



# **Evaluation of Antibiotic Resistant Enterobacterales in the Irish Environment**

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the degree of Doctor of Philosophy by:

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## List of Abbreviations

AMR	Antimicrobial resistance
AAC	Acetyltransferases
AME	Aminoglycoside modifying enzyme
ANT	Nucleotidyltransferases
APH	Phosphotransferases
AREST	Antimicrobial Resistance and the Environment - Sources, persistence, Transmission, and risk management
ARG	Antibiotic resistance gene
ATCC	American type culture collection
BDU	Bed Day Units
BIGSdb	Bacterial isolate genome sequence database
CGE	Center for Genomic Epidemiology
cgMLST	Core Genome Multi Locus Sequence Type
CLSI	Clinical & Laboratory Standards Institute
CPE	Carbapenemase-producing <i>Enterobacteriaceae</i>
CSO	Central Statistics Office
DDD	Defined daily dose
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DWTP	Drinking water treatment plant
<i>E. coli</i>	<i>Escherichia coli</i>
EARS-NET	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EPA	Environmental Protection Agency
ESBL	Extended-spectrum $\beta$ -lactamase
ESKAPE	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , and <i>Enterobacter species</i>
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GTP	Guanosine triphosphate
HALT	Healthcare-Associated Infections & Antimicrobial Use in Long-Term Care Facilities
HPRA	Health Products Regulatory Authority
HPSC	Health Protection Surveillance Centre
HSE	Health Service Executive

## Abbreviations

ICE	Integrative conjugative elements
ICW	Integrated constructed wetland
LAA	Local authority area
LPS	Lipopolysaccharide
LS1	Longitudinal Survey 1
LS2	Longitudinal Survey 2
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time of Flight
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
Minlen	Minimum length
MLST	Multi-locus sequence typing
MPN	Mean Probable Number
NCPERLS	National Carbapenemase-producing Enterobacterales Reference Laboratory Service, Ireland
PBPs	Penicillin binding proteins
PCR	Polymerase Chain Reaction
PE	Paired end
PIER	Public health Impact of Exposure to antibiotic resistance in Recreational waters
PNEC	Predicted no effect concentration
PP	Point prevalence
QRDR	Quinolone resistance-determining region
RMT	rRNA methyltransferases
RNA	Ribonucleic acid
RPP	Ribosome protection protein
rRNA	Ribosomal ribonucleic acid
ST	Sequence type
tRNA	Transfer ribonucleic acid
UWWD	Urban Wastewater Discharge
VTEC	Verotoxigenic <i>E. coli</i>
WHO	World Health Organization
WWTP	Wastewater treatment plant

## Declaration of Authorship

I, Brigid Hooban, certify that this thesis is all my own work and that I have not obtained a degree in this University or elsewhere using this work.

I declare that the entirety of this work was carried out while enrolled as a PhD candidate at the National University of Ireland Galway.

Signed: *Brigid Hooban*

Date: 02/04/2022

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## Abstract

Antibiotic resistance represents a significant public health challenge of global magnitude. In particular, infections caused by extended spectrum beta-lactamase (ESBL) and carbapenemase producing Enterobacterales (CPE) rank highly in respect to prevalence and clinical consequences. The environmental aspect of the One Health triad is not routinely monitored for antibiotic resistance, and as such represents an important knowledge gap relating to its role as a route of transmission of antibiotic resistance. Therefore, the aim of this research was to address this knowledge gap by evaluating the literature and assessing environmental samples on a national scale in Ireland for the presence of antibiotic resistance.

A scoping review was initially conducted to evaluate the literature examining the natural aquatic environment, free from direct point discharges, as a reservoir and potential route of dissemination of ESBL and carbapenemase producing Enterobacterales. This was followed by an initial point prevalence survey which included the collection and processing of 39 water and 25 sewage samples across the South, East and West of Ireland. This was subsequently followed by two additional sampling rounds in each area (118 water, 36 sewage samples) as part of the longitudinal study.

The scoping review included 41 studies ranging across 19 different countries. ESBL and carbapenemase genes were reported in 70.7% and 31.7% of studies respectively, revealing widespread dissemination across uncontaminated aquatic environments. A total of 630 Enterobacterales (487 water, 143 sewage) were isolated from the point prevalence and longitudinal survey sampling rounds. ESBL detection was widespread across the majority of samples collected. One or more CPE were isolated from 32 individual sampling sites (23 water, 9 sewage) across all three rounds. This included the detection of isolates harbouring *bla*<sub>OXA-48</sub> (n = 24), *bla*<sub>NDM</sub> (n = 16), *bla*<sub>KPC</sub> (n = 6), *bla*<sub>IMP</sub> (n=1) and

*bla*<sub>OXA-484</sub> (n = 1). Common sequence types detected included *E. coli* ST131, ST38 and ST10 along with *Klebsiella* ST405 and ST323. Highly similar isolates from different water samples, alongside water and sewage samples were identified using core genome MLST comparisons.

Carbapenemase producing Enterobacterales are considered a public health emergency in Ireland, and so their detection in the environment is a significant public health concern. The detection of ESBL and CPE across all seasons highlights the need for regular monitoring of the aquatic environment for the presence of antimicrobial resistant organisms to adequately inform policies to protect public health. Further areas of research which were identified in this thesis include evaluation of the risk of colonisation and infection due to exposure to the environmental resistome through ingestion of resistant bacteria in water via recreational exposure.

# Chapter 1: Introduction

## **1.1 Antibiotics and Mechanisms of Resistance**

Antibiotics play an essential role in the treatment of bacterial infections ranging from commonly encountered urinary tract infections, to life threatening cases of sepsis or meningitis. Various classes of antibiotics utilise a wide array of mechanisms in order to kill (bactericidal) or halt (bacteriostatic) bacterial replication (Pankey & Sabath 2004). Antibiotics can be categorised into different classes based on their structure and mechanism of action. Each antibiotic class works by targeting different bacterial components primarily including the cell wall, cell membrane, ribosomal sub-units or by interfering with DNA replication or folic acid metabolism (Kapoor et al., 2017). There are an array of antibiotics that are useful against infections caused by Enterobacterales, which are the microorganisms of interest within this research. These include beta-lactams, fosfomycin, fluoroquinolones, trimethoprim, aminoglycosides, tetracyclines, chloramphenicol and colistin.

Antibiotic resistance is a growing public health concern, which is estimated to be associated with 10 million deaths per year by 2050 unless action is taken (O' Neill, 2014). Bacteria have the capacity to rapidly evolve mechanisms of resistance following the introduction of new antibiotic agents, as outlined in Figure 1.2. Broadly speaking, mechanisms of resistance normally fall under three primary categories; modification of the target site, enzymatic inactivation of the antibiotic, or reduced intracellular accumulation by increased efflux or decreased uptake (Kapoor et al., 2017). Resistance against antibiotic agents may be due to intrinsic mechanisms, such as natural structural variations in the antibiotic target site, or acquired resistance via horizontal gene transfer (Peterson & Kaur 2018).

### **1.1.1 Beta-lactam antibiotics**

Beta-lactam antibiotics are among the earliest treatments for bacterial infections widely used in modern medicine, dating back to the initial discovery

of penicillin in 1928 (Fleming, 1929). Since then, many classes of antibiotics have been synthesised harbouring a beta-lactam ring that confer bactericidal effects by interfering with bacterial cell wall synthesis. The beta-lactam antibiotic class includes penicillins, monobactams, carbapenems and all generations of cephalosporins (Bush & Bradford 2016). These antibiotics exert their effect by targeting peptidoglycan cross-linking of bacterial cell wall formation, which affords strength and rigidity to the cell (Worthington & Melander, 2013). The peptidoglycan layer is primarily composed of repeating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, and the cross-linking reaction is catalysed by penicillin binding proteins (PBPs) (Vollmer et al., 2008). Beta-lactam antibiotics have a similar structure to that of the substrate of PBPs, creating competition for active site attachment (Worthington & Melander, 2013). Once the beta-lactam antibiotic transverses the bacterial membrane to the periplasmic space via porins in gram negative bacteria, it can successfully bind to the PBPs and inhibit transpeptidation (Tang et al., 2014). The ultimate outcome of this process is cell lysis.

Mechanisms of bacterial resistance against beta-lactam antibiotics range from intrinsic structural components including increased efflux pump activity and downregulation of porin channels, to the production of beta-lactamase enzymes that mediate direct hydrolysis of the antibiotic (Bush & Bradford 2016). Over time, beta-lactamases have evolved to target more potent and structurally advanced beta-lactam antibiotics. The earliest types of beta-lactamases discovered included the AmpC enzymes, dating back to 1940, when they were initially termed penicillinases (Abraham & Chain, 1940). AmpC enzymes can be categorised as Class C based on Ambler's structural classification scheme (Hall & Barlow, 2005), and group 1 within the Bush-Jacoby scheme, due to their inhibition and hydrolysis activity (Bush et al., 1995). In many instances, AmpCs can confer resistance to penicillins, the early generation cephalosporins and cephamycins including ceftiofuran (Meini et al., 2019). Examples of *ampC* genes

include *bla*<sub>CMY</sub>, *bla*<sub>DHA</sub> and *bla*<sub>ACT</sub>. Many Enterobacterales exhibit chromosomally encoded AmpC production such as *Enterobacter cloacae*, *Morganella morganii* and *Citrobacter freundii*, whilst other Enterobacterales including *Klebsiella pneumoniae* and *Salmonella* species lack chromosomal *ampC* genes (Tamma et al., 2019). A range of Amp proteins regulate AmpC production, including AmpR, AmpD and AmpG. AmpR reduces the levels of AmpC that is expressed. This mechanism is disrupted in the presence of beta-lactams, as products released following bacterial cell wall destruction competitively bind to AmpR, preventing its normal functioning and subsequently increasing AmpC production. AmpD participates in cleaving products from cell wall destruction preventing them from binding to AmpR, while AmpG functions as a transporter of these products into the cytoplasm where they can bind to AmpR (Meini et al., 2019). Although *E. coli* typically harbours an *ampC* gene within its chromosome, it is not inducible like most other species. Rather, AmpC is constitutively produced at low levels. Plasmid mediated *ampC* genes, which are typically continually expressed, are occasionally identified in *E. coli*, *Klebsiella* and *Salmonella* species (Tamma et al., 2019).

Cephalosporin antibiotics are currently categorised into five generations based on their spectrum of activity (Rusu & Lungu 2020). AmpC production at a low level is capable of conferring resistance to the first generations of cephalosporins such as cefazolin (Mizrahi et al., 2020). However, during upregulation this mechanism can reduce susceptibility to later generation cephalosporins. Following the introduction of third generation cephalosporins in the 1980s, the detection of plasmid mediated extended spectrum beta-lactamase (ESBL) enzymes soon followed in 1983 (Knothe et al., 1983). The earliest ESBLs included mutations in the broad spectrum beta-lactamases SHV-1, TEM-1 and TEM-2 which conferred resistance to generations of cephalosporins with extended spectrum of activity (Gniadkowski, 2001; Paterson & Bonomo, 2005). Generally, ESBLs are capable of hydrolysing

penicillins, aztreonam and up to the third generation of cephalosporins, but their action is impeded by beta-lactamase inhibitors (Paterson & Bonomo 2005). The majority of ESBLs belong to Bush-Jacoby group 2be and Ambler class A, with the exception of OXA enzymes which are categorised as group 2d and class D respectively (Dhillon & Clark 2012). Similarly to SHV and TEM beta-lactamases, only certain variants of OXA exert extended spectrum activity including OXA-10, 11, 14-19, 28, 31, 32, 35 and 45 (Paterson & Bonomo 2005). Although the carbapenem class of antibiotics remain successful at treating ESBL infections, the use of beta-lactamase inhibitors such as clavulanic acid is preferential (Harris et al., 2015; Muhammed et al., 2017). This is to protect the carbapenem antibiotics as they are considered one of the last lines of defence against beta-lactamase producing bacteria.

Beta-lactamases have evolved to produce carbapenemases, which are capable of hydrolysing almost all beta-lactam antibiotics (Queenan & Bush 2007). However, more recent studies have demonstrated significant restoration of susceptibility when carbapenem and beta-lactamase inhibitors are used in combination against carbapenemase producers (El Hafi et al., 2019). Similarly to ESBLs, the carbapenemase enzymes belong to more than 1 Ambler class. Many of the carbapenemases are categorised within Ambler class A, B and D, of which A and D possess a serine residue in the enzymes active site, while class B are metallo-beta-lactamases which harbour zinc ions in place of serine (Codjoe & Donkor, 2017). Ambler class A translates to Bush-Jacoby group 2f and includes *bla*<sub>KPC</sub>, *bla*<sub>IMI</sub>, *bla*<sub>SME</sub> and *bla*<sub>GES</sub> carbapenemases (Bush & Jacoby, 2010). Ambler class B (Bush-Jacoby group 3a) includes *bla*<sub>NDM</sub>, *bla*<sub>VIM</sub> and *bla*<sub>IMP</sub>, while Ambler class D (Bush-Jacoby group 2df) includes *bla*<sub>OXA-48</sub> and *bla*<sub>OXA-23</sub> amongst other OXA variants (Bush & Jacoby 2010, Codjoe & Donkor 2017). In Ireland, the carbapenemase genes detected in 2020 through colonisation screening and infections included *bla*<sub>OXA-48</sub> (n=606), *bla*<sub>KPC</sub> (n=144), *bla*<sub>OXA-181</sub> (n=46), *bla*<sub>NDM</sub> (n=42), *bla*<sub>VIM</sub> (n=38), *bla*<sub>OXA-244</sub> (n=18), *bla*<sub>IMP</sub> (n=11), *bla*<sub>IMI</sub> (n=5), *bla*<sub>OXA-484</sub>

(n=4), *bla*<sub>OXA-232</sub> (n=1), and *bla*<sub>NMC-A</sub> (n=1) (Health Service Executive, Unpublished). In 2018, the European Centre for Disease Prevention and Control (ECDC) performed a European wide assessment of the epidemiological spread of carbapenemase-producing *Enterobacteriaceae* (CPE), including all types of infections along with colonisation (ECDC, 2019). A total of 43% of participating countries ranked as regional or inter-regional spread (stage 4), including Ireland (Figure 1.1). This highlights the widespread dissemination of carbapenemase production within bacterial isolates capable of causing invasive infections.

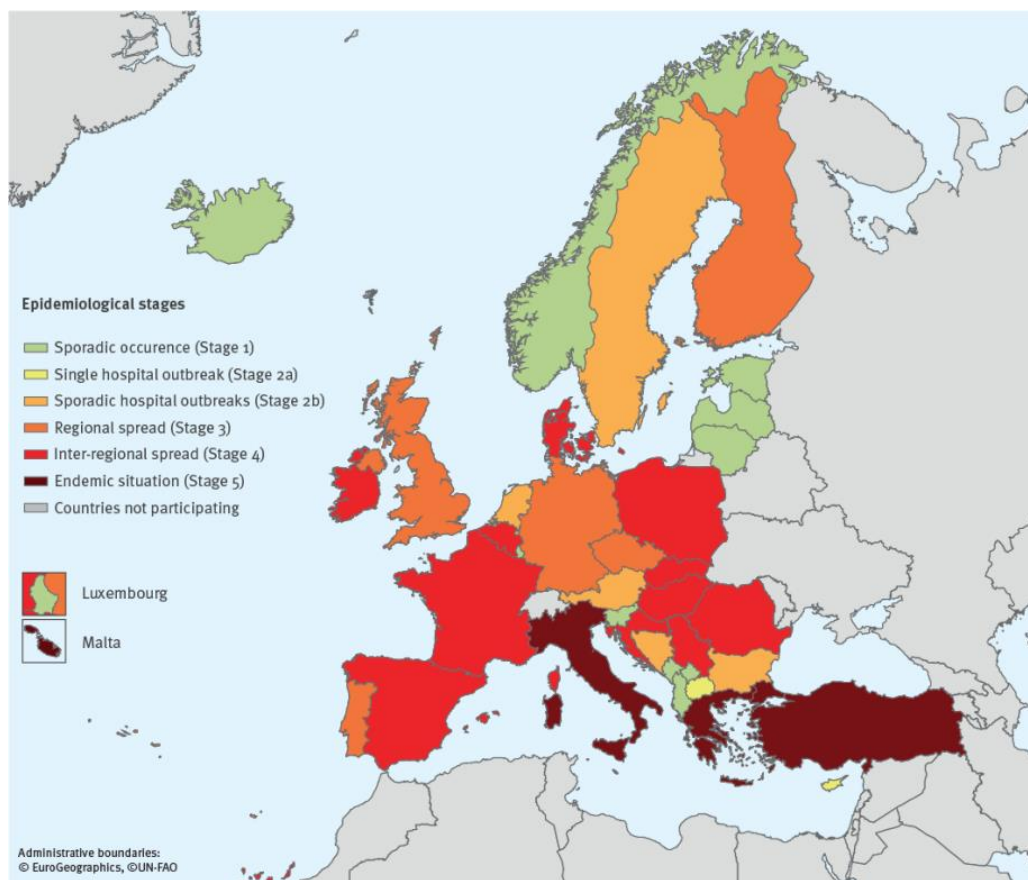


Figure 1.1: Epidemiological status of carbapenemase producing *Enterobacteriales* in Europe, including all types of infections and colonisation in 2018 (ECDC, 2019).

### 1.1.2 Fosfomycin

Fosfomycin operates by a similar mechanism to the beta-lactam antibiotics by inhibiting bacterial cell wall synthesis, but at an earlier stage. The antibiotic must

first transverse the bacterial cell membrane via membrane transporter proteins such as the glycerol-3-phosphate transporter and the hexose-6-phosphate transporter, both of which belong to the major facilitator superfamily (Cattoir & Guérin 2018). Once internalised, fosfomycin participates in competitive binding to the Cys115 residue within the active site of the MurA enzyme, involved in the early stages of peptidoglycan synthesis (Silver, 2017). Fosfomycin was initially discovered as a natural product of *Streptomyces fradiae* in 1969 (Hendlin et al., 1969). In recent years, fosfomycin has been increasingly chosen for treatment of multi-drug resistant infections due to its successful tissue penetration and the in vitro synergy that ensues when administered in combination with other antibiotics (Dijkmans et al., 2017). Combination therapy may include the use of ciprofloxacin, aminoglycosides, colistin or beta-lactam antibiotics along with fosfomycin (Ruiz Ramos & Salavert Lletí 2019).

Fosfomycin resistance can arise due to chromosomal mutations related to the MurA enzyme, or mutations resulting in the loss in activity of the transport systems required for bacterial cell entry (Ballester-Téllez et al., 2017). These mutations regularly occur in combination, but the additive effect does not always confer complete fosfomycin resistance. Alternatively, reduced susceptibility to fosfomycin may arise due to enzymes that directly inactivate fosfomycin, such as FosA (Ito et al., 2017). FosA inactivates the antibiotic by conjugating glutathione directly to the fosfomycin. The *fosA* gene may be plasmid or chromosomally encoded and is found more commonly in *Klebsiella*, *Enterobacter* and *Serratia* species. Other forms of these modifying enzymes include FosB and FosX (Silver et al., 2017).

### **1.1.3 Quinolones/Fluoroquinolones**

Another antibiotic class commonly used in the treatment of clinical infections are the quinolones/fluoroquinolones. The first antibiotic discovered that belonged to the quinolone class was nalidixic acid, isolated as a by-product of

chloroquine synthesis in the 1950s (Bisacchi 2015). The fluoroquinolone antibiotics evolved from the quinolone class in the 1980s, and differed by the presence of a nitrogen atom instead of the eight carbon atom, in addition to the occurrence of a fluorine atom (Redgrave et al., 2014). The first fluoroquinolone introduced into medical practice was flumequine followed by further generations including norfloxacin, ciprofloxacin, levofloxacin and moxifloxacin amongst others (Pham et al., 2019). The fluoroquinolone class of antibiotics operate by targeting enzymes involved in the process of DNA replication. These targets include the DNA gyrase (topoisomerase II) and topoisomerase IV, with DNA gyrase as the prime target in gram-negative bacteria (Hooper & Jacoby, 2005). Both of these enzymes possess the capacity to relax positively supercoiled DNA. However, DNA gyrase can also operate in an opposing manner of introducing negative supercoils into DNA that is in a relaxed state (Redgrave et al., 2014). Ultimately, these enzymes are essential for the role of catalysing DNA strand breakage and re-joining, which is necessary for DNA replication and control of gene expression. The fluoroquinolone antibiotics operate by trapping the DNA and topoisomerase complex at the strand breakage stage, preventing re-joining.

The most common mechanism of fluoroquinolone resistance is chromosomal mutations conferring alterations in the topoisomerase enzymes. The sites for these mutations can include the *gyrA*, *gyrB*, *parC* or *parE*, which involves amino acid substitutions in regions of the DNA called the quinolone resistance-determining region (QRDR) (Jacoby, 2015). These mutations can confer reduced affinity of the fluoroquinolone for the topoisomerase enzymes and subsequent fluoroquinolone resistance when combined with additional mutations in one or more of the four sites. In addition to chromosomal mutations, fluoroquinolone resistance can be attenuated by plasmid mediated resistance genes in combination with mutations in the QRDR (Redgrave et al., 2014; Kotb et al., 2019). The *qnr* genes are often identified on plasmids, which confer

resistance through protection of the quinolone target enzymes (Rezazadeh et al., 2016). There are approximately 100 *qnr* variants which are categorised into five families including *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS* (Nsofor et al., 2021). An additional plasmid mediated resistance gene is the *aac(6′)-Ib-cr* which encodes for an aminoglycoside acetyltransferase, that can inactivate some antibiotics belonging to the fluoroquinolone class (Redgrave et al., 2014). A less specific mechanism which confers a reduction in fluoroquinolone susceptibility is the efflux encoding genes; *oqxAB* and *qepA*. These can also be located on mobile genetic elements and work by expelling the antibiotic that enters the bacterial cell.

#### **1.1.4 Trimethoprim**

Trimethoprim is a commonly used antibiotic for the clinical treatment of urinary and respiratory tract infections, which also operates by interfering with internal cellular processes within bacteria (Masters et al., 2003). Trimethoprim competitively inhibits a crucial enzyme known as dihydrofolate reductase (DHFR), used as part of folic acid metabolism. The functional role of DHFR is to catalyse the reduction of dihydrofolic acid to tetrahydrofolic acid, which is subsequently used as a necessary component of nucleotides (purines) and bacterial DNA (Yoneyama & Katsumata 2006). Although DHFR also operates in human cells, the human enzyme is 50,000 to 100,000 times less sensitive to trimethoprim. Trimethoprim is commonly coupled with sulphonamides which act in a synergistic manner on a different enzyme known as dihydropteroate synthase in the same pathway (Kapoor et al., 2017).

Chromosomal mutations account for a small proportion of trimethoprim resistance, including increased production of DHFR, encoded by the *folA* gene as well as structural alterations to DHFR, reducing its affinity for trimethoprim (Sánchez-Osuna et al., 2020). More commonly encountered mechanisms of resistance to trimethoprim include mobile *dfrA* and *dfrB* resistance genes. The

*dfrA* gene is homologous to the *folA*, while *dfrB* encodes a structurally distinct DHFR enzyme (Toulouse et al., 2017). Both *dfr* genes encode DHFRs that can operate in the presence of trimethoprim. However, the *dfrB* gene is of significant clinical concern as it confers significantly increased trimethoprim resistance in comparison to *dfrA* (Lemay-St-Denis et al., 2021). The parallel use of trimethoprim and sulphonamides in clinical practice commonly selects for the presence of *sul* and *dfrA* genes on the same mobile genetic elements (Sánchez-Osuna et al., 2020).

### 1.1.5 Aminoglycosides

Many different antibiotic classes target the bacterial ribosomal subunits, successfully interfering with protein production necessary for survival. Aminoglycosides specifically target the 30S ribosomal subunit, by binding to the 16S ribosomal ribonucleic acid (rRNA) (Krause et al., 2016). Although not all antibiotics belonging to the aminoglycoside class bind to the same site within the 16S rRNA, they all exert a similar effect, changing the ribosomes conformation at the acceptor site, subsequently disrupting proofreading mechanisms (Ramirez & Tolmasky 2010). Streptomycin was the first antibiotic discovered belonging to the aminoglycoside class, isolated from a soil bacterium known as *Streptomyces griseus* (Waksman, 1948). Streptomycin operates by causing conformational changes to the 30S subunit and consequently misreading the rRNA (Demirci et al., 2013). Several other antibiotics belonging to the aminoglycoside class were introduced into clinical practice shortly after streptomycin with similar mechanisms of actions including kanamycin and gentamicin (Krause et al., 2016).

Aminoglycoside resistance can be conferred through modification of the 16S rRNA, increased efflux or reduced permeability by the bacterium, and/or by aminoglycoside modifying enzymes (AMEs) (Krause et al., 2016). The naming system commonly used for AMEs provides information in relation to the type of

enzyme modification (e.g. acetyltransferases), the site of modification (e.g. 6'), followed by a roman numeral referring to the resistance profile and a letter for protein designation (Ib) (Shaw et al., 1993). The first three letters can be AAC (acetyltransferases), ANT (nucleotidyltransferases) or APH (phosphotransferases) (Ramirez & Tolmasky 2010). All three catalyse some modification to the aminoglycoside antibiotic including acetylation of  $-NH_2$  groups (AAC), transfer of an AMP group from adenosine triphosphate (ANT) or transfer of a phosphate group (APH).

Modification of the 16S rRNA is achieved through enzyme methylation by 16S rRNA methyltransferases (RMTs) (Ramirez & Tolmasky 2010). This modification prevents the binding of the rRNA to the aminoglycosides. Plasmid-borne RMTs are encoded by *armA*, *rmtB1*, *rmtC* and many more genes.

### **1.1.6 Tetracyclines**

Tetracyclines are another group of antibiotics that also prevent protein production by targeting the 16S rRNA of the 30S ribosomal subunit (Markley & Wencewicz 2018). Once bound, the tetracycline prevents the attachment of tRNAs carrying amino acids to the acceptor site. Similarly to aminoglycosides, the first generation of tetracyclines were isolated from *Streptomyces* species (Chopra & Roberts 2001). These included chlortetracycline and oxytetracycline from *Streptomyces aureofaciens* and *Streptomyces rimosus* respectively. The generations of tetracyclines that followed have progressed from semi-synthetic second generation compounds including doxycycline and metacycline, to fully synthetic fourth generation antibiotics including ervacycline and omadacycline (Markley & Wencewicz 2018). Tetracyclines are the most commonly used antibiotic in veterinary medicine in Ireland accounting for 41.2% of antibiotic sales for veterinary purposes in 2020 (Health products regulatory authority 2021).

The most commonly encountered mechanisms of tetracycline resistance include ribosome protection proteins (RPPs) and efflux pumps (Markley & Wencewicz 2018). The majority of efflux pumps capable of expelling tetracycline from bacterial cells, reducing the internal concentrations, belong to the major facilitator superfamily (Grossmann, 2016). In gram-negative bacteria, Tet(A) and Tet(B) are the most commonly identified efflux pumps responsible for reducing susceptibility to tetracyclines. RPPs are GTPases that share homology with elongation factors (Markley & Wencewicz 2018). These proteins function by catalysing the release of tetracyclines using guanosine triphosphate (GTP) from the ribosome (Grossmann, 2016). Examples of commonly identified RPPs include Tet(O) and Tet(M).

### **1.1.7 Chloramphenicol**

Chloramphenicol is a further example of an antibiotic that operates by inhibiting the translational process at the 50S subunit of the ribosome. The target site of chloramphenicol is the acceptor site on the 50S subunit, preventing the transfer ribonucleic acid (tRNA) from binding (Svetlov et al., 2019). In the European Union chloramphenicol is strictly banned from administration to food producing animals, however, a fluorinated derivative of chloramphenicol, known as florfenicol, is used to treat respiratory infections in agriculture (De Smet et al., 2018). Similar to many antimicrobials, the discovery of chloramphenicol originated from the soil dwelling bacteria known as *Streptomyces venezuelae* (Ehrlich et al., 1947).

Resistance to chloramphenicol is often mediated by chloramphenicol acetyltransferases (CATs) (Schwarz et al., 2004). CATs inactivate the chloramphenicol antibiotic via acetylation, but have no effect on florfenicol due to the structural differences of a fluorine residue at the acceptor site (position C-3) in place of a hydroxyl group. Further less specific mechanisms, which can contribute to chloramphenicol resistance, include efflux systems, target site

mutations and reduction in cell permeability. (Fernández et al., 2012). Florfenicol resistance can be mediated by the *floR* gene which encodes for exporters (Lu et al., 2018a). Li et al. (2020) demonstrated that the *floR* resistance gene was the most common cause of florfenicol resistance in 97/106 isolates originating from chicken and geese faeces. However, the *floR* resistance gene has also been identified within human clinical isolates located on transferable plasmids (Lu et al., 2018a).

### 1.1.8 Colistin

Colistin is a primitive antibiotic that belongs to the polymixin family, and dates back to the late 1940s when its production by *Bacillus polymyxa* was initially discovered (Falagas et al., 2005). There are many different forms of polymixins, however, only colistin (polymixin E) and polymixin B are used for the treatment of infections (Andrade et al., 2020). Colistin is effective against gram-negative bacteria, but due to its nephrotoxic effects, its application in the clinical environment was gradually superseded by its use for veterinary purposes between 1970 and 1980. However, due to the recent shortages of effective antibiotics that can be used in the treatment of multi-drug resistant infections, the prescription of colistin has resurfaced in humans (Falagas et al., 2005). Colistin is one of the few antibiotics that operates by targeting the bacterial cell membrane, specifically the lipid A component of the lipopolysaccharide layer (Bialvaei & Samadi Kafil 2015). This binding results in the displacement of calcium and magnesium ions, subsequently leading to changes in the permeability of the cell membrane.

Colistin resistance can be due to structural changes in the bacterial lipopolysaccharide layer, the primary target of the antibiotic (Gogry et al., 2021). This resistance is intrinsic in certain bacterial species including *Proteus mirabilis* and *Serratia marcescens*. Acquired resistance through chromosomal modulations can also be observed in other gram negative bacteria such as

*Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter* species. In these species resistance can be conferred by chromosomal mutations in the *PhoPQ* and *PmrAB* genes encoding a two-component system responsible for modification of the lipopolysaccharide layer (Zafer et al., 2019). Alternatively, these pathogens may possess mobile colistin resistance (*mcr*) genes, which also confer resistance through modification of the lipid A. This mobile mechanism of resistance was only recently identified by Liu et al. (2016) in an isolate of swine faecal origin, potentially owing to the fact that colistin was previously used extensively in agriculture. To date 10 variants of the *mcr* gene have been identified, with *mcr-10* reported from a clinical strain isolated in 2016 (Wang et al., 2020a).

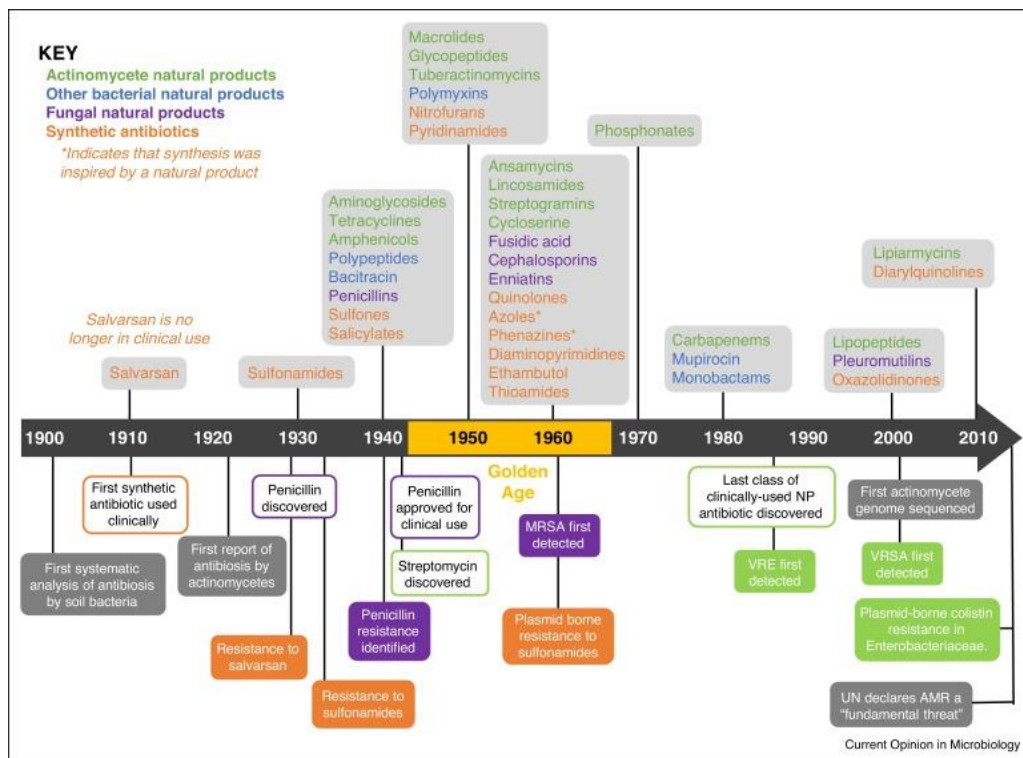


Figure 1.2: Timeline of the introduction of different antibiotics into clinical practice (Hutchings et al., 2019). The origins of the antibiotics (natural/synthetic) is along the top of the timeline. Below the timeline includes details of the initial discovery of some antibiotics along with the first resistance detection.

## 1.2 Enterobacterales

The presence of antibiotic resistance mechanisms only poses a risk to public health when it is combined with bacterial species capable of causing infections. Bacteria belonging to the Enterobacterales order regularly cause illness when displaced from regions in the body in which they exist as normal flora, such as lower respiratory tract infection, or when they are introduced into the body from an external source, e.g. nosocomial infections. The Enterobacterales order encompasses seven families including *Enterobacteriaceae*, *Erwiniaceae*, *Pectobacteriaceae*, *Yersiniaceae*, *Hafniaceae*, *Morganellaceae* and *Budviciaceae* (Adeolu et al., 2016). This order comprises of many pathogens including *Escherichia*, *Enterobacter*, *Salmonella*, *Shigella*, *Yersinia*, *Serratia* and *Proteus* which are all capable of causing multi-drug resistant infections.

All bacteria belonging to the Enterobacterales order share some common characteristics such as being Gram-negative bacilli that are facultative anaerobes (Adeolu et al., 2016). There are further attributes that are shared amongst members of the different families. For example, all *Enterobacteriaceae* possess catalase and lack oxidase production. However, at a genus level, different bacteria possess different properties that aid in their successful establishment of infection. According to the Health Protection Surveillance Centre (HPSC), key pathogens associated with concerning antibiotic resistance rates included *Escherichia coli* (*E. coli*) and *Klebsiella* that displayed resistance to fluoroquinolones, aminoglycosides, carbapenems (CPEs) and third generation cephalosporins (ESBLs) (HPSC, 2019b). These pathogens and associated resistance types are listed as being under regular surveillance by the European Antimicrobial Resistance Surveillance Network (EARS-Net), based on data collected and analysed on antibiotic resistance trends in Europe (ECDC, 2021a).

### 1.2.1 *Escherichia coli*

*Escherichia coli* is part of the normal flora in the gastrointestinal tract of both humans and animals and is therefore quantified as a marker of faecal pollution in the environment (Directive 2006/7/EC). *E. coli* is a pathogen that is responsible for causing a range of illnesses including infections associated with the urinary tract, gastrointestinal tract, surgical sites and less commonly cases of sepsis or meningitis (Sarowska et al., 2019). There are many different pathotypes of *E. coli* which comprise of different combinations of virulence factors, lending to their ability to cause infection in vivo. Of these pathotypes, there are six well characterised groups which include enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enterohaemorrhagic *E. coli* and diffusely adherent *E. coli* (Kaper et al., 2004).

Virulence factors play an important role in making *E. coli* such a commonly encountered pathogen in clinical infections. For example, uropathogenic *E. coli* harbour fimbriae allowing for adhesion and invasion of the host tissue, flagellum for motility, haemolysins for cytolysis activity along with siderophores for iron acquisition (Shah et al., 2019). Verotoxigenic *E. coli* (VTEC) are a particularly dangerous group of *E. coli* due to the production of the shiga toxins 1 and/or 2, potentially leading to haemolytic uraemic syndrome (O' Connor et al., 2021). According to the ECDC, Ireland had the highest country-specific notification rate of VTEC at 20.0 cases per 100,000 population in 2018 (ECDC, 2020b). The transmission of VTEC may be due to undercooked foods such as hamburgers, person to person transmission or by agricultural faecal contamination of natural water bodies including groundwater supplies (Kaper et al., 2004; Chique et al., 2021).

Along with different pathotypes, *E. coli* can be distinguished at a genetic level using multi-locus sequence typing (MLST). This includes examination of seven housekeeping genes (*adhA*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) for the alleles

at each of the loci (Pitout & Finn 2020). By employing this type of testing, certain clonal lineages are more commonly identified from sites of infection. Salipante et al. (2015) sequenced 312 urine and blood derived *E. coli* isolates, and identified that just six sequence types accounted for 51% of all isolates. These included ST131 and ST95 accounting for 16% and 11% respectively. ST127, ST73, ST69 and ST393 represented the remaining four most common types. Similarly, Matsui et al. (2020) examined 89 and 106 *E. coli* isolates from rectal swabs and urine samples respectively. Using multi-locus sequence typing (MLST), ST10 and ST131 were identified more commonly from faecal material, while ST95 and ST127 were more frequently recovered in urine samples. Finally, *E. coli* isolates from bloodstream infections also displayed a similar prevalence of clonal group ST131 accounting for 41/96 ESBL producing *E. coli* isolates (Mamani et al., 2019). These epidemic clonal groups are often linked to antibiotic resistance mechanisms, such as ESBL and fluoroquinolone resistance commonly encountered in ST131 isolates (Pitout & Finn 2020). Within the Irish healthcare system, ST131, followed by ST38 and ST10 were the most prevalence *E. coli* sequence types harbouring OXA-48 like genes, obtained from screening and infectious sites (Brehony et al., 2019).

### **1.2.2 *Klebsiella***

Worldwide, *Klebsiella* is recognised as a significant pathogen responsible for numerous invasive and multi-drug resistant infections. Similar to *E. coli*, *Klebsiella* is considered a normal commensal, found in the mucosal surfaces of the body including the gastrointestinal tract and the nasopharynx (Martin & Bachman 2018). However, hypervirulent strains are responsible for a significant portion of respiratory tract infections. To a lesser extent, *Klebsiella* is also accountable for urinary tract and bloodstream infections. *Klebsiella* is listed as one of the six 'ESKAPE' pathogens because it accounts for a considerable proportion of multi-drug resistant nosocomial infections (Rice, 2008). Recently, *Klebsiella* taxonomic classification has been updated so that new species

including *Klebsiella variicola* and *Klebsiella quasipneumoniae* are now recognised as separate species from *Klebsiella pneumoniae* (Rosenblueth et al., 2004; Brisse et al., 2014). All three species are significant pathogens that have previously been implicated in cases of sepsis, with equivalent 30-day mortality rates (Imai et al., 2019).

From a pathogenic perspective, *Klebsiella* has an array of virulence factors, the most important of which being the extracellular capsule (Rendueles, 2020). Studies have shown that the capsule reduces the activation of the immune response by masking antigens on the bacterial cell surface, providing protection against phagocytosis by immune cells such as macrophages (Khaertynov et al., 2018). The capsule also affords increased tolerance to antibiotics, reducing susceptibility to antibiotics that target processes within the bacterial cell (Rendueles, 2020). Further virulence factors employed by *Klebsiella*, which are common to many Enterobacterales, include fimbrial adhesions, siderophores and the lipopolysaccharide (LPS) layer (Zhu et al., 2021). The LPS layer is universal amongst all gram negative bacteria, which acts as an endotoxin (lipid A), whilst also providing protection against the bactericidal actions of the complement cascade (O antigen).

Through the increased use of sequencing in recent years, prominent *Klebsiella* sequence types have emerged as the causative agents of a significant proportion of multi-drug resistant infections. Sequence types ST258 and ST11 are extensively linked to the carriage of the *bla*<sub>KPC</sub> gene worldwide, conferring resistance to the last resort carbapenem antibiotics (Liu et al., 2018; DeLeo et al., 2014). *Klebsiella* species have also been linked to *bla*<sub>OXA-48-like</sub> gene carriage in Ireland, including sequence types ST1308, ST20, ST11 and ST37 (Brehony et al., 2019). More recently, the ECDC reported outbreaks of hypervirulent *Klebsiella* isolates belonging to ST23 frequently harbouring *bla*<sub>OXA-48</sub> across Europe (ECDC, 2021b). However, susceptibility testing data was not included to establish the resultant impact on phenotypic carbapenem susceptibility.

### 1.3 Horizontal Gene Transfer

Although there are prominent sequence types associated with multi-drug resistant and hypervirulent infections, bacteria have the ability to disseminate resistance genes to any species via horizontal gene transfer. This evolutionary trait plays a fundamental role in the increasing number of difficult to treat infections. Prior to 1990, most carbapenemase genes were considered chromosomally encoded and specific to one species (Queenan & Bush 2007). However, more recently carbapenemase genes have been identified on plasmids in circulation amongst many different bacterial species. For example, the very first detection of *bla*<sub>KPC-1</sub> was plasmid encoded within a clinical *Klebsiella pneumoniae* isolate (Yigit et al., 2001). More recently, the IncN plasmid was identified as the causative agent for an outbreak of *bla*<sub>KPC</sub> in multiple species including *E. coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae*, indicating horizontal gene transfer (Wozniak et al., 2021).

There are three main mechanisms which can be utilised by bacteria to successfully distribute antibiotic resistance genes. These processes include (i) transduction, whereby a bacteriophage introduces DNA into the bacterium, (ii) transformation, which is achieved when the bacterial cell uptakes free DNA and (iii) conjugation, where the genetic material is transferred from one bacterium to another through direct cell-to-cell contact (Partridge et al., 2018).

#### 1.3.1 Transduction

Bacteriophages are a type of virus primarily consisting of DNA or ribonucleic acid (RNA) enclosed in a protein capsid that specifically infect bacteria (Jebri et al., 2021). Initially the phages bind to complementary bacterial cell surface receptors, undergo adsorption and inject their genetic material into the recipient enabling viral reproduction. There are three processes of transduction that have been defined to date which include specialised, generalised and lateral transduction (Gómez-Gómez et al., 2019). Specialised transduction is

carried out by a lysogenic (temperate) phage, which uses the bacterium host cell to replicate without inferring cell lysis (Jebri et al., 2021). These temperate phages can only transfer specific bacterial DNA regions adjacent to the site of insertion. Generalised transduction can include the transfer of any region of the bacterial DNA, involving a lytic (virulent) or lysogenic phage (Balcazar et al., 2014). Finally, lateral transduction is mediated by lysogenic phages which can mobilise genes that are downstream of the insertion site (Gómez-Gómez et al., 2019).

Recently, there has been increased interest in using phages alongside antibiotics for the treatment of multi-drug resistant infections, due to the high specificity of these phages for bacterial cells (Principi et al., 2019). However, knowledge gaps relating to the risk of antibiotic resistance gene transmission in vivo preclude their introduction into clinical use in the near future. Unlike broad spectrum antibiotics, phages typically target only a limited number of strains belonging to one bacterial species which would also present challenges relating to empiric treatment (Jebri et al., 2021). Conversely, there are also many advantages of bacteriophages over antibiotic therapy including less significant alterations to the gut microbiome and lack of toxicity to human cells (Principi et al., 2019). Phages have been isolated from a range of sources including soil, marine environments and wastewater (Batinovic et al., 2019).

### **1.3.2 Transformation**

Transformation is the process in which bacteria can incorporate extracellular DNA into their genome (Wang et al., 2020b). This horizontal gene transfer mechanism can uptake chromosomal or plasmid DNA from the surrounding environment (Lerminiaux & Cameron 2019). DNA may be released extracellularly due to damage or lysis of bacterial cells and can persist for long periods of time depending on the surrounding conditions (Wang et al., 2020b). The recipient cell in the transformation process must be naturally competent

which is dictated by several genes located on the bacterial chromosome. Competence is considered transient in most bacterial species, which may be induced by progression through different growth phases or by selective pressures imposed by the presence of antibiotics (Lorenz & Wackernagel 1994). The main exception to this is *Neisseria gonorrhoeae*, which is reported to be consistently competent. Natural competence in Enterobacterales including *E. coli* and *Klebsiella* is poorly characterised, however, processes such as heat shock transformation can be used in vitro to study plasmids carrying antibiotic resistance genes (Lerminiaux & Cameron 2019; Hernández-García et al., 2019). The use of chlorine-based disinfection, which can be applied during wastewater treatment processes, can also stimulate transformation of plasmids (Zhang et al., 2021b). This may have significant implications for dissemination of resistance genes amongst bacteria.

### **1.3.3 Conjugation**

Conjugation is an extensively investigated mechanism of horizontal gene transfer, which involves genetic elements being transferred between bacterial cells, including plasmids and integrative conjugative elements within the bacterial chromosome (Graf et al., 2019). Transference is mediated by a type IV secretion system, which is composed of multiple subunits including a pilus needed for attachment to a recipient cell (Cabezón et al., 2017). Once attachment is complete, the pilus shortens to bring the bacterial cells in closer proximity (Graf et al., 2019). Pore formation occurs between the two cells, followed by attachment of a relaxosome to a singular strand of plasmid DNA and subsequent cutting of the DNA at the attachment site. This complex is transported through the pilus to the recipient cell, and the plasmid DNA is replicated in the donor and recipient cell so that both can participate as donors in further conjugation events.

Conjugation experiments *in vitro* have demonstrated the successful transference of carbapenemase genes such as *bla*<sub>OXA-48</sub> and *bla*<sub>NDM-1</sub> on different incompatibility type plasmids (Solgi et al., 2020; Chaalal et al., 2020). This mechanism makes it difficult to contain the dissemination of antibiotic resistance genes. However, several conjugative inhibitors such as bacteriophages specific for the bacterial pilus, antibodies targeting the relaxase protein and unsaturated fatty acids affecting the ATPase functioning are being investigated at present (Cabezón et al., 2017).

## **1.4 Mobile genetic elements**

There are different types of mobile genetic elements (MGEs) that are responsible for the movement of DNA within and between bacterial cells. Some MGEs, such as plasmids and integrative conjugative elements can utilise one or more of the horizontal gene transfer mechanisms to achieve intercellular dissemination (Partridge et al., 2018). Whilst other MGEs can relocate within or between DNA segments in the same bacterial cell (intracellular), including insertion sequences, integrons and transposons.

### **1.4.1 Plasmids**

Plasmids are extrachromosomal DNA segments that can replicate independently (San Millan, 2018). These circular or linear pieces of DNA can move horizontally between bacteria primarily using conjugation, and often facilitate the transfer of other mobile genetic elements such as insertion sequences, transposons and integrons (Partridge et al., 2018). Categorisation of plasmids was originally based on mating experimentation, in which plasmids that were unable to be maintained in the same cell belonged to one incompatibility group (Robertson et al., 2020). Plasmids belonging to the same incompatibility group were thought to be related due to similarities between the replication initiation systems preventing stable maintenance of both plasmids in one cell (Partridge et al., 2018). Recently, sequencing is the more

common method of plasmid identification using curated databases with extensive plasmid replicon sequences such as PlasmidFinder (Carattoli et al., 2014).

Different plasmid replicons are commonly associated with harbouring particular clinically significant antibiotic resistance genes (San Millan, 2018). For example, the plasmid pOXA-48 (IncL type) often carries the *bla*<sub>OXA-48</sub> gene which is strongly associated with *Klebsiella pneumoniae* ST11 and ST405. Similarly, the IncFII plasmids are often linked with *bla*<sub>CTX-M</sub> carriage and is often identifiable within *E. coli* ST131. Although there are prominent sequence types harbouring these antibiotic resistance encoding plasmids, the key concern is the ability of plasmids to disseminate between different bacterial species (Ludden et al., 2017; Wozniak et al., 2021).

#### **1.4.2 Integrative conjugative elements**

Integrative conjugative elements (ICE) are normally located in the bacterial chromosome, and are self-transmissible between different bacteria (Partridge et al., 2018). Each ICE contains a core set of genes responsible for excision from the chromosome, successful transfer via conjugation and site-specific integration in the recipient cell (Obi et al., 2018). Once a singular DNA strand has been mobilised to the recipient, the double-stranded element regenerates using DNA polymerase (Botelho & Schulenburg 2021). The ICE can then reintegrate into the recipient cell's chromosome where it can undergo replication.

Similar to other mobile genetic elements, ICEs can also harbour multiple resistance genes aiding in bacterial genome plasticity (Partridge et al., 2018). Unlike plasmids, there are large knowledge gaps relating to ICEs as vectors of antibiotic resistance genes, although ICEs are more abundant than plasmids within bacterial hosts (Botelho & Schulenburg 2021). In addition, ICEs display survival advantages in comparison to plasmids such as the ability to be

frequently mobilised between different bacterial taxa and their capability to survive during the cell division process. In a recent publication, He et al. (2021) identified the presence of an ICE harbouring *bla*<sub>NDM-1</sub>, along with 21 additional antibiotic resistance genes within a *Proteus mirabilis* isolate. In this study it was also demonstrated that conjugation experiments were successful, establishing the importance of ICEs in antibiotic resistance gene transfer.

### **1.4.3 Insertion sequences**

Insertion sequences are relatively short DNA segments that can autonomously relocate to different sites within the same or between different DNA segments in one bacterial cell (Partridge et al., 2018). Insertion sequences can work independently to transfer adjacent regions of DNA, or form a composite transposon whereby two similar insertion sequences are bound to either side of a DNA segment, enabling movement of the central region of DNA. This central region may contain one or more antibiotic resistance genes. It is possible for insertion sequences to move horizontally between bacterial cells with the aid of bacteriophages or plasmids (Vandecraen et al., 2017). Large copy numbers of insertion sequences can exist in one bacterial cell which enables significant diversification and evolution of the bacterial genome. Insertion sequences contain a transposase enzyme, which catalyses DNA cleavage and transfer of the DNA strand. Short terminal inverted repeats can be used to identify insertion sequences, but also play a role in the binding of transposases along with the other functionalities.

Insertion sequences are categorised based on the active site of the transposase and/or the mechanism of transposition (Partridge et al., 2018). This includes whether the insertion sequence undergoes excision and insertion into the recipient cell, or if it undergoes replication so that both donor and recipient cells contain the insertion sequence. Recent investigations have identified that some insertion sequences have stronger associations with antibiotic resistance genes

than others, such as those belonging to the IS6/26, IS1 and IS4/5 domains (Razavi et al., 2020).

#### 1.4.4 Transposons

Transposons are highly similar to insertion sequences, as both relocate DNA to different locations in a single bacterial cell, and are composed of a transposase gene and terminal inverted repeats (Partridge et al., 2018). However, transposons are larger in size, and the inverted repeats are located at each side of the mobilised DNA rather than two insertion sequences. There is overlap in the nomenclature between the two, as sequences that are closely related to insertion sequences have been designated as transposons and vice versa. Transposons can transfer DNA segments within the same DNA molecule such as the chromosome, or between different DNA components such as from the chromosome to a plasmid (Sultan et al., 2018). Some transposons are more commonly associated with certain antibiotic resistance gene carriage, such as Tn21 and *bla*<sub>OXA</sub> genes.

#### 1.4.5 Integrons

Integrons vary from insertion sequences and transposons in that they use site-specific recombination to relocate genes between DNA segments within a single bacterium (Partridge et al., 2018). The structure of an integron includes an integrase gene (*intI*), a recombination site (*aattI*) along with one or more promoters (Deng et al., 2015). Classification of integrons is based on the amino acid composition of the integrase enzyme, which includes class 1 (*intI1*), class 2 (*intI2*), class 3 (*intI3*) etc. The complete integron cannot be mobilised, but rather the gene cassettes that are located centrally within (Domingues et al., 2012). Due to their small size of typically a single gene and recombination site, multiple gene cassettes can be incorporated into one integron which may include the presence of antibiotic resistance genes. Class 1 integrons are commonly detected in gram-negative bacteria, and often contain streptomycin,

trimethoprim and beta-lactam resistance genes (Deng et al., 2015). Integrons are occasionally located within mobile genetic elements such as plasmids, which facilitates horizontal dissemination of antibiotic resistance genes. Classes of integrons may also be commonly associated with transposons, such as class 2 integrons and the Tn7 family of transposons.

## **1.5 Environmental antibiotic resistance**

There are two principal contributing factors to the large reservoir of antibiotic resistance, which constitutes the environmental resistome. Firstly, the natural production of antibiotics by environmental bacteria and fungi, that harbour genes encoding resistance mechanisms directed against the antibiotics they produce for protection (Allen et al., 2010). Secondly, the introduction of antibiotics to the environment via anthropogenic use, imposing selective pressures on bacteria, and subsequently promoting horizontal gene transfer of antibiotic resistance genes (Berglund, 2015). Anthropogenic influence is strongly interlinked with the 'One Health' concept, which recognises the nexus between the health of humans, animals and the environment (Kim & Cha 2021).

### **1.5.1 Natural environmental resistome**

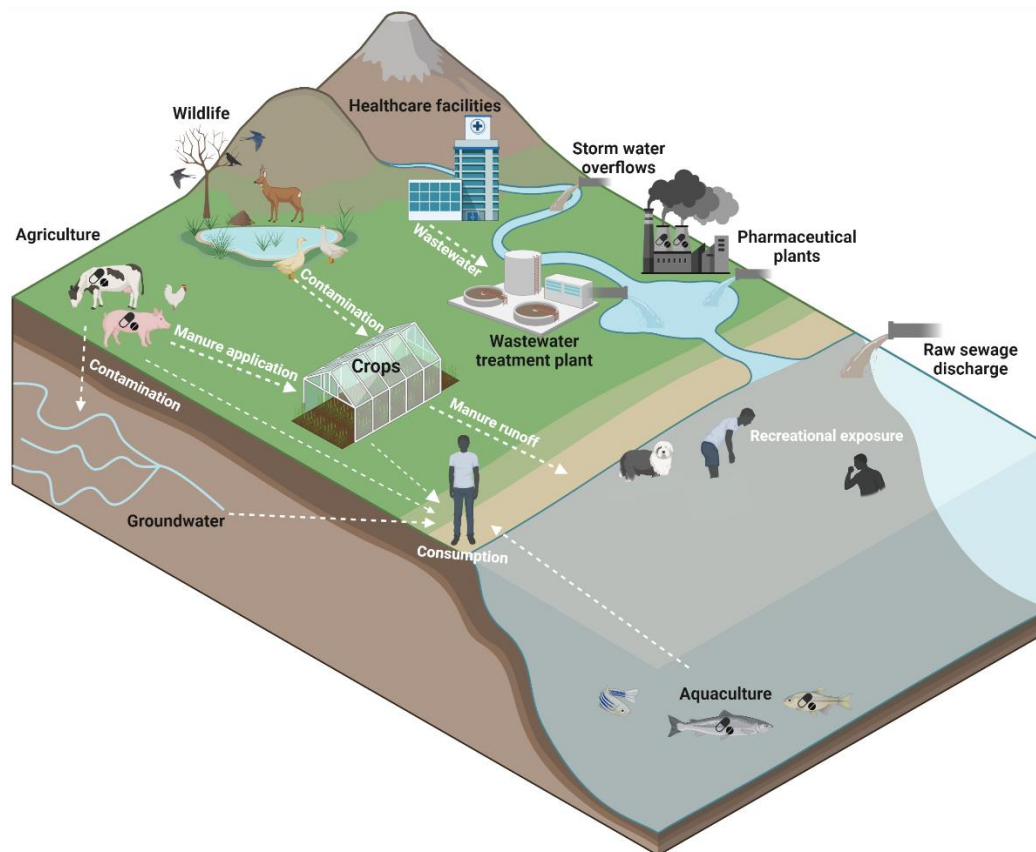
The natural environmental resistome was in existence long before the discovery and introduction of penicillin for clinical use (Fleming, 1929). In an attempt to understand the natural occurrence of different antibiotic resistance genes, many studies have examined 'pristine' sites which lack human or agricultural activity, with successful detection of clinically significant resistance genes (Perron et al., 2015; Van Goethem et al, 2018). The naturally occurring environmental resistome is discussed in more detail in Chapter 2, which incorporates a scoping review to evaluate the role of the natural environment in the dissemination of beta-lactamase genes.

### **1.5.2 Anthropogenic influence on the environmental resistome**

Apart from the natural resistome, antibiotic resistance stems from the administration of antibiotics in human and agricultural medicine, which selects for isolates that are capable of survival in the presence of antibiotics (Kim & Cha 2021). As a consequence, silent, unexpressed antibiotic resistance genes may become activated or horizontal gene transfer may occur between different bacterial strains. Premises such as hospitals and nursing homes have relatively high antibiotic consumption rates, and as such are significant contributors to the dissemination of antibiotics along with resistant bacteria via wastewater (HPSC, 2017; Bürgmann et al., 2018; HPSC, 2019a; Cahill et al., 2019). The unnecessary use of antibiotics further exacerbates the resistance crisis, such as their misuse in human medicine (Samreen et al., 2021). Antibiotic misuse in cases where antibiotics were administered for animal growth promotion was banned in the EU in 2006 as a significant step towards tackling the antibiotic resistance crisis (Regulation (EC) No 1831/2003). On a European scale, Ireland ranked 6<sup>th</sup> highest for the total consumption of antibiotics within the hospital and community sectors in 2020 (ECDC, 2021c). Ireland administered a defined daily dose (DDD) of 18.6 per 1000 inhabitants per day, which lay above the population weighted mean consumption (16.4) of the 27 participating EU countries. Of particular concern, Ireland was highlighted as one of the countries that displayed a statistically significant increase in the consumption of carbapenems between 2011 and 2020. From a veterinary perspective, the Health Products Regulatory Authority (2021) has reported an increase of 15.6 tonnes of antibiotics sold in 2020 in comparison to 2019, with third and fourth generation cephalosporins at their highest in years (0.36 tonnes). In comparison to other countries in the EU, along with the United Kingdom, Ireland ranked 13<sup>th</sup> of the 31 participating countries at a total of 103.9 tonnes of antimicrobials sold for veterinary use in 2020 (European Medicines Agency, 2021).

A multisectoral 'One Health' approach is necessary when tackling this global health crisis due to the overlap between antibiotics used in human and veterinary medicine, such as third-generation cephalosporins, along with their combined discharge through human and animal waste to the environment (McEwen & Collignon 2018). This was reflected in 'Ireland's Second One Health National Action Plan on Antimicrobial Resistance 2021-2025', which highlighted that the AREST project (Antimicrobial Resistance and the Environment -Sources, persistence, Transmission, and risk management) is playing an important role in advancing the understanding of the environmental resistome (Government of Ireland, 2021). Manure runoff from farms, alongside human wastewater discharges often comprise of antibiotic resistant bacteria and antibiotic resistance genes (Berglund, 2015). Antibiotics taken by humans result in the excretion of biologically active metabolites in wastewater via urine and faecal matter (Levison & Levison 2009). Antibiotics may persist during wastewater treatment depending on a range of factors including treatment applied, retention time, salinity and temperature (Singer et al., 2016). These antibiotics may enter receiving water bodies exceeding the predicted no effect concentration (PNEC), indicating potential resistance selection (Singer et al., 2019). This contamination alongside the natural resistome makes aquatic environments prime reservoirs for antibiotic resistance dissemination, which may be responsible for the proliferation of new resistance strains. In Ireland, the number of raw sewage discharges in 2020 was reported as 34, according to the Environmental Protection Agency (EPA, 2019; EPA, 2021). In the case of wastewater treatment plants in Ireland, a total of 12 areas failed to conform to current EUs Urban Waste Water treatment Directive in 2020 (Directive 91/271/EEC, 1991). These 12 plants collect 54% of the total wastewater load from large urban areas in Ireland. Overall, Ireland's wastewater management needs to be improved upon in order to protect the natural environment, particularly in the context of antibiotic resistance dissemination.

Further drivers of antibiotic resistance in the environment include the improper disposal of antibiotics, or discharges from pharmaceutical manufacturing plants (McEwen & Collignon 2018). Integrated constructed wetlands (ICWs) are also used in Ireland as a method of treating waste, in which wastewater is released directly into the environment (Sabri et al., 2021). ICWs have also been proven to be an effective alternative to conventional wastewater treatment plants in the United Kingdom, and include a series of cells which contain a range of aquatic plants that act as biofilters (Cooper et al., 2020). Subsequent environmental exposure of humans and animals may occur through drinking contaminated water, recreational activities near discharges and the ingestion of crops that have been treated with antibiotics or sludge further highlights the interconnections of the ‘One Health’ triad (Figure 1.3).



*Figure 1.3: Illustration of the interactions between humans, animals and the environment, which facilitate the dissemination and spread of antibiotic resistance. Created with BioRender.com*

Studies examining the presence of antibiotic resistance in the aquatic environment have identified clones that are causative agents of multi-drug resistant infections in the clinical and veterinary environment, further linking the three sectors. For example, *Klebsiella pneumoniae* ST258 which frequently harbours a *bla*<sub>KPC</sub> gene was recently identified in river water in Croatia (Jelić et al., 2019). As mentioned previously, this sequence type carrying *bla*<sub>KPC</sub> is commonly linked to mortality in the clinical environment due to untreatable infections (DeLeo et al., 2014). A recent study by Aslam et al. (2020) identified this KPC producing strain from clinical, environmental and veterinary (sludge) sources.

## **1.6 Public health concern regarding antibiotic resistance in recreational waters**

To date, there are significant knowledge gaps relating to the consequences of human exposure to antibiotic resistant bacteria during recreational water use. The limited number of studies that have attempted to address this are outlined further in Chapter 3. At present, there are no regulations at an EU level which state that the environment should be regularly examined for the presence of antibiotic resistance. However, the current bathing water directive outlines that sites classified as bathing waters must be monitored during the bathing season for the concentrations of *E. coli* and intestinal enterococci (CFU/100mL) (Directive 2006/7/EC). This directive and its limitations with regard to antibiotic resistance is discussed in more detail in Chapters 3 and 4.

## **1.7 Research aims and objectives**

This research was conducted as part of the EPA/HSE-funded AREST project. The overarching aims of this work were to assess natural water bodies both in direct receipt of discharges, and those free from known anthropogenic contamination, for the presence of antibiotic resistant Enterobacterales. In conjunction, wastewater samples were examined to identify possible sources responsible for

the introduction of multi-drug resistant Enterobacterales to the natural environment. The following objectives were fulfilled in order to address these primary aims.

1. Chapter 2: A scoping literature review was completed assessing the role of the natural aquatic environment in the dissemination of extended spectrum beta-lactamase and carbapenemase encoding genes. This review focused on water bodies that were not in direct receipt of point discharges in order to establish whether the aquatic environment may act as a natural reservoir of antibiotic resistance dissemination of clinical concern.
2. Chapter 3: A point prevalence survey was conducted by collecting water and sewage samples across three counties in Ireland located on the East, West and South coasts between 2018 - 2019. The sampling locations were chosen based on maps of potential sources of antibiotic resistance published by Chique et al. (2019). Phenotypic analysis was carried out in order to evaluate the resistance profile of isolates obtained and genotypic analysis was also completed which enabled a direct comparison of isolates from water and sewage sources.
3. Chapter 4: A longitudinal survey was completed based on choosing additional sampling points in areas of interest identified from the initial point prevalence survey. This paper included two rounds of sampling in each of the three counties between 2019 - 2020. Similar analysis was carried out to establish the resistance profiles along with the genomic similarities of isolates from waters and wastewaters.

## **Chapter 2: The Role of the Natural Aquatic Environment in the Dissemination of Extended Spectrum Beta-Lactamase and Carbapenemase Encoding Genes: A Scoping Review**

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## **Abstract**

The natural aquatic environment is a significant contributor to the development and circulation of clinically significant antibiotic resistance genes (ARGs). The potential for the aquatic environment to act as a reservoir for ARG accumulation in areas receiving anthropogenic contamination has been thoroughly researched. However, the emergence of novel ARGs in the absence of external influences, as well as the capacity of environmental bacteria to disseminate ARGs via mobile genetic elements remain relatively unchallenged. In order to address these knowledge gaps, this scoping literature review was established focusing on the detection of two important and readily mobile ARGs, namely, extended spectrum beta-lactamase (ESBL) and carbapenemase genes. This review included 41 studies from 19 different countries. A range of different water bodies including rivers (n=26), seawaters (n=6) and lakes (n=3), amongst others, were analysed in the included studies. ESBL genes were reported in 29/41 (70.7%) studies, while carbapenemase genes were reported in 13/41 (31.7%), including joint reporting in 9 studies. The occurrence of mobile genetic elements was evaluated, which included the detection of integrons (n=22), plasmids (n=18), insertion sequences (n=4) and transposons (n=3). The ability of environmental bacteria to successfully transfer resistance genes via conjugation was also examined in 11 of the included studies. The findings of this scoping review expose the presence of clinically significant ARGs in the natural aquatic environment and highlights the potential ability of environmental isolates to disseminate these genes among different bacterial species. As such, the results presented demonstrate how anthropogenic point discharges may not act as the sole contributor to the development and spread of clinically significant antibiotic resistances. A number of critical knowledge gaps in current research were also identified. Key highlights include the limited number of studies focusing on antibiotic resistance in uncontaminated aquatic environments as

well as the lack of standardisation among methodologies of reviewed investigations.

## 2.1 Introduction

Antibiotic resistance is recognised as a major threat to public health. Bacteria utilise a range of mechanisms to evade the effects of antibiotics leading to challenges in clinical infection treatments. These range from non-specific processes including increased efflux pump activity or downregulation of porin channels, to the production of enzymes that specifically target and inactivate antibiotics (Peterson and Kaur, 2018). As bacteria continue to adapt to the presence of antibiotics through the acquisition of antibiotic resistance genes (ARGs) via mobile genetic elements (MGEs), available antibiotics are becoming less effective. Due to the growing limitation of treatment options, older antibiotics such as colistin, which can cause negative side effects (Morrill et al., 2015), are being employed to treat infections caused by bacteria that are resistant to last resort antibiotics.

The increase in the proportion of serious infections associated with extended spectrum beta-lactamase (ESBL) and carbapenemase producing organisms is a significant clinical concern. ESBL enzymes have evolved by point mutations occurring in beta-lactamase genes such as *bla*<sub>TEM-1</sub>, *bla*<sub>TEM-2</sub> and *bla*<sub>SHV-1</sub> (Shaikh et al., 2015). The Ambler classification is used to categorise these beta-lactamases based on their amino acid sequence (Ambler, 1980). Many of the clinically significant ESBLs, including TEM, SHV and CTX-M variants, belong to Ambler Class A, (Liakopoulos et al., 2016). The most commonly detected carbapenemase enzymes belong to 3 Ambler classes; Class A (e.g. KPC), Class B (e.g. NDM, VIM, IMP) and Class D (e.g. OXA-48) (Fröhlich et al., 2019).

Both ESBL and carbapenemase genes are commonly associated with plasmid carriage enabling their widespread dissemination. The ability of bacteria to share genes between different DNA molecules, and further exchange MGEs

across different bacterial species is a key tool for survival and persistence amid environmental challenges. Mobility of ARGs is achieved by three mechanisms; (i) transduction (ii) transformation and (iii) conjugation. Some genetic elements classed as being mobile can only move within and between DNA located in the same bacterial cell (Partridge et al., 2018). These include insertion sequences and integrons. However, if these DNA segments harbour ARGs and relocate to a plasmid, then the bacterium has the ability to transfer these genes to another bacterium. Similarly, conjugative transposons can excise from DNA in one bacterial cell and move to another when there is direct cell-to-cell contact (Salyers et al., 1995).

The transfer of ARGs within plasmids via conjugation, often carried out in a controlled laboratory setting, can confirm the presence of resistance genes on MGEs, while also demonstrating the ease and speed at which bacteria can transfer ARGs (Yin et al., 2013). This highlights the danger of MGEs in comparison to intrinsic resistance whereby the rapid expansion of resistance genes across all types of bacterial species can occur rather than the inheritance in one species via vertical transmission. This ability enables highly virulent and pathogenic bacteria to acquire ARGs from harmless environmental isolates favoring their survival. The inherent complexity of horizontal gene transfer among bacteria precludes full comprehension of the mechanisms involved in ARG transfer. A recent review by Leclerc et al. (2019) highlighted several critical knowledge gaps relating to the movement of ARGs via horizontal gene transfer. This included the lack of studies examining horizontal gene transfer via transduction and transformation and the predominant use of one bacterial species (*Escherichia coli*) to demonstrate genetic transfer in the laboratory, when interspecies transfer is common. Overall, the extent and nature of gene transfer remains ambiguous, especially in vast environmental reservoirs.

Awareness surrounding the importance of employing a 'One Health' approach encompassing human, animal and environmental health when investigating and

tackling antibiotic resistance has increased in recent years. This is highlighted in the World Health Organization (WHO), 'Global Action Plan on Antimicrobial Resistance' (WHO, 2015). This report emphasizes the need for further research in terms of transmission routes of antibiotic resistance including food, water and the natural environment. Concomitant with the prominence of the 'One Health' concept, there has been an upsurge in research relating to the prevalence of antibiotic resistance in the environment. Nonetheless, information regarding the presence of naturally occurring ARGs in the absence of anthropogenic pressures, and how they are disseminated to potentially pathogenic species in the environment is lacking.

Many bacteria and fungi that are ubiquitous in nature can produce molecules with antibacterial properties, which are thought to play a role in communication and competition (Singer et al., 2006). The cephalosporin class of antibiotics originated from a fungus, *Acremonium chrysogenum*, which was isolated from a sewage outfall point in seawater in the late 1940's (Brakhage, 1998). Resistance to the third generation cephalosporins such as cefotaxime attributable to ESBL production was first reported in the early 1980's (Rawat and Nair, 2010). The majority of these ESBL enzymes including, SHV and TEM, were first discovered in the nosocomial setting; however, the lack of environmental research during the 1980's warrants the possibility of their presence in the environment being overlooked. Most microorganisms that produce antibiotics in the natural environment have the corresponding resistance genes present in their genome (Allen et al., 2010), which strengthens the possibility of some clinically significant ARGs originating from environmental organisms. In the case of the *bla*<sub>CTX-M</sub> genes, their origins have been traced to the chromosome of *Kluyvera* species which has been isolated from environmental waters and soils (Cantón et al., 2012a). The importance of soil bacteria as the progenitors of the *bla*<sub>CTX-M</sub> genes was further established by

Graham et al. (2016) who detected the presence of this ESBL gene in soils dating back to 1923, prior to antibiotic use in medicine in Denmark in the 1930s.

Similarly, the origins of the carbapenem class of antibiotics dates back to 1976 through the discovery of thienamycin from a soil bacterium known as *Streptomyces cattleya* (Papp-Wallace et al., 2011). The first reports of carbapenem hydrolysing enzymes known as ‘carbapenemases’ emerged in the early 1990s from clinical isolates (Cantón et al., 2012b). However, investigations into the origins of some of these genes revealed environmental strains as the progenitors. One example is the *bla*<sub>OXA-48</sub> carbapenemase encoding gene that was traced back to an environmental strain of *Shewanella* spp. (Tacão et al., 2018). This recent discovery emphasizes the importance of understanding the role of the environment in predicting emerging resistances, as well as the dissemination of known ARGs which negatively impact clinical treatment outcomes.

Recently, consideration has been given to the role of natural production of antibacterial molecules in the formation and spread of ARGs in the environment. Researchers are beginning to examine sites deemed to be ‘pristine’ in terms of anthropogenic influence. Anthropogenic influence encompasses all human activities that result in the contamination of the environment. Interestingly, different ARGs have been detected at these pristine locations. Van Goethem et al. (2018) recently reported a low abundance of Ambler class A, B and C beta-lactamases in remote Antarctic surface soils at a site categorised as ‘pristine’. However, according to Allen et al. (2010) it is challenging to label a geographical area as completely ‘pristine’, arguing that the only environments (globally) entirely devoid from human antibiotic prevalence existed in the pre-antibiotic era. This is due to the potential spread of antibiotic resistance from areas under anthropogenic pressures via wildlife and/or environmental transmission routes including regional-global wind and water circulation and cycles. However, research in remote environments free

from anthropogenic pressures is providing further insights into ARGs that are vertically inherited over multiple bacterial generations (Van Goethem et al., 2018), enabling bacterial survival in the presence of natural antibiotic production. Permafrost cores are a prime example of a remote environment, typically devoid of anthropogenic activity that can preserve and prevent migration of bacteria over many years. Perron et al. (2015) identified genes that could potentially confer low level antibiotic resistance in the permafrost core as well as genes conferring resistance to beta-lactams, aminoglycosides and tetracyclines in the upper active layers. These types of studies strengthen the theory that the development of antibiotic resistance may be a natural occurrence in the absence of anthropogenic based pressures.

The aim of this scoping literature review was to analyse the role of the natural aquatic environment in the transmission of clinically significant antibiotic resistances determinants, specifically ESBL and carbapenemase encoding genes. In this paper we define 'natural' aquatic environments as water bodies that are not in direct receipt of contaminating discharges, thus receiving minimal anthropogenic influence. In this context, the review aimed to identify, collate and analyse data from studies examining MGEs in antibiotic resistant isolates obtained from water bodies. Furthermore, current knowledge gaps for further research were identified and highlighted. The focus on water derives from the vital and constant interaction that both humans and animals have with aquatic systems, be it through recreational activities or consumption. This inextricable link constitutes an increased likelihood for the potential spread of antibiotic resistant organisms to both humans and animals, highlighting the importance of a 'One Health' approach.

## **2.2 Methods**

### **2.2.1 Research Question and Database Queries**

The following research question was formulated to focus and direct the scoping review:

“What role does the natural aquatic environment play in the transmission of ESBL and carbapenemase encoding genes via mobile genetic elements?”

The scoping review protocol was adapted from previously published papers (Andrade et al., 2018; Greig et al., 2015). In summary, a search string was formed based on the established research question, which comprised of a combination of relevant search terms adapted to each individual database (Supplementary Table A1). Databases employed in literature searches included PubMed, MEDLINE, EMBASE, Web of Science and Scopus. Searches were conducted on June 10<sup>th</sup> 2019. MeSH terms were applied when using the PubMed database in order to employ the medical vocabulary thesaurus. The ‘explode’ function was used in the MEDLINE/ EMBASE search string in order to search for narrower subject headings under the database’s hierarchy tree. The field tag ‘TS’ was applied to the Web of Science database to focus the search string on the topic of the articles. The search string was adapted to the Scopus database using TITLE-ABS-KEY to identify the search terms in titles, abstracts and keywords. Relevant subject areas and source types were also selected and applied in each database to limit the numbers of irrelevant articles retrieved. All articles obtained from the searches were exported to Endnote and duplicates were removed.

### **2.2.2 Additional sources**

Grey literature was examined by applying the general phrase ‘antibiotic resistance in the environment’ to the following databases: Trip ([www.tripdatabase.com](http://www.tripdatabase.com)), BASE ([www.base-search.net](http://www.base-search.net)), CDC ([www.cdc.gov](http://www.cdc.gov)),

ECDC ([www.ecdc.europa.eu](http://www.ecdc.europa.eu)) and Research Gate ([www.researchgate.net](http://www.researchgate.net)). Supplementary searches employing Google Scholar were also incorporated into the protocol. Bibliography screening of the final set of included papers was carried out in an attempt to identify additional relevant articles not captured within the original review protocol.

### **2.2.3 Screening Phase and Inclusion/ Exclusion Criteria.**

Phase 1 consisted of two independent reviewers screening the titles and abstracts of all retrieved articles against a pre-defined set of inclusion and exclusion criteria. Revision by a third independent reviewer was utilised to derive an outcome in cases where article inclusion/exclusion could not be agreed upon by the two independent reviewers. The inclusion and exclusion criteria applied during the screening phase are outlined in Table 2.1. Two main limitations and/or thresholds were set for article inclusion: (i) investigations published between 2008 and 2019 and (ii) full text provided in English. The publication year restriction was applied due to the upsurge in research related to the area of antibiotic resistance in the environment, evident following application of the search string to the Scopus database and analyses of the publication dates of retrieved articles. Additionally, this year range was employed because older methods of analysing DNA (e.g. pulse field electrophoresis) would not be comparable to more modern molecular methods such as whole genome sequencing. No geographical thresholds were implemented for article inclusion/exclusion.

Table 2.1: Inclusion and exclusion criteria applied to studies to determine eligibility.

<b>Inclusion criteria:</b>	<b>Exclusion criteria:</b>
1. Focuses on natural aquatic environments, including water bodies free from direct point source contaminant discharges, as a facilitating source in the transmission of carbapenemase and/or ESBL resistance genes.	1. Analyses water samples receiving direct point source contaminant discharges, (e.g. wastewater treatment plant discharge).
2. Detects the presence of mobile genetic elements or performs experimentation that demonstrates transferability of ESBL and/or carbapenemase resistance genes.	2. Detects the presence of ESBL and/or carbapenemase genes in isolates from the aquatic environment but does not investigate modes of dissemination of resistance genes.
3. Original research investigations.	3. Only examines transferability of other types of ARGs beyond ESBL and/ or carbapenemase genes.
4. Investigations involve microbial analysis of water samples.	4. Analysis of other types of environmental samples (e.g. soil).

Given the limited number of studies that tested water bodies reported to be strictly “free” from contamination, it was decided that ‘urban’ waters would be included if investigations did not mention the presence of point contaminant sources upstream or in close proximity of the sampling area. Evidently, inclusion of investigations with sample areas potentially under the influence of non-point sources increases the likelihood of contamination from anthropogenic sources. However, considering the uncertainty in terms of presence of local anthropogenic influence, it was deemed these investigations were relevant to the research question and included into the screening process. Papers featuring sample collection upstream and downstream of a point discharge were included, extracting data solely from upstream samples in order to accommodate inclusion criteria, i.e., no perceived local point source(s) of pollution. Following the initial title/abstract screening, phase 2 consisted of a

full text review conducted by two independent reviewers. Full text screening employed additional methodology criteria for article inclusion/exclusion:

- i) The genomic detection of ESBL and/or carbapenemase genes.
- ii) The genomic detection of MGEs or demonstration of transferability of resistance genes by experimentation.

In the case of articles where information on the variant of the beta-lactamase gene detected using genotypic methods was lacking, phenotypic screening such as antibiotic susceptibility testing was used to determine if the genes detected were true ESBL/ carbapenemase producers. As an example, the *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> genes have some variants allowing for classifications as ESBL producers (*bla*<sub>TEM-3</sub> and *bla*<sub>SHV-12</sub>) and some which are classified simply as beta-lactamases (*bla*<sub>TEM-1</sub>, *bla*<sub>SHV-1</sub>).

## 2.3 Results

### 2.3.1 Screening Stages for Article Inclusion

A total number of 1415 articles were identified and subject to the first screening stage following application of the search string to the 5 main databases including PubMed, Scopus, Web of Science, MEDLINE and EMBASE (Figure 2.1). An additional 5 peer-reviewed articles were identified from grey literature queries placing the total number of articles at 1420. Following initial title and abstract screening, 1313 articles were excluded, with a total of 107 articles subject to full text review. This was narrowed down to 34 following exclusion of 73 articles that failed to meet pre-established inclusion/exclusion criteria. The most common exclusion factor was local presence of point source discharges (n=24). A recurrent feature during this screening phase was the lack of detail provided in the results of several investigations which often resulted in article exclusion. Several investigations failed to link the results presented to specific site types analysed (e.g. polluted, pristine), but instead provided a general

summary of all resistance genes detected (e.g. Dhawde et al., 2018). In other cases, due to the extensive nature of the data generated, primarily a feature of investigations employing metagenomics, ARGs were often summarised to an overly generic level. In some instances, only the quantity of beta-lactamase resistance genes that were present in a given sample were discussed rather than providing details on the different types of resistance genes or their location within the chromosome or mobile genetic element (Garner et al., 2016). As such, it was not possible to determine the types of beta-lactamase genes detected. Bibliography screening of the 34 included papers following the pre-defined inclusion/ exclusion criteria identified an additional 14 potentially suitable articles. 6 of these papers were included for analysis increasing the total number of articles for inclusion to 40. A single article was identified during a general search using Google Scholar bringing the final number of included articles to 41.

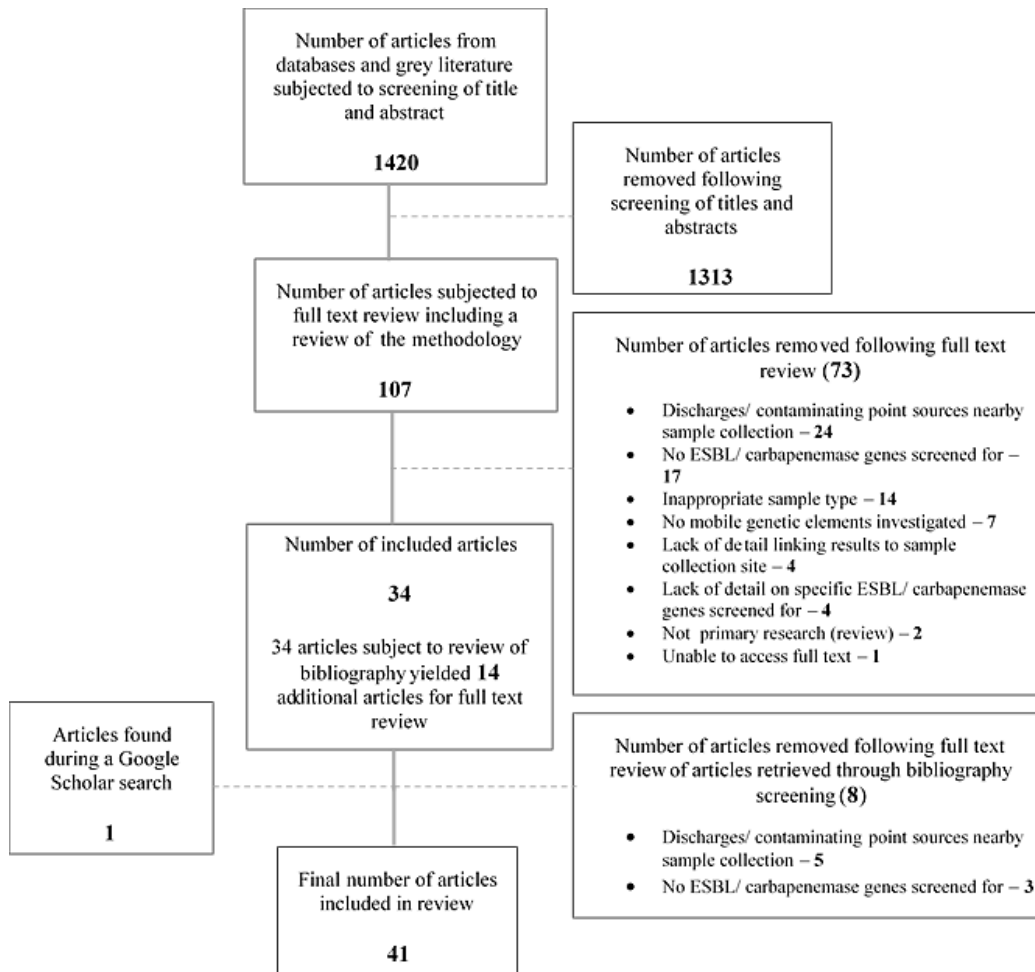


Figure 2.1: Flowchart outlining the process of elimination of articles based on inclusion/exclusion criteria set for this review.

### 2.3.2 Data Extraction

All 41 identified articles were subject to data extraction based on a number of pre-established data fields (Table 2.2). Primary data extraction fields included (i) country of origin for sample(s) analysed, (ii) type of sample analysed (e.g. seawater/river/lake), (iii) types of mobile genetic elements detected (e.g. plasmids, integrons, etc.), (iv) the application of conjugation transfer and whether it was successful at transferring the ARGs of interest, and (v) ESBL and/or carbapenemase gene variants detected. A range of data fields supplementing the information presented in Table 2.2 and pertaining local environments (e.g. local discharges downstream of sampling points), bacterial

species detected and further information on the types of mobile genetic elements detected (e.g. plasmid incompatibility groups, integron class) are outlined in Supplementary Table A2. Similarly, data relating to the methodology implemented in each investigation including initial collection volumes, processing and genomic screening for ARGs and MGEs are provided in Supplementary Table A3.

Table 2.2: Selected data extraction fields employed in the literature review protocol.

Reference	Country	Sample type (s)	Mobile genetic elements detected	Conjugation	ESBL detection	CPE detection
Zurfluh et al., 2015	Switzerland	River	Plasmid, transposon, insertion element, class 1 integron	Conjugation successful	N/A	<i>bla</i> <sub>VIM</sub> *
Ben Said et al., 2016	Tunisia	Rivers and lakes	Unknown	N/A	Not detected	N/A*
Lekunberri et al., 2017	Spain	River	Plasmid DNA	N/A	<i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>KPC</sub> , <i>bla</i> <sub>NDM</sub>
Adelowo et al., 2018	Nigeria	Ground-water	Class 1 integrons, plasmids, insertion sequences	N/A	<i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>SHV</sub>	N/A
Caltagirone et al., 2017	Italy	Rivers and ground-water	Plasmids	Conjugation successful	<i>bla</i> <sub>CTX-M-1, 28</sub> , <i>bla</i> <sub>SHV-12</sub>	Not detected*
Kim et al., 2008	South Korea	River	Class 1 integrons	Conjugation successful	<i>bla</i> <sub>TEM-52</sub> , <i>bla</i> <sub>OXA-4</sub> , <i>bla</i> <sub>CTX-M-14</sub>	N/A*
Stange et al., 2016	Germany	River	Class 1 and 2 integrons	N/A	<i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>SHV</sub>	N/A
Muraleedharan et al., 2019	United States	River	Plasmid	Conjugation successful	<i>bla</i> <sub>CTX-M-1</sub> , <i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>KPC-2</sub>
Olga et al., 2016	Greece	Stream water	Class 1 integron	N/A	Not detected	Not detected*
Wambugu et al., 2018	Kenya	River	Class 1 integron	N/A	<i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>CTX-M</sub>	N/A
Dolejská et al., 2009	Czech Republic	Pond	Class 1 integrons	N/A	Not detected	N/A*

## Chapter 2: Scoping review

Reference	Country	Sample type (s)	Mobile genetic elements detected	Conjugation	ESBL detection	CPE detection
Alouache et al., 2012	Algeria	Seawater	Unknown	N/A	Not detected	N/A*
Tafoukt et al., 2018	Algeria	River	Not detected	Conjugation unsuccessful	N/A	<i>bla</i> <sub>OXA-181</sub> , 199, 538*
Lepuschitz et al., 2019	Austria	River	Unknown	N/A	Not detected	Not detected*
Zarfel et al., 2017	Austria	River	Plasmids	N/A	<i>bla</i> <sub>CTX-M-15</sub> , 27, 14, 1, 2, 9, 14-like, <i>bla</i> <sub>SHV-12</sub> , <i>bla</i> <sub>GES-1</sub>	<i>bla</i> <sub>VIM-1</sub>
Charnock et al., 2014	Norway	Seawater	Class 1 integrons	N/A	Not detected	N/A
Jørgensen et al., 2017b	Norway	Seawater	Plasmids	N/A	<i>bla</i> <sub>CTX-M-1</sub> , 2, 9, 14, 15, 27, 55, <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>SHV-12</sub> , <i>bla</i> <sub>TEM-33-like</sub> , 52C	Not detected*
Jørgensen et al., 2017a	Norway	Seawater	Plasmids	N/A	<i>bla</i> <sub>CTX-M-1</sub>	N/A*
Kieffer et al., 2016	Portugal	River	Plasmids, Class 1 integrons	Conjugation successful	N/A	<i>bla</i> <sub>IMP-8</sub> , <i>bla</i> <sub>VIM-1</sub> , <i>bla</i> <sub>VIM-34</sub> *
Tacão et al., 2012	Portugal	Rivers	Class 1 integrons, insertion sequences	N/A	<i>bla</i> <sub>CTX-M</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>TEM</sub>	N/A*
Poirel et al., 2012a	Portugal	River	Plasmids, transposons	Conjugation successful	N/A	<i>bla</i> <sub>KPC-2</sub> *
Harnisz & Koreniewsk 2018	Poland	River	Class 2 integrons	N/A	Not detected	N/A
Osińska et al., 2016	Poland	River	Plasmid DNA	Conjugation successful	<i>bla</i> <sub>OXA</sub> , <i>bla</i> <sub>TEM</sub>	N/A*
Koczura et al., 2014	Poland	Lakes	Class 2 integrons	N/A	Not detected	N/A*
Osińska et al., 2017	Poland	River	Class 1 integrons	N/A	<i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>OXA</sub>	N/A*
Bajaj et al., 2016	India	River	Plasmids	Conjugation successful	<i>bla</i> <sub>CTX-M-15</sub>	N/A
Singh et al., 2018	India	River	Insertion sequence, class 1 integron	Conjugation successful	<i>bla</i> <sub>CTX-M-15</sub>	N/A

## Chapter 2: Scoping review

Reference	Country	Sample type (s)	Mobile genetic elements detected	Conjugation	ESBL detection	CPE detection
Akiba et al., 2016	India	Rivers	Plasmids	N/A	<i>bla</i> <sub>CTX-M-55, 15,</sub> <i>bla</i> <sub>OXA-1, 9,</sub> <i>bla</i> <sub>SHV-12</sub>	<i>bla</i> <sub>NDM-7*</sub>
Lamba et al., 2017	India	River	Class 1, 2 and 3 integrons	N/A	<i>bla</i> <sub>OXA, bla</sub> <sub>CTX-M,</sub> <i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>NDM</sub>
Fernandes et al., 2017	Brazil	Seawater	Plasmids	N/A	<i>bla</i> <sub>CTX-M-8, 1</sub>	N/A*
Sellera et al., 2017	Brazil	Seawater	Plasmid	N/A	<i>bla</i> <sub>CTX-M-15,</sub> <i>bla</i> <sub>OXA-17</sub>	<i>bla</i> <sub>KPC-2*</sub>
Francisco et al., 2019	Brazil	Rivers	Plasmids	N/A	<i>bla</i> <sub>CTX-M-15,</sub> <i>bla</i> <sub>SHV-11, bla</sub> <sub>OXA-1</sub>	<i>bla</i> <sub>KPC-2*</sub>
Nascimento et al., 2017	Brazil	Lakes	Plasmids, transposons	N/A	<i>bla</i> <sub>CTX-M-2, 15, 9</sub>	<i>bla</i> <sub>KPC-2*</sub>
Chen et al., 2010	China	River	Class 1 and 2 integrons	N/A	<i>bla</i> <sub>TEM, bla</sub> <sub>CTX-M,</sub> <i>bla</i> <sub>SHV</sub>	N/A*
Zhang et al., 2018	China	Bay	Class 1 integrons	N/A	<i>bla</i> <sub>TEM</sub>	N/A
Wang et al., 2018	China	River	Class 1 integrons	N/A	<i>bla</i> <sub>TEM</sub>	N/A*
Ye et al., 2017	China	River	Class 1 integrons	Conjugation successful	<i>bla</i> <sub>CTX-M-65, 55, 15,</sub> 14	N/A*
Yin et al., 2013	China	Lake	Class 1 and 2 integrons	Conjugation successful	<i>bla</i> <sub>TEM, bla</sub> <sub>SHV,</sub> <i>bla</i> <sub>CTX-M, bla</sub> <sub>OXA-1</sub>	N/A*
Wu et al., 2019a	China	Rivers	Class 1 integron	N/A	<i>bla</i> <sub>CTX-M, bla</sub> <sub>TEM</sub>	N/A
Zou et al., 2012	China	River	Plasmid	N/A	<i>bla</i> <sub>TEM, bla</sub> <sub>SHV</sub>	Not detected
Ouyang et al., 2015	China	River	Class 1 integron	N/A	<i>bla</i> <sub>SFO, bla</sub> <sub>SHV,</sub> <i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>IMP</sub>

*Unknown = Data not provided in the article.*

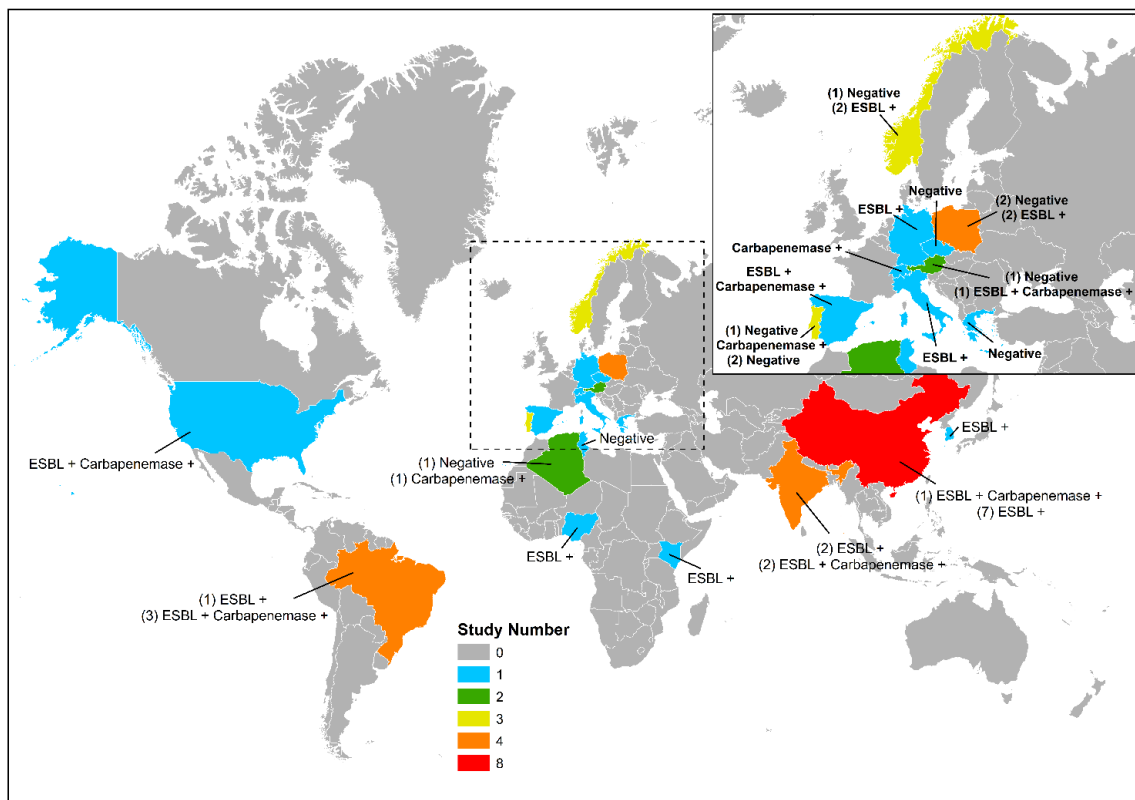
*Not detected = Screened for but not detected.*

*N/A = Not directly screened for using a targeted approach.*

*\* = Sequencing approach applied.*

### 2.3.3 Summary Analysis of Included Studies

The geographical distribution of included investigations is shown in Figure 2.2. Overall, studies derived from nineteen different countries across five continents. As such, the data presented is representative of water bodies at a global scale, but primarily comprises investigations based in Europe (n=18) and Asia (n=13). The remainder of identified investigations were based in Africa (n=5), South America (n=4) and North America (n=1). China featured as the country with most investigations (n=8) followed by Poland (n=4), India (n=4) and Brazil (n=4).



*Figure 2.2: Global distribution of investigations identified through the literature review protocol including positive detection of ESBL and/or carbapenemase genes. The number provided in brackets denotes the number of country-specific studies with the same detection outcome in terms of antibiotic resistance genes.*

Overall, the methodology employed for sample collection, processing and genomic screening varied substantially among investigations (Supplementary

Table A3). Sample volumes collected exhibited wide variations ranging from swab placement in water for 24 hours (Dolejská et al., 2009) to analysis of 7 L of water (Tacão et al., 2012). Concentration methods also varied significantly with filtration using a 0.45µm (n=14) and 0.22µm (n=6) being the most prevalent. However, a further 6 studies using a filtration approach failed to report filter pore size. Filtration was often followed by direct incubation of filters on agar plates, (n=12). PCR and subsequent sequencing (n=18) featured as the most prominent molecular method employed to detect ARGs and MGEs. Additional molecular detection methods included sole use of PCR (n=16) or a sequencing approach (n=5).

The bacterial species most commonly detected was *E. coli* with positive identification in a total of 24 investigations. *E. coli* detection was followed by *Klebsiella* spp. (n=11), *Enterobacter* spp. (n=6) and *Pseudomonas* spp. (n=6) (Supplementary Table A2). A range of natural water bodies were investigated in the reviewed studies. Inland waters were classified into lentic (i.e., stationary or still water), lotic (i.e., free-flowing water) and sub-surface/groundwater. Lentic systems included lakes (n=4) and ponds (n=1) whereas lotic systems encompassed rivers (n=28) and streams (n=1). Additionally, seawater (n=6) or bay waters (n=1) were classified as coastal/marine. Only two investigations evaluated sub-surface/groundwater for the presence of ARGs. A total of two included articles evaluated more than one type of water body, (rivers, lakes or groundwater).

In terms of ARG and MGE detection, a summary of the numbers and types of each are provided in Figure 2.3. A total of 33/41 (80.5%) investigations detected the presence of ESBL and/or carbapenemase genes in water samples. Specifically, 20/41 (48.8%) studies detected ESBL genes, 4/41 (9.8%) studies detected carbapenemase genes and 9/41 (22.0%) detected dual presence. The most commonly detected ESBL resistance gene was *bla*<sub>CTX-M</sub> (n=21), closely followed by *bla*<sub>TEM</sub> (n=18). Regarding the detection of carbapenemase

resistance genes, *bla*<sub>KPC</sub> was the most commonly detected gene with identification in 6 reviewed studies. This was followed by *bla*<sub>VIM</sub> (n=3), *bla*<sub>NDM</sub> (n=3) and *bla*<sub>IMP</sub> (n=2). In comparison to resistance gene detection, a larger proportion of studies (37/41; 90.2%) detected the presence of one or more MGE. This included class 1, 2 and 3 integrons (n=22), plasmids (n=18), insertion sequences (n=4) and transposons (n=3). In terms of integrons, class 1 was the most prominent (n=20) followed by class 2 (n=6) and class 3 (n=1). Regarding plasmid incompatibility groups, the most commonly detected types were IncFIB (n=10) followed by IncN (n=8). Furthermore, 11 studies also demonstrated successful conjugation transfer of ARGs.

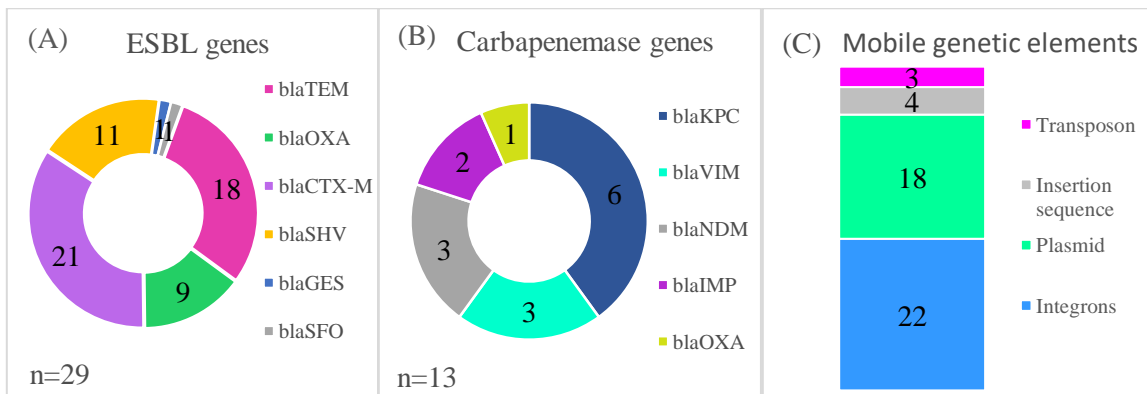


Figure 2.3: Number of studies detecting each type of (A) ESBL gene (B) carbapenemase gene and (C) mobile genetic element. The total number of studies detecting ESBLs and carbapenemases is also provided, with some studies reporting more than one type of ESBL (n=20) or carbapenemase (n=2) gene.

## 2.4 Discussion

This scoping literature review was established in order to examine existing scientific literature relating to the role of the natural aquatic environment in harbouring and transmitting ARGs of clinical importance. Here, an exclusive focus was placed on areas with “minimal” direct anthropogenic contamination. A key highlight from this investigation is the relative limited number of studies focusing on the nexus between the natural, unpolluted aquatic environments and the presence of antibiotic resistance. A significant feature identified

through the review protocol is a tendency for investigations to attempt to establish point sources as pollution agents; however, detection of resistance genes in uncontaminated areas is generally overlooked.

Overall, detection of ESBL and/or carbapenemase genes in 33/41 (80.5%) identified studies serves to demonstrate the importance of natural water bodies as large reservoirs of multiple ARGs. A high proportion of studies (37/41; 90.2%) demonstrated the presence of one or more MGE highlighting the potential dissemination of ARGs among environmental bacteria. Accordingly, the presented figures highlight the key role of the natural aquatic environment as a significant reservoir of ARGs.

#### **2.4.1 Synopsis of Identified Literature**

Publication dates of included articles ranged from 2008 to 2019, with the majority published between 2016 and 2019 (29/41; 70.7%). The upsurge in publications observed in recent years concurs with an increased interest by the research community in investigating the prevalence of antibiotic resistance in environmental settings (Kraemer et al., 2019). *E. coli* was the most commonly detected species in 24 studies followed by *Klebsiella* spp. (n=11), *Enterobacter* spp. (n=6) and *Pseudomonas* spp. (n=6) (Supplementary Table A2). These bacteria are often associated with clinical infections and as a result, some methodologies employed a selection bias using selective broths and agars, (see Supplementary Table A3). A range of natural water bodies were investigated in the reviewed studies, however, the majority of studies focused on lotic systems (n=29; 70.7%). The high incidence of studies based on these systems may be associated with the smaller dimensions of rivers/streams in comparison to larger marine and coastal water bodies, equating to a lesser dilutional effect on viable antibiotic resistant organisms. Additionally, lotic systems are often located within urban and agricultural landscapes, with river-sourced water often used as a domestic water supply and thus representing a potential

pathway for antibiotic resistance transmission to humans. As indicated above, based on the lack of studies investigating “pristine” aquatic environments, water bodies labelled as ‘urban’ were also included in the review protocol if no local point sources were described. A prevalent methodological approach among river-based investigations included collection of water samples in areas upstream and downstream of a discharge point in order to ascertain the influence of discharges in the proliferation of environmental ARGs. As mentioned above, only articles featuring sampling regimes based on collection of upstream samples were included in the review in order to accommodate inclusion criteria, i.e., no perceived local point source(s) of pollution.

The low number of investigations based on marine/coastal and lake environments that were free from anthropogenic contamination represents a key research gap considering their potential importance as environmental pathways for the transmission of antibiotic resistance to humans; particularly through recreational exposure (Leonard et al., 2018). Possible reasons for the low number of marine-based investigations observed may include lack of access to coastal sampling sites in landlocked countries or the potential for lower rates of bacterial survival in saltwater serving as a deterrent (Rozen and Belkin, 2001). In terms of lakes/ponds, their absence as prevalent landscape components in certain geographical settings may preclude their analysis. However, they do also pose a significant threat for the potential transmission of ARGs to humans (Bengtsson-Palme et al., 2014).

### **2.4.2 Antibiotic resistance gene detection**

A higher detection rate of ESBL (n=29) genes in comparison to carbapenemase genes (n=13) among included studies may be attributable to the fact that ESBL enzymes emerged approximately 10 years prior to carbapenemase detection. In addition, carbapenem antibiotics are classified as reserved/restricted in many

countries and therefore only used as a last resort, in an attempt to circumvent further resistance development. However, both third generation cephalosporins and carbapenems are classified as critically important antibiotics employed in veterinary medicine (WHO, 2016). Consequently, ESBL producing bacteria and to a lesser extent carbapenemase producers, are disseminated throughout most environments.

On a country-basis, Brazil had the highest number of detected carbapenemase enzymes, featuring in 3 out of a possible 4 studies. Interestingly, all 3 positive studies detected *bla*<sub>KPC-2</sub>. This carbapenemase gene was first detected in the USA in 1996 and is now considered as endemic in Brazil (Lee et al., 2016). Although China had the largest number of included studies, carbapenemase enzymes were only reported in 1/8 studies. In turn, China accounted for the highest detection rates of ESBLs among included investigations (8/8). Generally, ESBLs were widespread across countries and continents (Figure 2.2). This potentially reflects their widespread dissemination and/or natural occurrence in the aquatic environment as supported by several investigations (Swedan & Abu Alrub, 2019; de Oliveira et al., 2017; Yamashita et al., 2017; Adesoji & Ogunjobi, 2016).

The most commonly detected ESBL gene was *bla*<sub>CTX-M</sub> (n=21). This is unsurprising as the origins of this gene has been traced back to the environmental organism *Kluyvera*, previously isolated from water bodies (Cantón et al., 2012a). This was followed by *bla*<sub>TEM</sub> (n=18), *bla*<sub>SHV</sub> (n=11) and *bla*<sub>OXA</sub> (n=9) ESBL types. These findings concur with additional studies screening for ESBL genes in the environment. For example, Ranjbar & Sami. (2017) detected *bla*<sub>TEM</sub>, *bla*<sub>CTX</sub>, *bla*<sub>SHV</sub> and *bla*<sub>OXA</sub> ESBL types at a frequency of 37%, 27%, 27% and 25% respectively, in an investigation based on the analysis of river water. In terms of the carbapenemase encoding genes, *bla*<sub>KPC</sub> was the most commonly detected gene in 6/41 (14.6%) included studies. This was followed by *bla*<sub>VIM</sub> (n=3), *bla*<sub>NDM</sub> (n=3) and *bla*<sub>IMP</sub> (n=2). These enzymes were first identified in clinical isolates

(Khan et al., 2017; Yigit et al., 2001; Lauretti et al., 1999; Osano et al., 1994). However, their presence in the environment prior to reports in the nosocomial setting may have gone unidentified. Recent linkages of *bla*<sub>OXA-48</sub> with the environmental *Shewanella* species reported by Tacão et al. (2018) serves to highlight this possibility.

Positive detection of ESBL and/or carbapenemase genes in 33/41 (80.5%) investigations indicates the ubiquitous occurrence of these clinically significant ARGs in natural aquatic environments at a global scale. However, an important consideration in this area of research is publication bias against negative results. This bias could potentially inflate the high percentage of included studies with positive detections. Unfortunately, it is not a type of bias that can be controlled for within the scope of this review, but should be considered when interpreting the results. Reviewed investigations with negative detection of ESBLs and/or carbapenemases collected a range of different sample types including river water (n=2), lake water (n=1), river and lake water (n=1), seawater (n=2), stream water (n=1) and pond water (n=1). Large variations in methods employed observed among these articles may be associated with the lack of detection of ARGs. Small volumes of water were collected for analysis among some investigations. For example, Dolejská et al. (2009) employed the use of swabs placed in a pond for 24 hours as the initial sample collection technique while Ben Said et al. (2016) collected 5mL volumes of water for analysis (Supplementary Table A3). Additionally, certain processing techniques may have influenced the results. Harnisz and Korzeniewska. (2018) diluted samples with saline in contrast to the filtration and enrichment prior to culturing approach employed in other investigations (e.g. Bajaj et al., 2016). The large variation in methodologies employed demonstrates a lack of standardisation among environmental sampling and laboratory processing which prevents robust comparisons among reported results.

### 2.4.3 Mobile genetic element detection

In comparison to resistance gene detection, a larger proportion (37/41; 90.2%) of included studies detected the presence of one or more MGE. This included class 1, 2 and 3 integrons (n=22), plasmids (n=18), insertion sequences (n=4) and transposons (n=3) (Figure 2.3). Additionally, 11 studies demonstrated successful conjugation transfer of ARGs between different bacteria. The combination of these elements represent the potential for dissemination of resistance elements among aquatic bacteria, which can be attributed as a contributing factor in the detection of resistance in areas deemed to be 'free' from anthropogenic influence. The reported high level of detection emphasizes the ability of most bacteria to exchange genetic elements that are favourable to their survival across bacterial species, making this threat almost impossible to contain. This feature serves to highlight the challenges of containing and/or mitigating the environmental spread of ARGs.

The most commonly reported MGEs were integrons which have previously been detected in a wide variety of environments including soil, sediment, biofilms as well as waters irrespective of antibiotic contamination (Abella et al., 2015). Their ability to be transferred via insertion sequences, transposons and plasmids due to co-selection with resistance genes further amplifies their presence. In clinical environments class 1 and 3 integrons, and to a lesser extent class 2, are commonly detected in invasive bacterial isolates. As a result, these classes of integrons were largely screened for among included investigations. However, this approach may be underestimating the integron class diversity in aquatic environments, and in turn the ability of environmental species to disseminate ARGs, (Abella et al., 2015).

Plasmids were the second most commonly detected MGE, identified in 18 studies (Figure 2.3). These elements are responsible for dissemination of antibiotic resistance elements via conjugation, (San Millan, 2018). While

capable of harbouring and transmitting multiple gene types, ARGs have been closely linked with plasmids due to the clinical implications in terms of treatment. Several plasmid incompatibility groups were detected among reviewed investigations, including IncFIB (n=10) and IncN (N=8), which were the most commonly detected. Although not all studies confirmed the presence of resistance gene(s) on a plasmid, the potential transfer of the resistance gene from the chromosome to a plasmid and its' further dissemination across bacterial species via conjugation is possible. The successful conjugation transfer of resistance elements in the laboratory in 11/41 (26.8%) investigations strengthens this possibility of ARG propagation in aquatic environments. Notably, conjugation experiments were all performed using *E. coli* as the recipient with the majority using the J53 strain. The latter is in agreement with the review performed by Leclerc et al. (2019), highlighting the prevalence of *E. coli* as a common recipient for conjugation transfer and the general lack of investigations attempting interspecies transfer. However, in the review dataset, some of the environmental isolates used as conjugation donors were *Klebsiella* and/or *Acinetobacter* (Ye et al., 2017, Osińska et al., 2016, Yin et al., 2013). Hence, there was an attempt at demonstrating interspecies transfer in a limited number of cases.

#### **2.4.4 Sources of resistance**

A range of sources were linked with the incidence of ARGs in the aquatic environments sampled in the reviewed papers. For example, Jørgensen et al. (2017a) detected *bla*<sub>CTX-M-1</sub> and mentioned 'human bathing, boat toilets, farm animals, fertilizers or birds' as potential sources contributing to the presence of antibiotic resistance. Similarly, Nascimento et al., (2017) detected *bla*<sub>KPC</sub> and *bla*<sub>CTX-M</sub> types and hypothesized that nosocomial or domestic sewage had entered the lake system via a stream, despite treatment in a 'flotation treatment plant' prior to lake discharge. Beyond Ouyung et al. (2015), few

studies considered the possibility of naturally occurring antibiotic resistance in uncontaminated aquatic environments. In this study a 'pristine' site was sampled in a remote location which yielded the detection of 69 antibiotic resistance genes. The results presented were construed as indicative of ubiquitous antibiotic resistance in natural environments. However, the prevailing notion of ARG dissemination from regions under the influence of contaminating discharges to those without it, remains largely unchallenged. Accordingly, further research into intrinsic antibiotic resistance in environmental organisms and the dissemination of ARGs, inclusive of pathways, modes and extent, is urgently needed.

#### **2.4.5 Research highlights and recommendations**

The lack of standardised methodology adopted among identified investigations represents a significant knowledge gap and a challenge for the interpretation of collated data. In particular, issues with method sensitivity and the lack of valid comparisons to analyse reported results are apparent. As such, a strong argument is made for future investigations to adopt a more standardised methodological approach that is sensitive enough to detect low levels of antibiotic resistance genes. Similarly, future research should also take associated human risks into consideration, (e.g. potential consumption volumes during recreational activities and infectious dose of organisms). Evidently, methodology criteria applied in this review attempted to standardise articles with highly variable collection volumes and processing techniques as much as possible. In particular, the application of positive genomic detection criteria of ARGs and MGEs increased the comparability across studies, eliminating the need for comparison of phenotypic antibiotic resistance in one investigation to genomic ARG detection in another. In general, the lack of relevant journal articles identified limited the inclusion restrictions that could be applied in terms of sampling volumes and processing. Predominantly, the lack of

consistent monitoring of the environment for antibiotic resistance worldwide severely limits our knowledge in this area.

The insufficient detail provided on anthropogenic contamination sources in relation to water bodies in several reviewed investigations represents a second important research gap. Overall, it was not possible to ascertain if analysed water bodies were entirely “free” from contamination. In some studies the sampling sites were labelled as ‘pristine’ or in ‘areas of strict preservation’, but others list sampling points as ‘urban’ regions and so the likelihood of contamination is much greater (Supplementary table A2). This particular restraint means that this review could not be strictly confined to ‘pristine’ aquatic environments, but it does highlight the lack of research focus on the prevalence of antibiotic resistance in natural unpolluted aquatic environment. More research is required in the area as discovery of resistances of clinical significance in regions free from anthropogenic activity become more apparent. Additionally, more emphasis should be placed on investigations tracing ARG origins to environmental isolates and potentially screening the environment for novel ARGs. Future research should also highlight the role of MGEs in the dissemination of resistance elements rather than focusing primarily on contaminating sources.

In several investigations only certain strains of isolated bacteria were further characterised for the presence of antibiotic resistance using phenotypic and genotypic methods. These were generally restricted to highly virulent bacteria and those most often associated with clinical infections in humans. As such, some articles employed selective application of screening methods specific to detection of only certain types of bacteria, (e.g. Ye et al., 2017; Kieffer et al., 2016; Stange et al., 2016). This practice represents a major knowledge gap with the current role of ubiquitous, non-pathogenic bacteria in the transference of ARGs largely unaccounted for. Emphasis on antibiotic resistance profiles rather than species detected should be employed by scientists, considering MGEs that

harbour resistance genes spread throughout different bacterial species. Similarly, by confining environmental research to the most commonly identified MGEs within clinical environments, the potential for transmission of ARGs via MGEs present in natural aquatic environments is likely being underestimated.

Of key relevance is the high prevalence of phenotypic screening methods employed in the majority of reviewed investigations. This factor limited the detection of antibiotic resistance genes to those that reside within viable bacteria that are easily cultured. However, reportedly less than 1% of environmental bacteria can be easily cultured using standard laboratory techniques (Allen et al., 2010). Only four included studies performed PCR/sequencing of the sample without prior culturing of organisms (Supplementary Table A3) which enabled detection and analysis of fastidious bacteria. Overall, current research tends to exclude less clinically significant and fastidious environmental bacteria, which as a result, may go unnoticed as harbouring MGEs capable of spreading antibiotic resistance elements. Ideally, simultaneous application of both phenotypic culture methods and molecular analysis of environmental samples would need to be employed to generate complete resistance profiles inclusive of genetic composition as well as phenotypic expression.

## **2.5 Conclusions**

The results presented highlight the importance of aquatic environments as substantial reservoirs of ESBL and carbapenemase ARGs. As such, point contaminant sources may not be the sole contributors to the presence of antibiotic resistance in the aquatic environment. Additionally, collated data serves to demonstrate the potential for interspecies transference of ARGs among environmental bacteria to potentially pathogenic species. The following recommendations are made based on their potential to contribute to our

current understanding of both the prevalence and risk factors associated with ARGs in aquatic environments:

- More investigations are required involving routine screening of antibiotic resistance genes in water bodies, particularly those considered to be disassociated from direct point contaminant sources.
- A highly sensitive and standardised methodology which enables valid and robust comparisons among study outcomes.
- Incorporation of genomic screening and culture-based analyses aiming to mitigate the selective bias imposed by culturing.
- Detailed reporting in investigations, particularly in terms of study site characteristics (e.g. location of possible non-point and point sources).
- Implementation of replica aquatic environmental conjugation/ transformation/ transduction experiments demonstrating interspecies transfer.

Future research should focus on these key areas to strengthen the body of evidence which suggests that ARGs of clinical significance can potentially become widely disseminated by MGEs throughout uncontaminated aquatic environments. Outcomes from the reviewed investigations strengthen the need for a 'One Health' approach encompassing human, animal and environmental health when tackling the immense threat of a world without effective antibiotics.

## **Chapter 3: A Point Prevalence Survey of Antibiotic Resistance in the Irish Environment, 2018-2019**

Published by Environment International

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<https://doi.org/10.1016/j.envint.2021.106466>

## Abstract

Water bodies worldwide have proven to be vast reservoirs of clinically significant antibiotic resistant organisms. Contamination of waters by anthropogenic discharges is a significant contributor to the widespread dissemination of antibiotic resistance. The aim of this research was to investigate multiple different anthropogenic sources on a national scale for the role they play in the environmental propagation of antibiotic resistance. A total of 39 water and 25 sewage samples were collected across four local authority areas in the West, East and South of Ireland. In total, 211 Enterobacterales were isolated (139 water, 72 sewage) and characterised. A subset of isolates (n=60) were chosen for whole genome sequencing. Direct comparisons of the water versus sewage isolate collections revealed a higher percentage of sewage isolates displayed resistance to ceftazidime (46%) and ertapenem (32%), while a higher percentage of water isolates displayed resistance to tetracycline (55%) and ciprofloxacin (71%). Half of all isolates displayed extended spectrum beta-lactamase (ESBL) production phenotypically (n=105/211; 50%), with *bla*<sub>CTX-M</sub> detected in 99/105 isolates by PCR. Carbapenemase genes were identified in 11 isolates (6 sewage, 5 water). The most common variant was *bla*<sub>OXA-48</sub> (n=6), followed by *bla*<sub>NDM-5</sub> (n=2) and *bla*<sub>KPC-2</sub> (n=2). Whole genome sequencing analysis revealed numerous different sequence types in circulation in both waters and sewage including *E. coli* ST131 (n=15), ST38 (n=8), ST10 (n=4) along with *Klebsiella* ST405 (n=3) and ST11 (n=2). Core genome MLST (cgMLST) comparisons uncovered three highly similar *Klebsiella* isolates originating from hospital sewage and two nearby waters. The *Klebsiella* isolates from an estuary and seawater displayed 99.1% and 98.8% cgMLST identity to the hospital sewage isolate respectively. In addition, three pairs of *E. coli* isolates from different waters also revealed cgMLST similarities, indicating widespread dissemination and persistence of certain strains in the aquatic environment. These findings highlight the need for routine monitoring of water bodies used

for recreational and drinking purposes for the presence of multi-drug resistant organisms.

### 3.1 Introduction

Antibiotic resistance is recognised as one of the largest threats to the healthcare and agricultural sectors worldwide (Prestinaci et al., 2015). The correct and incorrect use of antibiotics in both human and veterinary medicine has been deemed a major contributor to the widespread development of antibiotic resistance. In recent years, a deeper appreciation of the 'One Health' concept has emerged, recognising the nexus between the health of humans, animals and the environment. Subsequently, the importance of the natural environment in the dissemination of antibiotic resistant organisms has been increasingly recognised (Hooban et al., 2020). This was emphasised in the 'Global Action Plan on Antimicrobial Resistance', released by the World Health Organization (WHO) in 2015 (WHO, 2015). Five key objectives that should be considered when tackling antibiotic resistance on a global scale were outlined in this report. According to objective 2, further research is needed on the development and spread of antibiotic resistance 'within and between humans and animals and through food, water and the environment'. More recently, the WHO released a priority pathogens list ranking carbapenem resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae*, as well as third generation cephalosporin resistant *Enterobacteriaceae* as of critical priority (WHO, 2017). These critical priority pathogens have recently been identified in the natural environment including carbapenem resistant *Acinetobacter* spp. in rivers (Kittinger et al., 2017), carbapenem resistant *Pseudomonas* in coastal waters (Paschoal et al., 2017) and carbapenemase producing Enterobacterales (CPE) in seawaters (Mahon et al., 2019).

The extent of the antibiotic resistance crisis is reflected on a European scale in recent reports. The European Antimicrobial Resistance Surveillance Network

(EARS-Net) publish annual data on antibiotic resistance among invasive isolates primarily from blood and cerebrospinal fluid. The latest figures revealed that carbapenem resistant *Acinetobacter*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* represented greater than 50% of invasive isolates identified in some southern European countries (European Centre for Disease Prevention and Control 2020a). Evidence of cross border human transmission clusters of carbapenemase producing *Klebsiella pneumoniae* has been established (Ludden et al., 2020), indicative of potential further widespread dissemination of carbapenem resistant bacteria across European countries. Recent figures released by the Health Protection Surveillance Centre (HPSC) in Ireland revealed *E. coli*, *Enterobacter cloacae* and *Klebsiella* spp. as the most common Enterobacterales identified from clinical samples to harbour carbapenemases (HPSC 2019e). Among the different carbapenemase enzymes detected through screening and clinical cases, OXA-48 ranked highest, followed by KPC, NDM, OXA-181/232 and VIM. The detection of CPE in hospital and municipal wastewater demonstrates this crucial pathway for transmission of these organisms to the environment (Zhang et al., 2020; Cahill et al., 2019; Jin et al., 2017). According to the Environmental Protection Agency (EPA) there are 35 raw sewage discharge points across Ireland where untreated sewage is being directly emitted to environmental waters (EPA, 2020). In cases where hospital effluent reaches a wastewater treatment plant prior to environmental discharge, these treatment processes are not designed for successful antibiotic resistance gene removal (Pazda et al., 2019).

At present, bathing waters are assessed over four bathing water seasons for the percentile values of *E. coli* and intestinal enterococci (CFU/100mL) for designation as excellent, good, sufficient or poor water quality (Directive 2006/7/EC). The designation of bathing water quality is based on cutoff values that differ for inland versus coastal waters. For example, inland waters harbouring  $\leq 200$ CFU/mL intestinal enterococci and  $\leq 500$ CFU/mL *E. coli* would

be designated as excellent bathing water status. In contrast, coastal waters must display  $\leq 100$ CFU/mL intestinal enterococci and  $\leq 250$ CFU/mL *E. coli* to also achieve excellent bathing water status. The current European Union (EU) bathing water directive requires monthly monitoring of the levels of *E. coli* and intestinal enterococci in waters used for recreational purposes. Under this directive there is no requirement for any further characterisation of the bacteria, such as antibiotic resistance profiling. In addition, just 250mL of water is collected, a relatively small volume to adequately represent vast bodies of water. Similarly, the drinking water directive (Directive 2020/2184) states that *E. coli* and enterococci levels should be 0/100mL in waters intended for human consumption.

In Ireland, waterborne illnesses represent a significant portion of gastrointestinal related infections. Cryptosporidiosis can often be linked to the inadequate treatment of drinking water supplies, with the most recent figures indicating 629 confirmed cases in Ireland in 2018 (HPSC 2019c). Chlorination alone is ineffective at killing *Cryptosporidium* and so water treatment systems contaminated with *Cryptosporidium* must include filtration and/or ultraviolet light for adequate disinfection (EPA, 2016). In Ireland, an estimated 170,000 households operate private wells with varying treatments employed (EPA, 2017). Private household wells are at risk of contamination with *Cryptosporidium* as well as faecal bacteria such as verotoxigenic *E. coli* (VTEC) through ingress mechanisms (Chique et al., 2021; Chique et al., 2020). The incidence of VTEC in Ireland was ten times the European average in 2017 with 923 notified cases (HPSC 2019d). Although the sources of these cases were not fully elucidated, 41% listed exposure to private well water. Similarly, many recreational activities result in the ingestion of water, making environmental monitoring of bathing waters an important public health concern. In the United Kingdom a recent study revealed surfers had higher carriage rates of *bla*<sub>CTX-M</sub> bearing *E. coli* (6.3%) in comparison to non-surfers (1.5%) (Leonard et al., 2018).

This raises the question of whether current EU monitoring criteria is adequate to protect public health interests. This concern was also emphasized by Mahon et al. (2017) who detected NDM-producing *Enterobacteriaceae* in a bathing water designated as sufficient quality.

With these important considerations in mind, the aim of this research was to examine the role of different sewage sources in the dissemination of antibiotic resistance to the natural aquatic environment. Natural water bodies including saltwater and freshwaters, as well as raw water supplying drinking water treatment plants were assessed. Antibiotic resistance profiling and next generation sequencing were carried out on Enterobacterales to examine the nexus between isolates collected from sewage and water samples. Resistant bacteria from different environments were also directly compared to analyse characteristics unique to each.

## **3.2 Materials and Methods**

### **3.2.1 Overview of sample collection sites**

Samples of sewage and water were collected from four local authority areas (LAAs) in the West, East and South of Ireland. LAAs are zones designated to different councils who are responsible for providing services such as housing, planning permission, road maintenance, environmental protection and development and maintenance of recreation facilities. Normally there are two councils designated per county; city councils for urbanised centres and county councils for larger rural areas.

#### **West**

Galway city and Galway county LAAs are located on the west coast of Ireland. The population is 78,668 in the city and 179,390 in the county according to the most recent central statistics office data (CSO, 2016). The area of Galway city is 50.0km<sup>2</sup> whereas Galway county is much larger at 5,796km<sup>2</sup>.

### **East**

Fingal local authority area is located in Dublin county. It has a population of 296,020 (CSO, 2016) despite being a small area (458km<sup>2</sup>). There is little agricultural activity in Fingal as identified from maps created by Chique et al. (2019) for the purposes of this project.

### **South**

Cork county local authority area is located in the south of Ireland with the largest population of 417,211 (CSO, 2016). It also has the largest area of all the local authority areas included in this study at 7,403km<sup>2</sup>. It has high agricultural activity and many discharge points along the coastline including 7 raw sewage discharges (EPA, 2020).

Sampling points of interest in each LAA under investigation were selected based on maps generated of potential contaminating sources of antibiotic resistance (Chique et al., 2019). Water bodies chosen for sample collection included 'hot spot' areas receiving discharges (storm water overflows, raw sewage discharges, primary and/or secondary wastewater treatment discharges). Where possible, 'cold spots' were also chosen which included waters receiving little or no contaminating discharges for comparison. Water bodies chosen for analysis included seawaters, rivers, lakes, estuaries and untreated water supplying drinking water treatment plants (Table 3.1). Contaminating sources chosen for inclusion comprised of hospital and nursing home sewage, airport sewage, as well as wastewater treatment plant influent and effluent across the LAAs (Table 3.2). Hospitals and nursing homes were selected based on the level of antibiotic usage data. In the case of long term care facilities, antibiotic usage data was obtained from the Healthcare-Associated Infections & Antimicrobial Use in Long-Term Care Facilities (HALT) study (HPSC, 2017). The levels of antibiotic usage among hospitals was obtained from the Hospital Antimicrobial Consumption Surveillance data (HPSC 2019f). Additional factors that influenced

the choice of nursing homes and hospitals included proximity to water sampling points and the willingness of the institute to participate. All sampling took place between November 2018 and July 2019. Rainfall data for the 24 hours prior to sample collection was recorded for each sampling point (Supplementary Table B7).

### **3.2.2 Water sample collection and processing**

Water sampling involved collection of 30L of water from pre-defined sampling sites. Collection was carried out using six sterile 5L containers and pooled prior to processing which took place no longer than 4-6 hours post collection.

Of the water samples, 5 sites were considered 'cold spots' due to the lack of contaminating discharges nearby. These included 3 seawaters located in Galway county (Beach J), Fingal (Beach P) and Cork (Beach U), as well as river A in Galway city and estuary C in Fingal (Table 3.1). The Colilert-18 test was performed on all water samples which included a 1/10 dilution on saltwater samples to aid bacterial survival. Water samples (30L) were filtered by applying the CapE method (Morris et al., 2016) using 0.45µm filters. The filters were enriched in 100mL of buffered peptone water and incubated at 42°C for 18-24 hours. The enrichment broth was subcultured on to three selective agars; CHROMagar™ mSuperCARBA™ (CHROMagar), *Brilliance*™ ESBL agar (Oxoid) and McConkey agar (Oxoid) with a 5µg ciprofloxacin disc (Oxoid) placed centrally on the agar surface. Growth within the zone of inhibition for the ciprofloxacin disc enabled the isolation of ciprofloxacin resistant isolates. A spread plating technique using 150µL neat and a 1/5 dilution (100 µL) of the enrichment broth were cultured on to the CHROMagar™ mSuperCARBA™ and *Brilliance*™ ESBL agars respectively. Dilutions were performed using sterile buffered peptone water. A swab was lawned on to the MacConkey agar surface with the subsequent addition of a ciprofloxacin disc (5µL). These plates were incubated at 37°C for 18-24 hours.

Table 3.1: Water sampling location details including sampling date and the number of each sample type in each local authority area.

Sample collection site	Sampling date	Local authority area	Number of samples per local authority area
Beach A	26 <sup>th</sup> Nov 2018	Galway city	4 seawater samples
Beach B	06 <sup>th</sup> Dec 2018		
Beach C	06 <sup>th</sup> Dec 2018		
Beach D	08 <sup>th</sup> Jan 2019		
Beach E	22 <sup>nd</sup> Jan 2019	Galway county	6 seawater samples
Beach F	22 <sup>nd</sup> Jan 2019		
Beach G	28 <sup>th</sup> Jan 2019		
Beach H	28 <sup>th</sup> Jan 2019		
Beach I	29 <sup>th</sup> Jan 2019		
Beach J	29 <sup>th</sup> Jan 2019		
Beach K	02 <sup>nd</sup> April 2019	Fingal	6 seawater samples
Beach L	02 <sup>nd</sup> April 2019		
Beach M	02 <sup>nd</sup> April 2019		
Beach N	02 <sup>nd</sup> April 2019		
Beach O	02 <sup>nd</sup> April 2019		
Beach P	03 <sup>rd</sup> April 2019		
Beach Q	13 <sup>th</sup> May 2019		
Beach R	13 <sup>th</sup> May 2019	Cork county	7 seawater samples
Beach S	14 <sup>th</sup> May 2019		
Beach T	15 <sup>th</sup> May 2019		
Beach U	16 <sup>th</sup> May 2019		
Beach V	16 <sup>th</sup> May 2019		
Beach W	16 <sup>th</sup> May 2019		
Total seawater samples: 23			
River A	04 <sup>th</sup> Dec 2018	Galway city	2 river water samples*
River B	04 <sup>th</sup> Dec 2018	Galway county	2 river water samples
River C	21 <sup>st</sup> Jan 2019		
River D	12 <sup>th</sup> Feb 2019	Cork county	1 river water sample
River E	15 <sup>th</sup> May 2019		
Total river water samples: 5			
Lake A	04 <sup>th</sup> Feb 2019	Galway county	2 lake water samples
Lake B	04 <sup>th</sup> Feb 2019	Cork county	1 lake water sample
Lake C	09 <sup>th</sup> May 2019		
Total lake water samples: 3			
Estuary A	04 <sup>th</sup> Dec 2018	Galway city	2 estuarine water samples
Estuary B	04 <sup>th</sup> Dec 2018	Fingal	2 estuarine water samples
Estuary C	03 <sup>rd</sup> April 2019		
Estuary D	03 <sup>rd</sup> April 2019		
Total estuarine water samples: 4			
DWTP influent A	29 <sup>th</sup> April 2019	Fingal	1 DWTP influent sample
DWTP influent B	14 <sup>th</sup> May 2019	Cork county	1 DWTP influent sample
DWTP influent C	22 <sup>nd</sup> July 2019	Galway city	1 DWTP influent sample
DWTP influent D	23 <sup>rd</sup> July 2019	Galway county	1 DWTP influent sample
Total drinking water treatment plant influents: 4			

DWTP: Drinking water treatment plant. \* Two of the river water samples (River A and B) were from different points along one river.

### 3.2.3 Sewage sample collection and processing

A total of 25 sewage samples were collected across the four local authority areas (Table 3.2). Sewage samples were collected by lowering a sterile 250mL glass bottle into a manhole/septic tank. Some nursing homes and hospitals had an open pipe flow system whereas others had an accumulative septic tank (Table 3.2). Sewage samples were directly cultured on CHROMagar™ mSuperCARBA™, *Brilliance*™ ESBL agar and McConkey agar with a ciprofloxacin disc (5µg) using a direct swab plating technique. These plates were incubated at 37°C for 18-24 hours.

*Table 3.2: Features of sewage samples collected including volume collected, sample type and further details about the institution.*

Sample collection site	Sampling date	Local authority area	Number of samples per local authority area	Volume collected	Sample type	Capacity	Treatment type	Antibiotic usage records (DDD per 100 BDU)
Hospital A1	26 <sup>th</sup> Nov 2018	Galway city	4 hospital sewage samples	74mL	Open pipe	220 beds	N/A	68.1 (2018) Combined data only available
Hospital A2	26 <sup>th</sup> Nov 2018			>250mL	Open pipe	220 beds	N/A	
Hospital B1	28 <sup>th</sup> Nov 2018			>250mL	Septic tank	664 beds	N/A	
Hospital B2	20 <sup>th</sup> Feb 2019			112mL	Septic tank	664 beds	N/A	
Hospital C	21 <sup>st</sup> Jan 2019	Galway county	2 hospital sewage samples	116mL	Open pipe	194 beds	N/A	98.5 (2018)
Hospital D	28 <sup>th</sup> Jan 2019			>250mL	Septic tank	50 beds	N/A	Unknown
Hospital E	16 <sup>th</sup> May 2019	Cork city	1 hospital sewage sample	250mL	Open pipe	332 beds	N/A	94.4 (2018)
Hospital F	18 <sup>th</sup> June 2019	Cork county	1 hospital sewage sample	250mL	Overflow bucket	54 beds	N/A	86.5 (2014)
<b>Total hospital sewage samples: 8</b>								
Nursing home A	6 <sup>th</sup> Dec 2018	Galway city	3 nursing home sewage samples	200mL	Open pipe	62 beds	N/A	13.6 (2016)
Nursing home B	09 <sup>th</sup> Jan 2019			60mL	Open pipe	26 beds	N/A	11.1 (2016)
Nursing home C	14 <sup>th</sup> Jan 2019			120mL	Septic tank	60 beds	N/A	Unknown
Nursing home D	21 <sup>st</sup> Jan 2019	Galway county	3 nursing home sewage samples	250mL	Septic tank	44 beds	N/A	12.2 (2016)
Nursing home E	22 <sup>nd</sup> Jan 2019			>250mL	Septic tank	42 beds	N/A	12.5 (2016)
Nursing home F	04 <sup>th</sup> Feb 2019			7mL	Open pipe	51 beds	N/A	Unknown
Nursing home G	04 <sup>th</sup> April 2019	Fingal	1 nursing home sewage sample	180mL	Open pipe	140 beds	N/A	12.4 (2016)
<b>Total nursing home sewage samples: 7</b>								
Airport A	04 <sup>th</sup> April 2019	Fingal	1 airport sewage sample	167mL	Open pipe	N/A	N/A	N/A
Airport B	18 <sup>th</sup> June 2019	Cork county	1 airport sewage sample	180mL	Open pipe	N/A	N/A	N/A
<b>Total airport sewage samples: 2</b>								
WWTP A	29 <sup>th</sup> April 2019	Fingal	2 WWTP samples	250mL (Influent and effluent)	Grab	65,000 PE	Secondary and UV	N/A

Sample collection site	Sampling date	Local authority area	Number of samples per local authority area	Volume collected	Sample type	Capacity	Treatment type	Antibiotic usage records (DDD per 100 BDU)
WWTP B	15 <sup>th</sup> May 2019	Cork county	2 WWTP samples	250mL (Influent and effluent)	Grab	20,500 PE	Secondary using Nereda	N/A
WWTP C	24 <sup>th</sup> July 2019	Galway city	2 WWTP samples	>250mL (Influent and effluent)	Grab	170,000 PE	Secondary	N/A
WWTP D	22 <sup>nd</sup> July 2019	Galway county	2 WWTP samples	>250mL (Influent and effluent)	Grab	13,500 PE	Secondary	N/A
Total wastewater treatment plants sampled: 4 (8 samples in total including influent and effluent)								

WWTP: Wastewater treatment plant, PE: Population equivalent. A total of 8 sewage samples were collected from 6 different hospitals. Sample collection at one hospital was repeated (hospital B1 and B2) and two samples were collected at different manholes on the same hospital grounds (hospital A1 and A2).

### 3.2.4 Identification and antibiotic susceptibility profiling of Enterobacterales

Isolates of interest were identified using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Bruker microflex). Enterobacterales that were identified were chosen for further analysis. Susceptibility testing to a range of antibiotics was carried out in accordance with EUCAST guidelines (EUCAST version 10.0, 2020). These included ampicillin (10µg), cefoxitin (30µg), cefpodoxime (10µg), ceftazidime (10µg), cefotaxime (5µg), ertapenem (10µg), meropenem (10µg), gentamicin (10µg), kanamycin (30µg), streptomycin (10µg), tetracycline (30µg), chloramphenicol (30µg), nalidixic acid (30µg), ciprofloxacin (5µg) and trimethoprim (5µg). In the case where no EUCAST breakpoints were available for nalidixic acid, streptomycin, tetracycline and kanamycin, CLSI breakpoints (CLSI version 30, 2020) were applied. Extended spectrum beta-lactamase (ESBL) production was confirmed using cefpodoxime alone and in combination with clavulanic acid (10µg/1µg). A greater than or equal to 5mm difference between the combination disc versus the cefpodoxime indicates ESBL production which is inhibited by the clavulanic acid. *Klebsiella pneumoniae* strain ATCC 700603 and *E. coli* strain ATCC 25922 were included in each batch to ensure quality control. The *Klebsiella* strain (ATCC 700603) harbours *bla*<sub>SHV-18</sub> on a pKQPS2

plasmid which is used to ensure that ESBL production is successfully being identified by the cefpodoxime and combination disc (Elliott et al., 2016). *E. coli* ATCC 25922 was obtained from a human sample in 1946 and is used to ensure that the zone of inhibition for each antibiotic tested is within its target range (Minogue et al., 2014).

### **3.2.5 Characterisation of beta-lactamase encoding genes using real time PCR**

ESBL producers were tested for the presence of *bla*<sub>CTX-M-group1</sub>, *bla*<sub>CTX-M-group2</sub> and *bla*<sub>CTX-M-group9</sub> by real time PCR (Birkett et al., 2007). Isolates that were intermediate/resistant to ertapenem and/or meropenem were tested for the presence of *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub> and *bla*<sub>NDM</sub> (Manchanda et al., 2011, Swayne et al., 2011). The assay applied for the detection of *bla*<sub>VIM</sub> and *bla*<sub>IMP</sub> derives from a duplex PCR that was designed by the National Carbapenemase-producing Enterobacterales Reference Laboratory Service, Ireland (Unpublished data; NCPERLS). The primer and probe sequences along with the protocol used for each assay is outlined in Supplementary Table B6.

### **3.2.6 Next generation sequencing**

Based on the antibiograms and PCR results, 60 isolates (42 *E. coli* and 18 *Klebsiella* spp.) were selected for whole genome sequencing. The selection process began by removal of possible duplicate isolates by comparing antibiograms of identical species from the same sample. Only *E. coli* and *Klebsiella* species were chosen for the next selection round. This was followed by separating isolates based on carbapenemase production, carbapenem resistance (with negative detection of a carbapenemase gene) and ESBL production. All carbapenemase producing Enterobacterales were chosen for sequencing, followed by a mixture of carbapenem resistant and ESBL producing Enterobacterales. Overall this encompassed 22 isolates from seawater, 11 from hospital sewage, 9 from estuarine waters, 6 from river waters, 4 from lake

waters, 3 from nursing home sewage, 3 from wastewater treatment plant influent and 2 from airport sewage. Sequenced isolates included 12 carbapenem resistant, 40 ESBL and 8 carbapenemase producing Enterobacterales. DNA extraction was carried out using the QIAamp® DNA Mini kit according to the kit protocol. The DNA concentration was determined using the Qubit fluorometer (original version) and the purity was evaluated using DeNovix DS-11 spectrophotometer/fluorometer by recording the 260/280 ratio. Sequencing was carried out using the Illumina NovaSeq 6000 platform in Oxford Genomics Centre. The high-throughput sequencing reads were assembled using Velvet assembly software (Zerbino and Birney, 2008, v1.2.10) using an automated genome assembly pipeline specifically designed for the de novo assembly of bacterial genomes. The pipeline implements VelvetOptimiser software (v2.2.4), developed to assist in the optimisation of each genome assembly based on sampling multiple K-mer lengths and searching for an optimum coverage cut off value (<https://github.com/tseemann/VelvetOptimiser>). All odd numbered K-mers were sampled from 71 to 151. The species of each assembled genome was confirmed using ribosomal MLST (Jolley et al., 2012). The assembled *E. coli* genomes were uploaded to the *Escherichia* database on PubMLST (<https://pubmlst.org/organisms/escherichia-spp/>), while the *Klebsiella* genomes are hosted on the public BIGSdb *Klebsiella* Pasteur MLST database (<http://bigsdb.pasteur.fr/klebsiella>). Sequencing reads were also uploaded to the European Nucleotide Archive (<https://www.ebi.ac.uk/ena>), project accession PRJEB21277. Individual isolate identifiers for each database are listed in Supplementary Table B5.

Bioinformatics analysis was carried out using the Center for Genomic Epidemiology (CGE) pipelines (ResFinder v3.2, VirulenceFinder v2.0, MLST v2.0 and PlasmidFinder v2.1) as well as the *Escherichia* database on PubMLST (Jolley et al., 2018). The CGE pipelines were used to identify acquired antibiotic

resistance genes and plasmids, as well as *E. coli* virulence genes. *Klebsiella* virulence genes were identified using the BIGSdb *Klebsiella* Pasteur MLST database v1.23.4. The 'GrapeTree' tool (Zhou et al., 2018) was used to visualise minimum spanning trees through core genome MLST comparisons. The genome comparator tool on the *Escherichia* database on PubMLST was used to perform direct allele comparisons at 2513 loci for *E. coli*. Similarly, the genome comparator tool on the BIGSdb *Klebsiella* Pasteur MLST database performed allele comparisons at 694 loci for *Klebsiella* isolates (Bialek-Davenet et al., 2014). In addition, sequenced isolates carrying *bla*<sub>OXA-48</sub> were selected for pOXA-48 plasmid comparisons (Brehony et al., 2019). This tool compared the pOXA-48 plasmid at 71 different loci to assess similarities between plasmids.

### 3.3 Results

A total of 39 water samples and 25 sewage samples were collected across four local authority regions in Ireland between November 2018 and July 2019. Overall, 211 Enterobacterales were isolated and characterised which included *E. coli* (n=145), *Klebsiella* spp. (n=28), *Enterobacter* spp. (n=18), *Citrobacter* spp. (n=12) and others (n=8). The breakdown of individual bacterial species identified across different sample types is provided in Supplementary Table B4. A total of 139 isolates were obtained from waters while 72 were isolated from sewage.

#### 3.3.1 *E. coli* quantification (MPN per 100mL)

The Colilert-18 test was carried out on all 39 water samples in order to determine the mean probable number (MPN) of *E. coli* per 100mL. The levels of *E. coli* for all water types combined with the approximate distances from sampling areas to point discharges are presented in Figure 3.1. This encompassed all water samples with the exception of drinking water treatment plant influents. Water samples included 5 cold spot locations with no nearby point discharges, 12 samples in close proximity to 1 point discharge, 13 samples

close to 2 point discharges and 3 sampling areas in close proximity to 3 or more discharges (Figure 3.1).

Seawaters were the most commonly collected sample type (n=23). Overall, 19/23 (83%) seawater samples harboured *E. coli* levels less than 250 MPN per 100mL which would infer excellent water quality according to the EU bathing water monitoring criteria (Directive 2006/7/EC). Beach F revealed the highest detectable *E. coli* levels amongst all seawater samples collected at 842 MPN per 100mL. A raw sewage discharge was located approximately 0.1km from the sampling point at Beach F (Figure 3.1). In comparison, estuarine waters revealed much higher levels of *E. coli*, with just 1/4 (25%) samples harbouring less than 250 (MPN per 100mL). The remaining three estuarine samples displayed *E. coli* levels between 520 to >2419.6 (MPN per 100mL). Estuary B revealed the single highest detectable levels of *E. coli* (>2419.6 MPN *E. coli* per 100mL) across all water sample types. This estuarine sample was collected during December and is located approximately 0.1km from a secondary urban wastewater discharge.

Freshwater samples harboured relatively low levels of *E. coli* with just 1/5 (20%) rivers and 0/3 lakes exhibiting levels greater than 250 *E. coli* (MPN per 100mL). Inland waters have a higher cut-off point of <500MPN per 100mL to infer excellent water quality status, implying that all inland waters sampled would be designated as excellent bathing water quality. Drinking water treatment plant influents which also originated from freshwater sources harboured *E. coli* levels varying between 16 to 115.3 MPN per 100mL (Figure 3.1).

In terms of cold spot locations, 4 out of 5 (60%) samples exhibited *E. coli* levels less than 50 MPN per 100mL. Estuary C was the only cold spot location that would not receive excellent water quality status with 520 *E. coli* (MPN per 100mL).

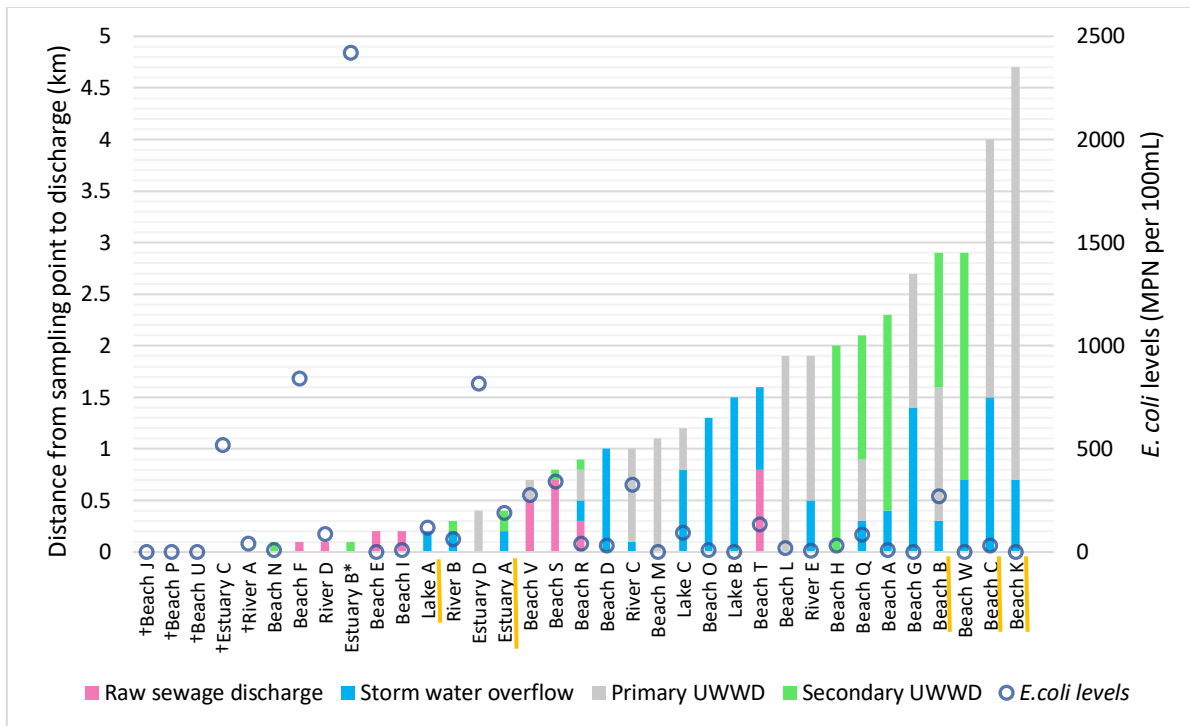


Figure 3.1: Stacked bar chart displaying distance from sampling points to discharge(s) (km) overlaid with *E. coli* detection levels (MPN per 100mL). The length of each coloured stacked bar is indicative of the distance from the sampling point to a contaminating point discharge. In the case of two or more coloured stacked bars, the length of each should be interpreted separately. †The five cold spot locations include Beach J, P, and U, estuary C and river A as they have no point discharges in close proximity. CPE positive sites are underlined with a yellow line. \*Estuary B indicates *E. coli* levels >2419.6. Supplementary Table B1 displays the raw data for this figure. UWWD = Urban wastewater discharge.

### 3.3.2 Antibiotic susceptibility testing

Two heatmaps were created using R displaying the antibiograms of individual isolates. Figure 3.2(A) encompasses isolates from Galway city and county, while Figure 3.2(B) comprises of isolates obtained from two geographically distinct local authority areas, Fingal and Cork county. The order in which the isolates were presented was determined by the similarities between the antibiotic susceptibility profiles. Analysis of the antibiograms revealed high levels of resistance to ampicillin, cefpodoxime, ciprofloxacin, nalidixic acid and to a lesser extent cefotaxime and ceftazidime, across both heatmaps. These resistances were present in both sewage and water isolates, evident from the stacked coloured bars indicative of sample types. The majority of isolates displayed

susceptibility to the carbapenem class of antibiotics, including ertapenem and meropenem. The antibiogram profiles were similar across isolates originating from both urban (Galway city and Fingal) and rural (Galway county and Cork county) local authority areas.

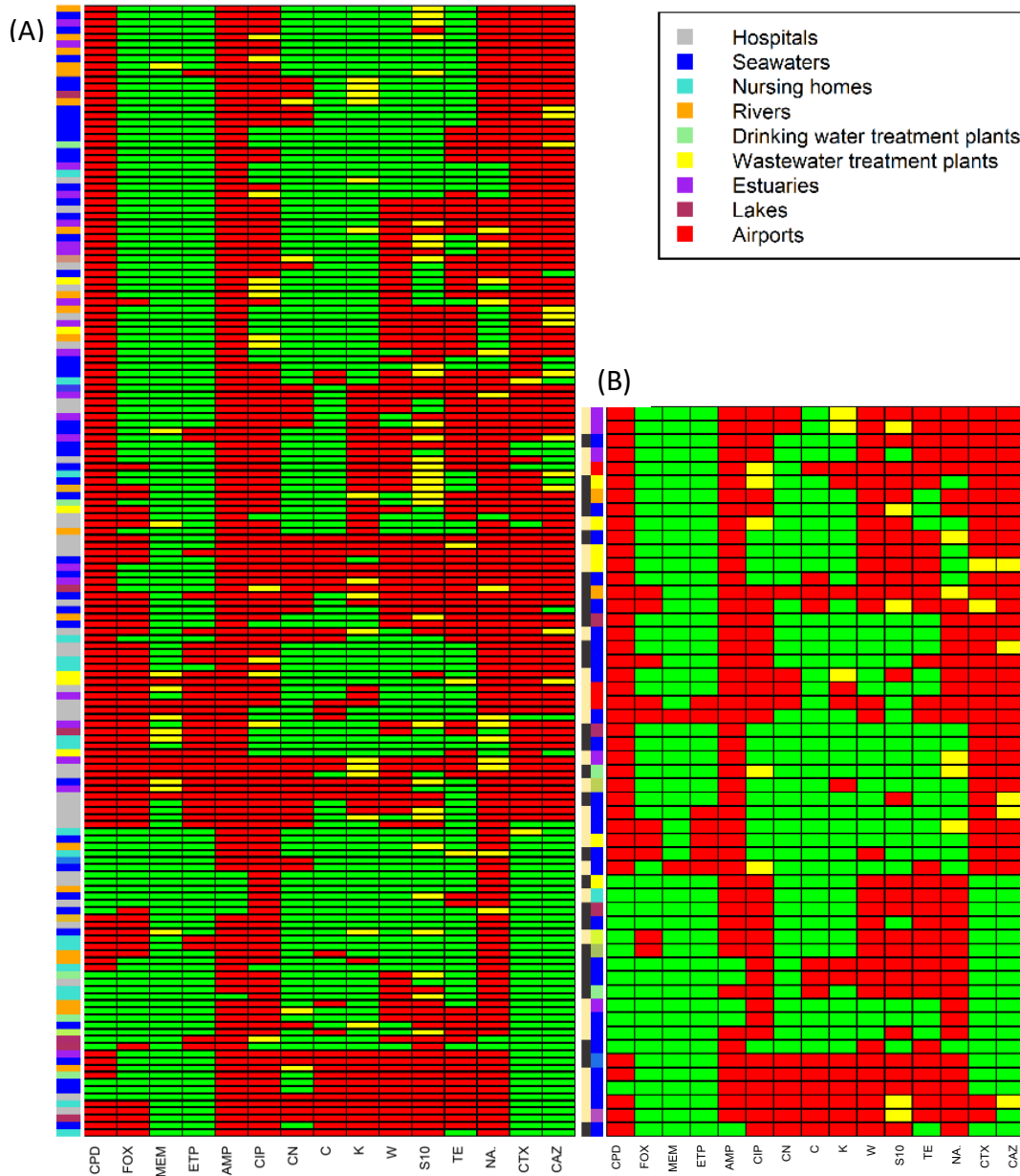


Figure 3.2: Heatmaps encompassing antibiogram profiles of isolates from different sample types. Red indicates resistance, yellow indicates an intermediate phenotype and green indicates susceptibility. Heatmap (A) includes isolates from Galway city and county, while (B) encompasses bacteria from Fingal and Cork. The second coloured bar

in heatmap (B) differentiates Fingal (cream) and Cork (black) isolates. CPD = Cefpodoxime, FOX = Cefoxitin, MEM = Meropenem, ETP = Ertapenem, AMP = Ampicillin, CIP = Ciprofloxacin, CN = Gentamicin, C = Chloramphenicol, K = Kanamycin, W = Trimethoprim, S = Streptomycin, TE = Tetracycline, NA = Naladixic acid, CTX = Cefotaxime, CAZ = Ceftazidime.

The antibiogram results were grouped into two categories; sewage and water isolates. The percentage of isolates that were resistant to each antibiotic from both groups (sewage 72 isolates and water 139 isolates) were calculated and directly compared (Figure 3.3). The sewage isolate collection displayed a higher percentage of isolates displaying a resistant phenotype to the majority of the antibiotics (10/15; 66.7%), when compared to isolates of water origin. However, there was little variation between the two groups, with less than 10% difference for 12/15 of the antibiotics. Cefoxitin (27% difference), ertapenem (20% difference) and gentamicin (13% difference) revealed the largest variation across these two groups, with all three displaying higher percentage resistances in sewage isolates. Of the four antibiotics that demonstrated higher levels of resistance in water isolates, ciprofloxacin (9% higher than sewage isolates) and tetracycline (5% higher than sewage isolates) revealed the largest differences between the two groups.

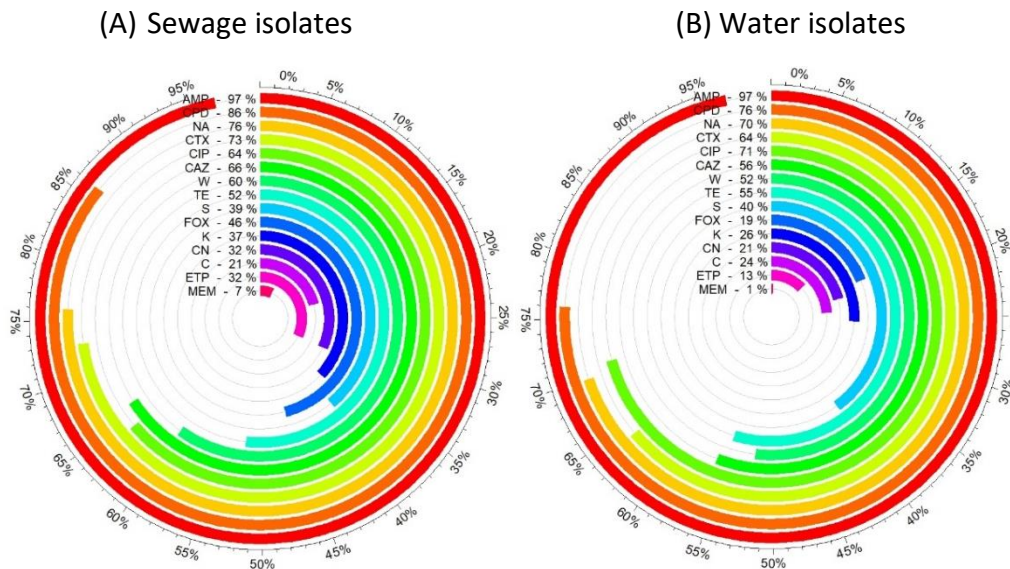


Figure 3.3: Percentage of isolates displaying resistance to a panel of 15 antibiotics for (A) sewage versus (B) water isolates. A total of 72 isolates were examined from sewage

sources, whereas 139 isolates were obtained from water bodies. AMP = Ampicillin, CPD = Cefpodoxime, NA = Naladixic acid, CTX = Cefotaxime, CIP = Ciprofloxacin, CAZ = Ceftazidime, W = Trimethoprim, TE = Tetracycline, S = Streptomycin, FOX = Cefoxitin, K = Kanamycin, CN = Gentamicin, C = Chloramphenicol, ETP = Ertapenem, MEM = Meropenem.

### 3.3.3 ESBL producing Enterobacterales

A total of 105 isolates were selected for real time PCR based upon the antibiogram results. Genotypic based screening of these isolates revealed the widespread detection of *bla*<sub>CTX-M</sub> groups in 99/105 (94%) Enterobacterales (Table 3.3). Positive *bla*<sub>CTX-M</sub> detection was identified in 77 isolates obtained from water samples and 22 isolates from sewage. The most commonly detected ESBL variants included *bla*<sub>CTX-M-group1</sub> (n=83) followed by *bla*<sub>CTX-M-group9</sub> (n=16). A total of 6 isolates that were confirmed phenotypically as ESBL producers tested negative for the *bla*<sub>CTX-M</sub> genes. In terms of the 'cold spot' locations, 2 out of 5 locations tested positive for the presence of one or more isolate harbouring *bla*<sub>CTX-M</sub> genes. These locations included an urban estuary and river which harboured *E. coli* levels of 520 and 40.8 (MPN per 100mL) respectively.

### 3.3.4 Carbapenem non-susceptible Enterobacterales

In total, 42 carbapenem resistant Enterobacterales were identified, 20% of the total Enterobacterales isolated. Eleven of the carbapenem resistant isolates tested positive for carbapenemase genes using real time PCR (Table 3.3). Carbapenemase encoding genes were identified in 3 seawater isolates, 4 hospital sewage isolates, 2 wastewater treatment plant (WWTP) influent isolates, 1 estuary and 1 lake water isolate. These included *bla*<sub>OXA-48</sub> (4 *Klebsiella pneumoniae*, 1 *E. coli* and 1 *Enterobacter kobei*), *bla*<sub>NDM</sub> (2 *E. coli*), *bla*<sub>KPC</sub> (1 *Klebsiella pneumoniae* and 1 *Citrobacter freundii*) and *bla*<sub>IMP</sub> (1 *Citrobacter youngae*). Six out of 11 CPE were resistant to both ertapenem and meropenem, whereas the remaining five displayed ertapenem resistance and an intermediate meropenem phenotype. Each CPE positive water body had a discharging source in close proximity, highlighted by the yellow underline in

Figure 3.1. These discharges ranged from storm water overflows to primary or secondary urban wastewater discharges. None of the 5 ‘cold spot’ locations tested positive for carbapenem resistance or carbapenemase production.

Examination of CPE detection in conjunction with the colilert results revealed that CPE were identified in water bodies that harboured *E. coli* levels ranging between 0 to 269 MPN per 100mL. According to the EU bathing water directive 2006 (Directive 2006/7/EC), these *E. coli* levels would lead to excellent/good water body classification.

Table 3.3: Summary table of ESBL and carbapenemase genes detected using real time PCR across different sample types.

Gene detected:	Seawaters (n=72)	Rivers (n=24)	Lakes (n=9)	Estuaries (n=27)	Hospitals (n=37)	Nursing homes (n=20)	Airports (n=3)	WWTP (n=14)	DWTP (n=9)	Total
<i>bla</i> <sub>CTX-M</sub> -group1	27	13	3	19	9	3	3	5	1	<b>83</b>
<i>bla</i> <sub>CTX-M</sub> -group2	0	0	0	0	0	0	0	0	0	<b>0</b>
<i>bla</i> <sub>CTX-M</sub> -group9	9	1	1	2	1	1	0	0	1	<b>16</b>
<i>bla</i> <sub>OXA-48</sub>	2	0	0	1	1	0	0	2	0	<b>6</b>
<i>bla</i> <sub>NDM</sub>	1	0	1	0	0	0	0	0	0	<b>2</b>
<i>bla</i> <sub>KPC</sub>	0	0	0	0	2	0	0	0	0	<b>2</b>
<i>bla</i> <sub>IMP</sub>	0	0	0	0	1	0	0	0	0	<b>1</b>
<i>bla</i> <sub>VIM</sub>	0	0	0	0	0	0	0	0	0	<b>0</b>

WWTP – Wastewater treatment plant, DWTP – Drinking water treatment plant. The number in brackets denotes the number of isolates tested from each sample type.

### 3.3.5 Whole genome sequencing analysis

A total of 60 isolates (42 *E. coli* and 18 *Klebsiella* spp.) were selected for whole genome sequencing based upon species identification, the phenotypic presence of carbapenem resistance and the genomic detection of ESBL and/or carbapenemase encoding genes. Sequenced isolates were assessed for multi locus sequence types (MLST), plasmid replicons, virulence genes and antibiotic resistance genes outlined in Table 3.4. A total of 15 *E. coli* isolates were identified as sequence type ST131. This was followed by ST38 (n=8), ST10 (n=4), ST1193 (n=2) and ST5584 (n=2). There were 11 additional sequence types detected that were not repeated among sequenced *E. coli*. The *Klebsiella*

sequence types varied more substantially across isolates with just two repeated types, ST405 (n=3) and ST11 (n=2). The remaining 13 *Klebsiella* sequence types were detected in just one isolate. Plasmid replicons were detected in 56/60 (93%) isolates, highlighting the potential of widespread dissemination of antibiotic resistance genes. The most commonly detected plasmid replicons included IncFIB (n=45), IncFII (n=39) and IncFIA (n=29) across *E. coli* and *Klebsiella* spp. from sewage and water sources. Many different virulence genes were identified in *E. coli* isolates. The virulence gene *celb* was unique to *E. coli* sewage isolates. In contrast, the virulence genes *vat*, *cma*, *ironN*, *cnf1*, *ireA*, and *ccl* were primarily found in *E. coli* originating from water bodies. The *Klebsiella* virulence genes identified revealed significant overlap between sewage and aquatic isolates. The main differences included the detection of *clb* gene variants solely in one hospital isolate and the detection of *iutA* in one isolate originating from lake water.

Sequencing data was also examined for the presence of different antibiotic resistance genes, with a particular focus on beta-lactamase gene detection (Table 3.4). The most commonly detected beta-lactamase gene was *bla*<sub>CTX-M</sub> (n=48) which could be further divided into *bla*<sub>CTX-M-15</sub> (n=34), *bla*<sub>CTX-M-14</sub> (n=7), *bla*<sub>CTX-M-27</sub> (n=4), *bla*<sub>CTX-M-1</sub> (n=1), *bla*<sub>CTX-M-24</sub> (n=1) and *bla*<sub>CTX-M-55</sub> (n=1). These results are in agreement with the PCR outcomes where *bla*<sub>CTX-M-group1</sub> and *bla*<sub>CTX-M-group9</sub> were the most prevalent variants, with no *bla*<sub>CTX-M-group2</sub> detections. Other commonly detected beta-lactamase genes included *bla*<sub>TEM-1</sub> (n=31), *bla*<sub>OXA-1</sub> (n=19) and *bla*<sub>SHV</sub> variants (n=16). The *bla*<sub>SHV</sub> genes included *bla*<sub>SHV-11</sub> (n=5), *bla*<sub>SHV-1</sub> (n=5), *bla*<sub>SHV-76</sub> (n=3), *bla*<sub>SHV-60</sub> (n=1), *bla*<sub>SHV-27</sub> (n=1) and *bla*<sub>SHV-62-like</sub> (n=1). Three different CPE genes were identified among sequenced isolates with positive detection of *bla*<sub>OXA-48</sub> in 4 isolates, *bla*<sub>NDM-5</sub> in 2 *E. coli* and *bla*<sub>KPC-2</sub> in 1 *Klebsiella pneumoniae*, also in agreement with detection from the PCR.

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Table 3.4: Summary of sequencing data including the number of *E. coli* and *Klebsiella* spp., sequence types, plasmid replicons, virulence genes and beta-lactamase genes detected.

Sample type	Species	Number of isolates	ST type	Plasmid replicons	Virulence genes	Beta-lactamase genes
Seawater	<i>E. coli</i>	18	131 (6), 38(3), 1193(2), 10(2), 3018, (1), 69(1), 540(1), 5584 (1), 167 (1)	IncFIB (14), IncFII (11), Col156 (12), IncFIA (9), Col(BSS12) (3), IncQ1 (2), IncB/O/K/Z (1), IncI-1 (1), IncFIC (1), IncX3 (1), IncI (1), IncX1 (1), IncM1 (1), Col(MG828) (1)	senB (11), iha (9), sat (8), gad (7), iss (7), eilA (6), air (6), capU (2), vat (2), nfaE (2), cma (1), iroN (1), astA (1), cnf1 (1), ireA (1), lpfA (1)	<i>bla</i> TEM-1 (10), <i>bla</i> CTX-M-15 (7), <i>bla</i> CTX-M-14 (4), <i>bla</i> OXA-1 (3), <i>bla</i> CTX-M-27 (2), <i>bla</i> NDM-5 (1), <i>bla</i> CTX-M-1 (1), <i>bla</i> CMY-42 (1), <i>bla</i> DHA-1 (1), <i>bla</i> CTX-M-24 (1)
	<i>Klebsiella</i> spp.	4	11(1), 17(1), 45(1), 405 (1)	IncFIB (4), IncFII (3), IncI (1), Col440I (1), IncFIA (1)	mrkA (4), mrkB (4), mrkC (4), mrkD (4), mrkF (4), mekH (4), mrkI (4), mrkJ (4), fyuA (3), irp1 (3), irp2 (3), ybtA (3), ybtE (3), ybtP (3), ybtQ (3), ybtS (3), ybtT (3), ybtU (3), ybtX (3), kfuA (1), kfuB (1), kfuC (1), kvgA (1), kvgS (1), mceA (1), mceB (1), mceC (1), mceD (1), mceE (1), mceG (1), mceH (1), mceI (1), mceJ (1)	<i>bla</i> CTX-M-15 (4), <i>bla</i> TEM-1 (3), <i>bla</i> OXA-1 (3), <i>bla</i> SHV-11 (2), <i>bla</i> OXA-48 (1), <i>bla</i> SHV-1 (1), <i>bla</i> SHV-76 (1)
River	<i>E. coli</i>	5	131 (1), 38 (1), 5584 (1), unknown (1), 1722 (1)	IncFIB (4), IncFII (3), IncFIA (2), Col156 (2), IncI-1 (1), ColRNAI (1), Col(BSS12) (1)	gad (4), sat (3), eilA (3), iha (2), senB (2), air (2), iss (1), cnf1 (1), nfaE (1), lpfA (1), ccl (1)	<i>bla</i> CTX-M-15 (5), <i>bla</i> TEM-1 (2), <i>bla</i> OXA-1 (1)
	<i>Klebsiella</i> spp.	1	1236 (1)	-	mrkA (1), mrkB (1), mrkC (1), mrkD (1), mrkF (1), mekH (1), mrkI (1), mrkJ (1), fyuA (1), irp1 (1), irp2 (1), ybtA (1), ybtE (1), ybtP (1), ybtQ (1), ybtS (1), ybtT (1), ybtU (1), ybtX (1)	<i>bla</i> CTX-M-15 (1), <i>bla</i> OXA-1 (1), <i>bla</i> SHV-1 (1)
Estuary	<i>E. coli</i>	5	131 (3), 38 (1), 10 (1)	IncFIB (4), IncFIA (3), IncFII (3), Col156 (2), Col(BSS12) (2), IncX1 (1), IncX4 (1), IncI2 (1), IncQ1 (1), IncFIC (1), IncI-1 (1), IncI2 (1), ColVC (1)	iss (4), sat (4), iha (3), nfaE (3), gad (3), senB (2), cma (1), iroN (1), air (1), eilA (1)	<i>bla</i> CTX-M-15 (4), <i>bla</i> TEM-1 (3), <i>bla</i> OXA-1 (2), <i>bla</i> CTX-M-14 (1)
	<i>Klebsiella</i> spp.	4	405 (1), 11 (1), 8 (1), 236 (1)	IncFIB (4), IncFII (3), IncFIA (2), IncI (1), IncR (1), Col440I (1)	mrkA (4), mrkB (4), mrkC (4), mrkD (4), mrkF (4), mekH (4), mrkI (4), mrkJ (4), fyuA (2), irp1 (2), irp2 (2), ybtA (2), ybtE (2), ybtP (2), ybtQ (2), ybtS (2), ybtT (2), ybtU (2), ybtX (2), kvgA (1), kvgS (1), mceA (1), mceB (1), mcsC (1), mceD (1), mceE (1), mceG (1), mceH (1), mceI (1), mceJ (1), kfuA (1), kfuB (1), kfuC (1)	<i>bla</i> CTX-M-15 (3), <i>bla</i> OXA-48 (1), <i>bla</i> OXA-1 (2), <i>bla</i> TEM-1 (2), <i>bla</i> DHA-1 (2), <i>bla</i> SHV-76 (1), <i>bla</i> SHV-11 (1), <i>bla</i> SHV-1-like (1), <i>bla</i> SHV-60 (1)
Lake	<i>E. coli</i>	2	131 (1), 11188 (1)	IncFIA (2), FII (2)	iss (3), air (2), senB (2), eilA (2), gad (2), iha (1), sat (1), lpfA (1)	<i>bla</i> CTX-M-27 (1), <i>bla</i> NDM-5 (1)
	<i>Klebsiella</i> spp.	2	111 (1), unknown (1)	IncFIB (2), IncN (2), IncFII (1), IncFIA (1), Col440I (1)	mrkA (2), mrkB (2), mrkC (2), mrkD (2), mrkF (2), mekH (2), mrkI (2), mrkJ (2), fyuA (1), irp1 (1), irp2 (1), kfuA (1), kfuB (1), kfuC (1), ybtA (1), ybtE (1), ybtP (1), ybtQ (1), ybtS (1), ybtT (1), ybtU (1), ybtX (1), iutA (1)	<i>bla</i> CTX-M-15 (1), <i>bla</i> SHV-11 (1), <i>bla</i> TEM-1 (1), <i>bla</i> TEM-1D-like (1), <i>bla</i> OKP-B-3-like (1)

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Sample type	Species	Number of isolates	ST type	Plasmid replicons	Virulence genes	Beta-lactamase genes
Hospital	<i>E. coli</i>	6	131 (2), 38 (1), 90 (1), 10 (1), 617 (1)	IncFIA (5), IncFII (5), IncFIB (5), Col156 (2), Inc1-I (1), IncN (1), IncHI2 (1), IncHI2A (1), Col (MG828) (1), p0111 (1)	iss (4), gad (3), iha (2), nfaE (2), sat (2), astA (2), air (1), eilA (1), senB (1), celB (1), capU (1)	<i>bla</i> CTX-M-15 (4), <i>bla</i> OXA-1 (3), <i>bla</i> TEM-1 (1), <i>bla</i> CTX-M-27 (1)
	<i>Klebsiella</i> spp.	5	5 (1), 101 (1), 258 (1), 405 (1), 1243 (1)	IncFIB (4), IncFII (4), IncFIA (1), IncN (1), IncM1 (1), IncR(1)	mrkA (5), mrkB (5), mrkD (5), mrkF (5), mrkH (5), mrkI (5), mrkJ (5), mrkC (4), kfuA (3), kfuB (3), kfuC (3), fyuA (3), irp1 (3), irp2 (3), ybtA (3), ybtE (3), ybtP (3), ybtQ (3), ybtS (3), ybtT (3), ybtU (3), ybtX (3), kvgA (1), kvgS (1), mceA (1), mceB (1), mceC (1), mceD (1), mceE (1), mceG (1), mceH (1), mceI (1), mceJ (1), clbA (1), clbB (1), clbC (1), clbD (1), clbE (1), clbF (1), clbG (1), clbH (1), clbI (1), clbJ (1), clbK (1), clbL (1), clbM (1), clbN (1), clbO (1), clbQ (1), clbR (1)	<i>bla</i> TEM-1 (4), <i>bla</i> OXA-1 (2), <i>bla</i> OXA-9-like (2), <i>bla</i> CTX-M-15 (1), <i>bla</i> KPC-2 (1), <i>bla</i> OXA-48 (1), <i>bla</i> SHV-1-like (1), <i>bla</i> SHV-76 (1), <i>bla</i> SHV-11 (1), <i>bla</i> CTX-M-14 (1), <i>bla</i> OXA-9 (1), <i>bla</i> SHV-1 (1), <i>bla</i> CTX-M-15-like (1), <i>bla</i> SHV-62-like (1)
Nursing home	<i>E. coli</i>	3	131 (2), 38 (1)	IncFIA (2), IncFII (2)	iss (3), gad (2), iha (2), sat (2), eilA (1), nfaE (1), air (1)	<i>bla</i> TEM-1 (2), <i>bla</i> OXA-1 (2), <i>bla</i> CTX-M-14-like (1)
Wastewater treatment plant	<i>E. coli</i>	1	38 (1)	IncY (1)	air (1), eilA (1), gad (1), iss (1)	<i>bla</i> CTX-M-15 (1), <i>bla</i> TEM-1 (1)
	<i>Klebsiella</i> spp.	2	967 (1), 1563 (1)	IncFIB (2), IncL (1)	mrkA (2), mrkB (2), mrkC (2), mrkD (2), mrkF (2), mekH (2), mrkI (2), mrkJ (2), kfuA (1), kfuB (1), kfuC (1)	<i>bla</i> LEN17 (1), <i>bla</i> OXA-48 (1), <i>bla</i> CTX-M-15 (1), <i>bla</i> SHV-27 (1)
Airport	<i>E. coli</i>	2	648 (1), 2614 (1)	IncFIB (2), IncFII (2), IncY (1), IncFIA (1), IncX1 (1), ColpVC (1), p0111 (1)	air (2), eilA (2), gad (2), lpfA (1)	<i>bla</i> CTX-M-15 (1), <i>bla</i> TEM-1 (1), <i>bla</i> CTX-M-55 (1)

*Carbapenemase genes are highlighted in blue. The bolded numbers in brackets denotes the number of isolates in which the characteristic was detected.*

Furthermore, different types of antibiotic resistance genes detected using ResFinder are outlined in Figure 3.4. The most commonly detected genes included *sul1* and *tet(A)*, identified in a total of 28 sequenced isolates. These were closely followed by positive detection of *strA*, *strB*, *sul2* and *mph(A)* in 26 isolates. Overall, 49 isolates (82%) harboured one or more aminoglycoside resistance gene. This was followed by sulfonamide (n=45; 75%), trimethoprim (n=44; 73%) and tetracycline (n=38; 63%) resistance encoding genes. The antibiotic class which had the lowest number of isolates harbouring one or more resistance genes against was fosfomycin (n=20; 33%).

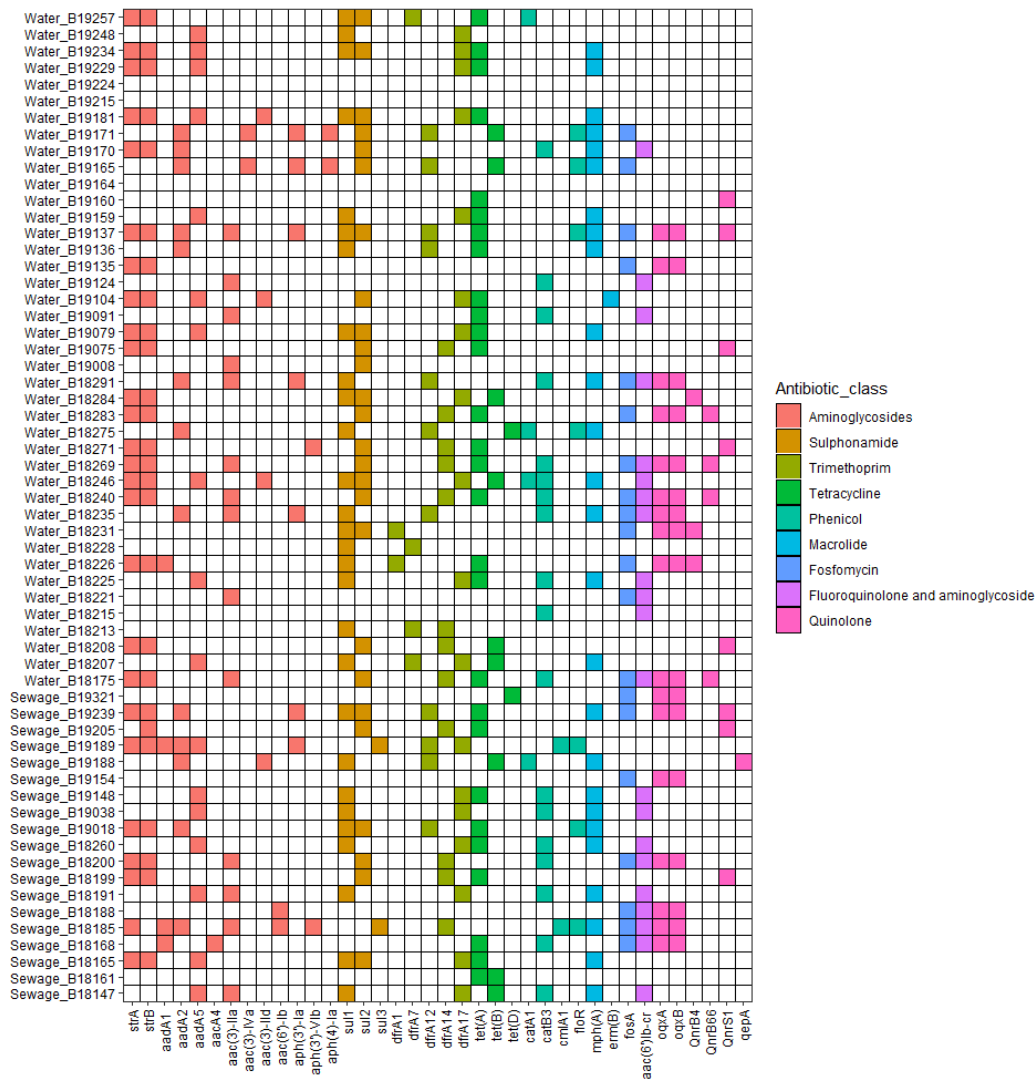


Figure 3.4: Antibiotic resistance gene detection among the 60 sequenced isolates. The colours correspond to the resistance conferred by the gene to a certain class of antibiotic.

The GrapeTree tool on the BIGSdb *E. coli* database was used to visualise core genome multi locus sequence type (cgMLST) comparisons across 42 sequenced *E. coli* isolates (Figure 3.5), irrespective of local authority area (LAA). This analysis revealed three pairs of highly similar isolates. The first pair of isolates (B19165, B19171) were identified at beach M and estuary C in Fingal LAA. These sites were located 3.4km apart and sample collection took place one day apart. The whole genome comparator tool on the BIGSdb database was used to perform pairwise allele alignments at 2513 loci between the submitted

genomes. This comparison revealed 99.8% allele similarity due to 4 missing loci in one of the isolates when compared with the other. The second pair of isolates (B18213, B18228) originated from river B and the receiving estuarine water (estuary A) in Galway city. These sites were located 1.08 kilometers apart and samples were collected on the same day. A minor discrepancy of two missing loci in one isolate that were present in the other indicated 99.9% cgMLST similarity. Interestingly, the third pair of isolates (B19160, B19075) were obtained from two geographically distinct water bodies (beach K and river C) over 135km apart. The whole genome comparator tool revealed 21 missing loci in one isolate compared to the other (99.2% similarity).

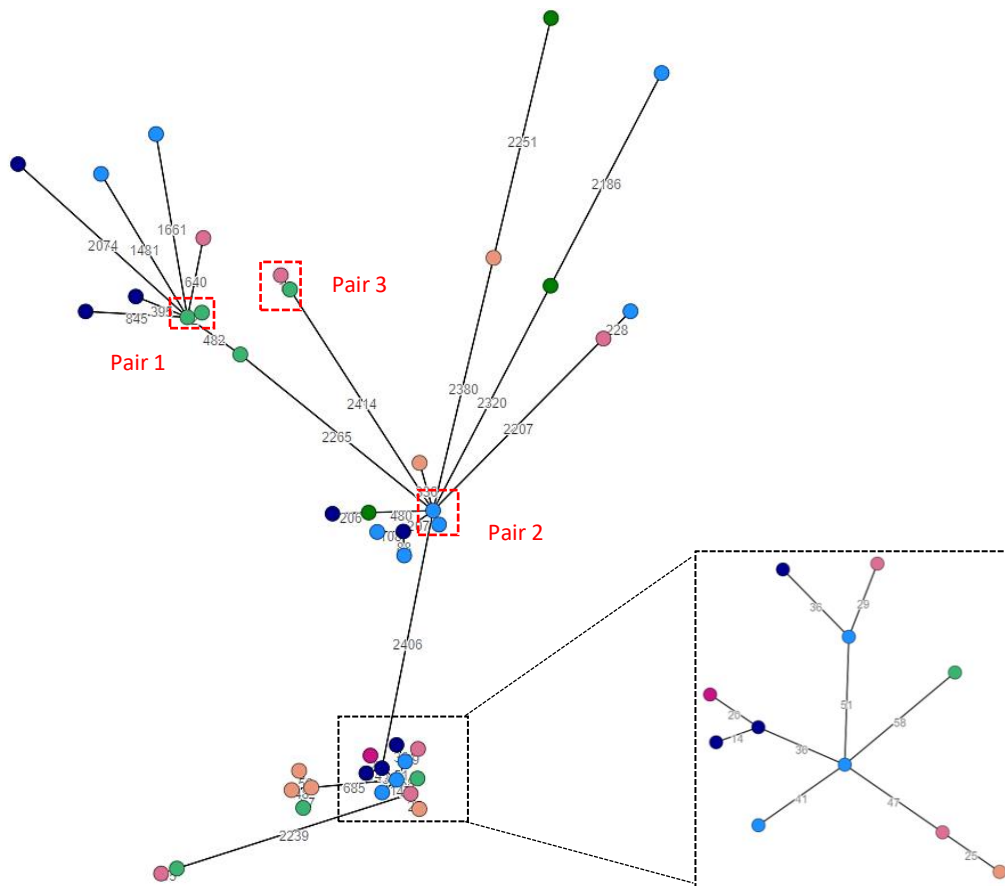


Figure 3.5: Core genome multi locus sequence type minimum spanning tree based on allele differences between 2513 loci in *E. coli* isolates. The circles are colour coded to indicate the local authority region: Galway city = blue; Galway county = pink; Fingal = green; Cork = orange. The darker colour indicates isolates from sewage origin while the

*lighter colours indicate water origin. The numbers between the nodes represents the number of locus allele differences between isolates.*

The GrapeTree tool was also applied to sequenced *Klebsiella* isolates (n=18) in Figure 3.6 using the *Klebsiella* BIGSdb database. This revealed the presence of two clusters of highly similar bacterial isolates. The first comprised of two isolates, B18291 (Beach C) and B18235 (Estuary A). These sites were located 2.8km apart. Whole genome comparisons using 694 core genome MLST loci uncovered just two loci allele variability between the two, revealing 99.7% similarity. In addition, pOXA-48 plasmid comparisons at 71 loci indicated identical plasmids (100% similarity) within these two isolates. Both isolates identified as sequence type ST11.

The second cluster comprised of two water isolates (B18240; Estuary B and B18175; Beach A) as well as one hospital sewage isolate (B18200; Hospital B). All three isolates are sequence type ST405. Whole genome comparisons revealed just 6-8 loci displaying variability between the sewage versus water isolates. This indicated 99.1% similarity between the *Klebsiella* obtained from the hospital sewage and estuary B, which are located 1.3km apart. Similarly, beach A is located just 1.6km from the hospital and 900m from the wastewater treatment plant receiving the hospital effluent. These isolates displayed 98.8% similarity.

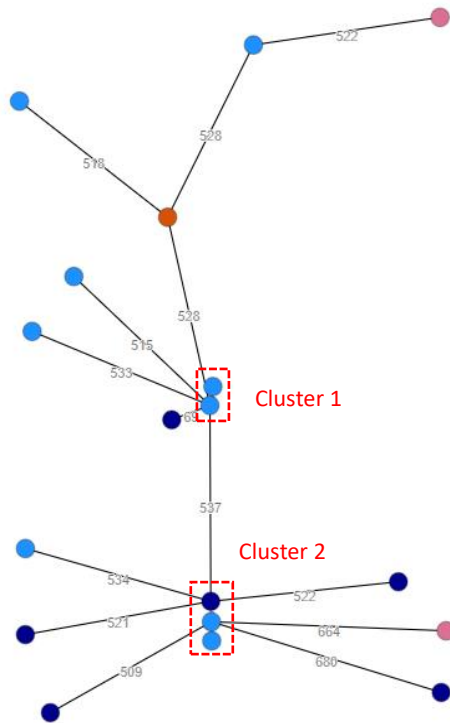


Figure 3.6: Core genome multi locus sequence type minimum spanning tree based on allele differences between 694 loci in *Klebsiella* isolates. The circles are colour coded to indicate the local authority region: Galway city = blue; Galway county = pink; Cork = orange. The darker colour indicates isolates from sewage origin while the lighter colours indicate water origin. The numbers between the nodes represents the number of locus allele differences between isolates.

### 3.4 Discussion

Aquatic environments worldwide have proven to be vast reservoirs of clinically significant antibiotic resistant bacteria (Mahon et al., 2019, Caltagirone et al., 2017, Fernando et al., 2016). Anthropogenic sources including hospital sewage (Cahill et al., 2019) and contaminating discharges such as wastewater treatment plant effluent (Pazda et al., 2019) have been well characterised for the role they play in the environmental dissemination of antibiotic resistance. Many studies that investigate links between sewage sources and water bodies do so by examining waters upstream and downstream of a single contaminating discharge (Harnisz & Korzeniewska 2018; Lekunberri et al., 2017). This approach can help determine the impact of the discharge on the presence of different

types of antibiotic resistant bacteria in circulation. However, these studies are often limited to one source over a relatively small area. In a recent review by Fouz et al. (2020), the role of sewage sources in the environmental dissemination of antimicrobial resistance was examined. This review identified 63 studies of interest, with just one examining different environmental waters and sewage sources on a national scale (Soge et al., 2009). However, this study focused primarily on antibiotic resistance in *Clostridium perfringens*. The lack of studies that analyse water and sewage samples on a national scale makes this a rare approach to investigating the environmental propagation of antibiotic resistance.

#### **3.4.1 Comparison of *E. coli* levels to distance from discharging sources**

As previously mentioned, seawaters and estuaries featured as harbouring the highest levels of *E. coli*, with freshwaters including rivers, lakes and drinking water treatment plant influents displaying much lower levels (Figure 3.1). Most of the waters that harboured high levels of *E. coli* had one or more discharges in close proximity. An exception to this was estuary C which had no known discharges nearby, yet displayed *E. coli* levels of 520 MPN per 100mL. The exceptionally high level of *E. coli* detected at this cold spot location prompted further investigation into this site. It was discovered that this estuary previously received storm overflow discharges from a wastewater pumping station prior to the introduction of a new wastewater treatment plant in this area in 2012. Additionally, sample collection took place in a shallow region of the estuary that was densely populated with swans, which could also potentially contribute to the high levels of *E. coli* via faecal matter. The remaining water bodies with no receiving discharges within a 1km radius (6 seawaters and 1 lake) displayed relatively low levels of *E. coli* (0-31 MPN per 100mL). However, the highly fluctuating nature of *E. coli* levels within large water bodies over a small time period limits the assumptions that can be drawn from a single sample collected

at one point in time (Wyer et al., 2018). This is one of the major limitations of current EU bathing water monitoring criteria which requires just one sample of water to be collected monthly across the bathing water season (Directive 2006/7/EC). To strengthen the evaluation of water suitability for public health, the colilert test could be modified to include the addition of antibiotic powders (Galvin et al., 2010). This would provide rapid results of the number of *E. coli* that are resistant to clinically significant antibiotics.

Although the colilert results revealed low *E. coli* levels at some sampling points with contaminating discharges in close proximity, this is likely to vary substantially especially in bad weather conditions in the instances of water bodies in close vicinity to storm water overflow points. Although the primary focus of this paper was centered upon contaminating discharges due to the large number of sites, there are other factors to be considered when examining the full picture. These include the salinity of the water body, as higher levels comparable to those found in seawater can decrease bacterial survival (Rozen & Belkin, 2001). In addition, the concentration of suspended solids in the water can influence the UV disinfection capabilities of the sun, (Palazón et al., 2017) along with wildlife and agricultural land use nearby all impacting upon *E. coli* levels. Heavy rainfall is another important factor that can promote slurry runoff from agricultural areas into nearby water bodies and activate storm water overflows. Comparison of rainfall levels in the 24 hours prior to sample collection presented no obvious relationship with the faecal indicator levels detected in each water sample (Supplementary Figure B1). However, the majority of sampling occasions had less than 3mm of rain (25/39) in the 24 hours prior to collection.

### **3.4.2 Antibiotic resistance detection**

Detection of viable multi-drug resistant bacteria was evident across all sample types. High numbers of isolates were resistant to beta-lactam antibiotics

including ampicillin, cefpodoxime and cefotaxime as well as nalidixic acid and ciprofloxacin (Figure 3.2). Sequencing analysis unveiled a large proportion of isolates harbouring resistance genes conferring these phenotypes. These included beta-lactamase (60/60), quinolone (15 water, 10 sewage; *oqxA*, *oqxB*, *qepA* and *Qnr* variants) and fluoroquinolone (9 sewage, 12 water; *aac(6')Ib-cr*) resistance genes (Figure 3.4). The fosfomycin resistance gene (*fosA*) was detected in a third of the sequenced isolates (7 sewage, 13 water). The majority of these isolates were *Klebsiella* spp. (n=18), with just two *E. coli* isolates testing positive for the gene. The widespread detection of this resistance gene in the environment is of significant clinical concern due to its combined use with colistin to treat carbapenem resistant infections (Benzerara et al., 2017). Previous detection of *fosA2* was reported in *Enterobacter cloacae* from a Canadian river (Xu et al., 2011).

ESBL encoding genes (*bla*<sub>CTX-M</sub>) were detected in 79/139 (56.8%) water isolates and 24/72 (33.3%) sewage isolates using real time PCR. These figures are in contrast with other recent studies such as Jørgensen et al. (2017b) who detected ESBL *E. coli* in 40% of recreational waters and 100% of wastewater samples. However, that paper had a relatively small sample size and distribution of sampling locations including sample collection from 4 different beaches and one wastewater treatment plant (influent) over 5 days. One possible reason for the lower percentage of isolates harbouring *bla*<sub>CTX-M</sub> genes in sewage versus water samples in this study may be caused by the presence of a carbapenemase gene masking the ESBL production phenotypically. This was the case for the sequenced sewage isolate B18185 which harboured *bla*<sub>CTX-M-14b</sub> and *bla*<sub>OXA-9</sub> along with *bla*<sub>OXA-48</sub>. Similarly, the sewage isolate B18188 harboured *bla*<sub>OXA-9</sub> and *bla*<sub>KPC-2</sub>. Both isolates displayed no indications of ESBL production phenotypically, although both harboured one or more ESBL encoding genes based on sequencing data. In addition, only the *bla*<sub>CTX-M</sub> genes were identified using PCR while the sequencing results revealed the presence of further ESBL

genes including *bla*<sub>SHV-27</sub>. This highlights the benefits of whole genome sequencing in obtaining the complete resistance gene profile.

### 3.4.3 Antibiotic resistance comparison between sewage and water isolates

Overall, sewage samples displayed higher percentages of isolates displaying resistance to the majority of antibiotics (10/15; 66.7%) (Figure 3.3). Tetracycline was an exception to this due to the higher percentage of water (55%) versus sewage (52%) isolates exhibiting resistance to tetracycline. This may be attributable to the use of tetracyclines in veterinary medicine, ranked as the most commonly sold antibiotic (39.5%) for veterinary use in 2018, according to the Health Products Regulatory Authority (HPRA, 2018). Interestingly, water samples also revealed a higher percentage of ciprofloxacin resistant isolates (71%) in comparison to sewage samples (64%). However, the fluoroquinolone class featured as one of the lowest antibiotic classes (0.8%) sold for veterinary purposes in Ireland in 2018. The *Qnr* gene, which is largely disseminated in the environment due to plasmid carriage, is believed to derive from a waterborne bacteria known as *Shewanella algae* (Poirel et al., 2012b). Amongst the sequenced isolates, the *Qnr* genes (*QnrS1* and *QnrB4*) were largely confined to waterborne isolates with the exceptions of one *Klebsiella* (B19239) and one *E. coli* isolate (B19199) from sewage sources. The most prevalent genes encoding fluoroquinolone resistance included the *oqxA* and *oqxB* genes. These efflux pump encoding genes are often identified in samples of animal origin, potentially due to the fact that they also confer resistance to olaquinox, an animal growth promoter (Hansen et al., 2005). Another potential factor contributing to higher levels of ciprofloxacin resistance in water isolates include its detection in municipal wastewater and its ability to persist for long periods of time in the environment (Kumar et al., 2019), imposing selective pressures on aquatic bacteria.

Sewage isolates displayed a higher percentage of resistant isolates to ceftazidime (27% difference) and ertapenem (20% difference), in comparison to aquatic isolates. The cephalosporin class (1.3%) was ranked as one of the lowest for veterinary use in Ireland (HPRA, 2018), suggesting less exposure of environmental bacteria to this antibiotic from agricultural runoff. In addition, the carbapenem class of antibiotics are highly conserved for severe multi-drug resistant clinical infections. These factors may be attributable to the large variation between the two groups.

#### **3.4.4 Virulence gene detection**

Comparison of virulence gene detection across all isolates revealed the presence of some genes among aquatic bacteria that were absent across sewage isolates. These included *vat*, *cma*, *iroN*, *cnf1*, *ireA*, and *ccl* in *E. coli*, and *iutA* in *Klebsiella* isolates. These genes encode toxins (*vat* and *cnf1*), iron acquisition elements (*iroN* and *ireA*) and colicins (*cma*), which are a type of bacteriocin produced by *E. coli* that is toxic to other *E. coli* strains (Feng et al., 2017). Similarly, the detection of *iutA* in a *Klebsiella* isolate from lake water encoding aerobactin receptor for iron uptake (Sobieszcańska, 2008) was unique to this aquatic bacteria. The presence of these genes highlights the pathogenic potential of these environmental isolates. Many of these virulence genes including *vat*, *cma*, *iroN*, *cnf1* and *ireA* were also detected by Blyton & Gordon. (2017) in *E. coli* isolated from chlorinated drinking water.

In contrast, just two virulence genes identified were unique to sewage isolates. This included *celb* detection in one *E. coli* isolated from sewage which is also a colicin needed for competition and survival (Feng et al., 2017). The colibactin cluster genes *clbA-R* (n=18) were also detected in *Klebsiella* from one hospital sewage source (Turton et al., 2018).

### 3.4.5 *E. coli* core genome MLST comparisons and sequence types detected

Among *E. coli* isolates, there were many repeated sequence types (STs) across aquatic and sewage sources (Table 3.4). The most common types included ST131 (n=15), ST38 (n=8) and ST10 (n=4). These STs also match those most commonly detected in the OXA-48 clinical isolates collection analysed by Brehony et al. (2019). Of the sequenced *E. coli* isolates, identification of three pairs of highly similar isolates was determined using core genome multi locus sequence typing (cgMLST) comparisons. However, all three pairs of isolates originated from water bodies. One of the isolate pairs was obtained from sites (beach M and estuary C) located 3.4km apart with collection taking place on two separate days. These isolates were both identified as sequence type ST10. This raises an interesting possibility that multi-drug resistant bacteria can replicate, disseminate and persist over large areas in the aquatic environment due to water movement. The second pair of *E. coli* isolates further highlights this possibility as the samples were collected on the same day from a fast-flowing river (B) and its receiving estuary (A) approximately 1km apart. These isolates were both ST38 which is commonly identified in clinical isolates (Brehony et al., 2019). The third pair of *E. coli* were located over 135km apart (beach K and river C) but displayed 99.2% cgMLST similarity. These water bodies do not directly connect to one another indicating the natural persistence of *E. coli* ST5584 in the aquatic environment rather than its dissemination from a particular anthropogenic source. Antibiotic resistance gene detection revealed the presence of *bla*<sub>CTX-M-15</sub>, *bla*<sub>TEM-1B</sub>, *tet(A)* and *QnrS1* common to both ST5584 *E. coli*. The *E. coli* isolate from river water harboured additional resistance genes including *strA*, *strB*, *sul2* and *dfrA14* in comparison to the *E. coli* from seawater. Both isolates also harboured identical virulence genes including *air*, *eilA* and *gad*. The concept of natural persistence of certain bacterial strains in the aquatic environment is relatively unchallenged due to the lack of studies that collect

water samples across an entire country. This finding identifies the need for further studies that compare the bacterial composition of water bodies on a larger scale.

#### **3.4.6 *Klebsiella* core genome MLST comparisons and sequence types detected**

ST11 (n=2) and ST405 (n=3) were the only sequence types repeated across *Klebsiella* isolates. Although the two ST11 isolates (B18235 & B18291) both originated from water bodies, a recent study by Brehony et al. (2019) identified 4 clinical *Klebsiella* isolates characterised as ST11, OXA-48 producers obtained from the Irish national CPE reference laboratory collection. Interestingly, the *Klebsiella* ST11 isolates obtained from different water bodies (beach C and estuary A) both harboured *bla*<sub>OXA-48</sub> with identical pOXA-48 plasmids. These sites are located 2.8km apart indicating widespread dissemination of the pOXA-48 plasmid in the aquatic environment. Sequence type ST11 is an important multi-drug resistant clone globally, which has previously been linked to *bla*<sub>OXA-48</sub> carriage in clinical isolates from Greece (Voulgari et al., 2013), Taiwan (Lu et al., 2018b) and Spain (Oteo et al., 2013). Furthermore, Beach C is the same location as the bathing water site examined by Mahon et al. (2019), who also previously reported OXA-48-like producing *K.pneumoniae* at this location. However, the sequence types are inconsistent, with ST101 reported by Mahon et al. (2019).

The second repeated *Klebsiella* sequence type, ST405 (n=3), comprised of one isolate from hospital sewage (B18200), and two isolates from a nearby seawater (B18175) and estuary (B18240). As mentioned previously, whole genome comparisons using core genome MLST revealed similarities between the isolates, with just 6-8 loci displaying variability between the sewage versus water isolates. The core genome MLST similarities indicates that these isolates are closely related, possibly belonging to a single sub-lineage. All three isolates harboured identical virulence gene profiles, plasmid detection as well as highly

similar antibiotic resistance genes. The two water isolates harboured an extra tetracycline (*tet(A)*) and fluoroquinolone resistance gene (*QnrB66*) which may be attributable to adaptation to their particular environment. This sequence type (ST405) is commonly reported to harbour *bla*<sub>OXA-48</sub> in clinical isolates (Miro et al., 2020, López-Camacho et al., 2018), although none of the ST405 isolates in this collection harboured this carbapenemase gene. A previous study by Mahon et al. (2017) identified indistinguishable bacterial isolates (NDM producing *E. coli*) from recreational waters, sewage and a clinical isolate using pulse field gel electrophoresis. The results of that study further demonstrates the link between wastewater and the dissemination of antibiotic resistant organisms to the natural aquatic environment. The detection of NDM producing *K. pneumoniae* from Beach A in that paper, was the same sampling location as Beach E listed in this study. Although no CPE were detected at beach E during this study.

#### **3.4.7 Carbapenemase gene detection among *E. coli* and *Klebsiella* isolates in comparison to previous studies.**

Three of the *E. coli* isolates in the current study harboured carbapenemase genes. These included two isolates from seawater (B18271; ST540 and B19159; ST167) and one from lake water (B19136; ST11188). One of the seawater isolates (B18271) harboured *bla*<sub>OXA-48</sub> whereas the other two tested positive for *bla*<sub>NDM-5</sub>. Two of these sequence types (ST540, ST167) have been identified harbouring these carbapenemase genes previously in clinical isolates from patients or hospital sewage samples (Gijón et al., 2020; Zou et al., 2020). The sequence type ST167 has also been recently identified harbouring *bla*<sub>NDM</sub> variants in river water in Switzerland and China (Bleichenbacher et al., 2020; Cheng et al., 2019). Bleichenbacher et al. (2020) identified the *bla*<sub>NDM-5</sub> gene on an IncFIA plasmid. This plasmid was also identified in isolate B19159 in this study, however long read sequencing would be required to establish the

position of the carbapenemase gene in this isolate. This is the first published report of *bla*<sub>NDM-5</sub> in Irish environmental waters.

The *Klebsiella* sequence types identified carrying carbapenemase encoding genes have been previously identified in clinical isolates worldwide. A prime example is the *Klebsiella pneumoniae* ST258 which is commonly linked with *bla*<sub>KPC-2</sub> carriage from clinical isolates across the world including New York (Chen et al., 2013), Brazil (Nicoletti et al., 2012), South Korea (Hong et al., 2013) and Belgium (Bogaerts et al., 2010). This isolate (B18188) was obtained from a hospital sewage sample. Two *Klebsiella* sequence types harboured *bla*<sub>OXA-48</sub>, including ST101 (B18185) from hospital sewage and ST1563 (B19321) from wastewater treatment plant influent. *Klebsiella* ST1563 has previously been reported harbouring *mcr-1* from pig rectal swabs in Portugal (Kieffer et al., 2017). However, to the authors knowledge this is the first report of *bla*<sub>OXA-48</sub> identification in a *Klebsiella* sequence type ST1563.

#### **3.4.8 Public health impacts**

At present little is known about the public health risks associated with human exposure to waters that serve as a reservoir of antibiotic resistant bacteria. A recent review by Amarasiri et al. (2020) identified a limited number of studies that examined different water exposure pathways including water sports and recreational water activities. This review highlighted work carried out by Leonard et al. (2018), which established higher colonisation rates of *bla*<sub>CTX-M</sub> bearing *E. coli* in surfers (6.3%) versus non-surfers (1.5%). Similarly, O'Flaherty et al. (2019) created a quantitative risk assessment model to identify the consequences of human exposure to antibiotic resistant *E. coli* in a bathing water site located in close vicinity to a wastewater treatment plant. Using a modelling approach, it was established that swimmers who ingest a sip of water could be exposed to between 0 and 72.94 CFU of antibiotic resistant *E. coli* per sip (21mL). An investigation by Coleman et al. (2012) also demonstrated higher

colonisation rates of antibiotic resistant *E. coli* in humans that consume water supplies that are contaminated with multi-drug resistant *E. coli*. This risk was quantified to be 1.26 times higher for consumers of contaminated versus uncontaminated water. Although further research is necessary to fully elucidate the risks to human health, establishment of a monitoring programme for antibiotic resistance in bathing waters is warranted.

#### **3.4.9 Natural occurrence of antibiotic resistance in the absence of anthropogenic pressures**

In recent years, evidence has emerged to suggest the ubiquitous occurrence of antibiotic resistance genes in the aquatic environment in the absence of contaminating discharges (Hooban et al., 2020). Therefore, five cold spot locations were chosen for assessment in this study; estuary C, beach J, beach P, beach U and river A. As mentioned previously, estuary C harboured an unusually high level of *E. coli* that surpassed levels identified in areas receiving discharges in close proximity. ESBL producers were also identified at this site, one of which was chosen for sequencing (B19171). This revealed the presence of multiple different antibiotic resistance genes including *bla*<sub>CTX-M-14</sub>. Similarly, river A also harboured ESBL producers, two of which were selected for sequencing (B18207, B18208). These isolates both tested positive for *bla*<sub>CTX-M-15</sub> along with many other resistance genes against different classes of antibiotics. The origins of the *bla*<sub>CTX-M</sub> genes have been traced back to *Kluyvera* species, which is an environmental organism (Cantón et al., 2012a). Although the three other cold spot locations tested negative for ESBLs and all five tested negative for carbapenem resistance, the isolates did display other forms of resistance. These included phenotypic resistance to penicillins, second generation cephalosporins, fluoroquinolones, aminoglycosides, tetracycline, trimethoprim and chloramphenicol. These findings demonstrate the presence of clinically significant antibiotic resistant bacteria circulating among water bodies receiving minimal anthropogenic contamination. This may be as a result of natural

evolution or due to widespread dissemination of resistance genes from bacteria in polluted regions using mobile genetic elements. The detection of plasmids across 40/43 (93.0%) water isolates further highlights this possibility of widespread dissemination.

### 3.5 Conclusion

In conclusion, the findings of this study demonstrate the significant number of multi-drug resistant bacteria circulating in wastewater and aquatic environments throughout Ireland. The widespread detection of bacterial isolates harbouring a multitude of antibiotic resistance genes highlights the limitations of current EU bathing water monitoring criteria. In particular, the detection of carbapenemase producing Enterobacterales in waters that would be classified as good/excellent status ascertains the importance of further characterisation of aquatic bacteria. Inclusion of antibiotic powder in the colilert test would overcome this limitation while still providing rapid results on the levels of antibiotic resistant *E. coli*. The detection of ESBL producing Enterobacterales in cold spot locations, as well as isolates displaying phenotypic resistance to different antibiotic classes highlights the natural resistome present and circulating in environments with minimal anthropogenic influence. Consideration of the natural resistome should be adapted by environmental scientists by incorporation of 'cold spot' locations when assessing anthropogenically impacted waters. The nexus between multi-drug resistant pathogens in wastewater and the natural aquatic environment was established using genotypic analysis that identified highly similar *Klebsiella* isolates originating from hospital sewage and two nearby waters. Furthermore, identification of genetically similar isolates from distant water bodies demonstrates the possibility of widespread propagation and persistence of certain strains of aquatic bacterial isolates. Additional national scale studies are needed to establish the persistence of strains in the aquatic environment.

## **Chapter 4: A Longitudinal Survey of Antibiotic-Resistant Enterobacterales in the Irish Environment, 2019-2020**

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## Abstract

The natural environment represents a complex reservoir of antibiotic resistant bacteria as a consequence of different wastewater discharges including anthropogenic and agricultural. Therefore, the aim of this study was to examine sewage and waters across Ireland for the presence of antibiotic resistant Enterobacterales. Samples were collected from the West, East and South of Ireland. Two periods of sampling took place between July 2019 and November 2020, during which 118 water (30L) and 36 sewage samples (200mL) were collected. Waters were filtered using the CapE method, followed by enrichment and culturing. Sewage samples were directly cultured on selective agars. Isolates were identified by MALDI-TOF and antibiotic susceptibility testing was performed in accordance with EUCAST criteria. Selected isolates were examined for *bla*<sub>CTX-M</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>KPC</sub> by real time PCR and whole genome sequencing (n=146). A total of 419 Enterobacterales (348 water, 71 sewage) were isolated from all samples. Hospital sewage isolates displayed the highest percentage resistance to many beta-lactam and aminoglycoside antibiotics. Extended spectrum beta-lactamase producers were identified in 78% of water and 50% of sewage samples. One or more carbapenemase producing Enterobacterales were identified at 23 individual sampling sites (18 water, 5 sewage). This included the detection of *bla*<sub>OXA-48</sub> (n=18), *bla*<sub>NDM</sub> (n=14), *bla*<sub>KPC</sub> (n=4) and *bla*<sub>OXA-484</sub> (n=1). All NDM-producing isolates harboured the *ble-MLB* bleomycin resistance gene. Commonly detected sequence types included *Klebsiella* ST323, ST17, and ST405 as well as *E. coli* ST131, ST38 and ST10. Core genome MLST comparisons detected identical *E. coli* isolates from wastewater treatment plant (WWTP) influent and nursing home sewage, and the surrounding waters. Similarly, one *Klebsiella pneumoniae* isolated from WWTP influent and the surrounding estuarine water were identical. These results highlight the need for regular monitoring of the aquatic environment for the

presence of antibiotic resistant organisms to adequately inform public health policies.

## 4.1 Introduction

Antibiotics are essential in the treatment of a range of infections responsible for morbidity and mortality in humans and animals. The introduction of more potent and structurally advanced antibiotics into clinical practice is continuously met with the evolution of new mechanisms of antibiotic resistance (Ventola, 2015). This continuous cycle makes it difficult to ensure successful treatment options are available in cases of multi-drug resistant infections. Mechanisms of antibiotic resistance vary widely from bacterial structural antibiotic target alterations, to the production of enzymes that directly cleave the antibiotic prior to bacterial cell entry (Reygaert, 2018). Often a culmination of mechanisms reduces bacterial susceptibility to a range of antibiotics. A significant factor that increases the difficulty in containing the spread of antibiotic resistance is the ability of bacteria to disseminate mobile genetic elements (e.g. plasmids), often harbouring a multitude of resistance genes (Partridge et al., 2018).

Different bacterial species and resistance to distinct antibiotic classes have varying priority rankings. According to the World Health Organisation (WHO), *Enterobacteriaceae* that are carbapenem resistant or extended spectrum beta-lactamase (ESBL) producing are of critical priority for the development of new and effective antibiotics (WHO, 2017). These ranked above vancomycin resistant *Enterococcus faecium* as well as methicillin and vancomycin resistant *Staphylococcus aureus*. On a European scale, an assessment was carried out on data from 2018 evaluating the incidence and distribution of carbapenemase producing *Enterobacteriaceae* (CPE), inclusive of infections and carriage (European Centre for Disease Prevention and Control, 2019). This report categorised the Republic of Ireland as having inter-regional spread, which is stage 4 on a 5-point scale. A further 11 European countries also reported inter-

regional spread including Spain, France and Croatia. A total of four European countries were classed as endemic (stage 5) including Italy, Greece, Turkey, and Malta.

Many reports in relation to antibiotic resistance reference clinical data, but there are large knowledge gaps in relation to the environment as a reservoir and potential transmission route of antibiotic resistance. Therefore, there is an increasing need to apply a One Health approach to the problem of antibiotic resistance. The One Health approach recognises the nexus between humans, animals, and the natural environment (Hernando-Amado et al., 2019). This was highlighted within objective 2 of the WHO's Global Action Plan on Antimicrobial Resistance, which stated the need for a deeper understanding of how antibiotic resistance circulates 'between humans and animals and through food, water and the environment' (WHO, 2015). This was echoed in Ireland's national action plan published in 2017 (Department of Health, 2017). Under the current EU bathing water monitoring criteria, waters are monitored during the bathing water season for the levels of intestinal enterococci and *E. coli* with no further bacterial characterisation (Directive 2006/7/EC). Therefore, consistent monitoring data relating to environmental antibiotic resistance is lacking, although it has been highlighted as a potential transmission route of multi-drug resistant pathogens to humans via recreational exposure (O'Flaherty et al., 2019, Leonard et al., 2018).

Environmental antibiotic resistance is largely a culmination of the natural resistome, present as a result of antibiotic production by environmental organisms (Hooban et al., 2020), and resistance introduced via wastewater from human and animal sources (He et al., 2020, Pazda et al., 2019). Accordingly, this study was carried out subsequent to the initial point prevalence survey (Hooban et al., 2021) in order to further evaluate the presence of antibiotic resistant Enterobacterales circulating in sewage and aquatic environments in Ireland. Water bodies receiving multiple anthropogenic discharges as well as those free

from them were included for comparison. The results of this study were evaluated against previous findings to examine the effects of seasonality on the presence of resistant organisms. The nexus between anthropogenic wastewater and the natural aquatic environment was established by comparing isolates obtained from both at a genetic level.

## **4.2 Materials and Methods**

### **4.2.1 Sample collection sites**

Two periods of sample collection were carried out across four local authority areas including Galway City, Galway County, Fingal and Cork County Council as previously described by Hooban et al. (2021). The first sampling period, known as longitudinal survey 1 (LS1), was carried out between August 2019 and January 2020, while the second period (longitudinal survey 2 (LS2)) began in February 2020 and was completed by November 2020. The culmination of both sampling campaigns resulted in the collection and processing of 118 water samples and 36 sewage samples (Supplementary Table C1). Sample collection points were chosen based on the findings of the point prevalence survey (Hooban et al., 2021). A total of 28 water and 7 sewage sampling points were retained across both sampling periods from the initial point prevalence survey. Additional sampling points were added in areas of interest in which carbapenem resistant, ESBL or carbapenemase producing Enterobacterales were previously detected in water bodies. This comprised of an additional 35 water sampling points, which included 27 locations that were sampled twice and 8 areas that were sampled once. The naming designation for these new locations were linked to previous collection sites through the addition of a numerical value following the sample name. For example, estuary C1 was an additional sample collected in close proximity to estuary C. Water samples included seawaters, lakes, rivers, estuaries, untreated water supplying drinking water treatment plants and an

estuarine lagoon. Sewage samples encompassed hospitals, nursing homes, airports and the influent and effluent of wastewater treatment plants.

#### **4.2.2 Collection and processing of water and sewage samples**

A total of 30L was collected for each water sample. Faecal indicator organisms were quantified using the Colilert-18 (IDEXX) test, for which seawater samples were diluted 1/10 to decrease the salt concentration, prolonging bacterial survival. The processing of water samples was carried out using filtration and enrichment as previously described (Hooban et al., 2021; Morris et al., 2016). Following enrichment, samples were cultured on CHROMagar™ mSuperCARBA™ (CHROMagar), Brilliance™ ESBL agar (Oxoid) and McConkey agar (Oxoid) with a 5µg ciprofloxacin disc (Oxoid) to screen for fluoroquinolone resistance. Due to the higher concentration of bacteria in sewage, direct swab plating was employed for wastewater samples on to the same agars. Bacterial species identification was achieved using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Bruker microflex).

#### **4.2.3 Antibiotic susceptibility testing**

Antibiotic susceptibility testing was performed on all Enterobacterales identified from sewage and water samples according to EUCAST guidelines (EUCAST version 11.0, 2021). Antibiotics included ampicillin (10µg), cefoxitin (30µg), cefpodoxime (10µg), cefpodoxime/clavulanic acid (10µg/1µg), ceftazidime (10µg), cefotaxime (5µg), ertapenem (10µg), meropenem (10µg), gentamicin (10µg), kanamycin (30µg), streptomycin (10µg), tetracycline (30µg), chloramphenicol (30µg), nalidixic acid (30µg), ciprofloxacin (5µg) and trimethoprim (5µg). EUCAST breakpoint were used to interpret the results with the exception of nalidixic acid, streptomycin, tetracycline and kanamycin for which CLSI breakpoints were applied. *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used in each batch as quality controls.

Antibiogram profiling was followed by real time PCR. Phenotypic ESBL producers were screened for the presence of ESBL genes including *bla*<sub>CTX-M-group1</sub>, *bla*<sub>CTX-M-group2</sub> and *bla*<sub>CTX-M-group9</sub> (Birkett et al., 2007). Real time PCR was also performed on isolates that displayed reduced susceptibility to the carbapenem antibiotics. This included testing for the presence of *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub> and *bla*<sub>NDM</sub> (Manchanda et al., 2011, Swayne et al., 2011). Additionally, the presence of *bla*<sub>VIM</sub> and *bla*<sub>IMP</sub> were also assessed using a duplex assay from the National Carbapenemase-producing *Enterobacteriales* Reference Laboratory Service, Ireland (Unpublished data). All primer and probe sequences, as well as the cycling conditions used are outlined in Supplementary Table B6. Potential duplicate isolates were removed at this point by comparing the antibiograms and PCR results of bacteria of the same species from the same sample.

#### 4.2.4 Whole genome sequencing

A total of 146 individual bacterial isolates were selected for whole genome sequencing. This encompassed 114 isolates originating from waters and 32 isolates from sewage sources. All carbapenemase producing *Enterobacteriales* were selected for sequencing irrespective of sample origin (n=37). The selection basis for the remaining 109 isolates included the creation of four heatmaps displaying antibiotic susceptibility data for all isolates from each of the four local authority areas. Using cluster analysis, isolates of the same species and highly similar antibiograms from waters and sewage sources were flagged. These isolates were separated out into carbapenem non-susceptible (resistant and intermediate) isolates, phenotypic ESBL producers and those without either type of beta-lactam resistance. This enabled the selection of further isolates of interest for sequencing including ESBL producers (87 *bla*<sub>CTX-M</sub> positive, 3 phenotypic ESBL producers but *bla*<sub>CTX-M</sub> negative) and carbapenem resistant (n=16) *Enterobacteriales*, in addition to a small subset of isolates that did not display ESBL production or reduced susceptibility to carbapenems (n=3).

DNA extraction was carried out using the EZ1 Advanced XL machine with the EZ1 DNA tissue kit (Qiagen) on 48 isolates. The majority (n=35) included a pre-enrichment, which involved the addition of 2-3 colonies from a pure culture to 2mL of buffered peptone water, incubated shaking at 37°C overnight. This enrichment broth was subsequently centrifuged at 7,500rpm for 5 minutes. The pellet was resuspended in buffer G2 and the protocol was followed according to the manufacturer's instructions. These isolates were sequenced using the Illumina NovaSeq 6000 in Oxfords Genomics Centre (PE150). The remaining 13 isolates had no pre-enrichment step and were sequenced using the Illumina MiSeq (PE300). Due to supply issues, DNA extraction was also carried out using the QIAamp DNA Mini Kit (Qiagen) for the majority of isolates (n=98). This process also included a pre-enrichment step as described previously. The concentrated pellet was resuspended in buffer ATL and the protocol was followed according to the manufacturer's instructions. These isolates were also sequenced using the Illumina NovaSeq 6000 platform (PE150).

The bacterial genomes were assembled and analysed bioinformatically using the tormes pipeline (v1.2.1) (Quijada et al., 2019). Trimmomatic (v0.39) was used to remove any sequencing adapters and filter the reads per quality (Leading: 25, Trailing: 25) and size (minlen: 125 for PE150, minlen: 225 for PE300) (Bolger et al., 2014). The average cleaned data size for the bacterial strains analysed on the Illumina Novaseq 6000 (n=133) was  $1.33 \pm 0.17$  Gb. The average cleaned data size for the bacterial strains analysed on the Illumina MiSeq (n=13) was  $0.31 \pm 0.06$  Gb. Bacterial genome assembly was carried out using SPAdes (v3.15.3) (Prjibelski et al., 2020). The assembled draft genomes were of an average depth of  $254 \pm 34x$  and  $59 \pm 11x$  for the bacterial strains sequenced on the Illumina Novaseq 6000 and the Miseq respectively. Taxonomic identification was calculated based on k-mers, using Kraken2 (v2.1.1) with the MiniKraken2 database (v.1) (Wood & Salzberg 2014). In the case of isolates with a low percentage identity at species level, the 16S gene was

isolated using ContEst16S (Lee et al., 2017) and identity was confirmed using BLAST Nucleotide with the default parameters. The assembled genomes were assigned multilocus sequenced types (MLST) using the tool mlst (v2.19.0) (Seemann, <https://github.com/tseemann/mlst>), based on scanning the contigs against the PubMLST database (Jolley et al., 2018). The assembled contigs were scanned for the presence of antibiotic resistance genes using the tool Abricate (v0.9.9) against the CARD (McArthur et al., 2013), ResFinder (Zankari et al., 2012) and ARG-ANNOT (Gupta et al., 2014) databases. Only hits with coverage and identity >90% were considered. Using Abricate, the assembled genomes were also screened against the PlasmidFinder (Carattoli et al., 2014) databases for the identification of plasmids. As before, only hits with >90% identity and coverage were kept. All databases used with Abricate were most recently updated on the 27<sup>th</sup> of March 2021 (Seemann, <https://github.com/tseemann/abicate>).

*E. coli* and *Klebsiella* isolates with repeated sequence types were compared using the genome comparator tool on the BIGSdb platform (Jolley et al., 2018). This tool performs pairwise allele alignments at 2513 loci for *E. coli* and 694 loci for *Klebsiella* between the submitted genomes. The 'GrapeTree' tool (Zhou et al., 2018) was used to visualise core genome MLST comparisons through creation of minimum spanning trees. All sequenced *E. coli* genomes are housed on the *Escherichia* database on PubMLST (v1) (<https://pubmlst.org/organisms/escherichia-spp/>), while the sequenced *Klebsiella* isolates were uploaded to the BIGSdb *Klebsiella* Pasteur MLST database (v1.30.0) (<http://bigsdbs.pasteur.fr/klebsiella>). The identifier number for each of the sequenced isolates on these databases, along with the European nucleotide archive database are outlined in Supplementary Table C8.

## 4.3 Results

### 4.3.1 Faecal indicator organisms

Examination of the Colilert results was carried out in conjunction with potential contributing factors including distance to discharges, average rainfall over a 7-day period prior to sample collection and agricultural activity (Figure 4.1, Supplementary Tables C2 & C3). The data was categorised as low (green), medium (yellow) and high (red) for each of the variables. Rainfall, coliforms and *E. coli* measurements were categorised based on low to high numerical values. In contrast, the distance to discharges were categorised based on risk, with smaller distances receiving high risk designation (red) versus longer distances being regarded as low risk (green). Agricultural activity was categorised at an electoral district level as low, medium and high based on maps of potential contributing sources of antibiotic resistance dissemination, created previously by Chique et al. (2019). The data generated during the initial point prevalence sampling campaign was also included for seasonal comparison (Hooban et al., 2021).

Cork local authority area harboured many discharges in close proximity to samples collected with three sampling sites identified as having 4 different discharges nearby. Although the rainfall levels were classified as low (<5mm) for both the point prevalence (PP) and longitudinal survey 2 (LS2) samples, the seasonal aspects largely differed. This was reflected in the overall lower levels of coliforms and *E. coli* detected in the PP samples collected in May/June 2019 versus the LS2 samples obtained in October/November 2019. Collection of the longitudinal survey 1 (LS1) samples took place in November 2020 with medium rainfall (5.0 - 9.9mm) for all samples. Direct comparison with the LS2 data revealed that the faecal indicator levels varied between the 14 sites collected across both periods. A total of 10 samples from the LS1 contained very high coliform levels ( $\geq 1000$  MPN/100mL) versus 6 collected during LS2.

Fingal had the lowest number of discharges deemed in close proximity to the sample collection sites. Seawater and estuarine waters were the only natural water types included in this region, with the latter harbouring much higher coliform and *E. coli* levels overall. Sample collection was carried out across different seasons with the PP survey completed in April 2019, LS1 in October 2019 and LS2 in September 2020. All samples within the PP and LS1 sample collection periods had low average rainfall in the week prior to sample collection. In contrast LS2 samples had low (n=4) and medium (n=8) rainfall levels. However, the medium rainfall only translated to categorically higher faecal indicator levels in two samples across the longitudinal survey periods.

Galway City had low agricultural activity and no raw sewage discharges in close proximity to any of the samples collected. However, it harboured the highest number of storm water overflows categorised as high risk ( $\leq 0.5\text{km}$ , n=10) across the four local authority areas. The average rainfall over seven days prior to sample collection was categorised as medium for each sample collected within LS1 (n=14) and the majority of samples collected in the PP survey (5/6), but varied from low (2/14) to high (4/14) in LS2. The coliform levels detected in 11/17 (65%) of the sites across one (n=9) or both (n=2) of the longitudinal survey sampling periods were categorised as high ( $\geq 1000$  MPN/100mL). PP samples were collected between November 2018 and January 2019, whereas the LS1 samples were collected in August 2019 and the LS2 in February/March 2020.

Galway County local authority area displayed low to medium agricultural activity and contained the highest number of raw sewage discharges in close proximity ( $\leq 0.5\text{km}$ ) to sampling points (n=4). Coliform and *E. coli* levels varied from low to high in samples located close to raw sewage discharges with low to medium rainfall across the three sampling periods. A seasonal approach was applied through collection of the PP samples in January/February 2019, LS1 in September 2019 and the LS2 in March and July 2020.

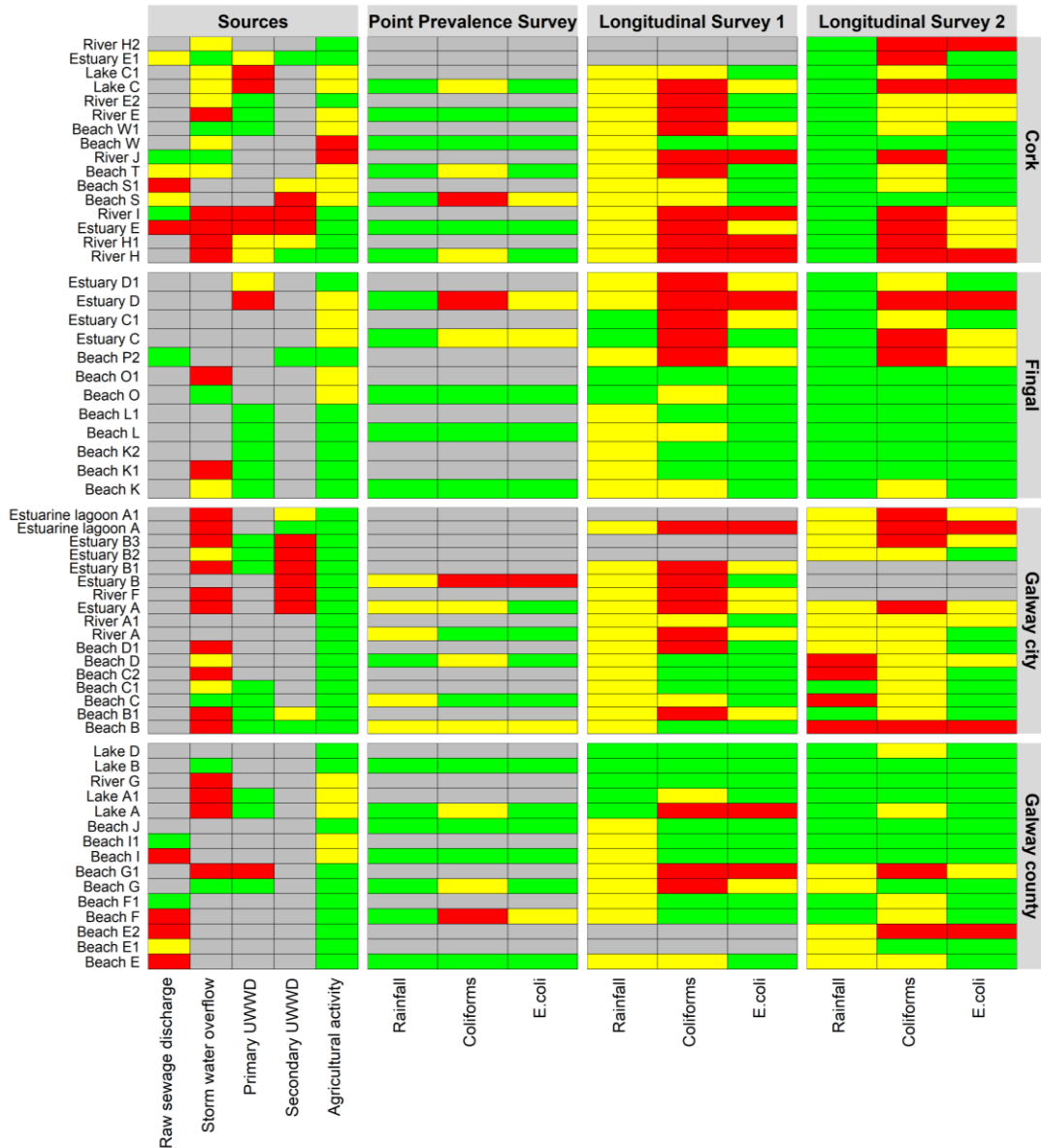


Figure 4.1: Tile plot displaying categorical data of potential contributing sources versus coliform and E. coli results. Grey tiles indicate the absence of data. Red tiles indicate  $\geq 1000$  MPN/100mL coliforms/E. coli,  $\geq 10$ mm rainfall average, high agricultural activity and  $\leq 0.5$ km from the sample collection point to a discharge/storm water overflow. Yellow tiles indicate between 251 - 999 MPN/100mL coliforms/E. coli, 5.0 - 9.9mm rainfall average, medium agricultural activity and between 0.6 - 1km from the sample collection point to a discharge/storm water overflow. Green indicates  $\leq 250$  MPN/100mL coliforms/E. coli,  $< 5$ mm rainfall average, low agricultural activity and  $\geq 1.1$ km distance from the sample collection point to a discharge/storm water overflow. UWWD = Urban wastewater discharge. Raw data relating to this figure is outlined in Supplementary Tables C2 & C3.

### 4.3.2 Antibiotic resistant Enterobacterales

A total of 419 antibiotic resistant Enterobacterales (348 water, 71 sewage) were isolated from all samples. The majority of isolates were *E. coli* (n=346) followed by *Klebsiella* spp. (n=40), *Enterobacter* spp. (n=13) and *Citrobacter* spp. (n=13). The remaining isolates included 3 *Morganella morganii*, 3 *Raoultella ornithinolytica* and one *E. albertii*.

Multi-drug antibiotic resistance was widely detected across all sample types (Figure 4.2). Isolates originating from hospital sewage displayed the highest percentage of resistance to the majority of beta-lactam antibiotics including cefpodoxime (86%), ceftazidime (71%), ertapenem (51%) and cefoxitin (40%). Similarly, resistance to nalidixic acid (80%), gentamicin (60%) and kanamycin (43%) featured highest amongst hospital sewage isolates. Estuarine waters ranked highest for the percentage of resistant isolates to cefotaxime (82%), ciprofloxacin (74%) and streptomycin (56%). Direct comparison of isolates from waters versus sewage (Supplementary Figure C1), revealed a higher percentage of water isolates displayed resistance to chloramphenicol (19% water, 9% sewage), streptomycin (41% water, 34% sewage) and tetracycline (50% water, 43% sewage). Conversely, sewage isolates showed predominant resistance to cefotaxime (76% sewage, 65% water) and ertapenem (36% sewage, 24% water). The lowest levels of resistance across all sample types was evident for meropenem (3% water, 6% sewage) and chloramphenicol (19% water, 9% sewage).



Figure 4.2: Percentage of isolates from each sample type that displayed a resistant phenotype to the panel of 15 antibiotics tested. DWTP = Drinking water treatment plant, WWTP = Wastewater treatment plant.

### 4.3.3 Extended spectrum beta-lactamase producing Enterobacterales

ESBL producers were identified by phenotypic ESBL production in 92/118 (78%) waters and 18/36 (50%) sewage samples. Many samples comprised of more than one ESBL producer, which included 185 Enterobacterales that harboured *bla*<sub>CTX-M-group1</sub> and 59 that contained *bla*<sub>CTX-M-group9</sub>. Whole genome sequencing of a selection of ESBLs (n=92) revealed that *bla*<sub>CTX-M-15</sub> (n=63) was the most frequently detected ESBL gene, followed by 17 isolates that harboured *bla*<sub>CTX-M-27</sub> (Figure 4.4). Additional *bla*<sub>CTX-M</sub> genes were also identified to a lesser extent including *bla*<sub>CTX-M-1</sub> (n=4), *bla*<sub>CTX-M-14</sub> (n=4), *bla*<sub>CTX-M-55</sub> (n=3), *bla*<sub>CTX-M-3</sub> (n=3), *bla*<sub>CTX-M-32</sub> (n=1) and *bla*<sub>CTX-M-9</sub> (n=1). Further ESBL types detected from sequenced isolates included *bla*<sub>SHV-12</sub> (n=5), *bla*<sub>SHV-106</sub> (n=3) and *bla*<sub>OXA-17</sub> (n=2).

### 4.3.4 Carbapenem resistant Enterobacterales

A total of 83 water (24%) and 31 sewage isolates (44%) displayed resistance to ertapenem alone or in combination with meropenem. One or more Enterobacterales harbouring a carbapenemase gene were isolated from 18 water and 5 sewage samples. The majority of these isolates contained *bla*<sub>OXA-48</sub> (n=18), followed by *bla*<sub>NDM</sub> (n=14), *bla*<sub>KPC</sub> (n=4) and *bla*<sub>OXA-484</sub> (n=1) (Figure 4.3). River water had the highest number of carbapenemase producing Enterobacterales detected (n=12) in 7 individual samples. NDM producers were solely detected in waters with the exception of one isolate from airport sewage.

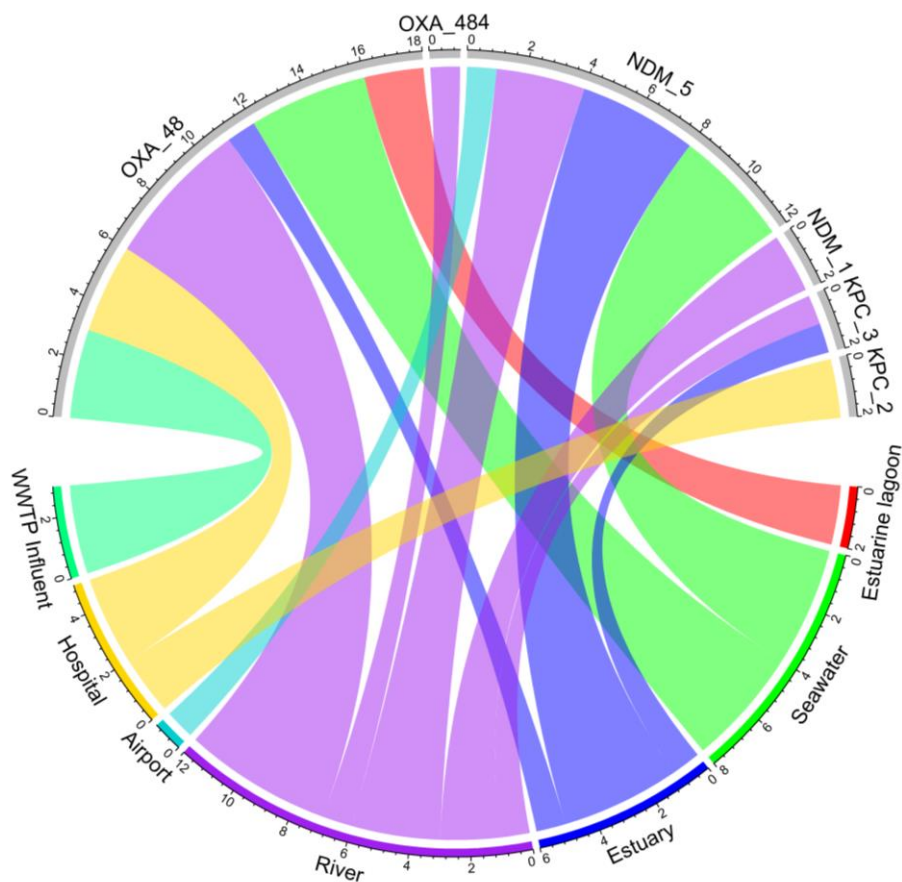


Figure 4.3: Carbapenemase genes detected within Enterobacterales isolated from different water and sewage sources. WWTP = Wastewater treatment plant

### 4.3.5 Additional antibiotic resistances detected using whole genome sequencing

A total of 146 bacterial isolates were sequenced encompassing 114 water and 32 sewage isolates. The antibiogram profiles along with the resistance genes identified using ResFinder were combined in Figure 4.4. The majority of isolates (n=138) harboured one or more beta-lactamase gene. This included most commonly *bla*<sub>CTX-M-15</sub> (48 water, 14 sewage), *bla*<sub>TEM-1B</sub> (43 water, 10 sewage) and *bla*<sub>OXA-1</sub> (15 water, 6 sewage). Comparison of the carbapenemase genes detected with the phenotypic data revealed that *bla*<sub>OXA-48</sub> (n=18) and *bla*<sub>OXA-484</sub> producers (n=1) displayed resistance to ertapenem in all isolates and an intermediate phenotype to meropenem for the majority of isolates (n=17). Antibiotics against which bacteria display an intermediate phenotype are not normally prescribed due to uncertainty surrounding therapeutic success. In contrast, *bla*<sub>NDM</sub> isolates displayed resistance to both ertapenem and meropenem in 13/14 isolates. Similarly, all four *bla*<sub>KPC</sub> producers showed dual resistance to both carbapenem antibiotics. All NDM producing isolates harboured the *ble-MLB* gene which confers bleomycin resistance, identified using the CARD database.

Sulphonamide resistance genes *sul1*, *sul2* and *sul3* were the next most common resistance type following the beta-lactamase genes, identified within 92 isolates. The majority of isolates also harboured one or more aminoglycoside (n=86) and trimethoprim (n=80) resistance gene. The presence of these resistance genes was reflected in the phenotypic data with 79/80 isolates that contained one or more *dfrA* genes displaying trimethoprim resistance. Thirteen different resistance genes were detected that confer reduced susceptibility to aminoglycosides. One pattern that emerged through comparison of the phenotypic and genotypic data was the presence the *aph(3'')-Ib* gene alone or in combination with the *aph(6)-Id* gene conferred streptomycin resistance in 49/52 isolates. However, many of these isolates also harboured additional

aminoglycoside resistance genes. The *tetA*, *tetB* and *tetD* genes were detected in 71 sequenced isolates, of which 70 displayed tetracycline resistance. The *fosA* gene which reduces susceptibility to fosfomicin was identified in 37 of the sequenced Enterobacterales. The majority of these isolates were *Klebsiella* spp. (n=27), followed by *Enterobacter* spp. (n=6), *E. coli* (n=3) and 1 *Raoultella*.

Some of the rarer antibiotic resistance genes detected included the *arr3* gene in one *Klebsiella pneumoniae* identified from hospital sewage and the *Inu(F)* gene in an *E. coli* from seawater, which confer resistance to rifamycin and lincomycin respectively. A total of four isolates also harboured *mcr9* including one *E. coli* from river water, along with two *Enterobacter* and one *Raoultella* isolate from sewage.

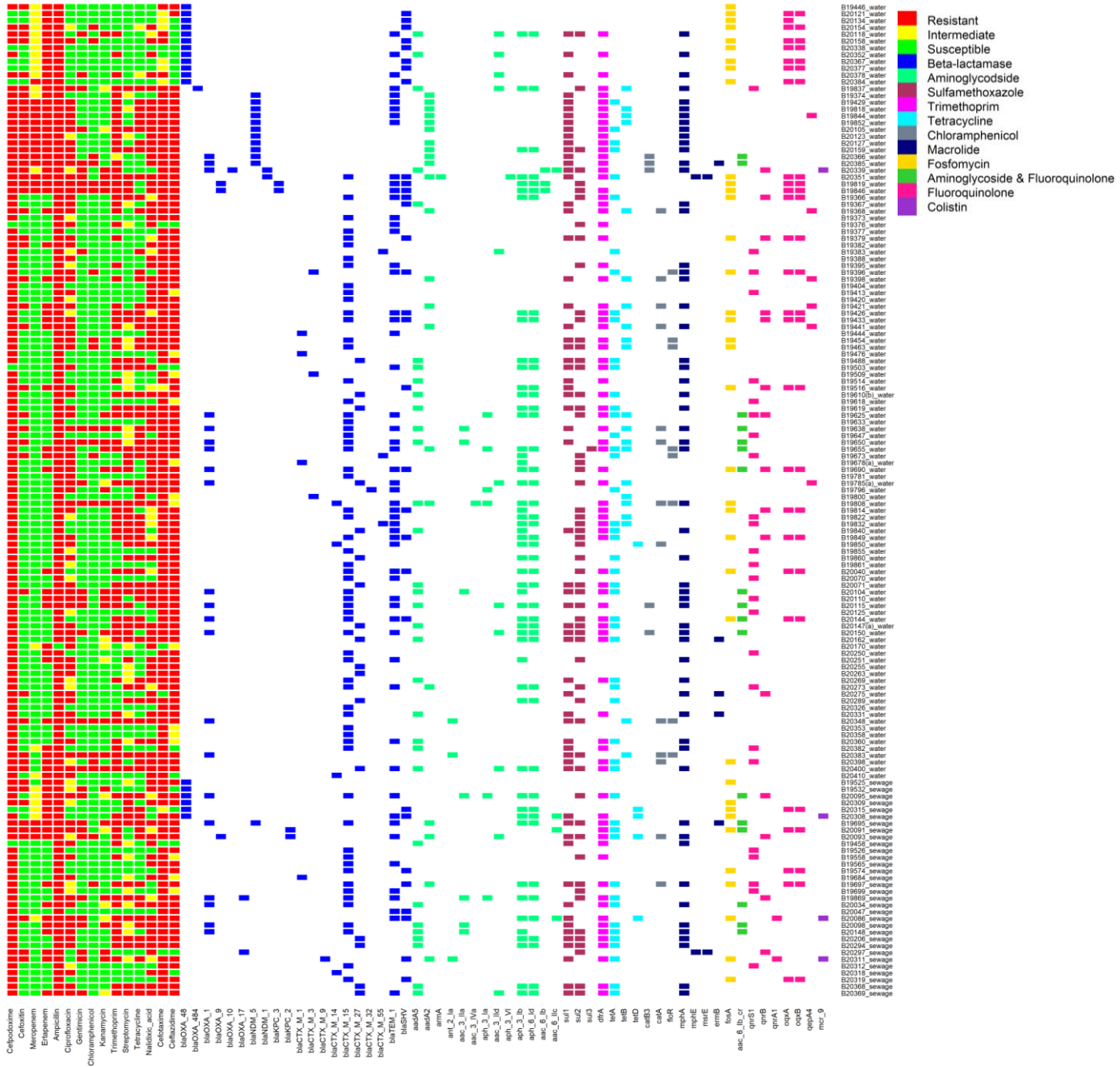


Figure 4.4: Antibiogram and resistance gene profile of the complete set of isolates chosen for whole genome sequencing (n=146). The *arr-3* and *lnu(F)* genes were detected in one isolate from hospital sewage (B20091) and one isolate from seawater (B20273) respectively, which are not included in this figure. *ant\_2\_1a*: *ant(2'')-Ia*, *aac\_3\_1Ia*: *aac(3)-Ila*, *aac\_3\_IVa*: *aac(3)-IVa*, *aph\_3\_1a*: *aph(3')-Ia*, *aac\_3\_IId*: *aac(3)-IId*, *aph\_3\_VI*: *aph(3')-VI*, *aph\_3\_Ib*: *aph(3'')-Ib*, *aph\_6\_Id*: *aph(6)-Id*, *aac\_6\_Ib*: *aac(6')-Ib*, *aac\_6\_Ic*: *aac(6')-Ic*.

### 4.3.6 Plasmid analysis

The majority of sequenced isolates harboured one or more plasmids (140/146). Plasmid replicon types detected included many different Inc and Col types, including Inc (FIA, FIB, FII) and Col (RNAI, MG828, 440I, 156). The most commonly detected plasmids across all sequenced isolates included IncFIB (n=104), followed by Col (n=96) and IncFII (n=84) (Table 4.1). *E. coli* isolates harboured the largest diversity of plasmids (n=18) with up to 9 plasmids detected within one *E. coli* isolate (B20115).

Table 4.1: Summarised results including number of isolates of individual species from different samples, sequence types and plasmids detected for all sequenced isolates.

Sample type	Species	No. of isolates	Sequence type	Plasmids
Seawater	<i>Escherichia coli/albertii</i>	38 – <i>E. coli</i> 1 – <i>E. albertii</i>	10 (3), 34 (2), 38 (2), 58 (1), 95 (1), 131 (8), 162 (2), 167 (2), 224 (1), 382 (1), 394 (1), 410 (2), 540 (1), 648 (3), 744 (1), 1015 (1), 1193 (1), 2659 (1), 4121 (1), 10302 (1), unknown (3)	IncFIB (26), 2 IncFIB (1), IncFII (22), 2 IncFII (1), Col (13), 2 Col (6), 3 Col (6), 4 Col (2), 5 Col (1), IncFIC(FII) (11), IncFIA (17), Incl (1), IncB/O/K/Z (1), 2 IncB/O/K/Z (1), IncX (3), IncR (1), IncY (5), IncP1 (1), IncN (1), Incl (1), 0 plasmids (2)
	<i>Klebsiella pneumoniae</i>	9	8 (1), 17 (3), 289 (2), 307 (1), 323 (1), 1933 (1)	IncFIB (8), 2 IncFIB (1), IncFIA (2), Col (3), 2 Col (1), IncFII (5), Incl (4), IncR (3)
	<i>Enterobacter hormaechei</i>	1	190 (1)	2 Col (1)
River	<i>Escherichia coli</i>	25	10 (3), 38 (3), 69 (1), 88 (1), 101 (2), 127 (1), 131 (2), 354 (1), 394 (2), 405 (3), 410 (2), 635 (1), 8530 (1), unknown (2)	IncFIB (16), 2 IncFIB (1), IncFII (13), 2 IncFII (1), Col (7), 2 Col (1), 3 Col (1) 4 Col (4), 5 Col (1), IncFIC(FII) (3), IncFIA (8), Incl (8), IncB/O/K/Z (4), IncX (2), IncY (3), IncQ (1), p0111 (3), 2 IncHI2 (1), RepA_pKPC-CAV1321 (1), pENTAS02 (1), 0 plasmids (2)
	<i>Klebsiella pneumoniae</i>	9	8 (1), 17 (3), 289 (2), 307 (1), 323 (1), 1933 (1)	IncFIB (4), 2 IncFIB (1), 3 IncFIB (1), IncFIA (2), Col (1), 2 Col (1), IncFII (1), 2 IncFII (1), IncHI (2), Incl (3), IncQ (1)
Estuary	<i>Escherichia coli</i>	18	10 (2), 69 (1), 88 (1), 131 (3), 167 (1), 405 (5), 410 (1), 648 (1), 1193 (1), 1294 (1), 4213 (1)	IncFIB (12), 2 IncFIB (1), IncFII (11), 2 IncFII (1), Col (4), 2 Col (5), 3 Col (4), 5 Col (1), IncFIC(FII) (4), IncFIA (9), Incl (6), IncX (2), p0111 (4)
	<i>Klebsiella pneumoniae</i>	5	17 (1), 231 (1), 309 (1), 323 (1), 5258 (1).	IncFIB (4), 2 IncFIB (1), IncFIA (2), Col (2), 2 Col (1), IncFII (2), 2 IncFII (1), IncHI (1), Incl (1), IncR (1)
Estuarine lagoon	<i>Escherichia coli</i>	3	38 (1), 131 (2)	IncFIB (2), IncFII (1), 2 IncFII (1), Col (1), 5 Col (1), IncFIA (1), IncX (1), 0 plasmids (1)
	<i>Enterobacter hormaechei</i>	1	190 (1)	Incl (1)
Lake	<i>Escherichia coli</i>	2	59 (1), 224 (1)	IncFIB (1), IncFII (1), 4 Col (1), IncFIC(FII) (1), Incl (1), IncB/O/K/Z (1)

Sample type	Species	No. of isolates	Sequence type	Plasmids
Drinking water treatment plant influent	<i>Escherichia coli</i>	3	131 (2), 348 (1)	IncFIB (3), IncFII (3), Col (2), IncFIA (3), IncL (1), IncY (1)
	<i>Klebsiella pneumoniae</i>	1	17 (1)	Col (1), IncFIA (1), IncFII (1)
Hospital sewage	<i>Escherichia coli</i>	5	69 (1), 131 (2), 2562 (1), 6280 (1)	IncFIB (4), IncFII (1), 2 IncFII (2), Col (2), 2 Col (1), 4 Col (1), IncFIC(FII) (1), IncFIA (2), IncL (1), 0 plasmids (1)
	<i>Klebsiella pneumoniae</i>	1	17 (1)	2 Col (1), 2 IncFIB (1), IncFII (1), IncR (1), IncN (1)
	<i>Klebsiella oxytoca</i>	3	2 (1), 88 (1), 135 (1)	IncFIB (1), IncFII (1), 2 Col (2), IncR (1), IncL (1)
	<i>Enterobacter kobei/ cloacae</i>	1 – <i>E. kobei</i> 1 – <i>E. cloacae</i>	<i>E. kobei</i> – 910 (1) <i>E. cloacae</i> – unknown (1)	IncFIA (1), IncFII (1), IncL (1), IncR (1), 2 Col (2), 2 IncHI2 (1), pKPC-CAV1321 (1), RepA_pKPC-CAV1321 (1)
	<i>Citrobacter freundii</i>	3	64 (1), 150 (1), 166 (1)	IncL (1), 3 Col (1), IncFII (1), 2 IncFIB (2), 2 IncFII (1), pKPC-CAV1321 (2)
Wastewater treatment plant influent	<i>Escherichia coli</i>	7	131 (4), 602 (1), 607 (1), 1193 (1)	IncFIB (5), IncFII (3), Col (4), 2 Col (2), IncFIC(FII) (2), IncFIA (5), IncL (2), IncX (1), IncR (1) IncY (1)
	<i>Klebsiella pneumoniae</i>	2	37 (1), 323 (1)	IncFIB (2), Col (1), IncFII (2), IncL (1), IncR (1)
	<i>Enterobacter cloacae/ hormaechei</i>	1 – <i>E. cloacae</i> 1 – <i>E. hormaechei</i>	<i>E. cloacae</i> – 910 (1) <i>E. hormaechei</i> – 133 (1)	IncFIA (1), IncFII (1), IncL (1), IncR (1), Col (2), 2 IncHI2 (1), pKPC-CAV1321 (2)
	<i>Raoultella ornithinolytica</i>	1	Unknown (1)	3 Col (1), IncFIB (1), IncFII (1), 2 IncHI2 (1), IncL (1), pKPC-CAV1321 (1)
Nursing Home	<i>Escherichia coli</i>	1	167 (1)	IncFIB (1), Col (1), IncFIC(FII) (1), IncFIA (1)
	<i>Klebsiella pneumoniae</i>	1	323 (1)	2 Col (1), IncFIA (1), IncFIB (1), IncFII (1)
Airport	<i>Escherichia coli</i>	3	38 (1), 394 (1), 131 (1)	IncFIB (2), IncFII (2), Col (2), IncFIC(FII) (1), IncFIA (1), IncB/O/K/Z (1),
	<i>Klebsiella pneumoniae</i>	1	76 (1)	IncFIA (1), IncFIB (1)

A number preceding the plasmid name indicates two different types, e.g. 2 IncQ: IncQ1 and IncQ2. The different plasmids that were identified using PlasmidFinder and summarised are listed. **Col:** Col(MG828), Col(pHAD28), Col440II, Col440I, ColRNAI, Col8282, ColE10, ColpVC, Col(BS512), Col(MP18), Col(Ye4449), Col156. **IncFIA:** IncFIA(HI1), IncFIA. **IncFIB:** IncFIB(K)(pCAV1099-114), IncFIB(K), IncFIB(pKPHS1), IncFIB(pNDM-Mar), IncFIB(pQil), IncFIB(K)\_Kpn3, IncFIB(pB171), IncFIB(pHCM2), IncFIB(pQil), IncFIB(AP001918). **IncFII:** IncFII(p14), IncFII(pECLA), IncFII(pKP91), IncFII(pRSB107), IncFII(Yp)\_Yersenia, IncFII(pECLA), IncFII\_pKP91, IncFII(29)\_pUTI89, FII(pBK30683), IncFII(pCRY), IncFII(pCoo), IncFII(pHN7A8), IncFII(pAMA1167-NDM-5), IncFII, IncFII\_pSFO. **IncHI:** IncHI1B(CIT)\_pNDM, IncHI1B(pNDM-MAR), IncHI1B(R27), IncHI1A(CIT)\_pNDM, IncHI1A. **IncHI2:** IncHI2A, IncHI2. **IncL:** IncL, IncL/M(pOXA-48). **IncB/O/K/Z:** IncB/O/K/Z\_1, IncB/O/K/Z\_2, IncB/O/K/Z\_4. **IncQ:** IncQ1, IncQ2. **IncX:** IncX1, IncX2, IncX3, IncX4.

Figure 4.5 displays the number of different plasmid types identified within isolates harbouring a carbapenemase gene. The majority of OXA-48 isolates contained the IncL plasmid (15/18). Of these, the IncL/M plasmid, commonly linked with OXA-48 carriage, was identified within three isolates. The *Raoultella*

*ornithinolytica* isolate (B20308), which harboured the *bla*<sub>OXA-48</sub> gene, contained a high number of plasmids, totaling 9. IncFIB was most commonly identified in Enterobacterales harbouring *bla*<sub>NDM</sub> (11/14), while IncFIB and IncFII were the only plasmids identified in all four KPC producing isolates.

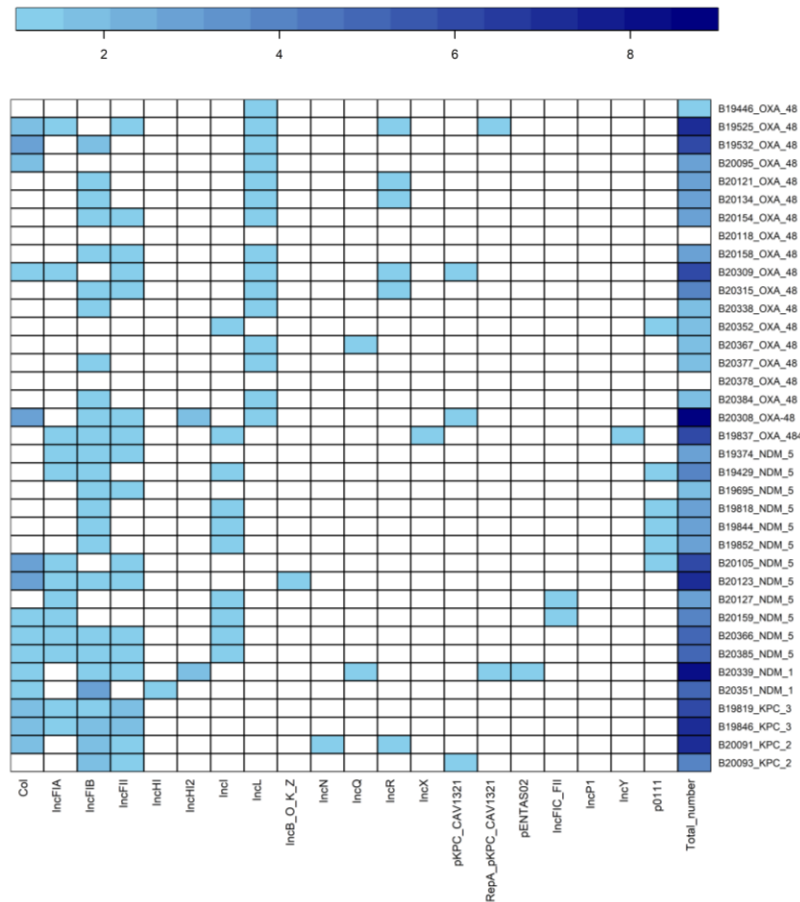


Figure 4.5: Heatmap displaying the number of each type of plasmid detected within Enterobacterales harbouring a carbapenemase gene.

### 4.3.7 Core genome MLST comparison

Core genome multilocus sequence type (cgMLST) comparisons were carried out on all *Escherichia* spp. (n=105) and *Klebsiella pneumoniae/variicola* (n=27) isolates that were selected for sequencing from the longitudinal survey periods. The sequenced isolates collected through the initial point prevalence sampling campaign (48 *E. coli* and 24 *Klebsiella* spp.) were also included for comparison (Hooban et al., 2021).

Analysis of the *Klebsiella* isolates revealed 31 different sequence types, of which 8 were repeated, highlighted by the circles in Figure 4.6. The most frequently detected sequence types included ST323 (n=7), ST17 (n=6), ST405 (n=4) and ST309 (n=3). Comparison of isolates at 694 core genome loci revealed clusters of isolates from different waters across Galway City indicated by light blue and Cork highlighted in light orange (Supplementary Table C6). Sequence type ST309 included three identical isolates (B20338, B20377, B20384) that all harboured *bla*<sub>OXA-48</sub> from waters which spanned a 9.8km distance in Cork. Similarly, the ST231 isolates were identical (B19819, B19846), and originated from aquatic samples collected over 24 hours from two sites in the same region in Cork. Both isolates harboured *bla*<sub>KPC-3</sub> and the collection sites were located 3.3km apart. The collection of isolates belonging to sequence type ST323 encompassed isolates from sewage (n=4) and waters (n=3) across Cork and Galway. The smaller cluster within this group included two genetically indistinguishable isolates with identical antibiograms from the same nursing home, collected 7 months apart (B19023, B19574). The larger cluster included identical *Klebsiella* from river H1 in Cork (B19849) as well as wastewater treatment plant influent (B19326) and an estuarine water (estuary B) in Galway City (B19426).

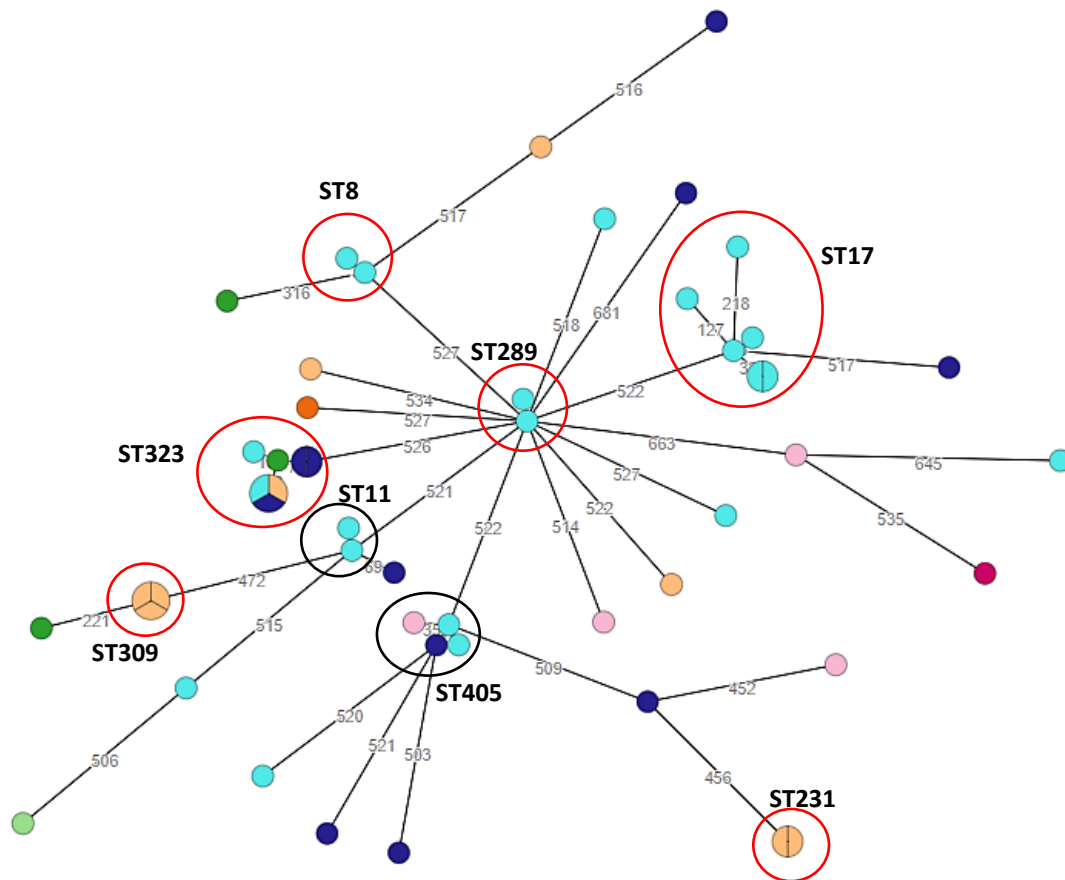


Figure 4.6: Minimum spanning tree depicting core genome multilocus sequence typing comparison across sequenced *Klebsiella* isolates ( $n=52$ ). The black circles indicate clusters that were previously identified in the point prevalence survey (Hooban et al., 2021), while the red circles highlight newly identified clusters. The circles are colour coded to indicate the local authority region: Galway city = blue; Galway county = pink; Fingal = green; Cork = orange. The darker colour indicates isolates from sewage origin while the lighter colours indicate water origin. The numbers between the nodes represents the number of locus allele differences between isolates.

The complete set of sequenced *E. coli* isolates ( $n=153$ ) were also compared using cgMLST (Figure 4.7). A total of 47 different sequence types (STs) were identified with 18 sequence types repeated by two or more isolates (Supplementary Table C7). Five of the isolates could not be matched with a known sequence type. The most commonly detected sequence types amongst *E. coli* included ST131 ( $n=39$ ) followed by ST38 ( $n=15$ ) and ST10 ( $n=12$ ). There were 9 clusters of two or more identical isolates based on cgMLST comparisons at 2513 loci. Just two isolates belonging to the large ST131 collection (B20400, B20369) were 100% identical and originated from a wastewater treatment plant

influent and a seawater located 3.2km apart in Cork. Similarly, another pair of identical isolates belonging to ST167 originated from sewage (nursing home E, B20148) and waters (estuary B2, B20104) in Galway City. However, the sampling locations were further apart, with a distance of 14.5km between them. A total of four isolates were identical within the ST410 cluster (B19368, B19398, B19421, B19441). These four isolates all originated from waters around Galway City and spanned four different collection points across 5.6km. There were two identical carbapenemase producers (B19818, B19852) collected from waters in a highly polluted region in Cork, located 3.8km apart. These isolates (ST405) both harboured *bla*<sub>NDM-5</sub> and sample collection took place one day apart.

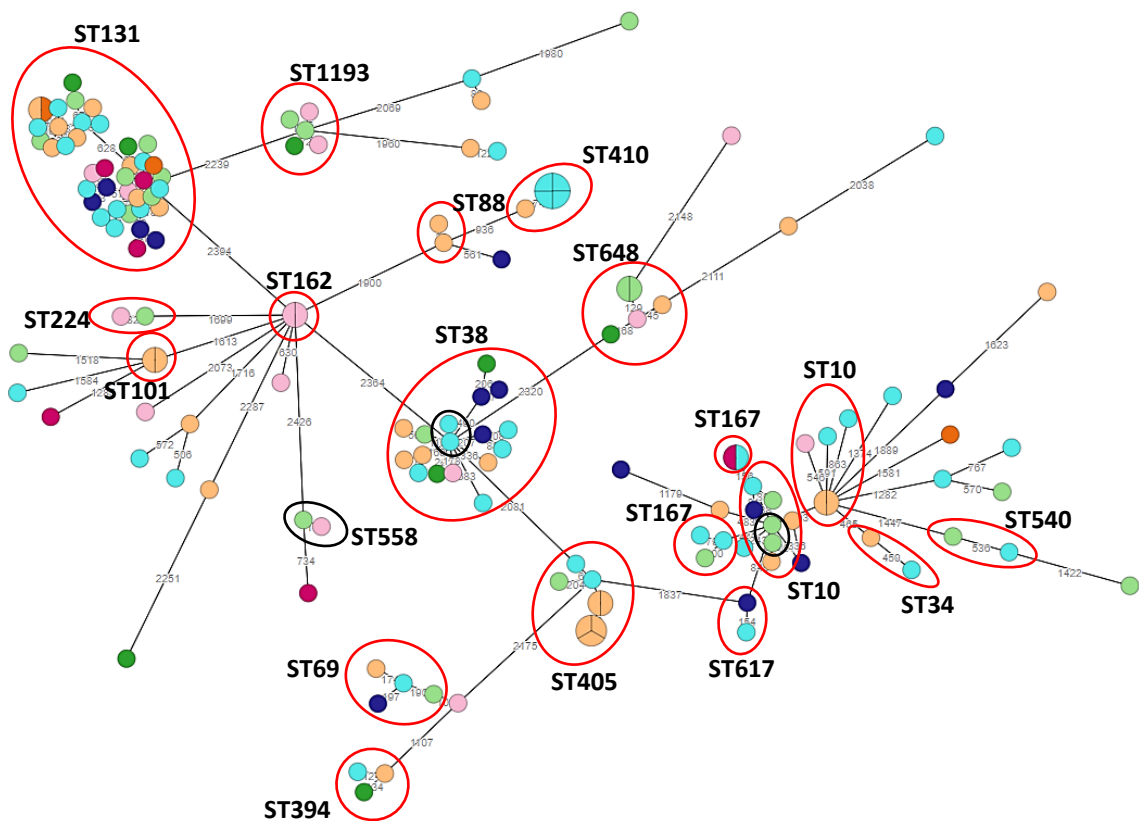


Figure 4.7: Minimum spanning tree portraying core genome multilocus sequence typing comparison across sequenced *Escherichia* isolates ( $n=153$ ). The black circles indicate clusters that were previously identified in the point prevalence survey (Hooban et al., 2021), while the red circles highlight newly identified clusters. The circles are colour coded to indicate the local authority region: Galway city = blue; Galway county = pink; Fingal = green; Cork = orange. The darker colour indicates isolates from sewage origin

*while the lighter colours indicate water origin. The numbers between the nodes represents the number of locus allele differences between isolates.*

## **4.4 Discussion**

### **4.4.1 Comparison across sampling periods**

Comparison of the longitudinal survey sampling periods to the initial point prevalence survey was carried out at a quantitative level through comparison of the coliforms and *E. coli* detected in waters (Figure 4.1). Overall, increased average rainfall over a 7-day period prior to sample collection augmented the coliform levels in some but not all sampling sites. The majority of samples had low (<5mm) to medium (5.0-9.9mm) rainfall, making it difficult to fully elucidate the significance of increased rainfall. Conflicting evidence has been previously reported in relation to the impact of heavy rainfall on coliform levels in natural waters. Sampson et al. (2006) reported no relationship between rainfall levels and the concentration of coliforms and *E. coli* detected at 15 beaches in the United States following at least 6mm of rain during a 'rainfall event' in the preceding 24 hours. These beaches were monitored between May to September over the course of two years (2003 - 2004). In contrast, Kim et al. (2013) demonstrated a correlation between the increased accumulative rainfall in the preceding 1 to 4 days, and the levels of faecal indicator organisms in 34 samples from two sites along the Han River in Korea. These samples were collected between July 2010 and February 2011. Various contributing factors may be responsible for these contrasting findings including the larger dilutional effect and higher salt concentration in seawater, the presence of faecal sources (anthropogenic, agricultural, wildlife) surrounding the sample collection site, and sample collection at varying times of the year.

During this study only four water samples were collected following a period of high rainfall ( $\geq 10$ mm), all located in Galway City. All four displayed higher levels of coliforms and *E. coli* when compared to other sample collection periods in which rainfall was categorised as low to medium. Notably, storm water

overflows were located within 1.5km of all four seawater samples. All four sites were regarded as low agricultural activity at an electoral district level, indicative that the higher coliform concentrations during wet conditions may be due to anthropogenic discharges. Previous studies have demonstrated that stormy weather promotes antibiotic resistance dissemination through activation of storm water overflows and agricultural runoff (Almakki et al., 2019; Eramo et al., 2017). An important consideration when interpreting faecal indicator bacterial levels is that *E. coli* concentrations can significantly vary throughout the day, previously demonstrated by Wyer et al. (2018). These samples were collected at one point in time, at a maximum of three times per site. Intensive sample collection at fewer sites during more extreme weather events is needed to fully establish the significance of increased rainfall at a particular site.

ESBL producers were present in the majority of samples across all sampling periods, indicative of its natural and widespread persistence in the environment across all seasons (Hooban et al., 2020). The *bla*<sub>CTX-M</sub> gene, detected across the majority of water samples, was previously traced back to the chromosome of *Kluyvera* species, which is ubiquitous in the natural environment (Cantón et al., 2012a). The majority of CPE from water samples originated from Cork and Galway city. Carbapenemase detection varied across different times of the year in Cork, with no detection in samples collected in May/June 2019 (Hooban et al., 2021). In contrast, different carbapenemase gene types were detected in both longitudinal survey periods collected in October/November in 2019 and 2020. The consistent detection of CPE was evident in samples collected from Galway City throughout different seasons. OXA-48 producing isolates were identified across all three periods in one or more water samples across the city. The longitudinal survey periods in Galway city both included the detection of NDM producers at 3 individual collection sites. To the authors knowledge, there have been little to no studies to date examining carbapenemase detection across different seasons in natural waters highlighting an important knowledge

gap. Natural water use for recreational purposes would continue throughout the year in Ireland. Therefore, the repeated presence of CPE throughout different seasons represents a public health concern in relation to increased colonisation rates due to ingestion of water (O'Flaherty et al., 2019, Leonard et al., 2018). Although the detection of antibiotic resistant organisms is beyond the scope of the current EU bathing water monitoring criteria (Directive 2006/7/EC), the repeated detection of CPE demonstrates the need for year round bathing water monitoring to protect public health. Farrell et al. (2021) also echoed the need for year long bathing water monitoring, and incorporation of antibiotic resistance detection to the bathing water directive based on a European wide scoping literature review.

#### **4.4.2 Antibiotic resistance across different sample types**

Multi-drug resistant isolates were detected in all sample types, inclusive of sewage and natural waters. Isolates from hospital sewage displayed the highest percentage of resistant isolates to most beta-lactam, aminoglycoside and quinolone antibiotics (Figure 4.2). Beta-lactams were the most frequently prescribed antibiotics in Ireland in 2019, with the penicillins used at a national average of 38.6 defined daily dose (DDD) per 100 bed-days used (BDU) within hospitals (Health Protection Surveillance Centre, 2020). Cephalosporins, monobactams and carbapenems were prescribed at an average of 9 DDD per 100BDU, increasing from 8.4 in 2018. Aminoglycosides and quinolones ranked lower at 3.7 and 3.3 DDD per 100BDU respectively.

Similarly to previous findings in the point prevalence survey (Hooban et al., 2021), tetracycline and ciprofloxacin resistance were observed at a higher percentage in water versus sewage isolates (Supplementary Figure C1). The possible explanation for this was outlined previously by Hooban et al. (2021), including the common use of tetracyclines in veterinary medicine (Health products regulatory authority, 2019) and the persistent nature of ciprofloxacin

in wastewater (Kumar et al., 2019). Ciprofloxacin was detected at levels exceeding the predicted no-effect concentrations (PNECs) in Irish wastewater treatment plant effluents previously (Rodriguez-Mozaz et al., 2020, Monahan et al., 2021). PNECs were established for individual antibiotics in an attempt to understand the maximum antibiotic concentration, above which, antibiotic resistance development may occur (Bengtsson-Palme & Larsson 2016). These values were estimated using the minimum inhibitory concentrations from EUCAST and can be used to define acceptable antibiotic concentrations in environmental discharges.

Interestingly, a higher percentage of water isolates also displayed resistance to chloramphenicol and streptomycin. Chloramphenicol was banned from veterinary medicine in Europe in 1994 and can only be administered to animals that will not be used for food purposes (European Food Safety Authority, 2018). However, it is naturally produced by *Streptomyces venezuelae*, which is ubiquitous in soil and the environment (Schwarz et al., 2004). Chloramphenicol acetyltransferases, encoded by *cat* genes, confer enzymatic inactivation of the antibiotic. In this study, the presence of *cat* genes were detected within 18 isolates (16 water, 2 sewage). However, just ten of these isolates displayed resistance to chloramphenicol. The *floR* gene which confers resistance to florfenicol, was detected in eight isolates, all originating from water (Lu et al., 2018a). The florfenicol antibiotic is a derivative of chloramphenicol which is solely used in animals, which may indicate agricultural runoff in close proximity to these sampling areas. Five of the eight strains harboured *floR* in the absence of a *cat* gene, of which four displayed chloramphenicol resistance.

Streptomycin is also derived from an environmental soil bacterium, *Streptomyces griseus* (de Lima Procópio et al., 2012), and therefore the natural resistome in the environment may account for some of the resistance seen amongst water isolates. Aminoglycosides only accounted for a small proportion of antibiotics (6.1%) used in veterinary medicine in Ireland in 2019 (Health

products regulatory authority, 2019). An interesting observation in relation to streptomycin was the presence of *aph(3'')-Ib* alone or in combination with *aph(6)-Id* (also known as *strB*) conferred streptomycin resistance in 49/52 isolates. However, these resistance genes were equally distributed across sequenced sewage and water isolates. This indicates that more non-specific mechanisms of resistance may be at play in the water isolates such as ribosomal mutations or efflux pump activity (Lyu et al., 2019).

#### 4.4.3 Antibiotic resistance of critical clinical concern

Some antibiotic resistance genes detected in environmental isolates are of significant clinical concern, including the detection of carbapenemase and fosfomycin resistance genes. According to clinical surveillance data from 2018, *bla<sub>OXA-48</sub>* was the most common carbapenemase gene detected in cases of colonisation and infection in Ireland (Health Protection Surveillance Centre, 2019e). This was echoed amongst environmental isolates, in which 18 harboured *bla<sub>OXA-48</sub>*, indicative that similar resistance types are in circulation across clinical and natural environments. OXA-48 conferred reduced susceptibility to ertapenem and meropenem in most isolates making them unsuitable for treatment (Figure 4.4). Additionally, 13 of the 18 isolates harboured the *fosA* gene, also reducing the potency of fosfomycin and further limiting potential treatment options. An OXA-48-like carbapenemase (*bla<sub>OXA-484</sub>*) was also detected in one *E. coli* isolate (B19837) from river water in Cork belonging to sequence type ST410. This isolate displayed a non-susceptible phenotype to all antibiotics tested apart from chloramphenicol. OXA-484 has not been reported in the most recent surveillance reports published by the carbapenemase reference laboratory in Ireland based on data from 2017 to quarter 2 of 2019 (Health Protection Surveillance Centre, 2019e). However, it's most closely related variants OXA-181/232 have been reported in 15 patients within the first half of 2019.

NDM was the second most commonly detected carbapenemase (Figure 4.3), with the majority of isolates collected from waters (13/14) and identified as *E. coli* (13/14). All the NDM producers also harboured the *ble-MBL* gene which confers bleomycin resistance. The presence of both genes on one operon, separated by just 3 base pairs was previously demonstrated by Dortet et al. (2012). The *ble-MBL* gene confers decreased susceptibility to bleomycin, which is used in cancer treatment. This combination is a significant clinical concern as the use of bleomycin in a hospital setting may select for NDM producing isolates in immunocompromised individuals. Bleomycin is naturally produced by *Streptomyces verticillus* isolated from soil (Umezawa et al., 1966), and therefore many environmental strains naturally express *ble* resistance genes (Shen et al., 2002). A metagenomics study was previously performed examining activated sludge, which revealed wastewater to be a large reservoir of bleomycin resistance genes (Mori, et al., 2008). Further investigations of soils and waters as reservoirs and transmission routes of bleomycin resistance genes are needed.

The final type of carbapenemase gene detected was *bla*<sub>KPC</sub>, found within two *Klebsiella* isolates from waters (KPC-3; B19819, B19846) and two isolates of different species from the same hospital sewage sample (KPC-2; B20091, B20093). Interestingly, the two KPC-3 producers from waters displayed identical antibiograms with resistance to all antibiotics except tetracyclines (Figure 4.4). Thus, these aquatic isolates would be exceptionally difficult to treat if they potentially caused an infection in humans or animals. Whole genome sequencing analysis revealed an array of antibiotic resistance genes within these isolates including beta-lactam, aminoglycoside, sulphonamide, fosfomycin and quinolone resistance genes. The KPC-2 producers had varying resistance profiles and also did not contain any overlapping plasmids potentially indicating chromosomal origin. KPC is the second most commonly identified

carbapenemase detected through surveillance of colonisation and infections in Irish hospitals (Health Protection Surveillance Centre, 2019e).

#### 4.4.4 Future antibiotic resistance gene threats

The natural aquatic environment represents ideal conditions where ubiquitous, non-pathogenic bacteria come in contact with clinically significant pathogens (Allen et al., 2010). This environment presents the opportunity for the potential transfer of antibiotic resistance genes from harmless bacteria to virulent and potentially pathogenic bacteria. A recent study by Zhang et al. (2021a) characterised different antibiotic resistance genes into current and future threats based on pathogenicity, human-associated enrichment, and the potential of gene transfer. A total of 5 different antibiotic resistance genes classified as emerging threats were detected in water and/ or sewage samples in this study. These included *bla*<sub>CMY-2</sub> (n=1), *bla*<sub>SHV</sub> (n=31), *aadA* (n=54), *erm(B)* (n=5) and *catA* (n=14) which confer beta-lactam, aminoglycoside, macrolide and chloramphenicol resistance respectively. According to Zhang et al. (2021a), some of these resistance genes have only recently been detected in the ESKAPE pathogens group, including SHV, ermB and catA. Amongst the environmental isolates, SHV was mainly detected in *Klebsiella pneumoniae* isolates (26/31), with the remainder including SHV-12 detection (5/31) in 2 *Enterobacter*, 2 *E. coli* and 1 *Raoultella* isolate. The detection of this beta-lactamase gene in environmental isolates belonging to the ESKAPE pathogens group further illustrates the need for more regular monitoring of the aquatic environment for the presence of clinically significant antibiotic resistant organisms. Frequent monitoring would aid in the establishment of the origins of different resistance genes and assist in the surveillance of future emerging threats. The findings in this paper illustrate how environmental monitoring would significantly benefit from genomics based analysis allowing for the detection of emerging resistance gene threats, rather than relying solely on a culture-based approach for resistance detection.

#### 4.4.5 Sequence types detected and core genome MLST comparisons

Examination of the sequence types detected revealed the presence of *E. coli* and *Klebsiella* strains associated with multi-drug resistance typically circulating in clinical environments. A prime example includes gut coloniser *E. coli* ST131 which is frequently linked to CTX-M production and fluoroquinolone resistance (Whitmer et al., 2019). This sequence type is commonly identified from urinary tract infections (Muller et al., 2021), but has also been isolated in cases of sepsis (Paramita et al., 2020). The majority of *E. coli* sequence type ST131 isolates that were selected for sequencing from this study harboured one CTX-M variant (23/24), highlighting the similarities amongst strains in the clinical and natural environments. A study by Hu et al. (2013) identified sequence types of CTX-M producing *E. coli* from a variety of sources including water, swine and humans. Interestingly, sequence types ST131 and ST38 were both solely detected from waters and human sources, potentially indicating anthropogenic activity as the source of these isolates in the environment. In the longitudinal survey sampling periods, ST38 was detected in 5 individual waters samples as well as one sample collected from airport sewage. Three of the ST38 isolates harboured an OXA-48, while one harboured NDM-5, both of which have previously been reported in this sequence type from human isolates (Abid et al., 2021, Brehony et al., 2019, Khan et al., 2018).

Core genome MLST comparisons identified two identical *E. coli* isolates belonging to the ST131 collection (B20400, B20369) originating from a wastewater treatment plant influent and a seawater in Cork, collected on the same day. These isolates shared identical resistance genes conferring reduced susceptibility to aminoglycosides, tetracyclines, trimethoprim and beta-lactam antibiotics. The close proximity of these sites (3.2km) and the location of the seawater sample downstream of the discharge from this plant potentially

indicates that this bacteria originated from human wastewater. However, it was not detected in the effluent sample collected on the same day from this wastewater treatment plant. An additional *E. coli* sequence type (ST167) contained two identical isolates from sewage (B20148; nursing home E) and water (B20104; estuary B2) in Galway City. This analysis indicates that the detection of this particular isolate in estuarine waters originated from human sewage sources. These two isolates also harboured identical antibiotic resistance genes, detected using ResFinder. Although *bla*<sub>CTX-M-15</sub> and *bla*<sub>OXA-1</sub> were the only beta-lactamase genes detected in these isolates, this sequence type is commonly associated with NDM carriage in the clinical environment (Wu et al., 2019b). However, two *E. coli* (B20127, B20159) from seawaters surrounding a wastewater treatment plant in Galway City belonging to ST167 harboured *bla*<sub>NDM-5</sub>. These two isolates differed from each other by 107 loci, and from the identical nursing home (B20148) and estuarine water (B20104) isolates by over 500 loci.

A significant pattern emerged in relation to *bla*<sub>NDM-5</sub> detection more frequently in *E. coli* sequence type ST405 in comparison to any other sequence type. A total of 7/9 isolates belonging to this sequence type harboured NDM-5, and all originated from aquatic samples. This sequence type was previously linked to NDM-5 carriage in clinical isolates reported from Italy (Bitar et al., 2017), Japan (Takayama et al., 2020) and India (Ranjan et al., 2016). Two of these aquatic isolates were identical (B19818, B19852) based on pairwise allele alignments at 2513 loci from a region in Cork receiving multiple anthropogenic discharges, potentially indicating human origin. These isolates were collected just on day apart and both harboured the same three plasmids including IncFIB, IncI Gamma, and p0111.

Similarly to the *E. coli*, many of the *Klebsiella* sequence types repeatedly detected from the environment mirror those that are commonly identified in cases of human carriage and infection. For example, 5 isolates belonging to

ST323, all harbouring *bla*<sub>CTX-M-15</sub>, *fosA6* and *fosA7*, were identified from 3 water and 2 sewage samples in this study. This *Klebsiella pneumoniae* sequence type was previously identified harbouring KPC-2 from a human sputum sample in China (Gong et al., 2018), and harbouring *bla*<sub>CTX-M-15</sub> from urine samples, along with rectal and wound swabs in Australia (Gorrie et al., 2018). Interestingly, two identical isolates (B19023, B19574) belonging to ST323 were isolated from the same nursing home effluent, collected over 8 months apart. This indicates potential long-term persistence of this bacterium in the gut of the nursing home resident(s), or alternatively persistence of the bacterium in the septic tank housed within the grounds. Repeated environmental contamination through runoff is also a possibility due to the location of the tank on a decline in respect to the nursing home. However, the tank normally remains covered so this possibility is less likely. Both isolates from this nursing home harboured identical resistance genes including *bla*<sub>CTX-M-15</sub>, *bla*<sub>SHV-187</sub>, *fosA6*, *fosA7*, *oqxA* and *oqxB*. Both also contained five identical plasmids including Col(pHAD28), Col440II, IncFIA(HI1), IncFIB(K) and IncFII(pKP91).

A total of 6 *Klebsiella pneumoniae* ST17 isolates were identified from different water samples. Two of these isolates were identical (B20121, B20134) and harboured *bla*<sub>OXA-48</sub>. Sample collection took place on the same day just 0.9km apart. Sequence type ST17 was previously reported in ESBL producing *Klebsiella pneumoniae* isolated from human faecal samples in Bulgaria (Markovska et al., 2021). Similarly, a clinical isolate characterized as 'extensively drug resistant' belonging to ST17, harbouring KPC from urine, was recently identified in Brazil (Nakamura-Silva et al., 2021). These clinical isolates highlight the pathogenic potential of this particular sequence type.

Sequence type ST309 was the fourth most common *Klebsiella* sequence type detected (B20338, B20377, B20384). Notably, all three *Klebsiella* ST309 isolates originated from polluted waters in different samples from Cork (River H2, River H, Estuary E1), harboured *bla*<sub>OXA-48</sub>, and were identical based on cgMLST

comparisons. Sample collection took place over 24 hours and the three sampling sites were located in close proximity to one another, with a distance of 9.7km between the two furthest points. All three isolates harboured IncFIB(K) and IncL plasmids. Reports of clinical ST309 *Klebsiella* isolates harbouring *bla*<sub>OXA-48</sub> have previously been published from Ireland (Brehony et al., 2019) and Yemen (Alsharapy et al., 2020). The ST309 isolates in this study were compared to those analysed by Brehony et al. (2019) using cgMLST comparisons. This comparison revealed that between 35 to 37 loci varied between the three isolates from this study and the two OXA-48 producing clinical isolates. This variability indicates significant differences in the core genomes between the clinical and environmental isolates.

A further two identical carbapenemase producers (B19819, B19846) were isolated from the same polluted region in Cork one year prior (2019). Sampling locations (Estuary E, River H) were located just 3.3km apart and sample collection took place one day apart (Supplementary Table C6). These *Klebsiella* isolates harboured *bla*<sub>KPC-3</sub> and belonged to sequence type ST231. Isolate B19846 harboured one additional plasmid (IncFIB(K)), totaling 7 (Figure 4.5), and also contained the *dfrA14* trimethoprim resistance gene which was not detected in B19819. This region in Cork receives multiple discharges including storm water overflows and raw sewage discharges from anthropogenic sources.

#### 4.4.6 Plasmid detection

The potential for widespread dissemination of antibiotic resistance genes of clinical concern amongst environmental isolates is high, due to the majority of sequenced isolates containing one or more plasmid (140/146). Due to the limitations of short read sequencing it could not be established which antibiotic resistance genes reside on plasmids. However, based on the literature, there are certain plasmids that are commonly linked to the carriage of different clinically significant antibiotic resistance genes. Of particular interest is the

carbapenemase resistance genes, and an example of such is the detection of *bla*<sub>OXA-48</sub> on the IncL plasmid. A total of 15 out of 18 of the OXA-48 isolates in this study also contained this plasmid (Figure 4.5). A previous study by Bleichenbacher et al. (2020) detected *bla*<sub>OXA-48</sub> on an IncL plasmid within *Raoultella ornithinolytica*, isolated from freshwater in Switzerland. In a previous study by Brehony et al. (2019), it was confirmed that 93/109 clinical isolates obtained from Irish hospitals contained both *bla*<sub>OXA-48</sub>, in addition to the backbone genes for the pOXA-48-like plasmid (IncL-type). Therefore, it may be the case that OXA-48 carriage in the 15 environmental isolates from this study are plasmid-borne. The potential for horizontal gene transfer of the IncL plasmid that harbours *bla*<sub>OXA-48</sub> has recently been proven to be high amongst clinical isolates in Germany (Hamprecht et al., 2019). Therefore, this plasmid type may harbour a significant proportion of the responsibility for the high number of OXA-48 producing isolates in Irish hospitals, rather than one particular clone. The detection of numerous OXA-48 isolates that harboured the IncL plasmid in this study further demonstrates the overlap between the clinical and natural environments. As mentioned previously, one *E. coli* isolate (B19837) characterised as ST410 from river water harboured an OXA-48-like gene (*bla*<sub>OXA-484</sub>). A recent study investigated the genetic characteristics of a clinical *E. coli* isolate which harboured *bla*<sub>OXA-484</sub> also belonging to ST410 (Sommer et al., 2021). They identified carriage of the *bla*<sub>OXA-484</sub> gene on the IncX3 plasmid, which was also detected in B19837, along with 6 other plasmids. This potentially indicates plasmid carriage of the carbapenemase resistance gene, however further testing is necessary to confirm this.

Comparably, the second most commonly detected carbapenemase gene (*bla*<sub>NDM</sub>) displayed a similar pattern in which the majority of isolates contained the IncFIB plasmid (11/14). Both *bla*<sub>NDM-1</sub> and *bla*<sub>NDM-5</sub> have previously been detected on IncFIB plasmids within isolates of clinical origin (Wu et al., 2019b). However, the *bla*<sub>NDM</sub> genes have also been commonly detected within other

plasmid replicon types including IncFIA, IncFIB, IncFII and IncX3. The location of the *bla*<sub>NDM-5</sub> gene has also recently been confirmed to reside within an IncFIB plasmid in an environmental isolate from natural waters in Switzerland (Bleichenbacher et al., 2020).

#### 4.4.7 Sampling cold spots

A total of 6 cold spot locations were sampled twice throughout the longitudinal survey periods to evaluate the natural resistome in the absence of anthropogenic activity. This encompassed water bodies that were free from known discharges upstream or in close proximity to sample sites, and low to medium agricultural activity (Figure 4.1). These sampling sites included estuary C and C1 in Fingal, river A and A1 in Galway City as well as beach J and lake D in Galway County. Extended spectrum beta-lactamase producing Enterobacterales were identified from the samples collected from Galway City and Fingal in line with the findings of the point prevalence survey. As discussed previously estuary C received storm water overflow discharges in the previous years and has a large wildlife presence (Hooban et al., 2021). Estuary C1 was located just 1.3km from estuary C. River A is located at the boundary, upstream of Galway City. However, many houses, boats and wildlife reside in the area. Four isolates were selected for sequencing from river A (B19404), estuary C (B19678(a)) and estuary C1 (B19673, B20289) across both sampling periods. Isolate B19404 displayed a limited resistance gene profile with the detection of *bla*<sub>CTX-M-15</sub> and efflux pump *mdf(A)*. In contrast, sequenced isolates from estuary C and C1 harboured *bla*<sub>CTX-M</sub> in addition to sulphonamide (*sul2*), tetracycline (*tet(A)*), quinolone (*qnrS1*), aminoglycoside (*aph(3'')-Ib*, *aph(6)-Id*) and chloramphenicol (*floR*) resistance genes. This is potentially owing to the fact that previous storm water overflow discharges fed into the water body. The remaining two cold spot locations within Galway County did not harbour any

ESBL producers. The location of these two sites were in more remote and less densely populated regions.

Comparison of the cold spot locations to the waters receiving anthropogenic discharges demonstrates the impact of anthropogenic activity on the dissemination of antibiotic resistance. The detection of carbapenem resistant and carbapenemase producing isolates solely in 'hot spot' locations indicates that these resistance types in the environment may be primarily of human origin. Similarly, the two most remote regions (beach J and lake D) were void of ESBL detection in comparison to the areas with a larger human presence. Moreover, no Enterobacterales were isolated across either sample collection round for beach J indicative that the natural presence of Enterobacterales is less likely in uncontaminated seawater.

## **4.5 Conclusion**

Information in relation to the aquatic environment as a reservoir and potential transmission route of antibiotic resistance is currently lacking, due to the absence of routine monitoring. To address this knowledge gap, this study investigated the natural resistome of different aquatic environments across Ireland on a longitudinal basis. The detection of extended spectrum beta-lactamase and carbapenemase producing Enterobacterales in waters across all seasons demonstrates the need for extended periods of bathing water monitoring, especially in countries where recreational water use is year round. A harmonised surveillance approach is necessary which encompasses screening for clinically significant, antibiotic resistance threats in order to adequately inform public health. Attempts to identify the sources of environmental antibiotic resistance were successful through cgMLST comparisons, which revealed identical isolates from different wastewaters and the surrounding waters. The influence of anthropogenic activity was further established through comparison of areas receiving multiple discharges to 'cold spot' locations. The

overall body of evidence accumulated from this study suggests that anthropogenic discharges play a significant role in the dissemination of antibiotic resistance to the natural environment. Further work is needed to fully elucidate the potential health implications of ingestion of these resistant organisms through recreational exposure.

## **Chapter 5: Discussion, Limitations and Future Work**

## 5.1 Discussion

The environmental component of the One Health triad is the most poorly characterised and often underestimated aspects in relation to antibiotic resistance dissemination. However, the research outlined in this thesis demonstrates the presence of multi-drug resistant Enterobacterales in a variety of natural waters, ranging from sites receiving multiple sources of anthropogenic pollution, to those free from known discharges. One of the key findings from this work was the repeated detection of carbapenemase producing Enterobacterales (CPE) in both waters and sewage sources across all seasons, as outlined in Chapters 3 and 4. The most prominent regions for repeated CPE detection in the aquatic environment included Galway city and Cork harbour, both of which are heavily polluted by storm water overflows, primary and secondary urban wastewater discharges, indicating anthropogenic origins (Chique et al., 2019). Evidence to indicate that CPE detected in the environment are of human origin is also justified by the fact that the types of carbapenemase genes detected reflect those in circulation in the clinical environment (Health Service Executive, Unpublished). These include *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub> and *bla*<sub>NDM</sub>. Furthermore, the absence of carbapenem resistance in isolates collected from locations lacking known discharges, such as raw sewage and storm water overflows, further demonstrates this link between human and environmental isolates in polluted regions. Although the origins of some aquatic isolates were successfully linked to isolates found in sewage sources such as nursing homes and wastewater treatment plant influents using cgMLST, this was not the case for any of the CPE isolates. Therefore, the origins of these CPE in environmental waters can be hypothesised, but not definitively established at a genetic level by this study.

Although Ireland ranked relatively high in relation to colonisation and infection caused by CPE in comparison to other European countries (ECDC, 2019), the vast

majority of CPE isolates are identified by screening (85-90%) rather than diagnostic specimens (10-15%) (HPSC, 2022). Alterations to the gut microbiota through colonisation with resistant bacteria has been previously linked to antibiotic usage, prolonged hospital stays and travel abroad (Angue et al., 2015; Tacconelli et al., 2009). However, to date there are knowledge gaps relating to colonisation by multi-drug resistant bacteria due to exposure to the natural aquatic environment. Of the studies that have examined this potential route of transmission to date, none have focused on CPE detection (Leonard et al., 2018; Amarasiri et al., 2020). The importance of addressing this knowledge gap is apparent from a public health perspective, due to the detection of two *Klebsiella* isolates from water bodies in this research that displayed a resistant phenotype to 14/15 of the antibiotics tested (Figure 4.4). These two *Klebsiella* isolates (B19819, B19846) both harboured *bla*<sub>KPC-3</sub>, along with a multitude of other resistance genes including further beta-lactamases (*bla*<sub>OXA-9</sub>, *bla*<sub>SHV-106</sub>, *bla*<sub>TEM-1A</sub>) and fluoroquinolone resistance genes (*oqx*A, *oqx*B). Therefore, treatment options would be extremely limited if these isolates were responsible for causing an infection. Tacconelli et al. (2009) has previously demonstrated that the same bacterial strains were responsible for colonisation and subsequent infection in a cohort of patients in hospital. Similarly, Tseng et al. (2018) also predicted an increased risk of multidrug resistant gram-negative infections following colonisation in a hospital setting.

The detection of CPE from wastewaters during this study included hospitals, wastewater treatment plant influents and one airport sewage sample. Hospital effluent has previously been identified as a significant source of CPE, which leads directly into the municipal wastewater system in Ireland, prior to reaching a wastewater treatment plant (Cahill et al., 2019). This is also the case in other countries such as Slovenia (Rozman et al., 2020). Recent studies have examined the potential of monitoring hospital sewage and wastewater treatment plant influents as a tool of mapping the prevalence of different types of micro-

organisms within the population. Flach et al. (2021) recently examined the value of wastewater monitoring at a hospital in Sweden, alongside conventional CPE screening of patients, as an efficient surveillance system for a large hospital population. Their data demonstrated strong links between the routine detection of CPE from patients, along with the detection of CPE and carbapenemase genes directly from sewage samples, including *bla*<sub>OXA-48-like</sub>, *bla*<sub>NDM</sub> and *bla*<sub>KPC</sub>. However, in the case of *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> there was no concordance evident due to high concentrations of the genes detected in sewage, but no corresponding patients were colonised with Enterobacterales harbouring these genes. This indicates the presence of these genes in non-Enterobacterales (Flach et al., 2021). Alongside wastewater monitoring, onsite treatment targeting pharmaceuticals, along with antibiotic resistant bacteria and genes would reduce the release of these antibiotic resistance enhancers into the sewage network, and ultimately the aquatic environment which receives effluent discharges. The benefits of onsite treatment have been demonstrated in the Netherlands, in which one hospital employed an onsite wastewater treatment system (Pharmafilter) using a combination of advanced technologies (Paulus et al., 2019). This study demonstrated superior removal of antibiotics and antibiotic resistance genes than convention activated sludge treatment at the existing wastewater treatment plants (Paulus et al., 2019). Onsite treatment at healthcare facilities would provide additional benefits in relation to the treatment of other hazardous waste including chemical, radioactive and pathological (WHO, 2014).

Wastewater surveillance has recently emerged as a successful method for tracking and predicting the presence of the SARS-CoV-2 virus within a sample of the population (Fitzgerald et al., 2021). The benefits of a successful wastewater surveillance system for CPE was recently evaluated by Blaak et al. (2021). This study examined wastewater treatment plant influent and effluent on a national scale in the Netherlands. Key findings included the detection of CPE at 89% of

WWTPs, including 87 influent and 53 effluents, demonstrating widespread colonisation with CPE (0.02%), along with dissemination of CPE in the effluent to environmental waters. A routine wastewater surveillance system can provide a greater representation of colonisation rates, in addition to routine hospital screening procedures. Benefits of this type of surveillance system may also include the detection of potential emerging antibiotic resistance threats. For example, the detection of *Klebsiella* ST1563 (B19321) in this research from wastewater treatment plant influent is the first published report of the *bla*<sub>OXA-48</sub> gene within this sequence type (Chapter 3).

The detection of the *bla*<sub>NDM-5</sub> gene within an *E. coli* isolate (B19695) belonging to sequence type ST38 from airport sewage on one occasion was also a noteworthy finding from this study. Recent publications outlining clinical isolates belonging to ST38 and also harbouring *bla*<sub>NDM-5</sub> were reported from East and South Asia, including China (Sun et al., 2019; Li et al., 2021), Bangladesh (Khan et al., 2018) and Taiwan (Huang et al., 2021). Although NDM is the fourth most commonly reported carbapenemase in relation to screening and cases of infection in Ireland (Health Service Executive, Unpublished; Vellinga et al., 2021), no information is provided with regards to the sequence type in Irish reports (Health Service Executive, Unpublished). However, to the authors knowledge, *E. coli* ST38 harbouring *bla*<sub>NDM-5</sub> has not been previously reported in published literature in Europe. The ECDC released a technical report focusing on cross-border transmission of CPE in 2011, which reported 18 cases of international importation of CPE following hospitalisation in another country (ECDC, 2011). Nordahl Petersen et al. (2015) previously reported a higher abundance and diversity of resistance genes carried on airplanes from South Asia compared to other regions of the world. Therefore, surveillance of airport wastewater along the sewage network could provide evidence that onsite treatment may be warranted as a preventative measure for the introduction of novel strains of carbapenemase carrying bacteria into Ireland.

Another important type of beta-lactamase producers that were repeatedly detected during this research included ESBL producing Enterobacterales. A small proportion of ESBLs were identified in environmental isolates with novel sequence types. These included isolates from natural waters, which harboured different *bla*<sub>CTX-M</sub> variants. The sequence types of these isolates did not match any previously designated types, and therefore were labelled as unknown. Natural water bodies can become significant reservoirs where bacterial strains, that are not typically in circulation in the clinical environment, may acquire antibiotic resistance genes via horizontal gene transfer (Allen et al., 2010). Consequently, new sequence types that were previously unrelated to ESBL gene carriage may emerge and become important human pathogens. Some antibiotic resistance genes have originated from bacterial strains prevalent in environmental reservoirs. For example, the origins of the *bla*<sub>CTX-M</sub> gene was linked to the chromosome of *Kluyvera*, which is ubiquitous in the environment and rarely associated with infection (Bush et al., 2018). Therefore, the acquisition of the *bla*<sub>CTX-M</sub> gene within these isolates of unknown sequence type may have resulted from anthropogenic influence, or due to the natural presence and dissemination of this gene amongst environmental isolates. The latter reason for the detection of beta-lactamase genes amongst environmental isolates is often omitted as a potential point of origin for resistance genes in aquatic strains, as outlined in Chapter 2.

Unlike CPE, there is evidence of colonisation with ESBL producing *E. coli* in people that frequently participate in recreational water activities (Leonard et al., 2018). ESBL detection was widespread across the majority of environmental samples in this study, indicative that it is abundant in the natural environment. This potentially increases the likelihood of colonisation due to recreational exposure. The detection of ESBL producing Enterobacterales in 'cold spot' locations further demonstrates how widespread it is in the aquatic

environment, while further justifying the potential of resistance gene dissemination from other ubiquitous strains.

In depth analysis of a selection of ESBL and carbapenemase producing isolates was achieved at a genetic level through the application of whole genome sequencing. This enabled a direct comparison of isolates of similar sequence type, by comparing a collection of gene loci within the core genome. This analysis revealed the presence of highly similar isolates across different water bodies, alongside similar isolates originating from water and sewage sources (Chapters 3 and 4). The detection of similar isolates across the aquatic environment may be as a consequence of anthropogenic contamination or due to horizontal gene transfer between environmental strains. As highlighted in Chapter 2, there are a lack of studies that examine mechanisms of horizontal gene transfer, including transduction and transformation, although extracellular DNA uptake and bacteriophage mediated transmission are hypothesized to play an important role in the diversification of environmental microbial ecosystems (Leclerc et al., 2019; von Wintersdorff et al., 2016). Moreover, knowledge relating to interspecies transfer is also lacking due to the predominant use of *E. coli* in mating assays. Although investigation of gene transfer mechanisms amongst aquatic isolates was beyond the scope of this research, it is an important knowledge gap that needs to be addressed. This was also echoed in the Irish research gap analysis relating to environmental dimensions of antimicrobial resistance, which highlighted the lack of information on the transfer of antibiotic resistance genes from environmental isolates to pathogenic bacteria (Cahill et al., 2021).

In Ireland, the presence of raw sewage discharges, failure of many wastewater treatment plants to reach EU standards alongside storm water discharge events from the sewage network creates perfect opportunities for environmental dissemination of antibiotic resistance. Over the duration of sample collection as part of this research (2018-2020), the number of raw sewage discharges in the

republic of Ireland reduced by 2, from 36 to 34 (EPA, 2019; EPA, 2021). Direct wastewater discharges contaminate the environment with antibiotic resistant bacteria of clinical concern (Mahon et al., 2017). In the case of wastewater that travels through the sewage network, discharges may occur prior to reaching the treatment plant, including storm water overflows due to heavy rainfall, breakdown of pumps, blockages or structural defects (EPA, 2021). A reported 27 of these types of incidents, occurred during the bathing water season alone in 2020. Furthermore, a total of 12 wastewater treatment plants in Ireland were deemed non-compliant with the EU's treatment standards in 2020, accounting for 54% of total wastewater in all urban areas (Directive 91/271/EEC, 1991). A previous meta-analysis of published literature revealed that the processing steps involved in wastewater treatment processes increase the overall concentrations of resistant bacteria post treatment, although overall bacterial numbers may be reduced (Harris et al., 2012). Due to these combination of factors, the detection of indistinguishable isolates from sewage and waters based on cgMLST was a possible outcome. Overall, Ireland's wastewater management system requires urgent intervention, in order to protect the natural environment.

Steps that could be taken include the removal of all raw sewage discharges by investing in new wastewater treatment plants, or redirecting sewage pipes to pre-existing plants where capacity permits. Irish water has outlined that it aims to achieve removal of all raw sewage discharges in Ireland by 2027 (EPA, 2021). Examination of storm water overflow events is needed to identify plants that require increased capacity. Alternatively, the removal of combined sewer systems which include wastewater and surface water run-off would alleviate capacity surplus which occurs during stormy weather. The failure of 12 plants to meet current EU treatment standards needs to be addressed, particular due to the fact that 9/12 failed secondary standards of treatment (Directive 91/271/EEC, 1991). Alternative approaches for wastewater treatment,

including the use of integrated constructed wetlands should be considered as a natural alternative for treatment. Prendergast et al. (2022) showed a statistically significant reduction in the number of resistant bacteria isolated from influent versus effluent samples from four integrated constructed wetlands in Ireland receiving farm wastewater. Similarly, additional treatment should be considered at pre-existing treatment plants such as the incorporation of anaerobic digestion. A recent study by Wang et al. (2021) demonstrated a reduction in antibiotic resistance genes using dry anaerobic co-digestion methods. Overall, wastewater treatment processes were not designed with the aim to specifically remove antibiotic resistant genes or antibiotic resistant bacteria (Uluseker et al., 2021). An important consideration when improving upon wastewater treatment is that it must be effective at removal of antibiotics, antibiotic resistant bacteria and antibiotic resistance genes, which are all excreted in human wastewater (Singer et al., 2019). Recent reviews have suggested a combination of treatment processes including activated sludge treatment using aerobic/anaerobic reactors followed by advanced processes including UV, oxidation and ozonation (Uluseker et al., 2021; Gao et al., 2022).

The direct discharge of effluent from wastewater treatment plants enables the potential dissemination of antibiotic resistant bacteria to environmental waters. At present, monitoring of the aquatic environment is limited to the bathing water season, during which the levels of intestinal enterococci and *E. coli* are measured on a monthly basis (Directive 2006/7/EC). The research presented in this thesis demonstrates that there is no relationship between the levels of faecal indicator organisms and the detection of clinically significant bacteria such as ESBL or carbapenemase producing Enterobacterales. A recent review by Farrell et al. (2021) highlighted many different bacterial, viral and protozoan organisms of public health concern in environmental waters worldwide. One important recommendation that arose from this review was the need for a wider range of testing employed as part of the bathing water directive, including

antibiotic resistance detection. As outlined in previous chapters, it is recommended that bathing water monitoring is extended year round in line with recreational usage in Ireland, and encompasses some form of characterisation of the resistome. A simple modification to the colilert-18 test in use at present, through addition of antibiotic powders, would provide valuable information on the quantity of resistant *E. coli* and intestinal enterococci present (Galvin et al., 2010). Ideally, a harmonised approach would be applied across EU countries for comparability purposes, something that is lacking in the field of environmental microbiology as highlighted in Chapter 2. Alternatively, screening for one or more genetic markers encoding antibiotic resistance of clinical concern could be considered as an indicator of widespread contamination with resistant organisms and/or antibiotic resistance genes. However, this approach would require additional equipment and expertise, along with the selection of appropriate genetic markers encompassing those that pose the most risk to public health. Potential markers of antibiotic resistant bacteria/gene contamination of the environment was discussed at length by Amarasiri et al. (2020). This review highlighted a lack of consensus amongst environmental scientists in relation to the optimal marker(s) for evaluation of the environmental resistome, including antibiotic pollution, antibiotic resistant bacteria and antibiotic resistance gene detection. Due to the variability in prescribing practices and prevalence of different resistance types across countries, a standardised surveillance marker may not be the optimal approach for environmental monitoring. However, comparable surveillance data such as the Tricycle protocol suggested by the WHO, using ESBL *E. coli* in a One Health approach to monitor resistance across humans, the food chain and the environment would provide valuable data on a global scale (WHO, 2021). Adopting this type of approach would enable further comparisons across all three sectors as part of the One Health triad, as surveillance programmes in

place for humans and animals are superior than environmental monitoring for antibiotic resistance at present.

The Irish government recently published 'Ireland's Second One Health National Action Plan on Antimicrobial Resistance (2021-2025)', which outlines plans to tackle the environmental dissemination of antibiotic resistance (Government of Ireland, 2021). These include the increased surveillance of the environment for antimicrobials, a national collection scheme for pharmaceutical waste and continued research, particularly in the area of human health risks due to environmental exposure to antibiotic resistance. Antibiotic stewardship amongst the human and agricultural sectors has become a focus for action in recent years, in an attempt to reserve the use of last resort antibiotics such as carbapenems (European Commission, 2017; Lloyd & Page 2018). However, contamination of the natural environment has not seen such significant improvements. The burden of antibiotic resistance was recently estimated in order to demonstrate the extent of this public health crisis globally (Antimicrobial Resistance Collaborators, 2022). It was estimated that 4.95 million deaths were associated with antibiotic resistance in 2019, of which 1.27 million deaths were directly attributed to antibiotic resistance. These figures demonstrate the detrimental consequences of antibiotic resistance.

Overall, this study established that the natural environment is a key reservoir of antibiotic resistance of clinical concern, along with providing evidence of genetic links between isolates of sewage and aquatic origin, demonstrating the influence of anthropogenic activity on resistance dissemination.

## **5.2 Limitations**

Although this research applied a valid approach in identifying antibiotic resistance across a range of water and sewage samples, some limitations relating to sample collection and subsequent processing employed must be considered. For example, collection of influent and effluent samples from

wastewater treatment plants included grab samples, which were collected at similar points in time. However, if composite sample collection took place to account for retention time, a more accurate comparison and stronger representation of pre and post treated wastewater could have been drawn from this study. Moreover, the number of wastewater treatment plants sampled was limited to one per local authority area, per sampling round (n=12), due to limitations placed on sample collection by the utility manager. More significant conclusions could have been drawn if there were an increased number of wastewater treatment plants sampled in each of the areas with varying processes and technology used to evaluate the effectiveness of different levels of treatment.

Similarly, sewage sample collection from healthcare sites was limited by the fact that participation was at the discretion of the hospital or long term care facility. Consequently, a significantly higher number of water samples were collected in comparison to sewage samples. In the predominant number of healthcare facilities, no onsite septic tank was present so sample collection took place at a manhole with an open flow pipe. These samples were therefore not an accurate representation of the population residing at these premises. The open flow pipe system often increased the difficulty in collecting an adequate sample volume, particularly at smaller nursing homes, resulting in variability in sample volume collection.

Further sampling related limitations included the lack of inclusion of animal faeces in this study, although it represents an important component of the One Health triad. Comparable Enterobacterales from animals in areas of interest potentially may have established the origins of further aquatic isolates through genetic comparisons. In particular, in areas that were densely populated with wildlife, it may have confirmed an additional source of resistance dissemination. Although no animal related data was presented in this thesis, there were studies based in Ireland that identified antibiotic resistant *E. coli* in wildlife including

deer and gull faeces, highlighting the capability of animals to act as vectors for resistance transmission (Carroll et al., 2015). As part of the overall AREST project, work is currently ongoing exploring how wildlife and animal manure applications are further contributors to the dissemination of antibiotic resistance (Tyrrell et al., 2019).

Another sampling related consideration in this study was the lack of extreme weather conditions during sample collection. The sampling schedule was initially designed to include sample collection throughout the four seasons in an attempt to elucidate the effects of different weather conditions on the presence of resistant Enterobacterales in the environment. However, when rainfall data was analysed in Chapters 3 and 4, the vast majority of samples did not have significant rainfall levels prior to sample collection. These limitations were highlighted, and the need for further studies which include more intensive sampling pre and post extreme weather events is necessary to draw any significant conclusions.

The final sampling related limitation included the delays experienced due to the SARS-CoV-2 pandemic. Sampling of the longitudinal survey round 2 was delayed by approximately four months due to University closures. The resulting implications meant that sampling in Fingal and Cork took place during similar months one year apart, between the two rounds of collection in the longitudinal survey. Additional consequences of the pandemic resulted in difficulty in sourcing supplies such as DNA extraction kits alongside delayed access to laboratories in Cork and Fingal due to local restrictions.

In relation to limitations associated with the subsequent processing of samples, there are two primary aspects that should be discussed. The first is the use of a culture based approach using selective agars. Due to the high numbers of samples processed, the application of selective agars was valid from a resources and time perspective in targeting bacteria and antibiotic resistance types of

significant clinical concern. However, by taking a holistic view, it provides limited knowledge in relation to the vast environmental resistome which includes other types of bacteria, bacteriophages and extracellular DNA that all have the potential to harbour resistance genes (Sivalingam et al., 2020; Sala-Comorera et al., 2021). An additional aspect relating to culture, is that not all viable cells are culturable, which is a mechanism that enables long term persistence in the environment, surviving adverse conditions (Bodor et al., 2020). This further illustrates that the antibiotic resistant Enterobacterales detected during this study, may represent only a small fraction of Enterobacterales harbouring resistance genes in the environment.

The second aspect of the methodology applied during this research that should be considered was the application of a qualitative approach in relation to antibiotic resistance detection. The collection of large volumes of water (30L) samples along with filtration and enrichment optimised the potential for detection of isolates of interest. For the purposes of this research, this approach was effective. However, this methodology prevented accurate quantification of resistant isolates present in the initial sample, and would not be a time efficient method that could be adopted by environmental health officers. Quantification of resistant organisms or genes present would provide additional information in relation to the risks to public health.

### 5.3 Future Work

Additional avenues of future work are ongoing at present using isolates obtained during the work carried out in this thesis. A selection of CPE isolates from the environment in Galway are being subjected to long read sequencing and hybrid genome assembly, using the short read sequencing carried out during this research. These include individual isolates harbouring *bla*<sub>OXA-48</sub> (n=12), *bla*<sub>NDM-5</sub> (n=7) and *bla*<sub>KPC-2</sub> (n=4). An additional group of isolates from a hospital, within a similar geographical area in Galway are also undergoing long read sequencing analysis and hybrid analysis in order to compare the two. These include isolates from clinical infections and colonisation, alongside hospital environmental strains collected from areas such as showers, sinks, toilets, etc. Hospital based isolates selected included Enterobacterales harbouring *bla*<sub>OXA-48</sub> (n=10), *bla*<sub>NDM-5</sub> (n=5) and *bla*<sub>KPC-2</sub> (n=3). This form of sequencing will allow direct comparison of the mobile genetic elements such as plasmids, along with identifying the location of the carbapenemase genes within the bacterial genome. Preliminary results are revealing links between the hospital and environmental isolates, consolidating the associations identified between human wastewater and aquatic environmental isolates ascertained using cgMLST during this research.

Further analysis is also being carried out on Enterobacterales harbouring the *mcr* gene. Chapter 4 outlined the detection of the *mcr-9* gene in 4 environmental isolates, which was the first published report of colistin resistance genes in the Irish environment. The *mcr-9* gene was first identified in a clinical *Salmonella* isolate in 2019 (Carroll et al., 2019). To date there is conflicting evidence published as to whether this *mcr* gene variant can confer colistin resistance. Tyson et al. (2020) tested over 100 *Salmonella* and *E. coli* isolates harbouring the *mcr-9* gene and concluded that all were susceptible to colistin (MIC  $\leq 1$   $\mu\text{g/ml}$ ). However, Kieffer et al. (2019) demonstrated that low concentrations of colistin could induce *mcr-9* gene expression and subsequently

confer resistance in a clinical *E. coli* isolate. The genetic sequence of the environmental isolates from Chapter 3 were subsequently re-examined due to the fact that the resistance gene database used at the time (ResFinder v3.2) did not include the most recent *mcr* gene variants. This revealed the presence of three additional *mcr* harbouring isolates (two *mcr-9*, one *mcr-8*), amounting to 7 in total from this research. One additional environmental isolate collected from an Irish integrated constructed wetland harbouring *mcr-8*, and one clinical isolate which contained the *mcr-9* gene were also included to undergo further analysis. This included testing the colistin minimum inhibitory concentration (MIC) in order to establish whether these genes (*mcr-8* and *mcr-9*) confer colistin resistance. Long read sequencing is also being carried out along with hybrid genomic assembly, to establish the location of the *mcr* gene and fully characterise the plasmids present.

Further work is also ongoing as part of the overall AREST project. In an attempt to further elucidate the risks of environmental recreational exposure to antibiotic resistant organisms, a risk ranking analysis is being conducted using Monte Carlo risk assessment methodologies. Additional sample collection took place at a beach in Galway which was in direct receipt of raw sewage, along with a freshwater agricultural stream. Previous detection of NDM producing *E. coli* at this beach was published by Mahon et al. (2017), which provides a graphical depiction of the site. Samples were collected on seven occasions across four months (January – April 2021). Sampling included seawater in direct receipt of the raw sewage discharge, a bathing area approximately 200m from the discharge and a freshwater stream leading on to the beach from agricultural land. In order to quantify the levels of resistant bacteria present, the Colilert-18 test was spiked with ciprofloxacin (1µg/mL), cefotaxime (2µg/mL) and ertapenem (0.5µg/mL). The concentrations of antibiotics used were chosen based off examination of the literature, and relate to MIC values above which isolates would be considered resistant by EUCAST or CLSI. This data is currently

being used in a quantitative risk ranking model to predict the potential of exposure and subsequent infection for water users.

An additional shotgun metagenomics study is also ongoing, using some of the samples that were collected to feed into the risk ranking model. A volume of 1L was sampled from the three locations outlined above in triplicate, on four dates between March and April 2021. Samples were subsequently filtered using 0.45µm pore sized filters. Direct DNA extraction was carried out from the filters using the DNeasy PowerWater kit (Qiagen) according to the protocol. DNA extracts were sent externally to BGI which completed the shotgun metagenomics sequencing. A subsequent sample collection round took place in December 2021 for seasonal comparison. These samples will be analysed using bioinformatics to establish the resistome, virulome and mobilome of these environmental waters. This approach addresses many of the limitations highlighted including the fact that the culture based approach represents a limited picture of the environmental resistome. Moreover, a quantitative approach can be applied using metagenomics which will help to further inform the risks to public health.

Apart from the AREST project, general further areas of research relating to environmental antibiotic resistance have been highlighted throughout this thesis which align with those identified in the recent research gap analysis (Cahill et al., 2021). In particular, validation of a standardised methodology which could be applied with more regular monitoring of the environment on an international scale for the detection of antibiotic resistance is needed. The EU bathing water directive should be updated to reflect and implement this type of monitoring on a European level. Further research is also urgently needed to evaluate the nexus between recreational exposure and colonisation/infection with resistant bacteria, in particular with carbapenem resistant strains for which the literature is currently lacking. The [PIER project](#) (public health impact of exposure to antibiotic resistance in recreational waters) is a colonisation study

ongoing which will address this knowledge gap by examining for the presence of ESBL and carbapenemase producing Enterobacterales. Regular water users will be compared with non-water users to establish the rates of colonisation. The PIER project aims to target areas which were sampled through the AREST project so that environmental and human isolates from similar regions can be compared at a genetic level.

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**Appendix A: Supplementary Material**  
**The role of the natural aquatic environment in the**  
**dissemination of extended spectrum beta-**  
**lactamase and carbapenemase encoding genes:**  
**A scoping review**

*Supplementary Table A1: Search string terms applied to databases including Pubmed, MEDLINE, EMBASE, Web of Science and Scopus.*

<b>Transmission</b>	<b>CPE/ ESBL resistance genes</b>	<b>Natural aquatic environment</b>	<b>NOT</b>
spread	carbapenem resistan*	river*	vibrio
genetic transfer	carbapenemase produc*	lake*	hospital environment
intermediary conjugation	ESBL extended spectrum beta lactamase	stream* sea	
transduction	resistance plasmid*	seawater	
transformation	beta lactam resistan*	pond*	
dissemination	class 1 integron*	marine environment	
horizontal gene transfer	mobile gene*  mobile genetic element CTX-M KPC NDM TEM VIM IMP OXA		

*Example search string employed in the Scopus database:*

TITLE-ABS-KEY ( transmission ) OR TITLE-ABS-KEY ( spread ) OR TITLE-ABS-KEY ( "genetic transfer" ) OR TITLE-ABS-KEY ( intermediary ) OR TITLE-ABS-KEY ( conjugation ) OR TITLE-ABS-KEY ( transduction ) OR TITLE-ABS-KEY ( transformation ) OR TITLE-ABS-KEY ( dissemination ) OR TITLE-ABS-KEY ( "horizontal gene transfer" ) AND TITLE-ABS-KEY ( "carbapenem resistan\*" ) OR TITLE-ABS-KEY ( "carbapenemase produc\*" ) OR TITLE-ABS-KEY ( esbl ) OR TITLE-ABS-KEY ( "extended spectrum beta lactamase" ) OR TITLE-ABS-KEY ( "resistance plasmid" ) OR TITLE-ABS-KEY ( "beta lactam resistance" ) OR TITLE-ABS-KEY ( "class 1 integron" ) OR TITLE-ABS-KEY ( "mobile gene" ) OR TITLE-ABS-KEY ( "mobile genetic element" ) OR TITLE-ABS-KEY ( "CTX M" ) OR TITLE-ABS-KEY ( kpc ) OR TITLE-ABS-KEY ( ndm ) OR TITLE-ABS-KEY ( tem ) OR TITLE-ABS-KEY ( vim ) OR TITLE-ABS-KEY ( imp ) OR TITLE-ABS-

KEY ( oxa ) AND TITLE-ABS-KEY ( "natural aquatic environment" ) OR TITLE-ABS-KEY ( river ) OR TITLE-ABS-KEY ( lake ) OR TITLE-ABS-KEY ( stream ) OR TITLE-ABS-KEY ( sea ) OR TITLE-ABS-KEY ( seawater ) OR TITLE-ABS-KEY ( pond ) OR TITLE-ABS-KEY ( "marine environment" ) AND NOT TITLE-ABS-KEY ( "hospital environment" ) AND NOT TITLE-ABS-KEY ( vibrio )

*Supplementary Table A2: Full data extracted from 41 included articles including details of sampling locations, bacterial species detected, mobile genetic elements identified or experimentation to test for mobility and ESBL and/ or carbapenemase genes detected.*

Citation	Sample location details	Bacterial species	ESBL resistance genes detected in bacterial species	Carbapenemase resistance genes detected in bacterial species	Mobile genetic elements/ experimentation to test for mobility of resistance genes
Ben Said et al., 2016	Rivers (35 samples, from 25 rivers), lakes (9 samples, from 9 lakes), and saline lakes (6 samples, from 6 saline lakes) in Tunisia. None connect to the WWTP effluent.	Unknown	No detection	N/A	Unknown
Harnisz & Korzeniewsk, 2018	River samples collected 600m upstream of WWTP discharge point in Olsztyn, Poland.	<i>Aeromonas</i> species	No detection	N/A	Class 2 integron ( <i>intI2</i> gene)
Lekunberri et al., 2017	River water samples were collected 900m upstream of a WWTP effluent discharge in the Ter river, Spain.	N/A - Completely genomic work no bacteria cultured or identified.	<i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>KPC</sub> <i>bla</i> <sub>NDM</sub>	Resistance genes identified in extracted plasmid DNA fractions
Osińska et al., 2016	River water collected 600m upstream of a WWTP discharge point in Olsztyn, Poland.	<i>Acinetobacter</i> sp.	<i>bla</i> <sub>OXA</sub>	N/A	bla genes detected in extracted plasmid DNA  Conjugation assay – successful transfer of all <i>bla</i> genes to <i>E. coli</i> J53
		<i>Aeromonas</i> sp.	<i>bla</i> <sub>TEM</sub>		
Adelowo et al., 2018	Groundwater (hand-dug well water and borehole water) collected within the vicinity of wastewater discharge points from hospitals in Nigeria.	<i>E. coli</i> (well water)	<i>bla</i> <sub>TEM</sub>	N/A	Class 1 integron ( <i>IntI1</i> ) MOB F11 (conjugative relaxase genes used to type/ categorise plasmid DNA) MOBP11
		<i>Enterobacter amnigenus</i> (well water)	<i>bla</i> <sub>TEM</sub>		Class 1 integron ( <i>IntI1</i> ) MOBF12

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		<i>E. coli</i> (well water)	Not detected		Class 1 integron ( <i>Int1</i> ) <i>ISEcp1</i> MOBF12
		<i>Shigella sonnei</i> (well water)	<i>bla</i> <sub>CTX-M-15</sub>		Class 1 integron ( <i>Int1</i> ) <i>ISEcp1</i> <i>IS26</i> MOBF12
		<i>E. coli</i> (borehole water)	<i>bla</i> <sub>TEM</sub>		Class 1 integron ( <i>Int1</i> ) MOB F11 MOBF12
		<i>E. coli</i> (borehole water)	<i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>CTX-M-15</sub>		Class 1 integron ( <i>Int1</i> ) <i>ISEcp1</i> <i>IS26</i>
Caltagirone et al., 2017	The Oltrepò Pavese area in Italy supports agricultural and industrial activities. Selected sites to include based off location of sources in the map (figure 1): River streams: 1T, 3T, 6T, 11T. Groundwater: 3P, 10P, 11P, 13P.	Site 1T – <i>E. coli</i>	Site 1T – <i>bla</i> <sub>CTX-M-1</sub>	Not detected	Plasmid incompatibility group detected: 1T – IncF (FIB), Inc FII (FII) 3T – IncP (P), IncF (FIB) 11T – IncN (N), Inc B/O (B/O), IncF (FIB) 6T – IncHI2 (HI2), IncN (N), IncF (FIA)  Successful conjugation transfer to <i>E. coli</i> : 1T – <i>bla</i> <sub>CTX-M-1</sub> , IncF (FIB) 3T – <i>bla</i> <sub>CTX-M-28</sub> , IncP (P), IncF (FIB). 11T – <i>bla</i> <sub>SHV-12</sub> , IncF (FIB)
		Site 3T – <i>E. coli</i>	Site 3T – <i>bla</i> <sub>CTX-M-28</sub>	Not detected	
		Site 11T – <i>E. coli</i>	Site 11T – <i>bla</i> <sub>SHV-12</sub>	Not detected	
		Site 6T – <i>Klebsiella oxytoca</i>	Site 6T – <i>bla</i> <sub>SHV-12</sub>	Not detected	
		Site 3P - Unknown	Not detected	Not detected	Unknown
		Site 10P - Unknown	Not detected	Not detected	Unknown
		Site 11P - Unknown	Not detected	Not detected	Unknown
		Site 13P - Unknown	Not detected	Not detected	Unknown
Charnock et al., 2014	Seawater from 26 beaches on the Oslo fjord in Norway. 'Although some beaches were in close proximity to residential areas, there were, with the exception of drains depositing excess surface water at 2 sites, no obvious sources of faecal contamination at any site.'	<i>E. coli</i>	Not detected ( <i>bla</i> <sub>TEM</sub> detected however phenotypic screening confirmed the absence of ESBL production).	N/A	Three isolates tested positive for class 1 integron: isolates 14 (near a residential area), 83 and 93 (both close to summer homes).
Chen et al., 2010	River water collected along the 2km stretch of the Yangtze River running through Chongqing, China.	<i>E. coli</i> (n=167, 52.4%) and <i>Klebsiella pneumoniae</i> (n=114, 35.8%).	Detection of one or more in 309 of 319 isolates (96.9%):	N/A	208 ESBL producers carried class 1 integrons. 3 ESBL producers carried class 2 integrons.

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		Detected 9 <i>Citrobacter freundii</i> , 6 <i>Enterobacter cloacae</i> , 4 <i>Citrobacter koseri</i> , and a couple more species less frequently.	<i>bla</i> <sub>TEM</sub> <i>bla</i> <sub>CTX-M</sub> <i>bla</i> <sub>SHV</sub>		Sequencing of integrons showed that no <i>bla</i> genes were detected in integron cassettes.
Bajaj et al., 2016	Multiple sampling sites along the urban river Yamuna flowing through the National Capital Territory of Delhi (India).	<i>E. coli</i>	<i>bla</i> <sub>CTX-M-15</sub> positive isolates: NG3, KP21, IP5N, NG32, NG 29, KK31, KK16, PA12, WB6, KKA.	N/A	Conjugation successful for isolates KP21, KK31, KK16, PA12, WB6, KKA to <i>E. coli</i> . Most plasmids from the conjugal transfer were the IncF group (FIB <i>inc/rep</i> replicon detected in transconjugants from KK16, KP21, KKA, PA12). IncN plasmids were observed in transconjugants from KK31 and WB6.
Kieffer et al., 2016	Water samples collected from the Ave River in the north of Portugal. 'Humans might be the source of this environmental contamination.'	<i>E. coli</i>	N/A	Isolates E201 and E203 harboured <i>bla</i> <sub>IMP-8</sub> . Isolates E61 and E202 harboured <i>bla</i> <sub>VIM-1</sub> and <i>bla</i> <sub>VIM-34</sub> .	Extracted plasmids (IncFIB) showed <i>bla</i> <sub>VIM-1</sub> and <i>bla</i> <sub>IMP-8</sub> were carried on a plasmid. All plasmids bearing carbapenemase resistance genes belonged to the IncFIB group. <i>bla</i> <sub>VIM-34</sub> was embedded in a class 1 integron in strain E202. Conjugation using <i>E. coli</i> J53 recipient was successful for 3 out of 4 isolates (E202 unsuccessful).
Zhang et al., 2018	Samples N7 and N10 of the Bohai Bay in China are 'rarely influenced by human activity'.	Unknown	<i>bla</i> <sub>TEM</sub> detected at sites N7 and N10 in both intracellular and extracellular DNA.	N/A	Class 1 integrons ( <i>Int1</i> ) were detected in all samples.
Alouache et al., 2012	A water sample was taken from Anse de Kouâli. This beach is part of a nature reserve in Algiers, Algeria.	Unknown	No detection	N/A	Unknown
Fernandes et al., 2017	11 different public beaches surrounding urban counties sampled in the south eastern Brazilian continental margin of São Paulo State. Santos is the major beachfront with the largest shipping terminal in Latin America. The beaches are labelled as 'anthropogenically affected'.	<i>E. coli</i>	<i>bla</i> <sub>CTX-M-8</sub>	N/A	Plasmids: I1, ColRNAI, X4
		<i>E. coli</i>	<i>bla</i> <sub>CTX-M-1</sub>		Plasmids: HI2, I1, N, X4

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Koczura et al., 2014	Lakes Kociołek and Skrynka are in areas of strict preservation in the national park south of Poznan in Poland.	N/A 'We did not culture any integron-bearing isolate from Skrzynka Lake'.	N/A	N/A	No integron-positive isolates were found in Lake Skrynka.
		<i>E. coli</i>	Not detected		Two integron-positive <i>E. coli</i> isolates ( <i>intI2</i> ) were detected in Lake Kociołek.
Sellera et al., 2017	Seawater sampled from a public beach in Brazil.	<i>Enterobacter cloacae</i>	<i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>OXA-17</sub>	<i>bla</i> <sub>KPC-2</sub>	IncFIB plasmid incompatibility group detected.
Singh et al., 2018	Samples of the river Yamuna which traverses the National Capital Territory of Delhi (India).	<i>E. coli</i>	<i>bla</i> <sub>CTX-M-15</sub> (10 <i>E. coli</i> strains)	N/A	The promoter regions of all <i>bla</i> <sub>CTX-M-15</sub> isolates contained the insertion sequence <i>ISEcp1</i> . <i>intI1</i> gene was detected in 30 strains. Conjugation was successful in transferability of <i>bla</i> <sub>CTX-M-15</sub> (in 10 strains), and class 1 integrons (in 9 strains) to <i>E. coli</i> J53.
Stange et al., 2016	River water samples collected from River Rhine near Düsseldorf, Germany.	<i>E. coli</i>	<i>bla</i> <sub>TEM</sub> (10.2%) <i>bla</i> <sub>SHV</sub> (1.9%)	N/A	Class 1 (9.8% of <i>E. coli</i> isolates) and 2 (3.8% of <i>E. coli</i> isolates) integrons detected.
		<i>Klebsiella</i> species	<i>bla</i> <sub>SHV</sub> (14.6%)		No class 1/2 integrons detected.
		<i>Lecleria</i> species	<i>bla</i> <sub>SHV</sub> (1.9%)		Class 1 integron detected in 2.1% of <i>Lecleria</i> species.
		<i>Enterobacter</i> species	Not detected		Class 1 integron detected in 4.2% of <i>Enterobacter</i> species.
		Unknown	<i>bla</i> <sub>TEM</sub> (4.2%)		Class 1 integron detection in 3.8% of unknown isolates.
Tação et al., 2012	9 sites were classified as unpolluted from 11 rivers integrated in the Vouga River basin, in central Portugal.	<i>E. coli</i>	<i>bla</i> <sub>CTX-M</sub>	N/A	Class 1 integron ( <i>intI1</i> ) gene detection Insertion sequence <i>ISEcp1</i>
		<i>E. coli</i>	<i>bla</i> <sub>TEM</sub> <i>bla</i> <sub>CTX-M</sub>		No class 1 integron detected Insertion sequence <i>ISEcp1</i>
		<i>Pseudomonas</i> sp.	<i>bla</i> <sub>CTX-M-15</sub>		Class 1 integron ( <i>intI1</i> ) gene detection Insertion sequence <i>ISEcp1</i>
Tafoukt et al., 2018	River water sampled from Soummam River in Béjaïa, Algeria.	Four strains of <i>Shewanella Xiamenensis</i>	N/A	<i>bla</i> <sub>OXA-181</sub> (2 isolates) <i>bla</i> <sub>OXA-199</sub> (1 isolate) <i>bla</i> <sub>OXA-538</sub> (1 isolate)	Conjugation of <i>bla</i> <sub>OXA</sub> genes with <i>E. coli</i> J53 was unsuccessful. Sequencing of <i>bla</i> <sub>OXA-538</sub> confirmed a chromosomal location.
Wang et al., 2018	River samples collected along the Weihe River, China. S13 is located far downstream of the polluted river but it	Range of phyla detected including Proteobacteria, Acinetobacteria	<i>bla</i> <sub>TEM</sub>	N/A	The <i>intI1</i> gene was detected in all water samples.

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	appears relatively free from contaminating point sources in the near vicinity in Figure 1.	, Cyanobacteria and Bacteroidetes.			
Ye et al., 2017	Water samples collected from different sites along the Pearl river, China. 'The water of Pearl River has been influenced by non-point and point pollution along with the rapid industrial, agriculture, and municipal development in this region.'	<i>E. coli</i>	<i>bla</i> <sub>CTX-M-65</sub>	N/A	<i>int11</i> positive Transconjugant negative (conjugation unsuccessful – <i>E. coli</i> recipient for all)
		<i>E. coli</i>	<i>bla</i> <sub>CTX-M-55</sub>		<i>int11</i> positive Transconjugant positive (conjugation successful)
		<i>E. coli</i>	<i>bla</i> <sub>CTX-M-15</sub>		<i>int11</i> positive Transconjugant negative (conjugation unsuccessful)
		<i>Klebsiella pneumoniae</i>	<i>bla</i> <sub>CTX-M-14</sub>		<i>int11</i> positive Transconjugant positive (conjugation successful)
		<i>Providencia rettger</i>	<i>bla</i> <sub>CTX-M-14</sub>		<i>int11</i> positive Transconjugant negative (conjugation unsuccessful)
Yin et al., 2013	Samples taken at 9 different points along Lake Taihu, a large shallow freshwater lake in China. It 'acts as a main source of drinking, irrigation and fishery water. With the extensive growth in agriculture and industry in the past few decades'.	<i>Pseudomonas aeruginosa</i> <i>Pseudomonas Acinetobacter Agrobacterium Stenotrophomonas Bacillus Brevundimonas Cupriavidus Shingomonas</i>	<i>bla</i> <sub>TEM</sub> n=14 (22.0%) <i>bla</i> <sub>SHV</sub> n=7 (12.5%) <i>bla</i> <sub>CTX-M</sub> n=5 (7.8%) <i>bla</i> <sub>OXA-1</sub> n=1 (1.6%)	N/A	30 of the 78 isolates were found to carry <i>int I</i> , of which 3 isolates harboured both <i>int I</i> and <i>int II</i> . 40 strains, were successfully able to transfer antibiotic resistance to <i>E. coli</i> through conjugation. 9 conjugants exhibited all resistance profiles of the donor strains.
		<i>Microbacterium Comamonas Flavobacterium</i>	Not detected		
Akiba et al., 2016	Water samples were collected from rivers in the Indian states of Bihar, Goa, Karnataka, Tamil Nadu.	<i>E. coli</i>	Not detected	<i>bla</i> <sub>NDM-7</sub>	Plasmid replicon A/C (two isolates)
		<i>E. coli</i>	<i>bla</i> <sub>CTX-M-55</sub>	Not detected	Plasmid replicon FIB, FIC, FII (two isolates)
		<i>E. coli</i>	<i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>OXA-1</sub> <i>bla</i> <sub>OXA-9</sub>	Not detected	Plasmid replicon FIIk (one isolate)
		<i>E. coli</i>	<i>bla</i> <sub>CTX-M-15</sub>	Not detected	Plasmid replicon Y (one isolate)
		<i>E. coli</i>	<i>bla</i> <sub>SHV-12</sub>	Not detected	Plasmid replicon FII (one isolate)
		<i>E. coli</i>	<i>bla</i> <sub>CTX-M-15</sub>	Not detected	Plasmid replicon I1 (one isolate)
Olga et al., 2016	Stream waters around Greece.	<i>Pseudomonas aeruginosa</i>	Not detected	Not detected	Isolate 317a was class 1 integron positive

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Jørgensen et al., 2017b	Recreational water samples collected from one freshwater and three saltwater beaches located close to Oslo, Norway. There is a nearby sewage plant.	<i>E. coli</i> (n=167)	<i>bla</i> <sub>CTX-M-1</sub> (13%) <i>bla</i> <sub>CTX-M-2</sub> (1%) <i>bla</i> <sub>CTX-M-9</sub> (4%) <i>bla</i> <sub>CTX-M-14</sub> (12%) <i>bla</i> <sub>CTX-M-15</sub> (48%) <i>bla</i> <sub>CTX-M-27</sub> (1%) <i>bla</i> <sub>CTX-M-55</sub> (6%) <i>bla</i> <sub>OXA-1</sub> (5%) <i>bla</i> <sub>SHV-12</sub> (2%) <i>bla</i> <sub>TEM-33-like</sub> (1%) <i>bla</i> <sub>TEM-52C</sub> (1%)	Not detected	Plasmid replicon types detected: IncFII, IncFIB, IncB/O/K/Z, Col, IncFIA, IncQ1, IncFIC, IncI1, IncY, IncFIA, IncI2, pO111, IncX4, IncN, IncX3.
Lepuschitz et al., 2019	5 river water samples were collected upstream of major Austrian cities.	<i>Klebsiella pneumoniae</i>	Not detected	Not detected	Unknown
Wambugu et al., 2018	Samples were taken from sections of the River Athi, Kenya, passing through virgin lands that have no human activity, agriculture or settlement.	<i>E. coli</i> (n=53)	<i>bla</i> <sub>TEM</sub> (2.4%) <i>bla</i> <sub>CTX-M</sub> (3.6%)	N/A	Class 1 integron gene detected ( <i>int1</i> ) (16.7%)
Francisco et al., 2019	Samples collected from two urban rivers in southeast Brazil.	Two strains of <i>Klebsiella pneumoniae</i>	In both strains: <i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>SHV-11</sub> <i>bla</i> <sub>OXA-1</sub>	In both strains: <i>bla</i> <sub>KPC-2</sub>	Plasmids detected in both strains: IncFIB (pQil) IncFII (K) IncFIB (K) IncN
Muraleedharan et al., 2019	Samples collected from two sites along the Clinton River in southeastern Michigan. 'The majority of this land is covered by forests, with a small fraction being used for agriculture. The remaining areas are mostly urban.'	<i>Rahnella aquatilis</i>	<i>bla</i> <sub>CTX-M-1</sub>	Not detected	Negative for conjugation transfer
		<i>Rahnella aquatilis</i>	<i>bla</i> <sub>CTX-M-1</sub>	Not detected	Negative for conjugation transfer
		<i>Aeromonas sobria</i>	<i>bla</i> <sub>CTX-M-1</sub>	Not detected	Negative for conjugation transfer
		<i>Aeromonas caviae</i>	<i>bla</i> <sub>CTX-M-1</sub>	Not detected	Negative for conjugation transfer
		<i>Aeromonas sobria</i>	<i>bla</i> <sub>CTX-M-1</sub>	Not detected	Negative for conjugation transfer
		<i>Bacillus megaterium</i>	<i>bla</i> <sub>CTX-M-1</sub>	Not detected	Negative for conjugation transfer
		<i>Leifsonia aquatic</i>	<i>bla</i> <sub>CTX-M-1</sub>	Not detected	Negative for conjugation transfer
		<i>Flavobacterium meningosepticum</i>	<i>bla</i> <sub>CTX-M-1</sub>	Not detected	Negative for conjugation transfer
		<i>Pseudomonas sp.</i>	<i>bla</i> <sub>CTX-M-1</sub>	Not detected	Negative for conjugation transfer
		<i>Streptococcus porcinus</i>	<i>bla</i> <sub>TEM</sub>	Not detected	Negative for conjugation transfer
		<i>Klebsiella pneumoniae</i>	Not detected	<i>bla</i> <sub>KPC-2</sub>	Conjugation successful to <i>E. coli</i> J53. PCR amplification confirmed the plasmid location of the <i>bla</i> <sub>KPC</sub> gene in transconjugants.
Wu et al., 2019a	Sampled three points along three rivers in China upstream of pollution sources: 'SZ-1' was upstream of combined sewage pump stations. 'DP-1' was upstream of a WWTP. 'Res' was a reservoir selected as an	Sampling site: SZ-1 Proteobacteria, Actinobacteria and Bacteroidetes were the predominant phyla.	<i>bla</i> <sub>CTX-M</sub> <i>bla</i> <sub>TEM</sub>	N/A	<i>int1</i> log <sub>10</sub> (copies/16S) -1.87

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	upstream site of the Huangpu river upstream from agriculturally influenced lands.	Sampling site: DP-1 Proteobacteria, Actinobacteria and Bacteroidetes were the predominant phyla.	<i>bla</i> <sub>CTX-M</sub> <i>bla</i> <sub>TEM</sub>		<i>int1</i> log <sub>10</sub> (copies/16S) -1.81
		Sampling site: Res Proteobacteria, Actinobacteria and Bacteroidetes were the predominant phyla.	<i>bla</i> <sub>CTX-M</sub> <i>bla</i> <sub>TEM</sub>		<i>int1</i> log <sub>10</sub> (copies/16S) -1.32
Osińska et al., 2017	River water was collected approximately 600m upstream from a WWD point in Poland.	<i>E. coli</i>	<i>bla</i> <sub>TEM</sub> <i>bla</i> <sub>OXA</sub>	N/A	<i>int1</i> positive isolates detected. No class 2 integrons detected.
Zarfel et al., 2017	The river Mur in Austria was sampled upstream of the urban WWTP. 'There are no cities bigger than 30,000 inhabitants upstream and no untreated urban, industrial and hospital wastewater is led into the river over the whole stretch upstream the sampling site. The land-scape is mainly used for small structured cattle breeding and grain farming.'	<i>E. coli</i> (70 isolates)	<i>bla</i> <sub>CTX-M-15</sub> <i>bla</i> <sub>CTX-M-27</sub> <i>bla</i> <sub>CTX-M-14</sub> <i>bla</i> <sub>CTX-M-1</sub> <i>bla</i> <sub>CTX-M-2</sub> <i>bla</i> <sub>CTX-M-14-like</sub> <i>bla</i> <sub>SHV-12</sub> <i>bla</i> <sub>CTX-M-9</sub>	Not detected	F, K, B/O, I1, FIB, FIA, N HI1, FIC, and Y plasmid incompatibility groups detected.
		<i>Klebsiella oxytoca</i> (1 isolate)	<i>bla</i> <sub>GES-1</sub>	Not detected	I1, FIB and F plasmid incompatibility groups detected.
		<i>Enterobacter cloacae</i> (2 isolates)	Not detected	<i>bla</i> <sub>VIM-1</sub>	Plasmid incompatibility groups HI2 and F were detected in both.
Dolejská et al., 2009	Samples were collected from Heřma-nický pond which is sourced from the river Vrbická Stružka in the Czech Republic. 'The pond serves as a nesting site for almost 250 species of species.' 'The pond is situated in a large industrial area densely inhabited with humans.'	<i>Salmonella E. coli</i>	'No ESBL-producing isolates were found in water samples'.	N/A	3 of the <i>E. coli</i> isolates from water yielded the <i>int1</i> gene. No class 2 integrons identified.

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Poirel et al., 2012a	Samples collected at different locations along one river in north Portugal. This paper does not mention any pollution sources but does say that it is possible that humans transmitted multi-drug resistant bacteria to the environment.	<i>E. coli</i>	N/A	<i>bla</i> <sub>KPC-2</sub>	IncF type plasmid harbouring <i>bla</i> <sub>KPC-2</sub> was transferred to <i>E. coli</i> J53 by conjugation. PCR mapping showed the presences of the <i>bla</i> <sub>KPC-2</sub> gene as part of a transposon Tn4401a.
Kim et al., 2008	Water samples were collected monthly from the Han River. This river passes through the middle of Seoul in South Korea and is the major source for drinking and irrigation water.	<i>E. coli</i>	<i>bla</i> <sub>TEM-52</sub> and <i>bla</i> <sub>OXA-4</sub> (5 isolates)	N/A	All class 1 integron positive
		<i>E. coli</i>	<i>bla</i> <sub>CTX-M-14</sub> (10 isolates)		All class 1 integron positive 7/10 isolates successfully transferred <i>bla</i> <sub>CTX-M-14</sub> by conjugation to <i>E. coli</i> J53 but a class 1 integron was not detected in transconjugants.
		<i>E. coli</i>	<i>bla</i> <sub>OXA-4</sub> (1 isolate)		Class 1 integron not detected
Zou et al., 2012	Water samples were collected from the Chengdu Fu River in China. This river is a drinking water source. Sampling points 1, 2 and 3 were selected for inclusion as they have less potential polluting sources nearby (Figure 1).	<i>E. coli</i>	<i>bla</i> <sub>TEM</sub> (11 isolates)  <i>bla</i> <sub>SHV</sub> (7 isolates)	Not detected	' <i>bla</i> <sub>TEM-1</sub> genes were present in the total plasmid'. The location of <i>bla</i> <sub>SHV</sub> was also confirmed on the plasmid.
Jørgensen et al., 2017a	Water samples collected from 1 freshwater and 3 saltwater beaches near Oslo. 'Humans bathing in the fjords, contamination from boat toilets, farm animals, fertilizers used in agriculture, or migrating birds are all possible sources.'	<i>E. coli</i>	ESBL positive samples were detected on 8 sampling occasions. <i>bla</i> <sub>CTX-M-1</sub> detected in two isolates.	N/A	IncF, IncI1, IncFIB, Col156, IncX4 and ColRNAI plasmid types detected in the two strains.
Ouyang et al., 2015	Source water that originated in a mountain and flows into the Jiulongjiang river in China was sampled as a pristine control. This source is located in a 'remote suburban mountain free of human activities'.	N/A (No culturing or identification)	<i>bla</i> <sub>SFO</sub> <i>bla</i> <sub>SHV</sub> <i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>IMP</sub>	Integrase 1 gene detection.
Lamba et al., 2017	A water sample was collected from the river Yamuna in New Delhi. Site Y1 was a 'pristine site' due to 'lack of sewage contamination'.	ESBL resistant strains included <i>Escherichia</i> spp., <i>Aeromonas</i> spp., <i>Pseudomonas</i> spp., and <i>Enterobacter</i> spp. Carbapenem resistant strains	<i>bla</i> <sub>OXA</sub> <i>bla</i> <sub>CTX-M</sub> <i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>NDM</sub>	Class 1, 2 and 3 integrons detected.

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		include <i>Escherichia</i> spp., <i>Klebsiella</i> spp., and <i>Pseudomonas</i> spp.			
Zurfluh et al., 2015	River water sample was collected from the Rhine River in Switzerland.	<i>Klebsiella pneumoniae</i>	N/A	<i>bla<sub>VIM</sub></i>	Successful conjugation transfer of IncN plasmid containing <i>bla<sub>VIM</sub></i> gene to <i>E. coli</i> . The plasmid contains a TN3-like composite transposon with an IS26-like insertion element. Within the transposon lies a class 1 integron.
Nascimento et al., 2017	Samples collected from three urban lakes and one reservoir in Southeastern Brazil. One of the lakes is inhabited by wildlife. 'The direct source of hospital contamination could not be evidenced'. The possibility of 'indirect contamination by nosocomial or domestic sewage through the Sapateiro stream could be raised'.	<i>Klebsiella pneumoniae</i>	<i>bla<sub>CTX-M-2</sub></i>	Not detected	N/A
		<i>E. coli</i>	<i>bla<sub>CTX-M-2</sub></i>	Not detected	N/A
		<i>Klebsiella pneumoniae</i>	Not detected	<i>bla<sub>KPC-2</sub></i>	The <i>bla<sub>KPC-2</sub></i> gene was carried on an IncN plasmid which also harboured a <i>Tn4401b</i> element. IncFIB, ColRNAI and IncFII plasmids were also identified.
		<i>E. coli</i>	<i>bla<sub>CTX-M-15</sub></i>	Not detected	IncFIB, IncX4 and IncFIA plasmid incompatibility groups identified.
		<i>Klebsiella pneumoniae</i>	<i>bla<sub>CTX-M</sub></i>	Not detected	N/A
		<i>Pseudomonas putida</i>	<i>bla<sub>CTX-M-9</sub></i>	Not detected	N/A

*Supplementary Table A3: Summary table of methods used in each of the papers including initial collection volumes and processing as well as genotypic examination for antibiotic resistance genes and mobile genetic elements.*

Citation	Volume of water collected and initial processing details	Molecular methods used to detect resistance genes/ mobile genetic elements and transfer experiments
Ben Said et al., 2016	Unknown volume of water collected. 5mL enriched in 270mL of buffered peptone water prior to plating on MacConkey agar with cefotaxime.	PCR and sequencing to detect genes encoding TEM, SHV, OXA-1 and CTX-M. PCR to detect <i>int1</i> and <i>int2</i> . Conjugation experiments.
Harnisz & Korzeniewsk, 2018	Unknown volume of water collected. Samples diluted with saline prior to pour plating on to TSA agar.	Used standard PCR and electrophoresis to identify <i>bla<sub>CTX-M</sub></i> , <i>bla<sub>SHV</sub></i> , <i>bla<sub>TEM</sub></i> and <i>bla<sub>OXA</sub></i> as well as two integrase genes.
Lekunberri et al., 2017	Six water replicates (1000mL) collected at 1 minute intervals. Water filtered using 0.22µm pore sized membrane. No culturing, direct DNA extraction from filter.	Real time PCR used to determine the copy number of beta-lactam genes including <i>bla<sub>KPC</sub></i> , <i>bla<sub>TEM</sub></i> and <i>bla<sub>NDM</sub></i> . Detected resistance genes in plasmid DNA fraction.
Osińska et al., 2016	Unknown volume of water collected. Water was filtered using a 0.45µm pore sized membrane or diluted with saline prior to plating on TSA agar with and without antibiotic supplementation.	PCR (for detection of <i>bla<sub>SHV</sub></i> , <i>bla<sub>TEM</sub></i> , <i>bla<sub>OXA</sub></i> and <i>bla<sub>CTX-M</sub></i> types) and sequencing of amplicons to detect resistance genes in the plasmid DNA. Conjugation experiments.

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Adelowo et al., 2018	Unknown volume of water collected. Samples were serially diluted prior to plating on antibiotic supplemented EMB and MHA agars.	Standard PCR used to detect <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>CTX-M</sub> and class 1, 2 and 3 integrons. Plasmids extracted and typed.
Caltagirone et al., 2017	Unknown volume of water collected. 100mL of groundwater and 1mL of river water was filtered using a 0.45µm pore sized membrane. Filters were placed directly on PCA, MacConkey and MCA agar containing antibiotics.	PCR used to screen for <i>bla</i> <sub>CTX-M</sub> variants, <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>KPC</sub> , <i>bla</i> <sub>VIM</sub> , <i>bla</i> <sub>IMP</sub> , <i>bla</i> <sub>NDM</sub> , <i>bla</i> <sub>OXA-48</sub> . PCR products sequenced. Plasmids sequenced. Conjugation experiments.
Charnock et al., 2014	2L of water collected. 100mL and 500mL were filtered (0.45µm pore size) and filters were placed on MacConkey agar. 1 mL of sample was directly plated.	DNA based microarray used to screen for 47 resistance and integrase genes including the class 1 integrase gene and all <i>bla</i> <sub>TEM</sub> genes.
Chen et al., 2010	3000mL of water collected. Membrane filter methods applied (unknown pore size). Filters incubated on MacConkey agar.	Extracted genomic and plasmid DNA and used PCR to detect <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>CTX-M</sub> , <i>bla</i> <sub>SHV</sub> and <i>bla</i> <sub>OXA</sub> genes. All PCR products were sequenced. Class 1, 2 and 3 integrase genes were amplified by multiplex PCR. 3 PCR products were transformed into <i>E. coli</i> and sequenced. Box-PCR used to determine <i>bla</i> genes type.
Bajaj et al., 2016	Sample collection described in previous paper. Unknown volume of water collected and 100mL was filtered (0.45 µm pore size). Filter enriched in McConkey broth, which was then plated on MacConkey agar.	Previous paper mentions PCR based screening for <i>bla</i> <sub>CTX-M</sub> , <i>bla</i> <sub>SHV</sub> and <i>bla</i> <sub>TEM</sub> . In this paper conjugation experiments performed and PCR- based replicon typing of transferred plasmids.
Kieffer et al., 2016	Unknown volume of water collected. 100mL of water was filtered (0.45 µm pore size). The filter was then placed on tryptone bile-X-glucuronide agar supplemented with an antibiotic.	PCR carried out to screen for carbapenemase genes (including <i>bla</i> <sub>IMP</sub> and <i>bla</i> <sub>VIM</sub> ) followed by sequencing. Conjugation assays performed and plasmids were typed.
Zhang et al., 2018	1L of water collected and filtered through two membrane filters (10µm followed by 0.22µm pore size). Filtrates were centrifuged and the supernatant was diluted using deionised water. Intracellular and extracellular DNA was extracted.	PCR and qPCR were used to quantitatively determine the levels of <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>TEM</sub> and <i>int1</i> .
Alouache et al., 2012	500mls of water collected. Membrane filtration technique used, (unknown pore size). Filters were incubated on Mueller Hinton with and without antibiotic supplementation and Tergitol medium.	PCR was used to screen for <i>bla</i> <sub>TEM</sub> and <i>bla</i> <sub>CTX-M</sub> . <i>bla</i> <sub>CTX-M</sub> PCR product was sequenced. Detection of insertion sequence ISE <sub>cp1B</sub> was performed by PCR. Conjugation experiments carried out and plasmids typed.
Fernandes et al., 2017	500mL of water was collected. 100mL was filtered (0.45 µm pore size). Filters were incubated on MacConkey agar, followed by placing them in MH broth. Broths were vortexed and an aliquot was streaked on antibiotic supplemented MacConkey agar.	DNA was sequenced using Illumina NextSeq which detected the presence of <i>bla</i> <sub>CTX-M-8</sub> , <i>bla</i> <sub>CTX-M-1</sub> and IncX4 plasmids. Plasmid sequences were aligned against a database using the MegaBLAST algorithm.
Koczura et al., 2014	Unknown volume of water collected followed by direct plating on brain-heart infusion agar.	Multiplex PCR was used to detect class 1, 2 and 3 integrase genes. Amplicons were sequenced. PCR was used to screen for <i>bla</i> <sub>OXA</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>GES</sub> , <i>bla</i> <sub>VEB</sub> , <i>bla</i> <sub>PER</sub> , <i>bla</i> <sub>DHA</sub> , <i>bla</i> <sub>CMY</sub> and <i>bla</i> <sub>CTX-M</sub> .

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Sellera et al., 2017	No information given on sample collection or initial processing.	One isolate was sequenced using Illumina MiSeq. Analysis included screening the sequence through ResFinder to detect ( <i>bla</i> <sub>KPC</sub> , <i>bla</i> <sub>CTX-M</sub> and <i>bla</i> <sub>OXA</sub> ). Plasmids types screened using PlasmidFinder.
Singh et al., 2018	Sample collection described in previous paper. Unknown volume of water collected. 100mL was filtered through a membrane (0.45 µm pore size). The filter was cut and enriched in MacConkey broth. Broth was plated on MacConkey agar.	PCR and electrophoresis used to detect <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>CTX-M-15</sub> in previous paper. PCR mapping of insertion sequences was carried out in this study. Also used PCR to detect integrase genes <i>intl1</i> , <i>intl2</i> , and <i>intl3</i> , and integron class 1 gene cassette. Conjugation experiments.
Stange et al., 2016	Unknown volume of water collected via grab samples of surface water. Direct plating on to lactose-TTC-agar.	PCR and electrophoresis used to detect integrons (classes 1 and 2) and beta-lactamase resistance genes including <i>bla</i> <sub>TEM</sub> and <i>bla</i> <sub>SHV</sub> .
Tacão et al., 2012	7 litres of water collected. Tested if waters were polluted/ unpolluted. Water was filtered through a membrane (0.45 µm pore size). The filters were placed onto MacConkey agar with and without antibiotic supplementation.	PCR was performed to detect ESBL genes encoding SHV, TEM, OXA, CTX-M, GES, VEB and PER. Integrase screening carried out for classes 1, 2 and 3. PCR amplicons were analysed using electrophoresis. Sequencing was done for the <i>bla</i> <sub>CTX-M</sub> gene fragments. The presence of insertion sequences in the genetic environment of <i>bla</i> <sub>CTX-M</sub> was screened for by PCR.
Tafoukt et al., 2018	Unknown volume of water collected. Direct plating on to MacConkey agar supplemented with an antibiotic.	Real time PCR was used to screen for beta lactamase genes including <i>bla</i> <sub>OXA</sub> variants, <i>bla</i> <sub>KPC</sub> , <i>bla</i> <sub>NDM-1</sub> , <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>SHV</sub> and <i>bla</i> <sub>CTX-M</sub> . Positive PCR amplicons were sequenced. Conjugation experiments. Whole genome sequencing of one isolate performed using Illumina Miseq.
Wang et al., 2018	Three 1-liter water samples were collected and filtered (0.22 µm pore size). The membrane was cut and mixed with ethanol prior to DNA extraction.	Droplet digital PCR was used to quantify ARGs including <i>bla</i> <sub>TEM</sub> and <i>intl1</i> . Performed high throughput sequencing on extracted DNA.
Ye et al., 2017	225mL of water was collected and enriched in 'Enterobacteria enrichment broth' prior to plating on violet red bile glucose agar.	PCR and sequencing were carried out to detect genes encoding TEM, SHV, OXA and CTX-M enzymes. The presence of <i>intl1</i> and <i>intl2</i> genes were examined by PCR. Conjugation experiments.
Yin et al., 2013	Unknown volume of water collected. Direct plating on to agars, (Luria-Bertani with and without antibiotic supplementation).	PCR carried out to detect beta lactamase and integrase genes, ( <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>CTX-M</sub> , class 1, 2 and 3 integrase genes). Conjugation experiments. Some isolates sequenced.
Akiba et al., 2016	Unknown volume of water collected. Water was diluted with PBS prior to plating on Chromocult coliform agar.	PCR carried out to test for beta lactamase genes including TEM, SHV, CTX-M, OXA, IMP, VIM-1, VIM-2 and NDM. Next generation sequencing performed on plasmids.
Olga et al., 2016	Unknown volume of water collected. Water filtered using membrane filtration technique, (unknown pore size). Does not give details on agar plates used.	PCR was used to screen for beta-lactamase genes encoding PER-1, OXA-2, VEB-1A, GES-1A, TEM-A, SHV-A, CTX-M variants, VIM-2, IMP, SIM-1, GIM-1, SPM-1 and NDM. PCR was used to identify the presence of class 1 and 2 integrase genes. Isolates positive for CTX-M group 9 and the class 1 integrase gene were sequenced.

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Jørgensen et al., 2017b	1L samples collected. 10-500mL was vacuum-filtered (unknown pore size). Filters were incubated on Brilliance agar and ChromID ESBL plates.	Multiplex PCR carried out to detect <i>bla</i> <sub>CTX-M</sub> genes. Microarray Check was carried out targeting <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>NDM</sub> and <i>bla</i> <sub>KPC</sub> . Some isolates from recreational waters chosen for sequencing on Illumina HiSeq.
Lepuschitz et al., 2019	500mL of water collected. 100mL per sample was filtered (unknown pore size) and enriched in BBL fluid thioglycollate medium. The enrichment was plated on to chromogenic media (chromID CARBA and chromID ESBL agar.	DNA sequenced on Illumina MiSeq. Plasmid finder and CARD pipelines used.
Wambugu et al., 2018	Unknown volume of water collected. 25mL was enriched using 225 mL of buffered peptone water prior to plating on MacConkey agar.	DNA extracted from colonies and PCR carried out to detect <i>bla</i> genes including <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>CTX-M</sub> and class 1 integrons.
Francisco et al., 2019	Sample collection described in previous paper. 500mL of water collected and 100mL was filtered (0.45 µm pore size). Filters were incubated on MacConkey agar, then place in MH broth, vortexed and streaked on to CHROMagar KPC.	DNA was sequenced for 3 isolates using Illumina MiSeq. Analysis included using ResFinder and PlasmidFinder.
Muraleedharan et al., 2019	750mL of water collected and filtered (0.45µm followed by 0.22µm). Log dilutions were plated on Tryptic soy agar and MacConkey agar with and without antibiotic supplementation.	qRT-PCR was used to quantitatively screen for the presence of <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>CTX-M-1</sub> and <i>bla</i> <sub>KPC</sub> . Conjugation experiments.
Wu et al., 2019a	600mL was collected in triplicate and filtered (0.22µm pore size). DNA extraction performed from filters.	Real time quantitative PCR used to screen for <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>CTX-M</sub> and <i>Int1</i> .
Osińska et al., 2017	500mL of water was collected and diluted with NaCl prior to plating. Selected samples were filtered (0.45µm pore size). Plating was on mFc agar with and without antibiotic supplementation.	Genomic DNA was extracted from bacterial colonies. Standard PCR was used to screen for <i>bla</i> genes including <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>CTX-M</sub> , <i>bla</i> <sub>OXA</sub> , <i>bla</i> <sub>TEM</sub> and integrase genes ( <i>int1</i> , <i>int2</i> ). Some PCR products were sequenced. Electrophoresis was used to visualize products.
Zarfel et al., 2017	Two 500mL water samples collected. 1000ml was filtered (0.45µm). Filters were incubated on chromID ESBL and chromID CARBA agar (2 filters on each).	PCR detected <i>bla</i> <sub>CTX-M-group 1</sub> , <i>bla</i> <sub>CTX-M-group 2</sub> , <i>bla</i> <sub>CTX-M-group 8</sub> , <i>bla</i> <sub>CTX-M-group 9</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>GES</sub> , and <i>bla</i> <sub>VEB</sub> . Multiplex PCR detected plasmid incompatibility groups.
Dolejská et al., 2009	Swabs placed at inflow and outflow points for 24 hours. Swabs enriched in buffered peptone water prior to plating on semisolid Rappaport-Vassiliadis agar for <i>Salmonella</i> detection and selective chromogenic agar for <i>E. coli</i> detection.	PCR carried out for detection of antibiotic resistance genes ( <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>OXA-1</sub> -like, <i>bla</i> <sub>OXA-2</sub> -like and <i>bla</i> <sub>CTX-M</sub> ) as well as class 1 and 2 integrase genes. PCR products further analysed by sequencing.
Poirel et al., 2012a	100mL of water was collected. Water was filtered (0.45µm pore size) prior to plating on an imipenem containing Drigalski plate.	PCR for antibiotic resistance genes including <i>bla</i> <sub>KPC-2</sub> followed by sequencing. PCR-based replicon typing and conjugation transfer of plasmid.
Kim et al., 2008	Unknown volume of water collected. Water was serially diluted before plating on MacConkey agar supplemented with an antibiotic.	PCR was carried out for ESBL beta-lactamase antibiotic resistance genes and class 1 integrons followed by sequencing. Conjugation experiments.
Zou et al., 2012	Water samples were collected in triplicate (250mL each). A 1.0mL aliquot was serially diluted prior to plating on nutrient agar plates.	PCR used to screen for <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>CTX-M</sub> , <i>bla</i> <sub>KPC</sub> and <i>bla</i> <sub>GES</sub> . PCR products were separated by electrophoresis. Plasmid DNA

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		extracted and electrophoresis was performed to determine sizes.
Jørgensen et al., 2017a	Unknown volume of water collected. Portions of ten 500mL volumes were vacuum filtered (unknown pore size). Filters grown on Brilliance agar and ChromID ESBL plates.	Whole genome sequencing performed on selected isolates using Illumina HiSeq.
Ouyang et al., 2015	Unknown volume of water collected in triplicate and filtered (0.22µm pore size). Filters were cut up and DNA extracted. Water samples were also serially diluted and spread plated on to R2A agar. Another 35mL aliquot was centrifuged and the cell pellet was resuspended in saline, serially diluted and plated on R2A agar supplemented with antibiotics.	High throughput qPCR was used to detect beta-lactamase genes and class 1 integrons.
Lamba et al., 2017	500mL replicate water samples taken. All samples were serially diluted prior to plating on selective media for faecal coliform, total coliform, ESBLs and carbapenem resistant bacteria.	Water samples were either filtered (0.22µm) or pelleted for DNA extraction. qPCR was used to screen for <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>OXA</sub> , <i>bla</i> <sub>CTX</sub> , <i>bla</i> <sub>NDM-1</sub> as well as class 1, 2 and 3 integrons.
Zurfluh et al., 2015	Sample collection described in previous paper. Unknown volume of water collected. 500mL was filtered (0.45µm pore size). Filters were enriched in EE broth prior to plating on Brilliance ESBL and Brilliance CRE agars.	Conjugation experiments. Southern blot analysis using <i>bla</i> <sub>VIM</sub> probe confirmed its location on the plasmid. PCR performed on transconjugants targeting <i>bla</i> <sub>VIM</sub> . Plasmid DNA extracted and sequenced.
Nascimento et al., 2017	500mL of water was collected. 100mL was filtered (0.45µm pore size). Filters were placed directly on MacConkey plates. Filters then removed, vortexed within MH broth which was then plated on MacConkey agar supplemented with antibiotics.	Plasmid mediated <i>bla</i> genes, ( <i>bla</i> <sub>CTX-M</sub> variants, <i>bla</i> <sub>KPC</sub> , <i>bla</i> <sub>NDM</sub> , <i>bla</i> <sub>OXA</sub> , <i>bla</i> <sub>IMP</sub> and <i>bla</i> <sub>VIM</sub> ) were identified by PCR and sequencing. Whole genome sequencing using NextSeq platform was performed on <i>bla</i> <sub>KPC-2</sub> and <i>bla</i> <sub>CTX-M-15</sub> positive isolates. ResFinder and PlasmidFinder used.

**Appendix B: Supplementary Material  
A Point Prevalence Survey of Antibiotic Resistance  
in the Irish Environment, 2018–2019**

*Supplementary Table B1: Water sampling location details, including distance from known potential contaminant discharges and E. coli levels.*

Sample collection site	Local authority area	Raw sewage discharge	Primary UWWD	Secondary UWWD	Storm water overflow	E. coli levels (MPN per 100mL)
Beach A	Galway city	-	-	1.9	0.4	10
Beach B	Galway city	-	1.3	1.3	0.3	269
Beach C	Galway city	-	2.5	-	1.5	30
Beach D	Galway city	-	-	-	1.0	31
Beach E	Galway county	0.2	-	-	-	0
Beach F	Galway county	0.1	-	-	-	842
Beach G	Galway county	-	1.3	-	1.4	0
Beach H	Galway county	-	-	2.0	-	31
Beach I	Galway county	0.2	-	-	-	10
Beach J	Galway county	-	-	-	-	0
Beach K	Fingal	-	4.0	-	0.7	0
Beach L	Fingal	-	1.9	-	-	20
Beach M	Fingal	-	1.1	-	-	0
Beach N	Fingal	-	-	0.1	-	10
Beach O	Fingal	-	-	-	1.3	10
Beach P	Fingal	-	-	-	-	0
Beach Q	Cork county	-	0.6	1.2	0.3	85
Beach R	Cork county	0.3	0.3	0.1	0.2	41
Beach S	Cork county	0.7	-	0.1	-	341
Beach T	Cork county	0.8	-	-	0.8	135
Beach U	Cork county	-	-	-	-	0
Beach V	Cork county	0.5	0.2	-	-	277
Beach W	Cork county	-	-	2.2	0.7	0
River A	Galway city	-	-	-	-	40.8
River B	Galway city	-	-	0.1	0.2	62
River C	Galway county	-	0.9	-	0.1	325.5
River D	Galway county	0.1	-	-	-	86.2
River E	Cork county	-	1.4	-	0.5	5.2
Lake A	Galway county	-	-	-	0.2	117.8
Lake B	Galway county	-	-	-	1.5	1
Lake C	Cork county	-	0.4	-	0.8	93.3
Estuary A	Galway city	-	-	0.2	0.2	189.9
Estuary B	Galway city	-	-	0.1	-	>2419.6
Estuary C	Fingal	-	-	-	-	520
Estuary D	Fingal	-	0.4	-	-	816
DWTP A	Fingal	?	?	?	?	29.2
DWTP B	Cork county	?	?	?	?	155.3
DWTP C	Galway city	?	?	?	?	17.3
DWTP D	Galway county	?	?	?	?	16

*Key: UWWD – Urban waste water discharge; DWTP – Drinking water treatment plant.*

*Red writing indicates carbapenemase detection at this sample location. A question mark was inserted for the drinking water treatment plants data as the exact location of sample collection is unknown prior to water being pumped into the plant.*

## Appendix B

*Supplementary Table B2: Total number of resistant isolates for each antibiotic from the different sample types.*

	CPD	FOX	MEM	ETP	AMP	CIP	CN	C	K	W	S	TE	NA	CTX	CAZ
<b>Hospitals</b> (36 isolates)	31	22	5	15	33	29	17	11	16	21	14	18	29	26	24
<b>Nursing homes</b> (19 isolates)	14	9	0	5	18	14	3	4	4	8	5	4	15	8	6
<b>Airports</b> (3 isolates)	3	1	0	1	3	2	2	1	2	2	1	2	3	3	3
<b>Wastewater treatment plants</b> (14 isolates)	12	6	2	4	14	5	0	0	2	10	8	10	6	11	9
<b>Drinking water treatment plants</b> (9 isolates)	5	0	0	0	9	6	0	3	3	4	3	6	7	4	3
<b>Seawaters</b> (72 isolates)	57	15	2	8	66	58	22	16	19	38	29	41	57	47	35
<b>Rivers</b> (23 isolates)	18	4	0	1	22	19	0	4	4	11	9	9	19	14	13
<b>Lakes</b> (8 isolates)	6	3	0	2	8	4	3	2	2	4	4	4	4	5	5
<b>Estuaries</b> (27 isolates)	25	5	1	6	26	20	10	6	8	18	10	17	16	24	21

*Supplementary Table B3: Percentage of resistant isolates for each antibiotic from the different sample types.*

	CPD (%)	FOX (%)	MEM (%)	ETP (%)	AMP (%)	CIP (%)	CN (%)	C (%)	K (%)	W (%)	S (%)	TE (%)	NA (%)	CTX (%)	CAZ (%)
<b>Hospitals</b> (36 isolates)	86.1	61.1	13.9	41.7	91.7	80.6	47.2	30.6	44.4	58.3	38.9	50	80.6	72.2	66.7
<b>Nursing homes</b> (19 isolates)	73.7	47.4	0	26.3	95.7	73.7	15.8	21.1	21.1	42.1	26.3	21.1	78.9	42.1	31.6
<b>Airports</b> (3 isolates)	100	33.3	0	33.3	100	66.7	66.7	33.3	66.7	66.7	33.3	66.7	100	100	100
<b>Wastewater treatment plants</b> (14 isolates)	85.7	42.9	14.3	28.6	100	35.7	0	0	14.3	71.4	57.1	71.4	42.9	78.6	64.3
<b>Drinking water treatment plants</b> (9 isolates)	55.6	0	0	0	100	66.7	0	33.3	33.3	44.4	33.3	66.7	77.8	44.4	33.3
<b>Seawaters</b> (72 isolates)	79.2	20.8	2.8	11.1	91.7	81.4	30.6	22.2	26.4	52.8	40.3	56.9	79.2	65.3	48.6
<b>Rivers</b> (23 isolates)	78.3	17.4	0	4.3	95.7	82.6	0	17.4	17.4	47.8	39.1	39.1	82.6	60.9	56.5
<b>Lakes</b> (8 isolates)	75	37.5	0	25	100	50	37.5	25	25	50	50	50	50	62.5	62.5
<b>Estuaries</b> (27 isolates)	92.6	18.5	3.7	22.2	96.3	74.1	37.0	22.2	29.6	66.7	37.0	63.0	59.3	88.9	77.8

Supplementary Table B4: Total number of bacterial species identified from different sample types.

	Seawaters (n=23)	Rivers (n=5)	Lakes (n=3)	Estuaries (n=4)	Hospitals (n=8)	Nursing homes (n=7)	Airports (n=2)	WWTP (n=8)	DWTP (n=4)	Total
<i>E. coli</i>	63	18	6	20	13	8	3	6	8	145
<i>Klebsiella pneumoniae</i>	4	2	2	6	5	3	-	3	-	25
<i>Klebsiella oxytoca</i>	-	-	-	-	1	1	-	-	-	2
<i>Klebsiella variicola</i>	-	-	-	-	-	-	-	1	-	1
<i>Enterobacter cloacae</i>	2	1	-	-	3	-	-	1	-	7
<i>Enterobacter kobei</i>	1	2	-	-	1	1	-	1	-	6
<i>Enterobacter asburiae</i>	-	-	-	-	1	3	-	-	-	4
<i>Enterobacter ludwigii</i>	1	-	-	-	-	-	-	-	-	1
<i>Citrobacter freundii</i>	-	-	-	-	5	1	-	-	-	6
<i>Citrobacter gillenii</i>	-	-	-	-	2	-	-	-	1	3
<i>Citrobacter braakii</i>	-	-	-	-	2	-	-	-	-	2
<i>Citrobacter youngae</i>	-	-	-	-	1	-	-	-	-	1
<i>Raoultella ornithinolytica</i>	-	-	-	1	2	-	-	-	-	3
<i>Morganella morganii</i>	1	-	-	-	-	1	-	2	-	4
<i>Yersinia enterocolitica</i>	-	-	-	-	-	1	-	-	-	1
<b>Total number of individual isolates:</b>	<b>72</b>	<b>23</b>	<b>8</b>	<b>27</b>	<b>36</b>	<b>19</b>	<b>3</b>	<b>14</b>	<b>9</b>	<b>211</b>

Key: WWTP – Wastewater treatment plant, DWTP – Drinking water treatment plant. The number in brackets next to the sample type indicates the total number of samples collected.

Supplementary Table B5: Isolate identifiers on the European nucleotide archive database  
(Project: PRJEB221277, Secondary Study Accession: ERPO23511)

Isolate ID	Aliases	Sample Accession	<i>Escherichia</i> database on PubMLST	<i>Klebsiella</i> Pasteur MLST database
B18147	WTCHG_781213_72455221	SAMEA6908008	243	-
B18161	WTCHG_781213_72465222	SAMEA6908009	244	-
B18164	WTCHG_781213_72475223	SAMEA6908010	-	<i>K. michiganensis</i> (not included in database)
B18165	WTCHG_781213_72485224	SAMEA6908011	245	-
B18168	WTCHG_781213_72495225	SAMEA6908012	-	12687
B18175	WTCHG_781213_72505226	SAMEA6908013	-	12688
B18185	WTCHG_781213_72515227	SAMEA6908014	-	12689
B18188	WTCHG_781213_72525228	SAMEA6908015	-	12690
B18191	WTCHG_781213_72535229	SAMEA6908016	246	-
B18199	WTCHG_781213_72545230	SAMEA6908017	247	-
B18200	WTCHG_781213_72555231	SAMEA6908018	-	12691
B18207	WTCHG_781213_72565232	SAMEA6908019	248	-
B18208	WTCHG_781213_72575233	SAMEA6908020	249	-
B18213	WTCHG_781213_72585234	SAMEA6908021	250	-
B18215	WTCHG_781213_72595235	SAMEA6908022	251	-
B18221	WTCHG_781213_72605236	SAMEA6908023	-	12692
B18225	WTCHG_781213_72615237	SAMEA6908024	252	-
B18226	WTCHG_781213_72625238	SAMEA6908025	-	12693
B18228	WTCHG_781213_72635239	SAMEA6908026	253	-
B18231	WTCHG_781213_72645240	SAMEA6908027	-	12694
B18235	WTCHG_781213_72655241	SAMEA6908028	-	12695
B18240	WTCHG_781213_72665242	SAMEA6908029	-	12696
B18246	WTCHG_781213_72675243	SAMEA6908030	254	-
B18260	WTCHG_781213_72685244	SAMEA6908031	255	-
B18269	WTCHG_781213_72695245	SAMEA6908032	-	12697
B18271	WTCHG_781213_72705246	SAMEA6908033	256	-
B18275	WTCHG_781213_72715247	SAMEA6908034	257	-
B18283	WTCHG_781213_72725248	SAMEA6908035	-	12698
B18284	WTCHG_781213_72735249	SAMEA6908036	258	-
B18291	WTCHG_781213_72745250	SAMEA6908037	-	12699
B19008	WTCHG_781213_72755251	SAMEA6908038	259	-
B19018	WTCHG_781213_72765252	SAMEA6908039	260	-
B19038	WTCHG_781213_72775253	SAMEA6908040	261	-

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B19075	WTCHG_781213_72795255	SAMEA6908042	262	-
B19079	WTCHG_781213_72805256	SAMEA6908043	263	-
B19091	WTCHG_781213_72815257	SAMEA6908044	264	-
B19104	WTCHG_781213_72825258	SAMEA6908045	265	-
B19124	WTCHG_781213_72835259	SAMEA6908046	266	-
B19135B1	WTCHG_781213_72845260	SAMEA6908047	-	12700
B19136	WTCHG_781213_72855261	SAMEA6908048	267	-
B19137	WTCHG_781213_72865262	SAMEA6908049	-	12701
B19148	WTCHG_781213_72885264	SAMEA6908051	268	-
B19154	WTCHG_781213_71935265	SAMEA6908052	-	12702
B19159	WTCHG_781213_71945266	SAMEA6908053	269	-
B19160	WTCHG_781213_71955267	SAMEA6908054	270	-
B19164	WTCHG_781213_71965268	SAMEA6908055	271	-
B19165	WTCHG_781213_71975269	SAMEA6908056	272	-
B19170	WTCHG_781213_71985270	SAMEA6908057	273	-
B19171	WTCHG_781213_71995271	SAMEA6908058	274	-
B19181	WTCHG_781213_72005272	SAMEA6908059	275	-
B19188	WTCHG_781213_72015273	SAMEA6908060	276	-
B19189	WTCHG_781213_72025274	SAMEA6908061	277	-
B19194	WTCHG_781213_72035275	SAMEA6908062	-	<i>K. michiganensis</i> (not included in database)
B19205	WTCHG_781213_72045276	SAMEA6908063	278	-
B19215	WTCHG_781213_72055277	SAMEA6908064	279	-
B19224	WTCHG_781213_72065278	SAMEA6908065	280	-
B19229	WTCHG_781213_72075279	SAMEA6908066	281	-
B19234	WTCHG_781213_72085280	SAMEA6908067	282	-
B19239	WTCHG_781213_72095281	SAMEA6908068	-	12703
B19248	WTCHG_781213_72105282	SAMEA6908069	283	-
B19257	WTCHG_781213_72115283	SAMEA6908070	284	-
B19321	WTCHG_781213_72145286	SAMEA6908073	-	12704
B19374	WTCHG_781213_72155287	SAMEA6908074	286	-

Supplementary Table B6: Primer and probe sequences for the real time PCR assays to detect beta-lactamase genes.

Gene target	Forward primer	Reverse primer	Probe	Probe	Probe	Reference
<i>bla</i> <sub>CTX-M</sub>	5'-ATG TGC AGY ACC AGT AAR GTK ATG GC- 3'	5'-ATC ACK CGG RTC GCC NGG RAT-3'	CTX-M-1 group probe: 5'-CCC GAC AGC TGG GAG ACG AAA CGT-3'	CTX-M-2 group probe: 5'-CAG GTG CTT ATC GCT CTC GCT CTG TT-3'	CTX-M-9 group probe: 5'-CTG GAT CGC ACT GAA CCT ACG CTG A-3'	Birkett et al., 2007
<i>bla</i> <sub>NDM</sub>	5'-GCT GGC GGT GGT GAC TC-3'	5'-GGC AAG CTG GTT CGA CAA C- 3'	<b>NDM probe:</b> 5'-TGG CAT AAG TCG CAA TCC CCG C-3'			Manchanda et al., 2011
<i>bla</i> <sub>KPC</sub>	5'-GCA GCG GCA GCA GTT TGT TGA TT-3'	5'-GTA GAC GGC CAA CAC AAT AGG TGC-3'	<b>KPC probe:</b> 5'-CAG TCG GAG ACA AAA CCG GAA CCT GC-3'			Swayne et al., 2011
<i>bla</i> <sub>OXA-48</sub>	5'-TTC GGC CAC GGA GCA AAT CAG-3'	5'-GAT GTG GGC ATA TCC ATA TTC ATC GCA-3'	<b>OXA-48 probe:</b> 5'-CTG GCT GCG CTC CGA TAC GTG TAA CTT ATT G-3'			Swayne et al., 2011
<i>bla</i> <sub>VIM</sub>	5'-GAT TGC YGA TGG TGT TTG G-3'	5'-TGA GAC CAT TGG ACG GGT AGA-3'	<b>VIM probe:</b> 5'-CGC ATA TGC CCA CGC RGT CGT TTG-3'			Unpublished data (NCPERLS)
<i>bla</i> <sub>IMP</sub>	<b>Forward primer 1:</b> 5'-GGT TTG TDG AGC GYG GCT AT- 3' <b>Forward primer 2:</b> 5'-GGT TTG TDG ARC GYG GCT AT- 3'	5'-TTC ATT TGT TAA TWC AGA TGC ATA YG- 3'	<b>IMP probe 1:</b> 5'-AAG GCA GCA TTT CCT CTC ATT TTC ATA GCG-3'	<b>IMP probe 2:</b> 5'-AAG GCA SYA TTT CCT CWC ATT TYC ATA GYG-3'		Unpublished data (NCPERLS)

NCPERLS: National Carbapenemase-producing Enterobacterales Reference Laboratory Service, Ireland.

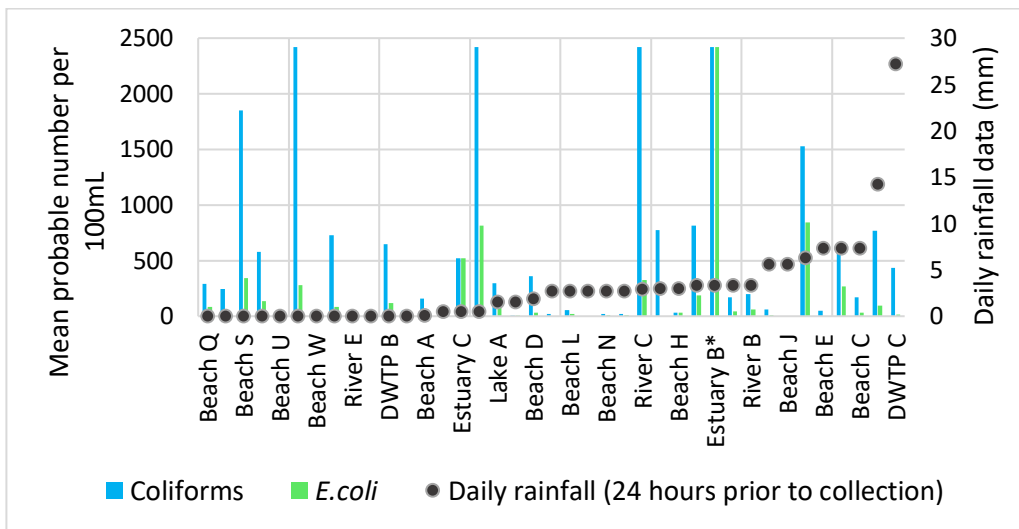
*Protocol for real time PCR reactions:*

1. Add 20 $\mu$ L of the reaction mixture (master-mix, primers, probes, water) to 5 $\mu$ L of the sample DNA.
2. Cycling conditions:
  - a. Heat to 50°C for 2 minutes
  - b. Heat to 95°C for 10 minutes
  - c. Run 35 cycles of 95°C for 15 seconds followed by 60°C for 1 minute

*Supplementary Table B7: Mean probably number of coliforms and E. coli with rainfall (mm) data from the 24 hours prior to sample collection.*

<b>Sample site</b>	<b>Coliforms (MPN per 100mL)</b>	<b>E. coli (MPN per 100mL)</b>	<b>Daily rainfall (mm) - 24 hours prior to collection</b>
Beach Q	288	85	trace
Beach R	246	41	0
Beach S	1,850	341	0
Beach T	576	135	trace
Beach U	0	0	0
Beach V	>2,419.60	277	0
Beach W	31	0	0
River D	727	86.2	0
River E	42	5.2	0
DWTP A	>2419.6	29.2	trace
DWTP B	648.8	115.3	0
DWTP D	48.7	16	trace
Beach A	161	10	0.1
Beach P	20	0	0.5
Estuary C	520	520	0.5
Estuary D	>2,419.60	816	0.5
Lake A	298.7	117.8	1.5
Lake B	5.2	1	1.5
Beach D	359	31	1.9
Beach K	20	0	2.7
Beach L	52	20	2.7
Beach M	0	0	2.7
Beach N	20	10	2.7
Beach O	20	10	2.7
River C	2,419.60	325.5	2.9
Beach G	775	0	3
Beach H	31	31	3
Estuary A	816.4	189.9	3.3
Estuary B	>2,419.60	2419.6	3.3
River A	172.3	40.8	3.3

River B	198.9	62	3.3
Beach I	63	10	5.6
Beach J	0	0	5.6
Beach F	1,529	842	6.3
Beach E	51	0	7.3
Beach B	613	269	7.3
Beach C	171	30	7.3
Lake C	770.1	93.3	14.2
DWTP C	435.2	17.3	27.2



Supplementary Figure B1: Mean probably number of *E. coli* and coliforms overlaid with rainfall (mm) data from the 24 hours prior to sample collection.

**Appendix C: Supplementary Material  
A Longitudinal Survey of Antibiotic-Resistant  
Enterobacterales in the Irish Environment,  
2019–2020**

*Supplementary Table C1: Sampling location details inclusive of isolates identifiers, sampling date and local authority area.*

Sample collection site	Sampling date		Isolate numbers		Local authority area
	Longitudinal survey 1	Longitudinal survey 2	Longitudinal survey 1	Longitudinal survey 2	
River H2	X	12.10.20	X	B20334 - B20339	Cork
Estuary E1	X	13.10.20	X	B20379 – B20385	
Lake C1	04.11.19	12.10.20	B19782 - B19785 (a)	B20345 – B20346	
Lake C	04.11.19	12.10.20	B19777 - B19781	B20341	
River E2	07.11.19	13.10.20	B19860 – B19863	B20358 – B20360	
River E	07.11.19	13.10.20	B19854 – B19857	B20353 – b20356	
Beach W1	05.11.19	14.10.20	B19808 – B19812	B20398 – B20401	
Beach W	05.11.19	14.10.20	B19803 – B19807	B20396	
River J	05.11.19	14.10.20	B19798 – B19801	B20392 – B20394	
Beach T	05.11.19	14.10.20	B19793 - B19796	B20388	
Beach S1	06.11.19	15.10.20	B19826 - B19828	B20405 – B20408	
Beach S	06.11.19	15.10.20	B19821 – B19822	B20403 – B20404	
River I	07.11.19	13.10.20	B19831 - B19837	B20362 - B20367	
Estuary E	06.11.19	15.10.20	B19813 - B19819	B20410 - B20413 (b)	
River H1	07.11.19	12.10.20	B19847 - B19852	B20348 - B20352	
River H	07.11.19	13.10.20	B19839 - B19846	B20374 - B20378	
DWTP B Influent	04.11.19	17.11.20	B19772 - B19775	B20419	
Hospital E	06.11.19	15.10.20	-	-	
WWTP B Influent	05.11.19	14.10.20	B19788 (a)	B20368 - B20371	
Estuary D1	07.10.19	07.09.20	B19625 - B19627	B20255 - B20257	Fingal
Estuary D	07.10.19	07.09.20	B19616 - B19620	B20250 - B20252	
Estuary C1	09.10.19	09.09.20	B19673	B20288 - B20290	
Estuary C	09.10.19	09.09.20	B19678 (a) - B19680	B20285	
Beach P2	07.10.19	07.09.20	B19631 (a) - B19634	B20259 - B20260	
Beach O1	09.10.19	09.09.20	B19690 – B19691	-	
Beach O	09.10.19	09.09.20	B19704 - B19706	-	
Beach L1	08.10.19	08.09.20	B19655 - B19656	B20278	
Beach L	08.10.19	08.09.20	B19650 – B19652	B20273 - B20275	
Beach K2	08.10.19	08.09.20	B19647	B20269 - B20271	
Beach K1	08.10.19	08.09.20	B19642 - B19643	B20268	
Beach K	08.10.19	08.09.20	B19636 - B19638	B20262 - B20264	
DWTP A Influent	07.10.19	29.09.20	B19610 (a) - B19613	B20326 - B20329	
Hospital G	21.11.19	10.09.20	B19865 - B19870	B20296 - B20297	
Airport A	11.10.19	10.09.20	B19695 - B19699	B20294	
WWTP A Influent	09.10.19	29.09.20	B19684 - B19688	B20315 - B20320	
Estuarine lagoon A1	X	17.02.20	X	B20113 - B20115	
Estuarine lagoon A	28.08.19	19.02.20	B19443 - B19446	B20118 - B20120	
Estuary B3	X	17.02.20	X	B20105 - B20111 (b)	
Estuary B2	X	17.02.20	X	B20101 - B20104	
Estuary B1	28.08.19	X	B19418 - B19421	X	
Estuary B	28.08.19	X	B19424 - B19429	X	
River F	28.08.19	X	B19437 - B19441	X	
Estuary A	28.08.19	11.02.20	B19431 - B19434	B20074 (a) - B20077	

Appendix C

River A1	27.08.19	11.02.20	B19407	B20063 - B20066	<b>Galway city</b>
River A	27.08.19	11.02.20	B19401 - B19404	B20056 - B20059	
Beach D1	26.08.19	11.02.20	B19371 - B19374	B20069 (a) - B20071	
Beach D	26.08.19	20.02.20	B19363 - B19368	B20144 - B20147 (b)	
Beach C2	26.08.19	20.02.20	B19387 - B19388	B20121 - B20126	
Beach C1	26.08.19	09.03.20	B19381 - B19383	B20149 - B20154	
Beach C	26.08.19	20.02.20	B19376 - B19379	B20134 - B20140 (b)	
Beach B1	27.08.19	09.03.20	B19412 - B19415	B20156 - B20159	
Beach B	27.08.19	20.02.20	B19394 - B19398	B20127 - B20133	
DWTP C Influent	28.01.20	05.10.20	B20040 - B20043	B20331 - B20333 (b)	
Hospital B	29.08.19	12.02.20	B19565 - B19567	B20091 - B20098	
Hospital A1	29.08.19	11.02.20	B19545 (a) - B19546	B20086 - B20090	
Hospital A2	29.08.19	11.02.20	B19554 - B19558	B20080 - B20081	
Nursing home C	29.08.19	X	B19573 (a) - B19574	X	
Nursing home A	29.08.19	X	B19536	X	
WWTP C Influent	28.01.20	28.09.20	B20047	B20300 (a) - B20305	
Lake D	11.09.19	22.07.20	B19493	B20221	<b>Galway county</b>
Lake B	10.09.19	21.07.20	B19498 - B19501	-	
River G	09.09.19	21.07.20	B19513 - B19516	B20215 - B20219	
Lake A1	09.09.19	21.07.20	B19503 - B19504	-	
Lake A	09.09.19	21.07.20	B19507 - B19510	B20210 - B20212	
Beach J	03.09.19	15.07.20	-	-	
Beach I1	04.09.19	15.07.20	B19490	-	
Beach I	04.09.19	15.07.20	B19488	B20189 - B20190	
Beach G1	03.09.19	14.07.20	B19452 - B19454	B20199 - B20201	
Beach G	03.09.19	14.07.20	B19463 - B19464	B20197	
Beach F1	04.09.19	22.07.20	B19475 - B19477	B20223 - B20225	
Beach F	04.09.19	22.07.20	B19481 - B19484	B20222	
Beach E2	X	11.03.20	X	B20170 - B20173	
Beach E1	X	11.03.20	X	B20168 - B20169	
Beach E	04.09.19	X	B19469 - B19471	X	
DWTP D Influent	27.01.20	28.09.20	-	B20321 (b) - B20323	
Hospital D	03.09.19	14.07.20	B19458	B20206	
Hospital C	10.09.19	21.07.20	B19524 - B19532	-	
Nursing home E	11.09.19	10.03.20	-	B20148	
WWTP D Influent	27.01.20	29.09.20	B20032 - B20034	B20308 - B20314	

WWTP = Wastewater treatment plant, DWTP = Drinking water treatment plant. Dates are written as day/month/year.

Supplementary Table C2: Raw data of potential contributing sources versus coliform and *E. coli* results across the point prevalence sampling rounds.

						PP survey		
	RSD	SWO	Primary UWWD	Secondary UWWD	Agricultural activity in ED	Rainfall	Coliforms	<i>E. coli</i>
<b>Cork</b>								
River H2	-	0.9	-	-	Low	X	X	X
Estuary E1	0.7	1.1	0.8	1.8	Low	X	X	X
Lake C1	-	0.9	0.3	-	Medium	X	X	X
Lake C	-	0.8	0.4	-	Medium	4.3	770.1	93.3
River E2	-	0.6	1.6	-	Low	X	X	X
River E	-	0.5	1.4	-	Medium	2.1	42	5.2
Beach W1	-	3.2	3	-	High	X	X	X
Beach W	-	0.7	-	-	High	0.0	31	<10
River J	3.6	3.5	-	-	High	X	X	X
Beach T	0.8	0.8	-	-	Medium	2.1	576	135
Beach S1	0.1	-	-	0.9	Medium	X	X	X
Beach S	0.7	-	-	0.1	Medium	3.9	1850	341
River I	2.1	0.3	0.4	0.1	Low	X	X	X
Estuary E	0.3	0.2	0.3	0.1	Low	3.9	246	41
River H1	-	0.2	0.8	1	Low	X	X	X
River H	-	0.3	0.6	1.2	Low	4.1	288	85
<b>Fingal</b>								
Estuary D1	-	-	0.8	-	Low	X	X	X
Estuary D	-	-	0.4	-	Medium	0.5	4352	816
Estuary C1	-	-	-	-	Medium	X	X	X
Estuary C	-	-	-	-	Medium	0.5	520	520
Beach P2	2.8	-	-	2.7	Low	X	X	X
Beach O1	-	0.4	-	-	Medium	X	X	X
Beach O	-	1.3	-	-	Medium	0.5	97	20
Beach L1	-	-	1.3	-	Low	X	X	X
Beach L	-	-	1.9	-	Low	0.4	52	20
Beach K2	-	-	1.5	-	Low	X	X	X
Beach K1	-	0.4	3.6	-	Low	X	X	X
Beach K	-	0.7	4	-	Low	0.4	20	<10
<b>Galway city</b>								
Estuarine lagoon A1	-	0.3	-	1	Low	X	X	X
Estuarine lagoon A	-	0.1	-	1.6	Low	X	X	X
Estuary B3	-	0.3	1.8	0.1	Low	X	X	X
Estuary B2	-	0.7	1.9	0.2	Low	X	X	X
Estuary B1	-	0.5	1.7	0.1	Low	X	X	X
Estuary B	-	-	-	0.1	Low	6.7	>2419.6	>2419.6
River F	-	0.1	-	0.1	Low	X	X	X
Estuary A	-	0.2	-	0.2	Low	6.7	816.4	198.9
River A1	-	-	-	-	Low	X	X	X
River A	-	-	-	-	Low	6.7	172.3	40.8
Beach D1	-	0.5	-	-	Low	X	X	X
Beach D	-	1	-	-	Low	0.6	359	31
Beach C2	-	0.5	-	-	Low	X	X	X
Beach C1	-	0.8	1.8	-	Low	X	X	X
Beach C	-	1.5	2.5	-	Low	5.6	171	30
Beach B1	-	0.5	1.3	0.6	Low	X	X	X

Beach B	-	0.3	1.3	1.3	Low	5.6	613	269
<b>Galway county</b>								
Lake D	-	-	-	-	Low	X	X	X
Lake B	-	1.5	-	-	Low	3.9	5.2	1
River G	-	0.5	-	-	Medium	X	X	X
Lake A1	-	0.5	2	-	Medium	X	X	X
Lake A	-	0.2	1.6	-	Medium	3.9	298.7	117.8
Beach J	-	-	-	-	Low	2.9	<10	<10
Beach I1	2	-	-	-	Medium	X	X	X
Beach I	0.2	-	-	-	Medium	2.9	63	10
Beach G1	-	0.4	0.5	-	Low	X	X	X
Beach G	-	1.4	1.3	-	Low	3.0	775	<10
Beach F1	2.4	-	-	-	Low	X	X	X
Beach F	0.1	-	-	-	Low	3.2	1529	842
Beach E2	0.1	-	-	-	Low	X	X	X
Beach E1	0.6	-	-	-	Low	X	X	X
Beach E	0.2	-	-	-	Low	4.4	51	<10

Key: RSD = Raw sewage discharge, SWO = Storm water overflow, UWWD = Urban wastewater discharge, ED = Electoral division, PP = Point prevalence. Rainfall data included the 7 day average, prior to the sample collection date. Red tiles indicate  $\geq 1000$  MPN/100mL coliforms/E. coli,  $\geq 10$ mm rainfall average, high agricultural activity and  $\leq 0.5$ km from the sample collection point to a discharge. Yellow tiles indicate between 251-999 MPN/100mL coliforms/E. coli, 5.0-9.9mm rainfall average, medium agricultural activity and between 0.6-1km from the sample collection point to a discharge. Green indicates  $\leq 250$  MPN/100mL coliforms/E. coli,  $< 5$ mm rainfall average, low agricultural activity and  $\geq 1.1$ km distance from the sample collection point to a discharge.

Supplementary Table C3: Raw data of potential contributing sources versus coliform and E. coli results across the two longitudinal survey sampling rounds.

						Longitudinal survey 1			Longitudinal survey 2		
	RSD	SWO	Primary UWWD	Secondary UWWD	Agricultural activity in ED	Rainfall	Coli-forms	E. coli	Rainfall	Coli-forms	E. coli
<b>Cork</b>											
River H2	-	0.9	-	-	Low	X	X	X	2.5	>24,1960	2280
Estuary E1	0.7	1.1	0.8	1.8	Low	X	X	X	2.2	1112	189
Lake C1	-	0.9	0.3	-	Medium	6.2	579.4	34.1	2.5	920	107.1
Lake C	-	0.8	0.4	-	Medium	6.2	>2419.6	770.1	2.5	>2419.6	1732.9
River E2	-	0.6	1.6	-	Low	5.6	2419.6	187.3	2.7	920.8	259.5
River E	-	0.5	1.4	-	Medium	5.6	2419.6	207.5	2.7	601.5	436
Beach W1	-	3.2	3	-	High	7.1	8164	327	1.9	860	211
Beach W	-	0.7	-	-	High	7.1	85	20	1.9	84	20
River J	3.6	3.5	-	-	High	6.5	15531	1850	2.5	1012	179
Beach T	0.8	0.8	-	-	Medium	6.5	1576	141	2.5	591	209
Beach S1	0.1	-	-	0.9	Medium	7.1	408	132	0.9	933	173
Beach S	0.7	-	-	0.1	Medium	7.1	331	146	0.9	63	<10
River I	2.1	0.3	0.4	0.1	Low	5.6	17329	3448	2.7	4611	504
Estuary E	0.3	0.2	0.3	0.1	Low	7.1	5172	910	0.9	6131	743
River H1	-	0.2	0.8	1	Low	5.6	17329	6867	2.5	3448	631
River H	-	0.3	0.6	1.2	Low	5.6	24196	4106	2.7	12997	3076
<b>Fingal</b>											
Estuary D1	-	-	0.8	-	Low	7.3	>2419.6	410.6	4.1	364	62

Appendix C

Estuary D	-	-	0.4	-	Medium	7.3	>2419.6	1299.7	4.1	24196	8164
Estuary C1	-	-	-	-	Medium	4.2	2419.6	344.8	4.6	332	73
Estuary C	-	-	-	-	Medium	4.2	1299.7	37.7	4.6	1483	355
Beach P2	2.8	-	-	2.7	Low	7.3	5794	884	4.1	19863	759
Beach O1	-	0.4	-	-	Medium	4.2	98	10	4.6	31	10
Beach O	-	1.3	-	-	Medium	4.2	281	107	4.6	10	10
Beach L1	-	-	1.3	-	Low	5	75	20	4.5	10	<10
Beach L	-	-	1.9	-	Low	5	598	108	4.5	201	52
Beach K2	-	-	1.5	-	Low	5	52	10	4.5	20	10
Beach K1	-	0.4	3.6	-	Low	5	63	<10	4.5	52	10
Beach K	-	0.7	4	-	Low	5	884	63	4.5	933	85
<b>Galway city</b>											
Estuarine lagoon A1	-	0.3	-	1	Low	X	X	X	7.1	3076	487
Estuarine lagoon A	-	0.1	-	1.6	Low	6.5	>24196	24196	7.7	8664	1450
Estuary B3	-	0.3	1.8	0.1	Low	X	X	X	7.1	1850	435
Estuary B2	-	0.7	1.9	0.2	Low	X	X	X	7.1	512	110
Estuary B1	-	0.5	1.7	0.1	Low	6.5	1860	408	X	X	X
Estuary B	-	-	-	0.1	Low	6.5	2419.6	62.1	X	X	X
River F	-	0.1	-	0.1	Low	6.5	>2419.6	285.1	X	X	X
Estuary A	-	0.2	-	0.2	Low	6.5	>2419.6	435.2	7.2	5172	3255
River A1	-	-	-	-	Low	5.6	488.4	9.7	7.2	920.8	488.4
River A	-	-	-	-	Low	5.6	1732.9	378.4	7.2	344.1	114.5
Beach D1	-	0.5	-	-	Low	5.7	1160	238	7.2	350	31
Beach D	-	1	-	-	Low	5.7	109	10	11	816	521
Beach C2	-	0.5	-	-	Low	5.7	63	10	11	556	109
Beach C1	-	0.8	1.8	-	Low	5.7	31	20	3.2	256	74
Beach C	-	1.5	2.5	-	Low	5.7	262	10	11	435	160
Beach B1	-	0.5	1.3	0.6	Low	5.6	1956	441	3.2	554	193
Beach B	-	0.3	1.3	1.3	Low	5.6	63	31	11	2143	1376
<b>Galway county</b>											
Lake D	-	-	-	-	Low	1.8	204.6	108.6	0.9	328.2	27.9
Lake B	-	1.5	-	-	Low	2.9	93.3	14.8	1.3	178.9	8.4
River G	-	0.5	-	-	Medium	3.6	124.6	18.7	1.3	74.9	13.1
Lake A1	-	0.5	2	-	Medium	3.6	275.5	65.1	1.3	172.3	19.7
Lake A	-	0.2	1.6	-	Medium	3.6	>2419.6	>2419.5	1.3	436	76.3
Beach J	-	-	-	-	Low	7.7	10	<10	1.5	31	10
Beach I1	2	-	-	-	Medium	7.2	63	20	1.5	10	<10
Beach I	0.2	-	-	-	Medium	7.2	185	97	1.5	146	63
Beach G1	-	0.4	0.5	-	Low	7.7	2755	1046	5.3	1223	262
Beach G	-	1.4	1.3	-	Low	7.7	1178	345	5.3	52	<10
Beach F1	2.4	-	-	-	Low	7.2	189	75	0.9	305	52
Beach F	0.1	-	-	-	Low	7.2	41	<10	0.9	262	31
Beach E2	0.1	-	-	-	Low	X	X	X	5.9	>24196	>24196
Beach E1	0.6	-	-	-	Low	X	X	X	5.9	52	10
Beach E	0.2	-	-	-	Low	6.9	727	98	5.9	789	160

Supplementary Table C4: Total number of isolates from each sample type displaying susceptible, intermediate or resistant phenotypes to each of the antibiotics.

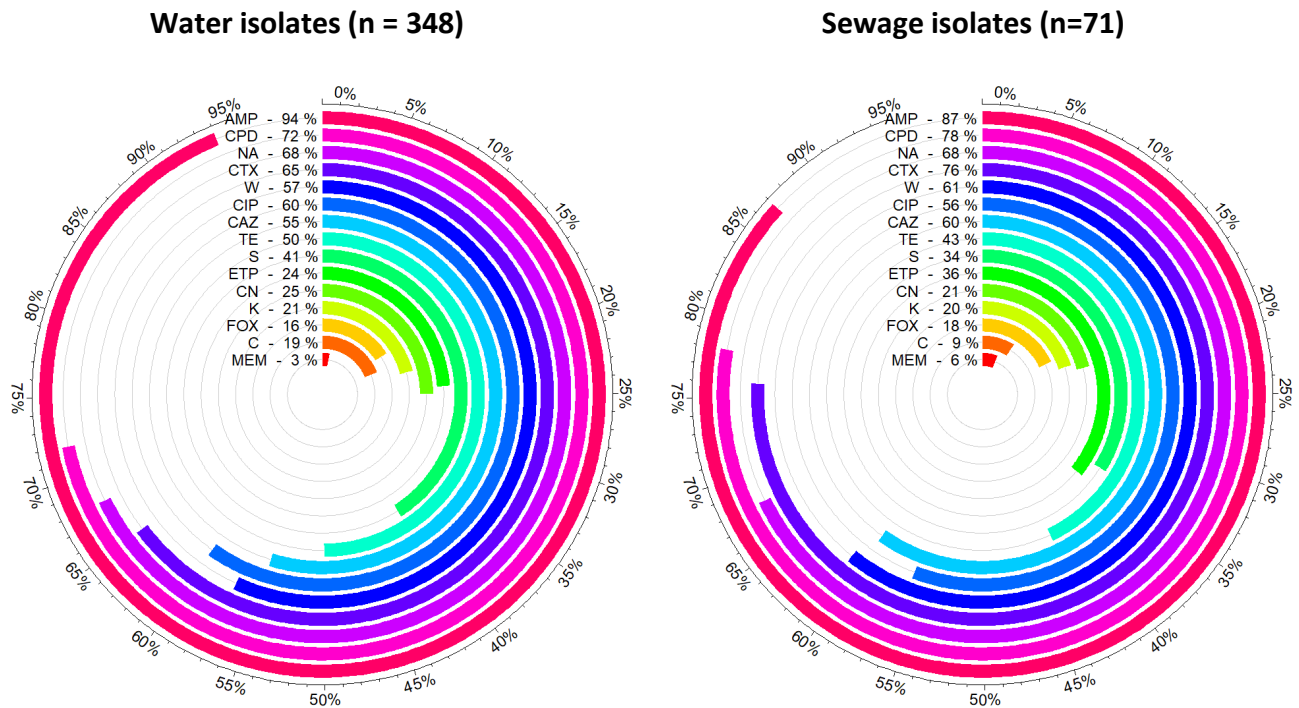
	CPD		CPD/CV		FOX		MEM			ETP		AMP		CIP			CN		C		K			W		S10			TE			NA			CTX			CAZ		
	S	R	<5 mm	>5 mm	S	R	S	I	R	S	R	S	R	S	I	R	S	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	
Seawater	25	127	59	93	122	30	141	7	4	118	34	8	144	31	25	96	118	34	112	40	102	18	32	68	84	55	41	56	66	3	83	33	9	110	35	4	113	40	10	102
Estuary	12	54	31	35	49	17	56	4	6	40	26	3	63	14	3	49	50	16	57	9	44	9	13	22	44	19	10	37	25	0	41	11	5	50	11	1	54	13	7	46
River	14	75	37	52	70	19	76	8	5	73	16	1	88	28	12	49	69	20	72	17	63	8	18	32	57	36	14	39	37	1	51	27	8	54	19	4	66	23	14	52
Lake	7	4	7	4	11	0	11	0	0	10	1	0	11	4	0	7	9	2	8	3	8	1	2	4	7	5	1	5	4	0	7	4	0	7	8	0	3	9	1	1
Estuarine lagoon	2	8	7	3	8	2	8	2	0	5	5	1	9	5	0	5	5	5	9	1	5	2	3	6	4	6	1	3	9	0	1	2	1	7	2	0	8	3	0	7
DWTP influent	7	13	10	10	18	2	20	0	0	19	1	3	17	8	1	11	17	3	17	3	16	1	3	10	10	6	7	7	9	0	11	6	1	13	9	0	11	9	0	11
Hospital	5	30	21	14	21	14	27	6	2	17	18	1	34	6	7	22	14	21	30	5	12	8	15	13	22	16	8	11	20	0	15	5	2	28	6	1	28	9	1	25
Nursing home	2	3	2	3	5	0	5	0	0	4	1	1	4	2	0	3	5	0	5	0	5	0	0	1	4	2	2	1	3	0	2	1	0	4	2	0	3	2	1	2
Airport	1	5	2	4	5	1	5	0	1	4	2	1	5	1	2	3	5	1	5	1	4	1	1	2	4	1	2	3	3	0	3	3	0	3	1	0	5	1	1	4
WWTP influent	4	21	10	15	21	4	21	4	0	15	10	3	22	8	4	13	23	2	24	1	16	4	5	16	9	11	5	9	15	0	10	9	1	15	4	1	20	7	3	15

S = Susceptible, I = Intermediate, R = Resistant, CPD = Cefpodoxime, CPD/CV = Cefpodoxime/clavulanic acid, FOX = Cefoxitin, MEM = Meropenem, ETP = Ertapenem, AMP = Ampicillin, CIP = Ciprofloxacin, CN = Gentamicin, C = Chloramphenicol, K = Kanamycin, W = Trimethoprim, S10 = Streptomycin, TE = Tetracycline, NA = Nalidixic acid, CTX = Cefotaxime, CAZ = Ceftazidime, DWTP = Drinking water treatment plant, WWTP = Wastewater treatment plant.

Supplementary Table C5: Percentage of isolates from each sample type displaying susceptible, intermediate or resistant phenotypes to each of the antibiotics.

	CPD		CPD/CV		FOX		MEM			ETP		AMP		CIP			CN		C		K			W		S10			TE			NA			CTX			CAZ		
	S	R	<5 mm	>5 mm	S	R	S	I	R	S	R	S	R	S	I	R	S	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	
Seawater	16	84	39	61	80	20	93	5	3	78	22	5	95	20	16	63	78	22	74	26	67	12	21	45	55	36	27	37	43	2	55	22	6	72	23	3	74	26	7	67
Estuary	18	82	47	53	74	26	85	6	9	61	39	5	95	21	5	74	76	24	86	14	67	14	20	33	67	29	15	56	38	0	62	17	8	76	17	2	82	20	11	70
River	16	84	42	58	79	21	85	9	6	82	18	1	99	31	13	55	78	22	81	19	71	9	20	36	64	40	16	44	42	1	57	30	9	61	21	4	74	26	16	58
Lake	64	36	64	36	100	0	100	0	0	91	9	0	100	36	0	64	82	18	73	27	73	9	18	36	64	45	9	45	36	0	64	36	0	64	73	0	27	82	9	9
Estuarine lagoon	20	80	70	30	80	20	80	20	0	50	50	10	90	50	0	50	50	90	10	50	20	30	60	40	60	10	30	90	0	10	20	10	70	20	0	80	30	0	70	
DWTP influent	35	65	50	50	90	10	100	0	0	95	5	15	85	40	5	55	85	15	85	15	80	5	15	50	50	30	35	35	45	0	55	30	5	65	45	0	55	45	0	55
Hospital	14	86	60	40	60	40	77	17	6	49	51	3	97	17	20	63	40	60	86	14	34	23	43	37	63	46	23	31	57	0	43	14	6	80	17	3	80	26	3	71
Nursing home	40	60	40	60	100	0	100	0	0	80	20	20	80	40	0	60	100	0	100	0	100	0	0	20	80	40	40	20	60	0	40	20	0	80	40	0	60	40	20	40
Airport	17	83	33	67	83	17	83	0	17	67	33	17	83	17	33	50	83	17	83	17	67	17	17	33	67	17	33	50	50	0	50	50	0	50	17	0	83	17	17	67
WWTP influent	16	84	40	60	84	16	84	16	0	60	40	12	88	32	16	52	92	8	96	4	64	16	20	64	36	44	20	36	60	0	40	36	4	60	16	4	80	28	12	60

*S* = Susceptible, *I* = Intermediate, *R* = Resistant, CPD = Cefpodoxime, CPD/CV = Cefpodoxime/clavulanic acid, FOX = Cefoxitin, MEM = Meropenem, ETP = Ertapenem, AMP = Ampicillin, CIP = Ciprofloxacin, CN = Gentamicin, C = Chloramphenicol, K = Kanamycin, W = Trimethoprim, S10 = Streptomycin, TE = Tetracycline, NA = Nalidixic acid, CTX = Cefotaxime, CAZ = Ceftazidime, DWTP = Drinking water treatment plant, WWTP = Wastewater treatment plant.



Supplementary Figure C1: Percentage of isolates from water and sewage sources displaying a resistance to the panel of antibiotics.

Supplementary Table C6: Details of clusters including sequence types of *Klebsiella* isolates detected, sampling information and outcomes of core genome multi locus sequence type comparisons.

Cluster	ST	Isolate IDs	Sample name	Date of collection	Local authority area	Distance between sites (km)	No. of loci variability	cgMLST similarity
1	8	B18231 B19379	Estuary A Beach C	4 <sup>th</sup> Dec '18 26 <sup>th</sup> Aug '19	Galway city Galway city	2.8	2	99.7%
2	11	B18235 B18291	Estuary A Beach C	04 <sup>th</sup> Dec '18 06 <sup>th</sup> Dec '18	Galway city Galway city	2.8	2	99.7%
3	17	B18283 B19366 B19433 B20040 B20121 B20134	Beach C Beach D Estuary A DWTP C Influent Beach C2 Beach C	06 <sup>th</sup> Dec '18 26 <sup>th</sup> Aug '19 28 <sup>th</sup> Aug '19 28 <sup>th</sup> Jan '20 20 <sup>th</sup> Feb '20 20 <sup>th</sup> Feb '20	Galway city Galway city Galway city Galway city Galway city Galway city	B20040 – 6.0 B20134 - 0.9	3 0	99.6% 100%
4	231	B19819 B19846	Estuary E River H	06 <sup>th</sup> Nov '19 07 <sup>th</sup> Nov '19	Cork Cork	3.3	0	100%

5	289	B20154 B20158	Beach C1 Beach B1	09 <sup>th</sup> Mar '20 09 <sup>th</sup> Mar '20	Galway city Galway city	1.8	1	99.9%
6	309	B20338  B20377 B20384	River H2  River H Estuary E1	12 <sup>th</sup> Oct '20  13 <sup>th</sup> Oct '20 13 <sup>th</sup> Oct '20	Cork  Cork Cork	B20377 – 5.6 B20384 – 9.6 B20384 – 4.5	0 0 0	100% 100% 100%
7	323	B19023  B19326  B19426 B19574 B19849  B20144  B20319	Nursing home C  WWTP C Influent  Estuary B Nursing home C River H1  Beach D  WWTP A Influent	14 <sup>th</sup> Jan '19  24 <sup>th</sup> Jul '19  28 <sup>th</sup> Aug '19 29 <sup>th</sup> Aug '19 07 <sup>th</sup> Nov '19  20 <sup>th</sup> Feb '20  29 <sup>th</sup> Sep '20	Galway city  Galway city  Galway city Galway city Cork  Galway city  Fingal	B19574 - 0.0 B20319 – 194.5 B19426 – 1.6 B20144 – 4.8 B20144 – 5.5 B20319 - 194.5 B19326 – 161.0 B19426 – 162.3 B20319 – 201.2 B19849 – 162.1	0 7 0 10 10 7 0 0 10 10	100% 99.0% 100% 98.6% 98.6% 99.0% 100% 100% 98.6% 98.6%
8	405	B18240 B18200  B18175 B19071	Estuary B Hospital B  Beach A River C	04 <sup>th</sup> Dec '18 28 <sup>th</sup> Nov '18  26 <sup>th</sup> Nov '18 21 <sup>st</sup> Jan '19	Galway city Galway city  Galway city Galway county	B18175 – 2.1 B18240 – 1.5 B18175 – 3.4	2 6 8	99.7% 99.1% 98.8%

*DWTP: Drinking water treatment plant influent, WWTP: Wastewater treatment plant influent, ST: Sequence type, cgMLST: core genome multi locus sequence type. Further details including distance between collection sites and outcomes of cgMLST comparisons were provided for isolates that were  $\geq 98.5\%$  similar (10 or less cgMLST loci that varied).*

*Supplementary Table C7: Details of clusters including sequence types of Escherichia isolates detected, sampling information and outcomes of cgMLST comparisons.*

Cluster	ST	Isolate IDs	Sample name	Date of collection	Local authority area	Distance between sites (km)	No. of loci variability	cgMLST similarity
1	10	B18161 B19104 B19165 B19171 B19376 B19413 B19655 B19832 B20110 B20353 B20358 B20382	Hospital A2 Beach G Beach M Estuary C Beach C Beach B1 Beach L1 River I Estuary B3 River E River E2 Estuary E1	26 <sup>th</sup> Nov '18 28 <sup>th</sup> Jan '19 02 <sup>nd</sup> Apr '19 03 <sup>rd</sup> Apr '19 26 <sup>th</sup> Aug '19 27 <sup>th</sup> Aug '19 08 <sup>th</sup> Oct '19 06 <sup>th</sup> Nov '19 17 <sup>th</sup> Feb '20 13 <sup>th</sup> Oct '20 13 <sup>th</sup> Oct '20 13 <sup>th</sup> Oct '20	Galway city Galway co Fingal Fingal Galway city Galway city Fingal Cork Cork Galway city Cork Cork Cork	B19171 – 3.3         B20358 – 0.7	4         2	99.8%         99.9%
2	34	B19395 B19796	Beach B Beach T	27 <sup>th</sup> Aug '19 05 <sup>th</sup> Nov '19	Galway city Cork	-	508	-
3	38	B19325 B18199 B18213 B18228 B19008 B19018 B19205 B19224 B19488 B19633 B19695	WWTP C Hospital B River B Estuary A Beach D Nursing home C WWTP A Influent Estuary E Beach I Beach P2 Airport A	24 <sup>th</sup> Jul '19 28 <sup>th</sup> Nov '18 04 <sup>th</sup> Dec '18 04 <sup>th</sup> Dec '18 08 <sup>th</sup> Jan '19 14 <sup>th</sup> Jan '19 29 <sup>th</sup> Apr '19 13 <sup>th</sup> May '19 04 <sup>th</sup> Sep '19 07 <sup>th</sup> Oct '19 11 <sup>th</sup> Oct '19	Galway city Galway city Galway city Galway city Galway city Galway city Fingal Cork Galway co Fingal Fingal	B18228 – 1.1	2	99.9%

Appendix C

		B19850 B20118 B20352 B20378	River H1 Estuarine lagoon A River H1 River H	07 <sup>th</sup> Nov '19 19 <sup>th</sup> Feb '20 12 <sup>th</sup> Oct '20 13 <sup>th</sup> Oct '20	Cork Galway city Cork Cork			
4	69	B18284 B19565 B19618 B19840	Beach C Hospital B Estuary D River H	06 <sup>th</sup> Dec '18 29 <sup>th</sup> Aug '19 07 <sup>th</sup> Oct '19 07 <sup>th</sup> Nov '19	Galway city Galway city Fingal Cork	-	-	-
5	88	B20348 B20383	River H1 Estuary E1	12 <sup>th</sup> Oct '20 13 <sup>th</sup> Oct '20	Cork Cork	-	34	-
6	101	B19855 B19861	River E River E2	07 <sup>th</sup> Nov '19 07 <sup>th</sup> Nov '19	Cork Cork	0.7	0	100%
7 (cluster 1)	131	B19164 B19229 B19234 B19248 B19367 B19373 B19388 B19444 B19610B B20294 B20331 B20369 B20400	Beach L River H Beach S Beach T Beach D Beach D1 Beach C2 Estuarine lagoon A DWTP A Airport A DWTP C Influent WWTP B Influent Beach W1	02 <sup>nd</sup> Apr '19 13 <sup>th</sup> May '19 14 <sup>th</sup> May '19 15 <sup>th</sup> May '19 26 <sup>th</sup> Aug '19 26 <sup>th</sup> Aug '19 26 <sup>th</sup> Aug '19 28 <sup>th</sup> Aug '19 07 <sup>th</sup> Oct '19 10 <sup>th</sup> Sep '20 05 <sup>th</sup> Oct '20 14 <sup>th</sup> Oct '20 14 <sup>th</sup> Oct '20	Fingal Cork Cork Cork Galway city Galway city Galway city Galway city Fingal Fingal Galway city Cork Cork	B20400 – 3.1	0	100%
8 (cluster 2)	131	B18191 B18215 B18225 B18246 B18260 B19038 B19079 B19091 B19148 B19181 B19215 B19860 B20034 B19619 B20115 B20098 B20071 B20147a B20206 B20150 B20360 B20368 B20255 B20263 B20289 B20318	Hospital B River B Estuary A Estuary B Nursing home A Nursing home D Beach E Beach F Hospital B Estuary D Lake C River E2 WWTP D Influent Estuary D Estuarine lagoon A1 Hospital B Beach D1 Beach D Hospital D Beach C1 River E2 WWTP B Influent River E Beach K Estuary C1 WWTP A Influent	28 <sup>th</sup> Nov '18 04 <sup>th</sup> Dec '18 04 <sup>th</sup> Dec '18 04 <sup>th</sup> Dec '18 06 <sup>th</sup> Dec '18 21 <sup>st</sup> Jan '19 22 <sup>nd</sup> Jan '19 22 <sup>nd</sup> Jan '19 20 <sup>th</sup> Feb '19 03 <sup>rd</sup> Apr '19 09 <sup>th</sup> May '19 07 <sup>th</sup> Nov '19 27 <sup>th</sup> Jan '20 07 <sup>th</sup> Oct '19 17 <sup>th</sup> Feb '20 12 <sup>th</sup> Feb '20 11 <sup>th</sup> Feb '20 20 <sup>th</sup> Feb '20 14 <sup>th</sup> July '20 09 <sup>th</sup> Mar '20 13 <sup>th</sup> Oct '20 14 <sup>th</sup> Oct '20 13 <sup>th</sup> Oct '20 08 <sup>th</sup> Sep '20 09 <sup>th</sup> Sep '20 29 <sup>th</sup> Sep '20	Galway city Galway city Galway city Galway city Galway city Galway co Galway co Galway co Galway city Fingal Cork Cork Galway co Fingal Galway city Galway city Galway city Galway city Galway co Galway city Cork Cork Cork Fingal Fingal Fingal	B20150 – 3.3	9	99.6%
9	162	B19454 B19463	Beach G1 Beach G	03 <sup>rd</sup> Sep '19 03 <sup>rd</sup> Sep '19	Galway co Galway co	B19463 – 1.3	1	99.96%
10	167	B19159 B20127 B20104 B20148 B20159	Beach K Beach B Estuary B2 Nursing home E Beach B1	02 <sup>nd</sup> Apr '19 20 <sup>th</sup> Feb '20 17 <sup>th</sup> Feb '20 10 <sup>th</sup> Mar '20 09 <sup>th</sup> Mar '20	Fingal Galway city Galway city Galway city Galway city	B20104 – 14.5	0	100%
11	224	B19503 B20275	Lake A1 Beach L	09 <sup>th</sup> Sep '19 08 <sup>th</sup> Sep '20	Galway co Fingal	-	102	-
12	394	B19377 B19699 B19781 B20250	Beach C Airport A Lake C Estuary C	26 <sup>th</sup> Aug '19 11 <sup>th</sup> Oct '19 04 <sup>th</sup> Nov '19 07 <sup>th</sup> Sep '20	Galway city Fingal Cork Fingal	-	-	-

13	405	B18275 B19429 B19673 B19818 B19844 B19852 B20105 B20366 B20385	Beach B Estuary B Estuary C1 Estuary E River H River H1 Estuary B3 River I Estuary E1	06 <sup>th</sup> Dec '18 2 <sup>th</sup> Aug '19 09 <sup>th</sup> Oct '19 06 <sup>th</sup> Nov '19 07 <sup>th</sup> Nov '19 07 <sup>th</sup> Nov '19 17 <sup>th</sup> Feb '20 13 <sup>th</sup> Oct '20 13 <sup>th</sup> Oct '20	Galway city Galway city Fingal Cork Cork Cork Galway city Cork Cork	B19844 – 3.3 B19852 – 0.3 B19818 – 3.6 B20385 – 235.6	4 4 0 3	99.8% 99.8% 100% 99.9%
14	410	B19368  B19398 B19421  B19441  B19837	Beach D  Beach B Estuary B1  River F  River I	26 <sup>th</sup> Aug '19  27 <sup>th</sup> Aug '19 28 <sup>th</sup> Aug '19  28 <sup>th</sup> Aug '19  06 <sup>th</sup> Nov '19	Galway city  Galway city Galway city  Galway city  Cork	B19398 – 4.2 B19421 – 5.6 B19441 – 1.2 B19441 – 0.6  B19398 – 1.4 B19368 – 5.2	0 0 0 0  0 0	100% 100% 100% 100%  100% 100%
15	540	B18271 B20273	Beach B Beach L	06 <sup>th</sup> Dec '18 08 <sup>th</sup> Sep '20	Galway city Fingal	-	656	-
16	617	B18183 B18147	Beach A Hospital A1	26 <sup>th</sup> Nov '18 26 <sup>th</sup> Nov '18	Galway city Galway city	-	173	-
17	648	B19188 B19638 B19650 B20170 B20410	Airport A Beach K Beach L Beach E2 Estuary E	04 <sup>th</sup> Apr '19 08 <sup>th</sup> Oct '19 08 <sup>th</sup> Oct '19 11 <sup>th</sup> Mar '20 15 <sup>th</sup> Oct '20	Fingal Fingal Fingal Galway co Cork	B19650 – 6.3 B19638 – 6.4	2 2	99.9% 99.9%
18	1193	B19124 B19170 B19678 (a) B19684 B20162	Beach I Beach N Estuary C WWTP A Influent Beach E	29 <sup>th</sup> Jan '19 02 <sup>nd</sup> Apr '19 09 <sup>th</sup> Oct '19 09 <sup>th</sup> Oct '19 11 <sup>th</sup> Mar '20	Galway co Fingal Fingal Fingal Galway co	-	-	-
19	5584	B19075 B19160	River C Beach K	21 <sup>st</sup> Jan '19 02 <sup>nd</sup> Apr '19	Galway co Fingal	-	22	-

*DWTP: Drinking water treatment plant influent, WWTP: Wastewater treatment plant influent, Galway co = Galway county. ST: Sequence type, cgMLST: core genome multi locus sequence type. Further details including distance between collection sites and outcomes of cgMLST comparisons were provided for isolates that were  $\geq 98.5\%$  similar.*

Supplementary Table C8: Isolate identifiers on the Escherichia database on PubMLST, Klebsiella Pasteur MLST database and European nucleotide achieve database (Project: PRJEB47048).

Isolate ID	Bacterial species	Experiment accession on European nucleotide achieve	Escherichia database on PubMLST	Klebsiella Pasteur MLST database
B19374 (WTCHG_781213_72155287)	<i>E. coli</i>	ERX4159066 (PRJEB21277)	286	-
B19366	<i>K. pneumoniae</i>	ERX6162348	-	15969
B19367	<i>E. coli</i>	ERX6172840	342	-
B19368	<i>E. coli</i>	ERX6174150	343	-
B19373	<i>E. coli</i>	ERX6174151	344	-
B19376	<i>E. coli</i>	ERX6174152	345	-
B19377	<i>E. coli</i>	ERX6174153	346	-
B19379	<i>K. pneumoniae</i>	ERX6174154	-	15970
B19382	<i>E. coli</i>	ERX6174155	347	-
B19383	<i>E. coli</i>	ERX6174156	348	-
B19388	<i>E. coli</i>	ERX6174157	349	-
B19395	<i>E. coli</i>	ERX6174158	350	-
B19396	<i>K. pneumoniae</i>	ERX6174159	-	15971
B19398	<i>E. coli</i>	ERX6174160	351	-
B19404	<i>E. coli</i>	ERX6174161	352	-
B19413	<i>E. coli</i>	ERX6174162	353	-
B19420	<i>E. coli</i>	ERX6174163	354	-
B19421	<i>E. coli</i>	ERX6174164	355	-
B19426	<i>K. pneumoniae</i>	ERX6174165	-	15972
B19429 (MiSeq)	<i>E. coli</i>	ERX6628602	436	-
B19433	<i>K. pneumoniae</i>	ERX6174166	-	15973
B19441	<i>E. coli</i>	ERX6174167	356	-
B19444	<i>E. coli</i>	ERX6174168	357	-
B19446 (MiSeq)	<i>E. hormaechei</i>	ERX6628603	-	-
B19454	<i>E. coli</i>	ERX6174169	358	-
B19458	<i>K. oxytoca</i>	ERX6174479	-	-
B19463	<i>E. coli</i>	ERX6174480	359	-
B19476	<i>E. albertii</i>	ERX6174481	360	-
B19488	<i>E. coli</i>	ERX6174482	361	-
B19503	<i>E. coli</i>	ERX6174483	362	-
B19509	<i>E. coli</i>	ERX6174484	363	-
B19514	<i>E. coli</i>	ERX6174485	364	-
B19516	<i>K. pneumoniae</i>	ERX6174486	-	15974

B19525 (MiSeq)	<i>E. kobei</i>	ERX6628604	-	-
B19526	<i>E. coli</i>	ERX6174487	365	-
B19532 (MiSeq)	<i>C. freundii</i>	ERX6628605	-	-
B19558	<i>E. coli</i>	ERX6174488	366	-
B19565	<i>E. coli</i>	ERX6174489	367	-
B19574	<i>K. pneumoniae</i>	ERX6174490	-	15975
B19610B	<i>E. coli</i>	ERX6174491	370	-
B19618	<i>E. coli</i>	ERX6174492	371	-
B19619	<i>E. coli</i>	ERX6174493	372	-
B19625	<i>E. coli</i>	ERX6174494	373	-
B19633	<i>E. coli</i>	ERX6174495	374	-
B19638	<i>E. coli</i>	ERX6174496	375	-
B19647	<i>E. coli</i>	ERX6174497	376	-
B19650	<i>E. coli</i>	ERX6174498	377	-
B19655	<i>E. coli</i>	ERX6174499	379	-
B19673	<i>E. coli</i>	ERX6174500	380	-
B19678A	<i>E. coli</i>	ERX6174501	381	-
B19684	<i>E. coli</i>	ERX6174502	382	-
B19690	<i>K. pneumoniae</i>	ERX6174503	-	15976
B19695 (MiSeq)	<i>E. coli</i>	ERX6628606	434	-
B19697	<i>K. pneumoniae</i>	ERX6174504	-	15977
B19699	<i>E. coli</i>	ERX6174505	383	-
B19781	<i>E. coli</i>	ERX6174506	384	-
B19785A	<i>E. coli</i>	ERX6174507	385	-
B19796	<i>E. coli</i>	ERX6174508	386	-
B19800	<i>E. coli</i>	ERX6628612	387	-
B19808	<i>E. coli</i>	ERX6628613	388	-
B19814	<i>K. pneumoniae</i>	ERX6628614	-	15955
B19818 (MiSeq)	<i>E. coli</i>	ERX6154922	437	-
B19819 (MiSeq)	<i>K. pneumoniae</i>	ERX6628607	-	15953
B19822	<i>E. coli</i>	ERX6628615	322	-
B19832	<i>E. coli</i>	ERX6628616	323	-
B19837	<i>E. coli</i>	ERX6628617	324	-
B19840	<i>E. coli</i>	ERX6628618	325	-
B19844 (MiSeq)	<i>E. coli</i>	ERX6628608	438	-
B19846 (MiSeq)	<i>K. pneumoniae</i>	ERX6628609	-	15954
B19849	<i>K. pneumoniae</i>	ERX6628619	-	15956
B19850	<i>E. coli</i>	ERX6628620	327	-
B19852 (MiSeq)	<i>E. coli</i>	ERX6628610	439	-

B19855	<i>E. coli</i>	ERX6628621	328	-
B19860	<i>E. coli</i>	ERX6628622	329	-
B19861	<i>E. coli</i>	ERX6628623	330	-
B19869	<i>K. oxytoca</i>	ERX6628624	-	-
B20034	<i>E. coli</i>	ERX6628625	331	-
B20040	<i>K. pneumoniae</i>	ERX6628626	-	15957
B20047	<i>E. coli</i>	ERX6628627	332	-
B20091	<i>K. pneumoniae</i>	ERX6628628	-	15958
B20095	<i>K. oxytoca</i>	ERX6628629	-	-
B20105	<i>E. coli</i>	ERX6628630	333	-
B20121	<i>K. pneumoniae</i>	ERX6628631	-	15959
B20123	<i>E. coli</i>	ERX6628632	334	-
B20127	<i>E. coli</i>	ERX6628633	335	-
B20134 (MiSeq)	<i>K. pneumoniae</i>	ERX6682737	-	16005
B20154	<i>K. pneumoniae</i>	ERX6628634	-	15960
B20070	<i>E. coli</i>	ERX6628635	398	-
B20071	<i>E. coli</i>	ERX6628636	400	-
B20086	<i>E. cloacae</i>	ERX6628637	-	-
B20098	<i>E. coli</i>	ERX6628638	396	-
B20104	<i>E. coli</i>	ERX6628639	393	-
B20110	<i>E. coli</i>	ERX6628640	394	-
B20115	<i>E. coli</i>	ERX6628641	395	-
B20118	<i>E. coli</i>	ERX6628642	410	-
B20125	<i>E. coli</i>	ERX6628643	399	-
B20144	<i>K. pneumoniae</i>	ERX6628644	-	15978
B20147a	<i>E. coli</i>	ERX6628645	401	-
B20148	<i>E. coli</i>	ERX6628646	405	-
B20150	<i>E. coli</i>	ERX6628647	404	-
B20158	<i>K. pneumoniae</i>	ERX6628648	-	15980
B20159	<i>E. coli</i>	ERX6628649	409	-
B20162	<i>E. coli</i>	ERX6628650	402	-
B20170	<i>E. coli</i>	ERX6628651	392	-
B20206	<i>E. coli</i>	ERX6628652	403	-
B20250	<i>E. coli</i>	ERX6628653	426	-
B20251	<i>E. coli</i>	ERX6628654	427	-
B20255	<i>E. coli</i>	ERX6628655	429	-
B20263	<i>E. coli</i>	ERX6628656	430	-
B20269	<i>E. coli</i>	ERX6628657	390	-
B20273	<i>E. coli</i>	ERX6628658	428	-

B20275	<i>E. coli</i>	ERX6628659	389	-
B20289	<i>E. coli</i>	ERX6628660	432	-
B20294	<i>E. coli</i>	ERX6628661	391	-
B20297	<i>C. freundii</i>	ERX6628662	-	-
B20309	<i>E. cloacae</i>	ERX6628663	-	-
B20311	<i>E. hormaechei</i>	ERX6628664	-	-
B20312	<i>E. coli</i>	ERX6628665	397	-
B20315	<i>K. pneumoniae</i>	ERX6628666	-	15986
B20318	<i>E. coli</i>	ERX6628667	433	-
B20319	<i>K. pneumoniae</i>	ERX6628668	-	15987
B20326	<i>E. coli</i>	ERX6628669	431	-
B20331	<i>E. coli</i>	ERX6628670	406	-
B20338	<i>K. pneumoniae</i>	ERX6628671	-	15983
B20339	<i>E. coli</i>	ERX6628672	415	-
B20348	<i>E. coli</i>	ERX6628673	416	-
B20351	<i>K. pneumoniae</i>	ERX6628674	-	15985
B20352	<i>E. coli</i>	ERX6628675	411	-
B20353	<i>E. coli</i>	ERX6628676	422	-
B20358	<i>E. coli</i>	ERX6628677	423	-
B20360	<i>E. coli</i>	ERX6628678	424	-
B20366	<i>E. coli</i>	ERX6628679	412	-
B20367	<i>K. pneumoniae</i>	ERX6628680	-	15984
B20368	<i>E. coli</i>	ERX6628681	425	-
B20369	<i>E. coli</i>	ERX6628682	419	-
B20377	<i>K. pneumoniae</i>	ERX6628683	-	15981
B20378	<i>E. coli</i>	ERX6628684	413	-
B20382	<i>E. coli</i>	ERX6628685	417	-
B20383	<i>E. coli</i>	ERX6628686	418	-
B20384	<i>K. pneumoniae</i>	ERX6628687	-	15982
B20385	<i>E. coli</i>	ERX6628688	414	-
B20398	<i>E. hormaechei</i>	ERX6628689	-	-
B20400	<i>E. coli</i>	ERX6628690	420	-
B20410	<i>E. coli</i>	ERX6628691	421	-
B20093 (MiSeq)	<i>C. freundii</i>	ERX6682736	-	-
B20308 (MiSeq)	<i>R. ornithinolytica</i>	ERX6682738	-	-

All isolates are found under Project: PRJEB47048 (Secondary Study Accession: ERP131289) on the ENA database, apart from B19374 which can be found under Project: PRJEB21277. All isolates were sequenced using the Illumina NovaSeq 6000 platform, apart from the 13 isolates listed with (MiSeq) in this table.

**Appendix D: Additional Publications, Conference  
Presentations and Awards**

## Publications

List of publications not included in this thesis:

- Chique, C., Cullinan, J., **Hooban, B.**, Morris, D. (2019) 'Mapping and Analysing Potential Sources and Transmission Routes of Antimicrobial Resistant Organisms in the Environment using Geographic Information Systems-An Exploratory Study', *Antibiotics*, 8(1). doi: 10.3390/antibiotics8010016.
- O' Connor, L., Brehony, C., Fitzhenry, K., **Hooban, B.**, Joyce, A., Cahill, N., Mahon, B., Hickey, P., Keane, S., Cormican, M., Morris, D. (2021) Widespread contamination of recreational seawaters, rivers and lakes with Shiga toxigenic *Escherichia coli*. *Proceedings of the International Academy of Ecology and Environmental Sciences*, 11(2).
- Farrell, M. L., Joyce, A., Duane, S., Fitzhenry, K., **Hooban, B.**, Burke, L. P., Morris, D. (2021). Evaluating the potential for exposure to organisms of public health concern in naturally occurring bathing waters in Europe: A scoping review. *Water Research*, 206. doi: 10.1016/j.watres.2021.117711

## Conferences – Oral Presentations

**B. Hooban**, K. Fitzhenry, A. Joyce, L. O'Connor, A. Chueiri, M. Farrell, M. Cormican, D. Morris. Longitudinal survey of antimicrobial resistant Enterobacterales in the Irish environment 2019-2020. Eccmid 2021. Online. Mini oral presentation. July 2021

**B. Hooban**, K. Fitzhenry, A. Joyce, L. O'Connor, G. Miliotis, A. Chueiri, M. L. Farrell, N. DeLappe, M. Cormican, D. Morris. Evaluation of environmental waters and sewage sources for the presence of antimicrobial resistant Enterobacterales in Ireland. One Health EJP 2021. Oral presentation. Online. June 2021

**B. Hooban**, K. Fitzhenry, A. Joyce, L. O'Connor, A. Chueiri, M. Farrell, M. Cormican, D. Morris. Detection of carbapenemase producing Enterobacterales in sewage and aquatic environments in the Republic of Ireland, 2019-2020. Environ 2021. Online. June 2021.

**B. Hooban**, K. Fitzhenry, A. Joyce, N. Cahill, R. Abbas Syed, L. O'Connor, M. Cormican, D. Morris. A Point Prevalence Survey of Antimicrobial Resistance in the Aquatic Environment, Ireland 2018-2019. Environ 2020. Online, October 2020.

**B. Hooban**, C. Chique, J. Cullinan, K. Burgess, F. Leonard, F. Walsh, E. Cummins, L. O'Connor, F. Brennan, R. Hendriksen, S. Fanning, M. Healy, B. Mc Mahon, X. Zhan, G. Duffy, L. Morrison, R. Gately, D. Crowley, S. Nolan, D. Prendergast, M. Cormican, D. Morris. The AREST Project - Antimicrobial Resistance and the Environment – Sources, persistence, Transmission and risk management. One Health European Joint Programme Annual Scientific Meeting, Dublin, May 22nd to 24th 2019.

**B Hooban**, B Wong Ngie Xiong, B Mahon, N Cahill, L O' Connor, P Hickey, S Keane & D Morris. Seawater as a Potential Transmission Route for Clinically Significant Antimicrobial Resistant Bacteria. Environ, Carlow, April 15th to 17th 2019.

## Conferences – Poster presentations

**B. Hooban**, K. Fitzhenry, A. Joyce, N. Cahill, R. Abbas Syed, L. O' Connor, M. Cormican, D. Morris. Evaluation of Antimicrobial Resistance in the Environment: Contaminating Sources, Wastewater Treatment and Natural Aquatic Reservoirs in Ireland. World One Health Congress 2020. Online, October 30th to November 3rd 2020.

**B. Hooban**, N. Cahill, A. Joyce, B. Wong Ngie Xiong, B. Mahon, L. O' Connor, P. Hickey, S. Keane, M. Cormican & D. Morris. Anthropogenic Pollution and Impact on Detection of Carbapenemase-Producing Enterobacterales in Recreational Waters. ESCAIDE, Stockholm, November 27th to 29th 2019.

**B, Hooban**, B. Wong Ngie Xiong, B. Mahon, N. Cahill, L. O' Connor, P. Hickey, S. Keane & D. Morris. Seawater as a Reservoir for Fluoroquinolone Resistant, Carbapenemase and Extended Spectrum Beta Lactamase Producing Enterobacterales. ASM Microbe, San Francisco, June 20th to 24th 2019.

## Awards & Achievements

- Winner of the 'Early Career Oral Presentation' at the One Health European Joint Programme Annual Scientific Meeting, June 2021.
- Winner of the 'Best Water Related Presentation' at Environ, June 2021.
- Recipient of the European Centre for Disease Prevention and Control 'ESCAIDE 2019 Funding Initiative' covering registration, travel and accommodation costs for the ESCAIDE conference in Stockholm, November 2019.
- Recipient of the Short-Term Mission Travel award from the Med-Vet-Net association to complete bioinformatics training in Oxford University (unable to avail of due to COVID travel restrictions).
- Finalist Threesis competition at the National University of Ireland Galway, December 2018.
- Youth Academy instructor teaching the course 'A world that we cannot see: the secret life of microbes', October 2019 – March 2021.