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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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1 Abstract

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

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

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

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

32 1 Introduction

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

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

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

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

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

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

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

114 Recently, consideration has been given to the role of natural production of antibacterial
115 molecules in the formation and spread of ARGs in the environment. Researchers are beginning
116 to examine sites deemed to be ‘pristine’ in terms of anthropogenic influence. Anthropogenic
117 influence encompasses all human activities that result in the contamination of the environment.
118 Interestingly, different ARGs have been detected at these pristine locations. Van Goethem et al.
119 (2018) recently reported a low abundance of Ambler class A, B and C beta-lactamases in remote
120 Antarctic surface soils at a site categorised as ‘pristine’. However, according to Allen et al.
121 (2010) it is challenging to label a geographical area as completely ‘pristine’, arguing that the

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

135 The aim of this scoping literature review was to analyse the role of the natural aquatic
136 environment in the transmission of clinically significant antibiotic resistances determinants,
137 specifically ESBL and carbapenemase encoding genes. In this paper we define ‘natural’ aquatic
138 environments as water bodies that are not in direct receipt of contaminating discharges, thus
139 receiving minimal anthropogenic influence. In this context, the review aimed to identify, collate
140 and analyse data from studies examining MGEs in antibiotic resistant isolates obtained from
141 water bodies. Furthermore, current knowledge gaps for further research were identified and
142 highlighted. The focus on water derives from the vital and constant interaction that both humans
143 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.

146 **2 Methods**

147 **2.1 Research Question and Database Queries**

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

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

151 The scoping review protocol was adapted from previously published papers (Andrade et al.,
152 2018; Greig et al., 2014). In summary, a search string was formed based on the established
153 research question, which comprised of a combination of relevant search terms adapted to each
154 individual database (Supplementary Table 1). Databases employed in literature searches included
155 PubMed, MEDLINE, EMBASE, Web of Science and Scopus. Searches were conducted on June
156 10th 2019. MeSH terms were applied when using the PubMed database in order to employ the
157 medical vocabulary thesaurus. The ‘explode’ function was used in the MEDLINE/ EMBASE
158 search string in order to search for narrower subject headings under the database’s hierarchy tree.
159 The field tag ‘TS’ was applied to the Web of Science database to focus the search string on the
160 topic of the articles. The search string was adapted to the Scopus database using TITLE-ABS-
161 KEY to identify the search terms in titles, abstracts and keywords. Relevant subject areas and
162 source types were also selected and applied in each database to limit the numbers of irrelevant
163 articles retrieved. All articles obtained from the searches were exported to Endnote and
164 duplicates were removed.

165 **2.2 Additional Sources**

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

173 **2.3 Screening Phase and Inclusion/ Exclusion Criteria.**

174 Phase 1 consisted of two independent reviewers screening the titles and abstracts of all retrieved
175 articles against a pre-defined set of inclusion and exclusion criteria. Revision by a third
176 independent reviewer was utilised to derive an outcome in cases where article
177 inclusion/exclusion could not be agreed upon by the two independent reviewers. The inclusion
178 and exclusion criteria applied during the screening phase are outlined in Table 1. Two main
179 limitations and/or thresholds were set for article inclusion: (i) investigations published between
180 2008 and 2019 and (ii) full text provided in English. The publication year restriction was applied
181 due to the upsurge in research related to the area of antibiotic resistance in the environment,
182 evident following application of the search string to the Scopus database and analyses of the
183 publication dates of retrieved articles. Additionally, this year range was employed because older
184 methods of analysing DNA (e.g. pulse field electrophoresis) would not be comparable to more
185 modern molecular methods such as whole genome sequencing. No geographical thresholds were
186 implemented for article inclusion/exclusion.

187 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).

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

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

204 In the case of articles where information on the variant of the beta-lactamase gene detected using

205 genotypic methods was lacking, phenotypic screening such as antibiotic susceptibility testing

206 was used to determine if the genes detected were true ESBL/ carbapenemase producers. As an

207 example, the *bla_{SHV}* and *bla_{TEM}* genes have some variants allowing for classifications as ESBL

208 producers (*bla_{TEM-3}* and *bla_{SHV-12}*) and some which are classified simply as beta-lactamases

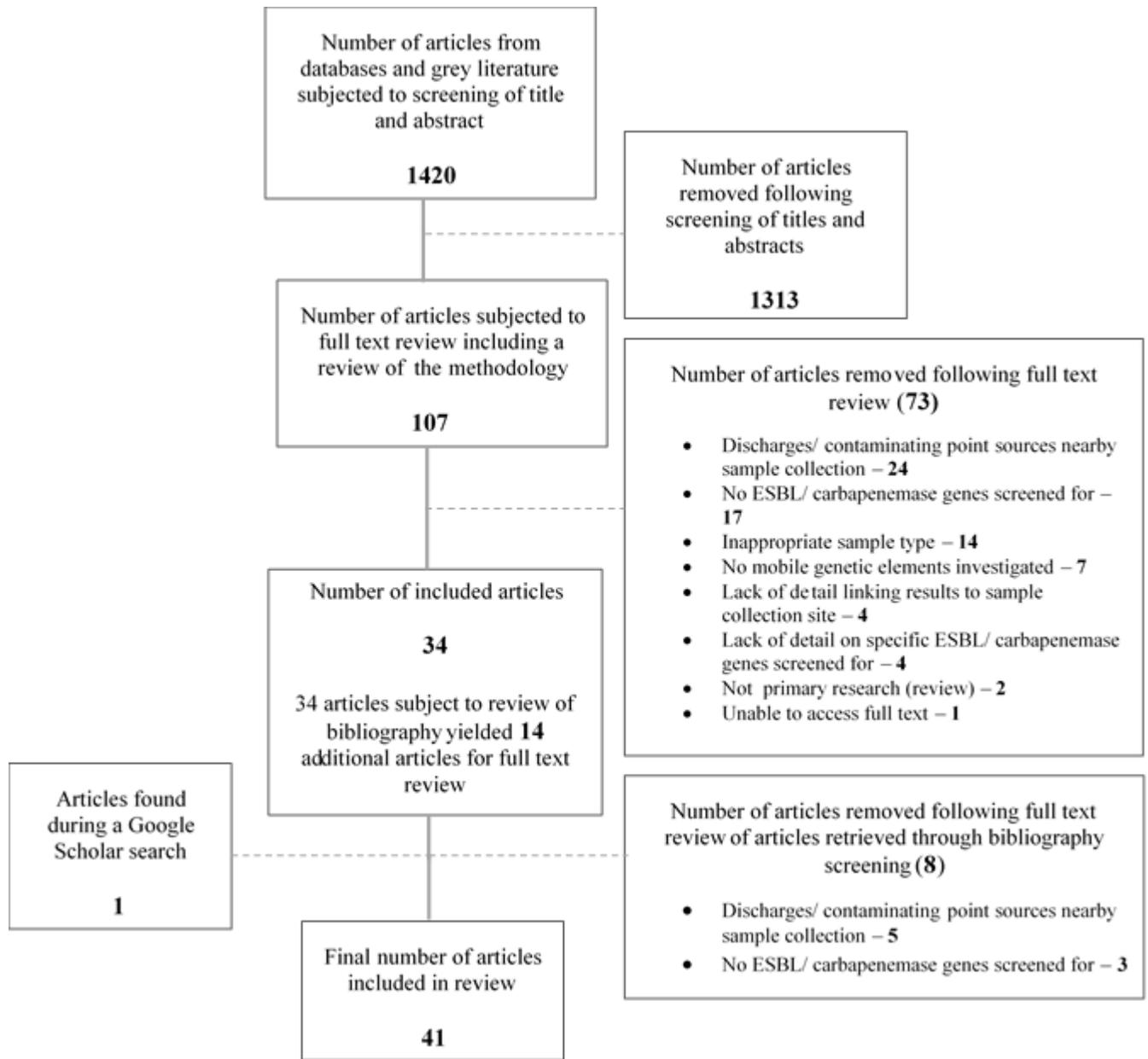
209 (*bla_{TEM-1}*, *bla_{SHV-1}*).

210 3 Results

211 3.1 Screening Stages for Article Inclusion

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

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



234
 235 Figure 1: Flowchart outlining the process of elimination of articles based on inclusion/exclusion
 236 criteria set for this review.

237 **3.2 Data Extraction**

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

250 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	<i>bla_{VIM}</i> *
Ben Said et al., 2016	Tunisia	Rivers and lakes	Unknown	N/A	Not detected	N/A*
Lekunberri et al., 2017	Spain	River	Plasmid DNA	N/A	<i>bla_{TEM}</i>	<i>bla_{KPC}</i> , <i>bla_{NDM}</i>
Adelowo et al., 2018	Nigeria	Ground-water	Class 1 integrons, plasmids, insertion sequences	N/A	<i>bla_{TEM}</i> , <i>bla_{CTX-M-15}</i> , <i>bla_{SHV}</i>	N/A
Caltagirone et al., 2017	Italy	Rivers and ground-water	Plasmids	Conjugation successful	<i>bla_{CTX-M-1, 28}</i> , <i>bla_{SHV-12}</i>	Not detected*
Kim et al., 2008	South Korea	River	Class 1 integrons	Conjugation successful	<i>bla_{TEM-52}</i> , <i>bla_{OXA-4}</i> , <i>bla_{CTX-M-14}</i>	N/A*
Stange et al., 2016	Germany	River	Class 1 and 2 integrons	N/A	<i>bla_{TEM}</i> , <i>bla_{SHV}</i>	N/A

Muraleedharan et al., 2019	United States	River	Plasmid	Conjugation successful	<i>bla</i> _{CTX-M-1} , <i>bla</i> _{TEM}	<i>bla</i> _{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	<i>bla</i> _{TEM} , <i>bla</i> _{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	<i>bla</i> _{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	<i>bla</i> _{CTX-M-15, 27, 14, 1, 2, 9, 14-like, bla_{SHV-12, bla_{GES-1}}}	<i>bla</i> _{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	<i>bla</i> _{CTX-M-1, 2, 9, 14, 15, 27, 55, bla_{OXA-1, bla_{SHV-12, bla_{TEM-33-like, 52C}}}}	Not detected*
Jørgensen et al., 2017a	Norway	Seawater	Plasmids	N/A	<i>bla</i> _{CTX-M-1}	N/A*
Kieffer et al., 2016	Portugal	River	Plasmids, Class 1 integrons	Conjugation successful	N/A	<i>bla</i> _{IMP-8, bla} _{VIM-1, bla} _{VIM-34*}
Tacão et al., 2012	Portugal	Rivers	Class 1 integrons, insertion sequences	N/A	<i>bla</i> _{CTX-M, bla} _{CTX-M-15, bla} _{TEM}	N/A*
Poirel et al., 2012	Portugal	River	Plasmids, transposons	Conjugation successful	N/A	<i>bla</i> _{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	<i>bla</i> _{OXA, bla} _{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	<i>bla</i> _{TEM, bla} _{OXA}	N/A*
Bajaj et al., 2016	India	River	Plasmids	Conjugation successful	<i>bla</i> _{CTX-M-15}	N/A
Singh et al., 2018	India	River	Insertion sequence, class 1 integron	Conjugation successful	<i>bla</i> _{CTX-M-15}	N/A
Akiba et al., 2016	India	Rivers	Plasmids	N/A	<i>bla</i> _{CTX-M-55, 15, bla} _{OXA-1, 9, bla} _{SHV-12}	<i>bla</i> _{NDM-7*}
Lamba et al., 2017	India	River	Class 1, 2 and 3 integrons	N/A	<i>bla</i> _{OXA, bla} _{CTX-M, bla} _{TEM}	<i>bla</i> _{NDM}
Fernandes et al., 2017	Brazil	Seawater	Plasmids	N/A	<i>bla</i> _{CTX-M-8, 1}	N/A*
Sellera et al., 2017	Brazil	Seawater	Plasmid	N/A	<i>bla</i> _{CTX-M-15, bla} _{OXA-17}	<i>bla</i> _{KPC-2*}
Francisco et al., 2019	Brazil	Rivers	Plasmids	N/A	<i>bla</i> _{CTX-M-15, bla} _{SHV-11, bla} _{OXA-1}	<i>bla</i> _{KPC-2*}
Nascimento et al., 2017	Brazil	Lakes	Plasmids, transposons	N/A	<i>bla</i> _{CTX-M-2, 15, 9}	<i>bla</i> _{KPC-2*}

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

251 Unknown = Data not provided in the article.

252 Not detected = Screened for but not detected.

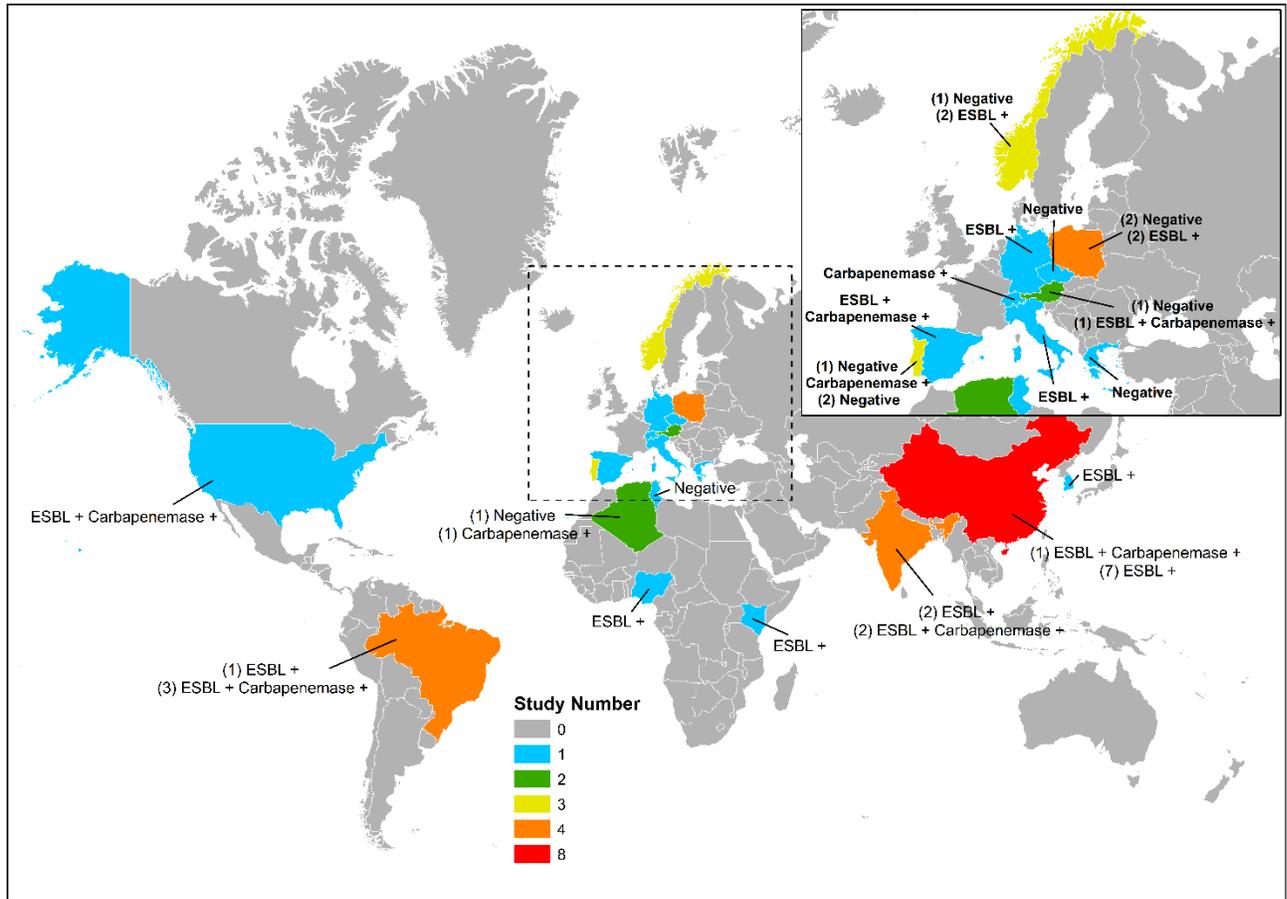
253 N/A = Not directly screened for using a targeted approach.

254 * = Sequencing approach applied.

255

256 3.3 Summary Analysis of Included Studies

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



263

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

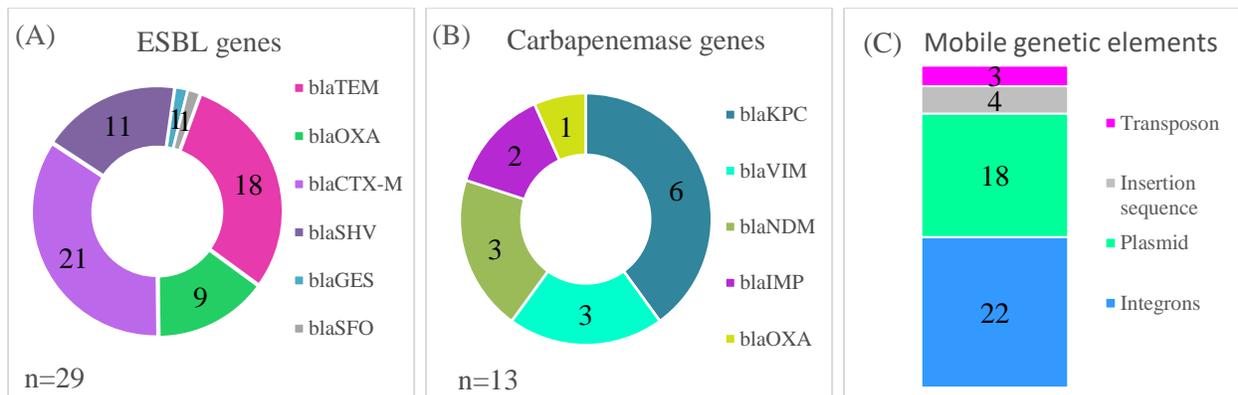
268 Overall, the methodology employed for sample collection, processing and genomic screening
 269 varied substantially among investigations (Supplementary Table 3). Sample volumes collected
 270 exhibited wide variations ranging from swab placement in water for 24 hours (Dolejská et al.,
 271 2009) to analysis of 7 L of water (Tação et al., 2012). Concentration methods also varied
 272 significantly with filtration using a 0.45µm (n=14) and 0.22µm (n=6) being the most prevalent.
 273 However, a further 6 studies using a filtration approach failed to report filter pore size. Filtration

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

278 The bacterial species most commonly detected was *E.coli* with positive identification in a total of
279 24 investigations. *E. coli* detection was followed by *Klebsiella* spp. (n=11), *Enterobacter* spp.
280 (n=6) and *Pseudomonas* spp. (n=6) (Supplementary Table 2). A range of natural water bodies
281 were investigated in the reviewed studies. Inland waters were classified into lentic (i.e.,
282 stationary or still water), lotic (i.e., free-flowing water) and sub-surface/groundwater. Lentic
283 systems included lakes (n=4) and ponds (n=1) whereas lotic systems encompassed rivers (n=28)
284 and streams (n=1). Additionally, seawater (n=6) or bay waters (n=1) were classified as
285 coastal/marine. Only two investigations evaluated sub-surface/groundwater for the presence of
286 ARGs. A total of two included articles evaluated more than one type of water body, (rivers, lakes
287 or groundwater).

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

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



302
 303 Figure 3: Number of studies detecting each type of (A) ESBL gene (B) carbapenemase gene and
 304 (C) mobile genetic element. The total number of studies detecting ESBLs and carbapenemases is
 305 also provided, with some studies reporting more the one type of ESBL (n=20) or carbapenemase
 306 (n=2) gene.

307 **4 Discussion**

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

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

322 **4.1 Synopsis of Identified Literature**

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

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

345

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

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

356 **4.2 Antibiotic resistance gene detection**

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

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

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

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

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

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

407 **4.3 Mobile genetic element detection**

408 In comparison to resistance gene detection, a larger proportion (37/41; 90.2%) of included
409 studies detected the presence of one or more MGE. This included class 1, 2 and 3 integrons
410 (n=22), plasmids (n=18), insertion sequences (n=4) and transposons (n=3) (Figure 3).

411 Additionally, 11 studies demonstrated successful conjugation transfer of ARGs between different
412 bacteria. The combination of these elements represent the potential for dissemination of
413 resistance elements among aquatic bacteria, which can be attributed as a contributing factor in
414 the detection of resistance in areas deemed to be 'free' from anthropogenic influence. The
415 reported high level of detection emphasizes the ability of most bacteria to exchange genetic
416 elements that are favourable to their survival across bacterial species, making this threat almost
417 impossible to contain. This feature serves to highlight the challenges of containing and/or
418 mitigating the environmental spread of ARGs.

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

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

442 transfer. However, in the review dataset, some of the environmental isolates used as conjugation
443 donors were *Klebsiella* and/or *Acinetobacter* (Ye et al., 2017, Osińska et al., 2016, Yin et al.,
444 2013). Hence, there was an attempt at demonstrating interspecies transfer in a limited number of
445 cases.

446 **4.4 Sources of resistance**

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

461 **4.5 Research highlights and recommendations**

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

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

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

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

491 In several investigations only certain strains of isolated bacteria were further characterised for
492 the presence of antibiotic resistance using phenotypic and genotypic methods. These were
493 generally restricted to highly virulent bacteria and those most often associated with clinical
494 infections in humans. As such, some articles employed selective application of screening
495 methods specific to detection of only certain types of bacteria, (e.g. Ye et al., 2017; Kieffer et al.,
496 2016; Stange et al., 2016). This practice represents a major knowledge gap with the current role
497 of ubiquitous, non-pathogenic bacteria in the transference of ARGs largely unaccounted for.
498 Emphasis on antibiotic resistance profiles rather than species detected should be employed by
499 scientists, considering MGEs that harbor resistance genes spread throughout different bacterial
500 species. Similarly, by confining environmental research to the most commonly identified MGEs
501 within clinical environments, the potential for transmission of ARGs via MGEs present in natural
502 aquatic environments is likely being underestimated.

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

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

515 **5 Conclusions**

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

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

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

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

539 **Author Contributions**

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

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