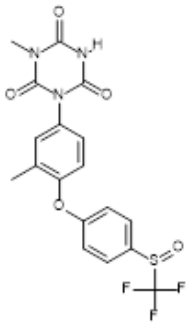
Roxarsone
ROX



Toltrazuril
TOL

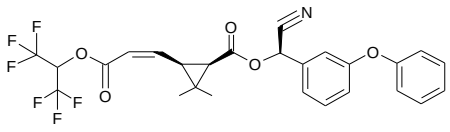


Toltrazuril sulphone
TOL-SO_2

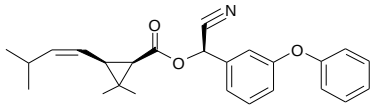


Toltrazuril sulphoxide
TOL-SO

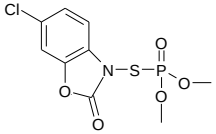
Figure A1-2 (b) *continued*



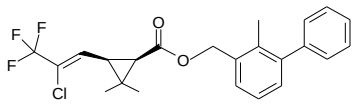
Acrinathrin
ACRIN



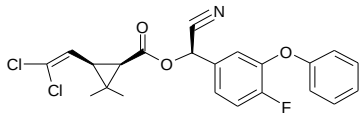
Allethrin
ALL



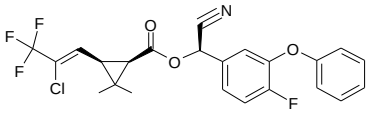
Azamethiphos
AZA



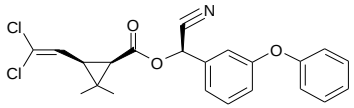
Bifenthrin
BIFE



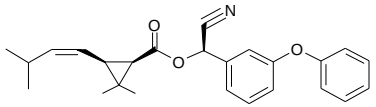
Cyfluthrin
CYFLU



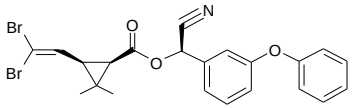
Cyhalothrin
CYHALO



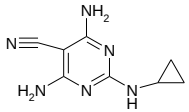
Cypermethrin
CYPER



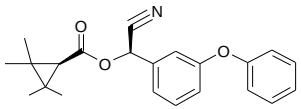
Cyphenothrin
CYPH



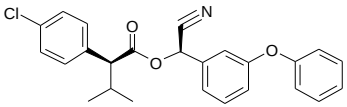
Deltamethrin
DELT



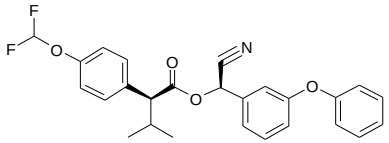
Dicyclanil
DIC



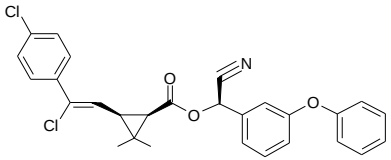
Fenpropathrin
FENP



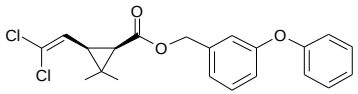
Fenvalerate
FENV



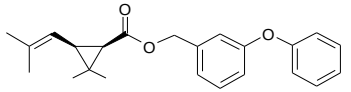
Flucythrinate
FLUC



Flumethrin
FLU

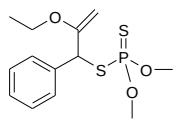


Permethrin
PERM

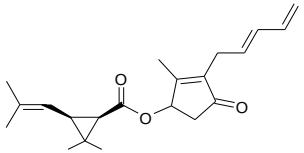


Phenothrin
PHENO

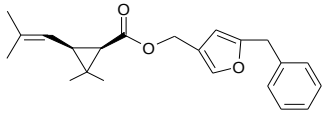
Figure A1-3 Structures of the pyrethroid insecticides



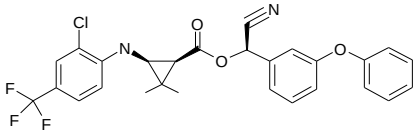
Phenthoate
PHEN



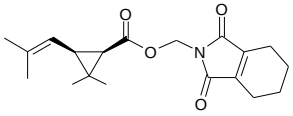
Pyrethrins I
PYR-1



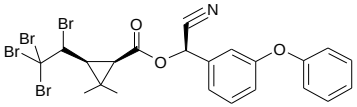
Resmethrin
RESM



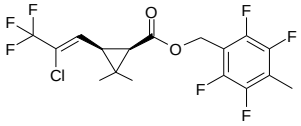
Tau-Fluvalinate
τ-FLUV



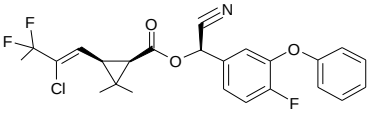
Tetramethrin
TET



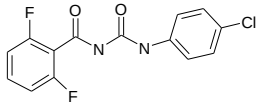
Tralomethrin
TRALO



Tefluthrin
TEF



Cyhalothrin
CYHAL



Diflubenzuron
DIFL

Figure A1-3 *continued*

Appendix 1B - Physicochemical properties

Table A1-1 Solubility definitions from (European Pharmacopoeia, 2007)

Solubility definition	**Parts of solvent required for one part of solute**	**Solubility range (mg mL^{-1})**
Very soluble (VS)	<1	>1000
Freely soluble (FS)	From 1 to 10	100–1000
Soluble	From 10 to 30	33–100
Sparingly soluble (SPS)	From 30 to 100	10–33
Slightly Soluble (SS)	From 100 to 1000	1–10
Very slightly soluble (VSS)	From 1, 000 to 10, 000	0.1–1

Table A1-2 Physicochemical properties data for pyrethroid insecticides

Pyrethroid	Abbreviation	S_w (mg L^{-1})	$logK_{ow}$	$logK_{oc}$
Acrinathrin	ACR	<0.02 [e]	6.73 [c d], 5.6 [e]	
allethrin	ALL	4.6 (25˚C) [b]	4.8 [b]	3.1 [g]
bifenthrin	BIFE	0.1 (25˚C) [b, e]	8.15 [d], 6.0 [e]	5.4 [g]
Cyfluthrin	CYFL	0.002 (20˚C) [b, e]	5.94[b], 5.74 [c], 5.9 [e]	5.1 [g]
λ-cyhalothrin	λ-CYH	0.003 (20˚C) [b, e]	9.9 [b], 6.85 [c, d], 6.9 [e]	5.5 [g]
Cypermethrin	CYPE	0.004 (20˚C) [b, e]	6.6 [b], 6.38[c, d], 6.6 [e]	5.0 [g]
Cyphenothrin	CYPH	-	6.3 [a]	-
Deltamethrin	DELT	<0.002 [a, e]	6.1[a], 6.18[c,d], 6.1[e]	6.0 [g]
Esfenvalerate	ESF	0.0002 (25˚C) [b, e]	6.1 [b], 6.76 [d], 4.0 [e]	3.7 [g]
Fenpropathrin	FENP	0.014 (25˚C) [b, e]	6.0 (20˚C) [b, e] 5.62 [d]	4.6 [g]
Fenvalerate	FENV	0.024–0.06 (22ºC) [f]	6.76 [c, d]	
Flucythrinate	FLUC	0.5 (21˚C) [b]	4.7 [b],6.56 [c]	
Flumethrin	FLUM	9.7×10^{-5} [b]	7.65 [b]	
τ-fluvalinate	FLUV	0.002 [b, e]	4.26 [b], 6.81 [c], 4.3 [e]	5.9 [g]
Halfenprox	HAL	-	8.35 [c]	
Permethrin	PERM	0.006 (20˚C) [b]	6.5 [b e], 7.43 [c], 6.18 [d],	4.9 [g]
Phenothrin	PHEN	2.0 (30˚C) [b]	6.0[a], 7.54[b]	
Prallethrin	PRAL	-	4.5[a]	
Resmethrin	RES	0.037 (25˚C) [b]	5.43 [b],7.11 [d], 5.4 [e]	5.0 [g]
Tefluthrin	TEF	0.002 (20˚C) [b]	6.5 [b], 7.19 [c]	
Tetramethrin	TETR	1.83	4.6 [b]	3.2 [g]
Tralomethrin	TRALO	0.08 [b, e]	4.5 [a], 7.6 [b, e]	
Transfluthrin	TRAN	-	5.5 [a]	
Pyrethrin I	PYR I	0.2 [b]	5.9 [b]	
Pyrethrin II	PYR II	9.0 [b]	4.3 [b]	

(a) extracted and adapted from Feo et al. (2010b) Table 1.

(b) extracted and adapted from Agency for Toxic Substances and Disease Registry (ATSDR, 2003)

(c) extracted and adapted from Ochiai et al. (2008) Table 1

(d) extracted and adapted from Van Hoeck et al. (2007) Table 1

(e) extracted and adapted from Albaseer et al. (2010) Table 1

(f) extracted and adapted from Oudou and Hansen (2002)Table 1.

(g) extracted and adapted from Hladik and Kuivila (2009)Table 3.

Appendix 1C - Data on occurrence levels in the environment

Table A1-3 Overview of publications relating to the occurrence of different EOC groups in groundwaters through Europe

EOC	Location	References
Pesticide	UK	(Lapworth et al., 2006; Lapworth et al., 2015)
	FRANCE	(Morvan et al., 2006; Baran et al., 2007; Lapworth et al., 2015)
	NORWAY	(Haarstad and Ludvigsen, 2007)
	NETHERLANDS	(ter Laak et al., 2012)
	PORTUGAL	(Goncalves et al., 2007; Andrade and Stigter, 2009)
	SPAIN	(Hildebrandt et al., 2008)
	GREECE	(Papastergioua and Papadopoulou-Mourkidou, 2001; Papadopoulou-Mourkidou et al., 2004)
	ITALY	(Guzzella et al., 2006)
Pharmaceuticals	UK	(Stuart et al., 2012; Stuart et al., 2014; Lapworth et al., 2015)
	FRANCE	(Rabiet M et al., 2006; Vulliet and Cren-Olive, 2011)
	GERMANY	(Heberer et al., 1998; Sacher, 2001; Heberer, 2002; Osenbruck et al., 2007; Strauch et al., 2007; Ternes et al., 2007; Reinstorf et al., 2008; Wolf et al., 2012)
Hormones and industrial compounds	UK	(Lapworth et al., 2006; Stuart et al., 2012; Lapworth et al., 2015)
	GERMANY	(Osenbruck et al., 2007; Strauch et al., 2007; Reinstorf et al., 2008)
Personal care products	UK	(Lapworth et al., 2006; Stuart et al., 2012)
	GERMANY	(Osenbruck et al., 2007; Ternes et al., 2007); (Strauch et al., 2007; Reinstorf et al., 2008)

(as partially adapted from Jurado et al. (2012))

Table A1-4 Summary of occurrences and related levels of anthelmintics in environmental samples

Anthelmintic**	Matrix	Levels detected (ng L^{-1})	MLOD/LOQ (ng L^{-1})	Ref
ABZ	Surface Water	<LOQ	0.22	(Zrncic et al., 2014)
FEB	(river)	1.09–1.49	1.07	
FBZ		0.32 (n = 1)	0.13	
FLU		1.32 (n = 1)	1.06	
LEV		3.8–39.43	0.72	
MBZ		n.d.	0.37	
MOXI		1.84 (n =1)	0.28	
OXI		1.16 (n = 1)	1.09	
PZQ		n.d.	0.70	
TCB		2.38 (n = 1)	0.07	
ABZ	River water	4–38	LOQs	(Sim et al., 2013)
FLU		2–1170	0.1 (ABZ)	
TBZ		<LOQ–22	0.1 (FLU)	
OXF		<LOQ–56	0.4 (TBZ)	
FBZ		<LOQ–63	0.1 (OXF)	
FBZ-SO_2		<LOQ–5	0.4 (FBZ)	
FBZ-NH_2		<LOQ–11	0.3 (FBZ-SO_2)	
FBZ-OH		<LOQ–11	0.1 (FBZ-NH_2)	
	Sea water	ABZ: 4–10	0.1(FBZ-OH)	
		FLU: 2–44		
		TBZ: 9		
		FBZ-NH_2: <LOQ–6		
		FBZ-OH: <LOQ–2		
	HTP Influent*	0–49400		
	HTP Effluent	<LOQ–12400		
	STP Influent	<MDL–4670		
	STP Effluent	<MDL–5630		
	HWTP Influent	<MDL–1390		
	HWTP effluent	<MDL–298		
	LWPT Influent	<MDL–241000		
	LWPT Effluent	4–1490		
FLU	River water	FLU: 5.3–39.3	1.6	(Wagil et al., 2015b)
FBZ	(Poland)	FEN: 7.1–87.7	1.7	

*HTP = Hospital Treatment Plant, STP = Sewage Treatment Plant, HWTP = Human waste treatment plans, LWTP = Livestock waste treatment plant

Table A1-4 *continued*

Anthelmintic	Matrix	Levels detected (ng L^{-1})	MLOD/LOQ (ng L^{-1})	Ref
FLU	WWTP Influent and effluent	19900–89700 55–671	<0.05	(Van De Steene and Lambert, 2008)
FLU	Influent Effluent River Water	0.5–221.2 <LOD–239.3 <LOD–20.2	<0.05/ 0.0 5	(Van De Steene et al., 2010)
FEB PZQ	Wastewater samples	50–60 3–400	0.1/ 1 0.1/ 1	(Babic et al., 2010)
IVER	Manure leachate water	800		(Raich-Montiu et al., 2008)
TBZ	River water WWTP effluent	3.9–27.3 32.9 ± 0.6	N/A	(Bartelt-Hunt et al., 2009)
TBZ	Stream water	15 ng/POCIS	N/A	(Alvarez et al., 2005)

** see Appendix A1, Figure A1-1 for description of abbreviations, MLOD = method limit of detection, MLOQ = method limit of quantification

Table A1-5 Summary of occurrences and related levels of anticoccidials in environmental samples.

Ionophore/ coccidiostat*	Matrix	Levels detected	MLOD/LOQ	Ref
DECO MAD MON SAL	River water (Spain)	13.6–40 ng L^{-1} 23 ng L^{-1} 14.6–16.7 ng L^{-1} 15.6–17.4 ng L^{-1}	6.2 / 12.5 ng L^{-1}	(Iglesias et al., 2012)
MON	Surface water / run-off	20–220 ng L^{-1}	15 ng L^{-1} (LOQ)	(Hao et al., 2006)
MON NAR SAL	River water	30–50 ng L^{-1} 40–60 ng L^{-1} 40 ng L^{-1}	30–50 ng L^{-1} (MDL)	(Cha et al., 2005)
AMP MON	Surface Run-off Livestock farm	10–288 ng L^{-1} 2–37 ng L^{-1}	8 ng L^{-1} (LOQ) 1 ng L^{-1} (LOQ)	(Song et al., 2007)
MON SAL NAR	Surface water	2–843 ng L^{-1} 2–13 ng L^{-1} 3–19 ng L^{-1}	1 ng L^{-1} (MDL) 2 ng L^{-1} 2 ng L^{-1}	(Thompson et al., 2009)
MON	Groundwater	40–390 ng L^{-1}	LOD 9 ng L^{-1}	(Watanabe et al., 2008)
MON	Groundwater Lagoon water	180–2350 ng L^{-1} 980–12900 ng L^{-1}	20 ng L^{-1} 100 ng L^{-1}	(Bartelt-Hunt et al., 2011)
MON SAL NAR	River water And Sediment	Water 36 ng L^{-1} 7 ng L^{-1} 38 ng L^{-1} Sediment 31.5 µg kg^{-1} 30.1 µg kg^{-1} 16.3 µg kg^{-1}	Water 1 ng L^{-1} LOQ Sediment 1 µg kg^{-1} LOQ	(Kim and Carlson, 2006)

* see Appendix A1, Figure A1-2 for description of abbreviations MDL and MLOD = method detection limits

Table A1-5 *continued*

Ionophore/ coccidiostat	Matrix	Levels detected	MLOD/LOQ	Ref
MON	Runoff from litter fertilised land	<MDL–2389 ng L^{-1}	22 ng L^{-1}	(Sun et al.,
SAL		<MDL– 9022 ng L^{-1}	38.8 ng L^{-1}	2013)
NAR		<MDL–358 ng L^{-1}	26.2 ng L^{-1}	
	Soil	5–101 µg kg^{-1} (SAL)	9 µg kg^{-1}	
MON	Poultry Litter	<MDL–4057 µg kg^{-1}	18.6 µg kg^{-1}	
SAL		<MDL-21878 µg kg^{-1}	25.1 µg kg^{-1}	
NAR		<MDL-3310 µg kg^{-1}	16.8 µg kg^{-1}	
TOL,	Pig manure	114.0 µg kg^{-1}	0.22 µg kg^{-1} (LOQ)	(Olsen et
TOL-SO		84.7 µg kg^{-1}	0.51 µg kg^{-1}	al., 2012)
TOL-SO_2		7.15 µg kg^{-1}	0.32 µg kg^{-1}	
	Agricultural soil	0.335 µg kg^{-1} (TOL-SO_2)	0.2 µg kg^{-1} (LOQ)	
MON	Agricultural soil	0.4 µg kg^{-1} (MON)	Soil LOQ	(Bak et al.,
SAL		2.2 µg kg^{-1} (NAR)	0.87 µg kg^{-1}	2013b)
NAR			0.64 µg kg^{-1}	
		0.4 µg kg^{-1} (SAL)		
	Sediment	0.7 µg kg^{-1} (NAR)	1.39 µg kg^{-1}	
			0.78 µg kg^{-1}	
		573.1 µg kg^{-1} (MON)		
	Poultry manure		1.01 µg kg^{-1}	
MON	River water	20 ng L^{-1} (MON)	15/49 ng L^{-1}	(Bak and
SAL				Björklund,
NAR	Soil	8(µg kg^{-1}MON)	1.34 µg kg^{-1}	2014)
		30 µg kg^{-1} (SAL)	1.39 µg kg^{-1}	(Bak et al.,
		18 µg kg^{-1} (NAR)	1.78 µg kg^{-1}	2013a)
	Sediment	13 µg kg^{-1} (Mon)	0.1 µg kg^{-1}	(Bak et al.,
		1-2 µg kg^{-1} (NAR)	0.2 µg kg^{-1}	2013b)
MON/ NAR	Sewage sludge	<LOQ–3.7 µg kg^{-1}	0.5/1 µg kg^{-1}	(Herrero et al., 2013)

* see Appendix A1, Figure A1-2 for description of abbreviations, MDL, LOD and MLOD = method detection limits

Table A1-6 Summary of occurrences and associated levels of pyrethroid insecticides in environmental samples

Insecticide*	matrix	Levels detected	MLOD/LOQ	Ref
RESM	Paddy field surface water	14–1450 ng L^{-1}	0.2/0.7 ng L^{-1}	(Aznar et al., 2016)
BIFE			0.4/1.2 ng L^{-1}	
FENP			0.4/1.3 ng L^{-1}	
CYHALO	Paddy field groundwater	6–688 ng L^{-1}	1.2/3.9 ng L^{-1}	
PERM			1.5/4.8 ng L^{-1}	
CYFLU			2.5/8.4 ng L^{-1}	
CYPER			5.4/18.3 ng L^{-1}	
FLUV			2.9/9.5 ng L^{-1}	
ESFEN			4.8/16 ng L^{-1}	
DELT			4.1/13.6 ng L^{-1}	
CYPER	River water	4.93–30.5 ng L^{-1}	<0.3/<1 ng L^{-1}	(Feo et al., 2010a)
CYPER	Surface water	0–1.89 ng L^{-1}	0.0005–0.015 ng L^{-1} (estimated)	(Xue et al., 2005)
FENV		0–3.22 ng L^{-1}		
DELT		0–6.28 ng L^{-1}		
CYPER	Pore Water	0–8.87 ng L^{-1}	0.0005–0.015 ng L^{-1}	
FENV		0–26.3 ng L^{-1}		
DELT		0–54.2 ng L^{-1}		
CYPER	Sediment	0–8.77 ng kg^{-1}	0.03–1.5 ng kg^{-1}	
FENV	Beijing reservoir	45.4–158 ng kg^{-1}		
DELT		78.6–301 ng kg^{-1}		
CYPER	Groundwater	22–90 ng L^{-1}		(Kumari et al., 2008)
DELT	India	17–61 ng L^{-1}		
CYPER	Soil	1–35 µg kg^{-1}		
FENV		1–22 µg kg^{-1}		
DELT		18–19 µg kg^{-1}		
BIFE	Sediment	3.6–6.5 µg kg^{-1}	0.33 µg kg^{-1}	(You et al., 2004)
CYH	Agricultural fields	2.6–16.8 µg kg^{-1}	0.22 µg kg^{-1}	
PERM		1.4– 459 µg kg^{-1}	0.55 µg kg^{-1}	
ESFEN		7.01 µg kg^{-1}	0.33 µg kg^{-1}	

* see Appendix A1, Figure A1-3 for description of abbreviations

Table A1-6 *continued*

Insecticide	matrix	Levels detected	MLOD/LOQ	Ref
ALL	Sediment	<MDL–20.4 µg kg^{-1}	0.74 9 µg kg^{-1}	(Woudneh and
BIFE	California	1.48–9.24 µg kg^{-1}	0.6 µg kg^{-1}	Oros, 2006)
CYFL		<MDL–17.6 µg kg^{-1}	0.34 µg kg^{-1}	
DELT/TRALO,		<MDL–2.73 µg kg^{-1}	0.27 µg kg^{-1}	
CYPER		<MDL–4.57 µg kg^{-1}	0.68 µg kg^{-1}	
FENV		<MDL–1.51 µg kg^{-1}	0.31 µg kg^{-1}	
FLUC		<0.35 µg kg^{-1}	0.34 µg kg^{-1}	
λ-CYH		<MDL–1.71 µg kg^{-1}	0.37 µg kg^{-1}	
PERM,		0.4–9.12 µg kg^{-1}	0.16 µg kg^{-1}	
PHEN		<MDL–0.395 µg kg^{-1}	0.17 µg kg^{-1}	
PRAL		<MDL–4.81 µg kg^{-1}	1.36 µg kg^{-1}	
RES		<MDL–0.592 µg kg^{-1}	0.45 µg kg^{-1}	
TETRA		<MDL–0.922 µg kg^{-1}	0.71 µg kg^{-1}	
Cis PERM	Sediment from river	0.58–309.5 µg kg^{-1}	0.001 µg kg^{-1}	(Yasin et al.,
Trans PERM	catchment	0.26–108.7 µg kg^{-1}	0.02 µg kg^{-1}	1996)
CYFL		<LOD–4.6 µg kg^{-1}	0.14 µg kg^{-1}	
CYPER	Soil	218 µg kg^{-1}	5 µg kg^{-1}	(Rissato et al.,
FENV		157 µg kg^{-1}	5 µg kg^{-1}	2005)
PERM	Sediments of	<MDL–459 µg kg^{-1}	1 µg kg^{-1}	(Weston et al.,
ESFEN	agriculture	<MDL –30 µg kg^{-1}		2004)
BIFE	influenced water	<MDL –21.0 µg kg^{-1}		
CYH	bodies	<MDL –16.8 µg kg^{-1}		

* see Appendix A1, Figure A1-3 for description of abbreviations, MLOD = method limit of detection, MLOQ = method limit of quantification

Appendix 1D - Summary tables of extraction and purification techniques for antiparasitic agents

Table A1-7 Summary of sample preparation for determination of anthelmintics in water samples

Anthelmintic*	Matrix	Pre-treatment	Extraction / Sorbent Type	Conditioning/ Load	Wash solvent	Elution Solvent	Detection System	Recovery (%)	Sensitivity (ngL^{-1})	Ref.
ABZ, FEN, FLU, FEB, MOX, LEV, MBZ, OXI, TCB, PZQ	Spiked surface river water (100 mL)	pH 7	SPE Oasis HLB (60 mg, 3 mL)	MeOH (5 mL) H_2O (5 mL) Load 100 mL	H_2O (5 mL)	MeOH (3 mL)	UHPLC-MS/MS	76.5–102.8 LEV: 42.8 MOX: 56.6	0.02–0.33	(Zrncic et al., 2014)
FLU	Surface water (SW) Influent (Inf), Effluent (Eff)	pH 7	SPE Speedisk phenyl (100 mg, 3 mL) Clean up with NH_2 SPE	MeOH (3 mL) H_2O (3 mL) Load 100 mL (SW), 1 mL (Inf), 10 mL (Eff) Eluate diluted with 4mL chloroform	H_2O: MeOH 60:40 *v/v*	MeOH (2×0.5 mL)	LC-MS/MS	90– 104.8	<0.05	(Van De Steene and Lambert, 2008)
FLU, FEN	Spiked water (500 mL)	pH 6	SPE Strata XC (200 mg, 3 mL)	MeOH (2 mL) H_2O (2 mL) Load 500 mL	1M HCl (5 mL) MeOH (4 mL)	MeOH: NH_4OH 95:5 *v/v* (4 mL)	LC-MS/MS	96.2/ 95.4	1.6 1.7	(Wagil et al., 2015b)
FEB, PZQ	Wastewater	pH 4 with HCl (0.1M)	SPE Strata-X (500 mg, 3mL)	MeOH (5mL) Water pH 4(5 mL) Load sample (100 mL)	Dried under vacuum	MeOH (2×5 mL)	LC-MS/MS	FEB:72.4–114 PZQ: 96–99.5	0.1	(Babic et al., 2010)
FLU	Seepage water (1000 mL)	NaCl (10g)	SPE Chromabond Easy (200 mg, 6 mL)	MeOH (5 mL) Load	-	MeOH with 1% acetic acid (8 mL)	LC-MS/MS	105	20 LOQ	(Weiss et al., 2008)

Table A1-7 *continued*

Anthelmintic*	Matrix	Pre-treatment	Extraction / Sorbent Type	Conditioning/ Load	Wash solvent	Elution Solvent	Detection System	Recovery (%)	Sensitivity (ngL^{-1})	Ref.
ABZ, FLU, TBZ, OXF, FBZ, FBZ-SO_2, FBZ-NH_2, FBZ-OH	River water, Sea water, Wastewater, Influent and effluent	-	Auto SPE HLB Disk	MTBE MeOH Water (pH)	Water	MeOH/MTBE (10%, v,v)(×2) MeOH (×3)	LC- MS/MS	50-120	0.1–0.4 (LOQ)	(Sim et al., 2013)
ABA, DOR, EMA,EPR, IVER, MOX, SEL	Surface water (500 mL)	pH 7	SPE Oasis HLB (200 mg, 6 mL)	n-heptane (6 mL) Acetone (2 mL) MeOH (6 mL) Water pH 7 (6 mL) Load 500 mL	MeOH (4 mL)	MeOH (2 × 2 mL) Acetone (2 × 2 mL)	LC-MS/MS	38–67	LOQ 2.5–14	(Krogh et al., 2008a)
[4] LEV, FEN, FEN-SO, FEN-SO_2, EPRINO		pH 3 (conc. HCl)	SPE Oasis HLB (500 mg, 6 mL)	MeOH (1:1, *v/v*) MeOH: H_2O water pH 3 Load 1L	Dried with flow of N_2 (10min)	MeOH: MeCN 1:1, *v/v*	LC-MS/MS	64.2–125.1 for all except EPRINO (35.4)	100	(Islam et al., 2013)
IVER	Surface water (11 mL)	pH 3 with formic acid	Hollow fibre liquid membrane	11 mL Sample pH adjusted to 3	-	n/a	LC- MS/MS	34 (tap) 28(lake)	250(Tap) 200 (Lake)	(Raich-Montiu et al., 2008)
ABZ, FLU, FEN, TBZ, BEN, CARB, fuberidazole	River, tab and well water		MISPE (DVB) 400 mg online 1000 mg offline	MeCN (50 mL) Milli-Q H_2O (50 mL) Load 500 mL	DMSO:MeCN 2: 98 *v/v* (5×1 mL)	Acetic Acid: MeOH, 50:50 *v/v* (12×1 mL)	HPLC-DAD	Online Tap :99–106 River: 95–104 Well: 89–105 Offline Tap: 99–106 River: 95–104 Well: 90–105	30–90 2–12	(Cacho et al., 2009)

Table A1-7 *continued*

Anthelmintic*	Matrix	Pre-treatment	Extraction / Sorbent Type	Conditioning/ Load	Wash solvent	Elution Solvent	Detection System	Recovery (%)	Sensitivity (ng L^{-1})	Ref.
IVER	Surface water (11 mL)	pH 3 with formic acid	Hollow fibre liquid membrane	11 mL Sample pH adjusted to 3	-	Fibre LD in mobile phase A	LC- MS/MS	34 (tap) 28 (lake)	250(Tap) 200 (Lake)	(Raich-Montiu et al., 2008)
IVER, EPRINO	Surface water	pH 7 ±0.5 with IN NaOH	Large vol. injection, Online C18	Sample: MeOH, 50:50, *v/v*)	n/a	n/a	LC-MS/MS	95.7–113.8	7/5	(Thompson et al., 2009)
TBZ, OXF, MBZ, ABZ, FEN	River, pond water	UA-CPE- sodium acetate, 7% (w/v), Triton X-114, 0.75% (w/v) 2 min ultra-sonication					HPLC- PDAD	81.6–111.4	5–100	(Santaladchaiyakit and Srijaranai, 2012)

*see Appendix 1A, Figure A1-1 for description of analyte abbreviations

Table A1-8 Summary of sample preparation techniques for the determination of anticoccidials in water samples

Ionophore/ Coccidiostat*	Matrix	Sample Preparation	Conditions	Detection System	Recovery (%)	Sensitivity/ LOD (ng L^{-1})	Ref.
DNC(NICARB), DICLAZ, ROB, MON, SAL, NAR, LAS, MAD	River water	SPE C_{18} (60 mg, 3 mL)	Samples filtered &acidified (0.1% HCOOH) Load: sample (200 mL) at 2 mL min^{-1} Wash: H_2O (5 mL) and air dried Elute: 5 mL MeOH acidified with 0.1% formic	LC MS/MS	85–90 Except ROB (60)	11–71	(Martinez-Villalba et al., 2009)
LAS, MON, NAR, SAL	River Water	SPE HLB (200 mg, 6 mL)	Sample (1L) adjusted to pH7 ±0.5 (HCl) Load: 1L @ 5–10 mL min^{-1} Elute: MeOH (6 mL)	LC- MS/MS	92–110	4–20	(Bak et al., 2013a)
AMP, MON	Surface water/runoff	SPE HLB (200 mg,6 mL)	Sample (30 mL) + MeOH (3 mL) Load: sample: MeOH mix Wash: Milli-Q H_2O (4 mL) Elute: MeOH with 2% formic acid (5 mL)	LC- MS/MS	89.4–113	8/1 (LOQ)	(Song et al., 2007)
MON	Surface water/runoff	SPE HLB (200 mg, 6 mL)	SPE conditioned: MeOH (6 mL), H_2O (6 mL) Load: sample: MeOH mix Wash: Milli-Q H_2O (4 mL) Elute: MeOH with 2% formic acid (5 mL)	LC- MS/MS	85.6–102.8	0.026 (water) 0.040 (river water)	(Zhang and Zhou, 2007)
MON, SAL, NAR	Surface RIVER water	SPE HLB (60 mg,3 mL)	Samples pH 7.5 with 0.01M NaOH SPE conditioned: MeOH (3 mL), 0.5M HCl (3 mL) and water (3 mL) Load: sample 120 mL Wash: H_2O (3 mL) Elute: MeOH (5 mL)	LC- MS/MS	96 ± 8.3	30–50	(Cha et al., 2005)

Table A1-8 *continued*

Ionophore/ Coccidiostat*	Matrix	Sample Preparation	Conditions	Detection System	Recovery (%)	Sensitivity/ LOD (ng L^{-1})	Ref.
LAS, MON, NAR, SAL	Surface water	Online enrichment (C18)	Sample (50 mL) adjusted to pH 7 (1M NaOH) Mixed with MeOH (2 ×25 mL rinses of sample container) resulting in a sample: MeOH (1;1 *v/v)*	Large volume injection - LC-MS/MS	96.7–114.2	1–2	(Thompson et al., 2009)
MON		SPE HLB (60 mg, 3 mL)	Load: sample 125 mL Wash: H_2O (3 mL) Elute: MeOH (5 mL)	LC- MS/MS	104±6.6	9	(Watanabe et al., 2008)
MON, SAL, NAR	river water	SPE HLB (60 mg,3 mL)	Load: sample 125 mL at 2 mL min^{-1} Wash: H_2O (3×3 mL) Elute: MeOH (2×2.5 mL)	LC- MS/MS	82.7–123.6	1–3 LOQ 1 (S/N)	(Kim and Carlson, 2006)
DEC, MAD, MON, NAR, SAL	Surface water	SPE Strata-X (60 mg,3 mL)	Sample (500 mL) acidified to pH 3 (HCl) Load: acidified sample (500 mL) Elute: vessel rinsed with MeOH (4 mL) applied to SPE and eluted with MeOH (4 mL)	LC- MS/MS	94–101 Mean	6.2 LOD MDL (7.5–17.3)	(Iglesias et al., 2012)
TOL, TOL-SO, TOL-SO_2	Surface water Tap water	SPE C_{18}(500 mg)	Sample(2L) pH adjusted to pH 3 (formic acid) and filtered (1.2 µm glass fibre filter). Load: 2 L at 1–2 mL min^{-1} and dried under vacuum Elute: acetonitrile (5 mL)	LC- MS/MS	96–123 (tap water) 100–116 (lake water)	0.06–0.13	(Olsen et al., 2012)

Table A1-8 *continued*

Ionophore/ Coccidiostat*	Matrix	Sample Preparation	Conditions	Detection System	Recovery (%)	Sensitivity/ LOD (ng L^{-1})	Ref.
MON, NAR, SAL, LAS, MAD	River water Sewage Influent (In) Effluent (Ef)	SPE HLB (150 mg)	Sample (250 mL, 500 mL and 1000 mL for influent, effluent and river water) SPE load: @ 10 mL min^{-1} Wash: ultra -pure H_2O (5 mL) and vacuum dried Elute: MeOH (10 mL)	LC- MS/MS	River: 85–97 In: 86–97 Ef: 87–100	River: 0.5–1 In: 2–10 Eff: 1–5	(Herrero et al., 2012)
MON, NAR, SAL	Run off	SPE HLB (500 mg, 6 mL) Followed by LLE with EtOAc	Sample (200 mL) filtered and pH adjusted (7–7.5) Load: sample (200 mL) at 2 mL min^{-1} Wash: H_2O (5 mL) and vacuum dry Elute: MeOH (2 × 3 mL) Extracts were dried to dryness, reconstituted in 0.5 mL buffer solution (10mM $Na_2 HPO_4$) and subsequent LLE with EtOAc (5 mL) (120rpm, 30mins)	LC- MS/MS	81–95	15.5–38.8	(Sun et al., 2013)

*see Appendix 1A, Figure A1-2 for description of analyte abbreviations

Table A1-9 Summary of sample preparation techniques for the determination of pyrethroid insecticides in water samples

Pyrethroid Insecticide*	Matrix	Sample Preparation	Conditions	Detection System	Recovery (%)	Sensitivity/ MLOD (ng L^{-1})	Ref.
ESF, BIFE, CYFL, FENV, λ-CYH, PERM	River Water	LLE	Extract with DCM (120 mL × 2) Solvent exchange with petroleum ether (3× 50 mL)	GC-ECD	76.5–103	1–3	(Mekebri et al., 2008)
BIFE, PERM, FENV, CYPER, DELT,	River water	mLLE	500 mL Sample extracted with 0.5 mL n-hexane. Organic layer injected directly into GC-MS	GC-MS	94–125.2	3–35	(Fernández-Gutiérrez et al., 1998)
BIFE, CYFL, λ-CYH, CYPER, ESF/FENV, FENP, FLUV, PERM, PHEN, RESM, TETR, TRALO/DELT	Tap and river water	UAEE	20 mL sample +1 mL chloroform Ultrasonic bath (35°C, 5mins) Centrifuge (3500 rpm, 5 min)	GC-NCI-MS	Tap water: 20–106 River water 30–100	0.03–35.8	(Feo et al., 2010a)
FENP, PERM, CYPER, DELT	Surface water	Auto SPE C18 (100 mg)	19 mL sample + 8 mL MeOH Load: 27 mL MeOH/sample solution Wash: MeOH:H_2O (30:70,*v/v*,1 mL) Elute: Toluene (1.5 mL)	Large volume injection-GC-ECD	33–104	1–8	(Van Der Hoff et al., 1996)
CYPER, FENV, DELT	water	SPE Oasis HLB (500 mg,6 mL)	1 L sample Condition: MeOH: MeCN 50:50, v/v (5 mL), MeOH (5 mL), ultra-pure H_2O (5 mL) Load: 4 mL min^{-1} Wash: ultra-pure H_2O (2 × 4 mL) Elute: MeOH: MeCN 50:50, v//v (3× 4 mL)	GC-μECD	70–89	0.0005–0.015	(Xue et al., 2005)

Table A1-9 *continued*

Pyrethroid Insecticide*	Matrix	Sample Preparation	Conditions	Detection System	Recovery (%)	Sensitivity/ MLOD (ng L^{-1})	Ref.
FENP, λ-CYH, DELT, FENV, PERM, FLUV, BIFE	Groundwater (GW) Sea water (SW)	SPE C_{18} (360 mg, 3 mL)	800 mL sample + 200 mL MeOH Load 1L sample: MeOH (80:20, *v/v*) at 10 mL min^{-1} Wash: 200 mL milli-Q water (seawater only) Dry: 30mins. under vacuum Elute: hexane (7 mL minimum)	LC- MS/MS	Groundwater 71.8–110 Sea water 80–115.6	Groundwater 0.2–0.5 Sea water 0.3–0.7	(Gil-Garcia et al., 2006)
TEF, TRANS, ALL, TETR, λ-CYH, CYPH, PERM, CYFL, CYPE, DELT	Tap water Groundwater River water	SPME (PDMS)	Sample (8 mL) + acetone (2% *v/v*) + sodium thiosulfate (0.02 % w/v) PDMS (100µm) 20mins @ 50ºC	TD- GC-µECD	Tap: 86.5–110 Groundwater 81.1–116 River: 76.7–136	0.05–2.18	(Casas et al., 2006)
FENP, λ-CYH, DELT, FENV, PERM, FLUV, BIFE	Groundwater	SPME (PDMS/DVB fibre)	Sample: MeCN (75:25, *v/v*) (3 mL) buffered to pH 3 with phosphate buffer Extracted:PDMS/DVB (60µm) dSPME, 1100 rpm, 65 ±2 ºC for 30 mins.	LC-PIF-FD	92–109	3–9	(Vazquez et al., 2008)
ALL, BIFE, FENP, λ-CYH, PERM, CYFL, CYPE, FLUV, FENV, DELT	Groundwater	Microwave assisted headspace SPME	Sample (20 mL) + 2 mL buffer (pH 4) PDMS (100µm) fibre with microwave irradiation (157W), 30ºC, 10 mins., 300 rpm	TD-GC-ECD	88.5–115.5	0.2–2.6	(Li et al., 2009)
BIFE, λ-CYH, CYPER, DELT, ESF, FENV, PERM FENP, ACRIN, RES	Water	SBSE (PDMS)	Stir bar coated with 25µl PDMS Sample (10 mL), MeOH (2 mL) extracted @900rpm, 60 mins., 25º C	TD-GC-MS	40–92	0.02–1.4	(Van Hoeck et al., 2007)

Table A1-9 *continued*

Pyrethroid Insecticide*	Matrix	Sample Preparation	Conditions	Detection System	Recovery (%)	Sensitivity/ MLOD (ng L^{-1})	Ref.
FENP, ACR, PERM (both cis & trans), CYPE, FENV, ESF, DELT	Groundwater	SBSE-LD (PDMS)	Sample (30 mL) with 5% MeOH modifier. PDMS (47µl) coated stir bar Extracted at 750rpm, 60mins. Liquid desorption into MeCN	SBSE–LD–LVI-GC–MS	81.8–105	1–2.5	(Serodio and Nogueira, 2005)
CYPER, FLUC, FENV, FLUV, λ-CYH, TEF, PERM, HAL	Water	Sequential SBSE	Stir bar 25µl PDMS 5 mL sample extracted (60mins, 1500rpm) Second SBSE: 30% NaCl dissolved in sample from above and extracted (60 min, 1500rpm)	TD-GC-MS	96–113	3.9–14 except cypermethrin (40)	(Ochiai et al., 2008)
λ-CYH, DELT, FENV PERM, FENP, BIFE	Surface Water	Online PC-LC (column switching) C18	12 mL sample + 8 mL MeCN (60:40 *v/v*) Columns pre-conditioned with MeCN:H_2O (80:20,*v/v*)	PC-LC-PIFD	93.1–118.6	10–30	(Martínez Galera et al., 2005)

* see Appendix 1A, Figure A1-3 for description of analyte abbreviations

Appendix 1E - Summary of instrumental detection methods for antiparasitics in environmental water matrices.

Table A1-10 Summary of instrumental method for the determination of anthelmintics in water samples

Anthelmintic	Matrix	Sample Prep	Final extract Volume/solvent	Instrument	Stationary Phase	Mobile Phase Composition	Ionisation	Sensitivity (ngL^{-1})	Ref.
ABZ, FEN, FLU, FEB, MOX, LEV, MBZ, OXI, TCB, PZQ	river water (100 mL)	SPE	1000µl MeOH: water (80:20, *v/v*)	UHPLC - MS/MS (QTRAP)	Waters UPLC BEH, C18, 2.1 × 100 mm, 1.7 µm	A: 10mM ammonium buffer with 0.1% formic B: MeCN with 0.1% formic	ESI +	0.02–0.33	(Zrncic et al., 2014)
FLU	Surface water, influent and effluent	SPE	1000µl water: MeCN (80:20, *v/v*)	HPLC-MS/MS	PFP column (Varian) (100×4.6 mm, 5µm) Metaguard pre-column (both Varian)	A: water: MeCN (95:5, *v/v*) + 2mM amm. acetate + acetic acid B: water: MeCN (5:95, *v/v*) + 2mM amm. acetate + acetic acid	ESI +	<0.05	(Van De Steene and Lambert, 2008)
FLU, FEN	Spiked water (500 mL)	SPE	1000µl mobile phase	LC-MS/MS	Gemini C18, (4.6 mm×150 mm, 5µm)	A: MeCN B: 1mM ammonium acetate: MeCN (90:10, *v/v*)	ESI +	1.6 1.7	(Wagil et al., 2015b)
FEB, PZQ	Wastewater	SPE	1000µl water: MeCN (50:50, *V/V*)	LC-MS/MS	Synergy Fusion C18 (50 mm×2.0 mm, 4µm)	A: 0.1% aq. formic B: 0.1% formic in MeCN	ESI +	0.1	(Babic et al., 2010)
ABZ, FLU, TBZ, OXF, FBZ, FBZ-SO2, FBZ-NH2, FBZ-OH	River water Sea water Wastewater Influent and effluent	Auto SPE	10% MeOH/MTBE and MeOH evaporated to 1000µl	LC-MS/MS	ZORBAX Eclipse XDB-C18 (4.6×150 mm, 3.5 µm)	A: 0.3% formic + 0.1% amm. formate in H_2O B: Acetonitrile/Methanol (50/50, *v/v*)	ESI +	0.1–0.4 (LOQ)	(Sim et al., 2013)

Table A1-10 *continued*

Anthelmintic	Matrix	Sample Prep	Final extract Volume/solvent	Instrument	Stationary Phase	Mobile Phase Composition	Ionisation	Sensitivity (ngL^{-1})	Ref.
ABA, DOR, EMA, EPR, IVER, MOX, SEL	Surface water (500 mL)	SPE	Eluted with MeOH and acetone, dried to 100µl and made to 500µl with water	LC-MS/MS	Zorbax Eclipse XDB-C8 (150×4.6 mm, 5µm) Agilent	A: MeCN B: 10mM ammonia pH 7.0 with formic acid	APCI +	LOQ 2.5–14	(Krogh et al., 2008a)
LEV, FEN, FEN-SO, FEN-SO_2, EPR	water	SPE	1000µl 20:80 (*v/v*) mobile phase A:B	LC-MS/MS	Atlantis T-3 (2.1×100 mm, 3µm) C_8 (4×2 mm) guard column	A: water: MeCN (90:10) +10mM amm. formate B: MeOH: MeCN (50:50, *v/v*) +10mM amm. formate	ESI +	100	(Islam et al., 2013)
FLU	Seepage water (1000 mL)	SPE)	1000µl MeOH: water (50:50, *v/v*)	LC- MS/MS	Phenomenex Polar 4×2 mm guard + Phenomenex Synergy Polar (150×2 mm, 80 Å)	A: water: MeOH (95:5*v/v*) with 0.05% formic B: water: MeOH (5:95,*v/v*) with 0.05% formic	ESI +	20 LOQ	(Weiss et al., 2008)
ABZ, FLU, FEN, TBZ, BEN, CARB, fuberidazole	River, tab and well water	MISPE	300µl MeCN	HPLC-Diode Array Detector	18-Kromasil column (250×4.6 mm, 5µm)	A: H_2O: acetic acid (4%) B: MeCN	n/a	Online 30–90 Offline 2–12	(Cacho et al., 2009)
IVER, EPRINO	Surface water	Large vol. injection, Online enrichment with C18	50 mL of diluted sample (50 mL sample diluted with 25 mL MeOH)	Large volume LC-MS/MS	Waters Xterra MS C_{18} (20×2.1 mm, 3.5 µm) C18 guard column (4×2 mm)	Quaternary gradient A: 0.1% *v/v* formic B: MeCN + 0.1% formic C: MeOH + 0.1% formic D: EtOAc	ESI +	7/5	(Thompson et al., 2009)

*see Appendix 1A Figure A1-1 for description of anthelmintic abbreviations.

Table A1-11 Summary of instrumental method for the determination of anticoccidials in water samples

Ionophore/ Coccidiostat*	Matrix	Sample Preparation	Final Extract Volume/solvent	Detection System	Stationary Phase	Mobile Phase	Ionisation Mode	Sensitivity (ng L^{-1})	Ref.
DNC (NICARB), DICLAZ, ROB, MON, SAL, NAR, LAS, MAD	River water	SPE C18	900 µl MeCN:H_2O (1:1, *v/v*)	LC- MS/MS	Thermo Hypersil Gold C18 (50×2.1 mm, 1.9 µm)	A: 0.1% formic in MeCN B: 0.1% formic aqueous	ESI + ESI –	11–71	(Martinez-Villalba et al., 2009)
LAS, MON, NAR, SAL	River Water	SPE HLB	1000 µL of a 30:70 *v/v* mobile phase A:B	LC- MS/MS	XTerra RP C18 column (2.1×100 mm, 3.5 µm) C_{18} column guard (2.1×20 mm, 3.5 µm) (Waters)	A: 95:5 Milli-Q water: MeCN + 10 mM formic + 50 µM NaCl. B: 5:95 Milli-Q water: MeCN +10 mM formic + 50 µM NaCl.	ESI +	4–20	(Bak et al., 2013a)
AMP, MON	Surface water/runoff	SPE HLB	Eluted with MeOH (2% formic)	LC- MS/MS	Thermo Hypersil Gold (50 mm×2.1 mm, 5 µm).	A: MeCN with 20mM heptafluorobutyric acid V: water with 20mM heptafluorobutyric acid	ESI +	8/1 (LOQ)	(Song et al., 2007)
MON	Surface water/runoff	SPE HLB	Eluted with MeOH (10 mL) and dried to 200 µl	LC- MS/MS	Waters Symmetry C_{18} (4.6×75 mm, 3.5 µm).	A: 0.1% formic aqueous B: MeCN C: MeOH	ESI +	0.026 (water) 0.040 (river water)	(Zhang and Zhou, 2007)
MON, SAL, NAR	Surface RIVER water	SPE HLB	Eluted MeOH, dried to 50µl and 70 µl mobile phase A added	LC- MS/MS	Xterra MS C_{18} (2.1×50 mm, 2.5 µm) Xterra MS C_{18} guard Column (2.1×4 mm)	A: 0.1% formic aqueous B: MeOH C: MeCN	ESI +	30–50	(Cha et al., 2005)

Table A1-11 *continued*

Ionophore/ Coccidiostat*	Matrix	Sample Preparation	Final Extract Volume/solvent	Detection System	Stationary Phase	Mobile Phase	Ionisation	Sensitivity (ng L^{-1})	Ref.
LAS, MON, NAR, SAL	Surface water	Online (C18)	50 mL sample diluted with 25mL MeOH	LVI- LC-MS/MS	Waters Xterra MS C_{18} (20×2.1 mm, 3.5µm) C18 guard cartridge (4 ×2 mm) (Phenomenex)	Quaternary gradient A: 0.1% *v/v* formic aq. B: MeCN + 0.1% formic C: MeOH + 0.1% formic D: EtOAc	ESI +	1–2	(Thompson et al., 2009)
MON	Groundwater	SPE HLB	Recon in 50 µL MeOH and 70 µL water with 0.1% formic	LC MS/MS	Gemini C18 column (50×2.0 mm, 5µm) (Phenomenex)	MeOH: water (0.1% formic) 80:20 isocratic	ESI +	9	(Watanabe et al., 2008)
MON, SAL, NAR	River water	SPE HLB	50 µl SPE extract with 70 µL of mobile phase A	LC MS/MS	XTerra MS RP C_{18} (Waters) (2.1×50 mm, 2.5 µm) C_{18} guard column (Phenomenex)	A: HPLC water + 0.1% formic acid B: MeOH (100%).	ESI +	1–3 LOQ	(Kim and Carlson, 2006)
DEC, MAD, MON, NAR, SAL	Surface water	SPE Strata-X	200 µl of 0.1% formic in MeOH	LC- MS/MS	Synergi Polar-RP 100 Å (50 × 2.0 mm, 2.5µm) with a Polar-RP security-guard cartridge (4.0×2.0 mm)	A: 0.1% formic in MeCN B: 0.1% formic aqueous	ESI +	7.5–17.3 MDL	(Iglesias et al., 2012)
TOL, TOL-SO, TOL-SO_2	Surface water	SPE C18	reconstitution in 200 µL mobile phase mixture (70:30, A:B)	LC- MS/MS	XTerra MS-C_{18}, (100 × 2.1 mm, 3.5 µm) + XTerraMS-C_{18} guard column (2.1×10 mm) (Waters)	A: H_2O: MeCN (95:5, *v/v*) with formic (0.1 %) B: MeCN: H_2O (95:5, *v/v*) with formic (0.1 %)	ESI (-)	0.06–0.13	(Olsen et al., 2012)

Table A1-11 *continued*

Ionophore/ Coccidiostat*	Matrix	Sample Preparation	Final Extract Volume/solvent	Detection System	Stationary Phase	Mobile Phase	Ionisation	Sensitivity (ng L^{-1})	Ref.
MON, NAR, SAL, LAS, MAD	River water Sewage Influent and effluent	SPE HLB	1000µl mL of water/MeOH/MeCN (2:1:1) with 0.1% HCOOH	LC- MS/MS	Ascentis Express RP-Amide (100×2.1 mm, 2.7 µm)	A: 0.1% formic aqueous B: MeOH: MeCN (50:50, *v/v*) 0.1% formic	ESI +	River: 0.5–1 INF 2–10 EFF 1–5	(Herrero et al., 2012)
MON, NAR, SAL	Run off	SPE Followed by EtOAc LLE	1000 µl methanol and 10 mM disodium phosphate (50/50, *v/v*).	LC- MS/MS	Ascentis RP-amide (2.1 mm×150 mm, 3 µm) (Supelco)	(A): H_2O: MeCN (75:25, *v/v*) with 0.1 formic (B): MeOH: MeCN (50/50, *v/v*)	ESI +	15.5–38.8	(Sun et al., 2013)

*see Appendix 1A Figure A1-2 for description of anticoccidial abbreviations

Table A1-12 Summary of instrumental method for the determination of insecticides in water samples

Pyrethroid Insecticide*	Matrix	Sample Preparation	Final Extract Volume/solvent	Instrument	Stationary Phase	Sensitivity (ng L^{-1})	Ref.
ESF, BIFE, CYFL, FENV, λ-CYH, PERM	River Water	LLE	Peroleum ether extracts concentrated to 1 mL and made to 2 mL with iso-octane	GC-μECD confirmation by GC-MS/MS	Two (DB5 and DB17MS) 60 m length, 0.25 mm I.D., 0.25 μm film thickness fused silica GC columns	1–3	(Mekebri et al., 2008)
BIFE, PERM, FENV, CYPER, DELT,	River water	mLLE	0.5 mL hexane with deuterated IS	GC-MS-SIM	HP- 5MS fused silica capillary (30 m×0.25 mm I.D., 0.25 μm film thickness) coated with cross-linked 5% phenyl-methyl silicone	3–35	(Fernández-Gutiérrez et al., 1998)
BIFE, CYFL, λ-CYH, CYPER, ESF/FENV, FENP, FLUV, PERM, PHEN, RESM, TETR, TRALO/DELT	Tap and river water	UAEE	100μL EtOAc	GC-NCI-MS	DB-5MS capillary column (15 × 0.25mm i.d., 0.1μm film thickness) containing 5% phenyl methyl siloxane	0.03–35.8	(Feo et al., 2010a)
FENP, PERM, CYPER, DELT	Surface water	Auto SPE C18(100 mg)	100μL toluene extract	LVI-GC-ECD Large vol. introduction	10 m×0.53 mm I.D. retention gap coupled to a 3m × 0.32 mm I.D., 0.17 μm film thickness HP-1 retaining pre-column with a 22 m×0.32 mm I.D., 0.17 μm film thickness HP-1 analytical GC column	1–8	(Van Der Hoff et al., 1996)
CYPER, FENV, DELT	water	SPE Oasis HLB (500 mg, 6 mL)	Reconstituted in 500μL acetone	GC-μECD	fused silica capillary column (HP-5, 30 m×0.25 mm i.d., 0.25 μm film thickness	0.0005–0.015	(Xue et al., 2005)
TEF, TRANS, ALL, TETR, λ-CYH, CYPH, PERM, CYFL, CYPE, DELT	Tap water Groundwater River water	SPME (PDMS)	N/A - Thermal desorption	Thermal desorption-GC-μECD	HP-5 (30m×0.32 mm,0.25μm film thickness)	0.05–2.18	(Casas et al., 2006)

Table A1-12 *continued*

Insecticide	Matrix	Sample Preparation	Final Extract Volume/solvent	Instrument	Stationary Phase	Sensitivity (ng L^{-1})	Ref.
FENP, λ-CYH, DELT, FENV, PERM, FLUV, BIFE	Groundwater (GW) Sea water	SPE C_{18} (360 mg, 3 mL)	500µL MeCN: Water (70: 30, *v/v*)	LC-ESI (+)-MS	Waters Symmetry C_{18} column (250×4.6 mm, 5 µm) Mob A: MeCN B: 50mM aqueous amm. acetate with 5% MeCN pH to 3.5 with formic	GW: 0.2–0.5 Sea: 0.3-0.7	(Gil-Garcia et al., 2006)
FENP, λ-CYH, DELT, FENV, PERM, FLUV, BIFE	Groundwater	SPME (PDMS/DVB)	Liquid desorbed into mobile phase	LC-PIF-FD	Symmetry C_{18} (Waters) (250×4.6 mm, 3.5 µm)	3–9	(Vazquez et al., 2008)
ALL, BIFE, FENP, λ-CYH, PERM, CYFL, CYPE, FLUV, FENV, DELT	Groundwater	Microwave assisted headspace SPME	N/A - Thermal desorption (TD)	TD-GC-ECD	fused silica DB-608 capillary column (30m×0.25 mm i.d., 0.25µm film)	0.2–2.6	(Li et al., 2009)
BIFE, λ-CYH, CYPE (4 isomers), DELT, ESF, FENV, PERM, FENP, ACR, RES	Water	SBSE (PDMS)	N/A - Thermal desorption	TD-GC-MS	HP-5MS fused silica capillary column (5% diphenyl, 95% dimethylsiloxane) 30 m × 0.25 mm I.D., 0.25 µm	0.02–1.4	(Van Hoeck et al., 2007)
FENP, ACR, PERM (both cis & trans), CYPE, FENV, ESF, DELT	Groundwater	SBSE-LD (PDMS)	80 µL EtOAc	LVI-GC–MS	TRB-5MS (30 m×0.25 mm ID, 0.25 µm) capillary column (5% diphenyl, 95% dimethylpolysiloxane)	1–2.5	(Serodio and Nogueira, 2005)
CYPER, FLUC, FENV, FLUV, λ-CYH, TEF, PERM, HAL	Water	Sequential SBSE	N/A - Thermal desorption	TD-GC-MS	HP-5MS fused silica capillary column (30m×0.25 mm i.d., 0.25µm film (Agilent)	3.9–14 CYPER (40)	(Ochiai et al., 2008)
λ-CYH, DELT, FENV, PERM, FENP, BIFE	Surface Water	Online PC-LC C18	50 mL sample: MeCN (60:40, *v/v*) Mobile phase MeCN/Water	PC-LC-PIFD	Enrichment: Hypersil Elite C_{18} (50×4.6 mm, 5µm) Separation: Symmetry C_{18} (250×4.6 mm, 3.5 µm)	10–30	(Martínez Galera et al., 2005)

*see Appendix 1A Figure A1-3 for pyrethroid insecticide abbreviation descriptions

Appendix 3A - Description of matrix matched calibration preparation for anticoccidials

Table A3-1 Preparation of matrix matched calibration, with corresponding sample concentrations

Spiking Vol. (µL)	Calibration Level	Concentration Ranges (ng L^{-1}) for Analyte Group[a]: A	B	C**	D	E	F
100	0.2 × L1	0.1	0.1	0.1	1	2	4
100	L1	0.5	0.5	0.5	5	10	20
100	L2	2.5	2.5	2.5	7.5	20	25
100	L3	10	10	10	10	40	50
100	L4	50	50	50	50	50	75
100	L5	100	75	75	100	75	100
100	L6	150	100	100	150	100	150
100	L7	200	125	125	200	125	200
100	L8	250	150	150	250	150	250
200	2 × L8	500	300	300	500	300	500

[a] Analytes within each concentration range group are as specified in Table 3-1
** group C analytes were spiked from a separate set of calibrants (1-8) which were prepared in MeCN +10% formic acid

Appendix 4A - Summary of anthelmintic detections as part of the spatial occurrence study

Table A4-1 Summary of anthelmintic compounds detected at each of the 23 sites recorded, with respective concentrations (ng L^{-1})

Site I.D.	Sample description		Anthelmintic detected (ng L^{-1})																	No. of analytes detected
	Matrix	Type	ABZ	ABZ-SO	ABZ-SO2	ABZ-NH_2-SO_2	FBZ	OXF	FBZ-SO_2	FLU-NH_2	MBZ-NH_2	TCB	TCB-SO	TCB-SO_2	TBZ	LEV	CLOS	OXY	RAF	
002	GW	Mon BH	n.d.	n.d.	n.d.	**2.5**	n.d.	n.d.	n.d.	**2.0**	**2.2**	n.d.	n.d.	n.d.	**1.1**	n.d.	n.d.	n.d.	n.d.	**4**
006	GW	Mon BH	n.d.	n.d.	n.d.	**1.5**	**1.2**	**1.0**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**3**
007	GW	Mon BH	n.d.	n.d.	n.d.	n.d.	**2.3**	**2.7**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**1.3**	n.d.	n.d.	n.d.	**3**
008	GW	Mon BH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**1.4**	n.d.	n.d.	n.d.	**1**
009	GW	Mon BH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**3.3**	n.d.	n.d.	**1**
010	GW	Mon BH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**1.9**	n.d.	n.d.	**1**
013	GW	Mon BH	n.d.	n.d.	n.d.	n.d.	**1.0**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**1.3**	n.d.	n.d.	n.d.	**2**
015	GW	Mon BH	n.d.	n.d.	n.d.	**1.7**	n.d.	n.d.	n.d.	**1.3**	**1.4**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**3**
032	GW	SP	n.d.	**5.2**	**4.5**	**4.4**	n.d.	**2.7**	**1.0**	n.d.	n.d.	n.d.	**>LOD**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**6**
071	GW	SP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**>LOD<LOQ**	n.d.	**1**
072	GW	SP*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**5.0**	n.d.	**1**
075	GW	SP	**1.2**	**3.2**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**2**
076	GW	Abstract BH	**4.9**	**8.5**	**7.3**	**5.0**	n.d.	n.d.	n.d.	n.d.	n.d.	**1.5**	n.d.	**>LOD**	n.d.	n.d.	n.d.	n.d.	n.d.	**6**
079	GW	Abstract BH	n.d.	n.d.	**22.2**	**5.2**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**2**
084	GW	SP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**2.3**	**>LOD<LOQ**	n.d.	**2**

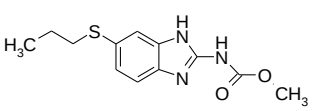
Table A4-1 *continued*

Site I.D.	Sample description		Anthelmintic detected (ng L^{-1})																No. of analytes detected
	Matrix	Type	ABZ	ABZ-SO	ABZ-SO2	ABZ-NH_2-SO_2	FBZ	OXF	FBZ-SO_2	FLU-NH_2	MBZ-NH_2	TCB	TCB-SO	TCB-SO_2	TBZ	LEV	CLOS	OXY	RAF	
094	GW	SP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**>LOD<LOQ**	n.d.	**1**
020	SW	river/stream	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**1.2**	n.d.	n.d.	n.d.	**1**
028	SW	SH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**>LOD<LOQ**	**2.2**	**2**
074	SW	SH	**<LOQ**	**1.1**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**2**
085	SW	river/stream	n.d.	**1.0**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**1**
089	SW	Drain	**40.6**	**21.5**	**5.6**	**6.8**	n.d.	n.d.	n.d.	n.d.	n.d.	**2.3**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**5**
099	SW	river/stream	n.d.	**1.6**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**4.7**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**2**
104	SW	river/stream	n.d.	n.d.	n.d.	**1.5**	n.d.	n.d.	n.d.	n.d.	n.d.	**2.4**	n.d.	n.d.	n.d.	n.d.	n.d.	**9.4**	n.d.	**3**
Frequency of detection (%)			**3.8**	**6.6**	**3.8**	**7.5**	**2.8**	**2.8**	**1.0**	**1.9**	**1.9**	**3.8**	**1.0**	**1.0**	**1.0**	**3.8**	**2.8**	**5.7**	**1.0**	

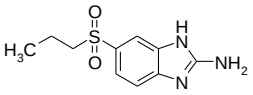
GW = Groundwater, SW = Surface water, Mon BH = Monitoring Borehole, Abstract BH = Abstraction Borehole SP = Spring, n.d. = not detected, >LOD implies compound detected at concentrations above the limit of detection (screening compounds only), >LOD<LOQ implies compounds was detected at levels that were not quantifiable, however were still above the limits of detection

SUPPLEMENTARY INFORMATION

Supplementary Information File SI-2.1



Albendazole
ABZ



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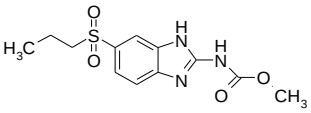

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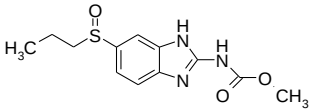

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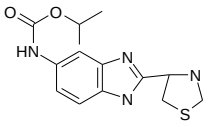

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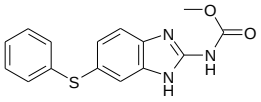

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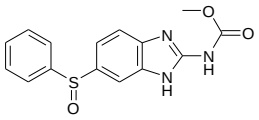
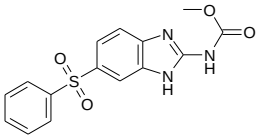
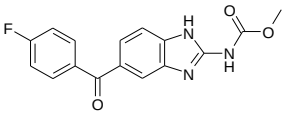


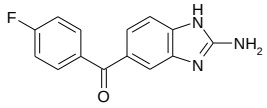
Oxfendazole
OXF



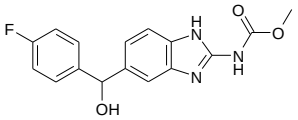
Fenbendazole sulphone
FBZ-SO₂



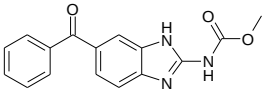
Flubendazole
FLU



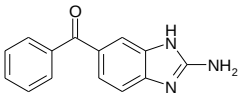
Amino-Flubendazole
FLU-NH₂



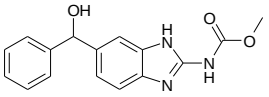
Hydroxy-Flubendazole
FLU-OH



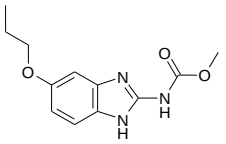
Mebendazole
MBZ



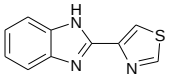
Amino-Mebendazole
MBZ-NH₂



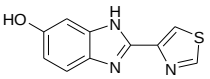
Hydroxy-Mebendazole
MBZ-OH



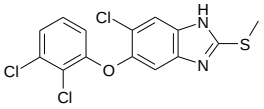
Oxibendazole
OXI



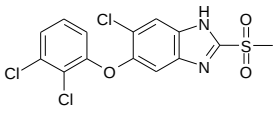
Thiabendazole
TBZ



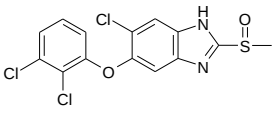
5-Hydroxy Thiabendazole
TBZ-OH



Triclabendazole
TCB

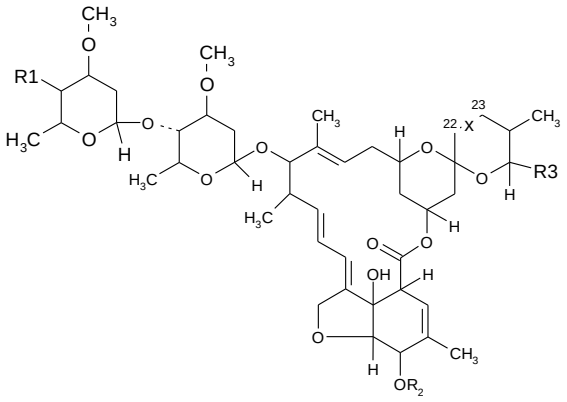


Triclabendazole sulphone
TCB-SO₂

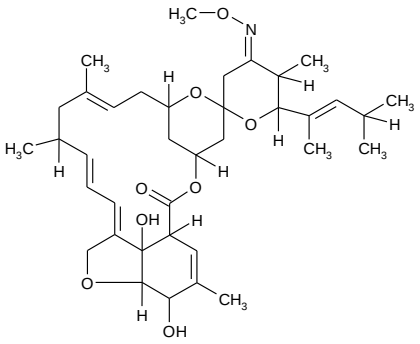


Triclabendazole sulphoxide
TCB-SO

Figure S2-1 (a) structures of benzimidazole anthelmintics

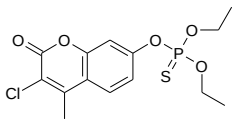


Avermectin	R1	R2	R3	C_{22}-x-CC_{23}
Abamectin B1a (ABA)	OH	H	$CHCH_3CH_2CH_3$	-CH=CH-
Doramectin (*DORA*)	OH	H	C_6H_{11}	-CH=CH-
Emamectin Benzoate B1a (*EMA*)	$C_6H_5COOHCH_3NH$	H	$CHCH_3CH_2CH_3$	-CH=CH-
Eprinomectin B1a (*EPRINO*)	$NHCOCH_3$	H	$CHCH_3CH_2CH_3$	-CH=CH-
Ivermectin B1a (*IVER*)	OH	H	$CHCH_3CH_2CH_3$	$-CH_2$-CH-

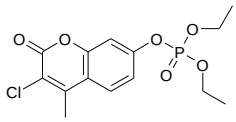


Moxidectin
MOXI

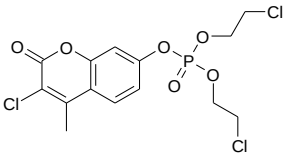
Figure S2-1 (b) structures of macrocyclic lactone anthelmintics from Tuck et al. (2016)



Coumaphos
COUMA

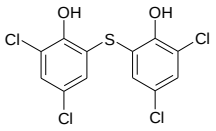


Coumaphos-oxon
COUMA-O

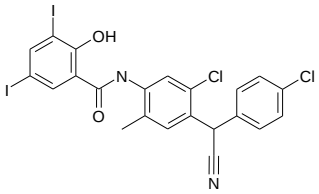


Haloxon
HALOX

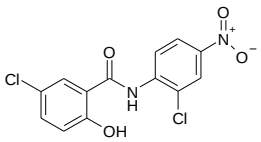
Figure S2-1 (c) structures of organophosphate anthelmintics



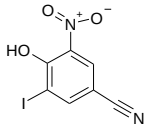
Bithionol
BITH



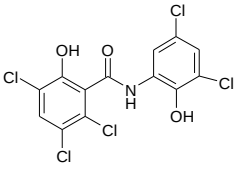
Closantel
CLOS



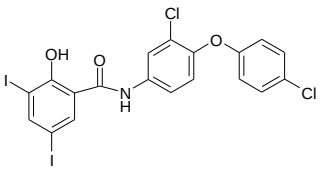
Niclosamide
NICLOS



Nitroxynil
NITROX



Oxyclozanide
OXY



Rafoxanide
RAFOX

Figure S2-1 (d) structures of salicylanilide and substituted phenol anthelmintics

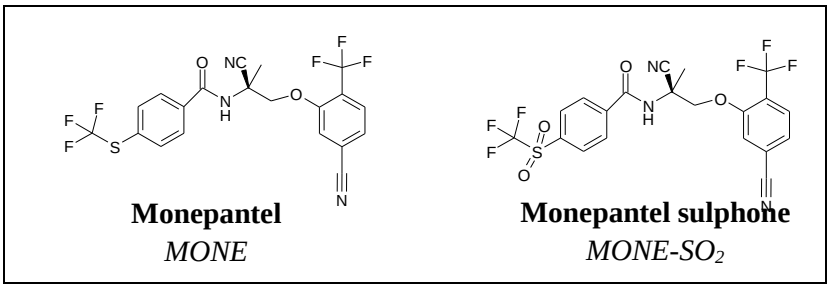


Figure S2-1 (e) structures of amino-acetonitrile derivative anthelmintics

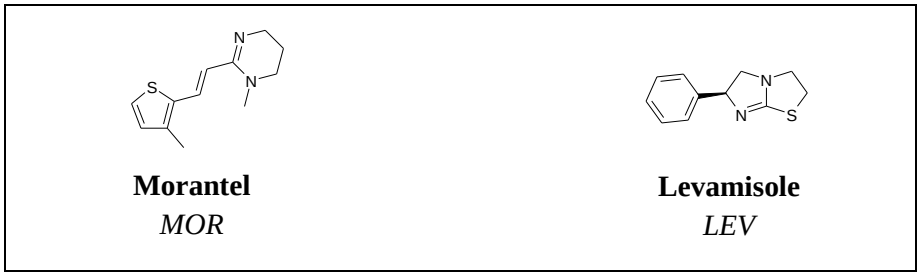


Figure S2-1 (f) structures of tetrahydropyrimidines (MOR) and imidazothiazole (LEV) anthelmintics

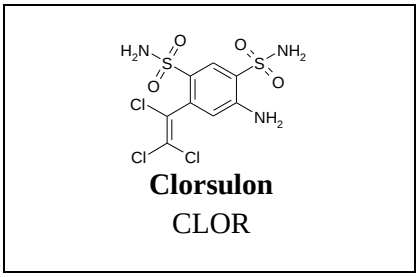


Figure S2-1 (g) structure of one miscellaneous anthelmintic (CLOR)

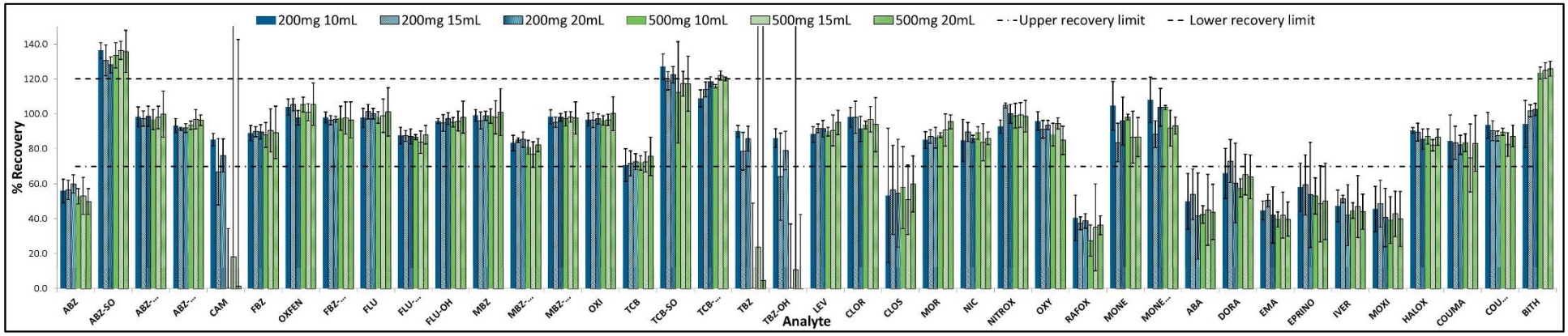


Figure S2-2 Mean recovery and precision (%RSD, presented as error bars) for assessment of sorbent mass (200 mg vs. 500 mg) each using three elution volume (10, 15 and 20 mL)

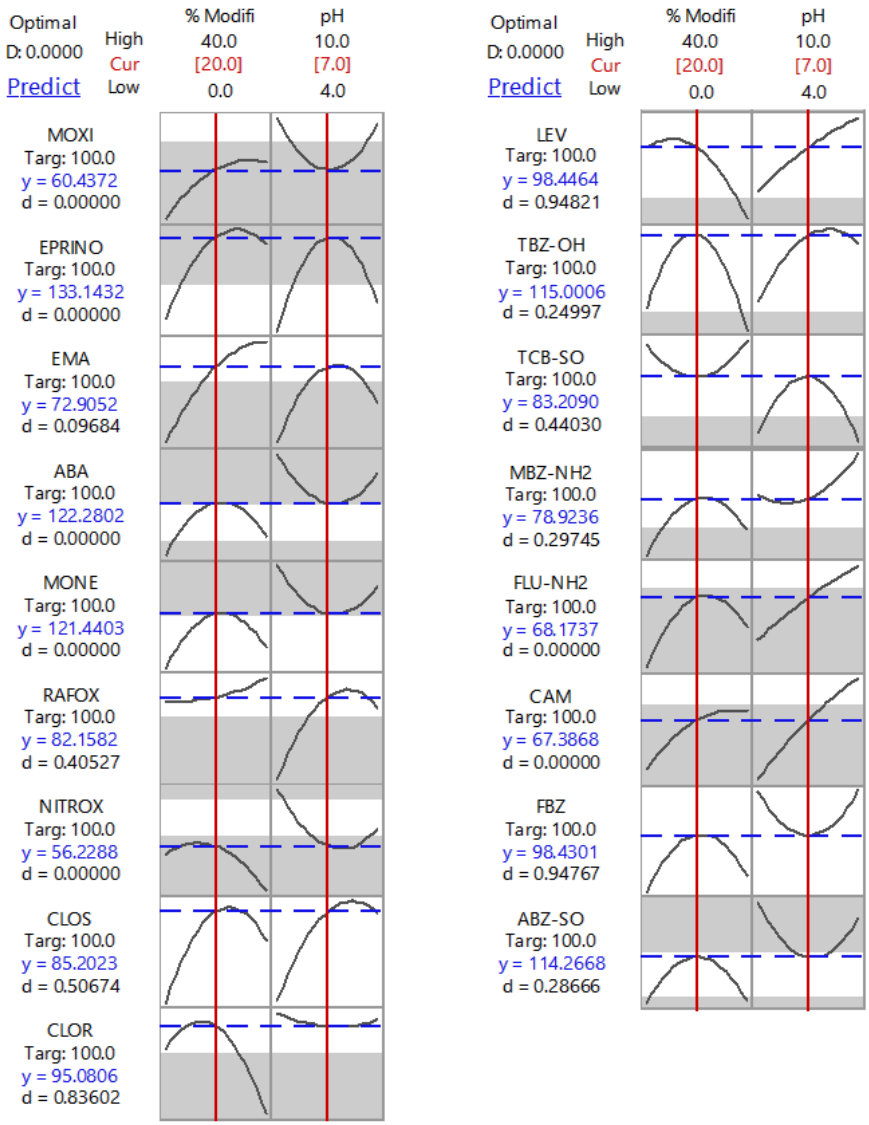


Figure S2-3(a) RSM optimiser graph for the 17 analytes selected for assessing the effect of percentage modifier (0–40%) and sample pH (4–10) on extraction

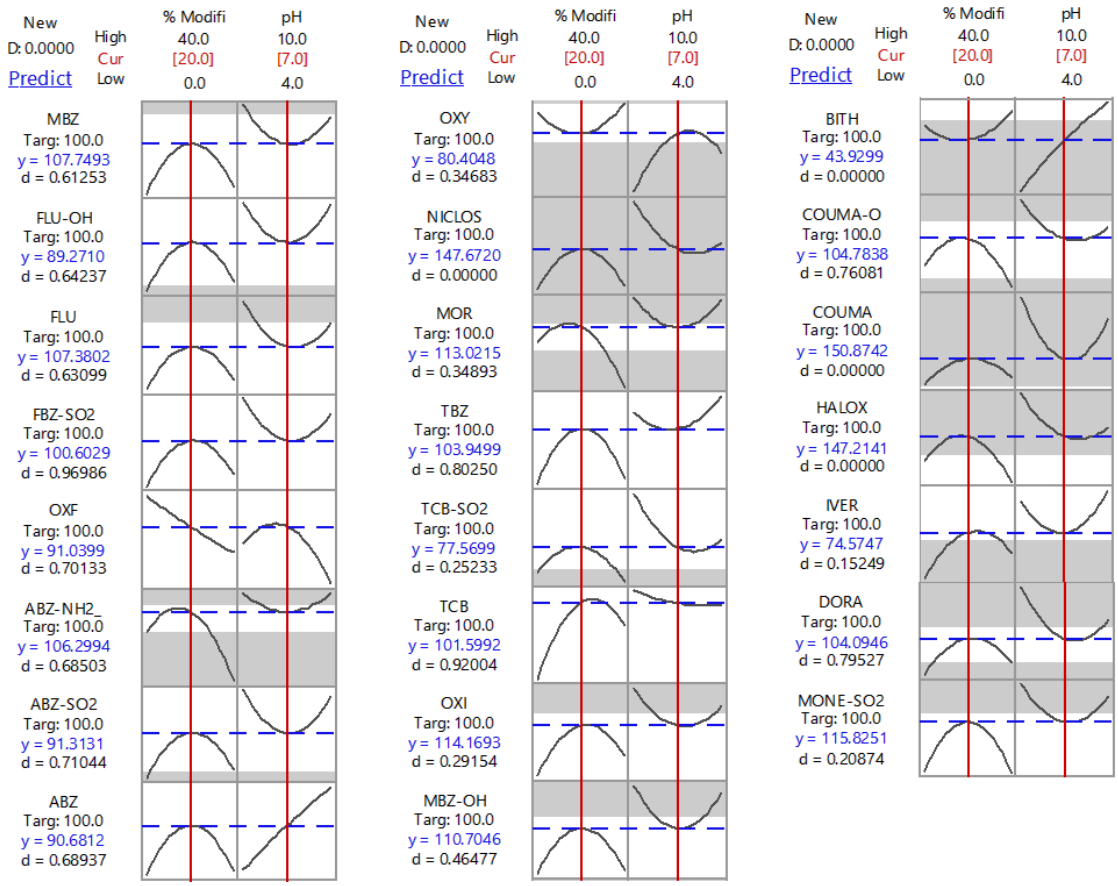


Figure S2-3(b) RSM optimiser graph demonstrating predicted recoveries for the remaining 23 analytes, under the selected optimum conditions for percentage modifier and sample pH (20% modifier and pH 7)

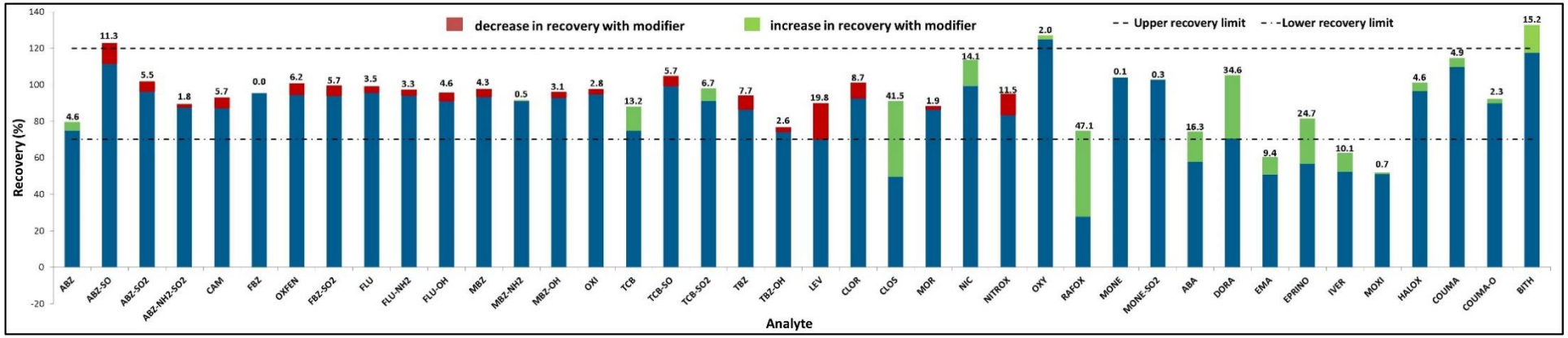


Figure S2-4 Increase (green bar) or decrease (red bar) in recoveries for all analytes, when the 20% MeOH sample modifier is incorporated, in comparison to the use of no modifier. The acceptable recovery range is as shown by the upper (120%) and lower (70%) recovery lines

Table S2-1 Physicochemical data for the studied anthelmintics (where available)

Class **Analyte**	**P/ TP**	**S_w** **mg L^{-1}**	**log K_{ow}**	**log K_{oc}**	**pK_a**
Benzimidazole					
Albendazole	P	10 [c], 46.39 [f]	3.07 [c] , 2.2–2.92 [e f]	2.94 [c]	3.37, 9.93 [c] 5.54, 13.11 [f]
Albendazole-sulphoxide	TP	62 [c]	1.2 [c], 0.83–0.94 [e]	-	3.5 [c], 9.8 [c] 7.8 [e]
Albendazole-sulphone	TP	-	0.9–1.01 [e]	-	-
Albendazole-amino-sulphone	TP	-	0.69–0.75 [e]	-	-
Cambendazole	P				
Fenbendazole	P	0.01–0.04[c], 6.38 [f]	1.95 [e] , 3.07–4.01 [e f]	3.37 [c]	5.12, 12.72 [f]
Oxfendazole	TP	407.2 [c f]	1.63[c], 1.88–2.13 [e f]	-	4.13, 11.79 [f]
Fenbendazole-sulphone	TP	-	2.13–3.30	-	-
Flubendazole	P	194.3 [c]	2.91 [c], 1.98–2.41[e]	3.05 [b c]	3.6, 9.9 [c]
Hydroxy-flubendazole	TP				
Amino-flubendazole	TP				
Mebendazole	P	10 [c], 50.05 [f]	2.71 [c], 2.44–2.52 [e]	3.00 [c]	3.5 [e], 9.2 [g]
Hydroxy-mebendazole	TP	-	2.22–2.61 [e]	-	9.8 [e]
Amino-mebendazole	TP	-	1.84–2.27[e]	-	5.5 [e]
Oxibendazole	P	-	1.86–2.63 [e]	-	4.6, 9.6 [g]
Triclabendazole	P	-	5.44 [b c] , 4.90– 6.66 [e]	-	2.5, 10.5 [e], 4.6 [g]
Triclabendazole-sulphoxide	TP		3.39–3.66 [e]	-	-
Triclabendazole-sulphone	TP	-	3.58–5.14 [e]	-	-
Thiabendazole	P	335.2 [b c f]	2.47 [c], 5.3–6.2 [e] 1.58–1.76 [e f]	2.69 [c]	2.5, 4.7 [e] , 5.22, 12.83 [f]
5-Hydroxy-thiabendazole	TP	30 [c]	1.29-1.37 [e]	-	4.5 [e]
Macro-cyclic lactones (Avermectins and Milbemycins)					
Abamectin	P	3.5×10^{-4} [b c]	4.0 [d]	3.72–4.48 [d]	-
Doramectin	P	-	4.1 [d]	3.88–4.94 [d]	-
Emamectin	P	-	5.0 [d]	4.39–5.86 [d]	7.6 [d] 4,2, 7.7[h]
Eprinomectin (Benzoate)	P	-	5.40 [d]	3.51–3.96 [d]	-
Ivermectin	P	-	3.22 [d]	3.60–4.41 [d]	-
Moxidectin	P	4 [a c]	4.77 [d], 5.67 [g]	3.90 [b c], 4.27–4.63 [d] 2.8, 12.6 [g]	-

Table S2-1 *continued*

Class Analyte	P/ TP	S_w mg L^{-1}	$logK_{ow}$	$logK_{oc}$	pK_a
Salicylanilides and substituted phenols					
Bithionol	P	0.2 [b c]	5.91 [b c]	4.67 [c]	4.83, 10.50 [c]
Closantel	P	1.5×10^{-5} [c]	8.11 [b c]	5.72 [c]	-
Niclosamide	P	10 [a c]	4.56 [b c]	3.58 [c]	-
Nitroxynil	P				
Oxyclozanide	P				
Rafoxanide	P	4.6×10^{-5} [b c]	8.14 [b c]	5.40 [c]	-
Tetrahydropyrimidines					
Morantel	P	1.5×10^{5} [a c] (tartrate)	3.69 [c]	2.9 [c]	
Imidazothiazoles					
Levamisole	P	1116 [c] $1x\ 10^{5}$ (HCl)	2.87 [b c]	1.88 [c]	-
Organophosphates					
Coumaphos	P	-	-	-	-
Coumaphos Oxon	P	-	-	-	-
Haloxon	P	-	-	-	-
Amino-acetonitrile derivatives					
Monepantel	P	-	-	-	-
Monepantel-sulphone	TP	-	-	-	-
Miscellaneous					
Clorsulon	P	-	-	-	-

(a) extracted from the Merck Index (O'Neill, 2001)

(b) calculated values using EPI Suite software (EPI WEB 4.0), 2009

(c) extracted from Horvat et al. (2012)Table 2.

(d) extracted from Krogh et al. (2008a)Table 1

(e) extracted from Danaher et al. (2007) Table 1

(f) extracted from Santaladchaiyakit and Srijaranai (2012)

(g) extracted from Zrncic et al. (2014) Table 1

(h) extracted from van der Velde-Koerts (2014)

P = parent compounds, TP = transformation product, SW = water solubility, $logK_{OW}$ = octanol water partition coefficient, $logK_{OC}$ = soil organic carbon – water partitioning coefficient, pKa = acid dissociation constant

Table S2-2 UHPLC-MS/MS conditions optimised and refined from Whelan et al. (2010)

Analyte	t_R (min)	MRM/F	M	Pre-ion (m/z)	Product Ions (*m/z*)	Dwell (s)	C (V)	CE (V)	IS
ABZ-NH_2SO_2-d_3	1.54	1	+	242.90	132.95	0.100	40	30	-
LEVA-d_5	1.55	1	+	210.10	183.05	0.100	40	21	-
ABZ-NH_2SO_2	1.57	1	+	240.05	**133.00**/198.00	0.100	40	26/18	ABZ-NH_2-SO_2-d_3
LEVA	1.58	1	+	205.10	122.90/**177.91**	0.100	40	28/20	LEVA-d_5
5-OH-TBZ	1.60	1	+	217.87	146.87/**190.85**	0.100	40	32/26	ABZ- NH_2-SO_2-d_3
TBZ-$^{13}C_6$	3.02	2	+	208.00	181.00	0.025	45	25	-
TBZ	3.03	2	+	201.90	130.90/**174.90**	0.025	45	32/25	TBZ-$^{13}C_6$
ABZ-SO-d_3	3.09	2	+	285.20	243.02	0.010	25	12	-
ABZ-SO	3.11	2	+	282.30	158.95/**240.00**	0.010	25	38/13	ABZ-SO-d_3
MBZ-NH_2	3.26	2	+	238.10	**104.90**/132.90	0.010	45	27/35	TCB-NH_2 (pos)
ABZ SO_2-d_3	3.42	2	+	301.00	158.95	0.010	35	38	-
ABZ-SO_2	3.44	2	+	298.20	**158.90**/266.00	0.010	35	35/20	ABZ-SO_2-d_3
FLU-NH_2	3.56	2	+	256.06	94.90/**122.95**	0.010	45	37/28	TCB-NH_2 (pos)
MOR	2.58,2.95	2	+	221.05	110.90/**122.90**	0.075	35	23/34	TBZ-$^{13}C_6$
NITROX	2.84	3	-	289.00	**126.85**/161.90	0.006	40	24/20	NITROX-$^{13}C_6$
NITROX-$^{13}C_6$	2.89	3	-	295.00	126.69	0.006	40	25	-
CLOR	3.10	3	-	379.90	**343.80**	0.006	18	12	SAL
	“	“	“	377.90	341.80	“	“	12	“
FBZ-SO-d_3	3.92	4	+	321.04	158.95	0.005	35	32	-
OXF	3.93	4	+	316.10	**159.05**, 191.09	0.005	35	30/24	FBZ-SO-d_3
MBZ-OH-d_3	4.07	4	+	301.15	16.05	0.008	35	32	-
MBZ-OH	4.09	4	+	298.25	160.05/**266.15**	0.010	35	36/22	MBZ-OH-d_3
FBZ-SO_2-d_3	4.27	4	+	335.05	299.90	0.010	35	23	-
FBZ-SO_2	4.28	4	+	331.9	158.90, **300.00**	0.010	35	36/21	FBZ-SO_2-d_3
FLU-OH	4.37	4	+	316.2	125.10, **160.05**	0.010	40	33/35	MBZ-OH-d_3

Table S2-2 *continued*

Analyte	t_R (min)	MRM/F	M	Pre-ion (m/z)	Product Ions (*m/z*)	Dwell (s)	C (V)	CE (V)	IS
CAM	4.58	4	+	302.96	216.85, **260.95**	0.008	35	26/18	FBZ-d_3
TFM	4.55	5	-	205.95	159.95	0.051	35	24	-
SAL	5.47	5	-	212.05	92.00	0.021	35	28	-
OXI-d_7	4.80	6	+	257.15	177.05	0.006	35	28	-
OXI	4.86	6	+	249.90	175.90/**218.00**	0.006	35	26/18	OXI-d_7
MBZ-d_3	5.00	6	+	299.15	105.05	0.006	40	33	-
MBZ	5.02	6	+	296.14	105.05/**264.10**	0.006	35	32/18	MBZ-d_3
FLU-d_3	5.24	6	+	318.15	123.00	0.006	40	36	-
FLU	5.26	6	+	313.80	123.00/**282.00**	0.006	40	35/24	FLU-d_3
ABZ-d_3	5.69	6	+	269.12	233.85	0.006	35	19	-
ABZ	5.70	6	+	266.07	**191.03**/234.00	0.006	35	32/13	ABZ-d_3
COUMA-O	5.93	7	+	347.01	**210.99**/291.20	0.005	30	29/22	FBZ-d_3
HALOX	6.08	7	+	414.90	**211.00**/272.95	0.005	35	35/32	ABZ-d_3
FBZ-d_3	6.12	7	+	303.00	267.95	0.005	35	22	-
FBZ	6.13	7	+	300.01	159.01/**268.01**	0.005	35	24/23	FBZ-d_3
TCB NH_2 (pos)	6.25	7	+	328.00	166.95	0.005	48	27	-
COUMA	6.80	8	+	363.02	227.05/**307.05**	0.008	35	25/16	ABZ-d_3
TCB	6.87	8	+	359.04	**274.07**/343.97	0.008	45	36/27	TCB-d_3
TCB-d_3	6.87	8	+	361.90	343.90	0.008	45	25	-
TCB-SO_2	6.12	9	-	389.00	244.16/**309.94**	0.006	55	38/35	TCB-NH_2(neg)
TCB-NH_2 (neg)	6.25	9	-	325.87	180.90	0.006	45	26	-
MONE-SO_2	6.50	9	-	504.00	165.85/**185.94**	0.006	15	50/15	CLOS-$^{13}C_6$
OXY	6.52	9	-	397.95	**176.00**/201.90	0.006	30	28/23	OXY-$^{13}C_6$
OXY-$^{13}C_6$	6.52	9	-	403.75	175.90	0.006	30	23	-
TCB SO	6.56	9	-	375.00	181.00/**213.00**	0.006	30	27/35	TCB-NH_2(neg)
MONE	6.72	9	-	472.00	166.00/**185.91**	0.006	15	45/13	CLOS-$^{13}C_6$

Table S2-2 *continued*

Analyte	t_R (min)	MRM/F	M	Pre-ion (m/z)	Product Ions (*m/z*)	Dwell (s)	C (V)	CE (V)	IS
NICLOS	6.77	9	-	324.95	**170.91**/288.89	0.006	35	26/17	SAL
BITH	6.99	10	-	352.90	**160.95**/191.95	0.005	28	27/22	RAFOX-$^{13}C_6$
CLOS	7.01	10	-	660.85	**126.90**/315.10	0.015	40	43/35	CLOS-$^{13}C_6$
CLOS-$^{13}C_6$	7.01	10	-	666.85	126.94	0.015	50	45	-
RAFOX	7.20	10	-	623.79	**126.87**/344.83	0.015	50	48/31	RAFOX-$^{13}C_6$
RAFOX-$^{13}C_6$	7.21	10	-	630.95	126.99	0.015	50	40	-
EMA	7.43	11	+	886.65	126.10/**158.10**	0.005	40	38/37	SEL
EPRINO	7.64	11	+	915.55	**144.15**/298.15	0.015	15	41/8	SEL
ABA	7.74	11	+	890.40	**305.15**/567.00	0.005	15	25/13	SEL
MOXI	7.92	11	+	640.30	498.10/**528.00**	0.005	15	11/9	SEL
DORA	7.94	11	+	916.60	**331.10**/593.10	0.005	15	25/13	SEL
SEL	8.14	11	+	770.40	333.30	0.005	40	22	-
IVER	8.21	11	+	892.40	**307.15**/569.10	0.005	20	26/14	SEL

t_R= Retention time, MRM/F = MRM window function where (1) 1.10 - 2.60 min (2) 1.90 - 3.90min (3)2.60 - 3.60min (4) 3.70 - 5.10min (5) 4.70 - 5.90 min (6) 4.50 - 6.00 min (7) 5.80 - 6.60min (8) 6.60 - 7.05min (9) 6.00 - 7.00min (10) 6.90 - 7.60min (11) 7.20 - 8.70min., M = ESI polarity mode; (+) = positive mode and (-) = negative mode , C= cone voltage, CE= collision energy, IS= internal standard. Product ion: quantifier shown in **bold**

Table S2-3 Summary of 13 experimental combinations, including 5 center points, generated using MiniTab, for response surface methodology assessing sample modifier (% MeOH) and pH conditions

Experiment	Modifier (%)	pH
1	20	7.0
2	20	7.0
3	20	7.0
4	0	4.0
5	20	5.5
6	20	7.0
7	40	10.0
8	20	7.0
9	0	10.0
10	20	8.5
11	40	4.0
12	10	7.0
13	30	7.0

Supplementary Information File SI-2.2

Anthelminthic Stability

According to 2002/657 (European Commission, 2002), insufficient stability of the analyte, or matrix components in a sample during storage, can give rise to significant deviations in the overall analytical result. Therefore, it is important to investigate such effects, to ensure the overall integrity of analytical results, throughout the entire analytical process. In general, investigation of stability often involves investigation of the analyte stability in solution during storage, as well as stability of analytes in matrix. As part of the method development work for the anthelmintics, a stability study was carried out to investigate the stability of the 40 anthelmintics throughout the analytical process, with an overall aim of informing sampling logistics and the logistics of the extraction methodology.

Croubels et al. (2003) emphasised the need for LC-UV detection for stability testing of veterinary drugs, to minimize day to day variations due to the instrumental determination. However, Berendsen et al. (2011) emphasized that the use of LC-MS/MS can be just as effective once measures are taken to eliminate the possibility of day to day instrumental variations. To achieve such, the authors incorporated a process whereby all stability samples are extracted and instrumentally analysed in one analytical run. For the matrix and intermediate stability experiments carried out as part of the current work, an approach similar to that proposed by (Berendsen et al., 2013) was adopted whereby stability samples were prepared and stored at different time points, in a manner which allowed all time points to be analysed in one day, at the end of the study at time point t=0. In addition to this, an added step was included to further reduce instrumental variation between samples. In this case, the internal standard (IS) was added to all samples at the end of the study, just prior to extraction and instrumental determination. By doing so, the IS level was consistent in all samples at the differing time points, thus when using response ratio i.e. calculated concentration, the IS corrects for any instrumental variations, however, does not correct for analyte variation due to the storage condition. Analytes were said to be stable if the change in concentration over time remained <15% in comparison to the original concentration (day 0). A criterion of 20% was accepted where analytes had their own internal standards.

Matrix Stability

Stability of analytes in matrix was assessed to inform the overall storage duration of samples following collection and prior to analysis. Two contrasting waters were selected based on dissolved organic carbon (DOC) content and water hardness (Table S2-4). For each water type, 32 aliquots (500 mL each) were weighed into amber glass bottles (1000 mL). In triplicate (n=3) the two different waters were fortified with analyte at two different concentration levels (low and high) corresponding to 20 ng L^{-1} and 200 ng L^{-1} for all analytes, except BITH, CLOR, MOR and OXY which were at a concentration of 40 and 400 ng L^{-1}, respectively. Samples were fortified at varying time points as below (Table S2-4) and stored in the cold room (4ºC) until analysis. All samples from all four time points for each respective water type were extracted as per the procedure previously described (Section 2.3) on the same day, t=0, and injected together as one batch, with injections of replicates in random order. For all time points, internal standard (25 µL) was added just prior to extraction on day t=0. Note time point four for water type 1 and 2 differ as it was necessary to stagger the storage duration, due to limitations in the number of samples that could physically be extracted in one day.

Table S2-4 Preparation of working calibrant solutions from six different intermediate standard solutions

Water	Hardness	DOC	Time points (days)
Type 1	Hard	High	0, 7, 14, 28
Type 2	Soft	Low	0, 7, 14, 21

DOC = Dissolved organic carbon
Soft water is given as water with <100 mg L^{-1} hardness as $CaCO_3$
Hard water is given as water with >250 mg L^{-1} hardness as $CaCO_3$
Low DOC is given as water with <1 mg L^{-1} DOC
High DOC is given as water with >10 mg L^{-1} DOC

Findings:

The results of the matrix stability are presented in Table S2-5 (Water Type 1) and S2-6 (Water Type 2). The majority of analytes appears to be stable in both water types for 7-14 days depending on the analyte. For water Type 1 (high DOC and hard water) at the lower concentrations (20/40 ng L^{-1}), 35 of 40 analytes were stable up to 28 days with no significant change in concentration determined (Table S2-5). The most significant change in concentration after 28 days was for BITH, RAFOX and ABZ with concentrations decreasing

to 39, 66 and 67% of the initial concentration, respectively. A similar trend was observed at the higher analyte concentrations (200/400 ng L^{-1}), with the stability of BITH slightly improved (59% at t=28), however still failed to meet the acceptance criteria (<20% variation). All analytes were stable up to 7 days, apart from RAFOX (79%), which fell just below the 20% acceptance criterion. ABZ, CLOR and RAFOX concentrations decreased to 67, 78 and 66% respectively, after 14 days in storage, indicating instability after the initial 7days.

Instability of analytes appeared to be more pronounced in this water type 1 with high DOC, hard water, as opposed to the water type 2 with low DOC, soft water. As evident from Table S2-6, stability of analytes at both concentration levels were much improved for water type 2, with much improved stability demonstrated for ABZ and BITH after 21 days, with both determined at >85% of the original concentration at t=0. Some irregularities were observed for the results for CAM, TCB-SO and TBZ, therefore stability of these analytes in water type 2 were inconclusive.

Overall, considering the results of both water types, and bearing in mind the difficulties in multi-residue analysis and thus the need for some compromise, it was decided that analysis of samples should be performed as soon as possible after collection, with any analysis and subsequent repeat analysis performed within 7 days, to ensure the most reliable results.

Table S2-5 Matrix stability results for anthelmintics in water type 1 (hard water with high DOC) at two concentrations, presented as the percentage of analyte remaining at different time points (0, 7, 14 and 28 days), by comparison to day 0 (t=0)

Analyte	Mean (n=3) % analyte remaining at time point (days) with RSD (%)															
	20/40 ng L^{-1}								200/400 ng L^{-1}							
	t= 0	RSD	t=7	RSD	t=14	RSD	t=28	RSD	t= 0	RSD	t=7	RSD	t=14	RSD	t=28	RSD
ABZ	100.0	4.9	83.2	6.7	68.0	8.3	65.6	6.6	100.0	6.1	89.0	2.5	66.8	7.5	67.0	4.5
ABZ-SO	100.0	5.3	118.5	3.8	108.6	5.8	123.1	9.3	100.0	1.7	118.2	4.9	115.5	4.9	124.2	4.9
ABZ-SO_2	100.0	3.9	104.3	1.1	98.3	6.4	105.6	10.5	100.0	4.1	100.5	2.4	98.2	3.1	102.0	1.4
ABZ-NH2-SO_2	100.0	2.2	112.8	4.8	105.1	3.0	117.1	8.6	100.0	2.5	108.0	0.4	106.2	5.1	110.7	3.3
CAM	100.0	4.8	108.5	5.9	107.6	2.1	115.2	7.9	100.0	2.9	102.4	1.3	98.9	2.2	102.1	0.9
FBZ	100.0	3.0	98.1	7.0	94.6	4.5	99.8	6.2	100.0	2.1	102.2	2.5	99.8	5.0	104.3	3.1
OXF	100.0	2.2	109.8	3.8	101.0	4.8	113.0	7.0	100.0	7.2	109.1	1.8	104.2	4.7	112.9	7.1
FBZ-SO_2	100.0	4.0	106.1	7.1	100.7	4.4	106.2	7.6	100.0	2.7	105.4	1.7	102.4	2.6	108.4	1.4
FLU	100.0	2.5	102.7	5.2	100.3	2.1	110.5	4.5	100.0	0.5	103.0	1.6	102.2	4.3	107.8	2.2
FLU-NH_2	100.0	5.3	108.5	5.8	97.3	8.5	108.5	4.0	100.0	1.5	107.1	3.3	100.0	3.2	107.5	5.2
FLU-OH	100.0	4.7	100.5	2.8	97.7	5.5	103.2	7.7	100.0	0.5	104.7	2.1	100.6	4.7	104.7	3.1
MBZ	100.0	6.3	111.4	7.2	107.0	4.9	119.4	3.4	100.0	3.0	107.7	2.2	106.3	3.8	114.1	5.0
MBZ-NH_2	100.0	6.0	107.6	8.2	97.2	4.4	108.9	7.5	100.0	2.8	106.4	1.0	101.6	4.5	107.7	3.6
MBZ-OH	100.0	6.6	108.6	3.4	99.7	5.0	108.3	4.2	100.0	1.7	104.9	0.9	99.8	4.6	102.9	1.3
OXI	100.0	3.3	104.1	5.1	99.4	4.2	106.8	4.2	100.0	2.4	104.6	3.7	99.9	2.8	105.1	1.7
TCB	100.0	5.0	98.8	9.2	91.5	5.7	90.6	5.2	100.0	1.3	96.7	4.9	92.2	4.0	89.4	3.4
TCB-SO	100.0	2.5	100.7	2.3	94.5	2.6	116.2	4.8	100.0	5.3	117.4	4.8	111.5	1.0	116.4	7.1
TCB-SO_2	100.0	5.7	93.4	4.4	97.9	1.6	94.3	3.7	100.0	2.5	105.9	5.2	104.4	3.5	106.7	4.8
TBZ	100.0	2.9	106.3	3.0	99.2	3.7	109.8	7.3	100.0	2.4	105.0	3.5	102.0	3.0	107.0	3.8
TBZ-OH	100.0	1.7	67.3	4.2	86.7	16.5	93.4	2.4	100.0	6.3	71.3	13.0	33.4	2.2	93.7	6.8
LEV	100.0	5.5	109.3	3.7	102.2	4.0	114.6	7.5	100.0	2.8	105.9	3.0	102.3	2.7	108.5	3.0
CLOR	100.0	11.6	93.1	16.4	88.5	41.2	74.5	5.8	100.0	8.3	101.6	4.8	97.5	7.1	101.3	4.5
CLOS	100.0	6.1	85.3	7.3	81.9	10.3	74.4	3.5	100.0	4.5	95.9	4.6	90.8	2.1	87.8	2.8
MOR	100.0	3.7	106.1	2.4	98.3	3.8	106.7	4.0	100.0	0.2	105.1	1.3	102.2	2.3	105.8	3.4
NICLOS	100.0	7.0	102.3	8.6	99.2	5.3	103.8	9.2	100.0	0.6	109.6	1.1	104.8	3.8	111.2	1.7
NITROX	100.0	17.1	93.7	12.7	76.2	2.5	91.3	7.3	100.0	9.2	107.5	7.4	104.1	3.7	105.3	7.7

Table S2-5 *continued*

Analyte	Mean (n=3) % analyte remaining at time point (days) with RSD (%)															
	20/40 ng L^{-1}								200/400 ng L^{-1}							
	t= 0	RSD	t=7	RSD	t=14	RSD	t=28	RSD	t= 0	RSD	t=7	RSD	t=14	RSD	t=28	RSD
OXYCLOZ	100.0	15.7	103.5	7.3	83.4	15.3	107.8	7.4	100.0	6.0	107.1	6.4	95.9	3.9	104.3	2.5
RAFOX	100.0	8.0	80.4	8.8	68.9	17.7	66.8	7.9	100.0	7.4	75.4	10.7	73.5	7.1	69.9	8.3
MONE	100.0	4.9	111.2	2.7	101.7	5.9	114.9	7.7	100.0	1.1	105.2	3.0	104.1	1.7	111.6	3.8
MONE-SO_2	100.0	3.3	108.4	2.9	101.2	3.5	110.4	5.2	100.0	0.1	108.1	2.0	110.0	2.2	115.0	4.5
ABA	100.0	9.0	107.3	0.0	103.7	14.5	107.5	1.0	100.0	0.9	115.6	5.9	118.3	3.5	132.1	12.6
DORA	100.0	9.1	99.3	4.6	88.1	18.1	119.3	10.3	100.0	9.0	115.9	6.0	109.5	3.8	119.6	8.3
EMA	100.0	8.3	115.6	10.2	99.2	5.7	111.8	5.0	100.0	1.2	107.5	3.2	103.6	6.2	107.3	5.9
EPRINO	100.0	10.6	125.0	14.8	116.1	2.3	135.1	7.7	100.0	6.5	109.4	3.2	109.0	1.3	115.7	4.3
IVER	100.0	5.9	99.6	12.4	84.2	4.8	101.1	6.3	100.0	5.0	120.9	3.6	99.1	7.3	113.8	3.9
MOXI	100.0	10.1	121.6	24.9	95.7	7.2	99.4	15.6	100.0	6.8	103.3	1.3	107.8	3.2	110.4	4.1
HALOX	100.0	6.0	98.4	11.4	93.7	12.4	97.6	2.6	100.0	3.7	103.2	0.7	93.2	2.1	91.4	1.0
COUMA	100.0	8.5	106.7	6.4	89.9	22.0	106.6	11.8	100.0	1.2	102.0	0.6	98.7	2.6	105.7	2.5
COUMA-O	100.0	4.0	105.6	4.2	97.2	7.4	104.8	9.6	100.0	4.1	103.3	2.1	102.5	4.9	101.7	5.9
BITH	100.0	12.5	59.4	21.5	50.1	26.3	39.0	13.1	100.0	4.5	101.0	3.7	73.5	13.1	59.4	6.0

t=0: storage for 0 days

t=7: storage for 7 days

t=14: storage for 14 days

t=28: storage for 28 days

Table S2-6 Matrix stability results for anthelmintics in water type 2 (soft water with low DOC) at two concentrations, presented as the percentage of analyte remaining at different time points (0, 7, 14 and 21 days), by comparison to day 0 (t=0)

Analyte	Mean (n=3) % analyte remaining at time point (days) with RSD (%)															
	20/40 ng L^{-1}								200/400 ng L^{-1}							
	t= 0	RSD	t=7	RSD	t=14	RSD	t=21	RSD	t= 0	RSD	t=7	RSD	t=14	RSD	t=21	RSD
ABZ	100.0	9.1	103.8	8.9	94.8	14.3	87.7	17.5	100.0	8.8	91.2	5.4	96.8	7.7	100.3	8.1
ABZ-SO	100.0	5.8	104.8	15.0	90.4	6.6	101.8	12.0	100.0	6.7	102.4	9.0	107.5	10.3	100.1	4.4
ABZ-SO_2	100.0	14.7	104.3	8.1	88.9	14.0	101.0	12.3	100.0	8.5	109.0	11.1	115.2	3.5	102.5	8.4
ABZ-NH2-SO_2	100.0	9.0	109.2	11.7	100.5	8.5	94.2	14.0	100.0	5.3	92.9	8.1	102.4	7.6	101.8	10.3
CAM	100.0	46.7	294.4	7.8	196.6	78.9	311.1	10.1	100.0	27.5	243.6	21.5	175.4	83.3	282.6	11.3
FBZ	100.0	11.5	113.2	1.1	107.2	21.6	99.6	2.7	100.0	6.1	94.5	12.1	87.6	21.7	113.2	9.9
OXF	100.0	4.9	101.3	8.1	97.1	3.5	100.4	8.9	100.0	3.4	94.9	9.7	101.8	4.4	99.1	8.4
FBZ-SO_2	100.0	8.8	105.7	11.7	95.9	11.1	99.8	9.1	100.0	6.1	96.1	15.5	102.2	6.9	104.2	9.2
FLU	100.0	8.9	97.8	12.3	97.4	6.4	93.7	4.5	100.0	7.3	95.3	11.9	99.0	7.9	98.0	9.8
FLU-NH_2	100.0	8.2	106.6	15.5	102.7	8.9	98.0	15.1	100.0	9.4	95.4	14.6	104.9	2.0	105.1	11.9
FLU-OH	100.0	6.9	96.1	11.5	95.0	7.7	92.1	17.8	100.0	5.3	95.1	11.3	103.2	5.7	102.1	9.4
MBZ	100.0	7.1	97.8	7.5	93.3	5.0	92.4	10.6	100.0	5.7	100.5	13.9	101.6	6.8	104.5	7.0
MBZ-NH_2	100.0	4.5	103.2	13.9	100.1	7.2	97.0	13.3	100.0	3.9	98.1	12.4	109.1	6.4	105.8	6.2
MBZ-OH	100.0	6.4	104.4	10.2	98.9	4.6	98.1	9.9	100.0	3.6	93.9	10.4	101.2	6.3	102.2	9.6
OXI	100.0	6.8	101.3	11.1	100.2	9.7	98.4	12.5	100.0	8.0	99.2	11.1	106.3	6.0	106.1	10.0
TCB	100.0	6.2	95.5	1.9	91.2	11.0	102.1	10.4	100.0	3.8	96.8	10.6	104.0	4.3	97.8	8.0
TCB-SO	100.0	35.0	543.8	27.4	368.3	80.0	569.7	30.9	100.0	41.5	303.7	27.5	209.3	83.3	314.4	7.9
TCB-SO_2	100.0	6.4	91.7	20.4	68.0	4.5	66.5	10.0	100.0	4.7	104.2	3.6	106.8	1.8	98.8	5.6
TBZ	100.0	56.3	372.2	20.6	209.5	81.1	309.6	8.1	100.0	44.4	259.4	17.3	183.8	86.3	298.8	10.7
TBZ-OH	100.0	13.8	136.0	6.0	100.5	49.7	126.4	16.5	100.0	2.3	113.7	5.2	88.2	55.4	116.5	1.7
LEV	100.0	5.7	105.2	9.1	100.2	7.0	97.1	13.3	100.0	7.8	97.1	7.1	104.7	6.0	105.8	9.5
CLOR	100.0	14.3	120.5	8.8	94.2	17.8	78.7	48.8	100.0	14.2	102.1	0.9	61.7	28.2	109.6	17.2
CLOS	100.0	9.7	89.2	1.7	87.4	3.2	78.2	1.0	100.0	4.9	97.1	4.1	79.7	22.1	88.5	0.8
MOR	100.0	9.2	103.4	8.2	100.6	3.3	96.9	9.0	100.0	9.6	92.4	3.4	106.8	5.4	103.5	12.3
NICLOS	100.0	5.4	98.6	8.0	99.8	7.9	93.7	12.6	100.0	2.9	91.5	13.3	94.0	4.5	96.7	10.4
NITROX	100.0	13.8	100.2	18.8	108.6	4.8	98.5	22.4	100.0	6.4	99.3	8.0	98.4	3.9	97.9	1.5

Table S2-6 *continued*

Analyte	Mean (n=3) % analyte remaining at time point (days) with RSD (%)															
	20/40 ng L^{-1}								200/400 ng L^{-1}							
	t= 0	RSD	t=7	RSD	t=14	RSD	t=21	RSD	t= 0	RSD	t=7	RSD	t=14	RSD	t=21	RSD
OXYCLOZ	100.0	19.6	107.1	43.8	107.8	16.8	105.0	22.3	100.0	9.5	80.1	15.1	93.9	6.9	98.9	17.2
RAFOX	100.0	10.6	82.2	10.9	67.3	15.8	77.5	21.0	100.0	7.8	93.3	6.1	93.4	9.5	84.9	12.8
MONE	100.0	4.7	99.5	11.8	96.5	5.9	91.3	7.8	100.0	2.2	91.5	8.3	95.9	3.8	95.9	11.3
MONE-SO_2	100.0	7.2	96.6	7.7	90.8	2.4	90.9	10.4	100.0	4.6	98.5	3.9	101.1	6.0	100.8	7.7
ABA	100.0	6.7	116.6	7.5	96.3	17.2	87.6	21.8	100.0	12.5	85.8	19.9	98.4	6.3	95.2	18.7
DORA	100.0	14.1	99.5	7.7	90.1	16.0	88.1	20.5	100.0	10.6	93.1	19.9	97.4	10.9	99.4	18.2
EMA	100.0	9.3	97.9	9.2	95.3	6.7	85.4	21.1	100.0	6.8	88.4	6.0	102.5	9.9	98.0	8.6
EPRINO	100.0	27.1	94.9	26.3	94.9	15.2	96.2	12.3	100.0	6.3	86.2	10.8	105.0	7.4	102.0	7.6
IVER	100.0	7.3	104.0	14.3	86.7	18.8	79.4	23.9	100.0	4.5	88.3	23.2	101.1	13.6	98.0	14.5
MOXI	100.0	27.0	99.9	25.5	102.1	19.3	77.6	26.4	100.0	6.4	88.0	14.8	97.6	14.4	96.2	21.4
HALOX	100.0	18.8	99.9	6.3	93.8	13.8	95.1	6.0	100.0	14.3	90.0	15.6	98.7	5.9	105.9	10.1
COUMA	100.0	16.6	105.4	2.2	95.0	6.2	95.6	14.3	100.0	4.6	93.9	9.9	100.7	8.3	104.2	15.1
COUMA-O	100.0	10.7	105.5	11.9	94.4	7.6	92.0	9.5	100.0	10.1	84.8	3.6	99.8	6.8	98.4	11.8
BITH	100.0	14.4	98.0	3.4	79.7	12.8	85.2	8.6	100.0	2.2	87.2	3.2	89.6	9.3	87.9	8.7

t=0: storage for 0 days

t=7: storage for 7 days

t=14: storage for 14 days

t=21: storage for 21 days

Intermediate Stability (Eluate)

The stability of analytes in eluate, post SPE, referred to as intermediate stability, was assessed over a 7 day period to determine how long eluate extracts could be stored for prior to evaporation. This was to verify whether samples could be extracted, then stored, and instrumentally analysed on an alternative day. Four different negative control samples, each of differing chemistry, were extracted (unfortified) in replicates of n=9 (500 mL) and carried through the extraction procedure, up until the post SPE stage (post elution). The eluates for each sample (10 mL aliquot, n=36 samples) were combined into one large composite eluate (360 mL), mixed thoroughly, and re-aliquoted as homogenous eluate aliquots (10 mL) into polypropylene tubes (15 mL). These eluate aliquots were stored at 4ºC and fortified at a low and high concentration as described for matrix stability, in replicates of n=3 at time points corresponding to 0, 1, 4 and 7 days storage post SPE extraction. All replicates, for each time point were fortified with internal standard (25 µL) on day 0, and the eluates underwent the final TurboVap LV evaporation step, with subsequent instrumental determination.

Findings:

For all analytes, as evident from table S2-7 below, even after 7 days stored at 4°C following the SPE extraction and prior to final evaporation, the difference in analyte concentration (compared to day 0) was typically <10%, both at the lower and higher spiking concentrations. The exception to this is abamectin (ABA), which shows degradation to approx. 75% of the original concentration after one day storage, after which the concentrations remained relatively constant, with 73% of analyte remaining after 7 days storage. This instability was only an issue for the lower spiked concentration of 40 ng L^{-1} for ABA, with the analyte demonstrating acceptable stability at the higher concentration of 400 ng L^{-1}, with 97% of the analyte remaining after 7 says. In practical terms, the use of the matrix matched calibration curves in addition to isotopically labelled internal standards will account for these slight losses during overnight storage. However, in order to maximise the analyte sensitivity, it was decided that where possible, samples will be evaporated and instrumentally determined on the same day as SPE extraction.

Table S2-7 Intermediate (eluate) stability of anthelmintics at two concentrations, presented as the percentage of analyte remaining at different time points (0, 1, 4 and 7 days), by comparison to day 0 (t=0)

Analyte	Mean (n=3) % analyte remaining at time point (days) with RSD (%)															
	20/40 ng L^{-1}								200/400 ng L^{-1}							
	t= 0	RSD	t=1	RSD	t=5	RSD	t=7	RSD	t= 0	RSD	t=1	RSD	t=5	RSD	t=7	RSD
ABZ	100.0	4.7	91.4	7.6	91.5	5.8	92.6	3.8	100.0	2.4	97.8	5.1	102.6	1.4	100.1	3.1
ABZ-SO	100.0	1.3	93.2	5.4	90.3	6.8	93.3	6.9	100.0	5.9	104.1	4.6	105.2	0.8	102.3	4.0
ABZ-SO_2	100.0	2.1	94.1	10.8	94.4	4.1	96.9	4.9	100.0	2.8	102.6	1.1	102.2	4.8	101.8	1.2
ABZ-NH2-SO_2	100.0	4.0	88.1	7.6	90.7	4.1	93.9	3.3	100.0	3.5	103.3	2.8	102.2	0.8	99.5	2.1
CAM	100.0	3.2	92.7	6.1	92.4	4.9	93.1	6.6	100.0	3.6	97.8	4.0	97.3	3.0	95.5	1.7
FBZ	100.0	4.6	96.1	11.5	95.0	1.7	91.0	2.1	100.0	2.6	100.3	4.2	103.5	5.7	97.6	4.2
OXF	100.0	6.5	92.0	7.5	94.8	2.5	94.1	2.2	100.0	3.9	100.1	3.9	102.4	2.7	99.3	5.8
FBZ-SO_2	100.0	3.2	92.9	9.4	92.0	7.0	94.5	4.9	100.0	4.4	99.0	5.1	101.4	1.8	98.3	1.6
FLU	100.0	4.9	90.1	9.2	94.1	4.8	91.6	2.0	100.0	8.4	99.4	4.6	104.7	2.7	99.8	5.2
FLU-NH_2	100.0	5.1	94.8	7.2	91.8	4.2	97.4	3.1	100.0	0.8	97.0	5.4	99.9	1.6	98.2	1.2
FLU-OH	100.0	4.3	90.1	10.0	91.3	6.1	93.6	9.7	100.0	6.1	97.9	3.5	100.4	2.9	98.5	1.8
MBZ	100.0	4.0	92.1	4.3	93.6	8.0	95.9	6.2	100.0	3.6	96.7	4.7	104.1	2.2	97.1	7.0
MBZ-NH_2	100.0	2.0	92.5	10.4	92.5	2.4	96.3	4.0	100.0	4.1	101.6	2.2	100.1	1.1	97.1	2.7
MBZ-OH	100.0	3.6	90.7	8.7	90.6	4.8	90.7	2.6	100.0	3.9	99.2	5.7	100.8	2.8	99.4	5.7
OXI	100.0	7.8	94.2	7.8	91.1	3.3	95.0	0.3	100.0	1.6	97.1	0.9	96.8	0.1	96.0	0.9
TCB	100.0	2.0	90.3	1.9	88.0	3.5	91.9	9.6	100.0	3.0	97.9	3.2	98.2	1.2	94.8	3.9
TCB-SO	100.0	4.8	109.5	6.1	110.3	2.0	98.8	15.6	100.0	9.6	97.4	4.8	104.9	7.6	99.9	4.4
TCB-SO_2	100.0	7.5	99.5	5.2	102.8	10.1	111.9	5.0	100.0	5.8	97.7	6.4	97.4	5.9	103.6	1.9
TBZ	100.0	3.1	89.5	7.7	90.9	1.7	92.1	3.2	100.0	3.9	100.6	2.1	103.3	2.8	97.9	2.3
TBZ-OH	100.0	2.0	94.7	4.3	95.0	3.6	94.5	0.7	100.0	2.7	101.2	2.0	100.0	0.3	97.3	1.7
LEV	100.0	5.7	90.5	7.6	90.3	3.6	92.5	3.2	100.0	4.9	100.6	3.0	101.9	0.8	97.4	1.2
CLOR	100.0	14.4	84.3	16.6	110.3	16.6	130.4	13.2	100.0	4.6	90.9	8.4	101.4	3.9	91.0	5.0
CLOS	100.0	3.6	97.6	7.5	88.9	8.7	94.8	3.1	100.0	2.6	98.7	5.6	96.9	2.8	97.3	2.6
MOR	100.0	4.0	90.8	7.6	90.3	5.8	90.1	1.0	100.0	3.3	102.9	3.2	102.7	1.4	99.5	2.1
NICLOS	100.0	7.0	91.8	7.2	93.0	5.9	95.7	7.8	100.0	7.0	101.2	6.2	99.8	3.7	99.0	2.6
NITROX	100.0	4.6	108.3	8.7	94.0	4.9	99.7	7.1	100.0	3.7	101.4	6.8	105.6	2.0	103.2	5.6

Table S2-7 *continued*

Analyte	Mean (n=3) % analyte remaining at time point (days) with RSD (%)															
	20/40 ng L^{-1}								200/400 ng L^{-1}							
	t= 0	RSD	t=1	RSD	t=5	RSD	t=7	RSD	t= 0	RSD	t=1	RSD	t=5	RSD	t=7	RSD
OXYCLOZ	100.0	7.7	97.2	3.6	92.7	9.3	92.2	0.9	100.0	9.4	101.5	6.3	104.9	4.9	104.3	4.4
RAFOX	100.0	5.8	94.9	8.4	91.6	7.5	95.1	5.0	100.0	2.3	99.1	1.7	95.7	3.8	98.0	4.9
MONE	100.0	6.9	86.9	6.5	91.0	8.1	94.9	2.4	100.0	5.3	97.2	3.5	103.1	3.1	97.6	2.3
MONE-SO_2	100.0	2.6	91.3	0.5	94.4	1.0	91.2	4.7	100.0	3.2	104.2	5.9	100.6	1.9	98.6	5.4
ABA	100.0	9.9	75.6	6.3	71.2	12.3	73.3	6.5	100.0	10.0	95.7	9.7	97.8	5.0	97.0	5.0
DORA	100.0	6.8	100.6	13.7	83.8	8.0	92.8	9.9	100.0	5.5	97.7	7.1	111.2	1.1	103.8	5.9
EMA	100.0	5.0	88.8	7.7	88.8	0.5	90.5	2.4	100.0	4.1	98.1	5.5	104.2	7.2	99.1	3.5
EPRINO	100.0	0.8	79.0	0.7	87.6	6.6	97.9	1.0	100.0	9.1	102.4	4.0	98.5	6.3	93.0	7.7
IVER	100.0	10.9	86.5	5.8	96.4	11.9	97.6	5.6	100.0	7.1	99.6	6.0	103.3	3.4	93.6	9.3
MOXI	100.0	10.8	99.1	14.0	96.1	10.7	97.8	8.8	100.0	7.9	100.4	8.9	99.8	4.8	96.9	6.2
HALOX	100.0	0.8	92.0	2.9	98.9	9.2	99.0	2.4	100.0	3.8	99.2	7.4	100.6	5.6	97.0	4.1
COUMA	100.0	6.0	90.8	9.0	96.2	0.7	91.4	4.2	100.0	6.5	101.2	10.3	100.1	8.2	93.7	4.4
COUMA-O	100.0	5.6	83.9	3.8	88.9	5.9	88.1	5.3	100.0	5.2	99.5	6.7	100.1	0.8	96.9	4.2
BITH	100.0	10.8	89.5	9.3	86.2	3.0	95.0	7.2	100.0	0.9	107.4	7.0	105.0	0.8	103.6	4.2

t=0: storage for 0 days

t=1: storage for 1 days

t=4: storage for 4 days

t=7: storage for 7 days

Final Extract stability

The stability of anthelmintic residues in the final DMSO extract was assessed during 5 weeks storage at +4°C and room temperature (RT) (+20°C). Four different negative control samples, each of differing chemistry, were extracted (unfortified) in replicates of n=12 (500 mL each) and carried through the extraction procedure, to the final DMSO extract (500 µL). All DMSO extracts (48 × 500 µL) were combined to form a large composite extract (24 mL), which was mixed and re-aliquoted (500 µL) into glass tubes (5mL). For each storage temperature (4°C vs. RT), DMSO extracts were fortified at two concentration levels (corresponding to 20 ng L^{-1} and 200 ng L^{-1} for analytes except BITH, CLOR, MOR and OXY, which were at twice these concentrations) in replicates of 3, at time points corresponding to 0, 2, 3 and 5 weeks. Internal standard (25 µL) was added to each sample at the same time as analyte fortification. Following fortification with analytes and IS at each time point, extracts were vortexed (60 s), evaporated under gentle flow of nitrogen to remove MeOH spiking solvent (125 µL), and filtered through 0.22 µm syringe filters into glass HPLC vials. Extracts were stored in HPLC vials to simulate a real life case, at the storage conditions in question (4°C and RT). All extracts for each time point was analysed on day 0 in one instrumental batch, in random order. Prior to instrumental determination, all extracts in HPLC vials were vortexed (60 s) prior to being placed in the auto-sampler.

The stability results for all anthelmintics spiked at the lower 20/40 ng L^{-1} concentration are as summarised in Table S2-8, while the results of the higher concentration are shown in Table S2-9. As a whole, the majority of analytes, at both concentration levels, were stable in final extract over the 5 week storage period, with the percentage of analyte remaining >85% of the original concentration. There was no apparent difference with storage at room temperature, compared to storage at 4°C for the majority. However, for a number of analytes, namely TCB-SO_2, CLOR, MONE and MONE-SO_2, there was an observed trend of analyte concentration increasing over time, and this was particularly an issue for the lower spiked concentration, and was more pronounced when stored at room temperature compared to 4°C. This effect may be caused by matrix components, or microbial activity, that is not adequately compensated for by the internal standards. Overall, considering these results, in order to avoid the of falsely inflated results for these analytes, storage of extracts is not recommended. However, stored extracts may be used for determination of all other analytes which shows no stability issues.

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Table S2-8 Final extract stability of anthelmintics spiked at 20/40 ng L^{-1} (n=3), presented as the percentage of analyte remaining at different time points (0, 1, 3 and 5 weeks), by comparison to day 0 (t=0)

Analyte	Mean (n=3) % analyte remaining at time point (weeks) with RSD (%) at 20/40 ng L^{-1}												
	t=0	RSD	Room Temperature (RT)						4 degree Celsius (4 °C)					
			t=1	RSD	t=3	RSD	t=5	RSD	t=1	RSD	t=3	RSD	t=5	RSD
ABZ	100.0	5.3	104.4	4.0	103.7	4.5	107.0	2.6	99.8	5.4	114.2	13.1	103.4	1.4
ABZ-SO	100.0	4.4	105.3	9.8	104.9	3.6	108.9	8.3	104.3	3.6	115.2	9.9	105.5	3.9
ABZ-SO_2	100.0	3.6	110.0	3.9	107.6	4.4	118.2	7.7	105.7	6.9	108.9	18.3	98.0	9.8
ABZ-NH_2-SO_2	100.0	1.9	101.8	5.6	100.7	3.0	103.9	1.4	98.0	1.8	108.6	15.1	100.7	1.3
CAM	100.0	1.0	103.2	4.2	103.6	0.8	106.6	6.0	100.4	2.3	108.8	10.1	105.0	1.5
FBZ	100.0	1.9	99.4	3.2	101.8	4.1	103.3	2.3	100.1	4.0	106.8	15.6	101.7	2.3
OXFEN	100.0	3.3	109.0	3.8	113.0	11.4	108.8	4.6	111.4	2.7	109.6	15.7	104.7	9.7
FBZ-SO_2	100.0	1.8	102.8	0.8	110.5	4.0	111.3	4.2	102.4	6.1	106.5	11.4	100.6	2.3
FLU	100.0	5.2	99.8	1.6	105.2	3.2	102.3	1.1	100.6	4.0	108.6	10.6	103.5	4.7
FLU-NH_2	100.0	8.6	101.7	5.5	102.6	4.5	109.8	3.2	103.0	0.7	111.7	15.9	100.0	1.7
FLU-OH	100.0	4.8	101.9	5.3	100.6	1.7	106.3	3.4	100.6	4.0	107.4	9.7	102.0	2.7
MBZ	100.0	3.2	97.7	6.5	98.5	6.7	106.5	1.6	101.3	1.3	103.6	14.0	101.9	4.7
MBZ-NH_2	100.0	6.2	103.2	3.6	108.1	2.8	113.3	5.1	104.2	3.2	113.5	12.9	102.0	2.5
MBZ-OH	100.0	5.3	99.8	3.4	101.0	2.4	102.1	2.1	97.5	3.9	108.6	16.5	101.6	3.4
OXI	100.0	4.3	103.8	1.4	103.2	3.8	100.0	2.6	104.5	1.4	108.1	16.8	100.5	1.6
TCB	100.0	2.6	101.7	3.5	98.6	4.2	103.4	3.6	98.1	3.1	111.1	15.3	98.2	5.9
TCB-SO	100.0	13.1	101.2	7.4	95.4	22.0	86.0	10.2	114.1	4.4	109.2	23.1	95.7	12.3
TCB-SO2	100.0	7.3	136.9	12.2	182.5	1.3	211.9	0.2	120.8	6.0	138.7	12.0	146.1	9.9
TBZ	100.0	1.2	100.8	1.4	101.2	2.5	103.4	1.8	101.5	0.8	112.0	13.3	101.5	1.9
TBZ-OH	100.0	3.1	105.9	1.3	107.5	6.8	110.6	2.9	104.4	1.6	117.7	15.5	110.9	1.8
LEV	100.0	1.7	99.4	1.5	100.7	0.8	102.9	1.9	101.1	1.2	109.8	11.9	101.8	0.9
CLOR	100.0	5.3	163.0	19.8	108.4	37.3	88.8	31.7	156.7	28.9	193.2	38.7	135.7	29.4
CLOS	100.0	1.7	101.7	0.8	110.9	4.3	129.1	5.9	100.5	2.2	107.3	13.7	104.3	1.8
MOR	100.0	0.9	98.1	3.1	94.2	2.8	95.9	1.8	98.5	1.0	107.8	13.0	99.3	1.2
NICLOS	100.0	5.2	98.3	1.7	104.8	7.5	104.6	4.0	101.5	5.3	110.0	13.7	101.6	4.1

Table S2-8 *continued*

Analyte	Mean (n=3) % analyte remaining at time point (weeks) with RSD (%) at 20/40 ng L^{-1} with RSD values												
	t=0	RSD	Room Temperature (RT)						4 degree Celsius (4 °C)					
			t=1	RSD	t=3	RSD	t=5	RSD	t=1	RSD	t=3	RSD	t=5	RSD
NITROX	100.0	7.6	107.0	11.4	111.1	2.3	107.6	3.4	101.7	14.0	105.7	21.0	107.4	13.5
OXYCLOZ	100.0	6.6	107.5	1.6	113.0	7.6	124.3	21.7	105.7	6.2	116.0	11.6	109.8	5.6
RAFOX	100.0	3.2	100.5	7.7	98.9	5.3	100.0	2.5	101.9	0.2	104.6	13.8	100.2	9.0
MONE	100.0	3.9	135.1	18.2	211.0	18.9	361.8	36.6	111.2	7.0	114.5	16.3	110.5	0.5
MONE-SO_2	100.0	6.5	135.9	16.6	222.9	21.2	351.3	35.7	111.5	5.4	120.5	14.5	112.0	3.3
ABA	100.0	11.1	104.5	4.5	104.0	0.8	102.3	8.5	101.4	9.9	100.6	29.4	83.9	6.0
DORA	100.0	7.0	95.7	2.9	95.3	9.9	93.8	15.8	86.7	4.9	89.6	18.3	97.4	10.7
EMA	100.0	3.3	103.0	4.4	96.4	6.6	101.4	7.8	99.7	2.2	104.7	7.7	100.1	3.1
EPRINO	100.0	4.3	93.4	16.9	94.5	5.4	100.0	5.9	100.7	11.7	91.6	6.8	98.8	1.8
IVER	100.0	2.5	120.4	9.3	104.6	1.8	120.2	11.4	106.6	13.8	116.8	21.0	103.7	8.3
MOXI	100.0	8.2	99.7	8.3	99.4	4.6	106.8	12.8	109.4	5.9	94.3	12.7	96.4	6.0
HALOX	100.0	12.0	97.9	9.7	90.3	6.3	86.5	6.6	100.2	7.0	109.2	15.4	104.3	3.4
COUMA	100.0	10.9	105.1	7.2	104.3	3.0	108.7	6.3	103.4	7.7	111.3	13.3	106.6	7.6
COUMA-O	100.0	6.6	101.0	4.3	96.3	2.8	99.0	1.6	96.8	5.5	105.5	14.2	99.5	9.9
BITH	100.0	9.0	100.1	12.6	97.3	7.8	93.7	9.8	99.8	2.5	108.3	21.3	97.6	6.0

t=0: storage for 0 days

t=1: storage for 1 week

t=3: storage for 3 weeks

t=5: storage for 5 weeks

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Table S2-9 Final extract stability of anthelmintics spiked at 200/400 ng L^{-1} (n=3), presented as the percentage of analyte remaining at different time points (0, 1, 3 and 5 weeks), by comparison to day 0 (t=0)

Analyte	Mean (n=3) % analyte remaining at time point (weeks) with RSD (%) at 200/400 ng L^{-1} with RSD values													
	t=0	RSD	Room Temperature (RT)						4 degree Celsius (4 °C)					
			t=1	RSD	t=3	RSD	t=5	RSD	t=1	RSD	t=3	RSD	t=5	RSD
ABZ	100.0	3.2	101.1	2.8	100.6	1.3	101.8	3.0	100.1	2.6	100.2	6.3	101.4	2.2
ABZ-SO	100.0	2.1	100.7	6.6	101.4	4.7	103.1	3.5	105.5	1.3	104.0	3.9	102.0	3.2
ABZ-SO2	100.0	1.8	98.6	4.6	98.3	1.9	103.4	3.5	98.9	1.1	101.4	2.8	104.2	2.5
ABZ-NH2-SO2	100.0	3.2	99.8	2.5	102.7	4.7	100.0	0.5	98.6	2.2	98.2	1.5	102.5	1.2
CAM	100.0	3.7	102.7	1.3	102.0	2.3	102.2	3.3	103.1	2.3	102.2	2.4	104.1	2.0
FBZ	100.0	1.2	100.0	2.9	100.4	2.3	102.1	2.4	100.3	1.4	99.9	0.8	101.3	0.4
OXFEN	100.0	5.6	97.3	3.6	102.7	4.8	100.9	10.1	103.8	4.4	100.9	2.3	102.6	5.0
FBZ-SO2	100.0	1.9	104.7	2.6	103.5	0.6	106.2	2.6	103.2	1.0	101.4	0.3	105.1	1.0
FLU	100.0	2.1	100.9	2.4	100.2	2.1	103.0	2.2	97.9	2.7	97.2	0.4	99.2	2.5
FLU-NH2	100.0	0.5	101.2	1.3	99.8	2.2	104.4	4.4	99.2	3.6	99.9	6.7	102.5	2.3
FLU-OH	100.0	1.1	101.9	3.8	98.1	1.3	104.5	2.9	106.4	3.5	106.2	3.5	104.4	5.0
MBZ	100.0	2.2	96.1	4.0	104.6	4.0	101.3	7.5	97.9	4.0	102.6	2.8	99.8	1.1
MBZ-NH2	100.0	0.7	101.2	2.8	102.6	1.6	104.9	0.8	101.4	4.8	103.1	7.4	105.1	1.0
MBZ-OH	100.0	1.4	95.6	2.0	99.3	2.1	101.3	2.5	104.1	3.6	101.5	3.1	100.7	2.5
OXI	100.0	2.2	100.9	2.8	99.4	1.0	102.2	2.4	96.8	1.3	99.3	2.3	100.3	1.9
TCB	100.0	2.2	102.9	2.0	103.0	2.5	104.0	4.3	100.6	2.3	102.0	1.8	102.7	2.7
TCB-SO	100.0	0.3	113.9	10.9	110.2	0.9	110.8	6.1	100.7	2.2	109.8	1.9	105.7	2.5
TCB-SO2	100.0	5.7	124.0	1.6	144.2	3.6	182.8	8.1	115.9	3.8	116.5	3.2	122.7	2.6
TBZ	100.0	1.0	99.8	0.6	97.3	2.6	101.2	1.1	98.3	1.2	99.2	1.8	99.8	2.4
TBZ-OH	100.0	2.2	101.3	2.4	112.8	11.1	109.4	1.4	106.0	1.7	109.0	0.2	112.9	0.9
LEV	100.0	1.7	101.2	1.3	101.6	1.0	101.8	1.2	100.5	1.5	101.8	1.2	102.8	1.0
CLOR	100.0	7.2	114.6	14.2	106.1	11.0	101.5	9.1	115.9	6.8	109.8	8.7	108.5	1.8
CLOS	100.0	1.9	101.8	4.7	104.3	4.2	104.7	4.5	103.4	5.8	101.6	2.6	102.0	2.5
MOR	100.0	1.8	99.2	2.3	95.6	3.1	96.1	0.4	98.3	2.0	98.0	2.0	98.7	2.7
NIC	100.0	2.2	100.5	0.8	101.5	3.0	105.0	1.9	99.8	2.1	98.9	1.1	102.8	1.3

Table S2-9 *continued*

Analyte	Mean (n=3) % analyte remaining at time point (weeks) with RSD (%) at 200/400 ng L^{-1} with RSD values													
	t=0	RSD	Room Temperature (RT)						4 degree Celsius (4 °C)					
			t=1	RSD	t=3	RSD	t=5	RSD	t=1	RSD	t=3	RSD	t=5	RSD
NITROX	100.0	5.2	98.3	1.7	104.8	7.5	104.6	4.0	101.5	5.3	110.0	13.7	101.6	4.1
OXY	100.0	1.7	101.2	3.6	95.6	5.2	99.9	1.1	90.6	1.9	94.6	2.9	95.2	1.1
RAFOX	100.0	2.5	98.1	1.3	102.2	1.7	98.7	2.3	97.0	3.8	101.2	0.3	102.4	3.2
MONE	100.0	1.3	160.0	12.4	197.3	31.5	282.0	21.8	106.1	6.0	107.6	4.3	112.9	2.3
MONE-SO2	100.0	0.3	154.8	13.5	196.2	32.7	290.5	20.1	101.7	9.4	102.6	0.7	110.9	4.4
ABA	100.0	4.2	107.6	5.7	104.2	8.4	104.4	10.9	101.0	11.2	96.9	7.8	99.4	7.5
DORA	100.0	2.6	98.0	2.4	96.6	3.6	95.4	3.5	89.5	1.5	88.1	12.3	94.1	5.2
EMA	100.0	3.9	96.8	2.4	96.1	1.3	93.8	5.9	97.0	3.5	100.0	1.4	95.9	2.7
EPRINO	100.0	4.6	107.3	4.4	108.0	1.4	107.1	7.2	102.8	2.9	109.5	9.9	100.5	2.1
IVER	100.0	1.4	104.5	3.0	101.1	2.3	104.7	3.0	100.3	1.2	100.9	4.4	103.0	1.0
MOXI	100.0	1.3	103.5	7.0	100.1	1.9	100.5	5.2	99.1	2.3	100.4	1.0	102.2	2.4
HALOX	100.0	1.3	98.2	3.4	90.4	2.0	89.2	3.0	103.4	3.0	103.9	3.5	107.1	1.4
COUMA	100.0	2.3	100.6	1.9	100.1	2.9	101.5	5.5	103.2	3.0	102.7	4.0	102.7	3.1
COUMA-O	100.0	2.8	100.4	1.0	99.5	2.9	100.3	1.9	104.1	2.9	99.9	1.0	103.5	1.9
BITH	100.0	2.8	97.1	2.7	100.2	1.6	96.8	1.9	94.7	5.9	97.4	5.1	100.4	1.7

t=0: storage for 0 days

t=1: storage for 1 week

t=3: storage for 3 weeks

t=5: storage for 5 weeks

Supplementary Information File SI-3.1

Table S3-1 Preparation of working calibrant solutions from six different intermediate standard solutions

Std. Cal	Volume of intermediate standard (mL)										Total Vol. MeCN (mL)	Final conc. in calibrant group[a:] (ng mL $^{-1}$)					
	WS A		WS B		WS C		WS D		WS E	WS F							
	1 µg mL^{-1}	25 µg mL^{-1}	1 µg mL^{-1}	25 µg mL^{-1}	1 µg mL^{-1}	25 µg mL^{-1}	1 µg mL^{-1}	25 µg mL^{-1}	25 µg mL^{-1}	25 µg mL^{-1}		A	B	C**	D	E	F
1	0.25		0.25		0.25		2.50		0.20	0.40	200	1.25	1.25	1.25	12.5	25	50
2	0.625		0.625		0.625		1.875		0.20	0.25	100	6.25	6.25	6.25	18.75	50	62.5
3		0.20		0.20		0.20		0.20	0.80	1.00	200	25	25	25	25	100	125
4	-	0.50	-	0.50	-	0.50	-	0.50	0.50	0.75	100	125	125	125	125	125	187.5
5	-	1.00	-	0.75	-	0.75	-	1.00	0.75	1.00	100	250	187.5	187.5	250	187.5	250
6	-	1.50	-	1.0	-	1.00	-	1.50	1.00	1.50	100	375	250	250	375	250	375
7	-	2.00	-	1.25	-	1.25	-	2.00	1.25	2.00	100	500	312.5	312.5	500	312.5	500
8		2.50		1.5		1.5		2.50	1.50	2.50	100	625	375	375	625	375	625

* All calibrants were prepared by dilution of the specified volume of each intermediate (A-B and D-F) in Acetonitrile (MECN) ** a second set of calibrants were prepared for group C analytes, with these calibrants prepared in MeCN + 10% formic acid (*v/v*) [a] Analytes within each group are specified in Table 3-1

Table S3-2 Validation criteria adhered to, with corresponding legislative guideline

Parameter	Performance Criteria	Guideline [a]
Identification		
-Points	Minimum of 4	2002/657
-Relative retention (RRT)	≤2.5%	2002/657
-Ion ratio tolerance (ΔR)	20–50%	2002/657
	30%	SANTE
Selectivity	Interferences: ≤ 10% lowest calibrant	2002/657
	Interferences: ≤ 30% lowest calibrant	SANTE
Linearity	Coefficient of determination R^2 ≥0.98	2002/657
	Residuals ± 20%	SANTE
Trueness(WL_R and WL_r)	70–120%	SANTE
Precision (RSD_{wR} and RSD_r)	≤ 20%	SANTE
Recovery	70–120%	SANTE
Limit of Detection (LOD)	S/N ≥3	SANTE
Limit of Quantification (LOQ)[b]	S/N ≥10**[b]	SANTE
Matrix Effects	Enhancement or suppression <20%[c]	SANTE
Retention Time, t_R	± 0.1 min	SANTE

WL_R = within lab reproducibility, WL_r = within lab repeatability, RSD_r = precision represented by relative standard deviation under repeatability conditions, $RSDw_R$ = precision represented by relative standard deviation under reproducibility conditions

[a] SANTE = SANTE/11813/2017 (European Commission, 2017)

2002/657 = European Commission Decision 2002/657/EC (European Commission, 2002)

*[b] LOQ taken as the lowest spiking level meeting the method performance criteria for trueness and precision with a minimum S/N of 10

[c] if more than 20% signal suppression or enhancement, matrix-effects need to be addressed in calibration

Supplementary Information File SI-3.2

Anticoccidial Matrix Stability

A limited stability study was carried out to provide insight on the acceptable storage duration of samples prior to extraction and instrumental determination for the anticoccidials, without compromising the analytical result. A composite water sample was produced by combining aliquots of 8 different unfiltered groundwater samples, of varying pH, DOC content and hardness (as $CaCO_3$). Stability was assessed by spiking aliquots of the water at differing time points (0, 7, 10 and 14 days), with spiking carried out in an order where all samples were extracted and instrumentally analysed in one analytical run. These time points were selected with consideration for the overall logistics of analysis and any subsequent re-analysis. In total, 32 aliquots (250 mL each) of the composite sample were weighed into amber glass bottles (500 mL). In triplicate (n=3) water aliquots were spiked with analytes at two different concentration levels as follows: low concentration (equivalent to CAL L2) corresponding to 2.5 ng L^{-1} (groups A-C), 7.5 ng L^{-1} (group D), 20 ng L^{-1} (group E) and 25 ng L^{-1} (Group F) and high concentration (equivalent to CAL L7) corresponding to 125 ng L^{-1} (Groups B, C, E) and 200 ng L^{-1} (Groups A,D and F) (See Table 3-1 for respective analytes in each group A-F). Once spiked, samples were stored in the cold room (4ºC) for the specified duration storage duration, until analysis. All samples from all four time points for each respective water type were extracted as per the procedure previously described (Section 3.2) on the same day, t=0, and injected together as one batch, with injections of replicates in random order. For all time points, internal standard (100 µL) was added just prior to extraction on day t=0. Analytes were deemed stable if the change in measures concentration in comparison to day 0 (t=0) was <15%.

The results of this stability study, at both concentration levels, are as summarised in Table S3-3 below. At the higher concentrations (125/200 ng L^{-1}), all analytes were stable for up to 14 days storage, except for halofuginone, which showed a decrease on concentration to 83% (t=14) of the initial concentration on day 0 (t=0). Acceptable stability was demonstrated for halofuginone at this higher concentration for up to 10 days storage. Some instability of several analytes, namely amprolium, monensin and salinomycin, was demonstrated at the lower concentrations (2.5/7.5/20/25 ng L^{-1}) after 10 days of storage, with analyse concentrations dropping to 82, 79 and 84 % respectively. However, all analytes showed acceptable stability for storage up to 7 days, and for this reason 7 days was selected as the overall maximum stage time for samples, without compromising the analytical integrity.

Table S3-3 Matrix stability results for 26 anticoccidial compounds in environmental water at two concentrations (equivalent to CAL L2 and L7), presented as the percentage of analyte remaining at different time points (0, 7, 10 and 14 days), by comparison to day 0 (t=0)

Analyte	Mean (n=3) % analyte remaining at time point (days) with RSD (%) at:															
	[LOW] (CAL L2: 2.5/7.5/20/25 ng L^{-1})								[HIGH] (CAL L7: 125/ 200 ng L^{-1})							
	t= 0	RSD	t=7	RSD	t=10	RSD	t=14	RSD	t= 0	RSD	t=7	RSD	t=10	RSD	t=14	RSD
Aklomide	100.0	4.4	100.4	6.1	101.4	9.7	99.8	0.8	100.0	1.6	98.9	2.4	97.1	5.0	104.5	6.8
Amprolium	100.0	2.5	89.3	2.6	82.1	3.7	83.8	2.4	100.0	5.8	97.4	1.5	97.2	3.1	92.2	9.8
ANOT	100.0	4.8	100.0	2.5	98.4	6.8	92.1	3.4	100.0	5.0	94.1	3.2	90.0	0.6	88.1	3.7
Arprinocid	100.0	0.6	97.1	2.5	96.0	2.9	95.4	1.3	100.0	2.9	99.5	0.4	95.5	3.8	94.4	2.6
Buquinolone	100.0	0.8	98.2	0.6	95.4	1.6	95.4	1.4	100.0	1.4	98.1	0.5	93.7	2.8	92.3	2.3
Clopidol	100.0	1.4	95.1	1.4	95.4	1.8	94.0	0.9	100.0	0.2	100.1	1.2	100.4	2.0	97.6	1.6
Cyromazine	100.0	1.1	99.1	1.9	96.2	0.9	101.0	3.8	100.0	1.4	101.3	1.0	99.2	1.5	94.3	8.4
Decoquinate	100.0	0.9	99.3	0.8	98.7	0.4	96.5	1.6	100.0	1.2	98.7	1.2	96.2	1.0	92.8	0.5
Diaveridine	100.0	2.1	96.3	2.4	93.4	2.2	90.1	4.0	100.0	1.1	100.9	1.5	98.4	1.8	95.1	2.8
Diclazuril	100.0	1.2	97.1	0.4	95.5	2.3	92.4	2.5	100.0	1.8	97.0	1.3	91.1	1.2	93.9	2.6
Dinitolmide	100.0	1.0	101.9	1.0	101.1	1.2	101.3	2.7	100.0	1.8	96.6	1.0	88.7	0.7	90.7	2.2
Ethopabate	100.0	2.5	101.4	1.4	97.5	0.7	101.4	2.4	100.0	1.6	98.0	1.7	91.1	0.8	90.6	1.6
Halofuginone	100.0	1.4	99.2	3.1	98.2	2.0	96.4	1.3	100.0	1.3	97.6	1.9	91.2	1.5	83.1	1.0
Lasalocid	100.0	1.3	97.3	2.6	89.3	1.6	90.2	1.1	100.0	1.4	100.7	3.3	103.2	0.9	99.1	1.0
Maduramicin	100.0	2.7	101.4	3.5	96.8	3.5	95.0	0.3	100.0	10.4	95.1	3.5	90.8	7.7	89.3	2.5
Monensin	100.0	4.5	87.1	3.4	78.9	1.5	73.4	2.5	100.0	2.1	95.5	0.9	90.4	1.0	103.2	1.6
Narasin	100.0	1.3	94.3	1.4	89.4	1.0	84.3	2.0	100.0	0.5	97.9	0.7	91.6	0.6	94.6	1.0
Nequinate	100.0	0.8	97.4	1.9	94.6	1.6	95.6	0.6	100.0	1.7	99.6	1.3	97.3	1.5	94.0	2.8
Nicarbazin	100.0	1.6	99.5	0.9	98.8	1.1	99.7	0.8	100.0	0.7	99.2	1.2	95.1	0.9	93.0	2.1
Nitromide	100.0	2.0	95.9	2.0	93.4	1.7	94.8	1.8	100.0	3.3	98.1	1.0	86.6	2.1	87.9	1.9
Robenidine	100.0	0.3	99.0	0.4	97.6	0.5	97.2	0.8	100.0	1.1	100.4	1.0	98.0	1.3	96.3	2.7
Salinomycin	100.0	3.0	91.4	2.6	84.1	0.6	78.5	2.2	100.0	2.2	97.6	0.6	89.6	0.5	98.1	0.9
Semduramicin	100.0	10.3	98.3	3.5	91.7	11.8	85.2	2.2	100.0	4.1	100.2	4.7	93.3	1.1	105.0	6.4
Toltrazuril	100.0	0.8	96.0	1.0	94.6	2.3	92.3	1.4	100.0	2.6	99.4	1.3	97.0	1.9	100.7	1.3
Toltrazuril sulphoxide	100.0	1.0	96.2	0.8	95.4	1.5	95.0	1.0	100.0	1.4	98.6	1.5	96.7	1.3	95.9	1.6
Toltrazuril sulphone	100.0	1.7	97.5	1.5	96.4	0.8	97.8	2.0	100.0	2.4	99.0	1.2	98.3	4.2	100.3	0.7

Supplementary Information File SI-4.1

Further detail and description of site properties used for the characterisation of groundwater sampling sites for anthelmintics, for the purpose of statistical analysis

- **Bedrock geology** was interpreted from the GSI Hydrostratigraphic Rock Units Group dataset (GSI, 2016a). This map is a reclassification of the 1:100,000 bedrock geology map, with over 1,200 bedrock Formations and Members grouped into 27 Rock Unit Groups, categorised based on their hydrogeological properties and other factors from the original bedrock geology datasets. These 27 Rock Unit group were further amalgamated into 6 lithological groups, as described by (Tedd et al., 2017) and are as summarised in Table S4-3 below.
- **GSI aquifer category** was determined using the Geological Survey of Ireland classification system. GSI's Aquifer classes are divided into three main groups based on their resource potential (Regionally Important, Locally Important or Poor Aquifers), and further subdivided based on the type of openings through which groundwater flows (DELG/EPA/GSI, 1999). For this study, the GSI Bedrock Aquifer (GSI, 2015b) and GSI Sand and Gravel Aquifer (GSI, 2015c) datasets were combined to form an overall classification system with 11 different Aquifer classes/categories as described in Table S4-3.
- **WFD flow regime**: The 11 GSI aquifer classes were amalgamated into four categories, to give what is referred to as the WFD Ireland Aquifer Categories, sometimes referred to as WFD flow regime or general groundwater types (Fitzsimons et al., 2005). This system groups the GSI aquifer categories together based on a number of similarities including hydrogeological properties and influence on surface water characterisation, as described in the WFD technical requirements for groundwater and related aspects,

guidance document GW1 (Working Group on Groundwater, 2001). The four WFD flow regime categories are karstic, productive fractures, poorly productive and intergranular, with the respective GSI aquifer categories which make up each of these, as listed summarised in Table S4-3.

- **Groundwater vulnerability** is classified into four main categories; Extreme (E), High (H), Moderate (M) and Low (L), based primarily on the subsoil permeability and thickness (depth to bedrock) (DELG/EPA/GSI, 1999). Data for groundwater vulnerability was obtained from the GSI Groundwater Vulnerability Map (1:40,000) dataset (GSI, 2015d). In this dataset, the Extreme vulnerability category is split into two categories, Extreme-X (X) and Extreme-E (E), resulting in an overall five different vulnerability classes. In this case, Extreme-E describes areas with 0-3m subsoil thickness and Extreme-X, covering areas of bedrock outcrop or shallow rock, with generally <1m soil/subsoil thickness (Daly, 2004).
- **Quaternary sediment**, interchangeably called Quaternary deposits, and commonly referred to as subsoils in Ireland, are surficial deposits made during the Quaternary age. The majority of Irelands bedrock is overlain by subsoils, and it is the properties of such subsoils that can influence the transport of contaminants to groundwater stored in the bedrock. Subsoil data were obtained from the GSI Quaternary Sediments Map (1:50,000) (GSI, 2016b) which contains detail of 53 different sediment classes, which were amalgamated into seven main genesis, based on their parent material (Table S4-4), using an approach similar to that described by (Fealy et al., 2009).

Table S4-1 Summary of site characteristics of the 11 karstic sites sampled as part of the temporal occurrence study

Site Name	Description		ZOC area (km^2)	Stocking density LU/Ha)	N ha^{-1} (kg/ha)	LPIS Crop type	Bedrock Geology[a]	Aquifer Category[a]	Flow[a]	Groundwater Vulnerability[a]	Quaternary Deposit[a]	Subsoil Permeability[a]	IFS soil type[a]
	Matrix	Type											
Clare A	GW	SP	63.6	0.854	62.4	Grass	DPBL	Rkc	Conduit	X	KaRck	DTB<3m	BminSW
Clare B	GW	SP	139.3	0.510	37.3	Grass	DPBL	Rkc	Conduit	X	KaRck	DTB<3m	BminSW
Clare C	GW	SP	426.2	0.683	50.6	Grass	DPBL	Rkc	Conduit	X	KaRck	DTB<3m	BminSW
Clare D	GW	SP	456.3	0.690	49.5	Grass	DPBL	Rkc	Conduit	X	KaRck	DTB<3m	BminSW
Ros A	GW	SP	24.9	1.238	87.1	Grass	DPBL	Rkc	Conduit	H	TLs	M	BminDW
Ros B	SW	SH	44.6	1.092	79.7	Grass	DPBL	Rkc	Conduit	H	TLs	DTB<3m	AminDW
Ros C	GW	SP	17.4	1.027	74.3	Grass	DPBL	Rkc	Conduit	E	TLs	DTB<3m	BminDW
Ros D	SW	SH	17.4	1.030	74.3	Grass	DPBL	Rkc	Conduit	E	TLs	DTB<3m	BminDW
Ros E	GW	SP	17.1	0.922	65.3	Grass	DPBL	Rkc	Conduit	E	TLs	DTB<3m	BminPD
Ros F	SW	SH	17.1	0.920	65.3	Grass	DPBL	Rkc	Conduit	E	TLs	DTB<3m	BminPD
Ros G	GW	SP	52.4	0.936	68.4	Grass	DPBL	Rkc	Conduit	H	TLs	M	AminPD

[a] predominant class within the ZOC

GW= Groundwater, SW = Surface water, SP = Spring, SH = Swallow hole/ sinking stream, LU = livestock unit, ha = hectare, LPIS = Land Parcel Information System, DPBL = Dinantian Pure Bedded Limestones, Rkc = Regionally Important Aquifer- Karstified (conduit dominant), X = Extreme (exposed), E = Extreme, H = High, KaRck = Karstified Rock, TLs = Tills derived from limestones, BminSW - Shallow well drained mineral (Mainly basic), BminDW - Deep well drained mineral (Mainly basic), BminPD - Mineral poorly drained (Mainly basic), AminPD - Mineral poorly drained (Mainly acidic)

Table S4-2 Assignment of 95 LPIS crop descriptions into the four land-use categories

Crop Description	LPIS land-use assignment	Crop Description	LPIS land-use assignment
Farm Road	Farmyard	Reed Canary Grass	Other
Farmyard	Farmyard	REPS 3 New Habitat	Other
Grass	Grass	REPS 4 New Habitat	Other
Grass Seed	Grass	REPS 4 New Woodland	Other
Grass Silage	Grass	REPS 4 Orchard	Other
Grass Year 1	Grass	REPS 4 Planted Buffer Zone	Other
Grass Year 2	Grass	Riparian Zone	Other
Grass Year 3	Grass	Rocky Outcrop	Other
Grass Year 4	Grass	Scrub	Other
Grass Year 5	Grass	Short Rotation Coppice	Other
Mixed Grazing	Grass	Unknown	Other
Permanent Pasture	Grass	Willow	Other
Rough Grazing	Grass	Woodland	Other
Species Rich Grassland	Grass	Arable Silage	Tillage
Switchgrass	Grass	Beans	Tillage
Trad. Sustainable Grazing	Grass	Camelina	Tillage
Traditional Hay Meadow	Grass	Clover	Tillage
100% Destocked Area	Other	Early Potatoes	Tillage
Access Road / Roadways	Other	Flax	Tillage
Arable Habitat	Other	Fodder Beet	Tillage
Bog	Other	Forage Rape	Tillage
Building	Other	Green Cover	Tillage
Designated Habitat	Other	Kale	Tillage
Fallow	Other	Linseed	Tillage
Flowers	Other	Lucerne	Tillage
Foliage	Other	Maincrop Potatoes	Tillage
Forestry	Other	Maize	Tillage
Forestry 2010	Other	Millet	Tillage
Forestry 2011	Other	Peas	Tillage
Forestry 2012	Other	Rye	Tillage
Forestry 2013	Other	Seed Potatoes	Tillage
Forestry 2014	Other	Spring Barley	Tillage
Forestry Eligible	Other	Spring Oats	Tillage
Forestry Setaside	Other	Spring Oilseed Rape	Tillage
Former REPS 3 New Habitat	Other	Spring Wheat	Tillage
Former REPS 4 New Habitat	Other	Sugar Beet	Tillage
Fruit	Other	Swede	Tillage
Gardens	Other	Triticale	Tillage
Habitat	Other	Turnips	Tillage
Invalid Crop	Other	Vegetables	Tillage
Lake / Waterway / Pond	Other	Wild Bird Cover	Tillage
Landscape Feature	Other	Winter Barley	Tillage
Linnet Habitat	Other	Winter Oats	Tillage
Miscanthus Sinensis	Other	Winter Oilseed Rape	Tillage
Nursery	Other	Winter Wheat	Tillage
Orchard	Other		
Planted Buffer Zone	Other		
Quarry	Other		
Re-generation	Other		
Recreational Area	Other		

Table S4-3 Summary of some of the main land use and physical hydrogeological site properties, used to characterise groundwater sites for statistical analysis, with the corresponding national dataset source

Property	**MP Type**	**Corine Land Cover**	**LPIS Land-Use**	**Bedrock Geology**	**Aquifer Category**	**Flow Regime**	**Groundwater vulnerability**	**Irish Forestry Soils (IFS)**	**Quaternary Sediments**	**Subsoil Permeability**
Data source	EPA	Corine Land Cover 2012 Digital Map	DAFM Land-Parcel Information System	Hydrostratigraphic Rock Units Group Map 1:100,000 (Digital) GSI	Groundwater Bedrock Aquifers Map 1:100,000 & Gravel Aquifers 1:50,000(Digital)	Amalgamated from GSI Aquifer Categories	Groundwater Vulnerability Map1:40,000 (Digital) GSI	IFS National Soil Map 1:50,000 (Digital) from the EPA	Quaternary Sediments Map 1:50,000 (Digital) GSI	Groundwater Subsoil Permeability Map 1:40,000 (Digital) GSI
Ref	(EPA, 2011)	(EPA Ireland, 2012)	(DAFM, 2014)	(GSI, 2016a) (Tedd et al., 2017)	(GSI, 2015b) (GSI, 2015c)	(Working Group on Groundwater, 2001)	(GSI, 2015d)	(Teagasc-EPA-GSI, 2006)	(GSI, 2016b).	(GSI, 2015a)
Classes	**Borehole** • Abstraction • Monitoring • **Spring** • **Turlough**	• **Arable** • **Non-arable (pasture)** • **Forest** • **Other** ○	95 crop descriptions amalgamated into 4 land-uses (Table S4-2) • **Farmyard** • **Grass** • **Tillage** • **Other**	27 rock units amalgamated into six lithological groups • **Sand and gravel** • **Impure limestone** • **Pure limestone** • **Non-calcareous sedimentary** • **Igneous** • **Metamorphic**	11 classes as follows*: • **Regionally Important** ○ Rk ○ Rkc ○ Rkd ○ Rf ○ Rg • **Locally Important** ○ Lm ○ Lk ○ Ll ○ Lg • **Poor Aquifer** ○ Pl ○ Pu	• Karstic conduit • Karstic diffuse • Fractured **WFD regimes** • Karstic ○ Rk, Rkc, Rkd & Lk • Productive fractured ○ Rf & Lm • Poorly productive ○ Ll, Pl and Pu • Intergranular (Rg & Lg)	• **X**- Extreme (exposed) • **E** – Extreme • **H**- High • **M**-Moderate • **L**-Low	**Type I:** Acid vs. Base **Type II:** Mineral vs Peat **Type III:** Deep vs. Shallow **Type IV:** Wet vs. Dry	**(Genesis)** • Alluvium • Irish Sea Tills • Karstified rock • Peat • Sand and Gravels • Tills • Bedrock at surface	• **High** • **Moderate** • **Low** • **DTB<3m ****

*Rk = Regionally Important Aquifer-Karstified , Rkc = Regionally Important Aquifer-Karstified (conduit flow), Rkd = Regionally Important Aquifer-Karstified (diffuse flow), Rf = Regionally Important Aquifer – Fissured bedrock, Lm = Locally Important Aquifer – Bedrock which is Generally Moderately Productive, Lk = Locally Important- Karstified, Ll = Locally Important Aquifer - Bedrock which is Moderately Productive only in Local Zones, Pl = Poor Aquifer - Bedrock which is Generally Unproductive except for Local Zones and Pu = Poor Aquifer - Bedrock which is Generally Unproductive Rg = Regionally Important Gravel Aquifers, Lg = Locally Important gravel aquifer

**Subsoil permeability could not be ranked for areas with less than 3 meters depth to bedrock, and were therefore assigned as DTB<3m

Table S4-4 List of the different Quaternary sediments throughout Ireland, with corresponding code, adapted from (Fealy et al., 2009)

Parent Material/ Quaternary Sediment	**Map Code**
Tills	
Sandstone and shale till (Cambrian/Precambrian)	TCSsS
Sandstone till (Lower Palaeozoic)	TLPSs
Shale till (Lower Palaeozoic)	TLPS
Sandstone and shale till (Lower Palaeozoic)	TLPSsS
Sandstone till (Lower Palaeozoic/Devonian)	TLPDSs
Sandstone till (Devonian)	TDSs
Sandstone till (Devonian/Carboniferous)	TDCSs
Sandstone and shale till (Devonian/Carboniferous)	TDCSsS
Limestone till (Carboniferous)	TLs
Shale and sandstone till (Namurian and Carboniferous)	TNCSSs
Shale and sandstone till (Namurian)	TNSSs
Chert till	TCh
Carboniferous sandstone and Chert till	TCSsCh
Quartzite till	TQz
Acid volcanic till	TAv
Granite till	TGr
Basic igneous till	TBi
Metamorphic till	TMp
Irish Sea Tills	
Sandstone and shale till (Cambrian/Precambrian) of Irish Sea Basin	IrSTCSsS
Sandstone till (Devonian) with matrix of Irish Sea Basin origin	IrSTDSs
Sandstone and shale till (Lower Palaeozoic) with matrix of Irish Sea Basin origin	IrSTLPSsS
Limestone till (Carboniferous) with matrix of Irish Sea Basin origin	IrSTLs
Sandstone till with matrix of Irish Sea Basin origin	IrSTSs
Acid volcanic till with matrix of Irish Sea Basin origin	IrSTAv
Glaciofluvial Sands and Gravels	
Acidic esker sands and gravels	AcEsk
Basic esker sands and gravels	BasEsk
Sandstone and shale sands and gravels (Cambrian/Precambrian)	GCSsS
Sandstone sands and gravels (Lower Palaeozoic)	GLPSs
Shale sands and gravels (Lower Palaeozoic)	GLPS
Sandstone and shale sands and gravels (Lower Palaeozoic)	GLPSsS
Sandstone sands and gravels (Lower Palaeozoic/Devonian)	GLPDSs
Sandstone sands and gravels (Devonian)	GDSs
Sandstone sands and gravels (Devonian/Carboniferous)	GDCSs
Limestone sands and gravels (Carboniferous)	GLs
Shale and sandstone sands and gravels (Namurian)	GNSSs
Chert sands and gravels	GCh
Quartzite sands and gravels	GQz
Granite sands and gravels	GGr
Basic igneous sands and gravels	GBi
Metamorphic sands and gravels	GMp
Alluvium	
Alluvium undifferentiated	A
Gravelly	Ag
Silty	As
Clayey	Ac
Peat	
Blanket peat	BktPt
Raised peat	RsPt
Fen peat	FenPt
Cutover peat	Cut

Bedrock at Surface	
Bedrock at surface	Rck
Bedrock at or near surface-Non calcareou1	RckNCa
Karstified Rock	
Karstified limestone bedrock at surface	KaRck
Bedrock at or near surface-Calcareous2	RckCa
Other	
Aeolian Sediments undifferentiated	Aeo
Blown sand	Ws
Blown sand in dunes	Wsd
Marl (Shell)	Mrl
Scree	Scree
Made ground	Made
Marsh	Marsh
Tidal marsh	TdlMr
Marine Deposits	
Raised beach sands and gravels	MGs
Beach sand	Mbs
Marine silts	Msi
Marine clays	Mc
Estuarine sediments (silts/clays)	Mesc
Glaciolacustrine deposits:	
Lake sediments undifferentiated	L
Sandy	Ls
Silty	Lsi
Clayey	Lc

Table S4-5 Statistical p-values for analysis of source factors (land-use) for detections defined for all anthelmintic compounds, benzimidazole compounds only, non-benzimidazole compounds, parent compounds only and transformation products (TPs) only.

Source Factor	*p* value with detections defined as:				
	All anthelmintics	**Benzimidazoles**	**Non-benzimidazoles**	**Parent Compound**	**Transformation Product**
Categorical variables					
Corine (Level 3 detail)	0.0880	0.0114	0.5030	0.2407	0.0758
Corine (amalgamated)	0.0913	0.0119	0.5110	0.2469	0.0779
LPIS land-use category	0.0880	0.0114	0.5030	0.2407	0.0758
Continuous variables					
% Agricultural land in ZOC	0.0093	0.1287	0.0143	0.0004	0.2144
% Grassland in ZOC	0.4980	0.0119	0.5545	0.8675	0.0860
% Tillage in ZOC	0.1088	0.0061	0.4921	0.2818	0.0479
% Farmyard in ZOC	0.3541	0.1255	0.9853	0.2764	0.0976
% Other in ZOC	0.3479	0.0976	0.7912	0.3283	0.2179
LU ha^{-1}*	0.9307	0.5149	0.6978	0.5813	0.8254
Density dairy cow	0.2617	0.5135	0.6339	0.5558	0.5276
Density suckler cow	0.9913	0.2504	0.4109	0.7074	0.4790
Density cattle 0-1yr	0.1449	0.5305	0.1049	0.2002	0.7314
Density cattle 1-2yrs	0.0767	0.5665	0.0577	0.1086	0.9978
Density cattle >2yrs	0.2519	0.1102	0.9249	0.3578	0.0578
Density of other cows	0.1732	0.0763	0.9055	0.3256	0.0626
Density ewes	0.0036	0.6595	0.0001	0.0012	0.6948
Density rams	0.0226[a]	0.8108	0.0086[a]	0.0138[a]	0.5672
Density of other sheep	0.0128	0.2816	0.0004	0.0042	0.4003
Nitrogen per Hectare (NPH)	0.4748	0.3658	0.6445	0.6282	0.6498

[a] Although $p<0.05$, interpretation of these results is not practical due to the outcome dependence on only a limited number of sites producing a questionable model fit

* LU = Livestock Unit per Hectare, calculated based on the area of each site ZOC

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Table S4-6 Statistical p values for analysis of pathway factors (hydrogeological factors) for detections defined for all anthelmintic compounds, benzimidazole compounds only, non-benzimidazole compounds, parent compounds only and transformation products (TPs) only.

Pathway Factor	*p* value with detections defined as:				
	All anthelmintics	Benzimidazoles	Non-benzimidazoles	Parent Compound	Transformation Product
Sampling point type	0.0419	0.0681	0.0564	0.0368	0.1622
Predominant Bedrock geology*	0.0524	0.0399	0.0897	0.0490	0.0720
Predominant GSI Aquifer Category *	0.2161	0.064	0.1675	0.0936	0.0346
% of Rkc aquifer in ZOC	0.8999	0.2568	0.4035	0.6083	0.3650
% of Rkd aquifer in ZOC	0.0971	0.6228	0.0516	0.0281	0.7716
% of Rf aquifer in ZOC	0.8675	0.5985	0.5985	0.7584	0.5556
% of Lm aquifer in ZOC	0.9491	0.6490	0.6490	0.8275	0.6010
% of Ll aquifer in ZOC	0.5344	0.0378	0.4299	0.9777	0.0181
% of Pl aquifer in ZOC	0.3624	0.6656	0.0239	0.1555	0.1165
% of Pu aquifer in ZOC	0.2354	0.0917	0.8038	0.1676	0.0756
Flow regime	0.1270	0.1373	0.1998	0.0618	0.2349
WFD Flow regime	0.2083	0.0768	0.6143	0.3178	0.1403
Predominant Groundwater vulnerability	0.3500	0.6737	0.5116	0.1136	0.7816
Groundwater vulnerability % X+E+H	0.4639	0.6257	0.7322	0.5769	0.6050
Groundwater vulnerability % E	0.1312	0.3151	0.1655	0.0879**	0.1732
Irish Forestry Soils Full II	0.0172	0.0794	0.0843	0.0806	0.2520
Irish Forestry Soils Type I	0.1531	0.1175	0.3869	0.1975	0.1971
Irish Forestry Soils Type II	0.8294	0.5776	0.5776	0.7272	0.5372
Irish Forestry Soils Type IIII	0.0266	0.0897	0.1072	0.1112	0.2882
Irish Forestry Soils Type IV	0.0198	0.2885	0.0593	0.0422	0.2227
Predominant Quaternary Genesis I	0.0142	0.0029	0.0891	0.1605	0.0738
Predominant Quaternary Genesis II	0.1073	0.0243	0.2381	0.4080	0.1995
Predominant Subsoil permeability	0.7646	0.0630	0.3997	0.8145	0.1710

* supplemented by a Fischer's exact test, due to non-convergence of the logistic regression

** odds ratio confidence intervals do not meet acceptance criteria for significant result

Rkc = Regionally Important Aquifer-Karstified (conduit flow), Rkd = Regionally Important Aquifer-Karstified (diffuse flow), Rf = Regionally Important Aquifer – Fissured bedrock, Lm = Locally Important Aquifer – Bedrock which is Generally Moderately Productive, Ll = Locally Important Aquifer - Bedrock which is Moderately Productive only in Local Zones, Pl = Poor Aquifer - Bedrock which is Generally Unproductive except for Local Zones and Pu = Poor Aquifer - Bedrock which is Generally Unproductive

Table S4-7 Summary of the anthelmintic compounds (and associated concentrations) detected at the four Clare sites (Clare A-D) during the 13-month temporal study

Month	Year	Anthelmintic detected (ng L^{-1})										
		ABZ	ABZ-SO	$ABZ\text{-}SO_2$	$ABZ\text{-}NH_2\text{-}SO_2$	FBZ	OXF	TCB	LEV	CLOS	OXY	IVER
Clare Site A												
November	2017	n.d.	n.d.	n.d.	3.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
December	2017	-	-	-	-	-	-	-	-	-	-	-
January	2018	n.d.	n.d.	n.d.	3.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	3	5.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
March	2018	n.d.	n.d.	6.7	6.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
April	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
May	2018	n.d.	n.d.	n.d.	11.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
June	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
July	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
August	2018	n.d.	n.d.	n.d.	30.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
September	2018	n.d.	n.d.	n.d.	11.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	3.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
November	2018	-	-	-	-	-	-	-	-	-		-
Clare Site B												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
December	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
March	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
April	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
June	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
July	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
August	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
September	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
November	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table S4-7 *continued*

Month	Year	Anthelmintic detected (ng L^{-1})										
		ABZ	ABZ-SO	$ABZ\text{-}SO_2$	$ABZ\text{-}NH_2\text{-}SO_2$	FBZ	OXF	TCB	LEV	CLOS	OXY	IVER
Clare Site C												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
December	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	n.d.	n.d.	n.d.	2.3	3.7	n.d.	n.d.	n.d.	n.d.	n.d.
March	2018	n.d.	n.d.	n.d.	n.d.	8.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
April	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
June	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
July	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
August	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
September	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
November	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Clare Site D												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ>LOD
December	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.9	n.d.	34.9
March	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.1	n.d.	n.d.	n.d.	47.5
April	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.9	13.6	n.d.	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
June	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
July	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10.4
August	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	50.3	9.5	n.d.	n.d.
September	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	25.8	4.7	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.3	n.d.	n.d.	10.0
November	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.4	n.d.	n.d.	<LOQ>LOD

<LOQ>LOD indicates the analyte was detected at levels that were not quantifiable, however were greater than the method detection limit, thus present

n.d. = not detected

Table S4-8 Summary of the anthelmintic compounds (and associated concentrations) detected at the seven Roscommon sites (Roscommon A-G), during the 13-month temporal study

Month	Year	Anthelmintic detected (ng L^{-1})										
		ABZ	ABZ-SO	$ABZ\text{-}SO_2$	$ABZ\text{-}NH_2\text{-}SO_2$	FBZ	OXF	TCB	LEV	CLOS	OXY	IVER
Roscommon Site A												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
December	2017	-	-	-	-	-	-	-	-	-	-	-
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.5	n.d.
March	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	17.4	n.d.
April	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
June	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
July	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
August	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
September	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
November	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Roscommon Site B												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
December	2017	-	-	-	-	-	-	-	-	-	-	-
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.2	n.d.	11.3	n.d.
March	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	27.3	n.d.
April	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.6	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.2	n.d.	10.0	n.d.
June	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.9	n.d.	n.d.	n.d.
July	2018	-	-	-	-	-	-	-	-	-	-	-
August	2018	-	-	-	-	-	-	-	-	-	-	-
September	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.3	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
November	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table S4-8 *continued*

Month	Year	Anthelmintic detected (ng L^{-1})										
		ABZ	ABZ-SO	ABZ-SO_2	ABZ-NH_2-SO_2	FBZ	OXF	TCB	LEV	CLOS	OXY	IVER
Roscommon Site C												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
December	2017	-	-	-	-	-	-	-	-	-	-	-
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	1.3	2.1	5.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
March	2018	6.2	14	39.6	9.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
April	2018	n.d.	5.1	18.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
June	2018	n.d.	9.3	13.4	21.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
July	2018	-	-	-	-	-	-	-	-	-	-	-
August	2018	n.d.	n.d.	20.9	27.3	n.d.	n.d.	20.0	n.d.	n.d.	n.d.	n.d.
September	2018	n.d.	n.d.	14.1	40.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	20.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
November	2018	n.d.	n.d.	n.d.	5.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Roscommon Site D												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
December	2017											
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	5.4	8.6	5.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
March	2018	n.d.	6.7	14.8	5.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
April	2018	n.d.	n.d.	n.d.	9.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10.6	n.d.	n.d.	n.d.	n.d.
June	2018	n.d.	n.d.	2.0	7.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
July	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.7	n.d.	n.d.	n.d.	n.d.
August	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
September	2018	n.d.	n.d.	n.d.	5.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
November	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table S4-8 *continued*

Month	Year	Anthelmintic detected (ng L^{-1})										
		ABZ	ABZ-SO	$ABZ\text{-}SO_2$	$ABZ\text{-}NH_2\text{-}SO_2$	FBZ	OXF	TCB	LEV	CLOS	OXY	IVER
Roscommon Site E												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.7	n.d.	n.d.	n.d.
December	2017	-	-	-	-	-	-	-	-	-	-	-
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.8	n.d.
March	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13.3	n.d.
April	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.7	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.7	8.1	n.d.	n.d.	n.d.
June	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.7	n.d.	n.d.	n.d.
July	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
August	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.3	19.6	n.d.	n.d.	n.d.
September	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.4	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.7	n.d.	n.d.	n.d.
November	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.9	n.d.	n.d.	n.d.
Roscommon Site F												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
December	2017	-	-	-	-	-	-	-	-	-	-	-
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10.8	n.d.
March	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.7	n.d.
April	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.9	n.d.	n.d.	n.d.
June	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.5	n.d.	n.d.	n.d.
July	2018	-	-	-	-	-	-	-	-	-	-	-
August	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.5	n.d.	n.d.	n.d.
September	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.5	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
November	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table S4-8 *continued*

Month	Year	Anthelmintic detected (ng L^{-1})										
		ABZ	**ABZ-SO**	**ABZ-SO_2**	**ABZ-NH_2-SO_2**	**FBZ**	**OXF**	**TCB**	**LEV**	**CLOS**	**OXY**	**IVER**
Roscommon Site G												
November	2017	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
December	2017	-	-	-	-	-	-	-	-	-	-	-
January	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
February	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
March	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
April	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
May	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
June	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
July	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
August	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	16.3	n.d.
September	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
October	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
November	2018	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

<LOQ>LOD indicates the analyte was detected at levels that were not quantifiable, however were greater than the method detection limit, thus present

n.d. = not detected

Supplementary Information File SI-4.2

Supplementary File SI-4.2 is provided in the format of an excel datasheet, which can be accessed using the following link:

https://drive.google.com/file/d/1_lPXtycSMCjetD7yVyGwa9zTwY7HnDQg/view?usp=sharing

Supplementary Information File SI-5.1

1. Further detail and description of the additional site properties and water quality parameters used for classification of sites for anticoccidials, for the purpose of statistical analysis

In addition to the site properties used in the final site selection (Section 5.2 of Chapter 5), several other physical site properties were also used for statistical analysis, to investigate any association between anticoccidial detections, and these site characteristics. Below is a more detailed description of these properties, subdivided as either land-use source factors, or physical hydrogeological pathway factors.

1.1 Land-use properties (source factors)

Land use was obtained from CORINE (Co-ORdinated INformation on the Environment) datasets, which consists of geo-spatial information on natural and built environments across Europe. Data for Ireland, was taken from the CORINE Land Cover (CLC) 2012 (Lyndon and Smith, 2014) dataset, downloaded as an ArcGis layer form from the EPA (EPA Ireland, 2012). The CLC 3-tiered hierarchy nomenclature system allows for categorisation of land-use data at three levels, each of differing detail : level 1 (5 classes), which can be further subdivided into 15 level-2 classes, and furthermore into an the most detailed level-3 system, comprising of 44 different classes. An overview of the different classes, of each of these levels is as shown in Figure S5-1 below. For more accurate statistical analysis the Corine land cover dataset was classified using an alternative approach which segregated the 44 “level 3” classes, into four amalgamated classes as described in Table 5-2 of the Chapter.

There are two main potential sources of anticoccidials from poultry activity; (a) from usage at the location of the poultry farm and/or (b) from spreading of poultry manure, which is very often transported away from the poultry farm location. Use of the Corine Land Cover data is too non-specific and not sensitive enough to adequately account for poultry activity. Therefore, in order to take poultry source factors into consideration when selecting sites, data were obtained from the Department of Agriculture, Fisheries and Marine (DAFM) (unpublished data) on poultry activity within the ZOC of each potential sampling site. These data was provided in the form of poultry premise locations (referred to hereafter as poultry farms), which is retained in a poultry premises register by the DAFM, as required under S.I.

No. 114 of 2014 (Government of Ireland, 2014b). Due to restrictions under the General Data Protection Regulations (GDPR)(European Parliament, 2016), this information could only be provided as individual location points, in the form of a GIS layer of location points, for each registered poultry farm in the Republic of Ireland at that time. Consequently, while each location point represented a poultry farm, the relative scale of the number of birds housed at each premises was not provided. As a result, a limitation of this dataset is that a location point could describe a farm housing anywhere from 1 to >100,000 birds. In addition to poultry farm locations, some limited information was also provided on poultry manure spreading. Poultry activity was therefore classified as the presence/ absence of poultry farm(s) and/ or poultry manure spreading within the ZOC, according to these DAFM datasets. It should be noted that the information is not definitive, given the potential for unregistered poultry premises.

1.2 Physical site characteristics (pathway factors)

MP type was classified as either borehole (BH) or spring (SP), from information provided as part of the national groundwater quality monitoring network (EPA, 2011). ZOC size (km^2) was calculated in ArcGis, from the ZOC boundaries provided (Section 5.2.1). For the purpose of statistical analysis, the number of poultry farms within the ZOC of each MP, was presented per unit area, to take account of the ZOC size. Bedrock geology was interpreted from the GSI Hydrostratigraphic Rock Units Group dataset (GSI, 2016a). This map is a reclassification of the 1:100,000 bedrock geology map, with over 1,200 bedrock Formations and Members grouped into 27 Rock Unit Groups, categorised based on their hydrogeological properties and other factors from the original bedrock geology datasets. A complete list of the 27 different bedrock unit groups are listed in Table S5-1, which were further amalgamated into 6 lithological groups, as described by (Tedd et al., 2017).

Two different soil classification systems were used, with different properties extracted out of each dataset. The Irish Forests Soils (IFS) Project (Bulfin et al., 2002) produced the IFS Map (1:50,000), which was accessed and downloaded through the EPA (Teagasc-EPA-GSI, 2006). This soil classification system initially subdivides mineral and organic soils that are further categorised based on the nature of the subsoil, drainage and depth, to produce 25 different soil classes (Table S5-2), as described by Fealy et al. (2009). For statistical analysis, these 25 classes were simplified into each of the principal components used in the classification system, with the data set dichotomised and analysed as described in Table 5-2

of the manuscript. The Irish Soils Information System (Creamer et al., 2007) National Map (1:250,000) was also obtained in the format of ArcGis file, accessible from the EPA (Teagasc-EPA, 2014). The soil series map is formed by soil associations, of which there are 61 throughout Ireland (Table S5-3). A soil association is a cartographic unit, which contains three or more soil series (derived from the same parent material), which occur in a particular pattern, but are difficult to differentiate at the given map scale (Simo et al., 2014). For this analysis, each MP was classified based on the primary soil series of the predominant soil association, within the ZOC. Data on soil drainage and texture was also extracted from this dataset and analysed as separate physical site properties

The majority of Irelands bedrock is overlain by sediments of Quaternary age, and it is the properties of such subsoils, that influences the transport of contaminants to bedrock aquifers. Data on Quaternary sediment types were obtained from the GSI Quaternary Sediments Map (1:50,000) (GSI, 2016b). Table S5-4 lists the 53 different quaternary sediment classes that fell within the 109 ZOCs, subdivided into each of the 8 geneses. Subsoil permeability was extracted from the GSI Groundwater Subsoil Permeability dataset (GSI, 2015a). This Subsoil permeability map (1:40,000), classifies how easily water can vertically percolate through the Quaternary sediments, with permeability ranked as High, Medium or Low where high permeability implies fast percolation, and low implies slow percolation. The permeability of subsoil can be used to imply how easily water-soluble contaminants may enter the groundwater body. However, a more useful measure of such is termed groundwater vulnerability. Groundwater vulnerability is defined as the intrinsic geological and hydrogeological characteristics that determine the ease with which groundwater may be contaminated by human activities (DELG/EPA/GSI, 1999). Vulnerability is classified into four main categories; Extreme (E), High (H), Moderate (M) and Low (L), based primarily on the subsoil permeability and thickness (depth to bedrock). Data for groundwater vulnerability was obtained from the GSI Groundwater Vulnerability Map (1:40,000) dataset (GSI, 2015d). In this dataset, the Extreme vulnerability category is split into two categories, Extreme-X (X) and Extreme-E (E), resulting in an overall five different vulnerability classes. In this case, Extreme-E describes areas with 0-3m subsoil thickness and Extreme-X, covering areas of bedrock outcrop or shallow rock, with generally <1m soil/subsoil thickness (Daly, 2004)

Figure S5-1 Corine Land Cover nomenclature 3-tiered Hierarchy showing the five level 1 classes, subdivided into 15 level 2 classes, and further detailed into an overall 44 level 3 classes. Extracted from (Lyndon and Smith, 2014)

Table S5-1 The 27 Rock Unit Groups, from the GSI Hydrostratigraphic Map

Rock Unit Group Description	Rock Unit Map Code
Basalts & other Volcanic rocks	BV
Cambrian Metasediments	CM
Devonian Kiltorcan-type Sandstones	DKS
Devonian Old Red Sandstones	DORS
Dinantian (early) Sandstones, Shales and Limestones	DESSL
Dinantian Dolomitised Limestones	DDL
Dinantian Lower Impure Limestones	DLIL
Dinantian Mixed Sandstones, Shales and Limestones	DMSS
Dinantian Mudstones and Sandstones (Cork Group)	DMSC
Dinantian Pure Bedded Limestones	DPBL
Dinantian Pure Unbedded Limestones	DPUL
Dinantian Sandstones	DS
Dinantian Shales and Limestones	DSL
Dinantian Upper Impure Limestones	DUIL
Granites & other Igneous Intrusive rocks	GII
Namurian Sandstones	NSH
Namurian Shales	NU
Namurian Undifferentiated	OM
Ordovician Metasediments	OV
Ordovician Volcanics	PM
Permo-Triassic Mudstone and Gypsum	PTMG
Permo-Triassic Sandstones	PTS
Precambrian Marbles	PM
Precambrian Quartzites, Gneisses & Schists	PQGS
Silurian Metasediments and Volcanics	SMV
Westphalian Sandstones	WSA
Westphalian Shales	WSH

Table S5-2 Description and Map code for the 25 different Irish Forest Soils (IFS) soil classes, adapted from (Fealy et al., 2009)

IFS Code	Description
Mineral	
AminDW	Deep well-drained mineral soil, Derived from mainly acidic parent materials
AminSW	Shallow well drained mineral soil derived from mainly acidic parent materials
AminPD	Deep poorly drained mineral soil derived from mainly acidic parent materials
AminSP	Shallow poorly drained mineral soil derived from mainly acidic parent materials
BminDW	Deep well drained mineral soil derived from mainly basic parent materials
BminSW	Shallow well drained mineral soil derived from mainly basic parent materials
BminPD	Poorly drained mineral soils derived from mainly basic parent materials
BminSP	Shallow poorly drained mineral soil derived from mainly basic parent materials
Peaty Mineral	
AminPDPT	Poorly drained mineral soils with peaty topsoil derived from acidic parent materials
AminSPPT	Peaty shallow poorly drained mineral soil derived from mainly acidic parent materials
AminSRPT	Shallow reasonable drained mineral soil derived from mainly acidic parent materials
BminPDPT	Peaty poorly drained mineral soils derived from mainly basic parent materials
BminSPPT	Peaty shallow poorly drained mineral soil derived from mainly basic parent materials
BminSRPT	Peaty shallow reasonable drained mineral soil derived from basic parent materials
Peat	
Cut	Blanket cutaway
BktPt	Blanket Mountain or lowlands
FenPt	Fen peat
Alluvium	
AlluvMIN	Mineral Alluvium
AlluvMRL	Alluvium from Marl type soils
Miscellaneous	
Lac	Lake
Scree	Scree
AeoUND	Aeolian undifferentiated
MarSands	Beach sand and gravels
MarSed	Marine/ Estuarine sediments
Water	Lake or Reservoir

Table S5-3 List of the 61 different Irish Soils Information System (SIS) soil association, with the associated series leader and description, reproduced from (Simo et al., 2014)

Soil Association	Series Leader	Description
0300a	Seafield	Podzols, Brown Podzolics and Groundwater Gleys with sandy textures associated to stoneless drift
0360a	Burren	Rendzinas and decalcified Lithosols on outcropping limestone, Luvisols and Brown earth associated with limestone bedrock and Peat
0360c	Crush	Rendzinas and Calcareous Brown Earth on calcareous gravels and limestone bedrock, with inclusions of Luvisols and Brown Earths on drift with limestones
0410a	Carrigvahanagh	Peat associated with Lithosols, Brown Podzolics, Podzols and Brown Earths over igneous and metamorphic stones, with inclusions of Groundwater Gleys
0410b	Bantry	Peat associated with Lithosols on sandstones and shale bedrock with inclusions of Podzols and Brown Podzolics on sandstone and shale bedrock
05LAK	Gurteen	Alluvial and drained alluvial soils of fine textures and base rich.
05MAR	Wexford Slob	Alluvial and drained alluvial soils on reclaimed coastal flats
05RIV	Boyne	Alluvial and drained alluvial soils on river floodplain with base rich and medium to coarse textures
0600a	Kilpierce	Poorly drained soils composed of Groundwater Gleys, Luvisols and Brown Earths soils, which are restricted to depressions and the less favourable slopes on drift with siliceous stones.
0650a	Mylerstown	Poorly drained soils consisting of Calcareous and Humic Calcareous Grondwater Gleys and Luvisols on drift with limestones
0660c	Puckane	Poorly drained soils composed by Humic Groundwater Gleys, Surface-water Gleys, on drift with siliceous stones and Peat, with inclusions of Humic Lithosols and Humic Brown Podzolics on drift with siliceous stones
0660d	Puckane	Poorly drained soils located in the uplands composed by Humic Groundwater Gleys, Podzols and Brown Podzolics on drift with siliceous stones and Peat
0660e	Ballywilliam	Poorly drained soils located in the uplands composed by Humic Groundwater Gleys, Brown Earths and Lithosols on drift with igneous and metamorphic stones with some inclusions of Brown Podzolics and Surface-water Gleys
0700a	Macamore	Surface-water Gleys in clayey marine drift; well drained sandy Brown Podzolics on outwash sands and gravels

Soil Association	Series Leader	Description
0700b	Kilrush	Surface-water Gleys, Brown Earths and Brown Podzolics on drift with siliceous stones, and inclusions of Groundwater Gleys
0700c	Drumkeeran	"Heavy" soils with clayey and fine textures. Association composed commonly of Surface-water Gleys and in lesser proportions, Luvisols, with inclusions of Brown Earths.
0700d	Straffan	Surfacewater and Luvisols commonly associated with fluvioglacial outwash gravels and Calcareous Brown Earths on lower slopes over limestones bedrock
0700f	Newport	Surface-water Gleys on lower slopes and Humic Brown Podzolics and Podzols in upper altitude, all on drift with siliceous stones
0700h	Kilrush	Surface-water Gleys associated with Luvisols, on drift with siliceous stones and Basin Peat
0760a	Gortaclareen	Mostly Surface-water Gleys associated with Brown Earths, over shale and slate bedrock and on drift with siliceous stones
0760c	Howardstown	Surface-water Gley and Luvisols in Clayey lowlands on drift with limestones with inclusions of Calcareous Brown Earth
0760e	Ballinamore	Humic Surface-water Gleys and Humic Groundwater Gleys , on drift with limestones, and Peat
0760f	Driminidy	Humic Surface-water Gleys and Humic Brown Earths on moderate slopes; upland with Blanket Peat with extensive bedrock with Histic Lithosols and Podzols.
0800a	Black Rock Mt.	Podzols on drift with igneous and metamorphic stones in uplands and Peat; and Lithosols over gneiss and schist on slopes
0800c	Ballycondon	Podzols and Brown Podzolics on drift with siliceous stones and sandstone bedrock and Peat
0843b	Knockastanna	Podzols and Brown Podzolics over shale and slate bedrock and Peat, with inclusions of Humic Rendzinas and Brown Earths on bedrock
0843e	Glenary	Podzols and Brown Podzolics on drift with siliceous stones and Peat
0843f	Glenary	Blanket Peat and Podzols in Mountainous areas, with outcropping rock and Humic Lithosols (on sandstones bedrock); interspersed with Stagno-Podzols and Surface-water Gleys, on drift with siliceous stones.
0900a	Cooga	Brown Podzolic soils with Groundwater Gleys on drift with siliceous stones an inclusion of Podzols and Brown Earths
0900b	Kiltealy	Brown Podzolics and Brown Earths predominate with altitudinal sequence of upland Peat, Lithosols and Podzols on drift with igneous and metamorphic stones
0900e	Ross Carbery	Brown Podzolics and Brown Earths mainly on drift with siliceous stones, Surface-water Gleys and Groundwater Gleys in lowland areas

Soil Association	Series Leader	Description
0900f	Clonin	Shallow Peat soils beside Lithosols, Podzols over sandstone bedrock and Brown Podzolics in upland on drift with siliceous stones
0900g	Cupidstownhill	Brown Ppodzolics and Brown earth on undulating shale/slate bedrock and on drift with siliceous stones
0900h	NBP4	Predominantly Brown Podzolic and Brown Earths and Rendzinas on gneiss and shist bedrock and on drift with igneous and metamorphic stones, with inclusions of Surface-water Gleys and Groundwater Gleys
0920a	Clonegall	Gleyic Brown Podzolics, Podzols and Brown Earths on drift with siliceous stones and inclusions of Groundwater Gleys
0960c	Borrisoleigh	Humic Brown Podzolics, gleyic and humic Brown Earths on a wet undulating on shale bedrock and on drift siliceous with inclusions of Groundwater Gleys and Humic Lithosols
0960d	Knockaceol	Altitudinal sequence of Humic Brown Podzolics, Podzols on sandstone bedrock and on drift siliceous and Peat with inclusions of Surface-water Gleys and Rendzinas
0960e	Knockboy	Brown Podzolics with Podzols on upper slopes and Surfacewater Gleys in depressions, on drift with siliceous stones
1000a	Elton	Luvisols associated with Surface-water Gleys, Stagnic Brown Earths and Calcareous Brown Earths, on drift with limestones
1000c	Elton	Luvisols associated to histic and humic Groundwater Gleys and Calcareous Brown Earths, on drift with limestones and Basin Peat
1000g	Elton	Heavier textures in the soils of this association. Association with Luvisols, Groundwater Gleys and Calcareous Brown Earths, on drift with limestones
1000x	Elton	Luvisols and Surfacewater Gleys on drift with mixed of limestones and siliceous stones
1030a	Crosstown	Luvisols, Surface-water Gleys and Stagnic Brown Earths on drift with siliceous stones, with inclusions of Groundwater Gleys
1030b	Rathowen	Luvisols, Surface-water Gleys, Groundwater Gleys on drift with limestones and Peat
1100a	Clonroche	Well drained Brown Earths on drift with siliceous stones in an undulating land with some Brown Podzolics on upper slopes. Surface-water Gleys and Groundwater Gleys found in depressions
1100c	Clashmore	Brown Earths and Luvisols on upper slopes; Surface-water Gleys and Grounwater Gleys in depressions
1100d	Ballyvorheen	Brown Earths, Brown Podzolics and Podzols, soils related to coarse textures on drift with siliceous stones

Soil Association	Series Leader	Description
1100e	Ballylanders	Brown Earths on lower slopes, Podzols on steeper slopes, Luvisols and Surface-water Gleys in depressions, related to fine soil textures on shale bedrock and on drift with siliceous stones.
1100h	Borris	Brown Earths and Brown Podzolics on slope on drift with igneous and metamorphic stones, inclusions of Surface-water Gleys
1100l	Kennycourt	Brown Earths, Luvisols and Grownd water Gleys on drift with limestones
1100m	Kill	Brown Earths, Brown Podzolics and Podzols on an undulating land with igneous and metamorphic stones, inclusions of Surface-water Gleys in depressions.
1100n	Clashmore	Brown Earths, Luvisols and Surface-water Gleys on drift with siliceous stones
1100q	Mullabane	Mostly Brown Earths and Calcareous Brown Earths on drift with limestones, associated with Luvisols and some inclusions of Rendzinas and Peat
1100s	Broomhill	Brown Earths and Brown Podzolics mainly on sandstones bedrock and some occurs on drift with siliceous stones, withinclusions of Podzols and Surface-water Gleys
1130a	Moord	Brown Earths and Surface-water Gleys associated to humic and gleyic diagnostic features on drift with siliceous stones and inclusions of Luvisols in a drumlin area.
1130b	Duarrigle	Brown Earths, Surface-water Gleys and Lithosols on shale/sandstone bedrock in an undulating area
1150a	Baggotstown	Calcareous Brown Earths, Brown Earths and Luvisols on calcareous gravels and on drift limestones, inclusions of Rendzinas
1150b	Ballincurra	Calcareous Brown Earths and Luvisols associated with Rendzinas and decalcified Lithosols, on limestones bedrock
1150c	Faoldroim	Calcareous Brown Earths, Brown Earths and Luvisols on drift with limestones, associated with Rendzinas and decalcified Lithosols on limestones bedrock and Peat
1160a	Ashgrove	Humic and gleyic Brown Earths and Surface-water Gleys, on drift with siliceous stones
1160c	Schull	Brown Earths, Brown Podzolics, Podzols and Surface-water Gleys associated to humic and gleyic diagnostic features on drift with siliceous stones

Table S5-4 List of the different Quaternary sediments throughout Ireland, with corresponding code, adapted from (Fealy et al., 2009)

Parent Material/ Quaternary Sediment	**Map Code**
Tills	
Sandstone and shale till (Cambrian/Precambrian)	TCSsS
Sandstone till (Lower Palaeozoic)	TLPSs
Shale till (Lower Palaeozoic)	TLPS
Sandstone and shale till (Lower Palaeozoic)	TLPSsS
Sandstone till (Lower Palaeozoic/Devonian)	TLPDSs
Sandstone till (Devonian)	TDSs
Sandstone till (Devonian/Carboniferous)	TDCSs
Sandstone and shale till (Devonian/Carboniferous)	TDCSsS
Limestone till (Carboniferous)	TLs
Shale and sandstone till (Namurian and Carboniferous)	TNCSSs
Shale and sandstone till (Namurian)	TNSSs
Chert till	TCh
Carboniferous sandstone and Chert till	TCSsCh
Quartzite till	TQz
Acid volcanic till	TAv
Granite till	TGr
Basic igneous till	TBi
Metamorphic till	TMp
Irish Sea Tills	
Sandstone and shale till (Cambrian/Precambrian) of Irish Sea Basin	IrSTCSsS
Sandstone till (Devonian) with matrix of Irish Sea Basin origin	IrSTDSs
Sandstone and shale till (Lower Palaeozoic) with matrix of Irish Sea Basin origin	IrSTLPSsS
Limestone till (Carboniferous) with matrix of Irish Sea Basin origin	IrSTLs
Sandstone till with matrix of Irish Sea Basin origin	IrSTSs
Acid volcanic till with matrix of Irish Sea Basin origin	IrSTAv
Glaciofluvial Sands and Gravels	
Acidic esker sands and gravels	AcEsk
Basic esker sands and gravels	BasEsk
Sandstone and shale sands and gravels (Cambrian/Precambrian)	GCSsS
Sandstone sands and gravels (Lower Palaeozoic)	GLPSs
Shale sands and gravels (Lower Palaeozoic)	GLPS

Parent Material/ Quaternary Sediment	**Map Code**
Sandstone and shale sands and gravels (Lower Palaeozoic)	GLPSsS
Sandstone sands and gravels (Lower Palaeozoic/Devonian)	GLPDSs
Sandstone sands and gravels (Devonian)	GDSs
Sandstone sands and gravels (Devonian/Carboniferous)	GDCSs
Limestone sands and gravels (Carboniferous)	GLs
Shale and sandstone sands and gravels (Namurian)	GNSSs
Chert sands and gravels	GCh
Quartzite sands and gravels	GQz
Granite sands and gravels	GGr
Basic igneous sands and gravels	GBi
Metamorphic sands and gravels	GMp
Alluvium	
Alluvium undifferentiated	A
Gravelly	Ag
Silty	As
Clayey	Ac
Peat	
Blanket peat	BktPt
Raised peat	RsPt
Fen peat	FenPt
Cutover peat	Cut
Bedrock at Surface	
Bedrock at surface	Rck
Bedrock at or near surface-Non calcareou1	RckNCa
Karstified Rock	
Karstified limestone bedrock at surface	KaRck
Bedrock at or near surface-Calcareous2	RckCa
Other	
Aeolian Sediments undifferentiated	Aeo
Blown sand	Ws
Blown sand in dunes	Wsd
Marl (Shell)	Mrl
Scree	Scree
Made ground	Made
Marsh	Marsh
Tidal marsh	TdlMr

Parent Material/ Quaternary Sediment	**Map Code**
Marine Deposits	
Raised beach sands and gravels	MGs
Beach sand	Mbs
Marine silts	Msi
Marine clays	Mc
Estuarine sediments (silts/clays)	Mesc
Glaciolacustrine deposits:	
Lake sediments undifferentiated	L
Sandy	Ls
Silty	Lsi
Clayey	Lc

Table S5-5 Summary of the EPA Water Quality Parameters (with associated methods of determination) measured at 98 sites, and used for statistical analysis

Water Quality Parameter	Units	Method of Determination	Instrumentation	LOQ
Field based Measurements				
pH	pH	-	YSI Professional Pro Plus Multiparameter	-
Temperature	°C	-	YSI Professional Pro Plus Multiparameter	-
Dissolved Oxygen	mg/L	-	YSI Professional Pro Plus Multiparameter	-
Conductivity	µS/cm	-	YSI Professional Pro Plus Multiparameter	-
ORP	mV	-	YSI Professional Pro Plus Multiparameter	-
Laboratory based Measurements				
Total Coliforms	No./100 mL	Footnote 1	IDEXX patented Defined Substrate Technology	-
Faecal Coliforms	No./100 mL	Footnote 1	IDEXX patented Defined Substrate Technology	-
pH	pH	Footnote 2	WTW METERS & PROBES	2
Conductivity	µS/cm	Footnote 3	WTW Conductivity Meter	15
Alkalinity	mg/l $CaCO_3$	Footnote 4	Discrete Analyser	10 mg/l
Colour	Hazen	Footnote 5	Discrete Analyser	5
Turbidity	NTU	Footnote 6	Turbidimeter, HACH models 2100N IS	0.5
Ammonium	mg (NH_4)/L	Footnote 7	Discrete Analyser	0.026
Nitrite as NO_2	mg (NO_2)/L	Footnote 7	Discrete Analyser	0.013
Nitrate	mg (N)/L	Footnote 7	Discrete Analyser	0.2
Total Phosphorus	mg (P)/L	Footnote 8	Ganimede P Analyser	0.01
Unfiltered MRP	mg (P)/L	Footnote 7	Discrete Analyser	0.01
Filtered MRP	mg (P)/L	Footnote 7	Discrete Analyser	0.01
Sulphate	mg/L	Footnote 11	Dionex Ion Chromatography	2
TOC	mg(C)/L	Footnote 9	HACH IL550 & Analytic Jena TOC Analysers	1
Aluminium	µg/L	Footnote 10	ICP-MS Perkin Elmer	10
Antimony	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Arsenic	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Barium	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Beryllium	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Boron	µg/L	Footnote 10	ICP-MS Perkin Elmer	10
Cadmium	µg/L	Footnote 10	ICP-MS Perkin Elmer	0.02
Calcium	mg/L	Footnote 10	ICP-MS Perkin Elmer	1
Chloride	mg/L	Footnote 7	Discrete Analyser	2
Chromium	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Cobalt	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Copper	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Fluoride	mg/L	Footnote 11	Dionex Ion Chromatography	0.2
Iron	µg/L	Footnote 10	ICP-MS Perkin Elmer	10
Lead	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Magnesium	mg/L	Footnote 10	ICP-MS Perkin Elmer	0.25

Table S5-5 *continued*

Water Quality Parameter	Units	Method of Determination	Instrumentation	LOQ
Manganese	µg/L	Footnote 10	ICP-MS Perkin Elmer	5
Mercury	µg/L	Footnote 10	ICP-MS Perkin Elmer	0.02
Molybdenum	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Nickel	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Potassium	mg/L	Footnote 10	ICP-MS Perkin Elmer	0.25
Silica	mg/L SiO_2	Footnote 7	Discrete Analyser	0.1
Sodium	mg/L	Footnote 10	ICP-MS Perkin Elmer	1
Strontium	µg/L	Footnote 10	ICP-MS Perkin Elmer	10
Uranium	µg/L	Footnote 10	ICP-MS Perkin Elmer	1
Zinc	µg/L	Footnote 10	ICP-MS Perkin Elmer	1

ORP = Oxidation-Reduction Potential, MRP = Molybdate Reactive Phosphorus, TOC = Total Organic Carbon

1. EPA in-house method based on ISO 9308-2:2012 Water quality — Enumeration of Escherichia coli and coliform bacteria

2. EPA in-house method based on BS EN ISO 10523:2012 Water Quality – Determination of Ph,

3. EPA in-house method based on IS EN 27888:1993 - Conductivity probe,

4. Bromophenol Blue Method,

5. EPA in-house method based on BS EN ISO 7887:2011 Water quality — Examination and determination of colour,

6. The nephelometric turbidity method is used,

7.EPA in-house method based on ISO 15923-1:2013, Water Quality – Determination of selected parameters by discrete analysis systems,

8. EPA in-house method based on I.S. EN ISO 6878:2004 Water Quality - Determination of phosphorus – Ammonium molybdate spectrometric method,

9. EPA in-house method based on ISO 8245:1999 Water quality — Guidelines for the determination of total organic carbon (TOC) and dissolved organic carbon (DOC),

10. EPA in-house method based on ISO 17294 -1: 2006 / ISO 27294-2:2016 (ICP-MS),

11. EPA in-house method based on ISO 10304-1:2009 (Ion Chromatography)

Table S5-6 Statistical p-values for logistic regression and Fisher's exact test (where logistic regression failed to converge), for detections defined for all anticoccidial compounds, ionophore compounds only, the ionophore monensin only and synthetic anticoccidial compounds only.

Site Characteristic	*p* value with detections defined as:			
	All anticoccidials	Ionophores	Monensin	Synthetic anticoccidial
Logistic regression				
Poultry Activity [a]	0.0083	0.0148	0.0450	0.1150
- Poultry farms per unit area [a]	0.0002	0.0002	<0.0001	0.3555
- Poultry manure spreading [a]	0.0005	0.0025	0.0014	0.0065
Groundwater vulnerability percentage	0.4171	0.5795	0.6752	0.783
Groundwater vulnerability % X+E+H	0.7616	0.3131	0.2796	0.6084
IFS Type I percentage	0.0681	0.1884	0.4351	0.9978
MP type	0.3929	0.3884	0.1621	0.9999
Predominant Bedrock geology*	0.9553	0.9269	0.8674	0.9267
Predominant Corine (amalgamated)	0.5871	0.772	0.808	0.9996
Predominant Corine (level 3)	0.9997	1.0000	1.0000	0.9144
Predominant Drainage	0.7288	0.3938	0.601	0.8109
Predominant Groundwater vulnerability	0.3828	0.4987	0.3801	0.9922
Predominant GSI Aquifer Category *	0.8522	0.7957	0.7876	0.7773
Predominant IFS (full)	0.6596	0.8386	0.9207	0.5825
Predominant IFS Type I	0.0555	0.1327	0.5982	0.8709
Predominant IFS Type II	0.6008	0.816	0.9113	0.7553
Predominant IFS Type III	0.5254	0.4437	0.6758	0.9876
Predominant IFS Type IV	0.5465	0.8482	0.7513	0.9202
Predominant Quaternary Genesis	0.985	0.943	0.9436	0.9999
Predominant Quaternary sediment	0.9133	0.9587	0.9997	0.4396
Predominant SIS Soils Association*	0.9913	0.9963	0.9978	0.9921
Predominant Subsoil permeability	0.8042	0.5904	0.9578	0.8805
Predominant Texture *	0.82	0.8057	0.8121	0.783
Predominant WFD flow regime	0.6826	0.7127	0.4392	0.6084
Fischer's Exact Test				
Predominant Bedrock geology	0.4503	0.292	0.1543	0.4037
Predominant GSI Aquifer Category	0.4119	0.3381	0.4696	-
Predominant SIS Soils Association	0.0335	-	0.2135	0.364
Predominant Texture	0.1166	0.0837	0.1102	0.4682

[a] source factors * supplemented by a Fischer's exact test, due to non-convergence of the logistic regression

Table S5-7 Statistical p values for analysis of water quality parameters for detections defined as all anticoccidial compounds, ionophore compounds only, the ionophore monensin only and synthetic anticoccidial compounds only

Water Quality Parameter	*p* value with detections defined as:			
	All anticoccidials	**Ionophores**	**Monensin**	**Synthetic anticoccidial**
Field based measurements				
pH [a]	0.0896	**0.0270**	**0.0066**	0.2514
Temperature [a]	0.8225	0.6073	0.7240	0.5429
Dissolved Oxygen [a]	0.8979	0.8649	0.5279	0.4194
Conductivity [a]	0.3706	0.5199	0.4211	0.5981
ORP [a]	0.5010	0.3309	0.6119	0.4258
Laboratory based measurements				
Total Coliforms [b]	0.4836	0.9542	0.9133	0.2355
Faecal Coliforms [b]	**0.0660**	0.7509	0.4700	0.1790
Conductivity [a]	0.2105	0.3278	0.2805	0.8388
Alkalinity [a]	0.9618	0.7835	0.6817	0.6312
Colour [c]	0.6103	0.3021	0.5479	0.7591
Turbidity [c]	0.2375	0.5128	0.8050	0.1025
Ammonium [c]	**0.0687**	**0.0266**	0.1720	0.1582
Nitrite as NO_2 [c]	0.9007	0.7727	0.6072	0.3748
Nitrate [b]	0.3007	0.1703	0.2984	0.3840
Total Phosphorus [b]	0.8943	0.8841	0.9755	0.9816
Unfiltered MRP [b]	0.5207	0.6387	0.5570	0.3855
Filtered MRP [b]	0.9311	0.7063	0.6500	0.6544
Sulphate [a]	0.6264	0.7342	0.4898	0.8383
Total Organic Carbon [b]	0.2993	0.2984	0.7652	0.1285
Aluminium [c]	0.6533	0.8381	0.9482	0.4840
Arsenic [c]	0.9735	0.9752	0.1509	0.2744
Barium [b]	0.1749	0.5762	0.6089	0.1308
Boron [b]	0.3077	0.4517	0.7276	0.9564
Cadmium [c]	0.9257	0.7821	0.7538	0.9258
Calcium [a]	0.1968	0.2463	0.1648	0.9854
Chloride [a]	0.3653	0.6232	0.4850	0.3390
Copper [b]	0.4216	0.4926	0.3661	0.5068
Fluoride [c]	0.7197	0.9145	0.7830	0.9910
Iron [c]	0.2375	0.5128	0.7157	0.1025

Water Quality Parameter	*p* value with detections defined as:			
	All anticoccidials	**Ionophores**	**Monensin**	**Synthetic anticoccidial**
Lead [b]	0.4433	0.6584	0.3754	0.9196
Magnesium [a]	0.8354	0.9977	0.5257	0.5362
Manganese [c]	0.7305	0.4256	0.8326	0.8668
Molybdenum [c]	0.9817	0.9825	0.3140	0.4434
Nickel [c]	0.5161	0.2759	0.3780	0.8381
Potassium [a]	0.7798	0.8616	0.7575	0.8243
Silica [a]	0.6987	0.6287	0.7968	0.5442
Sodium [a]	0.5463	0.8381	0.6801	0.7032
Strontium [a]	0.7657	0.8986	0.7154	0.8668
Uranium [b]	0.5499	0.3236	0.1268	0.9537
Zinc [b]	0.2419	0.1425	**0.0514**	0.2869

[a] fully or almost fully observed [b] censored values ranging from 30 to 70% censored c highly censored data with >70% censoring

Supplementary Information File SI-5.2

Supplementary File SI-5.2 is provided in the format of an excel datasheet, which can be accessed using the following link:

https://drive.google.com/file/d/1A8J5F_79jsMkdUC4yBv6aqbt9b_dX2gu/view?usp=sharing

Publications and Dissemination

1. Publications resulting from work carried out as part of this thesis

Mooney, D., Coxon, C., Richards, K. G., Gill, L., Mellander, P. E. & Danaher, M. 2019. Development and Optimisation of a Multiresidue Method for the Determination of 40 Anthelmintic Compounds in Environmental Water Samples by Solid Phase Extraction (SPE) with LC-MS/ MS Detection. *Molecules,* 24**,** 1978.

Mooney, D., Coxon, C., Richards, K. G., Gill, L. W., Mellander, P. E. & Danaher, M. 2020. A new sensitive method for the simultaneous chromatographic separation and tandem mass spectrometry detection of anticoccidials, including highly polar compounds, in environmental waters. *Journal of Chromatography A,* 1618, 460857.

Mooney, D., Richards, K. G., Danaher, M., Grant, J., Gill, L., Mellander, P. E. & Coxon, C. E. 2020. An investigation of anticoccidial veterinary drugs as emerging organic contaminants in groundwater. *Science of the Total Environmen*t, 746, 141116.

In preparation for submission to STOTEN:
Mooney, D., Richards, K. G., Danaher, M., Grant, J., Gill, L., Mellander, P. E. & Coxon, C. E. 2020. An analysis of the spatio-temporal occurrence of anthelmintic drug residues in groundwater.

2. Other key outputs and public outreach resulting from work carried out as part of this thesis

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. (2020). **Invited Speaker:** Emerging organic contaminants in Irish groundwater: the occurrence of veterinary pharmaceuticals *presented at IAH Ireland Technical Discussion, March 2020,* Geological Survey of Ireland, Beggars Bush, Dublin, **March 2020.**

Mooney, D., Coxon, C., Richards, K. G., Gill, L. W., Mellander, P. E. & Danaher, M. 2020. **Online Article:** Detecting the Undetected, in Teagasc T-Research, Volume 15: Number 1, Spring 2020, ISSN 1648-8917, pages 12-13, available at: https://www.teagasc.ie/media/website/publications/2020/TResearch_Spring2020.pdf

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. (2019). **Oral Presentation:** Emerging organic contaminants in Irish groundwater: investigating the occurrence of veterinary pharmaceuticals *at the Annual Teagasc Walsh Fellowships National Finals,* Teagasc Food Research Centre Ashtown, Dublin, **November 2019.**

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. (2019). **Poster Presentation:** Investigating the occurrence of anticoccidial agrochemicals in Irish groundwaters: preliminary findings *in proceedings of Catchment Science 2019, Agricultural Catchments Programme, Wexford, Ireland*, **November 2019**

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. (2019). **Invited Speaker:** Emerging organic contaminants in Irish groundwaters: investigating the occurrence of veterinary agrochemicals, presented to the Food Safety Authority of Ireland Chemical and Emerging Risks Team, FSAI Dublin, **October 2019**

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. (2019). **Oral Presentation:** Emerging organic contaminants in Irish groundwaters: investigating the occurrence of two commonly used groups of antiparasitic drugs in Irish agriculture, with determination by Solid Phase Extraction (SPE) and UHPLC-MS/MS detection *in Proceedings of the 17th International Conference on Chemistry and the Environment*, Thessaloniki, Greece, 16 – 20th **June 2019**

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. (2019). **Oral Presentation:** Agro-chemicals in Irish groundwaters: investigating the occurrence of veterinary drugs and their transformation products in *Book of Abstracts and Proceedings of The International Interdisciplinary Conference on Land Use and Water Quality (LuWQ), Agriculture and the Environment*, Aarhus, Denmark, 3 – 6th **June 2019**

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. (2018). **Oral Presentation:** Determination of 40 anthelmintic drug residues in environmental waters by solid phase extraction (SPE) with UHPLC-MS/MS detection. *In Book of Abstracts, 8th International Symposium on Hormone and Veterinary Drug Residue Analysis*, Ghent, Belgium, 22-25 **May 2018**, pp.35

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. (2018). **Oral Presentation:** Agro-chemicals in Irish groundwaters: preliminary findings of anthelmintic drug occurrence in Irish karst and fractured aquifers i*n Groundwater Matters: science and practice, Proceedings of 38th Annual Groundwater Conference*, International Association of Hydrogeologists (Irish Group), Tullamore, Offaly, , pp. IV-5, **April 2018**

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. 2017. **Poster Presentation:** Veterinary drug occurrence in Irish karst and fractured bedrock aquifers; preliminary findings in Groundwater Heritage and Sustainability, K. Posavek and T. Markovic (eds.), *Book of Abstracts at 44th Annual Congress of the International Association of Hydrogeologists (IAH*), pp. 325, Dubrovnik, Croatia, **September 2017**

Mooney, D., Danaher, M., Richards, K., Mellander, P.E., Gill, L., and Coxon, C. 2017 **Poster Presentation:** Emerging organic contaminants arising in rural environments-investigations in karst and fractured bedrock aquifers *in proceedings of ENVIRON 2017-The 27th Irish Environmental Research Colloquium*, Athlone Institute of Technology, Athlone, **April 2017**