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

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Abstract

The natural aquatic environment is a significant contributor to the development and circulation of clinically significant antibiotic resistance genes (ARGs). The potential for the aquatic environment to act as a reservoir for ARG accumulation in areas receiving anthropogenic contamination has been thoroughly researched. However, the emergence of novel ARGs in the absence of external influences, as well as the capacity of environmental bacteria to disseminate ARGs via mobile genetic elements remain relatively unchallenged. In order to address these knowledge gaps, this scoping literature review was established focusing on the detection of two important and readily mobile ARGs, namely, extended spectrum beta-lactamase (ESBL) and carbapenemase genes. This review included 41 studies from 19 different countries. A range of

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different water bodies including rivers (n=26), seawaters (n=6) and lakes (n=3), amongst others, were analysed in the included studies. ESBL genes were reported in 29/41 (70.7%) studies, while carbapenemase genes were reported in 13/41 (31.7%), including joint reporting in 9 studies. The occurrence of mobile genetic elements was evaluated, which included the detection of integrons (n=22), plasmids (n=18), insertion sequences (n=4) and transposons (n=3). The ability of environmental bacteria to successfully transfer resistance genes via conjugation was also examined in 11 of the included studies. The findings of this scoping review expose the presence of clinically significant ARGs in the natural aquatic environment and highlights the potential ability of environmental isolates to disseminate these genes among different bacterial species. As such, the results presented demonstrate how anthropogenic point discharges may not act as the sole contributor to the development and spread of clinically significant antibiotic resistances. A number of critical knowledge gaps in current research were also identified. Key highlights include the limited number of studies focusing on antibiotic resistance in uncontaminated aquatic environments as well as the lack of standardisation among methodologies of reviewed investigations.

Keywords: Aquatic Environment; Antibiotic Resistance; Extended Spectrum Beta-Lactamase; Carbapenemase; Mobile Genetic Elements.

Abbreviations: ARG, antibiotic resistance gene; ESBL, extended spectrum beta-lactamase; MGE, mobile genetic element; EARS-NET: European Antimicrobial Resistance Surveillance Network; ECDC, European Centre for Disease Prevention and Control; WHO: World Health Organization.

1 Introduction

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

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

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

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

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

76 the extent and nature of gene transfer remains ambiguous, especially in vast environmental
77 reservoirs.

78 Awareness surrounding the importance of employing a 'One Health' approach encompassing
79 human, animal and environmental health when investigating and tackling antibiotic resistance
80 has increased in recent years. This is highlighted in the World Health Organization (WHO),
81 'Global Action Plan on Antimicrobial Resistance' (WHO, 2015). This report emphasizes the
82 need for further research in terms of transmission routes of antibiotic resistance including food,
83 water and the natural environment. Concomitant with the prominence of the 'One Health'
84 concept, there has been an upsurge in research relating to the prevalence of antibiotic resistance
85 in the environment. Nonetheless, information regarding the presence of naturally occurring
86 ARGs in the absence of anthropogenic pressures, and how they are disseminated to potentially
87 pathogenic species in the environment is lacking.

88 Many bacteria and fungi that are ubiquitous in nature can produce molecules with antibacterial
89 properties, which are thought to play a role in communication and competition (Singer at al.,
90 2006). The cephalosporin class of antibiotics originated from a fungus, *Acremonium*
91 *chrysogenum*, which was isolated from a sewage outfall point in seawater in the late 1940's
92 (Brakhage, 1998). Resistance to the third generation cephalosporins such as cefotaxime
93 attributable to ESBL production was first reported in the early 1980's (Rawat and Nair, 2010).
94 The majority of these ESBL enzymes including, SHV and TEM, were first discovered in the
95 nosocomial setting; however, the lack of environmental research during the 1980's warrants the
96 possibility of their presence in the environment being overlooked. Most microorganisms that
97 produce antibiotics in the natural environment have the corresponding resistance genes present in
98 their genome (Allen et al., 2010), which strengthens the possibility of some clinically significant

ARGs originating from environmental organisms. In the case of the *bla*$_{\text{CTX-M}}$ genes, their origins have been traced to the chromosome of *Kluyvera* species which has been isolated from environmental waters and soils (Cantón et al., 2012a). The importance of soil bacteria as the progenitors of the *bla*$_{\text{CTX-M}}$ genes was further established by Graham et al. (2016) who detected the presence of this ESBL gene in soils dating back to 1923, prior to antibiotic use in medicine in Denmark in the 1930s.

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

Recently, consideration has been given to the role of natural production of antibacterial molecules in the formation and spread of ARGs in the environment. Researchers are beginning to examine sites deemed to be 'pristine' in terms of anthropogenic influence. Anthropogenic influence encompasses all human activities that result in the contamination of the environment. Interestingly, different ARGs have been detected at these pristine locations. Van Goethem et al. (2018) recently reported a low abundance of Ambler class A, B and C beta-lactamases in remote Antarctic surface soils at a site categorised as 'pristine'. However, according to Allen et al. (2010) it is challenging to label a geographical area as completely 'pristine', arguing that the

only environments (globally) entirely devoid from human antibiotic prevalence existed in the pre-antibiotic era. This is due to the potential spread of antibiotic resistance from areas under anthropogenic pressures via wildlife and/or environmental transmission routes including regional-global wind and water circulation and cycles. However, research in remote environments free from anthropogenic pressures is providing further insights into ARGs that are vertically inherited over multiple bacterial generations (Van Goethem et al., 2018), enabling bacterial survival in the presence of natural antibiotic production. Permafrost cores are a prime example of a remote environment, typically devoid of anthropogenic activity that can preserve and prevent migration of bacteria over many years. Perron et al. (2015) identified genes that could potentially confer low level antibiotic resistance in the permafrost core as well as genes conferring resistance to beta-lactams, aminoglycosides and tetracyclines in the upper active layers. These types of studies strengthen the theory that the development of antibiotic resistance may be a natural occurrence in the absence of anthropogenic based pressures.

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

144 inextricable link constitutes an increased likelihood for the potential spread of antibiotic resistant
145 organisms to both humans and animals, highlighting the importance of a ‘One Health’ approach.

2 Methods

2.1 Research Question and Database Queries

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

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

The scoping review protocol was adapted from previously published papers (Andrade et al., 2018; Greig et al., 2014). In summary, a search string was formed based on the established research question, which comprised of a combination of relevant search terms adapted to each individual database (Supplementary Table 1). Databases employed in literature searches included PubMed, MEDLINE, EMBASE, Web of Science and Scopus. Searches were conducted on June 10th 2019. MeSH terms were applied when using the PubMed database in order to employ the medical vocabulary thesaurus. The 'explode' function was used in the MEDLINE/ EMBASE search string in order to search for narrower subject headings under the database's hierarchy tree. The field tag 'TS' was applied to the Web of Science database to focus the search string on the topic of the articles. The search string was adapted to the Scopus database using TITLE-ABS-KEY to identify the search terms in titles, abstracts and keywords. Relevant subject areas and source types were also selected and applied in each database to limit the numbers of irrelevant articles retrieved. All articles obtained from the searches were exported to Endnote and duplicates were removed.

2.2 Additional Sources

Grey literature was examined by applying the general phrase 'antibiotic resistance in the environment' to the following databases: Trip (www.tripdatabase.com), BASE (www.base-search.net), CDC (www.cdc.gov), ECDC (www.ecdc.europa.eu) and Research Gate (www.researchgate.net). Supplementary searches employing Google Scholar were also incorporated into the protocol. Bibliography screening of the final set of included papers was carried out in an attempt to identify additional relevant articles not captured within the original review protocol.

2.3 Screening Phase and Inclusion/ Exclusion Criteria.

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

Table 1: Inclusion and exclusion criteria applied to studies to determine eligibility.

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

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

i) The genomic detection of ESBL and/or carbapenemase genes.

ii) The genomic detection of MGEs or demonstration of transferability of resistance genes by experimentation.

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

3 Results

3.1 Screening Stages for Article Inclusion

A total number of 1415 articles were identified and subject to the first screening stage following application of the search string to the 5 main databases including PubMed, Scopus, Web of Science, MEDLINE and EMBASE (Figure 1). An additional 5 peer-reviewed articles were identified from grey literature queries placing the total number of articles at 1420. Following initial title and abstract screening, 1313 articles were excluded, with a total of 107 articles subject to full text review. This was narrowed down to 34 following exclusion of 73 articles that failed to meet pre-established inclusion/exclusion criteria. The most common exclusion factor was local presence of point source discharges (n=24). A recurrent feature during this screening phase was the lack of detail provided in the results of several investigations which often resulted in article exclusion. Several investigations failed to link the results presented to specific site types analyzed (e.g. polluted, pristine), but instead provided a general summary of all resistance genes detected (e.g. Dhawde et al., 2018). In other cases, due to the extensive nature of the data generated, primarily a feature of investigations employing metagenomics, ARGs were often summarised to an overly generic level. In some instances, only the quantity of beta-lactamase resistance genes that were present in a given sample were discussed rather than providing details on the different types of resistance genes or their location within the chromosome or mobile genetic element (Garner et al., 2016). As such, it was not possible to determine the types of beta-lactamase genes detected. Bibliography screening of the 34 included papers following the pre-defined inclusion/ exclusion criteria identified an additional 14 potentially suitable articles. 6 of these papers were included for analysis increasing the total number of articles for inclusion to 40.

A single article was identified during a general search using Google Scholar bringing the final number of included articles to 41.

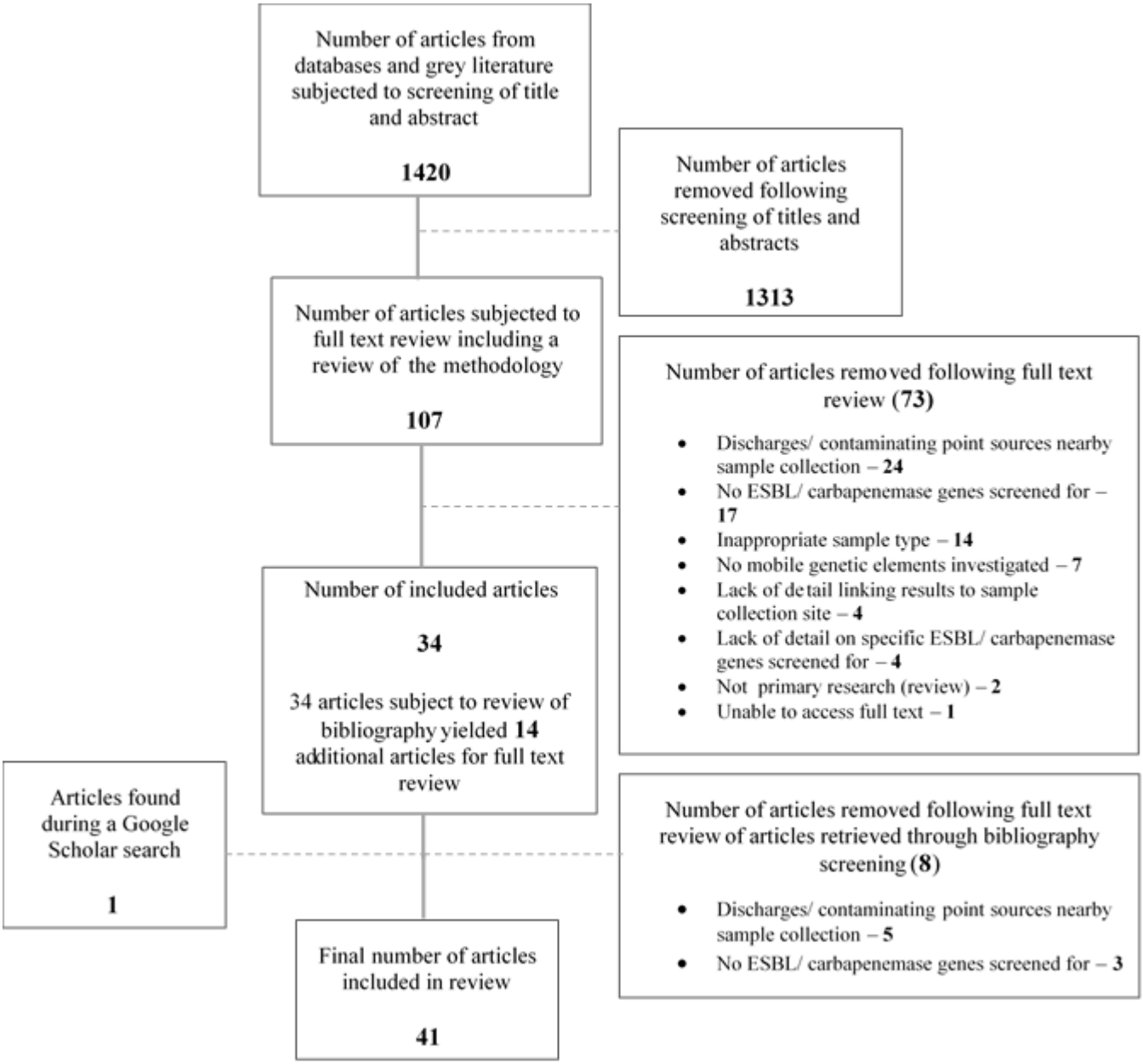

Figure 1: Flowchart outlining the process of elimination of articles based on inclusion/exclusion criteria set for this review.

3.2 Data Extraction

All 41 identified articles were subject to data extraction based on a number of pre-established data fields (Table 2). Primary data extraction fields included (i) country of origin for sample(s) analysed, (ii) type of sample analysed (e.g. seawater/river/lake), (iii) types of mobile genetic elements detected (e.g. plasmids, integrons, etc.), (iv) the application of conjugation transfer and whether it was successful at transferring the ARGs of interest, and (v) ESBL and/or carbapenemase gene variants detected. A range of data fields supplementing the information presented in Table 2 and pertaining local environments (e.g. local discharges downstream of sampling points), bacterial species detected and further information on the types of mobile genetic elements detected (e.g. plasmid incompatibility groups, integron class) are outlined in Supplementary Table 2. Similarly, data relating to the methodology implemented in each investigation including initial collection volumes, processing and genomic screening for ARGs and MGEs are provided in Supplementary Table 3.

Table 2: Selected data extraction fields employed in the literature review protocol.

Reference	**Country**	**Sample type (s)**	**Mobile genetic elements detected**	**Conjugation**	**ESBL detection**	**Carbapenemase detection**
Zurfluh et al., 2015	Switzerland	River	Plasmid, transposon, insertion element, class 1 integron	Conjugation successful	N/A	*bla*$_{VIM*}$
Ben Said et al., 2016	Tunisia	Rivers and lakes	Unknown	N/A	Not detected	N/A*
Lekunberri et al., 2017	Spain	River	Plasmid DNA	N/A	*bla*$_{TEM}$	*bla*$_{KPC}$, *bla*$_{NDM}$
Adelowo et al., 2018	Nigeria	Ground-water	Class 1 integrons, plasmids, insertion sequences	N/A	*bla*$_{TEM}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV}$	N/A
Caltagirone et al., 2017	Italy	Rivers and ground-water	Plasmids	Conjugation successful	*bla*$_{CTX-M-1, 28}$, *bla*$_{SHV-12}$	Not detected*
Kim et al., 2008	South Korea	River	Class 1 integrons	Conjugation successful	*bla*$_{TEM-52}$, *bla*$_{OXA-4}$, *bla*$_{CTX-M-14}$	N/A*
Stange et al., 2016	Germany	River	Class 1 and 2 integrons	N/A	*bla*$_{TEM}$, bla$_{SHV}$	N/A

Muraleedharan et al., 2019	United States	River	Plasmid	Conjugation successful	*bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{TEM}}$	*bla*$_{\text{KPC-2}}$
Olga et al., 2016	Greece	Stream water	Class 1 integron	N/A	Not detected	Not detected*
Wambugu et al., 2018	Kenya	River	Class 1 integron	N/A	*bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M}}$	N/A
Dolejská et al., 2009	Czech Republic	Pond	Class 1 integrons	N/A	Not detected	N/A*
Alouache et al., 2012	Algeria	Seawater	Unknown	N/A	Not detected	N/A*
Tafoukt et al., 2018	Algeria	River	Not detected	Conjugation unsuccessful	N/A	*bla*$_{\text{OXA-181, 199, 538*}}$
Lepuschitz et al., 2019	Austria	River	Unknown	N/A	Not detected	Not detected*
Zarfel et al., 2017	Austria	River	Plasmids	N/A	*bla*$_{\text{CTX-M-15, 27, 14, 1, 2, 9, 14-like}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{GES-1}}$	*bla*$_{\text{VIM-1}}$
Charnock et al., 2014	Norway	Seawater	Class 1 integrons	N/A	Not detected	N/A
Jørgensen et al., 2017b	Norway	Seawater	Plasmids	N/A	*bla*$_{\text{CTX-M-1, 2, 9, 14, 15, 27, 55}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{TEM-33-like, 52C}}$	Not detected*
Jørgensen et al., 2017a	Norway	Seawater	Plasmids	N/A	*bla*$_{\text{CTX-M-1}}$	N/A*
Kieffer et al., 2016	Portugal	River	Plasmids, Class 1 integrons	Conjugation successful	N/A	*bla*$_{\text{IMP-8}}$, *bla*$_{\text{VIM-1,}}$ *bla*$_{\text{VIM-34*}}$
Tacão et al., 2012	Portugal	Rivers	Class 1 integrons, insertion sequences	N/A	*bla*$_{\text{CTX-M}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM}}$	N/A*
Poirel et al., 2012	Portugal	River	Plasmids, transposons	Conjugation successful	N/A	*bla*$_{\text{KPC-2*}}$
Harnisz & Koreniewsk 2018	Poland	River	Class 2 integrons	N/A	Not detected	N/A
Osińska et al., 2016	Poland	River	Plasmid DNA	Conjugation successful	*bla*$_{\text{OXA}}$, *bla*$_{\text{TEM}}$	N/A*
Koczura et al., 2014	Poland	Lakes	Class 2 integrons	N/A	Not detected	N/A*
Osińska et al., 2017	Poland	River	Class 1 integrons	N/A	*bla*$_{\text{TEM}}$, *bla*$_{\text{OXA}}$	N/A*
Bajaj et al., 2016	India	River	Plasmids	Conjugation successful	*bla*$_{\text{CTX-M-15}}$	N/A
Singh et al., 2018	India	River	Insertion sequence, class 1 integron	Conjugation successful	*bla*$_{\text{CTX-M-15}}$	N/A
Akiba et al., 2016	India	Rivers	Plasmids	N/A	*bla*$_{\text{CTX-M-55, 15}}$, *bla*$_{\text{OXA-1, 9}}$, *bla*$_{\text{SHV-12}}$	*bla*$_{\text{NDM-7*}}$
Lamba et al., 2017	India	River	Class 1, 2 and 3 integrons	N/A	*bla*$_{\text{OXA}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$	*bla*$_{\text{NDM}}$
Fernandes et al., 2017	Brazil	Seawater	Plasmids	N/A	*bla*$_{\text{CTX-M-8, 1}}$	N/A*
Sellera et al., 2017	Brazil	Seawater	Plasmid	N/A	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-17}}$	*bla*$_{\text{KPC-2*}}$
Francisco et al., 2019	Brazil	Rivers	Plasmids	N/A	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{OXA-1}}$	*bla*$_{\text{KPC-2*}}$
Nascimento et al., 2017	Brazil	Lakes	Plasmids, transposons	N/A	*bla*$_{\text{CTX-M-2, 15, 9}}$	*bla*$_{\text{KPC-2*}}$

Chen et al., 2010	China	River	Class 1 and 2 integrons	N/A	*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$	N/A*
Zhang et al., 2018	China	Bay	Class 1 integrons	N/A	*bla*$_{TEM}$	N/A
Wang et al., 2018	China	River	Class 1 integrons	N/A	*bla*$_{TEM}$	N/A*
Ye et al., 2017	China	River	Class 1 integrons	Conjugation successful	*bla*$_{CTX-M-65, 55, 15, 14}$	N/A*
Yin et al., 2013	China	Lake	Class 1 and 2 integrons	Conjugation successful	*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{OXA-1}$	N/A*
Wu et al., 2019	China	Rivers	Class 1 integron	N/A	*bla*$_{CTX-M}$, *bla*$_{TEM}$	N/A
Zou et al., 2012	China	River	Plasmid	N/A	*bla*$_{TEM}$, *bla*$_{SHV}$	Not detected
Ouyang et al., 2015	China	River	Class 1 integron	N/A	*bla*$_{SFO}$, *bla*$_{SHV}$, *bla*$_{TEM}$	*bla*$_{IMP}$

Unknown = Data not provided in the article.
Not detected = Screened for but not detected.
N/A = Not directly screened for using a targeted approach.
* = Sequencing approach applied.

3.3 Summary Analysis of Included Studies

The geographical distribution of included investigations is shown in Figure 2. Overall, studies derived from nineteen different countries across five continents. As such, the data presented is representative of water bodies at a global scale, but primarily comprises investigations based in Europe (n=18) and Asia (n=13). The remainder of identified investigations were based in Africa (n=5), South America (n=4) and North America (n=1). China featured as the country with most investigations (n=8) followed by Poland (n=4), India (n=4) and Brazil (n=4).

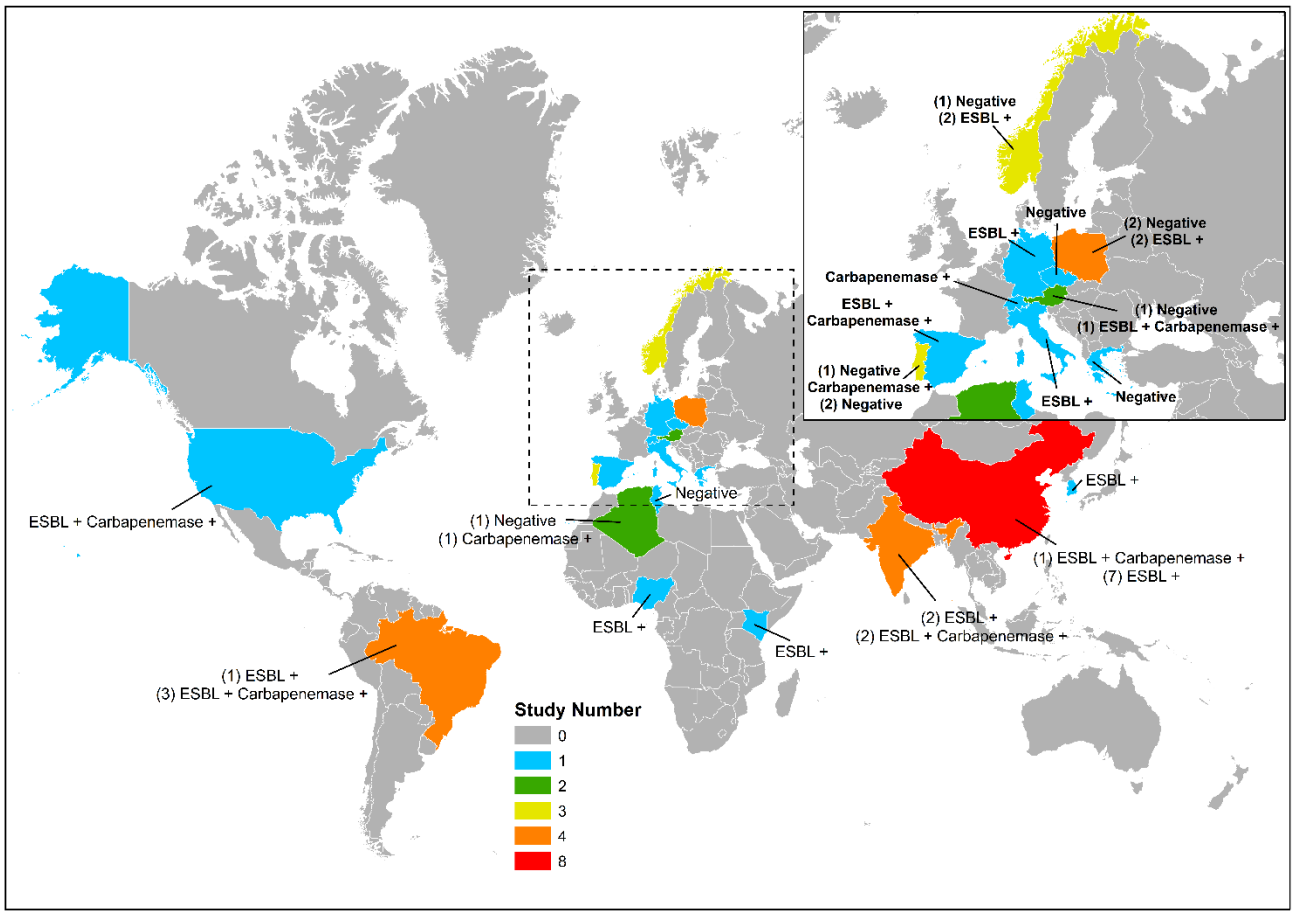

Figure 2: Global distribution of investigations identified through the literature review protocol including positive detection of ESBL and/or carbapenemase genes. The number provided in brackets denotes the number of country-specific studies with the same detection outcome in terms of antibiotic resistance genes.

Overall, the methodology employed for sample collection, processing and genomic screening varied substantially among investigations (Supplementary Table 3). Sample volumes collected exhibited wide variations ranging from swab placement in water for 24 hours (Dolejská et al., 2009) to analysis of 7 L of water (Tacão et al., 2012). Concentration methods also varied significantly with filtration using a 0.45µm (n=14) and 0.22µm (n=6) being the most prevalent. However, a further 6 studies using a filtration approach failed to report filter pore size. Filtration

was often followed by direct incubation of filters on agar plates, (n=12). PCR and subsequent sequencing (n=18) featured as the most prominent molecular method employed to detect ARGs and MGEs. Additional molecular detection methods included sole use of PCR (n=16) or a sequencing approach (n=5).

The bacterial species most commonly detected was *E.coli* with positive identification in a total of 24 investigations. *E. coli* detection was followed by *Klebsiella* spp. (n=11), *Enterobacter* spp. (n=6) and *Pseudomonas* spp. (n=6) (Supplementary Table 2). A range of natural water bodies were investigated in the reviewed studies. Inland waters were classified into lentic (i.e., stationary or still water), lotic (i.e., free-flowing water) and sub-surface/groundwater. Lentic systems included lakes (n=4) and ponds (n=1) whereas lotic systems encompassed rivers (n=28) and streams (n=1). Additionally, seawater (n=6) or bay waters (n=1) were classified as coastal/marine. Only two investigations evaluated sub-surface/groundwater for the presence of ARGs. A total of two included articles evaluated more than one type of water body, (rivers, lakes or groundwater).

In terms of ARG and MGE detection, a summary of the numbers and types of each are provided in Figures 3 and 4. A total of 33/41 (80.5%) investigations detected the presence of ESBL and/or carbapenemase genes in water samples. Specifically, 20/41 (48.8%) studies detected ESBL genes, 4/41 (9.8%) studies detected carbapenemase genes and 9/41 (22.0%) detected dual presence. The most commonly detected ESBL resistance gene was $bla_{\text{CTX-M}}$ (n=21), closely followed by bla_{TEM} (n=18). Regarding the detection of carbapenemase resistance genes, bla_{KPC} was the most commonly detected gene with identification in 6 reviewed studies. This was followed by bla_{VIM} (n=3), bla_{NDM} (n=3) and bla_{IMP} (n=2). In comparison to resistance gene detection, a larger proportion of studies (37/41; 90.2%) detected the presence of one or more

MGE. This included class 1, 2 and 3 integrons (n=22), plasmids (n=18), insertion sequences (n=4) and transposons (n=3). In terms of integrons, class 1 was the most prominent (n=20) followed by class 2 (n=6) and class 3 (n=1). Regarding plasmid incompatibility groups, the most commonly detected types were IncFIB (n=10) followed by IncN (n=8). Furthermore, 11 studies also demonstrated successful conjugation transfer of ARGs.

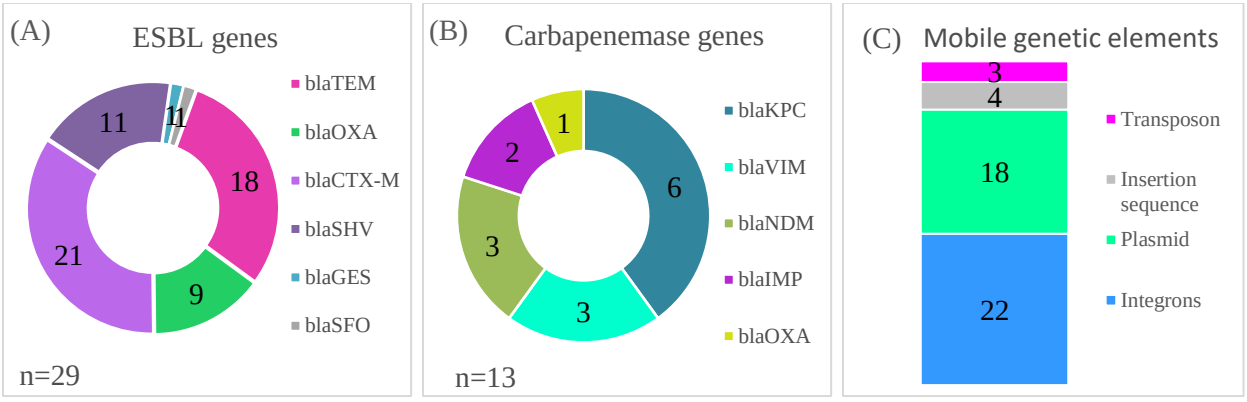

Figure 3: Number of studies detecting each type of (A) ESBL gene (B) carbapenemase gene and (C) mobile genetic element. The total number of studies detecting ESBLs and carbapenemases is also provided, with some studies reporting more the one type of ESBL (n=20) or carbapenemase (n=2) gene.

4 Discussion

This scoping literature review was established in order to examine existing scientific literature relating to the role of the natural aquatic environment in harboring and transmitting ARGs of clinical importance. Here, an exclusive focus was placed on areas with “minimal” direct anthropogenic contamination. A key highlight from this investigation is the relative limited number of studies focusing on the nexus between the natural, unpolluted aquatic environments and the presence of antibiotic resistance. A significant feature identified through the review protocol is a tendency for investigations to attempt to establish point sources as pollution agents; however, detection of resistance genes in uncontaminated areas is generally overlooked.

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

4.1 Synopsis of Identified Literature

Publication dates of included articles ranged from 2008 to 2019, with the majority published between 2016 and 2019 (29/41; 70.7%). The upsurge in publications observed in recent years concurs with an increased interest by the research community in investigating the prevalence of antibiotic resistance in environmental settings (Kraemer et al., 2019). *E.coli* was the most commonly detected species in 24 studies followed by *Klebsiella* spp. (n=11), *Enterobacter* spp. (n=6) and *Pseudomonas* spp. (n=6) (Supplementary Table 2). These bacteria are often associated

with clinical infections and as a result, some methodologies employed a selection bias using selective broths and agars, (see Supplementary Table 3). A range of natural water bodies were investigated in the reviewed studies, however, the majority of studies focused on lotic systems (n=29; 70.7%). The high incidence of studies based on these systems may be associated with the smaller dimensions of rivers/streams in comparison to larger marine and coastal water bodies, equating to a lesser dilutional effect on viable antibiotic resistant organisms. Additionally, lotic systems are often located within urban and agricultural landscapes, with river-sourced water often used as a domestic water supply and thus representing a potential pathway for antibiotic resistance transmission to humans. As indicated above, based on the lack of studies investigating "pristine" aquatic environments, water bodies labelled as 'urban' were also included in the review protocol if no local point sources were described. A prevalent methodological approach among river-based investigations included collection of water samples in areas upstream and downstream of a discharge point in order to ascertain the influence of discharges in the proliferation of environmental ARGs. As mentioned above, only articles featuring sampling regimes based on collection of upstream samples were included in the review in order to accommodate inclusion criteria, i.e., no perceived local point source(s) of pollution.

The low number of investigations based on marine/coastal and lake environments that were free from anthropogenic contamination represents a key research gap considering their potential importance as environmental pathways for the transmission of antibiotic resistance to humans; particularly through recreational exposure (Leonard et al., 2018). Possible reasons for the low number of marine-based investigations observed may include lack of access to coastal sampling sites in landlocked countries or the potential for lower rates of bacterial survival in saltwater

serving as a deterrent (Rozen and Belkin, 2001). In terms of lakes/ponds, their absence as prevalent landscape components in certain geographical settings may preclude their analysis. However, they do also pose a significant threat for the potential transmission of ARGs to humans (Bengtsson-Palme et al., 2014).

4.2 Antibiotic resistance gene detection

A higher detection rate of ESBL (n=29) genes in comparison to carbapenemase genes (n=13) among included studies may be attributable to the fact that ESBL enzymes emerged approximately 10 years prior to carbapenemase detection. In addition, carbapenem antibiotics are classified as reserved/restricted in many countries and therefore only used as a last resort, in an attempt to circumvent further resistance development. However, both third generation cephalosporins and carbapenems are classified as critically important antibiotics employed in veterinary medicine (WHO, 2016). Consequently, ESBL producing bacteria and to a lesser extent carbapenemase producers, are disseminated throughout most environments.

On a country-basis, Brazil had the highest number of detected carbapenemase enzymes, featuring in 3 out of a possible 4 studies. Interestingly, all 3 positive studies detected *bla*$_{\text{KPC-2}}$. This carbapenemase gene was first detected in the USA in 1996 and is now considered as endemic in Brazil (Lee et al., 2016). Although China had the largest number of included studies, carbapenemase enzymes were only reported in 1/8 studies. In turn, China accounted for the highest detection rates of ESBLs among included investigations (8/8). Generally, ESBLs were widespread across countries and continents (Figure 2). This potentially reflects their widespread dissemination and/or natural occurrence in the aquatic environment as supported by several

investigations (Swedan & Abu Alrub, 2019; de Oliveira et al., 2017; Yamashita et al., 2017; Adesoji & Ogunjobi, 2016).

The most commonly detected ESBL gene was *bla*$_{\text{CTX-M}}$ (n=21). This is unsurprising as the origins of this gene has been traced back to the environmental organism *Kluyvera*, previously isolated from water bodies (Cantón et al., 2012a). This was followed by *bla*$_{\text{TEM}}$ (n=18), *bla*$_{\text{SHV}}$ (n=11) and *bla*$_{\text{OXA}}$ (n=9) ESBL types. These findings concur with additional studies screening for ESBL genes in the environment. For example, Ranjbar & Sami. (2017) detected *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{OXA}}$ ESBL types at a frequency of 37%, 27%, 27% and 25% respectively, in an investigation based on the analysis of river water. In terms of the carbapenemase encoding genes, *bla*$_{\text{KPC}}$ was the most commonly detected gene in 6/41 (14.6%) included studies. This was followed by *bla*$_{\text{VIM}}$ (n=3), *bla*$_{\text{NDM}}$ (n=3) and *bla*$_{\text{IMP}}$ (n=2). These enzymes were first identified in clinical isolates (Khan et al., 2017; Yigit et al., 2001; Lauretti et al., 1999; Osano et al., 1994). However, their presence in the environment prior to reports in the nosocomial setting may have gone unidentified. Recent linkages of *bla*$_{\text{OXA-48}}$ with the environmental *Shewanella* species reported by Tacão et al. (2018) serves to highlight this possibility.

Positive detection of ESBL and/or carbapenemase genes in 33/41 (80.5%) investigations indicates the ubiquitous occurrence of these clinically significant ARGs in natural aquatic environments at a global scale. However, an important consideration in this area of research is publication bias against negative results. This bias could potentially inflate the high percentage of included studies with positive detections. Unfortunately, it is not a type of bias that can be controlled for within the scope of this review, but should be considered when interpreting the results. Reviewed investigations with negative detection of ESBLs and/or carbapenemases collected a range of different sample types including river water (n=2), lake water (n=1), river

and lake water (n=1), seawater (n=2), stream water (n=1) and pond water (n=1). Large variations in methods employed observed among these articles may be associated with the lack of detection of ARGs. Small volumes of water were collected for analysis among some investigations. For example, Dolejská et al. (2009) employed the use of swabs placed in a pond for 24 hours as the initial sample collection technique while Ben Said et al. (2016) collected 5mL volumes of water for analysis (Supplementary Table 3). Additionally, certain processing techniques may have influenced the results. Harnisz and Korzeniewska. (2018) diluted samples with saline in contrast to the filtration and enrichment prior to culturing approach employed in other investigations (e.g. Bajaj et al., 2016). The large variation in methodologies employed demonstrates a lack of standardisation among environmental sampling and laboratory processing which prevents robust comparisons among reported results.

4.3 Mobile genetic element detection

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

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

Plasmids were the second most commonly detected MGE, identified in 18 studies (Figure 3). These elements are responsible for dissemination of antibiotic resistance elements via conjugation, (San Millan, 2018). While capable of harboring and transmitting multiple gene types, ARGs have been closely linked with plasmids due to the clinical implications in terms of treatment. Several plasmid incompatibility groups were detected among reviewed investigations, including IncFIB (n=10) and IncN (N=8), which were the most commonly detected. Although not all studies confirmed the presence of resistance gene(s) on a plasmid, the potential transfer of the resistance gene from the chromosome to a plasmid and its' further dissemination across bacterial species via conjugation is possible. The successful conjugation transfer of resistance elements in the laboratory in 11/41 (26.8%) investigations strengthens this possibility of ARG propagation in aquatic environments. Notably, conjugation experiments were all performed using *E.coli* as the recipient with the majority using the J53 strain. The latter is in agreement with the review performed by Leclerc et al. (2019), highlighting the prevalence of *E.coli* as a common recipient for conjugation transfer and the general lack of investigations attempting interspecies

transfer. However, in the review dataset, some of the environmental isolates used as conjugation donors were *Klebsiella* and/or *Acinetobacter* (Ye et al., 2017, Osińska et al., 2016, Yin et al., 2013). Hence, there was an attempt at demonstrating interspecies transfer in a limited number of cases.

4.4 Sources of resistance

A range of sources were linked with the incidence of ARGs in the aquatic environments sampled in the reviewed papers. For example, Jørgensen et al. (2017a) detected $bla_{\text{CTX-M-1}}$ and mentioned 'human bathing, boat toilets, farm animals, fertilizers or birds' as potential sources contributing to the presence of antibiotic resistance. Similarly, Nascimento et al., (2017) detected bla_{KPC} and $bla_{\text{CTX-M}}$ types and hypothesized that nosocomial or domestic sewage had entered the lake system via a stream, despite treatment in a 'flotation treatment plant' prior to lake discharge. Beyond Ouyung et al. (2015), few studies considered the possibility of naturally occurring antibiotic resistance in uncontaminated aquatic environments. In this study a 'pristine' site was sampled in a remote location which yielded the detection of 69 antibiotic resistance genes. The results presented were construed as indicative of ubiquitous antibiotic resistance in natural environments. However, the prevailing notion of ARG dissemination from regions under the influence of contaminating discharges to those without it, remains largely unchallenged. Accordingly, further research into intrinsic antibiotic resistance in environmental organisms and the dissemination of ARGs, inclusive of pathways, modes and extent, is urgently needed.

4.5 Research highlights and recommendations

The lack of standardised methodology adopted among identified investigations represents a significant knowledge gap and a challenge for the interpretation of collated data. In particular,

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

The insufficient detail provided on anthropogenic contamination sources in relation to water bodies in several reviewed investigations represents a second important research gap. Overall, it was not possible to ascertain if analyzed water bodies were entirely “free” from contamination. In some studies the sampling sites were labelled as ‘pristine’ or in ‘areas of strict preservation’, but others list sampling points as ‘urban’ regions and so the likelihood of contamination is much greater (Supplementary table 2). This particular restraint means that this review could not be strictly confined to ‘pristine’ aquatic environments, but it does highlight the lack of research focus on the prevalence of antibiotic resistance in natural unpolluted aquatic environment. More research is required in the area as discovery of resistances of clinical significance in regions free

from anthropogenic activity become more apparent. Additionally, more emphasis should be placed on investigations tracing ARG origins to environmental isolates and potentially screening the environment for novel ARGs. Future research should also highlight the role of MGEs in the dissemination of resistance elements rather than focusing primarily on contaminating sources.

In several investigations only certain strains of isolated bacteria were further characterised for the presence of antibiotic resistance using phonotypic and genotypic methods. These were generally restricted to highly virulent bacteria and those most often associated with clinical infections in humans. As such, some articles employed selective application of screening methods specific to detection of only certain types of bacteria, (e.g. Ye et al., 2017; Kieffer et al., 2016; Stange et al., 2016). This practice represents a major knowledge gap with the current role of ubiquitous, non-pathogenic bacteria in the transference of ARGs largely unaccounted for. Emphasis on antibiotic resistance profiles rather than species detected should be employed by scientists, considering MGEs that harbor resistance genes spread throughout different bacterial species. Similarly, by confining environmental research to the most commonly identified MGEs within clinical environments, the potential for transmission of ARGs via MGEs present in natural aquatic environments is likely being underestimated.

Of key relevance is the high prevalence of phenotypic screening methods employed in the majority of reviewed investigations. This factor limited the detection of antibiotic resistance genes to those that reside within viable bacteria that are easily cultured. However, reportedly less than 1% of environmental bacteria can be easily cultured using standard laboratory techniques (Allen et al., 2010). Only four included studies performed PCR/sequencing of the sample without prior culturing of organisms (Supplementary Table 3) which enabled detection and analysis of fastidious bacteria. Overall, current research tends to exclude less clinically significant and

fastidious environmental bacteria, which as a result, may go unnoticed as harbouring MGEs capable of spreading antibiotic resistance elements. Ideally, simultaneous application of both phenotypic culture methods and molecular analysis of environmental samples would need to be employed to generate complete resistance profiles inclusive of genetic composition as well as phenotypic expression.

5 Conclusions

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

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

- Implementation of replica aquatic environmental conjugation/ transformation/ transduction experiments demonstrating interspecies transfer.

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

Author Contributions

BH and DM formulated the initial research question. BH and AJ developed the research question further by researching papers. BH created the search string and searched the databases. BH and AJ or KF carried out the screening of the articles including the first and second stages. DM acted as the third independent reviewer when article inclusion or exclusion could not be decided upon. BH extracted the data from the articles and drafted the paper. CC created the map (Figure 2) for the paper. DM, KF, AJ and CC edited and advised on the contents of the article.

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