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DESIGN – Detection of Environmental Sources of Infectious Disease in Groundwater Networks

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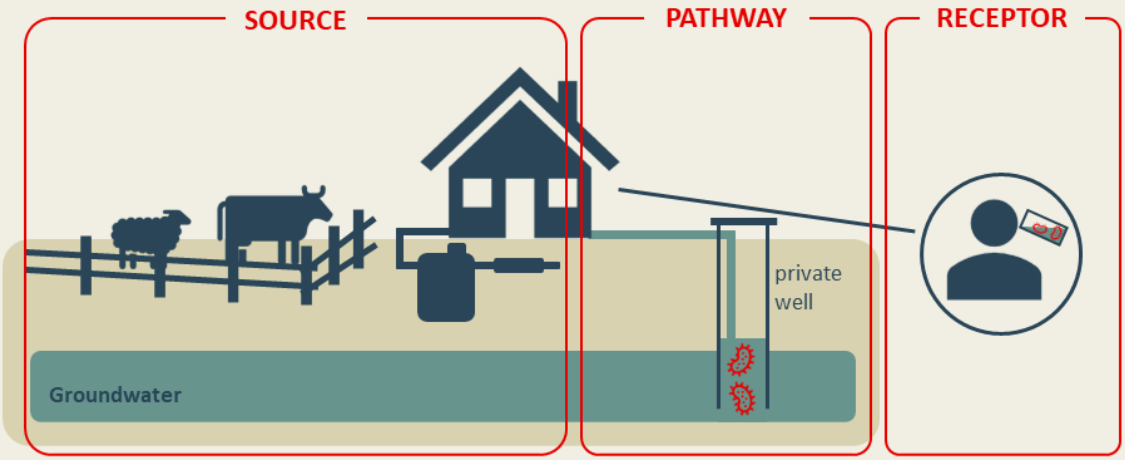

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Government of Ireland

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4. Office of Radiation Protection and Environmental Monitoring
5. Office of Communications and Corporate Services

The EPA is assisted by advisory committees who meet regularly to discuss issues of concern and provide advice to the Board.

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Lead organisations: University College Cork, Technological University Dublin, and National University of Ireland Galway

Identifying pressures

Infectious diseases associated with environmental change pose a significant challenge for public health, as their sources and transmission are frequently sporadic and associated mechanisms are not well understood. Ireland currently has the highest incidence of verotoxigenic *Escherichia coli* (VTEC) infection in the EU, with domestic private wells identified as a likely source of infection. Ireland also has a high incidence of the waterborne disease *cryptosporidiosis*; however, the role of groundwater in its transmission is unknown. This research provides the first quantitative assessment of VTEC and Cryptosporidium in domestic groundwater supplies in Ireland, identifying key risk factors associated with occurrence and providing recommendations to reduce the disease burden

Informing policy

This research project identified key challenges and recommendations related to waterborne disease exposure and transmission via domestic water wells, including immediate measures that could reduce the disease burden associated with these supplies. The Detection of Environmental Sources of Infectious Disease in Groundwater Networks (DESIGN) project will inform the development and implementation of policies such as the Healthy Ireland framework and the EU Drinking Water Directive by improving our understanding of pathogen sources, pathways and environmental fate in groundwater systems in Ireland. Moreover, the DESIGN project is facilitating the development of bespoke, spatio-temporal groundwater management policies, offering invaluable guidance for future planning and the remediation and mitigation of the microbiological contamination of Ireland's private water wells.

Developing solutions

Overall, the DESIGN project provided further insight into the prevalence, source and transport of VTEC and *Cryptosporidium* in groundwater supplies in Ireland. The research demonstrated a significantly higher burden of VTEC in (sampled) national groundwater resources than the global average. Moreover, a VTEC to generic E. coli ratio of 40% was calculated, which can be utilised by water service professionals, clinicians and hydrologists to estimate future risk and reduce the national disease burden. In this study, VTEC presence was significantly associated with decreasing well depth and increasing 30-day mean antecedent rainfall. The findings suggest a high risk of VTEC in *E. coli*-contaminated groundwater sources in Ireland, with multiple clinically relevant serogroups often present in heavily contaminated sources. Through geo-specific risk assessment of the heterogeneous nature of both aquifers and pathogen sources, areas of low, medium and high risk were identified. This will allow for spatio-temporal management strategies to be implemented in the context of changes in land use, climate and public infrastructure.

EPA RESEARCH PROGRAMME 2014–2020

DESIGN – Detection of Environmental Sources of Infectious Disease in Groundwater Networks

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EPA Research Report

Prepared for the Environmental Protection Agency

By

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This report is based on research carried out/data from 2019 to 2021. More recent data may have become available since the research was completed.

The EPA Research Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Contents

Acknowledgements **ii**

Disclaimer **ii**

Project Partners **iii**

List of Figures **vii**

List of Tables **ix**

Executive Summary **xi**

1 Introduction **1**

1.1 Project Context and Rationale 1

1.2 *Cryptosporidium* 3

1.3 Verotoxigenic *Escherichia coli* 5

1.4 Project Aims and Objectives 6

1.5 Report Structure 6

2 Scoping Literature Review **8**

2.1 Introduction 8

2.2 *Cryptosporidium* 8

2.3 Verotoxigenic *Escherichia Coli* 25

3 Verotoxigenic *Escherichia Coli* Contamination Status of Private Wells **44**

3.1 Introduction 44

3.2 Methodology 44

3.3 Results 48

3.4 Discussion and Conclusions 51

4 Preliminary Investigation of *Cryptosporidium* Contamination in Private Domestic Supplies **53**

4.1 Introduction 53

4.2 Methodology 53

4.3 Results 56

4.4 Discussion and Conclusions 57

5 Conclusions and Recommendations 59

5.1 Conclusions 59

5.2 Recommendations for Research Priorities 59

References 61

Abbreviations 72

List of Figures

Figure 2.1. Protocol employed for the *Cryptosporidium* systematic literature review, including the key phases (identification, screening, eligibility) leading to final study selection (inclusion) 9

Figure 2.2. Map illustrating the global distribution of *Cryptosporidium* studies included for review, with *Cryptosporidium* oocyst concentrations per country given in parentheses 11

Figure 2.3. Frequency of *Cryptosporidium* studies according to the number of supply sources and groundwater samples analysed within different sampling periods 15

Figure 2.4. (A) Frequency of use of *Cryptosporidium* detection methods per income category. (B) Frequency of use of detection methods employed per geographical region 16

Figure 2.5. (A) Timeline of *Cryptosporidium* detection methods employed following year of publication intervals. (B) Frequency of use of detection methods according to income categories within different year intervals 16

Figure 2.6. (A) Proportion of *Cryptosporidium* species detected via molecular methods and total number of positive samples per species. (B) Frequency (%) of *Cryptosporidium* species identified per income category (top) and region (bottom) 20

Figure 2.7. Protocol employed for the VTEC systematic literature review, including four distinct review phases (identification, screening, eligibility, inclusion) 26

Figure 2.8. Map illustrating the global distribution of VTEC studies included for review, with the number of VTEC-positive investigations per country given in parentheses 31

Figure 2.9. Frequency of VTEC studies included for review delineated by mean income level, urban/rural classification and geographical location 31

Figure 2.10. Frequency of VTEC studies included for review delineated by number of groundwater sources and samples analysed based on sampling period duration 32

Figure 2.11. VTEC to generic *E. coli* detection ratios, including (pooled) data specific to sampling strategy categories (“one-off” and “repeat”) 35

Figure 3.1. Location of sampled private groundwater wells and VTEC gene analysis findings in relation to GSI groundwater vulnerability classification 46

Figure 4.1. Location of private supplies analysed for *Cryptosporidium* (oocyst) presence during the first sampling phase (autumn 2019) in the south of Ireland 54

Figure 4.2. Location of private supplies analysed for *Cryptosporidium* (oocyst) presence during the second sampling phase (spring 2022) in mid-west Ireland 54

Figure 4.3. Location of private supplies analysed for *Cryptosporidium* (oocyst) presence (spring 2022) in relation to ED-level DWWTS density 57

List of Tables

Table 2.1.	Summary of data on key characteristics extracted from included *Cryptosporidium* studies ($n=37$)	12
Table 2.2.	Synthesis of pooled data from studies of *Cryptosporidium* groundwater samples ($n=3070$) and sources ($n=1797$) reporting positive *Cryptosporidium* contamination by selected study characteristic	17
Table 2.3.	Summary of selected characteristics within the "contamination" data extraction category based on studies with positive detection of *Cryptosporidium* in groundwater ($n=31$)	19
Table 2.4.	Summary of data on key characteristics extracted from included VTEC studies ($n=23$)	28
Table 2.5.	Synthesis of pooled data from studies of VTEC groundwater samples ($n=2230$) and sources ($n=1949$) reporting positive VTEC detection by selected study characteristic	33
Table 2.6.	Pooled generic *E. coli* and VTEC values according to geographical location	36
Table 2.7.	Summary of data on selected characteristics within the "VTEC contamination" data extraction category based on studies with positive detection of VTEC in groundwater ($n=9$)	37
Table 3.1.	Intrinsic, specific and infrastructure (ISI) risk factor data collated and assessed for association with groundwater supply contamination	47
Table 3.2.	Prevalence of *E. coli* in private groundwater wells	49
Table 3.3.	Detection of VTEC-associated virulence factors in groundwater wells	49
Table 3.4.	Detection of VTEC serogroup-specific markers in *stx1* and/or *stx2*-positive groundwater samples	49
Table 3.5.	Independent groundwater risk factors associated with well contamination by *E. coli* type and/or gene presence	50
Table 4.1.	Risk factor information pertaining to *Cryptosporidium* sampling sites ($n=40$)	55

Executive Summary

Private groundwater sources in Ireland provide domestic drinking water to approximately 750,000 people, mainly in rural areas characterised by a heavily dispersed yet locally dense settlement pattern. The spatial distribution of private groundwater supplies exhibits a clear urban–rural divide, accredited to the absence of piped infrastructure, in concurrence with decreased population density. Consequently, due to high rates of reliance on private groundwater sources in rural areas, combined with the ubiquity of private domestic wastewater treatment systems (DWWTSs) and pastoral agriculture, a temperate maritime climate, and diverse bedrock and quaternary geology, Ireland is intrinsically vulnerable with respect to groundwater susceptibility to contamination.

However, much work remains to be done to fully understand the transport mechanisms, sources and spatio-temporal patterns of groundwater-associated pathogenic microorganisms, including verotoxigenic *Escherichia coli* (VTEC) and *Cryptosporidium*, which represent a significant disease burden in Ireland. Waterborne transmission of VTEC infection through untreated private water wells has been documented as a likely infection pathway in rural Ireland, with both cattle density (i.e. faeces and/or manure/slurry application) and on-site DWWTSs identified as likely sources of groundwater contamination. Quantifying the relative contribution of the myriad of potential environmental sources and pathways of VTEC and *Cryptosporidium* contamination and their temporal significance (and variation) in Ireland remains a priority research area. Compounding this, there are currently no adequately sensitive methods for the routine identification and culture of either VTEC or *Cryptosporidium* from environmental samples, and, therefore, studies that differentiate the exposure pathways and sources are of paramount importance for establishing environmental management solutions that safeguard public health.

The aim of the DESIGN – Detection of Environmental Sources of Infectious Disease in Groundwater Networks – project was to identify the state of the art and best practice in the effective detection and identification of pathogen sources and what methods can be used to understand the contamination pathway for waterborne infectious diseases. Subsequently, building on existing research from project partners on groundwater source susceptibility, environmental fate models were created to model (i) the relative risk of the contamination of private wells with VTEC and (ii) the seasonal distribution of likely VTEC contamination. Moreover, a preliminary sampling regime was developed to explicitly assess the presence of *Cryptosporidium* in Irish groundwater and to identify sources and transport mechanisms.

Overall, the DESIGN project provided greater insight into the prevalence and transport of VTEC and *Cryptosporidium* in groundwater. Through international reviews, the study identified global baselines of 10–20% and 0.6–1.3% for *Cryptosporidium* and VTEC occurrence in groundwater supplies, respectively. In contrast, the DESIGN project demonstrated a significantly higher burden of VTEC in (sampled) national groundwater resources, with 19.2% ($n = 10/52$) of tested samples positive for VTEC marker genes *stx1* and /or *stx2*. Moreover, a VTEC to generic *E. coli* ratio of 40% was calculated, which can be utilised by water service professionals, clinicians and hydrologists to estimate future risk. In this study, VTEC presence was significantly associated with decreasing well depth and increasing 30-day mean antecedent rainfall. Serogroup O104 was associated with increased sheep density within the identified electoral divisions, and detection of *stx1/stx2* and *eae* (virulence) genes was associated with increased DWWTS density. The findings suggest a high risk of VTEC in *E. coli*-contaminated groundwater sources in Ireland, with multiple clinically relevant serogroups often present in heavily contaminated sources. However, owing to the relatively small sample size, further work is required to support these findings.

Groundwater samples ($n = 40$) were also sampled for *Cryptosporidium*, with no oocysts detected during the first sampling phase in winter 2019. However, in spring 2022, 10% of samples ($n = 2/20$) were positive for *Cryptosporidium*, with two oocysts/50 L detected in one sample and and four oocysts/50 L detected in the other. Preliminary analysis demonstrated no

association with hydrogeological variables, albeit that both supplies were located in “extreme” or “karst” groundwater vulnerability areas. Overall, although tentative, the results indicate that VTEC poses a significantly greater risk to private well users in Ireland than *Cryptosporidium*.

The DESIGN project may help to inform and guide the development of spatio-temporal groundwater management policies, offering invaluable guidance for future planning and the remediation and mitigation of the microbiological contamination of Ireland’s private water wells. Due to the heterogeneous nature of both aquifers and pathogen sources, results from the DESIGN project allow spatio-temporal management strategies to be implemented.

1 Introduction

1.1 Project Context and Rationale

Globally, groundwater is an important source of potable water. Estimates indicate that groundwater accounts for ≈95% of available global freshwater reserves – highlighting its key importance as a current and future drinking water source (Bradford and Harvey, 2017). Approximately 2.2 billion users rely on groundwater on a daily basis to fulfil domestic water demands (Murphy *et al*., 2017; Cuthbert *et al*., 2019). The importance of groundwater as a drinking water resource is mirrored in available statistics from Ireland, with approximately 15% of the population (≈750,000 people) (*c*.2011) relying on private (unregulated and often untreated) wells as a drinking water source (CSO, 2012).

Traditionally considered a microbiologically "safe" source of drinking water, owing largely to the effective and natural protection provided by overlying soil layers, evidence suggests that domestic groundwater resources are vulnerable to contamination from surface pollutant sources (Bain *et al*., 2014; Murphy *et al*., 2017; Chique *et al*., 2020). In particular, an emerging concern among relevant stakeholders is the likelihood of drinking water contamination with (infectious) microorganisms (i.e. enteric pathogens) responsible for gastrointestinal disease in humans, including bacteria, parasites and viruses (Bradford and Harvey, 2017). Despite prevailing notions regarding their high microbiological integrity, groundwater supplies have been increasingly implicated in epidemiological outbreaks, with a range of groundwater contamination ingress mechanisms or pathways identified (Muniesa *et al*., 2006; Guzman-Herrador *et al*., 2015; Murphy *et al*., 2017; Andrade *et al*., 2018; Chique *et al*., 2020). Therefore, under particular circumstances, consumption of ground-sourced drinking water may actually represent a latent pathogen transmission route, eventually resulting in gastroenteric disease. For example, a recent review by Murphy *et al*. (2017) estimated an annual global burden of 35.2–59.4 million cases of gastrointestinal disease linked to domestic groundwater supplies.

The role of groundwater in the active dissemination of pathogenic organisms and ensuing human exposure is particularly relevant in Ireland. Here, the spatial distribution of (private) groundwater supplies shows a distinct pattern, displaying a strong urban–rural divide that results in very high rates of (often exclusive) reliance on groundwater in rural settings. This largely results from a lack of piped water infrastructure outside urban areas, coupled with the low population density characteristic of rural Ireland. The last decade has seen a growing body of evidence highlighting potential links between key regional risk factors prevalent in rural settings, both in Ireland and elsewhere, and the incidence of human gastrointestinal disease (Avery *et al*., 2008; Denno *et al*., 2009; Brehony *et al*., 2018). In Ireland, the interplay between (waterborne) infectious disease and rurality is primarily attributed to the prevalence of environmental contamination sources (human and animal) and high rates of reliance on groundwater (McKeown and Garvey, 2011; ÓhAiseadha *et al*., 2017; O'Dwyer *et al*., 2018).

Both persistent and transient microbiological groundwater pollution have been well substantiated in Ireland (Bacci and Chapman, 2011; Hynds *et al*., 2012; O'Dwyer *et al*., 2018). The role of groundwater as a vector/pathway for gastrointestinal disease particularly concerns (parasitic) *Cryptosporidium* species and verotoxin-producing, or verotoxigenic, *Escherichia coli* (VTEC) bacteria – two of the most important waterborne pathogens in Ireland. Both pathogens are transmitted via the faecal–oral route, with environmental sources typically including livestock faeces (particularly cattle) and derivatives (e.g. manure), and domestic wastewater (Cho *et al*., 2013; Ahmed *et al*., 2015; Toledo *et al*., 2017; O'Leary *et al*., 2020). A synopsis of each pathogen, including perceived global burden, contamination sources and associated potential for (waterborne) transmission through groundwater networks, is provided in sections 1.2 and 1.3.

Specifically, Ireland has consistently reported the highest (clinical) incidence of VTEC infection among all European Economic Area (EEA) countries, that is, all countries in the EU plus Iceland, Lichtenstein and Norway, with a national incidence of 826 cases in 2020 (HPSC, 2021). For VTEC infections, the role of private

groundwater wells as a key transmission vector/pathway has been emphasised and directly linked with the incidence of clinical cases (Garvey *et al*., 2016; ÓhAiseadha *et al*., 2017; ECDC, 2019).

As regards cryptosporidiosis, available data indicate that confirmed cases (that is, crude incidence rates (CIRs)) have remained relatively stable in the last decade (ranging from 11.0 to 13.2 cases per 100,000) (HPSC, 2019a). While CIR values have remained consistent, a total of 210 cryptosporidiosis outbreaks have been reported in Ireland since it became a notifiable disease in 2004 (HPSC, 2019a). In particular, the 2007 Galway outbreak (242 confirmed cases), which was directly linked to inadequate treatment of drinking water, served as a stark indicator of the potential (local) health burden of cryptosporidiosis. The outbreak had an estimated total cost of €19 million (Chyzheuskaya *et al*., 2017), which also highlights the (high) financial burden of epidemiological outbreaks deriving from the contamination of drinking water supplies. An EPA pilot study focusing on 16 (publicly managed) groundwater drinking supplies detected *Cryptosporidium* in 62.5% (10/16) and 44% (64/146) of supplies and samples analysed, respectively (Read *et al*., 2015). Notably, all *Cryptosporidium*-positive supplies/samples originated from agricultural catchments and aquifers classified as "influenced" by surface water. The application of molecular (genotyping) tools enabled both the identification of *Cryptosporidium* species and tentative "host" allocation, with animal sources including cattle, sheep and other mammals (e.g. deer) featuring as the most likely contamination sources. To a lesser degree, potential human sources were also identified. The results thus clearly demonstrate the potential for contamination of groundwater-fed drinking water supplies by both human and (particularly) animal sources in Ireland.

Notwithstanding the available evidence linking groundwater supplies, pathogen incidence and gastrointestinal disease, to date no comprehensive investigations have focused on the prevalence of these *Cryptosporidium* and VTEC pathogens in Ireland. The relative contribution of the myriad of potential environmental sources and pathways of VTEC/*Cryptosporidium* groundwater contamination and their (spatio-temporal) variability remain a priority research area in Ireland. This is particularly relevant in the context of private groundwater supplies, which are often untreated and unregulated by public health authorities in Ireland and elsewhere (Hexemer *et al*., 2008; Kreutswiser *et al*., 2011; Mooney *et al*., 2020). A recent Irish study reported that regular well-water testing is undertaken in only 40% of cases, with 65% of private supply users exhibiting a "very low" perception of risk relating to potential supply contamination events (Andrade *et al*., 2019). Generally, private groundwater supplies show higher microbial contamination rates than public supplies, with the latter being more likely to be adequately maintained and subject to water treatment (Hynds *et al*., 2014a; Wallender *et al*., 2014). Compounding the (inherent) higher susceptibility to contamination accredited to private supplies is the potential role of Irish climatic conditions and current climate change projections. Available evidence, from both Ireland and elsewhere, emphasises the influence of higher precipitation rates on active/efficient environmental (catchment-wide) mobilisation of enteric pathogens (Hofstra, 2011; Lal *et al*., 2019). Specifically, persistent and heavy (antecedent) rainfall and ensuing efficiency in landscape hydrological "coupling" has been linked to increased incidence of (i) *E. coli* in Irish private wells and (ii) waterborne VTEC outbreaks (O'Dwyer *et al*., 2016, 2018). Importantly, Irish landscape settings, typically characterised by (annual) mild temperatures, wet subsoil environments and relatively low solar (ultraviolet; UV) radiation, are likely to provide highly favourable conditions for both (non-host) VTEC and *Cryptosporidium* survival and eventual mobilisation into domestic groundwater environments (King and Monis, 2007; Brennan *et al*., 2010). Climate change projections directly relevant to Ireland indicate that the frequency and severity of extreme rainfall events and flooding may increase substantially over the next century (Nolan, 2015; Forzieri *et al*., 2017). Accordingly, microbiological contamination of groundwater resources and epidemiological outbreaks associated with groundwater-sourced drinking water are likely to become recurrent features.

Overall, the DESIGN – Detection of Environmental Sources of Infectious Disease in Groundwater Networks – project was developed in line with available (strong) evidence for prevalent and recurrent pathogenic contamination of Irish groundwater supplies. To summarise, specific factors linking domestic groundwater and pathogen incidence in Ireland include (i) high rates of reliance on

groundwater sources; (ii) high livestock (e.g. cattle, sheep) density; (iii) high prevalence of (on-site) domestic wastewater treatment systems (DWWTSs), i.e. septic tanks; (iv) a maritime temperate climate characterised by mild temperatures, high precipitation and wet environments; and (v) hydrogeological settings (i.e. varied soil permeability rates).
The project is structured around key research components that seek to identify and integrate all of the abovementioned risk factors while addressing the urgent need to quantify the prevalence of these two pathogens in private groundwater supplies.

1.2 *Cryptosporidium*

Cryptosporidium species are gastrointestinal (protozoan) parasites exhibiting a wide global distribution. Recognised as a significant agent of gastrointestinal illness (cryptosporidiosis) in both humans and livestock, symptoms include acute watery diarrhoea, vomiting, nausea and fever, leading to dehydration and, in severe cases, death (Thomson *et al*., 2016). Cryptosporidiosis is a particular threat to those with compromised immune systems, such as children (aged < 5 years), the elderly and the immunodeficient (Huang and White, 2006; King *et al*., 2019). Cryptosporidiosis also has a complex and poorly understood association with malnutrition and the potential for prolonged effects following (symptomatic and asymptomatic) infection in children through faltered (stunted) growth (Checkley *et al*., 2015; Korpe *et al*., 2016). Khalil *et al*. (2018) estimated that, in 2016, cryptosporidiosis was the fifth most prevalent cause of diarrhoea among children aged < 5 years, causing more than 48,000 deaths and 4.2 million disability-adjusted life-years globally. Cryptosporidiosis is a recurrent global public health threat, attributed to multiple large disease outbreaks in developed regions (e.g. in Milwaukee in 1993; MacKenzie *et al*., 1995); however, the infection typically exhibits a significantly higher prevalence in low- and middle-income countries, due to the interaction of key socio-economic factors (Bouzid *et al*., 2018). Specific parameters of relevance in lower-income settings often include poor hygiene/sanitation facilities and a high prevalence of environmental contamination sources (e.g. high population/livestock density) (Delahoy *et al*., 2018). Globally, one of the biggest challenges for efficiently managing the impact/burden of cryptosporidiosis continues to be the lack of effective treatment following infection. Currently available therapeutic options (e.g. antiparasitics) are considered suboptimum, particularly for vulnerable individuals (Checkley *et al*., 2015).

Approximately 40 *Cryptosporidium* species have now been identified, several of which are associated with human infection and gastrointestinal disease (Feng *et al*., 2018). Overall, *Cryptosporidium parvum* and *C. hominis* are accredited with the majority of global human infection (Xiao and Feng, 2017). Transmission of *C. parvum* has been traditionally associated with animal (i.e. zoonotic) contamination sources (e.g. cattle), while *C. hominis* is more frequently associated with human (i.e. anthroponotic) sources (e.g. domestic wastewater), although modes of species (and species subtype) transmission are known to show regional variation (Xiao, 2010; King *et al*., 2019; Nader *et al*., 2019). As a result, potential contamination sources, dissemination/infection pathways and the relevance of *Cryptosporidium* species may all show marked geographical variability.

As a protozoan, *Cryptosporidium* relies on “oocysts”, the readily infectious and environmentally robust free-living life stage of the parasite, present in large numbers in the faeces of infected individuals, as a mode of environmental (host-to-non-host) transmission (Fayer, 2004). Essentially, following intestinal tract colonisation, reproduction takes place, leading to eventual shedding (through faeces) and environmental (oocyst) dissemination. Oocysts are composed of a thick-walled protective layer (protein capsid), and are thus highly adapted to the conditions posed by gastric environments. They are generally considered highly resistant to both physico-chemical degradation and chemical disinfection, although in non-host environments they are known for being sensitive to (high) temperate extremes, prolonged solar (UV) insolation and desiccation (Tzipori and Ward, 2002; King and Monis, 2007). In particular, *Cryptosporidium* oocysts are notorious for their capacity to withstand chemical disinfection, which often predominates among water treatment types in drinking water supplies. Importantly, oocysts have the ability to remain virulent (infectious) for prolonged periods outside (often mammalian) hosts, and have been associated with a very low infectious dose. Ingestion of < 10 oocysts may result in infection, with human transmission typically occurring via the faecal–oral route (DuPont *et al*., 1995; Chappell *et al*.,

2006; Ryan *et al*., 2016). Waterborne transmission of *Cryptosporidium* oocysts has been reported via both surface water and groundwater (Rose, 1997), with *Cryptosporidium* consistently ranked as the leading global cause (>50%) of waterborne outbreaks attributed to protozoan parasites (Karanis *et al*., 2007; Baldursson and Karanis, 2011; Efstratiou *et al*., 2017a; Murphy *et al*., 2017). Surface water is generally considered significantly more susceptible to pathogen ingress than groundwater (Moreira and Bondelind, 2017). Conversely, as indicated above, groundwater resources are often traditionally, and probably erroneously, perceived as an inherently (microbiologically) "safe" source of water for domestic usage because of the natural attenuation processes afforded by overlying contiguous subsoil layers (Bain *et al*., 2014; Murphy *et al*., 2017). Indeed, both field studies and outbreak investigations, including preliminary data from Ireland, indicate that groundwater-fed domestic water supply systems are a significant transmission vector of *Cryptosporidium* (Jin and Flury, 2002; Hynds *et al*., 2013; Read *et al*., 2015; Efstratiou *et al*., 2017a; Stokdyk *et al*., 2019). Furthermore, recent advances in *Cryptosporidium* biology, including new insights into genetics and life stage cycles, have led to its taxonomic reclassification from coccidian to gregarine (Karanis and Aldeyarbi, 2011; Aldeyarbi and Karanis, 2016; Thomson *et al*., 2016). As a result, *Cryptosporidium* species are now acknowledged for their capability to reproduce outside a living host, with the potential for environmental reproduction and dissemination (Clode *et al*., 2015; Ryan *et al*., 2016). In particular, *Cryptosporidium* species have been shown, at least in controlled laboratory settings (i.e. *in vitro*), to benefit from the environments provided by biofilms (Koh *et al*., 2013, 2014; Luo *et al*., 2017). The latter are essentially a cohesive mass of different (animal and plant) microorganisms, recognised by their ability to provide ecological refugia (e.g. food/energy, shelter), commonly occurring in aquatic environments and which may be particularly prevalent in domestic water supply infrastructure and distribution networks (Chaves-Simões and Simões, 2013). Biofilms have been tentatively linked with oocyst retention and gradual (pulse-like) release into drinking water supplies (Howe *et al*., 2002; Wolyniak *et al*., 2010; Chaves-Simões and Simões, 2013; Kelly *et al*., 2014). Accordingly, under certain conditions, *Cryptosporidium* species may have the ability to aggregate and freely multiply in engineered water supplies, hypothetically posing a significant threat to groundwater consumers through persistent *Cryptosporidium* dissemination, gastrointestinal disease and epidemiological outbreaks (Ryan *et al*., 2016; Thomson *et al*., 2016). For Ireland, the potential for *Cryptosporidium* environmental (i.e. non-host) reproduction, specifically within water supply infrastructure, is of key importance considering the high rates of reliance on private groundwater supplies, which are generally unregulated and often lack any type of effective treatment.

There has been no attempt to estimate the global importance of *Cryptosporidium* as an infectious agent and the role that domestic groundwater sources play in its dissemination. Evidently, a robust understanding of pathogen prevalence is essential to guide future research and policy in environmental quality and public health (Bradford and Harvey, 2017). A review by Murphy *et al*. (2017) focusing on gastrointestinal disease linked to groundwater sources suggests that *Cryptosporidium* has been identified as the aetiological agent in seven outbreaks between 1948 and 2016. However, epidemiological studies (via primary cases and outbreaks) represent only a fraction of the burden of cryptosporidiosis, with most cases and epidemiological outbreaks remaining unreported and undiagnosed, even in high-income countries with established surveillance systems, and particularly with respect to sporadic/endemic infection and localised/household clusters (Scallan *et al*., 2011; Checkley *et al*., 2015). Clearly, because of the prevalence of contamination sources, the lax to non-existent regulation and the higher likelihood of human exposure, lower-income settings would be expected to (globally) account for more unreported and undiagnosed cases/outbreaks than high-income countries. Overall, it is plausible that groundwater-sourced drinking water is a more important contributor to the global burden of cryptosporidiosis than expected, particularly if available figures are underestimates (Checkley *et al*., 2015; Khalil *et al*., 2018). In short, the relative prevalence of *Cryptosporidium* in global groundwater supplies remains largely understudied, thus representing a significant knowledge gap. Considering the current lack of effective (clinical) treatment for cryptosporidiosis, a concise and preventive strategy aimed at mitigating contamination of drinking water sources is imperative.

1.3 Verotoxigenic *Escherichia coli*

E. coli predominates among the microorganisms that constitute the natural gastrointestinal tract flora characterising mammals, including humans. *E. coli* are typically harmless, with gastrointestinal presence posing no significant human health risks. However, pathogenic *E. coli* variants (i.e. pathotypes), characterised by specific (intestinal) colonisation mechanisms and the presence of key virulence/ pathogenicity genes, can be major agents of gastrointestinal illness in humans (Croxen *et al*., 2013). Currently, six *E. coli* pathotypes, collectively known as diarrhoeagenic *E. coli*, are recognised as clinically important. VTEC strains are characterised by the production of verocytotoxins (*vt1*, *vt2*) similar to AB5-type Shiga toxins, and include enterohaemorrhagic *E. coli* strains (Bergan *et al*., 2012). Following host ingestion and (intestinal) colonisation, verotoxins may form a type of intestinal lesion (known as attaching and effacing lesions) that manifests itself in the form of gastrointestinal disease (Croxen *et al*., 2013). VTEC strains are further classified into "serotypes", which are identified based on the composition and distinct combinations of O and H antigens. To date, over 400 VTEC serotypes have been identified, only a subset of which has been linked to clinical symptoms and cases (Smith *et al*., 2014). Globally, *E. coli* O157 is the serotype most commonly associated with human clinical cases and epidemiological outbreaks. However, additional serogroups (i.e. non-O157) are increasingly being reported as pathogens of emerging clinical importance as monitoring and detection technologies improve (Ferens and Hodve, 2011; Gould *et al*., 2013; Luna-Gierke *et al*., 2014; Baranzoni *et al*., 2016). In humans, VTEC infection typically comprises a wide range of symptoms, from mild uncomplicated infection (or asymptomatic display) in healthy adults to severe haemorrhagic diarrhoea and haemorrhagic colitis (HC) among vulnerable subpopulations (Newell and La Ragione, 2018). Potential clinical complications include haemolytic–uraemic syndrome (HUS), renal failure and thrombotic thrombocytopenic purpura, all of which can prove fatal in some cases (3–10%) (Rahal *et al*., 2015).

VTEC transmission is often zoonotic, occurring via the faecal–oral route, with cattle the most frequently reported animal reservoir (via faeces) and environmental contamination source, but also potentially including other domesticated animals (e.g. sheep, pigs) and wildlife (Farrokh *et al*., 2013; Persad and LeJeune, 2014; Ahmed *et al*., 2015; Penakalapati *et al*., 2018). VTEC organisms are characterised by a relatively small infectious (threshold) dose (ID_{50} < 100 cells), with human infection and epidemiological outbreaks in many developed regions typically associated with consumption of contaminated water or food (Croxen *et al*., 2013; Saxena *et al*., 2015). Accordingly, VTEC infections and ensuing outbreaks represent a major global public health concern (ECDC, 2019). However, similar to cryptosporidiosis, the global human health burden of VTEC remains largely unknown because of the lack of "comprehensive" confirmed infection data, which is attributed to limited and/or resource-constrained surveillance systems in developing regions as well as underdiagnosis among healthy populations (Croxen *et al*., 2013; Rivas *et al*., 2016; Delahoy *et al*., 2018; Newell and La Ragione, 2018). Available estimates, which are conservative and likely to be significant underestimates, place the global burden of VTEC infection at 2.8 million cases per annum, in concurrence with 3890 cases of HUS, 270 cases of renal disease and 230 deaths (Majowicz *et al*., 2014).

Since their recognition as an aetiological agent in waterborne outbreaks in the early 1990s (Dev *et al*., 1991; Swerdlow *et al*., 1992), VTEC strains, and particularly serogroup O157, have been implicated in both sporadic cases and outbreaks of waterborne gastrointestinal infection via consumption of contaminated drinking water sources (Muniesa *et al*., 2006; Luna-Gierke *et al*., 2014; Saxena *et al*., 2015; Garvey *et al*., 2016; ECDC, 2019). VTEC strains have been reported in groundwater supplies and linked with multiple groundwater-related outbreaks (Muniesa *et al*., 2006; Hynds *et al*., 2014a; Guzman-Herrador *et al*., 2015; Saxena *et al*., 2015; Moreira and Bondelind, 2017; Murphy *et al*., 2017). For example, the Walkerton (Ontario) multi-aetiological outbreak was positively associated with a contaminated municipal groundwater supply, causing 2300 acute clinical cases and seven deaths, with *E. coli* O157 identified as one of two pathogens responsible (Hrudey *et al*., 2003).

Beyond the limited information that may be inferred from outbreak notifications, no significant "global" effort to ascertain the extent of VTEC prevalence in domestic groundwater supplies and subsequent human exposure has been undertaken. Collecting

these data is fundamental to accurately determining the level of exposure and public health burden attributable to groundwater sources contaminated by VTEC and is directly applicable to the development of guidelines aimed at reducing contamination and human exposure (Murphy *et al*., 2017; Newell and La Ragione, 2018). In short, there is an urgent need to (i) identify the global occurrence of VTEC in domestic groundwater supplies and (ii) identify and categorise local risk factors associated with VTEC contamination.

1.4 Project Aims and Objectives

The overarching goal of the DESIGN project was to inform groundwater management in Ireland by gaining insights into pathogenic prevalence in private domestic groundwater supplies and relevant contamination risk factors. Part of the project was based on the (meta-) analysis of (international) peer-reviewed literature, seeking to derive contamination baselines for both *Cryptosporidium* and VTEC in domestic groundwater supplies on global and regional scales. Such data are essential for enhancing the accuracy and consequent efficacy of predictive (environmental fate) modelling and assessment of health risks associated with groundwater sources. In addition, by collating and analysing available literature, the project aimed to identify local risk factors (e.g. infrastructural, environmental, socio-economic) linked to the pathogenic contamination of domestic groundwater environments. The DESIGN project also incorporated an essential field-based component, aiming to assess the (spatio-temporal) prevalence of *Cryptosporidium* and VTEC in private wells in Ireland, thus facilitating the identification of (field-validated) contamination risk factors and the development of predictive models. The latter is key for the local development of bespoke groundwater protection policy in a changing climate.

The six specific project objectives are outlined as follows:

1. Perform a synthesis of global literature (meta-analysis) to establish (i) state-of-the-art detection methods for the identification of environmental sources and (groundwater) contamination pathways for *Cryptosporidium* and VTEC and (ii) contamination baselines for both pathogens in domestic groundwater supplies.
2. Characterise the VTEC contamination status of private domestic supplies in Ireland and develop a novel VTEC to generic *E. coli* pathogenicity ratio using EPA capture, amplify, extract (CapE) protocols.
3. Based on field-validated VTEC data, create environmental fate models to assess the likely human health risks of the VTEC contamination of private groundwater supplies in Ireland (as defined as part of Objective 2).
4. Perform a preliminary investigation into *Cryptosporidium* (oocyst) prevalence in private water supplies in Ireland.
5. Based on research findings from Objectives 1–4 provide evidence-based recommendations applicable to policymaking and pertaining to groundwater management and public health.
6. Disseminate and communicate the interdisciplinary findings with relevant national and international stakeholders and catalyse the development of further research strategies as well as monitoring and mitigation measures.

1.5 Report Structure

The current introductory chapter presents an elementary background and context justifying the project structure and direction. This initial chapter also includes a brief overview of *Cryptosporidium* and VTEC as pathogenic agents, emphasising their relevance and potential implications for groundwater users. Subsequently, Chapter 2 presents the findings of a comprehensive (scoping) literature review with sections relating specifically to *Cryptosporidium* (section 2.2) and VTEC (section 2.3). Chapter 3 summarises the insights obtained from (spatio-temporal) VTEC analysis in private wells located in Ireland. Based on (field-validated) VTEC to generic *E. coli* detection ratios, an (updated) quantitative microbial risk assessment (QMRA) for VTEC contamination among private groundwater wells in Ireland is presented. Moreover, seasonal environmental fate models are presented based on VTEC prevalence data and current climate change projections for Ireland. Chapter 4 outlines key findings and insights obtained from a preliminary

(spatio-temporal) *Cryptosporidium* field-sampling campaign focusing on (private) groundwater supplies, including risk factor analysis denoting key local contamination risk variables. To conclude, Chapter 5 collates key findings presented in the results chapters (Chapters 2–4), providing concluding comments and key recommendations to relevant stakeholders and suggestions for future research.

2 Scoping Literature Review

2.1 Introduction

The literature review component of the DESIGN project was originally envisaged as an integrative study of *Cryptosporidium* and VTEC prevalence in domestic groundwater environments. However, based on the relatively large number of articles identified during initial trial (mock) searches, particularly in the case of *Cryptosporidium*, efforts were channelled into producing two individual (stand-alone) reviews. The production of tailored reviews was aimed at improving/enhancing the quality of data identified specific to each pathogen and, ultimately, better informing groundwater management. Accordingly, despite obvious similarities in the methodological approaches employed, Chapter 2 includes a section relating specifically to *Cryptosporidium* and a section relating specifically to VTEC.

Overall, the "global" scope employed and emphasis on elucidating local contamination risk factors are particularly pertinent given the myriad of local/regional conditions potentially influencing *Cryptosporidium* and/or VTEC prevalence and survival and ultimately their prevalence in groundwater. Similarly, the approach implemented follows reported regional variability in clinical cases and outbreaks linked to groundwater use (Watterworth *et al*., 2006; Avery *et al*., 2008; Bain *et al*., 2014; Saxena *et al*., 2015; Efstratiou *et al*., 2017a; Newell and La Ragione, 2018; Delahoy *et al*., 2018). The potential for (wide) regional variability in groundwater pathogenic prevalence and infection is perhaps best exemplified by the high rates of notifications of VTEC infections attributed to groundwater consumption in Ireland relative to EEA countries (Garvey *et al*., 2016; ECDC, 2019). The geographical approach implemented also follows substantial geostatistical evidence linking (notified) VTEC infections, high levels of reliance on groundwater and high prevalence of prospective contamination sources (e.g. livestock) within specific rural settings, at least concerning high-income and low-latitude regions (Strachan *et al*., 2006; Denno *et al*., 2009; ÓhAiseadha *et al*., 2017; Brehony *et al*., 2018).

2.2 *Cryptosporidium*

2.2.1 Literature review protocol

Primary research question, database sources and literature identification

The overarching "scoping" review protocol (Figure 2.1) was adapted from previous studies with similar scopes (Sargeant *et al*., 2006; Graham and Polizzotto, 2013; Hynds *et al*., 2014a; Andrade *et al*., 2018). A primary research question was developed to guide the review protocol as follows:

> What is the global prevalence of *Cryptosporidium* species in private "domestic" groundwater supplies, what are the commonly reported contamination sources/pathways and which detection methods are implemented for identification?

Literature searches were conducted on 1 June 2019 and confined to Scopus (https://www.scopus.com/) and Web of Science (https://www.webofknowledge.com/) bibliographic databases. Key search terms were based on a modified version of the population–outcome–agent (POA) model (Hynds *et al*., 2014a), which essentially consists of "umbrella" categories employed for search term classification in literature reviews. Initial trial searches using a separate "outcome" search term category (e.g. cryptosporidiosis, contamination, pollution, outbreak, presence, incidence) were found to significantly restrict the number of articles identified ($n = 80$). Accordingly, a combination of search terms from "agent" and "outcome" categories were chosen to expand the search scope. Database searches employed Boolean operators and positional operators ("AND", "OR", "WITH", "SAME", "ADJ") to focus/direct literature searches.

Literature screening and study selection

Phase 1 (identification) resulted in 539 records being identified, with de-duplication (removal of

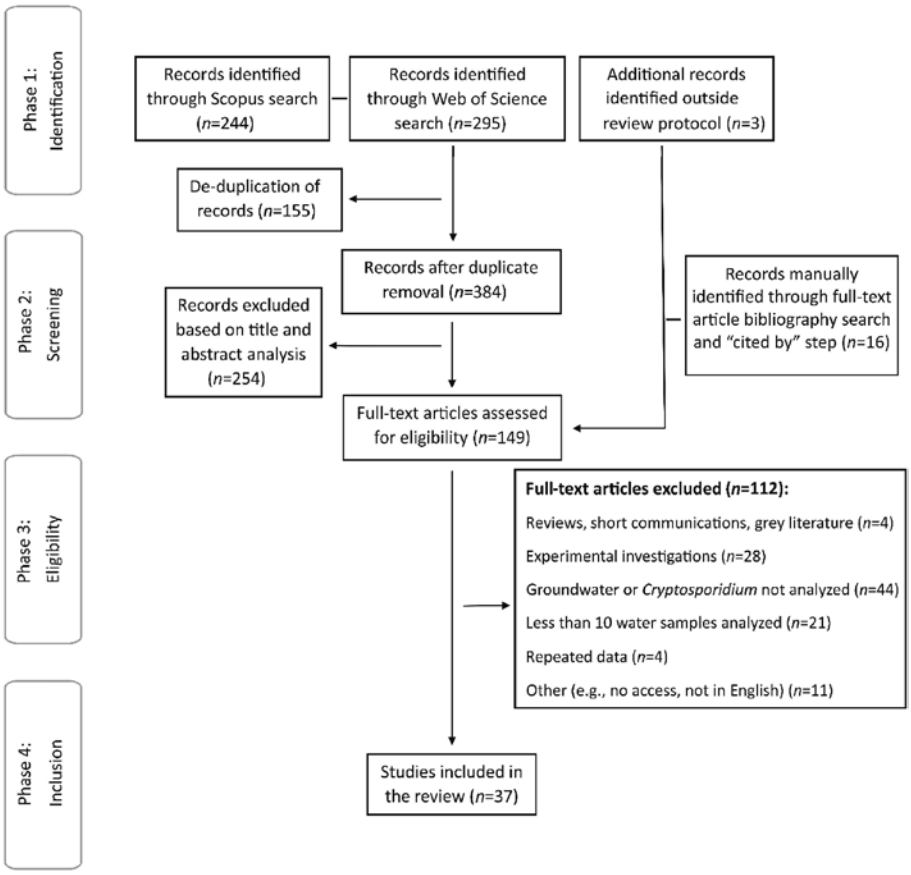


Figure 2.1. Protocol employed for the *Cryptosporidium* systematic literature review, including the key phases (identification, screening, eligibility) leading to final study selection (inclusion).

duplicate articles from the final database pool) reducing this number to 384 (Figure 2.1). Phases 2 and 3 (screening and eligibility) comprised an iterative record selection process guided by a set of pre-established "eligibility" criteria. These are specific requirements that each article must meet for inclusion in the final review dataset. First, screening of each article title and abstract was undertaken, leading to the exclusion of records deemed unsuitable as per the defined eligibility requirements. Subsequently, forward-selected articles were then subject to full-text analysis using the same eligibility approach (Phase 4). The literature review protocol exclusively focused on "original" (peer-reviewed) research articles. Conference proceedings, short communications and surveillance/academic reviews were therefore excluded. Based on established aims and objectives, the review protocol exclusively focused on investigations analysing "typical" infrastructure used to source domestic groundwater (e.g. boreholes, artesian wells (springs), drilled wells). Specifically, primary inclusion parameters included (i) original and full-text research articles; (ii) English-language articles; (iii) investigations based on groundwater sources intended for domestic consumption; (iv) analysis of *Cryptosporidium* (oocyst or genetic marker detection) in groundwater samples from relevant sources; and (v) investigations analysing > 10 individual water samples (Bain *et al*., 2014). The final parameter was adopted to exclude the results of small case studies that may exhibit bias in supply source selection, i.e. based on groundwater wells likely to be contaminated. Excluded records included (i) articles reviewing or analysing previously published data on *Cryptosporidium* groundwater occurrence (i.e. potential data replication), (ii) investigations focusing on non-domestic groundwater or (*in situ* and *ex situ*) experimental settings, including *Cryptosporidium* tracer/removal investigations (e.g. soil column transport, river bank filtration), (iii) studies analysing *Cryptosporidium* and failing to specify if detection occurred in relevant groundwater environments and (iv) articles that did not comprise extractable data. In the case of exclusion resulting from analysis of

previously published data, only original (earliest) published articles were considered for inclusion. There was no year of publication threshold employed for inclusion/exclusion, with uncertainty regarding article suitability (7/149; 4.7%) (Figure 2.1) addressed through discussion and reaching consensus among authors.

Data field extraction

Extracted data fields were organised into seven main categories, namely (i) bibliographic details; (ii) study setting (e.g. climatic zone, country income); (iii) study design (i.e. environmental, outbreak); (iv) groundwater supply (e.g. type, depth); (v) sampling design (e.g. "one-off" or "repeat" sampling); (vi) detection methods (e.g. microscopy, molecular detection); and (vii) contamination (e.g. incidence, reported ingress mechanism).

The final extraction dataset comprised 65 data fields. A recurrent feature of the literature screened was the lack of standardised reporting. In many instances, no extractable data were found to populate established data fields (e.g. hydrogeology, supply age), with variables thus categorised as "not reported". Based on the geographical distribution, a modified version of the World Health Organization (WHO, 2019) regional classification was used to categorise studies. These regions are South-East Asia, Africa, Europe, Greater Middle East, North America, Latin America and the Caribbean and Western Pacific. Similarly, each study was classified according to income levels (high, upper middle, lower middle, low) as defined by the United Nations (2017). Climate classifications followed the five main categories of the Köppen–Geiger classification system (Peel *et al*., 2007) and were based on specific geographical locations when described. If reported, climate allocation was based on a specific location or specific locations, with large-scale (e.g. regional, national) studies incorporating (multiple) climates assigned an "unknown" classification.

Settlement type classification was based on study site descriptions (rural, urban or mixed). Investigations classified as "not reported" generally involved sampling multiple unspecified locations in large-scale and geographically diverse (i.e. national, provincial) sampling campaigns. Groundwater supply sources were grouped into two main categories adapted from Bain *et al*. (2014), namely protected (boreholes, wells, tube wells, drilled wells, driven wells) and unimproved (artesian wells, dug wells) supply sources, reflecting their degree of design and construction. In several cases, classifications were tentative due to ambiguous structural descriptions. Groundwater supplies were also classified according to reported management approaches, including those under public regulation, individual household management (i.e. privately owned) or a combination of upkeep regimes. Excluding one investigation (with a sampling duration of 1 month), all repeated sampling designs (i.e. individual sources sampled on more than one occasion) involved a sampling duration of >6 months. Where possible, reported sampling periods were integrated with (averaged) monthly precipitation data from records extracted from the nearest (available) weather station. The World Meteorological Organization (WMO) global database (http://worldweather.wmo.int) was used to find representative weather stations (WMO, 2020), with each investigation allocated a category relative to (averaged) local precipitation statistics (i.e. high, low). As a result, precipitation classifications were relative to local monthly records. A range of investigations implemented varying volumes of source water for *Cryptosporidium* detection and were thus classified according to the minimum range reported. Several studies failed to report on the use of treatment systems (e.g. chlorination) and/or if groundwater samples were taken pre or post treatment. If treatment was reported, investigations had a tendency to focus on *Cryptosporidium* detection in groundwater prior to treatment (i.e. untreated). Only three investigations (8.1%) were based entirely on treated groundwater, with chlorination employed in each.

In all instances reported, *Cryptosporidium* oocyst counts were not adapted to study-specific oocyst recovery estimates (Ongerht, 2013, 2017). The later study by Ongerht (2017) refers to a methodological approach aimed at ascertaining the error likely to be associated with techniques employed in "concentrating" *Cryptosporidium* oocysts from environmental water samples. A limited number of investigations reported recovery estimates but were rather directed towards testing the efficiency of different concentration methods used. Groundwater contamination mechanisms were inferred from available study descriptions (if any), with four main groundwater contamination categories employed (Lee, 2005; Hynds *et al*., 2012; Andrade *et al*., 2018; Chique *et al*., 2020): (i) direct surface ingress, consisting of contaminants entering groundwater

supply units via surface structural components (well heads); (ii) groundwater recharge or subsoil layer filtration/migration of (surface-borne) contaminants and eventual groundwater deposition; (iii) direct underground migration, i.e. groundwater contamination originating from sources below soil surface (e.g. septic tanks); and (iv) inter-aquifer exchange, involving the contamination of groundwater (exclusively) through hydraulic interconnectivity. Similarly, contamination sources were grouped into two main categories: human (e.g. domestic wastewater) and animal. Likely contamination sources and ingress mechanisms were often tentative and ambiguously reported, with several investigations failing to identify specific sources/ pathways. Where reported, potential contamination sources were simplified into two main categories based on human and animal provenance regardless of ingress mechanism. The human category incorporates contamination from domestic/industrial wastewater infrastructure (e.g. septic tanks), solid waste and the land application of septage. The animal origin category comprises all sources associated with agricultural activity, including livestock faeces and manure/slurry spreading or storage.

2.2.2 Results

Included studies and their spatio-temporal distribution

A total of 37 relevant studies were identified during the review process and subject to data extraction and synthesis (Figure 2.1). Summary statistics extracted from included studies are provided in Tables 2.1–2.3. The geographical distribution of the studies identified, the number of studies per country and (aggregated) oocyst detection data are presented in Figure 2.2.

The literature included spans a period of 27 years (January 1992 to July 2019), with most investigations conducted between 2013 and 2019 (16/37; 43.2%) (Table 2.1). The majority of included studies derived

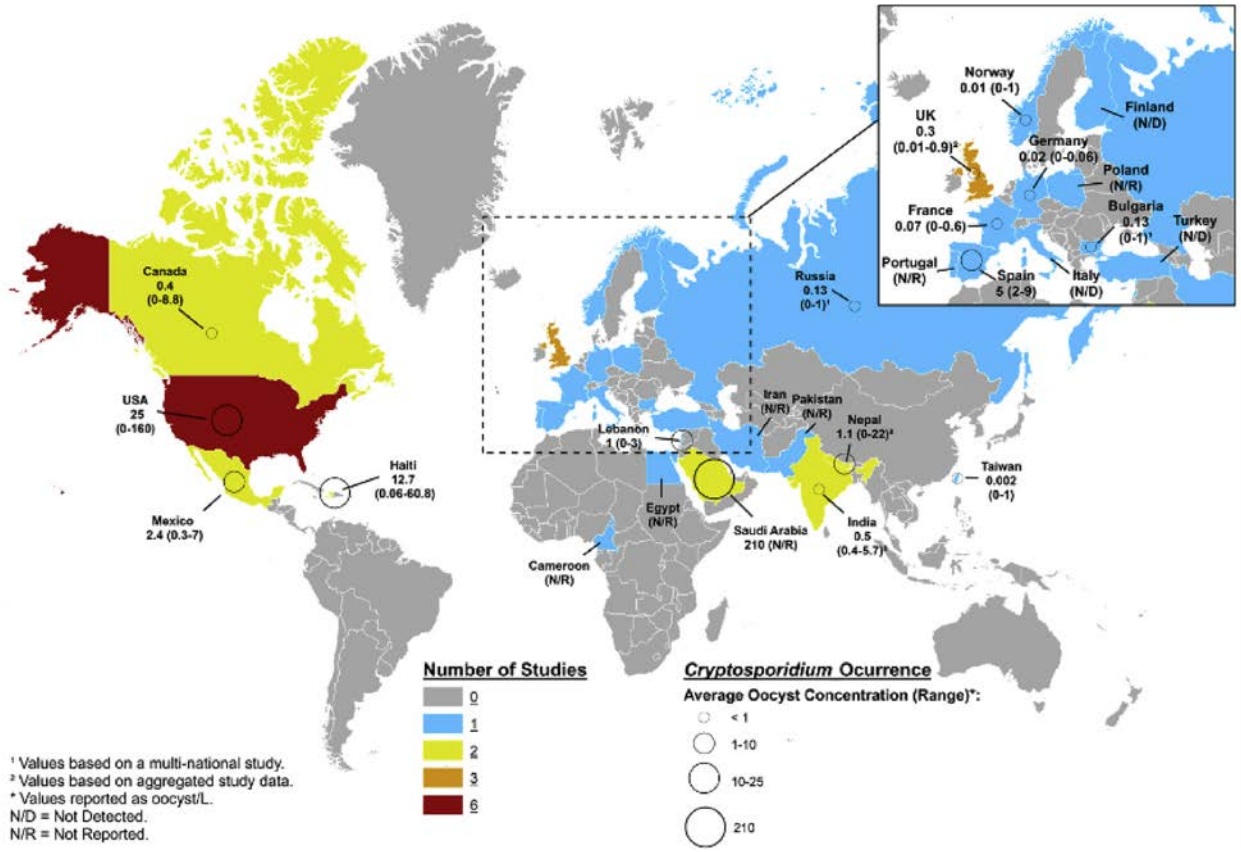


Figure 2.2. Map illustrating the global distribution of *Cryptosporidium* studies included for review, with *Cryptosporidium* oocyst concentrations per country given in parentheses. Average concentration (oocysts/L) and range values were aggregated at country level where applicable. N/D, countries with a single investigation not detecting *Cryptosporidium*; N/R, countries with a single investigation not reporting oocyst concentrations with positive *Cryptosporidium* detection.

Table 2.1. Summary of data on key characteristics extracted from included *Cryptosporidium* studies (*n* = 37)

Characteristic	Studies *n* (%)	*Cryptosporidium* study presence *n* (%)	Samples analysed *n* (%)
Publication year			
1992–2002	6 (16.2)	5 (83.3)	1083 (27)
2003–2012	15 (40.4)	11 (73.3)	920 (23)
2013–2019	16 (43.2)	15 (93.8)	2005 (50)
Study location			
Africa	1 (2.7)	1 (100)	28 (0.7)
South-East Asia	5 (13.5)	5 (100)	480 (12)
Europe	13 (35.1)	10 (76.9)	923 (23)
Latin America and the Caribbean	4 (10.8)	3 (75)	174 (4.3)
Greater Middle East	6 (16.2)	6 (100)	650 (16.2)
North America	8 (21.6)	6 (75)	1753 (43.7)
Country income level			
High	22 (59.5)	18 (81.8)	2749 (68.6)
Upper middle	6 (16.2)	5 (83.3)	334 (8.3)
Lower middle	5 (13.5)	5 (100)	738 (18.4)
Low	4 (10.8)	3 (75)	187 (4.7)
Setting			
Rural	23 (62.2)	20 (87)	2447 (61.1)
Urban	7 (18.9)	5 (71.4)	461 (11.5)
Mixed	5 (13.5)	4 (80)	641 (16)
N/R	2 (5.4)	2 (100)	459 (11.4)
Climate			
Arid	5 (13.5)	5 (100)	419 (10.5)
Tropical	5 (13.5)	4 (80)	584 (14.6)
Temperate	17 (45.9)	15 (88.2)	1090 (27.2)
Cold	7 (18.9)	4 (57.1)	1270 (31.7)
Unknown	3 (8.1)	3 (100)	645 (16.1)
Study design			
Environmental	33 (89.2)	29 (87.9)	3750 (93.6)
Outbreak	4 (10.8)	3 (75)	258 (6.4)
Hydrogeological description			
Yes	8 (21.6)	6 (75)	1509 (37.6)
No	29 (78.4)	25 (86.2)	2499 (62.4)
Supply type			
Public	9 (24.3)	7 (77.8)	643 (16)
Private	9 (24.3)	8 (88.9)	668 (16.7)
Mixed	9 (24.3)	7 (77.8)	1700 (42.4)
N/R	10 (27)	9 (90)	997 (24.9)
Source type			
Protected	29 (78.3)	26 (89.7)	3503 (87.4)
Unimproved	2 (5.4)	1 (50)	377 (9.4)
Protected/unimproved	3 (8.1)	2 (66.7)	60 (1.5)
N/R	3 (8.1)	2 (66.7)	68 (1.7)

Table 2.1. Continued

Characteristic	Studies *n* (%)	*Cryptosporidium* study presence *n* (%)	Samples analysed *n* (%)
Sampling strategy			
One-off	23 (62.2)	20 (87)	1633 (40.7)
Repeated	14 (37.8)	11 (78.6)	2375 (59.3)
Sampling season			
Dry	6 (16.3)	5 (83.3)	414 (10.3)
Wet	8 (21.6)	7 (87.5)	323 (8.1)
Both	14 (37.8)	11 (78.6)	2046 (51)
N/R	9 (24.3)	8 (88.8)	1225 (30.6)
Sampling period (months)			
≤1	5 (13.5)	4 (80)	302 (7.5)
2–12	14 (35.1)	11 (78.6)	685 (17.1)
13–24	6 (16.2)	4 (66.7)	695 (17.3)
≥25	4 (10.8)	4 (100)	1115 (27.8)
N/R	8 (21.6)	8 (100)	1211 (30.2)
Sources analysed (*n*)			
≤10	12 (32.4)	12 (100)	775 (19.3)
11–50	9 (24.3)	6 (66.7)	207 (5.2)
51–150	5 (13.5)	4 (80)	1562 (39)
≥151	5 (13.5)	5 (100)	1234 (30.8)
N/R	6 (16.2)	4 (66.7)	230 (5.7)
Sample treatment			
Chlorination	2 (5.4)	1 (50)	293 (7.3)
Varied	1 (2.7)	1 (100)	168 (4.2)
None[a]	19 (51.4)	16 (84.2)	2104 (52.5)
N/R	15 (40.5)	13 (86.7)	1443 (36)
Samples analysed (*n*)			
9–20	15 (40.5)	12 (80)	223 (5.6)
21–100	11 (29.7)	8 (80)	587 (14.6)
101–250	7 (18.9)	6 (85.7)	1349 (33.7)
≥251	4 (10.8)	4 (100)	1849 (46.1)
Sample volume (L)			
≤10	7 (18.9)	6 (85.7)	442 (11)
11–100	11 (29.7)	10 (90.9)	957 (23.9)
101–500	11 (29.7)	7 (63.6)	1201 (30)
≥501	3 (8.1)	3 (100)	885 (22.1)
N/R	5 (13.5)	5 (100)	523 (13)
Concentration method			
Membrane filtration	14 (37.8)	11 (78.6)	646 (16.1)
Cartridge filtration	13 (35.1)	10 (76.9)	1536 (38.3)
Ultra-filtration	5 (13.5)	5 (100)	1473 (36.8)
Centrifugation	3 (8.1)	3 (100)	90 (2.2)
Other	1 (2.7)	1 (100)	18 (0.4)
N/R	1 (2.7)	1 (100)	245 (6.1)

Table 2.1. Continued

Characteristic	Studies *n* (%)	*Cryptosporidium* study presence *n* (%)	Samples analysed *n* (%)
Detection method			
Light microscopy	5 (13.8)	5 (100)	571 (14.2)
IFA[b]	19 (51.3)	15 (78.9)	1907 (47.6)
PCR[c]	10 (27)	8 (80)	1489 (37.2)
IFA	11 (29.8)	9 (81.8)	1416 (35.3)
IMS-IFA	8 (21.6)	6 (75)	491 (12.2)
IFA + PCR	2 (5.4)	2 (100)	984 (24.6)
IMS-IFA + PCR	6 (16.2)	5 (83.3)	375 (9.4)
Other	5 (13.6)	4 (80)	171 (4.3)
Recovery efficiency[d]			
Yes[e]	3 (8.3)	–	102 (2.5)
No	33 (91.7)	–	3906 (97.5)
Oocyst viability[d]			
Yes	4 (11.1)	–	245 (6.1)
No	32 (88.9)	–	3763 (93.9)

Extracted data are based on *n* = 4008 groundwater samples and *n* = 1938 groundwater supplies.

[a]Includes studies analysing raw groundwater from a supply source with treatment undertaken.

[b]Calculation based on investigations implementing solely IFA as a detection method.

[c]Calculation based on all investigations implementing PCR as a component of the detection methodology.

[d]Excluding studies (*n* = 1) based entirely on PCR detection methods.

[e]Partial estimates with no evidence of sample-specific oocyst recovery efficiency.

IFA, immunofluorescence assay; IMS, immunomagnetic separation; N/R, not reported; PCR, polymerase chain reaction.

from Europe (13/37; 35.1%) and North America (8/37; 21.6%), with almost half the total number of groundwater samples associated with North American studies (1753/4008; 43.7%). By country, the USA (6/37; 16.2%) and the UK (3/37; 8.1%) accounted for the largest numbers of studies (Figure 2.2). Conversely, Africa was associated with only one study published during the reviewed time period (1/37; 2.7%) (Nsoh *et al.*, 2016). This geographical distribution is also reflected across income categories, with most studies (22/37; 59.5%) and samples (2749/4008; 68.6%) deriving from categorically high-income regions.

The majority of investigations followed an environmental "occurrence" or "prevalence" design (33/37; 89.2%), with only four studies being prompted by outbreaks of gastrointestinal illness (10.8%). All outbreak-related investigations originated from high-income countries (USA = 3; UK = 1). Most studies were based in distinctively rural settings (23/37; 62.2%), followed by urban (7/37; 18.9%) and "mixed" (i.e. peri-urban or following an urban–rural sampling gradient) (5/37; 13.5%) environments. Urban settings were concentrated in low- (*n* = 3), lower-middle- (*n* = 1) and upper-middle- (*n* = 3) income countries, with no urban investigations in high-income categories. Overall, 64.8% (24/37) of included studies were based in temperate (17/37; 45.9%) or cold (7/37; 18.9%) climates, equating to 58.9% (2360/4008) of samples analysed. Information relating to local (hydro-)geology settings was largely absent from reviewed literature. Overall, 78.4% (29/37) of investigations failed to report relevant hydrogeological data (e.g. bedrock, aquifer, sub(soil) type). Public, private and mixed supply types were equally represented in the included studies (nine each/37; 24.3%). Most investigations focused on groundwater sources with a presumed level of infrastructural protection (29/37; 78.3%), with only 5/37 (13.5%) studies (explicitly) incorporating supplies that were likely to be unimproved, consisting of artesian wells and dug wells. The latter primarily derived from low- and lower-middle-income countries, with a single study from a high-income region (Pitkänen *et al.*, 2015).

Sampling design and detection methods

The majority of investigations were based on "one-off" (i.e. prevalence) source sampling designs (23/37; 62.2%) (Table 2.1); however, most studies (irrespective of study design) incorporated both "dry" and "wet" periods (14; 40.5%), with average sampling campaigns lasting >4 months. A substantial proportion of investigations may be categorised as limited in scope, with several focusing on <10 sources (12/37; 32.4%), analysing 10–20 groundwater samples (15/37; 40.5%) and restricted to a sampling campaign of ≤1 year (19/37; 48.6%) (Table 2.1; Figure 2.3).

There was considerable variation in the volume of water sampled, with studies focusing on 11–100 L and 101–500 L (both 11/37; 29.7%) being the most common. Investigations analysing large volumes of water (>500 L) were rare (3/37; 8.1%) (Table 2.1). Microscopy and (polymerase chain reaction (PCR)-based) molecular procedures were the most commonly encountered detection methods. Immunofluorescence assay (IFA) was employed in 30/37 (81.1%) and 19/37 (51.3%) studies as an analytical component and stand-alone method, respectively. PCR was generally incorporated as a component of the methodology based on previous oocyst validation via microscopy. A total of 10/37 (27%) investigations implemented PCR techniques, but only one study (Hawash *et al*., 2015) was based entirely on PCR detection. Specifically, nested-PCR assays were the most common PCR technique employed (5/10; 50%), with most PCR-based investigations targeting multiple *Cryptosporidium* (species) genotypes. All molecular-based investigations targeted the two most commonly reported *Cryptosporidium* species, namely *C. parvum* and *C. hominis*. The investigation by Hawash *et al*. (2015) was the only one to employ PCR restricted to genetic markers at the genus level. Other detection methods focused on optical procedures, including electrochemiluminescence (ECL) (Lee *et al*., 2001) and fluorescence *in situ* hybridisation (FISH) (Khoury *et al*., 2016). A single study employed flow cytometry in conjunction with IFA (Khaldi *et al*., 2011). In the context of geographical/income distribution, application of IFA and PCR was concentrated in high-income regions, with light microscopy featuring as a detection method in only Africa and the Greater Middle East (Figures 2.4 and 2.5).

Cryptosporidium *incidence and reported contamination sources/pathways*

Cryptosporidium oocysts and/or genetic markers were positively identified in 31/37 (83.7%) of the reviewed studies (Table 2.1). Overall, pooled data, i.e. collated figures based on full/equivalent sample/source contamination reporting, indicate *Cryptosporidium* detection rates of 352/1797 (19.6%) and 407/3070 (13.3%) among the groundwater supply sources and samples analysed, respectively. Pooled values among study characteristics tended to range between 10% and 20%, with several categories characterised by relatively analogous values (e.g. urban settings) (Table 2.2). Several investigations reported positive

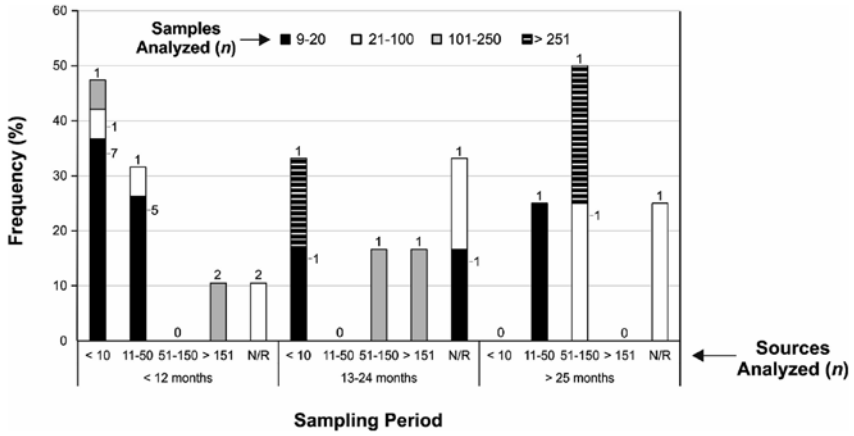


Figure 2.3. Frequency of *Cryptosporidium* studies according to the number of supply sources and groundwater samples analysed within different sampling periods. Investigations not reporting sampling period length (*n*=8) were excluded from the calculations presented. The number of investigations in each category is provided at the top or right-hand side of the stacked frequency bars. N/R, not reported.

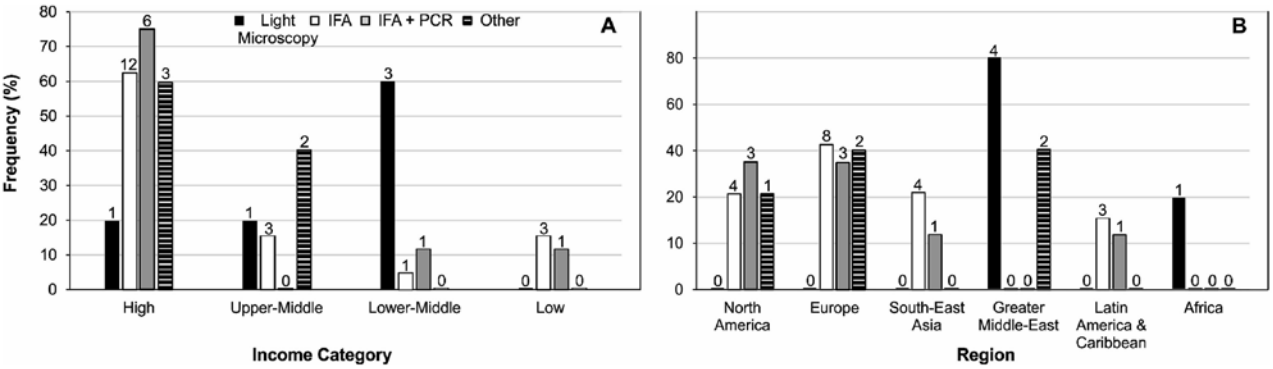


Figure 2.4. (A) Frequency of use of *Cryptosporidium* detection methods per income category. (B) Frequency of use of detection methods employed per geographical region. The "other" category comprises investigations employing ECL, FISH and flow cytometry, solely PCR-based investigations, and investigations using a combination of microscopy, IFA and PCR. The number of investigations in each category is provided at the top of the frequency bars.

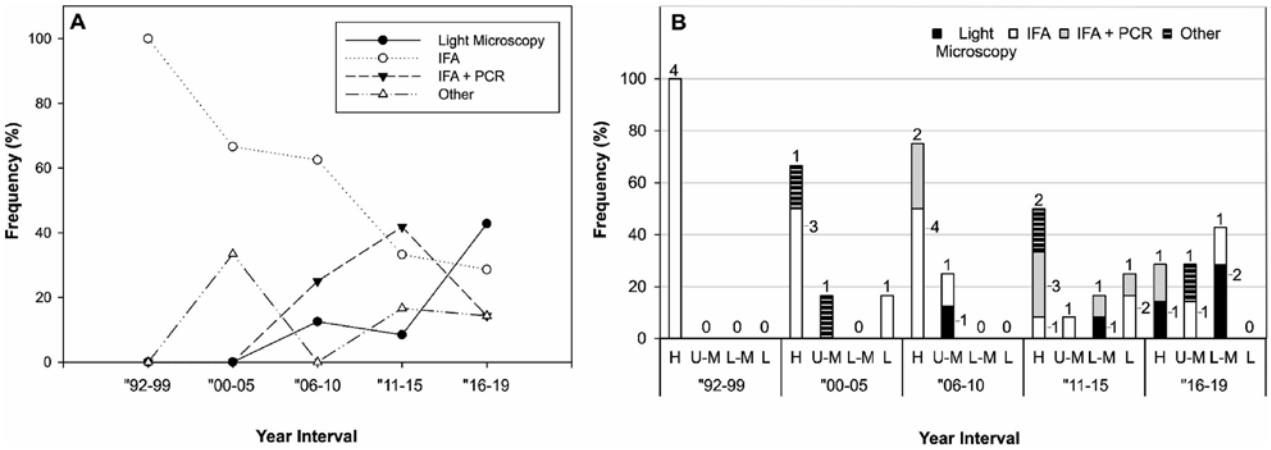


Figure 2.5. (A) Timeline of *Cryptosporidium* detection methods employed following year of publication intervals. (B) Frequency of use of detection methods according to income categories within different year intervals. H, high income; U-M, upper middle income; L-M, lower middle income; L, low income. The number of investigations in each category is provided at the top or right-hand side of the stacked frequency bars.

Cryptosporidium detection but failed to provide data on the number of contaminated supply sources ($n = 4$) and environmental samples ($n = 7$). There was a higher *Cryptosporidium* detection rate among studies conducted during 2003–2012 and 2013–2019 (Table 2.2), which hints at improved detection methodology. *Cryptosporidium* was ubiquitous across income, regional and climatic categories; however, higher sample/supply contamination rates were detected in upper-middle-income countries (20.8% and 31.3%). Geographically, estimated detection rates among samples were highest in the Greater Middle East (133/640; 20.8%). Europe accounted for the highest proportion of contaminated supply sources (66/248; 26.6%), followed by the Greater Middle East (123/488; 25.2%) and North America (107/476; 22.5%) (Table 2.2). Geographical patterns were partially reflected in climate trends, with cold and temperate regions accounting for most contaminated sources – 37.6% (102/271) and 31.6% (137/433), respectively. In the context of local environments, urban settings exhibited higher detection rates among samples and supply sources than were exhibited in rural and peri-urban areas, particularly in terms of samples (106/461; 23%). Private supplies had a higher sample/source contamination rate (16.6%; 16%) than public supplies (9.2%; 11.8%).

Table 2.2. Synthesis of pooled data from studies of *Cryptosporidium* groundwater samples (*n* = 3070) and sources (*n* = 1797) reporting positive *Cryptosporidium* contamination by selected study characteristic

Characteristic	Sample/source detection studies *n*	Positive samples/total samples *n* (%)	Positive sources/total sources *n* (%)
Publication year			
1992–2002	4/5	18/456 (3.9)	30/309 (9.7)
2003–2012	12/12	129/634 (20.3)	138/628 (22)
2013–2019	14/9	260/1980 (13.1)	184/860 (21.4)
Study location			
Africa	**1**/0	9/28 (32.1)	N/R
South-East Asia	**5**/4	54/480 (11.3)	46/443 (10.4)
Europe	11/10	68/716 (9.5)	66/248 (26.6)
Latin America and the Caribbean	3/3	27/159 (17)	10/142 (7)
Greater Middle East	5/3	133/640 (20.8)	123/488 (25.2)
North America	5/6	116/1047 (11.1)	107/476 (22.5)
Country income level			
High	18/26	130/1205 (10.8)	174/709 (24.5)
Upper middle	**6**/4	225/1083 (20.8)	87/278 (31.3)
Lower middle	3/3	34/1222 (2.8)	81/660 (12.3)
Low	3/3	18/92 (19.6)	10/150 (6.7)
Setting			
Rural	20/16	290/2319 (12.5)	228/1070 (21.3)
Urban	**7**/6	106/461 (23)	96/427 (22.5)
Mixed	3/2	11/290 (3.8)	15/166 (9)
N/R	0/**2**	N/R	13/134 (9.7)
Climate			
Arid	4/2	64/409 (15.6)	38/246 (15.4)
Tropical	**5**/4	62/584 (10.6)	53/556 (9.5)
Temperate	14/14	144/868 (16.6)	137/433 (31.6)
Cold	6/4	134/1191 (11.3)	102/271 (37.6)
Unknown	1/2	3/18 (16.7)	22/291 (7.6)
Supply type			
Public	8/7	58/633 (9.2)	19/161 (11.8)
Private	7/7	67/403 (16.6)	65/406 (16)
Mixed	8/7	248/1685 (14.7)	220/846 (26)
N/R	7/5	34/331 (10.3)	48/385 (12.5)
Source type			
Protected	24/21	361/2619 (13.8)	291/1358 (21.4)
Unimproved	**2**/**2**	37/377 (9.8)	37/377 (9.8)
Protected/unimproved	**3**/2	9/60 (15)	1/23 (4.3)
N/R	1/1	0/14 (0)	23/39 (59)
Sampling strategy			
One-off	20/16	245/1505 (16.3)	238/1200 (19.8)
Repeated	10/10	162/1565 (10.4)	114/597 (19.1)

Table 2.2. Continued

Characteristic	Sample/source detection studies *n*	Positive samples/total samples *n* (%)	Positive sources/total sources *n* (%)
Sampling season			
Dry	5/4	56/404 (13.9)	49/308 (15.9)
Wet	**8**/6	47/323 (14.6)	33/280 (11.8)
Both	12/9	228/2071 (11)	131/605 (21.7)
N/R	5/6	99/480 (20.6)	139/604 (23)
Sampling period (months)			
≤1	**5**/**5**	47/302 (15.6)	45/294 (15.3)
2–12	11/9	69/492 (15)	41/312 (13.1)
13–24	**6**/4	52/695 (7.5)	25/346 (7.2)
≥25	**4**/2	140/1115 (12.6)	102/241 (42.3)
N/R	4/6	99/466 (21.2)	139/604 (23)
Sources analysed (*n*)			
≤10	9/9	70/582 (12)	26/45 (57.8)
11–50	8/8	20/168 (11.9)	38/189 (20.1)
51–150	4/4	129/1192 (10.8)	112/504 (22.2)
≥151	4/**5**	164/898 (18.3)	176/1059 (16.6)
N/R	**6**/0	24/230 (10.4)	N/R
Samples analysed (*n*)			
9–20	13/11	61/198 (30.8)	24/117 (20.5)
21–100	9/4	63/469 (13.4)	55/196 (28.1)
101–250	6/**7**	168/1181 (14.2)	168/1042 (16.1)
≥251	2/**4**	115/1222 (9.4)	105/442 (23.8)

The study number column provides the number of investigations reporting extractable data in terms of samples (left) and supply sources (right) analysed, and corresponding contamination rates per categories, with values in bold indicating full (equivalent) reporting in the dataset. N/R, not reported.

Assessing the potential interplay between sampling design, detection and seasonality, extracted data indicated higher contamination rates among studies with a sampling campaign lasting >25 months (102/241; 42.3%) (Table 2.2). However, perhaps unexpectedly, a larger number of samples and supply sources analysed did not necessarily correlate with higher *Cryptosporidium* detection rates, as evidenced by high detection rates among supply sources in studies analysing <10 sources (26/45; 57.8%). Similarly, investigations analysing 10–20 groundwater samples reported the highest contamination rates (61/198; 30.8%). Studies undertaken during wet periods (24/115; 20.9%) and those incorporating both wet and dry seasons (154/813; 18.9%) accounted for the highest proportion of contaminated samples and sources, respectively. *Cryptosporidium* sample contamination rates were higher among (temporally restricted) "one-off" studies (16.3%) than among (more inclusive) "repeat" sampling studies (10.4%) (Table 2.2).

Several "*Cryptosporidium*-positive" studies failed to report (oocyst) concentration values (13/30; 43.3%). At a national level, where reported, the highest average oocyst counts were recorded in Saudi Arabia (210 oocysts/L) followed by the USA (25 oocysts/L) and Haiti (12.7 oocysts/L) (Figure 2.2). Specifically, Lee *et al*. (2001) reported the highest counts in an individual study (160 oocysts/L), which was linked to a critical failure in a municipal sewage lift station, the ensuing discharge of raw sewage to surface waters and the eventual groundwater source contamination via subsurface pathways. In total, 13/31 (41.9%) investigations reported likely *Cryptosporidium* ingress mechanisms, with groundwater recharge being the most commonly hypothesised pathway (12/13; 92.3%) (Table 2.3).

Table 2.3. Summary of selected characteristics within the "contamination" data extraction category based on studies with positive detection of *Cryptosporidium* in groundwater (*n* = 31)

Characteristic	Studies reporting/total number of studies (%)	Positive samples/ total samples *n* (%)	Positive sources/ total sources *n* (%)
Contamination ingress mechanisms[a]			
Groundwater recharge	12/13 (92.3)	192/1556 (12.3)	127/499 (25.4)
Direct surface ingress	3/13 (23)	7/31 (22.6)	9/27 (33.3)
Direct underground migration	3/13 (23)	4/151 (2.6)	24/47 (51)
Inter-aquifer exchange	1/13 (7.7)	N/R	N/R
N/R	18/31 (58.1)[b]	215/1348 (15.9)	191/1159 (16.5)
Contamination source[a]			
Animal origin	15/21 (71.4)	221/1807 (12.2)	198/802 (24.7)
Human origin	12/21 (57.1)	141/536 (26.3)	92/544 (16.9)
N/R	10/31 (32.2)[b]	74/730 (10.3)	62/542 (11.4)
Reported animal origin[a]			
Cattle	8/15 (53.3)	159/1445 (11)	115/427 (26.9)
Other livestock	3/15 (20)	11/98 (11.2)	32/48 (66.7)
Wild animals	1/15 (6.7)	N/R	23/39 (59)
***Cryptosporidium* spp. identified[a]**			
C. parvum	7/10 (70)	–	–
C. hominis	3/10 (30)	–	–
C. andersoni	2/10 (20)	–	–
FIO co-occurrence[a]			
Coliforms[c]	7/9 (77.7)	–	–
E. coli	7/10 (70)	–	–
Enterococcus spp.	2/4 (50)	–	–
Pathogen co-occurrence[a]			
Giardia spp.	13/26 (50)	–	–
Other	5/9 (55.5)	–	–

The study number column is based on the number of investigations analysing or reporting relevant data (e.g. source/ pathway attribution, PCR application and species, FIO/pathogen co-occurrence). Pooled synthesis data among selected characteristics are also provided.

[a]Multiple selection was available for data fields in this category, with percentages not amounting to 100%.

[b]Calculation based on the number of studies with positive detection of *Cryptosporidium* in groundwater (*n* = 31).

[c]Includes studies reporting total coliforms, faecal coliforms and thermotolerant coliforms.

FIO, faecal indicator organism; N/R, not reported.

Specific contamination sources were reported in 21/31 (67.7%) studies. Among these, sources of animal origin (15/21; 17.4%), and specifically of cattle origin (8/15; 53.3%), were reported as being the predominant local sources of *Cryptosporidium* contamination. Three studies tentatively linked on-site DWWTSs with *Cryptosporidium* occurrence, with one also highlighting the potential role of land-applied septage (O'Reilly *et al*., 2007). Furthermore, latrines were tentatively associated with *Cryptosporidium* presence in two investigations (Balderrama-Carmona *et al*., 2015; Odagiri *et al*., 2016). *C. parvum* was the most frequently encountered species identified through molecular procedures (74/84; 88.1%) (Figure 2.6). In contrast to other species, *C. parvum* was also widespread across geographical and income categories, albeit the scope of molecular detection was overall rather limited and highly concentrated in high-income regions (Figures 2.5 and 2.6).

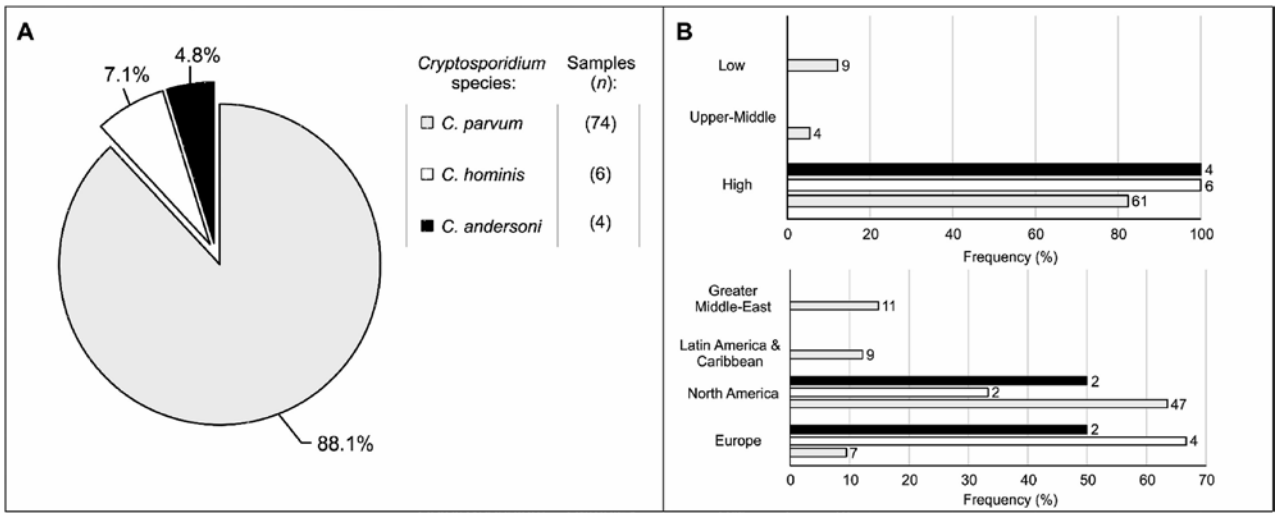


Figure 2.6. (A) Proportion of *Cryptosporidium* species detected via molecular methods and total number of positive samples per species. (B) Frequency (%) of *Cryptosporidium* species identified per income category (top) and region (bottom). Values are based on the total number of reported groundwater samples with positive *Cryptosporidium* species detection (*n* = 84). The total number of reported *Cryptosporidium* species in each category is provided at the end of the frequency bars.

2.2.3 Discussion

Global Cryptosporidium *prevalence and baseline contamination rates*

This scoping review provides unprecedented insights into the global prevalence of *Cryptosporidium* in domestic groundwater sources. To the authors' knowledge, this is the first global comprehensive scoping review of the presence of *Cryptosporidium* species in domestic groundwater sources. Accounting for the failure to publish "negative" results, a common feature characterising peer-reviewed literature, a pooled detection rate among supply sources of 19.6% suggests that *Cryptosporidium* species are frequently present in groundwater sources, representing a latent transmission vector and major health concern. As expected, the assumption that domestic groundwater supply systems are largely safe from *Cryptosporidium* contamination can therefore be challenged. Notably, estimated supply contamination rates (19.6%) are higher than those reported in the large-scale, albeit regionally restricted, field investigations by Hancock *et al*. (1998) (8%) and Moulton-Hancock *et al*. (2000) (7.6%).

Comparable values (14.7%) for protozoan supply source contamination have been reported in a review of North American groundwater systems by Hynds *et al*. (2014a). However, the current review integrates a much larger data pool, and is therefore likely to have generated more realistic detection rates for samples and supply sources. Therefore, based on pooled values (Table 2.2), a plausible baseline *Cryptosporidium* prevalence estimate of 10–20% among global groundwater supplies is proposed. In addition, based on the calculation of (often) analogous figures, it is also argued that values may provide category-specific insights (e.g. into protected, rural/urban and private sources), with prospective applications in water quality management and human health risk assessment. Importantly, a higher mean detection rate among samples was reported from "one-off" sampling designs (16.4%) than that calculated for studies focusing on "repeat" sampling (10.4%). Similarly, investigations analysing a limited number of samples (10–20; 30.8%) or supplies (< 10; 57.8%) exhibited higher detection rates. The unexpected figures presumably result from the "proof of concept" adopted in the reviewed investigations, i.e. studies designed to show *Cryptosporidium* "incidence" as opposed to actual (spatio-temporal) "prevalence". These selection biases have been increasingly reported as a feature in the scientific literature associated with microbiological contamination of groundwater systems (Hynds *et al*., 2014a; Andrade *et al*., 2018, 2020). Accordingly, it is argued that detection rates for samples and sources deriving from more inclusive "temporal" investigations (10.4% and

19.1%, respectively), which are probably subject to less bias (i.e. source/study area selection), provide a more accurate reflection of the *Cryptosporidium* prevalence baseline. A key consideration is that most of the data reviewed derive from environmental "prevalence" investigations rather than being prompted by epidemiological outbreaks (Table 2.2), highlighting regular *Cryptosporidium* occurrence among domestic groundwater sources. Furthermore, the lack of effective treatment reported within the included studies serves to highlight the overreliance of both users and authorities on the presumed integrity of groundwater supplies, with clear overarching health implications for groundwater consumers.

Geographically, extracted data show clear regional inequity among the included studies (Tables 2.1 and 2.2; Figures 2.2 and 2.4), with the focus on high-income regions representing a persistent issue and cause for concern in terms of the worldwide surveillance of cryptosporidiosis and other infectious diseases (Checkley *et al*., 2015; Efstratiou *et al*., 2017a; Murphy *et al*., 2017). Globally, cryptosporidiosis is significantly more prevalent in low-income countries (Bouzid *et al*., 2018; King *et al*., 2019), with concomitant levels of environmental incidence expected. However, the geographical relevance of groundwater as a transmission vector is yet to be ascertained. Here, collated data partially reflect expectations, with low detection rates among samples encountered in high-income countries (Table 2.2). Conversely, lower rates of source/sample contamination were observed in low- and lower-middle-income countries. However, it must be noted that the limited number of studies available in these categories reporting contamination data ($n = 3$) is highly restrictive and precludes major conclusions from being drawn. Moreover, there are limitations associated with the allocation of income categories, accounting for potentially significant country-specific variability. For example, Khoury *et al*. (2016) reported data from a refugee camp in an upper-middle-income country with local conditions realistically closer to a low-income region. Geographical gaps in the dataset are best represented by Africa, which, with 80% of its population (720 million people) relying on groundwater infrastructure (Macdonald *et al*., 2009) and a high burden of cryptosporidiosis (Limaheluw *et al*., 2019), features in a single investigation and has limited surveillance data (Table 2.1; Figure 2.2). However, data gaps are not exclusive to low-income regions. Much of the Pacific, Asia and Latin America are devoid of data (Table 2.1; Figure 2.2). At a country level, New Zealand, reported to have the highest incidence of waterborne outbreaks caused by parasitic protozoa between 2004 and 2016 (Baldursson and Karanis, 2011; Efstratiou *et al*., 2017a), lacks any reported data on *Cryptosporidium* groundwater incidence. Overall, global contamination estimates presented are constrained, and additional data are necessary to accurately discern a baseline for *Cryptosporidium* incidence and prevalence within groundwater systems at the regional or national level.

Detection methods, Cryptosporidium *occurrence and contamination sources*

The predominance of visually aided (IFA or light microscopy) oocyst identification, in concurrence with a paucity of oocyst recovery estimates (Table 2.1), represents a significant limitation in terms of risk factor identification and stakeholder communication (Efstratiou *et al*., 2017b). All extracted oocyst data are technically "raw" data in the absence of recovery efficiency measurements, and are thus more appropriately defined as "occurrence" rather than "concentration" data. While costly and labour-intensive, recovery estimates are recommended for accurate *Cryptosporidium* quantification, as detection methods typically recover a fraction of oocysts present, with recovery efficiency shown to vary both temporally and spatially (Ongerth, 2013, 2017). Accordingly, presented estimates may underrepresent the actual occurrence and concentration of oocysts in groundwater supplies. The importance of determining viability (i.e. infectivity) as a secondary objective in *Cryptosporidium* monitoring (Efstratiou *et al*., 2017b) is clearly observed in the dataset, with few investigations ($n = 4$) providing these data, thus representing a significant knowledge gap.

The prevalence of using IFA as a detection method (Table 2.1; Figure 2.5) largely derives from the prominence of standardised procedures for *Cryptosporidium* detection in environmental waters by the US Environmental Protection Agency (USEPA). In total, 15/37 (40.5%) of included studies followed (wholly or partially) the USEPA information collection rule procedure, successor methods 1622 and 1623, or international equivalents. Methodological practicalities

associated with these protocols that enable dual detection of *Cryptosporidium* oocysts and *Giardia* cysts resulted in high co-occurrence rates of the two protozoans (Table 2.3). From being exclusively based on IFA, detection methods have gradually diversified, with increasingly frequent use of both PCR and light microscopy (Figure 2.5). Comparison of the evolution of *Cryptosporidium* detection methods in domestic groundwater and surface waters (cf. Efstratiou *et al*., 2017b) indicates that PCR tools were used in groundwater monitoring less frequently and later (in the 2000s rather than the 1990s) than light microscopy. An opposite trend in the application of light microscopy is apparent, with it becoming prevalent in recent (post-2010) groundwater-based studies. The latter is partially attributed to increased numbers of groundwater investigations based in low-income countries (Figure 2.5). While light microscopy provides quantitative (oocyst) data at a reduced cost, it is limited by lower sensitivity and specificity than other methods, while requiring a high level of technical expertise for analysis and interpretation (Checkley *et al*., 2015; Adeyemo *et al*., 2018). Light microscopy has the potential to compromise the accuracy of oocyst occurrence data subject to user proficiency and laboratory standards. Methodological constraints can compromise the already limited data available from low-income countries.

IFA is a robust method for quantification of oocyst presence, characterised by high affinity and specificity, demonstrated to have a strong correlation with PCR results (Castro-Hermida *et al*., 2015 Stokdyk *et al*., 2019). However, similar to light microscopy, IFA approaches do not allow for species identification. Molecular characterisation of *Cryptosporidium* species and genotypes is essential to pinpoint contamination sources and potential host infectivity. Conversely, PCR-based tools are often characterised by relatively high sensitivity (Nichols, 2006; Efstratiou *et al*., 2017b), potentially explaining the increased *Cryptosporidium* detection rates encountered following increasing application of these methods in domestic groundwater studies (Table 2.2; Figure 2.5). Ideally, concomitant identification of species analysed from (catchment-based) stool samples and supply sources will provide a more robust framework for representative contamination source ascription. In this context, only 2/10 (20%) of the molecular-based studies employed a PCR-based approach to analyse both stool and supply sources, thus highlighting the limited extent of "robust" evidence linking contaminant sources with contaminated groundwater supplies and the urgent need for environmental prevalence investigations to employ integrative detection methods and sampling designs. Attempts to identify regional patterns in the distribution of *Cryptosporidium* species have been precluded by the high level of aggregation of species data in high-income regions (Figure 2.6). Again, this trend highlights the dearth of applicable data from low-income countries, with only a subset of regional investigations employing molecular detection methods (Figure 2.4). Only two studies employed PCR tools for *Cryptosporidium* subtype genotyping (2/10; 20%), which is concerning, as several clinically significant *C. parvum* subtypes (e.g. IIa, IIc) are considered largely anthroponotic (King *et al*., 2019), potentially resulting in incorrect source attribution. Both Stokdyk *et al*. (2019) and Lobo *et al*. (2009) reported subtype IIa as being prominent among identified *C. parvum* genotypes. As a result, the presence of subtype IIa precludes the identification of human or animal groundwater contamination sources, because of its capacity to employ both livestock and humans as hosts. Accordingly, studies that attribute contamination sources (Table 2.3), even if supported by molecular characterisation, should be interpreted with caution.

Hydrogeology and contamination pathways

As shown in Table 2.3, groundwater recharge, i.e. gradual gravitational percolation through pores in soil profiles and/or bedrock fractures, was the most frequently reported ingress mechanism (12/13; 92.3%). While pathway reporting is tentative, largely resulting from limitations associated with the methodological approaches employed (e.g. lack of microbial source tracking), this finding is unexpected considering general patterns attributed to microbial transport in the subsurface environment. For example, previous work has shown that source- and locally specific infrastructure and short-term (i.e. 5–10 day) antecedent rainfall are significantly predictive of bacterial presence, thus indicating localised preferential subsurface transport and direct source ingress as predominant contamination mechanisms (Hynds *et al*., 2012, 2014b; O'Dwyer *et al*., 2018). Importantly, in contrast to other pathogenic microorganisms (e.g. bacteria, viruses), *Cryptosporidium* oocysts are characterised by a

larger size (3–6 µm); therefore, mobilisation through soil profiles into aquifers (i.e. recharge), despite oocysts remaining viable/infectious, is considered less likely to occur (Robertson and Edberg, 1997). Notwithstanding this, experimental investigations have demonstrated the ability of oocysts to migrate through soil columns, although a substantial degree of soil retention (i.e. attenuation) has been observed (Mawdsley *et al*., 1996; Darnault *et al*., 2004; Boyer *et al*., 2008; Mohanram *et al*., 2010; Zopp *et al*., 2016). Despite retention, oocyst robustness and prolonged viability in conjunction with rainfall as an external stimuli and particular soil conditions (e.g. high soil–water saturation) may allow for subsequent oocyst release following soil attenuation. Coupled with the low infectivity dose (< 10 oocysts) characterising certain *Cryptosporidium* species, this soil "flushing" effect can represent another pathway for groundwater supply contamination. The potential importance of groundwater recharge is perhaps best demonstrated by the results presented by Stokdyk *et al*. (2019), with rates of *Cryptosporidium* contamination among groundwater wells classified as under the direct influence of surface water (highly vulnerable) being equivalent to the rates of *Cryptosporidium* contamination among groundwater wells without direct surface water contact. Hydrogeological properties can be highly influential in the prevalence of pathogens in groundwater, particularly within karst bedrock characterised by porous/permeable material and dynamic flow, and overlain by thin (or absent) soil horizons (Pronk *et al*., 2009; Zopp *et al*., 2016). Indeed, several studies have stressed the role of vulnerable karst bedrock environments in the transport of *Cryptosporidium* to groundwater supplies (e.g. Willocks *et al*., 1998; O'Reilly *et al*., 2006; Khaldy *et al*., 2011; Damiani *et al*., 2013). The current review found that the majority of identified studies that sampled wells located in karstic landscapes (6/7) reported positive *Cryptosporidium* detection. However, reporting of hydrogeological characteristics was largely absent from the literature (29/37; 78.4%), which contrasts with the presumed importance of recharge as a contamination pathway and denotes a critical lack of understanding of *Cryptosporidium* fate and ingress into groundwater supplies. Overall, only two investigations sampling groundwater sources across multiple hydrogeological settings described corresponding bedrock/aquifer properties. Pitkänen *et al*. (2015) found no *Cryptosporidium* contamination in sampled wells, while Stokdyk *et al*. (2019) reported *Cryptosporidium* presence across a range of geological settings with varying degrees of soil/geological vulnerability. Stokdyk *et al*. (2019) reported their results as being unexpected and potentially attributable to variables (e.g. precipitation, faulty infrastructure) beyond the scope of their study. Furthermore, Khaldi *et al*. (2011) underlined the potential for oocyst retention in karst conduits and subsequent remobilisation following intensive water pumping (extraction) in one groundwater supply. This was the only investigation exploring the potential links between groundwater supply exploitation and concentration of *Cryptosporidium* in groundwater.

Groundwater supply infrastructure

The geographically skewed distribution encountered (Figure 2.2) is attributed to the predominance of "protected" over "unimproved" source types in the dataset (Table 2.1), with the latter often more widespread in low-income regions (Bain *et al*., 2014). Here, adequate comparisons of contamination susceptibility among (tentative) supply types are rather precluded by the small number of unimproved sources analysed. From the limited number of investigations analysing both types of supply sources, Haramoto *et al*. (2011) and Shrestha *et al*. (2015) reported higher *Cryptosporidium* contamination in unimproved (and shallower) sources than in those described as possessing a higher degree of protection. Notwithstanding this, supply sources classified as "protected" exhibited high levels of *Cryptosporidium* prevalence in the dataset (21.4%), which is likely to represent a significant public health concern of key relevance to both groundwater consumers and regulatory bodies. Several of the investigations reviewed reported *Cryptosporidium* contamination of groundwater sources characterised by "adequate" levels of protection, stressing potential links between faulty infrastructure and subsoil percolation (e.g. Willocks *et al*., 1998; Kay *et al*., 2007; Gaut *et al*., 2008; Stokdyk *et al*., 2019). Specifically, CCTV surveillance was used to identify casing/liner cracking and soil water ingress into otherwise "structurally sound" sources in two studies (Willocks *et al*., 1998; Gaut *et al*., 2008). In addition, studies potentially linked the presence of *Cryptosporidium* in protected sources with general well deterioration (Khoury *et al*., 2016) and insufficient or absent well

head protection (Kay *et al*., 2007; Nsoh *et al*., 2016). Interestingly, Daniels *et al*. (2015) described higher contamination rates in deep wells (15%) than in shallow wells (5%), indicating that shallower structural depth is not necessarily positively associated with contamination. Overall, while some insights into the role of key structural components and supply characteristics in contamination can be obtained in some cases, the majority of investigations either failed to record relevant information on supply structure or chose to (partially or completely) omit this information from reporting. As a result, a key point from the current review is the urgent need for *Cryptosporidium* prevalence investigations, to explicitly record and report relevant characteristics of source structure and hydrogeological settings. In the context of supply management, pooled detection rates for sources and samples associated with private (16.6%; 16%) and public (9.2%; 11.8%) supplies (Table 2.2) are likely to result from differing levels of regulation and surveillance. In contrast to private supplies, which are largely unregulated, public sources are more likely to be maintained by local or regional regulatory authorities (Hexemer *et al*., 2008; Hynds *et al*., 2014a). Achieving analogous detection rates for samples and supply sources from private supplies may strengthen the efficacy of proposed baseline prevalence figures.

Influence of climate and seasonality

Cryptosporidiosis has been shown to exhibit distinct seasonal peaks across latitudes, due to (i) agricultural life cycles, (ii) human waste production cycles and spatial profiles and (iii) local climate patterns (e.g. action of rainfall for oocyst release from faecal material) (Sterk *et al*., 2016; Lal *et al*., 2019). Additional factors, often highly dependent on the season, such as human use of recreational waters and host susceptibility to infection, may represent concomitant infection drivers; however, precipitation and environmental prevalence of human/agricultural waste are generally regarded as key drivers of infection (Sorvillo *et al*., 1998; Mathieu *et al*., 2004; Lal *et al*., 2012, 2019). Our findings indicate that the elevated detection rates among samples/sources stem from studies that incorporated “wet” periods into sampling designs (Table 2.2). While it should be noted that the reported wet/dry classifications cannot account for inter-annual precipitation variability, the association with wet phases is significant considering the effects of increased overland flow, subsurface infiltration and recharge, and oocyst (re-)mobilisation as potential contamination pathways (Table 2.3). For certain regions, climate change projections have forecast periods of more intense rainfall, which would most likely result in higher rates of recharge and/or overland flow, depending on the season and previous subsoil conditions. This is particularly applicable to systems characterised by dynamic flows (e.g. karst) and shallow water tables (MacDonald *et al*., 2009; Green *et al*., 2011; Bradford and Harvey, 2017). Importantly, experimental investigations have shown a significant increase in *Cryptosporidium* oocyst release from soil-applied manure following high-intensity rainfall (Boyer *et al*., 2008; Forslund *et al*., 2011). For example, in surface waters, *Cryptosporidium* is reportedly more likely to be detected and occur at higher (oocyst) concentrations during and after extreme weather events (Young *et al*., 2015), highlighting the effects of increased land–water hydrological connectivity on oocyst mobilisation. Evidently, climate change is a multifaceted phenomenon affecting multiple processes associated with oocyst release, survival and transport (e.g. run-off, dispersion, dilution), potentially affecting *Cryptosporidium* species differentially, with marked regional variation (Robertson and Gjerde, 2007; Sterk *et al*., 2016).

Three key and intrinsically linked environmental variables, namely desiccation, temperature and solar (UV) radiation, are among the most critical factors affecting *Cryptosporidium* (oocyst) environmental survival and viability (King and Monis, 2007). In the collated dataset, the influence of temperature on oocyst survival may be reflected in the higher detection rates among sources associated with cold (37.6%) and temperate (31.6%) regions; however, it must be noted that data available from arid and tropical climates are severely limited (Tables 2.1 and 2.2). Oocysts are notoriously robust, and their survival often increases at temperatures below 15°C in water and soil, and they have been shown to survive in temperatures as low as –22°C and to remain viable in soils for prolonged periods, of a year or more (Fayer *et al*., 1998; Jenkins *et al*., 2002; Davies *et al*., 2004). Conversely, exposure to higher temperatures (>25°C) and concomitant desiccation are associated with increased oocyst degradation (Robertson *et al*., 1992; Olson *et al*., 1999; King *et al*., 2005), which could be relevant in terms of deactivation prior to (subsoil) mobilisation and

groundwater supply ingress. A higher *Cryptosporidium* prevalence in temperate and cold climates (Table 2.2) also serves to point out the potential nexus between larger livestock numbers and shifting rainfall patterns (Lake *et al*., 2007; Lal *et al*., 2019).

2.2.4 Key insights and recommendations

Prevalence figures (10–20%) derived from the investigations reviewed provide an unprecedented baseline for *Cryptosporidium* contamination of domestic groundwater sources globally, including specific figures derived from private supplies. Of key relevance is the probability of bias associated with "proof of concept" perceived from several investigations. Accordingly, detection rates among samples (10.4%) and supply sources (19.1%) from "temporal" investigations are likely to be most representative of *Cryptosporidium* prevalence baselines. The reportedly frequent occurrence of *Cryptosporidium* in domestic groundwater supply sources is evidently a topical health concern of direct relevance to public health authorities and groundwater consumers alike. Comparable detection rates in urban and rural settings indicate that groundwater sources are vulnerable to *Cryptosporidium* contamination regardless of (expected) land use and population density, although this may be largely subject to local income and associated settings. Estimated baselines are highly applicable in the formulation of preventive risk-based approaches in groundwater quality management, including QMRA and hydrodynamic (catchment) modelling. However, a key finding is the current uncertainty regarding *Cryptosporidium* (oocyst) transport and ingress into domestic groundwater supply sources. This information is essential for developing (contamination) mitigation strategies and this knowledge gap denotes a lack of cohesion among hydrogeological, epidemiological and public health subdisciplines.

The following specific recommendations are made based on their potential to guide and integrate groundwater and public health research towards an interdisciplinary approach:

1. Ensure explicit and homogeneous reporting in investigations, including of infrastructural, hydrogeological and environmental data.
2. Implement "temporal" and unbiased large-scale investigations incorporating different settings (e.g. hydrogeological, urban/rural).
3. Employ high-sensitivity and high-specificity molecular methods in studies, effectively linking (land–groundwater) species/subtype data, contamination sources/pathways and groundwater supplies.

2.3 Verotoxigenic *Escherichia Coli*

2.3.1 Literature review protocol

Primary research question, database sources and literature identification

The following research question was established at the outset of developing the VTEC review protocol:

> What is the global prevalence of verotoxigenic *Escherichia coli* (VTEC) in "domestic" groundwater supply sources, which methods are employed in its detection and what local risk factors are associated with VTEC contamination?

All database literature searches (Scopus, Web of Science) were conducted on 1 July 2019, 1 month after the *Cryptosporidium* literature review. Once again, trial searches found significant restrictions in the volume of retrieved database records (≈40). For this reason, a modified version of the POA model, excluding the "outcome" category and associated search terms, was adopted.

Literature screening and study selection

The previously described primary phases were employed for record identification (Phase 1), screening (Phase 2) and eligibility assessment (Phase 3) (Figure 2.7). A total of 444 records were retrieved from bibliographic database searches, with de-duplication decreasing this number to 302. Additional (relevant) articles not identified through the protocol developed were also captured using (i) a "snowball" approach and (ii) the "cite by" tool provided by Google Scholar (Figure 2.7).

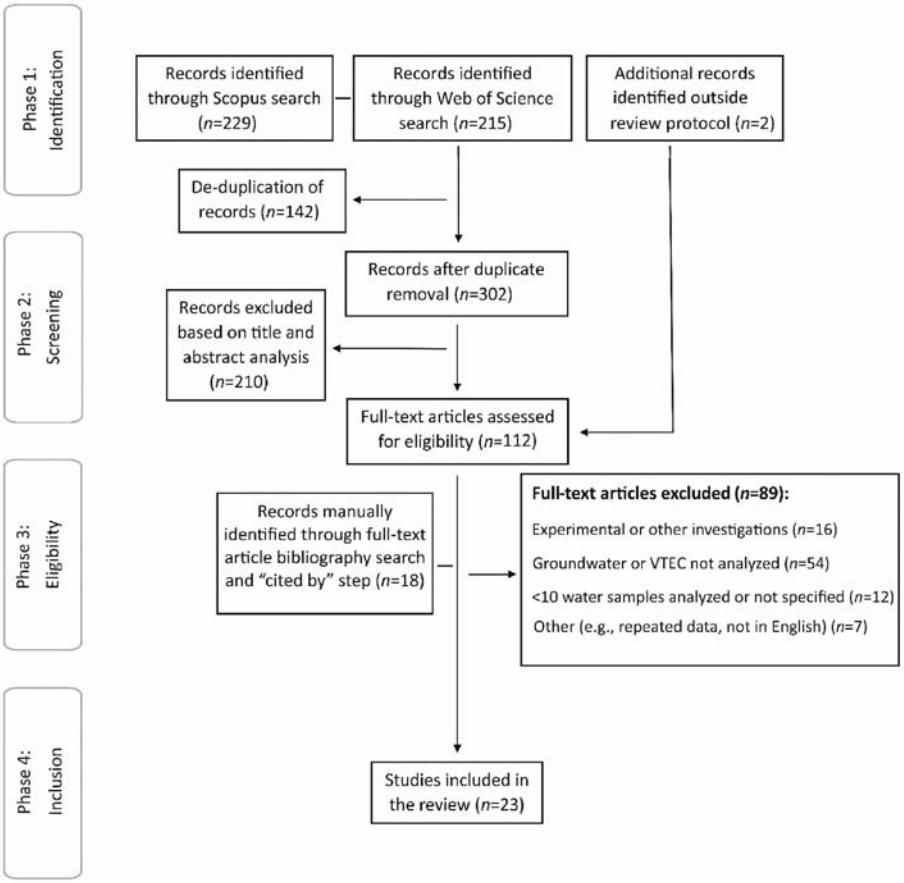


Figure 2.7. Protocol employed for the VTEC systematic literature review, including four distinct review phases (identification, screening, eligibility, inclusion).

Data field extraction

The WHO (2019) regional classification system was again used to assign studies to a corresponding geographical region. The regions included in this review were Asia, sub-Saharan Africa, Europe, North America, North Africa and the Middle East, Latin America and the Caribbean, and the Pacific. As with the *Cryptosporidium* review, mean income level and climate followed classification guidelines provided by the United Nations (2017) and Peel *et al*. (2007) (Köppen–Geiger system), respectively. The WMO (2020) weather station global database was again used to integrate each investigation and (averaged) local precipitation statistics (i.e. high, low). In addition, to gain insights into the (potential) influence of seasonal livestock management (e.g. summer grazing) on VTEC prevalence, investigations from higher latitudes (i.e. "temperate" and "cold" climates) were classified following "summer" sample collection descriptions in their experimental designs. Investigations based in "tropical" climates, i.e. lacking strong seasonality patterns, were omitted from "summer" classifications, and reported as "not available". Again, local settlement patterns were classified as "rural", "urban" or "mixed" (i.e. peri-urban). However, given the focus of investigations on specific agricultural settings, the rural category was subdivided according to the reported (primary) agricultural focus: (i) fresh produce (e.g. fruits, vegetables, grains) and (ii) livestock (dairy) production. Generally, groundwater supply type and/or accompanying infrastructural descriptions were largely ambiguous. Accordingly, once again, an attempt was made to (tentatively) classify groundwater sources into "protected" and "unimproved" (Bain *et al*., 2014).

Each investigation was classified according to the reported sampling point, namely (i) direct groundwater "source" (e.g. well tap) or (ii) point of "use" (e.g. household tap). The VTEC detection method employed was either amplification of verocytotoxin gene markers (*vt1*, *vt2*) or culture-based (presumptive) *E. coli* O157 detection, or a combination of both, i.e. presumptive VTEC detection followed by molecular confirmation. All cases of molecular

detection relied on the application of PCR assays. The PCR classifications employed were based on minimum information for publication of quantitative real-time PCR experiments guidelines (Bustin *et al*., 2009). Investigations seeking to establish links between (potential) environmental VTEC reservoirs and groundwater contamination using strain-typing tools were also incorporated into the data extraction protocol. The two techniques employed were pulsed-field gel electrophoresis (PFGE) and multiple-locus variable-number tandem-repeat analysis (MLVA) molecular typing. Groundwater contamination mechanisms and sources followed the same definitions and classification as established in section 2.2.1 (Lee, 2005; Hynds *et al*., 2012).

2.3.2 Results

Included studies, geography, local environments and inconsistent reporting

The VTEC review protocol identified 23 studies that complied with all inclusion criteria (Figure 2.7). A lack of consistent reporting and/or ambiguous reporting was observed among all established data extraction categories, and was particularly prevalent in terms of structural descriptions of the groundwater supply sources surveyed. Specifically, 65.2% of studies (15/23) included no extractable data for any pre-established infrastructural data fields. Overly generic structural supply unit descriptions (e.g. well depth) were observed in 17.4% (4/23) of studies. In turn, only two studies explicitly reported comprehensive infrastructural data in relation to analysed groundwater supplies (Pitkänen *et al*., 2011; Ferguson *et al*., 2012). Similarly, absent and/or ambiguous reporting was a consistent feature with regard to hydrogeological setting, with only five studies providing a relevant description (Table 2.4). Only one investigation included in the *Cryptosporidium* review featured in the VTEC dataset (see Odagiri *et al*., 2016), indicating that studies focusing on dual VTEC–*Cryptosporidium* groundwater contamination, at least among peer-reviewed literature, are rare.

The VTEC review dataset spanned a period of 16 years (2003 to 2019), with the majority of studies (13/23; 56.6%) being published during the 6-year period 2014–2019 (Table 2.4). The geographical distribution of reviewed studies, including (country-specific) VTEC detection rates among samples/supplies, is presented in Figure 2.8.

Sub-Saharan Africa was the region with the largest number of studies (7/23; 30.4%), followed by South-East Asia and North America, each accounting for 6/23 (26.1%) studies (Table 2.4). Of the analysed groundwater samples, approximately three-quarters (1819/2471; 73.6%) originated from investigations in South-East Asia (43.2%) and North America (30.4%). Studies conducted in countries characterised by high income constituted half of the dataset (12/23; 52.5%), with most groundwater samples also deriving from this category (1668/2471; 67.5%). A comparable number of investigations were conducted in lower-middle-income (6/23; 26.1%) and upper-middle-income (5/23; 21.7%) categories. Investigations based in rural settings were predominant (14/23; 60.9%) and constituted approximately half of groundwater samples analysed (1295/2471; 52.4%). Specifically, within the rural category, four (17.4%) and three (13%) investigations were based in agricultural environments primarily focusing on fresh produce and livestock production, respectively. Studies conducted in "mixed" (i.e. peri-urban) settings accounted for 4/23 (17.4%) of reviewed investigations. Identified studies based in urban settings were less common (3/23; 13%). The association between geographical study location, income category and local setting is presented in Figure 2.9. All investigations conducted in upper-middle-income economies originated from sub-Saharan Africa, primarily due to the high aggregation of studies conducted in South Africa ($n = 5$) (Figure 2.8). Most investigations conducted in high-income countries were based in rural environments (10/12; 83.3%) (Figure 2.10).

Study design, climate and seasonality

The dataset largely comprised investigations focusing on groundwater VTEC environmental "prevalence" (21/23; 91.3%) rather than those prompted by epidemiological outbreaks (2/23; 8.7%) (Table 2.4). The two infection-related investigations derived from outbreaks associated with (i) consumption of bagged spinach in the USA and Canada (Jay-Russell *et al*., 2014) and (ii) drinking water in a school camp in South Korea (Park *et al*., 2018). Geographically, studies were more frequently based in areas at high latitudes (Figure 2.8), resulting in the predominance of

Table 2.4. Summary of data on key characteristics extracted from included VTEC studies (*n* = 23)

Characteristic	Studies *n* (%)	VTEC study presence *n* (%)	Samples analysed *n* (%)
Publication year			
2003–2008	3 (13)	1 (33.3)	356 (14.4)
2009–2013	7 (30.4)	4 (57.1)	1091 (44.2)
2014–2019	13 (56.6)	4 (30.8)	1024 (41.4)
Study location			
Sub-Saharan Africa	7 (30.4)	5 (71.4)	379 (15.3)
Asia	6 (26.1)	2 (33.3)	1067 (43.2)
Europe	4 (17.4)	1 (25)	273 (11.1)
North America	6 (26.1)	1 (16.7)	752 (30.4)
Latin America and the Caribbean	0 (0)	N/A	N/A
North Africa and the Middle East	0 (0)	N/A	N/A
Pacific	0 (0)	N/A	N/A
Country income level			
High	12 (52.5)	3 (25)	1668 (67.5)
Upper middle	5 (21.7)	4 (80)	491 (19.9)
Lower middle	6 (26.1)	2 (33.3)	312 (12.6)
Low	0 (0)	N/A	N/A
Setting			
Rural	14 (60.9)	4 (28.6)	1295 (52.4)
Fresh produce	4 (17.4)	0 (0)	172 (7.1)
Livestock (dairy)	3 (13)	1 (33.3)	435 (17.6)
Other rural (unspecified)	7 (30.4)	2 (28.6)	688 (27.8)
Urban	3 (13)	2 (66.7)	135 (5.5)
Mixed	4 (17.4)	2 (50)	390 (15.8)
N/R	2 (8.7)	1 (50)	651 (26.3)
Climate			
Arid	2 (8.7)	1 (50)	67 (2.7)
Tropical	4 (17.4)	2 (50)	389 (15.7)
Temperate	10 (43.5)	3 (30)	831 (33.6)
Cold	4 (17.4)	2 (50)	467 (19)
Unknown	3 (13)	1 (33.3)	717 (29)
Study design			
Prevalence	21 (91.3)	8 (38.1)	2437 (98.6)
Outbreak	2 (8.7)	1 (50)	34 (1.4)
Hydrogeological description			
Yes	5 (21.7)	2 (40)	497 (20.1)
No	18 (78.3)	7 (38.9)	1974 (79.9)
Supply type			
Public	2 (8.7)	0 (0)	92 (3.7)
Private	11 (47.8)	5 (45.5)	841 (34)
Mixed	5 (21.7)	2 (40)	722 (29.2)
N/R	5 (21.7)	2 (40)	816 (33)

Table 2.4. Continued

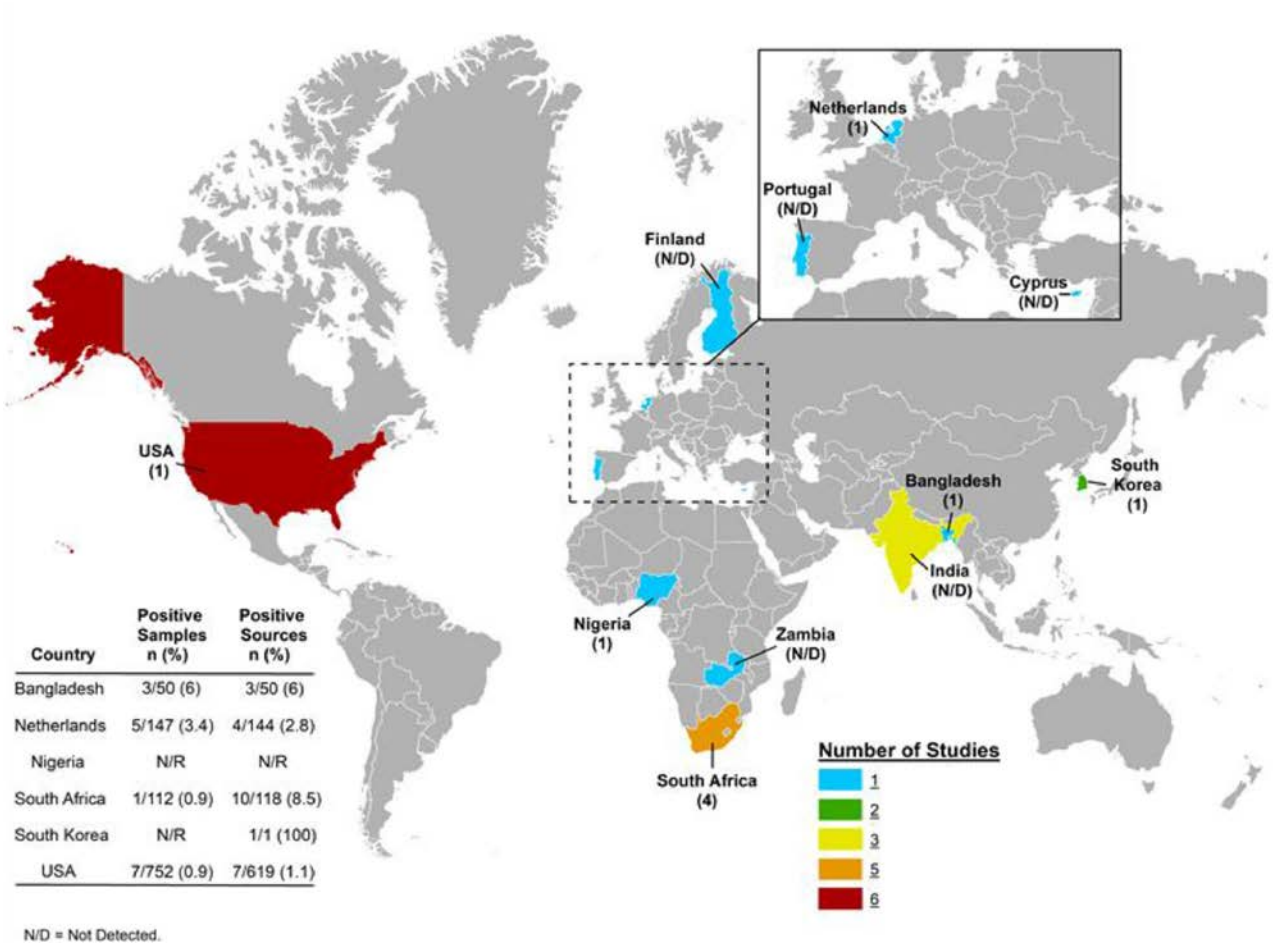
Characteristic	Studies *n* (%)	VTEC study presence *n* (%)	Samples analysed *n* (%)
Source type			
Protected	18 (78.3)	7 (38.9)	1518 (61.4)
Unimproved	0 (0)	N/A	N/A
Protected/unimproved	2 (8.7)	1 (50)	163 (6.6)
N/R	3 (13)	2 (66.7)	790 (32)
Sampling season			
Dry	1 (4.3)	0 (0)	16 (0.6)
Wet	7 (30.4)	5 (71.4)	724 (29.3)
Both	6 (26.1)	2 (33.3)	1219 (49.3)
N/R	9 (39.1)	2 (22.2)	512 (20.7)
Sampling strategy			
One-off	15 (65.2)	7 (46.7)	1638 (66.3)
Repeated	7 (30.5)	2 (28.6)	608 (24.6)
Mixed	1 (4.4)	0 (0)	225 (9.1)
Local precipitation			
Low	1 (4.4)	0 (0)	16 (0.6)
High	7 (30.4)	5 (71.4)	724 (29.3)
Both	6 (26.1)	2 (33.3)	1219 (49.3)
N/R	9 (39.1)	2 (22.2)	512 (20.7)
Sampling period (months)			
≤1	2 (8.7)	2 (100)	66 (2.7)
2–12	11 (47.8)	5 (45.5)	1600 (64.8)
≥12	4 (17.4)	0 (0)	445 (18)
N/R	6 (26.1)	2 (33.3)	360 (14.6)
Summer sampling			
Yes	11 (47.8)	3 (27.3)	1650 (66.8)
N/A	3 (13)	1 (33.3)	309 (12.5)
N/R	9 (39.1)	0 (0)	512 (20.7)
Sources analysed (*n*)			
≤10	3 (13)	1 (33.3)	94 (3.8)
11–50	9 (39.1)	4 (44.4)	442 (17.9)
≥51	8 (34.8)	3 (37.5)	1784 (72.2)
N/R	3 (13)	1 (33.3)	151 (6.1)
Samples analysed (*n*)			
10–20	4 (17.4)	1 (25)	62 (2.5)
21–50	5 (21.8)	2 (40)	201 (8.1)
51–150	9 (39.1)	5 (55.6)	751 (30.4)
≥151	5 (21.7)	1 (20)	1457 (59)
Point of sampling			
Source	11 (47.8)	4 (36.4)	1461 (59.1)
Point of use	4 (17.4)	3 (75)	534 (21.6)
Both	4 (17.4)	1 (25)	364 (14.7)
N/R	4 (17.4)	1 (25)	112 (4.5)

Table 2.4. Continued

Characteristic	Studies *n* (%)	VTEC study presence *n* (%)	Samples analysed *n* (%)
Sample volume (mL)			
100–250	11 (47.8)	5 (45.5)	1419 (57.4)
500	4 (17.4)	0 (0)	182 (7.4)
>1000	7 (30.4)	3 (42.9)	854 (34.6)
N/R	1 (4.4)	1 (100)	16 (0.6)
Concentration method			
Membrane filtration	16 (69.6)	7 (43.8)	2003 (81.1)
Cartridge filtration	1 (4.4)	0 (0)	42 (1.7)
Centrifugation[a]	3 (13)	2 (66.7)	157 (6.4)
IMS	1 (4.4)	0 (0)	18 (0.7)
N/R	2 (8.7)	0 (0)	251 (10.2)
Detection method			
PCR[b]	19 (82.6)	8 (42.1)	1568 (63.5)
E. coli positive isolates[c]	14 (73.4)	5 (35.7)	1150 (46.5)
All samples[c]	5 (26.3)	2 (40)	418 (16.9)
Culture[d]	4 (17.4)	1 (25)	903 (36.5)
Strain typing[e]			
Yes	3 (13)	2 (66.7)	181 (7.3)
PFGE	3 (13)	2 (66.7)	181 (7.3)
MLVA	1 (4.4)	0 (0)	18 (0.7)
No	20 (87)	7 (35)	2290 (92.7)
Sample treatment			
Chlorination	2 (8.7)	1 (50)	46 (1.9)
Varied	1 (4.4)	1 (100)	147 (5.9)
None	10 (43.5)	4 (40)	1014 (41)
N/R	10 (43.5)	3 (30)	1264 (51.2)

Extracted data are based on total of *n*=2471 groundwater samples and *n*=1998 groundwater supplies.
[a]Includes (*a priori*) groundwater sample *E. coli* detection using an IDEXX Colilert tray.
[b]Includes all investigations implementing PCR as a component of the detection methodology.
[c]Calculation based on the total number of investigations employing PCR (*n*=19).
[d]Includes investigations implementing solely culturing as a VTEC (*E. coli* O157:H7) detection method.
[e]Includes investigations employing both PFGE and MLVA.
N/A, not available; N/R, not reported.

"temperate" (10/23; 43.5%) and "cold" (4/23; 17.4%) climatic classifications (Table 2.4). "One-off" sampling regimes accounted for 15/23 (65.2%) studies and 1638/2471 (66.3%) analysed samples. In more than half of the identified investigations (13/23; 56.5%), sample collection was carried out during sampling months characterised by "high" local precipitation. Investigations carried out in these months are subdivided into investigations "fully" conducted within periods of inferred "high" precipitation (7/23; 30.4%) and those incorporating both "high" and "low" precipitation sampling periods (6/23; 26.1%). However, a substantial number of investigations provided insufficient information relating to the sampling regime employed, thus precluding integration of sampling months and (averaged) monthly precipitation (9/23; 39.1%). Temporally, 2/23 (8.7%) sampling campaigns were restricted to a "short" (1 month) period, with "longer" sampling campaigns, lasting >2 months (15/23; 65.2%), more frequently being employed (Table 2.4). In addition, most studies adopted sampling designs incorporating analysis of



Country	Positive Samples n (%)	Positive Sources n (%)
Bangladesh	3/50 (6)	3/50 (6)
Netherlands	5/147 (3.4)	4/144 (2.8)
Nigeria	N/R	N/R
South Africa	1/112 (0.9)	10/118 (8.5)
South Korea	N/R	1/1 (100)
USA	7/752 (0.9)	7/619 (1.1)

Figure 2.8. Map illustrating the global distribution of VTEC studies included for review, with the number of VTEC-positive investigations per country given in parentheses. Country-specific (pooled) VTEC detection rates among groundwater supply sources and samples are provided in the inset table (bottom left). Countries without positive VTEC detection were excluded from the inset table. N/D, not detected; N/R, not reported.

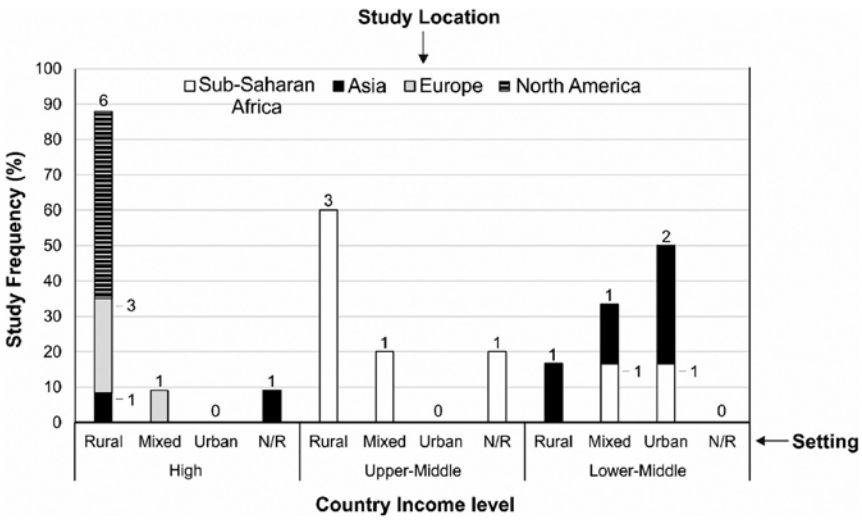


Figure 2.9. Frequency of VTEC studies included for review delineated by mean income level, urban/rural classification and geographical location. The number of investigations per category is provided at the top or right-hand side of the stacked bars. N/R, not reported.

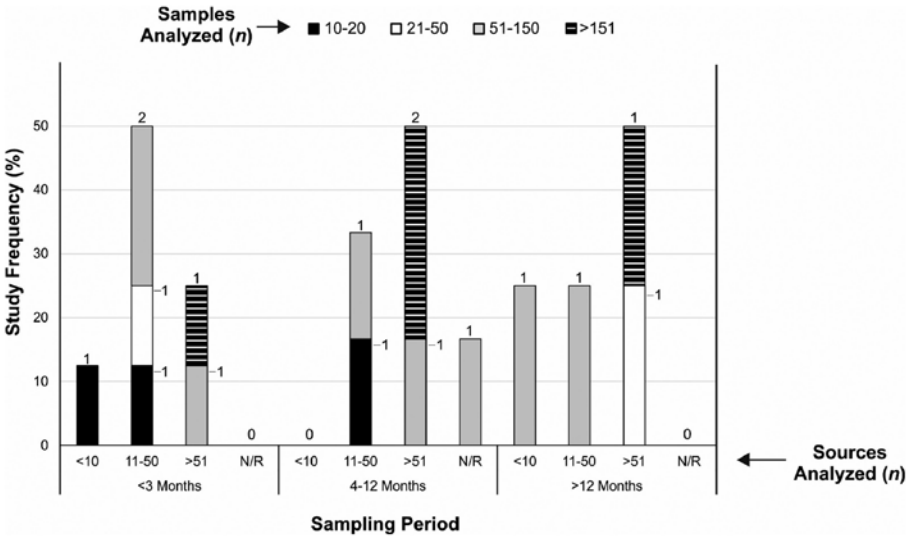


Figure 2.10. Frequency of VTEC studies included for review delineated by number of groundwater sources and samples analysed based on sampling period duration. Investigations not reporting the sampling period duration (*n* = 6) are excluded. The numbers of investigations per category are provided at the top or right-hand side of the stacked bars.

> 11 groundwater sources (17/23; 73.9%) (Table 2.4). Similarly, investigations focusing on > 51 groundwater samples were prevalent (14/23; 60.8%). Figure 2.10 summarises the scale of sampling design employed among the identified investigations, excluding those failing to report sampling campaign duration (*n* = 6).

Groundwater supply characteristics and detection methods

Approximately half of the identified studies focused on private groundwater supplies (11/23; 47.8%) (Table 2.4). Most studies (18/23; 78.3%) analysed groundwater supplies classified as "protected", accounting for a total of 1518/2471 (61.4%) samples analysed. Groundwater supplies categorised as "unimproved" featured in only two investigations. Few investigations reported VTEC analysis on groundwater samples that were subject to treatment (e.g. chemical, physical) (*n* = 3). However, several studies failed to describe if a treatment system was employed (*n* = 10). As shown in Table 2.4, there was a tendency for investigations to adhere to "source" sample collection points, with supplies directly sampled in 11/23 (47.8%) investigations. Conversely, the collection of samples from a domestic tap, i.e. following distribution, was reported in 4/23 (17.4%) studies. Approximately half of reviewed studies based VTEC analysis on sample volumes of 100–250 mL (11/23; 47.8%), with the preferred method employed for sample concentration being membrane filtration (16/23; 69.6%). Molecular identification involving PCR was the favoured detection method (19/23; 82.6%). Application of quantitative PCR (Bustin *et al*., 2009) was limited to 3/19 (15.9%) investigations. In total, 14/19 (73.4%) investigations performed PCR using *E. coli* previously isolated through culture/enrichment. A subset of PCR-based studies incorporated PCR analysis of all groundwater samples collected (5/19; 26.3%). Overall, the application of VTEC serotype-typing tools to identify (potential) environmental sources was infrequently employed (3/23; 13%) (Table 2.4).

Generic E. coli *and VTEC detection rates*

VTEC was cultured and/or genetic markers (*stx1*, *stx2*) identified in 9/23 (39.1%) studies, with overall sample- and source-specific detection rates being 0.6% (16/2471) and 1.3% (25/1998), respectively. The unexpectedly higher number of positive supply sources than of groundwater samples stems from the prevalence of inconsistent reporting in the review dataset, with several investigations failing to specify VTEC detection rates for sources/samples. Based on positive PCR detection in 8/19 (42.1%) reviewed studies, gene detection was reported in 0.8% (4/477) of groundwater samples and 6.9% (34/493) of supplies analysed (Table 2.5). A single study employing

Table 2.5. Synthesis of pooled data from studies of VTEC groundwater samples (*n* = 2230) and sources (*n* = 1949) reporting positive VTEC detection by selected study characteristic

Characteristic	VTEC sample/ source detection studies *n*	Positive samples/ total samples *n* (%)	Positive sources/ total sources *n* (%)
Publication year			
2003–2008	**3**/**3**	5/356 (1.4)	4/338 (1.2)
2009–2013	6/5	11/1067 (1)	11/977 (1.1)
2014–2019	9/10	0/807 (0)	10/634 (1.5)
Study location			
Sub-Saharan Africa	3/4	1/154 (0.6)	10/152 (6.6)
Asia	5/**6**	3/1051 (0.3)	4/998 (0.4)
Europe	**4**/3	5/273 (1.8)	4/180 (2.2)
North America	**6**/5	7/752 (0.9)	7/619 (1.1)
Country income level			
High	11/10	12/1652 (0.7)	12/1427 (0.8)
Upper middle	2/3	1/112 (0.9)	10/130 (7.7)
Lower middle	5/5	3/466 (0.6)	3/392 (0.8)
Setting			
Rural	12/9	8/1224 (0.7)	9/1002 (0.9)
Fresh produce	**4**/3	0/172 (0)	0/59 (0)
Livestock (dairy)	**3**/2	7/435 (1.6)	7/385 (1.8)
Other rural (unspecified)	6/7	1/697 (0.1)	2/638 (0.3)
Urban	2/2	3/110 (2.7)	3/56 (5.4)
Mixed	3/**4**	5/269 (1.9)	13/264 (4.9)
N/R	1/1	0/627 (0)	0/627 (0)
Climate			
Arid	1/1	0/12 (0)	0/12 (0)
Tropical	3/3	3/364 (0.8)	3/364 (0.8)
Temperate	9/9	6/710 (0.8)	14/554 (2.5)
Cold	3/**4**	7/451 (1.6)	8/392 (2)
Unknown	2/1	0/693 (0)	0/627(0)
Supply type			
Public	**2**/**2**	0/92 (0)	0/32 (0)
Private	9/9	15/800 (1.9)	15/631 (2.4)
Mixed	4/**5**	1/601 (0.2)	10/579 (1.7)
N/R	3/2	0/737 (0)	0/707 (0)
Source type			
Protected	15/13	11/1414 (0.8)	11/1137 (1)
Protected/unimproved	1/**2**	0/42 (0)	9/40 (22.5)
N/R	2/**3**	5/774 (0.6)	5/772 (0.6)
Sampling strategy			
One-off	11/11	11/1518 (0.7)	12/1420 (0.8)
Repeated	6/10	5/487 (1)	13/324 (4)
Mixed	**1**/**1**	0/205 (0)	0/205 (0)

Table 2.5. Continued

Characteristic	VTEC sample/ source detection studies *n*	Positive samples/ total samples *n* (%)	Positive sources/ total sources *n* (%)
Relative precipitation			
Low	**1/1**	0/16 (0)	0/16 (0)
High	5/6	15/683 (2.2)	15/649 (2.3)
Both	5/5	1/1098 (0.1)	10/970 (1)
N/R	7/6	0/433 (0)	0/314 (0)
Sampling period (months)			
≤1	1/**2**	3/50 (6)	4/51 (7.8)
2–12	9/9	13/1454 (0.9)	21/1371 (1.5)
≥12	**4/4**	0/445 (0)	0/311 (0)
N/R	4/6	0/281 (0)	0/216 (0)
Summer sampling			
Yes	9/10	13/1513 (0.9)	31/1351 (2.3)
N/A	2/2	3/284 (1.1)	3/284 (1.1)
N/R	7/6	0/433 (0)	0/314 (0)
Sources analysed (*n*)			
≤10	2/**3**	0/78 (0)	1/10 (10)
11–50	6/7	3/272 (1.1)	12/178 (6.7)
≥51	**8/8**	13/1784 (0.7)	12/1761 (0.7)
N/R	2/3	0/96 (0)	N/R
Samples analysed (*n*)			
10–20	3/**4**	0/46 (0)	1/32 (3.1)
21–50	3/2	0/152 (0)	0/102 (0)
51–150	7/7	9/575 (1.6)	17/378 (4.4)
≥151	**5/5**	7/1457 (0.5)	7/1437 (0.5)
Point of sampling			
Source	9/10	4/1315 (0.3)	22/1187 (1.9)
Point of use	3/**4**	12/518 (2.3)	12/516 (2.3)
Both	3/2	0/309 (0)	0/208 (0)
N/R	3/2	0/88 (0)	0/38 (0)

The VTEC sample/source detection studies column displays the number of investigations reporting extractable data in terms of samples (left) and supply sources (right) analysed, and corresponding (pooled) VTEC contamination rates, with values in bold indicating full (equivalent) VTEC contamination reporting in the dataset.

N/A, not available; N/R, not reported.

culture-based methods reported positive identification of (presumed) *E. coli* O157 in an unspecified number of groundwater sources/samples. Parallels between the (pooled) rates of detection of generic *E. coli* and VTEC in groundwater samples and study sampling strategy (i.e. "one-off" or "repeat") are presented in Figure 2.11. Pooled study data indicate a generic *E. coli* detection rate in groundwater samples of 315/1926 (16.4%) (Figure 2.11). Notwithstanding this, a total of 7/23 (30.4%) investigations did not explicitly report the study-specific generic *E. coli* detection rate. Again, this lack of reporting was associated with ambiguous/incomplete data collation and/or presentation (4/23; 17%), or was related to investigations not employing *E. coli* as a groundwater faecal indicator organism (FIO) (i.e. direct VTEC detection) (3/23; 13%). Overall, a VTEC to generic *E. coli* sample detection ratio of 15:152 (9.9%) was estimated (Figure 2.11A). Moreover, VTEC to generic *E. coli* ratios specific to "repeat" and "one-off"

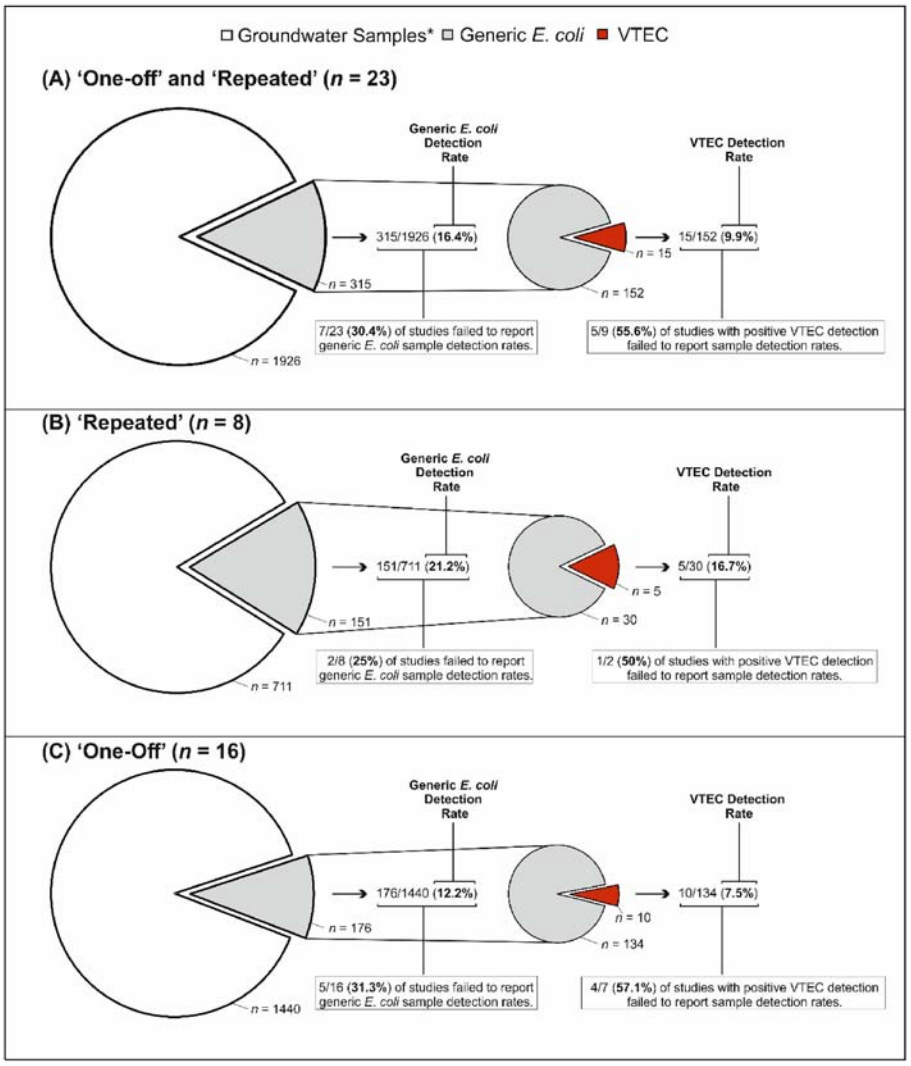


Figure 2.11. VTEC to generic *E. coli* detection ratios, including (pooled) data specific to sampling strategy categories ("one-off" and "repeat"). The total number (*n*) of investigations in each category is provided in panel titles, with the number of investigations failing to report relevant data to calculate detection rates (for both *E. coli* and VTEC) provided in text boxes. *Includes (pooled) groundwater samples from all investigations and corresponding sampling strategy categories as indicated.

investigations of 16.7% and 7.5%, respectively, were estimated (Figure 2.11B,C). In terms of geographical VTEC to generic *E. coli* detection ratios, Europe (19.2%) and North America (17.5%) exhibited the highest values (Table 2.6).

Pooled VTEC detection rates in groundwater samples and groundwater supply sources of 0.7% (16/2230) and 1.3% (25/1949) were calculated, respectively. Similar to generic *E. coli* incidence, several investigations failed to report sufficient data to calculate VTEC detection rates. Specifically, 5/9 (55.6%) VTEC-positive studies failed to specify the number of contaminated groundwater samples (Figure 2.11). Likewise, 3/9 (33.3%) investigations did not report the number of sampled groundwater supplies.

Potential drivers of VTEC contamination of groundwater

Geographically, the highest VTEC groundwater detection rates were reported in sub-Saharan Africa (10/152; 6.6%) (Table 2.5). This feature is clearly reflected in country-specific data (Figure 2.8), with South Africa reporting the highest VTEC detection rates in supply sources (8.5%). A local study (Abia *et al*., 2017) reported the highest VTEC (dataset) prevalence with respect to groundwater supplies (9/18;

Table 2.6. Pooled generic *E. coli* and VTEC values according to geographical location

Geographical location	*E. coli*/VTEC reporting studies *n*	Positive generic *E. coli*/ total generic *E. coli* samples *n* (%)	Positive VTEC/total VTEC samples *n* (%)
Study location			
Sub-Saharan Africa	4/1	167/182 (91.8)	0/4 (0)
Asia	3/3	82/737 (11.1)	3/82 (3.8)
Europe	**4/4**	26/273 (9.5)	5/26 (19.2)
North America	5/5	40/734 (5.5)	7/40 (17.5)
Country			
Bangladesh	1/1	20/50 (40)	3/20 (15)
Cyprus	1/1	10/30 (33.3)	0/10 (0)
Finland	1/1	4/80 (5)	0/4 (0)
India	1/1	16/60 (26.8)	0/16 (0)
Netherlands	1/1	4/147 (2.7)	5/0 (0)[a]
Nigeria	1/0	25/25 (100)	N/R
Portugal	1/1	8/16 (50)	0/8 (0)
South Africa	3/1	142/157 (90.5)	0/4 (0)
South Korea	1/1	46/627 (7.3)	0/46 (0)
USA	5/5	40/734 (5.5)	7/40 (17.5)
Zambia	0/0	N/R	N/R

The *E. coli*/VTEC reporting study column displays the number of investigations reporting extractable data in terms of generic *E. coli* (left) and VTEC (right) sample detection per (pooled) geographical location. Values in bold indicate full (equivalent) generic *E. coli*/VTEC sample reporting in the review dataset. All estimates presented incorporate values from both "one-off" and "repeat" investigations.

[a]Investigation reported VTEC detection in samples with no prior generic *E. coli* (FIO) detection (Schets *et al.*, 2005).

N/R, not reported.

50%). In turn, Won *et al*. (2013) reported the highest detection rate in groundwater samples (7/180; 3.9%), which were located in dairy farm environments in Ohio, USA. High detection rates in groundwater supplies in sub-Saharan Africa directly influence the high values observed in upper-middle-income categories (10/130; 7.7%) (Table 2.5; Figure 2.9). Extracted data also indicate that urban and peri-urban environments had higher supply VTEC contamination rates, at 5.4% and 4.9%, respectively. Conversely, rural settings had lower pooled VTEC detection rates in supplies and samples, at 0.7% and 0.9%, respectively. Trends in studies grouped by climate indicate that environments classified as "temperate" (14/554; 2.5%) and "cold" (8/392; 2%) accounted for the majority of contaminated supply sources; however, estimates are likely to have been constrained by the high-latitude focus of studies making up the dataset (Table 2.4). Conversely, no VTEC detection was reported in the single (data reporting) investigation based in an "arid" environment. Within the context of supply regulation/management, public supplies ($n = 2$) exhibited no contamination unlike those under private or alternative (i.e. "mixed" or "not reported") administration (Table 2.5). Overall, private (i.e. unregulated) groundwater samples (15/800; 1.9%) and supplies (15/631; 2.4%) were characterised by the highest VTEC detection rates. Moreover, while the number of investigations (explicitly) focusing on "unimproved" supplies was small ($n = 2$), higher supply-specific detection rates were calculated (9/40; 22.5%) for "unimproved" supplies than for supply sources classified as "protected" (11/1137; 1%).

Assessing the potential nexus between seasonality and sampling design, investigations focusing sampling efforts during periods of (normally) "high" precipitation reported higher detection rates for both groundwater samples (15/683; 2.2%) and supplies (15/649; 2.3%) relative to "low" precipitation periods, when no detections were reported across samples or sources (Table 2.5). The two investigations based on "temporally" limited sampling campaigns (< 1 month) reported the highest sample and supply VTEC

prevalence (6% and 7.8%, respectively). Similarly, VTEC detection rates in supply sources were higher (10%) in studies characterised by a more "limited" scope in terms of the number of sampled groundwater supply sources ($n \leq 10$). Pooled data indicate comparable groundwater source detection rates among investigations focusing on 10–20 (3.1%) and 51–150 (4.4%) groundwater samples. Investigations incorporating "repeated" sampling regimes reported higher VTEC detection rates in the water supply (13/324; 4%) than "one-off" investigations (12/1420; 0.8%) (Table 2.5). Similarly, as shown in Figure 2.11, higher rates of detection of generic *E. coli* in samples were reported in "repeated" (151/711; 21.2%) than in "one-off" (176/1440; 12.2%) investigations. An overall adjusted VTEC to generic *E. coli* detection ratio of 5:30 (16.7%) was calculated to account for the potential effects of "repeat" sampling on groundwater sample detection (see below).

Overall, the identification of contamination mechanisms, pathways and sources was often absent and/or subject to very ambiguous reporting. Only 3/9 (33.3%) investigations reporting positive VTEC detection described potential ingress mechanism(s) (Table 2.7). Specifically, groundwater recharge and direct surface ingress were the primary ingress mechanisms suggested, with each featuring in two

Table 2.7. Summary of data on selected characteristics within the "VTEC contamination" data extraction category based on studies with positive detection of VTEC in groundwater ($n = 9$)

Characteristic	Studies *n* (%)	Positive samples/total samples *n* (%)	Positive sources/total sources *n* (%)
Contamination ingress mechanisms[a]			
Groundwater recharge	2/3 (66.7)	3/50 (6)	12/68 (17.7)
Direct surface ingress	2/3 (66.7)	N/R	9/18 (50)
N/R	6/9 (66.7)[b]	13/427 (3)	13/425 (3.1)
Contamination source[a]			
Animal origin	5/6 (83.3)	13/427 (3)	20/442 (4.5)
Human origin	5/6 (83.3)	11/330 (3.3)	21/348 (6)
N/R	3/9 (33.3)[b]	N/R	1/1 (100)
Reported animal origin[a]			
Cattle	3/5 (60)	12/327 (3.7)	20/342 (5.9)
N/R	2/5 (40)	1/100 (1)	2/101 (2)
Reported human origin[a]			
Pit latrines	2/5 (40)	N/R	9/18 (50)
N/R	3/5 (60)	11/330 (3.3)	15/474 (3.2)
Serogroup identified[c]			
O157:H7	4/8 (50)	–	–
O103:H2	1/8 (12.5)	–	–
N/R	3/8 (37.5)	–	–
FIO co-occurrence[a]			
Coliforms[d]	2/4 (50)	–	–
E. coli	8/9 (88.9)	–	–
Enterococcus spp.	0/2 (0)	–	–

The study number column is based on the number of investigations analysing or reporting relevant data (e.g. reported source/pathway). Pooled synthesis data among selected characteristics are also provided.

[a]Multiple selection were available for data fields in this category, with percentages not amounting to 100%.

[b]Calculation based on the number of studies with positive detection of VTEC in groundwater ($n = 9$).

[c]Based on the total number of investigations with positive VTEC detection employing PCR ($n = 8$).

[d]Includes studies reporting total coliforms, faecal coliforms and thermotolerant coliforms.

N/R, not reported.

investigations, albeit largely based on tentative attribution (Table 2.7). Reported presumptive contamination sources were evenly distributed among "animal" (5/6; 83.3%) and "human" (5/6; 83.3%) categories, with cattle the only specific contamination source linked to VTEC groundwater supply contamination (3/5; 60%) (Table 2.7). In turn, human contamination sources were often (vaguely) associated with domestic waste effluents, with pit latrines explicitly linked to VTEC supply ingress in two out of five (40%) investigations. The attribution of contamination to specific sources was primarily based on source proximity/adjacency rather than actual "tracing". Two out of three investigations employing environmental VTEC strain typing reported the presence of VTEC. Through comparison of PFGE patterns obtained from human stool samples and drinking water isolates, Park *et al*. (2018) identified groundwater as the most likely source of a diarrhoeagenic *E*. *coli* outbreak (VTEC/ enteropathogenic *E. coli* (EPEC)). Similarly, Schets *et al*. (2005) compared (*E*. *coli* O157) PFGE patterns from groundwater isolates with collated records from different (potential) regional sources, identifying cattle as the likely contamination source. Beyond select investigations focusing on specific agricultural settings ($n = 7$), most investigations had a "broad", i.e. landscape-wide, approach towards VTEC detection without any (reported) explicit contamination sources (Tables 2.4 and 2.5). Five out of eight (62.5%) PCR-based investigations with positive VTEC detection specified the serogroups identified, with only O157 ($n = 4$) and O103 ($n = 1$) being reported (Table 2.7).

2.3.3 Discussion

Generic E. coli *and VTEC detection ratios*

One of the key deliverables of the literature review is the estimated VTEC to generic *E*. *coli* detection ratios (Figure 2.11), as these have potential (bespoke) applications in groundwater management. The ratios presented are highly applicable for the development of increasingly accurate QMRAs, specifically focusing on domestic groundwater supplies (Haas *et al*., 2014), primarily as a pathogen "contribution" or "loading" metric (i.e. model input) specific to groundwater-borne VTEC. To date, the development of QMRA for estimating the burden of groundwater VTEC has largely relied on generic *E*. *coli* to *E*. *coli* O157 ratios estimated from a range of environmental waters (e.g. Haas *et al*., 1999; Soller *et al*., 2010). Hence, it is argued that the ratios presented provide a more specific representation of VTEC incidence in groundwater environments. Notwithstanding this, as already outlined in section 2.2.3, it is critical to highlight the potential influence of selective sampling on the estimated values (Figure 2.11). Key data trends (e.g. sampling period, number of supplies analysed) suggest that studies with more "inclusive" (i.e. random rather than targeted) sampling designs reported lower levels of VTEC detection (Table 2.5), and, because of this, the likelihood of "bias" introduced by targeting susceptible supplies (i.e. "proof of concept") needs to be emphasised. Coupled with publication bias (i.e. failure to publish negative results), this is a factor that may compromise the integrity of some of the estimates presented. Accordingly, the ratios estimated from studies adopting "repeat" sampling (16.7%) may provide a more realistic estimate of the presence of both generic *E*. *coli* presence and VTEC in groundwater. The calculated "repeat" value is likely to represent a more robust metric that can be applied to accurately determine (human) VTEC exposure over time. VTEC to generic *E*. *coli* ratios deriving from "repeat" investigations are also less compromised by incomplete reporting, a factor that also potentially reinforces the robustness of estimated values (Figure 2.11). Comparison of estimated VTEC to generic *E*. *coli* detection ratios with those commonly reported in the literature may help to ascertain their potential validity. For example, previous QMRAs focusing on domestic water distribution systems (e.g. Howard *et al*., 2006; Machdar *et al*., 2013; Katukiza *et al*., 2014; Abia *et al*., 2016) have often employed an *E*. *coli* to VTEC detection ratio of 8% (Haas *et al*., 1999), as also employed as the *E*. *coli* to EPEC ratio (Shrestha *et al*., 2017), and comparable to the low range presented in this investigation (7.5–9.9%; Figure 2.11). In turn, Hynds *et al*. (2014c) used the lower bound probabilistic range of 1–16% as reported by Soller *et al*. (2010) for untreated drinking water. The range used by Soller *et al*. exhibits some agreement with the 16.7% ratio estimated based on "repeat" sampling here (Figure 2.11). While available figures are comparable, the likelihood of estimated VTEC to generic *E*. *coli* ratio values being (to a degree) inflated as a result of selective sampling needs to be highlighted. Clearly, the lack of clear and homogeneous reporting relating to the detection of both *E*. *coli* and VTEC in groundwater samples

(Figure 2.11; Table 2.5) represents a critical limitation of the studies identified.

VTEC incidence in groundwater and geographical data trends

Pooled data identified from the literature review were used to establish VTEC detection rates in groundwater samples and supplies of 0.7% and 1.3%, respectively. The unexpectedly lower detection rates estimated for groundwater samples than for supplies derive from inconsistent reporting of VTEC detection among investigations. Once more, given the potential influence of sampling "bias", values deriving from investigations employing "repeat" sampling designs (1–4%; Table 2.5) are likely to provide more "robust" VTEC prevalence estimates than values deriving from "one-off" sampling. It is possible that the former values represent global contamination baselines. Notably, an investigation published by Stokdyk *et al*. (2020) not included in the review dataset, as it was published outside the review time-span (1 July 2019), reporting on a considerable number of groundwater samples ($n = 834$) and supplies ($n = 145$), obtained VTEC detection rates in samples (0.4%) comparable to those presented here. Such analogous values may serve to support the VTEC detection rates presented; however, this study (Stokdyk *et al*., 2020) was both geographically restricted and exclusively focused on "public" supplies, with any comparisons thus requiring careful interpretation. Overall, the small number of investigations identified ($n = 23$) and the (relatively) limited number of groundwater samples/supplies constituting the review dataset (Table 2.4) represent both a key finding and a research gap, considering (i) the perceived importance of VTEC as a globally significant (waterborne) pathogen and the concomitant clinical burden and (ii) the reported links between domestic groundwater resources and risk of human infection (Muniesa *et al*., 2006; Majowicz *et al*., 2014; Guzman-Herrador *et al*., 2015; Moreira and Bondelind, 2017). Similarly, the lack of robust data on VTEC serotype prevalence in domestic groundwater wells (Table 2.7) constitutes another important finding, particularly considering the increasingly reported global incidence and clinical burden of non-O157 serotypes and potential for regional serotype variability (Gould *et al*., 2013; Luna-Gierke *et al*., 2014; Baranzoni *et al*., 2016). Geographically, the VTEC review dataset was characterised by evident regional data (knowledge) gaps (Figure 2.8), which are clearly more pronounced than those identified in the *Cryptosporidium* review (Figure 2.2). The lack of regional data represents a crucial limitation in terms of both reported geographical variations in notified VTEC infections and the potential importance of local/regional risk factors (Croxen *et al*., 2013; Newell and La Ragione, 2018). Regional *E. coli* and VTEC incidence data are essential for deriving geographically specific information and distributions. For example, Murphy *et al*. (2016) pooled available data from two North American studies to produce custom (QMRA) health risk assessments for Canadian groundwater supplies. Despite the poor geographical distribution encountered in the current study and potential bias in source selection, it is argued that some of the regional figures collated and VTEC to generic *E. coli* ratios estimated may find useful application in future (regional) investigations (Figure 2.8; Tables 2.5 and 2.6).

Local environments, settlement patterns and land use

Emphasising the importance of local risk factors is key considering the higher VTEC detection rates in groundwater supplies in urban (5.4%) and "mixed" (4.9%) settings than in rural settings (Table 2.5). To an extent, these findings contrast with available geostatistical evidence linking higher VTEC prevalence with rural environments in high-income regions (Schets *et al*., 2005; Denno *et al*., 2009; ÓhAiseadha *et al*., 2017; Brehony *et al*., 2018). Notably, the majority of reviewed investigations based in urban and "mixed" settings were concentrated in low-income regions in Asia and sub-Saharan Africa (Figure 2.9). It is likely that the data collated reflect the influence of local settings, as shaped by socio-economic parameters, on VTEC prevalence. For example, as a result of lax (or non-existent) regulations/guidelines and settlement "distribution" patterns characteristic of low-income settings, both cattle and latrines, reported herein as the only (tentative) contamination sources (Table 2.7), potentially co-exist in close proximity to domestic groundwater supplies (Graham and Polizzotto, 2013; Penakalapati *et al*., 2017; Burri *et al*., 2019). Available evidence from sub-Saharan Africa highlights the intensity of animal rearing in (peri-)urban settings, the prevalence of domestic wastewater sources, the incidence of animal origin VTEC strains and the high

contamination burden on groundwater resources (Kulabako *et al*., 2007; Adelana *et al*., 2008; Braune and Xu, 2010; Lupindu *et al*., 2014; Lapworth *et al*., 2017). In terms of cattle management practices, barn/feedlot confinement as opposed to traditional "pastoral" (i.e. free-roaming) rearing has been associated with the lower environmental prevalence of VTEC in developing countries (Callaway *et al*., 2009; Smith *et al*., 2011). However, the results presented suggest that a "dispersed" livestock (source) distribution and the associated potential for close interaction with groundwater supplies (receptor) increase the likelihood of the pathogenic contamination of supplies and human VTEC exposure. Highlighted geographical and land use trends may also reflect the regional importance of manure application (land spreading) in agriculture, a potentially key conduit for environmental VTEC dissemination (Fremaux *et al*., 2008; Ferens and Hodve, 2011; van Elsas *et al*., 2011). While accurate global data on manure soil application rates are not available, stocks and soil nitrogen data collated by the Food and Agriculture Organization of the United Nations (FAO) (http://www.fao.org/faostat) highlight the prevalent use of manure as a soil fertiliser in both Africa and Europe, which is potentially linked to estimated regional VTEC values (Table 2.5). Similarly, global spatial data projections on inorganic and organic fertiliser application emphasise the predominance of soil-applied manure among typical fertilisers in Africa (Potter *et al*., 2010).

Investigations based in agricultural environments and focusing on fresh produce ($n = 4$) reported no positive VTEC detection (Table 2.5), with a calculated rate of detection of generic *E. coli* in samples of 11.7% (18/154). Results are relevant in light of (global) evidence of microbial contamination in produce-growing regions and the potential for pathogen propagation via fresh produce (e.g. contaminated sprout outbreak in Germany in 2011: Buchholz *et al*., 2011; Pachepsky *et al*., 2011; Nguyen-the *et al*., 2016). Because of its animal origins, enhanced survival ability in soil/water and high prevalence in farming environments, VTEC is a leading agent in outbreaks linked to the consumption of fresh produce (Lal *et al*., 2012; Olaimat and Holley, 2012; Jung *et al*., 2014). Therefore, the results may (tentatively) indicate that domestic groundwater in produce-growing regions is relatively "safe". Conversely, studies based in rural environments with an explicit focus on cattle rearing exhibited higher VTEC detection rates than studies in other rural subcategories (Table 2.5). However, two investigations of supplies located adjacent to concentrated animal feeding operations (Economides *et al*., 2012; Li *et al*., 2015) reported positive (FIO) *E. coli* groundwater sample detection (8.6%) but no VTEC contamination. Similarly, Joung *et al*. (2013) analysed groundwater wells in relation to livestock carcass burial sites. All samples analysed ($n = 627$) were negative for VTEC, with rates of generic *E. coli* contamination calculated at 7.3%. The results presented seem to support the aforementioned inferences relating to the spatial distribution (i.e. confinement) of potential VTEC sources and a decreased likelihood of groundwater supply contamination.

Potential influence of climate and seasonality

Although based on limited data (in both frequency and scope), summary statistics highlight the potential influence of temperate and cold climatic settings on higher rates of VTEC incidence in supplies (2–2.5%) (Table 2.5). Pooled data may (at times discreetly) reflect the cumulative effects of lower temperatures, reduced UV solar radiation and higher precipitation on VTEC survival and transport, and thus on environmental prevalence. There is substantial evidence within the literature supporting enhanced generic *E. coli* and VTEC survival at lower temperatures (< 10°C) in both environmental and potable water, soils, plant material, and cattle faeces and derived effluents (e.g. manure, slurry) (John and Rose, 2005; Fremaux *et al*., 2008; Ma *et al*., 2011; van Elsas *et al*., 2011). Generally, increased stress and energy expenditure associated with higher temperatures limits *E. coli* persistence in the environment, with large temperature variation (±7°C) particularly impacting *E. coli* survival (Semenov *et al*., 2007). Previous experiments conducted on natural well water indicate increased VTEC survival at 5–10°C (Rice *et al*., 1992; Watterworth *et al*., 2006). Collated data trends may also be partially explained by reduced levels of solar UV radiation in temperate and cold settings increasing VTEC prevalence in soils, surface water and manures prior to mobilisation to subsoils and groundwater (LeJeune *et al*., 2001; Yaun *et al*., 2003, 2004; Fremaux *et al*., 2008). In addition, the effects of high rainfall on the release and propagation of faecal material and bacteria through enhanced

(landscape) hydrological connectivity (e.g. overland flow) have been well substantiated (Fremaux *et al*., 2008; Hofstra, 2011; McCarthy *et al*., 2012; Blaustein *et al*., 2016). Unsurprisingly, as highlighted before, VTEC outbreaks have been frequently linked with periods of heavy antecedent rainfall (Muniesa *et al*., 2006; O'Dwyer *et al*., 2016). These effects are suggested by the higher VTEC detection rates calculated from investigations focusing on periods of "high" precipitation (≈2%) (Table 2.5). *E. coli* may also benefit from the low-oxygen conditions provided by moist and waterlogged soil environments and often characteristic of (wet) temperate settings (Fremaux *et al*., 2008; Brennan *et al*., 2010; van Elsas *et al*., 2011). Overall, environmental factors potentially increasing VTEC survival prior to and post mobilisation into domestic groundwater find good agreement with those also accredited for *Cryptosporidium* (oocyst) survival and prevalence.

Seasonal livestock management, often comprising livestock over-wintering and summer grazing, has also been identified as a potential explanatory variable for temporal variations in VTEC prevalence (Calloway *et al*., 2009; Garvey *et al*., 2010; Smith *et al*., 2011). The summer grazing variable primarily relates to increased potential for VTEC dissemination associated with cattle dispersal, but may also reflect available evidence for summer peaks on actual *E. coli* O157 to H7 content in (cattle) faeces (Ferens and Hodve, 2011; Lal *et al*., 2012). The higher VTEC detection rate in groundwater supplies estimated from investigations incorporating "summer" sampling (2.3%) may indeed reflect the influence of seasonal cattle grazing on VTEC prevalence. However, any potential associations remain tentative as a result of ambiguous reporting and ensuing skewed data category distribution (see Table 2.5).

Contamination sources, pathways and hydrogeology

In addition to animal contamination sources, reviewed investigations point to the potential relevance of "human" contamination sources, and, specifically, latrines. With the exception of one study (Won *et al*., 2013), all investigations aligning contamination sources with "human" origin were derived from low-income settings ($n = 4$), thus reinforcing the potential underlying role of socio-economic drivers (Graham and Polizzotto, 2013). However, only three investigations reported potential ingress mechanisms (Table 2.7), all of which were largely grounded in inconclusive evidence, representing a critical knowledge gap. A major methodological limitation precluding the accurate identification of VTEC contamination sources was the lack of molecular VTEC strain "tracing" (e.g. PFGE, MLVA) employed in the reviewed studies (Table 2.4). The majority of studies tentatively identified VTEC sources based on proximity rather than by employing DNA typing to effectively link "source(s)" and "receptor(s)", thus representing a key limitation for the applicability of reported data. So, while cattle are generally reported as a major source of environmental VTEC contamination, their importance as a reservoir in developing regions, in the absence of data, is more uncertain (Mainga *et al*., 2018). Accordingly, other key animal (or human) VTEC reservoirs may remain unaccounted for (Ferens and Hovde, 2011; Ahmed *et al*., 2015). In general, an integrative research approach aimed at identifying VTEC contamination sources, pathways and potential risk factors is often omitted in favour of a simplistic focus on establishing VTEC groundwater incidence. This feature is also reflected in the recurrent omission of local (hydro)geological characteristics (Table 2.4). This is crucial considering the influence of parent material (i.e. bedrock) on key subsoil characteristics (e.g. porosity, permeability) and, in turn, *E. coli* survival and transport (i.e. retention, filtration) (Fremaux *et al*., 2008; Bolster *et al*., 2009; van Elsas *et al*., 2011). A limited number of investigations provided relevant hydrogeological descriptions (5/23; 21.7%). Ferguson *et al*. (2012) associated local geology with (VTEC) groundwater contamination through evidence-based links between shallow sandy aquifers, rainfall intensity and efficient groundwater recharge. Similarly, Li *et al*. (2015) associated alluvial sediment soil layers (3–30 m) with a high pollutant attenuation capacity and mitigation of VTEC transport into groundwater. Additional investigations reporting hydrogeological data did not directly address the potential relevance in terms of VTEC incidence. Therefore, the incorporation of hydrogeological parameters as potential risk factors in future investigations is strongly advised.

Groundwater supply type, infrastructure and administration

With noted exceptions (e.g. Ferguson *et al*., 2012), a significant majority of investigations failed to

describe sample collection protocols and well head characteristics (e.g. tap, pump characteristics), which would enable the identification of the effects (if any) of supply design/construction on VTEC incidence. Moreover, descriptions of source structural components (e.g. well casing) and other key features (e.g. depth, age) were also largely absent from reviewed investigations. The results draw parallels with the lack of supply descriptions previously observed in investigations focusing on *Cryptosporidium* incidence. This represents a clear limitation in the identification of risk factors associated with VTEC supply ingress and inappropriate/faulty structural components. For example, Ferguson *et al*. (2012) identified increased supply depth as a determining factor limiting VTEC contamination, attributed to effective pathogen retention with increasing well depth. Despite its potential importance, only 3/23 (13%) studies (explicitly) reported groundwater supply depth. Li *et al*. (2015) failed to detect VTEC irrespective of groundwater well depth, while Won *et al*. (2013) found no association between well depth and VTEC contamination, citing inadequate (private) supply maintenance as a possible mechanism for VTEC ingress.

The influence of varying levels of supply maintenance and surveillance could explain the higher VTEC detection values estimated for private supplies (Table 2.5). The results reaffirm the previously highlighted role of lax regulation (e.g. incorrect siting of private wells) and, potentially, lack of treatment for microbiological contamination of private supplies (Hexemer *et al*., 2008; Kreutswiser *et al*., 2011; Fox *et al*., 2016). Furthermore, higher VTEC detection rates among private supplies may also point to the prevalence of "direct" source contamination rather than widespread or aquifer-wide groundwater pollution. Pooled data based on (inferred) standards of supply construction and integrity indicate that "unimproved" supplies had elevated VTEC detection rates (22.5%) (Table 2.5). However, the findings require careful interpretation, as they largely reflect detection levels reported in a single study (Abia *et al*., 2017). Only two investigations explicitly analysed "unimproved" supplies, both of which derived from sub-Saharan Africa. Accordingly, any assessment of risk factors associated with supplies and levels of protection are precluded by (i) vague descriptions of groundwater supplies, (ii) the tentative nature of the supply classifications employed and (iii) the small number of investigations identified explicitly analysing "unimproved" supplies ($n = 2$).

2.3.4 Key insights and recommendations

Overall, a VTEC to generic *E. coli* sample detection ratio of 15:152 (9.9%) was derived from the investigations reviewed, providing a baseline for VTEC contamination rates in *E. coli*-contaminated domestic groundwater sources. Based on the relatively small number of investigations identified, the prevalence of inconsistent reporting and the limited number of data constituting the review dataset, a major recommendation deriving from this study relates to the pressing need for additional field-based studies/data on VTEC prevalence in domestic groundwater systems. Additional data are essential to improve established VTEC groundwater contamination baselines and accurately identify local contamination risk factors, which are, in turn, necessary to inform relevant multidisciplinary stakeholders. Similarly, investigations deriving from geographically diverse settings are required to obtain insights/data applicable within different (hydro)geological, climatic and (local) settlement (urban/rural) contexts. The following bespoke guidelines/recommendations are proposed, to improve the scope and integrity of, and insights obtained from, future VTEC groundwater investigations:

1. Implement explicit study reporting of (basic) summary statistics and methodology, particularly pertaining to (i) the incidence of *E. coli* and VTEC in groundwater analysed (*n* per sample/supply), (ii) the length of field sampling periods (e.g. month, year) and (iii) detailed descriptions of the groundwater sampling protocols employed (e.g. point of sampling).
2. Employ more temporally inclusive (i.e. "repeat") sampling strategies/designs in investigations, with the aim of providing more robust estimates of VTEC groundwater prevalence over time.
3. Identify and describe the groundwater supply type(s) analysed (e.g. hand-dug well, driven well), key infrastructural components (e.g. cap, casing), associated structural parameters (e.g. depth, age) and relevant (i.e. local) supply hydrogeological characteristics.

4. Implement molecular (VTEC) strain-typing techniques (e.g. PFGE, MLVA, whole-genome sequencing) to enable the effective elucidation of (catchment groundwater supply) VTEC contamination sources, pathways and ingress mechanisms.
5. Carry out more investigations employing tools that enable the identification of the VTEC serotypes present within domestic groundwater systems.

There are clear similarities among the key findings and recommendations derived from the *Cryptosporidium* literature review (see section 2.2.4) and the VTEC literature review. These include bias in source selection, inconsistent reporting, failure to (adequately) identify contamination sources and pathways, and lack of and/or ambiguous supply source descriptions. Many of these issues have been highlighted as recurrent and ubiquitous in a range of literature meta-analyses relating to microbiological contamination of groundwater (Hynds *et al*., 2014a; Andrade *et al*., 2018, 2020), which ultimately denotes a lack of integration among key disciplines.

3 Verotoxigenic *Escherichia Coli* Contamination Status of Private Wells

3.1 Introduction

Despite high VTEC notification rates and available geostatistical evidence linking high rates of reliance on groundwater with human VTEC exposure, no comprehensive study has analysed the incidence and prevalence of VTEC in private domestic groundwater supplies in Ireland. A number of studies have presented source–pathway–receptor models for private wells in relation to (FIO) generic *E. coli* or, in turn, have attempted to identify relevant (*E*. *coli*) contamination risk factors (e.g. Hynds *et al*., 2014b; O'Dwyer *et al*., 2014, 2017, 2018). However, the prevalence of VTEC strains in untreated groundwater supplies in Ireland remains largely unsubstantiated. Based on the application of QMRA tools, Hynds *et al*. (2014c) provided some insights into the (potential) burden of VTEC infection associated with private domestic supplies, predicting an overall CIR of 28.3/100,000 well users per year. This rate is noteworthy, as it is approximately six times higher than previously reported CIRs in Ireland and up to 40 times higher than reported EU means (ECDC, 2019). Notwithstanding this, obtaining (VTEC) field-validated data in Ireland remains an urgent necessity. Accordingly, this project aimed to identify VTEC to generic *E*. *coli* detection ratios, which will allow for the establishment of actual (private well) prevalence and improve current QMRA predictions and allow the implementation of predictive modelling. The approach adopted is highly relevant, as it represents the first Irish study providing a PCR-confirmed pathogenicity ratio based on a standard FIO. In addition to molecular VTEC identification (i.e. PCR-based assays that investigate the presence of the *eae*, *vtx1*, *vtx2* genes), the project also sought to identify the incidence of serogroups (e.g. *E*. *coli* O157), to ascertain the clinical importance of groundwater-borne VTEC. Indirectly, given the prevalence of groundwater treatment encountered among the supplies surveyed, the results presented may also provide some (limited) insights into the efficiency of groundwater treatment types in Ireland.

The specific tasks of this field-based VTEC project component were as follows:

1. Conduct a spatio-temporal analysis of VTEC (including serogroups) prevalence in private domestic groundwater supplies.
2. Update the current QMRA model (Hynds *et al*., 2014c) based on new field-validated data, providing an updated spatio-temporal estimate of the potential human health risks of the VTEC contamination of private domestic groundwater supplies unique to Ireland.
3. Develop seasonal (spring, summer, autumn, winter) environmental fate models assessing private well (VTEC) contamination risk relative to meteorological variables in light of climate change projections.

3.2 Methodology

3.2.1 Study area

The study area comprises two neighbouring administrative regions (counties) on the west coast of Ireland, Counties Galway (including the Galway–Mayo border) and Clare. The population of County Galway (excluding Galway city) is 179,390 (population density 29.4/km^2) and that of County Clare is 118,817 (population density 34.4/km^2), both significantly lower than the national average (population density 70/km^2). Both counties are characterised by primarily rural populations (Galway – 78.0%; Clare – 60.7%); however, numerous spatial divisions have moderate to high urban influence, principally centred around Galway and Limerick cities (CSO, 2019). Mean annual precipitation (1173.6 vs 977.6 mm) and relative humidity (76.8% vs 71.9%) are both significantly higher than the national average due to proximity to the Atlantic (Met Éireann, 2022). The quaternary geology of the area is heterogeneous, with Dinantian limestone tills dominating in east Galway's fertile agricultural pastureland and extending to east Clare, while much of south-west Galway and north Clare

are characterised by epikarstified limestone geology and absent (outcrop) or thin (< 3 m; subcrop) overlying subsoils. The study area's groundwater vulnerability classification, as assessed by Geological Survey Ireland (GSI) guidelines (Fitzsimons and Misstear, 2006), typically mirrors the quaternary geological profile within the region, with epikarstified areas being classified as extremely vulnerable. Land use is primarily for pastoral agriculture, and there is a high density of cattle and sheep in both areas.

3.2.2 Groundwater sampling and microbiological analysis

Participant recruitment and well sampling was undertaken during two 5-week phases, in late September and October 2019 (Phase 1) and during the same period in 2021 (Phase 2). Participants were recruited via dissemination of a study information sheet and expression of interest requests by email and via social media, with subsequent "snowball" distribution by recipients. Potential participants were offered free microbiological water testing in return for access to untreated drinking water from a private groundwater source. All participants received a microbial water quality report, including interpretations and recommendations, within 1 week of result availability. Groundwater vulnerability and agricultural and population factors were not taken into account in site selection. Registration was by return email (Phase 1) and electronic registration form (Phase 2), both of which captured informed consent. Emails were initially targeted to employees of the University of Galway (Phase 1) and well owners who lived within 90 minutes of the laboratory in Galway city (Phase 2). Overall, 52 participants contributed to the study. Phase 1 examined 21 wells (19 in Galway and 2 in Mayo), while Phase 2 examined 31 different wells (13 in Galway and 18 in Clare; Figure 3.1).

Untreated (raw) water samples were taken directly from a pre-disinfected (70% ethanol) household tap after a 2-min flushing period. Samples (30 L) were collected in 6 × 5-L sterile polypropylene containers, transported immediately back to the laboratory and processed within 4 hours of collection by the CapE method (Morris *et al*., 2016). Briefly, 100 mL of each sample was used for most probable number (MPN) enumeration of indicator organisms (coliforms and *E. coli*) using the Colilert-18 Quanti-Tray 2000 (IDEXX, Technopath, Limerick, Ireland) in accordance with the manufacturer's instructions. The remaining volume of each 30-L sample was passed through a modified IDEXX Filta-Max system (IDEXX Laboratories, Inc., Westbrook, ME, USA) with an in-line high-pressure stainless steel 142-mm filter holder (Millipore Corp., Bedford, MA, USA) attached to the outlet hose of the sampling rig for bacterial capture. Samples were pumped through one (or more, when pores became blocked) 0.45-µm MF-Millipore membrane filter (Merck Millipore Ltd, Cork, Ireland) to capture microorganisms, which were subsequently enriched overnight at 42°C in 100 mL of buffered peptone water. The Filta-Max system was flushed with 15–20 L of deionised water between samples to prevent carry-over, and disinfected via instillation with Virkon overnight. Contamination controls were included on each sampling day, consisting of filtering 10 L of deionised water through the system and subsequent enrichment of the 0.45-µm MF-Millipore membrane filter. Following the overnight enrichment of microorganisms, DNA was extracted from cells as follows: 1 mL of enrichment broth was centrifuged at 21,000,000 relative centrifugal force (RCF) for 10 min and the pellet was resuspended in 200 µL of sterile molecular-grade water; cells were then lysed by heating to 95°C for 10 min and centrifuging at 21,000 RCF for 10 min; the supernatant containing well sample DNA was transferred to a clean microcentrifuge tube and stored at –20°C (Morris *et al*., 2016).

3.2.3 Molecular detection of VTEC-associated genes

Three multiplex real-time PCR assays were used to screen for the presence of VTEC gene targets in all wells (Nielsen and Andersen, 2003; Perelle *et al*., 2004, 2005). The first assay detected the *stx1*/*stx2* Shiga toxin genes and the *eae* gene, which encodes intimin. Samples identified as *stx1*- or *stx2*-positive were defined as VTEC positive and subsequently serotyped for O157, O26, O103, O111, O145 and O104 serogroup gene targets using the remaining two multiplex real-time PCRs, as set out in ISO 13136 (ISO, 2012). Primers and probes were sourced from Integrated DNA Technologies. All assays used a 20-µL reaction mix consisting of primers at a final concentration of 0.5 µM, probes at a concentration of 0.2 µM, 1 × TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 2.5 µL of TaqMan Exogenous

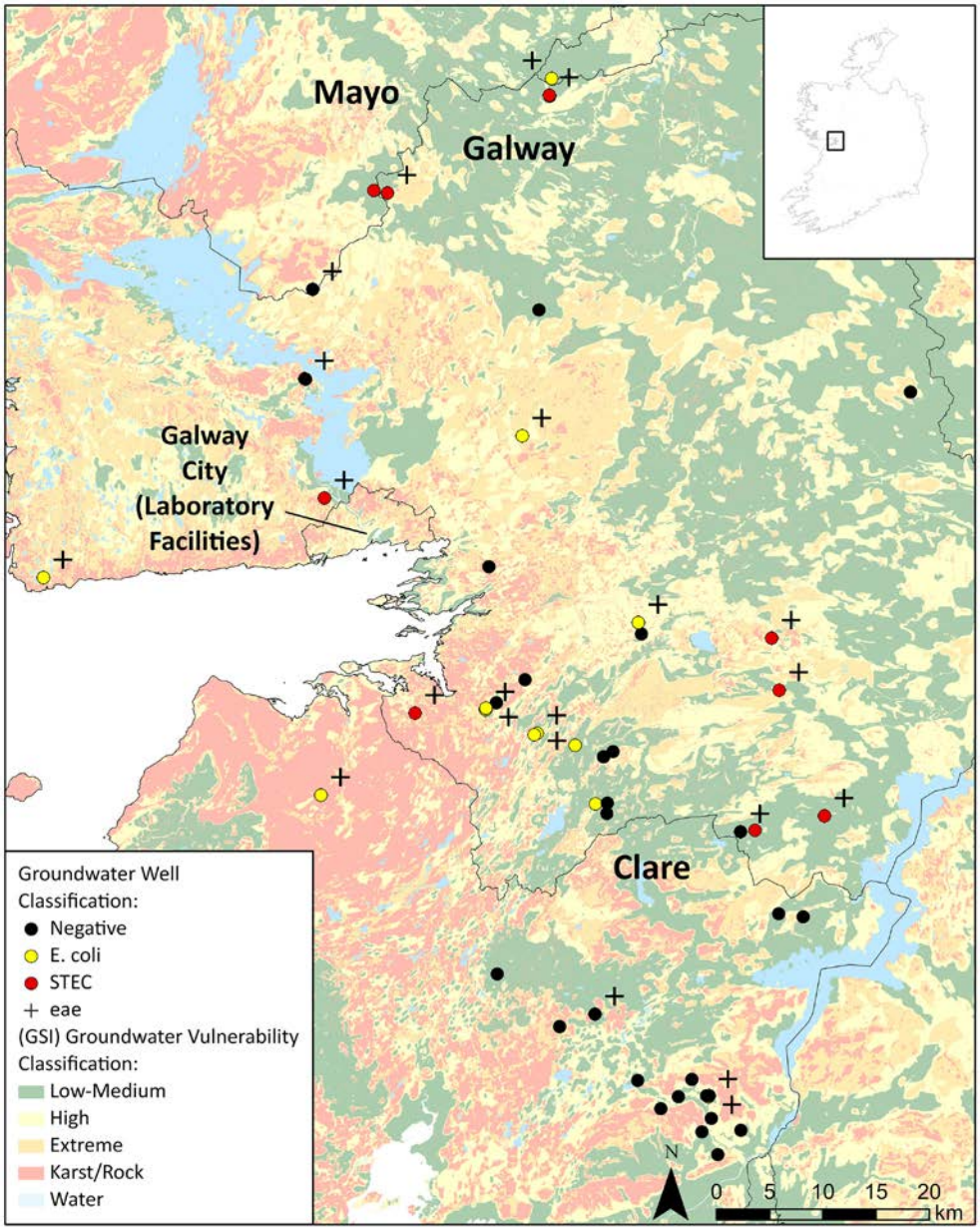


Figure 3.1. Location of sampled private groundwater wells and VTEC gene analysis findings in relation to GSI groundwater vulnerability classification. The "*E. coli*" classification (yellow circles) indicates *E. coli* detection by Colilert-18 test. The "VTEC/STEC" classification (red circles) indicates that either the Shiga toxin *stx1* or *stx2* gene was detected. The "+*eae*" classification indicates that the *eae* gene was detected.

internal positive control (IPC) reagents, 0.5 µL of IPC DNA and 5 µL of well sample DNA. A no-template control containing 5 µL of deionised water and appropriate negative controls containing a 5-µL sample of bacterial DNA not containing any of the VTEC gene targets were included in each PCR assay. Reactions were performed using an ABI 7500 Fast PCR system (Applied Biosystems) as follows: one cycle of 50°C for 2 min, one cycle of 95°C for 20 s, 40 cycles of 95°C for 3 s/60°C for 30 s, followed by one cycle of cooling at 50°C. Results were interpreted with reference to experimental and sample contamination controls.

3.2.4 Risk factor extraction and delineation

Three risk factor categories, intrinsic, specific and infrastructural, were recorded for each domestic groundwater source based on a previously developed predictive model for groundwater *E. coli* contamination in Ireland (O'Dwyer *et al*., 2018).

Intrinsic factors related to hydrological, geological and hydrogeological conditions; specific factors related to human and animal faecal contamination sources; and infrastructural factors related to the design, construction and locational characteristics of wells. Data for 27 variables were sourced and spatio-temporally linked to each sampled groundwater supply (Table 3.1). The geographical coordinates of each sampling point were derived from participant postcodes (Eircode) or, if wells were located >20 m from the household, using global positioning system devices, and were integrated into a geographical information system (GIS) environment for spatial interpolation. All spatio-temporal analyses were conducted on ESRI ArcGIS Pro version 2.9.1.

Hydrogeological data comprising *"intrinsic"* variables (e.g. groundwater vulnerability) were sourced from datasets collated by GSI (Table 3.1). Rainfall data were extracted from a high-resolution gridded (1 km^2) annual (2019–2021) dataset produced by the Irish

Table 3.1. Intrinsic, specific and infrastructure (ISI) risk factor data collated and assessed for association with groundwater supply contamination

Risk factor	Data source	Measurement level
Intrinsic		
Subsoil permeability	GSI	Ordinal
Groundwater vulnerability		
Aquifer type		Dichotomous
Karst bedrock (yes/no)		
Groundwater recharge coefficient		Continuous
5-day mean rainfall (mm)[a]	Met Éireann	
10-day mean rainfall (mm)[a]		
30-day mean rainfall (mm)[a]		
5-day max rainfall (mm)[a]		
10-day max rainfall (mm)[a]		
30-day max rainfall (mm)[a]		
Specific		
SA population density (persons/km^2)	CSO[b]	Continuous
SA DWWTS density (units/km^2)		
SA livestock density (head/km^2)	CSO (2012b)	
SA cattle density (head/km^2)		
SA sheep density (head/km^2)		
Infrastructural		
Well age (y)	Questionnaire	Continuous
Well depth (m)		
Well type	Questionnaire/inspection	Dichotomous
Well head protected by manhole	Inspection	
Well head sealed with casing cap		
Well head 150 mm above ground level		
DWWTS within recommended offset distance (30/40 m)[c]		
DWWTS upslope of well		
Farm pollution source within recommended offset distance (25 m)[d]		
Farm pollution source upgradient of well		
Well head fenced off (>3 m) from grazing animals		

[a]Rainfall data calculated in mm per WFD-designated groundwater body.

[b]Census of Ireland 2016 (CSO, 2023).

[c]Offset distance as recommended by IGI guidelines for groundwater well siting and completion (IGI, 2007).

Meteorological Service (Met Éireann). The grid dataset comprises total daily rainfall values (mm) derived from 24-hour weather station records, which were geo-statistically interpolated for the full extent of Ireland. A methodological description of dataset construction, including quality control and interpolation guidelines, is provided by Walsh (2016). Briefly, a GIS workflow was created to spatio-temporally link daily rainfall grid values to the "cumulative" area of Water Framework Directive (WFD) (Directive 2000/60/EC) designated groundwater bodies. Sampled groundwater supplies were spatially linked to their corresponding WFD catchment area, permitting calculation of antecedent rainfall values (mean and cumulative total) pertinent to each groundwater sample (lagged 5-, 10- and 30-day intervals based on sampling date) as per Andrade *et al*. (2022).

"Specific" risk factor data (e.g. septic tank density) were extracted from the Central Statistics Office (CSO) Census of Ireland 2016 (CSO, 2023) and Census of Agriculture 2010 (CSO, 2012b) datasets. CSO 2016 census data were spatially linked to 18,488 pre-defined geopolitical subdivisions called "small areas" (SAs) (mean area = 3.8 km^2). These represent the smallest legally defined administrative areas within Ireland and are the highest resolution geographical unit available for statistical compilation. Conversely, Census of Agriculture 2010 data (CSO, 2012b) are available at only a coarser electoral district (ED) resolution (mean area = 20.6 km^2). To provide more accurate livestock density estimates, geoinformatics tools were used to combine the latest (Ireland) Coordination of Information on the Environment (CORINE) land use dataset (EPA, 2018) with the agricultural census data, to calculate livestock density (head/km^2) in the land area under rural use. As above, all "specific" risk factor variables were spatially linked to each sampled groundwater source at SA level.

"Infrastructural" data were collated via a participant questionnaire (completed with all well owners) and/or by visual inspection by the researchers during sampling, as indicated in Table 3.1. Specifically, each well head was assessed for compliance with the Institute of Geologists of Ireland (IGI) guidelines for groundwater well siting and completion (IGI, 2007). The setback distance and gradient of the well head from potential contamination sources (e.g. DWWTSs, grazing livestock) were also measured. Well head assessment was undertaken by recording/measuring the presence or absence of (i) a well-drained concrete or block-built manhole with cover, (ii) a protective casing protruding ≥ 150 mm above ground level and (iii) a vermin-proof casing cap. All infrastructural variables, excluding well depth and age, were treated as dichotomous (presence/absence) variables for risk factor analysis. A full data codebook is available in the supplementary materials (Table S2).

3.2.5 Statistical analysis

Prior to analyses, all independent variables were evaluated for normality via quantile–quantile plots and a series of Shapiro–Wilk tests. Several variables exhibited non-normal distributions; thus, non-parametric approaches were employed for all subsequent analyses. Risk factor assessment was undertaken via a series of bivariate hypotheses testing for dependent variables of interest, including presence of *E. coli*; VTEC; VTEC + *eae*; the *stx1, stx2, eae* genes; and each of the six main serogroup markers. Mann–Whitney *U* or chi-squared tests were employed as appropriate based on independent variable types (i.e. dichotomous, categorical, ordinal, continuous). All statistical analyses were performed using SPSS Statistics 27 (IBM) and a threshold of $p \leq 0.05$ was used for statistical significance, as per convention.

3.3 Results

3.3.1 Prevalence of E. coli *in groundwater*

During the first sampling phase (in 2019), 52.4% of supplies ($n = 11$) tested positive for *E. coli*, with a mean concentration of 59.2 MPN/100 mL (median 7.5 MPN/100 mL) (Figure 3.1, Table 3.2). During the second sampling period (in 2021), the prevalence of *E. coli* in groundwater was lower, with only 29% of supplies ($n = 9$) testing positive. The mean concentration of *E. coli* in groundwater during this sampling phase was 2.9 MPN/100 mL (median 8.4 MPN/100 mL). Overall, 38.5% of wells tested positive for *E. coli,* with a median concentration of 8.5 MPN/100 mL.

3.3.2 Detection of VTEC-associated virulence factors in groundwater

Shiga toxin genes *stx1* or *stx2* were detected in 8/11 (72.7%) and 0/9 (0%) *E. coli*-positive groundwater

Table 3.2. Prevalence of *E. coli* in private groundwater wells

Sampling phase	*E. coli* detected *n* (%)	Mean *E. coli* MPN/100 mL *n*[a] (SD)	Median *E. coli* MPN/100 mL *n*[b]
Phase 1 (Sept/Oct 2019)	11 (52.4)	59.2 (198.1)	7.5
Phase 2 (Sept/Oct 2021)	9 (29.0)	2.9 (7.5)	8.4
Total	20 (38.5)	25.6 (129.0)	8.5

[a]Mean MPN/100 mL of all wells sampled during the period.
[b]Median MPN/100 mL of *E. coli*-positive wells during the period.
SD, standard deviation.

samples from 2019 and 2021, respectively, equating to an overall VTEC to generic *E. coli* detection ratio of 8:20 (40%) (Tables 3.3 and 3.4). Overall, Shiga toxin genes were detected in 10/52 wells (19.2%), including two wells where *stx2* genes were detected in the absence of viable *E. coli,* indicating the presence of dead or viable but non-culturable VTEC cells, or free or inducible *stx*-containing phage. A significant difference was found, with respect to *stx1* and/or *stx2* detection rate(s), between wells sampled in 2019 (9/21; 42.9%) and those sampled during the same period in 2021 (1/31; 3.2%). However, similar rates of *eae* gene detection were encountered during each year: 9/21 (42.9%) in 2019 and 13/31 (41.9%) in 2021. One-third (33.3%) of wells sampled in 2019 and 15.4% of wells overall were positive for both a Shiga toxin gene and the *eae* gene. Nine of 10 *stx1*- and/or *stx2*-positive samples (90%) were also positive for one or more gene targets for the most common VTEC serogroups associated with human infection; multiple serogroup markers were detected in 4/10 samples, with O145 ($n=6$), O157 ($n=5$) and O103 ($n=4$) being most prevalent. Multiple VTEC serogroup markers were detected in samples from wells with *E. coli*

Table 3.3. Detection of VTEC-associated virulence factors in groundwater wells

Sampling phase	*stx1* *n* (%)	*stx2* *n* (%)	*stx1* and/or *stx2* *n* (%)	*eae* *n* (%)	*stx1* and/or *stx2* and *eae* *n* (%)
Phase 1 (Sept/Oct 2019)[a]	5 (23.8)	6 (28.6)	9 (42.9)	9 (42.9)	7 (33.3)
Phase 2 (Sept/Oct 2021)[b]	0 (0)	1 (3.2)	1 (3.2)	13 (41.9)	1 (3.2)
Total[c]	5 (9.6)	7 (13.5)	10 (19.2)	22 (42.3)	8 (15.4)

[a]21 wells.
[b]31 wells.
[c]52 wells.

Table 3.4. Detection of VTEC serogroup-specific markers in *stx1* and/or *stx2*-positive groundwater samples

Phase	Well	*stx1*	*stx2*	*eae*	*E. coli* MPN/100 mL	VTEC serogroup(s)
1	1	+	+	–	12.1	O104
1	2	–	+	+	1	O157
1	7	+	+	+	59.8	O103, O111, O145, O26
1	10	+	–	–	6.3	O145
1	13	+	–	+	920.8	O103, O104, O145, O157, O26
1	14	–	+	+	18.9	O103, O145, O157
1	15	–	+	+	214.3	O103, O145, O157
1	17	–	+	+	0	O145
1	18	+	–	+	1	O157
2	29	–	+	+	0	None

+, gene detected; –, gene not detected.

contamination at concentrations of > 19 MPN/100 mL (r_s = 0.901; p = < 0.001).

3.3.3 *Bivariate risk factor analysis of microbial contamination*

In the "intrinsic" category of independent variables (potential risk factors), the presence of generic *E. coli* was statistically significantly associated with increased (i.e. above average) 5-day and 10-day mean and cumulative antecedent rainfall, and underlying karstic bedrocks (Table 3.5). Detection of generic *E. coli* was also associated with the presence of an adjacent (< 100 m) DWWTS upgradient of the well head (χ^2 = 11.124; p = 0.001), but was not significantly associated with detection of VTEC (p = 0.242) or pathogenic *E. coli* (*eae*-positive) (p = 0.202). VTEC presence (U = 2.126; p = 0.034) and VTEC + *eae* presence (U = 2.157; p = 0.030) were both associated with increased 30-day mean antecedent rainfall. The presence of VTEC O157 was associated with increased 30-day cumulative antecedent rainfall (U = 2.522; p = 0.008), whereas detection of serogroup O145 and, independently, *eae* was associated with

Table 3.5. Independent groundwater risk factors associated with well contamination by *E. coli* type and/or gene presence

Dependent variable	Independent variable	Standardised test statistic	Significance (p-value)
E. coli	DWWTS upgradient of well	11.124[a]	0.001[c]**
	Karst bedrock	5.194[a]	0.041[d]*
	Cattle density (head/km^2)	2.296[b]	0.022[c]*
	10-day mean rainfall (mm)	2.833[b]	0.005[c]*
	5-day mean rainfall (mm)	2.475[b]	0.013[c]*
	10-day max. rainfall (mm)	3.228[b]	0.001[c]**
	5-day max. rainfall (mm)	2.550[b]	0.011[c]*
VTEC	Well depth (m)	–2.444[b]	0.024[d]*
	30-day mean rainfall (mm)	2.126[b]	0.034[c]*
VTEC + *eae*	DWWTS density (units/km^2)	2.246[b]	0.023[d]*
	30-day mean rainfall (mm)	2.157[b]	0.030[d]*
stx1	10-day max. rainfall (mm)	1.957[b]	0.048[d]*
eae	5-day mean rainfall (mm)	1.965[b]	0.049[c]*
	10-day max. rainfall (mm)	2.168[b]	0.030[c]*
	DWWTS density (units/km^2)	2.243[b]	0.025[c]*
O145	10-day mean rainfall (mm)	2.574[b]	0.010[d]*
	5-day mean rainfall (mm)	2.574[b]	0.010[d]*
	10-day max. rainfall (mm)	2.574[b]	0.010[d]*
	5-day max. rainfall (mm)	2.574[b]	0.010[d]*
O157	30-day max. rainfall (mm)	2.522[b]	0.008[d]**
O104	Sheep density (head/km^2)	2.089[b]	0.044[d]*

[a]**Pearson's chi-squared test.**

[b]**Mann–Whitney *U*-test.**

[c]**Asymptotic significance is displayed.**

[d]**Exact significance is displayed.**

***p < 0.05; **p < 0.01.**

increased short-term (5- to 10-day) mean/cumulative antecedent rainfall (Table 3.5).

In the "specific" risk factor category, higher mean cattle density was associated with the presence of generic *E. coli* (mean rank 22.69 vs 32.6 head/km^2; $U = 2.296$; $p = 0.022$), with no significant association found between cattle density and the presence of VTEC ($p = 0.265$), *stx2* ($p = 0.075$) or *eae* ($p = 0.067$). The presence of *eae* was, however, statistically associated with a higher mean DWWTS density (mean rank 22.47 vs 32.0 units/km^2; $U = 2.243$; $p = 0.025$), as was the presence of VTEC + *eae* ($U = 2.226$; $p = 0.023$). There was a statistically significant association between mean sheep density and the presence of VTEC O104 (mean rank 4.5 vs 9.5 head/km^2; $U = 2.089$; $p = 0.044$). Analyses of "infrastructural" variables revealed a significant inverse association between mean well depth and presence of VTEC (mean rank 19.44 vs 10.0 m; $U = –2.243$; $p = 0.024$), with this association not found for generic *E. coli* ($p = 0.706$).

3.4 Discussion and Conclusions

Ireland consistently reports the highest incidence of laboratory-confirmed VTEC infection in the EU, with latest figures suggesting that national CIRs are approximately eight times the EU average (Boudou *et al*., 2021). A review of Irish VTEC outbreak data for the period 2004–2012 found that the second most significant transmission route was waterborne spread from untreated or poorly treated private water supplies (55/219; 25.1%) (Garvey *et al*., 2016). However, to date, there has been no direct investigation of VTEC prevalence in private groundwater supplies in Ireland. Accordingly, the current study sought to identify and elucidate the presence of VTEC in private groundwater supplies in mid-west Ireland, a region characterised by particularly high rates of VTEC enteritis (Boudou *et al*., 2021).

A marked difference was found between sampling Phases 1 (2019) and 2 (2021) with respect to *E. coli* detection rate (52% vs 29%), *E. coli* mean concentration (59 MPN/100 mL vs 3 MPN/100 mL) and VTEC detection based on the presence of *stx1*/*stx2* genes (43% vs 3%) (Table 3.2). While some of these differences may be attributed to a slight change in study area, sampling Phases 1 and 2 were undertaken in analogous regions with respect to hydrogeological setting, contaminant source density and local climate; thus, location is unlikely to be the only factor driving these deviations. A recent study by Latchmore *et al*. (2022) reported that *E. coli* detection rates in private drinking wells across Ontario, Canada, decreased by approximately 50–80% in the period from March to December 2020, i.e. a period characterised by COVID-19 non-pharmaceutical interventions (travel restrictions, stay-at-home orders, etc.), compared with a "normal" period (based on detection rates from 2016 to 2019). Previous studies (e.g. Shani *et al*., 2013; Zhu *et al*., 2020) have reported that extended and/or increased pumping (and subsequent drawdown (i.e. change in water table level)), as might be expected during COVID-19 stay-at-home orders because of increased numbers of residents within households for longer periods, reduce the effective microbial load gaining entry to groundwater sources as a result of hydraulic and ecological factors. These factors may include extended subsurface pathways due to spatially increased zones of contribution, resulting in increased attenuation via filtration (longer pathways), natural die-off (increased time of travel) and ecological predation. Similarly, decreased connectivity between contaminant sources (e.g. on-site sanitation, grazing pastures) and the water table, due to increased drawdown, may lead to decreased subsurface pathogen loading (Latchmore *et al*., 2022) and may explain the marked decrease in *stx1*/*stx2* detection rates found during the current study (i.e. 43% vs 3%). Moreover, the lower detection rates in 2021 may be associated with an increase in soil temperature during this sampling campaign; average soil temperatures (at 10 cm depth) in the region were recorded as 13.4°C during Phase 2, 2.8°C higher than during Phase 1 (10.6°C, October 2019) (Met Éireann, 2022). Previous studies suggest that cooler temperatures promote survival of VTEC in freshwater (Rice *et al*. 1992; Watterworth *et al*., 2006), which may also be a contributing factor to the lower detection rates reported, although further research is required to confirm this.

QMRA has been identified as a method of choice for estimating burdens of disease associated with waterborne illness, and particularly with respect to private (unregulated) supplies, as they are typically characterised by a lack of periodic monitoring, governmental oversight and effective treatment (Petterson and Ashbolt, 2016; Owens *et al*., 2020). To date, development of QMRAs for estimating the

burden of groundwater-borne VTEC has largely relied on VTEC to generic *E. coli* ratios (defined as "the estimated laboratory-confirmed pathogenic VTEC to *E. coli* ratio") estimated from a range of aquatic systems (e.g. Soller *et al*., 2010; Haas *et al*., 2014). The present study found a VTEC to generic *E. coli* detection ratio of 4:10 (40%), which is double the recent estimate for groundwater wells in Europe and four times the mean global detection ratio estimate (see Chique *et al*., 2021). Moreover, in a recent review of global groundwater studies, Chique *et al*. (2021) identified mean global private groundwater sample-specific and source-specific (i.e. well-specific) VTEC detection rates of 0.6% (16/2471) and 1.3% (25/1998), respectively. The present study, although admittedly much smaller in scale and more limited in geographical spread than these previous studies, reports a much higher overall groundwater sample- and source-specific VTEC detection rate, of 19.2% (10/52), which, to our knowledge, represents the VTEC highest detection rate reported to date.

The dominantly detected Shiga toxin gene was *stx2,* which was present in 7 of 10 positive wells; this 70% detection rate closely mirrors that of *stx2* associated with notified VTEC enteritis in Ireland (317/894, 73.4%), according to the latest available annual epidemiological report (HPSC, 2019b). However, over half of human infection isolates with *stx2* also carried *stx1* (339/656, 51.6%), while only 2 of 10 wells (20%) were positive for both *stx* genes in the current study. As 43.1% of human infection-associated VTEC isolates positive for both *stx1* and *stx2* in Ireland belong to the O26 serogroup (HPSC, 2019b), the present authors consider that this difference is likely to be due to low levels of VTEC O26 being identified because of the timing of sampling campaigns, as discussed below.

The *eae* gene was detected in 80% of wells positive for Shiga toxin genes *stx1* and/or *stx2*. A recent scientific opinion on the pathogenicity assessment of VTEC carried out by the European Food Safety Authority (EFSA) Panel on Biological Hazards concluded that carriage of *eae* can be considered a general indicator of a VTEC strain with a high potential to cause severe infection (characterised by HUS, hospitalisation or HC) (Koutsoumanis *et al*., 2020). However, *eae* is not considered a definitive marker for severe infection. Not all intimin-positive strains cause severe infection. Neither is *eae* a requisite for severe infection; several alternative enterocyte attachment mechanisms are also implicated. Moreover, the aforementioned panel's analysis of the European Centre for Disease Prevention and Control's European Surveillance System VTEC data from 2012–2017 found that *stx2*-carrying, *eae*-positive, VTEC enteritis-causing strains were associated with higher rates of HUS (160/904, 17.7%), hospitalisations (314/748, 42%) and HC (366/1240, 29.5%) than both *stx1*-carrying, *eae*-positive strains (HUS 1.2%, hospitalisations 27.4%, HC 19.3%) and *stx2*-carrying, *eae*-negative strains (HUS 2.7%, hospitalisations 24.3%, HC 11.7%). The pathogenicity assessment study found that, along with *eae*, specific *stx2* subtypes (*stx2a, stx2c* and *stx2d*) are better indicators of the likelihood of severe infection than serogroup designation, leading the EFSA to recommend that standard molecular characterisation is revised to include *stx* subtyping (Koutsoumanis *et al*., 2020). Herein, the *stx2* + *eae* gene combination was detected in 6/52 (11.5%) wells, demonstrating a high likelihood of clinically relevant VTEC presence. This virulence gene combination (virulotype) was the most frequently reported in severe human VTEC cases in the EU/EEA in 2019 (ECDC, 2021) and 2020 (ECDC, 2022), the most recent year for which data are available.

4 Preliminary Investigation of *Cryptosporidium* Contamination in Private Domestic Supplies

4.1 Introduction

This project component was grounded in (i) the potential importance of *Cryptosporidium* as a (waterborne) enteric pathogen in Ireland and (ii) the complete ambiguity relating to its prevalence in private groundwater supplies. In contrast to VTEC, which relies on some (basic) insights obtained from (generic) *E. coli* incidence and application of QMRA tools, the association between *Cryptosporidium* and domestic private supplies within Ireland still remains uncertain. To date, no research has been undertaken on the prevalence of *Cryptosporidium* in private (unregulated) domestic groundwater supplies. This is a major research gap considering that available data derived from public groundwater supplies located in agricultural settings suggest that the likelihood of (oocyst) groundwater ingress and contamination is relatively high. Given the multiple local risk factors potentially associated with *Cryptosporidium* contamination of groundwater supplies, a spatio-temporal approach was implemented to investigate its prevalence, the role of different (local) settings and the potential influence of seasonality.

4.2 Methodology

4.2.1 Supply source selection, study design and field sampling

A similar approach to that outlined in section 3.2 was employed in terms of supply source identification and ensuing selection. Supply owners were identified via social media and/or email invitation through University College Cork (UCC) and National University of Ireland Galway list servers. However, in this case, postal addresses were obtained from each well owner, with geographical coordinates geo-referenced and integrated into a GIS environment with ED-level CSO livestock density (head/km^2) and septic tank density (km^2) data, along with and a range of GSI hydrogeological data (e.g. groundwater vulnerability). Subsequently, and where possible subject to availability, groundwater supplies were selected based on different local parameters, but primarily based on (dispersed) spatial distribution, (high) livestock density and groundwater susceptibility to (surface) contaminants. This approach was taken to prioritise sampling in heterogeneous settings with a (perceived) higher likelihood of both (i) *Cryptosporidium* environmental prevalence and (ii) groundwater supply contamination. Field sampling was divided into two distinct phases to account for seasonal variation and reported cryptosporidiosis notifications within Ireland. The first sampling phase was conducted in October 2019 (autumn) with a delayed (due to the COVID-19 pandemic) second phase restricted to February/March 2022 (spring). Sampling phases had different geographical scopes centred in Counties Cork and Kerry (autumn 2019) and Tipperary and Limerick (spring 2022), to account for (potential) regional variability (Figures 4.1 and 4.2). In total, 40 groundwater supplies were surveyed (one sample collected from each) and evenly distributed between the two phases ($n = 20$ per phase). At all times, sample collection prior to treatment (if available) was given preference. For *Cryptosporidium* analysis, sample volume varied per location, with a minimum limit of 10 L. *Cryptosporidium* samples were concentrated directly from the tap into a housing unit containing a Filta-Max filter (IDEXX). An additional 500 mL of groundwater (stored in sterile bottles) intended for FIO analysis was also obtained from each supply. Filta-Max filters and (500 mL) bottles were placed in a cool box and transported to the laboratory facilities of City Analysts Ltd (Shannon, Co. Clare) and the School of Biological, Earth and Environmental Sciences at UCC, and processed within 24 and 4 hours, respectively.

4.2.2 Risk factor variables and spatio-temporal geo-database

As above, three main risk factor categories were used to group local variables (source infrastructure, contaminant sources, hydrogeological setting). This enabled the establishment of a “spatio-temporal” database of potential risk factors associated with the *Cryptosporidium* contamination of private supplies,

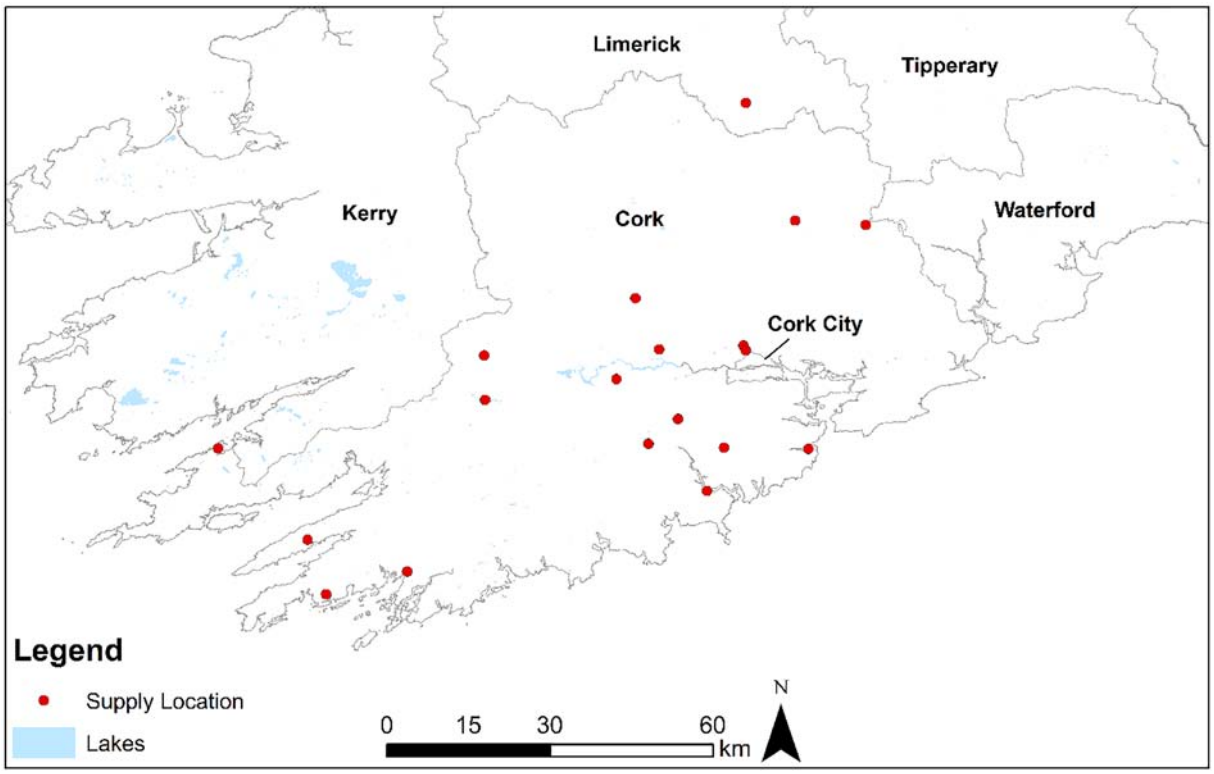


Figure 4.1. Location of private supplies analysed for *Cryptosporidium* (oocyst) presence during the first sampling phase (autumn 2019) in the south of Ireland.

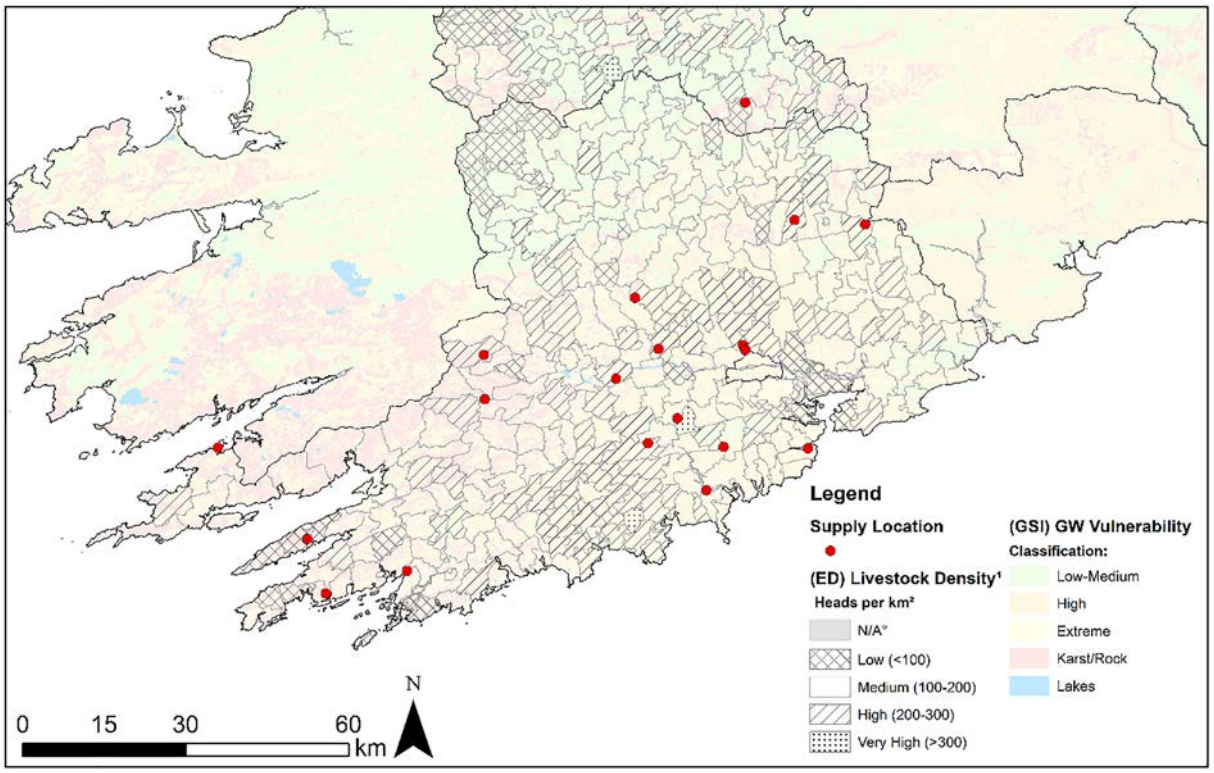


Figure 4.2. Location of private supplies analysed for *Cryptosporidium* (oocyst) presence during the second sampling phase (spring 2022) in mid-west Ireland.

effectively integrating the two sampling phases. Variables comprised a combination of local features compiled during field surveys and data extracted from (spatial) national datasets (Table 4.1). Supply infrastructural data (e.g. supply age, depth) and local contamination sources (e.g. presence of septic tanks, livestock) were directly noted in the field through observation and/or supply owners. As outlined in

Table 4.1. Risk factor information pertaining to *Cryptosporidium* sampling sites (*n*=40)

Supply ID	Supply	Cover	Well head location	Livestock density	Septic tank density (units/km^2)	Groundwater vulnerability
1	Borehole	Steel	Below	Medium	10	Extreme
2	Borehole	Plastic	At ground	High	11	Rock/karst
3	Borehole	N/R	Below	Very high	12	Extreme
4	Borehole	N/R	N/R	High	10	Extreme
5	Borehole	Concrete	Above	Medium	9	Extreme
6	Borehole	Steel	Above	Low	4	Extreme
7	Borehole	Steel	Above	Medium	7	Rock/karst
8	Borehole	Concrete	At ground	Medium	9	Rock/karst
9	Hand-dug well	Concrete	At ground	Medium	9	Rock/karst
10	Borehole	Concrete	Below	High	15	Extreme
11	Hand-dug well	Stone	At ground	Medium	4	Rock/karst
12	Borehole	Steel	Below	High	3	Extreme
13	Borehole	None	At ground	Medium	4	Rock/karst
14	Borehole	Wood house	At ground	High	7	Extreme
15	Borehole	Concrete	Above	High	9	Rock/karst
16	Borehole	Concrete	Below	High	12	Extreme
17	Borehole	Stone	Above	High	6	Extreme
18	Borehole	Concrete	Above	High	7	Rock/karst
19	Borehole	Concrete	Above	High	6	Extreme
20	Borehole	Steel	Above	High	15	Extreme
21	Borehole	Concrete	Below	Medium	11	High
22	Borehole	Concrete	Above	Medium	6	Rock/karst
23	Hand-dug well	Concrete	At ground	Low	9	Extreme
24	Borehole	Stone	At ground	Medium	21	Moderate
25	Borehole	Steel	Below	Medium	7	Extreme
26	Borehole	None	At ground	Low	15	Moderate
27	Borehole	Wood house	Below	Low	7	Rock/karst
28	Borehole	Concrete	At ground	Low	7	Rock/karst
29	Borehole	Concrete	At ground	Medium	19	Moderate
30	Borehole	Plastic	Above	Medium	7	High
31	Hand-dug well	N/R	Below	High	10	Extreme
32	Hand-dug well	N/R	Above	Low	5	Extreme
33	Borehole	Concrete	Below	Medium	4	High
34	Borehole	Steel	At ground	High	5	Extreme
35	Borehole	Steel	Below	Medium	7	High
36	Borehole	Concrete	At ground	Low	8	Rock/karst
37	Borehole	Concrete	At ground	Medium	6	Rock/karst
38	Borehole	Stone	Above	Medium	8	Extreme
39	Borehole	Steel	Below	Medium	5	High
40	Borehole	Concrete	Above	High	5	High

N/R, not reported.

section 3.2, datasets from the CSO, GSI and Met Éireann were accessed to extract key agricultural and wastewater infrastructure and meteorological variables.

4.2.3 *Faecal indicator organism analysis*

FIO (coliforms and *E. coli*) analysis relied on Colilert-18 Quanti-Tray 2000 (IDEXX) culture kits using 100 mL of sampled groundwater.

4.2.4 **Cryptosporidium** *(oocyst) separation, concentration and identification*

Samples were processed in accordance with the guidelines of the European Communities (Drinking Water) Regulations, 2000 (S.I. No. 439/2000), with oocyst identification relying on the IFA method. Briefly, Filta-Max filters were decompressed, and contents eluted using (IDEXX) wash station apparatus. Contents were then concentrated by employing filtration/centrifugation, with oocyst separation from environmental debris achieved through immunomagnetic separation using labelled dynabeads. Subsequently, extracted material was stained using specific fluorescently labelled antibodies and DAPI (4′,6-diamidino-2-phenylindole), with (oocyst) identification relying on fluorescent microscopy.

4.2.5 *Statistical and risk factor analysis*

Risk factor analysis was undertaken in the same way as described in section 3.2.5.

4.3 Results

4.3.1 *Infrastructural characteristics of supplies and sources of contamination*

The main structural characteristics of the supplies analysed are summarised in Table 4.1. The majority of supplies analysed were classified as boreholes (35/40; 88%), with hand-dug wells being uncommon (5/40; 12%). The majority of supply sources surveyed had some sort of structural cover protection, mostly concrete and steel covers (26/40; 65%). Only one supply lacked any type of protective cover. Similarly, approximately half of supplies analysed had a protective cap (22/40; 55%). In terms of well head elevation relative to ground level, many were above ground, a factor that may be associated with lower contamination risk. Cattle were the most common type of livestock (observed/reported) in the vicinity of supplies (34/40; 85%), followed by pigs (4/40; 10%) and horses (2/40; 10%). Only one groundwater sample was collected following treatment (UV), with the majority of samples subject to *Cryptosporidium* (oocyst) detection being samples of raw (untreated) groundwater. At the ED level, estimated livestock density for the supplies analysed was generally "medium" (100–200 head/km^2) (18/40; 45%), followed by "high" (200–300 head/km^2) (14/40; 35%) (Figure 4.2). In turn, the number of septic tanks was generally small (≤ 10 units/km^2). All supplies analysed were located in areas considered of high groundwater vulnerability comprising surface rock/karst (15/40; 38%) or classified as having "extreme" vulnerability (18/40; 40%) (Figure 4.2).

4.3.2 *Faecal indicator organism prevalence*

Faecal coliforms were highly prevalent, with positive detection in 30/40 (75%) supplies analysed. In turn, lower detection rates were observed for *E. coli* (17/40; 42.5%). Overall, FIO results indicate frequent microbiological ingress and contamination among surveyed supplies. Other than the prevalence of animal sources in close proximity (< 30 m) to several supplies, no tangible association between (additional) contamination risk factors and FIO incidence was apparent.

4.3.3 **Cryptosporidium** *(oocyst) prevalence*

All groundwater samples collected during the first sampling phase ($n = 20$) were negative for *Cryptosporidium* oocysts. The negative results contrast with the abundance of potential animal sources (cattle) of *Cryptosporidium* and the high FIO incidence. The results may be (tentatively) linked to the "appropriate" levels of structural supply source protection encountered. During the second sampling phase, 2 of the 20 (10%) samples collected were positive for the presence of *Cryptosporidium* oocysts, with two oocysts/50 L and four oocysts/50 L detected, respectively.

4.3.4 Cryptosporidium *contamination risk factors*

Based on the findings of the second sampling phase, some insight into contamination risk factors can be ascertained. Although the sample size is small (supplies: $n = 20$; positive supplies: $n = 2$), both contaminated wells were situated in extreme and karst groundwater vulnerability areas (Figure 4.3). Moreover, a moderate level of agriculture was associated with the contaminated supplies. No risk factors were statistically associated with *Cryptosporidium* occurrence at the 95% confidence level; however, DWWTS density (per km^2) was associated with the contaminated supplies at the 90% confidence level ($U = 1.781$; $p = 0.072$).

4.4 Discussion and Conclusions

Overall, the *Cryptosporidium* contamination rates were low compared with those reported in international literature. The lack of positive samples during the first sampling phase (October 2019) indicates that the seasonal trend in cryptosporidiosis is potentially mimicked in the subsurface environment. International research has indicated that *Cryptosporidium* in groundwater exhibits distinct seasonal peaks across latitudes and that this is due to (i) agricultural life

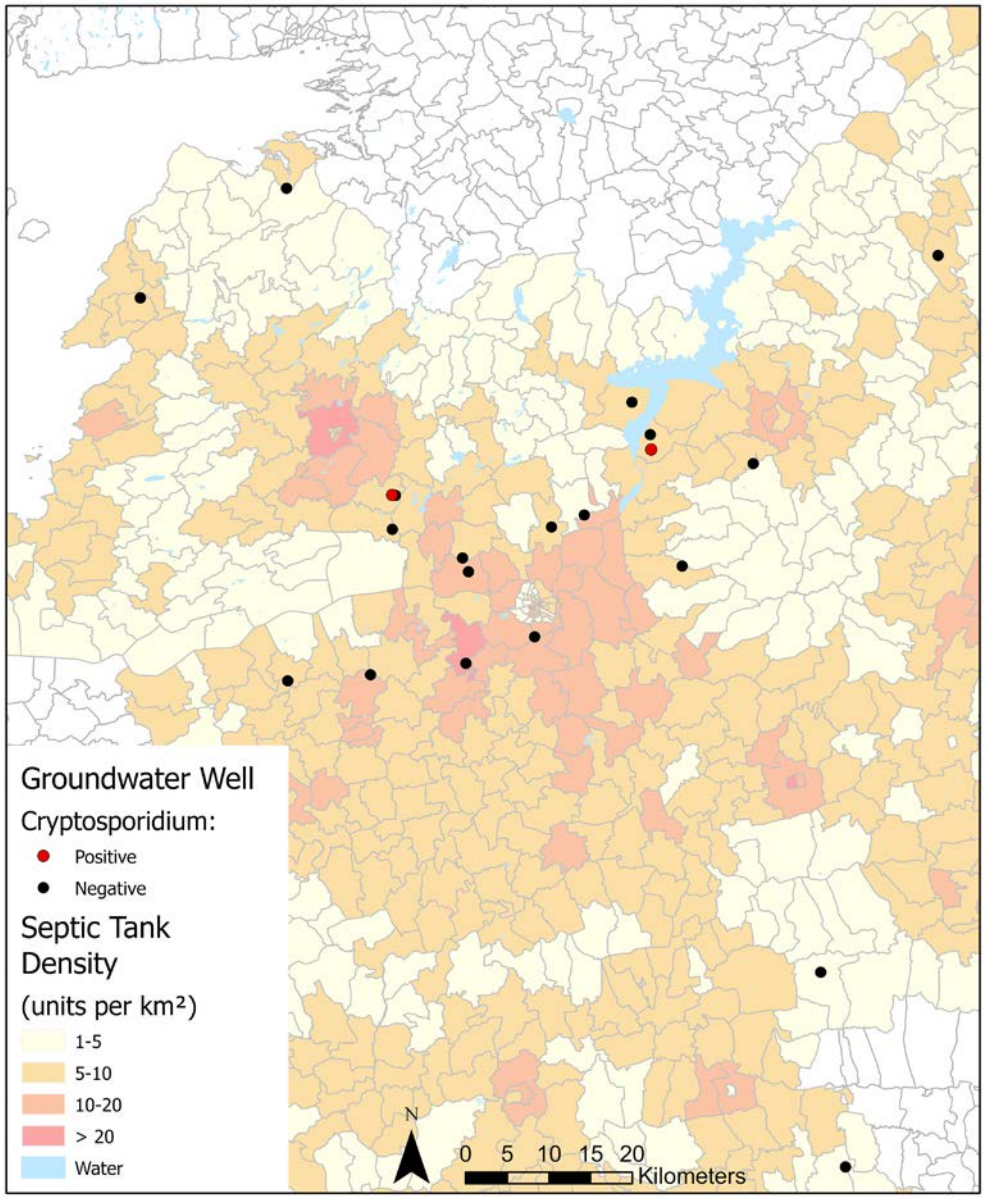


Figure 4.3. Location of private supplies analysed for *Cryptosporidium* (oocyst) presence (spring 2022) in relation to ED-level DWWTS density.

cycles, (ii) human waste production cycles and spatial profiles, and (iii) local climate patterns (e.g. action of rainfall in facilitating oocyst release from faecal material) (Sterk *et al*., 2016; Lal *et al*., 2019). From a hydrogeological viewpoint, aquifers overlain with limited subsoil are at increased risk of contamination, despite groundwater recharge (i.e. gradual gravitational percolation through pores in the soil matrix and/or bedrock fractures) being the most frequently reported ingress mechanism (in 12/13 studies; 92.3%) internationally. *Cryptosporidium* oocysts are characterised by their relatively large size (3–6 µm); thus, mobilisation through the subsurface profile to aquifers (i.e. recharge), despite oocysts remaining viable/infectious, is considered less likely to occur than with bacterial species (Robertson and Edberg, 1997). However, in areas where the subsoil layer is thin or absent, direct ingress into the aquifer is possible. Risk factor analysis, albeit rudimentary considering the small sample size, showed no association with hydrogeological, agricultural or infrastructural variables. However, there was a statistically significant association with DWWTS density at the 90% confidence level ($p = 0.072$). Previous studies tentatively linked on-site DWWTSs with *Cryptosporidium* occurrence in groundwater outside Ireland (O'Reilly *et al*., 2006), so this will be an important area of research moving forward. A key limitation of the current study is the lack of differentiation between *Cryptosporidium* species, as differentiating between species could provide further insight into contaminant source attribution and enhance risk factor identification.

5 Conclusions and Recommendations

5.1 Conclusions

This report provides a comprehensive overview of the state of the art of global research into the prevalence of two primarily waterborne pathogens in groundwater, namely VTEC and *Cryptosporidium*. This report also provides a detailed summary of findings arising from two field-based sampling activities quantifying the risks of exposure to both pathogens from Irish groundwater-derived drinking water supplies. Research in Ireland to date has concentrated on identifying risk factors associated with FIO (primarily *E. coli*); thus, the findings of the DESIGN project increase our knowledge and understanding of the potential impacts of contaminated groundwater on public health. Globally, results indicate that *Cryptosporidium* is a relatively common contaminant of groundwater resources, with both rural and urban areas equally susceptible. In contrast, VTEC is seldom found in groundwater resources internationally, with relatively few studies reporting significant issues with this pathogen. In Ireland, however, findings from the DESIGN project indicate that the opposite to is true, with a high incidence of VTEC-associated genes (*vtx1*, *vtx2*) being detected in groundwater supplies, with relatively few (5%, $n = 2/40$) supplies being positive for *Cryptosporidium*. Some evidence suggests an association between septic tank density and the contamination of groundwater with VTEC; however, this evidence should be interpreted with caution, as sample numbers in the project were quite small ($n = 51$). The most prominent finding of the project is the development of a VTEC to generic *E. coli* ratio, which was calculated as 40%, almost four times higher than the global average (9.9%). Further studies, of a more comprehensive nature, using a harmonised approach across time and space, are needed to build on these data. Little or no research has been carried out in Ireland in, for example, areas of low groundwater vulnerability, the east of the country and non-consolidated aquifer settings.

5.2 Recommendations for Research Priorities

5.2.1 Short term

- Develop a *Cryptosporidium* sampling regime with higher spatio-temporal resolution, to gain more insight into both the prevalence and the seasonality of the *Cryptosporidium* contamination of private groundwater wells in Ireland.
- Conduct a nationwide analysis of VTEC in groundwater supplies using both genetic and phenotypic analyses, to ascertain immediate and emergent risks.
- Develop a “microbial vulnerability” risk map for the island of Ireland, differentiating microbial from chemical contaminant transport in the subsurface environment.
- Carry out further research into potential solutions to mitigate the transfer of pathogens from animal and human sources to groundwater environments, to protect public health.
- Develop a nationwide multimedia information campaign targeted at private well users, to inform them of the risks of drinking contaminated water and how to protect their supplies.
- Analyse the impact of climate change on the dynamics of groundwater contamination in Ireland.

5.2.2 Medium term

- Develop and implement standardised and rapid methodologies for sample collection, testing and reporting with regard to pathogens in groundwater environments. Adopting a standardised approach will facilitate data sharing and geospatial analyses, supporting the development of validated risk assessment tools.
- Perform further research to differentiate between sources of contamination, allowing for more impactful policy development targeting appropriate sectors (e.g. agriculture or wastewater infrastructure).

- Prioritise the role of groundwater in the transmission and dissemination of other waterborne pathogens (e.g. campylobacter, norovirus) in national research strategies.
- Examine and implement behavioural interventions through enhanced communication with private well users, health professionals and those working in the animal and environmental sectors.

5.2.3 Long term

- Develop and implement guidelines relating to the appropriate management and surveillance of wastewaters and effluents, and their interaction with groundwater.
- Develop a centralised knowledge database in which information on groundwater quality data can be shared. Such a database would be hugely beneficial in assessing and monitoring groundwater resources outside the National Groundwater Monitoring Programme.
- Develop a strategic infrastructural plan to connect households most at risk to the public water supply.

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Abbreviations

CapE	Capture, amplify, extract
CIR	Crude incidence rate
CSO	Central Statistics Office
DESIGN	Detection of Environmental Sources of Infectious Disease in Groundwater Networks
DWWTS	Domestic wastewater treatment system
ECL	Electrochemilumisescence
ED	Electoral district
EEA	European Economic Area
EFSA	European Food Safety Authority
EPEC	Enteropathogenic *Escherichia coli*
EU	European Union
FIO	Faecal indicator organism
FISH	Fluorescence *in situ* hybridisation
GIS	Geographical information system
GSI	Geological Survey Ireland
HC	Haemorrhagic colitis
HUS	Haemolytic uraemic syndrome
IFA	Immunofluorescent assay
IGI	Institute of Geologists of Ireland
IMS	Immunomagnetic separation
IPC	Internal positive control
MLVA	Multiple-locus variable-number tandem-repeat analysis
MPN	Most probable number
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
POA	Population–outcome–agent
QMRA	Quantitative microbial risk assessment
RCF	Relative centrifugal force
SA	Small area
UCC	University College Cork
UV	Ultraviolet
VNTR	Variable-number tandem repeat
VT	Verocytotoxin
VTEC	Verotoxigenic *Escherichia coli*
WFD	Water Framework Directive
WMO	World Meteorological Organization

An Ghníomhaireacht Um Chaomhnú Comhshaoil

Tá an GCC freagrach as an gcomhshaol a chosaint agus a fheabhsú, mar shócmhainn luachmhar do mhuintir na hÉireann. Táimid tiomanta do dhaoine agus don chomhshaol a chosaint ar thionchar díobhálach na radaíochta agus an truaillithe.

Is féidir obair na Gníomhaireachta a roinnt ina trí phríomhréimse:

Rialáil: Rialáil agus córais chomhlíonta comhshaoil éifeachtacha a chur i bhfeidhm, chun dea-thorthaí comhshaoil a bhaint amach agus díriú orthu siúd nach mbíonn ag cloí leo.

Eolas: Sonraí, eolas agus measúnú ardchaighdeáin, spriocdhírithe agus tráthúil a chur ar fáil i leith an chomhshaoil chun bonn eolais a chur faoin gcinnteoireacht.

Abhcóideacht: Ag obair le daoine eile ar son timpeallachta glaine, táirgiúla agus dea-chosanta agus ar son cleachtas inbhuanaithe i dtaobh an chomhshaoil.

I measc ár gcuid freagrachtaí tá:

Ceadúnú

- Gníomhaíochtaí tionscail, dramhaíola agus stórála peitril ar scála mór;
- Sceitheadh fuíolluisce uirbigh;
- Úsáid shrianta agus scaoileadh rialaithe Orgánach Géinmhodhnaithe;
- Foinsí radaíochta ianúcháin;
- Astaíochtaí gás ceaptha teasa ó thionscal agus ón eitlíocht trí Scéim an AE um Thrádáil Astaíochtaí.

Forfheidhmiú Náisiúnta i leith Cúrsaí Comhshaoil

- Iniúchadh agus cigireacht ar shaoráidí a bhfuil ceadúnas acu ón GCC;
- Cur i bhfeidhm an dea-chleachtais a stiúradh i ngníomhaíochtaí agus i saoráidí rialáilte;
- Maoirseacht a dhéanamh ar fhreagrachtaí an údaráis áitiúil as cosaint an chomhshaoil;
- Caighdeán an uisce óil phoiblí a rialáil agus údaruithe um sceitheadh fuíolluisce uirbigh a fhorfheidhmiú
- Caighdeán an uisce óil phoiblí agus phríobháidigh a mheasúnú agus tuairisciú air;
- Comhordú a dhéanamh ar líonra d'eagraíochtaí seirbhíse poiblí chun tacú le gníomhú i gcoinne coireachta comhshaoil;
- An dlí a chur orthu siúd a bhriseann dlí an chomhshaoil agus a dhéanann dochar don chomhshaol.

Bainistíocht Dramhaíola agus Ceimiceáin sa Chomhshaol

- Rialacháin dramhaíola a chur i bhfeidhm agus a fhorfheidhmiú lena n-áirítear saincheisteanna forfheidhmithe náisiúnta;
- Staitisticí dramhaíola náisiúnta a ullmhú agus a fhoilsiú chomh maith leis an bPlean Náisiúnta um Bainistíocht Dramhaíola Guaisí;
- An Clár Náisiúnta um Chosc Dramhaíola a fhorbairt agus a chur i bhfeidhm;
- Reachtaíocht ar rialú ceimiceán sa timpeallacht a chur i bhfeidhm agus tuairisciú ar an reachtaíocht sin.

Bainistíocht Uisce

- Plé le struchtúir náisiúnta agus réigiúnacha rialachais agus oibriúcháin chun an Chreat-treoir Uisce a chur i bhfeidhm;
- Monatóireacht, measúnú agus tuairisciú a dhéanamh ar chaighdeán aibhneacha, lochanna, uiscí idirchreasa agus cósta, uiscí snámha agus screamhuisce chomh maith le tomhas ar leibhéil uisce agus sreabhadh abhann.

Eolaíocht Aeráide & Athrú Aeráide

- Fardail agus réamh-mheastacháin a fhoilsiú um astaíochtaí gás ceaptha teasa na hÉireann;
- Rúnaíocht a chur ar fáil don Chomhairle Chomhairleach ar Athrú Aeráide agus tacaíocht a thabhairt don Idirphlé Náisiúnta ar Ghníomhú ar son na hAeráide;
- Tacú le gníomhaíochtaí forbartha Náisiúnta, AE agus NA um Eolaíocht agus Beartas Aeráide.

Monatóireacht & Measúnú ar an gComhshaol

- Córais náisiúnta um monatóireacht an chomhshaoil a cheapadh agus a chur i bhfeidhm: teicneolaíocht, bainistíocht sonraí, anailís agus réamhaisnéisiú;
- Tuairiscí ar Staid Thimpeallacht na hÉireann agus ar Tháscairí a chur ar fáil;
- Monatóireacht a dhéanamh ar chaighdeán an aeir agus Treoir an AE i leith Aeir Ghlain don Eoraip a chur i bhfeidhm chomh maith leis an gCoinbhinsiún ar Aerthruailliú Fadraoin Trasteorann, agus an Treoir i leith na Teorann Náisiúnta Astaíochtaí;
- Maoirseacht a dhéanamh ar chur i bhfeidhm na Treorach i leith Torainn Timpeallachta;
- Measúnú a dhéanamh ar thionchar pleananna agus clár beartaithe ar chomhshaol na hÉireann.

Taighde agus Forbairt Comhshaoil

- Comhordú a dhéanamh ar ghníomhaíochtaí taighde comhshaoil agus iad a mhaoiniú chun brú a aithint, bonn eolais a chur faoin mbeartas agus réitigh a chur ar fáil;
- Comhoibriú le gníomhaíocht náisiúnta agus AE um thaighde comhshaoil.

Cosaint Raideolaíoch

- Monatóireacht a dhéanamh ar leibhéil radaíochta agus nochtadh an phobail do radaíocht ianúcháin agus do réimsí leictreamaighnéadacha a mheas;
- Cabhrú le pleananna náisiúnta a fhorbairt le haghaidh éigeandálaí ag eascairt as taismí núicléacha;
- Monatóireacht a dhéanamh ar fhorbairtí thar lear a bhaineann le saoráidí núicléacha agus leis an tsábháilteacht raideolaíochta;
- Sainseirbhísí um chosaint ar an radaíocht a sholáthar, nó maoirsiú a dhéanamh ar sholáthar na seirbhísí sin.

Treoir, Ardú Feasachta agus Faisnéis Inrochtana

- Tuairisciú, comhairle agus treoir neamhspleách, fianaise-bhunaithe a chur ar fáil don Rialtas, don tionscal agus don phobal ar ábhair maidir le cosaint comhshaoil agus raideolaíoch;
- An nasc idir sláinte agus folláine, an geilleagar agus timpeallacht ghlan a chur chun cinn;
- Feasacht comhshaoil a chur chun cinn lena n-áirítear tacú le hiompraíocht um éifeachtúlacht acmhainní agus aistriú aeráide;
- Tástáil radóin a chur chun cinn i dtithe agus in ionaid oibre agus feabhsúchán a mholadh áit is gá.

Comhpháirtíocht agus Líonrú

- Oibriú le gníomhaireachtaí idirnáisiúnta agus náisiúnta, údaráis réigiúnacha agus áitiúla, eagraíochtaí neamhrialtais, comhlachtaí ionadaíocha agus ranna rialtais chun cosaint chomhshaoil agus raideolaíoch a chur ar fáil, chomh maith le taighde, comhordú agus cinnteoireacht bunaithe ar an eolaíocht.

Bainistíocht agus struchtúr na Gníomhaireachta um Chaomhnú Comhshaoil

Tá an GCC á bainistiú ag Bord lánaimseartha, ar a bhfuil Ard-Stiúrthóir agus cúigear Stiúrthóir. Déantar an obair ar fud cúig cinn d'Oifigí:

1. An Oifig um Inbhunaitheacht i leith Cúrsaí Comhshaoil
2. An Oifig Forfheidhmithe i leith Cúrsaí Comhshaoil
3. An Oifig um Fhianaise agus Measúnú
4. An Oifig um Chosaint ar Radaíocht agus Monatóireacht Comhshaoil
5. An Oifig Cumarsáide agus Seirbhísí Corparáideacha

Tugann coistí comhairleacha cabhair don Ghníomhaireacht agus tagann siad le chéile go rialta le plé a dhéanamh ar ábhair imní agus le comhairle a chur ar an mBord.

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