2017

Evaluation of levels of antibiotic resistance in groundwater-derived E. coli isolates in the Midwest of Ireland and elucidation of potential predictors of resistance

Jean O'Dwyer

Paul Hynds

Matthieu Pot

See next page for additional authors

Authors
Jean O'Dwyer, Paul Hynds, Matthieu Pot, Catherine Adley, and Michael Ryan

Evaluation of levels of antibiotic resistance in groundwater-derived E. coli isolates in the Midwest of Ireland and elucidation of potential predictors of resistance

Article *in* Hydrogeology Journal · June 2017

DOI: 10.1007/s10040-017-1546-8

5 authors, including:

Jean O'Dwyer
University College Cork

Paul D Hynds
Technological University Dublin - City Campus

Matthieu Pot
Institut Pasteur de Guadeloupe

C. Adley
University of Limerick

Evaluation of levels of antibiotic resistance in groundwater-derived *E. coli* isolates in the Midwest of Ireland and elucidation of potential predictors of resistance

Authors: Jean O 'Dwyer[1]*^, Paul Hynds[2], Matthieu Pot[3], Catherine C. Adley[4], Michael P. Ryan[5]^

Address:

[1]Deptartment of Biological Sciences, School of Natural Sciences, University of Limerick, Limerick, Ireland V94 T9PX,

[2] Environmental Health and Sustainability Institute, Dublin Institute of Technology, Bolton Street, Dublin 7

[3]Ecole Supérieure d'Ingénieurs en Agroalimentaire de Bretagne atlantique (ESIAB), Technopôle Brest-Iroise, 29280 Plouzané, France.

[4]Microbiology Laboratory, Dept. of Chemical Sciences, School of Natural Sciences, University of Limerick, Limerick, Ireland V94 T9PX,

[5]Industrial Biochemistry Programme, Dept. of Chemical Sciences, School of Natural Sciences, University of Limerick, Limerick, Ireland V94 T9PX

^These authors contributed equally to this work.

***Corresponding author:**

Jean O'Dwyer

Department of Life Sciences

School of Natural Sciences

University of Limerick

Limerick

Ireland

E-mail: Jean.ODwyer@ul.ie

Keywords: Groundwater monitoring, Groundwater quality, Antibiotic resistance, Health, Ireland

NOTE TO COPYEDITOR – PLEASE INSERT THE FOLLOWING AS A FIRST-PAGE FOOTNOTE:

Published in the special issue “Hydrogeology and Human Health”

Abstract

Antibiotic-resistant (pathogenic and non-pathogenic) organisms and genes are now acknowledged as significant emerging aquatic contaminants with potentially adverse human and ecological health impacts, and thus require monitoring. This study is the first to investigate levels of resistance among Irish groundwater (private wells) samples; *Escherichia coli* isolates were examined against a panel of commonly prescribed human and veterinary therapeutic antibiotics, followed by determination of the causative factors of resistance. Overall, 42 confirmed *E. coli* isolates were recovered from a groundwater sampling cohort. Resistance to the human panel of antibiotics was moderate; nine (21.4 %) *E. coli* isolates demonstrated resistance to one or more human antibiotics. Conversely, extremely high levels of resistance to veterinary antibiotics were found, with all isolates presenting resistance to one or more veterinary antibiotics. Particularly high levels of resistance (93 %) were found with respect to the aminoglycoside class of antibiotics. Results of statistical analysis indicate a significant association between the presence of human (multiple) antibiotic resistance (p = 0.002 - 0.011) and both septic tank density and the presence of vulnerable sub-populations (<5 years). For the veterinary antibiotics, results point to a significant relationship (p = <0.001) between livestock (cattle) density and the prevalence of multiple antibiotic resistant *E. coli*. Groundwater continues to be an important resource in Ireland, particularly in rural areas; thus, results of this preliminary study offer a valuable insight into the prevalence of antibiotic resistance in the hydrogeological environment and establish a need for further research with a larger geological diversity.

1. Introduction

Human and veterinary antibiotics are a group of micro-organic compounds which are increasingly being identified as contaminants in sediments, groundwater, surface water, and recreational waterbodies (Kümmerer 2003, Coleman *et al*. 2013, Wellington *et al*. 2013, Frey *et al*. 2015, Ma *et al*. 2015). Previous studies have reported multiple sources associated with the release of these (and other) non-metabolized organic contaminants to the aquatic environment including hospital effluents (Galvin *et al*. 2010), municipal sludge and effluents (Glassmeyer *et al*. 2005, Watkinson *et al*. 2009), domestic wastewater treatment systems (Godfrey *et al.* 2007), landfill (Peng *et al.* 2014), and agricultural manure application and storage (Colemen *et al.* 2013; Frey *et al.* 2015).

The presence of non-metabolized antibiotics and/or their by-products in the natural environment have been shown to result in qualitative and quantitative effects on resident non-pathogenic and pathogenic organisms, resulting in the development and selection of resistant (and multi resistant) bacterial strains through a process known as horizontal gene transfer and the clonal spread of this now resistant bacteria (Kummerer, 2003; Wellington *et al.* 2013; Williams-Nguyen *et al.* 2016). The primary human health concerns associated with the presence of antibiotics and antibiotic resistant bacteria (ARBs) in drinking water are two-fold, namely (i) potential direct toxic effects of the antibiotic drug, and (ii) accumulation/proliferation of specific types of resistance that could potentially lead to treatment failure (Williams-Nguyen *et al.* 2016). Holmes *et al*. (2007) speculate that antibiotic concentrations in waterbodies located in developed regions are typically low and therefore, direct toxic effects to humans are unlikely. Conversely, chronic low-dose exposure to antibiotics may result in proliferation of ARB and/or antibiotic resistant genes (ARG), potentially resulting in treatment failure when bacterial infections do occur (irrespective of the pathogen source) (Wellington *et al.* 2013; Fernandes *et al.* 2015). Furthermore, recent evidence suggests that human gastroenteric infection caused by antibiotic resistant pathogens are associated with increased severity, morbidity and mortality rates, resulting in higher healthcare costs (CDCP, 2013). Of particular concern is the proliferation of antibiotic resistance among vulnerable populations (i.e. <5 years, >65 years), among whom bacterial infections and subsequent complications are more prevalent (Parry & Palmer, 2000).

A recent national baseline reconnaissance of pharmaceuticals and other emerging organic contaminants in US groundwater found that 31.5% of samples were positive for the presence of a veterinary or human antibiotics, the most prevalent of which was sulfamethoxazole (23.4% of samples) (Barnes *et al.* 2008). Frey *et al*. (2015) recently reported on the presence of *Campylobacter* tetracycline antibiotic resistant genes (*tet*(O)) in Canadian groundwater following land application of animal manures, with antibiotic by-products found up to 56 days post-application. Similarly, Li *et al*. (2015) report that 63.6% and 86.1% of *Escherichia coli* and *Enterococcus* isolates from alluvial groundwater samples associated with an irrigated agricultural region in California were resistant to ≥3 antibiotics. While the majority of studies examining the presence of antibiotics and/or antibiotic-resistant bacteria/genes in groundwater bodies have been undertaken in North America, a number of similar European studies have been published in the international literature. For example, Morasch (2013) reports on the presence of multiple micro-pollutants including antibiotic compounds (sulfamethoxazole, norfloxacin, azithromycin, and trimethoprim) in a karst system in the Swiss Jura. This is particularly relevant within the Irish context, due to the predominance of karstified limestones, which underlie approximately 50% of the country. Morasch (2013) confirmed that the point of ingress during the 10-month monitory period was a swallow hole draining an agricultural plain, while the likely source of human antibiotics were domestic wastewater treatment systems. Similarly, Cabeza et al. (2012) report on the presence of pharmaceuticals including three antibiotics (sulfamethoxazole, sulfamethazine, and trimethoprim) in a deep confined aquifer over an extended period (3 years) in the Llobregat Delta (Barcelona, Spain); while the authors note that the aquifer was artificially recharged by tertiary treated wastewater (TTW) via injection wells thus representing a source of at least some of the pharmaceuticals present, 10 pharmaceuticals were confirmed as not being derived from injected TTW, and were likely derived from agricultural activities and/or infiltration of poorly treated wastewater. López-Serna et al. (2013) have shown that natural bank filtration from a river receiving significant volumes of WWTP effluent from a large urban conurbation (Barcelona, Spain) was a highly influential source of contamination, with adjacent groundwater exhibiting high ranges of micro-organic compounds (including six antibiotics), and in some cases, in higher concentrations than those from the river itself. Further afield, Maran et al. (2016) have recently examined the antimicrobial susceptibility profile of bacteria from wells located in two Brazilian regions; in all, 26.7% and 64.4% of bacteria detected in groundwater samples (*n* = 45) exhibited antibiotic-resistance and multi-drug resistance, respectively, with 91.1% of resistance associated with β-lactam antibiotics.

In Ireland, Galvin *et al*. (2010) found that effluent downstream of a hospital in the west of Ireland was characterised by significantly increased concentrations of antimicrobial resistant *E. coli* than was found upstream. Thus, there is considered to be a high likelihood of the presence of antibiotics and antimicrobial-resistant organisms in the Irish subsurface environment. The same study found that wastewater treatment did not eliminate all antimicrobial resistant organisms; *E. coli* resistant to cefotaxime, ciprofloxacin, and cefoxitin were present in treated effluents. Recently, Coxon (2014) describes a number of international studies focusing on the presence and transport mechanisms of antibiotics and antibiotic-resistant organisms in natural groundwaters, highlighting the point that, to date, no similar studies have been undertaken in the Republic of Ireland.

The Republic of Ireland is characterised by a high (and long standing) reliance on unregulated, private water wells and (regulated) on-site domestic wastewater treatment, a dispersed yet locally dense rural settlement pattern (Scott & Murray, 2009), a unique agricultural profile, and diverse (and frequently vulnerable) localised (hydro)geological settings. Previous studies have shown that private wells in the Republic of Ireland are a significant source of pathogenic and non-pathogenic organisms, therefore representing a public health concern (Bacci & Chapman, 2012; Hynds *et al.* 2012; Hynds *et al.* 2014; O'Dwyer *et al.* 2014; O'hAiseadha *et al.* 2017). Moreover, a recent Eurobarometer survey has shown that Irish antibiotic use is significantly above the European mean; 43% of surveyed Irish residents reported antibiotic usage during the previous 12-month period, compared with a European mean of 35% (European Commission, 2013).

The current study is the first to investigate the presence of antimicrobial-resistant bacteria in Irish groundwater, and the role of anthropogenic (i.e. sources) and natural (i.e. pathways) drivers on levels of encountered resistance. Antibiotic susceptibility testing was carried out on groundwater derived *E. coli* isolates, followed by geo-spatial data extraction and analyses for elucidation of the sources and transport mechanisms associated with antimicrobial presence in Irish groundwater. Lapworth *et al*. (2012) predict that the number of emerging contaminants with defined drinking water standards, environmental quality standards, groundwater threshold values, and/or monitory requirements will increase substantially over the coming decade; this is particularly likely with respect to antibiotics, thus, improving our understanding of current spatial and temporal patterns should be prioritised in order to inform future groundwater investigations and monitoring strategies.

2. Materials and Methods

2.1 Study and sampling site description

The study area is located in the Midwestern region of Ireland, encompassing three administrative counties, namely: Limerick, Clare and North Tipperary, extending 8,248 km²; 11% of the total area of the Republic of Ireland (Figure 1).

This region was selected for examination due to its high reliance on private water supplies (23,014 (17.3%), CSO 2012), and its proximity to appropriate laboratory facilities. The region is geologically diverse (Figure 2), and is variously underlain by bedded and un-bedded Dinantian limestone and Devonian sandstone derived bedrocks, in addition to volcanics and shale deposits.

Regional subsoils are similarly diverse; limestone, sandstone and shale tills are predominant in North Tipperary and Limerick, while large regions of County Clare are characterised by karstified outcrop/subcrop and therefore lacks substantial subsoil deposits. Climatologically, the Midwestern region is characterised by a higher annual rainfall and relative humidity than the national average due to its coastal Atlantic location (Met Eireann 2016).

Altogether, 125 untreated private groundwater wells were sampled three times (*n* = 375) during October 2011- October 2012 (O'Dwyer *et al.* 2014). The water samples were collected from households sought through a national online forum and thus are a random representation of the study area; no selection bias in terms of geology, infrastructure, time of year etc. was applied. The mean household size was 3.71 persons (S.D = 1.31) with 54.4% (*n* = 68/125) and 14.4% (18/126) of households comprising a resident ≤5 years, and ≥65 years, respectively. The majority of sampled supplies were a bored well (*n* = 98/125, 78.4%), with the remaining 21.6% (27/125) of supplies being of the shallow hand-dug type; which is a higher reliance that the national average, estimated at around

10% (Hynds et al. 2012). Well age was recorded as part of the groundwater sampling process; 38.4% (n = 48/125) of sampled wells were >25 years, while 20% (n = 25/125) of wells were <10 years.

2.2 Sampling and recovery of *E.coli* isolates

Water samples were taken from an (untreated) household tap subsequent to sterilization using 70% ethanol and allowing the water to run for 60 seconds to ensure samples were not taken from the distribution system. Given the maximum volume of sample (100 mL) required for zero-dilution bacteriological analysis, well water samples were collected in-situ in disposable 120 mL sterile vessels containing sodium thiosulphate to negate any residual chlorine present. All samples were stored in a cool environment and analysed within 4 hours. The most probable number (MPN) of *E. coli* was enumerated using a standard ISO approved (ISO, 93083:1998) commercial culture kit (Colilert, IDEXX Laboratories Inc., Westbrook Maine).

Colilert reagent contains two carbon sources which are selectively metabolised by most coliforms and *E. coli* present in the sample. The resulting metabolic reaction causes a discernible colour change; yellow for total coliforms (TC) and fluorescent under a UV light source for *E. coli*. Samples were transferred to a 51 "well" tray, heat sealed and incubated for 24 hours at 37°C. In all, 73 (n = 73/125, 54/8%) private groundwater supplies tested positive for the presence of *E. coli* over the sampling period. (O'Dwyer *et al.* 2014). *E. coli* positive samples were recovered via pipetting 100μL of sample into 5 mL of Maximum Recovery Diluent (MRD) (Sigma-Aldrich, Saint-Louis, MO, USA), followed by stirred aerobic incubation at 30°C for 1 hr at 145 rpm. Recovered *E.coli* isolates were subsequently cultured using an analogous method to that presented by Anderson & Sobsey (2006), with reference strain *E. coli* ATCC 25922 cultured in parallel, thus permitting appropriate colony selection. Gram staining was carried out for all selected colonies. Presumptive *E.coli* colonies isolated from MacConkey plates were purified and isolated on a Nutrient Agar (Oxoid, Basingstoke, Hampshire, UK), followed by confirmation using API® 20E test strips (bioMerieux, Hazelwood, MO, USA). In total, 42 confirmed *E. coli* isolates (pure cultures) were recovered from the 75 *E. coli* positive groundwater samples, the locations of which are presented in Figure 2.

2.3 Antimicrobial susceptibility analyses

Antimicrobial susceptibility analyses were carried out for panels of both human (*n* = 13, Table 1) and veterinary antibiotics (*n* = 8, Table 2) using the (susceptibility) disk diffusion method (CLSI 2013, EUCAST 2016). The human panel of antibiotics are those are tested for frequently under the Enterobacteriaceae using the EUCAST method. The veterinary panel of antibiotics are those that are tested for routinely by the Veterinary Laboratory Service of the Department of Agriculture Food and the Marine in the Republic of Ireland. Agar plates were lawned with a pure *E. coli* culture after which commercially prepared disks, each of which are pre-impregnated with a standard concentration of a specific antibiotic, were evenly dispensed and lightly pressed onto the agar surface. Following overnight incubation, the bacterial growth around each disc was examined and recorded. If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" will be observed around that particular disk. However, if the isolate is resistant, varying degrees of growth will remain. All susceptibility analyses were carried out on Müller-Hinton (MH) agar (Oxoid, Basingstoke, Hampshire, UK) in accordance with the Clinical and Laboratory Standards Institute (CLSI), and European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards. Analytical results for the human panel were interpreted using the EUCAST criteria for *Enterobacteriaceae* (EUCAST 2016) (Table 1) thus corresponding with recommended practice in Europe (Kahlmeter *et al.* 2003), while the veterinary panel were interpreted using the CLSI criteria for *Enterobacteriaceae* (CSLI, 2013) (Table 2), which is in line with current Irish veterinary practice (DAFM, 2014). Isolates are considered sensitive (S) when growth of the organism is inhibited by the antibiotic to a diameter accepted by the specific criteria employed (EUCAST or CSLI). Where the organism has grown to a diameter within the threshold of resistance, but is not yet classified as resistant, it is considered as exhibiting intermediate resistance (IR). Resistance (R) is characterised when the organism is not effectively inhibited by the antibiotic according to the zone diameters outlined. Zone diameters for the disc susceptibility tests were measured with Vernier callipers, with all analyses carried out in triplicate. *E. coli* ATCC 25922 was included as a reference strain (control) for all susceptibility analyses. All results were found to occur within recommended limits.

Results of antimicrobial susceptibility analyses were categorised on the basis of presence/absence of antibiotic resistance to each individual antibiotic within the diagnostic panel. These results provide an overview of general levels of sensitivity and resistance among groundwater *E. coli* to a broad range of commonly employed antibiotics. Subsequently, resistance and sensitivity were categorised based on antibiotic classification (i.e. chemical structure); antibiotics grouped within a structural classification are typically characterised by similar biochemical and molecular mechanisms, in addition to analogous patterns of efficacy, toxicity, and allergic potential, and may thus provide an overview of the antibiotic resistance mechanisms in the subsurface environment.

2.4 Spatially-derived variables and data sources

Site-specific and regional hydrogeological, agricultural and infrastructural features that could be sources and/or pathways of resistance were identified through literature review. Data sources were identified, extracted and collated via a Geographical Information System (ESRI ArcMap 10) in order to develop a spatially linked database associated with all confirmed *E. coli* isolates. Data pertaining to household composition, and particularly the presence of potentially vulnerable household residents (≤ 5 years and ≥ 65 years), were recorded via a self-administered questionnaire which was completed in concurrence with groundwater sampling. Completed questionnaires provided information regarding both household demographics and infrastructural parameters relative to the sampled household groundwater source. The frequency (and regional density) of domestic wastewater treatment system (DWWTS) (i.e. septic tank) reliance were extracted from the CSO Census of Ireland 2011 dataset, followed by spatial indexing to one of 3,400 pre-defined Census enumeration divisions termed "Electoral Divisions" which are the smallest legally defined administrative areas in the State for which Small Area Population Statistics (SAPS) are published. The Census of Agriculture (Central Statistics Office, 2012) was finalised in 2009 for all agricultural holdings in the Republic of Ireland with a "farmed area" ≥ 1hectare (2.47 acres), in compliance with Regulation (EC) No. 1166/2008; equivalent censuses were conducted in all EU member states during 2009/2010 (Central Statistics Office, 2012). Census data were extracted spatially aggregated and used to calculate livestock (cattle) populations and associated densities for each Electoral Division. Hydrogeological parameters including groundwater vulnerability and aquifer type were spatially extracted using Geological Survey of Ireland (GSI) mapping resources, while local subsoil permeability data/layers were similarly extracted and assigned using An Teagasc (Agriculture and Food Development Authority) mapping data. All extracted variables were GIS-derived (i.e. assigned to distinct sampling point via direct GPS coordinates or joined and related to the corresponding electoral division) using The Economic and Social Research Institutes (ESRI) ArcMap10. Census data (both agricultural and infrastructural: DWWTs density) were spatially assigned at the Electoral Division level, while hydrogeological data were assigned to the specific GPS coordinates recorded upon sample collection.

2.5 Statistical Analysis and Data Categorisation.

All variables were assessed for normality using the Kolmogorov-Smirnov test, in concurrence with Q-Q plots. Resistance profiles (dependent variable(s)) were dichotomised into (i) Antibiotic Resistance (AR), whereby an isolate either exhibits resistance to ≥1 antibiotic (AR Present) or demonstrates no AR (AR Absent), and (ii) Multiple Antibiotic Resistance (MAR), whereby an isolate exhibits resistance to ≥5 antibiotics (MAR Present) (McKeon, 1995) or does not (MAR Absent). Resistance to ≤ 5 antibiotics was utilised to facilitate a more definitive segregation across the groups. The Mann-Whitney U test was used to test for associations between non-parametric continuous independent variables (Cattle Density, DWWTS Density, Household Size) and AR/MAR classification. Pearson's $\chi 2$ tests were used to test for associations between categorical independent variables (i.e. Presence of potentially vulnerable household resident (≤ 5 or ≥ 65 years of age), Subsoil Permeability, Groundwater Vulnerability, Aquifer Importance) and the dependent variables (AR (Pres/Abs), MAR (Pres/Abs)). Logistic regression (LR) models were constructed using sensitive/resistant profiles of *E. coli* isolates during the study period as the dichotomous dependent variable. For the human panel of antibiotics, AR (present/absent) was used as the dependent variable, whereas for the veterinary panel, MAR (present/absent) was utilised. The collinearity diagnostic test for tolerance (<0.1) and the variance inflation factor (VIF) (>10) were used to assess collinearity between independent variables prior to regression modelling. The independent variables for logistic regression modelling, as with the previous analysis, were selected based on plausibility, as documented in international literature. The “forced entry” method was used: all variables were tested simultaneously, with backward elimination of variables that contributed least to the model. The Hosmer–Lemeshow test and Nagelkerke's R^2 were used to assess model goodness-of-fit and effect size, respectively. SPSS® 22 was employed for all statistical analyses with confidence set at 95% ($p < 0.05$) by convention.

3. Results

3.1 Antibiotic susceptibility

Overall, 42 *E. coli* isolates from groundwater derived private water supplies in the Midwest of Ireland were subjected to a comprehensive suite (n = 21) of antibiotic susceptibility analyses. Susceptibility of isolates to the human antibiotic panel varied considerably between individual antibiotics (Table 1). Bacteria displayed the greatest level of resistance to ampicillin and tircacillin/clavulanic-acid, with 14.3% (n = 10) of isolates demonstrating either Intermediate Resistance (IR) (n = 2) or Resistance (R) (n = 8) to these two antibiotics. As shown (Table 1), resistance was also encountered with respect to piperacillin/tazobactam, cefpodoxime, ciprofloxacin, norfloxacin, nitrofurantoin and trimethoprim.

Table 1: Human antibiotics, criteria of resistance, MIC values and no. of resistant isolates

Antibiotic	Zone diameter breakpoint (mm)		Mean (±SD) Zone of Inhibition (mm)	No. (%) of R isolates	No. (%) of IR isolates	Total no. (%) of R & IR isolates
	S ≥	R <				
Penicillins						
Ampicillin (AMP, 10 μg)	14	14	17.33 (6.22)	6 (14.29)	0 (0)	6 (14.29)
Piperacillin/Tazobactam (TZP, 30/6 μg)	20	17	23.14 (3.38)	2 (4.76)	2 (4.76)	4 (9.52)
Tircacillin/clavulanic acid (TIM,75/10μg)	23	23	25.55 (4.39)	6 (14.29)	0 (0)	6 (14.29)
Cephalosporins						
Cefepime (CPM, 30 μg)	24	21	31.3 (2.26)	0 (0)	0 (0)	0 (0)
Cefotaxime (CTX, 5 μg)	20	17	26.31 (2.48)	0 (0)	0 (0)	0 (0)
Cefpodoxime (CPD, 10 μg)	21	21	21.38 (3.41)	1 (2.38)	0 (0)	1 (2.38)
Ceftazidime (CAZ, 10 μg)	22	19	25.1 (2.22)	0 (0)	1 (2.38)	1 (2.38)
Carbapenem						
Meropenem (MEM, 10 μg)	22	16	25.83 (2.29)	0 (0)	1 (2.38)	1 (2.38)
Fluoroquinolones						
Ciprofloxacin (CIP, 5 μg)	22	19	28.6 (2.48)	2 (4.76)	0(0)	2 (4.76)
Norfloxacin (NOR, 10 μg)	22	19	28.93 (4.19)	2 (4.76)	1 (2.38)	3 (7.14)
Monobactam						
Aztreonam (ATM, 30 μg)	24	21	26.86 (4.22)	0 (0)	0(0)	0 (0)
Miscellaneous						
Nitrofurantoin (F, 100 μg)	11	11	21.62 (6.70)	1 (2.38)	0(0)	1 (2.38)
Trimethoprim (W, 5 μg)	18	15	30.83 (1.85)	1 (2.38)	2 (4.76)	3 (7.14)

S= Susceptible, R= Resistant, IR=Intermediate Resistance, SD= Standard Deviation

Higher levels of resistance were found within the veterinary panel of antibiotics then to the human panel (Table 2) with all *E. coli* isolates (n = 42) presenting resistance to one or more veterinary antibiotic. Significantly, resistance (R) to streptomycin and neomycin was encountered among 92.9% and 61.9% of isolates, respectively; upon inclusion of intermediate resistance, total resistance (R & IR) increased to 95.2% and 90.5% for streptomycin and neomycin, respectively. Resistance to amoxycillin/clavulanate (16.7%), enrofloxacin (11.9%) and ceftiofur (11.9%) was also prevalent among *E. coli* isolates (Table 2).

Table 2: Veterinary antibiotics, criteria of resistance, MIC values and no. of resistant isolates

Antibiotic	Zone diameter breakpoint (mm)			Mean (±SD) Zone of Inhibition (mm)	No. (%) of R isolates	No. (%) of IR isolates	Total no. (%) of R & IR isolates
	S ≥	I	R <				
β-Lactam/ β-Lactamase Inhibitors							
Amoxycillin/ Clavulanate (AMC, 20/10µg)	18	14-17	13	17.76 (5.98)	7 (16.66)	8 (19.05)	15 (35.71)
Cephalosporins							
Ceftiofur (EFT, 30 µg)	21	18-20	17	24.64 (4.33)	5 (11.9)	4 (9.52)	9 (21.43)
Cefpodoxime (CPD, 10 µg)	18	-	17	21.38 (3.41)	1 (2.38)	3 (7.14)	4 (9.52)
Aminoglycosides							
Neomycin (N, 30 µg)	17	13-16	12	19.6 (8.86)	26 (61.9)	12 (28.6)	-
Streptomycin (S, 10 µg)	15	12-14	11	8.5 (3.50)	39 (92.86)	1 (2.38)	40 (95.23)
Tetracycline							
Tetracycline (TE, 30 µg)	19	15-18	14	22.79 (8.1)	4 (9.52)	4 (9.52)	8 (19.05)
Folate Pathway Inhibitor							
Sulfamethoxazole& Trimethoprim SXT (10 µg)	16	11-15	10	29.7 (7.68)	1 (2.38)	2 (4.76)	3 (7.14)
Fluoroquinolones							
Enrofloxacin (Enr, 5 µg)	23	17-22	16	18.10 (9.70)	5 (11.9)	3 (7.14)	8 (19.05)

S= Susceptible, R= Resistant, IR=Intermediate Resistance, SD= Standard Deviation

With respect to the antibiotic classification (Table 3), highest levels of resistance were encountered for the human panel among the penicillin class, with 12.7% of isolates demonstrating either IR (*n* = 2) or R (*n* = 14) to this group. Resistance to other antibiotic classes was less prevalent, although resistance to the fluoroquinolones was exhibited by five (11.9%) isolates (R = 4; IR = 1).

Table 3: Human antibiotic resistance profiles by antibiotic class:

Antibiotic	No. (%) of R isolates	No. (%) of IR isolates	No. (%) of S isolates	No. (%) of R & IR isolate	Total
Penicillins	14 (11.1)	2 (1.59)	110 (87.3)	16 (12.7)	126[1]
Cephalosporins	1 (0.6)	1 (0.6)	166 (99.4)	2 (1.2)	168[2]
Carbapenem	0 (0)	1 (2.38)	41 (97.62)	1 (2.4)	42
Fluoroquinolones	4 (4.76)	1 (1.19)	79 (94.04)	5 (6.0)	84[3]
Monobactam	0 (0)	0 (0)	42 (100)	0 (0.0)	42
Miscellaneous	1 (2.38)	0 (0)	41 (97.62)	1 (2.4)	42

[1] Three antibiotic assessed, [2] Four antibiotics assessed, [3] Two antibiotics assessed.

Yet again, a higher level of resistance was encountered with respect to group/class resistance within the veterinary panel (Table 4); resistance to the aminoglycoside group of antibiotics was widespread, with 93% of isolates demonstrating resistance or intermediate resistance. Extensive resistance to β-Lactams / was also observed with over a third of isolates (35.5%) exhibiting resistance or intermediate resistance. Similarly, R/IR to fluoroquinolones was high with almost 22% of isolates exhibiting resistance.

Table 4: Veterinary antibiotic resistance profiles by antibiotic class

Antibiotic	No. (%) of R isolates	No. (%) of IR isolates	No. (%) of S isolates	No. (%) of R & IR isolate	Total
β-Lactam/ β-Lactamase inhibitors	7 (16.66)	8 (19.04)	27 (64.29)	15 (35.47)	42
Cephalosporins	6 (7.14)	7 (8.33)	71 (84.52)	13 (15.48)	84[1]
Aminoglycosides	65 (77.4)	13 (15.48)	6 (7.14)	78 (92.86)	84[1]
Tetracyclines	4 (9.52)	4 (9.52)	34 (80.95)	8 (19.05)	42
Folate Pathway Inhibitor	1 (2.38)	2 (4.76)	39 (92.86)	3 (7.14)	42
Fluoroquinolones	5 (11.9)	4 (9.52)	33 (78.57)	9 (21.43)	42

[1] Two antibiotics assessed

3.2 Factors associated with antibiotic resistance

In order to elucidate the potential sources and pathways associated with the presence of antimicrobial resistant *E. coli* in the Irish groundwater environment, bivariate and multivariate statistical analyses were undertaken to quantify levels of association between antibiotic resistance profiles (i.e. ((M)AR Pres/Abs) and spatially derived predictor variables.

As shown (Table 5), in all cases, an increased local DWWTS reliance; i.e. the number of systems per ED, and the presence of household residents ≤5 years was found to correspond with the presence of AR (≥1 antibiotic) to the human antibiotic panel. Conversely, areas within the study region characterised by lower levels of DWWTS reliance and/or an absence of children ≤5 years, were found to exhibit little or no AR to the human antibiotic panel.

Table 5: Tests of association between spatially and specifically derived predictors and human AR/MAR in *E. coli* isolates (*n* = 42). P-values in italics are statistically significant.

Case Type	Resistance	*n*	Mean Septic Tank/ED[1]	Mean Cattle/ED[1]	Children Under 5[2]	Adults over 65[2]	Mean Household Size
AR							
	Yes	9	223.00	2643.33	7	1	4
	No	33	135.76	3622.1	2	7	3.64
	Sig.	-	*p = 0.010*	p= 0.181	*p= 0.022*	p = 0.662	p = 0.450
MAR							
	Yes	7	250.86	3538.31	11	1	4.57
	No	35	135.17	2783.14	7	7	3.54
	Sig.	-	*p = 0.002*	p = 0.407	*p <0.001*	p = 0.598	p = 0.073

[1] Mann Whitney U
[2] Pearson's X^2

For example, private wells with isolates exhibiting human AR were associated with a mean DWWTS frequency of 223.00/ED, compared with 135.76/ED in areas where no antibiotic resistant isolates were recovered (p = 0.011). This trend was particularly pronounced when MAR was employed as the dependant variable; MAR isolates were associated with a mean DWWTS frequency of 250.86/ED (p = 0.002). Similarly, a significant association was found between the presence of a young (≤5 years) household resident and both AR (p = 0.022) and MAR (p = <0.001) prevalence within the human panel. No association was found between human AR and cattle density, the presence of elderly (>65 years) household residents or total household size.

High levels of encountered veterinary AR (Table 6) resulted in an inability to carry out bivariate tests of association due to a lack of variation within the sample population; i.e. there was not enough of both resistant and non-resistant isolates for meaningful differences to be established. Accordingly, tests of association were only carried out for MAR isolates. No significant association was found with respect to DWWTS reliance or household composition. However, as might be expected, a significant association was found between cattle density/ED and the prevalence of MAR among *E. coli* isolates (p = 0.001). Domestic wells with isolates exhibiting MAR were associated with a mean cattle population of 3,861.71/ED, compared with 1,858.36/ED in areas where no MAR isolates were recovered.

Table 6: Tests of association between spatially and specifically derived predictors and MAR in *E. coli* isolates (*n* = 42)

MAR Resistance	*n*	Mean Septic Tank density[1]	Cattle density[1]	Children Under 5[2]	Adults over 65[2]	Mean People in Household[1]
Yes	31	157.87	3861.71	11	1	3.65
No	11	144.82	1858.36	7	7	3.91
Sig.	-	p = 0.315	*p = 0.001*	p = 0.443	p = 0.463	p = 0.073

[1] Mann Whitney U [2] Pearson's X^2, P-values in italics are statistically significant.

In assessing the potential influence of hydrogeological parameters on subsurface occurrence and transport of antibiotic resistant bacteria, extracted soil and bedrock characteristics were considered including: subsoil permeability, groundwater vulnerability, and aquifer type (bedrock or sand/gravel). No significant associations were found between extracted hydrogeological variables and the incidence of AR and MAR across both the human and the animal veterinary panel.

To further elucidate potential predictors of antibiotic resistance within *E. coli* isolates in the study area, Logistic regression was undertaken. Results of Hosmer-Lemeshow GOF tests (p >0.05) indicate both logistic regression models were well calibrated. As shown (Table 7), the final model for the human panel of antibiotics included two variables, namely DWWTS (septic tank) density (p 0.049) and the presence of persons less than 5 years of age in the household (p = 0.034) thus corresponding with results of bivariate analyses (Table 5). Interpretation of resulting odds ratios indicate an increased likelihood of AR among *E. coli* isolates occurring in parallel with increasing DWWTS density (OR = 1.009) and the presence of younger populations in the home (OR = 11.667). For the veterinary panel of antibiotics, cattle density (p = 0.011) was associated with MAR in *E. coli* isolates indicating an increased rate of MAR in areas with greater cattle densities.

Table 7: Logistic regression models for antibiotic resistance (AR, MAR) in *E. coli* isolates from groundwater against a human and veterinary antibiotic panel

Model	Predictor	B	P	OR	95% CI
Human Panel (AR)	DWWTS density,	0.009	0.049	1.009	1.001-1.012
	Persons $\leq$ 5	2.457	0.034	11.667	6.231-48.258
Veterinary Panel (MAR)	Cattle density	0.001	0.011	1.001	1.001-1.003

B, Coefficient of the predictor variables, P, Significance, OR, Odds ratio, CI, confidence interval, DWWTS, domestic wastewater treatment systems (septic tanks)

4. Discussion

The current study represents the first to examine the presence and extent of antibiotic resistance among *E. coli* isolates derived from groundwater wells in the Republic of Ireland, and subsequently assesses a number of potentially associated variables, which may be used to infer antibiotic sources and/or transport mechanisms. Previous research has shown the two primary sources of antibiotic resistant bacteria and resistance genes in the natural environment are human sewage and animal manure, with storm water, wild animals, birds, pets, and aquatic life also acting as contributory sources, albeit to a lesser degree (Servais & Passerat 2009). Accordingly, both human and veterinary antibiotic panels were employed for susceptibility analysis in the current study.

4.1 Resistance to Human Panel of Antibiotics:

Resistance to the human panel of antibiotics was found to be moderate with 9 (21.4%) *E. coli* isolates demonstrating some level of resistance to $\geq$1 human antibiotic; the highest levels of resistance were associated with the penicillins, while notable levels of resistance also found among the fluoroquinolones and cephalosporins. As might be expected, the most frequently occurring resistance phenotypes were associated with the 1st and 2nd generation broad spectrum antimicrobials including ampicillin (14.3%), a β-lactam antibiotic first introduced in 1948 (Hauser, 2013). Typically, broad spectrum antibiotics are more frequently prescribed for non-fatal acute infection and thus, these antibiotics are characterised by a higher prevalence of resistance within human populations. Interestingly, in the current study, levels of resistance to the 4th generation antibiotic tircacillin/clavulanic-acid (14.3%) (first introduced in 1985) occurred at a similar level to ampicillin resistance. Thispotentially demonstrates ineffective β-lactamase enzymatic blocking within the *E. coli* isolates (Drawz & Bonomo, 2010); a mechanism incorporated into antibiotics to delay the development of resistance. Notably, resistance was also found within the fluoroquinolone class of antibiotics, with 4.8% (*n* = 4) of isolates exhibiting some level of resistance. This represents a particular concern, as this antibiotic class is frequently employed in the treatment of salmonellosis, an enteric infection with potentially high human health effects within specific subpopulations including the elderly, the young, and the immunocompromised, with hospitalisation often required (Ryan *et al.* 2011); while salmonella has not yet been identified within Irish groundwater bodies, it has been identified internationally (Murphy *et al.* 2017).

4.2 Resistance to Veterinary panel of Antibiotics:

In contrast to the human panel, higher levels of antibiotic resistance were found against the veterinary panel of antibiotics, with all isolates presenting resistance to at least one antibiotic. Particularly high levels of resistance (93%) occurred within the aminoglycoside class; high levels of resistance to aminoglycoside antibiotics (e.g. streptomycin and neomycin) in *E. coli* isolates have been reported in food-producing animals (cattle, sheep, and pigs) in Europe (Bywater *et al*. 2004, Hendriksen *et al*. 2008). Tetracycline resistance was also prevalent in *E. coli* isolates (19.1%), which was expected as tetracycline has been extensively used as a therapeutic agent and growth promoter in animal feeds since its approval in 1948 (McEwen and Fedorka-Cray 2002) and may thus infiltrate the subsurface environment. It should also be stated that the possibility exists that antibiotic resistance could be present naturally within the bacterial fauna in the soil (Forsberg et al. 2012, Durso et al., 2016). The use of tetracycline as a growth promoter in animal feeds is no longer authorised within the European Union (HPRA 2013), however, bacterial tetracycline resistance has been reported over a decade (126 months) after cessation as a feed additive or therapeutic agent within swine flocks (Langlois *et al*. 1983). Furthermore, tetracycline is still routinely prescribed for veterinary use in Ireland, with the Health Products Regulatory Authority (formerly Irish Medicine Board) reporting that tetracycline antibiotics accounted for 36% of all antibiotics sold in Ireland for veterinary use in 2013 (HPRA, 2013).

4.3 Sources of antibiotic resistance:

Within the environment, the prevalence of antibiotic resistance is attributable to numerous factors, including source and pathway dynamics, soil type, and excretion rates associated with un-metabolized antibiotics themselves. The majority of therapeutic antibiotics are water-soluble and therefore about 90% of the dose may be excreted in urine, while up to 75% may be released in animal faeces (Halling-Sørensen, 2001). In the current study, strong statistical relationships were found between the presence of both human AR (p =0.011) and human MAR (p =0.002) and DWWTS reliance per Electoral Division, indicating that regions characterised by a higher density of on-site treatment systems are associated with the presence of antibiotic resistant *E. coli*, thus corroborating previous studies which have shown that dissemination of resistance bacteria in the environment are a direct result of waste water treatment (Sapkota *et al*. 2007; Watkinson *et al*. 2007; Michael *et al*. 2013), and poorly functioning septic tank systems (Biswal, Mazza *et al*. 2014). This was corroborated through regression analysis which indicated that AR to the human panel of antibiotics increased in line with an increase in DWWTS density per ED. Furthermore, a significant association was found between households comprising children $\leq$5 years of age and the presence of both human AR (p = 0.022) and human MAR (p <0.001). Regression analysis also supported this result, with households with small children over eleven times more likely to have AR *E. coli* in their water supply.

A recent longitudinal study undertaken in Ireland reports that 18.4% of children aged ≤3 years were prescribed ≥3 courses of antibiotics in the previous 12 months, with two-thirds (66%) of three-year-olds having received at least one course of antibiotics during the previous 12 months (Williams *et al*. 2013). The high antibiotic prescription rate among the ≤ 3year population, in parallel with the association found between AR and MAR indicates that high levels of antibiotic prescription and usage among young children is a significant source of antibiotic resistance in the Irish subsurface environment. Further research is required to elucidate the relationship between antibiotic usage and environmental antibiotic resistance (ARGs, ARBs, and ARPs), stratified by both population demographic and local/regional infrastructure i.e., the prevalence of private water wells and septic tank utilisation.

With reference to veterinary antibiotics, Sapotka *et al*. (2007) have previously reported that land application of manure may result in environmental/aquatic transport of bacteria resistant to veterinary antibiotics. Antibiotics used for veterinary purposes are excreted by treated animals, thus leading to their widespread presence in soils via grazing livestock or manures used as agricultural fertiliser (Jørgensen *et al*. 2000). Results from the current study suggest a significant association (p = <0.001) exists between cattle density/ED and the prevalence of veterinary MAR *E. coli* isolates. This is supported by international literature with Chee-Sanford *et al*. (2001) reporting tetracycline-resistance genes in groundwater close to swine production facilities in the United States.

4.4 Environmental Fate of Antibiotic Resistance

The presence and fate of antibiotics (and pathogenic bacteria) in the subsurface environment is not just a factor of the antibiotic source, but is also highly dependent upon local physical–chemical properties, prevailing climatic conditions, and hydrogeological setting, in addition to a variety of other local/regional environmental factors. In the current study, no significant associations were found between extracted local hydrogeological parameters and the prevalence of either AR or MAR within both antibiotic panels. However, it should be noted that the current research formed part of a larger overall study which sought to investigate the susceptibility of differing subsurface environments to faecal contamination (O'Dwyer *et al.* 2014). Accordingly, due to the overarching objectives of the primary study, *E. coli* isolates were primarily sampled from regions with characteristically high levels of susceptibility to groundwater contamination, and are as such indicative of specific hydrogeological characteristics considered conducive to contamination. For example, results from the aforementioned study have shown that the presence of *E. coli* can be predicted relative to aquifer type and the presence of karst features; bedrock aquifers with karst geomorphology were more conducive to *E. coli* contamination. Accordingly, the relatively small number of *E. coli* isolates characterised by human/veterinary AR/MAR, in concurrence with the high level of homogeneity associated with the study samples represent the primary study limitations, particularly with respect to elucidation of subsurface occurrence and movement. Further work is thus required to address these limitations, in addition to examining antibiotic resistance in the environment using soil microbiota that are routinely present in the subsurface environment as the indicator organism rather than *E. coli* which theoretically should not be present in groundwater derived potable water supplies. Further work should include a more prolific examination of the pathways conducive to antibiotic resistance in the subsurface examination; larger sample numbers with more diverse sources of bacteria and more varied bedrock lithologies to increase data variability.

The current study, while limited in its hydrogeological scope, is invaluable insofar as it is the first to present irrefutable evidence of the presence and extent of antibiotic resistance in the Irish groundwater environment, which represents the primary daily source of drinking water for ≈750,000 people, in addition to many more on a transient basis. Moreover, all isolates were sampled from groundwater sources for domestic human consumption, thus the presence of, in some cases multiple antibiotic resistance, cannot be overstated; it has been established that water contaminated with antibiotic resistant *E. coli* has been associated with the carriage of resistant *E. coli* in humans (Coleman *et al.* 2012). As groundwater continues to be an important source of potable water for a significant proportion of the Irish population, the results presented provide an invaluable benchmark to highlight the need for, and provide guidance for further research into antibiotic resistance in the subsurface environment.

5. Conclusion

Our findings suggest that AR *E. coli* are not uncommon to the environment of rural groundwater supplies in Ireland. *E. coli* isolates were examined against a panel of commonly prescribed human and veterinary therapeutic antibiotics and it was found that resistance to the veterinary panel in particular is an area of concern. Resistance was found to exist to the first generation antibiotics corroborating international literature. However, resistance was also noted against newer generations of antibiotics which are a cause for concern (as they are potentially used as a last defence) because of possible colonization of the gastrointestinal tract and/or conjugal transfer of antibiotic resistance in aquatic and other environments. Potential pathways and causative factors of resistance were also assessed in this study and it was found that potential high concentrative sources of antibiotic residues (DWWTSs, Cattle) were associated with the prevalence of both AR and MAR as well as the presence of young children which receive more frequent doses of antibiotics relative to the rest of the population. The *E.coli* isolates in this study were taken from a cohort of samples in which groundwater vulnerability was assessed. As such, there was reduced hydrogeological variability within the samples as they were taken from already vulnerable environments; there was no significant association found between hydrogeological parameters and resistance to any antibiotics. This is undoubtedly a limitation of the study. Further work should focus on prevalence of resistance within defined and preselected hydrological environments using more ubiquitous indicators (e.g. antibiotic resistance genes) to facilitate knowledge transfer of resistance transport in the subsurface environment. Nevertheless, this study has provided valuable insight into previously uncharacterised antibiotic resistance in the Irish groundwater environment and provides a benchmark for future studies.

Acknowledgments

The authors wish to acknowledge the efforts of the associate editor, editor and two anonymous reviewers for their helpful suggestions in an earlier draft of this work This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

References

Bacci F, Chapman DV. (2011) Microbiological assessment of private drinking water supplies in Co. Cork, Ireland. J Water Health 9(4), 738-751.

Barnes KK, Kolpin DW, Furlong E, Zaugg SD, Meyer MT, Barber LB. (2008) A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States Groundwater. Sci Total Environ 402(2), 192-200.

Biswal BK, Mazza A, Masson L, Gehr R, Frigon D. (2014) Impact of wastewater treatment processes on antimicrobial resistance genes and their co-occurrence with virulence genes in *Escherichia coli*. Water Res 50: 245-253.

Bywater R, Deluyker H, Deroover E, De Jong A, Marion H, McConville M, Rowan T, Shryock T, Shuster D, Thomas V (2004). A European survey of antimicrobial susceptibility among zoonotic and commensal bacteria isolated from food-producing animals. J Antimicrob Chemother 54(4): 744-754.

Cabeza Y, Candela L, Ronen D, Teijon G. (2012) Monitoring the occurrence of emerging contaminants in treated wastewater and groundwater between 2008 and 2010. The Baix Llobregat (Barcelona, Spain). Jour Hazard Mater 239-240: 32-9

Center for Disease Control and Prevention. (2013). Antibiotic Resistance Threats in the United States 2013. Technical Paper CS239559-B

Central Statistics Office (2012) Census of Agriculture 2010 - Final Results. Dublin.
Central Statistics Office (2012) This is Ireland. http://www.cso.ie/en/census/census2011reports/census2011thisisirelandpart1/ Central Statistics Office: Dublin (Accessed 8th August 2016).

Chee-Sanford JC, Aminov R.I,. Krapac I, Garrigues-Jeanjean N, Mackie RI (2001) Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. Appl Environ Microbiol 67(4): 1494-1502.

CLSI (2013) Performance Standards for Antimicrobial Susceptibility Testing; 18th Informational Supplement, M100–S23. Wayne, PA: Clinical and Laboratory Standards Institute.

Coleman BL, Louie M, Salvadori MI, McEwen SA, Neumann N, Sibley K, Irwin RJ,. Jamieson FB,. Daignault D, Majury A (2013) Contamination of Canadian private drinking water sources with antimicrobial resistant *Escherichia coli*. Water Res 47(9): 3026-3036.

Coxon C (2014). Water Quality in Irish Karst Aquifers. Proceedings of 34th Annual Groundwater Conference, IAH Irish Group. Tullamore, Co Offaly, Ireland.

DAFM (2014) All-island Animal Disease Surveillance Report 2014. Department of Agrigulture, Food and the Marine, Dublin 2, Ireland.

https://www.agriculture.gov.ie/media/migration/animalhealthwelfare/labservice/rvlreports/2014_All_Island_Disease_Surveillance_Report.pdf

Drawz SM, Bonomo RA (2010) Three decades of β-lactamase inhibitors. Clin Microbiol Re. 23(1), 160-201.

Durso LM, Wedin DA, Gilley JE, Miller DN, Marx DB (2016) Assessment of Selected Antibiotic Resistances in Ungrazed Native Nebraska Prairie Soils. J Environ Qual 45: 454-462 DOI 10.2134/jeq2015.06.0280

Fernandes AM, Balasegaram S, Willis C, Wimalarathna HM, Maiden MC, McCarthy ND. (2015) Partial failure of milk pasteurization as a risk for the transmission of Campylobacter from cattle to humans. Clin Infect Dis 61:903–909.

European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2016). Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0. http://www.eucast.org

European Commission. (2013). Special Eurobarometer 407. Antimicrobial Resistance

Friesema I, Van De Kassteele J, De Jager C, Heuvelink A, Van Pelt W (2011) Geographical association between livestock density and human Shiga toxin-producing *Escherichia coli* O157 infections. Epidemiol Infect 139(07): 1081-1087.

Frey SK, Topp E, Khan IU, Ball BR, Edwards M, Gottschall N, Sunohara M, . Lapen DR (2015) Quantitative *Campylobacter* spp., antibiotic resistance genes, and veterinary antibiotics in surface and ground water following manure application: Influence of tile drainage control. Sci Total Environ 532: 138-153.

Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G (2012) The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. Science 337: 1107-1111

Galvin S, Boyle F, Hickey P, Vellinga A, Morris D, Cormican M (2010) Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. Appl Environ Microbiol 76(14): 4772-4779.

Godfrey E, Woessner WW, Benotti MJ. 2007 Pharmaceuticals in On-site Sewage Effluent and Ground Water, Western Montana. Ground Water 45:263.

Glassmeyer ST, Furlong ET,. Kolpin DW, Cahill JD,. Zaugg SD, Werner SL, Meyer MT,. Kryak DD (2005) Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination. Environ Sci Technol 39(14): 5157-5169.

Haller MY, Müller SR, McArdell CS, Alder AC, Suter MJF. (2002) Quantification of veterinary antibiotics (sulfonamides and trimethoprim) in animal manure by liquid chromatography–mass spectrometry. J Chromatogr 952(1): 111-120.

Halling-Sørensen B. (2001) Inhibition of aerobic growth and nitrification of bacteria in sewage sludge by antibacterial agents. Arch Environ Contam Toxicol 40(4): 451-460.

Hauser AR (2012) *Antibiotic basics for clinicians: The ABCs of choosing the right antibacterial agent*. Lippincott Williams & Wilkins.

Hendriksen RS, Mevius DJ, Schroeter A, Teale C, Meunier D, Butaye P, Franco A, Utinane A, Amado A, Moreno M. (2008) Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. Acta Vet Scand 50(1): 1.

Hirsch R, Ternes T, Haberer K, Kratz K-L. (1999) Occurrence of antibiotics in the aquatic environment. Sci Total Environ 225(1): 109-118.

Holmes P, Boxall A, Johnson K, Assem L, Levy LS (2007) *Evaluation of the potential risks to consumers from indirect exposure to veterinary medicines, Final report*. Institute of Environmental and Health and the Central Science Laboratory for the Department for Environmental Food and Rural Affairs, Bedfordshire, United Kingdom.

HPRA (2013) Report on consumption of veterinary antibiotics in Ireland during 2013. Health Products RegulatoryAuthority

Hynds P, Misstear BD, Gill LW, Murphy HM. (2014) Groundwater source contamination mechanisms: Physicochemical profile clustering, risk factor analysis and multivariate modelling. J Contam Hydrol159: 47-56.

Hynds PD, Misstear BD, Gill LW. (2012) Development of a microbial contamination susceptibility model for private domestic groundwater sources. Water Resour Res, 48(12).

ISO (1998) Water Quality, Standards for the enumeration of microorganism as per IDEXX methods, ISO 9308-3:1998/2010.

Jørgensen S E, Halling-Sørensen B. (2000) Drugs in the environment. Chemosphere 40(7): 691-699.

Kahlmeter G, Brown DF, Goldstein FW, MacGowan AP, Mouton JW, Osterlund A, Rodloff A, Steinbakk M, Urbaskova P, Vatopoulos A. (2003) European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. J Antimicrob Chemother 52: 145-148.

Kümmerer K. (2003) Significance of antibiotics in the environment. J Antimicrob Chemother 52(1): 5-7.

Langlois B, Cromwell G, Stahly T, Dawson K, Hays V (1983) Antibiotic resistance of fecal coliforms after long-term withdrawal of therapeutic and subtherapeutic antibiotic use in a swine herd. Appl Environ Microbiol 46(6): 1433-1434.

Lapworth DJ, Baran N, Stuart ME, Ward RS. (2012) Emerging organic contaminants in groundwater: a review of sources, fate and occurrence. Environ Pollut 163: 287-303.

Li X, Watanabe N, Xiao C, Harter T, McCowan B, Liu Y, Atwill ER. (2014) Antibiotic-resistant *E. coli* in surface water and groundwater in dairy operations in Northern California. Environ Monit Assess 186(2): 1253-1260.

López-Serna R, Jurado A, Vázquez-Suñé E, Carrera J, Petrovic M, Barcelo D. (2013) Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain. Environ Pollut 174: 305–315

Ma Y, Li M, Wu M, Li Z and Liu X (2015) Occurrences and regional distributions of 20 antibiotics in water bodies during groundwater recharge. Sci Total Environ 518: 498-506.

Maran NH, do Amaral Crispim B, Ramirez-Iahnn S, Pires de Araujo R, Burufatti Grisolia A, Pires de Oliveira K. (2016) Depth and Well Type Related to Groundwater Microbiological Contamination; Int J. Environ Res Public Health13(10)

Morasch B. (2013) Occurrence and dynamics of micropollutants in a karst aquifer. Environ Pollut 173: 133–137

Met Eireann (2016) Climate of Ireland. The Iirsh Meterological Office. [Accessed Online] http://www.met.ie/climate/climate-of-ireland.asp (Accessed 8th August 2016).

McEwen SA, Fedorka-Cray PJ. (2002) Antimicrobial use and resistance in animals. Clin Infect Dis 34(3): 93-106.

McKeon D., Calabrese JP, Bissonnette GK. (1995) Antibiotic resistant gram-negative bacteria in rural groundwater supplies. Water Res 29(8):1902-1908.

Michael I, Rizzo L. McArdell CS, Manaia C M, Merlin C, SchwartzT, Fatta-Kassinos D. (2013) Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. Water Res 47(3): 957-995.

Murphy HM, Prioleau MD, Borchardt MA, Hynds PD. (2017). Groundwater and Enteric Disease: A Global Review of the Epidemiological Evidence 1948-2013. Hydrogeol J Special Issue

O'Dwyer J, Dowling A, Adley CC. (2014) Microbiological assessment of private groundwater-derived potable water supplies in the Mid-West Region of Ireland. J Water Health 12(2): 310-317.

ÓhAiseadha C, Hynds PD, Fallon U, O'Dwyer J. (2017) A geo-statistical investigation of agricultural and infrastructural risk factors associated with primary verotoxigenic *E. coli* (VTEC) infection in the Republic of Ireland, 2008–2013. Epidemiol Infect 145(1) :95-105.

Parry SM, Palmer SR (2000) The public health significance of VTEC 0157. J Appl Microbiol 88:S1-9.

Peng X, Ou W, Wang C, Wang Z, Huang Q, Jin, J, Tan J. (2014) Occurrence and ecological potential of pharmaceuticals and personal care products in groundwater and reservoirs in the vicinity of municipal landfills in China. Sci Total Environ 490: 889-898.

Pruden A, Pei R, Storteboom H, Carlson KH. (2006) Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. Environ Sci Technol 40(23): 7445-7450.

Ribeiro AF, Laroche E, Hanin G, Fournier M, Quillet L, Dupont J-P, Pawlak B. (2012) Antibiotic-resistant *Escherichia coli* in karstic systems: a biological indicator of the origin of fecal contamination? FEMS Microbiol Ecol 81(1): 267-280.

Ryan MP, Dillon C Adley CC. (2011) Nalidixic acid-resistant strains of Salmonella showing decreased susceptibility to fluoroquinolones in the midwestern region of the Republic of Ireland due to mutations in the gyrA gene. J Clin Microbiol 49(5): 2077-2079.

Sapkota AR, Curriero FC, Gibson KE, Schwab K.J (2007) Antibiotic-resistant enterococci and fecal indicators in surface water and groundwater impacted by a concentrated swine feeding operation. Environ Health Perspect 115(7): 1040-1045.

Sayah RS., Kaneene JB, Johnson Y, Miller R. (2005) Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic-and wild-animal fecal samples, human septage, and surface water. Appl Environ Microbiol 71(3): 1394-1404.

Scott M, Murray M. (2009) Housing rural communities: Connecting rural dwellings to rural development in Ireland. Hous Stud 24(6)755–774.

Servais P, Passerat J. (2009) Antimicrobial resistance of fecal bacteria in waters of the Seine river watershed (France). Sci Total Environ 408(2): 365-372.

Strachan NJC, Dunn GM, Locking ME, Reid TMS, Ogden ID. (2006) *Escherichia coli* O157: Burger bug or environmental pathogen? Int J Food Microbiol 112(2): 129-137.

Taylor NG, Verner-Jeffreys DW, Baker-Austin C. (2011) Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol Evol 26(6): 278-284.

Walsh F, Ingenfeld A, Zampicolli M, Hilber-Bodmer M, Frey J, Duffy B. (2011) Real-time PCR methods for quantitative monitoring of streptomycin and tetracycline resistance genes in agricultural ecosystems. J Microbiol Methods 86(2): 150-155.

Watkinson A, Murby E, Kolpin D, Costanzo S. (2009) The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. Sci Total Environ 407(8): 2711-2723.

Wellington EM,. Boxall AB, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W. (2013) The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. Lancet Infect Dis 13(2): 155-165.

Williams-Nguyen J, Sallach JB, Bartelt-Hun S., Boxall AB, Durso LM, McLain J E, Zilles JL. (2016). Antibiotics and antibiotic resistance in agroecosystems: State of the science. J Environ Qual 45(2): 394-406.

Williams J, Murray A, McCrory C, McNally S. (2013) Growing Up in Ireland national longitudinal study of children: development from birth to three years infant cohort, Department of Children and Youth Affairs.

FIGURE CAPTIONS:

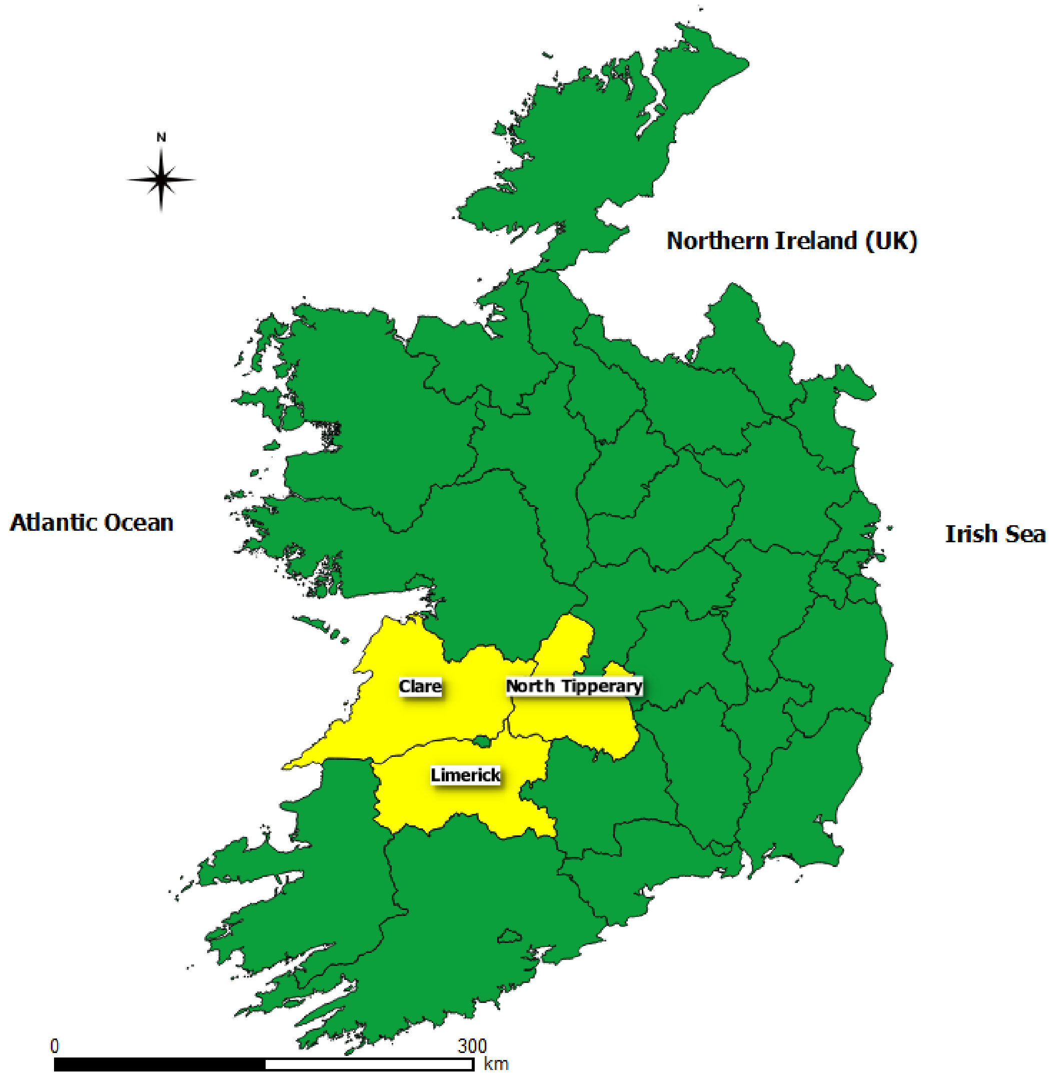

Figure 1: Location of the sampling area within the Republic of Ireland: Counties Limerick, Clare and North Tipperary (shown in yellow).

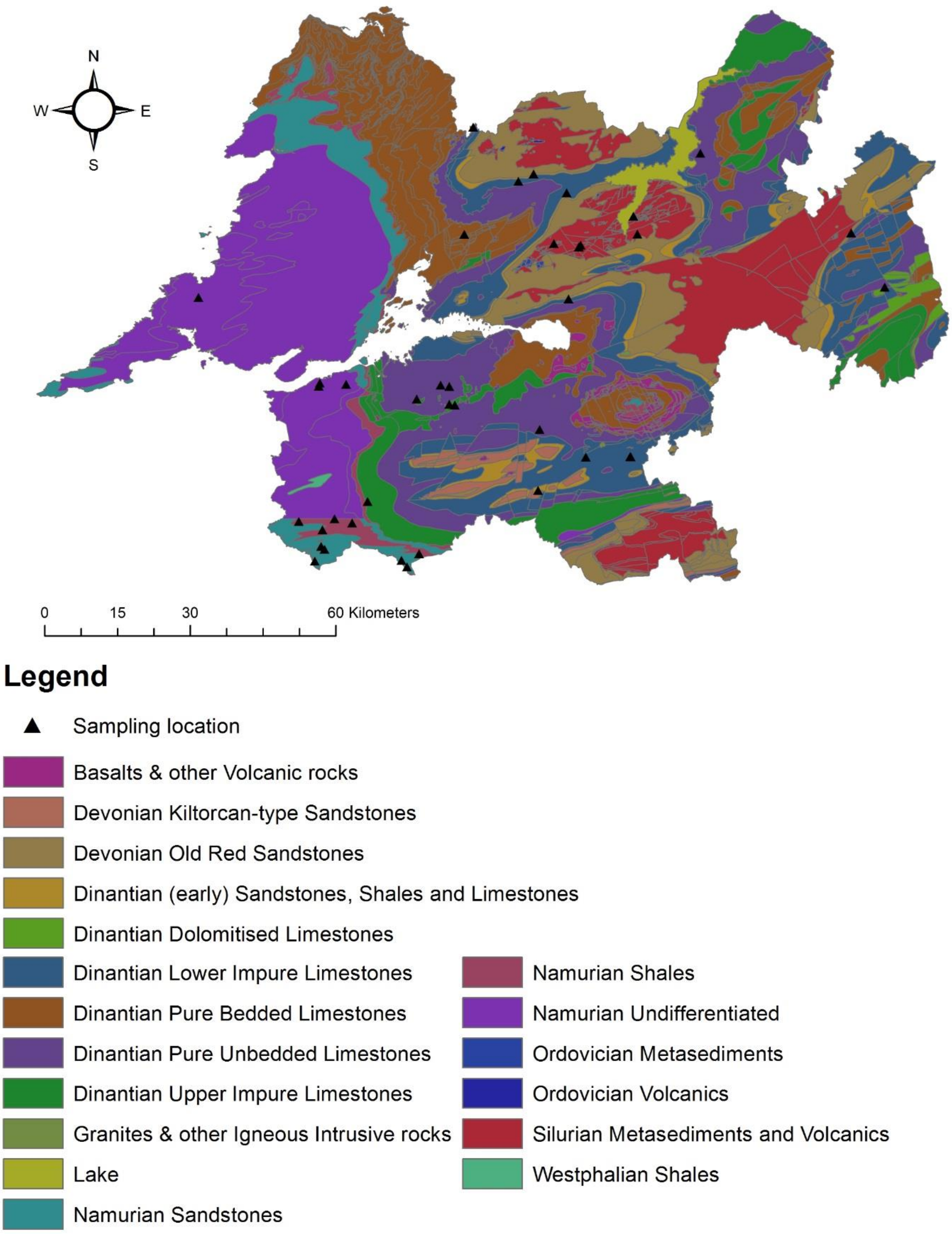

Figure 2: Geographical distribution of sampled sites and the geology of the research area