A Point Prevalence Survey of Antibiotic Resistance in the Irish Environment, 2018–2019

Brigid Hooban, Kelly Fitzhenry, Niamh Cahill, Aoife Joyce, Louise O' Connor, James E. Bray, Sylvain Brisse, Virginie Passet, Raza Abbas Syed, Martin Cormican, et al.

Environment International 152 (2021) 106466

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

A Point Prevalence Survey of Antibiotic Resistance in the Irish Environment, 2018–2019

Brigid Hooban [a,b,*], Kelly Fitzhenry [a,b], Niamh Cahill [a,b], Aoife Joyce [a,b], Louise O' Connor [a,b], James E. Bray [c], Sylvain Brisse [d], Virginie Passet [d], Raza Abbas Syed [a], Martin Cormican [a,b,e], Dearbháile Morris [a,b]

[a] Antimicrobial Resistance and Microbial Ecology Group, School of Medicine, National University of Ireland, Galway
[b] Centre for One Health, Ryan Institute, National University of Ireland, Galway
[c] Department of Zoology, University of Oxford, Oxford, United Kingdom
[d] Biodiversity and Epidemiology of Bacterial Pathogens, Institut Pasteur, Paris, France
[e] Health Service Executive, Ireland

ARTICLE INFO

Handling Editor: Frederic Coulon

Keywords:
Antibiotic Resistance
Aquatic Environment
Wastewater
Carbapenemase
Extended Spectrum Beta-Lactamase

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 cefoxitin (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 $bla_{\text{CTX-M}}$ detected in 99/105 isolates by PCR. Carbapenemase genes were identified in 11 isolates (6 sewage, 5 water). The most common variant was $bla_{\text{OXA-48}}$ (n=6), followed by $bla_{\text{NDM-5}}$ (n=2) and $bla_{\text{KPC-2}}$ (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.

Abbreviations: ESBL, extended spectrum beta-lactamase; CPE, carbapenemase producing Enterobacterales; EARS-NET, European Antimicrobial Resistance Surveillance Network; WHO, World Health Organization; HPSC, Health Protection Surveillance Centre; EPA, Environmental Protection Agency; EU, European Union; LAA, Local Authority Area, CSO, Central Statistics Office; HALT, Healthcare-Associated Infections & Antimicrobial Use in Long-Term Care Facilities; DWTP, Drinking water treatment plant; WWTP, Wastewater treatment plant; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time of Flight; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CLSI, Clinical & Laboratory Standards Institute; NCPERLS, National Carbapenemase-producing Enterobacterales Reference Laboratory Service, Ireland; CGE, Center for Genomic Epidemiology; MPN, Mean Probable Number; UWWD, Urban Wastewater Discharge; cgMLST, Core Genome Multi Locus Sequence Type; MLST, Multi Locus Sequence Type; HPRA, Health Products Regulatory Authority.

* Corresponding author.
E-mail address: b.hooban1@nuigalway.ie (B. Hooban).

https://doi.org/10.1016/j.envint.2021.106466
Received 27 November 2020; Received in revised form 12 February 2021; Accepted 13 February 2021
Available online 8 March 2021

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 2019). 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 harbor carbapenemases (HPSC 2019a). Amongst 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/100 mL) 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 harboring $\leq$ 200 CFU/100 mL intestinal *Enterococci* and $\leq$ 500 CFU/100 mL *E. coli* would be designated as excellent bathing water status. In contrast, coastal waters must display $\leq$ 100 CFU/100 mL intestinal *Enterococci* and $\leq$ 250 CFU/100 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 250 mL 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 0/100 mL 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 at 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 $bla_{\text{CTX-M}}$ 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 of 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.

2. Materials and methods

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 centers 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 50.0 km^2, whereas Galway county is much larger at 5,796 km^2.

East

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

South

Table 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	No. of samples per local authority area
Beach A	26th Nov 2018	Galway city	4 seawater samples
Beach B	06th Dec 2018		
Beach C	06th Dec 2018		
Beach D	08th Jan 2019		
Beach E	22nd Jan 2019	Galway county	6 seawater samples
Beach F	22nd Jan 2019		
Beach G	28th Jan 2019		
Beach H	28th Jan 2019		
Beach I	29th Jan 2019		
Beach J	29th Jan 2019		
Beach K	02nd April 2019	Fingal	6 seawater samples
Beach L	02nd April 2019		
Beach M	02nd April 2019		
Beach N	02nd April 2019		
Beach O	02nd April 2019		
Beach P	03rd April 2019		
Beach Q	13th May 2019	Cork county	7 seawater samples
Beach R	13th May 2019		
Beach S	14th May 2019		
Beach T	15th May 2019		
Beach U	16th May 2019		
Beach V	16th May 2019		
Beach W	16th May 2019		
Total seawater samples: 23			
River A	04th Dec 2018	Galway city	2 river water samples*
River B	04th Dec 2018		
River C	21st Jan 2019	Galway county	2 river water samples
River D	12th Feb 2019		
River E	15th May 2019	Cork county	1 river water sample
Total river water samples: 5			
Lake A	04th Feb 2019	Galway county	2 lake water samples
Lake B	04th Feb 2019		
Lake C	09th May 2019	Cork county	1 lake water sample
Total lake water samples: 3			
Estuary A	04th Dec 2018	Galway city	2 estuarine water samples
Estuary B	04th Dec 2018		
Estuary C	03rd April 2019	Fingal	2 estuarine water samples
Estuary D	03rd April 2019		
Total estuarine water samples: 4			
DWTP influent A	29th April 2019	Fingal	1 DWTP influent sample
DWTP influent B	14th May 2019	Cork county	1 DWTP influent sample
DWTP influent C	22nd July 2019	Galway city	1 DWTP influent sample
DWTP influent D	23rd 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.

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 7,403 km^2. 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 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 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 (Hennessy et al., 2017). The levels of antibiotic usage among hospitals was obtained from the Hospital Antimicrobial Consumption Surveillance data (HPSC 2019b). 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 h prior to sample collection was recorded for each sample (supplementary Table 7).

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 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 0.45 μm filters. The filters were enriched in 100 mL 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 5 μg 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 150 μL 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 McConkey agar surface with the subsequent addition of a ciprofloxacin disc (5 μL). These plates were incubated at 37 °C for 18–24 hours.

2.3. Sewage sample collection and processing

A total of 25 sewage samples were collected across the four local authority areas (Table 2). Sewage samples were collected by lowering a sterile 250 mL glass bottle in to a manhole/septic tank. Some nursing homes and hospitals had an open pipe flow system whereas others had an accumulative septic tank (Table 2). Sewage samples were directly cultured on CHROMagar™ mSuperCARBA™, *Brilliance*™ ESBL agar and

Table 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	26th Nov 2018	Galway city	4 hospital sewage samples	74 mL	Open pipe	220 beds	N/A	68.1 (2018) Combined data only available
Hospital A2	26th Nov 2018			>250 mL	Open pipe	220 beds	N/A	
Hospital B1	28th Nov 2018			>250 mL	Septic tank	664 beds	N/A	
Hospital B2	20th Feb 2019			112 mL	Septic tank	664 beds	N/A	
Hospital C	21st Jan 2019	Galway county	2 hospital sewage samples	116 mL	Open pipe	194 beds	N/A	98.5 (2018)
Hospital D	28th Jan 2019			>250 mL	Septic tank	50 beds	N/A	Unknown
Hospital E	16th May 2019	Cork city	1 hospital sewage sample	250 mL	Open pipe	332 beds	N/A	94.4 (2018)
Hospital F	18th June 2019	Cork county	1 hospital sewage sample	250 mL	Overflow bucket	54 beds	N/A	86.5 (2014)
Total hospital sewage samples: 8								
Nursing home A	6th Dec 2018	Galway city	3 nursing home sewage samples	200 mL	Open pipe	62 beds	N/A	13.6 (2016)
Nursing home B	09th Jan 2019			60 mL	Open pipe	26 beds	N/A	11.1 (2016)
Nursing home C	14th Jan 2019			120 mL	Septic tank	60 beds	N/A	Unknown
Nursing home D	21st Jan 2019	Galway county	3 nursing home sewage samples	250 mL	Septic tank	44 beds	N/A	12.2 (2016)
Nursing home E	22nd Jan 2019			>250 mL	Septic tank	42 beds	N/A	12.5 (2016)
Nursing home F	04th Feb 2019			7 mL	Open pipe	51 beds	N/A	Unknown
Nursing home G	04th April 2019	Fingal	1 nursing home sewage sample	180 mL	Open pipe	140 beds	N/A	12.4 (2016)
Total nursing home sewage samples: 7								
Airport A	04th April 2019	Fingal	1 airport sewage sample	167 mL	Open pipe	N/A	N/A	N/A
Airport B	18th June 2019	Cork county	1 airport sewage sample	180 mL	Open pipe	N/A	N/A	N/A
Total airport sewage samples: 2								
WWTP A	29th April 2019	Fingal	2 WWTP samples	250 mL (Influent and effluent)	Grab	65,000 PE	Secondary and UV	N/A
WWTP B	15th May 2019	Cork county	2 WWTP samples	250 mL (Influent and effluent)	Grab	20,500 PE	Secondary using Nereda	N/A
WWTP C	24th July 2019	Galway city	2 WWTP samples	>250 mL (Influent and effluent)	Grab	170,000 PE	Secondary	N/A
WWTP D	22nd July 2019	Galway county	2 WWTP samples	>250 mL (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).

McConkey agar with a ciprofloxacin disc (5 μg) using a direct swab plating technique. These plates were incubated at 37 °C for 18–24 hours.

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 (10 μg), cefoxitin (30 μg), cefpodoxime (10 μg), ceftazidime (10 μg), cefotaxime (5 μg), ertapenem (10 μg), meropenem (10 μg), gentamicin (10 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg) and trimethoprim (5 μg). 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 (10 μg/1μg). A greater than or equal to 5 mm 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) harbors $bla_{\text{SHV-18}}$ 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).

2.5. Characterisation of beta-lactamase encoding genes using real time PCR

ESBL producers were tested for the presence of $bla_{\text{CTX-M-group1}}$, $bla_{\text{CTX-M-group2}}$ and $bla_{\text{CTX-M-group9}}$ 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_{VIM}, bla_{IMP}, $bla_{\text{OXA-48}}$, bla_{KPC} and bla_{NDM} (Manchanda et al., 2011, Swayne et al., 2011). The assay applied for the detection of bla_{VIM} and bla_{IMP} 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 6.

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 (Qiagen) according 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 5.

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 $bla_{\text{OXA-48}}$ 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. 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 4. A total of 139 isolates were obtained from waters while 72 were isolated from sewage.

3.1. E. coli quantification (MPN per 100 mL)

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 100 mL. The levels of *E. coli* for all water types combined with the approximate distances from sampling areas to point discharges are presented in Fig. 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 (Fig. 1).

Seawaters were the most commonly collected sample type (n = 23). Overall, 19/23 (83%) seawater samples harbored *E. coli* levels less than 250 MPN per 100 mL 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 100 mL. A raw sewage

Table 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
$bla_{CTX-M-group1}$	27	13	3	19	9	3	3	5	1	**83**
$bla_{CTX-M-group2}$	0	0	0	0	0	0	0	0	0	**0**
$bla_{CTX-M-group9}$	9	1	1	2	1	1	0	0	1	**16**
bla_{OXA-48}	2	0	0	1	1	0	0	2	0	**6**
bla_{NDM}	1	0	1	0	0	0	0	0	0	**2**
bla_{KPC}	0	0	0	0	2	0	0	0	0	**2**
bla_{IMP}	0	0	0	0	1	0	0	0	0	**1**
bla_{VIM}	0	0	0	0	0	0	0	0	0	**0**

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

discharge was located approximately 0.1 km from the sampling point at Beach F (Fig. 1). In comparison, estuarine waters revealed much higher levels of *E. coli*, with just 1/4 (25%) samples harboring less than 250 (MPN per 100 mL). The remaining three estuarine samples displayed *E. coli* levels between 520 to > 2419.6 (MPN per 100 mL). Estuary B revealed the single highest detectable levels of *E. coli* (>2419.6 MPN *E. coli* per 100 mL) across all water sample types. This estuarine sample was collected during December and is located approximately 0.1 km from a secondary urban wastewater discharge.

Freshwater samples harbored 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 100 mL). Inland waters have a higher cut-off point of less than 500 MPN per 100 mL 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 harbored *E. coli* levels varying between 16 and 115.3 MPN per 100 mL (Fig. 1).

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

3.2. Antibiotic susceptibility testing

Two heatmaps were created using R displaying the antibiograms of individual isolates. Fig. 2(A) encompasses isolates from Galway city and county, while Fig. 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.

The antibiogram results were grouped in to 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 (Fig. 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.

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_{CTX-M} groups in 99/105 (94%) Enterobacterales (Table 3). Positive bla_{CTX-M} detection was identified in 77 isolates obtained from water samples and 22 isolates from sewage. The most commonly detected ESBL variants included $bla_{CTX-M-group1}$ (n = 83) followed by $bla_{CTX-M-group9}$ (n = 16). A total of 6 isolates that were confirmed phenotypically as ESBL producers tested negative for the bla_{CTX-M} genes. In terms of the 'cold spot' locations, 2 out of 5 locations tested positive for the presence of one of more isolate harboring bla_{CTX-M} genes. These locations included an urban estuary and river which harbored *E. coli* levels of 520 and 40.8 (MPN per 100 mL) respectively.

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). 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_{OXA-48} (4 *Klebsiella pneumoniae*, 1 *E. coli* and 1 *Enterobacter kobei*), bla_{NDM} (2 *E. coli*), bla_{KPC} (1 *Klebsiella pneumoniae* and 1 *Citrobacter freundii*) and bla_{IMP} (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 Fig. 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 harbored *E. coli* levels ranging between 0 and 269 MPN per 100 mL. According to the EU bathing water directive 2006 (Directive 2006/7/EC), these *E. coli* levels would lead to excellent/good water body classification.

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

Table 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	***E. coli***	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(BS512) (**3**), IncQ1 (**2**), IncB/O/K/Z (**1**), Inc1-I (**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**), *IpfA* (**1**)	$bla_{\text{TEM-1}}$ (**10**), $bla_{\text{CTX-M-15}}$ (**7**), $bla_{\text{CTX-M-14}}$ (**4**), $bla_{\text{OXA-1}}$ (**3**), $bla_{\text{CTX-M-27}}$ (**2**), ***bla*$_{\text{NDM-5}}$** (**1**), $bla_{\text{CTX-M-1}}$ (**1**), $bla_{\text{CMY-42}}$ (**1**), $bla_{\text{DHA-1}}$ (**1**), $bla_{\text{CTX-M-24}}$ (**1**)
	***Klebsiella* spp.**	4	11(**1**), 17(**1**), 45 (**1**), 405 (**1**)	IncFIB (**4**), IncFII (**3**), IncL (**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**)	$bla_{\text{CTX-M-15}}$ (**4**), $bla_{\text{TEM-1}}$ (**3**), $bla_{\text{OXA-1}}$ (**3**), $bla_{\text{SHV-11}}$ (**2**), ***bla*$_{\text{OXA-48}}$** (**1**), $bla_{\text{SHV-1}}$ (**1**), $bla_{\text{SHV-76}}$ (**1**)
River	***E. coli***	5	131 (**1**), 38 (**1**), 5584 (**1**), unknown (**1**), 1722 (**1**)	IncFIB (**4**), IncFII (**3**), IncFIA (**2**), Col156 (**2**), IncI1-I (**1**), ColRNAI (**1**), Col(BS512) (**1**)	*gad* (**4**), *sat* (**3**), *eilA* (**3**), *iha* (**2**), *senB* (**2**), *air* (**2**), *iss* (**1**), *cnf1* (**1**), *nfaE* (**1**), *IpfA* (**1**), *ccl* (**1**)	$bla_{\text{CTX-M-15}}$ (**5**), $bla_{\text{TEM-1}}$ (**2**), $bla_{\text{OXA-1}}$ (**1**)
	***Klebsiella* 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**)	$bla_{\text{CTX-M-15}}$ (**1**), $bla_{\text{OXA-1}}$ (**1**), $bla_{\text{SHV-1}}$ (**1**)
Estuary	***E. coli***	5	131 (**3**), 38 (**1**), 10 (**1**)	IncFIB (**4**), IncFIA (**3**), IncFII (**3**), Col156 (**2**), Col(BS512) (**2**), IncX1 (**1**), IncX4 (**1**), IncI2 (**1**), IncQ1 (**1**), IncFIC (**1**), Inc1-I (**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**)	bla$_{\text{CTX-M-15}}$ (**4**), $bla_{\text{TEM-1}}$ (**3**), $bla_{\text{OXA-1}}$ (**2**), $bla_{\text{CTX-M-14}}$ (**1**)
	***Klebsiella* spp.**	4	405 (**1**), 11 (**1**), 8 (**1**), 236 (**1**)	IncFIB (**4**), IncFII (**3**), IncFIA (**2**), IncL (**1**), IncR (**1**), Col4401 (**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**)	$bla_{\text{CTX-M-15}}$ (**3**), ***bla*$_{\text{OXA-48}}$** (**1**), $bla_{\text{OXA-1}}$ (**2**), $bla_{\text{TEM-1}}$ (**2**), $bla_{\text{DHA-1}}$ (**2**), $bla_{\text{SHV-76}}$ (**1**), $bla_{\text{SHV-11}}$ (**1**), $bla_{\text{SHV-1-like}}$ (**1**), $bla_{\text{SHV-60}}$ (**1**)
	E. coli	2	131 (**1**), 11,188 (**1**)	IncFIA (**2**), FII (**2**)	*iss* (**3**), *air* (**2**), *senB* (**2**), *eilA* (**2**), *gad* (**2**), *iha* (**1**), *sat* (**1**), *IpfA* (**1**)	$bla_{\text{CTX-M-27}}$ (**1**), ***bla*$_{\text{NDM-5}}$** (**1**)
Lake	***Klebsiella* 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**)	$bla_{\text{CTX-M-15}}$ (**1**), $bla_{\text{SHV-11}}$ (**1**), $bla_{\text{TEM-1}}$ (**1**), $bla_{\text{TEM-1D-like}}$ (**1**), $bla_{\text{OKP-B-3-like}}$ (**1**)
	E. coli	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**)	$bla_{\text{CTX-M-15}}$ (**4**), $bla_{\text{OXA-1}}$ (**3**), $bla_{\text{TEM-1}}$ (**1**), $bla_{\text{CTX-M-27}}$ (**1**)
Hospital	***Klebsiella* 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**)	$bla_{\text{TEM-1}}$ (**4**), $bla_{\text{OXA-1}}$ (**2**), $bla_{\text{OXA-9-like}}$ (**2**), $bla_{\text{CTX-M-15}}$ (**1**), ***bla*$_{\text{KPC-2}}$** (**1**), ***bla*$_{\text{OXA-48}}$** (**1**), $bla_{\text{SHV-1-like}}$ (**1**), $bla_{\text{SHV-76}}$ (**1**), $bla_{\text{SHV-11}}$ (**1**), $bla_{\text{CTX-M-14}}$ (**1**), $bla_{\text{OXA-9}}$ (**1**), $bla_{\text{SHV-1}}$ (**1**), $bla_{\text{CTX-M-15-like}}$ (**1**), $bla_{\text{SHV-62-like}}$ (**1**)
Nursing home	***E. coli***	3	131 (**2**), 38 (**1**)	IncFIA (**2**), IncFII (**2**)	*iss* (**3**), *gad* (**2**), *iha* (**2**), *sat* (**2**), *eilA* (**1**), *nfaE* (**1**), *air* (**1**)	$bla_{\text{TEM-1}}$ (**2**), $bla_{\text{OXA-1}}$ (**2**), $bla_{\text{CTX-M-14-like}}$ (**1**)
	E. coli	1 2	38 (**1**) 967 (**1**), 1563 (**1**)	IncY (**1**) IncFIB (**2**), IncL (**1**)	*air* (**1**), *eilA* (**1**), *gad* (**1**), *iss* (**1**)	$bla_{\text{CTX-M-15}}$ (**1**), $bla_{\text{TEM-1}}$ (**1**)

(*continued on next page*)

Table 4 (*continued*)

Sample type	Species	Number of isolates	ST type	Plasmid replicons	Virulence genes	Beta-lactamase genes
Wastewater treatment plant	***Klebsiella* spp.**				*mrkA* (**2**), *mrkB* (**2**), *mrkC* (**2**), *mrkD* (**2**), *mrkF* (**2**), *mekH* (**2**), *mrkI* (**2**), *mrkJ* (**2**), *kfuA* (**1**), *kfuB* (**1**), *kfuC* (**1**)	bla_{LEN17} (**1**), **bla_{OXA-48}** (**1**), $bla_{CTX-M-15}$ (**1**), bla_{SHV-27} (**1**)
Airport	***E. coli***	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**), *IpfA* (**1**)	$bla_{CTX-M-15}$ (**1**), bla_{TEM-1} (**1**), $bla_{CTX-M-55}$ (**1**)

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

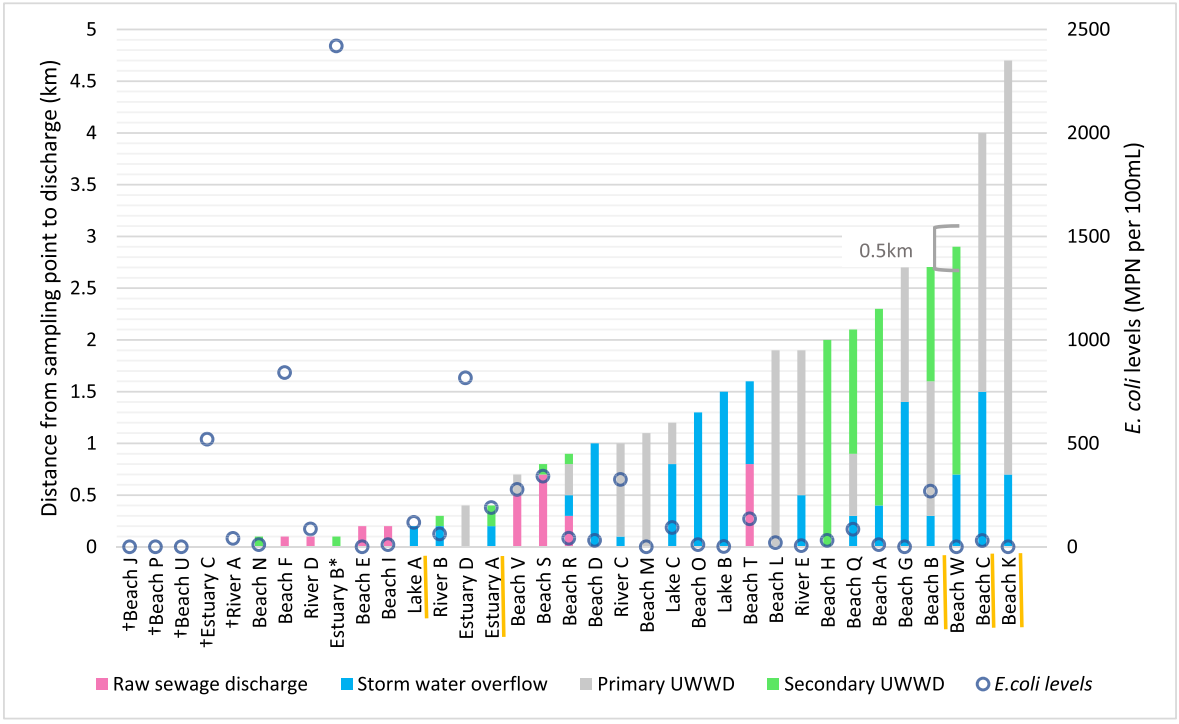

Fig. 1. Stacked bar chart displaying distance from sampling points to discharge(s) (km) overlaid with *E. coli* detection levels (MPN per 100 mL). The length of each coloured stacked bar is indicative of the distance from the sampling point to a contaminating point discharge. In the case of two or more 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 MPN/100 mL. Supplementary Table 1 displays the raw data for this figure. UWWD = Urban wastewater discharge. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were assessed for multi locus sequence types (MLST), plasmid replicons, virulence genes and antibiotic resistance genes outlined in Table 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, iroN, 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 sewage 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 4). The most commonly detected beta-lactamase gene was bla_{CTX-M} (n = 48) which could be further divided in to $bla_{CTX-M-15}$ (n = 34), $bla_{CTX-M-14}$ (n = 7), $bla_{CTX-M-27}$ (n = 4), $bla_{CTX-M-1}$ (n = 1), $bla_{CTX-M-24}$ (n = 1) and $bla_{CTX-M-55}$ (n = 1). These results are in agreement with the PCR outcomes where $bla_{CTX-M-group1}$ and $bla_{CTX-M-group9}$ were the most prevalent variants, with no $bla_{CTX-M-group2}$ detections. Other commonly detected beta-lactamase genes included bla_{TEM-1} (n = 31), bla_{OXA-1} (n = 19) and bla_{SHV} variants (n = 16). The bla_{SHV} genes included bla_{SHV-11} (n = 5), bla_{SHV-1} (n = 5), bla_{SHV-76} (n = 3), bla_{SHV-60} (n = 1), bla_{SHV-27} (n = 1) and $bla_{SHV-62-like}$ (n = 1). Three different CPE genes were identified among sequenced isolates with positive detection of bla_{OXA-48} in 4 isolates, bla_{NDM-5} in 2 *E. coli* and bla_{KPC-2} in 1 *Klebsiella pneumoniae*, also in agreement with detection from the PCR.

Furthermore, different types of antibiotic resistance genes detected using ResFinder are outlined in Fig. 4. The most commonly detected genes included *sul1* and *tet(A),* identified in a total of 28 sequenced

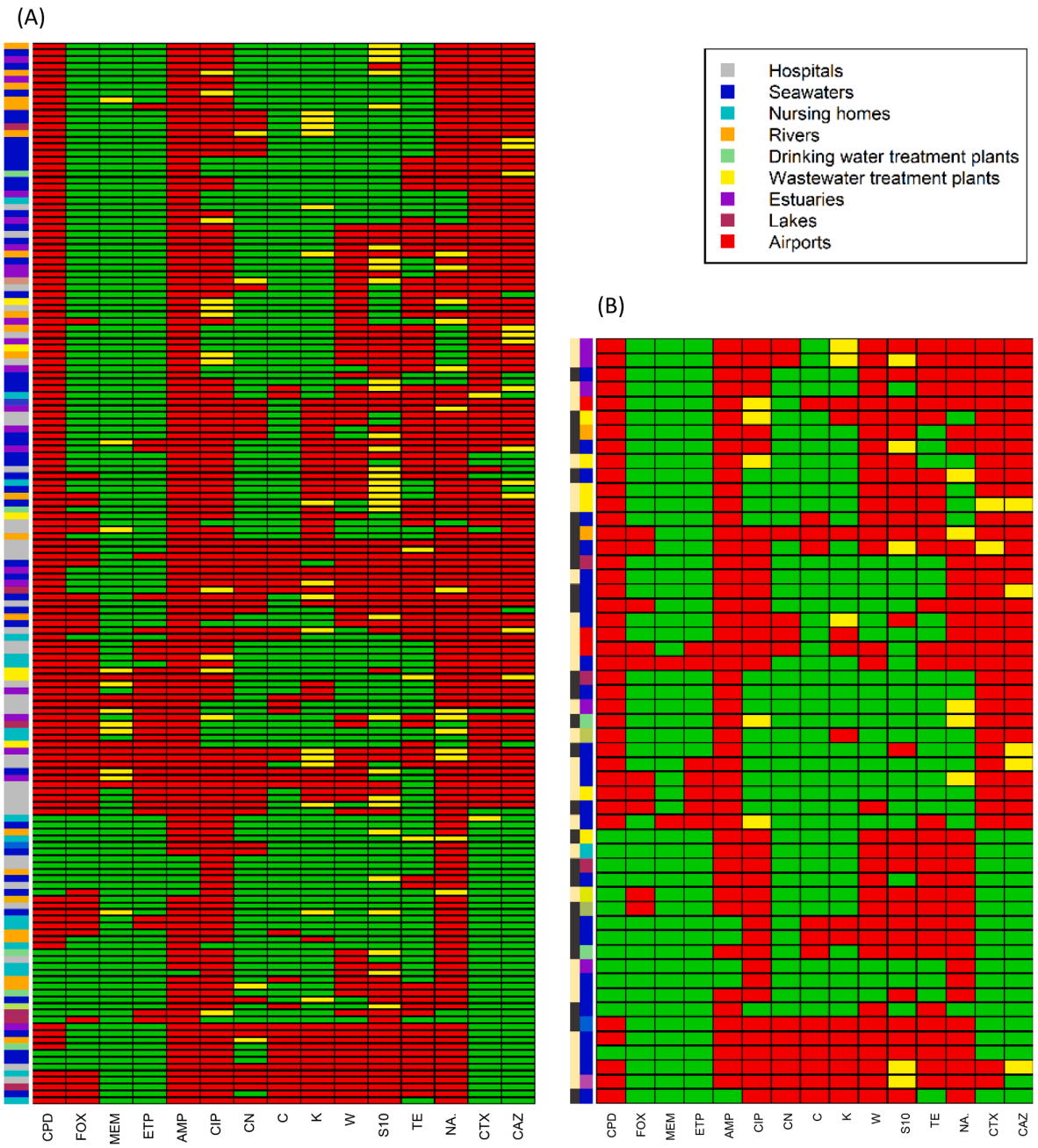

Fig. 2. Heatmaps encompassing antibiogram profiles of isolates from different sample types. Red indicates resistance, yellow indicates an intermediate phenotype and green indicates susceptibility. Heatmap (A) includes isolates from Galway city and county, while (B) encompasses bacteria from Fingal and 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

isolates. These were closely followed by positive detection of *strA*, *strB*, *sul2* and *mph(A)* in 26 isolates. Overall, 49 isolates (82%) harbored 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 harboring one or more resistance genes against was fosfomycin (n = 20; 33%).

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 (Fig. 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 3.4 km 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 reveled 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 km 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

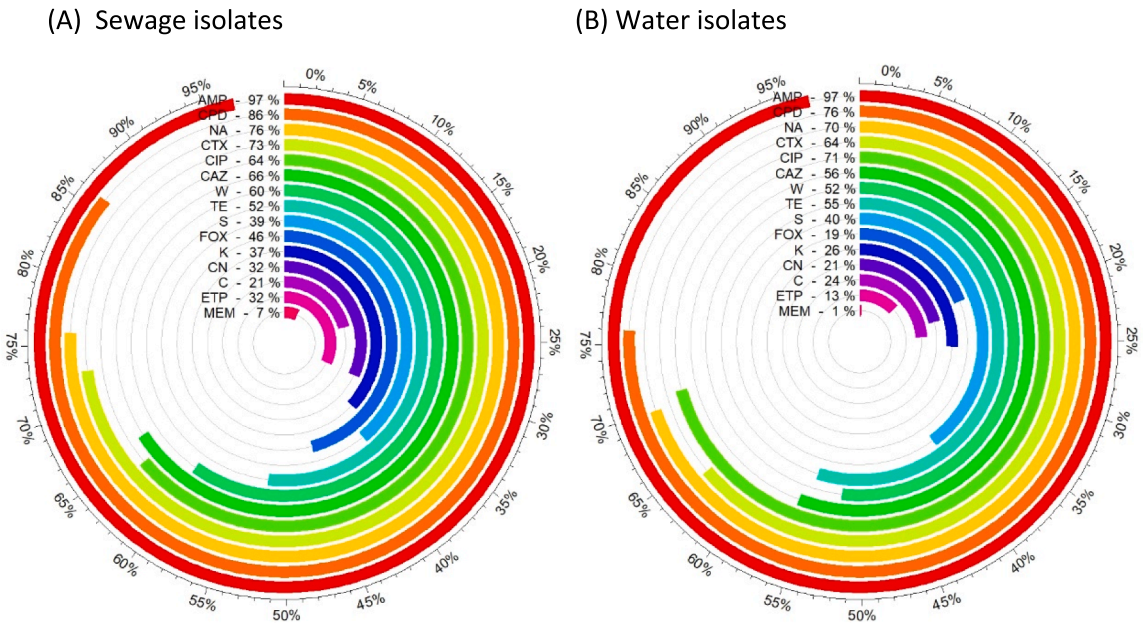

Fig. 3. Percentage of isolates displaying resistance to a panel of 15 antibiotics for (A) sewage versus (B) water isolates. A total of 72 isolates were examined from sewage sources, whereas 139 isolates were obtained from water bodies. AMP = Ampicillin, CPD = Cefpodoxime, NA = Naladixic acid, CTX = Cefotaxime, CIP = Ciprofloxacin, CAZ = Ceftazidime, W = Trimethoprim, TE = Tetracycline, S = Streptomycin, FOX = Cefoxitin, K = Kanamycin, CN = Gentamicin, C = Chloramphenicol, ETP = Ertapenem, MEM = Meropenem.

(beach K and river C) over 135 km apart. The whole genome comparator tool revealed 21 missing loci in one isolate compared to the other (99.2% similarity).

The GrapeTree tool was also applied to sequenced *Klebsiella* isolates (n = 18) in Fig. 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 2.8 km 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 1.3 km apart. Similarly, beach A is located just 1.6 km from the hospital and 900 m from the wastewater treatment plant receiving the hospital effluent. These isolates displayed 98.8% similarity.

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.

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

As previously mentioned, seawaters and estuaries featured as harboring the highest levels of *E. coli,* with freshwaters including rivers, lakes and drinking water treatment plant influents displaying much lower levels (Fig. 1). Most of the waters that harbored 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 100 mL. The exceptionally high level of *E. coli* detected at this cold spot location prompted further investigation in to 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 1 km radius (6 seawaters and 1 lake) displayed relatively low levels of *E. coli* (0–31 MPN per 100 mL). 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.

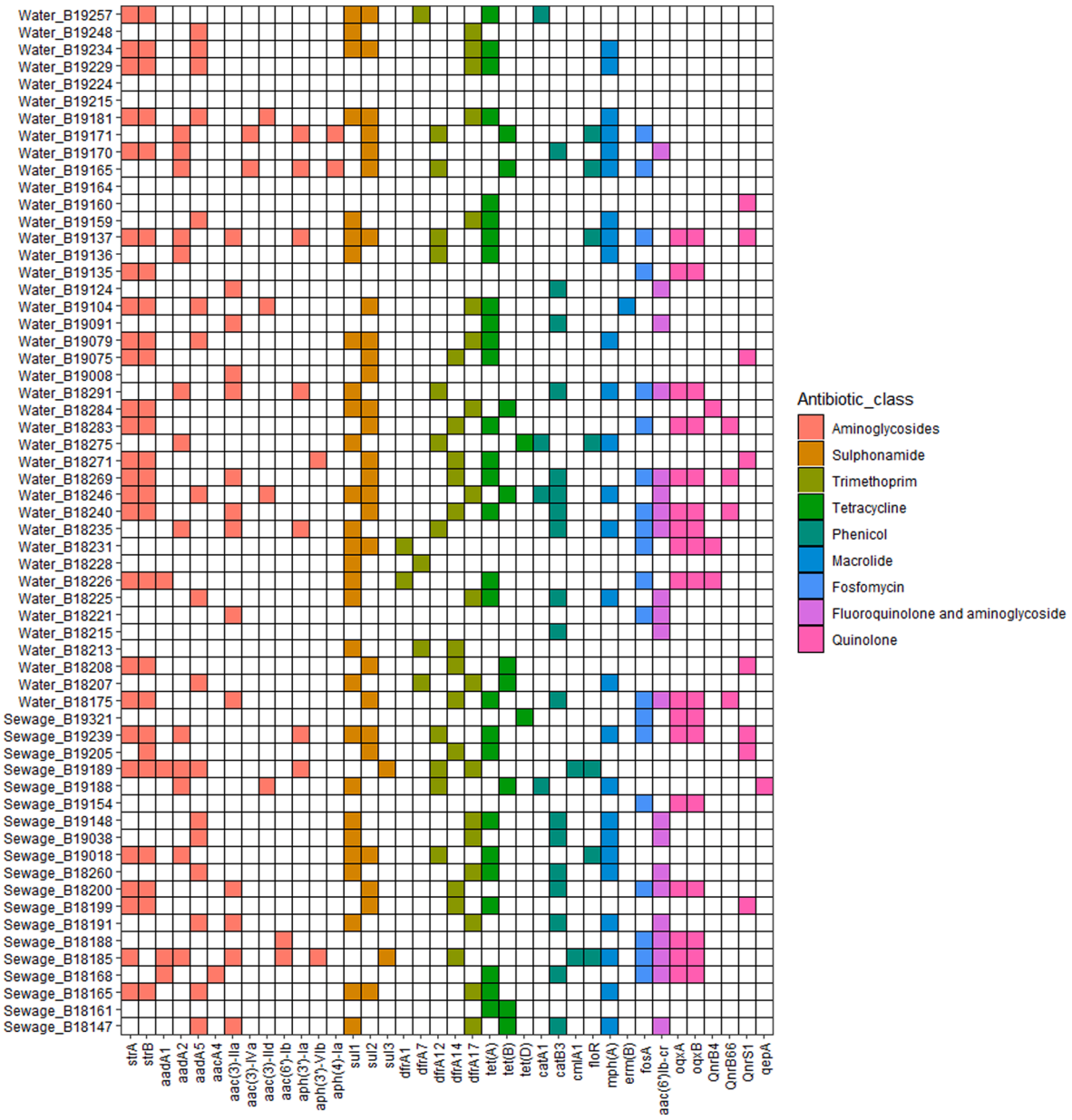

Fig. 4. Antibiotic resistance gene detection among the 60 sequenced isolates. The colours correspond to the resistance conferred by the gene to a certain class of antibiotic.

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 in to 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 Fig. 1). However, the majority of sampling occasions had less than 3 mm of rain (25/39) in the 24 hours prior to collection.

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 (Fig. 2). Sequencing analysis unveiled a large proportion of isolates harboring 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 (Fig. 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.,

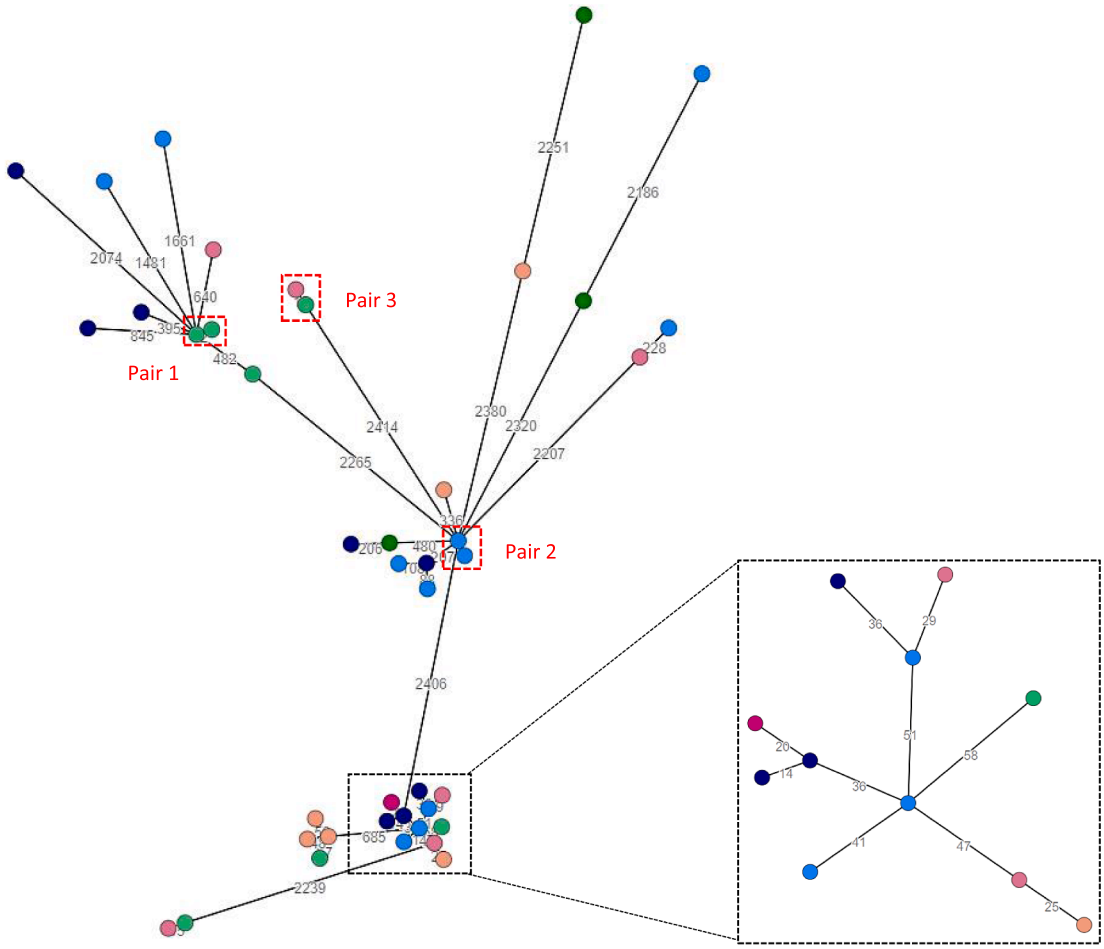

Fig. 5. Core genome multi locus sequence type minimum spanning tree based on allele differences between 2513 loci in *E. coli* isolates. The circles are colour coded to indicate the local authority region: Galway city = blue; Galway county = pink; Fingal = green; Cork = orange. The darker colour indicates 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2017). Previous detection of *fosA2* was reported in *Enterobacter cloacae* from a Canadian river (Xu et al., 2011).

ESBL encoding genes ($bla_{\text{CTX-M}}$) 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. (2017) 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 harboring $bla_{\text{CTX-M}}$ 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 harbored $bla_{\text{CTX-M-14b}}$ and $bla_{\text{OXA-9}}$ along with $bla_{\text{OXA-48}}$. Similarly, the sewage isolate B18188 harbored $bla_{\text{OXA-9}}$ and $bla_{\text{KPC-2}}$. Both isolates displayed no indications of ESBL production phenotypically, although both harbored one or more ESBL encoding genes based on sequencing data. In addition, only the $bla_{\text{CTX-M}}$ genes were identified using PCR while the sequencing results revealed the presence of further ESBL genes including $bla_{\text{OXA-9}}$ and $bla_{\text{SHV-27}}$. This highlights the benefits of whole genome sequencing in obtaining the complete resistance gene profile.

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%) (Fig. 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., 2012). 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 olaquindox, 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

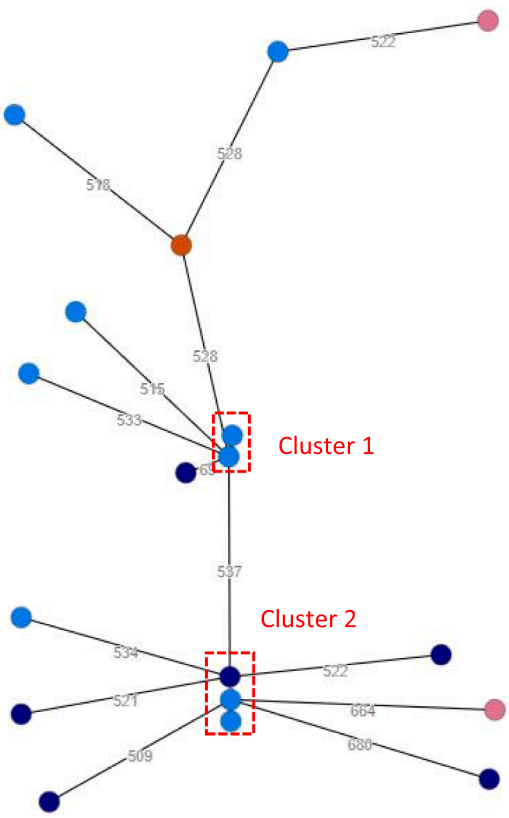

Fig. 6. Core genome multi locus sequence type minimum spanning tree based on allele differences between 694 loci in *Klebsiella* isolates. The circles are colour coded to indicate the local authority region: Galway city = blue; Galway county = pink; Cork = orange. The darker colour indicates isolates from sewage origin while the lighter colours indicate water origin. The numbers between the nodes represents the number of locus allele differences between isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cefoxitin (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.

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 (Sobieszczańska 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., 2016).

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 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 3.4 km 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 1 km 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 135 km 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 $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, *tet(A)* and *QnrS1* common to both ST5584 *E. coli*. The *E. coli* isolate from river water harbored additional resistance genes including *strA*, *strB*, *sul2* and *dfrA14* in comparison to the *E. coli* from seawater. Both isolates also harbored 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.

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 harbored $bla_{\text{OXA-48}}$ with identical pOXA-48 plasmids. These sites are located 2.8 km 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 $bla_{\text{OXA-48}}$ carriage in clinical isolates from Greece (Voulgari et al., 2012), Taiwan (Lu et al., 2018) 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 harbored identical virulence gene profiles, plasmid detection as well as highly similar antibiotic resistance genes. The two water isolates harbored 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 harbor $bla_{\text{OXA-48}}$ in clinical isolates (Miro et al., 2020, López-Camachoa et al., 2018), although none of the ST405 isolates in this collection harbored 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.

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 harbored 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) harbored $bla_{\text{OXA-48}}$ whereas the other two tested positive for $bla_{\text{NDM-5}}$. Two of these sequence types (ST540, ST167) have been identified harboring 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 harboring bla_{NDM} variants in river water in Switzerland and China (Bleichenbacher et al., 2020; Cheng et al., 2019). Bleichenbacher et al. (2020) identified the $bla_{\text{NDM-5}}$ 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 $bla_{\text{NDM-5}}$ 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 $bla_{\text{KPC-2}}$ 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., 2009). This isolate (B18188) was obtained from a hospital sewage sample. Two *Klebsiella* sequence types harbored $bla_{\text{OXA-48}}$, including ST101 (B18185) from hospital sewage and ST1563 (B19321) from wastewater treatment plant influent. *Klebsiella* ST1563 has previously been reported harboring *mcr-1* from pig rectal swabs in Portugal (Kieffer et al., 2017). However, to the authors knowledge this is the first report of $bla_{\text{OXA-48}}$ identification in a *Klebsiella* sequence type ST1563.

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. (2019) 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 $bla_{\text{CTX-M}}$ 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 (21 mL). 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.

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 harbored 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 $bla_{\text{CTX-M-14}}$. Similarly, river A also harbored ESBL producers, two of which were selected for sequencing (B18207, B18208). These isolates both tested positive for $bla_{\text{CTX-M-15}}$ along with many other resistance genes against different classes of antibiotics. The origins of the $bla_{\text{CTX-M}}$ genes have been traced back to *Kluyvera* species, which is an environmental organism (Cantón et al., 2012). 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 38/41 (93%) water isolates further highlights this possibility of widespread dissemination.

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 harboring 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.

CRediT authorship contribution statement

Brigid Hooban: Investigation, Formal analysis, Writing - original draft, Visualization. **Kelly Fitzhenry:** Investigation, Writing - review & editing. **Niamh Cahill:** Investigation, Writing - review & editing. **Aoife Joyce:** Investigation, Writing - review & editing. **Louise O' Connor:** Conceptualization, Resources, Writing - review & editing. **James E. Bray:** Formal analysis, Writing - review & editing. **Sylvain Brisse:** Formal analysis, Writing - review & editing. **Virginie Passet:** Formal analysis, Writing - review & editing. **Raza Abbas Syed:** Investigation, Writing - review & editing. **Martin Cormican:** Conceptualization, Writing - review & editing. **Dearbháile Morris:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Acknowledgements

This project is jointly funded by the Environmental Protection agency, under the EPA Research Programme 2014-2020, and the Health Service Executive (2017-HW-LS-1). The EPA Research Programme is a Government of Ireland initiative funded by the Department of Communications, Climate Action and Environment. It is administered by the Environmental Protection Agency, which has the statutory function of co-ordinating and promoting environmental research.

A portion of this work was also financially supported by the Med-VetKlebs project, a component of the European Joint Programme One Health EJP, which has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No. 773830.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106466.

References

Amarasiri, M., Sano, D., Suzuki, S., 2020. Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: Current knowledge and questions to be answered. Critical Reviews in Environmental Science and Technology 50, 2016–2059.

Benzerara, Y., Gallah, S., Hommeril, B., Genel, N., Decré, D., Rottman, M., et al., 2017. Emergence of Plasmid-Mediated Fosfomycin-Resistance Genes among Escherichia coli Isolates. France. Emerging infectious diseases 23, 1564–1567. https://doi.org/10.3201/eid2309.170560.

Bialek-Davenet, S., Criscuolo, A., Ailloud, F., Passet, V., Jones, L., Delannoy-Vieillard, A.-S., et al., 2014. Genomic definition of hypervirulent and multidrug-resistant Klebsiella pneumoniae clonal groups. Emerging infectious diseases 20, 1812–1820. https://doi.org/10.3201/eid2011.140206.

Birkett, C.I., Ludlam, H.A., Woodford, N., Brown, D.F.J., Brown, N.M., Roberts, M.T.M., et al., 2007. Real-time TaqMan PCR for rapid detection and typing of genes encoding CTX-M extended-spectrum β-lactamases. https://doi.org/10.1099/jmm.0.46909-0.

Bleichenbacher, S., Stevens, M.J.A., Zurfluh, K., Perreten, V., Endimiani, A., Stephan, R., et al., 2020. Environmental dissemination of carbapenemase-producing Enterobacteriaceae in rivers in Switzerland. Environmental Pollution 265, 115081. https://doi.org/10.1016/j.envpol.2020.115081.

Blyton, M.D.J., Gordon, D.M., 2017. Genetic Attributes of E. coli Isolates from Chlorinated Drinking Water. e0169445 e169445 PloS one 12. https://doi.org/10.1371/journal.pone.0169445.

Bogaerts, P., Montesinos, I., Rodriguez-Villalobos, H., Blairon, L., Deplano, A., Glupcyzynski, Y., 2009. Emergence of clonally related Klebsiella pneumoniae isolates of sequence type 258 producing KPC-2 carbapenemase in Belgium. Journal of Antimicrobial Chemotherapy 65, 361–362. https://doi.org/10.1093/jac/dkp453.

Brehony, C., McGrath, E., Brennan, W., Tuohy, A., Whyte, T., Brisse, S., et al., 2019. An MLST approach to support tracking of plasmids carrying OXA-48-like carbapenemase. Journal of Antimicrobial Chemotherapy 74, 1856–1862. https://doi.org/10.1093/jac/dkz136.

Cahill, N., O'Connor, L., Mahon, B., Varley, Á., McGrath, E., Ryan, P., et al., 2019. Hospital effluent: A reservoir for carbapenemase-producing Enterobacterales? Science of The Total Environment 672, 618–624. https://doi.org/10.1016/j.scitotenv.2019.03.428.

Caltagirone, M., Nucleo, E., Spalla, M., Zara, F., Novazzi, F., Marchetti, V.M., et al., 2017. Occurrence of Extended Spectrum β-Lactamases, KPC-Type, and MCR-1.2-Producing Enterobacteriaceae From Wells, River Water, and Wastewater Treatment Plants in Oltrepò Pavese Area, Northern Italy. Frontiers in microbiology 8. https://doi.org/10.3389/fmicb.2017.02232.

Cantón, R., Gonzalez-Alba, J.M., Galán, J.C., 2012. CTX-M Enzymes: Origin and Diffusion. Frontiers in Microbiology 3, 110. https://doi.org/10.3389/fmicb.2012.00110.

Central Statistics Office. 2016. https://statbank.cso.ie/px/pxeirestat/Statire/SelectVarVal/Define.asp?maintable=E2001&PLanguage=0 (Accessed 27/07/2020).

Chen, L., Chavda, K.D., Melano, R.G., Jacobs, M.R., Levi, M.H., Bonomo, R.A., et al., 2013. Complete Sequence of a blaKPC-2-Harboring IncFIIK1 Plasmid from a Klebsiella pneumoniae Sequence Type 258 Strain. Antimicrob Agents Chemother 57, 1542–1545. https://doi.org/10.1128/AAC.02332-12.

Cheng, P., Li, F., Liu, R., Yang, Y., Xiao, T., Ishfaq, M., et al., 2019. Prevalence and molecular epidemiology characteristics of carbapenem-resistant Escherichia coli in Heilongjiang Province. China. Infection and drug resistance 12, 2505–2518. https://doi.org/10.2147/IDR.S208122.

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, 16. https://doi.org/10.3390/antibiotics8010016.

Chique, C., Hynds, P., Burke, L.P., Morris, D., Ryan, M.P., O'Dwyer, J., 2021. Contamination of domestic groundwater systems by verotoxigenic escherichia coli (VTEC), 2003–2019: A global scoping review. Water Research 188, 116496.

Chique, C., Hynds, P.D., Andrade, L., Burke, L., Morris, D., Ryan, M.P., et al., 2020. Cryptosporidium spp. in groundwater supplies intended for human consumption – A descriptive review of global prevalence, risk factors and knowledge gaps. Water Research 176, 115726.

CLSI, 2020. CLSI M100-ED30:2020 Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition.

Coleman, B.L., Salvadori, M.I., McGeer, A.J., Sibley, K.A., Neumann, N.F., Bondy, S.J., et al., 2012. The role of drinking water in the transmission of antimicrobial-resistant E. coli. Epidemiol Infect 140, 633–642.

Elliott, A.G., Ganesamoorthy, D., Coin, L., Cooper, M.A., Cao, M.D., 2016. Complete Genome Sequence of Klebsiella quasipneumoniae subsp. similipneumoniae Strain ATCC 700603. Genome announcements 4, e00438–e516.

Environmental Protection Agency 2016. Economic Assessment of the Waterborne Outbreak of Cryptosporidium hominis in Galway, 2007. Report No. 177. https://www.epa.ie/pubs/reports/research/water/EPA%20-%20Research%20Report%20177_Essentra%20web.pdf.

Environmental Protection Agency 2017. Focus on Private Water Supplies. https://www.epa.ie/pubs/reports/water/drinking/Focus%20on%20Private%20SuppliesV6.pdf.

Environmental Protection Agency 2020. Urban Waste Water Treatment in 2019. https://www.epa.ie/pubs/reports/water/wastewater/Urban%20waste%20water%20treatment%20in%202019%20Report%20%20web%20version.pdf.

EUCAST, 2020. European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, valid from 2020-01-01. https://eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf.

European Centre for Disease Prevention and Control, 2019. Antimicrobial resistance in the EU/EEA (EARS-NET). Annual Epidemiological Report for 2019. https://www.ecdc.europa.eu/sites/default/files/documents/surveillance-antimicrobial-resistance-Europe-2019.pdf.

Feng, Y., Mannion, A., Madden, C.M., Swennes, A.G., Townes, C., Byrd, C., et al., 2017. Cytotoxic Escherichia coli strains encoding colibactin and cytotoxic necrotizing factor (CNF) colonize laboratory macaques. Gut Pathog. 9, 2017. https://doi.org/10.1186/s13099-017-0220-y. eCollection.

Fernando DM, Tun HM, Poole J, Patidar R, Li R, Mi R, et al. Detection of Antibiotic Resistance Genes in Source and Drinking Water Samples from a First Nations Community in Canada. 2016. Applied and Environmental Microbiology. DOI: 10.1128/AEM.00798-16.

Fouz, N., Pangesti, K.N.A., Yasir, M., Al-Malki, A.L., Azhar, E.I., Hill-Cawthorne, G.A., et al., 2020. The Contribution of Wastewater to the Transmission of Antimicrobial Resistance in the Environment: Implications of Mass Gathering Settings. Tropical medicine and infectious disease 5, 33. https://doi.org/10.3390/tropicalmed5010033.

Galvin, S., Boyle, F., Hickey, P., Vellinga, A., Morris, D., Cormican, M., 2010. Enumeration and characterization of antimicrobial-resistant Escherichia coli bacteria in effluent from municipal, hospital, and secondary treatment facility sources. Appl Environ Microbiol 76, 4772–4779. https://doi.org/10.1128/AEM.02898-09.

Gijón D, Tedim AP, Valverde A, Rodríguez I, Morosini MI, Coque TM, et al. Early OXA-48-Producing Enterobacterales Isolates Recovered in a Spanish Hospital Reveal a Complex Introduction Dominated by Sequence Type 11 (ST11) and ST405 Klebsiella Pneumoniae Clones. mSphere 2020; 5. DOI: 10.1128/mSphere.00080-20.

Hansen, L.H., Sørensen, S.J., Jørgensen, H.S., Jensen, L.B., 2005. Affiliations expand The Prevalence of the OqxAB Multidrug Efflux Pump Amongst Olaquindox-Resistant

Escherichia Coli in Pigs. Microbial drug resistance (Larchmont, N.Y.) 11. https://doi.org/10.1089/mdr.2005.11.378.
Harnisz, M., Korzeniewska, E., 2018. The prevalence of multidrug-resistant Aeromonas spp. in the municipal wastewater system and their dissemination in the environment. Science of The Total Environment 626, 377–383. https://doi.org/10.1016/j.scitotenv.2018.01.100.
Hennessy S, Murphy H, Burns, K. Point Prevalence Survey of Healthcare-Associated Infections & Antimicrobial Use in Long-Term Care Facilities (HALT): May 2016. 2017. https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/infectioncontrolandhai/surveillance/hcaiinlongtermcarefacilities/haltreports/2016report/File,16218,en.pdf.
Health Products Regulatory Authority, 2018. Report on Consumption of Veterinary Antibiotics in Ireland during 2018. http://www.hpra.ie/docs/default-source/default-document-library/report-on-consumption-of-veterinary-antibiotics-in-ireland-during-2018.pdf?sfvrsn=0.
Health Protection Surveillance Centre, 2019a. Enhanced Surveillance of Carbapenemase-Producing Enterobacterales (CPE), 2018. https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/strategyforthecontrolofantimicrobialresistanceinirelandsari/carbapenemresistantenterobacteriaceaecre/surveillanceofcpeinireland/cpeannualreports/CPE%20Enhanced%20Surveillance%20Report%202018.pdf.
Health Protection Surveillance Centre, 2019b. Hospital Antimicrobial Consumption Surveillance. https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/europeansurveillanceofantimicrobialconsumptionesac/PublicMicroB/SACHC/Report1.html.
Health Protection Surveillance Centre, 2019c. Annual Epidemiological Report. Cryptosporidiosis in Ireland, 2018. https://www.hpsc.ie/a-z/gastroenteric/cryptosporidiosis/publications/epidemiologyofcryptosporidiosisinirelandannualreports/Crypto%20Annual%20Report%202018.pdf.
Health Protection Surveillance Centre, 2019d. Annual Epidemiological Report. VTEC infection in Ireland, 2017. https://www.hpsc.ie/a-z/gastroenteric/vtec/publications/annualreportsonepidemiologyofverotoxigenicecoli/VTEC%20infection%20in%20Ireland%202017.pdf.
Hong, S.K., Yong, D., Kim, K., Hong, S.S., Hong, S.G., Khosbayar, T., et al., 2013. First Outbreak of KPC-2-Producing Klebsiella pneumoniae Sequence Type 258 in a Hospital in South Korea. J Clin Microbiol. 51, 3877–3879. https://doi.org/10.1128/JCM.01730-13.
Hooban, B., Joyce, A., Fitzhenry, K., Chique, C., Morris, D., 2020. The role of the natural aquatic environment in the dissemination of extended spectrum beta-lactamase and carbapenemase encoding genes: A scoping review. Water Research 180, 115880. https://doi.org/10.1016/j.watres.2020.115880.
Jin, L., Wang, R., Wang, X., Wang, Q., Zhang, Y., Yin, Y., et al., 2017. Emergence of mcr-1 and carbapenemase genes in hospital sewage water in Beijing. China. Journal of Antimicrobial Chemotherapy 73, 84–87. https://doi.org/10.1093/jac/dkx355.
Jolley, K.A., Bliss, C.M., Bennett, J.S., Bratcher, H.B., Brehony, C., Colles, F.M., Wimalarathna, H., Harrison, O.B., Sheppard, S.K., Cody, A.J., Maiden, M.C.J., 2012. Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain. Microbiology. 158, 1005–1015. https://doi.org/10.1099/mic.0.055459-0.
Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Research 2018 3:124 2018; 3. DOI: 10.12688/wellcomeopenres.14826.1.
Jørgensen, S.B., Søraas, A.V., Arnesen, L.S., Leegaard, T.M., Sundsfjord, A., Jenum, P.A., 2017. A Comparison of Extended Spectrum β-Lactamase Producing Escherichia Coli From Clinical, Recreational Water and Wastewater Samples Associated in Time and Location. PloS one 12. https://doi.org/10.1371/journal.pone.0186576.
Kieffer, N., Aires-de-Sousa, M., Nordmann, P., Poirel, L., 2017. High Rate of MCR-1–Producing Escherichia coli and Klebsiella pneumoniae among Pigs. Portugal. Emerging Infectious Disease journal 23, 2023. https://doi.org/10.3201/eid2312.170883.
Kittinger C, Kirschner A, Lipp M, Baumert R, Mascher F, Farnleitner AH, et al. Antibiotic Resistance of Acinetobacter spp. Isolates from the River Danube: Susceptibility Stays High. International journal of environmental research and public health 2017; 15: 52. DOI: 10.3390/ijerph15010052.
Kumar, M., Jaiswal, S., Sodhi, K.K., Shree, P., Singh, D.K., Agrawal, P.K., et al., 2019. Antibiotics bioremediation: Perspectives on its ecotoxicity and resistance. Environment International 124, 448–461. https://doi.org/10.1016/j.envint.2018.12.065.
Lekunberri, I., Villagrasa, M., Balcázar, J.L., Borrego, C.M., 2017. Contribution of bacteriophage and plasmid DNA to the mobilization of antibiotic resistance genes in a river receiving treated wastewater discharges. Science of The Total Environment 601–602, 206–209. https://doi.org/10.1016/j.scitotenv.2017.05.174.
Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, A.K., et al., 2018. Exposure to and colonisation by antibiotic-resistant E. coli in UK coastal water users: Environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environment International 114, 326–333. https://doi.org/10.1016/j.envint.2017.11.003.
López-Camacho, E., Paño-Pardo, J.R., Ruiz-Carrascoso, G., Wesselink, J.-J., Lusa-Bernal, S., Ramos-Ruiz, R., et al., 2018. Population structure of OXA-48-producing Klebsiella pneumoniae ST405 isolates during a hospital outbreak characterised by genomic typing. Journal of Global Antimicrobial Resistance 15, 48–54. https://doi.org/10.1016/j.jgar.2018.06.008.
Lu, M.C., Tang, H.L., Chiou, C.S., Wang, Y.C., Chiang, M.K., Lai, T.C., 2018. Clonal Dissemination of Carbapenemase-Producing Klebsiella Pneumoniae: Two Distinct Sub-Lineages of Sequence Type 11 Carrying Bla KPC-2 and Bla OXA-48. International journal of antimicrobial agents 52. https://doi.org/10.1016/j.ijantimicag.2018.04.023.
Ludden, C., Lötsch, F., Alm, E., Kumar, N., Johansson, K., Albiger, B., et al., 2020. Cross-border spread of blaNDM-1- and blaOXA-48-positive Klebsiella pneumoniae: a European collaborative analysis of whole genome sequencing and epidemiological data, 2014 to 2019. Eurosurveillance 25, 20. https://doi.org/10.2807/1560-7917.ES.2020.25.20.2000627.
Mahon, B.M., Brehony, C., Cahill, N., McGrath, E., O'Connor, L., Varley, A., et al., 2019. Detection of OXA-48-like-producing Enterobacterales in Irish recreational water. Science of The Total Environment 690, 1–6. https://doi.org/10.1016/j.scitotenv.2019.06.480.
Mahon, B.M., Brehony, C., McGrath, E., Killeen, J., Cormican, M., Hickey, P., et al., 2017. Indistinguishable NDM-producing Escherichia coli isolated from recreational waters, sewage, and a clinical specimen in Ireland, 2016 to 2017. Eurosurveillance 2017. DOI: 10.2807/1560-7917.ES.2017.22.15.30513.
Manchanda, V., Rai, S., Gupta, S., Rautela, R.S., Chopra, R., Rawat, D.S., et al., 2011. Development of TaqMan Real-Time Polymerase Chain Reaction for the Detection of the Newly Emerging Form of Carbapenem Resistance Gene in Clinical Isolates of Escherichia Coli, Klebsiella Pneumoniae, and Acinetobacter Baumannii. Indian journal of medical microbiology 29. https://doi.org/10.4103/0255-0857.83907.
Minogue, T.D., Daligault, H.A., Davenport, K.W., Bishop-Lilly, K.A., Broomall, S.M., Bruce, D.C., et al., 2014. Complete Genome Assembly of Escherichia coli ATCC 25922, a Serotype O6 Reference Strain. Genome Announce 2. https://doi.org/10.1128/genomeA.00969-14.
Miro, E., Rossen, J.W.A., Chlebowicz, M.A., Harmsen, D., Brisse, S., Passet, V., et al., 2020. Core/Whole Genome Multilocus Sequence Typing and Core Genome SNP-Based Typing of OXA-48-Producing Klebsiella pneumoniae Clinical Isolates From Spain, 2961 2961 Frontiers in microbiology 10. https://doi.org/10.3389/fmicb.2019.02961.
Morris, D., Kavanagh, S., Carney, K., MacDomhnaill, B., Cormican, M., 2016. CapE (capture, amplify, extract): A rapid method for detection of low level contamination of water with Verocytotoxigenic Escherichia coli (VTEC). Science of The Total Environment 563–564, 267–272. https://doi.org/10.1016/j.scitotenv.2016.04.075.
Nicoletti, A.G., Fehlberg, L.C.C., Picão, R.C., Machado Ade, O., Gales, A.C., 2012. Clonal Complex 258, the Most Frequently Found Multilocus Sequence Type Complex in KPC-2-Producing Klebsiella pneumoniae Isolated in Brazilian Hospitals. Antimicrob Agents Chemother. 56, 4563–4564. https://doi.org/10.1128/AAC.00219-12.
Official Journal of the European Union. DIRECTIVE 2006/7/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006L0007&from=EN.
Official Journal of the European Communities. Directive (EU) 2020/2184 of the European Parliament and of the Concil of 16 December 2020 on the quality of water intended for human consumption. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020L2184&from=EN.
O'Flaherty, E., Solimini, A., Pantanella, F., Cummins, E., 2019. The potential human exposure to antibiotic resistant-Escherichia coli through recreational water. Sci Total Environ 650, 786–795.
Oteo, J., Hernánnnnndez, J.M., Espasa, M., Fleites, A., Sáez, D., Bautista, V., et al., 2013. Emergence of OXA-48-producing Klebsiella Pneumoniae and the Novel Carbapenemases OXA-244 and OXA-245 in Spain. The Journal of antimicrobial chemotherapy 68. https://doi.org/10.1093/jac/dks383.
Palazón, A., López, I., Aragonés, L., Villacampa, L., Navarro-González, F.J., 2017. Modelling of Escherichia Coli Concentrations in Bathing Water at Microtidal Coasts. The Science of the total environment 593–594. https://doi.org/10.1016/j.scitotenv.2017.03.161.
Paschoal, R.P., Campana, E.H., Corrêa, L.L., Montezzi, L.F., Barrueto, L.R.L., da Silva, I. R., et al., 2017. Concentration and Variety of Carbapenemase Producers in Recreational Coastal Waters Showing Distinct Levels of Pollution. Antimicrob Agents Chemother 61. https://doi.org/10.1128/AAC.01963-17.
Pazda, M., Kumirska, J., Stepnowski, P., Mulkiewicz, E., 2019. Antibiotic resistance genes identified in wastewater treatment plant systems – A review. Science of The Total Environment 697, 134023. https://doi.org/10.1016/j.scitotenv.2019.134023.
Poirel, L., Cattoir, V., Nordmann, P., 2012. Plasmid-Mediated Quinolone Resistance; Interactions between Human, Animal, and Environmental Ecologies. Frontiers in Microbiology 3, 24. https://doi.org/10.3389/fmicb.2012.00024.
Prestinaci, F., Pezzotti, P., Pantosti, A., 2015. Antimicrobial resistance: a global multifaceted phenomenon. Pathogens and global health 109, 309–318. https://doi.org/10.1179/2047773215Y.0000000030.
Rozen, Y., Belkin, S., 2001. Survival of enteric bacteria in seawater. FEMS Microbiology Reviews 25, 513–529. https://doi.org/10.1111/j.1574-6976.2001.tb00589.x.
Sobieszczańska, B.M., 2008. Distribution of genes encoding iron uptake systems among enteroaggregative Escherichia coli strains isolated from adults with irritable bowel syndrome. Clinical Microbiology and Infection 14, 1083–1086. https://doi.org/10.1111/j.1469-0691.2008.02093.x.
Soge, O.O., Tivoli, L.D., Meschke, J.S., Roberts, M.C., 2009. A conjugative macrolide resistance gene, mef(A), in environmental Clostridium perfringens carrying multiple macrolide and/or tetracycline resistance genes. J Appl Microbiol 106, 34–40. https://doi.org/10.1111/j.1365-2672.2008.03960.x.
Swayne, R.L., Ludlam, H.A., Shet, V.G., Woodford, N., Curran, M.D., 2011. Real-time TaqMan PCR for rapid detection of genes encoding five types of non-metallo- (class A and D) carbapenemases in Enterobacteriaceae. International Journal of Antimicrobial Agents 38, 35–38. https://doi.org/10.1016/j.ijantimicag.2011.03.010.
Turton JF, Payne Z, Coward A, Hopkins KL, Turton JA, Doumith M, et al. Virulence Genes in Isolates of Klebsiella Pneumoniae From the UK During 2016, Including Among Carbapenemase Gene-Positive Hypervirulent K1-ST23 and 'Non-

Hypervirulent' Types ST147, ST15 and ST383. Journal of medical microbiology 2018; 67. DOI: 10.1099/jmm.0.000653.

Voulgari, E., Zarkotou, O., Ranellou, K., Karageorgopoulos, D.E., Vrioni, G., Mamali, V., et al., 2012. Outbreak of OXA-48 Carbapenemase-Producing Klebsiella Pneumoniae in Greece Involving an ST11 Clone. The Journal of antimicrobial chemotherapy 68. https://doi.org/10.1093/jac/dks356.

World Health Organization, 2015. Global Action Plan on Antimicrobial Resistance. https://apps.who.int/iris/bitstream/handle/10665/193736/9789241509763_eng.pdf?sequence=1.

World Health Organization, 2017. GLOBAL PRIORITY LIST OF ANTIBIOTIC-RESISTANT BACTERIA TO GUIDE RESEARCH, DISCOVERY, AND DEVELOPMENT OF NEW ANTIBIOTICS. https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf.

Wyer, M.D., Kay, D., Morgan, H., Naylor, S., Clark, S., Watkins, J., et al., 2018. Within-day variability in microbial concentrations at a UK designated bathing water: Implications for regulatory monitoring and the application of predictive modelling based on historical compliance data, 100006 100006 Water research X 1. https://doi.org/10.1016/j.wroa.2018.10.003.

Xu, H., Miao, V., Kwong, W., Xia, R., Davies, J., 2011. Identification of a novel fosfomycin resistance gene (fosA2) in Enterobacter cloacae from the Salmon River. Canada. Lett Appl Microbiol 52, 427–429. https://doi.org/10.1111/j.1472-765X.2011.03016.x.

Zerbino and Birney, 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Research. 18 (5), 821–829. https://doi.org/10.1101/gr.074492.107.

Zhang, L., Ma, X., Luo, L., Hu, N., Daun, J., Tang, Z., et al., 2020. The Prevalence and Characterization of Extended-Spectrum β-Lactamase- and Carbapenemase-Producing Bacteria from Hospital Sewage, Treated Effluents and Receiving Rivers. International Journal of Environmental Research and Public Health 17, 1183. https://doi.org/10.3390/ijerph17041183.

Zhou, Z., Alikhan, N.-F., Sergeant, M.J., Luhmann, N., Vaz, C., Francisco, A.P., et al., 2018. GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens. Genome research 28, 1395–1404. https://doi.org/10.1101/gr.232397.117.

Zou, H., Jia, X., Liu, H., Li, S., Wu, X., Huang, S., 2020. Emergence of NDM-5-Producing Escherichia coli in a Teaching Hospital in Chongqing, China: IncF-Type Plasmids May Contribute to the Prevalence of blaNDM–5. Frontiers in Microbiology 11, 334. https://doi.org/10.3389/fmicb.2020.00334.