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Spatiotemporal Modelling of Antimicrobial Resistance in the Irish Subsurface Environment

Thesis presented by

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for the degree of

Doctor of Philosophy

University College Cork

School of Biological, Earth and Environmental Sciences

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2023

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Signature: Luisa Albuquerque de Andrade

Date: 31·01·2023

Dedication

Dedico esta dissertação aos meus pais, meus maiores apoiadores desde sempre, Ana e Silvio,

aos meus irmãos com quem eu posso contar sempre, Clarice e Humberto,

à Cillian, meu Lindo, meu Parceiro,

à minhas avós: Anita, Letícia e Bebel,

às minhas tias, tios, primas e primos tão queridos,

aos meus amigos/friends, who supported and helped me throughout this entire journey and even gave me a place to stay when most needed – I am thinking of you Camila, Fernanda, Hannah, Juliana, and Larissa,

to my supervisors, for their support, guidance, reassurance, and for always having my back

and especially to Jean, for always making me feel better after our chats no matter how tough the times - thank you!

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The support and comradeship of my fellow postgraduate students, with a special thanks to my now friends **Hannah Binner** and **Larissa Macedo**, and

All my friends and family, without whom I would never have finished this – *muito obrigada!*

"A beleza não está nem na luz da manhã nem na sombra da noite, está no crepúsculo, nesse meio tom, nessa incerteza."

"Beauty is not in the morning light nor in the night's shadows, it is in the twilight, in this halftone, this uncertainty"

– Lygia Fagundes Telles [Venha Ver o Por do Sol]

Abstract

Antimicrobial resistance represents a significant public health risk, with global estimates indicating that as many as 1.27 million deaths were directly attributable to antimicrobial resistant infections in 2019. The adverse human health effects attributed to the continuous rapid spread of antimicrobial resistance are particularly pressing in the aftermath of the COVID-19 pandemic, which highlighted societies' acute susceptibility to infectious diseases in the absence of effective treatment options. The proliferation of Antimicrobial Resistant Bacteria (ARB) was also acknowledged as the key emerging issue of environmental concern by the United Nations Environmental Programme in 2017. Despite this, the natural environment's role in the spread of ARB-related infections remains unclear. In this context, ARB presence in groundwater resources represents a noteworthy concern, as these can act as prospective reservoirs, while offering a direct exposure route to the over 2.2 billion groundwater consumers worldwide. In the Republic of Ireland (RoI), private groundwater wells supply approximately 11% of the population, with these users already associated with increased incidence rates of waterborne infections. Indeed, Irish private wells have been linked to persistent microbiological contamination attributable to inadequate on-site domestic wastewater treatment systems and the widespread agricultural activities associated with the Irish rural landscape; both of which are also potential sources of ARB. Thus, and in combination with a diverse (hydro)geological profile and a temperate climate characterised by the absence of a dry season, the RoI may present the 'perfect storm' for groundwater contamination by ARB and subsequent adverse human health effects. To-date, however, just one spatiotemporally limited study exists which investigates this issue within the RoI,

leading to significant knowledge gaps regarding the spatial and temporal dynamics of ARB contamination in Irish groundwater environments.

Accordingly, the current research blends systematic literature review and pooled analyses, traditional hydrogeological fieldwork, geo-referencing, molecular microbiology, analytical chemistry, genome sequencing, and statistical modelling to quantify occurrence rates and elucidate the potential drivers of ARB contamination in groundwater resources. The overarching aim of this research is to better understand ARB presence in Irish groundwater supplies and inform appropriate source-protection strategies, in line with Sustainable Development Goals 3 ("good health and well-being") and 6 ("clean water and sanitation for all"). Research findings indicate that groundwater resources may represent a global reservoir/pathway for ARB, with 80.2% ± 29.0 of groundwater-derived bacteria from studies in the systematic review exhibiting resistance/intermediate resistance to at least one antimicrobial. In the RoI specifically, the analyses of monitoring data from a 10-year period showed *Escherichia coli* contamination in 66.7% of monitored (regulated) water supplies nationwide at least once, with pastoral agriculture (a well-known potential source of ARB in addition to faecal bacteria contamination) identified as a key risk factor. Additionally, fieldwork data identified antimicrobial resistance in 16.7% of *Escherichia coli* (8/48) but none of the *Pseudomonas aeruginosa* (0/6) collected from 132 geographically dispersed (unregulated) private wells in the RoI sampled during autumn 2019 and summer 2021. Fieldwork results also revealed that the presence of resistant *E. coli* was significantly associated with increased local cattle density (OR = 1.028; p = 0.037), having likely been transported into wells via surface run-off followed by direct wellhead ingress. Moreover, lack of statistical significance

($p \geq 0.084$) between overarchingly low concentrations of co-selective (i.e., metal/metalloid) parameters and antimicrobial resistance gene abundance in *E. coli* isolates lead to the hypothesis that *E. coli* may have acquired resistance prior to entering these groundwater supply environments. With that, findings suggest contamination of rural groundwater resources with ARB in the RoI as being highly preventable by ensuring appropriate source protection measures. Research results bridge the gap between hydrology, geoscience, and heath, promote capacity building, and inform the development of effective policy for groundwater management and antimicrobial resistance action plans into the future.

Thesis Overview

This is a Doctor of Philosophy (PhD) thesis organised by publication. I am a first author and key contributor to all presented work. My contributions to each manuscript are clearly stated in the preface section in Contributor Roles Taxonomy (CRediT) format, with individual author contributions specified at the end of each manuscript in line with journal layout requirements.

This thesis contains six chapters. **Chapter 1** is a general introduction to antimicrobial resistance and its emergence/dissemination with a particular focus on groundwater environments. The key concepts surrounding antimicrobial resistance are also described in this chapter, in addition to the relevance of the Republic of Ireland (RoI) as a study location, the two bacterial species assessed (i.e., *Escherichia coli* and *Pseudomonas aeruginosa*), and the general study aim and objectives. Chapters 2 to 5 present the primary research outputs. **Chapter 2** consists of a systematic literature review and pooled analyses of antimicrobial resistant and intermediately-resistant bacteria in groundwater resources, carried out to quantify the global extent of the issue and identify research trends and knowledge gaps (published in *Water Research*; impact factor = 13.40). **Chapter 3** presents the spatiotemporal exploration of *E. coli* incidence within regulated groundwater supplies in the RoI using publicly available National Groundwater Monitoring Programme datasets gathered over a 10-year period (published in *Science of the Total Environment*; impact factor = 10.754). In **Chapter 4**, current rates of antimicrobial resistant bacteria in 132 unregulated groundwater supplies in rural Ireland (i.e., private wells) are elucidated, as well as key hydrogeological, meteorological, anthropogenic, and infrastructural factors

associated with their presence (published in *Environmental Pollution*; impact factor = 9.98). **Chapter 5** presents a genotypic assessment of groundwater-derived *E. coli* and expounds on whether private wells in agriculturally-affected areas may represent (co-)selective environments for antimicrobial resistance (under review in *Environmental Pollution*; impact factor = 9.98). The thesis is brought to a close with **Chapter 6**, where key conclusions and research implications are discussed.

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Chapter 1

General Introduction

1.1 The issue of Antimicrobial Resistance

Antimicrobial (including antibiotic) drugs are compounds used to treat microbial (e.g., bacterial) infections in humans, animals and plants, and work by inhibiting pathogen reproduction (Pursel, 2020). They have also been historically employed as growth promoters to increase meat production; however, this practice has been banned in many countries, including all European Union members since 2006 (Cogliani et al., 2011). Antimicrobial resistance occurs when an organism undergoes spontaneous mutational adaptations or acquire external genetic traits which restrict or hinder an antimicrobial agent's mode(s) of action (Munita et al., 2016). This is considered a natural evolutionary response, with evidence of antimicrobial-resistant bacteria pre-dating the development and commercialisation of antimicrobial drugs (D'Costa et al., 2011). However, anthropogenic behaviours, including the historical over-use (with hundreds of thousands of tons sold per annum) and inappropriate disposal of human and veterinary antimicrobials have resulted in the addition of significant selective pressures both in clinical and non-clinical settings (i.e., the natural environment) (Van Boeckel et al., 2014; Bell et al., 2014). The overarching legacy and ongoing consequence of these widespread practices have resulted in rapid global dissemination of antimicrobial resistance, commonly attributed to the horizontal transfer of resistance determinants (i.e., antimicrobial resistance genes; ARGs) between organisms and species inhabiting the same environments.

Three classifications are used to classify an organism's response to a specific antimicrobial drug; namely: susceptible (i.e., where bacterial growth is inhibited by

the antimicrobial at recommended therapeutic concentrations), intermediately-resistant (i.e., where the antimicrobial is ineffective at inhibiting growth at recommended and commonly used therapeutic concentrations becoming efficacious only where the drug is physiologically concentrated) and resistant (i.e., where the antimicrobial does not prohibit bacterial growth at achievable therapeutic concentrations) (CLSI, 2020). The human health implications of antimicrobial treatment losses (which may occur due to resistant and/or intermediately-resistant pathogens) are well documented, with increasing notifications of treatment failure for both human and veterinary infectious diseases (Wright, 2010; Opatowski et al., 2019) leading to more severe, prolonged, and fatal infections, and prohibitive healthcare costs (Laxminarayan et al., 2013). Recent global estimates indicate that in 2019 alone, as many as 1.27 million deaths were directly attributable to infections caused by antimicrobial resistant bacteria (ARB) (Murray et al., 2022). To combat this, key global recommendations are to (i) limit the selective pressures posed by antimicrobial-rich environments via the rational use of antimicrobials in both human and veterinary medicine, and (ii) increase efforts to develop and guarantee widespread access to novel antimicrobial classes (Infectious Diseases Society of America, 2011; Laxminarayan et al., 2013, WHO, 2022).

1.2 Antimicrobial Resistant Bacteria: emerging groundwater contaminants

Groundwater is the world's largest reservoir of freshwater, and the primary domestic drinking water source for approximately 2.2 billion people worldwide (Murphy et al., 2017). Therefore, groundwater resources can, when contaminated, represent a potentially important pathway for the dissemination of ARB and

subsequent infections. Indeed, provision of direct and indirect human (and animal) exposure is possible via groundwater consumption (Coleman et al., 2012) and through its use in agricultural irrigation (Siebert et al., 2010). Compounding this, small-scale private groundwater abstractions remain widely unregulated, thus presenting a favourable and buffered environment for harbouring diverse microbiological contaminants (including pathogens and ARB), as well as pollutants which may present co-selective pressures for antimicrobial resistance acquisition and/or persistence (e.g., trace metals, biocides, and disinfectants) (Wu et al., 2018; Zhang et al., 2018).

ARB contamination has already been acknowledged as the most important of six emerging issues of environmental concern by the United Nations Environmental Programme (UNEP, 2017). With regards to groundwater environments specifically, no reviews were available prior to this research which had quantified the extent of this issue. However, reports from as early as 1976 had already identified presence of ARB (≈ 49% of the 321 total and faecal coliform isolates tested) within groundwater supplies in New Zealand, for example (Cooke, 1976). More recently, varying levels of ARB contamination have been reported in drinking wells across the globe – e.g., Canada (Coleman et al., 2013), Guinea-Bissau (Machado and Bordalo, 2014), Ireland (O'Dwyer et al., 2017), United States (Kim et al., 2018), and Nigeria (Akaniro et al., 2019), to name a few. The widespread nature of this groundwater contamination issue highlights the importance of better understanding ARB presence and dissemination within these environments. Research outputs presented here can then be used to inform source-protection strategies, particularly in the case of drinking

resources, in line with Sustainable Development Goals 3 ("good health and well-being") and 6 ("clean water and sanitation for all") (United Nations, 2014).

1.3 The Republic of Ireland as a study area

Groundwater reliance in the Republic of Ireland (RoI) is relatively high, with over 180,000 wells used for domestic and industrial/agricultural use across the country (EPA, 2021). While domestic supplies include regulated community systems, most abstractions are household-based and unregulated (i.e., private wells), located in rural areas where public supply networks are not available. These private wells currently provide drinking water to 11% of the Irish population (DEHLG, 2021) and have been shown to be at an increased risk of microbiological and chemical contamination (Hynds et al., 2014; O'Dwyer et al., 2018), with instances of contamination facilitated by overarching lack of appropriate well construction and maintenance (Hynds et al., 2013; Schuitema et al., 2020). As private wells in the RoI are mostly located in rural settlements, they are highly exposed to contaminants arising from both widespread agricultural practices (i.e., animal origin) and on-site domestic wastewater treatment systems (DWWTSs) (i.e., human origin) (Bacci and Chapman 2011; Hynds et al., 2012; 2014; O'Dwyer et al. 2014); both of which are also potential sources of antimicrobials and ARB (Frey et al., 2015; O'Dwyer et al., 2017).

The RoI is also characterised by a diverse bedrock geology, with the majority of aquifers used for domestic abstraction being unconfined (i.e., not overlain by impermeable rock or clay and having the water table as upper boundary; see **Figure 1.1**) and thus more vulnerable to increased recharge caused by rainfall (as these can lead to shallower water tables and more exposed aquifer settings). Moreover, the

theoretical risk of contamination via groundwater recharge in the RoI has been ranked by the Geological Survey of Ireland (GSI) in partnership with the Environment Protection Agency (EPA) and approximately 63.5% of the country's land cover is classified as of 'high', 'extreme' or 'extreme-karst/rock' groundwater vulnerability (DEHLG/EPA/GSI, 1999; GSI, 2017). Briefly, groundwater vulnerability classifications are determined based on depth to bedrock, subsoil permeability, and (in the case of gravel aquifers) the unsaturated zone thickness (DEHLG/EPA/GSI, 1999) (**Table 1.1**). Groundwater vulnerability is highest in karst limestone aquifers, which represent approximately 19% of the country's land cover (mostly in the northwest region; **Figure 1.2)**. In these aquifers rapid groundwater flow through conduit, fissure and cave systems do not allow for sufficient retention times required for attenuation of microbiological and chemical contaminants (i.e., these are not sufficiently filtered out during transport through by the porous media). Limited contaminant attenuation due to rapid flow in fractured networks can also be present in productive fractured bedrock and poor bedrock aquifers, which are found in approximately 78% of the country's territory (**Figure 1.2**). The lowest groundwater vulnerability, covering just under 13.8% of land in the RoI (GSI, 2017), is typically described as areas where low permeability layers of clay soil and subsoil reduce contaminant vertical migration and recharge (Tynan, 2014).

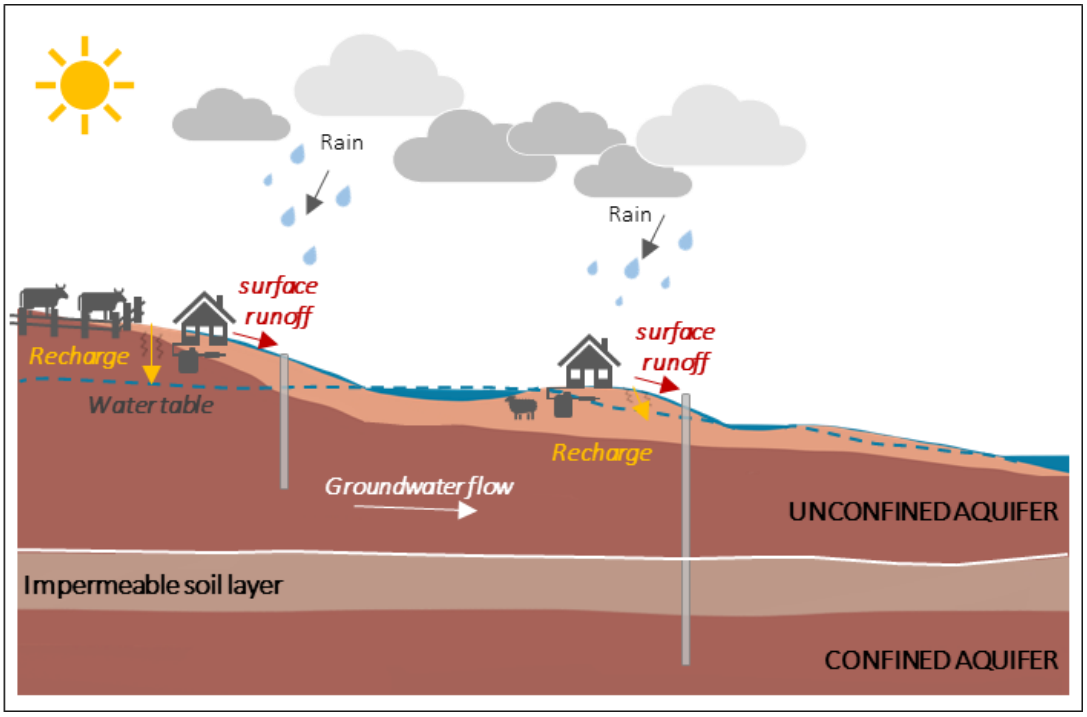


Figure 1.1: Schematic showing groundwater wells in a confined and unconfined aquifers and highlighting the water movement in the surface and subsurface (i.e., surface run-off, recharge, and groundwater flow)

Table 1.1: Groundwater vulnerability mapping criteria for the Republic of Ireland (simplified from DEHLG/EPA/GSI, 1999)

Depth to rock	Bedrock and karst aquifer types			All sand and gravel aquifer types
	sand or gravel subsoil (i.e., high permeability)	sandy subsoil (i.e., moderate permeability)	clay/clayey or peat subsoil (i.e., low permeability)	
0 - 3 m	Extreme	Extreme	Extreme	Extreme
3 - 5 m	High	High	High	High
5 - 10 m	High	High	Moderate	High
> 10 m	High	Moderate	Low	High

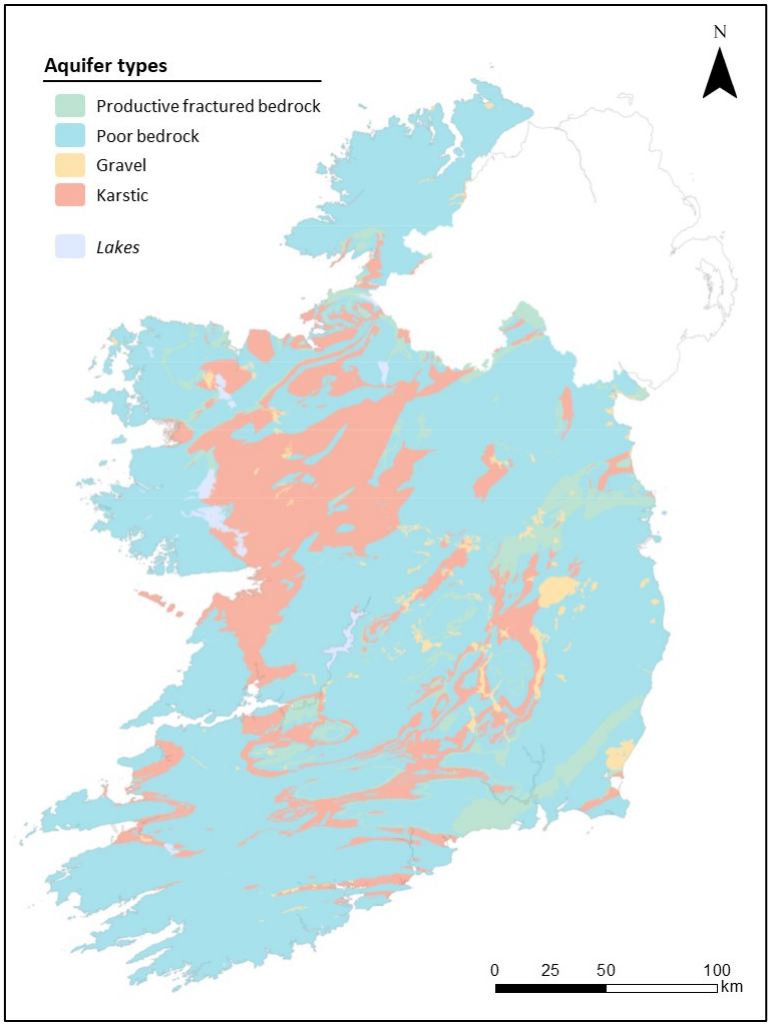


Figure 1.2: Map of the Republic of Ireland showing the nationwide distribution of the four main aquifer types (i.e., productive fractured bedrock, poor bedrock, gravel, and karstic). Source: GSI (2017)

In addition to a high percentage of 'highly vulnerable' hydrogeological settings, the RoI is characterised by a maritime temperate climate without a distinct dry season (Peel, 2007). These climatic conditions, in which days with little to no rainfall are infrequent, increase the risk of groundwater contamination due to their direct effect in triggering recharge and surface run-off (see **Figure 1.1**) and resulting in the transport of potential contaminants from the surface into groundwater supplies and aquifers (Lee, 2005; Hynds et al., 2012). Indeed, increased antecedent rainfall has been extensively linked to instances of microbiological groundwater contamination in national (Hynds et al., 2012; O'Dwyer et al., 2014; Andrade et al., 2022) and

international literature (Engstrom et al., 2015; Murphy et al., 2020). Thus, groundwater supplies in the RoI can represent a clear source-pathway-receptor model for ARB – i.e., the movement of human and/or agricultural waste into groundwater supplies via infiltration, recharge, and/or surface run-off followed by direct wellhead ingress (all of which can be triggered by rainfall events; Lee, 2005; Hynds et al., 2012)and subsequent exposure to human (and animal) receptors via consumption. Accordingly, the RoI represents a relevant case study to examine the occurrence and likely drivers of ARB within a rural setting, while also allowing for transferability of findings. Compounding this, and as a considerable proportion of the Irish population relies on unregulated groundwater supplies for consumption (i.e., potential receptors) (Hynds et al., 2013, Mooney et al., 2021), the risk of direct human exposure to ARB, where present, also represents a key concern. Hence, findings from this RoI-based study can also be used to provide evidence-based source protection advice and inform antimicrobial resistance mitigation policies to safeguard human health.

1.4 Rationale for organism selection

It has been well-established that distinct organisms (e.g., bacterial species) respond differently to available antimicrobial drugs. Gram-negative bacteria, for example, characterised by a thinner cell wall than their gram-positive counterparts (Silhavy et al., 2010), are typically more vulnerable to (i.e., more likely to acquire antimicrobial resistance determinants from) antimicrobial drug interventions, exposure to sub-therapeutical antimicrobial levels, and co-selective substances such as metals/metalloids (Qing et al., 2007; Steinbuch and Fridman, 2016; Gupta and

Datta, 2019). Additionally, specific antimicrobial modes of action can be entirely ineffective against particular bacterial species due to intrinsic resistance, owed to certain genetic and/or physiological characteristics (Christaki et al., 2020).

As such, it is important that studies incorporate analyses of more than one bacterial species to gain insights into the (possibly differing) origins and transport pathways of distinct antimicrobial resistant organisms into groundwater environments. Additionally, assessment of two or more species provides a more holistic picture of local groundwater and source-specific resistomes and aids in increasing the accuracy of ARB presence/absence measurement in that particular environment. Detailed descriptions of the two species incorporated in the current study (i.e., *Escherichia coli* and *Pseudomonas aeruginosa*) are provided below (see also **Table 1.2**).

Table 1.2: Summary of key Escherichia coli and P. aeruginosa characteristics

Characteristics	Studied bacteria	
	E. coli	***P. aeruginosa***
Family	*Enterobacteriaceae*	*Pseudomonadaceae*
Gram classification	Gram-negative	Gram-negative
Primary habitat	Intestinal flora of warm-blooded animals	Soil, water, and vegetation
Occurrence in Irish groundwater environments	Found in 28.9 (Hynds et al., 2012) to 51.4% of groundwater supplies (O'Dwyer et al., 2018)	No baseline data available
Role as a water quality parameter	Employed as general microbiological parameter for drinking water quality monitoring in the EU (Council Directive, 2015) parametric threshold = 0/100 ml (tap water) and 0/250 ml (bottled water)	Listed as a microbiological parameter with approved enumeration methodology (Council Directive, 2015)
Associated (serious) waterborne infections	VTEC-enteritis (also referred to as STEC-enteritis) These can cause severe damage to intestines and kidneys and lead to serious complications such as haemolytic uremic syndrome (HUS), which is sometimes fatal	Serious *P. aeruginosa* infections from ingestion are rare, however this is still possible particularly in immunocompromised persons (e.g., those with cystic fibrosis*). Additionally, topical contact can lead to skin rashes, and ear, eye and urinary tract infections
Detection and enumeration methods	Membrane Filtration (ISO 9308-1, 2014) and Most Probable Number methods (ISO 9308-2, 2012; e.g., Colilert)	Membrane Filtration (ISO 16266, 2006) and Most Probable Number methods (ISO 16266-2, 2018; e.g., Pseudalert)
Inclusion in previous groundwater-based antimicrobial resistance research	38 studies (54.3%; Andrade et al., 2020)	12 studies (17.1%; Andrade et al., 2020)
WHO priority pathogen list	carbapenem-resistant and ESBL-producing strains	carbapenem-resistant strains
Key issue associated with resistance acquisition	Ubiquitous in the human (and animal) gut, and can serve as a pathway for host colonisation with resistant determinants (Coleman et al., 2012)	Susceptible to a limited number of treatment options, due to intrinsic resistance to a wide variety of antimicrobials (Breidenstein et al., 2011)
Mean rates of antimicrobial resistance in groundwater-based studies	79.8% ± 30.1 (Andrade et al., 2020)	100.0%** (Andrade et al., 2020)

VTEC = Verotoxigenic *E. coli*; STEC = Shiga toxin-producing *E. coli*; ESBL = Extended spectrum beta-lactamase;
* Cystic fibrosis is an inherited disorder that causes damage to the lungs, digestive system, and other organs, and of which Ireland has the largest incidence in the world (approximately 1:1353 people; Farrel et al., 2007);
**data only available for studies in low to middle income countries and which included antimicrobials to which *P. aeruginosa* is intrinsically resistant (Andrade et al., 2020).

1.4.1 Escherichia coli

Escherichia coli (*E. coli*) is a gram-negative coliform bacterial species, member of the *Enterobacteriaceae* family, and an integral component of human and other warm-blooded animals' intestinal microflora. *E. coli* is also the most abundant facultative anaerobic organism in the human gut (Kaper et al., 2004). Most *E. coli* strains are non-pathogenic and co-exist with their mammalian host, however, several serotypes can be pathogenic, causing enteric diseases and/or extra-intestinal infections (e.g., urinary tract infections and meningitis) (Kaper et al., 2004). The relevance of the *E. coli* species in (ground)water contamination studies is linked to their use as a faecal indicator organism, due to its ubiquitous presence in faeces and limited proliferation in natural environments, in combination with the wide availability of simple and validated methods for its detection and quantification in water (ISO 9308-2, 2012; ISO 9308-1, 2014). This species is also a noteworthy organism within antimicrobial resistance research as carbapenem-resistant and extended spectrum beta-lactamase-producing (ESBL-producing) *E. coli,* together with the entire *Enterobacteriaceae* family, have been listed as *critical* in the World Health Organization's priority pathogens list of antibiotic-resistant bacteria (WHO, 2017).

1.4.2 Pseudomonas aeruginosa

Pseudomonas aeruginosa (*P. aeruginosa*) are gram-negative bacteria belonging to the *Pseudomonadaceae* family. They are opportunistic pathogens frequently associated with chronic respiratory infections in patients suffering from cystic fibrosis, as well as urinary tract infections, ear infections, etc., particularly in immunocompromised individuals (Gellatly and Hancock, 2013). *P. aeruginosa* is

typically associated with nosocomial infections (i.e., hospital-acquired) (Gellatly and Hancock, 2013; Cabot et al., 2016), however its relevance as a pathogenic contaminant outside clinical settings is also recognised. This is seen, for example, in the inclusion of approved methodologies for its enumeration in the European Union (EU) Drinking water directive (Council Directive, 2015); namely ISO 16266 (2006) and ISO 16266-2 (2018). *P. aeruginosa* can also proliferate in a wide range of environments, including freshwater and soil, due to its metabolically versatility (Cabot et al., 2016). Inherent versatility (and in particular the presence of endogenous ARGs and multidrug efflux transporters) is also responsible for the species' intrinsic resistance to myriad antimicrobial agents, including aminoglycosides, fluoroquinolones, and beta-lactams (Breidenstein et al., 2011; Poole, 2011). This may account, in part, for *P. aeruginosa* infections having the some of the highest fatality rates of all gram-negative bacterial infections (Aliaga et al. 2002), with loss of available (and already limited) treatment options being particularly worrisome. Carbapenem-resistant *P. aeruginosa*, in particular, features as 'critical' in the WHO priority pathogens list of antibiotic-resistant bacteria (WHO, 2017). The human health significance of this species in the context of antimicrobial resistance surveillance and research is further highlighted by the provision of *P. aeruginosa*-specific susceptibility breakpoints in the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020).

1.5 Research aim and objectives

The principal research aim was to assess the role of groundwater resources as reservoirs for ARB and identify risk factors associated with ARB occurrence. To achieve this, the following five specific objectives were undertaken:

1. Review the rates of ARB contamination within groundwater resources globally, determining potential trends and knowledge gaps. [**Chapter 2**]

2. Assess the spatiotemporal variability in faecal bacteria contamination within monitored (regulated) groundwater supplies in the RoI, as these organisms may harbour antimicrobial resistance. [**Chapter 3**]

3. Measure occurrence rates of ARB in (unregulated) groundwater supplies in the RoI, incorporating spatially (i.e., geographically spread) and temporally diverse sampling (i.e., collected at different time-periods). [**Chapter 4**]

4. Identify key hydrogeological, meteorological, anthropogenic and/or infrastructural factors associated with the presence of ARB in Irish drinking groundwater resources, highlighting their likely source(s) and pathway(s). [**Chapter 4**]

5. Investigate the impact of localised stressors (i.e., metal/metalloid co-contaminants) on the incidence of bacteria-encoded resistance genes within private groundwater resources in the RoI. [**Chapter 5**]

6. Provide recommendations for policy and groundwater management strategies to reduce the public health burden of exposure to antimicrobial resistance in untreated groundwater. [**Chapter 4** and **Chapter 5**]

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Chapter 2

Groundwater resources as a global reservoir for antimicrobial-resistant bacteria

Preface

Title: Groundwater resources as a global reservoir for antimicrobial-resistant bacteria

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Status: Published

LA's (CRediT) contribution: Conceptualization, Methodology, Investigation, Formal analysis, Data visualization, Writing – original draft, Writing – review & editing

Relevance: This is a systematic review and pooled-analysis of antimicrobial resistant bacteria (ARB), including intermediate resistance, within global groundwater resources. Importantly, the review and analyses carried out and reported here informed the subsequent work undertaken for this dissertation (see **Chapter 3**) by highlighting the need to incorporate re-sampling methods, collection and reporting of hydrogeological, meteorological and infrastructural data, and analyses of distinct bacterial species (with *E. coli* and *P. aeruginosa* standing out as strategic options).

Significance: This paper was the first to quantitatively synthesise reported instances of groundwater resource contamination with antimicrobial resistant bacteria across the globe. It identified that, where present, 80.2% ± 29.0 (median = 100% (IQR = 59.9 - 100.0%)) of groundwater-borne bacteria were resistant (here the terminology includes intermediate resistance) to at least one antimicrobial. Moreover, trends such as higher ARB occurrence rates in urban areas and during dry weather periods, were identified, and recommendations made for future studies.

Abstract

Antimicrobial resistance represents one of our most significant global health threats, with increasing incidences noted in both clinical and environmental settings. As such, identifying and understanding the sources and pathways for antimicrobial-resistant bacteria (ARB) is critical. The current study presents the first systematic review and pooled analysis of ARB occurrence in global groundwater supplies, which are used as primary drinking water sources by 2.2 billion people worldwide and are recurrently linked to significant outbreaks of infection. Seventy peer-reviewed studies were identified and included; findings reveal that 80.2% ± 29.0 (median = 100% (interquartile range = 59.9 – 100.0%)) and 57.2% ± 36.8 (57.7% (22.2 – 100.0%)). of groundwater isolates in the included studies were resistant to ≥ 1 and ≥ 3 antimicrobials, respectively. Where bacteria were present, ARB were identified in 76.9% ± 33.7 (100.0% (42.7 – 100.0%)) of individual wells and springs. Our results leave little doubt that groundwater represents a major global reservoir for ARB, however significant research is required to establish environmental determinants and mechanisms mediating their occurrence.

Highlights

- Global analysis of antimicrobial-resistant bacteria in groundwater sources.
- Where reported, 31.4% ± 32.6 of studied sources harboured resistant bacteria.
- In total, 80.2% ± 29.0 of aggregated isolates were antimicrobial-resistant.
- Overall, 57% ± 36.8 of isolates were resistant to ≥3 antimicrobials.

- Results highlight groundwater is a noteworthy source of antimicrobial resistance.

Keywords: Groundwater; Antimicrobial Resistance; Drinking water; Environment and Health; Risk Factors.

2.1 Introduction

Antimicrobial resistance is now widely recognised as a global public health threat, requiring multi-sectorial preventative and mitigative interventions (Bradford and Harvey, 2017; WHO, 2018; Larsson et al., 2018). Anthropogenic influences and behaviours, including the misuse/overuse of human and veterinary antimicrobials has resulted in the addition of significant selective pressures to the naturally occurring resistance within and between bacterial species (Van Boeckel et al., 2014; Bell et al., 2014; O'Neill, 2016). As such, a global increase in acquired resistance traits has been noted among bacterial isolates, including clinically significant species (e.g., *Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterobacteriaceae* spp.; WHO, 2017) with the incidence of multidrug resistance (i.e., resistance to ≥ 3 antimicrobials) increasing both spatially and temporally (Munita and Arias, 2016). Cases of treatment failure of both human and veterinary infectious diseases are increasingly documented (Wright, 2010; Opatowski et al., 2019), which results in higher healthcare costs, more severe and prolonged infections, and rising rates of morbidity and mortality (Laxminarayan et al., 2013). Recent estimates by the European Antimicrobial Resistance Surveillance Network (EARS-Net) indicate that approximately 33,000 deaths are attributable to antimicrobial-resistant bacterial infections per annum within the EU/EEA, comparable with the combined human

health burden of influenza, tuberculosis, and HIV/AIDS in the same region (Cassini et al., 2019). Accordingly, antimicrobial resistant infections have received significant attention within both the media and research community over the past two decades, with an extensive body of research existing in clinical settings, coinciding with the development of several antimicrobial resistance action plans at varying scales (European Commission, 2011; WHO, 2015). More recently, however, the role of the natural aquatic environment as a source and transmission pathway for antimicrobial-resistant bacteria (ARB) has been acknowledged as an area of growing concern (Sanderson et al., 2018).

Human and veterinary antimicrobials are increasingly released to the environment at sub-therapeutic concentrations via myriad sources of domestic, agricultural, industrial, and clinical/hospital origin. These microbiologically diverse, pharmaceutically dilute media may readily catalyse the development of ARB within the natural environment, inevitably resulting in their ingress to both surface and groundwater sources (Van Schaik, 2015). However, at present, more research is still needed to better understand the occurrence and transport of ARB to and in natural waterbodies. This is particularly true with regard to groundwater wells and aquifers, which currently supply approximately 27.8% (2.2 billion people) of the global population with domestic drinking water (Murphy et al., 2017).

Microbial contamination of groundwater and its adverse public health effects have been well substantiated within the scientific literature. A recent review by Murphy et al. (2017) presents clear epidemiological evidence of disease transmission due to groundwater contamination at a global scale, with an

estimated 35.2 to 59.4 million cases of acute gastrointestinal infection potentially attributable to groundwater consumption per year. Thus, the potential implications of groundwater-borne ARB pose a significant threat to public health, allied with an already high global burden of infection. A recent study in the Republic of Ireland found that wastewater systems, livestock density and the presence of children in a household were significantly associated with the presence of antimicrobial resistant *Escherichia coli* (*E. coli*) in private groundwater supplies (O'Dwyer et al., 2017). As such, the ubiquity of contaminant sources, in concurrence with the presence of bacteria and sub-therapeutic antimicrobial concentrations, suggests that vulnerable groundwater systems may be a significant and frequently overlooked reservoir for ARB (Wellington et al., 2013). Indeed, research carried out with 878 Canadian individuals has shown that consumers of *E. coli* contaminated groundwater are 1.26 times more likely to be colonised by antimicrobial resistant *E. coli* than non-consumers (OR = 1.26, p = 0.027) (Coleman et al., 2012).

Nonetheless, there is presently no consensus regarding the role of groundwater in the global dissemination of ARB. The extent of this threat is further complicated by the nuances of groundwater contamination mechanisms, which are typically determined and/or driven by numerous environmental and source-specific risk factors such as source design, location and maintenance, local hydrogeological setting, and shifting climatic and landuse patterns (Hynds et al., 2012; Wallender et al., 2014; Atherholt et al., 2017; Andrade et al., 2018). Accordingly, the current study sought to further understand the occurrence, distribution, and potential drivers of ARB in groundwater sources (i.e., wells and springs) via a global pooled analysis of peer-reviewed studies. Findings can be used to support evidence-based

risk management strategies to inform non-clinical considerations in existing antimicrobial resistance action plans and guide future research strategies.

2.2 Methods

2.2.1 Literature identification

Identification of scientific articles examining the occurrence of ARB in groundwater sources was conducted with an overarching systematic review protocol based upon pre-established guidelines (Pullin et al., 2018) and adapted from previous studies (Eftim et al., 2017; 2018; Nappier et al., 2019). Defined search terms (**Table 2.1**) were used to search Scopus, Web of Science, Pub Med, and ProQuest databases on October 8th, 2018. Employed "Outcome" search terms, and particularly the antimicrobials and bacterial species selected, were based on the global priority list of ARB (WHO, 2017). Manual supplementary searches were additionally performed between October 15th and November 12th, 2018. These comprised the examination of article bibliographies and studies citing articles which were identified via database search and marked as "provisionally included" upon title and abstract assessment (n = 215).

Table 2.1: Search terms employed in the current study

Element	Description	Search terms				
Population	All non-saline groundwater	Groundwater, Ground Water, Aquifer, Subsoil, Subsurface, Borehole, Bore Well, Bored Well, Dug Well, Well Water, Water Well				
Outcome(s)	Occurrence of antimicrobial-resistant (or susceptible) bacteria	Antimicrobial, Antibiotic, Antibacterial, Bacteriostatic, Bactericidal, Penicillin, Cephalosporin, Carbapenem, (Fluoro)quinolone, β lactam, Aminoglycoside, Tetracycline, Vancomycin, Clarithromycin, Ampicillin, Sulphonamide	*AND*	Resistance, Resistant, Susceptible, Susceptibility, Sensitive, Sensitivity	*AND*	Bacteria, Bacterial, Microbe, Microbial, Organism, Pathogen, *E. coli*, *Escherichia coli*, *Pseudomonas*, *Enterococcus*, *Enterococci*, *Campylobacter*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Shigella*, *Klebsiella*, *Enterobacter*, *Enterobacteriaceae*

2.2.2 Study selection

Studies uncovered during the identification phase (i.e., database and supplementary searches) were independently screened by two authors using explicit eligibility criteria (**Table 2.2**) via title and abstract assessment. Articles without an available full text were excluded, with eligibility disagreements resolved via provisional inclusion. All "provisionally included" and manually identified studies had full-texts manually (i.e., no computer-assisted techniques employed) and independently assessed (eligibility assessment) using the presented criteria (**Table 2.2**). Disagreements at this stage were resolved via author panel consensus.

Excluded studies were those that: i) reviewed previously published studies, ii) incorporated experimentally controlled elements in their study design (i.e., spiking or laboratory-based (micro-, meso-) groundwater environments), iii) examined thermal/hot springs, iv) did not study water samples derived from groundwater sources (e.g., surface and marine water), v) combined resistance data from multiple

environmental media, vi) did not examine ≥ 10 distinct groundwater samples (including articles where sampling number was not reported), vii) did not analyse antimicrobial resistance/susceptibility in bacterial isolates from groundwater (including studies where bacteria were not found), viii) did not report number of isolates tested, ix) did not report bacterial resistance (or susceptibility) to each antimicrobial tested against (**Figure 2.1**).

Table 2.2: Eligibility (inclusion/exclusion) criteria employed

Inclusion Criteria	Exclusion Criteria
Study type: All peer-reviewed articles excluding reviews	**Study type:** Academic reviews; grey literature
Language: English	**Language:** non-English
Population: Naturally occurring groundwater environments; groundwater sources (i.e., wells and springs)	**Population:** Artificial groundwater media (i.e., lab-based); pre-packaged water; Thermal/Hot springs; surface water bodies; maritime aquatic environments; wastewater treatment plants; soil; saline, brackish, or soil water.
Exposure: Pre-existing environmental exposures (i.e., prior to study)	**Exposure:** Any controlled exposure (i.e., spiking)
Event/Outcome: Antimicrobial-resistant (or susceptible) bacteria found in groundwater resources	**Event/Outcome:** Absence of bacterial contamination in groundwater; absence of antimicrobial resistance profiling of groundwater isolates
Study design: Analysis of ≥ 10 groundwater samples; results including percentages of bacterial isolates resistant/susceptible to each antimicrobial tested against.	**Study design:** Analysis of < 10 groundwater samples (includes number of groundwater samples not reported); results combined for different sampled environments; results combined for all antimicrobials tested against; number of isolates tested not reported.
Period: Any	**Period:** -

2.2.3 Study Inclusion

During full-text assessment (eligibility phase), studies included were those that assessed 10 or more groundwater samples and explicitly stated the percentage of bacterial isolates resistant or susceptible to each antimicrobial agent tested against. In all, 76 studies were excluded due to reporting on < 10 groundwater samples, including studies where sample number was not reported (n = 12) (exclusion criteria

vi). Forty-six studies reported composite occurrence rates of ARB in combined study environments (e.g., merged findings from groundwater, surface water and/or wastewater), and as such were excluded under exclusion criteria (v). Studies that did not provide an adequate description of water sample origin; that is, referred solely to “tap water” (n = 14) were excluded under exclusion criteria (iv) (i.e., groundwater source not analysed) and where bacterial isolates were not identified in groundwater samples (n = 4), articles were excluded under exclusion criteria (vii), as antimicrobial resistance could not be determined, this criteria also comprised studies that provided an assessment of antimicrobial resistance through genotypical methods only (i.e., presence/absence of resistance genes via qPCR or digital droplet qPCR analyses). As such, 70 of 1,864 identified studies (identification phase) were deemed eligible for inclusion following the full review process (**Figure 2.1**).

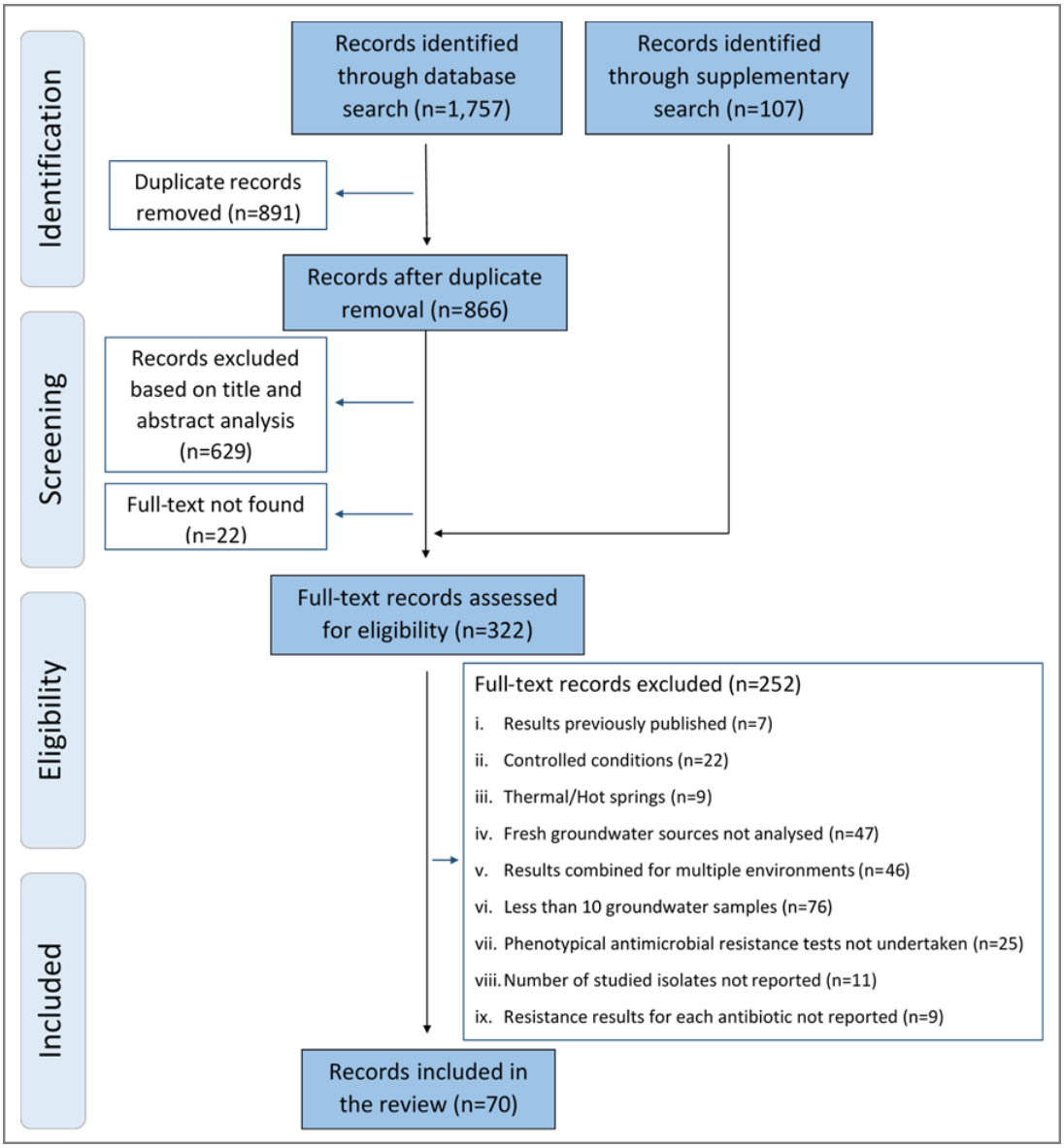


Figure 2.1: Systematic review protocol employed during the current study, including literature identification, screening, eligibility assessment, and final study inclusion.

2.2.4 Critical appraisal of study validity

Included articles were independently evaluated by two authors using a critical appraisal tool adapted from Bain et al. (2014). In it, a score ranging from 0 to 14 was attributed to each study according to the number of affirmative responses to the fourteen pre-established criteria. Based on it, articles were classified as presenting low (score ≤ 5), medium (score of 6 to 8) or high (score ≥ 9) validity. Individual article

assessments are presented in **Supplementary Data**. All disagreements were resolved by a consensus following a discussion between authors.

2.2.5 Data extraction

Relevant data pertaining to each included study were extracted to MS Excel 2016. Extracted variables were classified and exported under six primary categories; namely, bibliographic details, study region (e.g., country, location within country and settlement type), groundwater characteristics (e.g., source type, well type, ownership, uses and treatment presence), sampling regime (e.g., number of samples, re-sampling, length of sampling regime), analytic elements (e.g., bacterial species tested, antimicrobial agents tested against, method and criteria were used to assess susceptibility/resistance), and resistance profile (e.g., percentage of ARB and MRB amongst tested isolates and percentage of resistance to each antimicrobial agent tested against). It is important to note that with regards to studies in which re-sampling was employed, data pertaining to different sampling rounds were merged and extracted as single outcomes, with "re-sampling" and "one-off" used just to classify two contrasting approaches employed across identified studies. As each study only provides one outcome to the analysis, independence between observations from each analytical unit (i.e., study) can be assumed.

Antimicrobial resistance results were extracted according to the standards (i.e., EUCAST, CLSI, etc.) and interpretations employed in each published manuscript, inhibition zone and/or minimal inhibitory concentration results were not routinely specified and, as such, could not be uniformly re-assessed. Moreover, as intermediate resistance indicates that an antimicrobial is ineffective at

recommended and commonly used therapeutic concentrations (Rodloff et al., 2008), potentially resulting in treatment failure, it was considered as resistance, as per other studies (Reinthaler et al., 2003; O'Dwyer et al., 2017).

Where key variables were unclear or not explicitly documented, article authors were contacted for clarification and/or articles were analysed for identifiable characteristics, and thus classified. Where classification was not possible, variables were categorised as "not reported". Sample sizes were categorised as small (< 30 samples), medium (30 – 99 samples) or large (≥ 100), as previously defined by Bain et al. (2014), with a maximum threshold established whereby data from one study comprising > 5,000 samples were not extracted to prevent geographical and/or analytical bias (Coleman et al., 2013); however, data reported from a smaller sample (n = 657) in this study were included in analyses. As such, the maximum sample number from a single included study was 939 (Akoachere et al., 2013).

Studies were further classified according to globally established characteristics relating to the study regions. Countries were classified as low, lower-middle, upper-middle, and high income based upon World Bank classification (World Bank, 2018). Specific study areas' primary (arid, cold, polar, temperate, and tropical) and secondary (e.g., dry summer, dry winter, without dry season, monsoon, and rainforest) climates were determined based on the Köppen-Geiger climate classification (Peel et al., 2007), and used to ascertain sampling season (i.e., summer, winter, spring, autumn) and period (i.e., wet or dry).

Moreover, where possible, occurrence rates of antimicrobial resistance (≥ 1 antimicrobial) and multidrug resistance (≥ 3 antimicrobials) were calculated for all

isolates reported in a study (i.e., number of resistant and multidrug resistant isolated, respectively, divided by the total number of isolates recovered from all groundwater sources and samples examined within that study). This approach was used as even when studies employed re-sampling in their methodology (i.e., more than one sample taken from the same groundwater sources at different times), antimicrobial resistance results were often integrated during reporting. With regards to occurrence rates of ARB amongst groundwater sources (i.e., wells and/or springs) or the specific sources in which bacterial isolates were found, these comprised the percentage of sources that harboured ARB at least once, as reported in each manuscript (i.e., number of sources where ARB were found at least once divided by total number of tested sources or by the number of sources in which bacteria were found at least once, respectively).

2.2.6 Multiple Antimicrobial Resistance Index

Multiple Antimicrobial Resistance (MAR) indices were calculated to standardise the rates of antimicrobial resistance reported across each study (**Equation 2.1**; Krumperman, 1983). MAR indices provide a single measure of antimicrobial resistance and account for the number of antimicrobial agents tested against, thus avoiding potential bias (i.e., elevated ARB occurrence rates are typically found when more antimicrobials are incorporated in a study design).

$$MAR\ index = \frac{y}{n \times x} \qquad \text{[Equation 2.1]}$$

where y is the aggregate antimicrobial resistance score of all isolates tested (i.e., the sum of isolates resistant to each antimicrobial), n is the number of isolates

tested, and x is the number of antimicrobial agents tested against (Krumperman, 1983).

A single MAR index was calculated for each study, irrespective of temporal methodologies employed, and included in the analyses. These were used to ascertain the overall findings regarding antimicrobial resistance in groundwater bacteria on a study-by-study basis, thus permitting cross-study comparisons. Secondly, for descriptive purposes only, discrete MAR indices were calculated for each study within isolates of the same species or genus and within different antimicrobial classes. These were not included in analyses, but merely used to a) identify the relative rates of antimicrobial resistance associated with each bacterial genus, and b) determine the differences between resistance rates within and between different antimicrobial classes.

2.2.7 Data analyses

Three dependent variables were calculated and used to quantify presence of ARB and/or Multidrug Resistant Bacteria (MRB) in groundwater and represent the main findings from each included study (i.e., studies were the analytic unit throughout analyses), namely: i) occurrence rates of antimicrobial resistance (≥ 1 antimicrobial) amongst all groundwater isolates tested; ii) occurrence rates of multidrug resistance (≥ 3 antimicrobials) amongst all groundwater isolates tested; and iii) calculated study-specific MAR indices.

Dependent variables were not normally distributed and could not be normalized using standard (transformation) techniques, so non-parametric Mann-

Whitney U and Kruskal-Wallis tests were employed to identify categorical associations between dependant and independent variables (i.e., the extracted characteristics outlined in **Table 2.3**). Where significance was found ($p < 0.05$) and the independent variable described three or more levels of measurement, Dunn's (non-parametric) pairwise post-hoc tests were used. Mean MAR indices were further calculated for each country and discretized into ranges (e.g., 0.000-0.100, 0.100-0.200, …) using all included studies, with all studies equally weighted.

Table 2.3: External, source-specific, and study-specific characteristics and their sub-categories used as independent variables in the non-parametric statistical tests.

Categories		Sub-categories	Non-parametric test
External			
1.	Economic classification [a]	Low; Lower middle; Upper middle; High income	Kruskal-Wallis
2.	Climate [b]	Tropical; Arid; Cold; Temperate	Kruskal-Wallis
3.	Sampling period [c]	Wet; Dry	Mann-Whitney U
4.	Settlement type [d]	Urban; Rural	Mann-Whitney U
5.	Waste source adjacent to study site [e]	Human waste; Animal waste	Mann-Whitney U
Source-specific			
6.	Source type	Well; Spring	Mann-Whitney U
7.	Well type	Hand-dug; Bored	Mann-Whitney U
Study-specific			
8.	Sampling regime	One-off sampling; Re-sampled sources	Mann-Whitney U
9.	Sample size	Under 30; 30 to 99; 100 and over	Kruskal-Wallis
10.	Length of sampling period	Under 6 months; 6 to 12 months; more than 12 months	Kruskal-Wallis

[a] economic classification according to the World Bank (2018); [b] climate classified according to Peel et al. (2007); [c] wet and dry as defined in Peel et al. (2007); [d] urban settlements include urban, sub-urban or peri-urban regions; [e] human waste encompasses septic tanks and wastewater treatment plants, and animal waste encompasses animal grazing fields and agricultural fields where manure is spread.

2.3 Results

2.3.1 External, groundwater-specific and study-specific characteristics

A total of 70 relevant studies were included for data extraction and pooled analyses. A summary of all included studies is presented in **Table 2.4**, with detailed

study characteristics and full reference list provided in **Supplementary Data**. Relevant studies were conducted in geographically, climatically, and economically diverse regions and spanned a 42-year period (1976-2018), with 81.4% (n = 57) of identified articles published since 2010. All inhabited continents as well as four of the five climatic zones were represented. Overall, 51.4% (n = 36) of identified studies were undertaken in lower-middle income countries, followed by countries classified by high (21.4%; n = 15) and upper-middle (21.4%; n = 15) incomes. There were two primary sampling regimes identified, with repeat (temporal/seasonal) sampling employed in 32.9% (n = 23) of studies, while 52.9% (n = 37) performed one-off ('snapshot') cross-sectional sampling. Where sampling seasons could be ascertained (n = 58), it was exclusively undertaken during wet or dry seasons in 41.4% (n = 24) and 32.7% (n = 19) of studies, respectively; however, just 10.3% of studies (n = 6) explicitly reported this information.

Overall, 52.9% (n = 37) of studies were conducted in categorically rural areas, while 27.1% (n = 19) were undertaken in urban regions and the remaining 5.7% (n = 4) in "mixed settlements" (i.e., both urban and rural areas surveyed). Approximately two thirds (64.3%; n = 45) of studies reported the presence of faecal waste sources adjacent to study sites; 79.2% (n = 38) of human and 48.9% (n = 22) of animal origin (e.g., septic tanks, wastewater treatment plants, animal grazing fields and manure application). Wells and springs were examined in 91.4% (n = 64) and 12.9% (n = 9) of included studies, respectively, with 4.3% (n = 3) reporting concurrent analyses of both source types. Well construction was explicitly defined in 23 studies, of which 60.9% (n = 14) were hand-dug and 39.1% (n = 9) were bored. A significant paucity of data was encountered with respect to reporting of numerous source-specific elements

(e.g., supply depth, age, and operational condition/performance), with just 18.6% (n = 13) of included studies describing one or more of these. Similarly, just 17.1% (n = 12) of studies reported local hydrogeological characteristics (e.g., aquifer type, subsoil type, depth and permeability, bedrock geology and groundwater vulnerability). Sampled groundwater sources were used for human consumption in 57 of the 60 studies where groundwater usage was reported (95%), with an absence of water treatment before consumption noted in 76.9% of the 13 studies that reported this information (n = 10).

A total of 8,741 groundwater samples were collected across all included studies, ranging from 10 to 939 per study (mean ± SD = 125 ± 189), with 7,157 identified groundwater isolates examined for resistance against 89 distinct antimicrobial agents. *E. coli* was the most frequently analysed bacteria (54.3% of studies; n = 38), followed by *Pseudomonas* spp. (21.4%; n = 15). Combined, these corresponded to almost half of the isolates tested across all studies, with 2,737 and 824 tested isolates, respectively. The penicillin antimicrobial class was most frequently incorporated in study designs (94.3%; n = 66), followed by aminoglycosides (87.1%; n = 61), fluoroquinolones (81.4%; n = 57), cephalosporins (72.9%; n = 51), and tetracyclines (71.4%; n = 50).

Table 2.4: Summary of principal characteristics extracted, with study (n = 70) and groundwater sample (n = 8,741) number associated with each corresponding sub-category

Characteristics	Studies n (%)	Samples n (%)	Characteristics	Studies n (%)	Samples n (%)
Publication year			**Source type**		
Pre-1990	6 (8.6)	446 (5.1)	Wells	61 (87.1)	7,981 (91.3)
1990-1999	0 (0.0)	0 (0.0)	*Dug*	*13 [21.3]*	*2,123 [26.6]*
2000-2009	7 (10.0)	696 (8.0)	*Bored*	*9 [14.8]*	*1,236 [15.5]*
2010-2018	57 (81.4)	7,599 (86.9)	*Mixed*	*9 [14.8]*	*1,526 [19.1]*
			Not reported	*30 [49.1]*	*3,096 [38.8]*
Continent			Springs	6 (8.6)	423 (4.8)
Africa	32 (45.7)	3,941 (45.1)	Mixed	3 (4.3)	337 (3.9)
Asia	22 (31.4)	2,645 (30.3)			
Central & South America	6 (8.6)	436 (5.0)	**Water supply**		
North America	4 (5.7)	1,032 (11.8)	Private	17 (24.3)	2,893 (33.1)
Europe	5 (7.1)	658 (7.5)	Public	1 (1.4)	108 (1.2)
Oceania	1 (1.4)	29 (0.3)	Mixed	2 (2.9)	275 (3.1)
			Not reported	50 (71.4)	5,465 (62.5)
Economic classification [a]					
Low income	3 (4.3)	149 (1.7)	**Primary settlement type** [e]		
Lower-middle income	36 (51.4)	4,466 (51.1)	Rural	37 (52.9)	4,328 (49.5)
Upper-middle income	15 (21.4)	1,194 (13.7)	Urban	19 (27.1)	2,858 (32.7)
High income	16 (22.9)	2,932 (33.5)	Mixed	4 (5.7)	975 (11.2)
			Not reported	10 (14.3)	580 (6.6)
Climate [b]					
Tropical	25 (35.7)	3,506 (40.1)	**Waste adjacent to GW sites** [f]		
Arid	15 (21.4)	850 (9.7)	Human waste	23 (32.9)	3,301 (37.8)
Temperate	24 (34.3)	3,193 (36.5)	Animal waste	7 (10.0)	1,326 (15.2)
Cold	6 (8.6)	1,192 (13.6)	Animal and human waste	15 (21.4)	2,489 (28.5)
Polar	0 (0.0)	0 (0.0)	Not reported	25 (35.7)	1,625 (18.6)
Sampling period [c]			**Treatment before use**		
Wet	24 (34.3)	2,677 (30.6)	Yes	3 (4.3)	223 (2.6)
Dry	19 (27.1)	1,185 (13.6)	No	11 (15.7)	1,027 (11.7)
Both	15 (21.4)	3,857 (44.1)	Mixed [g]	4 (5.7)	1,680 (19.2)
Not identifiable	12 (17.1)	1,022 (11.7)	Not reported	52 (74.3)	5,811 (66.5)
Sample size [d]			**Human consumption**		
Small (< 30)	13 (18.6)	-	Yes	57 (81.4)	7,036 (80.5)
Medium (30 - 99)	34 (48.6)	-	No	3 (4.3)	299 (3.4)
Large (≥ 100)	23 (32.8)	-	Not reported	10 (14.3)	1,406 (16.1)
Length of sampling period			**Antimicrobials tested against**		
0 - 6 months	23 (32.9)	1,511 (17.3)	1 – 5	2 (2.9)	145 (1.7)
7 - 12 months	15 (21.4)	3,009 (34.4)	6 – 10	35 (50.0)	4,660 (53.3)
> 12 months	6 (8.6)	1,907 (21.8)	11 –15	20 (28.6)	2,589 (29.6)
Not reported	26 (37.1)	2,314 (26.5)	16 – 20	13 (18.6)	1,347 (15.4)
Sampling regime			**Type of bacteria**		
One-off sampling	37 (52.9)	2,920 (33.4)	Gram-positive	8 (11.4)	461 (5.3)
Re-sampled sources	23 (32.9)	4,405 (50.4)	Gram-negative	49 (70.0)	6,412 (73.4)
Not reported	10 (14.3)	1,416 (16.2)	Both	12 (17.1)	1,793 (20.5)
			Not specified	1 (1.4)	75 (0.9)
Validity score					
Low (≤ 5)	5 (7.1)	245 (2.8)			
Medium (6 – 8)	19 (27.1)	2,377 (27.2)			
High (≥ 9)	46 (65.7)	6,119 (70.0)			

[a] Countries economically classified according to the Work Bank (2018); [b] Climate classification according to Peel et al. (2007); [c] as defined in Peel et al. (2007); [d] Sample size classified following approach in Bain et al. (2014); [e] Urban settlements include urban, sub-urban and/or peri-urban regions; [f] Human waste encompasses septic tanks and wastewater treatment plants, and animal waste encompasses animal grazing fields, agricultural fields where manure is spread, etc.; [g] "Mixed" treatment before use encompasses studies in which treated and untreated supplies were combined during analysis

2.3.2 Critical appraisal of study validity

Study validity scores obtained varied greatly among included studies, spanning from 5 to 13, however most studies were considered of medium to high quality (i.e., ≥ 6; 92.8%; n = 65). The frequency of studies in each quality category are summarised in **Table 2.4** and complete analysis can be found in **Supplementary Data**.

2.3.3 Antimicrobial-resistant bacteria in global groundwater

Where reported (n = 20 studies; 28.6%), ARB were identified in 31.4% ± 32.6 (median = 20.2% (interquartile range (IQR) = 7.36 – 46.6%)) of studied groundwater sources and in 76.9% ± 33.7 (100.0% (42.7 – 100.0%)) of sources where bacteria were present. Additionally, 80.2% ± 29.0 (100.0% (59.8 – 100.0%)) and 57.2% ± 36.8 (57.7% (22.2 – 100.0%)) of pooled groundwater isolates (3,456 and 1,403 isolates, respectively) were resistant to ≥ 1 (ARB; n = 55; 78.6%) and ≥ 3 antimicrobials (MRB; n = 32; 45.7%), respectively.

As shown (**Figure 2.2**), consistently high occurrence rates of antimicrobial resistance were found among *Pseudomonas* (99.9% ± 0.2; range = 90.0 – 100.0%) *Klebsiella* (99.8% ± 0.6; 97.8 – 100.0%) and *Enterobacter* spp. (99.2% ± 2.2; 92.9 – 100.0%), as well as others which were reported in fewer studies (i.e., < 7), with *Pseudomonas* spp. also exhibiting high rates of multidrug resistance (96.6% ± 5.8; 88.3 – 100.0%). Moreover, *Pseudomonas* spp. (0.544 ± 0.237) were associated with some of the highest calculated MAR indices, across the 15 studies that examined the genus (**Figure 2.2**). MAR indices calculated within antimicrobial classes (**Table 2.5**) indicate that 1st generation Cephalosporins (0.594 ± 0.325), Glycopeptides (0.549 ± 0.378), 2nd generation Cephalosporins (0.529 ± 0.316) and Sulphonamides (0.517 ±

0.315) were associated with the highest mean MAR Indices. Conversely, lowest mean values were associated with Carbapenems (0.040 ± 0.078) and 4th generation Cephalosporins (0.150 ± 0.236).

A calculated mean MAR index of 0.352 ± 0.207 represents the level of ARB presence in global groundwater during the total review period (i.e., between 1976 and 2018; **Figure 2.3**), and of 0.359 ± 0.207 from 2010 to 2018 (not shown). Just one study exhibited a calculated MAR index of zero (i.e., Traoré et al., 2015). MAR index values were highest in Kenya ($\bar{x}$ ± SD= 0.549 ± 0.208), Nepal (0.545), China (0.537 ± 0.041), Morocco (0.528), Saudi Arabia (0.516) and South Africa (0.513 ± 0.122). At a continental level, MAR indices were highest in Africa (0.423 ± 0.202) and Asia (0.370 ± 0.201), followed by Central and South America (0.281 ± 0.098), Oceania (0.173 ± 0.000), Europe (0.138 ± 0.083) and North America (0.110 ± 0.045).

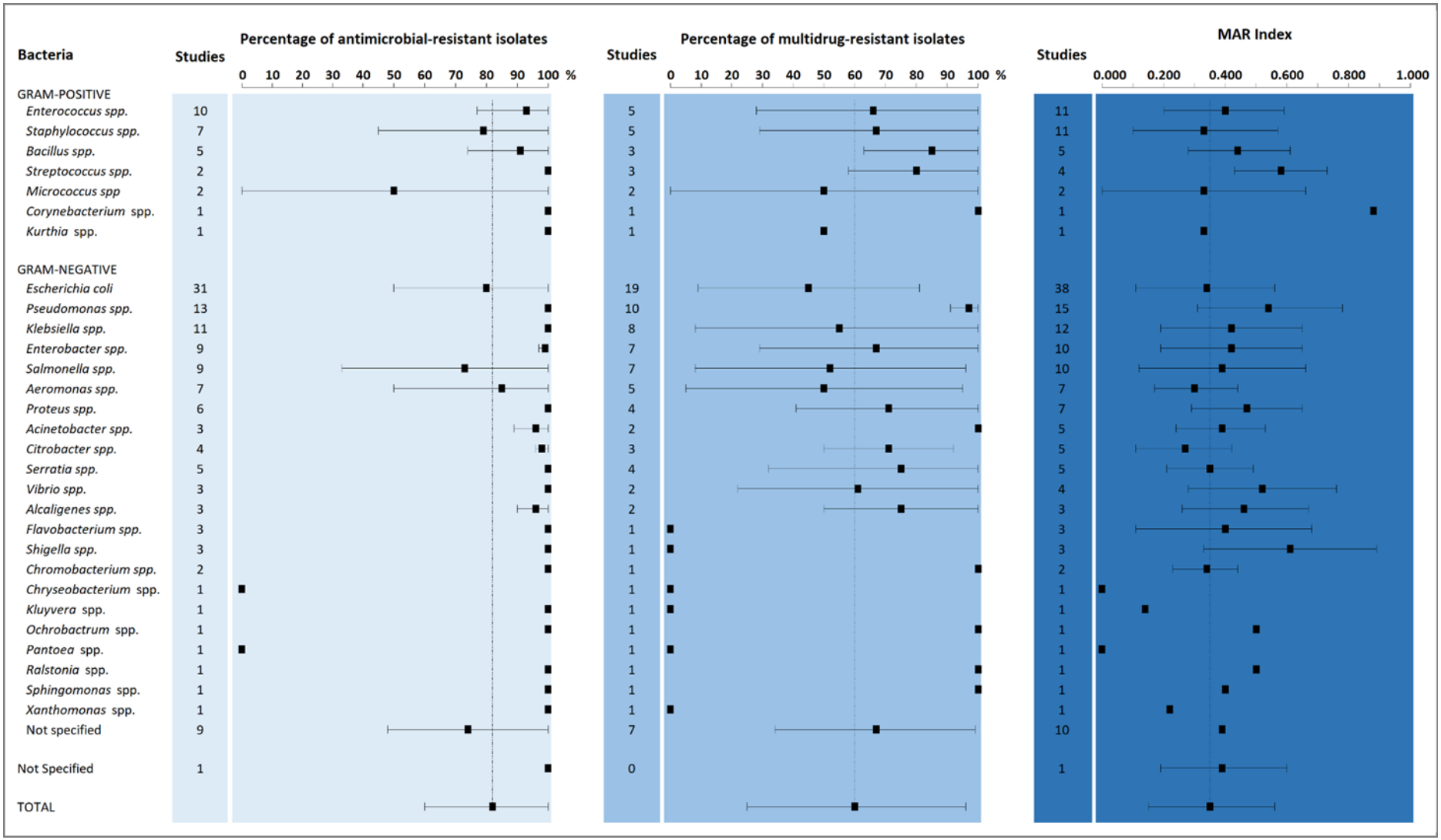


Figure 2.2: Forest plot of i) antimicrobial resistance, ii) multidrug resistance and iii) calculated multiple antimicrobial resistance (MAR) Indices in groundwater isolates from each genus examined

Table 2.5: Summary statistics of MAR indices calculated within antimicrobial classes and bacterial species

Bacteria	Antimicrobial class Mean ± SD (n)													TOTAL
	Aminoglycosides[1]	Carbapenems[2]	Cephalosporins				Fluoroquinolones[7]	Glycopeptides[8]	Lincosamides[9]	Macrolides[10]	Penicillins[11]	Sulfonamides[12]	Tetracyclines[13]	
			1st gen[3]	2nd gen[4]	3rd gen[5]	4th gen[6]								
Gram-positive														
Enterococcus spp.	0.344 ± 0.268 (7)	-	0.333 ± 0.333 (2)	0.923 ± 0.000 (1)	0.615 ± 0.440 (3)	-	0.188 ± 0.190 (6)	0.433 ± 0.350 (7)	0.692 ± 0.000 (1)	0.466 ± 0.245 (8)	0.518 ± 0.302 (10)	0.600 ± 0.400 (2)	0.417 ± 0.262 (9)	**0.396 ± 0.198 (11)**
Staphylococcus spp.	0.250 ± 0.314 (10)	-	0.451 ± 0.399 (3)	0.380 ± 0.302 (3)	0.283 ± 0.349 (7)	-	0.270 ± 0.390 (7)	0.285 ± 0.184 (3)	0.424 ± 0.348 (5)	0.208 ± 0.273 (4)	0.350 ± 0.255 (10)	0.629 ± 0.000 (1)	0.293 ± 0.194 (7)	**0.334 ± 0.235 (11)**
Bacillus spp.	0.243 ± 0.388 (5)	-	0.286 ± 0.286 (2)	0.583 ± 0.083 (2)	0.322 ± 0.349 (4)	-	0.333 ± 0.471 (3)	-	0.889 ± 0.079 (3)	0.545 ± 0.455 (2)	0.554 ± 0.246 (5)	0.000 ± 0.000 (1)	0.071 ± 0.071 (2)	**0.443 ± 0.163 (5)**
Streptococcus spp.	0.544 ± 0.390 (3)	-	-	0.683 ± 0.111 (2)	0.646 ± 0.256 (3)	-	0.545 ± 0.386 (3)	0.606 ± 0.000 (1)	0.714 ± 0.000 (1)	0.412 ± 0.412 (2)	0.561 ± 0.174 (4)[c #]	0.861 ± 0.000 (1)	0.585 ± 0.068 (3)	**0.581 ± 0.146 (4)**
Micrococcus spp.	0.406 ± 0.406 (2)	-	-	0.469 ± 0.000 (1)	0.328 ± 0.328 (2)	-	0.422 ± 0.422 (2)	-	0.813 ± 0.000 (1)	0.000 ± 0.000 (1)	0.286 ± 0.286 (2)	-	0.000 ± 0.000 (1)	**0.332 ± 0.332 (2)**
Gram-negative														
Escherichia coli	0.270 ± 0.302 (35)	0.051 ± 0.107 (7)[a]	0.599 ± 0.284 (5)	0.421 ± 0.333 (15)	0.375 ± 0.391 (19)[a]	0.036 ± 0.055 (4)	0.212 ± 0.226 (34)	0.486 ± 0.367 (3)	-	0.402 ± 0.288 (4)	0.486 ± 0.335 (36)	0.394 ± 0.281 (8)	0.428 ± 0.277 (28)	**0.339 ± 0.226 (38)**
Pseudomonas spp.	0.322 ± 0.371 (15)	0.151 ± 0.157 (4)[a*]	0.950 ± 0.000 (1)	0.666 ± 0.356 (6)	0.478 ± 0.380 (10)	0.185 ± 0.185 (2)	0.332 ± 0.371 (13)	0.194 ± 0.000 (1)	-	0.750 ± 0.204 (3)	0.614 ± 0.298 (15)	0.780 ± 0.137 (4)	0.643 ± 0.342 (10)	**0.544 ± 0.237 (15)**
Klebsiella spp.	0.307 ± 0.321 (12)	0.000 ± 0.000 (1)[a*]	0.586 ± 0.268 (3)	0.428 ± 0.384 (5)	0.261 ± 0.360 (7)[a*]	0.000 ± 0.000 (1)	0.260 ± 0.358 (11)	0.080 ± 0.080 (2)	-	0.750 ± 0.250 (2)	0.666 ± 0.229 (12)	0.956 ± 0.000 (1)	0.442 ± 0.285 (8)	**0.423 ± 0.231 (12)**
Enterobacter spp.	0.329 ± 0.406 (10)	0.000 ± 0.000 (1)[a]	0.798 ± 0.236 (3)	0.484 ± 0.358 (5)	0.276 ± 0.246 (6)[a]	0.000 ± 0.000 (1)	0.272 ± 0.367 (9)	-	-	0.542 ± 0.208 (2)	0.591 ± 0.212 (10)	0.857 ± 0.000 (1)	0.404 ± 0.342 (6)	**0.424 ± 0.229 (10)**
Salmonella spp.	0.300 ± 0.353 (9)	0.000 ± 0.000 (1)[a]	0.902 ± 0.000 (1)	1.000 ± 0.000 (1)	0.338 ± 0.413 (7)[a]	-	0.134 ± 0.246 (9)[b]	1.000 ± 0.000 (2)	-	0.802 ± 0.217 (3)	0.577 ± 0.410 (10)	0.427 ± 0.357 (3)	0.308 ± 0.372 (8)	**0.391 ± 0.271 (10)**
Aeromonas spp.	0.011 ± 0.018 (7)	0.000 ± 0.000 (2)	0.586 ± 0.309 (3)	0.813 ± 0.000 (1)	0.159 ± 0.268 (5)	0.000 ± 0.000 (1)	0.000 ± 0.000 (6)	1.000 ± 0.000 (1)	1.000 ± 0.000 (1)	0.778 ± 0.314 (3)	0.730 ± 0.331 (7)	1.000 ± 0.000 (1)	0.100 ± 0.200 (5)	**0.304 ± 0.135 (7)**
Proteus spp.	0.384 ± 0.432 (7)	-[4]	0.555 ± 0.016 (2)	0.524 ± 0.278 (4)	0.494 ± 0.271 (4)[a]	-	0.366 ± 0.317 (6)	-	-	0.500 ± 0.000 (1)	0.524 ± 0.274 (7)	1.000 ± 0.000 (1)	0.683 ± 0.331 (4)	**0.471 ± 0.182 (7)**
Acinetobacter spp.	0.117 ± 0.194 (5)	0.000 ± 0.000 (1)[a*]	0.467 ± 0.033 (2)	0.708 ± 0.257 (3)	0.594 ± 0.427 (4)	0.000 ± 0.000 (1)	0.263 ± 0.327 (4)	-	0.750 ± 0.000 (1)	1.000 ± 0.000 (1)	0.563 ± 0.343 (5)	0.833 ± 0.000 (1)	0.000 ± 0.000 (2)	**0.388 ± 0.151 (5)**
Citrobacter spp.	0.000 ± 0.000 (5)	0.000 ± 0.000 (1)	0.180 ± 0.055 (2)	0.563 ± 0.438 (2)	0.250 ± 0.433 (4)	0.000 ± 0.000 (1)	0.000 ± 0.000 (4)	-	-	0.625 ± 0.000 (1)	0.505 ± 0.346 (5)	0.588 ± 0.000 (1)	0.123 ± 0.102 (3)	**0.269 ± 0.155 (5)**
Serratia spp.	0.200 ± 0.400 (5)	0.333 ± 0.000 (1)[a]	0.536 ± 0.036 (2)	0.667 ± 0.333 (2)	0.111 ± 0.157 (3)[a]	0.000 ± 0.000 (1)	0.167 ± 0.289 (4)	-	-	0.250 ± 0.250 (2)	0.652 ± 0.129 (5)	0.429 ± 0.000 (1)	0.000 ± 0.000 (3)	**0.351 ± 0.138 (5)**
Vibrio spp.	0.359 ± 0.178 (4)	-	-	0.415 ± 0.000 (1)	0.360 ± 0.454 (3)	-	0.000 ± 0.000 (2)	1.000 ± 0.000 (1)	-	0.590 ± 0.410 (2)	0.820 ± 0.312 (4)	0.875 ± 0.125 (2)	0.491 ± 0.425 (4)	**0.520 ± 0.237 (4)**
Alcaligenes spp.	0.389 ± 0.437 (3)	-	0.750 ± 0.000 (1)	0.500 ± 0.000 (1)	0.250 ± 0.250 (2)	-	0.375 ± 0.375 (2)	-	-	0.000 ± 0.000 (1)	0.757 ± 0.236 (3)	0.417 ± 0.000 (1)	0.250 ± 0.250 (2)	**0.461 ± 0.205 (3)**
Flavobacterium spp.	0.250 ± 0.204 (3)	-	0.500 ± 0.000 (1)	0.500 ± 0.000 (1)	0.500 ± 0.500 (2)	-	0.250 ± 0.250 (2)	-	0.750 ± 0.000 (1)	0.000 ± 0.000 (1)	0.611 ± 0.283 (3)	0.333 ± 0.000 (1)	0.000 ± 0.000 (2)	**0.397 ± 0.282 (3)**
Shigella spp.	0.567 ± 0.419 (3)	-	-	0.833 ± 0.000 (1)	0.458 ± 0.458 (2)	-	0.375 ± 0.375 (2)	-	-	1.000 ± 0.000 (1)	0.617 ± 0.238 (3)	1.000 ± 0.000 (1)	0.500 ± 0.500 (2)	**0.607 ± 0.273 (3)**
Chromobacterium spp.	0.000 ± 0.000 (2)	1.000 ± 0.000 (1)	0.667 ± 0.000 (1)	-	0.500 ± 0.000 (1)	0.000 ± 0.000 (1)	0.000 ± 0.000 (1)	-	-	-	0.750 ± 0.250 (2)	0.000 ± 0.000 (1)	0.000 ± 0.000 (1)	**0.339 ± 0.106 (2)**
TOTAL[§]	**0.253 ± 0.274 (61)**	**0.040 ± 0.078 (16)**	**0.594 ± 0.325 (14)**	**0.529 ± 0.316 (26)**	**0.326 ± 0.291 (38)**	**0.150 ± 0.236 (8)**	**0.178 ± 0.199 (57)**	**0.549 ± 0.342 (15)**	**0.493 ± 0.325 (7)**	**0.493 ± 0.325 (21)**	**0.492 ± 0.313 (66)**	**0.517 ± 0.315 (18)**	**0.393 ± 0.285 (50)**	**0.352 ± 0.207 (70)**

MAR Index = Multiple antimicrobial resistance index = aggregate antimicrobial resistance score of all isolates tested / (number of isolates tested x number of antimicrobials tested against) (Krumperman, 1983). = mean MAR index ≤0.200; = mean MAR index from 0.201 to 0.400 ; = mean MAR index from 0.401 to 0.600; = mean MAR index from 0.601 to 0.800, and = mean MAR index from 0.801 to 1.000; SD = Standard deviation; n = number of studies; [1] Amikacin, Gentamicin, Kanamycin, Neomycin, Netilmicin, Paromomycin, Streptomycin and/or Tobramycin (61 studies); [2] Biapenem, Ertapenem, Imipenem and/or Meropenem (16 studies); [3] Cefadroxil, Cefalexin, Cefazolin, Cefradine and/or Cephaloridine (14 studies); [4] Cefaclor, Cephalothin, Cefamandole, Cefoxitin and/or Cefuroxime (26 studies); [5] Cefixime, Cefixime, Cefoperazone, Cefoperazone-sulbactam, Cefotaxime, Cefotaxime-clavulanic Acid, Cefpodoxime, Cefsulodin, Ceftazidime, Ceftiofur and/or Ceftriaxone (28 studies); [6] Cefepime (8 studies); [7] Ciprofloxacin, Enrofloxacin, Levofloxacin, Moxifloxacin, Nalidixic acid, Nitrofurantoin, Norfloxacin, Ofloxacin, Pefloxacin and/or Sparfloxacin (57 studies); [8] Teicoplanin and/or Vancomycin (15 studies); [9] Clindamycin and/or Lincomycin (7 studies); [10] Erythromycin and/or Roxithromycin (21 studies); [11] Amoxicillin, Amoxicillin-clavulanic acid, Ampicillin, Ampicillin-sulbactam, Ampiclox, Carbenicillin, Cloxacillin, Mecillinam, Methicillin, Oxacillin, Piperacillin, Piperacillin-tazobactam, Ticarcillin and/or Ticarcillin-clavulanic acid (66 studies); [12] Sulfamethoxazole, Sulfanilamide, Sulphafurazole and/or Sulfabenzamide-sulfacetamide-sulfathiazole (i.e. "Triple Sulfa") (18 studies); [13] Chlortetracycline, Doxycycline, Oxytetracycline and/or Tetracycline (50 studies); [a] Priority 1 (i.e. critical) in the global priority list of antimicrobial-resistant bacteria (WHO, 2017); [b] Priority 2 (i.e. high) in the global priority list of antimicrobial-resistant bacteria (WHO,2017); [c] Priority 3 (i.e. medium) in the global priority list of antimicrobial-resistant bacteria (WHO, 2017); * The only *Pseudomonas*, *Klebsiella* and *Acinetobacter* species included in the global critical priority list of antimicrobial-resistant bacteria are *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Acinetobacter baumannii*, respectively (WHO, 2017); # The only *Streptococcus* species included in the global medium priority list of antimicrobial-resistant bacteria is *Streptococcus pneumoniae* (WHO, 2017); [§] The total calculated MAR Indices were obtained per study, amalgamating results for all bacteria tested (included ones of unspecified genus) and using Equation 1. All studies were weighted equally.

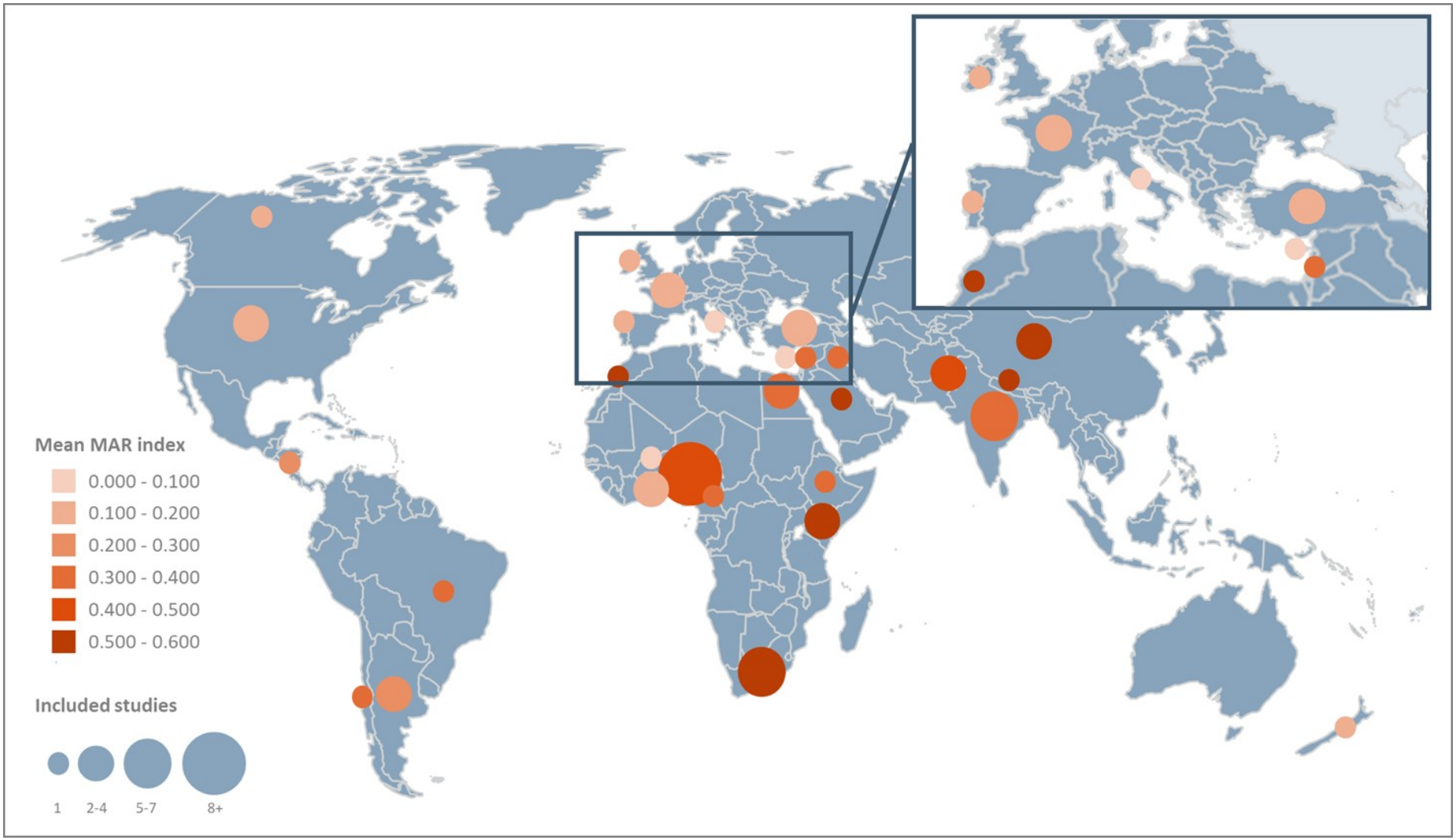
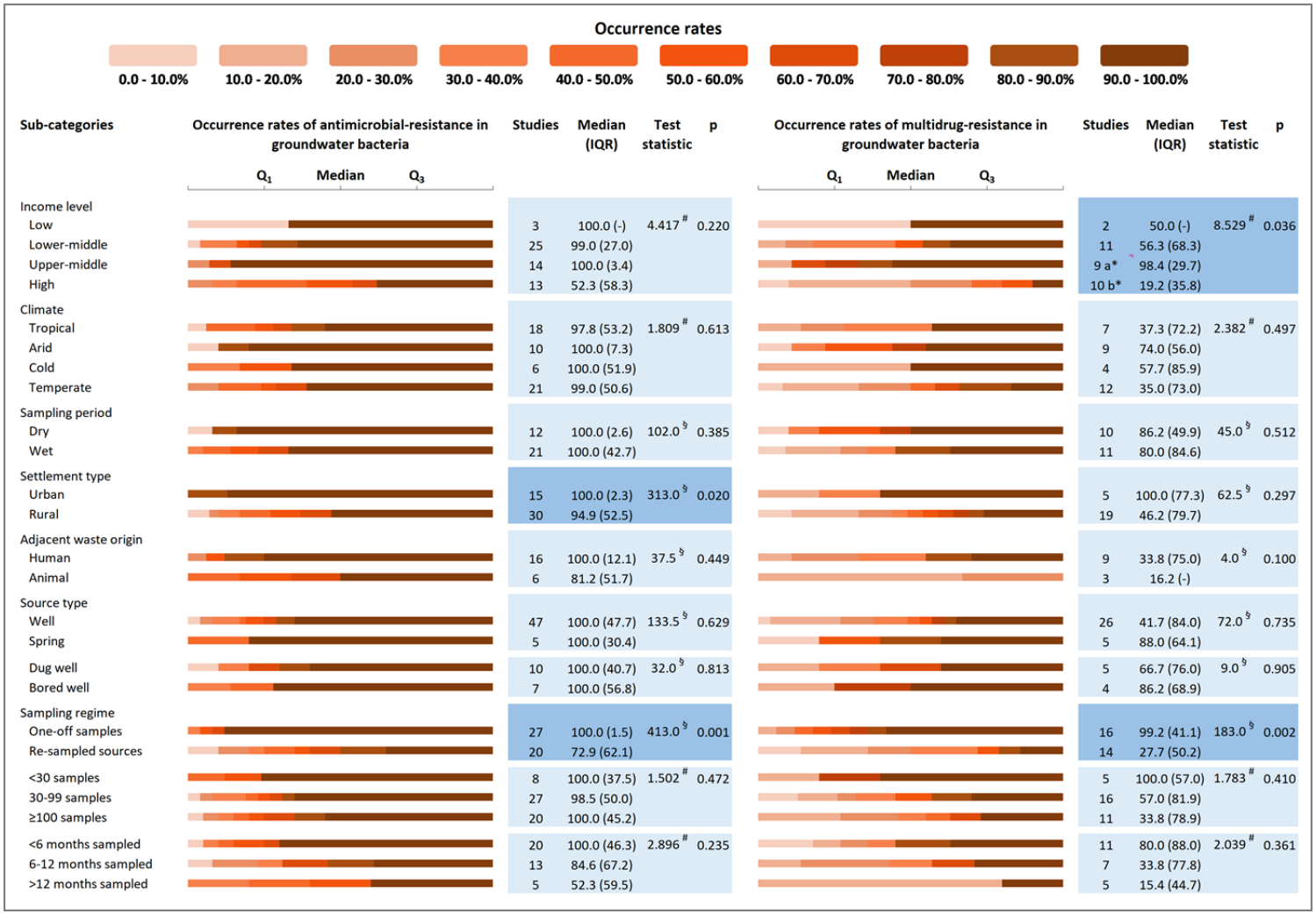


Figure 2.3: Mean Multiple Antimicrobial Resistance (MAR) Indices in global groundwater from 1976-2018 (n = 70) in each included country

2.3.4. Potential drivers of antimicrobial-resistant bacteria in groundwater

As shown (**Figure 2.4**), occurrence rates of antimicrobial resistance in groundwater bacteria were significantly higher in urban settlements ($p = 0.020$), with rates of MRB in groundwater from high income countries significantly lower than those from upper-middle income countries ($p = 0.035$). Studies that employed one-off sampling regimes yielded significantly higher percentages of both antimicrobial and multidrug resistance in groundwater bacteria when compared to regimes where re-sampling was employed ($p = 0.001$ and $p = 0.002$, respectively).

MAR indices were significantly lower in groundwater from high-income countries when compared to upper-middle ($p = 0.018$) and lower-middle ($p = 0.002$) income countries (**Figure 2.5**), and significantly higher in arid versus temperate regions ($p = 0.021$) and in samples collected during dry versus wet seasons ($p = 0.014$). Significantly higher MAR indices were also observed in urban settlements ($p = 0.001$) and when waste sources adjacent to groundwater supplies were predominantly of human (e.g., septic tanks and wastewater treatment plants) as opposed to animal (e.g., animal grazing and manure spreading) origin ($p = 0.005$). No significant differences were found when comparing MAR index distributions with sources type (i.e., wells versus springs) or source construction (i.e., hand-dug versus bored wells). One-off sampling regimes (n = 37) yielded significantly higher MAR indices in groundwater when compared to regimes where re-sampling was employed (n = 23) ($p = 0.003$).



Sub-categories	Occurrence rates of antimicrobial-resistance in groundwater bacteria	Studies	Median (IQR)	Test statistic	p	Occurrence rates of multidrug-resistance in groundwater bacteria	Studies	Median (IQR)	Test statistic	p
	Q_1 Median Q_3					Q_1 Median Q_3				
Income level										
Low		3	100.0 (-)	4.417 #	0.220		2	50.0 (-)	8.529 #	0.036
Lower-middle		25	99.0 (27.0)				11	56.3 (68.3)		
Upper-middle		14	100.0 (3.4)				9 a*	98.4 (29.7)		
High		13	52.3 (58.3)				10 b*	19.2 (35.8)		
Climate										
Tropical		18	97.8 (53.2)	1.809 #	0.613		7	37.3 (72.2)	2.382 #	0.497
Arid		10	100.0 (7.3)				9	74.0 (56.0)		
Cold		6	100.0 (51.9)				4	57.7 (85.9)		
Temperate		21	99.0 (50.6)				12	35.0 (73.0)		
Sampling period										
Dry		12	100.0 (2.6)	102.0 §	0.385		10	86.2 (49.9)	45.0 §	0.512
Wet		21	100.0 (42.7)				11	80.0 (84.6)		
Settlement type										
Urban		15	100.0 (2.3)	313.0 §	0.020		5	100.0 (77.3)	62.5 §	0.297
Rural		30	94.9 (52.5)				19	46.2 (79.7)		
Adjacent waste origin										
Human		16	100.0 (12.1)	37.5 §	0.449		9	33.8 (75.0)	4.0 §	0.100
Animal		6	81.2 (51.7)				3	16.2 (-)		
Source type										
Well		47	100.0 (47.7)	133.5 §	0.629		26	41.7 (84.0)	72.0 §	0.735
Spring		5	100.0 (30.4)				5	88.0 (64.1)		
Dug well		10	100.0 (40.7)	32.0 §	0.813		5	66.7 (76.0)	9.0 §	0.905
Bored well		7	100.0 (56.8)				4	86.2 (68.9)		
Sampling regime										
One-off samples		27	100.0 (1.5)	413.0 §	0.001		16	99.2 (41.1)	183.0 §	0.002
Re-sampled sources		20	72.9 (62.1)				14	27.7 (50.2)		
<30 samples		8	100.0 (37.5)	1.502 #	0.472		5	100.0 (57.0)	1.783 #	0.410
30-99 samples		27	98.5 (50.0)				16	57.0 (81.9)		
≥100 samples		20	100.0 (45.2)				11	33.8 (78.9)		
<6 months sampled		20	100.0 (46.3)	2.896 #	0.235		11	80.0 (88.0)	2.039 #	0.361
6-12 months sampled		13	84.6 (67.2)				7	33.8 (77.8)		
>12 months sampled		5	52.3 (59.5)				5	15.4 (44.7)		

Figure 2.4: Summary of descriptive and non-parametric test results for occurrence rates of i) antimicrobial (n = 55) and ii) multidrug resistance (n = 32) amongst groundwater isolates tested. Q_1 = 25th percentile; Q_3 = 75th percentile; IQR = Interquartile range = $Q_3 - Q_1$; $p \geq 0.1$; $p < 0.05$; # Kruskal-Wallis test; § Mann-Whitney U test; * Dunn's pairwise tests (each similar letter denotes a subset of each category whose distribution do not differ significantly from each other at the $p < 0.05$ level and different letters indicate statistically significant differences at the $p < 0.05$ level).

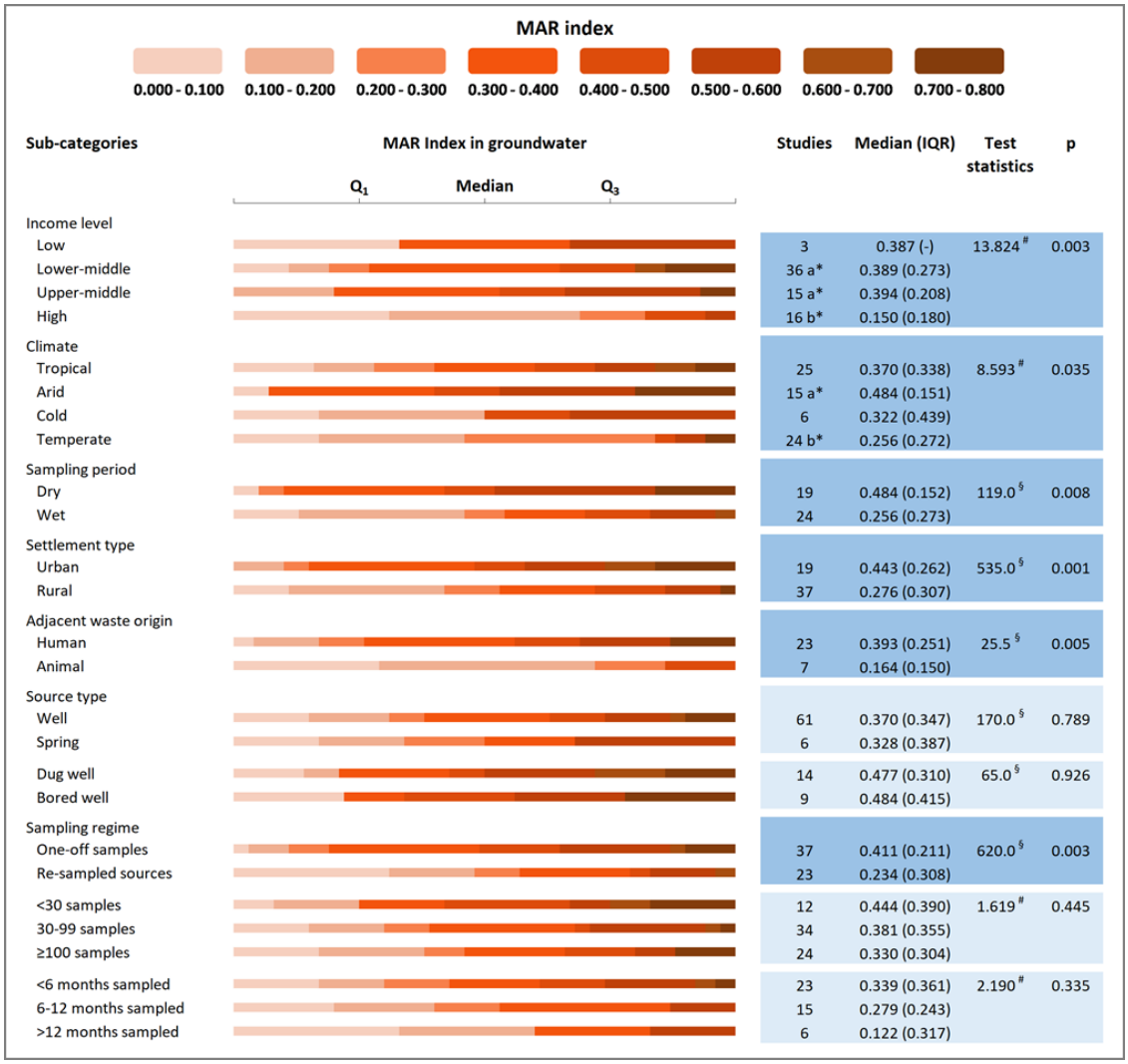


Figure 2.5: Summary of descriptive and non-parametric test results for the calculated Multiple Antimicrobial Resistance (MAR) indices in groundwater (iii; n = 70). Q_1 = 25th percentile; Q3 = 75th percentile; IQR = Interquartile range = $Q_3 - Q_1$; $p \geq 0.05$; $p < 0.05$; # Kruskal-Wallis test; § Mann-Whitney U test; * Dunn's pairwise tests (each similar letter denotes a subset of each category whose distribution do not differ significantly from each other at the p < 0.05 level and different letters indicate statistically significant differences at the p < 0.05 level).

2.4 Discussion

Antimicrobial resistance is a widely recognised global public health threat with growing evidence of its spread beyond clinical settings now available within the scientific literature (Bradford and Harvey, 2017; Larsson et al., 2018; Opatowski et al., 2019). Most recently, the role of the natural aquatic environment in the dissemination of ARB has gained interest (Suzuki et al., 2017; Sanderson et al., 2018), with groundwater resources being particularly relevant given the reliance of 2.2

billion people around the world on this source of drinking water (Murphy et al., 2017). Moreover, due to the widely held belief that groundwater is an intrinsically 'clean' water source, many sources lack treatment, in spite of well document incidences of faecal contamination as a result of agricultural and wastewater sources (Schets et al., 2005; Hynds et al., 2014; Murphy et al., 2017). As such, the risks posed via consumption of contaminated groundwater are significant, with a recent United Nations (UN) report referring to discharge of contaminated wastes to the environment and inadequate access to clean water as key drivers of antimicrobial resistance (Interagency Coordination Group, 2019). However, despite the importance of groundwater for human consumption and its potential role in the global resistome, to date no comprehensive synthesis of previous studies of ARB in the subsurface has been undertaken. The current study sought to address this gap in the scientific literature.

Occurrence rates of ARB amongst groundwater-derived isolates were consistently high across the seventy included studies, which were undertaken in geographic, climatologic, and socioeconomically diverse regions (**Figure 2.2**; **Table 2.4**). In the pooled analysis, studies where isolates were not obtained were excluded and hence our findings are relevant to groundwater sources where bacteria are present, which, where reported, accounted for 31.4% ± 32.6 (median = 20.2% (IQR = 7.36 – 46.6%)) of studied groundwater sources. Results show that four fifths (80.2% ± 29.0; 100.0% (59.8 – 100.0%)) of aggregated groundwater isolates were resistant to one or more antimicrobial agents, with ARB identified in 76.9% ± 33.7 (100.0% (42.7 – 100.0%)) of individual groundwater sources where bacteria were present. These findings seem to suggest that in the minority of groundwater sources where

bacteria were present these were often resistant to at least one antimicrobial. Just one study, undertaken in Burkina Faso, reported no ARB in examined groundwater sources (Traoré et al., 2015). The high mean MAR index values calculated across differing regions (**Figure 2.3**), when considered in concurrence with high occurrence rates of MRB (57.2% ± 36.8; 57.7% (22.2 – 100.0%)), further highlights the presence of multidrug resistance as a particular issue within groundwater sources. Compounding this, myriad ARB listed on the World Health Organisation priority list as *critical* (WHO, 2017) were specifically noted across the included studies (**Table 2.5**); a high proportion of which were used for human consumption (e.g., Ribeiro et al., 2014; Maran et al., 2016; O'Dwyer et al., 2017). Accordingly, pooled results point to groundwater as an environment characterised by the presence of ARB and MRB and highlight its potential role in the spread of antimicrobial resistance both within the environment and directly to humans via consumption of, frequently untreated, drinking water (Coleman et al., 2012).

Similarly, the abovementioned concurrently high incidence of ARB and MRB, and elevated pooled mean MAR index (0.352 ± 0.207), suggests the presence of significant selective pressures within groundwater systems, potentially resulting from extended residence times along subsurface pathways with exposure to sub-therapeutic concentrations of antimicrobial residues. Continuous release of human and veterinary antimicrobials to the natural environment has been shown to facilitate their ingress to both surface and groundwater sources (Van Schaik, 2015); however, occurrence rates of antimicrobial resistance in surface water bacteria found in middle- and high-income countries (O'Flaherty and Cummins, 2017) are lower than those encountered in the current study. Unlike surface water, extended residence

times associated with subsurface systems may lead to prolonged bacterial exposure to antimicrobial residues (at low concentrations), resistance genes and ARB within a relatively confined and oftentimes buffered (e.g., UV, pH and temperature) environment (Van Schaik, 2015; Williams-Nguyen et al., 2016). Compounding this, bacterial isolates may present high rates of resistance prior to subsurface ingress, with a recent study reporting that tetracycline-resistant *E. coli* strains were more mobile than susceptible strains in saturated porous media (Walczak et al., 2011). However, there is limited research exploring this hypothesis in diverse soil and aquifer types. Indeed, further research is required which combines microbial source tracking, advanced hydrogeological modelling, antimicrobial susceptibility testing and pharmaceutical residue concentration measurements to facilitate a greater understanding of the intricacies of microbial transport and resistance acquisition by these microbes in the subsurface.

More generally, pooled analyses suggest a paucity of research specifically addressing the mechanisms mediating the occurrence of ARB and MRB in groundwater, particularly with respect to varying climates, anthropogenic practices, hydrogeological settings, and groundwater source types. This represents a common limitation within blended groundwater and microbiological studies; due to their multidisciplinary nature, important source-pathway-receptor information are oftentimes omitted, overlooked or unreported (Hynds et al., 2014). For example, just 8.6%, 17.1% and 18.6% of included studies explicitly reported meteorological, hydrogeological, and detailed source-specific data, respectively. Accordingly, in combination with the inherently high degree of variability in reporting identified across identified studies, more robust quantitative examination (i.e., meta-

regression) and (multi-)collinearity diagnostics could not be undertaken as part of the current study, which may partially account (due to the multiple instances of missing data) for the lack of statistical significance between some external factors and ARB/MRB occurrence in groundwater (**Figure 2.4**). Moreover, owing to the nature of the research reporting within identified studies, it was not possible to calculate "treatment" effects (effect sizes), thus a meta-analysis could not be undertaken, as studies could not be weighted using any meaningful outcome measure. As such, retrospective pooled analyses with the analytical units (i.e., individual study findings) unweighted was employed to identify significant trends in this research field (**Figure 2.4** and **Figure 2.5**). Results suggest that studies undertaken in high income countries reported significantly lower MAR indices compared to lower and upper middle-income countries (p = 0.003), with similar results found for MRB occurrence (i.e., lower MRB rates reported in groundwater from high income countries; p = 0.036). This is likely due to lower antimicrobial diversity, lack of sanitation, poor hygiene practices and reduced antimicrobial stewardship in areas characterised by lower mean incomes (Morgan et al., 2011; Ayukekbong et al., 2017); however, the asymmetry in study numbers from each area (i.e., just 22.9% in high income countries; **Table 2.4**) likely impacted study findings.

Climatologically, pooled analysis indicates that groundwater samples collected during dry periods yielded significantly higher MAR indices (p = 0.008) than during wet periods, directly contrasting previous findings in surface water environments (Sanderson et al., 2018). While precipitation is roundly acknowledged as a primary driver of groundwater contamination (Hynds et al., 2012; Andrade et al., 2018), in the context of antimicrobial resistance, the evidence is less compelling. For example,

drier periods may enable higher concentration of antimicrobial residues within the subsurface (Dhar et al., 2008), thus leading to increased bacterial exposure to sub-therapeutic levels of antimicrobial residues. Moreover, as temperature is directly correlated with bacterial proliferation, even marginal increases in subsurface temperatures may increase bacterial loading *in situ* (John and Rose, 2005). However, it is important to note that in the absence of specific hydrogeological information, interpreting the impact of seasonality on ARB and MRB occurrence is largely speculative in this instance, further reiterating the need for a more holistic approach to multidisciplinary groundwater research. Socio-geographically, groundwater sources located in categorically urban (as opposed to rural) regions were associated with significantly higher MAR indices (p = 0.001; **Figure 2.5**). Similarly (and likely collinear with this previous finding), supplies predominantly adjacent to human waste sources (e.g., septic tanks and wastewater treatment plants) were characterised by a significantly higher (p = 0.005) mean MAR index than those adjacent to animal waste sources (e.g., animal grazing and manure spreading). This is possibly driven by the antimicrobial residual concentration differentials between urban and rural areas. For example, while antimicrobial concentrations in manure are typically orders of magnitude higher than those encountered in wastewaters (Arikan et al., 2009; Sabri et al., 2018), the spatiotemporal exposure is inconsistent, due to the diffuse nature of the source. Conversely, wastewater treatment infrastructures typically result in point source contamination mechanisms, providing a consistent source of low-dose antimicrobial residues. Moreover, transmission (infiltration) of common veterinary antimicrobials in the subsurface may be confined and thus spatially limited. Transport of tetracycline compounds, in particular, are restricted to

fast preferential and macropore flow or require facilitation of co-transport with mobile colloids, such as dissolved organic matter (Thiele-Bruhn, 2003). Nevertheless, an increase in the global occurrence of ARB and MRB in groundwater sources is expected due to current and future population growth, urbanisation, lack of sanitation, inappropriate wastewater treatment, and the misuse and over-use of antimicrobials, irrespective of geographic location.

Focusing on the identification of factors driving ARB and MRB occurrence, significant research challenges also exist due to inherent complexities associated with the genetic acquisition of antimicrobial resistance, in addition to the multifaceted hydrogeological mechanisms governing subsurface contamination. For example, while MAR indices calculated within carbapenems, which are generally used as 'last line' drugs (Van Boeckel et al., 2014), were found to be significantly lower than other classes, other *last line* antimicrobials, such as glycopeptides (Van Boeckel et al., 2014), were associated with relatively high mean MAR index values (**Table 2.4**). Accordingly, antimicrobial usage itself may not be a primary driver of MRB or ARB in groundwater, despite remaining an undeniably important factor in the broader antimicrobial resistance crisis. This is in line with findings from Collignon et al. (2018) that antimicrobial consumption was not significantly associated with the global indices for antimicrobial resistance.

At the genus level, there was significant variance observed in the rates of encountered antimicrobial resistance (**Figure 2.2** and **Table 2.5**). Ubiquitous members of the soil microbiome *Pseudomonas* spp., *Klebsiella* spp. and *Enterobacter* spp. exhibited some of the highest rates of antimicrobial resistance at 99.9% ± 0.2

(range = 90.0 – 100.0%), 99.8% ± 0.6 (97.8 – 100.0%), and 99.2% ± 2.2 (92.9 – 100.0%), respectively. These findings are particularly significant as both Pseudomonas and Klebsiella spp. are considered to be ubiquitously "soil-resident" bacteria, and as such, may characterise the local and/or source specific resistome more accurately than faecal indicator and/or (opportunistic) pathogens, which are often absent. However, it should be noted that a proportion of this resistance is attributable to intrinsic resistance, and thus, should not be interpreted as a wholly anthropogenic phenomenon in all cases. For example, *Pseudomonas aeruginosa* is intrinsically resistant to many antimicrobial agents accredited to the low permeability of its outer membrane (Livermore, 1984), the expression of various efflux pumps with wide substrate specificity (Livermore, 2001) and the naturally occurring chromosomal AmpC beta-lactamase (Nordmann & Guibert, 1998). A more compelling assessment of acquired resistance focuses on *E. coli*, which was the most frequently examined bacterial species across the studies, with pooled isolates exhibiting below average, but considerably high, rates of resistance to ≥ 1 (79.8% ± 30.1; median = 100.0% (IQR = 50.0 – 100.0%)) and ≥ 3 (45.4% ± 36.0; 32.0% (13.7 – 100.0%)) antimicrobial agents. Mechanisms explaining between-species variation could not be determined in the current study, as it may reflect locally specific circumstances. However, drawing from previous field and modelling work (Hynds et al., 2012), findings may suggest higher rates of ARB ingress and resistance acquisition taking place in the subsurface via traditional (i.e. non-preferential) recharge, with lower rates of antimicrobial resistance associated with rapid entry (i.e., surface run-off followed by direct wellhead ingress and/or preferential recharge) into groundwater sources. As such, it is recommended that future studies perform antimicrobial susceptibility assays,

where possible, using both faecal and soil/water-resident bacteria to accurately establish the extent of resistance within microbial communities in groundwater, and their most likely ingress mechanisms. Moreover, as molecular methods of antimicrobial resistance characterisation (specific gene targeting) increase in popularity, it is important that isolate-specific phenotypic (i.e., culture-based) research be undertaken to permit a greater understanding of the specific public health significance of environmental exposures to ARB.

As with any literature findings-based study, it is important to acknowledge that data used for analyses are limited to previous reports; and thus, the potential for reporting bias exists and should be highlighted (i.e., studies targeting susceptible areas or groundwater sources where ARB contamination is suspected). Over half (52.9%) of identified studies included in this review based their findings on "one-off" (i.e., non-temporal) groundwater sampling regimes and were associated with significantly higher MAR indices (p = 0.003). Moreover, a widespread lack of explicit methodology for achieving sample representativeness was identified during validity appraisal (**Supplementary Data**). As such, the occurrence rates of ARB and MRB in global groundwater garnered from this review may represent an overestimate, with true values likely more accurately reflected via means calculated from longitudinal studies only (i.e., ARB = 65.5% ± 33.4, and MRB = 38.9 ± 32.0). However, the issue of temporal and spatial representativeness still represents a significant concern. Baseline sampling and analytical procedures should be developed and routinely employed via unbiased and robust spatiotemporal studies, following consistent methodologies, in diverse (hydro)geological and climatic settings.

The current study is the first to integrate findings from international literature regarding the occurrence of ARB and MRB in groundwater sources. While this research is undoubtedly topical, identified studies highlight a long-standing issue, with the earliest relevant research available within the scientific literature (Cooke, 1976) reporting antimicrobial resistance in 48.9% of 321 total coliform isolates (species unspecified) from groundwater sources in New Zealand. As such, and as groundwater remains an important source of drinking water for a considerable proportion of the global population, the results presented show that groundwater is a significant environment where antimicrobial resistance can be disseminated, and highlight the need for further research looking at the occurrence of ARB and MRB in the subsurface environment.

2.5 Conclusion

Study results highlight groundwater as a noteworthy source of global ARB and MRB, and the pressing need for more representative studies (i.e., "baseline" work) on this topic. Additionally, lack of more robust methodologies and of key relevant data were identified as persistent issues among published work, making it difficult to ascertain a comprehensive global perspective, which is vital to quantify the risks associated with groundwater consumption and antimicrobial resistance. Thus, a key recommendation of this research is the requirement for the scientific community to refine their approach to groundwater- and health-related research. Specifically, it is imperative that future studies employ:

- hydrogeological, meteorological, climatic, and detailed source-specific data measurement and reporting,

- longitudinal, rather than one-off, sampling methodologies,
- spatial distribution (as an attempt to achieve representativeness), and
- separate assessment of both faecal and soil-resident bacteria to establish the presence, origin, and ingress mechanisms of ARB in groundwater systems.

The importance of efforts to understand and prevent the spread of antimicrobial resistance cannot be overstated. In an era characterised by significant global challenges, including antimicrobial resistance and anthropogenic climate change, consolidation of robust research approaches and systematic design of sampling programmes to help answer pressing global questions is not only warranted, but necessary. Regardless, findings of this study offer valuable insights into the extent and significance of groundwater as a potential source of ARB and provides guidance for future research to guide policy development, action plans and remediation efforts/technologies to safeguard public health into the future.

Author contributions

L.A. and M.K. performed the initial review of literature. All authors (L.A., M.K., P.H., J.W., A.M. and J.OD.) determined final inclusion. Statistical analyses were carried out by L.A. in consultation with J.OD. and P.H. All authors were involved in the writing of the manuscript, with J.OD., P.H. and J.W. providing final approval for submission.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Data

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Critical appraisal of included studies

Study	Critical appraisal criteria														Score
	i.	ii.	iii.	iv.	v.	vi.	vii.	viii.	ix.	x.	xi.	xii.	xiii.	xiv.	
1	yes	no	no	yes	no	no	yes	yes	no	no	no	yes	yes	no	6
2	yes	no	yes	yes	yes	no	yes	yes	yes	no	no	yes	no	yes	9
3	yes	no	no	yes	no	yes	yes	yes	yes	no	no	no	no	yes	7
4	yes	no	no	yes	no	no	yes	yes	yes	no	no	no	no	yes	6
5	yes	no	yes	yes	yes	no	yes	yes	yes	no	no	no	no	yes	8
6	yes	no	no	yes	no	no	yes	yes	no	yes	yes	yes	no	yes	8
7	yes	no	no	yes	yes	yes	yes	yes	no	yes	yes	yes	no	yes	10
8	yes	no	no	yes	yes	yes	yes	yes	yes	yes	no	no	no	yes	9
9	yes	no	yes	yes	no	no	yes	yes	yes	yes	yes	no	no	yes	9
10	yes	no	no	yes	no	no	yes	yes	no	yes	no	yes	no	yes	6
11	yes	no	no	yes	no	no	yes	yes	no	yes	no	yes	no	yes	6
12	yes	no	no	yes	no	yes	yes	yes	no	yes	yes	yes	no	yes	9
13	yes	yes	no	yes	no	yes	yes	yes	no	no	no	no	no	yes	7
14	yes	no	no	yes	yes	no	yes	yes	yes	yes	yes	yes	no	yes	10
15	yes	no	no	yes	no	no	yes	yes	yes	yes	yes	no	no	yes	8
16	yes	no	no	yes	no	no	yes	yes	no	no	no	no	no	yes	5
17	yes	no	no	yes	no	yes	yes	yes	yes	yes	yes	yes	yes	yes	11
18	yes	no	no	yes	no	yes	yes	yes	yes	yes	yes	yes	yes	yes	11
19	yes	no	no	yes	yes	yes	yes	yes	no	yes	yes	yes	yes	yes	11
20	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	12
21	yes	no	yes	yes	no	no	yes	yes	yes	no	no	yes	no	yes	8
22	yes	no	no	yes	yes	yes	yes	yes	yes	no	no	no	yes	yes	9
23	yes	no	yes	yes	yes	no	yes	yes	yes	yes	yes	yes	no	yes	11
24	yes	no	no	yes	no	no	yes	yes	no	no	no	no	no	yes	5
25	yes	no	no	yes	no	yes	yes	yes	no	yes	no	no	no	yes	7
26	yes	no	yes	yes	yes	yes	yes	yes	no	no	no	yes	no	yes	9
27	yes	no	no	yes	no	no	yes	yes	yes	yes	yes	yes	no	yes	9
28	yes	no	yes	yes	yes	no	yes	yes	yes	no	no	no	no	yes	8
29	yes	no	yes	yes	yes	no	yes	yes	yes	yes	no	yes	no	yes	10
30	yes	no	yes	yes	yes	yes	yes	yes	no	no	no	yes	yes	yes	10
31	yes	no	yes	yes	yes	yes	yes	yes	yes	no	no	yes	yes	yes	11
32	yes	no	yes	yes	yes	yes	yes	yes	no	yes	no	yes	no	yes	10
33	yes	no	no	yes	no	yes	yes	yes	yes	yes	no	yes	yes	yes	10
34	yes	no	no	yes	yes	yes	yes	yes	no	no	no	no	yes	yes	8
35	yes	no	no	yes	no	no	yes	yes	no	no	no	no	no	yes	5
36	yes	no	yes	yes	yes	no	yes	yes	no	no	no	yes	no	yes	8
37	yes	no	no	yes	no	no	yes	yes	yes	yes	yes	yes	no	yes	9
38	yes	no	no	yes	yes	yes	yes	yes	no	no	no	no	yes	yes	8
39	yes	no	no	yes	yes	no	yes	yes	yes	yes	yes	yes	no	yes	10
40	yes	no	no	yes	no	yes	yes	yes	yes	yes	yes	yes	yes	yes	11
41	yes	no	no	yes	no	no	yes	yes	no	yes	yes	yes	no	yes	8
42	yes	no	no	yes	yes	no	yes	yes	no	yes	no	yes	yes	yes	9
43	yes	no	no	yes	no	no	yes	yes	yes	yes	yes	yes	no	yes	9
44	yes	no	no	yes	yes	no	yes	yes	no	yes	yes	yes	no	yes	9
45	yes	no	yes	yes	yes	no	yes	yes	no	yes	yes	yes	no	yes	10
46	yes	no	no	yes	yes	yes	yes	yes	no	yes	no	yes	no	yes	9
47	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	12
48	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes	yes	no	yes	11
49	yes	no	no	yes	no	yes	yes	yes	no	no	no	no	no	yes	6
50	yes	no	no	yes	no	no	yes	yes	yes	yes	no	no	no	yes	7
51	yes	no	yes	yes	no	no	yes	yes	yes	yes	yes	yes	no	yes	10
52	yes	no	no	yes	yes	no	yes	yes	yes	yes	no	no	yes	yes	9
53	yes	no	yes	yes	no	yes	yes	yes	no	yes	no	yes	yes	yes	10
54	yes	no	no	yes	yes	yes	yes	yes	no	yes	yes	yes	no	yes	10
55	yes	no	no	yes	yes	no	yes	yes	yes	yes	yes	yes	yes	yes	11
56	yes	no	no	yes	no	no	yes	yes	no	no	no	no	no	yes	5
57	yes	no	no	yes	no	no	yes	yes	yes	yes	yes	yes	no	yes	9
58	yes	yes	no	yes	yes	yes	yes	yes	no	yes	no	no	yes	yes	10
59	yes	no	no	yes	yes	yes	yes	yes	no	yes	no	no	yes	yes	9
60	yes	no	no	yes	no	no	yes	yes	no	no	no	no	no	yes	5
61	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	no	yes	13
62	yes	no	yes	yes	no	no	yes	yes	yes	yes	yes	yes	no	yes	10
63	yes	no	no	yes	yes	yes	yes	yes	no	yes	yes	yes	no	yes	10
64	yes	yes	no	yes	yes	yes	yes	yes	no	yes	no	yes	no	yes	10
65	yes	no	no	yes	yes	yes	yes	yes	yes	yes	yes	yes	no	yes	11
66	yes	no	no	yes	yes	yes	yes	yes	no	yes	yes	yes	yes	yes	11
67	yes	yes	no	yes	yes	yes	yes	yes	no	yes	yes	yes	no	yes	11
68	yes	no	no	yes	no	yes	yes	yes	yes	yes	yes	yes	yes	yes	11
69	yes	no	yes	yes	no	no	yes	yes	yes	yes	yes	yes	no	yes	10
70	yes	no	yes	yes	no	no	yes	yes	yes	no	no	yes	no	yes	8

i) Was election of samples described? (includes selection of type and use of source); ii) Was approach to achieve representativeness detailed? (are samples representative of the area?); iii) Was sampling randomized?; iv) Was geographic region reported?; v) Were seasons and months of sampling reported?; vi) Were quality control procedures specified or referred to?; vii) Are methods of microbial analysis described or referenced?; viii) Are methods of antibiotic susceptibility testing described or referenced? ix) Was the point at which water was sampled well defined? x) Were sample handling procedures described? (including collection, transportation and duration); xi) Does sample handling and processing meet the following criteria: transport on ice or between 2–8oC, analysis within 6 hours of collection, and specified incubation temperature?; xii) Was the microbial analysis done in an accredited laboratory?; xiii) Did trained technicians/lab technicians conduct water quality assessments or the analyses? Was that specified?; xiv) Was the study subject to peer review or external review prior to publication?

Characteristics of included studies

Study	Year of Publication	Country	Economic classification*	Climate	Calendar months in sampling period	Sampling period	Sampling regime	Sample size	Primary settlement type	Waste source adjacent to study site	Source type	Well type	Water supply	Water used for human consumption?	Treatment before use	Number of antibiotics tested against	Sources contaminated with antimicrobial-resistant bacteria	Groundwater isolates analysed	Antimicrobial-resistant isolates	Multidrug-resistant isolates	MAR index
1	1976	New Zealand	High income	Temperate		Wet		Small	Rural	Mixed	Mixed			yes		11		321	157 (48.9%)		0.173
2	1987	Nigeria	Lower middle income	Tropical	3	Both	One-off sampling	Medium	Rural	Human	Wells			yes		6		36	18 (50.0%)	10 (27.8%)	0.255
3	1988	Egypt	Lower middle income	Arid		Dry	Re-sampling	Medium		Human	Wells			yes	Yes	5		101	82 (81.2%)	32 (31.7%)	0.394
4	1988	Nigeria	Lower middle income	Tropical	9	Both	One-off sampling	Medium	Urban		Wells			yes		10		145	141 (97.2%)		0.386
5	1989	Nigeria	Lower middle income	Tropical	7		Re-sampling	Large	Urban		Wells			yes		10		144	124 (86.1%)		0.234
6	1988	United States	High income	Cold	16	Wet	Re-sampling	Large	Rural	Both	Wells		Private	yes	No	12		65	34 (52.3%)	10 (15.4%)	0.088
7	2003	Turkey	Upper middle income	Temperate	2	Dry		Large	Urban		Wells					8		66	66 (100.0%)		0.339
8	2006	United States	High income	Temperate	10	Wet	Re-sampling	Large	Rural	Animal	Wells			no		12		101	63 (62.4%)	24 (23.8%)	0.169
9	2007	Pakistan	Lower middle income	Arid		Dry		Large	Urban		Wells			yes		12		39			0.701
10	2007	Turkey	Upper middle income	Cold		Wet		Large	Rural	Animal	Springs	-		yes		7		55	55 (100.0%)		0.164
11	2007	Nigeria	Lower middle income	Tropical			One-off sampling	Small	Urban		Wells					10		196	196 (100.0%)		0.443
12	2008	Argentina	High income	Temperate		Wet		Medium	Rural		Wells			no		6		30			0.100
13	2009	India	Lower middle income	Arid	2	Dry	One-off sampling	Large			Wells			yes		11		8	8 (100.0%)	8 (100.0%)	0.750
14	2010	Morocco	Lower middle income	Arid	12	Dry	Re-sampling	Large	Mixed	Both	Wells			yes		18		1183	208 (100.0%)	768 (88.7%)	0.528
15	2010	Nigeria	Lower middle income	Tropical	3		Re-sampling	Large	Rural	Both	Wells	Dug	Mixed	yes	No	14		74	60 (100.0%)		0.432
16	2011	Saudi Arabia	High income	Arid		Dry	Re-sampling	Medium			Springs	-				7		26		15 (57.7%)	0.516
17	2011	South Africa	Upper middle income	Arid		Dry	One-off sampling	Small	Rural	Human	Wells	Bored	Private	yes	No	9		139	139 (100.0%)		0.484
18	2011	South Africa	Upper middle income	Arid		Dry	One-off sampling	Small	Rural		Wells	Bored		yes	No	10		127	64 (100.0%)	94 (74.0%)	0.326
19	2011	Nigeria	Lower middle income	Tropical	6	Wet	Re-sampling	Medium	Urban		Wells	Dug	Private	yes	No	13		70	70 (100.0%)		0.603
20	2012	Nicaragua	Lower middle income	Tropical	8	Dry	One-off sampling	Medium	Rural	Human	Wells			yes		9	8 (21.6%)	174			0.279
21	2012	Cyprus	High income	Temperate	48	Both	Re-sampling	Large	Rural	Animal	Wells	Bored		yes		11		37	16 (43.2%)	6 (16.2%)	0.086
22	2012	France	High income	Temperate	3	Wet	Re-sampling	Medium	Rural	Both	Springs	-		yes	Yes	16	6 (100.0%)	555	223 (40.2%)	51 (9.2%)	0.059
23	2012	India	Lower middle income	Tropical	2	Wet	One-off sampling	Medium	Mixed	Human	Wells	Dug	Private	yes		12		163			0.169
24	2012	Ethiopia	Low income	Temperate		Wet	Re-sampling	Medium	Urban	Human	Wells	Dug	Private	yes	Mixed	10		15	15 (100.0%)	15 (100.0%)	0.387

25	2012	Egypt	Lower middle income	Arid		Dry	One-off sampling	Medium	Rural		Wells			yes	No	9		87	84 (96.6%)	49 (56.3%)	0.383
26	2012	Pakistan	Lower middle income	Temperate	4	Wet	Re-sampling	Medium		Human	Springs	-				14		100	99 (99.0%)	88 (88.0%)	0.380
27	2012	Nigeria	Lower middle income	Tropical			One-off sampling	Small		Human	Wells	Dug	Private	yes		11		106	79 (100.0%)		0.792
28	2012	Nigeria	Lower middle income	Tropical	5	Wet	Re-sampling	Medium	Rural		Wells			yes		10		51	33 (64.7%)	19 (37.3%)	0.182
29	2013	Cameroon	Lower middle income	Tropical	10	Both	Re-sampling	Large	Urban	Human	Wells	Dug	Private		Mixed	8		130	110 (84.6%)	44 (33.8%)	0.341
30	2013	South Africa	Upper middle income	Arid		Dry	One-off sampling	Small	Rural		Wells	Bored		yes		8	22 (100.0%)	179	179 (100.0%)	179 (100.0%)	0.534
31	2013	Canada	High income	Cold	19	Wet		Large	Mixed	Mixed	Wells	Mixed	Private	yes	Mixed	6	233 (35.5%)	657	233 (35.5%)	90 (13.7%)	0.131
32	2013	United States	High income	Temperate	4	Wet		Small	Rural	Animal	Wells					10	1 (50.0%)	2	1 (50.0%)		0.050
33	2013	Chile	High income	Temperate			One-off sampling	Large	Rural		Wells			yes		8	78 (100.0%)	78	78 (100.0%)	36 (46.2%)	0.322
34	2013	Turkey	Upper middle income	Temperate	17	Both		Medium	Urban	Human	Wells			yes		15		43	42 (97.7%)	5 (11.6%)	0.113
35	2013	India	Lower middle income	Tropical				Medium	Mixed		Wells	Bored		yes		9		3	1 (33.3%)		0.074
36	2013	Nepal	Low income	Temperate	8	Wet	One-off sampling	Medium	Urban		Mixed					8		25	25 (100.0%)		0.545
37	2013	Nigeria	Lower middle income	Tropical		Both	One-off sampling	Large	Urban	Human	Wells			yes		10		184			0.195
38	2014	France	High income	Temperate	3	Wet	Re-sampling	Large	Rural	Both	Springs	-		yes	Yes	16	5 (100.0%)	267	267 (100.0%)	254 (95.1%)	0.276
39	2014	India	Lower middle income	Arid	2	Dry	Re-sampling	Medium	Urban	Human	Wells	Mixed	Mixed	yes		20		27	27 (100.0%)		0.393
40	2014	South Africa	Upper middle income	Arid		Dry	One-off sampling	Small	Rural		Wells	Bored		yes		6		165			0.708
41	2014	Kenya	Lower middle income	Temperate				Large	Urban	Human	Wells	Bored		yes		8		54	54 (100.0%)		0.757
42	2014	India	Lower middle income	Tropical	12	Both	Re-sampling	Medium	Rural	Both	Wells	Dug		yes		12		35	13 (37.1%)	5 (14.3%)	0.071
43	2014	Nigeria	Lower middle income	Tropical			One-off sampling	Small	Urban		Wells	Dug	Private	yes		11		108	108 (100.0%)	108 (100.0%)	0.624
44	2014	Nigeria	Lower middle income	Tropical	6	Dry	Re-sampling	Medium	Urban	Human	Wells	Dug		yes		11		12			0.530
45	2015	Ghana	Lower middle income	Tropical	12	Both	Re-sampling	Large	Rural	Both	Wells	Dug		yes		8		26	2 (7.7%)		0.019
46	2015	China	Upper middle income	Cold	3	Dry	One-off sampling	Medium		Human	Springs	-		yes		18		16	16 (100.0%)	16 (100.0%)	0.552
47	2015	Burkina Faso	Low income	Arid	5	Dry	Re-sampling	Medium	Rural	Both	Wells			yes		12		1			0.000
48	2015	China	Upper middle income	Cold	2	Wet	One-off sampling	Large	Rural		Wells			yes		19		10	10 (100.0%)	10 (100.0%)	0.579
49	2015	Nigeria	Lower middle income	Tropical	3		One-off sampling	Medium			Wells	Mixed		yes		9		68	67 (98.5%)		0.575
50	2015	Pakistan	Lower middle income	Arid		Dry	One-off sampling	Large	Rural	Human	Wells		Private	yes	No	12		30			0.411
51	2015	Nigeria	Lower middle income	Tropical			One-off sampling	Medium	Urban	Human	Wells	Dug	Private	yes		8		56	56 (100.0%)	16 (34.0%)	0.797
52	2016	Brazil	Upper middle income	Temperate	4	Wet	One-off sampling	Medium	Rural	Both	Wells	Mixed	Private	yes		27		45	42 (93.3%)	36 (80.0%)	0.333

53	2016	Kenya	Lower middle income	Tropical			One-off sampling	Large		Human	Wells	Mixed		yes		8	79 (100.0%)	77	77 (100.0%)		0.341
54	2016	China	Upper middle income	Cold	2	Wet	One-off sampling	Large	Rural	Human	Wells		Private	yes		16	5 (100.0%)	23	23 (100.0%)		0.481
55	2016	Nigeria	Lower middle income	Tropical	3	Both	One-off sampling	Medium	Rural		Wells			yes	No	8	7 (33.3%)	21	7 (33.3%)		0.048
56	2016	Iraq	Upper middle income	Arid		Dry	One-off sampling	Medium			Wells				No	19	8 (100.0%)	8	8 (100.0%)	8 (100.0%)	0.382
57	2016	Ghana	Lower middle income	Tropical	7	Both	One-off sampling	Small	Urban	Both	Wells			yes		9	4 (100.0%)	6	6 (100.0%)	6 (100.0%)	0.370
58	2017	Portugal	High income	Temperate	4	Both	One-off sampling	Small	Rural	Animal	Wells	Mixed	Private	no		16		83		10 (12.0%)	0.114
59	2017	Italy	High income	Temperate	12	Both	Re-sampling	Large		Human	Wells		Public	yes		19		9	2 (22.2%)	2 (22.2%)	0.058
60	2017	India	Lower middle income	Temperate		Wet	One-off sampling	Medium	Urban	Human	Wells	Dug		yes		12		16	16 (100.0%)		0.536
61	2017	Ireland	High income	Temperate	12	Wet	Re-sampling	Large	Rural	Both	Wells	Mixed	Private	yes		20		42	42 (100.0%)		0.181
62	2017	Nigeria	Lower middle income	Tropical	8	Wet	One-off sampling	Medium	Rural	Human	Wells	Mixed		yes		16		45	45 (100.0%)	45 (100.0%)	0.375
63	2017	India	Lower middle income	Temperate	25	Both	One-off sampling	Medium	Rural	Both	Wells	Mixed		yes		10		16			0.369
64	2017	Argentina	High income	Temperate	3	Wet	One-off sampling	Medium	Rural	Animal	Wells			yes		6	9 (100.0%)	12	12 (100.0%)		0.236
65	2017	Turkey	Upper middle income	Temperate	12	Both	Re-sampling	Medium	Rural		Wells		Private			2	11 (100.0%)	7	2 (28.6%)		0.143
66	2018	Lebanon	Upper middle income	Temperate	11	Both	One-off sampling	Large	rural		Mixed	Dug		yes	No	11	8 (66.7%)	12	8 (66.7%)	8 (66.7%)	0.394
67	2018	Argentina	High income	Temperate	1	Wet	One-off sampling	Medium	rural	Animal	Wells					6	8 (100.0%)	8	8 (100.0%)		0.417
68	2018	South Africa	Upper middle income	Arid	16	Dry	One-off sampling	Medium	Rural		Wells	Bored		yes		13		122	122 (100.0%)	120 (98.4%)	0.511
69	2018	Nigeria	Lower middle income	Tropical		Wet	One-off sampling	Small	Urban	Both	Wells	Bored		yes	Mixed	10		164	131 (100.0%)	115 (94.3%)	0.445
70	2018	Nigeria	Lower middle income	Tropical			One-off sampling	Medium	Rural	Human	Wells	Dug	Private	yes	No	10		51			0.522

Antimicrobials, their frequency, and mean resistance rates in included studies

Antibiotic	Studies, n (%)	% of resistance, mean ± SD
Aminoglycoside		
1 Amikacin	15 (21.4%)	9.7 ± 18.3
2 Gentamicin	49 (70.0%)	23.9 ± 29.2
3 Kanamycin	12 (17.1%)	30.3 ± 31.7
4 Neomycin	5 (7.1%)	24.5 ± 33.3
5 Netilmicin	5 (7.1%)	5.9 ± 3.7
6 Paromomycin	1 (1.4%)	5.9 ± 0.0
7 Streptomycin	28 (40.0%)	36.9 ± 32.7
8 Tobramycin	5 (7.1%)	7.1 ± 13.1
Carbapenem		
9 Ertapenem	2 (2.9%)	5.6 ± 5.6
10 Imipenem	12 (17.1%)	6.9 ± 11.3
11 Meropenem	8 (11.4%)	2.3 ± 5.2
Cephalosporin		
First generation		
12 Cefadroxil	2 (2.9%)	84.0 ± 9.9
13 Cefalexin	4 (5.7%)	62.1 ± 26.6
14 Cefazolin	4 (5.7%)	60.8 ± 30.8
15 Cefradine	2 (2.9%)	36.7 ± 30.0
16 Cephaloridine	2 (2.9%)	62.6 ± 15.4
Second generation		
17 Cefaclor	3 (4.3%)	69.1 ± 21.8
18 Cephalothin	13 (18.6%)	48.2 ± 34.8
19 Cefamandole	1 (1.4%)	60.2 ± 0.0
20 Cefoxitin	3 (4.3%)	59.4 ± 21.8
21 Cefuroxime	14 (20.0%)	59.1 ± 35.8
Third generation		
22 Cefixime	3 (4.3%)	54.2 ± 32.5
23 Cefoperazone	1 (1.5%)	31.3 ± 0.0
Third generation		
24 Cefoperazone-sulbactam	2 (2.9%)	37.2 ± 3.9
25 Cefotaxime	17 (24.3%)	23.8 ± 28.2
26 Cefotaxime-clavulanic acid	1 (1.4%)	18.8 ± 0.0
27 Cefpodoxime	1 (1.4%)	9.5 ± 0.0
28 Cefsulodin	1 (1.4%)	84.3 ± 0.0
29 Ceftazidime	21 (30.0%)	35.2 ± 36.0
30 Ceftiofur	1 (1.4%)	21.4 ± 0.0
31 Ceftriaxone	18 (25.7%)	36.0 ± 30.3
Fourth generation		
32 Cefepime	8 (11.4%)	15.0 ± 23.6
Fluoroquinolone		
33 Ciprofloxacin	41 (58.6%)	12.1 ± 17.2
34 Enrofloxacin	4 (5.7%)	20.9 ± 13.3
35 Levofloxacin	5 (7.1%)	20.0 ± 27.2
36 Moxifloxacin	3 (4.3%)	0.9 ± 1.2
37 Nalidixic acid	25 (35.7%)	26.9 ± 25.3
38 Nitrofurantoin	13 (18.6%)	45.0 ± 31.9
39 Norfloxacin	12 (17.1%)	16.8 ± 22.1
40 Ofloxacin	14 (20.0%)	21.3 ± 24.4
41 Pefloxacin	5 (7.1%)	23.6 ± 17.1
42 Sparfloxacin	1 (1.4%)	19.6 ± 0.0
Glycopeptide		
43 Teicoplanin	3 (4.3%)	27.5 ± 20.5
44 Vancomycin	15 (21.4%)	56.7 ± 36.7
45 Tigecycline	1 (1.4%)	83.3 ± 0.0
Lincosamide		
46 Clindamycin	3 (4.3%)	50.0 ± 40.8
Lincosamide		
47 Lincomycin	4 (5.7%)	72.9 ± 3.2
Macrolide		
48 Erythromycin	21 (30.0%)	56.6 ± 28.3
49 Roxithromycin	1 (1.4%)	100.0 ± 0.0
Miscellaneous		
50 Chloramphenicol	45 (64.3%)	21.4 ± 23.7
51 Colistin (Polymyxin E)	7 (10.0%)	39.0 ± 33.9
52 Daptomycin	1 (1.4%)	0.0 ± 0.0
53 Florfenicol	2 (2.9%)	11.3 ± 5.4
54 Fosfomycin	3 (4.3%)	21.5 ± 18.8
55 Metronidazole	1 (1.4%)	100.0 ± 0.0
56 Novobiocin	1 (1.4%)	28.6 ± 0.0
57 Polymyxin B	3 (4.3%)	54.4 ± 40.5
58 Rifampin	3 (4.3%)	48.3 ± 11.7
59 Spectinomycin	1 (1.4%)	4.6 ± 0.0
60 Trimethoprim	12 (17.1%)	38.1 ± 35.5
61 Trimethoprim-sulfamethoxazole	33 (47.1%)	32.7 ± 33.1
Monobactam		
62 Aztreonam	6 (6.6%)	38.9 ± 40.7
Oxazolidinones		
63 Linezolid	2 (2.9%)	0.0 ± 0.0
Penicillin		
64 Amoxicillin	17 (24.3%)	56.5 ± 31.5
65 Amoxicillin-clavulanic acid	26 (37.1%)	37.1 ± 30.2
66 Ampicillin	50 (71.4%)	57.2 ± 33.3
Penicillin		
67 Ampicillin-sulbactam	4 (5.7%)	39.2 ± 38.1
68 Ampiclox	2 (2.9%)	21.8 ± 21.8
69 Carbenicillin	2 (2.9%)	23.9 ± 1.0
70 Cloxacillin	7 (10.0%)	63.2 ± 27.6
71 Mecillinam	1 (1.4%)	0.0 ± 0.0
72 Methicillin	3 (4.3%)	85.2 ± 15.8
73 Oxacillin	3 (4.3%)	48.5 ± 30.2
74 Penicillin	13 (18.6%)	79.2 ± 29.2
75 Piperacillin	6 (8.6%)	33.3 ± 40.1
76 Piperacillin-tazobactam	8 (11.4%)	5.2 ± 7.7
77 Ticarcillin	2 (2.9%)	51.6 ± 43.9
78 Ticarcillin-clavulanic acid	4 (5.7%)	31.8 ± 39.6
Polypeptide		
79 Bacitracin	1 (1.4%)	13.3 ± 0.0
Streptogramins		
80 Quinupristin-dalfopristin	1 (1.4%)	0.0 ± 0.0
Sulfonamide		
81 Sulfamethoxazole	10 (14.3%)	58.0 ± 31.8
82 Sulfanilamide	1 (1.4%)	63.4 ± 0.0
83 Sulphafurazole	3 (4.3%)	67.9 ± 13.9
84 Triple-sulfa	1 (1.4%)	7.7 ± 0.0
Tetracycline		
85 Chlortetracycline	1 (1.4%)	62.4 ± 0.0
86 Doxycycline	4 (5.7%)	54.3 ± 38.3
87 Oxytetracycline	1 (1.4%)	28.6 ± 0.0
88 Tetracycline	47 (67.1%)	38.8 ± 27.3

Chapter 3

Spatiotemporal dynamics of *Escherichia coli* presence and magnitude across a national groundwater monitoring network, Republic of Ireland, 2011-2020

Preface

Title: Spatiotemporal dynamics of *Escherichia coli* presence and magnitude across a national groundwater monitoring network, Republic of Ireland, 2011-2020

Authors: **Luisa Andrade**, Martin Boudou, Paul Hynds, Carlos Chique, John Weatherill, and Jean O'Dwyer

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LA's (CRediT) contribution: Conceptualization, Methodology, Writing - original draft, Writing – review & editing.

Relevance: This paper analysed an extensive spatially distributed time-series (2011-2020) of monitored (regulated) groundwater supplies in the RoI (n = 3,808 water samples) to identify *E. coli* detection rates and whether they relate to potential (extra-)local drivers. Mixed effects logistic regression modelling indicate pastural agriculture and karst geology as key risk factors for *E. coli* occurrence, with the first also representing a potential source of antimicrobial resistance. This study also shows the ubiquity and magnitude of groundwater contamination with faecal bacteria (i.e., *E. coli* found in 66.7% of supplies at least once) and when taken in conjunction with the high incidence of resistance within groundwater bacteria globally (see Chapter 2), it also highlights groundwater resources in the RoI as potential reservoirs for ARB.

Significance: The current study provides the first national-scale quantitative assessment of temporal microbial contamination within (regulated) groundwater supplies in the RoI.

Abstract

Groundwater is a vital drinking water resource and its protection from microbiological contamination is paramount to safeguard public health. The Republic of Ireland (RoI) is characterized by the highest incidence of verocytotoxigenic *Escherichia coli* (VTEC) enteritis in the European Union (EU), linked to high reliance on unregulated groundwater sources (~16% of the population). Yet, the spatiotemporal factors influencing the frequency and magnitude of microbial contamination remain largely unknown, with past studies typically constrained through reliance on spatiotemporally limited sampling campaigns. Accordingly, the current investigation sought to analyse an extensive spatially distributed time-series (2011-2020) of groundwater monitoring data in the RoI. The dataset, compiled by the Environmental Protection Agency (EPA), showed *high* contamination rates, with 66.7% (88/132) of supplies testing positive for *E. coli,* and 29.5% (39/132) exceeding concentrations of 10MPN/100ml at least once during the 10-year monitoring period. Seasonal decomposition analyses indicate that *E. coli* detection rates peak during late autumn/early winter, coinciding with increases in annual rainfall, while gross contamination peaks in spring (May) and late-summer (August), likely reflecting seasonal shifts in agricultural practices. Mixed effects logistic regression modelling indicates that monitoring sources located in karst limestone are statistically associated with *E. coli* presence (OR = 2.76, p = 0.03) and gross contamination (OR = 2.54, p = 0.037) when compared to poorly productive aquifers (i.e., transmissivity below $10m^2/d$). Moreover, mm rises in 5-day and 30-day antecedent rainfall increased the likelihood of *E. coli* contamination (OR = 1.027, p < 0.001 and OR = 1.005, p = 0.016, respectively), with the former also being associated with gross contamination (OR = 1.042, p < 0.001). As such, it is inferred that preferential flow and direct ingress of surface runoff are the most likely ingress mechanisms associated with *E. coli* groundwater supply contamination. The results presented

are expected to inform policy change around groundwater source protection and provide internationally transferable tools for predicting bacterial contamination of groundwater sources in geologically heterogeneous regions.

Highlights

- Novel microbial assessment of a national groundwater monitoring scheme.
- *E. coli* detected in 66.7% of monitoring wells (88/132) at least once in 10 years.
- *E. coli* present > 10MPN/100 ml in 29.5% of monitoring wells (39/132) within 10-year period.
- Pastural agriculture and karst geology key risk factors for *E. coli* occurrence.

Keywords: Groundwater Contamination, *Escherichia coli*, Environmental Fate modelling, Hydrodynamics

3.1 Introduction

Globally, groundwater accounts for 95% of available freshwater, with approximately 31.5% (2.2 billion people) of the world's population relying on a groundwater supply for daily consumption (Murphy et al., 2017). In the Republic of Ireland (RoI), groundwater abstractions constitute approximately 25% of all drinking water with 16% of the national population (~750,000 people) relying on private groundwater sources for daily domestic use. While traditionally presumed to represent a "safe" water resource, largely due to the contaminant attenuation capacity of overlying subsoil layers, groundwaters are still susceptible to microbiological contamination (Bradford and Harvey, 2017). Agricultural activities such as livestock rearing and slurry/manure spreading, which vary spatially and temporally, and sewage disposal from domestic, municipal, or industrial sources are amongst the most

common sources of these organic contaminants (Andrade et al, 2018; Chique et al, 2020ab). Ingress of animal or human faecal matter represents a key threat to groundwater quality and public health, as these materials may contain pathogenic microorganisms such as *Salmonella* spp., *Escherichia coli*, *Cryptosporidium* spp., *Campylobacter* spp., and noroviruses (Hynds et al., 2014a; Murphy et al., 2017; Kauppinen et al., 2018; Chique et al., 2020ab). For example, pathogenic *E. coli* are widely recognized as a key aetiological agent of human infection, causing mild- to severe- gastroenteritis, and have been widely reported in domestic groundwater sources from low- to high-income regions and across all climate classifications (Ferguson et al, 2012; Croxen et al, 2013; Odagiri et al, 2016; Murphy et al., 2017; Chique et al, 2020b). Crude incidence rates of verocytotoxigenic *Escherichia coli* (VTEC) enteritis in Ireland, an enteric infection characterised by high rates of waterborne transmission and a spectrum of severity ranging from mild diarrhoea to haemorrhagic colitis and haemolytic uremic syndrome (Hunter and Thompson, 2005, Karmali et al., 2010), are currently the highest in Europe (ECDC, 2019). Recent work by ÓhAiseadha et al. (2017) report that the incidence of VTEC O157 infection is statistically associated with the use of private (household-based) groundwater supplies (OR 6.869; $p < 0.01$); a type of supply frequently linked with low rates of appropriate microbiological treatment (Hynds et al., 2013; Mustacchio et al., 2020). Further, ongoing monitoring demonstrates that regulated groundwater abstractions serving small private and public buildings also regularly exhibit poor water quality, including frequent contamination with faecal indicator organisms (EPA, 2020).

Microbial contamination of groundwater is a multifaceted issue, potentially influenced by a range of interconnected risk factors and (pre/post) conditions, such as prevailing weather (including extreme weather events), soil moisture, local hydrogeological setting, hazard proximity (setback distance and gradient), and source-specific infrastructural characteristics

(e.g., well design, maintenance) (Hynds et al, 2012; Wallender et al, 2014; Atherholt et al, 2017; Andrade et al., 2018). The significance of risk factors, both individually and in combination, may be subject to considerable spatiotemporal variability (O'Dwyer et al, 2018; 2021), adding a layer of complexity to correctly attributing contamination sources and mechanisms. Hydro-meteorological variability plays a central role in the spread of pathogenic microorganisms throughout the natural environment, significantly affecting transport and eventual ingress to both aquifers and supplies (Hynds et al., 2012; Murphy et al., 2017; Moreira and Bondelind, 2017; Andrade et al., 2018). For example, extended periods of rainfall have been shown to promote the release of pathogens from faecal material, and its subsequent transport via surface runoff and subsurface percolation (Fremaux et al, 2008; Blaustein et al., 2016). In the Irish context, clear links have been established between *E. coli* detection in groundwater sources and antecedent (5-10 day) rainfall ($p < 0.05$) (Hynds et al, 2014b; O'Dwyer et al, 2014; 2018; 2021), due to enhanced (sub-)surface hydrological connectivity (e.g., overland flow, preferential infiltration/recharge). Likewise, heavy rainfall events, have been associated ($p < 0.01$) with outbreaks of VTEC enteritis in the RoI (O'Dwyer et al, 2016). Taken together, the interaction between rainfall (frequency, duration and intensity), enhanced hydrological connectivity, environmental dissemination of faecally-derived microorganisms, and public health represent an increasing concern. This is further heightened in light of mounting evidence of (global) climate change and the increased likelihood of more frequent and intense rainfall events (Arnell and Gosling, 2016; Forzieri et al, 2017).

Spatially distributed time-series data obtained from groundwater monitoring networks provide essential feedback for ascertaining the pathways and mechanisms associated with groundwater contamination (Hancock et al, 1998; Lee and Kwon 2016; Little et al, 2016;

Rayne et al, 2018; McGrogry et al, 2017), and play a key role in informing policy aimed at safeguarding public health (Rohde et al, 2017). Thus, the EU Water Framework Directive (WFD) (Directive 2000/60/EC) developed a new foundation for European water resource management and policy-making by emphasising active water quality monitoring to identify and achieve pre-determined water quality targets. In the RoI, long-term groundwater monitoring is managed by the Environmental Protection Agency (EPA) with assistance from the Geological Survey of Ireland (GSI) through the National Groundwater Monitoring Programme (NGMP), which has routinely monitored the chemical and microbial quality of Irish groundwater since the early 1990s. To date, however, robust temporal and spatiotemporal analyses of monitoring data have yet to be undertaken at a national scale.

Accordingly, the current study sought to explore the spatiotemporal incidence of *E*. *coli* in Irish groundwater, a region characterised by high concurrent rates of groundwater reliance and waterborne enteric infection, using the publicly available NGMP dataset. Specifically, and with a marked "mechanistic" focus, the objectives of this study were to: (*i*) examine groundwater *E*. *coli* detection rate across Ireland as they relate to potential (extra-)local drivers (e.g., hydrogeological setting, local anthropogenic pressures, antecedent rainfall, seasonality) and (*ii*) statistically attribute likely *E*. *coli* contamination mechanisms via delineated modelling of *E. coli* magnitude and seasonally-specific temporal periods. Ultimately, findings may be used to inform relevant stakeholders, policy-makers, and regulatory frameworks, develop increasingly evidence-based groundwater protection guidelines and leverage an active (long-term) groundwater monitoring network.

3.2 Methods

3.2.1 Groundwater Monitoring Data

The NGMP comprises 284 geographically distributed monitoring sites, selected to represent variations in hydrogeological setting, contamination sources, pathways and pressures across the country (EPA, 2020). Monitoring sites include 132 pumping boreholes (i.e., drilled wells) which serve as public or private groundwater supplies, 72 springs or infiltration galleries, 70 observation boreholes constructed for water quality surveillance monitoring (i.e., not employed for human use), 8 surface water monitoring points, and 2 unclassified abstractions. The current study focused exclusively on data derived from groundwater supplies (n = 132) (**Figure 3.1**), as these are regularly pumped and thus likely representative of national abstractions for human consumption. Almost all sites (n = 128, 97%) are defined as active drinking water supplies, with the remaining 4 supplies employed for agricultural/industrial use.

The supplies monitored as part of the NGMP are geographically distributed and located in hydrogeologically diverse sites, with all groundwater vulnerability classifications being represented. The existing groundwater vulnerability classification used in the RoI is based on multiple hydrogeological factors, such as the thickness and permeability of overlaying subsoil layers, type of aquifer, presence of an unsaturated zone, and (in the case of karst areas) recharge type (DoEHLG/EPA/GSI, 1999). The current vulnerability typology includes five categories: “Low”, “Moderate”, “High”, “Extreme” and “Extreme X (Karst/Rock with little to no overlaying soil layers)”; these serve as indicators for the theoretical risk of contaminant ingress from surface-based sources via groundwater recharge (DoEHLG/EPA/GSI, 1999).

Previous Irish studies report vulnerability classifications as a useful predictor for the presence of microbial groundwater contaminants (Hynds et al., 2014; O'Dwyer et al., 2018).

Data from a 10-year monitoring period (2011 to 2020, inclusive) were used in the current study, with supplies sampled a mean of 3 (St. Dev ± 1) times annually, totalling in a mean of 29 (St. Dev ± 6) samples per supply. Water samples were collected from pre-treatment (raw water) well taps, with *E. coli* presence and magnitude determined via the most probable number (MPN, multiple tube) method (ISO 9308-2:2012). Samples where *E. coli* concentrations exceeded 10 per 100 ml (10 MPN/100 ml) were regarded as "grossly contaminated", which is consistent with the classification historically employed for thermotolerant coliforms in the Irish groundwater context (EPA, 2006; Bacci and Chapman, 2011). Monthly time series of *E. coli* detection rate across the monitoring network and, where *E. coli* were present, detection rates above 10 MPN/100ml were developed.

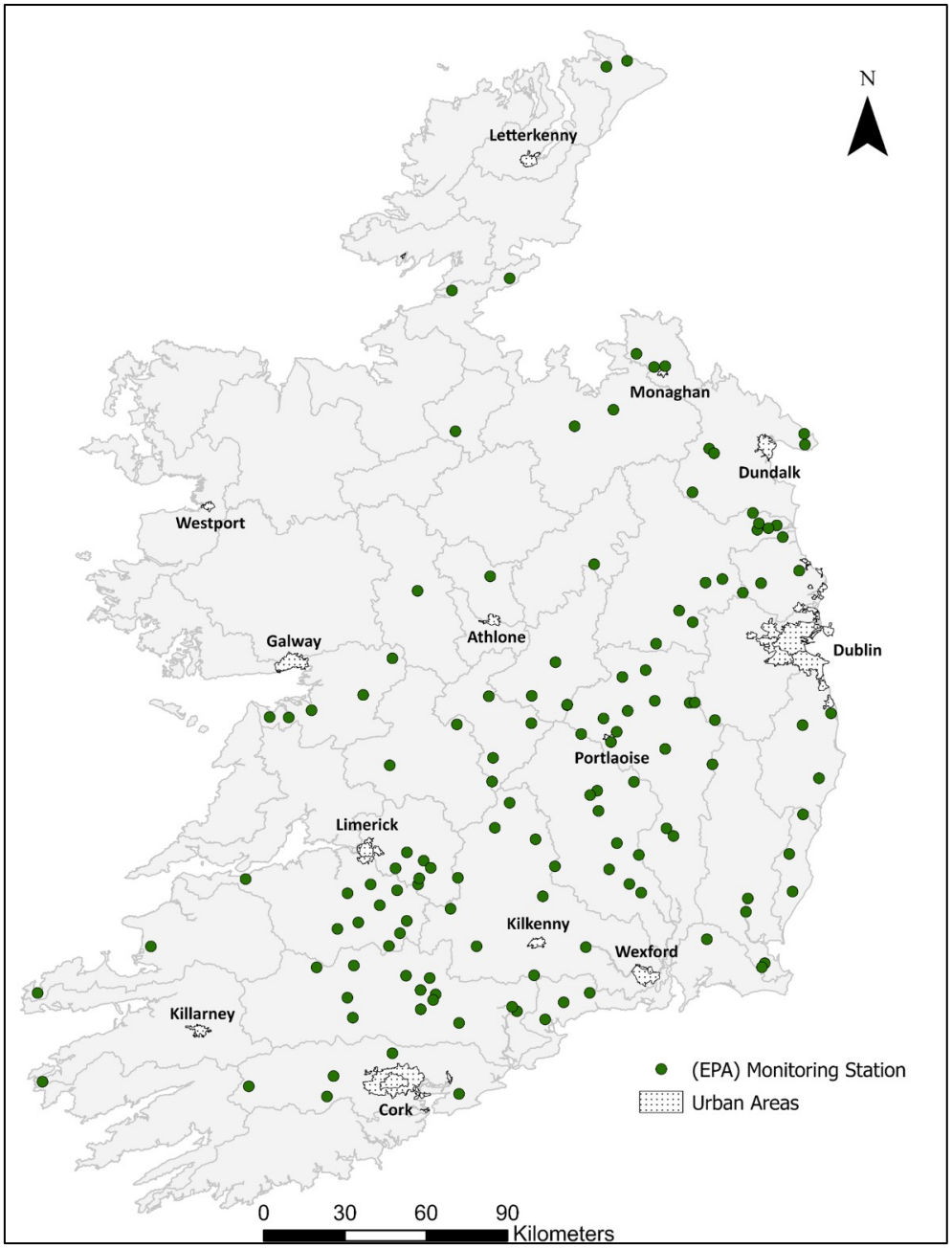


Figure 3.6: Map of the Republic of Ireland showing EPA monitoring stations included in the current study (n= 132)

3.2.2 Seasonal Decomposition

Seasonal decomposition was employed to identify seasonal and overarching temporal trends in monthly detection rates of (i) *E. coli* incidence (i.e., presence) and, where present, (ii) incidence greater than 10 MPN/100 ml (i.e., indicative of gross contamination, Bacci and Chapman, 2011). Analyses were performed on the complete dataset (irrespective of location/hydrogeological setting) and for three subsets based on mapped groundwater

vulnerability classification (i.e., “Low”/”Moderate”, “High” and “Extreme”/“Extreme X – Rock/Karst”).

During the study period several months were characterised by an absence of monitoring, ranging from one to seven consecutive months, thus requiring preliminary imputation prior to decomposition. Multiple imputation techniques were explored, with the “Seasonally Decomposed Missing Value Imputation” (SDMVI) approach (Moritz and Bartz-Beielstein, 2017) considered the most appropriate as it retains the seasonal signal within the original time-series. Briefly, SDMVI de-seasonalizes the time-series, re-adding the predicted seasonal signal post-imputation (i.e., imputation is based on simulated data residuals and trend). Imputation was performed at a monthly resolution using the “*imputeTS*” (v3.2) package in R Studio (v 4.0.5). Seasonal decomposition of the imputed time series was undertaken via the seasonal-trend decomposition method (STL) based on the LOESS (Locally Estimated Scatterplot Smoothing) technique. The STL method decomposes the time-series data into three distinct components (signals): (i) seasonal variation (based on a 12-month cycle), (ii) overall trend over time during the entire time series and (iii) residuals (remainders), with the original (raw) dataset equal to the sum of all three components based on an additive model. STL analyses were run in R version 4.0.5 using the *STL()* function from the *“forecast”* package (Hyndman et al., 2020).

3.2.3 Mixed-Effects Modelling

Outcomes and predictors

Mixed-effects modelling was employed to identify significant model inputs as they relate to modelled outcomes (*E. coli* presence and magnitude), thus permitting interpolation

of likely (generalised) contamination mechanisms. This approach was used to account for repeat sampling and subsequent "non-independence" i.e., analytical time-series deriving from an individual groundwater supply could not be considered statistically independent from sample to sample. Modelled outcomes were converted to binary format, with a logistic link function thus employed throughout. *E. coli* presence (> 0 MPN/100 ml) was taken as being indicative of recent faecal ingress, and where present, concentrations above 10 MPN/100 ml were reclassified as being indicative of "gross contamination" (gross/not gross).

All modelled potential predictors i.e., parameters potentially influencing the presence and/or magnitude of *E. coli* in monitored groundwater supplies, are summarised in **Table 3.1**, these include groundwater vulnerability classification, density of nearby faecal sources, land-cover, antecedent rainfall, etc. Cattle and domestic wastewater treatment system (DWWTS) density variables were collated from census data available at the Electoral Division (ED) level, which is the smallest legally defined administrative area in the RoI (n = 3,440), and at the Small Area (SA) level, which are delineated areas comprising between 80 and 120 dwellings (n = 18,641; CSO, 2011). Land-cover classifications were extracted from the CORINE (Co-ORdinated INformation on the Environment) dataframe (CORINE, 2012; 2018). As such, monitored supplies located in the same Electoral Divisions were assigned the same estimated values. Landscape subcategories were derived from a recent reclassification of physiographic units in the RoI (Carlier et al., 2021). Cumulative antecedent rainfall (mm) parameters were calculated using daily historical data from the nearest Met Éireann synoptic station (Met Éireann, 2021); in cases where the nearest station lacked daily readings, data from the second nearest weather station were used in calculations (vis. Howard et al., 2003; Hynds et al., 2012). A time series plot showing mean monthly precipitation across the country over the 10-

year study period was also produced. All georeferenced data were extracted in ArcMap v10.6 using sample source coordinates.

Table 3.1: Potential predictors of E. coli ingress into groundwater supplies, and their sources delineated into one of four categories, namely supply-specific, hydrogeological, land-use, topographical and hydrometeorological

Predictors	Data source
Supply-specific parameters	
Supply type ("Public supply well", "Group water scheme well", "Private well", "Industrial/agricultural well")	EPA (2020)
Supply depth (m)	EPA (2020)
Hydrogeological parameters	
Aquifer type ("Fissured bedrock", "Karst", "Poorly productive bedrock", "Gravel")	GSI (2020)
Aquifer importance ("Regionally important", "Locally important", "Poor")	GSI (2020)
Groundwater vulnerability ("Low", "Moderate", "High", "Extreme", "Extreme X – Rock/karst")	GSI (2020)
Bedrock type ("Limestone", "Sandstone", "Igneous", "Schist/gneiss")	GSI (2020)
Land-use and topographical parameters	
Cattle density (heads/km^2)	CSO (2011)
Domestic Wastewater Treatment System (DWWTS) density (units/km^2)	CSO (2011)
Land-cover ("Pasture", "Non-pasture")	CORINE (2012; 2018)
Landscape ("Intensified Lowlands", "Semi-intensified Lowlands", "Semi-intensified Elevated", "Extensive Lowlands", "Extensive Uplands", "Extensive Bedrock Plains", "Peatlands", "Marshlands – Estuarine")	Carlier et al., 2021
Mean Small Area slope (m/m)	Authors
Hydrometeorological parameters	
1-day antecedent rainfall (mm)	Met Éireann (2021)
5-day antecedent rainfall (mm)	Met Éireann (2021)
10-day antecedent rainfall (mm)	Met Éireann (2021)
30-day antecedent rainfall (mm)	Met Éireann (2021)
60-day antecedent rainfall (mm)	Met Éireann (2021)
90-day antecedent rainfall (mm)	Met Éireann (2021)

Model training and testing

Multilevel mixed-effects logistic models (Have et al., 1998) were used to examine associations between predictors (**Table 3.1**) and (i) *E. coli* presence (irrespective of season), (ii) gross contamination among *E. coli*-positive samples (irrespective of season), (iii) *E. coli* contamination during historically wetter months (October to March; Met Éireann, 2021), and

(iv) *E. coli* contamination during historically drier months (April to September; Met Éireann, 2021). All statistical analyses were conducted in STATA/MP 14.2 using the *melogit* command and adjusted for sample correlation within the random nesting factor (well ID). All models were trained on 80% of the dataset (balanced based on outcome proportion), and tested on the remaining 20% to avoid model over-fitting and increase parsimony (Hynds et al., 2012). Each groundwater sample represented one observation (i.e., long data format) and all predictors (**Table 3.1**) were coded as fixed effects at the same hierarchical level, on condition that no strong inter-variable correlations were present (i.e., (multi)collinearity). Inter-variable correlations among predictors of interest were manually assessed prior to model development using Spearman's rho; where rho ≥ 0.5, sensitivity analyses were performed by including both input variables separately, with the best fitting model selected (based on model sensitivity and explained variance as indicated by pseudo-R-squared; McKelvey and Zavoina, 1975). One predictor (well depth) was missing for > 10% of observations and thus excluded from analyses.

3.3 Results

3.3.1 E. coli detection

Summary statistics describing the 132 monitored groundwater supplies and all water samples tested for *E. coli* during the study period (n = 3,808) are presented in **Table 3.2**. Approximately three quarters of monitored supplies (n = 101; 76.5%) were located in areas classified as "pasture" (viz. CORINE 2018), with mean calculated cattle and DWWTS densities of 123 ± 54 and 7.7 ± 3 per km^2, respectively. Monitored wells were located in areas classified as "High" (n = 62; 47.0%), "Moderate" (n = 23; 17.4%), "Low" (n = 22; 16.7%), "Extreme" (n = 17; 12.9%) and "Extreme X (Rock/Karst)" (n = 8; 6.0%) groundwater vulnerability, respectively.

Locally important, regionally important, and poor aquifers were associated with 61 (46.2%), 60 (45.5%), and 11 (8.3%) supplies, respectively; 45 supplies (34.1%) were associated with low productivity bedrocks, followed by 38 (28.8%) in karst, 37 (28.0%) in productive fissured bedrock and 12 (9.1%) in gravel aquifers. The mean well depth, where reported (n =101; 76.5%), was 55.7 ± 32.6 m ranging from 7.3 to 182 m. The distribution of hydrogeological and land-use parameters associated with monitored groundwater supply locations are presented in Supplementary Materials.

Overall, 66.7% (88/132) of monitored supplies tested positive for *E. coli* at least once during the 10-year study period (this is geographically represented in **Figure 3.2**), with 29.5% (39/132) displaying concentrations above 10MPN/100 ml at least once in the same period. Detection rates of *E. coli* (> 0 MPN/100ml) and detection above 10 MNP/100 ml across all groundwater samples were of 12.8% (n = 486/3,808) and 4.5% (n = 171/3,808), respectively. "Gross" concentrations (> 10 MNP/100 ml) were found in 35.2% (n = 171/486) of *E. coli*-contaminated samples. No substantial differences were found in *E. coli* detection rates between historically drier (13.0%; n = 296/2,273) and wetter months (12.4%; n = 190/1,535). A mean *E. coli* detection rate of 12.6% (± 2.9%) was found in samples collected each year, ranging from 8.9% (2013) to 19.1% (2014), with concentrations above 10MPN/100ml found in a mean of 4.4% (± 1.6%) of samples annually, ranging from 2.8% (2020) to 8.6% (2014) (**Table 3.3**). Likewise, mean *E. coli* detection rates were monotonic with respect to delineated groundwater vulnerability category (**Table 3.3**), with 10-year mean detection rates of 24.7%, 11.7% and 9.3% among boreholes in Extreme, High and Moderate/Low vulnerability categories, respectively. However, this pattern was not evident among samples > 10 MPN/100ml, with Extreme and High vulnerability areas exhibiting similar mean detection

rates (36.3% and 38%, respectively), while Moderate/Low vulnerability areas displayed significantly lower rates of "gross" contamination (15%).

Table 3.2: Characteristics of monitored groundwater supplies (n=132) and samples collected from 2011 to 2020 (n=3,808) as part of the EPA groundwater monitoring programme

Characteristic	MS, n (%)	Samples, n (%)	Characteristic	MS, n (%)	Samples, n (%)
Year			**Aquifer type**		
2011	130 (98.5)	474 (12.4)	Poorly productive bedrock	54 (41.0)	1,555 (40.8)
2012	129 (97.7)	489 (12.8)	Productive fissured bedrock	32 (24.2)	940 (24.7)
2013	127 (96.2)	384 (10.1)	Karst	32 (24.2)	928 (24.4)
2014	127 (96.2)	372 (9.8)	Gravel	14 (10.6)	385 (10.1)
2015	126 (95.5)	362 (9.5)			
2016	120 (90.9)	358 (9.4)	**Groundwater Vulnerability**		
2017	123 (93.2)	365 (9.6)	Low	22 (16.7)	642 (16.9)
2018	123 (93.2)	366 (9.6)	Moderate	23 (17.4)	632 (16.6)
2019	122 (92.4)	357 (9.4)	High	62 (47.0)	1,784 (46.8)
2020	120 (90.9)	281 (7.4)	Extreme	17 (12.9)	504 (13.2)
			Extreme X (Rock/Karst)	8 (6.0)	245 (6.4)
Wet vs. dry months					
Wet months (Oct – Mar)	130 (98.4)	1,535 (40.3)	**Supply depth**		
Dry months (Apr – Sep)	130 (98.4)	2,273 (59.7)	Under 30 m	23 (17.4)	667 (17.5)
			31 to 60 m	32 (24.2)	908 (23.8)
Land-cover			61 to 90 m	25 (18.9)	688 (18.1)
Pasture	101 (76.5)	2,943 (77.3)	Over 90 m	21 (15.9)	593 (15.6)
Agriculture/arable land	15 (11.4)	460 (12.1)	Not reported	31 (23.5)	952 (25.0)
Urban/industrial area	12 (9.1)	282 (7.4)			
Vegetation/natural cover	4 (3.0)	122 (3.2)	**Supply use**		
			Drinking	128 (97.0)	3,709 (97.4)
Cattle density			Agriculture/Industry	4 (3.0)	99 (2.6)
Low (≤50 head/km^2)	13 (9.8)	382 (10.0)			
Low-Medium (51-100 head/km^2)	32 (24.2)	975 (25.6)	**Water supply type**		
Medium (101 - 200 head/km^2)	73 (55.3)	2,069 (54.3)	Public Supply well	116 (87.9)	3,343 (87.8)
High (201-300 head/km^2)	8 (6.1)	246 (6.5)	Group Water Scheme well	9 (6.8)	275 (7.2)
Very high (> 300 head/km^2)	1 (0.7)	32 (0.8)	Private Supply	3 (2.3)	91 (2.4)
NA (0 units/km^2)	4 (3.0)	104 (2.7)	Industrial supply	3 (2.3)	67 (1.8)
			Agricultural supply	1 (0.7)	32 (0.8)
DWWTS density					
Low (<5 units/km^2)	24 (18.2)	676 (17.7)	***E coli* presence**		
Medium (5 – 15 units/km^2)	106 (80.3)	3,0989(81.1)	*E. coli* absent	44 (33.3)	3,322 (87.2)
High (> 15 units/km^2)	2 (1.5)	43 (1.2)	*E. coli* present	88 (66.7)	486 (12.8)
Aquifer importance			***E coli* > 10 MPN/100 ml**		
Regionally important	54 (40.9)	1,569 (41.2)	*E. coli* absent	44 (33.3)	3,322 (87.2)
Locally important	64 (48.5)	1,811 (47.6)	*E. coli* present <10 MPN/100 ml	49 (37.1)	315 (8.3)
Poor	14 (10.6)	428 (11.2)	*E. coli* > 10 MPN/100 ml	39 (29.5)	171 (4.5)

MS = Monitored Supplies; DWWTS = Domestic Wastewater Treatment System

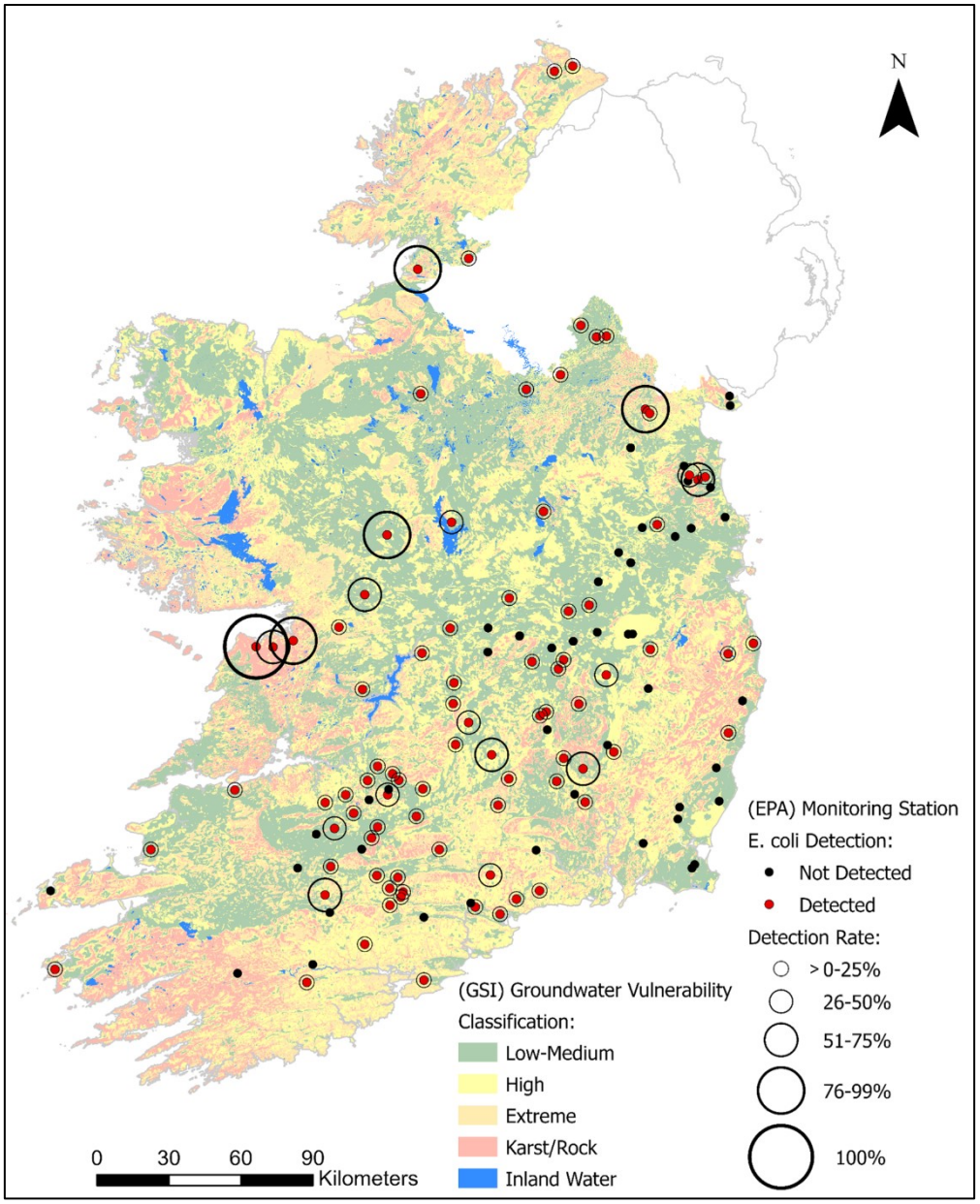


Figure 3.7: ***Map of the Republic of Ireland showing E. coli detection and detection rates in monitoring stations from 2011 to 2020. Detection rates denote the percentage of contaminated samples amongst all samples collected during the study period.***

Table 3.3: Annual detection rates of E. coli and gross (> 10 MPN/100 ml) E. coli contamination within Low-Moderate (L-M), High (H) and Extreme (E-X) groundwater vulnerability categories

	***E. coli* detection rates**				***E. coli* detection > 10 MPN/100 ml among contaminated samples**			
Year	**L-M**	**H**	**E-X**	**Total**	**L-M**	**H**	**E-X**	**Total**
2011	8.6	6.6	26.5	12.1	23.6	21.3	42.4	30.6
2012	10.5	11.6	28.8	14.1	28.1	30	39.9	30.5
2013	7.3	7.2	19.5	9.4	<1	29.8	26.4	18.5
2014	17.1	18.6	41.9	21.7	16.9	50.3	57.9	44.2
2015	8.4	17.1	31.5	18	25.5	41.1	36.5	42.2
2016	11	10.6	26.3	14.9	21.2	39.4	17.9	22.4
2017	8	14.5	13.1	11.6	14.2	31.8	35.9	22.1
2018	8.7	12.3	11.9	10.7	12.5	48.1	58.9	32.7
2019	5.3	9	21.2	10.9	8.3	37.7	24.4	23
2020	8.3	9	26.5	11.1	<1	51	22.7	24.5
2011 – 2020 (mean)	**9.3**	**11.7**	**24.7**	**13.5**	**15.0**	**38.0**	**36.3**	**29.1**

Seasonal Decomposition

Seasonal decomposition identified seasonal patterns of *E. coli* detection and, where present, detection > 10 MPN/100 ml over the 10-year study period (**Figure 3.3**). Mean precipitation in the RoI during the study period is shown in **Figure 3.4**. Annual precipitation patterns in Ireland are characterised by increased precipitation volumes from October to March, while "dry" periods are typically more frequently reported from April to September. Two hydrologically extreme events were reported during that time-frame: the generalised winter floods of December 2015 to March 2016, and the June-July 2018 drought. As shown (Figure 3a), *E. coli* detection rates annually peak in late autumn/early winter (October to December), which coincides with an increase in monthly precipitation in most years (**Figure 3.4**). The proportion of samples characterised by *E. coli* > 10 MPN/100 ml exhibits a bi-modal pattern (**Figure 3.3b**), with an initial peak during late spring (peaking in May) followed by a secondary (higher) peak in late Summer/early Autumn (August/September). Long-term *E. coli*

detection trends indicate a notable increase during 2014, followed by a decrease over the two subsequent years, culminating in a relatively stable annual trend from 2018 onwards.

Decomposed seasonal patterns among delineated groundwater vulnerability subsets (Low/Moderate, High, and Extreme/karst) indicate distinct, albeit divergent, annual cycles (**Figure 3.5a** and **Figure 3.5b**). *E. coli* detection rates in "Extreme-Karst" and "High" vulnerability areas show a relatively similar bi-modal, with initial peaks (≈ 20%) occurring in Summer (May – July), followed by a lower secondary peak (≈ 12%) in Autumn (September-October). Conversely, detection rates in Low-Moderate vulnerability exhibited a significantly lower level of seasonal variance (-15% - +12%), with just one noticeable peak during August-September. Decomposed seasonal detection rates > 10 MPN/100ml approximately mirrored detection rates > 0 MPN/100ml in both Extreme/Karst and Low vulnerability areas, likely pointing to a single dominant contamination mechanism and higher mean *E. coli* concentration when contaminated. This was not found among sources located in categorically High vulnerability areas i.e., detection rates pertaining to > 0 MPN/100ml and > 10 MPN/100ml were not similar, with the seasonal signal for detection rates > 10 MPN/100ml showing a low level of variance (± 5%) throughout the year.

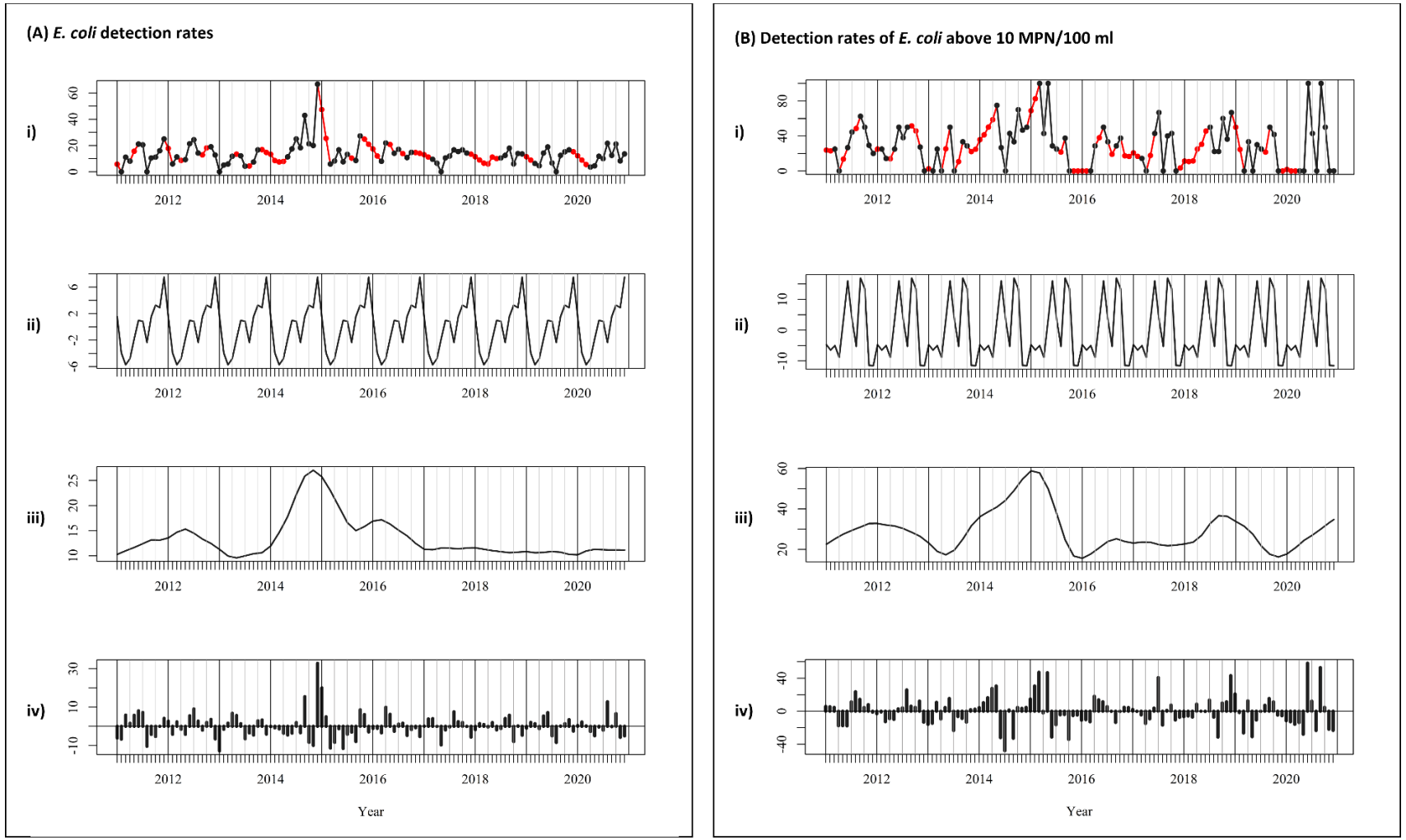


Figure 3.8: (A) STL seasonal decomposition of E. Coli detection rate (%) from January 2011 to December 2020 – (i) (Black - measured data; Red simulated data) – (ii) seasonal variations – (iii) Long-term trend – (iv) residuals. (B) STL seasonal decomposition of detection rates for E. coli > 10 MPN/100ml from January 2011 to December 2020 – (i) Raw data, in black actual data, in red, simulated data – (ii) seasonal variations – (iii) Long-term trend – (iv) residuals

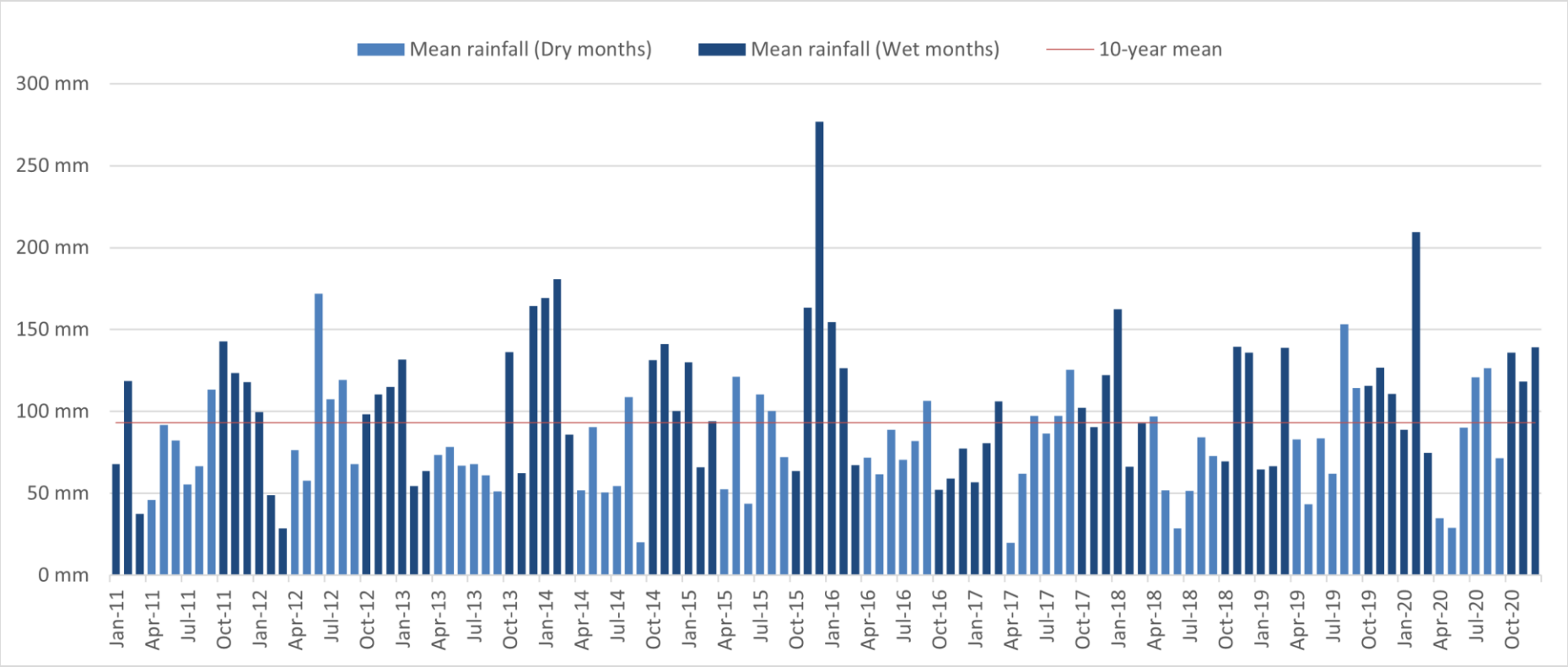


Figure 3.9: Mean monthly precipitation in the Republic of Ireland from 2011 to 2020, highlighting historically 'wetter' and 'dryer' months and the 10-year mean monthly precipitation

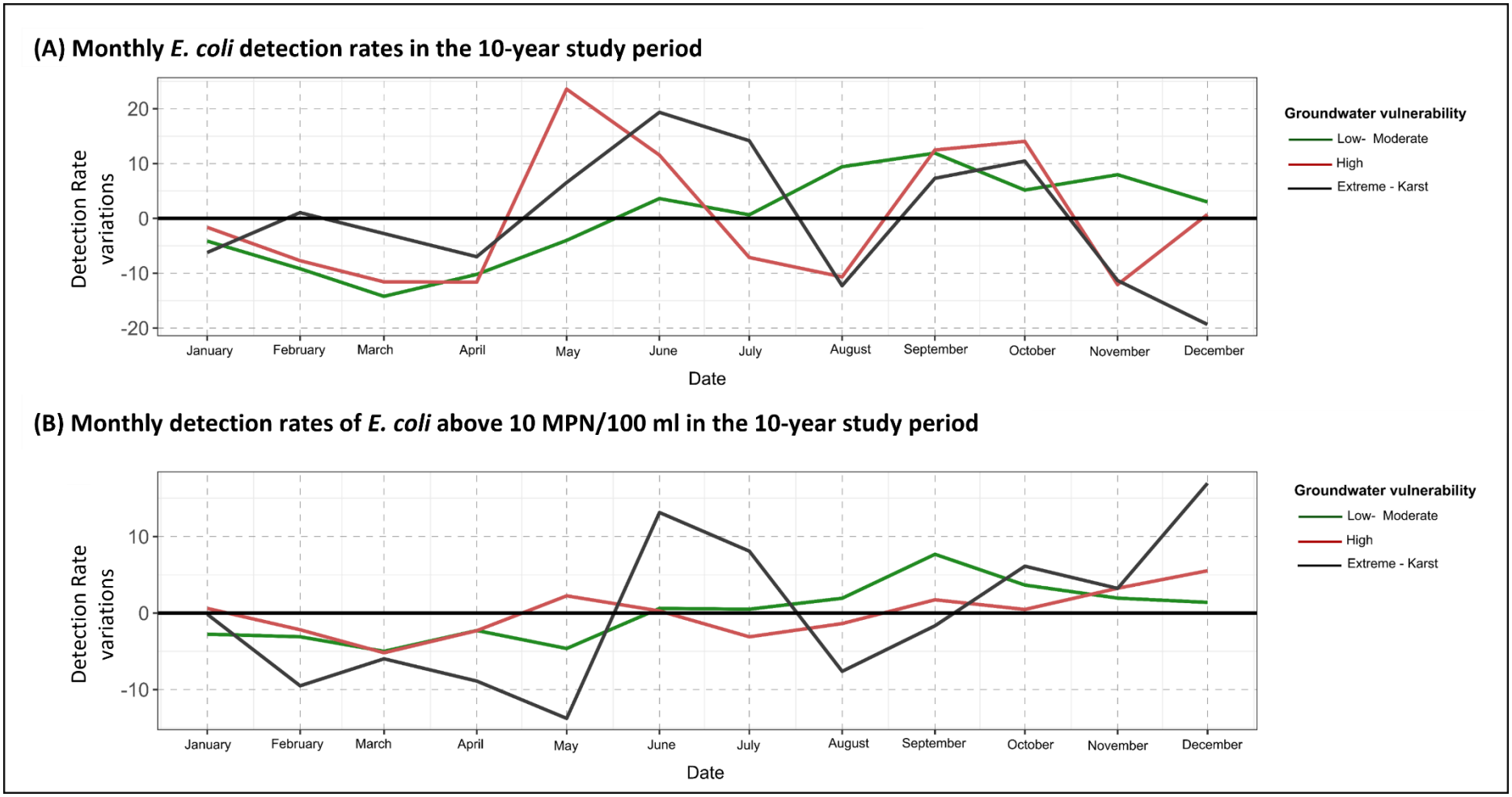


Figure 3.10: (A) STL seasonal decomposition of E. coli detection rate (%) from January 2011 to December 2020 based on delineated groundwater vulnerability category (B) STL seasonal decomposition of detection rates for E. coli > 10 MPN/100ml from January 2011 to December 2020 based on delineated groundwater vulnerability category. Note that decomposed patterns are based around variance from the long-term (10-year) delineated means from Table 4 e.g., in 4A, 0 = 11.7% for High vulnerability (Red line)

3.3.2 E. coli contamination modelling

Trained mixed-effects models and associated confusion matrices for *E. coli* presence and gross *E. coli* contamination are presented in **Table 3.5** and **Table 3.6**, while models and associated confusion matrices specific to historically "wet" and "dry" months are presented in **Table 3.7** and **Table 3.8**. Strong inter-predictor correlations were found within all models between aquifer type and aquifer importance, and cattle and DWWTS densities at the ED and SA levels, as well as multiple combinations of cumulative rainfall data; only best-fit models (based on model sensitivity and accuracy, pseudo r-squared and predictor significance) have been presented.

E. coli contamination models

The *E. coli* contamination model (**Table 3.5a**) achieved a validation accuracy of 92.0%, which was comparable with the training set accuracy of 91.8%, with moderate validation and training sensitivities (i.e., true positive prediction) of 52.6 and 53.2%, respectively, owing to the skewed nature of the dataset; i.e., significantly more negative results than positive. As shown, the odds of *E. coli* presence were over 4-times higher among group water schemes than public water supply wells (OR = 4.59, 95% CI = 1.16 – 18.16), with the odds of *E. coli* presence significantly higher in karst areas (2.76, 1.10 – 6.95) and lower in areas underlain by productive fracture/ bedrocks (0.29, 0.10 – 0.83). Groundwater vulnerability classification was also highly predictive of *E. coli* presence, with 3- and 14-times higher odds of detection in areas classified as "Extreme" (3.12, 1.01 – 9.60) and "Extreme X (Rock/Karst)" (14.37, 3.36 – 61.40) than those in "High" groundwater vulnerability areas, respectively. A significant contrast was also observed with regards to land-use; supplies located in pasture-classified areas were associated with 4-times higher odds of *E. coli* presence (4.24, 1.47 – 12.34).

Increases in 5- and 30-day cumulative rainfall significantly increased in the odds of *E. coli* presence, with odds ratios of 1.027 (95%CI = 1.017 – 1.038) and 1.005 (1.001 – 1.009), respectively.

In the gross *E. coli* contamination model (**Table 3.5b**), which only considered observations (samples) where *E. coli* was present (training subset = 359), model validation and training accuracies of 78% and 73.3% were achieved, with relatively modest sensitivities of 35.7% and 38.8%, respectively. A lower number of significant risk factors were identified. As for *E. coli* presence, supplies located in karst areas exhibited significantly higher odds of gross *E. coli* contamination than those in poorly productive bedrock aquifers (2.54, 1.06 – 6.09), with 5-day antecedent rainfall identified as a significant risk factor; a 1mm increase in 5-day cumulative precipitation equated to a 4% increase in the odds of gross *E. coli* contamination (1.04, 1.024 – 1.061). Conversely, a 10mm increase in 90-day cumulative rainfall concurred with a 4% decrease in the odds of gross *E. coli* contamination (0.996, 0.993 – 0.999). The odds of gross *E. coli* contamination were significantly lower in "Moderate" vulnerability areas when compared with "High" groundwater vulnerability areas (0.26, 0.09 – 0.80).

Table 3.5: Mixed-effects logistic regression models predicting (A) Escherichia coli presence within a training subset of all water supply samples tested for E. coli and (B) gross E. coli presence (> 10 MPN/100 ml) within a training subset of all E. coli-positive samples. Models employ EPA Groundwater Monitoring Programme samples collected from 2011-2020.

Model predictors	(A) *E. coli* detection (n=3,079)			(B) Gross *E. coli* detection (n=359)		
	N	OR (95% CI)	*p*	N	OR (95% CI)	*p*
Water supply type						
Public supply wells	2,688	Reference				
Group water scheme wells	226	**4.586 (1.158 – 18.161)**	**0.030**			
Private supply	79	0.465 (0.021 – 10.389)	0.629			
Agricultural/industrial supply	86	2.631 (0.227 – 30.432)	0.439			
Aquifer type						
Poorly productive bedrock	1,261	Reference		156	Reference	
Karst	744	**2.764 (1.098 – 6.95)**	**0.031**	160	**2.537 (1.057 – 6.090)**	**0.037**
Productive fractured bedrock	756	**0.287 (0.099 – 0.826)**	**0.021**	32	0.761 (0.202 – 2.861)	0.686
Gravel	318	0.597 (0.142 – 2.508)	0.481	11	0.912 (0.149 – 5.590)	0.921
Groundwater vulnerability						
Low	523	0.507 (0.153 – 1.679)	0.266	34	0.607 (0.154 – 2.395)	0.476
Moderate	498	1.301 (0.458 – 3.696)	0.621	**59**	**0.263 (0.087 – 0.798)**	**0.018**
High	1,459	Reference		128	Reference	
Extreme	400	**3.116 (1.012 – 9.597)**	**0.048**	70	1.223 (0.416 – 3.596)	0.715
Extreme X (Rock/karst)	199	**14.372 (3.364 – 61.398)**	**<0.001**	68	0.851 (0.286 – 2.534)	0.773
Landcover						
Non-pasture	687	Reference				
Pasture	2,392	**4.255 (1.467 – 12.341)**	**0.008**			
5-day antecedent rainfall	3,079	**1.027 (1.017 – 1.038)**	**<0.001**	359	**1.042 (1.024 – 1.061)**	**<0.001**
30-day antecedent rainfall	3,079	**1.005 (1.001 – 1.009)**	**0.016**			
90-day antecedent rainfall				359	**0.996 (0.993 – 0.9999)**	**0.045**
Pseudo R^2		0.568			0.278	
Pseudo R^2 (fixed effects only)		0.409			0.200	

Note: Missing values represent those which did not add to model accuracy and were omitted.

Table 3.6 Training and testing sensitivity and accuracy for (a) E. coli and (b) gross E. coli detection models

		E. coli detection model			Gross *E. coli* detection model		
		Predicted	Observed	% Correct	Predicted	Observed	% Correct
Model dataset	*E. coli* absent	2,622	2,690	97.5%	213	230	92.6%
	E. coli present	207	389	53.2%	50	129	38.8%
	Total	2,829	3,079	**91.9%**	263	359	**73.2%**
Validation dataset	*E. coli* absent	620	632	98.1%	82	85	96.5%
	E. coli present	51	97	52.6%	15	42	35.7%
	Total	671	729	**92.0%**	97	127	**76.4%**

E. coli contamination in wet versus dry months

Mixed-effects modelling of *E. coli* contamination during historically wetter (October to March) and drier months (April to September) are shown in **Table 3.7**, with calculated accuracy and sensitivity for testing and training subsets highly comparable for both models (**Table 3.8**). In the *drier months* model, supplies located in karst aquifers had higher odds of *E. coli* presence than those in poorly productive bedrocks (2.72, 1.02 – 7.28), while no significance was found in the *wetter months* model. Increased odds of *E. coli* presence in "Extreme" compared to "High" groundwater vulnerability areas was found in *drier months* model only (4.24, 1.26 – 14.26). Pasture-classified areas were associated with an increased odds of *E. coli* presence in both models, with the equivalent odds ratio slightly higher in wetter months (5.87, 1.14 – 30.27). Differences in hydrometeorological trends in both models are also noted: 10-day cumulative rainfall was associated with higher odds of *E. coli* presence in wetter months (1.02, 1.011 – 1.032), while 5- and 30-day cumulative rainfall increased the odds of *E. coli* contamination during drier months (1.03, 1.012 – 1.042 and 1.01, 1.005 – 1.017, respectively).

Table 3.7: Mixed-effects logistic regression models predicting Escherichia coli presence within a training subset of samples collected during (A) historically wetter (Oct-Mar) and (B) historically drier (Apr-Sept) months. Models employ EPA Groundwater Monitoring Programme samples collected from 2011-2020.

Model predictors	(A) *E. coli* detection in wetter months (n=1,210)			(B) *E. coli* detection in drier months (n=1,791)		
	N	**OR (95% CI)**	***p***	**N**	**OR (95% CI)**	***p***
Water supply type						
Public supply wells				1,572	Reference	
Group water scheme wells				130	**4.947 (1.176 – 20.809)**	**0.029**
Private supply				41	0.550 (0.0186 – 16.217)	0.729
Agricultural/industrial supply				48	4.793 (0.304 – 75.589)	0.265
Aquifer type						
Poorly productive bedrock	491	Reference		737	Reference	
Karst	290	2.477 (0.684 – 8.976)	0.167	428	**2.725 (1.020 – 7.282)**	**0.046**
Productive fractured bedrock	303	**0.137 (0.026 – 0.723)**	**0.019**	436	**0.257 (0.080 – 0.824)**	**0.022**
Gravel	126	0.817 (0.118 – 5.642)	0.838	190	0.387 (0.075 – 1.984)	0.255
Groundwater vulnerability						
Low	200	0.740 (0.129 – 4.241)	0.736	285	0.983 (0.275 – 3.513)	0.979
Moderate	202	1.839 (0.416 – 8.129)	0.421	302	1.616 (0.524 – 4.982)	0.403
High	574	Reference		843	Reference	
Extreme	156	3.800 (0.762 – 18.953)	0.103	248	**4.242 (1.262 – 14.261)**	**0.019**
Extreme X (Rock/karst)	78	**19.264 (2.372 – 140.608)**	**0.005**	113	**20.176 (4.336 – 93.887)**	**<0.001**
Landcover						
Non-pasture	281	Reference			Reference	
Pasture	929	**5.872 (1.139 – 30.270)**	**0.034**	1,391	**5.111 (1.576 – 16.578)**	**0.007**
5-day antecedent rainfall				1,791	**1.027 (1.012 – 1.041)**	**<0.001**
10-day antecedent rainfall	1,210	**1.021 (1.011 – 1.032)**	**<0.001**			
30-day antecedent rainfall				1,791	**1.011 (1.005 – 1.017)**	**<0.001**
Pseudo R^2		0.638			0.576	
Pseudo R^2 (fixed effects only)		0.466			0.438	

Note: Missing values represent those which did not add to model accuracy and were omitted.

Table 8: Training and testing sensitivity and accuracy for E. coli detection models in (a) wetter and (b) drier months

		Model for *E. coli* in wetter months			Model for *E. coli* in drier months		
		Predicted	Observed	% Correct	Predicted	Observed	% Correct
Model dataset	*E. coli* absent	1,034	1,064	97.2%	1,524	1,554	98.1%
	E. coli present	80	146	54.8%	122	237	51.5%
	Total	1,114	1,210	**92.1%**	1,646	1,791	**91.9%**
Validation dataset	*E. coli* absent	275	281	97.9%	414	723	97.9%
	E. coli present	25	44	56.8%	30	59	50.8%
	Total	300	325	**92.3%**	444	482	**92.1%**

3.4 Discussion

The presented study provides the first national-scale quantitative assessment of a temporally monitored groundwater supply network, comprising 3,808 water samples, over a ten-year period in the Republic of Ireland. Overall, contamination rates were high with over 66% of monitoring wells (88/132) positive for *E. coli* at least once during the sampling period, with concentrations exceeding 10MPN/100ml on at least one occasion in 29.5% of wells (39/132). Results are indicative of the persistent and spatiotemporally diverse issue of groundwater quality in the ROI which is particularly pertinent due to the high reliance on groundwater within the state (~16%). Previous studies focusing on domestic groundwater wells have reported contamination rates ranging from 28.9% (Hynds et al., 2012) to 51.4% (O'Dwyer et al., 2018), albeit emerging international research suggests potential environmental adaptation of *E. coli* to groundwater environments and thus overestimation of persistent contamination events (Touchon et al., 2020; Tropea et al., 2021). Regardless, reported *E. coli* contamination levels highlight a significant concern associated with groundwater contamination across the island of Ireland. In particular, the extent of gross contamination reported across the monitored sites poses a specific risk to public health, with the infectious dose for pathogenic strains of *E. coli* (e.g. STEC/VTEC) as low as 10 CFU per 100ml (FSAI, 2018); notably, Ireland has the highest incidence rate of VTEC in the EU, with private wells identified as a key risk factor (O'hAiseadha et al., 2017).

Time-series analyses via seasonal decomposition indicate that *E. coli* detection rates peak during late autumn/early winter, coinciding with an increase in the

frequency and intensity of rainfall across the country, with precipitation levels typically at their highest during December and January (Met Eireann, 2021). Notably, the decomposed incidence of gross (> 10 MPN/100ml) contamination demonstrates a late spring/early summer peak (May) followed by a secondary peak in summer (August), likely reflecting agricultural practices, which in Ireland are dominated by dairy and beef farming (i.e., pasture-based) with cattle left out to pasture in late Spring and calves weaned to pasture by late Summer (Teagasc, 2020). Delineation of monitoring sources by groundwater vulnerability category indicates that the differential timing associated with *E. coli* detection rate and the incidence of gross contamination may be elucidated via differing mechanisms based on hydrogeological setting. Significantly higher rates of *E. coli* detection occur in extreme/karst areas during winter months, reflective of the relative ease of microbial transport through shallow or absent layers of high-permeability subsoils, and subsequently, limited attenuation capacities i.e., rapid response to precipitation events and overarching precipitation patterns (Daly and Warren, 1998). Conversely, increased bacterial loading (> 10 MPN/100ml) is more frequent in high vulnerability areas during late spring/early summer, suggesting that while karst areas are more frequently contaminated due to their inherent vulnerability, higher bacterial loading occurred in areas overlain by moderate (shallow) and high (deep) permeability subsoils, perhaps indicative of the activities which predominate in high vulnerability areas, i.e., increased agricultural density where there are appreciable subsoil deposits to sustain pastural agriculture (Shaloo et al.,2004); in essence, the increased permeability which allows for productive grassland also allows for subsurface ingress of microbial contamination at increased rates (Foppen et al., 2006). Moreover, potentially higher

temperatures during late summer and early autumn may also lead to an increase in *E. coli* numbers detected (John & Rose, 2005), which warrants further research. Across the ten-year study period, a marked increase in E. coli detection rates occurred during 2014 – 2015, although this was potentially more pronounced due to low data availability and limited observations during this time-period. Bias might also have been introduced by the data imputation**.** When assessing mean meteorological conditions across the monitoring timeframe, there are no stark deviations in rainfall from the ten-year trend for this specific period, albeit, this does not consider localised precipitation events, which may be explanatory. A potential consideration may be the abolition of milk quotas in Ireland in 2014, which rapidly resulted in a 38% increase in national dairy herd (Farming Independent, 2020) and subsequently increased faecal loading in agricultural areas, however, if this did have an impact, it was short-lived and is not replicated in subsequent years in the seasonal decomposition models.

Mixed effects logistic regression modelling was employed to identify key risk factors for both the occurrence of *E. coli* and gross *E. coli* contamination across the groundwater monitoring network. Mirroring the seasonal decomposition profile, extreme and/or karst areas were statistically associated with both *E. coli* presence and gross contamination (**Table 3.3**); findings indicate that *E. coli* contamination is over 19 times more likely in karstic environments, thus concurring with previous work which has shown that groundwater monitoring locations in karst limestone areas consistently exhibit significantly higher levels of microbiological pollution (Bradley et al., 2015), reflecting the inherently vulnerable nature of dynamic flow systems, in concurrence with the lack of attenuation capacity associated with shallow soils or

subsoils (Pronk et al., 2009). This is further substantiated through recent national epidemiological models which highlight three primary hotspots of waterborne infectious disease (verotoxigenic *E. coli*), all of which occur in karst areas (Cleary et al., 2021).

Microbial contamination of groundwater is particularly associated with diffuse sources of contamination, particularly agriculture, in carboniferous limestone regions (Drew et al., 1996; Wallender et al., 2014). In Ireland, the agricultural sector is characteristically diverse, ranging from tillage to pasture, however, results from the current study point to livestock farming as the primary agricultural driver of *E. coli* presence in Irish groundwater systems, with monitoring wells located in areas dominated by pastural agriculture over 4 times more likely to harbour *E. coli*. It is estimated that 80% of organic wastes generated in Ireland are derived from agricultural activities (Daly, 2000), which are often applied directly to land with no pre-treatment undertaken, thus potentially infiltrating both surface and groundwater resources. This relationship between livestock-based agricultural practices and groundwater contamination is well substantiated within international literature (Schets et al., 2005; Close et al., 2008; Kabore et al., 2010; Lenaker et al., 2017, Chique et al., 2020, 2021), with a recent global review of verotoxigenic *E. coli* (VTEC) in groundwater noting that cattle-dominated rural areas exhibited higher VTEC detection rates in comparison to other rural sub-categories (Chique et al., 2021). Notably, Ireland has repeatedly reported the highest crude incidence rates of VTEC enteritis within the EU, with cases of this primarily waterborne infection statistically associated with utilisation of private groundwater supplies (O'hAiseadha et al., 2017). Interestingly, pastural agricultural activities were not predictive of gross

contamination, suggesting that while agriculture is a definitive source of bacteria, and thus predictive of *E. coli* presence, the magnitude of contamination (i.e. gross contamination) is predicated upon hydrogeological processes which allow for easier ingress through the subsurface, and specifically the presence of karst aquifer systems which were 2.537 times more likely to have gross *E. coli* contamination.

Antecedent cumulative rainfall associated with a variety of temporal periods demonstrated a level of predictive capacity for both *E. coli* presence and the incidence of gross contamination within presented models. Rainfall influences both vertical and horizontal movement of bacteria at the soil interface, moderating the rate and concentration at which microorganisms may enter groundwater (Unc and Goss, 2003). Periods of prolonged rainfall have been shown to increase the discharge and microbial concentration of agricultural runoff, transporting microbiologically diverse materials (surface runoff, associated sediments, and faecal matter) to rivers, coastal waters, and groundwater wells (Semenza and Menne, 2009). Several previous studies have found a significant relationship between antecedent rainfall (mm) and the presence faecal indicators and disease outbreaks (Howard et al., 2003; Hynds et al., 2012, 2014a; O'Dwyer et al., 2016); for example, Howard et al. (2003) reported that increased rainfall over the 48-hr pre-sampling period increased the likelihood of thermotolerant coliform and faecal streptococci presence in shallow groundwater in Kampala, Uganda, while O'Dwyer et al. (2016) report an association between heavy rainfall events and outbreaks of VTEC in Ireland. In the current study, five-day and 30-day antecedent precipitation increased the likelihood of *E. coli* contamination, with the former also being associated with gross contamination. The relatively short timeframe between cumulative rainfall and gross contamination likely serves as a

relative proxy for extreme vulnerability areas whereby rainfall can transport organically rich runoff to the subsurface through direct ingress (Hynds et al., 2014). Compounding this, further elucidation of gross contamination signifies potentially two predominant contaminant mechanisms during categorically dry periods, with traditional recharge represented through statistical association with 30-day rainfall and potential preferential flow suggested through the association with 5-day rainfall. While prolonged periods with no rainfall are infrequent in Ireland (**Figure 3.4**), recent research by O'Dwyer et al. (2021) suggests that during reduced rainfall, there can be a hydrodynamic shift towards relatively rapid preferential flow of water (and contaminants) within the subsurface. In contrast, during categorically *wet* periods, 10-day rainfall is associated with *E. coli* occurrence, likely indicative of overland flow and direct ingress mediated by soil saturation, although further research is required to determine exact mechanisms.

3.5 Limitations and Recommendations

The authors consider that due to a number of inherent data constraints within the presented models/time series, careful consideration is required in the interpretation of presented findings. Importantly, the pre-existing groundwater monitoring datasets utilised in this study possess several gaps, particularly at a temporal scale. Thus, event peaks and the impact of hydrology on *E. coli* detection levels are difficult to ascertain with any great certainty and may be, in some instances, overestimated. Accordingly, a key recommendation of this research is the modification of the national groundwater monitoring regime to ensure a temporally representative dataset whereby hydro(geo)logical processes can be inferred more

accurately. This could be achieved, for example, by implementing a monthly (or at least seasonal) sampling schedule which would enhance our understanding of the seasonal patterns of *E. coli* contamination and allow for the development of tailored recommendations to ensure that groundwater sources are appropriately protected year-round. Moreover, the spatial distribution of the monitoring data is also problematic, with large areas (particularly the West) of the country distinctly unrepresented. While the monitoring programme is designed to represent major aquifer settings rather than uniform spatial resolution, the lack of monitored groundwater supplies in certain areas precludes comparison and/or control across similar aquifers which are under different land uses settings and meteorological conditions. As such, a review of the spatial distribution of the monitoring network is also recommended and an increase in the number of (regularly pumped) groundwater supplies should be considered. Finally, the reporting of site-specific data, such as supply depth and age, nearby sources of contamination, local topography, etc. - is also recommended, as these would allow a more comprehensive analysis of the risk factors contributing to the incidence and/or frequency of *E. coli* contamination at a high spatial resolution.

3.6 Conclusion

Groundwater contamination is a significant issue, which poses potential public health threats for groundwater consumers. The Republic of Ireland represents the 'perfect storm' with regard to groundwater pollution, with a relatively high groundwater reliance in concurrence with an intensive agricultural sector and a diverse hydrogeological profile. The presented study analysed data from a large-scale

national monitoring regime to assess the spatiotemporal quality of groundwater and identify key risk factors. Mixed effects logistic regression modelling indicates that monitoring sources located in karst limestone are statistically associated with *E. coli* presence (OR = 2.76, p = 0.03) and gross contamination (OR = 2.54, p = 0.037) when compared to poorly productive aquifers. Moreover, 5-day and 30-day antecedent rainfall increased the likelihood of *E. coli* contamination (OR = 1.027, p < 0.001 and OR = 1.005, p = 0.016, respectively), with the former also being associated with gross contamination (OR = 1.042, p < 0.001). The models developed represent potentially useful and internationally transferable tools for predicting bacterial contamination of groundwater monitoring wells in geologically heterogeneous regions with high groundwater contamination rates. When viewed through the lens of the characteristic agricultural, infrastructural, and hydrogeological profile of rural Ireland and other similar regions, findings serve to highlight the ubiquity and magnitude of groundwater source hazards in addition to the inherent complexities associated with effective source protection and remediation.

Author contributions

Luisa Andrade: Conceptualization, Methodology, Writing - original draft. **Martin Boudou**: Data curation. **Paul Hynds**: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Funding acquisition, Supervision. **Carlos Chique**: Data Visualisation, Writing – original draft. **John Weatherill**: Writing - review & editing, Supervision. **Jean O'Dwyer**: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Data

Maps showing the location of monitoring sites in relation to hydrogeological, land-use and agricultural variables

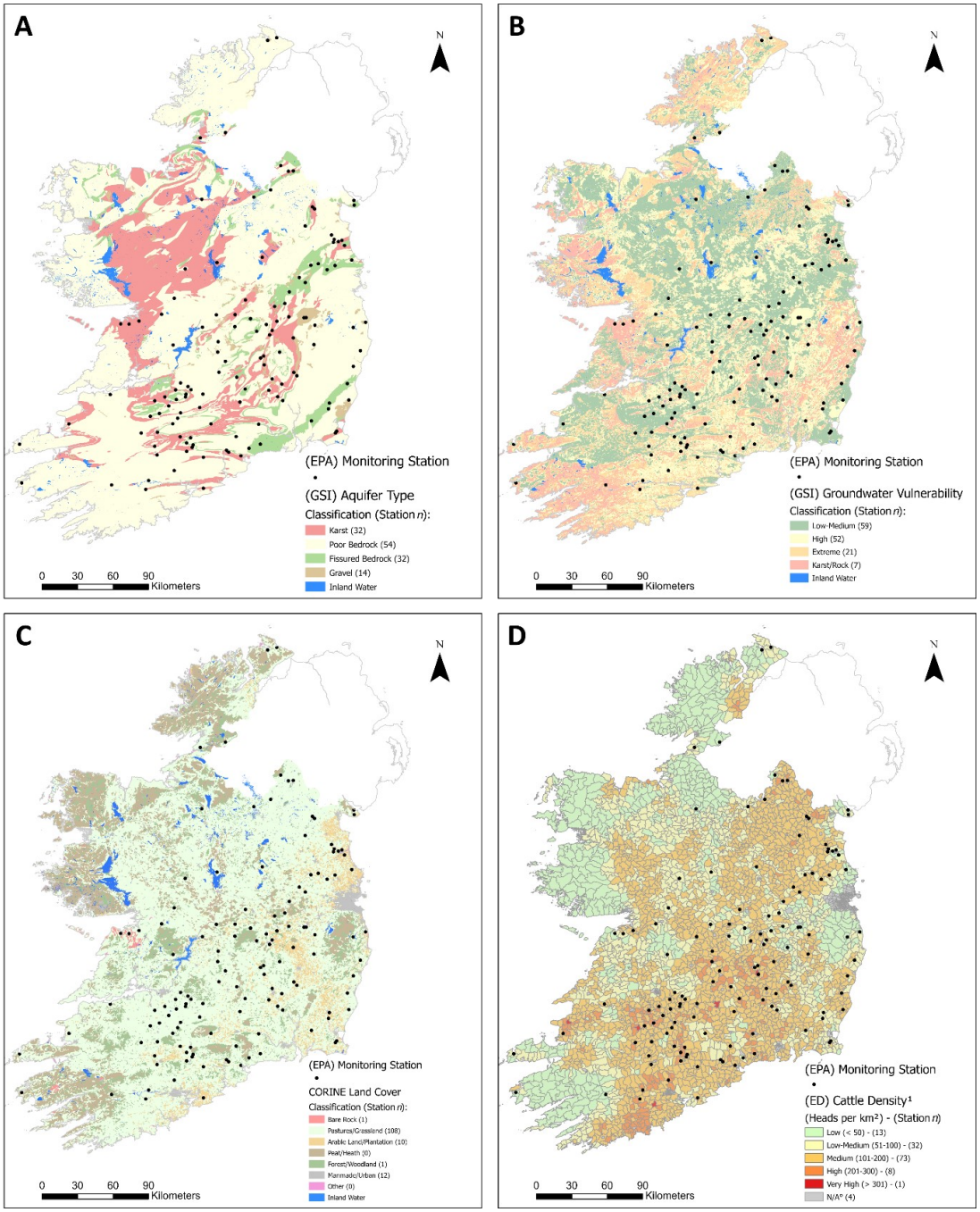
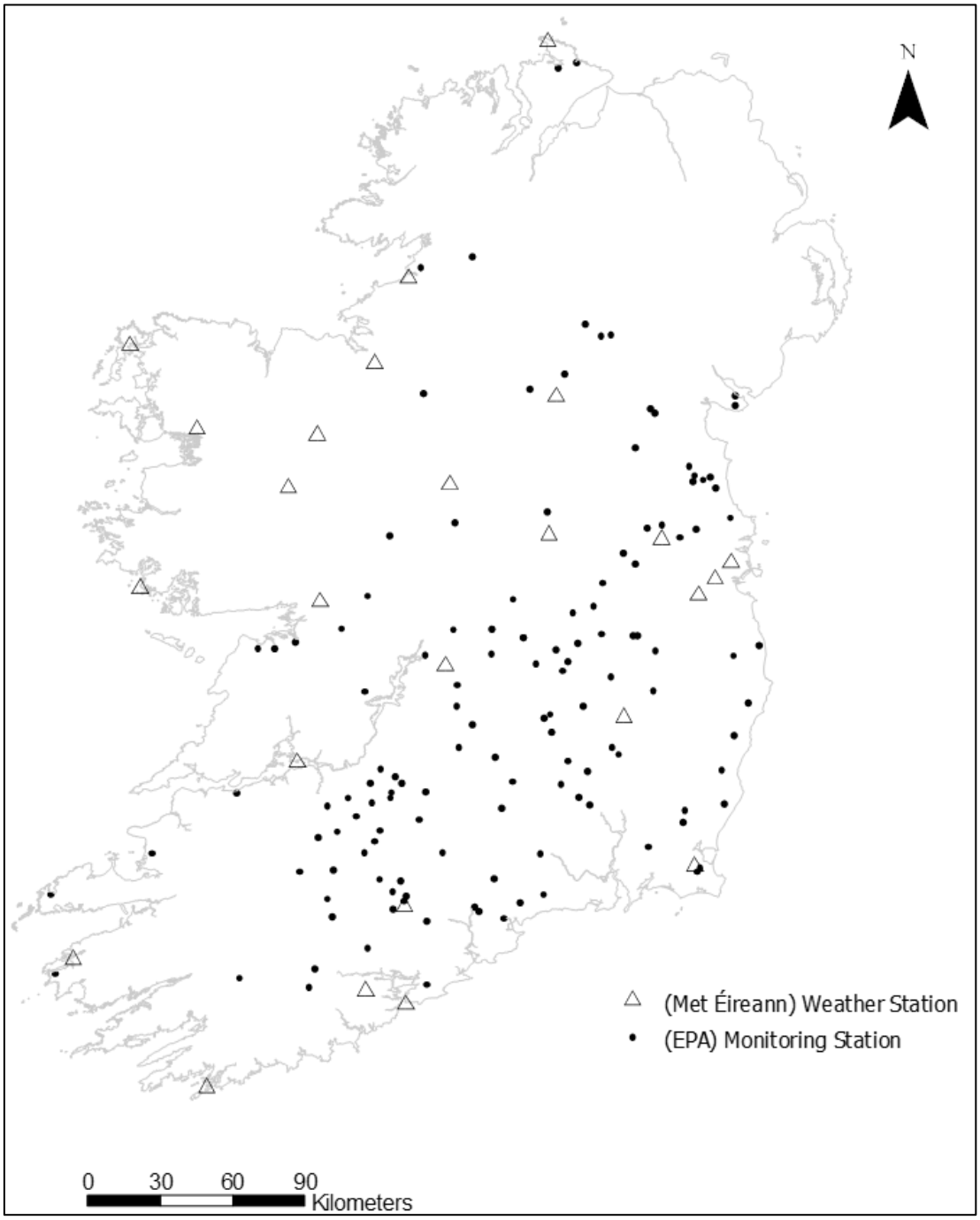


Figure 11: Republic of Ireland maps illustrating the location of groundwater monitoring stations modelled in relation to hydrogeological, land-use and agricultural variables. The number of monitoring stations falling into distinct categories are also provided in each map. (A) Map of selected GSI aquifer types. (B) Map illustrating GSI groundwater vulnerability estimates. (C) Simplified map of CORINE Land Cover categories. (D) Map incorporating CSO (ED) cattle density – ° Livestock data not available at the ED level, ¹ estimates based on total cattle numbers reported at ED level

Map showing the location of monitored groundwater suppliesin relation to weather stations



Chapter 4

The antimicrobial resistance profiles of *Escherichia coli* and *Pseudomonas aeruginosa* isolated from private groundwater wells in the Republic of Ireland

Preface

Title: The antimicrobial resistance profiles of *Escherichia coli* and *Pseudomonas aeruginosa* isolated from private groundwater wells in the Republic of Ireland

Authors: **Luisa Andrade**, Carlos Chique, Paul Hynds, John Weatherill, and Jean O'Dwyer

Journal: Environmental Pollution

Status: Published

LA's (CRediT) contribution: Conceptualization, Methodology, Investigation, Formal analysis, Data Visualisation, Writing - original draft, Writing – review & editing

Relevance: This paper examines current rates of antimicrobial resistant bacteria (*E. coli* and *P. aeruginosa*) and key hydrogeological, meteorological, anthropogenic and/or infrastructural factors associated with their presence in 132 private wells in the Republic of Ireland. The multivariate models produced reveal that cattle farming and direct wellhead ingress are likely the predominant source and ingress mechanism, respectively, associated with the presence of antimicrobial resistant bacteria within rural supplies in the country.

Significance: The current study was just the second to investigate the occurrence of antimicrobial resistant bacteria in groundwater sources in Ireland, and the first to incorporate repeat sampling and the analysis of two bacterial species. To the author's knowledge, this is also the first study to investigate the presence of *P. aeruginosa* in Irish groundwater samples. Overall, resistance was found in 16.3% of *E. coli* and none of the *P. aeruginosa*, resulting in 6.1% of the studied wells (primarily used for human consumption) harbouring antimicrobial resistant bacteria at least once.

Abstract

The role of the natural environment in the dissemination of antimicrobial resistant bacteria has been increasingly recognised in the literature. However, knowledge surrounding the critical factors and mechanisms mediating their occurrence is still limited, particularly in presumably *pristine* groundwater environments. In the Republic of Ireland (RoI), a country characterised by high groundwater reliance, household-based (unregulated) wells provide drinking water to 11% of the population and are generally located in rural areas, where the risk of microbiological contamination is high. Indeed these private supplies are highly susceptible to widespread use of manure in agricultural practices and high reliance on domestic wastewater treatment systems; both of which are also potential sources of antimicrobials and antimicrobial resistant bacteria. Accordingly, the current research sought to elucidate current rates of antimicrobial resistant bacteria and the principal factors associated with their presence in private wells in the RoI. A total of 250 samples (from 132 wells nationwide) were assessed for the presence of faecal (*Escherichia coli*) and environmental (*Pseudomonas aeruginosa*) bacteria, detected in 21.6 and 4.8% of samples, respectively. Single isolates from each contaminated sample were then tested phenotypically against antimicrobials used in human and veterinary medicine. Findings show that while 16.7% of *E. coli* (n = 8/48) were categorically resistant to ≥ 1 antimicrobial, with a further 79.2% classified as intermediately resistant, no categorical resistance was found among *P. aeruginosa* isolates (n = 0/6), with just one intermediately-resistant isolate detected. Multivariate regression modelling indicates significantly higher odds of resistant *E. coli* detection in concurrence with elevated cattle density (OR = 1.028, p = 0.032),

aligning with findings of highest resistance rates to veterinary antimicrobials (e.g., streptomycin = 14.6%, tetracycline = 12.5%, and ampicillin = 12.5%). Multivariate model results also suggest overland flow culminating in direct wellhead ingress as a primary ingress mechanism for resistant *E. coli*. Study findings may inform groundwater source protection initiatives and antimicrobial resistance surveillance moving forward.

Highlights

- 132 private groundwater wells in rural Ireland sampled/assessed in 2019 and 2021.
- *E. coli* and *P. aeruginosa* detected in 21.6% and 4.8% of samples, respectively.
- Antimicrobial resistance present in 16.7% of *E. coli* and none of the *P. aeruginosa*.
- Resistant *E. coli* associated with veterinary antimicrobials and cattle density.
- Direct wellhead ingress viewed as principal contamination mechanism for resistant *E. coli*.

Keywords: Antimicrobial Resistance, Groundwater, Private Wells, *Escherichia coli*, *Pseudomonas aeruginosa*

4.1 Introduction

The global public health burden of antimicrobial resistance and its rapid dissemination has been extensively recognised in recent decades, with evidence now available of resistance genes and/or resistant bacteria presence in non-clinical environments, including but not limited to, aquatic systems (Belding and Boopathy,

2018; Hooban et al., 2020; 2022), rural and urban soils (Montealegre et al., 2018; Zhao et al., 2019), food crops (Cerqueira et al., 2019), and uncultivated vegetation (Obermeier et al., 2021). The colonisation of these diverse ecosystems with antimicrobial resistant bacteria highlights their widespread incidence and are concurrent with increased cases of more severe, prolonged, and/or fatal infections, which may previously have been easily treatable (Opatowski et al., 2019; Center for Disease Control, 2019). Recent surveillance reports in the United States and Europe, for example, indicate that annually, over 35,000 and 33,000 deaths, respectively, are attributable to antimicrobial-resistant infections (Cassini et al., 2019; Center for Disease Control, 2019). The problem related to antimicrobial resistance stems from the rapid spread of resistance caused by widespread misuse and overuse of antimicrobials (Huttner et al., 2013), which has not been matched by ongoing development of antimicrobial drugs with new modes of action (Miethke et al., 2021). Indeed, the World Health Organization (WHO) recently reported that of the eleven antibiotics approved in the United States and Europe since 2017, just two represent novel classes of antimicrobial drugs (World Health Organization, 2020).

Recently, a global systematic review highlighted the role of groundwater as an important reservoir for antimicrobial resistant bacteria (Andrade et al., 2020), despite studies from as early as 1976 reporting the issue (Cooke, 1976). Widely believed to be relatively *pristine* environments, groundwater resources have the potential, when contaminated, to act as both a source and pathway for antimicrobial resistance amongst bacterial populations (Sanderson et al., 2018) while also providing a direct exposure route to humans (Coleman et al., 2012). In the groundwater context, the risk of human exposure is particularly relevant, as over 2.2 billion people worldwide

rely on groundwater wells for domestic use and as principal source of drinking water (Murphy et al., 2017). Additionally, indirect exposed due to the extensive use of groundwater resources in agricultural irrigation is increasing (Siebert et al., 2010). Moreover, small-scale groundwater abstractions remain widely unregulated causing many sources to lack appropriate treatment and maintenance (Murphy et al., 2017; McDowell et al., 2020), and thus, be at a high risk of microbiological contamination (Hynds et al., 2014; Lee and Murphy, 2020; Mapili et al., 2020). These groundwater resources also present a favourable and sheltered environment for harbouring diverse microbiological contaminants (including resistant bacteria) as well as pollutants which may pose co-selective pressures for acquisition of antimicrobial resistance (e.g., trace metals, biocides, and disinfectants) (Wu et al., 2018; Zhang et al., 2018). Because of the significant global burden of infection associated with groundwater resources (Murphy et al., 2017), the increased risks of therapeutic failure in the case of antimicrobial resistant groundwater-borne infections represent a direct and substantial threat to public health. Acute gastrointestinal infection associated with groundwater contamination, for example, are estimated to surpass 35.2 million cases per year globally (Murphy et al., 2017). Outbreaks of infection due to consumption of contaminated groundwater resources are also well documented (He et al., 2009; Eurien et al., 2021). Despite this, limited knowledge is available on the external factors mediating the occurrence of antimicrobial resistant bacteria in groundwater resources, particularly in respect to local/regional weather, anthropogenic activities, local hydrogeology, and groundwater source types, thus preventing development of effective monitoring and mitigation strategies.

Accordingly, the current study employed a combination of hydrogeological fieldwork and sampling, microbiological analyses, and statistical modelling to identify: i) current rates of resistance among groundwater bacteria in rural settings, ii) key factors associated with the presence of resistant bacteria, and iii) potential origin(s) and ingress mechanism(s) associated with resistant bacteria occurrence (i.e., likely sources and pathways). For this, two bacteria of human significance and from separate primary habitats (i.e., faecal, and environmental) were assessed: *Escherichia coli*, a well-known (zoonotic and anthropogenic) faecal indicator organism commonly used in groundwater-based antimicrobial resistance investigations (Andrade et al., 2020), and Pseudomonas aeruginosa, an opportunistic pathogen that (unlike *E. coli*) can proliferate in soil and freshwater (Cabot et al., 2016). Notably, the Pseudomonas genus has been recently identified as the bacteria with highest rates of resistance and intermediate resistance in global groundwater resources (Andrade et al., 2020). In this context, potential loss of an already limited number of treatment options for *P. aeruginosa*-caused infections (Lister et al., 2009) is particularly worrisome, as *P. aeruginosa* infections have the highest fatality rates of all gram-negative pathogens (Aliaga et al., 2002). Overall, the choice of bacterial species was principally predicated on the potential to gain insights into differing resistance origins and transport pathways in groundwater supplies, as well as to obtain a clear picture of local and source-specific resistomes, following key recommendations by Andrade et al. (2020). The authors consider that the RoI represents an appropriate case study for transferability of findings as a region characterised by diverse hydrogeology, intensive agricultural practices, a dispersed rural population (≈ 27 people/km^2; Central Statistics Office, 2016a) with a high reliance on private groundwater systems

(Central Statistics Office, 2016b), and a temperate climate without a distinct dry season (Peel et al., 2007). Hence, study findings provide an important baseline for quantifying antimicrobial resistant bacteria in groundwater resources, particularly in comparison with other more *exposed* environments such as surface water and soil ecosystems, or locations near health-care facilities and/or densely populated areas. With the ever-increasing threat of antimicrobial resistance, the results of this study provide an important steppingstone to assist in informing groundwater source protection initiatives to safeguard human health and support policy in antimicrobial resistance Action Plans.

4.2 Methods

4.2.1 Study area

The study was undertaken in rural settings in the RoI, a region of high groundwater reliance with over 180,000 private supplies in operation for domestic and industrial/agricultural use in 2019 (EPA, 2021a). Private groundwater abstractions for domestic use in the RoI comprise community systems (i.e., Private Group Water Schemes) which are regulated by the relevant local authorities, and small-scale household wells yielding under 10 m^3 of water per day (i.e., private wells) which are exempt from State regulations (EPA, 2013) in accordance with the European Union (EU) drinking water directive (Council Directive 2020/2184, 2020). Overall, private wells provide drinking water to approximately 11% of the Irish population (Department of Housing Local Government and Heritage, 2021) located in rural settlements (Central Statistics Office, 2016a).

The bedrock geology of the RoI is diverse (**Figure 4.1**), primarily comprised of sedimentary bedrock formations (e.g., limestone, sandstone, shale) (Geological Survey Ireland, 2017). Hydrogeologically, most aquifers used for groundwater abstraction in the RoI are unconfined (i.e., not overlain by impermeable rock or clay and having the water table as upper boundary), with groundwater vulnerability classifications (i.e., the categorisation of the theoretical risk of groundwater contamination via recharge; Department of the Environment and Local Government/Environmental Protection Agency/Geological Survey of Ireland, 1999) ranging from 'low' to 'extreme' (**Figure 4.1**). Briefly, groundwater vulnerability classifications are based on (i) depth to bedrock, (ii) subsoil permeability and, (iii) thickness/characterisation of the unsaturated zone (for sand and gravel aquifers) (Department of the Environment and Local Government/Environmental Protection Agency/Geological Survey of Ireland, 1999). Groundwater vulnerability is highest in karst limestone aquifers, where rapid groundwater flow through conduit, fissure and cave systems hinder the contaminant attenuation and subsurface retention which allow for microbial die-off. Rapid flow with limited attenuation is also associated with the interconnected fractures, fissures and, joints (i.e., fractured networks) common in Irish bedrock aquifers. The lowest groundwater vulnerability is typically seen where thick impermeable layers of clay soil and subsoil prevent contaminant percolation and recharge (Tynan, 2014).

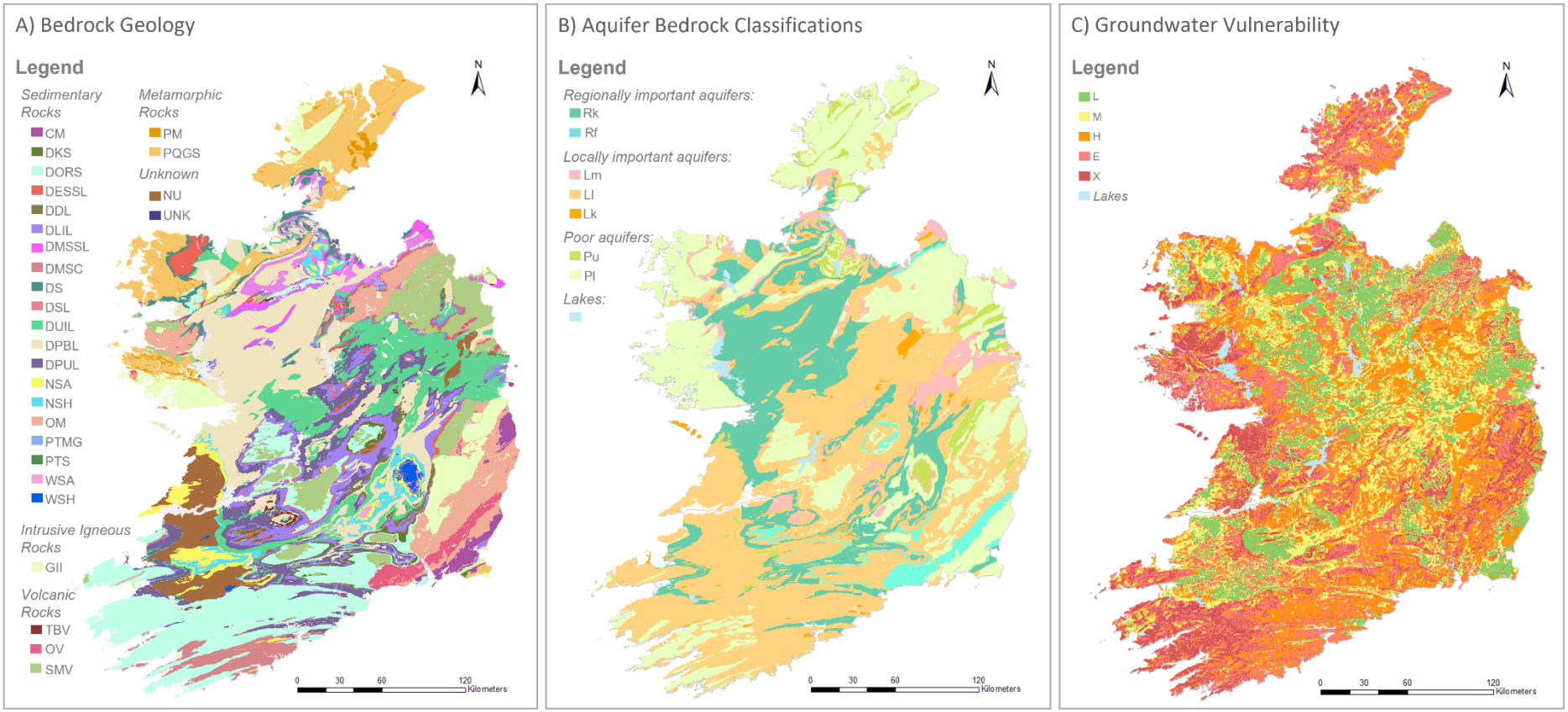


Figure 4.1: Republic of Ireland maps showing the geographical distribution of Bedrock Geology (A), Aquifer Bedrock (B), and Groundwater Vulnerability (C) classifications. *A) CM=Cambrian Metasediments, DKS=Devonian (Kiltorcan-type) Sandstones, DORS=Devonian (Old Red) Sandstones, DESSL=Dinantian (early) Sandstones, Shales and Limestones, DDL=Dinantian (Dolomitised) Limestones, DLIL=Dinantian (Lower Impure) Limestones, DMSSL=Dinantian Mixed Sandstones, Shales and Limestones, DMSC=Dinantian Mudstones and Sandstones, DS=Dinantian Sandstones, DSL=Dinantian Shales and Limestones, DUIL=Dinantian (Upper Impure) Limestones, DPBL=Dinantian (Pure Bedded) Limestones, DPUL=Dinasntian (Pure Unbedded) Limestones, NSA=Namurian Sandstones, NSH=Namurian Shales, OM=Ordovician Metasediments, PTMG=Permo-Triassic Mudstones and Gypsum, PTS=Permo-Triassic Sandstones, WSA=Westphalian Sandstones, WSH=Westphalian Shales, GII=Granites (and other Igneous Intrusive Rocks), TBV=Tertiary basalts (and other Volcanic Rocks), OV=Ordovician Volcanics, SMV=Silurian Metasediments and Volcanics, PM=Precambrian Marbles, PQGS=Precambrian Quartzites, Gneisses and Schists, NU=Namurian Undifferentiated, UNK=Unknown. B) Rk=Karstified, Rf=Fissured Bedrock, Lm=Generally Moderately Productive, Ll=Moderately Productive Only in Local Zones, Lk=Locally Important Karstified aquifer, Pu=Generally Unproductive Except for Local Zones, Pl=Generally Unproductive. C) L=Low , M=Moderate, H=High, E=Extreme, X=Extreme (Rock/Karst). Source: Geological Survey Ireland (2017)*

4.2.2 Sample collection

Private (household) wells were the focus of the current study, with supply selection and inclusion based on responses to a nationwide recruitment effort (e.g., via university mailing lists and social media campaigns). This was an exploratory study, with no specific focus based on bedrock geology, geographical location, supply infrastructure, etc. Samples were taken from household taps, generally located outside of the home and prior to any microbial treatment, during two distinct sample collection periods: Round 1 from September to November 2019, and Round 2 from June to August 2021. Samples were collected in 100 ml sterile vessels (IDEXX Laboratories Inc., Westbrook, Maine) after being allowed to run at high flow for a minimum of 60 s (≥ 2 L) to ensure the water did not come from the distribution system. After collection, samples were transported to the laboratory at 4°C for analysis within 6 h.

4.2.3 Laboratory analyses

Bacterial enumeration and isolation

The most probable number (MPN) of *E. coli* and *P. aeruginosa* in each water sample was determined via standard ISO approved (International Organization for Standardization, 1998) commercial culture kits (Colilert and Pseudalert, IDEXX Laboratories Inc., Westbrook, Maine). Briefly, following separate addition of the IDEXX reagents, samples were transferred to 51-well IDEXX quanti-trays, heat sealed, and incubated aerobically for 24 h at 37°C. The Colilert and Pseudalert reagents indicate *E. coli* and *P. aeruginosa* presence, respectively, through fluorescence under

a UV light source. The MPN for each sample was ascertained by counting the number of positive *wells* and referring to the respective IDEXX tables, an enumeration methodology with similar uncertainty (i.e., ± 20%) to other established methods such as genetic-based (e.g., quantitative polymerase chain reaction; qPCR) and membrane filtration techniques (Harmel et al., 2016).

A single *E. coli* or *P. aeruginosa*-positive sub-sample (i.e., *well*) was randomly selected from each bacterial-positive quanti-tray and recovered by pipetting 100 µL of sample onto a MacConkey agar plate and incubating it for 24 h at 37°C. Subsequently, single presumptive *E. coli* and *P. aeruginosa* colonies were isolated, with reference strains *E. coli* ATCC® 25,922 and *P. aeruginosa* ATCC® 25,924 (Oxoid, Basingstoke, Hampshire, UK) cultured in parallel, respectively, to aid in appropriate colony selection. In cases where sub-sample incubation did not return appropriate colonies, a different sub-sample was attempted, this process was repeated until no viable sub-samples were available.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion technique (Hudzicki, 2009). Commercially prepared discs and Müller-Hinton (MH) Agar (Oxoid, Basingstoke, Hampshire, UK) were used in accordance with Clinical and Laboratory Standards Institute (CLSI) standards (CLSI, 2018; 2020). Briefly, pure bacterial colonies were suspended in 0.85% Saline solution with turbidity adjusted to the 0.5 McFarland standard. Each inoculum was evenly distributed over the dried surface of 100 mm MH agar plates, this was followed by the placement of a maximum of six antimicrobial discs, and incubation at 37°C for 16–18 h (CLSI, 2020). Bacterial

growth around each disc (i.e., zone of inhibition diameters) were then measured and recorded.

The antimicrobials evaluated in the study are presented in **Table 4.1**. Antimicrobial usage classifications were based on approval status of these medications for human or veterinary in the RoI by the Health Products Regulatory Authority in Ireland (HPRA, 2020a; 2020b). Overall, *E. coli* and *P. aeruginosa* isolates were tested against panels of 18 and 9 antimicrobials, respectively. Breakpoints for the antimicrobial zones of inhibition were interpreted according to M100 CLSI guidelines (CLSI, 2020) for Enterobacterales and Pseudomonas aeruginosa (VET01S in the case of Enrofloxacin; CLSI 2018). Isolates were subsequently classified as Resistant (R), Intermediate (I) (i.e., clinically efficacious only where the drug is physiologically concentrated; CLSI, 2020), or Susceptible (S) to each antimicrobial. Quality control strains, namely *E. coli* ATCC® 25,922 and *P. aeruginosa* ATCC® 25,924 were used in concurrence with all *E. coli* and *P. aeruginosa* susceptibility analyses, respectively, in accordance with best practice and following CLSI (2020) guidelines.

Table 4.1: Antimicrobial agents included in the current study, their license status for human and veterinary use in the Republic of Ireland (RoI), and the organisms with established breakpoints in the Clinical and Laboratory Standards Institute (CLSI) guidelines

Antimicrobial	Use in human medicine in RoI [a]	Use in veterinary medicine in RoI [a]	Organisms with established breakpoints [b]
Penicillins			
Ampicillin (AMP, 10 µg)	License withdrawn in 2014	Currently in use	*E. coli*
Amoxicillin-Clavulanic acid (AMC, 20/10 µg)	Currently in use	Currently in use	*E. coli*
Piperacillin (PRL, 100 µg)	License withdrawn in 2002	Never licensed	*Both*
Piperacillin-Tazobactam (TZP, 100/10 µg)	Currently in use	Never licensed	*Both*
Cephalosporins			
Cefazolin (KZ, 30 µg)	License withdrawn in 1992	License withdrawn in 2005	*E. coli*
Ceftazidime (CAZ, 30 µg)	Currently in use	Never licensed	*Both*
Ceftiofur (EFT, 30 µg)	Never licensed	Currently in use	*E. coli*
Cefepime (FEP, 30 µg)	Currently in use	Never licensed	*Both*
Aminoglycosides			
Streptomycin (S, 10 µg)	License withdrawn in 1987	Currently in use	*E. coli*
Gentamicin (CN, 10 µg)	Currently in use	Currently in use	*Both*
Carbapenems			
Imipenem (IPM, 10 µg)	Currently in use	Never licensed	*Both*
Fluoroquinolones			
Ciprofloxacin (CIP, 5 µg)	Currently in use	Never licensed	*Both*
Enrofloxacin (ENR, 5 µg)	Never licensed	Currently in use	*Both*
Monobactams			
Aztreonam (ATM, 30 µg)	Currently in use	Never licensed	*Both*
Tetracyclines			
Tetracycline (TE, 30 µg)	Currently in use	Currently in use	*E. coli*
Folate Pathway Inhibitors			
Trimethoprim (W, 5 µg)	Currently in use	License withdrawn in 2001	*E. coli*
Trimethoprim-Sulfamethoxazole (SXT, 1.25/23.75 µg)	Currently in use	Currently in use	*E. coli*
Phenicols			
Chloramphenicol (C, 30 µg)	Currently in use	Never licensed	*E. coli*

[a] Medical and veterinary use and license status in the Republic of Ireland (RoI) according to HPRA (Health Products Regulatory Authority in Ireland) (HPRA, 2020a; 2020b); [b] Breakpoints available for *Enterobacteriaceae* spp. and/or *Pseudomonas aeruginosa* according to the CLSI (2018; 2020)

4.2.4 Environmental and infrastructural risk factors

The potential risk factors which could influence both bacterial presence and resistance profiles (viz. Hynds et al., 2012; Coleman et al., 2013; O'Dwyer et al., 2014; 2017) were collected on-site and via geo-referenced datasets. Individual risk factors

were categorised into (i) infrastructural, (ii) supply-/household-specific, and (iii) local parameters (**Table 4.2**). Geo-referenced spatial data available through the Geological Survey Ireland (GSI, 2017) and Central Statistics Office (CSO) Census of Ireland (CSO, 2012) were assigned to each supply using ESRI® ArcGIS Pro® version 2.9.1. based on supply-specific GPS coordinates. These included hydrogeological parameters, and the estimated density of anthropogenic activities/facilities within pre-delineated land sections of 80–120 dwellings, known as Small Areas (SA) (CSO, 2016b). In the case of agricultural data, GIS tools were employed to *aggregate* the 'coarser' resolution data (available only at Electoral Division level; mean area = 20.6 km^2) into the SA level (mean area = 3.8 km^2), while adjusting it to the spatial dimensions of representative land-use types (e.g., pastures; CORINE, 2018) rather than employing an all-inclusive land-use approach (e.g., inland waters, urban areas).

The 5-, 10-, 30- and 60-day (cumulative) antecedent rainfall values (in mm) were calculated by adding daily rainfall totals for the relevant number of days up to sample collection, which were recorded at the nearest synoptic station; historical rainfall data were provided by Met Eireann (the Irish National Meteorological Service; Met Eireann, 2021). Site surveys were carried out immediately after sample collection and covered a 100 m radius surrounding the well. These included determining distances and gradient differences between supplies and the nearest livestock and domestic wastewater treatment system (DWWTS - e.g., septic tank) and measuring the casing clearance (i.e., the distance from the well chamber floor to the top of the casing; see **Supplementary Data**). The Irish Environmental Protection Agency (EPA, 2020) recommends minimum setback distances to livestock grazing areas and DWWTSs of 3 and 30 m, respectively, to theoretically allow time/space for

sufficient contaminant attenuation, and the Institute of Geologists of Ireland (Institute of Geologists of Ireland, 2007) established a minimum casing clearance of 150 mm to prevent direct contaminant ingress through the wellhead; these thresholds were therefore used in the current study. Information of well depth and antimicrobial use within the household were acquired via well-owner questionnaire (provided in **Supplementary Data**).

Table 4.2: Environmental and infrastructural parameters which may influence resistance profiles (risk factors) and corresponding data origin

Measured Risk Factors	Data origin
Environmental parameters I (local)	
Groundwater vulnerability (low/moderate/high/extreme/extreme-rock/karst)	GSI Mapping
Aquifer importance (poor/locally important/regionally important)	GSI Mapping
Aquifer type (karst/gravel/fractured bedrock/poorly productive bedrock)	GSI Mapping
Karst aquifer (yes/no)	GSI Mapping
Sheep density at the Small Area [a] level (heads/km^2)	CSO
Cattle density at the Small Area [a] level (heads/km^2)	CSO
Livestock density at the Small Area [a] level (heads/km^2)	CSO
DWWTS density at the Small Area [a] level (number/km^2)	CSO
5-day rainfall (mm)	Met Éireann
10-day rainfall (mm)	Met Éireann
30-day rainfall (mm)	Met Éireann
60-day rainfall (mm)	Met Éireann
Environmental parameters II (supply- and household-specific)	
Livestock within 3 m of the well [a] (yes/no)	On-site measurement
Gradient difference to nearest livestock (below/same/above the well)	On-site measurement
DWWTS within 30 m of the well [a] (yes/no)	On-site measurement
Gradient difference to nearest DWWTS (below/same/above the well)	On-site measurement
Antimicrobials used in the household in the last 3 months (yes/no)	Well-owner questionnaire
Antimicrobials used in the household in the last 6 months (yes/no)	Well-owner questionnaire
Infrastructural parameters	
Supply type (drilled/hand-dug)	On-site survey
Supply depth (m)	On-site measurement
Presence of sealed cap (yes/no)	On-site survey
Presence of protective casing (yes/no)	On-site survey
Wellhead cracks present (yes/no)	On-site survey
Casing clearance of at least 150 mm [c] (yes/no)	On-site measurement

DWWTS=Domestic Wastewater Treatment System; [a] Small Area refers to areas comprising between 80 and 120 dwellings (CSO, 2016b); [b] 3 and 30 m are the minimum recommended distances between a water well and the

nearest livestock grazing area and DWWTS in the RoI, respectively (EPA, 2021b); [c] 150 mm is the minimum recommended casing clearance for water wells in the RoI (IGI, 2007, Bacci and Chapman, 2011)

4.2.5 Statistical analyses

Potential risk factors (**Table 4.2**) associations with bacterial detection (i.e., *E. coli* and *P. aeruginosa* > 0 MPN/100 ml, separately) and presence of categorically resistant bacteria in groundwater samples were preliminary assessed using bivariate statistical techniques. Chi-Square (X^2) tests were used to identify associations between categorical independent variables and each categorical (binary) outcome, and Mann–Whitney U tests were used in the case of continuous independent variables (e.g., antecedent rainfall, supply depth, and sheep, cattle and DWWTS densities).

Bacterial detection (*E. coli* and *P. aeruginosa*) and presence of resistant bacteria outcomes were further analysed using multivariate binary logistic regression; independence between repeat samples was assumed, owing to the large period between sampling campaigns (i.e., over 18 months between Rounds 1 and 2) and the dynamic nature of microbiological groundwater contamination (Andrade et al., 2022). Independent variables (**Table 4.2**) with strong inter-variable correlations (i.e., Spearman's |rho| ≥ 0.5, $p < 0.05$) were included separately, with the best fitting model selected based on the Nagelkerke R^2 (i.e., the proportion of outcome variation explained by the independent variables). Collinearity was found between "aquifer importance" and "aquifer type" variables, "livestock density" and both "cattle density" and "sheep density" variables, and for all combinations of antecedent rainfall periods (i.e., 5-, 10-, 30- and 60-day rainfall). Six variables: "gradient difference to nearest livestock", "gradient differences to nearest DWWTS", "supply

depth", "presence of protective casing", "antimicrobials used in last 3 months", and "antimicrobials used in last 6 months" were excluded from regression models due to missing data in > 10% of observations, with sensitivity analyses indicating that their inclusion did not result in meaningful changes to model accuracy. All independent variables which fitted inclusion criteria were simultaneously entered into the models (i.e., "forced entry" method) and retained regardless of statistical significance. The *second best-fit* models identified are included in **Supplementary Data**. All statistical analyses were undertaken using IBM SPSS version 28.

4.3 Results

4.3.1 Supply characteristics

A total of 132 private wells located in 21 of the 26 administrative counties in the RoI, were sampled in the current study. Of those, 132 were sampled and analysed from September to November 2019 (Round 1) and 118 were re-sampled from June to August 2021 (Round 2). Summary statistics describing these wells and associated samples are presented in **Table 4.3**. Most wells in the current study were drilled (88.6%; n = 117), with a mean overall supply depth of 40.9 ± 27.7 m and a mean casing clearance of 155 ± 224 mm, where recorded (75.0 and 95.4% of wells, respectively). Overall, 26.4% (n = 48) of wells were located in areas classified as having "high" groundwater vulnerability, followed by "extreme" (27.3%; n = 36), "moderate" (12.9%; n = 17), "extreme-rock/karst" (12.1%; n = 16) and "low" (11.4%; n = 15) classifications; with 81.8% (n = 108) of wells in areas of poorly productive bedrock aquifers, 12.9% (n = 17) in karst, and just 3% (n = 4) and 2.3% (n = 3) in fractured bedrock and unconsolidated (glaciofluvial) aquifers, respectively. In total, 14.4% (n =

19) of wells were located within 30 m of the nearest DWWTS and 18.9% (n = 25) were situated within 3 m of livestock. The calculated mean ± standard deviation DWWTS density at the Small Area level was 8 ± 5 units/km^2, with mean cattle and sheep densities of 161 ± 59 and 95 ± 137 heads/km^2, respectively. Of the 132 well owners in the study, 84.8% (n = 112) confirmed that the well water was used for household consumption. Antimicrobials were used in 17.2% (n = 43) and 30.8% (n = 77) of households in the three and six months prior to sample collection, respectively.

Table 4.3: Environmental and infrastructural characteristics of the 132 wells (and 250 samples) in the current study

Characteristic	Wells, n (%)	Samples, n (%)	Characteristic	Wells, n (%)	Samples, n (%)
Groundwater vulnerability			**Supply type**		
Low	15 (11.4)	30 (12.0)	Drilled	117 (88.6)	223 (89.2)
Moderate	17 (12.9)	34 (13.6)	Hand-dug	15 (11.4)	27 (10.8)
High	48 (36.4)	90 (36.0)			
Extreme	36 (27.3)	65 (26.0)	**Supply depth**		
Extreme-rock/karst	16 (12.1)	31 (12.4)	≤10 m	15 (11.4)	27 (10.8)
			11-30 m	30 (22.7)	59 (23.6)
Aquifer importance			31-60 m	38 (28.8)	76 (30.4)
Poorly	37 (28.0)	72 (28.8)	61-90 m	9 (6.8)	18 (7.2)
Locally	76 (57.6)	142 (56.8)	> 90 m	7 (5.3)	14 (5.6)
Regionally	19 (14.4)	36 (14.4)	Not recorded	33 (25.0)	56 (22.4)
Aquifer type			**Sealed cap**		
Poorly productive bedrock	108 (81.8)	204 (81.6)	Yes	46 (34.8)	89 (35.6)
Karst	17 (12.9)	33 (13.2)	No	79 (59.8)	148 (59.2)
Fractured bedrock	4 (3.0)	7 (2.8)	Not recorded	7 (5.3)	13 (5.2)
Gravel	3 (2.3)	6 (2.4)			
			Protective PVC casing		
Sheep density [a]			Yes	32 (24.2)	61 (24.4)
≤50 head/km^2	71 (53.8)	134 (53.6)	No	75 (56.8)	142 (56.8)
51-100 head/km^2	19 (14.4)	35 (14.0)	Not recorded	25 (18.9)	47 (18.8)
101-200 head/km^2	22 (16.7)	43 (17.2)			
201-300 head/km^2	8 (6.1)	15 (6.0)	**Wellhead cracking**		
> 300 head/km^2	12 (9.1)	23 (9.2)	Yes	10 (7.6)	18 (7.2)
			No	118 (89.4)	224 (89.6)
Cattle density [a]			Not recorded	4 (3.0)	8 (3.2)
≤50 head/km^2	8 (6.1)	15 (6.0)			
51-100 head/km^2	10 (7.6)	19 (7.6)	**Casing clearance**		
101-200 head/km^2	83 (62.9)	159 (63.6)	<150 mm	80 (60.6)	149 (59.6)
201-300 head/km^2	27 (20.5)	49 (19.6)	≥150 mm	46 (34.8)	89 (35.6)
> 300 head/km^2	4 (3.0)	8 (3.2)	Not recorded	6 (4.5)	12 (4.8)
DWWTS density [a]			**Water use in the household**		
≤5 units/km^2	42 (31.8)	79 (31.6)	Drinking	112 (84.9)	216 (86.4)
6-15 units/km^2	83 (62.9)	159 (63.6)	Other	14 (10.6)	25 (10.0)
> 15 units/km^2	7 (5.3)	12 (4.8)	Not recorded	6 (4.5)	9 (3.6)
Nearest livestock			**Antimicrobials used in last 3 months**		
≤3 m	25 (18.9)	48 (19.2)	Yes	-	43 (17.2)
4-100 m	92 (69.7)	172 (68.8)	No	-	177 (70.8)
> 100 m	15 (11.4)	30 (12.0)	Not recorded	-	30 (12.0)
Nearest DWWTS			**Antimicrobials used in last 6 months**		
≤30 m	19 (14.4)	35 (14.0)	Yes	-	77 (30.8)
31-100 m	100 (75.8)	190 (76.0)	No	-	143 (57.2)
> 100 m	13 (9.8)	25 (10.0)	Not recorded	-	30 (12.0)

DWWTS=Domestic Wastewater Treatment System

4.3.2 E. coli and P. aeruginosa presence

E. coli was detected in 21.6% of groundwater samples (n = 54; Round 1 = 26.5% and Round 2 = 16.1%) and 33.3% of wells (n = 44) at least once, while *P. aeruginosa*

presence was limited to 4.8% of samples (n = 12; Round 1 = 6.1% and Round 2 = 3.4%) and 8.3% of wells (n = 11). Instances of re-occurring *E. coli* and *P. aeruginosa* contamination (i.e., a single species detected in the same site more than once) were found in 9.8% (n = 13) and 0.7% (n = 1) of wells, respectively, and co-occurrence (i.e., both species recovered from the same site) was identified in 6.8% wells (n = 9). As shown in **Figure 4.2**, bacterial contamination was geographically spread across the RoI, being identified in 17 of the 21 counties. Such instances of contamination were immediately reported to supply owners in concurrence with recommendations on how to safeguard their health, such as with the installation of microbiological treatments (e.g., ultra-violet and reverse osmosis treatment systems). Regarding local hydrogeology, noteworthy findings include an absence of *P. aeruginosa* contamination in areas of "low" groundwater vulnerability, with highest incidence rates found in "extreme" vulnerability areas (17.2%; n = 5 samples). Conversely, similar *E. coli* contamination rates were found across all groundwater vulnerability classifications, ranging from 21.2 to 26.3%. The independent parameters significantly associated with *P. aeruginosa*, and *E. coli* detection (as well as presence of resistant bacteria) are presented in the **Supplementary Data** (i.e., bivariate analyses results).

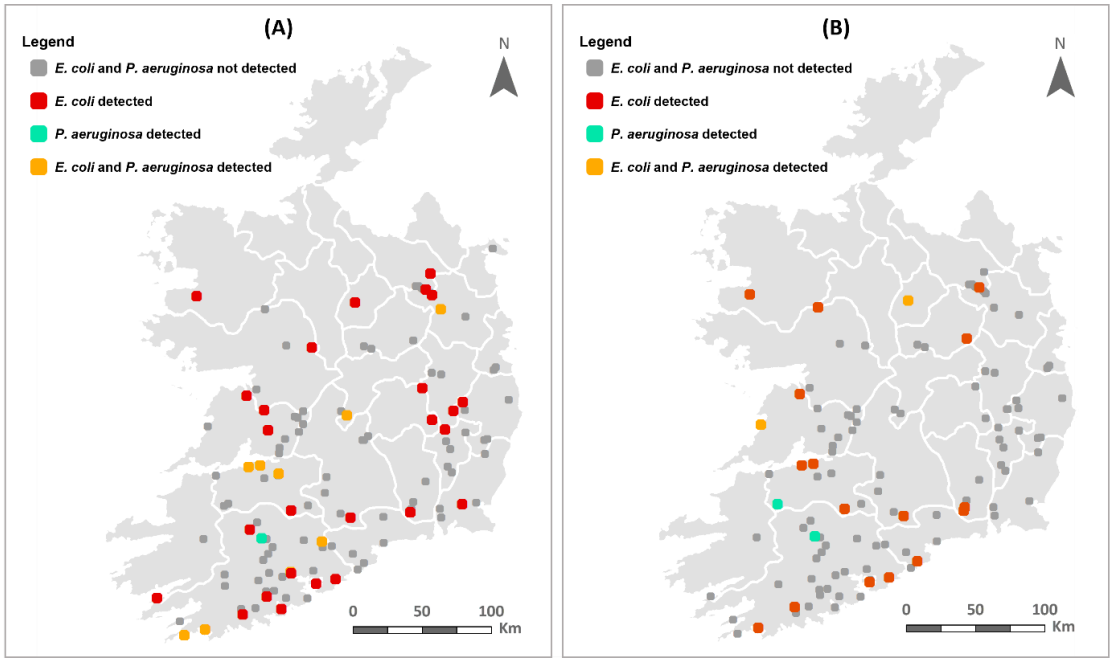


Figure 4.2: Republic of Ireland maps showing the geographical distribution of sampled wells and contamination status in Round 1 (autumn 2019; A), and Round 2 (summer 2021; B)

4.3.3 Antimicrobial resistance profiles

The phenotypic susceptibility results for 48 *E. coli* isolates (each from separate wells) are shown in **Table 4.4**, while those for *P. aeruginosa* isolates (n = 6) are described in text. *E. coli* and *P. aeruginosa* isolates could not be recovered from six Round 1 samples and were not included for further analyses. Consequently, just three resistance profiles were obtainable for co-occurring *E. coli* and *P. aeruginosa* isolates (i.e., detected in the same samples), with no resistance found in either species. As shown in **Table 4.4**, four *E. coli* isolates from each Round were resistant to one or more antimicrobials, resulting in a total resistance rate of 16.7% (n = 8), with 12.5% of isolates (n = 6; three from each Round) being resistant to ≥ 3 antimicrobials (i.e., multidrug-resistant) and a further 79.2% (n = 38) being classified as intermediately resistant. Conversely, none of the recovered *P. aeruginosa* isolates were categorically resistant to the tested antimicrobials. Thus, 3.2% (n = 8) of groundwater samples and

6.1% (n = 8) of wells harboured antimicrobial resistant bacteria (i.e., *E. coli*), with no repeated instances of resistant *E. coli* contamination.

E. coli isolates exhibited highest levels of resistance to streptomycin, with a total resistance rate of 14.6% (n = 7); intermediate classifications were also most prevalent in relation to this antimicrobial, found in a further 81.3% (n = 39) of the isolates tested. The second highest resistance rates were to tetracycline and ampicillin, both corresponding to 12.5% of *E. coli* isolates (n = 6), followed by 10.4% (n = 5) to piperacillin; an additional 8.3% (n = 4) and 4.2% (n = 2) of isolates were intermediately-resistant to ampicillin and piperacillin, respectively. Resistance was also found to chloramphenicol (4.2%; n = 2), trimethoprim (2.1%; n = 1), and trimethoprim-sulfamethoxazole (2.1%; n = 1). It is worth noting that despite no resistance found to cephazolin, 54.2% of *E. coli* isolates (n = 26) were intermediately-resistant to it. Moreover, intermediate resistance was found to amoxicillin-clavulanic acid (10.4%; n = 5), ciprofloxacin (6.3%; n = 3), enrofloxacin (6.3%; n = 3), cefepime (4.2%; n = 2), and ceftiofur (2.1%; n = 1). All groundwater-derived *E. coli* isolates were susceptible to imipenem, piperacillin-tazobactam, ceftazidime, gentamicin, or aztreonam. In the case of *P. aeruginosa*, one Round 2 isolate exhibited intermediate resistance to enrofloxacin.

Table 4.4: Susceptibility results for 48 groundwater-derived E. coli isolates collected in autumn 2019 (Round 1, n = 29) and summer 2021 (Round 2, n = 19)

Antimicrobial	Current medical use in RoI [a]	Round 1 (n = 29)		Round 2 (n = 19)		Total (n = 48)	
		R isolates No. (%)	I isolates No. (%)	R isolates No. (%)	I isolates No. (%)	R isolates No. (%)	I isolates No. (%)
Penicillins							
Ampicillin	Veterinary	3 (10.3)	2 (6.9)	3 (15.8)	2 (10.5)	6 (12.5)	4 (8.3)
Amoxicillin-Clavulanic acid	Both	0	2 (6.9)	0	3 (15.8)	0	5 (10.4)
Piperacillin	Human*	3 (10.3)	1 (3.4)	2 (10.5)	1 (5.3)	5 (10.4)	2 (4.2)
Piperacillin-Tazobactam	Human	0	0	0	0	0	0
Cephalosporins							
Cefazolin	Both*	0	15 (51.7)	0	11 (57.9)	0	26 (54.2)
Ceftazidime	Human	0	0	0	0	0	0
Ceftiofur	Veterinary	0	1 (3.4)	0	0	0	1 (2.1)
Cefepime	Human	0	2 (6.9)	0	0	0	2 (4.2)
Aminoglycosides							
Streptomycin	Veterinary	4 (13.8)	23 (79.3)	3 (15.8)	16 (84.2)	7 (14.6)	39 (81.3)
Gentamicin	Both	0	0	0	0	0	0
Carbapenems							
Imipenem	Human	0	0	0	0	0	0
Fluoroquinolones							
Ciprofloxacin	Human	0	2 (6.9)	0	1 (5.3)	0	3 (6.3)
Enrofloxacin	Veterinary	0	1 (3.4)	0	2 (10.5)	0	3 (6.3)
Monobactams							
Aztreonam	Human	0	0	0	0	0	0
Tetracyclines							
Tetracycline	Both	3 (10.3)	0	3 (15.8)	0	6 (12.5)	0
Folate Pathway Inhibitors							
Trimethoprim	Human	1 (3.4)	0	0	0	1 (2.1)	0
Trimethoprim-Sulfamethoxazole	Both	1 (3.4)	0	0	0	1 (2.1)	0
Phenols							
Chloramphenicol	Human	1 (3.4)	0	1 (5.3)	0	2 (4.2)	0
Total		4 (13.8)	22 (75.9)	4 (21.1)	15 (78.9)	8 (16.7)	38 (79.2)

[a] Use and license status for human and veterinary medicine in the RoI according to the Health Products Regulatory Authority in Ireland (HPRA, 2020a; 2020b); * No longer licensed for medical use in the RoI (HPRA, 2020a: 2020b); RoI = Republic of Ireland; R = Resistant based on Clinical and Laboratory Standards Institute (CLSI) Zone Diameter breakpoints (CLSI 2018; 2020); I = Intermediate based on CLSI's Zone Diameter breakpoints (CLSI 2018; 2020)

4.3.4 Multivariate logistic regression modelling

Binary logistic regression models assessing the detection of (i) *P. aeruginosa*, (ii) *E. coli*, (iii) and resistant *E. coli* are presented in **Table 4.5**, with Nagelkerke R^2 of 0.504, 0.285, and 0.475, respectively. *Second best-fit* models are presented in the Supplementary Data. Overall, the *P. aeruginosa* model showed no significant associations with hydrogeological or agricultural parameters, despite lower sheep density at the Small Area level approaching significance (at the 90% confidence level) with higher odds of *P. aeruginosa* presence (OR = 0.959, p = 0.056). Environmental risk factors significantly associated with higher odds of *P. aeruginosa* detection included presence of DWWTS within 30 m of the well (OR = 14.046, p = 0.026) and (above mean) increases to 5-day rainfall (OR = 1.069, p = 0.005). Hand-dug wells were 19-times more likely to harbour *P. aeruginosa* than drilled wells (OR = 18.902, p = 0.020), and the presence of wellhead cracking led to a one-hundred-fold increase in the odds of detection (OR = 100.256, p = 0.009). The *second best-fit P. aeruginosa* model, where 5 and 10-day rainfall parameters were interchanged, displayed the same significant patterns, while also signalling 10-day rainfall increases as being significantly associated with higher odds of *P. aeruginosa* presence (OR = 1.036, p = 0.005; see **Supplementary Data**).

In the case of *E. coli*, the final model showed over three times higher odds of detection in karst aquifers (OR = 3.903, p = 0.009), with 1 mm (above mean) increases to 60-day rainfall also significantly associated with 8% higher odds of *E. coli* presence (OR = 1.008, p = 0.008). Conversely, the resistant *E. coli* model showed that detection odds were significantly higher in areas of "low" groundwater vulnerability than those

in “high” (OR = 333.333, p = 0.022), “extreme” (OR = 25.641, p = 0.049) and “extreme-rock/karst” (OR = 37.037, p = 0.041) groundwater vulnerability areas. Regarding potential contamination sources, significantly higher odds of *E. coli* presence were associated with DWWTS located within 30 m of the well (OR = 5.090, $p < 0.001$), and with higher cattle density at the Small Area level (OR = 1.010, p = 0.011); while just cattle density was significantly associated with higher odds of resistant *E. coli* presence (OR = 1.028, p = 0.037). The odds of *E. coli* and resistant *E. coli* detection were both significantly higher in hand-dug versus drilled wells (OR = 7.199 with $p < 0.001$ and OR = 41.071 with p = 0.050, respectively). Moreover, significance at the 90% confidence level indicated that absence of an appropriately sealed cap could more than double the odds of *E. coli* detection (OR = 2.212; p = 0.068) and lead to a six-fold increase in the odds of resistant *E. coli* detection (OR = 6.452, p = 0.098). The *second best-fit* models for *E. coli* and resistant *E. coli*, while emphasising the same significant parameters, also showed that 10-day antecedent rainfall increases were associated with significantly higher odds of *E. coli* detection (OR = 1.016, p = 0.008).

Table 4.5: Mmultivariate binary logistic regression models for presence/absence of (i) Pseudomonas aeruginosa, (ii) Escherichia coli, and (ii)* Resistant *Escherichia coli

Risk Factors	*P. aeruginosa* detection (n = 233)		*E.coli* detection (n = 233)		Resistant [a] *E.coli* detection (n = 227)	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Groundwater vulnerability						
Low	0.000 (0.000–.)	0.997	Reference		Reference	
Moderate	0.187 (0.010–3.460)	0.260	0.446 (0.094–2.109)	0.308	0.000 (0.000–.)	0.997
High	0.320 (0.028–3.657)	0.359	0.569 (0.164–1.972)	0.374	**0.003 (0.000–0.423)**	**0.022***
Extreme	0.134 (0.010–1.893)	0.137	1.247 (0.349–4.461)	0.734	**0.039 (0.002–0.990)**	**0.049***
Extreme-karst	Reference		0.946 (0.225–3.972)	0.940	**0.027 (0.001–0.858)**	**0.041***
Aquifer type						
Karst vs Non-karst (Ref)	6.309 (0.628–63.364)	0.118	**3.903 (1.41–10.78)**	**0.009***	0.397 (0.017–9.368)	0.567
Sheep density [b]						
Increases of 1 head/km^2	0.959 (0.918–1.001)	0.056**	1.001 (0.998–1.004)	0.519	0.991 (0.977–1.005)	0.196
Cattle density [b]						
Increases of 1 head/km^2	1.000 (0.983–1.017)	0.983	**1.010 (1.002–1.018)**	**0.011***	**1.028 (1.002–1.056)**	**0.037***
DWWTS density [b]						
Increases of 1 unit/km^2	0.994 (0.884–1.118)	0.920	1.025 (0.961–1.094)	0.455	0.973 (0.709–1.335)	0.866
Nearest livestock ≤3 m						
Present vs. Absent (Ref)	0.067 (0.002–1.979)	0.118	1.071 (.412–2.785)	0.888	4.977 (0.632–39.193)	0.127
Nearest DWWTS ≤30 m						
Present vs. Absent (Ref)	**14.046 (1.38–142.96)**	**0.026***	**5.090 (1.93–13.40)**	**<0.001***	0.000 (0.000–.)	0.998
5-day rainfall						
Increases of 1 mm	**1.069 (1.020–1.121)**	**0.005***	-	-	-	-
60-day rainfall						
Increases of 1 mm	-	-	**1.008 (1.002–1.014)**	**0.008***	1.009 (0.993–1.025)	0.287
Supply type						
Hand-dug vs. Drilled (Ref)	**18.902 (1.59–225.13)**	**0.020***	**7.199 (2.25–23.02)**	**<0.001***	**41.071 (1.001-1685.5)**	**0.050***
Sealed cap						
Present vs. Absent (Ref)	0.358 (0.037–3.422)	0.372	0.452 (0.193–1.060)	0.068**	0.155 (0.017–1.410)	0.098**
Wellhead cracks						
Present vs. Absent (Ref)	**100.26 (3.11-3227.9)**	**0.009***	2.847 (0.75–10.78)	0.124	0.000 (0.000–.)	0.998
Casing clearance ≥150 mm						
Present vs. Absent (Ref)	0.731 (0.118–4.519)	0.736	0.603 (0.27–1.33)	0.211	1.323 (0.104–16.872)	0.829
Nagelkerke R^2	0.504		0.285		0.475	

[a] Categorically resistant in accordance with CLSI (2018; 2020); [b] Sheep, cattle and DWWTS densities calculated per Small Area, which are delineated land sections in the RoI territory which comprise 80 to 120 dwellings (CSO, 2016b); DWWTS=Domestic Wastewater treatment System; CI=Confidence Interval; * *p*-value≤0.05; ** 0.05<*p*-value≤0.1

4.4 Discussion

The current study is the second to investigate the occurrence of antimicrobial resistant bacteria in groundwater sources in the RoI (succeeding O'Dwyer et al., 2017), and the first to comprise a country-wide (geographically dispersed) sampling network while also including the analyses of bacterial species of distinct primary habitats. To the author's knowledge, this study is also the first to analyse groundwater samples for the presence of *P. aeruginosa* in the RoI. In total, 132 private wells (250 samples) were assessed from 21 out of the 26 administrative counties in the country, with samples collected at two time periods: September to November 2019 (Round 1) and June to August 2021 (Round 2); notably no sampling was carried out in spring or winter months. *E. coli*, a well-known faecal indicator organism, and *P. aeruginosa*, a bacteria that can proliferate in soil and freshwater, were used to assess current rates of antimicrobial resistant bacteria, and identify key associated anthropogenic and environmental sources/pathways, as well as infrastructural risk factors.

Overall, observed instances of *P. aeruginosa* contamination were low, detected in just 8.3% of private wells (n = 11), while *E. coli* were found in 33.3% of wells at least once (n = 44). *E. coli* detection fell within the range reported by previous studies of private groundwater quality in the RoI, which described rates of 28.9% (Hynds et al., 2012) to 51.4% (O'Dwyer et al., 2018). No RoI-specific baseline data are available on the incidence of *P. aeruginosa* in groundwater sources; however, the lower incidence identified here (i.e., 8.3%), as well as in some other international literature (Bamigboye et al., 2020; Ayad et al., 2021), may be explained by the lower abundance

of this species in rural soil and freshwater when compared to other Pseudomonads (Chatterjee et al., 2017) or to samples from urban environments (Crone et al., 2020). The 48 *E. coli* and six *P. aeruginosa* isolates recovered in the current study underwent phenotypic antimicrobial susceptibility analyses, with results varying substantially between both species. Resistance (categorised according to CLSI, 2018, 2020) was found in 16.7% of recovered *E. coli* (75.0% of which were resistant to ≥ 3 antimicrobials) and in none of the *P. aeruginosa* isolates.

The lack of resistance and low incidence (n = 1) of intermediate resistance among *P. aeruginosa* isolates deviates from international review findings (Andrade et al., 2020), where groundwater-derived Pseudomonas spp. displayed highest mean rates of resistance and/or intermediate resistance globally (mean ± standard deviation = 99.9% ± 0.2). Notably, however, overall resistance/intermediate resistance rates in the review were significantly higher in urban rather than rural settings (Andrade et al., 2020), with most previous Pseudomonads-specific literature focusing on groundwater resources in urban and densely populated areas. Compounding this, *P. aeruginosa* is intrinsically resistant to many antimicrobial agents (Livermore, 1984; Nordmann and Guibert, 1998). Indeed, *P. aeruginosa* susceptibility breakpoints are not available for most veterinary antimicrobials included in this study (see **Table 4.2**). A recent study in rural settings found moderate rates of resistance among *P. aeruginosa* from hydrocarbon-contaminated groundwater supplies in Hungary (i.e., 31.6%) (Kaszab et al., 2021); it must be noted, however, that the presence of petroleum hydrocarbons has been shown to cause co-selection of antimicrobial resistance genes in soil and water (Chen et al., 2017; Wang et al., 2017).

Conversely, current *E. coli* resistance rates (i.e., 16.7%) were higher than in studies from other developed countries. A Canadian study examining private groundwater supplies in Alberta and Ontario, for example, found that 10.5% and 3.7% of groundwater-derived *E. coli* were resistant to ≥ 1 and ≥ 3 antimicrobials, respectively (Coleman et al., 2013). Present findings are also at the high end of resistance within the international context, with 95.8% (n = 46) of all tested *E. coli* being classified as resistant and/or intermediate resistance to at least one antimicrobial; a higher rate than the global mean of 79.8% ± 30.1 identified for groundwater-derived *E. coli* (Andrade et al., 2020). Notwithstanding, resistance rates identified here were significantly lower than those detected in the mid-west of the RoI by O'Dwyer et al. (2017), who found that all groundwater-derived *E. coli* (n = 42) were resistant to at least one antimicrobial, primarily due to Aminoglycoside resistance identified in over 92% of isolates (contrasting with 14.6% in the current study; n = 7). The disparity seen here can be contextualised in view of reported disagreements between *E. coli* isolates' phenotypic and genotypic resistance to streptomycin in international literature (Kozak et al., 2009; Allen et al., 2011).

Nevertheless, and similar to findings reported by O'Dwyer et al. (2017), streptomycin was associated with the highest resistance rates among the *E. coli* isolates, followed by tetracycline (12.5%; n = 6) and ampicillin (12.5%; n = 6), all commonly used veterinary antimicrobials (Martin et al., 2020). In fact, these three have been listed as the most frequently employed antimicrobials in Irish bovine (i.e., dairy) agricultural operations (Gibbons et al., 2014), with another Irish study reporting that *E. coli* isolated from cattle were most frequently resistant, to streptomycin, tetracycline, sulfonamide, and ampicillin (Karczmarczyk et al., 2011).

Resistance among the groundwater-derived *E. coli* was also found, albeit in lower rates, to piperacillin (10.4%; n = 5), chloramphenicol (4.2%; n = 2), trimethoprim (2.1%; n = 1), and trimethoprim-sulfamethoxazole (2.1%; n = 1), which are antimicrobials exclusively used among humans in the RoI. However, as resistance found to piperacillin was always concurrent with that to ampicillin (both broad-spectrum beta-lactam antimicrobials) with all isolates being susceptible to piperacillin-tazobactam (the only form of therapeutic piperacillin currently available in the RoI, see **Table 4.1**), the authors consider that the piperacillin resistance found was likely conferred by beta-lactamase resistance genes which also confer resistance to ampicillin (Hussain et al., 2021). The significantly lower rates of resistance to the other screened human antimicrobials may be attributable to the current study being undertaken in relatively sparsely populated rural settings, with previous research by Moore et al. (2020) noting an increase in antimicrobial resistance among faecal bacteria from groundwaters in closer proximity to highly populated areas, such as tourist resorts. Indeed, *E. coli* and resistant *E. coli* regression models (**Table 4.5**) show that while *E. coli* presence was significantly associated with both human (DWWTS present within 30 m of well; OR = 5.090, $p < 0.001$) and pastoral agricultural sources (local cattle density in heads/km^2; OR = 1.010, $p = 0.011$), only cattle density was significantly associated with the detection of resistant *E. coli* (OR = 1.028, $p = 0.037$). Consequently, and in combination with the lack of resistance among *P. aeruginosa*, an environmental bacteria which has been linked with human activities in the present investigation as well as previous work (Crone et al., 2020), from study findings resistance pressures arising from human sources appear to be considerably less prominent than agricultural sources in rural and sparsely populated environments.

This is in spite of the presence of nearby point-sources of contamination such as DWWTSs (e.g., septic tanks) (Hynds et al., 2014; O'Dwyer et al., 2018), which are historically under-regulated by the State (C-188/08, 2009; C-374/11, 2012) and, when inspected, typically have a high rate of individual system failure - reported in 54% of the 806 septic tanks inspected in 2020 (EPA, 2021c).

Associations between livestock-based agriculture and antimicrobial resistant bacteria in rural groundwater resources have been similarly reported nationally (O'Dwyer et al., 2017) and internationally (Coleman et al., 2013; Gambero et al., 2018). This is particularly relevant as cattle (beef and dairy) accounts for over 59% of the RoI's agricultural outputs in 2021 (CSO, 2021). National antimicrobial usage data is not yet collected at farm levels in the RoI (Martin et al., 2020), however research conducted in the country reports high antimicrobial administration in cattle as part of dairy farming activities (Gibbons et al., 2014) and these supply 60% of the national beef (Morrison, 2020). Hence, the seasonal nature of the calving season, which takes place in spring (February to April) and represents the period of highest antimicrobial prescription (> 63%) (Gibbons et al., 2014), may partially account for the higher rates of resistance in Round 2 (summer 2021; 21.1%) than Round 1 (autumn 2019; 13.8%). This increase may also be attributable to national veterinary antimicrobial prescriptions having increased since the COVID-19 pandemic, with a recent surveillance report indicating that sales in 2020 were higher than those from the previous eight (pre-pandemic) years (HPRA, 2021). With regards to human antimicrobial use, the lower impact inferred is further supported by the only intermediate resistance classification among *P. aeruginosa* isolates being to a veterinary antimicrobial (i.e., enrofloxacin); despite increased odds of *P. aeruginosa*

detection at close proximity to DWWTS (i.e., ≤ 30 m; OR = 14.046, p = 0.026), and lack of significant associations with animal sources of contamination. Of note, significance at a 90% confidence level pointed to lower sheep densities being associated with higher odds of *P. aeruginosa* detection (OR = 1.043; p = 0.056); however, this may be in part attributable to sheep grazing areas having thinner and less organically rich topsoils, which have been shown to negatively influence the detection of *Pseudomonas* species (Dignam et al., 2018).

Key differences with respect to likely bacterial transport pathways can be inferred via comparison of final multivariate models. In the case of *E. coli*, for example, transport to contaminated groundwater supplies would seem to be attributable to a few ingress mechanisms: i) rapid aquifer recharge in karst regions (OR = 3.903, p = 0.009), ii) "generalised" (traditional) recharge, owing to above mean increases in longer-term (60-day) rainfall being significantly associated with higher odds of *E. coli* presence (OR = 1.008, p = 0.008), and iii) direct ingress at the wellhead, substantiated by higher odds of *E. coli* detection approaching significance in the absence of an appropriately sealed cap (OR = 2.847, p = 0.068) and being significantly associated with increases in shorter-term (10-day) rainfall (OR = 1.016, p = 0.008; seen in the *second best-fit* model with a marginally lower R^2). The final resistant *E. coli* model, however, (as well as others attempted with the different rainfall periods) showed no significant relationship with cumulative rainfall, while significantly higher odds of detection were observed in areas of low groundwater vulnerability (where impermeable layers of clay soil and subsoil prevent contaminant percolation and recharge), and where the well cap was not appropriately sealed (significant at the 90% confidence level; OR = 6.452, p = 0.098). These results point to the primary

ingress mechanism associated with presence of resistant *E. coli* in Irish rural supplies being overland flow followed by direct wellhead ingress, contrasting with *traditional* recharge suggestions posed by Andrade et al. (2020) based on the analyses of data from rural and urban settings combined. In line with the proposed theory, an association with shorter-term (5- and/or 10-day) cumulative rainfall might be expected, as it is a key driver of overland flow; however, the lack of a significant relationship may be justified by a greater impact of rainfall incidence/frequency rather than intensity especially as detection of resistant *E. coli* was significantly higher in low groundwater vulnerability areas, where thicker impermeable layers allow for higher rates of overland flow. Direct ingress at the wellhead and/or rapid infiltration (i.e., preferential flow) may also be the principal transport mechanisms of *P. aeruginosa*, owing to significantly higher odds of detection related to increases in shorter-term antecedent rainfall (i.e., 5-day; OR = 1.069, p = 0.005) and presence of wellhead cracking (OR = 100.256, p = 0.009), combined with lack of *P. aeruginosa* detection in "low" groundwater vulnerability areas. Notably, in all multivariate models (i.e., *P. aeruginosa*, *E. coli* and resistant *E. coli*) the odds of detection were significantly higher for hand-dug than drilled wells (OR = 18.902, 7.199, and 41.071, respectively), thus confirming the inherent microbial susceptibility of hand-dug wells (Hynds et al., 2012; Maran et al., 2016) and indicating higher likelihood of localised (i.e., confined to the well) rather than aquifer-wide contamination. It is also worth noting that lack of repeat (i.e., re-occurring) antimicrobial resistant bacteria presence between sampling rounds points to the fluctuating nature of this type of contamination.

As is the case with many exploratory environmental and groundwater contamination studies, limitations must be acknowledged owing to the outcome variances not being fully explained by the independent variables in the models (i.e., Nagelkerke R^2 ranging from 28.0 to 50.8%). In the case of resistant *E. coli* modelling, in particular, this is likely attributable to the added complexity of antimicrobial resistance acquisition in the environment, potentially influenced by a range of factors outside of the scope of this research such as presence of chemical co-pollutants (e.g., toxic metals, biocides and disinfectants) and biofilm formation (Wu et al., 2018; Zhang et al., 2018), excessive organic matter (Chee-Sanford et al., 2009) and even microplastics (Imran et al., 2019). Indeed, additional research is needed to investigate the role of these localised pressures in the acquisition and transport of resistance within groundwater environments. The focus of this investigation, however, was to identify the likely impact of larger-scale environmental and infrastructural determinants in facilitating groundwater contamination with antimicrobial resistant bacteria, as well as suggest preventative source protection measures. To this end, the current study is valuable for highlighting distinctions in overall resistance rates between *E. coli* and *P. aeruginosa*, as well as their likely sources and transport pathways. Results also show that specific well construction elements play an important role in preventing antimicrobial resistance (and indeed bacterial) contamination and their associated health impacts, such as (1) opting for a *drilled* as opposed to a *hand-dug* well, (2) ensuring minimum setback distances to sources of faecal (and potentially antimicrobial) contamination (e.g., septic tanks and livestock activities), (3) fitting an appropriately sealed cap, and (4) appropriately maintaining the wellhead (i.e., keeping it clean, dry, and crack-free).

It is also important to emphasise that despite the low overall detection of antimicrobial resistant bacteria (i.e., present in 3.2% of samples and 6.1% of wells at least once, with isolates classified as intermediately-resistant in an additional 15.2% of samples and 25.8% of wells), their occurrence in drinking water resources presents substantial risks to human health. Groundwater resources' role in disease transmission is well-substantiated within the literature, with estimates of up to 59.4 million annual cases of acute gastrointestinal infection linked to groundwater consumption globally (Murphy et al., 2017) and high incidence of verotoxigenic *E. coli* (VTEC) enteritis being significantly associated with consumption of private well water in the RoI (OhAiseadha et al., 2017). Thus, increased groundwater resource colonisation with antimicrobial resistant bacteria can represent a meaningful contribution to the higher morbidity and mortality associated with "harder-to-treat" infections globally. Accordingly, and given the importance of reducing the spread of antimicrobial resistance within and outside of clinical settings, further efforts are needed to mitigate presence of antimicrobial resistant bacteria in rural groundwater resource environments.

4.5 Conclusion

The results presented here reiterate the noteworthy role of groundwater - even those in rural settings (where population density is lower, non-point agricultural sources of contamination are dominant, and sizable point sources are less common) - as potential reservoirs for antimicrobial resistant bacteria. While no resistance was found among *P. aeruginosa* isolates, an environmental bacteria linked to intense human activities, phenotypic susceptibility results showed that 16.7% of *E. coli* were

resistant to one or more antimicrobials - detected in 6.1% of wells in this study. The likely source of antimicrobial resistant *E. coli* in the investigated supplies was cattle farming, with highest resistance rates found to veterinary antimicrobials and multivariate modelling results indicating presence of resistant *E. coli* as significantly associated with higher cattle density. Hence, out-patient use of human antimicrobials in rural areas of the RoI may present a comparatively small contribution to the spread of resistance. Moreover, as detection of resistant *E. coli* was significantly more likely in areas of low groundwater vulnerability and where supplies were not fitted with an appropriately sealed cap, overland flow followed by direct wellhead ingress is hypothesised as the primary ingress mechanism associated with their presence. Findings also provide empirical evidence to support groundwater source protection policies, such as reinforcing the increased safety offered by drilled rather than hand-dug wells, and the importance of an appropriately sealed well cap and minimum well setback distances to faecal contamination sources (e.g., DWWTSs and livestock activities). Importantly, however, the outcome variance in the multivariate model produced was only partially explained by the independent variables included. As such, further work is needed to investigate existing pressures within these 'protected' environments (e.g., co-contaminants, physico-chemical parameters, etc.) in the hopes of identifying likely antimicrobial resistance acquisition trends and appropriate prevention frameworks. Nevertheless, this study is valuable for demonstrating key differences in resistance rates within distinct bacterial species, while identifying likely sources and transport pathways of antimicrobial resistant *E. coli*. Moreover, despite the low overall detection rates found, the presence of antimicrobial resistant bacteria in groundwater resources used for human

consumption shows that there exists a substantial risk to public health and can serve as a benchmark for future groundwater studies.

Author contributions

Luisa Andrade: Conceptualization, Methodology, Investigation, Formal analysis, Data Visualisation, Writing - original draft. **Carlos Chique:** Data curation, Data Visualisation, Writing - review & editing. **Paul Hynds:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Supervision. **John Weatherill:** Writing - review & editing, Supervision. **Jean O'Dwyer:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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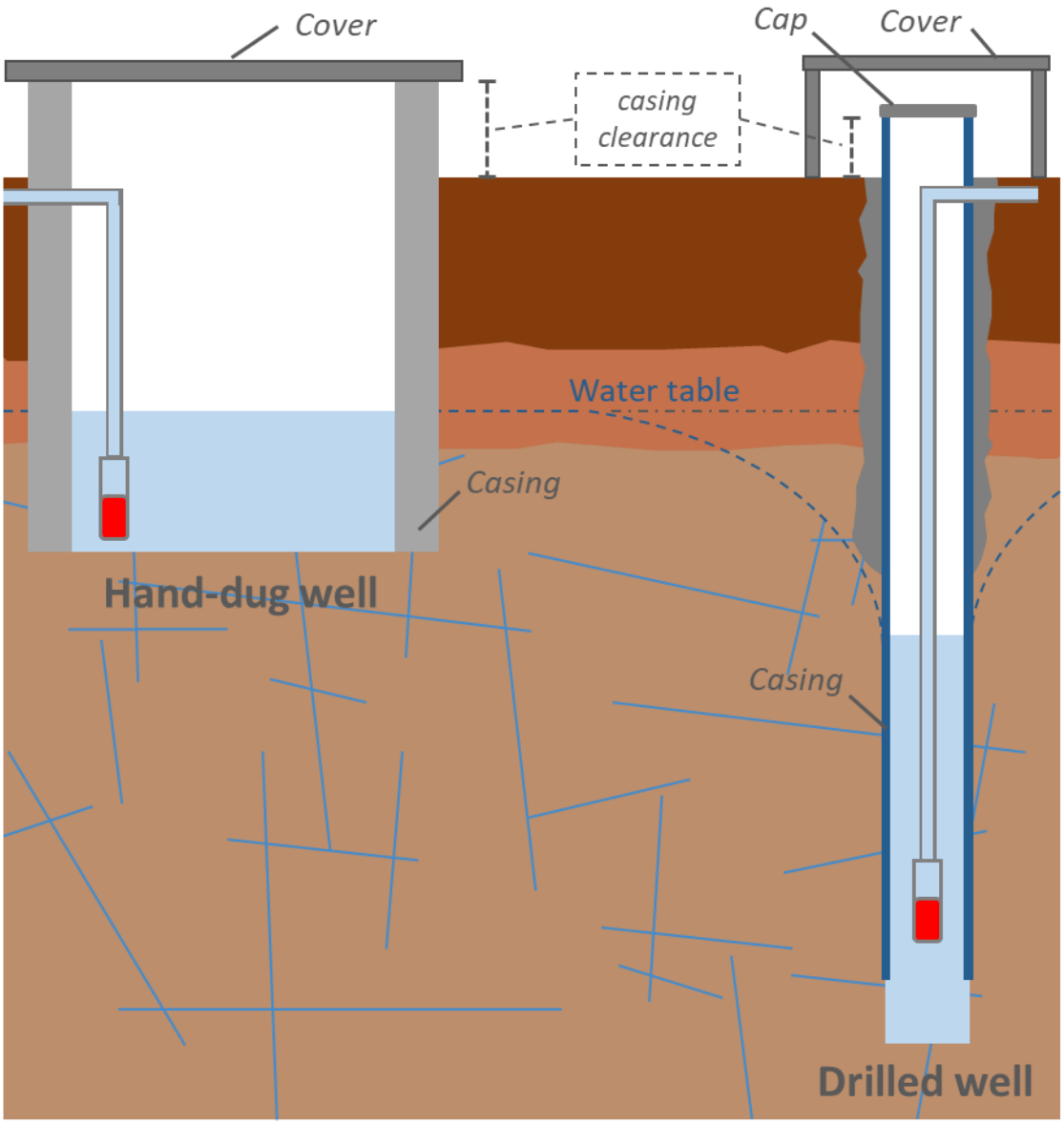
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Supplementary Data

Typical hand-dug and drilled wells and their key infrastructural characteristics



Well-owner questionnaire

SMARTIE — Spatiotemporal multiscale Modelling of Antimicrobial Resistance in the Irish subsurface Environment

UCC — Coláiste na hOllscoile Corcaigh, Éire — University College Cork, Ireland

Please fill out the entire questionnaire

EIRCODE:

1. Is well water used for drinking in your household **currently**?
   Please mark your answer with an "x" in the shaded area.

☐ Yes ☐ No

2. Please fill out below the household member age and amount of well water consumed by **all current members** of your household (including yourself).
   If a household member does not drink from the well water source, please indicate the amount of water consumed as ***0 (zero).***

Household member	*Age*	*Number of 330ml glasses of water consumed from your well per day, **not in tea, coffee or food (just water)***
1 (You)		
2		
3		
4		
5		
6		
7		
8		
9		
10		

3. To the best of your knowledge, how deep is your well? *Please indicate well depth in either feet **or** metres.*

In feet:

OR in metres:

4. If you are unsure of your exact well depth, please mark which of the below ranges best describes your well's depth.
   Mark with an "x" in the shaded area.

☐ Up to 15 ft (Up to 5m) ☐ 100 to 200 ft (30 to 60 m)

☐ 15 to 30 ft (5 to 10 m) ☐ 200 to 300 ft (60 to 90 m)

☐ 30 to 100 ft (10 to 30 m) ☐ Over 300 ft (Over 90 m)

5. To the best of your knowledge, how many courses of antibiotics were consumed in your household in the two time periods shown below?
   Please fill out the number in the shaded area below.

In the past 3 months: In the past year:

In the past 6 months: In the past 18 months:

SMARTIE — Spatiotemporal multiscale Modelling of Antimicrobial Resistance in the Irish subsurface Environment

UCC — Coláiste na hOllscoile Corcaigh, Éire — University College Cork, Ireland

6. To the best of your knowledge, when was the last time that someone in your household consumed antibiotics? *Please, mark the appropriate answer with an "x" and answer the number of weeks, months or years in the corresponding shaded area.*

☐ There is someone taking them now

☐ A few weeks ago - How many weeks?

☐ A few months ago – How many months?

☐ A few years ago – How many years?

7. What was the antibiotic (or antibiotics) referred to in the above question (question 8)?
   Please write you answer in the shaded area below. If you cannot remember or find that information anywhere, please write "Don't know".

Antibiotic(s):

8. Are any of the following treatments applied **between your well and the outdoor well tap** (i.e. the tap where your sample will be collected from)?
   Please, mark with an "x" in the shaded area as many options as are applicable.

☐ UV lights ☐ Filter – which type?

☐ Reverse Osmosis ☐ Nothing

☐ Water softener ☐ Other:

9. Have you undertaken any **modifications** to your well since the last sampling visit in Autumn 2019?
   Please mark with an "x" all that apply.

☐ Added/changed **well cap** ☐ Added a **treatment system.** Please specify:

☐ Added/changed **well cover** ☐ Nothing

☐ Added/changed well **pump/pipe work** ☐ Other:

10. If you did modify your well since the last sampling visit in Autumn 2019, please describe the changes in more detail below.

Risk factor analyses

Measured Risk Factors	Presence/absence of *P. aeruginosa*			Presence/absence of *E. coli*			Presence/absence of Resistant [#] *E. coli*		
	N	Variable Coefficient	*p*-value	N	Variable Coefficient	*p*-value	N	Variable Coefficient	*p*-value
Local parameters									
Groundwater vulnerability [a]	250	**10.92**	**0.027***	250	1.13	0.890	244	7.69	0.108
Aquifer importance [a]	250	0.91	0.636	250	5.31	0.070**	244	0.12	0.942
Aquifer type (karst vs non-karst) [a]	250	0.13	0.716	250	**4.89**	**0.027***	244	0.01	0.931
Sheep density [b]	250	**822.50**	**0.013***	250	4961.00	0.482	244	**533.50**	**0.036***
Cattle density [b]	250	1372.50	0.820	250	**6223.00**	**0.048***	244	1267.50	0.099**
Livestock density [b]	250	**844.50**	**0.017***	250	5403.00	0.813	244	832.5	0.570
DWWTS density [b]	250	1676.00	0.310	250	**6501.00**	**0.010***	244	1183.5	0.222
5-day rainfall [b]	250	1860.00	0.075**	250	6119.50	0.079**	244	1232.50	0.142
10-day rainfall [b]	250	1945.00	0.034*	250	6401.50	0.018*	244	1099.50	0.428
60-day rainfall [b]	250	1706.00	0.255	250	6246.00	0.043*	244	1192.00	0.206
Season (autumn/summer) [a]	250	0.97	0.324	250	**3.990**	**0.046***	244	0.01	0.925
Supply- and Supply-specific parameters									
Livestock under 3 m from well [a]	250	0.96	0.327	250	0.02	0.886	244	**5.02**	**0.025***
DWWTS under 30 m from the well [a]	250	1.27	0.260	250	8.14	**0.004***	244	1.29	0.255
Gradient difference to nearest DWWTS [a]	225	5.07	0.079**	225	1.78	0.410	224	1.25	0.536
Infrastructural parameters									
Supply type [a]	250	**7.63**	**0.006***	250	11.43	**<0.001***	244	2.35	0.125
Supply depth [b]	194	659.50	0.131	194	2728.50	0.081**	189	652.00	0.634
Casing clearance above 150 mm [a]	238	0.089	0.765	238	3.117	0.077**	232	2.14	0.143

[a] Chi-square X^2 test; [b] Mann-Whitney U test; [#] Classified as 'Resistant' based on breakpoints by the Clinical and Laboratory Standards Institute (CLSI 2018; 2020); * *p*-value ≤ 0.05; ** 0.05 < *p*-value ≤ 0.1

Second best-fit multivariate binary logistic regression models

Risk Factors	*P. aeruginosa* detection (n=233)		*E.coli* detection (n=233)		Resistant [a] *E.coli* detection (n=227)	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Groundwater vulnerability						
Low	0.000 (0.000 – .)	0.997	Reference		Reference	
Moderate	0.114 (0.00 – 2.328)	0.158	0.457 (0.098 – 2.122)	0.317	0.000 (0.000 – .)	0.997
High	0.317 (0.030 – 3.413)	0.344	0.539 (0.158 – 1.841)	0.324	**0.004 (0.000 – 0.423)**	**0.021***
Extreme	0.130 (0.009 – 1.799)	0.128	0.981 (0.277 – 3.470)	0.976	**0.031 (0.001 – 0.826)**	**0.038***
Extreme-karst	Reference		0.806 (0.188 – 3.464)	0.772	**0.020 (0.000 – 0.829)**	**0.040***
Aquifer type						
Karst vs. Non-karst (Ref)	2.036 (0.211 – 19.599)	0.538	**3.661 (1.335 – 10.036)**	**0.012***	0.370 (0.016 – 8.609)	0.536
Sheep density [b]						
Increases of 1 head/m^2	0.965 (0.929 – 1.002)	0.065**	1.001 (0.998 – 1.003)	0.69	0.990 (0.976 – 1.004)	0.153
Cattle density [b]						
Increases of 1 head/m^2	1.000 (0.983 – 1.017)	0.989	**1.008 (1.000 – 1.015)**	**0.040***	1.025 (1.000 – 1.051)	0.051**
DWWTS density [b]						
Increases of 1 unit/m^2	0.997 (0.888 – 1.119)	0.955	1.020 (0.955 – 1.090)	0.551	0.959 (0.681 – 1.350)	0.810
Nearest livestock ≤3 m						
Present vs. Absent (Ref)	0.100 (0.004 – 2.324)	0.152	1.145 (0.442 – 2.970)	0.780	4.453 (0.580 – 34.182)	0.151
Nearest DWWTS ≤30 m						
Present vs. Absent (Ref)	**13.897 (1.413 – 13.692)**	**0.024***	**4.745 (1.819 – 12.377)**	**0.001***	0.000 (0.000 – .)	0.998
10-day rainfall						
Increases of 1 mm	**1.036 (1.011 – 1.062)**	**0.005***	**1.016 (1.004 – 1.027)**	**0.008***	1.016 (0.983 – 1.051)	0.336
Supply type						
Hand-dug vs. Drilled (Ref)	**17.067 (1.571 – 185.433)**	**0.020***	**7.385 (2.323 – 23.473)**	**<0.001***	32.675 (0.877 – 1217.422)	0.059**
Sealed cap						
Present vs. Absent (Ref)	0.517 (0.065 – 4.103)	0.533	0.489 (0.210 – 1.141)	0.098**	0.155 (0.017 – 1.402)	0.097**
Wellhead cracks						
Present vs. Absent (Ref)	**39.439 (1.676 – 928.320)**	**0.023***	2.440 (0.642 – 9.271)	0.190	0.000 (0.000 – .)	0.998
Casing clearance ≥150 mm						
Present vs. Absent (Ref)	0.782 (0.121 – 5.052)	0.796	0.625 (0.285 – 1.374)	0.243	1.198 (0.096 – 14.910)	0.888
Nagelkerke R^2	0.503		0.282		0.471	

[a] Categorically resistant in accordance with CLSI (2018; 2020); [b] Sheep, cattle and DWWTS densities calculated per Small Area, which are delineated land sections in the RoI territory which comprise 80 to 120 dwellings (CSO, 2016a); [c] classification cut-off=0.5; [c] classification cut-off=0.4; DWWTS = Domestic Wastewater treatment System; CI = Confidence Interval; * p-value ≤ 0.05; ** 0.05< p-value ≤ 0.1

Chapter 5

Assessing antimicrobial and metal resistance genes in *Escherichia coli* from domestic groundwater supplies in rural Ireland

Preface

Title: Assessing antimicrobial and metal resistance genes in *Escherichia coli* from domestic groundwater supplies in rural Ireland

Authors: **Luisa Andrade**, Michael P. Ryan, Liam Burke, Paul Hynds, John Weatherill, and Jean O'Dwyer

Journal: Environmental Pollution

Status: Under review

LA's (CRediT) contribution: Conceptualization, Methodology, Investigation, Formal analysis, Data Curation, Visualisation, Writing - original draft, Writing – review & editing

Relevance: This research paper combines phenotypic and genotypic analyses of 48 *E. coli* isolates from domestic and rural groundwater supplies in the RoI, and further investigates these environments' co-selective potential. The analytical and statistical findings presented indicate low likelihood of (co-)selective pressures and suggest that bacteria acquired antimicrobial (and metal) resistance genes prior to supply ingress.

Significance: It was showed that, owing to a lack of (co-)selective pressures, presence of antimicrobial resistant bacteria within the studied groundwater environments and subsequent human (and animal) exposure are likely highly preventable through appropriate groundwater management practices. Findings also emphasise the importance of investigating environments of human significance regardless of their presumed level of contamination with antimicrobial resistant bacteria and (co-)selective compounds.

Abstract

Natural ecosystems can become significant reservoirs and/or pathways for antimicrobial resistance (AMR) dissemination, with the potential to affect nearby microbiological, animal, and ultimately human communities. This is further accentuated in environments that provide direct human exposure, such as drinking water. To date, however, few studies have investigated AMR dissemination potential and the presence of co-selective stressors (e.g., metals/metalloids) in groundwater environments of human health significance. Accordingly, the present study analysed samples from rural (drinking) groundwater supplies (i.e., private wells) in the Republic of Ireland, where land use is dominated by livestock grazing activities. In total, 48 *Escherichia coli* isolates tested phenotypically for antimicrobial susceptibility in an earlier study were further subject to whole genome sequencing (WGS) and corresponding water samples were further analysed for trace metal/metalloid concentrations. Eight isolates (i.e., 16.7%) were genotypically resistant to antimicrobials, confirming prior phenotypic results through the identification of ten antimicrobial resistance genes (ARGs); namely: *aph(3'')-Ib* (*strA*; n=7), *aph(6)-Id* (*strA*; n=6), *blaTEM* (n=6), *sul2* (n=6), *tetA* (n=4), *floR* (n=2), *dfrA5* (n=1), *tetB* (n=1), and *tetY* (n=1). Additional bioinformatic analysis revealed that all ARGs were plasmid-borne, except for two of the six *sul2* genes, and that 31.2% of all tested isolates (n=15) and 37.5% of resistant ones (n=3) carried virulence genes. Study results also found no significant relationships between metal concentrations and ARG abundance. Additionally, just one genetic linkage was identified between ARGs and a metal resistance gene (MRG), namely *merA*, a mercury-resistant gene found on the same plasmid as *blaTEM, dfrA5, strA*, *strB*, and *sul2* in the only isolate of inferred porcine

(as opposed to bovine) origin. Overall, findings suggest that ARG (and MRG) acquisition may be occurring prior to groundwater ingress, and are likely a *legacy issue* arising from agricultural practices.

Highlights

- 48 rural groundwater-derived *E. coli* genotypically assessed.
- *E. coli*-contaminated groundwater samples analysed for trace metal concentrations.
- Antimicrobial resistance genes found in 8 *E. coli* isolates.
- Metal resistance genes found in all *E. coli* isolates irrespective of metal presence.
- Antimicrobial and metal resistance genes likely acquired prior to well ingress.

Keywords: Antimicrobial Resistant Bacteria, Antimicrobial Resistance Genes, Metal Resistance Genes, Groundwater, *Escherichia coli*, Trace metals

5.1 Introduction

The continuing global rise of antimicrobial resistance (AMR) has been seen through multiple reports of increased morbidity and mortality from resistant infections (Murray et al., 2022; Cassini et al., 2019), and is now widely recognised as a global public health threat (World Health Organization, 2022a). Briefly, the principal known causes of AMR are bacterial mutation/heritable genetic modifications and, more commonly (Bennet, 2008; Von Wintersdorff et al., 2016), acquisition of new resistance determinants (i.e., antimicrobial resistance genes; ARGs). Both can occur and are amplified in response to selective environments, such as in the presence of

elevated concentrations of antimicrobial drugs (i.e., antimicrobials) (Bennet, 2008; Christaki et al., 2020). Conjugation is arguably the most important mechanism of AMR acquisition (Christaki et al., 2020), it can occur when a bacterium comes into direct physical contact with other ARG-carrying bacteria, particularly when the ARGs are located in mobile genetic elements (MGEs – e.g., plasmids, transposons, integrating conjugative elements etc.) (Partridge et al., 2018) as opposed to chromosomal placement. Upon acquisition, newly ARG-carrying bacteria can, when pathogenic, cause harder-to-treat (resistant) infections, requiring alternative therapeutic options which may not always be available. Thus, "One Health" mitigative efforts to enforce the rational use of antimicrobials in human and veterinary medicine and increase development (and widespread access to) novel antimicrobial classes are key priorities (Infectious Diseases Society of America, 2011; Laxminarayan et al., 2020, WHO, 2022a).

The occurrence of AMR pre-dates the discovery and commercialisation of antimicrobials (D'Costa et al., 2011), however historic over-prescription and excessive/inappropriate antimicrobial use have led to it heightened dissemination. This culminates in the increased detection of antimicrobial resistant bacteria and genes within natural environments (Larsson and Flach, 2022), particularly those heavily impacted by human activities such as agriculture, aquaculture, and ineffective waste and wastewater treatment/disposal (Kraemer et al. 2019). Compounding this, anthropogenic and geogenic contaminants which are present in the environment (in concurrence with antimicrobials) can promote/facilitate processes that indirectly select (i.e., co-select) for AMR. Potentially toxic metals, for example, some of which also possess antimicrobial properties (Poole, 2017; Gharpure et al., 2020), have been

extensively reported as co-selective for AMR (Seiller et al., 2012; Pal et al., 2017; Imran et al., 2019; Nguyen et al., 2019), including in environments unaffected by large-scale antimicrobial use (Wardwell et al., 2009; Hobman and Crossman, 2015). This poses further challenges in reducing the environmental dissemination of AMR, as metals are ubiquitous in the natural environment (occurring as mineral phases, mobile cations, and surface complexes) with concentrations of µg/L to mg/L and mg/kg, while antimicrobials are typically detected at trace to ultra-trace levels (ng/L to µg/L and µg/kg); even in areas adjacent to potential sources, such as agriculture (Zhao et al., 2018; Veiga-Gómez et al., 2021).

The key mechanisms by which co-selection can happen have been defined as cross- and co-resistance. Cross-resistance occurs when a single (physiological) mechanism such as an *efflux pump* provides resistance to both the co-selective compound and the antimicrobial drug (Chapman, 2003; Baker-Austin et al., 2006), while co-resistance describes genes conferring resistance to a co-selective compound (e.g., metal resistance genes; MRGs) and antimicrobial(s) (i.e., ARGs) being located next to each other on a single MGE or chromosome (Chapman, 2003). In both cases, presence of co-selective compounds, such as metals, disinfectants, biocides, etc., at toxic concentrations may pose extended pressures for the evolution (i.e., acquisition and/or persistence) of antibiotic resistance in the local microbiome (Nguyen et al., 2019). Metals have been shown to also increase the level of tolerance to antimicrobials via resistance gene co-regulation (Baker-Austin et al., 2006). It is important to note, however, that not all metals are equally toxic to bacteria. Metals (including metalloids) such as silver (Ag), arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb), for example, have limited biological function and are considered toxic

to most bacteria and other microorganisms, even at (very) low concentrations (Nies, 1999; Naik and Dubey, 2013). Conversely, cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), and zinc (Zn) are metals which can be vital to bacterial physiological functions, only becoming toxic at higher concentrations - and further subject to the element's bioavailability (Nies, 1999).

Multiple studies focused on surface water, soil, and effluents report associations between increased metal concentrations and heightened environmental abundance of ARGs (McKinney et al., 2010; Graham et al., 2011; Knapp et al., 2011; Ji et al., 2012; Hu et al., 2016; Knapp et al., 2017; Zhao et al., 2017; Zhao et al., 2019; Komijani et al., 2021) and, to a lesser extent, increases in ARG-carrying (Martins et al., 2014) and phenotypically resistant bacteria (Stepanauskas et al., 2005; Wright et al., 2006). Additionally, associations have been reported between MRG and ARG abundance in livestock waste lagoons (McKinney et al., 2010) and between specific MRG-ARG combinations encoded by environment-derived bacteria (Martins et al., 2014; Pal et al., 2015; Chen et al., 2019). Notably, however, studies tend to focus on more "exposed" and heavily (metal) contaminated environments as noted by large-scale reviews carried out by Imran et al. (2019) and Nguyen et al. (2019), such as surface water and soils directly impacted by manure application, industrial waste, and urbanization. This results in less knowledge availability on presumably 'cleaner' groundwater environments, where exposure to metal and antimicrobial contamination is limited, but which can provide direct dissemination pathways for AMR to humans (and animals) via consumption. Additionally, most co-selection groundwater-based studies examine ARG occurrence at a community level (i.e., present in total DNA extracted from groundwater) (viz. Li et al., 2018; Zainab et al.,

2020; Zou et al., 2021; Liu et al., 2022; Kampouri et al., 2023), which may limit their ability to quantify the overarching human (and animal) health threats. The current study sought to address both gaps in the literature by (i) investigating co-selective pressures in rural groundwater resources used for drinking in the Republic of Ireland (RoI), and (ii) incorporating the analyses of groundwater-derived *Escherichia coli* through whole genome sequencing (WGS), while also quantifying isolates' pathogenic potential (e.g., virulence genes - which are the those that confer pathogenicity).

5.2 Methods

5.2.1 Study sites and groundwater sample collection

This was an exploratory study of rural (domestic) groundwater supplies (i.e., private wells) in the RoI, a country characterised by high rural-reliance on domestic groundwater abstractions (i.e., private wells) (EPA, 2021), and with a rural profile marked by widespread livestock grazing activities and high populational reliance on domestic wastewater treatment systems (DWWTSs; e.g., septic tanks) (CSO, 2016). Overall, 250 samples were collected from 132 private wells, selected based on responses to a nationwide recruitment effort, and sampled during two distinct periods: autumn 2019 (Round 1; n=132) and summer 2021 (Round 2; n=118). Site locations were geographically distributed across the country and located >1 km away from hospital, industrial (including mining areas), and large-scale domestic waste and wastewater disposal points. See Andrade et al. (2023) for a complete description of study sites and their geographic locations.

Groundwater samples were collected prior to any microbiological treatment, following ethanol disinfection of sample taps and after being allowed to run at high flow for a minimum of 60 seconds in order to purge any stagnant water within the distribution network. Samples intended for *E. coli* enumeration and subsequent WGS analyses were collected into 100 ml sterile vessels (IDEXX Laboratories Inc., USA) and transported to the laboratory at 4^{o}C for analysis within 6 hours. Samples for trace metal analysis were filtered onsite using 0.45 µm pore size inline syringe filters (Sartorius Stedim Biotech, Germany) and collected into 60 ml factory clean HDPE bottles (Thermo Fisher, UK) containing 0.5 mL of 20% ultrapure nitric acid (67-70%, Optima™, Ultra Trace Elemental Analysis; Fisher Chemical, UK) to buffer samples' pH to <4. Temperature, pH, and specific electrical conductivity were measured immediately prior to sample collection using a pre-calibrated multiparameter in-situ water quality sonde (Manta+20, Eureka Water Probes, USA) with readings recorded after stabilisation to <5%. The specific well type (i.e., drilled versus hand-dug) was recorded following sample collection.

5.2.2 Laboratory procedures

Bacterial enumeration, whole genome sequencing, and bioinformatic analyses

The most probable number (MPN) of *E. coli* in each water sample was determined using standard ISO approved (ISO, 1998) commercial culture kits (Colilert; IDEXX Laboratories Inc., Westbrook, Maine) and 51-'well' IDEXX quanti-trays (IDEXX Laboratories Inc., Westbrook, Maine) according to the manufacturer's instructions (see Andrade et al., 2023 for full description). WGS of recovered *E. coli* isolates was carried out by MicrobesNG (University of Birmingham, UK). Briefly,

standard Nextera protocols (Adey et al., 2010) were used for strain recovery and 2 × 250 bp paired-end reads HiSeq Illumina Miseq technology, allowing for a 30x genome coverage. Reads were adapter-trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15 (Bolger et al., 2014) and a de-novo assembly of reads was performed using SPAdes (Bankevich et al., 2012). The reads were then mapped back to the resultant contigs using the Burrows-Wheeler Aligner mem (Li and Durbin, 2010), with automated annotation performed using Prokka (Seemann, 2014).

Genome sequence FASTA (.faa) files produced were analysed using several open-source bioinformatic platforms. Firstly, the EZBioCloud ANI calculator tool (https://www.ezbiocloud.net/tools/ani) was employed to confirm isolate species by comparing isolate genomes to that of an established reference strain (i.e., *E. coli* K-12), with 'identity' ≥95% used to designate a match (Yoon et al., 2017). Subsequently, the Galaxy platform (ABRicate tool v1.0.1 - https://usegalaxy.org/; Afgan et al., 2018) was utilised, applying a minimum 'identity' and 'coverage' of 80%, to identify antibiotic resistance genes (ARGs) (ResFinder and CARD), *E. coli* virulence genes (Ecoli.VF), and plasmids (PlasmidFinder) present, as well as the isolate's serotype (EcOH). Additionally, the IntegronFinder tool (v2.0.2; Néron et al., 2022) was used through the Galaxy Europe platform (https://usegalaxy.eu/) to detect integrons, with an applied e-value cut-off of 1e−5.

The metal resistance genes (MRGs) present were identified by subjecting genome sequences to a BLASTx search (v2.2.26; Altschul et al., 1997) against the BacMet 'Experimentally Confirmed' database (v2.0 - http://bacmet.biomedicine.gu.se/index.html; Pal et al., 2014), with an applied e-

value cut-off of 1e−5 and minimum 'identity' and 'positives' of 80%. The Clermont Typing (v21.03 - http://clermontyping.iame-research.center/index.php; Beghain et al., 2018) and Multi Locus Sequence Typing (MLST v2.0.9 - http://www.cbs.dtu.dk/services/mlst; Larsen et al., 2012) pipelines were used to ascertain isolates' phylotypes and sequence types (STs), respectively. Finally, the 'Platon' tool (Schwengers et al., 2020) was utilised to detect likely chromosome and plasmid contigs. These were then scanned using ResFinder/CARD and subjected to BLASTx BacMet searches, as described above, to infer which ARGs and/or MRGs are located on plasmids versus on the bacterial chromosome. The genomic context of the ARGs and MRGs found on plasmid-related contigs in the same isolate were further investigated using BLASTn (Altschul et al., 1990).

Trace metal analysis

In groundwater samples where *E. coli* were detected, total dissolved trace metals (including metalloids) analysis was carried out in the School of Chemistry (University College Cork, Ireland) using inductively coupled argon plasma-mass spectrometry (ICP-MS) (Agilent 7700x, Agilent Technologies, United Kingdom). The typical limit of quantification was <0.001 µg/L and the isotopes used to quantify the selected elements were: ^{107}Ag, ^{75}As, ^{59}Co, ^{111}Cd, ^{52}Cr, ^{63}Cu, ^{56}Fe, ^{55}Mn, ^{60}Ni, ^{208}Pb, ^{121}Sb, and ^{66}Zn.

5.2.3 Statistical analyses

Associations between each ARG and the specific MRGs detected in the groundwater-derived *E. coli* (i.e., all bivariate ARG-MRG combinations) were

examined using the Fisher's exact test, except in cases where just one 'positive' outcome was detected. Additionally, Mann–Whitney U tests were employed to assess associations between median metal concentrations and presence of the relevant MRG(s), and Kruskal–Wallis H tests were used to identify individual metal concentrations in their associations with ARG and MRG abundance (i.e., using the quantities of ARGs and MRGs found in each isolate as dependent variables, respectively). All statistical analyses were undertaken using IBM SPSS version 28, with confidence level set at 95% (i.e. *p*-value < 0.05).

5.3 Results

5.3.1 Descriptive characteristics of groundwater supplies and samples

As described elsewhere (Andrade et al., 2023), *E. coli* isolates were identified in 54 groundwater samples, with 48 isolates recovered and re-occurrence (i.e., repeat *E. coli* presence) identified in 13 wells. Thus, the *E. coli* analysed in the current study originated from 35 distinct groundwater wells (See **Figure 5.1** for their locations). Most *E. coli* positive wells were drilled (77.1%; n = 27), with the remainder classified as shallow hand-dug wells (22.9%; n = 8).

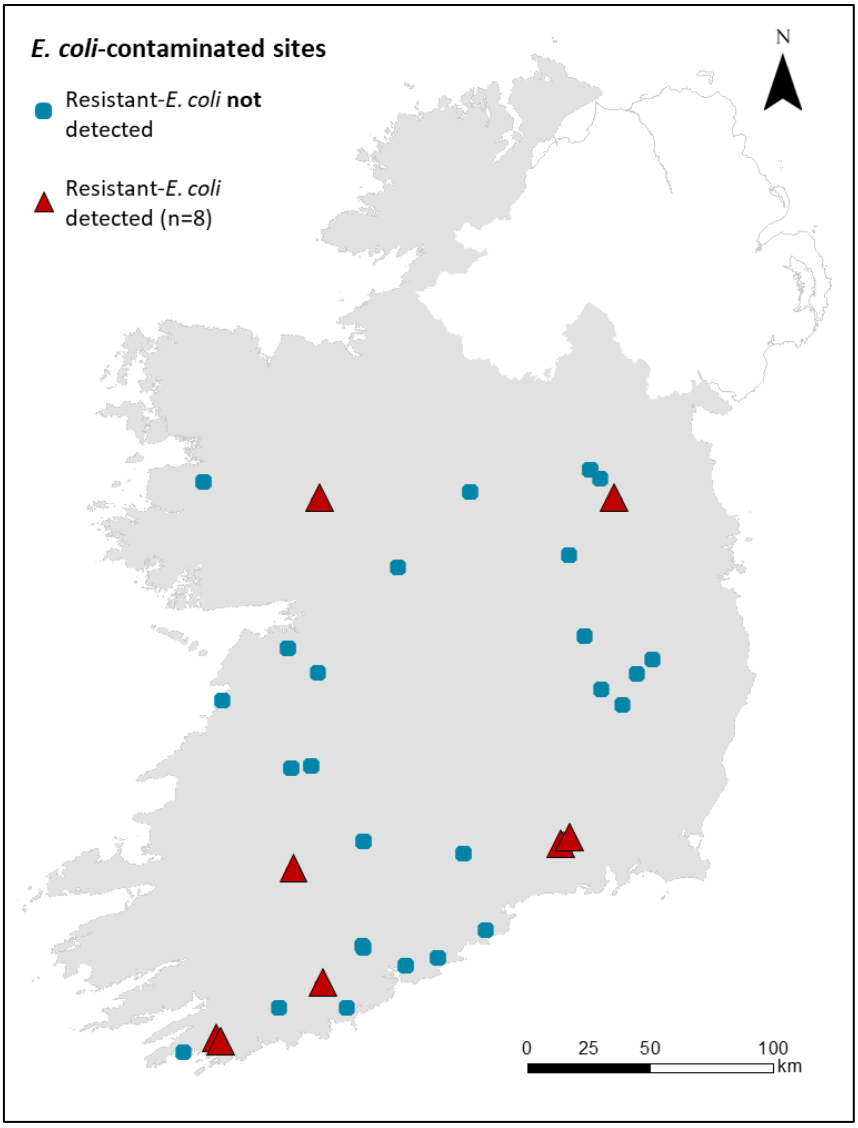


Figure 5.1: Map of Ireland showing the 35 groundwater wells where E. coli was detected and cultured for subsequent genotypic analyses (n = 48) and highlighting the wells harbouring antimicrobial-resistant E. coli (n = 8)

Further analyses of the 48 *E. coli*-positive groundwater samples indicated temperatures of 11.5 to 18.6°C, pH of 5.8 to 7.5, and electrical conductances of 142.0 to 943.6 µS/cm, respectively, with no outliers found. A median of 5.30 MPN/100 ml (interquartile range = 2.0 to 26.4) was identified for *E. coli*; see **Table 5.1** for quantity distributions. As also shown in **Table 5.1**, highest median trace metal concentrations were found for zinc (median = 32.52 µg/L, IQR = 7.68-109.86), followed by copper (6.57 µg/L; 2.70-21.50), manganese (1.38 µg/L, <0.00-15.21), nickel (0.24 µg/L,

<0.001-0.64), arsenic (0.19 µg/L, 0.07-0.44), and chromium (0.14 µg/L, 0.08-0.27), the remaining elements (i.e., cobalt, cadmium, silver, iron, and lead) all had median concentrations lower than 0.05 µg/L. Dissolved trace metal concentrations remained within safe drinking water levels except for manganese, which exceeded the recommended 80 µg/L in four samples (WHO, 2022b).

Table 5.1: Statistical summary of metal (µg/L) and E. coli (MPN/100 ml) concentrations in all E. coli isolates (n = 48) and those in which antimicrobial resistance was detected (n = 8)

	Metals (µg/L)												*E. coli* (MPN/ 100 ml)
	Ag	**As**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**Mn**	**Ni**	**Pb**	**Sb**	**Zn**	
(A) All *E. coli* isolates													
Minimum	0*	0*	0*	0*	0.02	0.65	0*	0*	0*	0*	0.01	0*	1.0
1st quartile	0*	0.07	0*	0.02	0.08	2.70	0*	0*	0*	0*	0.04	7.68	2.0
Median	0*	0.19	0.01	0.04	0.14	6.57	0*	1.38	0.24	0*	0.07	32.52	5.3
3rd quartile	0*	0.44	0.03	0.14	0.27	21.50	0*	15.22	0.64	0.33	0.13	109.86	26.5
95th percentile	0.03	1.59	0.27	0.74	0.35	72.89	16.46	98.07	4.37	0.69	0.37	186.75	> 200.5#
Maximum	0.07	9.97	0.45	5.11	0.56	289.62	4602^	319.56	7.61	1.92	0.53	693.25	> 200.5#
(B) Resistant *E. coli*													
Minimum	0*	0*	0*	0.01	0.05	0.65	0*	0*	0*	0*	0.01	0*	1.0
1st quartile	0*	0.06	0.01	0.02	0.07	5.12	0*	0.76	0*	0*	0.07	12.80	3.1
Median	0*	0.15	0.02	0.03	0.12	6.61	0*	4.84	0.19	0.01	0.10	66.61	4.8
3rd quartile	0.01	0.32	0.11	0.06	0.17	15.49	0.22	23.18	0.36	0.08	0.19	131.09	62.4
95th percentile	0.06	1.30	0.36	0.19	0.33	22.86	1.32	108.27	2.98	0.28	0.48	178.21	> 200.5#
Maximum	0.07	1.62	0.37	0.23	0.36	24.05	1.57	152.85	4.28	0.33	0.53	191.17	> 200.5#

* metal concentration below the limit of quantification; # *E. coli* enumeration above the maximum detection limit of 200.5 MPN/100 ml;
^ outlier; Ag=silver; As=arsenic; Co=cobalt; Cu=copper; Cr=chromium; Cd=cadmium; Fe=Iron; Mn = manganese; Ni=nickel; Pb=lead; Sb=antimony; Zn = zinc

5.3.2 Description of the groundwater-derived E. coli

Antimicrobial resistance

AMR was genotypically identified in 16.7% of the *E. coli* isolates (n=8), with 12.5% (n=6) classified as multi-drug resistant (i.e., resistant to ≥3 antimicrobials

belonging to different classes; Magiorakos et al., 2012) confirming phenotypic findings in Andrade et al. (2023) (**Figure 5.2**). No repeated instances (i.e., re-occurrence) of resistant-*E. coli* contamination were detected. As shown in **Figure 5.2** genes were identified which conferred resistance to antimicrobials from the Aminoglycosides (14.%; n=7), Tetracyclines (12.5%; n=6), Beta-Lactams (12.5%; n=6), Sulfonamides (12.5%; n=6), Amphenicols (4.2%; n=2), and Diaminopyrimidine (2.1%; n=1) antimicrobial classes. Namely, *aph(3'')-Ib* (n=7; encoded by *strA*), *aph(6)-Id* (n=6; encoded by *strB*), *blaTEM* (n=6), *tetA* (n=4), *floR* (n=2), *dfrA5* (n=1), *tetB* (n=1), and *tetY* (n=1). When comparing genotypic findings with and phenotypic data from Andrade et al. (2023) a single discrepancy was found whereby one isolate was phenotypically classified as intermediately (not categorically) resistant to piperacillin despite presence of the *blaTEM* gene (see **Figure 5.2**).

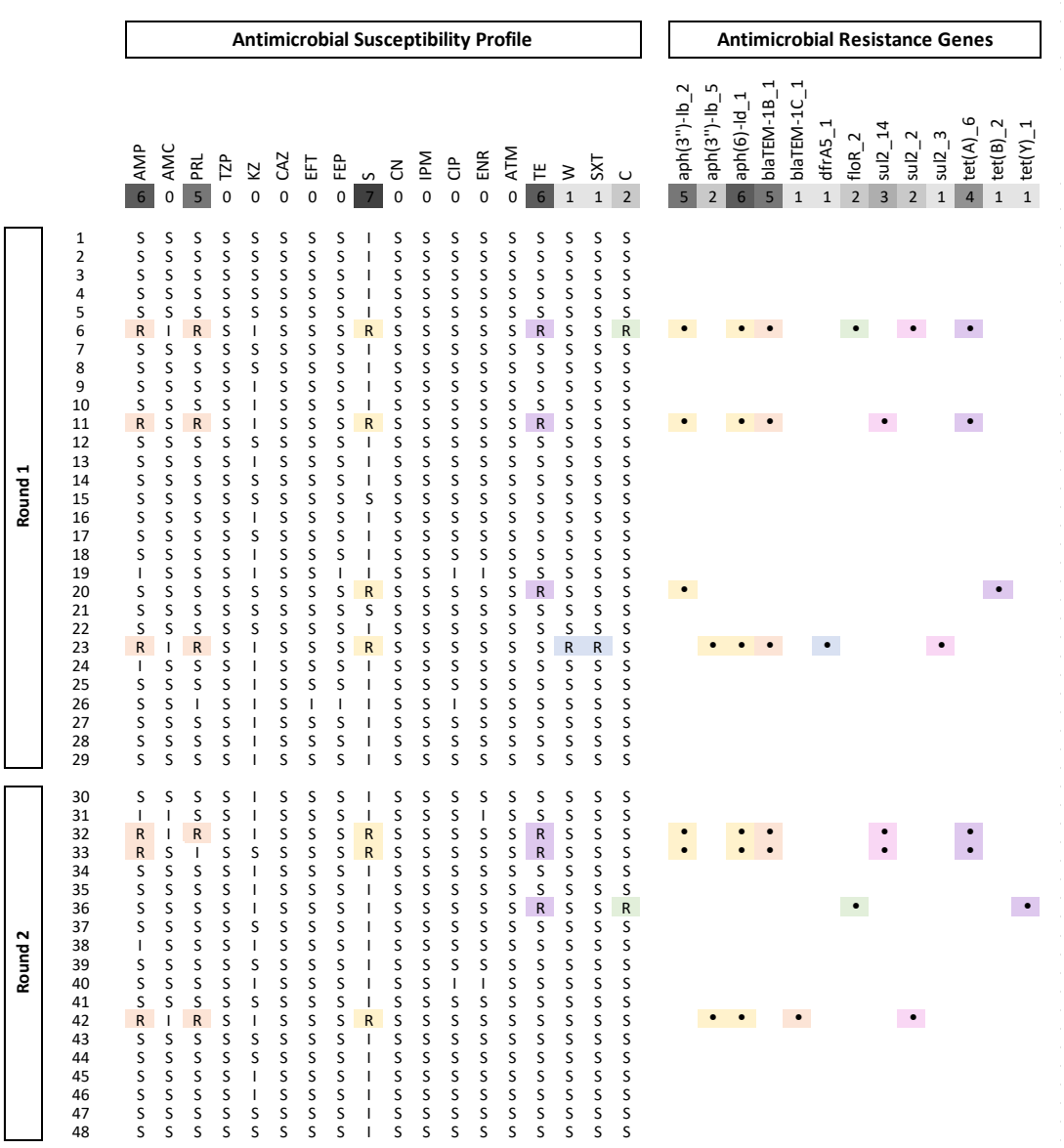


Figure 5.2: Antibiogram and resistance gene profiles for all groundwater-derived E. coli from both sampling rounds (n = 48). Colour groupings indicate genotypic and phenotypic resistance to corresponding antimicrobials and antimicrobial classes. R=resistant; I=Intermediately resistant; S=susceptible; AMP = ampicillin; AMC=amoxicillin-clavulanic acid; PRL=piperacillin; TZP = piperacillin-tazobactam; KZ=cefazolin; CAZ=ceftazidime; EFT=ceftiofur; FEP = cefepime; S=streptomycin; CN = gentamycin; IPM=imipenem; CIP = ciprofloxacin; ENR=enrofloxacin; ATM=aztreonam; TE=tetracycline; W=trimethoprim; SXT= trimethoprim-sulfamethoxazole; C=chloramphenicol

Metal resistance

As shown (**Table 5.2**), eleven MRGs were identified among the sequenced *E. coli* isolates, of which the three most abundant were the zinc-resistance gene *zntA* (97.9%; n = 47), the nickel- and cobalt-resistance gene *rcnA* (93.8%; n = 45), and the arsenic-resistance gene *arsB* (81.3%; n = 39). Other identified MRGs included the

arsenic-resistance genes *arsC*, and *arsA* found in 4.2 and 2.1% of isolates, respectively, the copper-resistance genes *pcoA*, *pcoB*, *pcoC*, and *pcoD* carried by 6.3, 4.2, 2.1 and 2.1% of isolates, respectively, the mercury-resistance gene *merA* (2.1%; n = 1), and the zinc-resistance gene *rcnB* (2.1%; n = 1). All tested isolates carried at least one MRG, with *arsB-rcnA-zntA* being the most common combination (68.8%; n = 33), followed by *rcnA-zntA* (14.6%; n = 7); all other MRG combinations occurred just once (see **Supplementary data**).

Table 5.2: Summary of whole genome sequence results for all tested isolates (n = 48)

Sequence types (n)	Phylotypes (n)	Plasmid replicons (n)	Integrase (n)	Virulence genes (n)	ARGs (n)	MRGs (n)
155 (**3**), 906 (**3**), 1079 (**3**), 10 (**2**), 58 (**2**), 187 (**2**), 196 (**2**), 95 (**1**), 101 (**1**), 129 (**1**), 164 (**1**), 297 (**1**), 345 (**1**), 401 (**1**), 642 (**1**), 681 (**1**), 685 (**1**), 753 (**1**), 765 (**1**), 1056 (**1**), 1086 (**1**), 1122 (**1**), 1131 (**1**), 1152 (**1**), 1170 (**1**), 1621 (**1**), 1643 (**1**), 2002 (**1**), 2536 (**1**), 2538 (**1**), 2853 (**1**), 4382 (**1**), 5597 (**1**), 5662 (**1**), 8621 (**1**), unknown (**3**)	B1 (**30**), E (**5**), A (**4**), B2 (**4**), D (**4**), A/B1 (**1**)	*IncFIB* (**34**), *ColRNAI* (**16**), *IncFIA* (**13**), *IncFII* (**8**), *Col156* (**8**), *IncI1* (**7**), *IncFIC* (**5**), *IncB/O/K/Z* (**4**), *IncY* (**4**), *Col(MG828)* (**1**), *IncP1* (**1**), *IncR* (**1**), *Col(Ye4449)* (**1**), *IncHI1A* (**1**), *IncHI1B* (**1**), *Col440I* (**1**), *IncX1* (**1**), *IncX3* (**1**), *p0111* (**1**), NONE (**7**)	*IntI1* (**1**)	*astA* (**10**), *ctd* (**4**), *cnf2* (**3**), *hylA* (**2**), *subA* (**1**), *espL2* (**1**), *stx2* (**1**)	*strA* (**7**), *strB* (**6**), *blaTEM* (**6**), *sul2* (**6**), *tetA* (**4**), *floR* (**2**), *tetB* (**1**), *tetY* (**1**), *dfrA5* (**1**)	*zntA* (**47**), *rcnA* (**45**), *arsB* (**39**), *pcoA* (**3**), *pcoC* (**2**), *arsC* (**2**), *arsA* (**1**), *pcoC* (**1**), *pcoD* (**1**), *rcnB* (**1**), *merA* (**1**)

ARG=antimicrobial resistance gene; MRG=metal resistance gene

Genetic Elements

Approximately 85% of sequenced *E. coli* isolates harboured one or more plasmids (85.4%; n = 41 – see **Table 5.2**), of which 90.2% carried IncF (n = 37/41) and 46.3% carried Col (n = 19/41) replicon types, while just one isolate was identified as harbouring a Class 1 integrase: *intI1* (see isolate 23 in **Table 5.3**). Isolate 23 was collected from a hand-dug well in a pig farm; the only one in the current study. Among

the antimicrobial-resistant isolates, all of which carried plasmid(s), the most frequently detected replicons were *IncFIB* (n = 7/8), *IncI1* (n = 5/8) and *ColRNAI* (n = 4/8). With regards to the genomic locations of ARGs in the eight antimicrobial-resistant isolates, Platon findings show that most were in plasmid contigs, except for *sul2,* which were located in chromosome contigs in two of the six isolates where it was present (as shown in **Table 5.3**). Conversely, all MRGs from the resistant *E. coli*, except one (i.e., *merA* in isolate 23), were in chromosome contigs. Further investigation of the composite transposon identified in isolate 23 found that it was the same (100% identity score using Blastn analysis) as a transposon found on an *E.coli* plasmid (pRes13-Lact-PEB17-18) from pig faeces (Poulin-Laprade et al., 2021). Comparison of the plasmid sequence to the sequence of isolate 23 shows high levels of similarity (including a contig containing the mercury resistance operon). This indicates that in Isolate 23, the five identified ARGs (i.e., *dfrA5_1*, *blaTEM-1B_1*, *aph(6)-Id_1*, *aph(3'')-Ib_5*, and *sul2_3*) and *merA* MRG are co-located on the same plasmid, such was not the case for any of the other isolates in this study.

Table 5.3: Summary of whole genome sequence results related to the antimicrobial* resistant *E. coli isolates (n = 8)

N	ST	Phylotype	Plasmid Replicons	Integrase	VGs	ARGs		MRGs	
						chromosome	plasmid	chromosome	plasmid
6	155	B1	*IncI1*, *IncFIA*, *IncFIB*	-	-	-	*floR_2*, *aph(6)-Id_1*, *aph(3'')-Ib_2*, *sul2_2*, *tet(A)_6*, *blaTEM-1B_1*	*rcnA*, *arsB*, *arsC*, *zntA*	-
11	164	B1	*IncFIA*, *IncFIB*, *IncI1*, *ColRNAI*	-	-	*su2_14*	*tet(A)_6*, *aph(3'')-Ib_2*], *aph(6)-Id_1*, *blaTEM-1B_1*	*rcnA*, *arsB*, *zntA*	-
20	58	B1	*IncI1*, *IncFIB*	-	*astA*, *cdt*, *cnf2*	-	*tet(B)_2*, *aph(3'')-Ib_2*	*arsB*, *rcnA*, *zntA*	-
23	58	B1	*IncFII*, *IncFIB*, *Col156*, *ColRNAI*, *Col(MG828)*	*intI1*	-	-	*dfrA5_1*, *blaTEM-1B_1*, *aph(6)-Id_1*, *aph(3'')-Ib_5*, *sul2_3* [50]	*arsB*, *arsC*, *zntA*, *rcnA*	*merA*
32	1079	B1	*IncI1*, *IncY*, *IncFIB*	-	-	*su2_14*	*tet(A)_6*, *aph(3'')-Ib_2*, *aph(6)-Id_1*, *blaTEM-1B_1*	*rcnA*, *arsB*, *zntA*	-
33	10	A	*IncI1*, *IncFIB*, *ColRNAI*	-	*cdt*, *cnf2*	-	*tet(A)_6*, *sul2_14*, *aph(6)-Id_1*, *aph(3'')-Ib_2*, *blaTEM-1B_1*	*rcnA*, *zntA*, *arsB*	-
36	685	A	*IncFIB*, *IncB/O/K/Z*, *ColRNAI*	-	-	-	*floR_2*, *tet(Y)_1*	*zntA*, *rcnA*, *arsB*	-
42	95	B2	*IncB/O/K/Z* [13]	-	*cdtABC*	-	*sul2_2*, *aph(3'')-Ib_5*, *blaTEM-1C_1*, *aph(6)-Id_1*	*rcnA*, *zntA*	-

N = isolate number (see also Fig. 3); ST = serotype; ARG = antimicrobial resistance gene; MRG = metal resistance gene; VG = virulence gene

Sequence types, phylotypes and virulence

Overall, 36 serotypes (STs) were detected among the *E. coli* isolates in the current study. The most prevalent STs were ST155 (n = 3), ST906 (n = 3) and ST1079 (n = 3), followed by ST10 (n = 2), ST58 (n = 2), ST187 (n = 2) and ST196 (n = 2); the 29 remaining types were not repeated (see **Table 5.2**). Three isolates of different unknown STs were also detected. STs identified for the eight resistant isolates were ST10, ST58 (n = 2), ST95, ST155, ST164, ST685, and ST1079 (**Table 5.3**). Isolates belonging to phylogroup B1 were most prevalent across all *E. coli* (62.5%; n = 30), followed by 10.4% in phylogroup E (n = 5) and 8.3% (n = 4) in A, B2 and D lineages, with a single isolate exhibiting an ambiguous classification (i.e., belonging to both A and B2 phylogroups). In the case of antimicrobial-resistant isolates, phylogroup B1 was once again the most prevalent (62.5%; n = 5/8), followed by groups A (25%; n = 2/8) and B2 (12.5%; n = 1/8).

One or more virulence genes were detected in 31.2% of the 48 tested isolates (n = 15) and 37.5% of the resistant-isolates (n = 3). As shown (**Table 5.2**), the most prevalent of these were *astA* (20.8%; n = 10), followed by *ctd* (8.3%; 4) *cnf2* (6.3%; 3), *hlyA* (4.2%; n = 2), and *subA*, *stx2* and *espL2* (each corresponding to 2.1%; n = 1). Notably, the shiga toxin virulence gene *stx2* was present in a single isolate belonging to phylogroup B1 (ST8621), this shiga toxin-producing *E. coli* (STEC) was susceptible to all antimicrobials tested and did not harbour ARGs. Another (also susceptible) B1 isolate (ST765) carried the *espL2* virulence gene and is thus considered an enterohemorrhagic *Escherichia coli* (EHEC; Bugarel et al., 2011). Both the STEC and EHEC isolates were found in drilled wells and carried *arsB*, *rcnA*, and *zntA* MRGs.

5.3.3 Assessing co-selective pressures for antimicrobial resistance

ARG and MRG co-occurrence in groundwater E. coli

A single isolate contained ARGs and an MRG (*merA*) co-located on the same plasmid (i.e., one co-resistant plasmid; Chapman, 2003). Additionally, as shown (**Table 5.4**), significant associations were identified between co-occurrence of the *arsC* MRG and *aph(3'')-Ib* (*strA*; *p*-value = 0.019), *aph(6')-Id* (*strB*; *p* = 0.013), *blaTEM* (*p* = 0.013), *dfrA5* (*p* = 0.042) and *sul2* (*p* = 0.013). Bivariate associations containing *drfA5*, *tet(Y)*, *pcoC*, *pcoD*, *merA*, and/or *rcnB* were not presented in **Table 5.4** as these genes were only detected once across all studied isolates.

Table 5.4: Fisher's exact test results for each ARG-MRG pair in the groundwater-derived E. coli

MRGs	ARGs						
	strA [a]	***strB*** [b]	***blaTEM***	***floR***	***sul2***	***tetA***	***tetB***
arsA	0.854	0.875	0.875	0.958	0.875	0.917	0.979
arsB	0.608	0.688	0.688	0.657	0.688	0.423	0.813
arsC	**0.019***	**0.013***	**0.013***	0.082**	**0.013***	0.161	0.958
pcoA	0.616	0.664	0.664	0.878	0.664	0.766	0.938
pcoB	0.727	0.763	0.763	0.918	0.763	0.839	0.958
rcnA	0.616	0.664	0.664	0.878	0.664	0.766	0.938
zntA	0.854	0.875	0.875	0.958	0.875	0.917	0.979

* $p<0.05$ (highlighted in bold); ** $0.05<p<0.1$; ARG = antimicrobial resistance gene, MRG = metal resistance gene; *drfA5*, *tet(Y)*, *pcoC*, *pcoD*, *merA*, and *rcnB* not included as they were carried by a single isolate; [a] encodes *aph(3'')-Ib*; [b] encodes *aph(6)-Id*

Associations between trace metal concentrations and ARG/MRG abundance in groundwater E. coli

Bivariate statistical test results (**Table 5.5**) were shown to approach significance in the associations between ARG abundance and arsenic concentrations (*p* = 0.084). No significance or approaching significance was found for relationships between

trace metal levels and the respective MRGs (Mann-Whitney, data not shown) or overall MRG abundance (Kruskal-Wallis, see **Table 5.5**).

Table 5.5: Kruskal-Wallis test results assessing metal magnitude associations with abundance of ARGs and MRGs in the groundwater-derived E. coli

Trace metals	ARG abundance		MRG abundance	
	Variable Coefficient	*p*-value	Variable Coefficient	*p*-value
Silver (Ag)	4.46	0.347	3.83	0.574
Arsenic (As)	8.22	0.084**	2.46	0.783
Cadmium (Cd)	3.18	0.528	4.61	0.465
Cobalt (Co)	2.83	0.587	2.36	0.797
Chromium (Cr)	5.89	0.207	5.53	0.355
Copper (Cu)	0.70	0.951	4.36	0.498
Iron (Fe)	1.05	0.902	9.16	0.103
Manganese (Mn)	7.21	0.125	2.46	0.783
Nickel (Ni)	0.66	0.956	4.18	0.524
Lead (Pb)	1.62	0.805	2.86	0.722
Antimony (Sb)	7.48	0.112	5.72	0.335
Zinc (Zn)	2.09	0.720	3.52	0.621

** $0.05 < p < 0.1$; ARG = antimicrobial resistance gene; MRG = metal resistance genes

5.4 Discussion

AMR has been recognised as a key global health issue by the WHO (WHO, 2014; WHO; 2022a) and primary emerging issue of environmental concern by the United Nations Environment Programme (UNEP, 2017). With that, extensive research has been undertaken to better understand the conditions facilitating/permitting the occurrence and dissemination of AMR, such as presence of co-selective contaminants (e.g., metals/metalloids) in soil (Knapp et al., 2011; Hu et al., 2017), surface (Wright et al., 2006; Martins et al., 2014; Xu et al., 2016) and groundwater environments (Zou et al., 2021; Liu et al., 2022). However, recent reviews highlight the need for more studies which investigate 'cleaner' environments of human health significance and

that measure co-selective pressures on specific bacterial isolates (Pal et al., 2017; Imran et al., 2019; Nguyen et al., 2019). The present study sought to fill the first gap by studying domestic groundwater resources in the RoI, which are present in agricultural areas with no noteworthy point-sources of antimicrobial/metal pollution (e.g., hospitals, wastewater treatment plants, mines, and industrial sites). The *E. coli* isolates detected were tested for encoded ARGs and MRGs, and water samples were analysed for levels of potentially co-selective elements (i.e., metals/metalloids). Statistical analyses were further employed to identify potential relationships between metal concentrations and abundance of MRGs and ARGs in the groundwater-derived *E. coli*, and between ARG-MRG co-occurrence.

Of the 48 *E. coli* isolates examined, 16.7% (n=8) and 12.5% (n=6) harboured ≥ 1 and ≥ 3 ARGs, respectively, amounting to ten distinct ARGs: *aph(3'')-Ib* (*strA*; n=7), *aph(6)-Id* (*strB*; n=6), *blaTEM* (n=6), *sul2* (n=6), *tetA* (n=4), *floR* (n=2), *dfrA5* (n=1), *tetB* (n=1), and *tetY* (n=1). Conversely, all tested isolates (n=48) harboured one or more MRGs (e.g., *zntA*=47, *rcnA*=45, *arsB*=39, and others). This is unsurprising, as metal concentrations are typically much higher than that of antimicrobial residues in natural environments associated with agricultural activities (Guo et al., 2018; Veiga-Gómez et al., 2021). Of all MRGs found among the resistant-*E. coli,* just one was located in a plasmid (i.e., *merA*), implying that, for the most part, MRG-carrying resistant isolates have adapted to metal-contaminated environments (Remenar et al., 2018). This is not reflected, however, in the dissolved metal concentrations reported here (see **Table 5.1B**), with maximum values (not taking bioavailability or particulate mass into account) being lower than what are considered toxic to *E. coli* (i.e., Ag=5 µg/L, Cd=100 µg/L, Co=350 µg/L, Cu=400 µg/L, Ni=550 µg/L, Zn=200 µg/L;

Hoegler and Hecht, 2018). Compounding this, no statistical significance was found between metal concentrations and presence of the relevant MRG(s) or overall MRG abundance in the tested isolates. As such, findings suggests that MRG acquisition is likely taking place outside of these groundwater environments. Within agricultural settings, manure-treated soils and grasslands, for example, can provide sufficiently selective environments to drive metal resistance (Peng et al., 2019; Shi et al., 2019).

Two studies have previously described minimum cadmium, cobalt, copper, lead, nickel, and zinc concentrations correlated with increased detection of antimicrobial resistance in bacteria (i.e., minimum co-selective concentration; MCC) within surface water environments (Wright et al., 2006; Stepanauskas et al., 2005). Based on these, Seiler and Berendonk (2012) determined MCCs for these metals in water (i.e., Cd=0.03 µg/L, Co=0.05 µg/L, Cu=1.5 µg/L, Ni=0.29 µg/L, Pb=0.15 µg/L; Zn=19.61 µg/L). Notably, median concentrations found in the 8 water samples containing resistant-*E. coli* exceeded MCCs for all metals except Cadmium (**Table 5.1B**). However, as MCC exceedances were also found in 39/40 water samples harbouring susceptible-*E. coli* (see **supplementary materials**) and in the absence of significant associations between metal/metalloid concentrations and encoded ARG abundance (**Table 5.5**) findings suggests that metal co-selection is unlikely to be taking place. Statistical analyses (**Table 5.3**) also indicate that *arsC*, an arsenate reductase gene (Hobman and Crossman, 2015) detected in two isolates, was significantly associated with the presence of five separate ARGs: *aph(3'')-Ib* (*strA*; p=0.019), *aph(6)-Id* (*strB*; p=0.013), *blaTEM* (p=0.013), *sul2* (p=0.013), and *dfrA5* (p=0.045). It is important to highlight, however, that association does not mean causation, and may merely indicate that *arsC* and the listed ARGs have a shared

origin, with arsenic identified as an unintentional livestock feed pollutant in the RoI (Mac Monagail, et al., 2018), in addition to geogenic soil sources in recharge waters. Moreover, a single isolate (isolate 23) displayed genetic associations between encoded mercury-resistance gene *merA* and five ARGs. The low sample numbers and very low number of positive AMR outcomes preclude definitive conclusions, however, and further investigations are required to examine these relationships. Undeniably, focusing solely on specific bacterial species may provide a limited picture of the local resistome (e.g., other bacterial species may be present which carry ARGs even in the absence of resistant-*E. coli*). Thus, a combined analytical approach employing (1) targeted quantitative polymerase chain reaction (qPCR) of ARGs within groundwater samples in addition to (2) WGS of local bacterial isolates (following phenotypic testing) is recommended to provide an increasingly holistic assessment of AMR and existing (co-)selective pressures in these environments. Briefly, qPCR results can provide a complete picture of the local resistome, while bacterial-specific genome sequences allow for the identification of co-resistance. It is worth mentioning that while metagenomics is a useful tool for determining the gene types and relative abundances in environmental samples, it may not be the optimal method in this case due to its high cost and the lower DNA yields expected from drinking (and supposedly 'clean') groundwater samples. Additionally, the authors highlight the importance of establishing MCCs specific to *E. coli*, as this species is the most widely used in water quality monitoring and surveillance, including in the field of groundwater contamination with AMR (Andrade et al., 2020).

Andrade et al. (2023) recently identified significant positive associations between presence of phenotypic AMR in tested *E. coli* and increased cattle density at

the local level and, from this, hypothesised cattle farming as the likely origin of resistant-*E. coli*. This is further supported by the genotypic results for the same isolates presented here (**Figure 5.2**), with prevalent ARGs being among the most abundant in previous studies of cattle-derived *E. coli* in the RoI (Karczmarczyk et al., 2011a; 2011b), namely: *aph(3'')-Ib* (*strA*), *aph(6)-Id* (*strB*), *blaTEM*, *sul2*, and *tetA*. All five prevalent genes confer resistance to veterinary antimicrobials, with streptomycin, tetracycline, and ampicillin antimicrobials, in particular, among the most commonly prescribed in bovine agriculture in RoI (Gibbons et al., 2013). Thus, and in light of the apparent absence of metal co-selection, it is plausible to suppose that ARG-acquisition is taking place inside these mammalian hosts and ARG-carrying isolates are transported to groundwater via faecal ingress (e.g., direct wellhead ingress of contaminated surface run-off; Andrade et al., 2023) owing, for example, to inappropriate faeces storage, manure/slurry spreading, and animal excretion while grazing. However, specific investigations quantifying and examining the impact of both trace metal/metalloids and antimicrobial residue concentrations in *E.coli*-borne ARG abundance are needed to confirm this.

Examining the antimicrobial resistant STs (see **Table 3**), it is also apparent that two of the seven have been previously associated exclusively with animals (including livestock), namely: ST164 (Wang et al., 2020), and ST1079 (Barth et al., 2016; Apostolakos at al., 2017), while the other five have been found in both humans and farm animals (i.e., poultry, pigs, horses, and/or cattle) – i.e., ST10 (Manges et al., 2015), ST58 (Reid et al., 2022), ST95 (Manges et al., 2015), ST155 (Aworh et al, 2021), and ST685 (Souverein et al., 2016; Apostolakos at al., 2017). Additionally, 86.5% of resistant-*E. coli* isolates (n=7/8) belonged to A or B1 phylogenetic classification

groups, which were also the most common in *E. coli* from Irish cattle – accounting for 90, 86, and 79% in cattle from healthy farm herds (Karczmarczyk et al., 2011a), those with clinical mastitis symptoms (Keane, 2018), and from veterinary hospitals (Karczmarczyk et al., 2011b), respectively. *E. coli* belonging to phylogroup B1 have also been most frequently detected from surface waters affected by livestock agriculture internationally (Berthe et al., 2013; Tymensen et al., 2015). Although phylogenetic groups B2 and D have a greater frequency and diversity of virulence traits (Johnson et al., 2001), phylogroups A and B1 have also been isolated from humans, accounting for 46, 40 and 33% of human-derived *E. coli* in France (Escobar-paramo et al., 2004), Sweden (Nowrouzian et al., 2005) and Canada (Tropea et al., 2021), respectively, as well as other animals (Tenaillon et al., 2010; Tropea et al., 2012). Indeed, isolate number 23, which belonged to phylogroup B1 (ST58), was collected from a hand-dug well on a pig farm (the only one in the present study) and, thus, is likely of porcine (not bovine) origin. In line with this finding, it is worth noting that antimicrobial prescription rates for pig farming is the highest of all livestock activities in the RoI (despite cattle being the predominant livestock sector), with data from 2016 research and surveillance (combined) showing that the pig sector used over 42% of all veterinary antimicrobials sold that year (Martin et al., 2020).

Resistant-*E. coli* presence within groundwater sources used for human consumption, particularly in the absence of microbiological treatment such as is the case here, can lead to potential adverse health consequences. These are even more pronounced when considering that 31.2% of all *E. coli* isolates (n=15) and 37.5% of the resistant-*E. coli* (n=3) carried virulence genes (i.e., have the potential to cause infections). This similar distribution of virulence genes between AMR and non-AMR

strains may relate to the low relative incidence of virulence in so-called commensal intestinal *E. coli* strains from phylogenetic groups A and B1, (Clermont et al, 2011, Cocchi et al, 2007) which comprised 7 of 8 (87.5%) AMR isolates. Susceptible pathogens included one ETEC (ST765 carrying the *espL2* virulence gene) and one STEC (ST8621 carrying the *stx2* virulence gene) isolate, with STEC recently shown to be associated with human sources of contamination (i.e., septic tanks) in a recent study of private wells in the RoI (Burke et al., 2023). Notedly, ST10, ST95 and ST58 seen in four resistant isolates (i.e., 50%) are among the most reported extraintestinal pathogenic *E. coli* globally (Manges et al., 2019). Such highly successful clones are adapted to colonisation and infection and thus can contribute significantly to the dissemination and increased health burden of AMR to humans and animals.

A previous study carried out in Canada reported that those using groundwater supplies harbouring resistant-*E. coli* were 1.26 times more likely to become colonised with resistant-*E. coli* (i.e., carry them in their gastrointestinal tract; Coleman et al., 2012), thus furthering the spread of AMR beyond the groundwater environment, as once in the human gut the ARG-carrying plasmids can be shared with other commensal, and potentially pathogenic, bacteria (Christaki et al., 2020). Human and animal hosts are also at an increased risk of resistant infection(s) upon colonisation with antimicrobial resistant bacteria (Tacconelli et al., 2009). This further highlights the importance of One Health studies which investigate environments of human significance regardless of their contamination level, as they may still pose significant health risks to individuals and be noteworthy contributors to the spread of AMR (Larsson and Flach, 2022). Such is also true for countries/regions with presumably larger sources of AMR and/or metals, as purposely focusing sampling solely in high

susceptibility areas may lead to skewed "worst case scenario" findings and, as such, provide unrealistic baseline data.

With this being an exploratory study of a lesser-contaminated environment a key limitation stems from the low number of *E. coli*-positive samples (i.e., 48 in total, and just 8 ARG-carrying isolates) which restricted statistical analyses and interpretation of findings. Another limitation is the absence of antimicrobial residue analyses, owing to resource constraints, which precluded the analyses of selective pressures. Finally, it must be acknowledged, that further limitations stem from lack of information regarding ARG abundance/diversity at a community level (i.e., present in total DNA extracted from groundwater) such as could be obtained through targeted qPCR, and which would have allowed for a more complete picture of the local resistome. It is evident that heavily contaminated environments have a more pronounced impact in the overall dissemination of AMR, however the current study highlights that even presumably 'cleaner' groundwater environments, when of human significance, are still relevant reservoirs for antimicrobial resistant bacteria. Indeed, the assumption that resistance acquisition may be taking place outside of these groundwater supplies means that this route of AMR exposure is highly preventable by ensuring appropriate source protection measures, such as those outlined in Andrade et al. (2023).

5.5 Conclusion

The present study analysed data from 48 *E. coli*-harbouring domestic groundwater supply samples in rural areas of the RoI. Findings show no significant association between (overarchingly low) metal concentrations and abundance of

ARG (or MGE) carried by the groundwater *E. coli* and suggest that the bacteria may have acquired the resistance genes prior to groundwater ingress. Genomic analysis results also show just one case of co-resistance (i.e. ARGs and MRGs located on the same plasmid) and no instances of re-occurring resistant-*E. coli* contamination. Therefore, these environments may be less likely to become long-term (i.e., continuous) AMR reservoirs, which is a key concern for more exposed fresh and marine water environments. However, while co-selective pressures may not be present, rural groundwater supplies are still noteworthy pathways for the dissemination of AMR insofar as they are commonly used for human consumption both in the RoI and abroad. A key recommendation from the current study is that future research combines qPCR with WGS of specific bacterial isolates, where possible, to better quantify AMR outcomes and (co)-selective relationships. The authors also emphasise the importance of carrying out such investigations in groundwater environments of human significance worldwide and regardless of their presumed level of exposure to antimicrobial drugs, antimicrobial resistant bacteria, and/or co-selective contaminants, as gathered information can help inform holistic/One Health AMR action plans and safeguard human (and animal) health.

Author contributions

Luisa Andrade: Conceptualization, Methodology, Investigation, Formal analysis, Data Curation, Visualisation, Writing - original draft, Writing – review & editing. **Michael P. Ryan**: Formal analysis, Validation, Resources, Writing – Review & Editing. **Liam Burke**: Data Curation, Formal analysis, Resources, Writing – Review & Editing. **Paul Hynds**: Conceptualization, Writing - review & editing, Funding

acquisition, Supervision. **John Weatherill**: Conceptualization, Writing - review & editing, Supervision, Funding acquisition. **Jean O'Dwyer**: Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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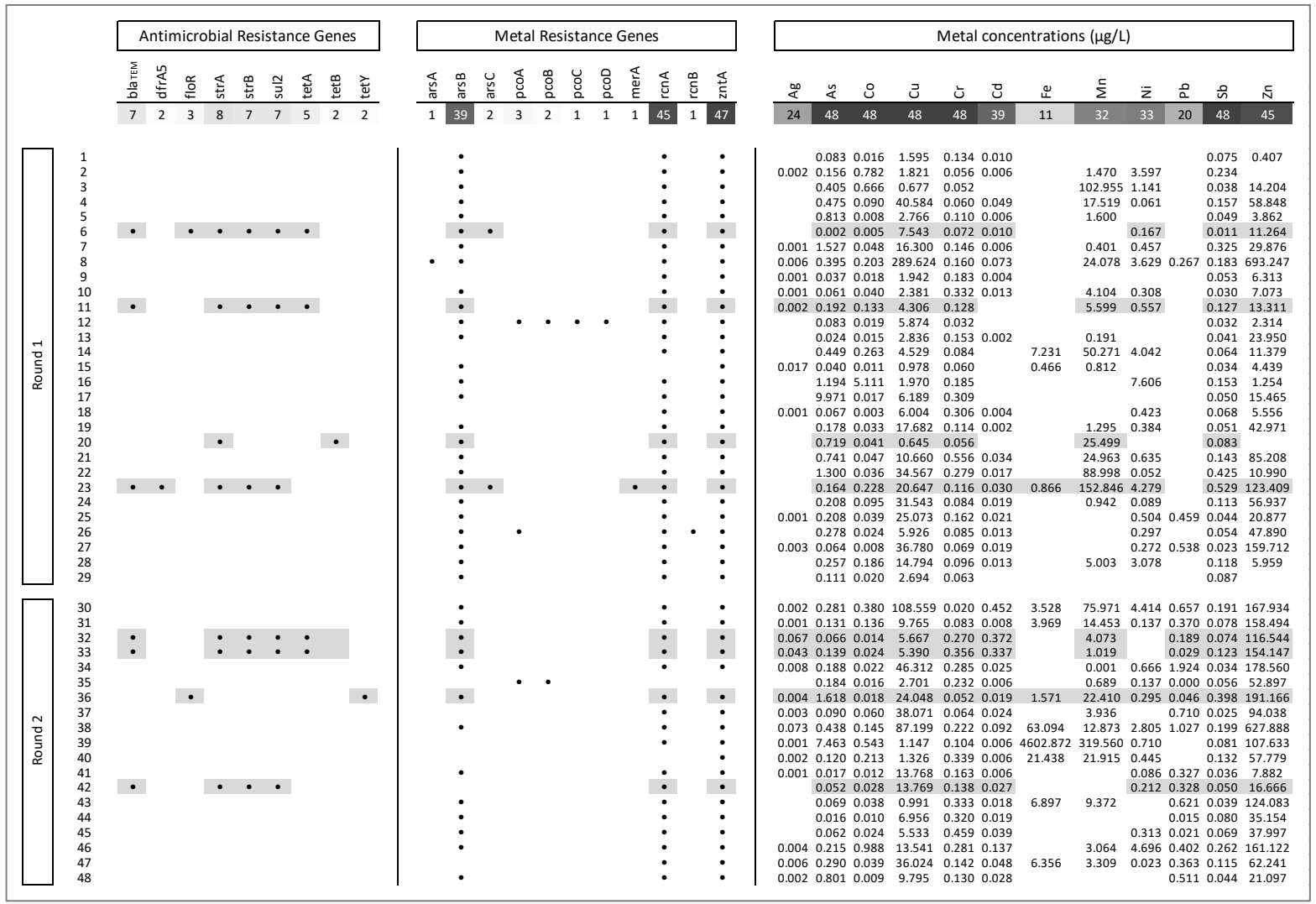
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Supplementary Data

Antimicrobial and metal resistance genes carried by E. coli isolates and metal concentrations in the E. coli-contaminated groundwater samples



		Antimicrobial Resistance Genes									Metal Resistance Genes										Metal concentrations (μg/L)											
		bla_{TEM}	dfrA5	floR	strA	strB	sul2	tetA	tetB	tetY	arsA	arsB	arsC	pcoA	pcoB	pcoC	pcoD	merA	rcnA	rcnB	zntA	Ag	As	Co	Cu	Cr	Cd	Fe	Mn	Ni	Pb	Sb	Zn
		7	2	3	8	7	7	5	2	2	1	39	2	3	2	1	1	1	45	1	47	24	48	48	48	48	39	11	32	33	20	48	45
Round 1	1											•							•		•		0.083	0.016	1.595	0.134	0.010					0.075	0.407
	2											•							•		•	0.002	0.156	0.782	1.821	0.056	0.006		1.470	3.597		0.234	
	3											•							•		•		0.405	0.666	0.677	0.052			102.955	1.141		0.038	14.204
	4											•							•		•		0.475	0.090	40.584	0.060	0.049		17.519	0.061		0.157	58.848
	5											•							•		•		0.813	0.008	2.766	0.110	0.006		1.600			0.049	3.862
	6	•		•	•	•	•	•				•	•						•		•		0.002	0.005	7.543	0.072	0.010			0.167		0.011	11.264
	7											•							•		•	0.001	1.527	0.048	16.300	0.146	0.006		0.401	0.457		0.325	29.876
	8										•	•							•		•	0.006	0.395	0.203	289.624	0.160	0.073		24.078	3.629	0.267	0.183	693.247
	9											•							•		•	0.001	0.037	0.018	1.942	0.183	0.004					0.053	6.313
	10											•							•		•	0.001	0.061	0.040	2.381	0.332	0.013		4.104	0.308		0.030	7.073
	11	•			•	•	•	•				•							•		•	0.002	0.192	0.133	4.306	0.128			5.599	0.557		0.127	13.311
	12											•		•	•	•	•		•		•		0.083	0.019	5.874	0.032						0.032	2.314
	13											•							•		•		0.024	0.015	2.836	0.153	0.002		0.191			0.041	23.950
	14											•							•		•		0.449	0.263	4.529	0.084		7.231	50.271	4.042		0.064	11.379
	15											•									•	0.017	0.040	0.011	0.978	0.060		0.466	0.812			0.034	4.439
	16											•							•		•		1.194	5.111	1.970	0.185				7.606		0.153	1.254
	17											•							•		•		9.971	0.017	6.189	0.309						0.050	15.465
	18																		•		•	0.001	0.067	0.003	6.004	0.306	0.004			0.423		0.068	5.556
	19											•							•		•		0.178	0.033	17.682	0.114	0.002		1.295	0.384		0.051	42.971
	20				•				•			•							•		•		0.719	0.041	0.645	0.056			25.499			0.083	
	21											•							•		•		0.741	0.047	10.660	0.556	0.034		24.963	0.635		0.143	85.208
	22											•							•		•		1.300	0.036	34.567	0.279	0.017		88.998	0.052		0.425	10.990
	23	•	•		•	•	•					•	•					•	•		•		0.164	0.228	20.647	0.116	0.030	0.866	152.846	4.279		0.529	123.409
	24											•							•		•		0.208	0.095	31.543	0.084	0.019		0.942	0.089		0.113	56.937
	25											•							•		•	0.001	0.208	0.039	25.073	0.162	0.021			0.504	0.459	0.044	20.877
	26											•		•					•	•	•		0.278	0.024	5.926	0.085	0.013			0.297		0.054	47.890
	27											•							•		•	0.003	0.064	0.008	36.780	0.069	0.019			0.272	0.538	0.023	159.712
	28											•							•		•		0.257	0.186	14.794	0.096	0.013		5.003	3.078		0.118	5.959
	29											•							•		•		0.111	0.020	2.694	0.063						0.087	
Round 2	30											•							•		•	0.002	0.281	0.380	108.559	0.020	0.452	3.528	75.971	4.414	0.657	0.191	167.934
	31											•							•		•	0.001	0.131	0.136	9.765	0.083	0.008	3.969	14.453	0.137	0.370	0.078	158.494
	32	•			•	•	•	•				•							•		•	0.067	0.066	0.014	5.667	0.270	0.372		4.073		0.189	0.074	116.544
	33	•			•	•	•	•				•							•		•	0.043	0.139	0.024	5.390	0.356	0.337		1.019		0.029	0.123	154.147
	34											•							•		•	0.008	0.188	0.022	46.312	0.285	0.025		0.001	0.666	1.924	0.034	178.560
	35													•	•								0.184	0.016	2.701	0.232	0.006		0.689	0.137	0.000	0.056	52.897
	36			•						•		•							•		•	0.004	1.618	0.018	24.048	0.052	0.019	1.571	22.410	0.295	0.046	0.398	191.166
	37											•							•		•	0.003	0.090	0.060	38.071	0.064	0.024		3.936		0.710	0.025	94.038
	38											•							•		•	0.073	0.438	0.145	87.199	0.222	0.092	63.094	12.873	2.805	1.027	0.199	627.888
	39											•							•		•	0.001	7.463	0.543	1.147	0.104	0.006	4602.872	319.560	0.710		0.081	107.633
	40																		•		•	0.002	0.120	0.213	1.326	0.339	0.006	21.438	21.915	0.445		0.132	57.779
	41											•							•		•	0.001	0.017	0.012	13.768	0.163	0.006			0.086	0.327	0.036	7.882
	42	•			•	•	•					•							•		•		0.052	0.028	13.769	0.138	0.027			0.212	0.328	0.050	16.666
	43											•							•		•		0.069	0.038	0.991	0.333	0.018	6.897	9.372		0.621	0.039	124.083
	44											•							•		•		0.016	0.010	6.956	0.320	0.019				0.015	0.080	35.154
	45											•							•		•		0.062	0.024	5.533	0.459	0.039			0.313	0.021	0.069	37.997
	46											•							•		•	0.004	0.215	0.988	13.541	0.281	0.137		3.064	4.696	0.402	0.262	161.122
	47																		•		•	0.006	0.290	0.039	36.024	0.142	0.048	6.356	3.309	0.023	0.363	0.115	62.241
	48											•							•		•	0.002	0.801	0.009	9.795	0.130	0.028				0.511	0.044	21.097

Chapter 6

Conclusions and Recommendations

6.1 Key findings

The presented research investigates the role and potential determinants of groundwater resources as reservoirs for antimicrobial resistant bacteria (ARB), with a focus on supplies used for consumption in the RoI. This was achieved through a combination of robust systematic review and pooled analyses of international literature (**Chapter 2**), the analyses of RoI-specific monitoring data for (regulated) groundwater supplies over a 10-year period provided by the National Groundwater Monitoring Programme (**Chapter 3**), and the collation and analyses of samples from 132 geographically dispersed field sites (i.e., unregulated groundwater supplies) in the RoI (**Chapters 4** and **5**). Throughout this body of work several research questions (RQs) have been answered, these are detailed below.

6.1.1 Can groundwater resource contamination with ARB be considered a global issue? (RQ1)

The systematic review and pooled analysis (**Chapter 2**) reveals that antimicrobial resistance (AMR) rates amongst groundwater-derived isolates were consistently high across the seventy included studies, which were undertaken in geographically, climatologically, and socioeconomically diverse regions across the globe. Indeed, 80.2% ± 29.0 (median = 100% (IQR = 59.9 - 100.0%)) of groundwater isolates were resistant or intermediately-resistant to ≥ 1 antimicrobial agent, while 57.2% ± 36.8 (57.7% (22.2 – 100.0%)) were classified as resistant or intermediately-resistant to ≥ 3 antimicrobials. These findings, in combination with mounting evidence of widespread bacterial contamination of groundwater resources (Hynds et al., 2014; Pandey et al., 2014; **Chapter 3**) which can lead to outbreaks of infection

worldwide (Murphy et al., 2017), leave little doubt as to the global significance and public health issues posed by ARB contamination of groundwater resources. Findings presented in **Chapter 2** also suggest that this groundwater contamination issue is significantly more pronounced in lower-income countries, supplies located in urban and peri-urban settlements, and dry, arid regions. However, a lack of consistent hydrogeological, meteorological, climatic, and infrastructural data reporting precluded identification of more in-depth global trends and key risk factors.

6.1.2 Is ARB contamination of groundwater supplies an issue in Ireland? (RQ2)

Chapter 3 highlighted that over a 10-year monitoring period, faecal bacteria (i.e., *E. coli*) were detected at least once in 66.7% of regulated groundwater supplies in the RoI. Such conclusion gives rise to the possibility of resistance presence, as *E. coli*, antimicrobials, and ARB have shared sources: human and animal faeces, and with pastoral agriculture, in particular, having been found a key risk factor for *E. coli* presence. This possibility is further highlighted in light of systematic review findings (**Chapter 2**). The review found that when present, 80.2% ± 29.0 (median = 100% (IQR = 59.9 - 100.0%)) of groundwater bacteria at a global level were resistant or intermediately resistant to at least one antimicrobial. Compounding this, RoI-specific mid-west focused research by O'Dwyer et al. (2017) found that all *E. coli* deriving from unregulated supplies (i.e., private wells) were resistant to at least one antimicrobial. The more geographically representative research described in **Chapter 4**, however, found ARB in just 6.1% of the 132 (unregulated) private wells sampled and in 3.2% of the 250 samples collected.

Despite the relatively low detection of ARB described in **Chapters 4** (and indeed **Chapter 5**), their occurrence in unregulated drinking water resources still represent substantial risks to human health. The potential adverse health effects are even more pronounced considering 31.2% of all *E. coli* isolates (n = 15) and 37.5% of the resistant *E. coli* (n = 3) harboured virulence genes (i.e., have the potential to cause infections) (**Chapter 5**), with human (and animal) hosts at an increased risk of resistant infection(s) upon colonisation with an ARB (Christaki et al., 2020). Additionally, human and animal colonisation with resistant *E. coli* from exposure to contaminated groundwater supplies contributes to the spread of AMR beyond the groundwater environment, as once in the human gut the ARGs can be transferred to other commensal, and potentially pathogenic, bacteria (Christaki et al., 2020). Therefore, ARB presence within these domestic supplies certainly represents an environmental as well as a human (and animal) health issue in the RoI.

6.1.3 Which factors may be influencing ARB occurrence in Irish groundwater supplies? (RQ3)

The logistic regression model presented in **Chapter 4** indicates three principal determinants significantly influencing the occurrence of ARB in the studied groundwater supplies. These were (1) local groundwater vulnerability, (2) local cattle density, and (3) well type. In summary, the risk of ARB contamination is higher for (i) supplies located in "low" as opposed to "high" (OR = 333.333, p = 0.022), "extreme" (OR = 25.641, p = 0.049) and "extreme-rock/karst" (OR = 37.037, p = 0.041) groundwater vulnerability areas, (ii) supplies in areas of above-mean cattle density

(i.e., above 161 heads/km^2) (OR = 1.028, p = 0.037), and (iii) hand-dug as opposed to drilled supplies (OR = 41.071, p = 0.050).

6.1.4 What is/are the likely source(s) of ARB to Irish (rural) groundwater supplies? (RQ4)

The most likely source of antimicrobial resistant *E. coli* in the investigated supplies was cattle farming, with multivariate modelling results presented in **Chapter 4** indicating that while *E. coli* presence (54/250 samples) was significantly associated with both human (DWWTS present within 30 m of well; OR = 5.090, p < 0.001) and pastoral agricultural sources (cattle density; OR = 1.010, p = 0.011), only cattle density was significantly associated with the presence of resistant *E. coli* (8/250; OR = 1.028, p = 0.037). It is important to highlight, however, that genotypic analyses later showed one resistant isolate as of inferred porcine (as opposed to bovine) origin (**Chapter 5**).

Associations between livestock-based agriculture and antimicrobial resistant bacteria is also corroborated by highest resistance rates found to veterinary antimicrobials (i.e., streptomycin = 14.6%, tetracycline = 12.5%, and ampicillin = 12.5%), and encoded ARGs and resistant *E. coli* serotypes representing those commonly isolated from bovine and livestock animals in Ireland and abroad (as shown in **Chapter 5**). Lack of resistance among groundwater-derived *P. aeruginosa* (12/250 samples), which have been linked with human activities in **Chapter 4** as well as previous work (Crone et al., 2020), also confirms that ARB from human sources are 20considerably less prominent groundwater contaminants in the sparsely populated environments of rural Ireland (i.e., ≈ 27 people/km^2; CSO, 2016).

6.1.5 What is/are the likely pathway(s) of ARB into the studied supplies? (RQ5)

The likely resistant *E. coli* transport pathways were inferred through interpretation of final multivariate models presented in **Chapter 4**. Significantly higher likelihood of ARB presence were observed in areas characterised by "low" groundwater vulnerability (where low permeability layers of clay soil and subsoil can prevent contaminant percolation and recharge), and where the well cap was not appropriately sealed (significant at the 90% confidence level; OR = 6.452, p = 0.098). Results point to the primary ingress mechanism (i.e., pathway) associated with presence of resistant *E. coli* in Irish rural supplies being overland flow followed by direct wellhead ingress, contrasting with the *traditional* recharge hypothesis posed in **Chapter 2** which was based on combined worldwide data from rural and urban settings.

It is also worth noting that the odds of *P. aeruginosa*, *E. coli*, and resistant *E. coli* detection were significantly higher for hand-dug than drilled wells (OR = 18.902, 7.199, and 41.071, respectively), thus confirming the inherent contamination susceptibility of hand-dug wells (Hynds et al., 2012; Maran et al., 2016) and indicating higher likelihood of localised (i.e., confined to the well) rather than widespread aquifer contamination. Moreover, lack of repeat (i.e., re-occurring) ARB presence between sampling rounds points to the event-based nature of this mechanism.

6.1.6 Are the studied groundwater environments reservoirs for AMR? (RQ6)

Results presented in **Chapter 5** indicate that the rural (domestic) groundwater supplies under investigation unlikely to become *long-term* (i.e., continuous) AMR reservoirs, which is a key concern for more exposed fresh and marine water

environments (Di Cesare et al. 2015; Squadrone et el., 2020) and may still be the case within urban groundwater resources – found to be significantly more contaminated with ARB and MRB than those in rural settlements (**Chapter 2**). This is substantiated by the lack of significant association between (overarchingly low) concentrations of co-selective contaminants (i.e., metals/metalloids) and abundance of *E. coli*-encoded ARGs (**Chapter 5**), coupled with a supposed low likelihood of antimicrobial residues presence within these rural groundwater supplies at sufficient selective concentrations (see studies in similar environments: Kivits, et al., 2018; Gros et al., 2021; Veiga-Gómez et al., 2021). However, despite the potential absence of (co-)selective pressures, presence of ARB in 6.1% of the private wells investigated in **Chapter 4** shows that these (unregulated) rural supplies can still be considered potential AMR reservoirs, even if just for a limited time.

6.1.7 Can human exposure to AMR via groundwater resources be prevented? (RQ7)

The conclusion that ARB contamination is more pronounced than selective (i.e., antimicrobial residues) or co-selective (i.e., metal/metalloids) compounds indicates that AMR exposure via these (domestic) groundwater supplies is preventable through appropriate source protection practices (and installation of microbial water treatment systems). The models presented in **Chapter 4**, for example, present three key ARB-preventing measures: (i) favouring drilled (rather than hand-dug) wells, (ii) ensuring appropriate setback distances to potential sources of contamination (i.e., DWWTS and livestock agriculture), and (iii) fitting an appropriately sealed cap. As an additional layer of protection, microbiological water treatment options are also available at the required (domestic) scale, such as UV treatment and reverse osmosis.

Of those, reverse osmosis presents the superior option, as it can remove most ARGs present in addition to ARB (Slipko et al., 2019).

6.2 Limitations

It is important that research conclusions are considered in the context of existing, and at times inherent, research limitations; thus, these have been explicitly described as part of the discussion sub-sections within each publication chapter with key aspects reiterated here. In **Chapter 2**, inherent limitations stem from the potential for bias within the data reported in the included peer-reviewed research, such as studies targeting groundwater sources where/when ARB contamination was suspected. Studies' validity appraisal was carried out to minimise this issue, however it is still conceivable that occurrence rates of ARB and MRB in global groundwater garnered from this review may represent an overestimation, particularly as there was a noted lack of explicit methodology descriptions for achieving sample representativeness. Concerning **Chapter 3** key constraints arose from the noted gaps in the spatial and temporal distribution of the available monitoring data. This precluded the definite identification of temporal peaks and the hydrogeological impacts on *E. coli* detection levels - which may have been, in some instances, overestimated. With regards to **Chapter 4**, it must be acknowledged that the variances in the resistant *E. coli* presence outcome were not fully explained by the independent variables in the regression model (i.e., Nagelkerke R^2 = 0.475). This is likely attributable to the absence of potentially significant environment-specific factors outside of the scope of this research, such as presence of chemical co-pollutants (e.g., antimicrobial residues, toxic metals, biocides, and disinfectants),

biofilm forming organisms, heightened organic matter levels, microplastics, etc. In **Chapter 5** absence of antimicrobial residue analyses (owing to resource constraints) represented a clear limitation. Additionally, data analyses in **Chapter 4** and **Chapter 5** were limited by the small sample size and low number of 'positive' outcomes for *E. coli* and resistant *E. coli* presence (i.e., 48/250 and 8/250, respectively) which restricted statistical analyses and, at times, interpretation of findings.

6.3 Future research

As shown in the current research, local (macro) scale physiography and hydrometeorology (e.g., hydrogeology, climate/weather, land-use patterns, supply infrastructure, etc.) and supply-scale (micro) parameters (e.g., organic matter content, local microbiological diversity, presence of selective and co-selective contaminants, biofilm characteristics, etc.) can impact groundwater contamination with ARB. So, identifying the varying levels of influence attributable to each specific risk factor can help inform the development of successful source-protection interventions specific to the study region. From this, a key recommendation for future research is that more baseline investigations are carried out at diverse groundwater settings across the globe. These should seek to employ (i) risk factor measurements (to enable the identification of the determinants facilitating groundwater contamination with ARB), (ii) a longitudinal and/or a spatially distributed sampling regime (to achieve some level of representativeness and avoid under- or over-estimations of ARB contamination rates), and (iii) the assessment, where possible, of more than one bacterial species (to increasing the accuracy of ARB presence/absence measurements). With regards to supply-scale (micro) parameters,

in-depth research is also needed to investigate the potential of specific geogenic and/or anthropogenic groundwater contaminants (e.g., antimicrobial residues, metals, metalloids, disinfectants, microplastics, biofilms, etc.) to drive acquisition/persistence of resistance determinates (i.e., ARGs) within groundwater supplies and/or aquifers systems.

Outlined and discussed below are also a few field-specific topics that also require further research, and which have been identified through the current work.

6.3.1 Measuring AMR in groundwater resources

As described in **Chapters 4** and **5**, the AMR outcome investigated was ARB presence within groundwater supplies, and specifically presence of resistant *E. coli* and resistant *P. aeruginosa,* through both phenotypic (i.e., disc diffusion susceptibility testing; **Chapter 4**) and genotypic methods (i.e., whole genome sequencing; **Chapter 5**). However, there are alternative approaches for measuring AMR outcomes which are also valuable and must be acknowledged. An increasingly common approach, for example, is measuring ARG occurrence/abundance at a community level (i.e., presence in total DNA extracted from the water sample) (viz. Li et al., 2018; Zainab et al., 2020; Zou et al., 2021; Liu et al., 2022; Kampouri et al., 2022). This can be done, for example, through molecular methods (e.g., quantitative real-time PCR (qPCR), digital droplet PCR, etc., which provide gene diversity, copy number, and expression level).

The two approaches (i.e., samples analysed for specific ARB versus overall ARG presence/abundance) have advantages and disadvantages which must be considered in result interpretations. In the case of overall ARG outcomes, for example, it must

be considered that a proportion of ARGs identified may be inactive or located outside of a living cell, and in both cases the links to adverse human health consequences are unclear. Nonetheless, a key advantage from this approach is the measure of ARG abundance/diversity within all bacterial communities present. Regarding specific ARB presence (the approach used here) the biggest disadvantage lies in the limited picture of the local resistome, which can lead to the underestimation of overall ARB presence (e.g., other bacterial species may carry active ARGs/be phenotypically resistant even in the absence of resistant *E. coli*). Despite this, such approach is extremely valuable for providing a direct exposure risks to human health – i.e., presence of culturable bacteria carrying resistance phenotypes (i.e., active ARGs) in drinking water environments conveys a clear risk of consumer colonisation with ARB (Coleman et al., 2012) and ultimately a higher likelihood of acquiring resistant-infection(s) (Tacconelli et al., 2009). Additionally, while overall ARG presence/abundance may better represent *real-life* conditions, co-selection studies using this as an AMR outcome are considered less informative than those focused on specific species, as only the latter approach allows for the identification of cross-resistance (e.g., MRG and ARGs collocated within the same isolate and in the same plasmid) (Pal et al., 2017). Thus, where possible, it is recommended that these two approaches are combined and that phenotypic susceptibility methods are employed for a holistic assessment of AMR/ARB presence and abundance and of the effects of existing (co-)selective pressures.

Additionally, when assessing the likelihood for specific groundwater environment's to harbour (co-)selective pressures further research is needed which considers the potential for (co-)selective agents (e.g., antimicrobials, metals, and/or

disinfectants) to adsorb onto local sediments or become otherwise focally concentrated in microenvironments such as biofilms. In such cases the overall environment may not be (co-)selective, but (co-)selection could be taking place on a local but catalytic basis, which would not be elucidated by water analyses alone.

6.3.2 Modelling ARB presence in groundwater resources

As outlined in **Chapter 4**, multivariate logistic regression was employed to identify the risk factors potentially associated with presence/absence of *P. aeruginosa*, *E. coli*, and resistant *E. coli* within groundwater supply samples collected in autumn 2019 and summer 2021. These models, which assume independence of samples, were employed as there is insufficient evidence for sample dependence (in terms of source inputs, ingress mechanisms, and physico-chemical conditions) to justify repeat measures/mixed effects modelling. Microbial, and particularly *E. coli*, groundwater contamination typically occurs as discrete episodic events (e.g., Latchmore et al., 2022), with no evidence of *E. coli* adaptation/naturalisation within Irish groundwater environments. A recent provincial-scale study of private groundwater supplies in Canada, for example, has reported median die off rates in the region of 11 to 18 days for consolidated and unconsolidated aquifer media, respectively (Latchmore et al., 2022). In the RoI, *E. coli* is known to have a limited lifespan of up to a maximum of 100 days in low temperature (ca. 10°C) groundwater environments (this forms the basis of all risk-based management strategies for protection of public supply wells in the country; DEHLG/EPA/GSI, 1999). Hence, isolates obtained 18 months apart in the present study are, from an ecological perspective, entirely distinct (independent). Furthermore, unobserved changes in

both anthropogenic (e.g., supply infrastructure, groundwater abstraction rates, wastewater inputs, agricultural practices and faecal matter deposition patterns) and hydrogeological parameters (e.g., water table fluctuations, flow path connectivity, groundwater recharge/discharge cycles, soil moisture content and hydrochemistry) over such an extended intervening period are expected to significantly affect the composition of microbiomes within groundwater supplies. Indeed, this inherent variability/uncertainty associated with groundwater composition and quality leads many studies to produce seasonally-specific models from the same sample network (Shrestha et al., 2014; Latchmore et al., 2020; Abiriga et al. 2021), with rapid faecal transport through preferential flow paths in subsoils and bedrock fracture networks shown to be highly transient or seasonal in nature (e.g., Sorensen et al., 2018; 2021).

In certain circumstances, such as time-series datasets that employ suitable temporal resolution and continuity of repeat sampling, statistical models accounting for sample dependence are justified (see **Chapter 3**). Such models may be employed, for example, where several samples per case/source are taken based on a systematic sample strategy (e.g., seasonally, monthly, annually over many years, etc.), and where variance and uncertainty associated with input parameters can be quantified. However, this is not the case in **Chapter 4** as a maximum of two samples were taken from each source with a large temporal gap and many external confounders - including COVID-19 lockdowns, which added a significant level of uncertainty and variability to some anthropogenic and hydrogeological parameters such as pumping rates, DWWTS usage, water tables, etc. Ultimately, however, study designs which allow for more advanced statistical methodologies such as the ones described in

Chapter 3 are recommended for future ARB research to further elucidate the complex spatiotemporal patterns affecting groundwater contamination with AMR.

6.3.3 Identifying AMR/ARB in aquifer systems

The research presented in this dissertation has specifically focused on the presence of ARB in groundwater supplies (i.e., wells and springs). However, it is crucial to note that there exists a broader, complex network of groundwater environments that also warrants investigation (i.e., aquifer systems), particularly as these can connect surrounding environments, such as surface waters, top- and subsoil, groundwater supplies, bedrocks, etc., and allow for the transport of pollutants between them. **Chapter 4** findings indicate that a key ARB transport pathway into the studies groundwater supplies is surface run-off followed by direct wellhead ingress, as opposed to recharge (preferential or otherwise). However, it is possible that, where present, AMR determinants such as ARB, ARG and residues are transported from groundwater supplies to aquifer systems and along the groundwater pathway affecting downgradient gradient areas where, hypothetically, no potential sources exist (vis. Li et al., 2015).

It must be acknowledged, however, that fieldwork data showed lack of resistant *E. coli* contamination in neighbouring wells and that significantly higher odds of its presence were found in hand-dug rather than drilled wells (OR = 41.071), thus pointing to a higher likelihood of localised (i.e., confined to the well) rather than widespread aquifer contamination. Indeed, lack of repeat (i.e., re-occurring) ARB presence also points to the event-based nature of this mechanism. That said, the transport of ARB within the subsurface was beyond the scope of the current

dissertation and additional work is needed to identify the occurrence of additional and potentially long-term groundwater transport pathways.

The identification and quantification of AMR persistence/continuity across hydrogeological formations forms an important piece of the puzzle in recognising the vulnerability of groundwater resources at increased distances from potential contamination sources (including those used for drinking and other key human activities). Research on the transport and source tracking of antimicrobial resistant determinants within the subsurface is still limited, however. Accordingly, this is highlighted here as an important field for future research.

6.4 Policy recommendations

Results from **Chapters 4** highlight key determinants for ARB contamination of groundwater supplies in the RoI, showing that exposure to this type of contamination may be prevented. To achieve this, strategies can be developed which target (i) source(s) of ARB (e.g., by lowering antimicrobial use), or (ii) the groundwater pathways for ARB dissemination (e.g., by promoting appropriate groundwater source-protection practices and/or the introducing appropriate treatments).

6.4.1 Reducing sources of AMR/ARB

As outlined in **Chapter 1**, the overprescription of antimicrobial drugs in human medicine (Binder et al. 1999) and their intensive use in agricultural activities (Bech et al. 2014) represent the origin and principal ongoing contributor to the current AMR problem. Indeed, the rational use of antimicrobials in both human and veterinary medicine is the first global recommendations to limit the selective pressures posed

by antimicrobial-rich environments and lower AMR dissemination (Infectious Diseases Society of America, 2011; WHO, 2022). Concerning AMR contamination of groundwater resources, veterinary antimicrobial use in livestock (cattle) farming, in particular, is highlighted as the principal AMR source within the investigated rural supplies in the RoI (**Chapter 4**). Notably, the RoI represents a unique agricultural landscape characterised by low crop production and widespread pastoral/livestock grazing areas covering over 90% of the country's agricultural area (O'Mara et al., 2021).

Despite the sheer magnitude of veterinary antimicrobial use in the RoI (> 103.9 tonnes in 2020; Health Products Regulatory Authority (HPRA), 2021) and its widespread influence in AMR dissemination through the natural environment both in the RoI (O'Dwyer et al., 2017; **Chapter 4**) and abroad (Coleman et al., 2013; Gambero et al., 2018), however, many countries (including the RoI) lack strict policies to control their use. In the RoI specifically, for example, no limits have been established for antimicrobial usage on farms as long as they are prescribed by a veterinarian, and surveillance efforts are limited to country-wide annual sales rather than on a farm-by farm basis (Martin et al., 2020). Thus certifying that the miss-use as growth promoters and/or prophylactic treatments can neither be measured nor reprimanded. This may change, however, with European Union regulations now dictating the use of veterinary medicinal products and medicated feed of its member-states (EU Regulation 2019/4; 2019/6). In the context of this evolving policy landscape, a direct recommendation from the present research to significantly reduce sources of AMR into groundwater, and indeed many other neighbouring environments, is to place tighter regulations for antimicrobial use on farms, via the

implementation of policies that limit/control and supervise their application. Indeed, the same recommendations apply for other non-EU countries with significant livestock industries. Additionally (and importantly), top-down approach must be supported by effective bottom-up communication strategies to ensure custodians (i.e., farmers and livestock veterinaries) are motivated to change their practices in line with novel regulations (Regan et al., 2023).

As described previously, a holistic plan for AMR prevention must also include the reduction of antimicrobial use in human medicine. Notedly, despite not being significantly associated with ARB presence in **Chapter 5**, human antimicrobial use remains the primary source of AMR in other environments (including some groundwater resources – viz. **Chapter 2**). Solutions to lower antimicrobial use in human medicine at a local and global level are certainly not easy, however, and the need for effective bottom-up efforts may be even stronger than in the reduction of antimicrobial use on farms. An international systematic review of interventions for promoting prudent antimicrobial prescriptions, for example, has identified educational efforts targeting medical professionals as significantly effective, while also highlighting the importance of developing educational programs suitable for other non-medical groups - both adults and children (Lee et al., 2015). Furthering this, the World Health Organization (WHO) emphasises the need for coordinated action by individuals, health-care professionals, the healthcare industry, and policy makers at both a local and global scale (WHO, 2015). Indeed, actionable steps will vary substantially for each stakeholder group, however a common denominator is that these must all be appropriately communicated/disseminated to the relevant target audiences and encouraged, where feasible, through policy regulations.

6.4.2 Preventing groundwater as a pathway for ARB dissemination

The mitigation of ARB exposure through contact with contaminated groundwaters can also be attained by limiting the formation of a pathway and, as such, breaking source-pathway-receptor linkages. This can be done either by preventing the contaminants from entering the groundwater resources, treating the water prior to consumption, or (ideally) both. Findings presented in **Chapter 4** outline three specific groundwater source protection measures which were shown to lower the likelihood of ARB presence. These included (i) favouring drilled (not hand-dug) wells, (ii) certifying that the well is at minimum setback distances to potential sources of faecal contamination, and (iii) ensuring that the well cap is appropriately sealed. Source treatment as an additional layer of protection is also highlighted in the current dissertation. For small-scale private groundwater abstraction these are domestic-scale water treatments systems - preferably reverse osmosis, which is efficient at both ARB and ARG removal (Slipko et al., 2019).

In fact, the source protection and treatment recommendations outlined here are already in line with, and as such provide additional scientific evidence for, well construction and maintenance guidelines already available to private well users in the RoI (EPA, 2021). It is important to highlight, however, that these small (domestic) scale groundwater abstractions are not regulated (EPA, 2013; EU Council Directive 2020/2184, 2020), resulting in each owner being responsible for their own private well water quality. Thus, a key policy recommendation from this research is that the evidence-based guidance to mitigate contamination and subsequent human exposure to ARB (and other contaminants) via groundwater resources is appropriately communicated to promote bottom-up uptake, in line with expert

recommendations (viz. Mooney et al., 2020; Andrade et al., 2021). Additionally, a top-down approach such as through a country-wide registration of existing wells, inclusion of private well requirements as part of property transactions, etc. may also be effective at motivating behavioural changes and, as such, warrant further consideration.

6.5 Concluding remarks

Based on study findings, and in line with the principal research aim and five research objectives previously outlined (**Chapter 1**), five main conclusions can be drawn:

1. Groundwater environments may present noteworthy reservoirs in the dissemination of antimicrobial resistant bacteria globally and in Ireland.
2. Cattle farming represents the most likely source of antimicrobial-resistant *E. coli* in groundwater supplies in rural Ireland,
3. *E. coli* is likely already resistant to antimicrobials prior to entering these private wells,
4. Surface run-off followed by direct wellhead ingress is the most likely transport pathway for antimicrobial-resistant *E. coli* into the studied groundwater supplies, and
5. Human exposure to AMR via these (rural) groundwater supplies can be reduced through lowering antimicrobial use on farms and implementing appropriate groundwater source protection measures; namely: favouring drilled wells, ensuring that the well is at minimum setback distances to potential sources of

contamination (e.g., DWWTS and livestock agriculture), and fitting an appropriately sealed cap.

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Appendix A

Published chapters

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Andrade, L., Chique, C., Hynds, P., Weatherill, J. and O'Dwyer, J., 2023. The antimicrobial resistance profiles of Escherichia coli and Pseudomonas aeruginosa isolated from private groundwater wells in the Republic of Ireland. *Environmental Pollution*, *317*, p.120817.

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Groundwater resources as a global reservoir for antimicrobial-resistant bacteria

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ABSTRACT

Antimicrobial resistance represents one of our most significant global health threats, with increasing incidences noted in both clinical and environmental settings. As such, identifying and understanding the sources and pathways for antimicrobial-resistant bacteria (ARB) is critical. The current study presents the first systematic review and pooled analysis of ARB occurrence in global groundwater supplies, which are used as primary drinking water sources by 2.2 billion people worldwide and are recurrently linked to significant outbreaks of infection. Seventy peer-reviewed studies were identified and included; findings reveal that 80.2% ± 29.0 and 57.2% ± 36.8 of aggregated groundwater isolates were resistant to ≥1 and ≥3 antimicrobials, respectively. Where bacteria were present, ARB were identified in 76.9% ± 33.7 of individual wells and springs. Our results leave little doubt that groundwater represents a major global reservoir for ARB, however significant research is required to establish environmental determinants and mechanisms mediating their occurrence.

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1. Introduction

Antimicrobial resistance is now widely recognised as a global public health threat, requiring multi-sectorial preventative and mitigative interventions (Bradford and Harvey, 2017; WHO, 2018; Larsson et al., 2018). Anthropogenic influences and behaviours, including the misuse/overuse of human and veterinary antimicrobials has resulted in the addition of significant selective pressures to the naturally occurring resistance within and between bacterial species (Van Boeckel et al., 2014; Bell et al., 2014; O'Neill, 2016). As such, a global increase in acquired resistance traits has been noted among bacterial isolates, including clinically significant species (e.g. *Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterobacteriaceae* spp.; WHO, 2017) with the incidence of multi-drug resistance (i.e., resistance to ≥ 3 antimicrobials) increasing both spatially and temporally (Munita and Arias, 2016). Cases of treatment failure of both human and veterinary infectious diseases are increasingly documented (Wright, 2010; Opatowski et al., 2019), which results in higher healthcare costs, more severe and prolonged infections, and rising rates of morbidity and mortality (Laxminarayan et al., 2013). Recent estimates by the European Antimicrobial Resistance Surveillance Network (EARS-Net) indicate that approximately 33,000 deaths are attributable to antimicrobial-resistant bacterial infections per annum within the EU/EEA, comparable with the combined human health burden of influenza, tuberculosis and HIV/AIDS in the same region (Cassini et al., 2019). Accordingly, antimicrobial resistant infections have received significant attention within both the media and research community over the past two decades, with an extensive body of research existing in clinical settings, coinciding with the development of several antimicrobial resistance Action Plans at varying scales (European Commission, 2011; WHO, 2015). More recently, however, the role of the natural aquatic environment as a source and

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transmission pathway for antimicrobial-resistant bacteria (ARB) has been acknowledged as an area of growing concern (Sanderson et al., 2018).

Human and veterinary antimicrobials are increasingly released to the environment at sub-therapeutic concentrations via myriad sources of domestic, agricultural, industrial and clinical/hospital origin. These microbiologically diverse, pharmaceutically dilute media may readily catalyse the development of ARB within the natural environment, inevitably resulting in their ingress to both surface and groundwater sources (Van Schaik, 2015). However, at present, more research is still needed to better understand the occurrence and transport of ARB to and in natural waterbodies. This is particularly true with regard to groundwater wells and aquifers, which currently supply approximately 31.5% (2.2 billion people) of the global population with domestic drinking water (Murphy et al., 2017).

Microbial contamination of groundwater and its adverse public health effects have been well substantiated within the scientific literature. A recent review by Murphy et al. (2017) presents clear epidemiological evidence of disease transmission due to groundwater contamination at a global scale, with an estimated 35.2 to 59.4 million cases of acute gastrointestinal infection potentially attributable to groundwater consumption per year. Thus, the potential implications of groundwater-borne ARB pose a significant threat to public health, allied with an already high global burden of infection. A recent study in the Republic of Ireland found that wastewater systems, livestock density and the presence of children in a household were significantly associated with the presence of antimicrobial resistant *Escherichia coli* (*E. coli*) in private groundwater supplies (O'Dwyer et al., 2017). As such, the ubiquity of contaminant sources, in concurrence with the presence of bacteria and sub-therapeutic antimicrobial concentrations, suggests that vulnerable groundwater systems may be a significant and frequently overlooked reservoir for ARB (Wellington et al., 2013). Indeed, research carried out with 878 Canadian individuals has shown that consumers of *E. coli* contaminated groundwater are 1.26 times more likely to be colonised by antimicrobial resistant *E. coli* than non-consumers (Coleman et al., 2012). Nonetheless, there is presently no consensus regarding the role of groundwater in the global dissemination of ARB. The extent of this threat is further complicated by the nuances of groundwater contamination mechanisms, which are typically determined and/or driven by numerous environmental and source-specific risk factors (e.g. source design, location and maintenance, local hydrogeological setting, and shifting climatic and landuse patterns), their permutations and spatiotemporal distributions (Hynds et al., 2012; Wallender et al., 2014; Atherholt et al., 2017; Andrade et al., 2018). Accordingly, the current study sought to further understand the occurrence, distribution and potential drivers of ARB in groundwater sources (i.e. wells and springs) via a global pooled analysis of peer-reviewed studies. Findings can be used to support evidence-based risk management strategies to inform non-clinical considerations in existing antimicrobial resistance Action Plans and guide future research strategies.

2. Methods

2.1. Literature identification

Identification of scientific articles examining the occurrence of ARB in groundwater sources was conducted with an overarching systematic review protocol based upon pre-established guidelines (Pullin et al., 2018) and adapted from previous studies (Eftim et al., 2017; Nappier et al., 2019). Defined search terms (Table 1) were used to search Scopus, Web of Science, Pub Med and ProQuest databases on October 8th, 2018. Employed "Outcome" search terms, and particularly the antimicrobials and bacterial species selected, were based on the global priority list of ARB (WHO, 2017). Manual supplementary searches were additionally performed between October 15th and November 12th, 2018. These comprised the examination of article bibliographies and studies citing articles which were identified via database search and marked as "provisionally included" upon title and abstract assessment ($n = 215$).

2.2. Study selection

Studies uncovered during the identification phase (i.e. database and supplementary searches) were independently screened by two authors using explicit eligibility criteria (Table 2) via title and abstract assessment. Articles without an available full text were excluded, with eligibility disagreements resolved via provisional inclusion. All "provisionally included" and manually identified studies had full-texts manually (i.e., no computer-assisted techniques employed) and independently assessed (eligibility assessment) using the presented criteria (Table 2). Disagreements at this stage were resolved via author panel consensus.

Excluded studies were those that: (i) reviewed previously published studies, (ii) incorporated controlled elements in their study design (i.e., spiking or laboratory-based (micro-, meso-) groundwater environments), (iii) examined thermal/hot springs, (iv) did not study water samples derived from groundwater sources (e.g., surface and marine water), (v) combined resistance data from multiple environmental media, (vi) did not examine $\geq$ 10 groundwater samples (including articles where sampling number was not reported), (vii) did not analyse antimicrobial resistance/susceptibility in bacterial isolates from groundwater (including studies where bacteria were not found), (viii) did not report number of isolates tested, ix) did not report bacterial resistance (or susceptibility) to each antimicrobial tested against (Fig. 1).

2.3. Study inclusion

During full-text assessment (eligibility phase), studies included were those that assessed 10 or more groundwater samples and

Table 1
Search terms employed in the current study.

Element	Description	Search terms				
Population	All non-saline groundwater	Groundwater, Ground Water, Aquifer, Subsoil, Subsurface, Borehole, Bore Well, Bored Well, Dug Well, Well Water, Water Well				
Outcome(s)	Occurrence of antimicrobial-resistant (or susceptible) bacteria	Antimicrobial, Antibiotic, Antibacterial, Bacteriostatic, Bactericidal, Penicillin, Cephalosporin, Carbapenem, (Fluoro)quinolone, β lactam, Aminoglycoside, Tetracycline, Vancomycin, Clarithromycin, Ampicillin, Sulphonamide	AND	Resistance, Resistant, Susceptible, Susceptibility, Sensitive, Sensitivity	AND	Bacteria, Bacterial, Microbe, Microbial, Organism, Pathogen, *E. coli*, *Escherichia coli*, *Pseudomonas*, *Enterococcus*, *Enterococci*, *Campylobacter*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Shigella*, *Klebsiella*, *Enterobacter*, *Enterobacteriaceae*

Table 2
Eligibility (inclusion/exclusion) criteria employed.

Inclusion Criteria	Exclusion Criteria
Study type: All peer-reviewed articles excluding reviews **Language:** English **Population:** Naturally occurring groundwater environments; groundwater sources (i.e. wells and springs) **Exposure:** Pre-existing environmental exposures (i.e. prior to study) **Event/Outcome:** Antimicrobial-resistant (or susceptible) bacteria found in groundwater resources **Study design:** Analysis of ≥10 groundwater samples; results including percentages of bacterial isolates resistant/susceptible to each antimicrobial tested against. **Period:** Any	**Study type:** Academic reviews; grey literature **Language:** non-English **Population:** Artificial groundwater media (i.e. lab-based); pre-packaged water; Thermal/Hot springs; surface water bodies; maritime aquatic environments; wastewater treatment plants; soil; saline, brackish, or soil water. **Exposure:** Any controlled exposure (i.e. spiking) **Event/Outcome:** Absence of bacterial contamination in groundwater; absence of antimicrobial resistance profiling of groundwater isolates **Study design:** Analysis of <10 groundwater samples (includes number of groundwater samples not reported); results combined for different sampled environments; results combined for all antimicrobials tested against; number of isolates tested not reported. **Period:**

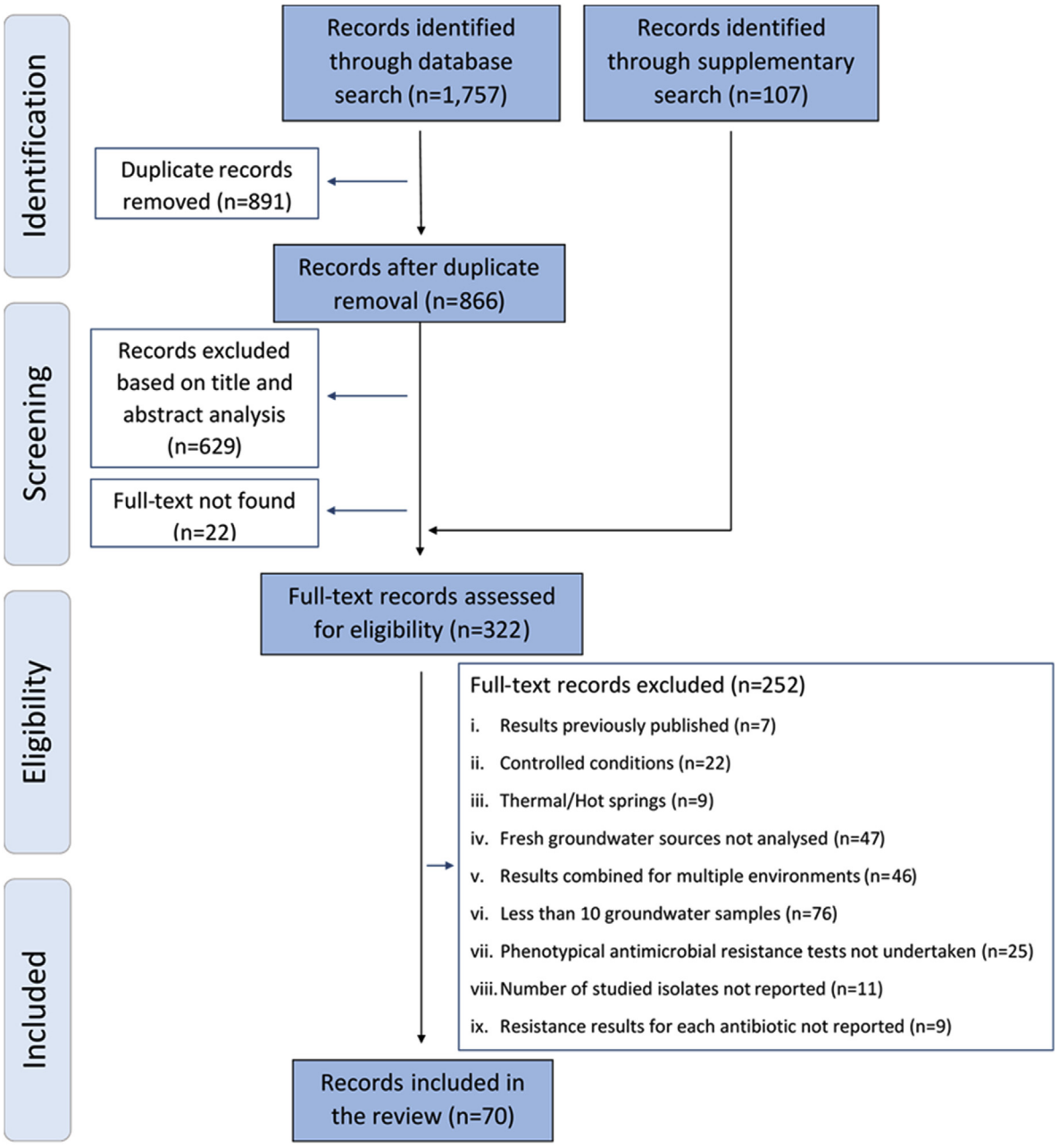


Fig. 1. Systematic review protocol employed during the current study, including literature identification, screening, eligibility assessment, and final study inclusion.

explicitly stated the percentage of bacterial isolates resistant or susceptible to each antimicrobial agent tested against. In all, 76 studies were excluded due to reporting on < 10 groundwater samples, including studies where sample number was not reported (n = 12) (exclusion criteria vi). Forty-six studies reported composite occurrence rates of ARB in combined study environments (e.g., merged findings from groundwater, surface water and/or wastewater), and as such were excluded under exclusion criteria (v). Studies that did not provide an adequate description of water sample origin; that is, referred solely to "tap water" (n = 14) were excluded under exclusion criteria (iv) (i.e. groundwater source not analysed) and where bacterial isolates were not identified in groundwater samples (n = 4), articles were excluded under exclusion criteria (vii), as antimicrobial resistance could not be determined, this criteria also comprised studies that provided an assessment of antimicrobial resistance through genotypical methods only (i.e. presence/absence of resistance genes via qPCR or digital droplet qPCR analyses). As such, 70 of 1864 identified studies (identification phase) were deemed eligible for inclusion following the full review process (Fig. 1).

2.4. Critical appraisal of study validity

Included articles were independently evaluated by two authors using a critical appraisal tool adapted from Bain et al. (2014). In it, a score ranging from 0 to 14 was attributed to each study according to the number of affirmative responses to the fourteen pre-established criteria. Based on it, articles were classified as presenting low (score ≤ 5), medium (score of 6–8) or high (score ≥ 9) validity. Individual article assessments are presented in Supplementary Table 1. All disagreements were resolved by a consensus between authors.

2.5. Data extraction

Relevant data pertaining to each included study were extracted to MS Excel 2016. Extracted variables were classified and exported under six primary categories; namely, bibliographic details, study region (e.g., country, location within country and settlement type), groundwater characteristics (e.g., source type, well type, ownership, uses and treatment presence), sampling regime (e.g., number of samples, re-sampling, length of sampling regime), analytic elements (e.g. bacterial species tested, antimicrobial agents tested against, method and criteria were used to assess susceptibility/resistance), and resistance profile (e.g., percentage of ARB and MRB amongst tested isolates and percentage of resistance to each antimicrobial agent tested against). It is important to note that with regards to studies in which re-sampling was employed, data pertaining to different sampling rounds were merged and extracted as single outcomes, with "re-sampling" and "one-off" used just to classify two contrasting approaches employed across identified studies. As each study only provides one outcome to the analysis, independence between observations from each analytical unit (i.e. study) can be assumed. Moreover, due to the large periodicity associated with repeat groundwater sampling rounds (e.g. where groundwater sources are sampled many months apart), observations may be treated independently due to the fluid (acute) nature of groundwater contamination (Bjerg and Christensen, 1992; Morvan et al., 2006; Pacheco Castro et al., 2018).

Antimicrobial resistance results were extracted according to the standards (i.e. EUCAST, CLSI, etc.) and interpretations employed in each published manuscript, inhibition zone and/or minimal inhibitory concentration results were not routinely specified and as such, could not be uniformly re-assessed. Moreover, as intermediate resistance indicates that an antimicrobial is ineffective at recommended and commonly used therapeutic concentrations (Rodloff et al., 2008), potentially resulting in treatment failure, it was considered as resistance, as per other studies (Reinthaler et al., 2003; O'Dwyer et al., 2017).

Where key variables were unclear or not explicitly documented, article authors were contacted for clarification and/or articles were analysed for identifiable characteristics, and thus classified. Where classification was not possible, variables were categorised as "not reported". Sample sizes were categorised as small (<30 samples), medium (30–99 samples) or large (≥100), as previously defined by Bain et al. (2014), with a maximum threshold established whereby data from one study comprising > 5000 samples were not extracted to prevent geographical and/or analytical bias (Coleman et al., 2013); however, data reported from a smaller sample (n = 657) in this study were included in analyses. As such, the maximum sample number from a single included study was 939 (Akoachere et al., 2013).

Studies were further classified according to globally established characteristics relating to the study regions. Countries were classified as low, lower-middle, upper-middle, and high income based upon World Bank classification (World Bank, 2018). Specific study areas' primary (arid, cold, polar, temperate and tropical) and secondary (e.g., dry summer, dry winter, without dry season, monsoon and rainforest) climates were determined based on the Köppen-Geiger climate classification (Peel et al., 2007), and used to ascertain sampling season (i.e., summer, winter, spring, autumn) and period (i.e., wet or dry).

Moreover, where possible, occurrence rates of antimicrobial resistance (≥1 antimicrobial) and multidrug resistance (≥3 antimicrobials) were calculated for all isolates reported in a study (i.e. number of resistant and multidrug resistant isolated, respectively, divided by the total number of isolates recovered from all groundwater sources and samples examined within that study). This approach was used as even when studies employed re-sampling in their methodology (i.e. more than one sample taken from the same groundwater sources at different times), antimicrobial resistance results were often integrated during reporting. With regards to occurrence rates of ARB amongst groundwater sources (i.e. wells and/or springs) or the specific sources in which bacterial isolates were found, these comprised the percentage of sources that harboured ARB at least once, as reported in each manuscript (i.e. number of sources where ARB were found at least once divided by total number of tested sources or by the number of sources in which bacteria were found at least once, respectively).

2.6. Multiple antimicrobial resistance index

Multiple Antimicrobial Resistance (MAR) indices were calculated to standardise the rates of antimicrobial resistance reported across each study (Equation (1); Krumperman, 1983). MAR indices provide a single measure of antimicrobial resistance and control for the number of antimicrobial agents tested against, thus avoiding potential bias (i.e. elevated ARB occurrence rates are typically found when more antimicrobials are incorporated in a study design).

$$MAR\ \ index = \frac{y}{n \times x} \qquad \text{[Equation. 1]}$$

where y is the aggregate antimicrobial resistance score of all isolates tested (i.e., the sum of isolates resistant to each antimicrobial), n is the number of isolates tested, and x is the number of antimicrobial agents tested against (Krumperman, 1983).

A single MAR index was calculated for each study, irrespective of temporal methodologies employed, and included in the analyses. These were used to ascertain the overall findings regarding

antimicrobial resistance in groundwater bacteria on a study by study basis, thus permitting cross-study comparisons (see Fig. 5). Secondly, for descriptive purposes only, discrete MAR indices were calculated for each study within isolates of the same species or genus and within different antimicrobial classes. These were not included in analyses, but merely used to (a) identify the relative rates of antimicrobial resistance associated with each bacterial genus, and (b) determine the differences between resistance rates within and between different antimicrobial classes (see Table 5).

2.7. Data analyses

Three dependent variables were calculated and used to quantify presence of ARB and/or Multidrug Resistant Bacteria (MRB) in groundwater and represent the main findings from each included study (i.e. studies were the analytic unit throughout analyses), namely: (i) occurrence rates of antimicrobial resistance (≥1 antimicrobial) amongst all groundwater isolates tested; (ii) occurrence rates of multidrug resistance (≥3 antimicrobials) amongst all groundwater isolates tested; and (iii) calculated study-specific MAR indices.

Dependent variables were not normally distributed and could not be normalized using standard (transformation) techniques, thus non-parametric Mann-Whitney U and Kruskal-Wallis tests were employed to identify categorical associations between dependant and independent variables (i.e. the extracted characteristics outlined in Table 3). Where significance was found ($p < 0.05$) and the independent variable described three or more levels of measurement, Dunn's (non-parametric) pairwise post-hoc tests were used. Mean MAR indices were further calculated for each country and discretized into ranges (e.g. 0.000–0.100, 0.100–0.200, …) using all included studies, with all studies equally weighted.

3. Results

3.1. External, groundwater-specific and study-specific characteristics

A total of 70 relevant studies were included for data extraction and pooled analyses. A summary of all included studies is presented in Table 4, with detailed study characteristics and full reference list provided in Supplementary Materials 2. Relevant studies were conducted in geographically, climatically and economically diverse regions and spanned a 42-year period (1976–2018), with 81.4% (n = 57) of identified articles published since 2010. All inhabited continents as well as four of the five climatic zones were represented. Overall, 51.4% (n = 36) of identified studies were undertaken in lower-middle income countries, followed by countries classified by high (21.4%; n = 15) and upper-middle (21.4%; n = 15) incomes. There were two primary sampling regimes identified, with repeat (temporal/seasonal) sampling employed in 32.9% (n = 23) of studies, while 52.9% (n = 37) performed one-off ('snapshot') sampling. Where sampling seasons could be ascertained (n = 58), it was exclusively undertaken during wet and dry seasons in 41.4% (n = 24) and 32.7% (n = 19) of studies, respectively; however, just 10.3% of studies (n = 6) explicitly reported this information.

Overall, 52.9% (n = 37) of studies were conducted in categorically rural areas, while 27.1% (n = 19) were undertaken in urban regions and the remaining 5.7% (n = 4) in "mixed settlements" (i.e. both urban and rural areas surveyed). Approximately two thirds (64.3%; n = 45) of studies reported the presence of waste sources adjacent to study sites; 79.2% (n = 38) of human and 48.9% (n = 22) of animal origin (e.g. septic tanks, wastewater treatment plants, animal grazing fields and manure application). Wells and springs were examined in 91.4% (n = 64) and 12.9% (n = 9) of included studies, respectively, with 4.3% (n = 3) reporting concurrent analyses of both source types. Well construction was explicitly defined in 23 studies, of which 60.9% (n = 14) were hand-dug and 39.1% (n = 9) were bored. A significant paucity of data was encountered with respect to reporting of numerous source-specific elements (e.g. supply depth, age and operational condition/performance), with just 18.6% (n = 13) of included studies describing one or more of these. Similarly, just 17.1% (n = 12) of studies reported local hydrogeological characteristics (e.g. aquifer type, subsoil type, depth and permeability, bedrock geology and groundwater vulnerability). Sampled groundwater sources were used for human consumption in 57 of the 60 studies where groundwater usage was reported (95%), with an absence of water treatment before consumption noted in 76.9% of the 13 studies that reported this information (n = 10).

A total of 8741 groundwater samples were collected across all included studies, ranging from 10 to 939 per study (mean ± SD = 125 ± 189), with 7157 identified groundwater

Table 3
External, source-specific and study-specific characteristics and their sub-categories used as independent variables in the non-parametric statistical tests.

Categories		Sub-categories	Non-parametric test Sub
Environmental			
1.	Economic classification [a]	Low; Lower middle; Upper middle; High income	Kruskal-Wallis
2.	Climate [b]	Tropical; Arid; Cold; Temperate	Kruskal-Wallis
3.	Sampling period [c]	Wet; Dry	Mann-Whitney U
4.	Settlement type [d]	Urban; Rural	Mann-Whitney U
5.	Waste source adjacent to study site [e]	Human waste; Animal waste	Mann-Whitney U
Source-specific			
6.	Source type	Well; Spring	Mann-Whitney U
7.	Well type	Hand-dug; Bored	Mann-Whitney U
Study-specific			
8.	Sampling regime	One-off sampling; Re-sampled sources	Mann-Whitney U
9.	Sample size	Under 30; 30 to 99; 100 and over	Kruskal-Wallis
10.	Length of sampling period	Under 6 months; 6–12 months; more than 12 months	Kruskal-Wallis

[a] Economic classification according to the World Bank (2018).
[b] Climate classified according to Peel et al. (2007).
[c] Wet and dry as defined in Peel et al. (2007).
[d] Urban settlements include urban, sub-urban or peri-urban regions.
[e] Human waste encompasses septic tanks and wastewater treatment plants, and animal waste encompasses animal grazing fields and agricultural fields where manure is spread.

Table 4
Summary of principal characteristics extracted, with study (n = 70) and groundwater sample (n = 8741) number associated with each corresponding sub-category.

Characteristics	Studies	Samples
	n (%)	n (%)
Publication year		
Pre-1990	6 (8.6)	446 (5.1)
1990–1999	0 (0.0)	0 (0.0)
2000–2009	7 (10.0)	696 (8.0)
2010–2018	57 (81.4)	7599 (86.9)
Continent		
Africa	32 (45.7)	3941 (45.1)
Asia	22 (31.4)	2645 (30.3)
Central and South America	6 (8.6)	436 (5.0)
North America	4 (5.7)	1032 (11.8)
Europe	5 (7.1)	658 (7.5)
Oceania	1 (1.4)	29 (0.3)
Economic classification [a]		
Low income	3 (4.3)	149 (1.7)
Lower-middle income	36 (51.4)	4466 (51.1)
Upper-middle income	15 (21.4)	1194 (13.7)
High income	16 (22.9)	2932 (33.5)
Climate [b]		
Tropical	25 (35.7)	3506 (40.1)
Arid	15 (21.4)	850 (9.7)
Temperate	24 (34.3)	3193 (36.5)
Cold	6 (8.6)	1192 (13.6)
Polar	0 (0.0)	0 (0.0)
Sampling period [c]		
Wet	24 (34.3)	2677 (30.6)
Dry	19 (27.1)	1185 (13.6)
Both	15 (21.4)	3857 (44.1)
Not identifiable	12 (17.1)	1022 (11.7)
Sample size [d]		
Small (<30)	13 (18.6)	–
Medium (30–99)	34 (48.6)	–
Large (≥100)	23 (32.8)	–
Length of sampling period		
0–6 months	23 (32.9)	1511 (17.3)
7–12 months	15 (21.4)	3009 (34.4)
> 12 months	6 (8.6)	1907 (21.8)
Not reported	26 (37.1)	2314 (26.5)
Sampling regime		
One-off sampling	37 (52.9)	2920 (33.4)
Re-sampled sources	23 (32.9)	4405 (50.4)
Not reported	10 (14.3)	1416 (16.2)
Validity score		
Low (≤5)	5 (7.1)	245 (2.8)
Medium (6–8)	19 (27.1)	2377 (27.2)
High (≥9)	46 (65.7)	6119 (70.0)
Source type		
Wells	61 (87.1)	7981 (91.3)
Dug	*13 [21.3]*	*2123 [26.6]*
Bored	*9 [14.8]*	*1236 [15.5]*
Mixed	*9 [14.8]*	*1526 [19.1]*
Not reported	*30 [49.1]*	*3096 [38.8]*
Springs	6 (8.6)	423 (4.8)
Mixed	3 (4.3)	337 (3.9)
Water supply		
Private	17 (24.3)	2893 (33.1)
Public	1 (1.4)	108 (1.2)
Mixed	2 (2.9)	275 (3.1)
Not reported	50 (71.4)	5465 (62.5)
Primary settlement type [e]		
Rural	37 (52.9)	4328 (49.5)
Urban	19 (27.1)	2858 (32.7)
Mixed	4 (5.7)	975 (11.2)
Not reported	10 (14.3)	580 (6.6)
Waste adjacent to GW sites [f]		
Human waste	23 (32.9)	3301 (37.8)
Animal waste	7 (10.0)	1326 (15.2)
Both animal and human waste	15 (21.4)	2489 (28.5)
Not reported	25 (35.7)	1625 (18.6)
Treatment before use		
Yes	3 (4.3)	223 (2.6)
No	11 (15.7)	1027 (11.7)
Mixed [g]	4 (5.7)	1680 (19.2)
Not reported	52 (74.3)	5811 (66.5)
Human consumption		
Yes	57 (81.4)	7036 (80.5)
No	3 (4.3)	299 (3.4)
Not reported	10 (14.3)	1406 (16.1)
Antimicrobials tested against		
1–5	2 (2.9)	145 (1.7)
6–10	35 (50.0)	4660 (53.3)
11–15	20 (28.6)	2589 (29.6)
16–20	13 (18.6)	1347 (15.4)
Type of bacteria		
Gram-positive	8 (11.4)	461 (5.3)
Gram-negative	49 (70.0)	6412 (73.4)
Both	12 (17.1)	1793 (20.5)
Not specified	1 (1.4)	75 (0.9)

[a] Countries economically classified according to the Work Bank (2018).
[b] Climate classification according to Peel et al. (2007).
[c] As defined in Peel et al. (2007).
[d] Sample size classified following approach in Bain et al. (2014).
[e] Urban settlements include urban, sub-urban and/or peri-urban regions.
[f] Human waste encompasses septic tanks and wastewater treatment plants, and animal waste encompasses animal grazing fields, agricultural fields where manure is spread, etc.
[g] "Mixed" treatment before use encompasses studies in which treated and untreated supplies were combined during analysis.

isolates examined for resistance against 89 distinct antimicrobial agents. *E. coli* was the most frequently analysed bacteria (54.3% of studies; n = 38), followed by *Pseudomonas* spp. (21.4%; n = 15). Combined, these corresponded to almost half of the isolates tested across all studies, with 2737 and 824 tested isolates, respectively. The Penicillin antimicrobial class was most frequently incorporated in study designs (94.3%; n = 66), followed by Aminoglycosides (87.1%; n = 61), Fluoroquinolones (81.4%; n = 57), Cephalosporins (72.9%; n = 51), and Tetracyclines (71.4%; n = 50).

3.2. Critical appraisal of study validity

Study validity scores obtained varied greatly among included studies, spanning from 5 to 13, however most studies were considered of medium to high quality (i.e. ≥6; 92.8%; n = 65). The frequency of studies in each quality category are summarized in Table 3 and complete analysis can be found in Supplementary Materials.

3.3. Antimicrobial-resistant bacteria in global groundwater

Where reported (n = 20 studies; 28.6%), ARB were identified in 31.4% ± 32.6 of studied groundwater sources and in 76.9% ± 33.7 of sources where bacteria were present. Additionally, 80.2% ± 29.0 and 57.2% ± 36.8 of pooled groundwater isolates (3456 and 1403 isolates, respectively) were resistant to ≥ 1 (ARB; n = 55; 78.6%) and ≥3 antimicrobials (MRB; n = 32; 45.7%), respectively.

Table 5
Summary statistics of MAR indices calculated within antimicrobial classes and bacterial species.

Bacteria	Aminoglycosides[1]	Carbapenems[2]	Cephalosporins				Fluoroquinolones[7]	Glycopeptides[8]	Lincosamides[9]	Macrolides[10]	Penicillins[11]	Sulfonamides[12]	Tetracyclines[13]	TOTAL
			1st gen[3]	2nd gen[4]	3rd gen[5]	4th gen[6]								
	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)
Gram-positive														
Enterococcus spp.	0.344 ± 0.268 (7)	-	0.333 ± 0.333 (2)	0.923 ± 0.000 (1)	0.615 ± 0.440 (3)	-	0.188 ± 0.190 (6)	0.433 ± 0.350 (7)	0.692 ± 0.000 (1)	0.466 ± 0.245 (8)	0.518 ± 0.302 (10)	0.600 ± 0.400 (2)	0.417 ± 0.262 (9)	**0.396 ± 0.198 (11)**
Staphylococcus spp.	0.250 ± 0.314 (10)	-	0.451 ± 0.399 (3)	0.380 ± 0.302 (3)	0.283 ± 0.349 (7)	-	0.270 ± 0.390 (7)	0.285 ± 0.184 (3)	0.424 ± 0.348 (5)	0.208 ± 0.273 (4)	0.350 ± 0.255 (10)	0.629 ± 0.000 (1)	0.293 ± 0.194 (7)	**0.334 ± 0.235 (11)**
Bacillus spp.	0.243 ± 0.388 (5)	-	0.286 ± 0.286 (2)	0.583 ± 0.083 (2)	0.322 ± 0.349 (4)	-	0.333 ± 0.471 (3)	-	0.889 ± 0.079 (3)	0.545 ± 0.455 (2)	0.554 ± 0.246 (5)	0.000 ± 0.000 (1)	0.071 ± 0.071 (2)	**0.443 ± 0.163 (5)**
Streptococcus spp.	0.544 ± 0.390 (3)	-	-	0.683 ± 0.111 (2)	0.646 ± 0.256 (3)	-	0.545 ± 0.386 (3)	0.606 ± 0.000 (1)	0.714 ± 0.000 (1)	0.412 ± 0.412 (2)	0.561 ± 0.174 (4) [c,#]	0.861 ± 0.000 (1)	0.585 ± 0.068 (3)	**0.581 ± 0.146 (4)**
Micrococcus spp.	0.406 ± 0.406 (2)	-	-	0.469 ± 0.000 (1)	0.328 ± 0.328 (2)	-	0.422 ± 0.422 (2)	-	0.813 ± 0.000 (1)	0.000 ± 0.000 (1)	0.286 ± 0.286 (2)	-	0.000 ± 0.000 (1)	**0.332 ± 0.332 (2)**
Gram-negative														
Escherichia coli	0.270 ± 0.302 (35)	0.051 ± 0.107 (7) [a]	0.599 ± 0.284 (5)	0.421 ± 0.333 (15)	0.375 ± 0.391 (19) [a]	0.036 ± 0.055 (4)	0.212 ± 0.226 (34)	0.486 ± 0.367 (3)	-	0.402 ± 0.288 (4)	0.486 ± 0.335 (36)	0.394 ± 0.281 (8)	0.428 ± 0.277 (28)	**0.339 ± 0.226 (38)**
Pseudomonas spp.	0.322 ± 0.371 (15)	0.151 ± 0.157 (4) [a,*]	0.950 ± 0.000 (1)	0.666 ± 0.356 (6)	0.478 ± 0.380 (10)	0.185 ± 0.185 (2)	0.332 ± 0.371 (13)	0.194 ± 0.000 (1)	-	0.750 ± 0.204 (3)	0.614 ± 0.298 (15)	0.780 ± 0.137 (4)	0.643 ± 0.342 (10)	**0.544 ± 0.237 (15)**
Klebsiella spp.	0.307 ± 0.321 (12)	0.000 ± 0.000 (1) [a,*]	0.586 ± 0.268 (3)	0.428 ± 0.384 (5)	0.261 ± 0.360 (7) [a,*]	0.000 ± 0.000 (1)	0.260 ± 0.358 (11)	0.080 ± 0.080 (2)	-	0.750 ± 0.250 (2)	0.666 ± 0.229 (12)	0.956 ± 0.000 (1)	0.442 ± 0.285 (8)	**0.423 ± 0.231 (12)**
Enterobacter spp.	0.329 ± 0.406 (10)	0.000 ± 0.000 (1) [a]	0.798 ± 0.236 (3)	0.484 ± 0.358 (5)	0.276 ± 0.246 (6) [a]	0.000 ± 0.000 (1)	0.272 ± 0.367 (9)	-	-	0.542 ± 0.208 (2)	0.591 ± 0.212 (10)	0.857 ± 0.000 (1)	0.404 ± 0.342 (6)	**0.424 ± 0.229 (10)**
Salmonella spp.	0.300 ± 0.353 (9)	0.000 ± 0.000 (1) [a]	0.902 ± 0.000 (1)	1.000 ± 0.000 (1)	0.338 ± 0.413 (7) [a]	-	0.134 ± 0.246 (9) [b]	1.000 ± 0.000 (2)	-	0.802 ± 0.217 (3)	0.577 ± 0.410 (10)	0.427 ± 0.357 (3)	0.308 ± 0.372 (8)	**0.391 ± 0.271 (10)**
Aeromonas spp.	0.011 ± 0.018 (7)	0.000 ± 0.000 (2)	0.586 ± 0.309 (3)	0.813 ± 0.000 (1)	0.159 ± 0.268 (5)	0.000 ± 0.000 (1)	0.000 ± 0.000 (6)	1.000 ± 0.000 (1)	1.000 ± 0.000 (1)	0.778 ± 0.314 (3)	0.730 ± 0.331 (7)	1.000 ± 0.000 (1)	0.100 ± 0.200 (5)	**0.304 ± 0.135 (7)**
Proteus spp.	0.384 ± 0.432 (7)	- [a]	0.555 ± 0.016 (2)	0.524 ± 0.278 (4)	0.494 ± 0.271 (4) [a]	-	0.366 ± 0.317 (6)	-	-	0.500 ± 0.000 (1)	0.524 ± 0.274 (7)	1.000 ± 0.000 (1)	0.683 ± 0.331 (4)	**0.471 ± 0.182 (7)**
Acinetobacter spp.	0.117 ± 0.194 (5)	0.000 ± 0.000 (1) [a,*]	0.467 ± 0.033 (2)	0.708 ± 0.257 (3)	0.594 ± 0.427 (4)	0.000 ± 0.000 (1)	0.263 ± 0.327 (4)	-	0.750 ± 0.000 (1)	1.000 ± 0.000 (1)	0.563 ± 0.343 (5)	0.833 ± 0.000 (1)	0.000 ± 0.000 (2)	**0.388 ± 0.151 (5)**
Citrobacter spp.	0.000 ± 0.000 (5)	0.000 ± 0.000 (1)	0.180 ± 0.055 (2)	0.563 ± 0.438 (2)	0.250 ± 0.433 (4)	0.000 ± 0.000 (1)	0.000 ± 0.000 (4)	-	-	0.625 ± 0.000 (1)	0.505 ± 0.346 (5)	0.588 ± 0.000 (1)	0.123 ± 0.102 (3)	**0.269 ± 0.155 (5)**
Serratia spp.	0.200 ± 0.400 (5)	0.333 ± 0.000 (1) [a]	0.536 ± 0.036 (2)	0.667 ± 0.333 (2)	0.111 ± 0.157 (3) [a]	0.000 ± 0.000 (1)	0.167 ± 0.289 (4)	-	-	0.250 ± 0.250 (2)	0.652 ± 0.129 (5)	0.429 ± 0.000 (1)	0.000 ± 0.000 (3)	**0.351 ± 0.138 (5)**
Vibrio spp.	0.359 ± 0.178 (4)	-	-	0.415 ± 0.000 (1)	0.360 ± 0.454 (3)	-	0.000 ± 0.000 (2)	1.000 ± 0.000 (1)	-	0.590 ± 0.410 (2)	0.820 ± 0.312 (4)	0.875 ± 0.125 (2)	0.491 ± 0.425 (4)	**0.520 ± 0.237 (4)**
Alcaligenes spp.	0.389 ± 0.437 (3)	-	0.750 ± 0.000 (1)	0.500 ± 0.000 (1)	0.250 ± 0.250 (2)	-	0.375 ± 0.375 (2)	-	-	0.000 ± 0.000 (1)	0.757 ± 0.236 (3)	0.417 ± 0.000 (1)	0.250 ± 0.250 (2)	**0.461 ± 0.205 (3)**
Flavobacterium spp.	0.250 ± 0.204 (3)	-	0.500 ± 0.000 (1)	0.500 ± 0.000 (1)	0.500 ± 0.500 (2)	-	0.250 ± 0.250 (2)	-	0.750 ± 0.000 (1)	0.000 ± 0.000 (1)	0.611 ± 0.283 (3)	0.333 ± 0.000 (1)	0.000 ± 0.000 (2)	**0.397 ± 0.282 (3)**
Shigella spp.	0.567 ± 0.419 (3)	-	-	0.833 ± 0.000 (1)	0.458 ± 0.458 (2)	-	0.375 ± 0.375 (2)	-	-	1.000 ± 0.000 (1)	0.617 ± 0.238 (3)	1.000 ± 0.000 (1)	0.500 ± 0.500 (2)	**0.607 ± 0.273 (3)**
Chromobacterium spp.	0.000 ± 0.000 (2)	1.000 ± 0.000 (1)	0.667 ± 0.000 (1)	-	0.500 ± 0.000 (1)	0.000 ± 0.000 (1)	0.000 ± 0.000 (1)	-	-	-	0.750 ± 0.250 (2)	0.000 ± 0.000 (1)	0.000 ± 0.000 (1)	**0.339 ± 0.106 (2)**
TOTAL[§]	**0.253 ± 0.274 (61)**	**0.040 ± 0.078 (16)**	**0.594 ± 0.325 (14)**	**0.529 ± 0.316 (26)**	**0.326 ± 0.291 (38)**	**0.150 ± 0.236 (8)**	**0.178 ± 0.199 (57)**	**0.549 ± 0.342 (15)**	**0.493 ± 0.325 (7)**	**0.493 ± 0.325 (21)**	**0.492 ± 0.313 (66)**	**0.517 ± 0.315 (18)**	**0.393 ± 0.285 (50)**	**0.352 ± 0.207 (70)**

MAR Index = Multiple antimicrobial resistance index = aggregate antimicrobial resistance score of all isolates tested / (number of isolates tested x number of antimicrobials tested against) (Krumperman, 1983). = mean MAR index ≤0.200; = mean MAR index from 0.201 to 0.400 ; = mean MAR index from 0.401 to 0.600; = mean MAR index from 0.601 to 0.800, and = mean MAR index from 0.801 to 1.000. SD = Standard deviation, n = number of studies.

[1] Amikacin, Gentamicin, Kanamycin, Neomycin, Netilmicin, Paromomycin, Streptomycin and/or Tobramycin; 61 studies, [2] Biapenem, Ertapenem, Imipenem and/or Meropenem; 16 studies, [3] Cefadroxil, Cefalexin, Cefazolin, Cefradine and/or Cephaloridine; 14 studies, [4] Cefaclor, Cephalothin, Cefamandole, Cefoxitin and/or Cefuroxime; 26 studies, [5] Cefixime, Cefixime, Cefoperazone, Cefoperazone-sulbactam, Cefotaxime, Cefotaxime-clavulanic Acid, Cefpodoxime, Cefsulodin, Ceftazidime, Ceftiofur and/or Ceftriaxone; 28 studies, [6] Cefepime; 8 studies, [7] Ciprofloxacin, Enrofloxacin, Levofloxacin, Moxifloxacin, Nalidixic acid, Nitrofurantoin, Norfloxacin, Ofloxacin, Pefloxacin and/or Sparfloxacin; 57 studies, [8] Teicoplanin and/or Vancomycin; 15 studies, [9] Clindamycin and/or Lincomycin; 7 studies, [10] Erythromycin and/or Roxithromycin; 21 studies, [11] Amoxicillin, Amoxicillin-clavulanic acid, Ampicillin, Ampicillin-sulbactam, Ampiclox, Carbenicillin, Cloxacillin, Mecillinam, Methicillin, Oxacillin, Piperacillin, Piperacillin-tazobactam, Ticarcillin and/or Ticarcillin-clavulanic acid; 66 studies, [12] Sulfamethoxazole, Sulfanilamide, Sulphafurazole and/or Sulfabenzamide-sulfacetamide-sulfathiazole (i.e. "Triple Sulfa"); 18 studies, [13] Chlortetracycline, Doxycycline, Oxytetracycline and/or Tetracycline; 50 studies.

[a] Priority 1 (i.e. critical) in the global priority list of antimicrobial-resistant bacteria[7]
[b] Priority 2 (i.e. high) in the global priority list of antimicrobial-resistant bacteria[7]
[c] Priority 3 (i.e. medium) in the global priority list of antimicrobial-resistant bacteria[7]
* The only *Pseudomonas*, *Klebsiella* and *Acinetobacter* species included in the global critical priority list of antimicrobial-resistant bacteria are *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Acinetobacter baumannii*, respectively[7]
# The only *Streptococcus* species included in the global medium priority list of antimicrobial-resistant bacteria is *Streptococcus pneumoniae*[7]
§ The total calculated MAR Indices were obtained per study, amalgamating results for all bacteria tested (included ones of unspecified genus) and using Equation 1. All studies were weighted equally

As shown (Fig. 2), consistently high occurrence rates of antimicrobial resistance were found among *Pseudomonas* (99.9% ± 0.2), *Klebsiella* (99.8% ± 0.6) and *Enterobacter* spp. (99.2% ± 2.2), as well as others which were reported in fewer studies (i.e. <7), with *Pseudomonas* spp. also exhibiting high rates of multidrug resistance (96.6% ± 5.8). Moreover, *Pseudomonas* spp. (0.544 ± 0.237) were associated with some of the highest calculated MAR indices, across the 15 studies that examined the genus (Fig. 2). MAR indices calculated within antimicrobial classes (Table 5) indicate that 1st generation Cephalosporins (0.594 ± 0.325), Glycopeptides (0.549 ± 0.378), 2nd generation Cephalosporins (0.529 ± 0.316) and Sulphonamides (0.517 ± 0.315) were associated with the highest mean MAR Indices. Conversely, lowest mean values were associated with Carbapenems (0.040 ± 0.078) and 4th generation Cephalosporins (0.150 ± 0.236).

A calculated mean MAR index of 0.352 ± 0.207 represents the level of ARB presence in global groundwater during the total review period (i.e. between 1976 and 2018; Fig. 3), and of 0.359 ± 0.207 from 2010 to 2018 (not shown). Just one study exhibited a calculated MAR index of 0.000 (zero) (i.e. Traoré et al., 2015). MAR index values were highest in Kenya (x ± SD = 0.549 ± 0.208), Nepal (0.545), China (0.537 ± 0.041), Morocco (0.528), Saudi Arabia (0.516) and South Africa (0.513 ± 0.122). At a continental level, MAR indices were highest in Africa (0.423 ± 0.202) and Asia (0.370 ± 0.201), followed by Central and South America (0.281 ± 0.098), Oceania (0.173 ± 0.000), Europe (0.138 ± 0.083) and North America (0.110 ± 0.045).

3.4. Potential drivers of antimicrobial-resistant bacteria in groundwater

As shown (Fig. 4), occurrence rates of antimicrobial resistance in groundwater bacteria were significantly higher in urban settlements ($p = 0.020$), with rates of MRB in groundwater from high income countries significantly lower than those from upper-middle income countries ($p = 0.035$). Studies that employed one-off sampling regimes yielded significantly higher percentages of both antimicrobial and multidrug resistance in groundwater bacteria when compared to regimes where re-sampling was employed ($p = 0.001$ and $p = 0.002$, respectively).

MAR indices were significantly lower in groundwater from high-income countries when compared to upper-middle ($p = 0.018$) and lower-middle ($p = 0.002$) income countries (Fig. 5), and significantly higher in arid versus temperate regions ($p = 0.021$) and in samples collected during dry versus wet seasons ($p = 0.014$). Significantly higher MAR indices were also observed in urban settlements ($p = 0.001$) and when waste sources adjacent to groundwater supplies were predominantly of human (e.g. septic tanks and wastewater treatment plants) as opposed to animal (e.g. animal grazing and manure spreading) origin ($p = 0.005$). No significant differences were found when comparing MAR index distributions with sources type (i.e. wells versus springs) or source construction (i.e. hand-dug versus bored wells). One-off sampling regimes ($n = 37$) yielded significantly higher MAR indices in groundwater when compared to regimes where re-sampling was employed ($n = 23$) ($p = 0.003$).

4. Discussion

Antimicrobial resistance is a widely recognised global public health threat with growing evidence of its spread beyond clinical settings now available within the scientific literature (Bradford and Harvey, 2017; Larsson et al., 2018; Opatowski et al., 2019). Most recently, the role of the natural aquatic environment in the dissemination of ARB has gained interest (Suzuki et al., 2017; Sanderson et al., 2018), with groundwater resources being

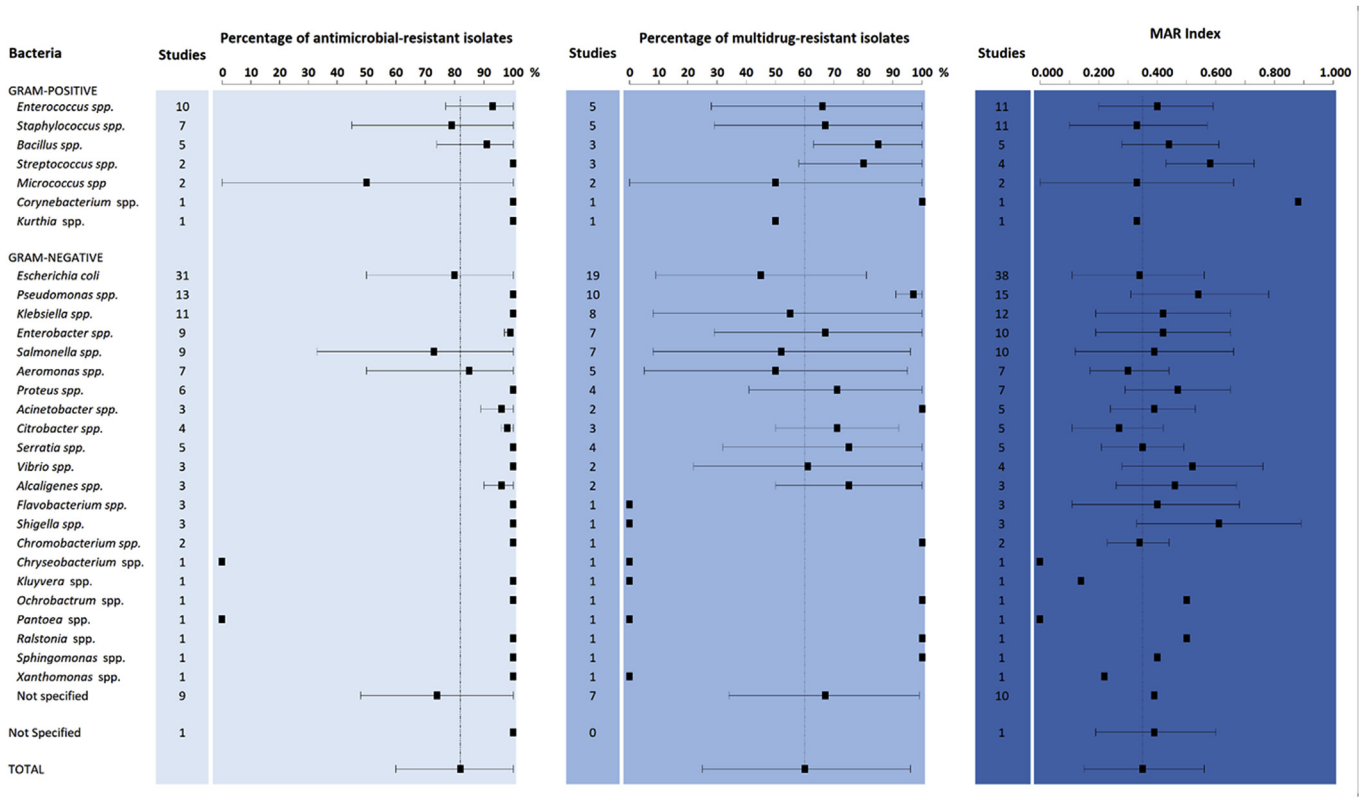


Fig. 2. Forest plot of (i) antimicrobial resistance, (ii) multidrug resistance and (iii) calculated multiple antimicrobial resistance (MAR) Indices in groundwater isolates from each genus examined.

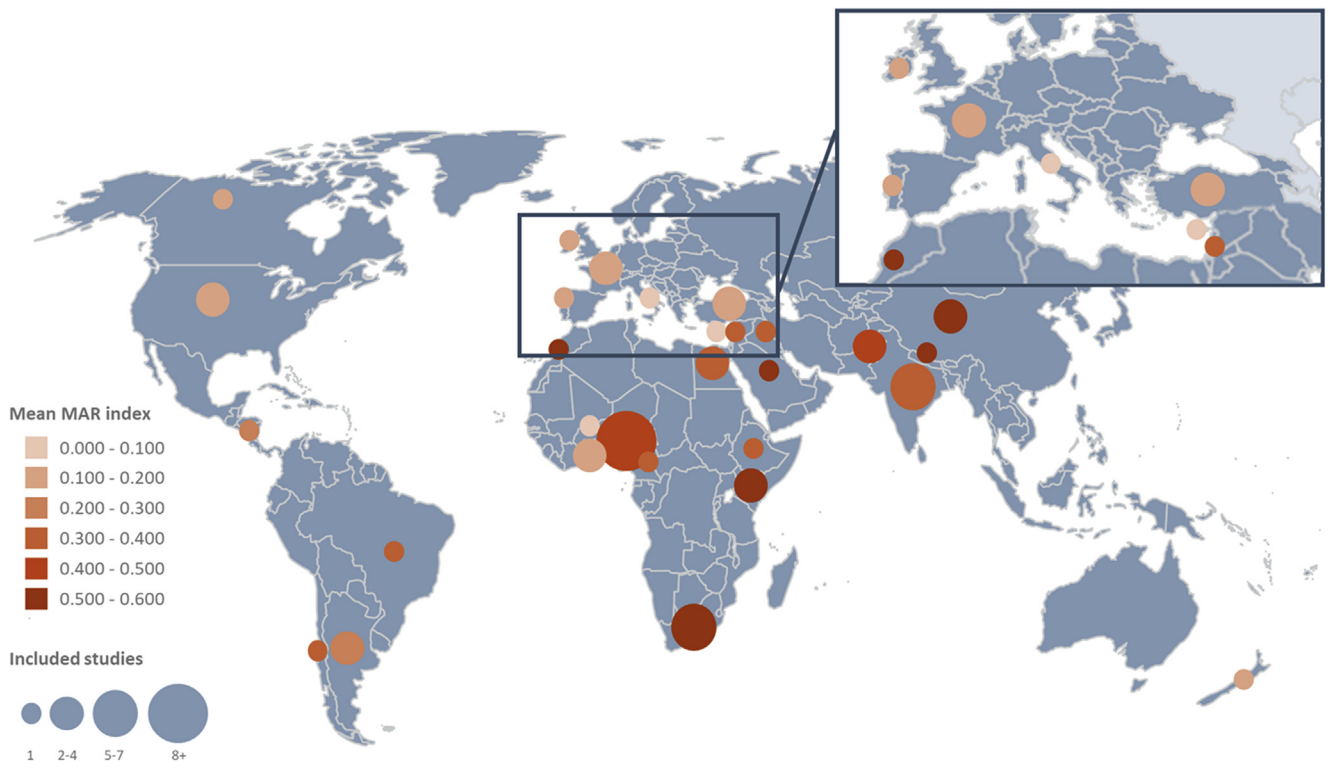


Fig. 3. Mean Multiple Antimicrobial Resistance (MAR) Indices in global groundwater from 1976 to 2018 (n = 70) in each included country.

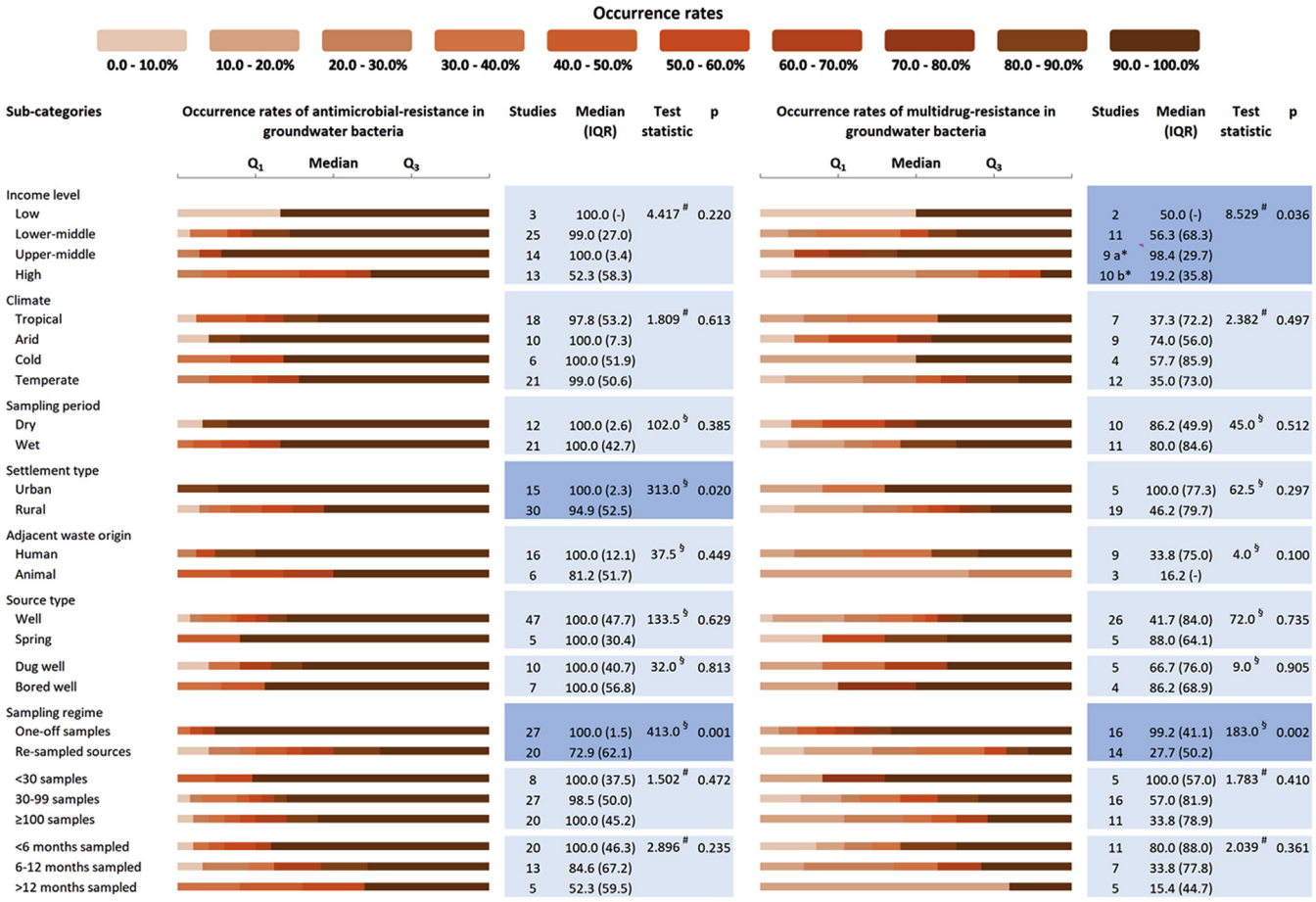


Fig. 4. Summary of descriptive and non-parametric test results for occurrence rates of (i) antimicrobial (n = 55) and (ii) multidrug resistance (n = 32) amongst groundwater isolates tested. Q_1 = 25th percentile; Q_3 = 75th percentile; IQR = Interquartile range = $Q_3 - Q_1$; $p \geq 0.1$; $p < 0.05$; # Kruskal-Wallis test; § Mann-Whitney *U* test; * Dunn's pairwise tests (each similar letter denotes a subset of each category whose distribution do not differ significantly from each other at the $p < 0.05$ level and different letters indicate statistically significant differences at the $p < 0.05$ level).

particularly relevant given the reliance of 2.2 billion people around the world on this source of drinking water (Murphy et al., 2017). Moreover, due to the widely held belief that groundwater is an intrinsically 'clean' water source, many sources lack treatment, in spite of well document incidences of faecal contamination as a result of agricultural and wastewater sources (Schets et al., 2005; Hynds et al., 2014; Murphy et al., 2017). As such, the risks posed via consumption of contaminated groundwater are significant, with a recent United Nations (UN) report referring to discharge of contaminated wastes to the environment and inadequate access to clean water as key drivers of antimicrobial resistance (IACG, 2019). However, despite the importance of groundwater for human consumption and its potential role in the global resistome, to date no comprehensive synthesis of previous studies of ARB in the subsurface has been undertaken. The current study sought to address this gap in the scientific literature.

Occurrence rates of ARB amongst groundwater-derived isolates were consistently high across the seventy included studies, which were undertaken in geographic, climatologic and socioeconomically diverse regions (Fig. 2; Table 4). In the pooled analysis, studies where isolates were not obtained were excluded and hence our findings are relevant to groundwater sources where bacteria are present, which, where reported, accounted for 31.4% ± 32.6 of studied groundwater sources. Results show that four fifths (80.2% ± 29.0) of aggregated groundwater isolates were resistant to one or more antimicrobial agents, with ARB identified in 76.9% ± 33.7 of individual groundwater sources where bacteria were present. These findings seem to suggest that in the minority of groundwater sources were bacteria were present they were often resistant to at least one antimicrobial. Just one study, undertaken in Burkina Faso, reported no ARB in examined groundwater sources (Traoré et al., 2015). The high mean MAR index values calculated across differing regions (Fig. 3), when considered in concurrence with high occurrence rates of MRB (57.2% ± 36.8), further highlights the presence of multidrug resistance as a particular issue within groundwater sources. Compounding this, myriad ARB listed on the World Health Organisation priority list as 'critical' (WHO, 2017) were specifically noted in across included studies (Table 5); a high proportion of which were used for human consumption (e.g. Ribeiro et al., 2014; Maran et al., 2016; O'Dwyer et al., 2017). Accordingly, pooled results point to groundwater as an environment characterised by the presence of ARB and MRB, and highlight its potential role in the spread of antimicrobial resistance both within the environment and directly to humans via consumption of, frequently untreated, drinking water (Coleman et al., 2012).

Similarly, the abovementioned concurrently high incidence of ARB and MRB, and elevated pooled mean MAR index (0.352 ± 0.207), suggests the presence of significant selective pressures within groundwater systems, potentially resulting from extended residence times along subsurface pathways with exposure to sub-therapeutic concentrations of antimicrobial residues. Continuous release of human and veterinary antimicrobials to the

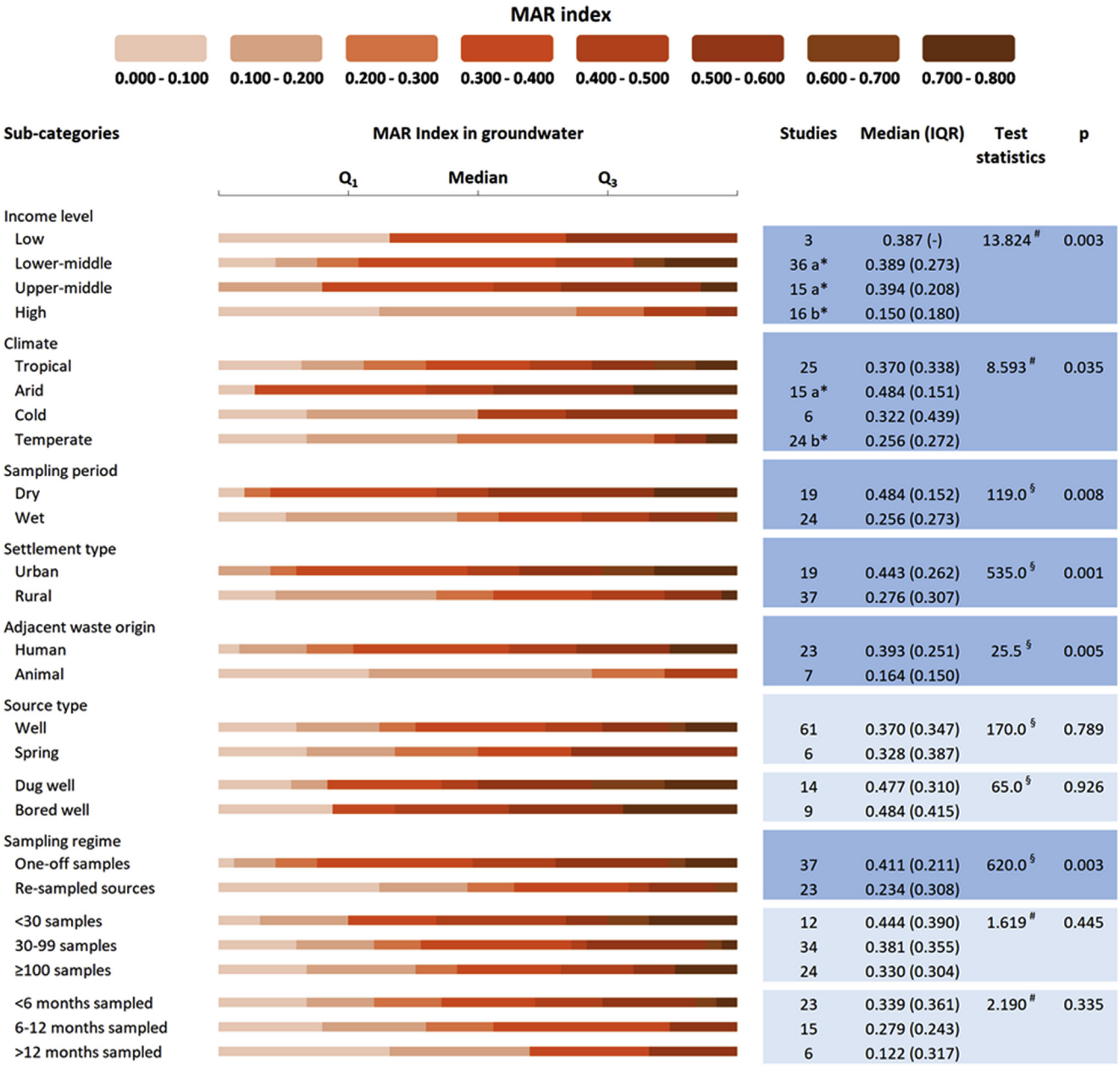


Fig. 5. Summary of descriptive and non-parametric test results for the calculated Multiple Antimicrobial Resistance (MAR) indices in groundwater (iii; n = 70). Q_1 = 25th percentile; Q3 = 75th percentile; IQR = Interquartile range = $Q_3 - Q_1$; ▫ $p \geq 0.05$; ■ $p < 0.05$; # Kruskal-Wallis test; § Mann-Whitney *U* test; * Dunn's pairwise tests (each similar letter denotes a subset of each category whose distribution do not differ significantly from each other at the $p < 0.05$ level and different letters indicate statistically significant differences at the $p < 0.05$ level).

natural environment has been shown to facilitate their ingress to both surface and groundwater sources (Van Schaik, 2015); however, occurrence rates of antimicrobial resistance in surface water bacteria found in middle- and high-income countries (O'Flaherty and Cummins, 2017) are lower than those encountered in the current study. Unlike surface water, extended residence times associated with subsurface systems may lead to prolonged bacterial exposure to antimicrobial residues (at low concentrations), resistance genes and ARB within a relatively confined and oftentimes buffered (e.g. UV, pH and temperature) environment (Van Schaik, 2015; Williams-Nguyen et al., 2016). Compounding this, bacterial isolates may present high rates of resistance prior to subsurface ingress, with a recent study reporting that tetracycline-resistant *E. coli* strains were more mobile than susceptible strains in saturated porous media (Walczak et al., 2011), and thus can be characterised by higher rates of transport in the subsurface. However, there is limited research exploring this hypothesis in diverse soil and aquifer types. Indeed, further research is required which combines microbial source tracking, advanced hydrogeological modelling, antimicrobial susceptibility testing and pharmaceutical residue concentrations to facilitate a greater understanding of the intricacies of microbial transport and resistance acquisition by these microbes in the subsurface.

More generally, pooled analyses suggest a paucity of research specifically addressing the mechanisms mediating the occurrence of ARB and MRB in groundwater, particularly with respect to

varying climates, anthropogenic practices, hydrogeological settings, and groundwater source types. This represents a common limitation within blended groundwater and microbiological studies; due to their multidisciplinary nature, important source-pathway-receptor information are oftentimes omitted, overlooked or unreported (Hynds et al., 2014). For example, just 8.6%, 17.1% and 18.6% of included studies explicitly reported meteorological, hydrogeological, and detailed source-specific data, respectively. Accordingly, in combination with the inherently high degree of variability in reporting identified across identified studies, more robust quantitative examination (i.e., meta-regression) and (multi-) collinearity diagnostics could not be undertaken as part of the current study, which may be partially accountable (due to the multiple instances of missing data) for the lack of statistical significance between some external factors and ARB/MRB occurrence in groundwater (Fig. 4). Moreover, owing to the nature of the research reporting within identified studies, it was not possible to calculate "treatment" effects (effect sizes), thus a meta-analysis could not be undertaken, as studies could not be weighted using any meaningful outcome measure. As such, retrospective pooled analyses with the analytical units (i.e. individual study findings) weighted equally was employed to identify significant trends in this research field (Figs. 4 and 5). Result suggest that studies undertaken in high income countries reported significantly lower MAR indices compared to lower and upper middle income countries ($p = 0.003$), with similar results found for MRB occurrence (i.e. lower MRB rates reported in groundwater from high income countries; $p = 0.036$). This is likely due to lower antimicrobial diversity, lack of sanitation, poor hygiene practices and reduced antimicrobial stewardship in areas characterised by lower mean incomes (Morgan et al., 2011; Ayukekbong et al., 2017); however, the asymmetry in study numbers from each area (i.e. just 22.9% in high income countries; Table 4) likely impacted study findings.

Climatologically, pooled analysis indicates that groundwater samples collected during dry periods yielded significantly higher MAR indices ($p = 0.008$) than during wet periods, directly contrasting previous findings in surface water environments (Sanderson et al., 2018). While precipitation is roundly acknowledged as a primary driver of groundwater contamination (Hynds et al., 2012; Andrade et al., 2018), in the context of antimicrobial resistance, the evidence is less compelling. For example, drier periods may enable higher concentration of antimicrobial residues within the subsurface (Dhar et al., 2008), thus leading to increased bacterial exposure to sub-therapeutic levels of antimicrobial residues. Moreover, as temperature is directly correlated with bacterial proliferation, even marginal increases in subsurface temperatures may increase bacterial loading in-situ (John and Rose, 2005). However, it is important to note that in the absence of specific hydrogeological information, interpreting the impact of seasonality on ARB and MRB occurrence is largely speculative in this instance, further reiterating the need for a more "holistic" approach to multidisciplinary groundwater research. Socio-geographically, groundwater sources located in categorically urban (as opposed to rural) regions were associated with significantly higher MAR indices ($p = 0.001$; Fig. 5). Similarly (and likely collinear with this previous finding), supplies predominantly adjacent to human waste sources (e.g. septic tanks and wastewater treatment plants) were characterised by a significantly higher ($p = 0.005$) mean MAR index than those adjacent to animal waste sources (e.g. animal grazing and manure spreading). This is possibly driven by the antimicrobial residual concentration differentials between urban and rural areas. For example, while antimicrobial concentrations in manure are typically orders of magnitude higher than those encountered in wastewaters (Arikan et al., 2009; Sabri et al., 2018), the spatiotemporal exposure is inconsistent, due to the diffuse nature of the source. Conversely, wastewater treatment infrastructures typically result in point source contamination mechanisms, providing a consistent source of low-dose antimicrobial residues. Moreover, transmission (infiltration) of common veterinary antimicrobials in the subsurface may be confined and thus spatially limited. Transport of tetracycline compounds, in particular, are restricted to fast preferential and macropore flow or require facilitation of co-transport with mobile colloids, such as dissolved organic matter (Thiele-Bruhn, 2003). Nevertheless, an increase in the global occurrence of ARB and MRB in groundwater sources is expected due to current and future population growth, urbanisation, lack of sanitation, inappropriate wastewater treatment, and the misuse and over-use of antimicrobials, irrespective of geographic location.

Focusing on the identification of factors driving ARB and MRB occurrence, significant research challenges also exist due to inherent complexities associated with the genetic acquisition of antimicrobial resistance, in addition to the multifaceted hydrogeological mechanisms governing subsurface contamination. For example, while MAR indices calculated within Carbapenems, which are generally used as "last line" drugs (Van Boeckel et al., 2014), were found to be significantly lower than other classes, several similarly prescribed antimicrobials, such as Glycopeptides (Van Boeckel et al., 2014), were associated with relatively high mean MAR index values (Table 4). Accordingly, antimicrobial usage itself may not be a primary driver of MRB or ARB in groundwater, despite remaining an undeniably important factor in the broader antimicrobial resistance crisis. This is in line with findings from Collignon et al. (2018) that antimicrobial consumption was not significantly associated with the global indices for antimicrobial resistance.

At the genus level, there was significant variance observed in the rates of encountered antimicrobial resistance (Fig. 2; Table 5). Ubiquitous members of the soil microbiome *Pseudomonas* spp., *Klebsiella* spp. and *Enterobacter* spp. exhibited some of the highest rates of antimicrobial resistance at 99.9% ± 0.2, 99.8% ± 0.6 and 99.2% ± 2.2, respectively. These findings are particularly significant as both Pseudomonas and Klebsiella spp. are considered to be ubiquitously "soil-resident" bacteria, and as such, may characterise the local and/or source specific resistome more accurately than faecal indicator and/or (opportunistic) pathogens, which are often absent. However, it should be noted that a proportion of this resistance is attributable to 'intrinsic resistance', and thus, should not be interpreted as a wholly anthropogenic phenomenon in all cases. For example, *Pseudomonas aeruginosa* is intrinsically resistant to many antimicrobial agents accredited to the low permeability of its outer membrane (Livermore, 1984), the expression of various efflux pumps with wide substrate specificity (Livermore, 2001) and the naturally occurring chromosomal AmpC blactamase (Nordmann and Guibert, 1998). A more compelling assessment of acquired resistance focuses on *E. coli*, which was the most frequently examined bacterial species across the studies, with pooled isolates exhibiting below average, but considerably high, rates of resistance to ≥1 (79.8 ± 30.1) and ≥3 (45.4 ± 36.0) antimicrobial agents. Mechanisms explaining between-species variation could not be determined in the current study, as it may reflect locally specific circumstances. However, drawing from previous field and modelling work (Hynds et al., 2012), findings may suggest higher rates of ARB ingress and resistance acquisition taking place in the subsurface via "traditional" recharge, with lower rates of antimicrobial resistance associated with rapid entry (i.e. runoff at wellhead and/or preferential flow) into groundwater sources. As such, it is recommended that future studies perform antimicrobial susceptibility assays, where possible, using both faecal and soil-resident bacteria to accurately establish the extent of resistance

within microbial communities in groundwater, and their most likely ingress mechanisms. Moreover, as molecular methods of antimicrobial resistance characterisation (specific gene targeting) increase in popularity, it is important that isolate-specific phenotypic (i.e. culture-based) research be undertaken to permit a greater understanding of the specific public health significance of environmental exposures to ARB."

As with any literature findings-based study, it is important to acknowledge that data used for analyses are limited to previous reports; and thus, the potential for reporting bias exists and should be highlighted (i.e. studies targeting susceptible areas or groundwater sources where ARB contamination is suspected). Over half (52.9%) of identified studies included in this review based their findings on "one-off" (i.e. non-temporal) groundwater sampling regimes, and were associated with significantly higher MAR indices ($p = 0.003$). Moreover, a widespread lack of explicit methodology for achieving sample representativeness was identified during validity appraisal (Supplementary Materials 1). As such, the occurrence rates of ARB and MRB in global groundwater garnered from this review may represent an overestimate, with true values likely more accurately reflected via means calculated from temporal studies only (i.e. ARB = 65.5% ± 33.4 and MRB = 38.9 ± 32.0). However, the issue of temporal and spatial representativeness still represents a significant concern. Baseline sampling and analytical procedures should be developed and routinely employed via unbiased and robust spatiotemporal studies, following consistent methodologies, in diverse (hydro)geological and climatic settings.

The current study is the first to integrate findings from international literature regarding the occurrence of ARB and MRB in groundwater sources. While this research is undoubtedly topical, identified studies highlight a long-standing issue, with the earliest relevant research available within the scientific literature (Cooke, 1976) reporting antimicrobial resistance in 48.9% of 321 total and faecal coliform isolates (species unspecified) from groundwater sources in New Zealand. As such, and as groundwater remains an important source of potable water for a considerable proportion of the global population, the results presented show that groundwater is a significant environment where antimicrobial resistance can be spread, and highlight the need for further research looking at the occurrence of ARB and MRB in the subsurface environment.

5. Conclusion

Study results highlight groundwater as a noteworthy source of global ARB and MRB, and the pressing need for more representative studies (i.e. "baseline" work) on this topic. Additionally, lack of more robust methodologies and of key relevant data were identified as persistent issues among published work, making it difficult to ascertain a comprehensive global perspective, which is vital to quantify the risks associated with groundwater consumption and antimicrobial resistance. Thus, a key recommendation of this research is the requirement for the scientific community to refine their approach to groundwater- and health-related research. Specifically, it is imperative that future studies employ:

- hydrogeological, meteorological, climatic and detailed source-specific data measurement and reporting;
- temporal, rather than one-off, sampling methodologies;
- spatial distribution (as an attempt to achieve representativeness); and
- assessment of both faecal and soil-resident bacteria to establish the presence, origin and ingress mechanisms of antimicrobial resistant bacteria in groundwater systems.

The importance of efforts to understand and prevent the spread of antimicrobial resistance cannot be overstated. In an era characterised by significant global challenges, including antimicrobial resistance and anthropogenic climate change, consolidation of robust research approaches and systematic design of sampling programmes to help answer pressing global questions is not only warranted, but necessary. Regardless, findings of this study offer valuable insights into the extent and significance of groundwater as a potential source of ARB, and provides guidance for future research to guide policy development, action plans and remediation efforts/technologies to safeguard public health into the future.

Author contributions

L.A. and M.K. performed the initial review of literature. All authors (L.A., M.K., P.H., J.W., A.M. and J.OD.) determined final inclusion. Statistical analyses were carried out by L.A. in consultation with J.OD. and P.H. All authors were involved in the writing of the manuscript, with J.OD., P.H. and J.W. providing final approval for submission.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.115360.

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Spatiotemporal dynamics of *Escherichia coli* presence and magnitude across a national groundwater monitoring network, Republic of Ireland, 2011–2020

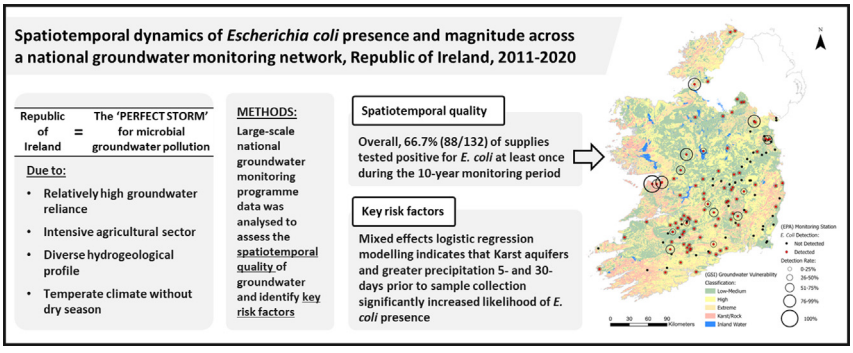
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HIGHLIGHTS

- Novel microbial assessment of a national groundwater monitoring scheme
- *E. coli* detected in 66.7% of monitoring wells (88/132) at least once in 10 years
- *E. coli* present >10MPN/100 ml in 29.5% of monitoring wells (39/132) within 10-year period
- Pastural agriculture and karst geology key risk factors for *E. coli* occurrence

GRAPHICAL ABSTRACT



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Hydrodynamics

ABSTRACT

Groundwater is a vital drinking water resource and its protection from microbiological contamination is paramount to safeguard public health. The Republic of Ireland (RoI) is characterised by the highest incidence of verocytotoxigenic *Escherichia coli* (VTEC) enteritis in the European Union (EU), linked to high reliance on unregulated groundwater sources (~16% of the population). Yet, the spatio-temporal factors influencing the frequency and magnitude of microbial contamination remain largely unknown, with past studies typically constrained to spatio-temporally 'limited' sampling campaigns. Accordingly, the current investigation sought to analyse an extensive spatially distributed time-series (2011 − 2020) of groundwater monitoring data in the RoI. The dataset, compiled by the Environmental Protection Agency (EPA), showed 'high' contamination rates, with 66.7% (88/132) of supplies testing positive for *E. coli,* and 29.5% (39/132) exceeding concentrations of 10MPN/100 ml (i.e. gross contamination) at least once during the 10-year monitoring period. Seasonal decomposition analyses indicate that *E. coli* detection rates peak during late autumn/early winter, coinciding with increases in annual rainfall, while gross contamination peaks in spring (May) and late-summer (August), likely reflecting seasonal shifts in agricultural practices. Mixed effects logistic regression modelling indicates that monitoring sources located in karst limestone are statistically associated with *E. coli* presence (OR = 2.76, p = 0.03) and gross contamination (OR = 2.54, p = 0.037) when compared to poorly productive aquifers (i.e., transmissivity below 10m^2/d). Moreover, 5-day and 30-day antecedent rainfall increased the likelihood of *E. coli* contamination (OR = 1.027, $p < 0.001$ and OR = 1.005, p = 0.016, respectively), with the former also being associated with gross contamination (OR = 1.042, $p < 0.001$). As such, it is inferred that preferential flow and direct

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ingress of surface runoff are the most likely ingress mechanisms associated with *E. coli* groundwater supply contamination. The results presented are expected to inform policy change around groundwater source protection and provide insight for the development of groundwater monitoring programmes in geologically heterogeneous regions.

1. Introduction

Globally, groundwater accounts for 95% of available freshwater, with approximately 31.5% (2.2 billion people) of the world's population relying on a groundwater supply for daily consumption (Murphy et al., 2017). In the Republic of Ireland (RoI), groundwater abstractions constitute approximately 25% of all drinking water with 16% of the national population (~750,000 people) relying on private groundwater sources for daily domestic use. While traditionally presumed to represent a "safe" water resource, largely due to the contaminant attenuation capacity of overlying subsoil layers, groundwaters are still susceptible to microbiological contamination (Bradford and Harvey, 2017). Agricultural activities such as livestock rearing and slurry/manure spreading, which vary spatially and temporally, and sewage disposal from domestic, municipal or industrial sources are among the most common sources of these organic contaminants (Andrade et al., 2018; Chique et al., 2020a, 2020b). Ingress of animal or human faecal matter represents a key threat to groundwater quality and public health, as these materials may contain pathogenic microorganisms such as *Salmonella* spp., *E. coli*, *Cryptosporidium* spp., *Campylobacter* spp., and noroviruses (Hynds et al., 2014a; Murphy et al., 2017; Kauppinen et al., 2018; Chique et al., 2020a, 2020b). For example, pathogenic *E. coli* are widely recognized as a key aetiological agent of human infection, causing mild- to severe- gastroenteritis, and have been widely reported in domestic groundwater sources from low- to high-income regions and across all climate classifications (Ferguson et al., 2012; Croxen et al., 2013; Odagiri et al., 2016; Murphy et al., 2017; Chique et al., 2020b). Crude incidence rates of verocytotoxigenic *Escherichia coli* (VTEC) enteritis in Ireland, an enteric infection characterised by high rates of waterborne transmission and a spectrum of severity ranging from mild diarrhoea to haemorrhagic colitis and haemolytic uremic syndrome (Hunter and Thompson, 2005, Karmali et al., 2010), are currently the highest in Europe (ECDC, 2019). Recent work by Ohaiseadha et al. (2017) report that the incidence of VTEC O157 infection is statistically associated with the use of private (household-based) groundwater supplies (OR 6.869; $p < 0.01$); a type of supply frequently linked with low rates of appropriate microbiological treatment (Hynds et al., 2013). Further, ongoing monitoring demonstrates that regulated groundwater abstractions serving small private and public buildings also regularly exhibit poor water quality, including frequent contamination with faecal indicator organisms (EPA, 2021a).

Microbial contamination of groundwater is a multifaceted issue, potentially influenced by a range of interconnected risk factors and (pre/post) conditions, such as prevailing weather (including extreme weather events), soil moisture, local hydrogeological setting, hazard proximity (setback distance and gradient), and source-specific infrastructural characteristics (e.g., well design, maintenance) (Hynds et al., 2012; Wallender et al., 2014; Atherholt et al., 2017; Andrade et al., 2018). The significance of risk factors, both individually and in combination, may be subject to considerable spatio-temporal variability (O'Dwyer et al., 2018, 2021), adding a layer of complexity to correctly attributing contamination sources and mechanisms. Hydro-meteorological variability plays a central role in the spread of pathogenic microorganisms throughout the natural environment, significantly affecting transport and eventual ingress to both aquifers and supplies (Hynds et al., 2012; Murphy et al., 2017; Moreira and Bondelind, 2017; Andrade et al., 2018). For example, extended periods of rainfall have been shown to promote the release of pathogens from faecal material, and its subsequent transport via surface runoff and subsurface percolation (Fremaux et al., 2008; Blaustein et al., 2016). In the Irish context, clear links have been established between *E. coli* detection in groundwater sources and antecedent (5–10 day) rainfall ($p < 0.05$) (Hynds et al., 2014b; O'Dwyer et al., 2014, 2018, 2021), due to enhanced (sub-)surface hydrological connectivity (e.g., overland flow, preferential infiltration/recharge). Likewise, heavy rainfall events, have been associated ($p < 0.01$) with outbreaks of VTEC enteritis in the RoI (O'Dwyer et al., 2016). Taken together, the interaction between rainfall (frequency, duration and intensity), enhanced hydrological connectivity, environmental dissemination of faecally-derived microorganisms, and public health represent an increasing concern. This is further heightened in light of mounting evidence of (global) climate change and the increased likelihood of more frequent and intense rainfall events (Arnell and Gosling, 2016; Forzieri et al., 2017).

Spatially distributed time-series data obtained from groundwater quality monitoring networks provide essential feedback for ascertaining the pathways and mechanisms associated with groundwater contamination (Hancock et al., 1998; Lee and Kwon, 2016; Little et al., 2016; Rayne et al., 2019; McGrory et al., 2017), and play a key role in informing policy aimed at safeguarding public health (Rohde et al., 2017). Thus, the EU Water Framework Directive (WFD) (Directive 2000/60/EC) developed a new foundation for European water resource management and policy-making by emphasising active water quality monitoring to identify and achieve pre-determined water quality targets. In the RoI, long-term groundwater monitoring is managed by the Environmental Protection Agency (EPA) with assistance from the Geological Survey of Ireland (GSI) through the National Groundwater Monitoring Programme (NGMP), which has routinely monitored the chemical and microbial quality of Irish groundwater since the early 1990s. To date, however, robust temporal and spatiotemporal analyses of monitoring data have yet to be undertaken at a national scale.

Accordingly, the current study sought to explore the spatiotemporal incidence of *E. coli* in Irish groundwater, a region characterised by high concurrent rates of groundwater reliance and waterborne enteric infection, using the publicly available NGMP dataset. Specifically, and with a marked "mechanistic" focus, the objectives of this study were to: (*i*) examine groundwater *E. coli* detection rate across Ireland as they relate to potential (extra-)local drivers (e.g., hydrogeological setting, local anthropogenic pressures, antecedent rainfall, seasonality) and (*ii*) statistically attribute likely *E. coli* contamination mechanisms via delineated modelling of *E. coli* magnitude and seasonally-specific temporal periods. Ultimately, findings may be used to inform relevant stakeholders, policy-makers, and regulatory frameworks, develop increasingly evidence-based groundwater protection guidelines and leverage an active (long-term) groundwater monitoring network.

2. Methods

2.1. Groundwater monitoring data

The NGMP comprises 284 geographically distributed monitoring sites, selected to represent variations in hydrogeological setting, contamination sources, pathways and pressures across the country (EPA, 2021b). Monitoring sites include 132 pumping boreholes (i.e., drilled wells) which serve as public or private groundwater supplies, 72 springs or infiltration galleries, 70 observation boreholes constructed for water quality surveillance monitoring (i.e., not employed for human use), 8 surface water monitoring points, and 2 unclassified abstractions. The current study focused exclusively on data derived from groundwater supplies ($n = 132$) (Fig. 1), as these are regularly pumped and thus likely representative of national abstractions for human consumption. Almost all sites ($n = 128$, 97%) are defined as active drinking water supplies, with the remaining 4 supplies employed for agricultural/industrial use.

The supplies monitored as part of the NGMP are geographically distributed and located in hydrogeologically diverse sites, with all groundwater

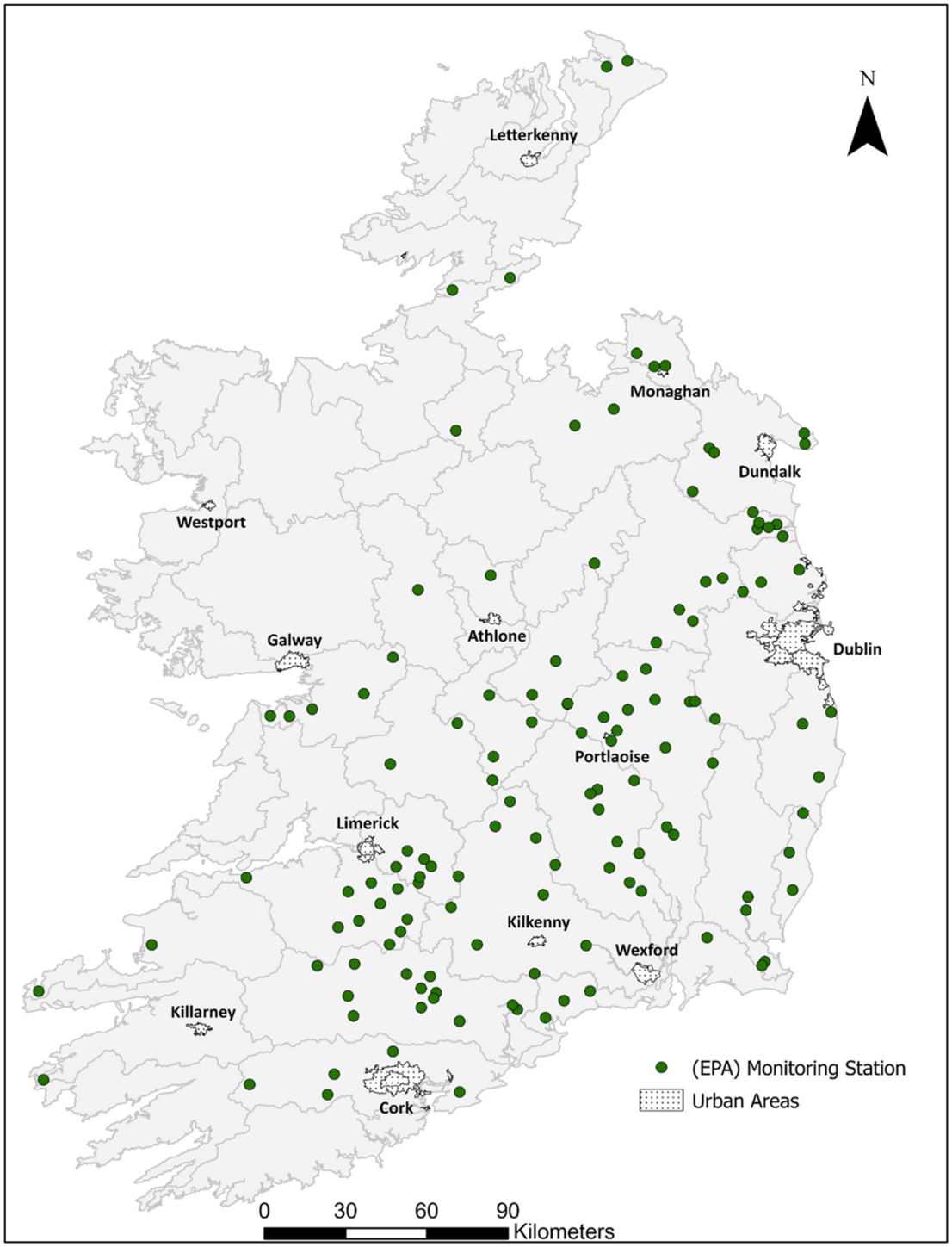


Fig. 1. Map of the Republic of Ireland showing EPA monitoring stations included in the current study ($n = 132$).

vulnerability classifications being represented. The existing groundwater vulnerability classification used in the RoI is based on multiple hydrogeological factors, such as the thickness and permeability of overlaying subsoil layers, type of aquifer, presence of an unsaturated zone, and (in the case of karst areas) recharge type (DoEHLG/EPA/GSI, 1999). The current vulnerability typology includes five categories: "Low", "Moderate", "High", "Extreme" and "Extreme X (Karst/Rock with little to no overlaying soil layers)"; these serve as indicators for the theoretical risk of contaminant ingress from surface-based sources via groundwater recharge (DoEHLG/EPA/GSI, 1999). Previous Irish studies report vulnerability classifications as a useful predictor for the presence of microbial groundwater contaminants (Hynds et al., 2014a, 2014b; O'Dwyer et al., 2018).

Data from a 10-year monitoring period (2011 to 2020, inclusive) were used in the current study, with supplies sampled a mean of 3 (St. Dev ± 1) times annually, totalling in a mean of 29 (St. Dev ± 6) samples per supply. Water samples were collected from pre-treatment (raw water) well taps, with *E. coli* presence and magnitude determined via the most probable number (MPN, multiple tube) method (ISO 9308-2:2012). Samples where *E. coli* concentrations exceeded 10 per 100 ml (10 MPN/

100 ml) were regarded as "grossly contaminated", which is consistent with the classification historically employed for thermotolerant coliforms in the Irish groundwater context (EPA, 2006; Bacci and Chapman, 2011). Monthly time series of *E. coli* detection rate across the monitoring network and, where *E. coli* were present, detection rates above 10 MPN/100 ml were developed.

2.2. Seasonal decomposition

Seasonal decomposition was employed to identify seasonal and overarching temporal trends in monthly detection rates of (i) *E. coli* incidence (i.e., presence) and, where present, (ii) incidence greater than 10 MPN/100 ml (i.e., indicative of gross contamination, Bacci and Chapman, 2011). Analyses were performed on the complete dataset (irrespective of location/hydrogeological setting) and for three subsets based on mapped groundwater vulnerability classification (i.e., "Low"/"Moderate", "High" and "Extreme"/"Extreme X – Rock/Karst").

During the study period several months were characterised by an absence of monitoring, ranging from one to seven consecutive months, thus requiring preliminary imputation prior to decomposition. Multiple imputation techniques were explored, with the "Seasonally Decomposed Missing Value Imputation" (SDMVI) approach (Moritz and Bartz-Beielstein, 2017) considered the most appropriate as it retains the seasonal signal within the original time-series. Briefly, SDMVI de-seasonalizes the time-series, re-adding the predicted seasonal signal post-imputation (i.e., imputation is based on simulated data residuals and trend). Imputation was performed at a monthly resolution using the "*imputeTS*" (v3.2) package in R Studio (v 4.0.5). Seasonal decomposition of the imputed time series was undertaken via the seasonal-trend decomposition method (STL) based on the LOESS (Locally Estimated Scatterplot Smoothing) technique. The STL method decomposes the time-series data into three distinct components (signals): (i) seasonal variation (based on a 12-month cycle), (ii) overall trend over time during the entire time series and (iii) residuals (remainders), with the original (raw) dataset equal to the sum of all three components based on an additive model. STL analyses were run in R version 4.0.5 using the *STL()* function from the *"forecast"* package (Hyndman et al., 2020).

2.3. Mixed-effects modelling

2.3.1. Outcomes and predictors

Mixed-effects modelling was employed to identify significant model inputs as they relate to modelled outcomes (*E. coli* presence and magnitude), thus permitting interpolation of likely (generalised) contamination mechanisms. This approach was used to account for repeat sampling and subsequent "non-independence" i.e., analytical time-series deriving from an individual groundwater supply cannot be considered statistically independent from sample to sample. Modelled outcomes were converted to binary format, with a logistic link function thus employed throughout. *E. coli* presence (>0 MPN/100 ml) was taken as being indicative of recent faecal ingress, and where present, concentrations above 10 MPN/100 ml were reclassified as being indicative of "gross contamination".

All modelled potential predictors i.e., parameters potentially influencing the presence and/or magnitude of *E. coli* in monitored groundwater supplies, are summarised in Table 1, these include groundwater vulnerability classification, density of nearby faecal sources, land-cover, antecedent rainfall, etc. Cattle and domestic wastewater treatment system (DWWTS) density variables were collated from census data available at the Electoral Division (ED) level, which is the smallest legally defined administrative area in the RoI ($n = 3440$), and at the Small Area (SA) level, which are delineated areas comprising between 80 and 120 dwellings ($n = 18{,}641$; CSO, 2011). Land-cover classifications were extracted from the CORINE (Co-ORdinated INformation on the Environment) dataframe (CORINE, 2012; CORINE, 2018). As such, monitored supplies located in the same Electoral Divisions were assigned the same estimated values. Landscape subcategories were derived from a recent reclassification of physiographic units in the RoI (Carlier et al., 2021). Cumulative antecedent rainfall (mm) parameters were calculated using daily historical data from the nearest Met Éireann synoptic station (Met Éireann, 2021); in cases where the nearest station lacked daily readings, data from the second nearest weather station were used in calculations (vis. Hynds et al., 2012). A time series for mean monthly precipitation across the country over the 10-year study period was also produced and analysed via the seasonal-trend decomposition method (STL). All georeferenced data were extracted in ArcMap v10.6 using sample source coordinates.

Table 1
Potential predictors of *E. coli* ingress into groundwater supplies, and their sources delineated into one of four categories, namely supply-specific, hydrogeological, land-use, topographical and hydrometeorological.

Predictors	Data source
Supply-specific parameters	
Supply type ("Public supply well", "Group water scheme well", "Private well", "Industrial/agricultural well")	EPA (2021a)
Supply depth (m)	EPA (2021a)
Hydrogeological parameters	
Aquifer type ("Fissured bedrock", "Karst", "Poorly productive bedrock", "Gravel")	GSI (2017a)
Aquifer importance ("Regionally important", "Locally important", "Poor")	GSI (2017a)
Groundwater vulnerability ("Low", "Moderate", "High", "Extreme", "Extreme X – Rock/karst")	GSI (2017b)
Bedrock type ("Limestone", "Sandstone", "Igneous", "Schist/gneiss")	GSI (2017a)
Land-use and topographical parameters	
Cattle density (heads/km^2)	CSO (2011)
Domestic Wastewater Treatment System (DWWTS) density (units/km^2)	CSO (2011)
Land-cover ("Pasture", "Non-pasture")	CORINE (2012); CORINE (2018)
Landscape ("Intensified Lowlands", "Semi-intensified Lowlands", "Semi-intensified Elevated", "Extensive Lowlands", "Extensive Uplands", "Extensive Bedrock Plains", "Peatlands", "Marshlands – Estuarine")	Carlier et al., 2021
Mean Small Area slope (m/m)	Authors
Hydrometeorological parameters	
1-day antecedent rainfall (mm)	Met Éireann (2021)
5-day antecedent rainfall (mm)	Met Éireann (2021)
10-day antecedent rainfall (mm)	Met Éireann (2021)
30-day antecedent rainfall (mm)	Met Éireann (2021)
60-day antecedent rainfall (mm)	Met Éireann (2021)
90-day antecedent rainfall (mm)	Met Éireann (2021)

2.3.2. Model training and testing

Multilevel mixed-effects logistic models (Vermunt, 2005) were used to examine associations between predictors (Table 1) and (i) *E. coli* presence (irrespective of season), (ii) gross contamination among *E. coli*-positive samples (irrespective of season), (iii) *E. coli* contamination during historically wetter months (October to March; Met Éireann, 2021), and (iv) *E. coli* contamination during historically drier months (April to September; Met Éireann, 2021). All statistical analyses were conducted in STATA/MP 14.2 using the *melogit* command and adjusted for sample correlation within the random nesting factor (well ID). All models were trained on 80% of the dataset (balanced based on outcome proportion), and tested on the remaining 20% to avoid model over-fitting and increase parsimony (Hynds et al., 2012). Each groundwater sample represented one observation (i.e., long data format) and all predictors (Table 1) were coded as fixed effects at the same hierarchical level, on condition that no strong inter-variable correlations were present (i.e., (multi)collinearity). Inter-variable correlations among predictors of interest were manually assessed prior to model development using Spearman's rho; where rho ≥ 0.5, sensitivity analyses were performed by including both input variables separately, with the best fitting model selected (based on model sensitivity and explained variance as indicated by pseudo-R-squared; McKelvey and Zavoina, 1975). One predictor (supply depth) was missing for >10% of observations and thus excluded from analyses.

3. Results

3.1. E. coli detection

Summary statistics describing the 132 monitored groundwater supplies and all water samples tested for *E. coli* during the study period (n = 3808) are presented in Table 2. Approximately three quarters of monitored supplies (n = 101; 76.5%) were located in areas classified as "pasture" (viz. CORINE, 2018), with mean calculated cattle and DWWTS densities of 123 ± 54 and 7.7 ± 3 per km^2, respectively. Monitored wells were located in areas classified as "High" (n = 62; 47.0%), "Moderate" (n = 23; 17.4%), "Low" (n = 22; 16.7%), "Extreme" (n = 17; 12.9%) and "Extreme X (Rock/Karst)" (n = 8; 6.0%) groundwater vulnerability, respectively. Locally important, regionally important, and poor aquifers were associated with 61 (46.2%), 60 (45.5%), and 11 (8.3%) supplies, respectively; 45 supplies (34.1%) were associated with low productivity bedrocks, followed by 38 (28.8%) in karst, 37 (28.0%) in productive fissured bedrock and 12 (9.1%) in gravel aquifers. The mean well depth, where reported (n = 101; 76.5%), was 55.7 ± 32.6 m ranging from 7.3 to 182 m. The distribution of hydrogeological and land-use parameters associated with monitored groundwater supply locations are presented in Supplementary Materials.

Overall, 66.7% (88/132) of monitored supplies tested positive for *E. coli* at least once during the 10-year study period (this is geographically represented in Fig. 2), with 29.5% (39/132) displaying concentrations above 10MPN/100 ml at least once in the same period. Detection rates of *E. coli* (>0 MPN/100 ml) and detection above 10 MNP/100 ml across all groundwater samples were of 12.8% (n = 486/3808) and 4.5% (n = 171/3808), respectively. "Gross" concentrations (>10 MNP/100 ml) were found in 35.2% (n = 171/486) of *E. coli*-contaminated samples. A mean *E. coli* detection rate of 12.6% (± 2.9%) was found in samples collected each year, ranging from 8.9% (2013) to 19.1% (2014), with concentrations above 10MPN/100 ml found in a mean of 4.4% (± 1.6%) of samples annually, ranging from 2.8% (2020) to 8.6% (2014) (Table 3). Likewise, mean *E. coli* detection rates were monotonic with respect to delineated groundwater vulnerability category (Table 3), with 10-year mean detection rates of 24.7%, 11.7% and 9.3% among boreholes in Extreme, High and Moderate/Low vulnerability categories, respectively. However, this pattern was not evident among samples >10 MPN/100 ml, with Extreme and High vulnerability areas exhibiting similar mean detection rates (36.3% and 38%, respectively), while Moderate/Low vulnerability areas displayed significantly lower rates of "gross" contamination (15%).

3.1.1. Seasonal decomposition

Seasonal decomposition identified seasonal patterns of *E. coli* detection and, where present, detection >10 MPN/100 ml over the 10-year study period (Fig. 3). Annual precipitation patterns in Ireland are characterised by increased precipitation volumes from October to March, while "dry" periods are typically more frequently reported from April to September (Fig. 4). Two hydrologically extreme events were reported during that time-frame: the nationwide winter floods of December 2015 to March 2016, and the June–July 2018 drought (seen in Fig. 4). As shown (Fig. 3a), *E. coli* detection rates annually peak in late autumn/early winter (October to December), which coincides with an increase in monthly precipitation in most years (Fig. 4). The proportion of samples characterised by *E. coli* > 10 MPN/100 ml exhibits a bi-modal pattern (Fig. 3b), with an initial peak during late spring (peaking in May) followed by a secondary (higher) peak in late Summer/early Autumn (August/September). Long-term *E. coli* detection trends indicate a notable increase during 2014, followed by a decrease over the two subsequent years, culminating in a relatively stable annual trend from 2018 onwards.

Decomposed seasonal patterns among delineated groundwater vulnerability subsets (Low/Moderate, High, and Extreme/karst) indicate distinct, albeit divergent, annual cycles (Fig. 5a and b). *E. coli* detection rates in Extreme-Karst" and "High" vulnerability areas show a relatively similar bi-modal, with initial peaks (≈20%) occurring in Summer (May – July), followed by a lower secondary peak (≈12%) in Autumn (September–October). Conversely, detection rates in Low-Moderate vulnerability

Table 2
Characteristics of monitored groundwater supplies (n = 132) and samples collected from 2011 to 2020 (n = 3808) as part of the EPA groundwater monitoring programme.

Characteristic	MS, n (%)	Samples, n (%)
Year		
2011	130 (98.5)	474 (12.4)
2012	129 (97.7)	489 (12.8)
2013	127 (96.2)	384 (10.1)
2014	127 (96.2)	372 (9.8)
2015	126 (95.5)	362 (9.5)
2016	120 (90.9)	358 (9.4)
2017	123 (93.2)	365 (9.6)
2018	123 (93.2)	366 (9.6)
2019	122 (92.4)	357 (9.4)
2020	120 (90.9)	281 (7.4)
Wet vs. dry months		
Wet months (Oct – Mar)	130 (98.4)	1535 (40.3)
Dry months (Apr – Sep)	130 (98.4)	2273 (59.7)
Land-cover		
Pasture	101 (76.5)	2943 (77.3)
Agriculture/arable land	15 (11.4)	460 (12.1)
Urban/industrial area	12 (9.1)	282 (7.4)
Vegetation/natural cover	4 (3.0)	122 (3.2)
Cattle density		
Low (≤50 head/km^2)	13 (9.8)	382 (10.0)
Low-Medium (51–100 head/km^2)	32 (24.2)	975 (25.6)
Medium (101–200 head/km^2)	73 (55.3)	2069 (54.3)
High (201–300 head/km^2)	8 (6.1)	246 (6.5)
Very high (>300 head/km^2)	1 (0.7)	32 (0.8)
NA (0 units/km^2)	4 (3.0)	104 (2.7)
DWWTS density		
Low (<5 units/km^2)	24 (18.2)	676 (17.7)
Medium (5–15 units/km^2)	106 (80.3)	30,989(81.1)
High (>15 units/km^2)	2 (1.5)	43 (1.2)
Aquifer importance		
Regionally important	54 (40.9)	1569 (41.2)
Locally important	64 (48.5)	1811 (47.6)
Poor	14 (10.6)	428 (11.2)
Aquifer type		
Poorly productive bedrock	54 (41.0)	1555 (40.8)
Productive fissured bedrock	32 (24.2)	940 (24.7)
Karst	32 (24.2)	928 (24.4)
Gravel	14 (10.6)	385 (10.1)
Groundwater Vulnerability		
Low	22 (16.7)	642 (16.9)
Moderate	23 (17.4)	632 (16.6)
High	62 (47.0)	1784 (46.8)
Extreme	17 (12.9)	504 (13.2)
Extreme X (Rock/Karst)	8 (6.0)	245 (6.4)
Supply depth		
Under 30 m	23 (17.4)	667 (17.5)
31 to 60 m	32 (24.2)	908 (23.8)
61 to 90 m	25 (18.9)	688 (18.1)
Over 90 m	21 (15.9)	593 (15.6)
Not reported	31 (23.5)	952 (25.0)
Supply use		
Drinking	128 (97.0)	3709 (97.4)
Agriculture/Industry	4 (3.0)	99 (2.6)
Water supply type		
Public Supply well	116 (87.9)	3343 (87.8)
Group Water Scheme well	9 (6.8)	275 (7.2)
Private Supply	3 (2.3)	91 (2.4)
Industrial supply	3 (2.3)	67 (1.8)
Agricultural supply	1 (0.7)	32 (0.8)
E coli presence		
E. coli absent	44 (33.3)	3322 (87.2)
E. coli present	88 (66.7)	486 (12.8)
E coli > 10 MPN/100 ml		
E. coli absent	44 (33.3)	3322 (87.2)
E. coli present <10 MPN/100 ml	49 (37.1)	315 (8.3)
E. coli > 10 MPN/100 ml	39 (29.5)	171 (4.5)

MS = Monitored Supplies; DWWTS = Domestic Wastewater Treatment System.

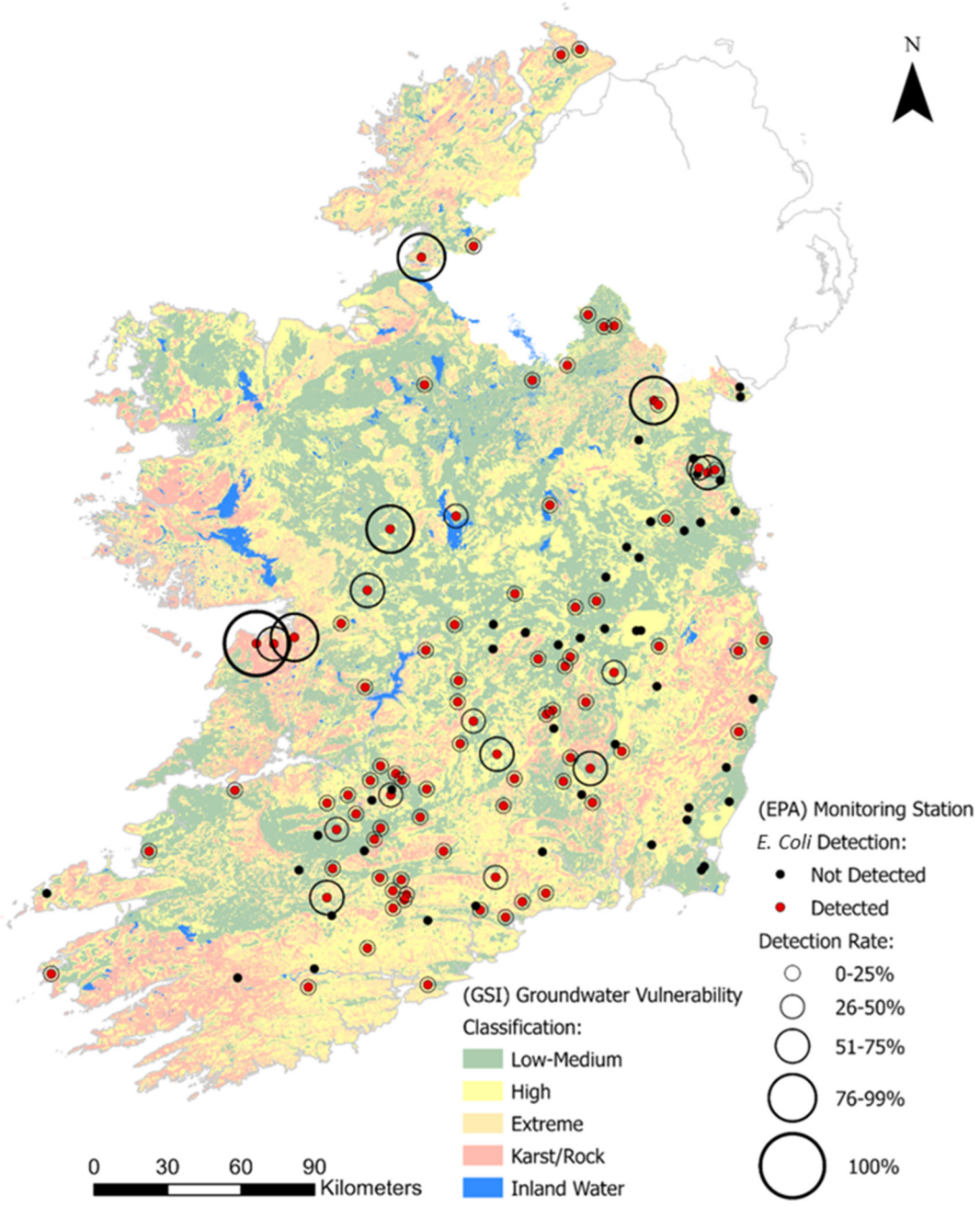


Fig. 2. Map of the Republic of Ireland showing *E. coli* detection and detection rates in monitoring stations from 2011 to 2020. Detection rates denote the percentage of contaminated samples among all samples collected during the study period.

exhibited a significantly lower level of seasonal variance (−15% - +12%), with just one noticeable peak during August–September. Decomposed seasonal detection rates >10 MPN/100 ml approximately mirrored detection rates >0 MPN/100 ml in both Extreme/Karst and Low vulnerability areas, likely pointing to a single dominant contamination mechanism and higher mean *E. coli* concentration when contaminated. This was not found among sources located in categorically High vulnerability areas i.e., detection rates pertaining to >0 MPN/100 ml and > 10 MPN/100 ml were not similar, with the seasonal signal for detection rates >10 MPN/100 ml showing a low level of variance (±5%) throughout the year.

3.2. E. coli contamination modelling

Trained mixed-effects models and associated confusion matrices for *E. coli* presence and gross *E. coli* contamination are presented in Tables 4 and 5, while models and associated confusion matrices specific to historically "wet" and "dry" months are presented in Tables 6 and 7. Strong inter-predictor correlations were found within all models between aquifer type and aquifer importance, and cattle and DWWTS densities at the ED and SA levels, as well as multiple combinations of cumulative rainfall data; only best-fit models (based on model sensitivity and accuracy, pseudo r-squared and predictor significance) have been presented.

3.2.1. E. coli contamination models

The *E. coli* contamination model (Table 4a) achieved a validation accuracy of 92.0%, which was comparable with the training set accuracy of 91.8%, with moderate validation and training sensitivities (i.e., true positive prediction) of 52.6 and 53.2%, respectively, owing to the skewed nature of the dataset; i.e. significantly more negative results than positive. As shown, the odds of *E. coli* presence were over 4-times higher among

Table 3
Annual detection rates of *E. coli* and gross (>10 MPN/100 ml) *E. coli* contamination within Low-Moderate (L-M), High (H) and Extreme (*E*-X) groundwater vulnerability categories.

	E. coli detection rates (%)				*E. coli* detection > 10 MPN/100 ml among contaminated samples (%)			
Year	L-M	H	E-X	Total	L-M	H	E-X	Total
2011	8.6	6.6	26.5	12.1	23.6	21.3	42.4	30.6
2012	10.5	11.6	28.8	14.1	28.1	30	39.9	30.5
2013	7.3	7.2	19.5	9.4	<1	29.8	26.4	18.5
2014	17.1	18.6	41.9	21.7	16.9	50.3	57.9	44.2
2015	8.4	17.1	31.5	18	25.5	41.1	36.5	42.2
2016	11	10.6	26.3	14.9	21.2	39.4	17.9	22.4
2017	8	14.5	13.1	11.6	14.2	31.8	35.9	22.1
2018	8.7	12.3	11.9	10.7	12.5	48.1	58.9	32.7
2019	5.3	9	21.2	10.9	8.3	37.7	24.4	23
2020	8.3	9	26.5	11.1	<1	51	22.7	24.5
2011–2020 (mean)	**9.3**	**11.7**	**24.7**	**13.5**	**15.0**	**38.0**	**36.3**	**29.1**

group water schemes than public water supply wells (OR = 4.59, 95% CI = 1.16–18.16), with the odds of *E. coli* presence significantly higher in karst areas (2.76, 1.10–6.95) and lower in areas underlain by productive fracture/ bedrocks (0.29, 0.10–0.83). Groundwater vulnerability classification was also highly predictive of *E. coli* presence, with 3- and 14-times higher odds of detection in areas classified as "Extreme" (3.12, 1.01–9.60) and "Extreme X (Rock/Karst)" (14.37, 3.36–61.40) than those in "High" groundwater vulnerability areas, respectively. A significant contrast was also observed with regards to land-use; supplies located in pasture-classified areas were associated with 4-times higher odds of *E. coli* presence (4.24, 1.47–12.34). Increases in 5- and 30-day cumulative rainfall significantly increased in the odds of *E. coli* presence, with odds ratios of 1.027 (95%CI = 1.017–1.038) and 1.005 (1.001–1.009), respectively.

In the gross *E. coli* contamination model (Table 4b), which only considered observations (samples) where *E. coli* was present (training subset = 359), model validation and training accuracies of 78% and 73.3% were achieved, with relatively modest sensitivities of 35.7% and 38.8%, respectively. A lower number of significant risk factors were identified. As for *E. coli* presence, supplies located in karst areas exhibited significantly higher odds of gross *E. coli* contamination than those in poorly productive bedrock aquifers (2.54, 1.06–6.09), with 5-day antecedent rainfall identified as a significant risk factor; a 1 mm increase in 5-day cumulative precipitation equated to a 4% increase in the odds of gross *E. coli* contamination (1.04, 1.024–1.061). Conversely, a 10 mm increase in 90-day cumulative rainfall concurred with a 4% decrease in the odds of gross *E. coli* contamination (0.996, 0.993–0.999). The odds of gross *E. coli* contamination were significantly lower in "Moderate" vulnerability areas when compared with "High" groundwater vulnerability areas (0.26, 0.09–0.80).

3.2.2. *E. coli* contamination in wet versus dry months

Mixed-effects modelling of *E. coli* contamination during historically wetter (October to March) and drier months (April to September) are shown in Table 6, with calculated accuracy and sensitivity for testing and training subsets highly comparable for both models (Table 7). In the 'drier months' model, supplies located in karst aquifers had higher odds of *E. coli* presence than those in poorly productive bedrocks (2.72, 1.02–7.28), while no significance was found in the 'wetter months' model. Increased odds of *E. coli* presence in "Extreme" compared to "High" groundwater vulnerability areas was found in 'drier months' model only (4.24, 1.26–14.26). Pasture-classified areas were associated with an increased odds of *E. coli* presence in both models, with the

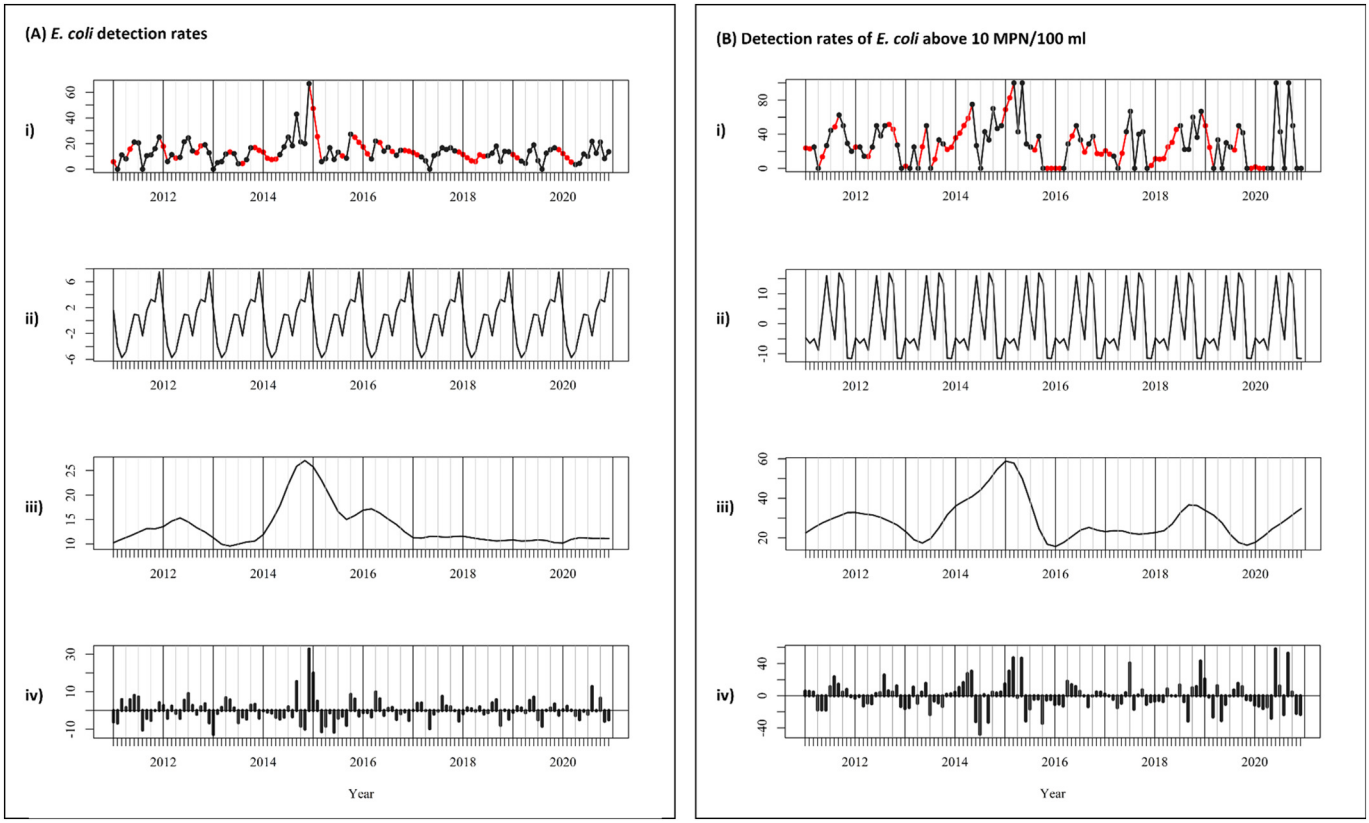


Fig. 3. (A) STL seasonal decomposition of *E. Coli* detection rate (%) from January 2011 to December 2020 – (i) raw measured data in Black and simulated data in Red – (ii) seasonal variations – (iii) long-term trend – (iv) residuals. (B) STL seasonal decomposition of detection rates for *E. coli* >10 MPN/100 ml from January 2011 to December 2020 – (i) raw measured data in Black and simulated data in Red – (ii) seasonal variations – (iii) long-term trend – (iv) residuals.

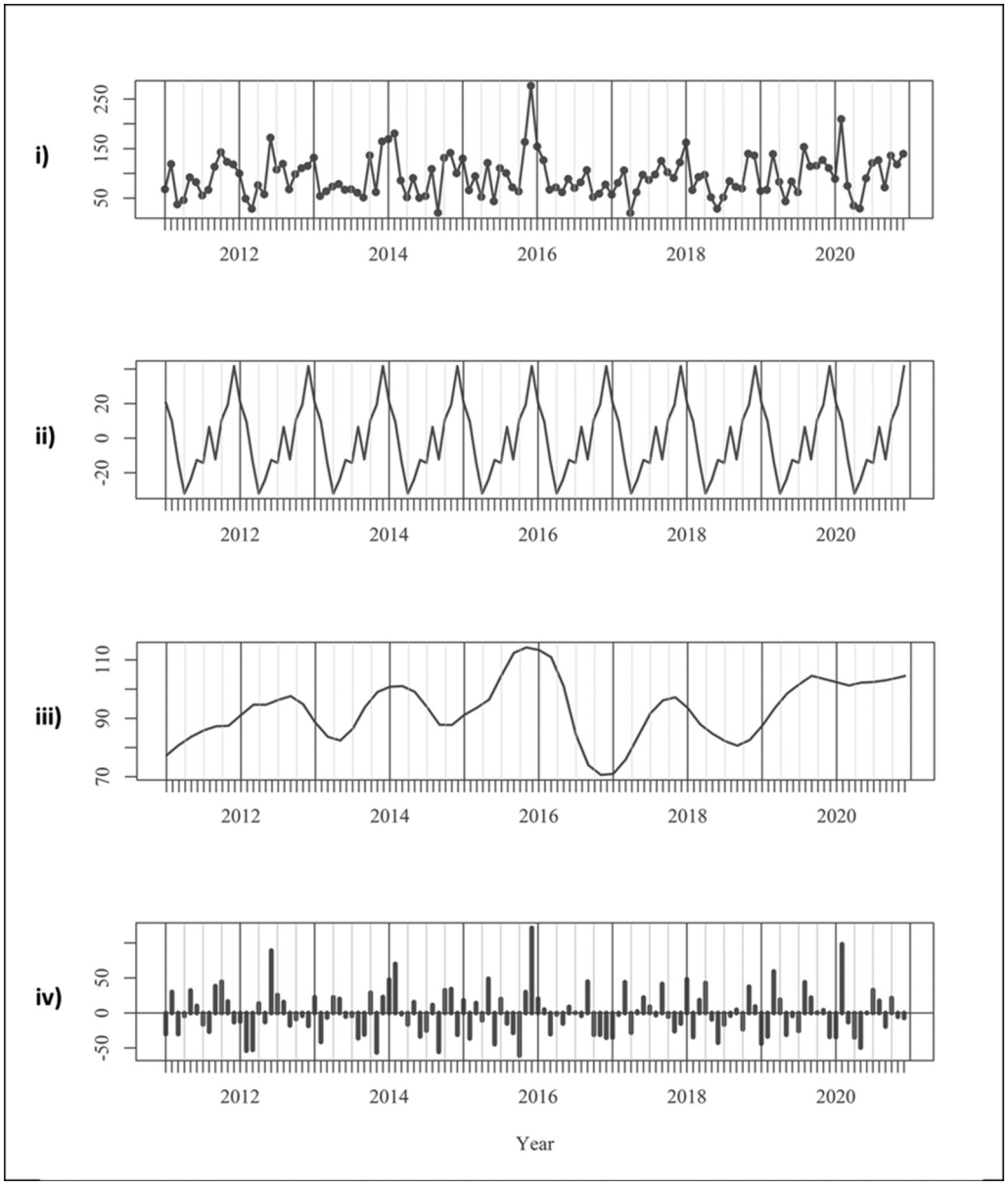


Fig. 4. STL seasonal decomposition of mean monthly rainfall data across the Republic of Ireland from January 2011 to December 2020 – (i) raw data – (ii) seasonal variations – (iii) long-term trend – (iv) residuals.

equivalent odds ratio slightly higher in wetter months (5.87, 1.14–30.27). Differences in hydrometeorological trends in both models are also noted: 10-day cumulative rainfall was associated with higher odds of *E. coli* presence in wetter months (1.02, 1.011–1.032), while 5- and 30-day cumulative rainfall increased the odds of *E. coli* contamination during drier months (1.03, 1.012–1.042 and 1.01, 1.005–1.017, respectively).

4. Discussion

The presented study provides the first national-scale quantitative assessment of a temporally monitored groundwater supply network, comprising 3808 water samples, over a ten-year period in the Republic of Ireland. Overall, contamination rates were high with over 66% of monitoring wells (88/132) positive for *E. coli* at least once during the sampling period, with concentrations exceeding 10MPN/100 ml on at least one occasion in 29.5% of wells (39/132). Results are indicative of the persistent and spatiotemporally diverse issue of groundwater quality in the ROI which is particularly pertinent due to the high reliance on groundwater within the state (~16%). Previous studies focusing on domestic groundwater wells have reported contamination rates ranging from 28.9% (Hynds et al., 2012) to 51.4% (O'Dwyer et al., 2018), albeit emerging international research suggests potential environmental adaptation of *E. coli* to groundwater environments and thus overestimation of persistent contamination events (Touchon et al., 2020; Tropea et al., 2021). Regardless, reported *E. coli* contamination levels highlight a significant concern associated with groundwater contamination across the island of Ireland. In particular, the extent of gross contamination reported across the monitored sites poses a specific risk to public health, with the infectious dose for pathogenic strains of *E. coli* (e.g. STEC/VTEC) as low as 10 CFU per 100 ml (FSAI, 2018); notably, Ireland has the highest incidence rate of VTEC in the EU, with private wells identified as a key risk factor (Ohaiseadha et al., 2017).

Time-series analyses via seasonal decomposition indicate that *E. coli* detection rates peak during late autumn/early winter, coinciding with an increase in the frequency and intensity of rainfall across the country, with precipitation levels typically at their highest during December and January (Met Éireann, 2021). Notably, the decomposed incidence of gross (>10 MPN/100 ml) contamination demonstrates a late spring/early summer peak (May) followed by a secondary peak in summer (August), likely

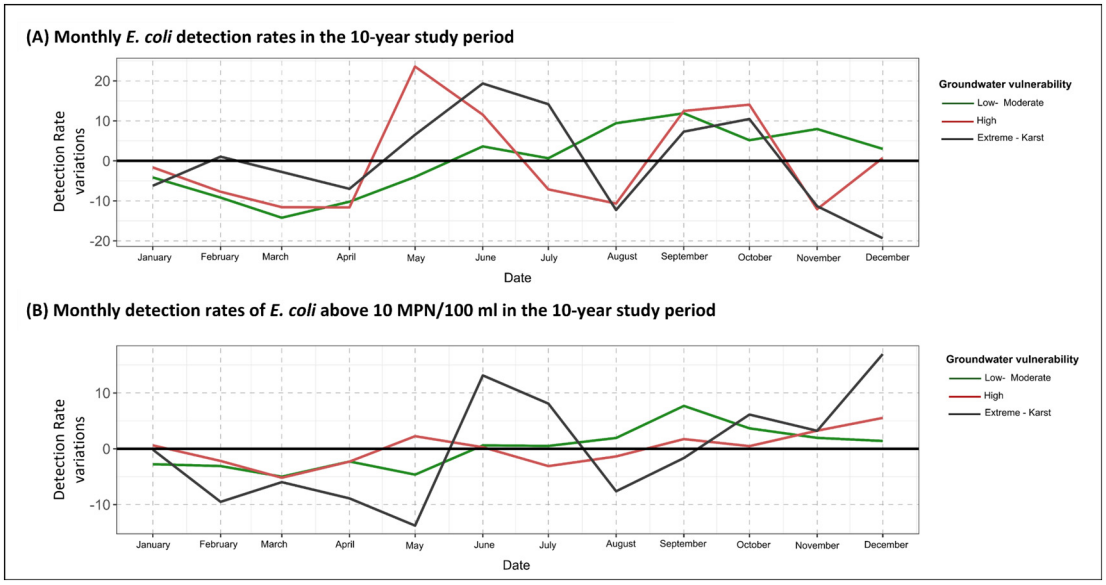


Fig. 5. (A) STL seasonal decomposition of *E. coli* detection rate (%) from January 2011 to December 2020 based on delineated groundwater vulnerability category (B) STL seasonal decomposition of detection rates for *E. coli* >10 MPN/100 ml from January 2011 to December 2020 based on delineated groundwater vulnerability category. Note that decomposed patterns are based around variance from the long-term (10-year) delineated means from Table 4 e.g., in 4A, 0 = 11.7% for High vulnerability (Red line).

reflecting agricultural practices, which in Ireland are dominated by dairy and beef farming (i.e., pasture-based) with cattle left out to pasture in late Spring and calves weaned to pasture by late Summer (Teagasc, 2020). Delineation of monitoring sources by groundwater vulnerability category indicates that the differential timing associated with *E. coli* detection rate and the incidence of gross contamination may be elucidated via differing mechanisms based on hydrogeological setting. Significantly higher rates of *E. coli* detection occur in extreme/karst areas during winter months, reflective of the relative ease of microbial transport through shallow or absent layers of high-permeability subsoils, and subsequently, limited attenuation capacities i.e., rapid response to precipitation events and overarching precipitation patterns (Daly and Warren, 1998). Conversely, increased bacterial loading (>10 MPN/100 ml) is more frequent in high vulnerability areas during late spring/early summer. This finding suggests that while (extremely vulnerable) karst areas are more frequently contaminated, higher bacterial loading typically occurs in areas overlain by moderate (shallow) and high (deep) permeability subsoils. In essence, results may be indicative of the activities which predominate in high vulnerability areas, where

Table 4
Mixed-effects logistic regression models predicting (A) *Escherichia coli* presence within a training subset of all water supply samples tested for *E. coli* and (B) gross *E. coli* presence (>10 MPN/100 ml) within a training subset of all *E. coli*-positive samples. Models employ EPA Groundwater Monitoring Programme samples collected from 2011 to 2020.

Model predictors	(A) *E. coli* detection (n = 3079)			(B) Gross *E. coli* detection (n = 359)		
	N	OR (95% CI)	p	N	OR (95% CI)	p
Water supply type						
Public supply wells	2688	Reference				
Group water scheme wells	226	**4.586 (1.158–18.161)**	**0.030**			
Private supply	79	0.465 (0.021–10.389)	0.629			
Agricultural/industrial supply	86	2.631 (0.227–30.432)	0.439			
Aquifer type						
Poorly productive bedrock	1261	Reference		156	Reference	
Karst	744	**2.764 (1.098–6.95)**	**0.031**	160	**2.537 (1.057–6.090)**	**0.037**
Productive fractured bedrock	756	**0.287 (0.099–0.826)**	**0.021**	32	0.761 (0.202–2.861)	0.686
Gravel	318	0.597 (0.142–2.508)	0.481	11	0.912 (0.149–5.590)	0.921
Groundwater vulnerability						
Low	523	0.507 (0.153–1.679)	0.266	34	0.607 (0.154–2.395)	0.476
Moderate	498	1.301 (0.458–3.696)	0.621	**59**	**0.263 (0.087–0.798)**	**0.018**
High	1459	Reference		128	Reference	
Extreme	400	**3.116 (1.012–9.597)**	**0.048**	70	1.223 (0.416–3.596)	0.715
Extreme X (Rock/karst)	199	**14.372 (3.364–61.398)**	**<0.001**	68	0.851 (0.286–2.534)	0.773
Landcover						
Non-pasture	687	Reference				
Pasture	2392	**4.255 (1.467–12.341)**	**0.008**			
5-day antecedent rainfall	3079	**1.027 (1.017–1.038)**	**<0.001**	359	**1.042 (1.024–1.061)**	**<0.001**
30-day antecedent rainfall	3079	**1.005 (1.001–1.009)**	**0.016**			
90-day antecedent rainfall				359	**0.996 (0.993–0.9999)**	**0.045**
Pseudo R^2	0.568			0.278		
Pseudo R^2 (fixed effects only)	0.409			0.200		

Note: Missing values represent those which did not add to model accuracy and were omitted.
Values in bold indicate statisitical significance (i.e. $p < 0.05$).

Table 5
Training and testing sensitivity and accuracy for (a) *E. coli* and (b) gross *E. coli* detection models.

		E. coli detection model			Gross *E. coli* detection model		
		Predicted	Observed	% Correct	Predicted	Observed	% Correct
Model dataset	*E. coli* absent	2622	2690	97.5%	213	230	92.6%
	E. coli present	207	389	53.2%	50	129	38.8%
	Total	2829	3079	**91.9%**	263	359	**73.2%**
Validation dataset	*E. coli* absent	620	632	98.1%	82	85	96.5%
	E. coli present	51	97	52.6%	15	42	35.7%
	Total	671	729	**92.0%**	97	127	**76.4%**

Values in bold indicate statisitical significance (i.e. $p < 0.05$).

Table 6
Mixed-effects logistic regression models predicting *Escherichia coli* presence within a training subset of samples collected during (A) historically wetter (Oct-Mar) and (B) historically drier (Apr-Sept) months. Models employ EPA Groundwater Monitoring Programme samples collected from 2011 to 2020.

Model predictors	(A) *E. coli* detection in wetter months (n = 1210)			(B) *E. coli* detection in drier months (n = 1791)		
	N	OR (95% CI)	p	N	OR (95% CI)	p
Water supply type						
Public supply wells				1572	Reference	
Group water scheme wells				130	**4.947 (1.176–20.809)**	**0.029**
Private supply				41	0.550 (0.0186–16.217)	0.729
Agricultural/industrial supply				48	4.793 (0.304–75.589)	0.265
Aquifer type						
Poorly productive bedrock	491	Reference		737	Reference	
Karst	290	2.477 (0.684–8.976)	0.167	428	**2.725 (1.020–7.282)**	**0.046**
Productive fractured bedrock	303	**0.137 (0.026–0.723)**	**0.019**	436	**0.257 (0.080–0.824)**	**0.022**
Gravel	126	0.817 (0.118–5.642)	0.838	190	0.387 (0.075–1.984)	0.255
Groundwater vulnerability						
Low	200	0.740 (0.129–4.241)	0.736	285	0.983 (0.275–3.513)	0.979
Moderate	202	1.839 (0.416–8.129)	0.421	302	1.616 (0.524–4.982)	0.403
High	574	Reference		843	Reference	
Extreme	156	3.800 (0.762–18.953)	0.103	248	**4.242 (1.262–14.261)**	**0.019**
Extreme X (Rock/karst)	78	**19.264 (2.372–140.608)**	**0.005**	113	**20.176 (4.336–93.887)**	**<0.001**
Landcover						
Non-pasture	281	Reference			Reference	
Pasture	929	**5.872 (1.139–30.270)**	**0.034**	1391	**5.111 (1.576–16.578)**	**0.007**
5-day antecedent rainfall				1791	**1.027 (1.012–1.041)**	**<0.001**
10-day antecedent rainfall	1210	**1.021 (1.011–1.032)**	**<0.001**			
30-day antecedent rainfall				1791	**1.011 (1.005–1.017)**	**<0.001**
Pseudo R^2	0.638			0.576		
Pseudo R^2 (fixed effects only)	0.466			0.438		

Note: Missing values represent those which did not add to model accuracy and were omitted.
Values in bold indicate statisitical significance (i.e. $p < 0.05$).

increased permeability allows for subsurface ingress of microbial contamination at increased rates (Foppen and Schijven, 2006) while stimulating grassland productivity, needed to sustain intense pastural agriculture (Shalloo et al., 2004). Moreover, potentially higher temperatures during late summer and early autumn may also lead to an increase in *E. coli* numbers detected (John and Rose, 2005), which warrants further research. Across the ten-year study period, a marked increase in *E. coli* detection rates occurred during 2014–2015, although this was potentially more pronounced due to low data availability and limited observations during this time-period. Bias might also been introduced by the data imputation. When assessing mean meteorological conditions across the monitoring timeframe, there are no stark deviations in rainfall from the ten-year trend for this specific period, albeit, this does not consider localised precipitation events, which may be explanatory. A potential consideration may be the abolition of milk quotas in Ireland in 2014, which rapidly resulted in a 38% increase in national dairy herd (Farming Independent, 2020) and subsequently increased faecal loading in agricultural areas, however, if this did have an impact, it was short-lived and is not replicated in subsequent years in the seasonal decomposition models.

Mixed effects logistic regression modelling was employed to identify key risk factors for both the occurrence of *E. coli* and gross *E. coli* contamination across the groundwater monitoring network. Mirroring the seasonal

Table 7
Training and testing sensitivity and accuracy for *E. coli* detection models in (a) wetter and (b) drier months.

		Model for *E. coli* in wetter months			Model for *E. coli* in drier months		
		Predicted	Observed	% Correct	Predicted	Observed	% Correct
Model dataset	*E. coli* absent	1034	1064	97.2%	1524	1554	98.1%
	E. coli present	80	146	54.8%	122	237	51.5%
	Total	1114	1210	**92.1%**	1646	1791	**91.9%**
Validation dataset	*E. coli* absent	275	281	97.9%	414	723	97.9%
	E. coli present	25	44	56.8%	30	59	50.8%
	Total	300	325	**92.3%**	444	482	**92.1%**

decomposition profile, extreme and/or karst areas were statistically associated with both *E. coli* presence and gross contamination (Table 3); findings indicate that *E. coli* contamination is over 19 times more likely in karstic environments, thus concurring with previous work which has shown that groundwater monitoring locations in karst limestone areas consistently exhibit significantly higher levels of microbiological pollution (Bradley et al., 2015), reflecting the inherently vulnerable nature of dynamic flow systems, in concurrence with the lack of attenuation capacity associated with shallow soils or subsoils (Pronk et al., 2009). This is further substantiated through recent national epidemiological models which highlight three primary hotspots of waterborne infectious disease (verotoxigenic *E. coli*), all of which occur in karst areas (Cleary et al., 2021).

Microbial contamination of groundwater is particularly associated with diffuse sources of contamination, particularly agriculture, in carboniferous limestone regions (Drew et al., 1996; Wallender et al., 2014). In Ireland, the agricultural sector is characteristically diverse, ranging from tillage to pasture, however, results from the current study point to livestock farming as the primary agricultural driver of *E. coli* presence in Irish groundwater systems, with monitoring wells located in areas dominated by pastural agriculture over 4 times more likely to harbour *E. coli*. It is estimated that 80% of organic wastes generated in Ireland are derived from agricultural activities (Daly and Warren, 1998), which are often applied directly to land with no pre-treatment undertaken, thus potentially infiltrating both surface and groundwater resources. This relationship between livestock-based agricultural practices and groundwater contamination is well substantiated within international literature (Schets et al., 2005; Close et al., 2008; Kabore et al., 2010; Lenaker et al.,2017, Chique et al., 2020a, 2020b), with a recent global review of verotoxigenic *E. coli* (VTEC) in groundwater noting that cattle-dominated rural areas exhibited higher VTEC detection rates in comparison to other rural sub-categories (Chique et al., 2020b). Notably, Ireland has repeatedly reported the highest crude incidence rates of VTEC enteritis within the EU, with cases of this primarily waterborne infection statistically associated with utilisation of private groundwater supplies (Ohaiseadha et al., 2017). Interestingly, pastural agricultural activities were not predictive of gross contamination, suggesting that while agriculture is a definitive source of bacteria, and thus predictive of *E. coli* presence, the magnitude of contamination (i.e. gross contamination) is predicated upon hydrogeological processes which allow for easier ingress through the subsurface, and specifically the presence of karst aquifer systems which were 2.5 times more likely to have gross *E. coli* contamination.

Antecedent cumulative rainfall associated with a variety of temporal periods demonstrated a level of predictive capacity for both *E. coli* presence and the incidence of gross contamination within presented models. Rainfall influences both vertical and horizontal movement of bacteria at the soil interface, moderating the rate and concentration at which microorganisms may enter groundwater (Unc and Goss, 2003). Periods of prolonged rainfall have been shown to increase the discharge and microbial concentration of agricultural runoff, transporting microbiologically diverse materials (surface runoff, associated sediments and faecal matter) to rivers, coastal waters, and groundwater wells (Semenza and Menne, 2009). Several previous studies have found a significant relationship between antecedent rainfall (mm) and the presence faecal indicators and disease outbreaks (Howard et al., 2003; Hynds et al., 2012, 2014a; O'Dwyer et al., 2016); for example, Howard et al. (2003) reported that increased rainfall over the 48-h pre-sampling period increased the likelihood of thermotolerant coliform and faecal streptococci presence in shallow groundwater in Kampala, Uganda, while O'Dwyer et al. (2016) report an association between heavy rainfall events and outbreaks of VTEC in Ireland. In the current study, five-day and 30-day antecedent precipitation increased the likelihood of *E. coli* contamination, with the former also being associated with gross contamination. The relatively short timeframe between cumulative rainfall and gross contamination likely serves as a relative proxy for extreme vulnerability areas whereby rainfall can transport organically rich runoff to the subsurface through direct ingress (Hynds et al., 2014a, 2014b). Compounding this, further elucidation of gross contamination signifies potentially two predominant contaminant mechanisms during categorically dry periods, with traditional recharge represented through statistical association with 30-day rainfall and potential preferential flow suggested through the association with 5-day rainfall. While prolonged periods with no rainfall are infrequent in Ireland (Fig. 4), recent research by O'Dwyer et al. (2021) suggests that during reduced rainfall, there can be a hydrodynamic shift towards relatively rapid preferential flow of water (and contaminants) within the subsurface. In contrast, during categorically 'wet' periods, 10-day rainfall is associated with *E. coli* occurrence, likely indicative of overland flow and direct ingress mediated by soil saturation, although further research is required to determine exact mechanisms.

5. Limitations and recommendations

The authors consider that due to a number of inherent data constraints within the presented models/time series, careful consideration is required in the interpretation of presented findings. Importantly, the pre-existing groundwater monitoring datasets utilised in this study possess several gaps, particularly at a temporal scale. Thus, event peaks and the impact of hydrology on *E. coli* detection levels are difficult to ascertain with any great certainty and may be, in some instances, overestimated. Accordingly, a key recommendation of this research is the modification of the national groundwater monitoring regime to ensure a temporally representative dataset whereby hydro(*geo*)logical processes can be inferred more accurately. It is recommended that a monthly sampling regime be implemented to increase confidence in the monitoring programme and the suitability of collected data for further analysis. Moreover, a monthly sample collection schedule would enhance our understanding of the seasonal patterns of *E. coli* contamination and allow for the development of tailored recommendations to ensure that groundwater sources are appropriately protected year-round and crucially, allow for assessments of climatic change on water resources. Similarly, the spatial distribution of the monitoring data could also be optimised, with large areas (particularly the West) of the country distinctly unrepresented. While the monitoring programme is designed to represent major aquifer settings rather than uniform spatial resolution, the lack of monitored groundwater supplies in certain areas precludes comparison and/or control across similar aquifers which are under different land uses settings and meteorological conditions. As such, a review of the spatial distribution of the monitoring network is also recommended, with the recruitment of additional (regularly pumped) groundwater supplies, particularly in the north and north-west of the country. Finally, the reporting of site-specific data, such as supply depth and age, nearby sources of contamination, local topography, etc. is also recommended, facilitating comprehensive analyses of the risk factors contributing to the incidence and/or frequency of *E. coli* contamination and enable prioritisation of sites where source protection and infrastructural investments are needed to ensure drinking-water safety, in line with the Water Safety Planning approach promoted by the World Health Organisation (WHO, 2022).

6. Conclusion

Groundwater contamination is a significant issue, which poses potential public health threats for groundwater consumers. The Republic of Ireland represents the 'perfect storm' with regard to groundwater pollution, with a relatively high groundwater reliance in concurrence with an intensive agricultural sector and a diverse hydrogeological profile. The presented study analysed data from a large-scale national monitoring regime to assess the spatiotemporal quality of groundwater and identify key risk factors. Mixed effects logistic regression modelling indicates that monitoring sources located in karst limestone are statistically associated with *E. coli* presence (OR = 2.76, $p = 0.03$) and presence above 10 MPN/100 ml (i.e. gross contamination; OR = 2.54, $p = 0.037$) when compared to poorly productive aquifers. Moreover, 5-day and 30-day antecedent rainfall increased the likelihood of *E. coli* contamination (OR = 1.027, $p < 0.001$ and OR = 1.005, $p = 0.016$, respectively), with the former also being associated with gross contamination (OR = 1.042, $p < 0.001$). Findings support the need for in-depth analyses of national groundwater monitoring data in

order to (i) increase our understanding of groundwater contamination in hydrogeologically heterogeneous regions and (ii) optimise the monitoring network. When viewed through the lens of the characteristic agricultural, infrastructural, and hydrogeological profile of rural Ireland and other similar regions, findings serve to highlight the ubiquity and magnitude of groundwater source hazards in addition to the inherent complexities associated with effective source protection and remediation.

CRediT authorship contribution statement

Luisa Andrade: Conceptualization, Methodology, Writing - original draft. Martin Boudou: Data curation. Paul Hynds: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Funding acquisition, Supervision. Carlos Chique: Data Visualisation, Writing – original draft. John Weatherill: Writing - review & editing, Supervision. Jean O'Dwyer: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.156311.

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The antimicrobial resistance profiles of *Escherichia coli* and *Pseudomonas aeruginosa* isolated from private groundwater wells in the Republic of Ireland☆

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ABSTRACT

The role of the natural environment in the dissemination of antimicrobial resistant bacteria has been increasingly recognised in the literature. However, knowledge surrounding the critical factors and mechanisms mediating their occurrence is still limited, particularly in relatively 'pristine' groundwater environments. In the Republic of Ireland (RoI), a country characterised by high groundwater reliance, household-based (unregulated) wells provide drinking water to 11% of the population. These private wells are generally located in rural areas, where the risk of microbiological contamination is high due to intensive agricultural practices and high reliance on domestic wastewater treatment systems; both of which are also potential sources of antimicrobials and antimicrobial resistant bacteria. Accordingly, the current research sought to elucidate current rates of antimicrobial resistant bacteria and the principal factors associated with their presence in private wells in the RoI. A total of 250 samples (from 132 wells nationwide) were assessed for the presence of faecal (*Escherichia coli)* and environmental (*Pseudomonas aeruginosa*) bacteria, with single isolates from each contaminated sample tested phenotypically against 18 and 9 antimicrobials, respectively. Findings show that while 16.7% of *E. coli* (n = 8/48) were categorically resistant to ≥1 antimicrobial, with a further 79.2% classified as intermediately resistant, no categorical resistance was found among *P. aeruginosa* isolates (n = 0/6), with just one intermediately resistant isolate detected. Multivariate regression modelling indicates significantly higher odds of resistant *E. coli* detection in concurrence with elevated cattle density (OR = 1.028, $p = 0.032$), aligning with findings of highest resistance rates to veterinary antimicrobials (e.g., streptomycin = 14.6%, tetracycline = 12.5%, and ampicillin = 12.5%). Multivariate model results also suggest overland flow culminating in direct wellhead ingress as a primary ingress mechanism for resistant *E. coli*. Study findings may inform groundwater source protection initiatives and antimicrobial resistance surveillance moving forward.

1. Introduction

The global public health burden of antimicrobial resistance and its rapid dissemination has been extensively recognised in recent decades, with evidence now available of resistance genes and/or resistant bacteria presence in non-clinical environments, including but not limited to, aquatic systems (Belding and Boopathy, 2018; Hooban et al., 2020; 2022), rural and urban soils (Montealegre et al., 2018; Zhao et al., 2019), food crops (Cerqueira et al., 2019), and uncultivated vegetation (Obermeier et al., 2021). The colonisation of these diverse ecosystems with antimicrobial resistant bacteria highlights their widespread incidence and are concurrent with increased cases of more severe, prolonged, and/or fatal infections, which may previously have been easily treatable (Opatowski et al., 2019; Center for Disease Control, 2019). Recent surveillance reports in the United States and Europe, for example, indicate that annually, over 35,000 and 33,000 deaths,

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respectively, are attributable to antimicrobial-resistant infections (Cassini et al., 2019; Center for Disease Control, 2019). The problem related to antimicrobial resistance stems from the rapid spread of resistance caused by widespread misuse and overuse of antimicrobials (Huttner et al., 2013), which has not been matched by ongoing development of antimicrobial drugs with new modes of action (Miethke et al., 2021). Indeed, the World Health Organization (WHO) recently reported that of the eleven antibiotics approved in the United States and Europe since 2017, just two represent novel classes of antimicrobial drugs (World Health Organization, 2020).

Recently, a global systematic review highlighted the role of groundwater as an important reservoir for antimicrobial resistant bacteria (Andrade et al., 2020), despite studies from as early as 1976 reporting the issue (Cooke, 1976). Widely believed to be relatively 'pristine' environments, groundwater resources have the potential, when contaminated, to act as both a source and pathway for antimicrobial resistance amongst bacterial populations (Sanderson et al., 2018) while also providing a direct exposure route to humans (Coleman et al., 2012). In the groundwater context, the risk of human exposure is particularly relevant, as over 2.2 billion people worldwide rely on groundwater wells for domestic use and as principal source of drinking water (Murphy et al., 2017). Additionally, indirect exposed due to the extensive use of groundwater resources in agricultural irrigation is increasing (Siebert et al., 2010). Moreover, small-scale groundwater abstractions remain widely unregulated causing many sources to lack appropriate treatment and maintenance (Murphy et al., 2017; McDowell et al., 2020), and thus, be at a high risk of microbiological contamination (Hynds et al., 2014; Lee and Murphy, 2020; Mapili et al., 2020). These groundwater resources also present a favourable and sheltered environment for harbouring diverse microbiological contaminants (including resistant bacteria) as well as pollutants which may pose co-selective pressures for acquisition of antimicrobial resistance (e.g., trace metals, biocides, and disinfectants) (Wu et al., 2018; Zhang et al., 2018). Because of the significant global burden of infection associated with groundwater resources (Murphy et al., 2017), the increased risks of therapeutic failure in the case of antimicrobial resistant groundwater-borne infections represent a direct and substantial threat to public health. Acute gastrointestinal infection associated with groundwater contamination, for example, are estimated to surpass 35.2 million cases per year globally (Murphy et al., 2017). Outbreaks of infection due to consumption of contaminated groundwater resources are also well documented (He et al., 2009; Eurien et al., 2021). Despite this, limited knowledge is available on the external factors mediating the occurrence of antimicrobial resistant bacteria in groundwater resources, particularly in respect to local/regional weather, anthropogenic activities, local hydrogeology, and groundwater source types, thus preventing development of effective monitoring and mitigation strategies.

Accordingly, the current study employed a combination of hydrogeological fieldwork and sampling, microbiological analyses, and statistical modelling to identify: i) current rates of resistance among groundwater bacteria in rural settings, ii) key factors associated with the presence of resistant bacteria, and iii) potential origin(s) and ingress mechanism(s) associated with resistant bacteria occurrence (i.e., likely sources and pathways). For this, two bacteria of human significance and from separate primary habitats (i.e., faecal, and environmental) were assessed: *Escherichia coli,* a well-known (zoonotic and anthropogenic) faecal indicator organism commonly used in groundwater-based antimicrobial resistance investigations (Andrade et al., 2020), and *Pseudomonas aeruginosa*, an opportunistic pathogen that (unlike *E. coli*) can proliferate in soil and freshwater (Cabot et al., 2016). Notably, the *Pseudomonas* genus has been recently identified as the bacteria with highest rates of resistance and intermediate resistance in global groundwater resources (Andrade et al., 2020). In this context, potential loss of an already limited number of treatment options for *P. aeruginosa*-caused infections (Lister et al., 2009) is particularly worrisome, as *P. aeruginosa* infections have the highest fatality rates of all gram-negative pathogens (Aliaga et al. 2002). Overall, the choice of bacterial species was principally predicated on the potential to gain insights into differing resistance origins and transport pathways in groundwater supplies, as well as to obtain a clear picture of local and source-specific resistomes, following key recommendations by Andrade et al. (2020). The authors consider that the RoI represents an appropriate case study for transferability of findings as a region characterised by diverse hydrogeology, intensive agricultural practices, a dispersed rural population (≈27 people/km^2; Central Statistics Office, 2016a) with a high reliance on private groundwater systems (Central Statistics Office, 2016b), and a temperate climate without a distinct dry season (Peel et al., 2007). Hence, study findings provide an important baseline for quantifying antimicrobial resistant bacteria in groundwater resources, particularly in comparison with other more 'exposed' environments such as surface water and soil ecosystems, or locations near health-care facilities and/or densely populated areas. With the ever-increasing threat of antimicrobial resistance, the results of this study provide an important steppingstone to assist in informing groundwater source protection initiatives to safeguard human health and support policy in antimicrobial resistance Action Plans.

2. Methods

2.1. Study area

The study was undertaken in rural settings in the RoI, a region of high groundwater reliance with over 180,000 private supplies in operation for domestic and industrial/agricultural use in 2019 (EPA, 2021a). Private groundwater abstractions for domestic use in the RoI comprise community systems (i.e., Private Group Water Schemes) which are regulated by the relevant local authorities, and small-scale household wells yielding under 10 m^3 of water per day (i.e., private wells) which are exempt from State regulations (EPA, 2013) in accordance with the European Union (EU) drinking water directive (DIRECTIVE 2020/2184, 2020). Overall, private wells provide potable water to approximately 11% of the Irish population (Department of Housing Local Government and Heritage, 2021) located in rural settlements (Central Statistics Office, 2016a).

The bedrock geology of the RoI is diverse (Fig. 1), primarily comprised of sedimentary bedrock formations (e.g., limestone, sandstone, shale) (Geological Survey Ireland, 2017). Hydrogeologically, most aquifers used for groundwater abstraction in the RoI are unconfined, with groundwater vulnerability classifications (i.e., the categorisation of the theoretical risk of groundwater contamination via recharge; Department of the Environment and Local Government/Environmental Protection Agency/Geological Survey of Ireland, 1999) ranging from low to extreme (Fig. 1). Briefly, groundwater vulnerability classifications are based on (i) depth to bedrock, (ii) subsoil permeability and, (iii) thickness/characterisation of the unsaturated zone (for sand and gravel aquifers) (Department of the Environment and Local Government/Environmental Protection Agency/Geological Survey of Ireland, 1999). Groundwater vulnerability is highest in karst limestone aquifers, where rapid groundwater flow through conduit, fissure and cave systems hinder the contaminant attenuation and subsurface retention which allow for microbial die-off. Rapid flow with limited attenuation is also associated with the interconnected fractures, fissures and, joints (i.e., fractured networks) common in Irish bedrock aquifers. The lowest groundwater vulnerability is typically seen where thick impermeable layers of clay soil and subsoil prevent contaminant percolation and recharge (Tynan, 2014).

2.2. Sample collection

Private (household) wells were the focus of the current study, with supply selection and inclusion based on responses to a nationwide recruitment effort (e.g., via university mailing lists and social media

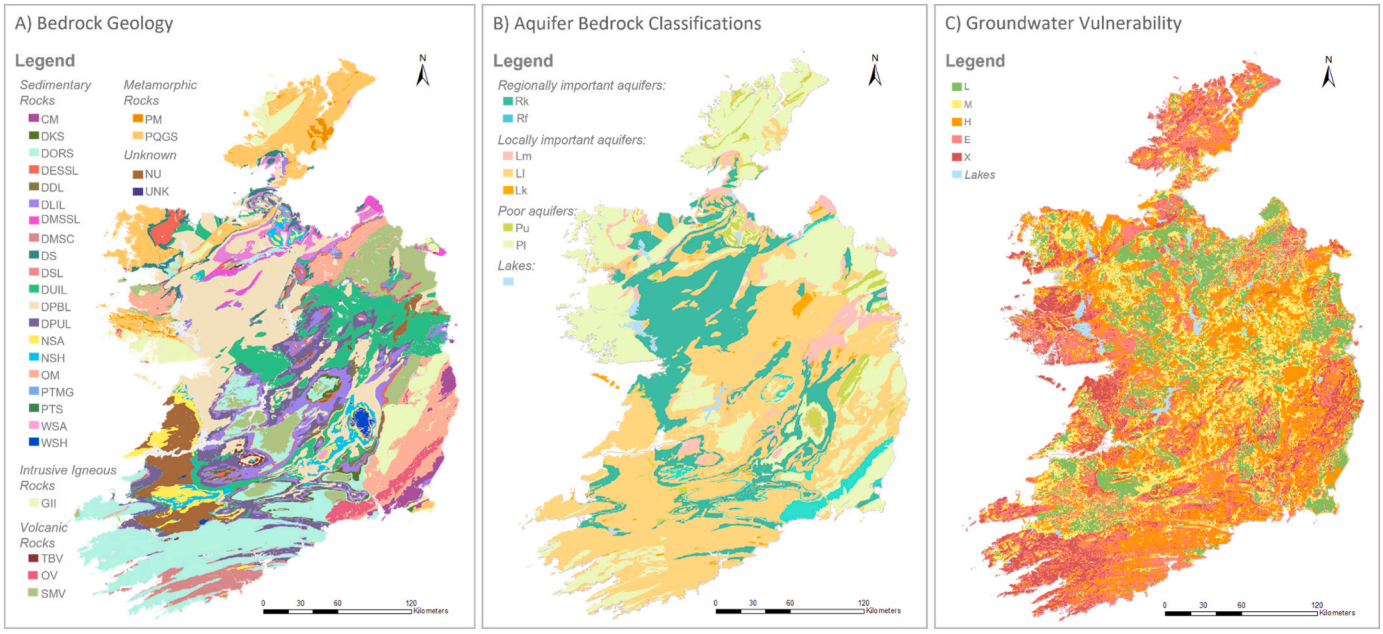


Fig. 1. Republic of Ireland maps showing the geographical distribution of Bedrock Geology (A), Aquifer Bedrock (B), and Groundwater Vulnerability (C) classifications. A) CM=Cambrian Metasediments, DKS = Devonian (Kiltorcan-type) Sandstones, DORS = Devonian (Old Red) Sandstones, DESSL = Dinantian (early) Sandstones, Shales and Limestones, DDL = Dinantian (Dolomitised) Limestones, DLIL = Dinantian (Lower Impure) Limestones, DMSSL = Dinantian Mixed Sandstones, Shales and Limestones, DMSC = Dinantian Mudstones and Sandstones, DS = Dinantian Sandstones, DSL = Dinantian Shales and Limestones, DUIL = Dinantian (Upper Impure) Limestones, DPBL = Dinantian (Pure Bedded) Limestones, DPUL = Dinasntian (Pure Unbedded) Limestones, NSA=Namurian Sandstones, NSH=Namurian Shales, OM=Ordovician Metasediments, PTMG=Permo-Triassic Mudstones and Gypsum, PTS=Permo-Triassic Sandstones, WSA=Westphalian Sandstones, WSH=Westphalian Shales, GII = Granites (and other Igneous Intrusive Rocks), TBV = Tertiary basalts (and other Volcanic Rocks), OV=Ordovician Volcanics, SMV=Silurian Metasediments and Volcanics, PM=Precambrian Marbles, PQGS=Precambrian Quartzites, Gneisses and Schists, NU=Namurian Undifferentiated, UNK=Unknown. B) Rk = Karstified, Rf=Fissured Bedrock, Lm = Generally Moderately Productive, Ll = Moderately Productive Only in Local Zones, Lk = Locally Important Karstified aquifer, Pu=Generally Unproductive Except for Local Zones, Pl = Generally Unproductive. C) L = Low, M = Moderate, H=High, E = Extreme, X = Extreme (Rock/Karst). Source: Geological Survey Ireland (2017)

campaigns). This was an exploratory study, with no specific focus based on bedrock geology, geographical location, supply infrastructure, etc. Samples were taken from household taps, generally located outside of the home and prior to any microbial treatment, during two distinct sample collection periods: Round 1 from September to November 2019, and Round 2 from June to August 2021. Samples were collected in 100 ml sterile vessels (IDEXX Laboratories Inc., Westbrook, Maine) after being allowed to run at high flow for a minimum of 60 s (≥2 L) to ensure the water did not come from the distribution system. After collection, samples were transported to the laboratory at 4 °C for analysis within 6 h.

2.3. Laboratory analyses

2.3.1. Bacterial enumeration and isolation

The most probable number (MPN) of *E. coli* and *P. aeruginosa* in each water sample was determined via standard ISO approved (International Organization for Standardization, 1998) commercial culture kits (Colilert and Pseudalert, IDEXX Laboratories Inc., Westbrook, Maine). Briefly, following separate addition of the IDEXX reagents, samples were transferred to 51-well IDEXX quanti-trays, heat sealed, and incubated aerobically for 24 h at 37 °C. The Colilert and Pseudalert reagents indicate *E. coli* and *P. aeruginosa* presence, respectively, through fluorescence under a UV light source. The MPN for each sample was ascertained by counting the number of positive 'wells' and referring to the respective IDEXX tables, an enumeration methodology with similar uncertainty (i.e., ±20%) to other established methods such as genetic-based (e.g., quantitative polymerase chain reaction; qPCR) and membrane filtration techniques (Harmel et al., 2016).

A single *E. coli* or *P. aeruginosa*-positive sub-sample (i.e., 'well') was randomly selected from each bacterial-positive quanti-tray and recovered by pipetting 100 μL of sample onto a MacConkey agar plate and incubating it for 24 h at 37 °C. Subsequently, single presumptive *E. coli* and *P. aeruginosa* colonies were isolated, with reference strains *E. coli* ATCC® 25,922 and *P. aeruginosa* ATCC® 25,924 (Oxoid, Basingstoke, Hampshire, UK) cultured in parallel, respectively, to aid in appropriate colony selection. In cases where sub-sample incubation did not return appropriate colonies, a different sub-sample was attempted, this process was repeated until no viable sub-samples were available.

2.3.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion technique (Hudzicki, 2009). Commercially prepared discs and Müller-Hinton (MH) Agar (Oxoid, Basingstoke, Hampshire, UK) were used in accordance with Clinical and Laboratory Standards Institute (CLSI) standards (CLSI, 2018, 2020). Briefly, pure bacterial colonies were suspended in 0.85% Saline solution with turbidity adjusted to the 0.5 McFarland standard. Each inoculum was evenly distributed over the dried surface of 100 mm MH agar plates, this was followed by the placement of a maximum of six antimicrobial discs, and incubation at 37 °C for 16–18 h (CLSI, 2020). Bacterial growth around each disc (i.e., zone of inhibition diameters) were then measured and recorded.

The antimicrobials evaluated in the study are presented in Table 1. Antimicrobial usage classifications were based on approval status of these medications for human or veterinary in the RoI by the Health Products Regulatory Authority in Ireland (HPRA, 2020a; 2020b). Overall, *E. coli* and *P. aeruginosa* isolates were tested against panels of 18 and 9 antimicrobials, respectively. Breakpoints for the antimicrobial zones of inhibition were interpreted according to M100 CLSI guidelines (CLSI, 2020) for *Enterobacterales* and *Pseudomonas aeruginosa* (VET01S in the case of Enrofloxacin; CLSI 2018). Isolates were subsequently

Table 1
Antimicrobial agents included in the current study, their license status for human and veterinary use in the Republic of Ireland (RoI), and the organisms with established breakpoints in the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Antimicrobial	Use in human medicine in RoI [a]	Use in veterinary medicine in RoI [a]	Organisms with established breakpoints [b]
Penicillins			
Ampicillin (AMP, 10 μg)	License withdrawn in 2014	Currently in use	*E. coli*
Amoxicillin-Clavulanic acid (AMC, 20/10 μg)	Currently in use	Currently in use	*E. coli*
Piperacillin (PRL, 100 μg)	License withdrawn in 2002	Never licensed	*Both*
Piperacillin-Tazobactam (TZP, 100/10 μg)	Currently in use	Never licensed	*Both*
Cephalosporins			
Cefazolin (KZ, 30 μg)	License withdrawn in 1992	License withdrawn in 2005	*E. coli*
Ceftazidime (CAZ, 30 μg)	Currently in use	Never licensed	*Both*
Ceftiofur (EFT, 30 μg)	Never licensed	Currently in use	*E. coli*
Cefepime (FEP, 30 μg)	Currently in use	Never licensed	*Both*
Aminoglycosides			
Streptomycin (S, 10 μg)	License withdrawn in 1987	Currently in use	*E. coli*
Gentamicin (CN, 10 μg)	Currently in use	Currently in use	*Both*
Carbapenems			
Imipenem (IPM, 10 μg)	Currently in use	Never licensed	*Both*
Fluoroquinolones			
Ciprofloxacin (CIP, 5 μg)	Currently in use	Never licensed	*Both*
Enrofloxacin (ENR, 5 μg)	Never licensed	Currently in use	*Both*
Monobactams			
Aztreonam (ATM, 30 μg)	Currently in use	Never licensed	*Both*
Tetracyclines			
Tetracycline (TE, 30 μg)	Currently in use	Currently in use	*E. coli*
Folate Pathway Inhibitors			
Trimethoprim (W, 5 μg)	Currently in use	License withdrawn in 2001	*E. coli*
Trimethoprim-Sulfamethoxazole (SXT, 1.25/23.75 μg)	Currently in use	Currently in use	*E. coli*
Phenicols			
Chloramphenicol (C, 30 μg)	Currently in use	Never licensed	*E. coli*

[a] Medical and veterinary use and license status in the Republic of Ireland (RoI) according to HPRA (Health Products Regulatory Authority in Ireland) (HPRA, 2020a; 2020b).
[b] Breakpoints available for *Enterobacteriaceae* spp. and/or *Pseudomonas aeruginosa* according to the CLSI (2018; 2020).

classified as Resistant (R), Intermediate (I) (i.e., clinically efficacious only where the drug is physiologically concentrated; CLSI, 2020), or Susceptible (S) to each antimicrobial. Quality control strains, namely *E. coli* ATCC® 25,922 and *P. aeruginosa* ATCC® 25,924 were used in concurrence with all *E. coli* and *P. aeruginosa* susceptibility analyses, respectively, in accordance with best practice and following CLSI (2020) guidelines.

2.4. Environmental and infrastructural risk factors

The potential risk factors which could influence both bacterial presence and resistance profiles (viz. Hynds et al., 2012; Coleman et al., 2013; O'Dwyer et al., 2014; 2017) were collected on-site and via geo-referenced datasets. Individual risk factors were categorised into (i) infrastructural, (ii) supply-/household-specific, and (iii) local parameters (Table 2). Geo-referenced spatial data available through the Geological Survey Ireland (GSI, 2017) and Central Statistics Office (CSO) Census of Ireland (CSO, 2012) were assigned to each supply using ESRI® ArcGIS Pro® version 2.9.1. based on supply-specific GPS coordinates. These included hydrogeological parameters, and the estimated density of anthropogenic activities/facilities within pre-delineated land sections of 80–120 dwellings, known as Small Areas (SA) (CSO, 2016b). In the case of agricultural data, GIS tools were employed to "aggregate" the 'coarser' resolution data (available only at Electoral Division level; mean area = 20.6 km^2) into the SA level (mean area = 3.8 km^2), while adjusting it to the spatial dimensions of representative land-use types (e.g., pastures; CORINE, 2018) rather than employing an all-inclusive land-use approach (e.g., inland waters, urban areas).

The 5-, 10-, 30- and 60-day (cumulative) antecedent rainfall values (in mm) were calculated by adding daily rainfall totals for the relevant number of days up to sample collection, which were recorded at the nearest synoptic station; historical rainfall data were provided by Met Éireann (the Irish National Meteorological Service; Met Éireann, 2021). Site surveys were carried out immediately after sample collection and covered a 100 m radius surrounding the well. These included determining distances and gradient differences between supplies and the nearest livestock and domestic wastewater treatment system (DWWTS - e.g., septic tank) and measuring the casing clearance (i.e., the distance from the well chamber floor to the top of the casing; see Supplementary Data). The Irish Environmental Protection Agency (EPA, 2020) recommends minimum setback distances to livestock grazing areas and DWWTSs of 3 and 30 m, respectively, to theoretically allow time for sufficient contaminant attenuation, and the Institute of Geologists of Ireland (Institute of Geologists of Ireland, 2007) established a minimum casing clearance of 150 mm to prevent direct contaminant ingress through the wellhead; these thresholds were therefore used in the current study. Information of well depth and antimicrobial use within the household were acquired via well-owner questionnaire (provided in Supplementary Data).

2.5. Statistical analyses

Potential risk factors (Table 2) associations with bacterial detection (i.e., *E. coli* and *P. aeruginosa* > 0 MPN/100 ml, separately) and presence of categorically resistant bacteria in groundwater samples were preliminary assessed using bivariate statistical techniques. Chi-Square (X^2) tests were used to identify associations between categorical independent variables and each categorical (binary) outcome, and Mann–Whitney U tests were used in the case of continuous independent variables (e.g., antecedent rainfall, supply depth, and sheep, cattle and DWWTS densities).

Bacterial detection (*E. coli* and *P. aeruginosa*) and presence of resistant bacteria outcomes were further analysed using multivariate binary logistic regression; independence between repeat samples was assumed, owing to the large period between sampling campaigns (i.e., over 18

months between Rounds 1 and 2) and the dynamic nature of microbiological groundwater contamination (Andrade et al., 2022). Independent variables (Table 2) with strong inter-variable correlations (i.e., Spearman's $|rho| \geq 0.5$, $p < 0.05$) were included separately, with the best fitting model selected based on the Nagelkerke R^2 (i.e., the proportion of outcome variation explained by the independent variables). Collinearity was found between "aquifer importance" and "aquifer type" variables, "livestock density" and both "cattle density" and "sheep density" variables, and for all combinations of antecedent rainfall periods (i.e., 5-, 10-, 30- and 60-day rainfall). Six variables: "gradient difference to nearest livestock", "gradient differences to nearest DWWTS", "supply depth", "presence of protective casing", "antimicrobials used in last 3 months", and "antimicrobials used in last 6 months" were excluded from regression models due to missing data in >10% of observations, with sensitivity analyses indicating that their inclusion did not result in meaningful changes to model accuracy. All independent variables which fitted inclusion criteria were simultaneously entered into the models (i.e., "forced entry" method) and retained regardless of statistical significance. The second 'best-fit' models identified are included in Supplementary Data. All statistical analyses were undertaken using IBM SPSS version 28.

Table 2
Environmental and infrastructural parameters which may influence resistance profiles (risk factors) and corresponding data origin.

Measured Risk Factors	Data origin
Environmental parameters I (local)	
Groundwater vulnerability (low/moderate/high/extreme/extreme-rock/karst)	GSI Mapping
Aquifer importance (poor/locally important/regionally important)	GSI Mapping
Aquifer type (karst/gravel/fractured bedrock/poorly productive bedrock)	GSI Mapping
Karst aquifer (yes/no)	GSI Mapping
Sheep density at the Small Area [a] level (heads/km^2)	CSO
Cattle density at the Small Area [a] level (heads/km^2)	CSO
Livestock density at the Small Area [a] level (heads/km^2)	CSO
DWWTS density at the Small Area [a] level (number/km^2)	CSO
5-day rainfall (mm)	Met Éireann
10-day rainfall (mm)	Met Éireann
30-day rainfall (mm)	Met Éireann
60-day rainfall (mm)	Met Éireann
Environmental parameters II (supply- and household-specific)	
Livestock within 3 m of the well [b] (yes/no)	On-site measurement
Gradient difference to nearest livestock (below/same/above the well)	On-site measurement
DWWTS within 30 m of the well [b] (yes/no)	On-site measurement
Gradient difference to nearest DWWTS (below/same/above the well)	On-site measurement
Antimicrobials used in the household in the last 3 months (yes/no)	Well-owner questionnaire
Antimicrobials used in the household in the last 6 months (yes/no)	Well-owner questionnaire
Infrastructural parameters	
Supply type (drilled/hand-dug)	On-site survey
Supply depth (m)	On-site measurement
Presence of sealed cap (yes/no)	On-site survey
Presence of protective casing (yes/no)	On-site survey
Wellhead cracks present (yes/no)	On-site survey
Casing clearance of at least 150 mm [c] (yes/no)	On-site measurement

DWWTS = Domestic Wastewater Treatment System.

[a] Small Area refers to areas comprising between 80 and 120 dwellings (Central Statistics Office, 2016b).

[b] 3 and 30 m are the minimum recommended distances between a water well and the nearest livestock grazing area and DWWTS in the RoI, respectively (EPA, 2021a).

[c] 150 mm is the minimum recommended casing clearance for water well in the RoI (Institute of Geologists of Ireland, 2007; Bacci and Chapman, 2011).

3. Results

3.1. Supply characteristics

A total of 132 private wells located in 21 of the 26 administrative counties in the RoI, were sampled in the current study. Of those, 132 were sampled and analysed from September to November 2019 (Round 1) and 118 were re-sampled from June to August 2021 (Round 2). Summary statistics describing these wells and associated samples are presented in Table 3. Most wells in the current study were drilled (88.6%; n = 117), with a mean overall supply depth of 40.9 ± 27.7 m and a mean casing clearance of 155 ± 224 mm, where recorded (75.0 and 95.4% of wells, respectively). Overall, 26.4% (n = 48) of wells were located in areas classified as having "high" groundwater vulnerability, followed by "extreme" (27.3%; n = 36), "moderate" (12.9%; n = 17), "extreme-rock/karst" (12.1%; n = 16) and "low" (11.4%; n = 15) classifications; with 81.8% (n = 108) of wells in areas of poorly productive bedrock aquifers, 12.9% (n = 17) in karst, and just 3% (n = 4) and 2.3% (n = 3) in fractured bedrock and unconsolidated (glaciofluvial) aquifers, respectively. In total, 14.4% (n = 19) of wells were located within 30 m of the nearest DWWTS and 18.9% (n = 25) were situated within 3 m of livestock. The calculated mean ± standard deviation DWWTS density at the Small Area level was 8 ± 5 units/km^2, with mean cattle and sheep densities of 161 ± 59 and 95 ± 137 heads/km^2, respectively. Of the 132 well owners in the study, 84.8% (n = 112) confirmed that the well water was used for household consumption. Antimicrobials were used in 17.2% (n = 43) and 30.8% (n = 77) of households in the three and six months prior to sample collection, respectively.

3.2. E. coli and P. aeruginosa presence

E. coli was detected in 21.6% of groundwater samples (n=54; Round 1 = 26.5% and Round 2 = 16.1%) and 33.3% of wells (n = 44) at least once, while *P. aeruginosa* presence was limited to 4.8% of samples (n=12; Round 1 = 6.1% and Round 2 = 3.4%) and 8.3% of wells (n = 11). Instances of re-occurring *E. coli* and *P. aeruginosa* contamination (i.e., a single species detected in the same site more than once) were found in 9.8% (n = 13) and 0.7% (n = 1) of wells, respectively, and co-occurrence (i.e., both species recovered from the same site) was identified in 6.8% wells (n = 9). As shown in Fig. 2, bacterial contamination was geographically spread across the RoI, being identified in 17 of the 21 counties. Such instances of contamination were immediately reported to supply owners in concurrence with recommendations on how to safeguard their health, such as with the installation of microbiological treatments (e.g., ultra-violet and reverse osmosis treatment systems). Regarding local hydrogeology, noteworthy findings include an absence of *P. aeruginosa* contamination in areas of "low" groundwater vulnerability, with highest incidence rates found in "extreme" vulnerability areas (17.2%; n = 5 samples). Conversely, similar *E. coli* contamination rates were found across all groundwater vulnerability classifications, ranging from 21.2 to 26.3%. The independent parameters significantly associated with *P. aeruginosa*, and *E. coli* detection (as well as presence of resistant bacteria) are presented in the Supplementary Data (i.e., bivariate analyses results).

3.3. Antimicrobial resistance profiles

The phenotypic susceptibility results for 48 *E. coli* isolates are shown in Table 4, while those for *P. aeruginosa* isolates (n = 6) are described in text. *E. coli* and *P. aeruginosa* isolates could not be recovered from six Round 1 samples and were not included for further analyses. Consequently, just three resistance profiles were obtainable for co-occurring *E. coli* and *P. aeruginosa* isolates (i.e., detected in the same samples), with no resistance found in either species. As shown in Table 4, four *E. coli* isolates from each Round were resistant to one or more antimicrobials, resulting in a total resistance rate of 16.7% (n = 8), with 12.5%

Table 3
Environmental and infrastructural characteristics of the 132 wells (and 250 samples) in the current study.

Characteristic	Wells, n (%)	Samples, n (%)	Characteristic	Wells, n (%)	Samples, n (%)
Groundwater vulnerability			**Supply type**		
Low	15 (11.4)	30 (12.0)	Drilled	117 (88.6)	223 (89.2)
Moderate	17 (12.9)	34 (13.6)	Hand-dug	15 (11.4)	27 (10.8)
High	48 (36.4)	90 (36.0)			
Extreme	36 (27.3)	65 (26.0)	**Supply depth**		
Extreme-rock/karst	16 (12.1)	31 (12.4)	≤10 m	15 (11.4)	27 (10.8)
			11–30 m	30 (22.7)	59 (23.6)
Aquifer importance			31–60 m	38 (28.8)	76 (30.4)
Poorly	37 (28.0)	72 (28.8)	61–90 m	9 (6.8)	18 (7.2)
Locally	76 (57.6)	142 (56.8)	>90 m	7 (5.3)	14 (5.6)
Regionally	19 (14.4)	36 (14.4)	Not recorded	33 (25.0)	56 (22.4)
Aquifer type			**Sealed cap**		
Poorly productive bedrock	108 (81.8)	204 (81.6)	Yes	46 (34.8)	89 (35.6)
Karst	17 (12.9)	33 (13.2)	No	79 (59.8)	148 (59.2)
Fractured bedrock	4 (3.0)	7 (2.8)	Not recorded	7 (5.3)	13 (5.2)
Gravel	3 (2.3)	6 (2.4)			
			Protective PVC casing		
Sheep density			Yes	32 (24.2)	61 (24.4)
≤50 heads/km^2	71 (53.8)	134 (53.6)	No	75 (56.8)	142 (56.8)
51-100 heads/km^2	19 (14.4)	35 (14.0)	Not recorded	25 (18.9)	47 (18.8)
101-200 heads/km^2	22 (16.7)	43 (17.2)			
201-300 heads/km^2	8 (6.1)	15 (6.0)	**Wellhead cracking**		
>300 heads/km^2	12 (9.1)	23 (9.2)	Yes	10 (7.6)	18 (7.2)
			No	118 (89.4)	224 (89.6)
Cattle density			Not recorded	4 (3.0)	8 (3.2)
≤50 heads/km^2	8 (6.1)	15 (6.0)			
51-100 heads/km^2	10 (7.6)	19 (7.6)	**Casing clearance**		
101-200 heads/km^2	83 (62.9)	159 (63.6)	<150 mm	80 (60.6)	149 (59.6)
201-300 heads/km^2	27 (20.5)	49 (19.6)	≥150 mm	46 (34.8)	89 (35.6)
>300 heads/km^2	4 (3.0)	8 (3.2)	Not recorded	6 (4.5)	12 (4.8)
DWWTS density			**Water use in the household**		
≤5 units/km^2	42 (31.8)	79 (31.6)	Drinking	112 (84.9)	216 (86.4)
6–15 units/km^2	83 (62.9)	159 (63.6)	Other	14 (10.6)	25 (10.0)
>15 units/km^2	7 (5.3)	12 (4.8)	Not recorded	6 (4.5)	9 (3.6)
Nearest livestock			**Antimicrobials used in last 3 months**		
≤3 m	25 (18.9)	48 (19.2)	Yes	–	43 (17.2)
4–100 m	92 (69.7)	172 (68.8)	No	–	177 (70.8)
>100 m	15 (11.4)	30 (12.0)	Not recorded	–	30 (12.0)
Nearest DWWTS			**Antimicrobials used in last 6 months**		
≤30 m	19 (14.4)	35 (14.0)	Yes	–	77 (30.8)
31–100 m	100 (75.8)	190 (76.0)	No	–	143 (57.2)
>100 m	13 (9.8)	25 (10.0)	Not recorded	–	30 (12.0)

of isolates (n = 6; three from each Round) being resistant to ≥ 3 antimicrobials (i.e., multidrug-resistant) and a further 79.2% (n = 38) being classified as intermediately resistant. Conversely, none of the recovered *P. aeruginosa* isolates were categorically resistant to the tested antimicrobials. Thus, 3.2% (n = 8) of groundwater samples and 6.1% (n = 8) of wells harboured antimicrobial resistant bacteria (i.e., *E. coli*), with no repeated instances of resistant *E. coli* contamination.

E. coli isolates exhibited highest levels of resistance to streptomycin, with a total resistance rate of 14.6% (n = 7); intermediate classifications were also most prevalent in relation to this antimicrobial, found in a further 81.3% (n = 39) of the isolates tested. The second highest resistance rates were to tetracycline and ampicillin, both corresponding to 12.5% of *E. coli* isolates (n = 6), followed by 10.4% (n = 5) to piperacillin; an additional 8.3% (n = 4) and 4.2% (n = 2) of isolates were intermediately resistant to ampicillin and piperacillin, respectively. Resistance was also found to chloramphenicol (4.2%; n = 2), trimethoprim (2.1%; n = 1), and trimethoprim-sulfamethoxazole (2.1%; n = 1). It is worth noting that despite no resistance found to cephazolin, 54.2% of *E. coli* isolates (n = 26) were intermediately resistant to it. Moreover, intermediate resistance was found to amoxicillin-clavulanic acid (10.4%; n = 5), ciprofloxacin (6.3%; n = 3), enrofloxacin (6.3%; n = 3), cefepime (4.2%; n = 2), and ceftiofur (2.1%; n = 1). All groundwater-derived *E. coli* isolates were susceptible to imipenem, piperacillin-tazobactam, ceftazidime, gentamicin, or aztreonam. In the case of *P. aeruginosa*, one Round 2 isolate exhibited intermediate resistance to enrofloxacin.

3.4. Multivariate logistic regression modelling

Binary logistic regression models assessing the detection of (i) *P. aeruginosa*, (ii) *E. coli*, (iii) and resistant *E. coli* are presented in Table 5, with Nagelkerke R^2 of 0.504, 0.285, and 0.475, respectively. Second 'best-fit' models are presented in the Supplementary Data. Overall, the *P. aeruginosa* model showed no significant associations with hydrogeological or agricultural parameters, despite lower sheep density at the Small Area level being significantly associated (at the 90% confidence level) with higher odds of *P. aeruginosa* presence (OR = 0.959, p = 0.056). Environmental risk factors significantly associated with higher odds of *P. aeruginosa* detection included presence of DWWTS within 30 m of the well (OR = 14.046, p = 0.026) and (above mean) increases to 5-day rainfall (OR = 1.069, p = 0.005). Hand-dug wells were 19-times more likely to harbour *P. aeruginosa* than drilled wells

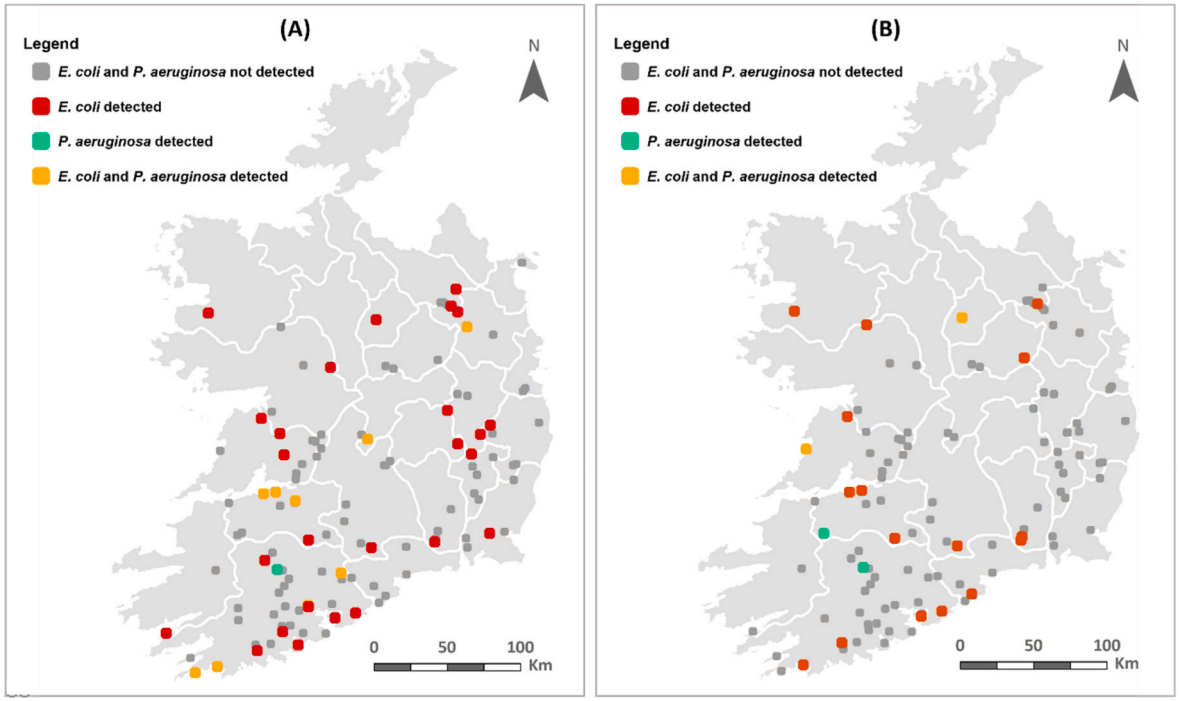


Fig. 2. Republic of Ireland maps showing the geographical distribution of sampled wells and contamination status in Round 1 (autumn 2019; A), and Round 2 (summer 2021; B).

(OR = 18.902, $p = 0.020$), and the presence of wellhead cracking led to a one-hundred-fold increase in the odds of detection (OR = 100.256, $p = 0.009$). The second 'best-fit' *P. aeruginosa* model, where 5 and 10-day rainfall parameters were interchanged, displayed the same significant patterns, while also signalling 10-day rainfall increases as being significantly associated with higher odds of *P. aeruginosa* presence (OR =

Table 4
Susceptibility results for 48 groundwater-derived *E. coli* isolates collected in autumn 2019 (Round 1, n = 29) and summer 2021 (Round 2, n = 19).

Antimicrobial	Current medical use in RoI [a]	Round 1 (n = 29)		Round 2 (n = 19)		Total (n = 48)	
		R isolates No. (%)	I isolates No. (%)	R isolates No. (%)	I isolates No. (%)	R isolates No. (%)	I isolates No. (%)
Penicillins							
Ampicillin	Veterinary	3 (10.3)	2 (6.9)	3 (15.8)	2 (10.5)	6 (12.5)	4 (8.3)
Amoxicillin-Clavulanic acid	Both	0	2 (6.9)	0	3 (15.8)	0	5 (10.4)
Piperacillin	Human*	3 (10.3)	1 (3.4)	2 (10.5)	1 (5.3)	5 (10.4)	2 (4.2)
Piperacillin- Tazobactam	Human	0	0	0	0	0	0
Cephalosporins							
Cefazolin	Both*	0	15 (51.7)	0	11 (57.9)	0	26 (54.2)
Ceftazidime	Human	0	0	0	0	0	0
Ceftiofur	Veterinary	0	1 (3.4)	0	0	0	1 (2.1)
Cefepime	Human	0	2 (6.9)	0	0	0	2 (4.2)
Aminoglycosides							
Streptomycin	Veterinary	4 (13.8)	23 (79.3)	3 (15.8)	16 (84.2)	7 (14.6)	39 (81.3)
Gentamicin	Both	0	0	0	0	0	0
Carbapenems							
Imipenem	Human	0	0	0	0	0	0
Fluoroquinolones							
Ciprofloxacin	Human	0	2 (6.9)	0	1 (5.3)	0	3 (6.3)
Enrofloxacin	Veterinary	0	1 (3.4)	0	2 (10.5)	0	3 (6.3)
Monobactams							
Aztreonam	Human	0	0	0	0	0	0
Tetracyclines							
Tetracycline	Both	3 (10.3)	0	3 (15.8)	0	6 (12.5)	0
Folate Pathway Inhibitors							
Trimethoprim	Human	1 (3.4)	0	0	0	1 (2.1)	0
Trimethoprim- Sulfamethoxazole	Both	1 (3.4)	0	0	0	1 (2.1)	0
Phenols							
Chloramphenicol	Human	1 (3.4)	0	1 (5.3)	0	2 (4.2)	0
Total		4 (13.8)	22 (75.9)	4 (21.1)	15 (78.9)	8 (16.7)	38 (79.2)

[a] Use and license status for human and veterinary medicine in the RoI according to the Health Products Regulatory Authority in Ireland (HPRA, 2020a; 2020b); * No longer licensed for medical use in the RoI (HPRA, 2020a: 2020b); RoI = Republic of Ireland; R = Resistant based on Clinical and Laboratory Standards Institute (CLSI) Zone Diameter breakpoints (CLSI, 2018; 2020); I = Intermediate based on CLSI's Zone Diameter breakpoints (CLSI, 2018; 2020).

Table 5
Final multivariate binary logistic regression models for presence/absence of (i) *Pseudomonas aeruginosa*, (ii) *Escherichia coli*, and (ii) Resistant *Escherichia coli*

Risk Factors	*P. aeruginosa* detection (n = 233)		*E.coli* detection (n = 233)		Resistant [a] E.coli detection (n = 227)	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	p-value
Groundwater vulnerability						
Low	0.000 (0.000–.)	0.997	Reference		Reference	
Moderate	0.187 (0.010–3.460)	0.260	0.446 (0.094–2.109)	0.308	0.000 (0.000–.)	0.997
High	0.320 (0.028–3.657)	0.359	0.569 (0.164–1.972)	0.374	0.003 (0.000–0.423)	0.022*
Extreme	0.134 (0.010–1.893)	0.137	1.247 (0.349–4.461)	0.734	0.039 (0.002–0.990)	0.049*
Extreme-karst	Reference		0.946 (0.225–3.972)	0.940	0.027 (0.001–0.858)	0.041*
Aquifer type						
Karst vs Non-karst (Ref)	6.309 (0.628–63.364)	0.118	**3.903 (1.413–10.782)**	**0.009***	0.397 (0.017–9.368)	0.567
Sheep density [b]						
Increases of 1 head/m^2	0.959 (0.918–1.001)	0.056**	1.001 (0.998–1.004)	0.519	0.991 (0.977–1.005)	0.196
Cattle density [b]						
Increases of 1 head/m^2	1.000 (0.983–1.017)	0.983	**1.010 (1.002–1.018)**	**0.011***	1.028 (1.002–1.056)	0.037*
DWWTS density [b]						
Increases of 1 unit/m^2	0.994 (0.884–1.118)	0.920	1.025 (0.961–1.094)	0.455	0.973 (0.709–1.335)	0.866
Nearest livestock ≤3 m						
Present vs. Absent (Ref)	0.067 (0.002–1.979)	0.118	1.071 (.412–2.785)	0.888	4.977 (0.632–39.193)	0.127
Nearest DWWTS ≤30 m						
Present vs. Absent (Ref)	**14.046 (1.380–142.963)**	**0.026***	**5.090 (1.934–13.401)**	**<0.001***	0.000 (0.000–.)	0.998
5-day rainfall						
Increases of 1 mm	**1.069 (1.020–1.121)**	**0.005***	–	–	–	–
60-day rainfall						
Increases of 1 mm	-	-	**1.008 (1.002–1.014)**	**0.008***	1.009 (0.993–1.025)	0.287
Supply type						
Hand-dug vs. Drilled (Ref)	**18.902 (1.587–225.127)**	**0.020***	**7.199 (2.252–23.016)**	**<0.001***	41.071 (1.001–1685.502)	0.050*
Sealed cap						
Present vs. Absent (Ref)	0.358 (0.037–3.422)	0.372	0.452 (0.193–1.060)	0.068**	0.155 (0.017–1.410)	0.098**
Wellhead cracks						
Present vs. Absent (Ref)	**100.256 (3.114–3227.933)**	**0.009***	2.847 (0.752–10.782)	0.124	0.000 (0.000–.)	0.998
Casing clearance ≥150 mm						
Present vs. Absent (Ref)	0.731 (0.118–4.519)	0.736	0.603 (0.273–1.331)	0.211	1.323 (0.104–16.872)	0.829
Nagelkerke R^2	0.504		0.285		0.475	

* *p*-value≤0.05; ** 0.05 < *p*-value≤0.1
[a] Categorically resistant in accordance with CLSI (2018; 2020).
[b] Sheep, cattle and DWWTS densities calculated per Small Area, which are delineated land sections in the RoI territory which comprise 80 to 120 dwellings (Central Statistics Office, 2016b); DWWTS = Domestic Wastewater treatment System; CI=Confidence Interval.

1.036, $p = 0.005$; see Supplementary Data).

In the case of *E. coli*, the final model showed over three times higher odds of detection in karst aquifers (OR = 3.903, $p = 0.009$), with 1 mm (above mean) increases to 60-day rainfall also significantly associated with 8% higher odds of *E. coli* presence (OR = 1.008, $p = 0.008$). Conversely, the resistant *E. coli* model showed that detection odds were significantly higher in areas of "low" groundwater vulnerability than those in "high" (OR = 333.333, $p = 0.022$), "extreme" (OR = 25.641, $p = 0.049$) and "extreme-rock/karst" (OR = 37.037, $p = 0.041$) groundwater vulnerability areas. Regarding potential contamination sources, significantly higher odds of *E. coli* presence were associated with DWWTS located within 30 m of the well (OR = 5.090, $p < 0.001$), and with higher cattle density at the Small Area level (OR = 1.010, $p = 0.011$); while just cattle density was significantly associated with higher odds of resistant *E. coli* presence (OR = 1.028, $p = 0.037$). The odds of *E. coli* and resistant *E. coli* detection were both significantly higher in hand-dug versus drilled wells (OR = 7.199 with $p < 0.001$ and OR = 41.071 with $p = 0.050$, respectively). Moreover, significance at the 90% confidence level indicated that absence of an appropriately sealed cap could more than double the odds of *E. coli* detection (OR = 2.212; $p = 0.068$) and lead to a six-fold increase in the odds of resistant *E. coli* detection (OR = 6.452, $p = 0.098$). The second 'best-fit' models for *E. coli* and resistant *E. coli,* while emphasising the same significant parameters, also showed that 10-day antecedent rainfall increases were associated with significantly higher odds of *E. coli* detection (OR = 1.016, $p = 0.008$).

4. Discussion

The current study is the second to investigate the occurrence of antimicrobial resistant bacteria in groundwater sources in the RoI (succeeding O'Dwyer et al., 2017), and the first to comprise a country-wide (geographically dispersed) sampling network while also including the analyses of bacterial species of distinct primary habitats. To the author's knowledge, this study is also the first to analyse groundwater samples for the presence of *P. aeruginosa* in the RoI. In total, 132 private wells (250 samples) were assessed from 21 out of the 26 administrative counties in the country, with samples collected at two time periods: September to November 2019 (Round 1) and June to August 2021 (Round 2); notably no sampling was carried out in spring or winter months. *E. coli,* a well-known faecal indicator organism, and *P. aeruginosa*, a bacteria that can proliferate in soil and freshwater, were used to assess current rates of antimicrobial resistant bacteria and identify key associated anthropogenic and environmental sources/-pathways, as well as infrastructural risk factors.

Overall, observed instances of *P. aeruginosa* contamination were low, detected in just 8.3% of private wells (n = 11), while *E. coli* were found in 33.3% of wells at least once (n = 44). *E. coli* detection fell within the range reported by previous studies of private groundwater quality in the RoI, which described rates of 28.9% (Hynds et al., 2012) to 51.4% (O'Dwyer et al., 2018). No RoI-specific baseline data are available on the incidence of *P. aeruginosa* in groundwater sources; however, the lower incidence identified here (i.e., 8.3%), as well as in some other international literature (Bamigboye et al., 2020; Ayad et al., 2021), may be

explained by the lower abundance of this species in rural soil and freshwater when compared to other Pseudomonads (Chatterjee et al., 2017) or to samples from urban environments (Crone et al., 2020). The 48 *E. coli* and six *P. aeruginosa* isolates recovered in the current study underwent phenotypic antimicrobial susceptibility analyses, with results varying substantially between both species. Resistance (categorised according to CLSI, 2018, 2020) was found in 16.7% of recovered *E. coli* (75.0% of which were resistant to ≥ 3 antimicrobials) and in none of the *P. aeruginosa* isolates.

The lack of resistance and low incidence (n = 1) of intermediate resistance among *P. aeruginosa* isolates deviates from international review findings (Andrade et al., 2020), where groundwater-derived *Pseudomonas* spp. displayed highest mean rates of resistance and/or intermediate resistance globally (mean ± standard deviation = 99.9% ± 0.2). Notably, however, overall resistance/intermediate resistance rates in the review were significantly higher in urban rather than rural settings (Andrade et al., 2020), with most previous *Pseudomonads*-specific literature focusing on groundwater resources in urban and densely populated areas. Compounding this, *P. aeruginosa* is intrinsically resistant to many antimicrobial agents (Livermore, 1984; Nordmann and Guibert, 1998). Indeed, *P. aeruginosa* susceptibility breakpoints are not available for most veterinary antimicrobials included in this study (see Table 2). A recent study in rural settings found moderate rates of resistance among *P. aeruginosa* from hydrocarbon-contaminated groundwater supplies in Hungary (i.e., 31.6%) (Kaszab et al., 2021); it must be noted, however, that the presence of petroleum hydrocarbons has been shown to cause co-selection of antimicrobial resistance genes in soil and water (Chen et al., 2017; Wang et al., 2017).

Conversely, current *E. coli* resistance rates (i.e., 16.7%) were higher than in studies from other developed countries. A Canadian study examining private groundwater supplies in Alberta and Ontario, for example, found that 10.5% and 3.7% of groundwater-derived *E. coli* were resistant to ≥1 and ≥3 antimicrobials, respectively (Coleman et al., 2013). Present findings are also at the high end of resistance within the international context, with 95.8% (n = 46) of all tested *E. coli* being classified as resistant and/or intermediate resistance to at least one antimicrobial; a higher rate than the global mean of 79.8% ± 30.1 identified for groundwater-derived *E. coli* (Andrade et al., 2020). Notwithstanding, resistance rates identified here were significantly lower than those detected in the mid-west of the RoI by O'Dwyer et al. (2017), who found that all groundwater-derived *E. coli* (n = 42) were resistant to at least one antimicrobial, primarily due to Aminoglycoside resistance identified in over 92% of isolates (contrasting with 14.6% in the current study). The disparity seen here can be contextualised in view of reported disagreements between *E. coli* isolates' phenotypic and genotypic resistance to streptomycin in international literature (Kozak et al., 2009; Allen et al., 2011).

Nevertheless, and similar to findings reported by O'Dwyer et al. (2017), streptomycin was associated with the highest resistance rates among the *E. coli* isolates, followed by tetracycline (12.5%) and ampicillin (12.5%), all commonly used veterinary antimicrobials (Martin et al., 2020). In fact, these three have been listed as the most frequently employed antimicrobials in Irish bovine (i.e., dairy) agricultural operations (Gibbons et al., 2014), with another Irish study reporting that *E. coli* isolated from cattle were most frequently resistant, to streptomycin, tetracycline, sulfonamide, and ampicillin (Karczmarczyk et al., 2011). Resistance among the groundwater-derived *E. coli* was also found, albeit in lower rates, to piperacillin (10.4%), chloramphenicol (4.2%), trimethoprim (2.1%), and trimethoprim-sulfamethoxazole (2.1%), which are antimicrobials exclusively used among humans in the RoI. However, as resistance found to piperacillin was always concurrent with that to ampicillin (both broad-spectrum β-lactam antimicrobials) with all isolates being susceptible to piperacillin-tazobactam (the only form of therapeutic piperacillin currently available in the RoI, see Table 1), the authors consider that the piperacillin resistance found was likely conferred by beta-lactamase resistance genes which also confer resistance to ampicillin (Hussain et al., 2021). The significantly lower rates of resistance to the other screened human antimicrobials may be attributable to the current study being undertaken in relatively sparsely populated rural settings, with previous research by Moore et al. (2020) noting an increase in antimicrobial resistance among faecal bacteria from groundwaters in closer proximity to highly populated areas, such as tourist resorts. Indeed, *E. coli* and resistant *E. coli* regression models (Table 5) show that while *E. coli* presence was significantly associated with both human (DWWTS present within 30 m of well; OR = 5.090, $p < 0.001$) and pastoral agricultural sources (cattle density; OR = 1.010, $p = 0.011$), only cattle density was significantly associated with the detection of resistant *E. coli* (OR = 1.028, $p = 0.037$). Consequently, and in combination with the lack of resistance among *P. aeruginosa,* an environmental bacteria which has been linked with human activities in the present investigation as well as previous work (Crone et al., 2020), study findings confirm that resistance pressures arising from human sources are considerably less prominent than agricultural sources in rural and sparsely populated environments. This is in spite of the presence of nearby point-sources of contamination such as DWWTSs (e.g., septic tanks) (Hynds et al., 2014; O'Dwyer et al., 2018), which are historically under-regulated by the State (C-188/08, 2009; C-374/11, 2012) and, when inspected, typically have a high rate of individual system failure - reported in 54% of the 806 septic tanks inspected in 2020 (EPA, 2021b).

Associations between livestock-based agriculture and antimicrobial resistant bacteria in rural groundwater resources have been similarly reported nationally (O'Dwyer et al., 2017) and internationally (Coleman et al., 2013; Gambero et al., 2018). This is particularly relevant as cattle (beef and dairy) accounts for over 59% of the RoI's agricultural outputs in 2021 (CSO, 2021). National antimicrobial usage data is not yet collected at farm levels in the RoI (Martin et al., 2020), however research conducted in the country reports high antimicrobial administration in cattle as part of dairy farming activities (Gibbons et al., 2014) and these supply 60% of the national beef (Morrison, 2020). Hence, the seasonal nature of the calving season, which takes place in spring (February to April) and represents the period of highest antimicrobial prescription (>63%) (Gibbons et al., 2014), may partially account for the higher rates of resistance in Round 2 (summer 2021; 21.1%) than Round 1 (autumn 2019; 13.8%). This increase may also be attributable to national veterinary antimicrobial prescriptions having increased since the COVID-19 pandemic, with a recent surveillance report indicating that sales in 2020 were higher than those from the previous eight (pre-pandemic) years (HPRA, 2021). With regards to human antimicrobial use, the lower impact inferred is further supported by the only intermediate resistance classification among *P. aeruginosa* isolates being to a veterinary antimicrobial (i.e., enrofloxacin); despite increased odds of *P. aeruginosa* detection at close proximity to DWWTS (i.e., ≤30 m; OR = 14.046, $p = 0.026$), and lack of significant associations with animal sources of contamination. Of note, significance at a 90% confidence level pointed to lower sheep densities being associated with higher odds of *P. aeruginosa* detection (OR = 1.043; $p = 0.056$); however, this may be in part attributable to sheep grazing areas having thinner and less organically rich topsoils, which have been shown to negatively influence the detection of *Pseudomonas* species (Dignam et al., 2018).

Key differences with respect to likely bacterial transport pathways can be inferred via comparison of final multivariate models. In the case of *E. coli*, for example, transport to contaminated groundwater supplies would seem to be attributable to a few ingress mechanisms: i) rapid aquifer recharge in karst regions (OR = 3.903, $p = 0.009$), ii) "generalised" (traditional) recharge, owing to above mean increases in longer-term (60-day) rainfall being significantly associated with higher odds of *E. coli* presence (OR = 1.008, $p = 0.008$), and iii) direct ingress at the wellhead, substantiated by higher odds of *E. coli* detection approaching significance in the absence of an appropriately sealed cap (OR = 2.847, $p = 0.068$) and being significantly associated with increases in shorter-term (10-day) rainfall (OR = 1.016, $p = 0.008$; seen in the second 'best-

fit' model with a marginally lower R^2). The final resistant *E. coli* model, however, (as well as others attempted with the different rainfall periods) showed no significant relationship with cumulative rainfall, while significantly higher odds of detection were observed in areas of low groundwater vulnerability (where impermeable layers of clay soil and subsoil prevent contaminant percolation and recharge), and where the well cap was not appropriately sealed (significant at the 90% confidence level; OR = 6.452, $p = 0.098$). These results point to the primary ingress mechanism associated with presence of resistant *E. coli* in Irish rural supplies being overland flow followed by direct wellhead ingress, contrasting with 'traditional' recharge suggestions posed by Andrade et al. (2020) based on the analyses of data from rural and urban settings combined. In line with the proposed theory, an association with shorter-term (5- and/or 10-day) cumulative rainfall might be expected, as it is a key driver of overland flow; however, the lack of a significant relationship may be justified by a greater impact of rainfall incidence/frequency rather than intensity especially as detection of resistant *E. coli* was significantly higher in low groundwater vulnerability areas, where thicker impermeable layers allow for higher rates of overland flow. Direct ingress at the wellhead and/or rapid infiltration (i.e., preferential flow) may also be the principal transport mechanisms of *P. aeruginosa,* owing to significantly higher odds of detection related to increases in shorter-term antecedent rainfall (i.e., 5-day; OR = 1.069, p = 0.005) and presence of wellhead cracking (OR = 100.256, 0.009), combined with lack of *P. aeruginosa* detection in "low" groundwater vulnerability areas. Notably, in all multivariate models (i.e., *P. aeruginosa, E. coli* and resistant *E. coli*) the odds of detection were significantly higher for hand-dug than drilled wells (OR = 18.902, 7.199, and 41.071, respectively), thus confirming the inherent microbial susceptibility of hand-dug wells (Hynds et al., 2012; Maran et al., 2016) and indicating higher likelihood of localised (i.e., confined to the well) rather than aquifer-wide contamination. It is also worth noting that lack of repeat (i.e., re-occurring) antimicrobial resistant bacteria presence between sampling rounds points to the fluctuating nature of this type of contamination.

As is the case with many exploratory environmental and groundwater contamination studies, limitations must be acknowledged owing to the outcome variances not being fully explained by the independent variables in the models (i.e., Nagelkerke R^2 ranging from 28.0 to 50.8%). In the case of resistant *E. coli* modelling, in particular, this is likely attributable to the added complexity of antimicrobial resistance acquisition in the environment, potentially influenced by a range of factors outside of the scope of this research such as presence of chemical co-pollutants (e.g., toxic metals, biocides and disinfectants) and biofilm formation (Wu et al., 2018; Zhang et al., 2018), excessive organic matter (Chee-Sanford et al., 2009) and even microplastics (Imran et al., 2019). Indeed, additional research is needed to investigate the role of these localised pressures in the acquisition and transport of resistance within groundwater environments. The focus of this investigation, however, was to identify the likely impact of larger-scale environmental and infrastructural determinants in facilitating groundwater contamination with antimicrobial resistant bacteria, as well as suggest preventative source protection measures. To this end, the current study is valuable for highlighting distinctions in overall resistance rates between *E. coli* and *P. aeruginosa*, as well as their likely sources and transport pathways. Results also show that specific well construction elements play an important role in preventing antimicrobial resistance (and indeed bacterial) contamination and their associated health impacts, such as (1) opting for a 'drilled' as opposed to a 'hand-dug' well, (2) ensuring minimum setback distances to sources of faecal (and potentially antimicrobial) contamination (e.g., septic tanks and livestock activities), (3) fitting an appropriately sealed cap, and (4) appropriately maintaining the wellhead (i.e. keeping it clean, dry, and crack-free).

It is also important to emphasise that despite the low overall detection of antimicrobial resistant bacteria (i.e., present in 3.2% of samples and 6.1% of wells at least once, with isolates classified as intermediately resistant in an additional 15.2% of samples and 25.8% of wells), their occurrence in drinking water resources presents substantial risks to human health. Groundwater resources' role in disease transmission is well-substantiated within the literature, with estimates of up to 59.4 million annual cases of acute gastrointestinal infection linked to groundwater consumption globally (Murphy et al., 2017) and high incidence of verotoxigenic *E. coli* (VTEC) enteritis being significantly associated with consumption of private well water in the RoI (ÓhAiseadha et al., 2017). Thus, increased groundwater resource colonisation with antimicrobial resistant bacteria can represent a meaningful contribution to the higher morbidity and mortality associated with "harder-to-treat" infections globally. Accordingly, and given the importance of reducing the spread of antimicrobial resistance within and outside of clinical settings, further efforts are needed to mitigate presence of antimicrobial resistant bacteria in rural groundwater resource environments.

5. Conclusion

The results presented here reiterate the noteworthy role of groundwater - even those in rural settings (where population density is lower, non-point agricultural sources of contamination are dominant, and sizable point sources are less common) - as potential reservoirs for antimicrobial resistant bacteria. While no resistance was found among *P. aeruginosa* isolates, an environmental bacteria linked to intense human activities, phenotypic susceptibility results showed that 16.7% of *E. coli* were resistant to one or more antimicrobials - detected in 6.1% of wells in this study. The likely source of antimicrobial resistant *E. coli* in the investigated supplies was cattle farming, with highest resistance rates found to veterinary antimicrobials and multivariate modelling results indicating presence of resistant *E. coli* as significantly associated with higher cattle density. Hence, outpatient use of human antimicrobials in rural areas of the RoI present a comparatively small contribution to the spread of resistance. Moreover, as detection of resistant *E. coli* was significantly more likely in areas of low groundwater vulnerability and where supplies were not fitted with an appropriately sealed cap, overland flow followed by direct wellhead ingress is hypothesised as the primary ingress mechanism associated with their presence. Findings also provide empirical evidence to support groundwater source protection policies, such as reinforcing the increased safety offered by drilled rather than hand-dug wells, and the importance of an appropriately sealed well cap and minimum well setback distances to faecal contamination sources (e.g., DWWTSs and livestock activities). Importantly, however, the outcome variance in the multivariate model produced was only partially explained by the independent variables included. As such, further work is needed to investigate existing pressures within these 'protected' environments (e.g., co-contaminants, physico-chemical parameters, etc.) in the hopes of identifying likely antimicrobial resistance acquisition trends and appropriate prevention frameworks. Nevertheless, this study is valuable for demonstrating key differences in resistance rates within distinct bacterial species, while identifying likely sources and transport pathways of antimicrobial resistant *E. coli*. Moreover, despite the low overall detection rates found, the presence of antimicrobial resistant bacteria in groundwater resources used for human consumption shows that there exists a substantial risk to public health and can serve as a benchmark for future groundwater studies.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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