

Title	Groundwater resources as a global reservoir for antimicrobial- resistant bacteria
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Publication date	2019-12-03
Original Citation	Andrade, L., Kelly, M., Hynds, P., Weatherill, J., Majury, A. and O'Dwyer, J. (2019) 'Groundwater resources as a global reservoir for antimicrobial-resistant bacteria', Water Research, 170, 115360 (13pp). doi: 10.1016/j.watres.2019.115360
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.watres.2019.115360
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Download date	2025-08-26 22:11:00
Item downloaded from	https://hdl.handle.net/10468/9459



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Groundwater resources as a global reservoir for antimicrobial-resistant bacteria

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PII: S0043-1354(19)31134-0

DOI: https://doi.org/10.1016/j.watres.2019.115360

Reference: WR 115360

To appear in: Water Research

Received Date: 23 July 2019

Revised Date: 25 October 2019

Accepted Date: 30 November 2019

Please cite this article as: Andrade, L., Kelly, M., Hynds, P., Weatherill, J., Majury, A., O'Dwyer, J., Groundwater resources as a global reservoir for antimicrobial-resistant bacteria, *Water Research* (2020), doi: https://doi.org/10.1016/j.watres.2019.115360.

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1	Title: Groundwater resources as a global reservoir for antimicrobial-resistant bacteria
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#### 26 Abstract

27 Antimicrobial resistance represents one of our most significant global health threats, with increasing incidences noted in both clinical and environmental settings. As such, identifying and 28 29 understanding the sources and pathways for antimicrobial-resistant bacteria (ARB) is critical. The 30 current study presents the first systematic review and pooled analysis of ARB occurrence in global 31 groundwater supplies, which are used as primary drinking water sources by 2.2 billion people 32 worldwide and are recurrently linked to significant outbreaks of infection. Seventy peer-reviewed studies were identified and included; findings reveal that 80.2% ± 29.0 and 57.2% ± 36.8 of 33 34 aggregated groundwater isolates were resistant to  $\geq 1$  and  $\geq 3$  antimicrobials, respectively. Where bacteria were present, ARB were identified in 76.9% ± 33.7 of individual wells and springs. Our 35 36 results leave little doubt that groundwater represents a major global reservoir for ARB, however significant research is required to establish environmental determinants and mechanisms mediating 37 38 their occurrence. 39 Keywords: Groundwater; Antimicrobial Resistance; Drinking water; Environment and Health; Risk 40 Factors. 41

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#### 44 1. Introduction

45 Antimicrobial resistance is now widely recognised as a global public health threat, requiring 46 multi-sectorial preventative and mitigative interventions (Bradford and Harvey, 2017; WHO, 2018; 47 Larsson et al., 2018). Anthropogenic influences and behaviours, including the misuse/overuse of 48 human and veterinary antimicrobials has resulted in the addition of significant selective pressures to 49 the naturally occurring resistance within and between bacterial species (Van Boeckel et al., 2014; 50 Bell et al., 2014; O'Neill, 2016). As such, a global increase in acquired resistance traits has been 51 noted among bacterial isolates, including clinically significant species (e.g. Pseudomonas aeruginosa, 52 Staphylococcus aureus and Enterobacteriaceae spp.; WHO, 2017) with the incidence of multidrug 53 resistance (i.e., resistance to  $\geq$  3 antimicrobials) increasing both spatially and temporally (Munita and 54 Arias, 2016). Cases of treatment failure of both human and veterinary infectious diseases are increasingly documented (Wright, 2010; Opatowski et al., 2019), which results in higher healthcare 55 costs, more severe and prolonged infections, and rising rates of morbidity and mortality 56 57 (Laxminarayan et al., 2013). Recent estimates by the European Antimicrobial Resistance Surveillance 58 Network (EARS-Net) indicate that approximately 33,000 deaths are attributable to antimicrobial-59 resistant bacterial infections per annum within the EU/EEA, comparable with the combined human 60 health burden of influenza, tuberculosis and HIV/AIDS in the same region (Cassini et al., 2019). 61 Accordingly, antimicrobial resistant infections have received significant attention within both the 62 media and research community over the past two decades, with an extensive body of research 63 existing in clinical settings, coinciding with the development of several antimicrobial resistance 64 Action Plans at varying scales (European Commission, 2011; WHO, 2015). More recently, however, 65 the role of the natural aquatic environment as a source and transmission pathway for 66 antimicrobial-resistant bacteria (ARB) has been acknowledged as an area of growing concern 67 (Sanderson et al., 2018).

Human and veterinary antimicrobials are increasingly released to the environment at sub therapeutic concentrations via myriad sources of domestic, agricultural, industrial and

clinical/hospital origin. These microbiologically diverse, pharmaceutically dilute media may
readily catalyse the development of ARB within the natural environment, inevitably resulting in
their ingress to both surface and groundwater sources (Van Schaik, 2015). However, at present,
more research is still needed to better understand the occurrence and transport of ARB to and in
natural waterbodies. This is particularly true with regard to groundwater wells and aquifers,
which currently supply approximately 31.5% (2.2 billion people) of the global population with
domestic drinking water (Murphy et al., 2017).

Microbial contamination of groundwater and its adverse public health effects have been 77 78 well substantiated within the scientific literature. A recent review by Murphy et al. (2017) 79 presents clear epidemiological evidence of disease transmission due to groundwater contamination at a global scale, with an estimated 35.2 to 59.4 million cases of acute 80 81 gastrointestinal infection potentially attributable to groundwater consumption per year. Thus, the 82 potential implications of groundwater-borne ARB pose a significant threat to public health, allied 83 with an already high global burden of infection. A recent study in the Republic of Ireland found that 84 wastewater systems, livestock density and the presence of children in a household were significantly associated with the presence of antimicrobial resistant Escherichia coli (E. coli) in private 85 86 groundwater supplies (O'Dwyer et al., 2017). As such, the ubiquity of contaminant sources, in 87 concurrence with the presence of bacteria and sub-therapeutic antimicrobial concentrations, suggests that vulnerable groundwater systems may be a significant and frequently overlooked 88 89 reservoir for ARB (Wellington et al., 2013). Indeed, research carried out with 878 Canadian 90 individuals has shown that consumers of *E. coli* contaminated groundwater are 1.26 times more 91 likely to be colonised by antimicrobial resistant *E. coli* than non-consumers (Coleman et al., 2012). 92 Nonetheless, there is presently no consensus regarding the role of groundwater in the global 93 dissemination of ARB. The extent of this threat is further complicated by the nuances of 94 groundwater contamination mechanisms, which are typically determined and/or driven by 95 numerous environmental and source-specific risk factors (e.g. source design, location and

maintenance, local hydrogeological setting, and shifting climatic and landuse patterns), their
permutations and spatiotemporal distributions (Hynds et al., 2012; Wallender et al., 2014;
Atherholt et al., 2017; Andrade et al., 2018). Accordingly, the current study sought to further
understand the occurrence, distribution and potential drivers of ARB in groundwater sources (i.e.
wells and springs) via a global pooled analysis of peer-reviewed studies. Findings can be used to
support evidence-based risk management strategies to inform non-clinical considerations in existing
antimicrobial resistance Action Plans and guide future research strategies.

103 2. Methods

#### 104 2.1 Literature identification

105 Identification of scientific articles examining the occurrence of ARB in groundwater sources was conducted with an overarching systematic review protocol based upon pre-established 106 guidelines (Pullin et al., 2018) and adapted from previous studies (Efim et al., 2017; 2018; Nappier et 107 108 al., 2019). Defined search terms (Table 1) were used to search Scopus, Web of Science, Pub Med and ProQuest databases on October 8<sup>th</sup>, 2018. Employed "Outcome" search terms, and particularly the 109 antimicrobials and bacterial species selected, were based on the global priority list of ARB (WHO, 110 2017). Manual supplementary searches were additionally performed between October 15<sup>th</sup> and 111 November 12<sup>th</sup>, 2018. These comprised the examination of article bibliographies and studies citing 112 113 articles which were identified via database search and marked as "provisionally included" upon title 114 and abstract assessment (n=215).

115

#### 117 Table 1: Search terms employed in the current study

Element	Description	Search terms				
<b>P</b> opulation	All non-saline groundwater	Groundwater, Ground Water, Aq Dug Well, Well Water, Water We	quifer, S ell	Subsoil, Subsurfac	e, Boreh	ole, Bore Well, Bored Well,
<b>O</b> utcome(s)	Occurrence of antimicrobial- resistant (or susceptible) bacteria	Antimicrobial, Antibiotic, Antibacterial, Bacteriostatic, Bactericidal, Penicillin, Cephalosporin, Carbapenem, (Fluoro)quinolone, β lactam, Aminoglycoside, Tetracycline, Vancomycin, Clarithromycin, Ampicillin, Sulphonamide	AND	Resistance, Resistant, Susceptible, Susceptibility, Sensitive, Sensitivity	AND	Bacteria, Bacterial, Microbe, Microbial, Organism, Pathogen, E. coli, Escherichia coli, Pseudomonas, Enterococcus, Enterococc Campylobacter, Salmonella, Staphylococcus, Streptococcus, Shigella, Klebsiella, Enterobacter, Enterobacteriaceae

#### 119 2.2 Study selection

Studies uncovered during the identification phase (i.e. database and supplementary searches) 120 121 were independently screened by two authors using explicit eligibility criteria (Table 2) via title and 122 abstract assessment. Articles without an available full text were excluded, with eligibility disagreements resolved via provisional inclusion. All "provisionally included" and manually identified 123 124 studies had full-texts manually (i.e., no computer-assisted techniques employed) and independently 125 assessed (eligibility assessment) using the presented criteria (Table 2). Disagreements at this stage 126 were resolved via author panel consensus. 127 Excluded studies were those that: i) reviewed previously published studies, ii) incorporated 128 controlled elements in their study design (i.e., spiking or laboratory-based (micro-, meso-) 129 groundwater environments), iii) examined thermal/hot springs, iv) did not study water samples 130 derived from groundwater sources (e.g., surface and marine water), v) combined resistance data 131 from multiple environmental media, vi) did not examine  $\geq 10$  groundwater samples (including

- 132 articles where sampling number was not reported), vii) did not analyse antimicrobial
- 133 resistance/susceptibility in bacterial isolates from groundwater (including studies where bacteria

- 134 were not found), viii) did not report number of isolates tested, ix) did not report bacterial resistance
- 135 (or susceptibility) to each antimicrobial tested against (Figure 1).
- 136 Table 2: Eligibility (inclusion/exclusion) criteria employed

Inclusion Criteria	Exclusion Criteria
Study type: All peer-reviewed articles excluding reviews	Study type: Academic reviews; grey literature
Language: English	Language: non-English
<b>Population:</b> Naturally occurring groundwater environments; groundwater sources (i.e. wells and springs)	<b>Population:</b> Artificial groundwater media (i.e. labbased); pre-packaged water; Thermal/Hot springs; surface water bodies; maritime aquatic environments; wastewater treatment plants; soil; saline, brackish, or soil water.
<b>Exposure:</b> Pre-existing environmental exposures (i.e. prior to study)	<b>Exposure:</b> Any controlled exposure (i.e. spiking)
<b>Event/Outcome:</b> Antimicrobial-resistant (or susceptible) bacteria found in groundwater resources	<b>Event/Outcome:</b> Absence of bacterial contamination in groundwater; absence of antimicrobial resistance profiling of groundwater isolates
<b>Study design:</b> Analysis of ≥ 10 groundwater samples; results including percentages of bacterial isolates resistant/susceptible to each antimicrobial tested against.	<b>Study design:</b> Analysis of < 10 groundwater samples (includes number of groundwater samples not reported); results combined for different sampled environments; results combined for all antimicrobials tested against; number of isolates tested not reported.
Period: Any	Period: -

## 137

## 138 2.3 Study Inclusion

139	During full-text assessment (eligibility phase), studies included were those that assessed 10 or
140	more groundwater samples and explicitly stated the percentage of bacterial isolates resistant or
141	susceptible to each antimicrobial agent tested against. In all, 76 studies were excluded due to
142	reporting on < 10 groundwater samples, including studies where sample number was not reported
143	(n=12) (exclusion criteria vi). Forty-six studies reported composite occurrence rates of ARB in
144	combined study environments (e.g., merged findings from groundwater, surface water and/or
145	wastewater), and as such were excluded under exclusion criteria (v). Studies that did not provide an
146	adequate description of water sample origin; that is, referred solely to "tap water" (n=14) were
147	excluded under exclusion criteria (iv) (i.e. groundwater source not analysed) and where bacterial

- isolates were not identified in groundwater samples (n=4), articles were excluded under exclusion
- 149 criteria (vii), as antimicrobial resistance could not be determined, this criteria also comprised studies
- 150 that provided an assessment of antimicrobial resistance through genotypical methods only (i.e.
- 151 presence/absence of resistance genes via qPCR or digital droplet qPCR analyses). As such, 70 of
- 152 1,864 identified studies (identification phase) were deemed eligible for inclusion following the full
- 153 review process (Figure 1).



154 155 156

Figure 1: Systematic review protocol employed during the current study, including literature identification, screening, eligibility assessment, and final study inclusion.

#### 158 2.4. Critical appraisal of study validity

Included articles were independently evaluated by two authors using a critical appraisal tool
adapted from Bain et al. (2014). In it, a score ranging from 0 to 14 was attributed to each study
according to the number of affirmative responses to the fourteen pre-established criteria. Based on
it, articles were classified as presenting low (score ≤ 5), medium (score of 6 to 8) or high (score ≥ 9)
validity. Individual article assessments are presented in Supplementary Table 1. All disagreements
were resolved by a consensus between authors.

#### 165 2.5 Data extraction

Relevant data pertaining to each included study were extracted to MS Excel 2016. Extracted 166 167 variables were classified and exported under six primary categories; namely, bibliographic details, study region (e.g., country, location within country and settlement type), groundwater 168 characteristics (e.g., source type, well type, ownership, uses and treatment presence), sampling 169 170 regime (e.g., number of samples, re-sampling, length of sampling regime), analytic elements (e.g. 171 bacterial species tested, antimicrobial agents tested against, method and criteria were used to 172 assess susceptibility/resistance), and resistance profile (e.g., percentage of ARB and MRB amongst tested isolates and percentage of resistance to each antimicrobial agent tested against). It is 173 174 important to note that with regards to studies in which re-sampling was employed, data pertaining 175 to different sampling rounds were merged and extracted as single outcomes, with "re-sampling" and 176 "one-off" used just to classify two contrasting approaches employed across identified studies. As 177 each study only provides one outcome to the analysis, independence between observations from 178 each analytical unit (i.e. study) can be assumed. Moreover, due to the large periodicity associated 179 with repeat groundwater sampling rounds (e.g. where groundwater sources are sampled many 180 months apart), observations may be treated independently due to the fluid (acute) nature of 181 groundwater contamination (Bjerg & Christensen, 1992; Morvan et al., 2006; Pacheco Castro et al., 182 2018).

Antimicrobial resistance results were extracted according to the standards (i.e. EUCAST, CLSI, etc.) and interpretations employed in each published manuscript, inhibition zone and/or minimal inhibitory concentration results were not routinely specified and as such, could not be uniformly reassessed. Moreover, as intermediate resistance indicates that an antimicrobial is ineffective at recommended and commonly used therapeutic concentrations (Rodloff et al., 2008), potentially resulting in treatment failure, it was considered as resistance, as per other studies (Reinthaler et al., 2003; O'Dwyer et al., 2017).

190 Where key variables were unclear or not explicitly documented, article authors were 191 contacted for clarification and/or articles were analysed for identifiable characteristics, and thus 192 classified. Where classification was not possible, variables were categorised as "not reported". Sample sizes were categorised as small (< 30 samples), medium (30 – 99 samples) or large ( $\geq$  100), as 193 194 previously defined by Bain et al. (2014), with a maximum threshold established whereby data from 195 one study comprising > 5,000 samples were not extracted to prevent geographical and/or analytical 196 bias (Coleman et al., 2013); however, data reported from a smaller sample (n=657) in this study 197 were included in analyses. As such, the maximum sample number from a single included study was 939 (Akoachere et al., 2013). 198

Studies were further classified according to globally established characteristics relating to the study regions. Countries were classified as low, lower-middle, upper-middle, and high income based upon World Bank classification (World Bank, 2018). Specific study areas' primary (arid, cold, polar, temperate and tropical) and secondary (e.g., dry summer, dry winter, without dry season, monsoon and rainforest) climates were determined based on the Köppen-Geiger climate classification (Peel et al., 2007), and used to ascertain sampling season (i.e., summer, winter, spring, autumn) and period (i.e., wet or dry).

206 Moreover, where possible, occurrence rates of antimicrobial resistance (≥ 1 antimicrobial) and
 207 multidrug resistance (≥ 3 antimicrobials) were calculated for all isolates reported in a study (i.e.

208 number of resistant and multidrug resistant isolated, respectively, divided by the total number of 209 isolates recovered from all groundwater sources and samples examined within that study). This 210 approach was used as even when studies employed re-sampling in their methodology (i.e. more 211 than one sample taken from the same groundwater sources at different times), antimicrobial 212 resistance results were often integrated during reporting. With regards to occurrence rates of ARB 213 amongst groundwater sources (i.e. wells and/or springs) or the specific sources in which bacterial 214 isolates were found, these comprised the percentage of sources that harboured ARB at least once, 215 as reported in each manuscript (i.e. number of sources where ARB were found at least once divided 216 by total number of tested sources or by the number of sources in which bacteria were found at least 217 once, respectively).

#### 218 2.6 Multiple Antimicrobial Resistance Index

Multiple Antimicrobial Resistance (MAR) indices were calculated to standardise the rates of antimicrobial resistance reported across each study (Equation 1; Krumperman, 1983). MAR indices provide a single measure of antimicrobial resistance and control for the number of antimicrobial agents tested against, thus avoiding potential bias (i.e. elevated ARB occurrence rates are typically found when more antimicrobials are incorporated in a study design).

224 
$$MAR index = \frac{y}{n \times x}$$
 [Equation.

where y is the aggregate antimicrobial resistance score of all isolates tested (i.e., the sum of isolates resistant to each antimicrobial), n is the number of isolates tested, and x is the number of antimicrobial agents tested against (Krumperman, 1983).

A single MAR index was calculated for each study, irrespective of temporal methodologies employed, and included in the analyses. These were used to ascertain the overall findings regarding antimicrobial resistance in groundwater bacteria on a study by study basis, thus permitting crossstudy comparisons (see Figure 5). Secondly, for descriptive purposes only, discrete MAR indices were calculated for each study within isolates of the same species or genus and within different

11

1]

233	antimicrobial classes. These were not included in analyses, but merely used to a) identify the relative
234	rates of antimicrobial resistance associated with each bacterial genus, and b) determine the
235	differences between resistance rates within and between different antimicrobial classes (see Table
236	5)

237 2.7 Data analyses

238 Three dependent variables were calculated and used to quantify presence of ARB and/or 239 Multidrug Resistant Bacteria (MRB) in groundwater and represent the main findings from each 240 included study (i.e. studies were the analytic unit throughout analyses), namely: i) occurrence rates 241 of antimicrobial resistance (≥ 1 antimicrobial) amongst all groundwater isolates tested; ii) occurrence rates of multidrug resistance (≥ 3 antimicrobials) amongst all groundwater isolates 242 tested; and iii) calculated study-specific MAR indices. 243 Dependent variables were not normally distributed and could not be normalized using 244 standard (transformation) techniques, thus non-parametric Mann-Whitney U and Kruskal-Wallis 245 246 tests were employed to identify categorical associations between dependant and independent 247 variables (i.e. the extracted characteristics outlined in Table 3). Where significance was found (p < 1

0.05) and the independent variable described three or more levels of measurement, Dunn's (nonparametric) pairwise post-hoc tests were used. Mean MAR indices were further calculated for each

country and discretized into ranges (e.g. 0.000-0.100, 0.100-0.200, ...) using all included studies, with
all studies equally weighted.

# Table 3: External, source-specific and study-specific characteristics and their sub-categories used as independent variables in the non-parametric statistical tests.

Cat	egories	Sub-categories	Non-parametric test Sub
Envi	onmental		
1.	Economic classification <sup>a</sup>	Low; Lower middle; Upper middle; High income	Kruskal-Wallis
2.	Climate <sup>b</sup>	Tropical; Arid; Cold; Temperate	Kruskal-Wallis
3.	Sampling period <sup>c</sup>	Wet; Dry	Mann-Whitney U
4.	Settlement type <sup>d</sup>	Urban; Rural	Mann-Whitney U
5.	Waste source adjacent to study site <sup>e</sup>	Human waste; Animal waste	Mann-Whitney U
Sour	ce-specific		
6.	Source type	Well; Spring	Mann-Whitney U
7.	Well type	Hand-dug; Bored	Mann-Whitney U
Stud	y-specific		
8.	Sampling regime	One-off sampling; Re-sampled sources	Mann-Whitney U
9.	Sample size	Under 30; 30 to 99; 100 and over	Kruskal-Wallis
10.	Length of sampling period	Under 6 months; 6 to 12 months; more than 12 months	Kruskal-Wallis

<sup>a</sup> economic classification according to the World Bank (2018); <sup>b</sup> climate classified according to Peel et al. (2007); <sup>c</sup> wet and dry as defined in Peel et al. (2007); <sup>d</sup> urban settlements include urban, sub-urban or peri-urban regions; <sup>e</sup> human waste encompasses septic tanks and wastewater treatment plants, and animal waste encompasses animal grazing fields and agricultural fields where manure is spread.

255

#### 256 3. Results

#### 257 **3.1. External, groundwater-specific and study-specific characteristics**

258 A total of 70 relevant studies were included for data extraction and pooled analys
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summary of all included studies is presented in Table 4, with detailed study characteristics and full

- 260 reference list provided in Supplementary Materials 2. Relevant studies were conducted in
- 261 geographically, climatically and economically diverse regions and spanned a 42-year period (1976-
- 262 2018), with 81.4% (n=57) of identified articles published since 2010. All inhabited continents as well
- as four of the five climatic zones were represented. Overall, 51.4% (n=36) of identified studies were
- undertaken in lower-middle income countries, followed by countries classified by high (21.4%; n=15)
- and upper-middle (21.4%; n=15) incomes. There were two primary sampling regimes identified, with
- repeat (temporal/seasonal) sampling employed in 32.9% (n=23) of studies, while 52.9% (n=37)
- 267 performed one-off ('snapshot') sampling. Where sampling seasons could be ascertained (n=58), it

268	was exclusively undertaken during wet and dry seasons in 41.4% (n=24) and 32.7% (n=19) of studies,
269	respectively; however, just 10.3% of studies (n=6) explicitly reported this information.
270	Overall, 52.9% (n=37) of studies were conducted in categorically rural areas, while 27.1% (n=
271	19) were undertaken in urban regions and the remaining 5.7% ( $n=4$ ) in "mixed settlements" (i.e.
272	both urban and rural areas surveyed). Approximately two thirds (64.3%; n=45) of studies reported
273	the presence of waste sources adjacent to study sites; 79.2% (n=38) of human and 48.9% (n=22) of
274	animal origin (e.g. septic tanks, wastewater treatment plants, animal grazing fields and manure
275	application). Wells and springs were examined in 91.4% (n=64) and 12.9% (n=9) of included studies,
276	respectively, with 4.3% (n=3) reporting concurrent analyses of both source types. Well construction
277	was explicitly defined in 23 studies, of which 60.9% (n=14) were hand-dug and 39.1% (n=9) were
278	bored. A significant paucity of data was encountered with respect to reporting of numerous source-
279	specific elements (e.g. supply depth, age and operational condition/performance), with just 18.6%
280	(n=13) of included studies describing one or more of these. Similarly, just 17.1% (n=12) of studies
281	reported local hydrogeological characteristics (e.g. aquifer type, subsoil type, depth and
282	permeability, bedrock geology and groundwater vulnerability). Sampled groundwater sources were
283	used for human consumption in 57 of the 60 studies where groundwater usage was reported (95%),
284	with an absence of water treatment before consumption noted in 76.9% of the 13 studies that
285	reported this information (n=10).

A total of 8,741 groundwater samples were collected across all included studies, ranging from 10 to 939 per study (mean ± SD = 125 ± 189), with 7,157 identified groundwater isolates examined for resistance against 89 distinct antimicrobial agents. *E. coli* was the most frequently analysed bacteria (54.3% of studies; n=38), followed by *Pseudomonas* spp. (21.4%; n=15). Combined, these corresponded to almost half of the isolates tested across all studies, with 2,737 and 824 tested isolates, respectively. The Penicillin antimicrobial class was most frequently incorporated in study

## designs (94.3%; n=66), followed by Aminoglycosides (87.1%; n=61), Fluoroquinolones (81.4%; n=57),

## 293 Cephalosporins (72.9%; n=51), and Tetracyclines (71.4%; n=50).

# 294Table 4: Summary of principal characteristics extracted, with study (n=70) and groundwater sample (n=8,741) number295associated with each corresponding sub-category

Characteristics	Studies n (%)	Samples n (%)	Characteristics	Studies n (%)	Samples n (%)
Publication year			Source type		
Pre-1990	6 (8.6)	446 (5.1)	Wells	61 (87.1)	7,981 (91.3)
1990-1999	0 (0.0)	0 (0.0)	Dug	13 [21.3]	2,123 [26.6]
2000-2009	7 (10.0)	696 (8.0)	Bored	9 [14.8]	1,236 [15.5]
2010-2018	57 (81.4)	7,599 (86.9)	Mixed	9 [14.8]	1,526 [19.1]
			Not reported	30 [49.1]	3,096 [38.8]
Continent			Springs	6 (8.6)	423 (4.8)
Africa	32 (45.7)	3,941 (45.1)	Mixed	3 (4.3)	337 (3.9)
Asia	22 (31.4)	2,645 (30.3)			
Central and South America	6 (8.6)	436 (5.0)	Water supply		
North America	4 (5.7)	1,032 (11.8)	Private	17 (24.3)	2,893 (33.1)
Europe	5 (7.1)	658 (7.5)	Public	1 (1.4)	108 (1.2)
Oceania	1 (1.4)	29 (0.3)	Mixed	2 (2.9)	275 (3.1)
			Not reported	50 (71.4)	5,465 (62.5)
Economic classification <sup>a</sup>					
Low income	3 (4.3)	149 (1.7)	Primary settlement type <sup>e</sup>		
Lower-middle income	36 (51.4)	4,466 (51.1)	Rural	37 (52.9)	4,328 (49.5)
Upper-middle income	15 (21.4)	1,194 (13.7)	Urban	19 (27.1)	2,858 (32.7)
High income	16 (22.9)	2,932 (33.5)	Mixed	4 (5.7)	975 (11.2)
			Not reported	10 (14.3)	580 (6.6)
Climate <sup>b</sup>					
Tropical	25 (35.7)	3,506 (40.1)	Waste adjacent to GW sites <sup>f</sup>		
Arid	15 (21.4)	850 (9.7)	Human waste	23 (32.9)	3,301 (37.8)
Temperate	24 (34.3)	3,193 (36.5)	Animal waste	7 (10.0)	1,326 (15.2)
Cold	6 (8.6)	1,192 (13.6)	Both animal and human waste	15 (21.4)	2,489 (28.5)
Polar	0 (0.0)	0 (0.0)	Not reported	25 (35.7)	1,625 (18.6)
Sampling period <sup>c</sup>			Treatment before use		
Wet	24 (34.3)	2,677 (30.6)	Yes	3 (4.3)	223 (2.6)
Dry	19 (27.1)	1,185 (13.6)	No	11 (15.7)	1,027 (11.7)
Both	15 (21.4)	3,857 (44.1)	Mixed <sup>g</sup>	4 (5.7)	1,680 (19.2)
Not identifiable	12 (17.1)	1,022 (11.7)	Not reported	52 (74.3)	5,811 (66.5)
Somulo sizo <sup>d</sup>			Human concumption		
Sample Size	12 (19 6)			E7 (01 A)	7 026 (90 E)
Madium (20, 00)	15 (10.0)	-	Tes No.	57 (61.4) 2 (4 2)	7,050 (80.5)
1 - 100	34 (40.0) 22 (22 9)	-	Not reported	3 (4.3) 10 (14 2)	299 (3.4)
Large (2 100)	25 (52.8)	-	Not reported	10 (14.5)	1,400 (10.1)
Length of sampling period			Antimicrobials tested against		
0-6 months	23 (32.9)	1.511 (17.3)	1-5	2 (2.9)	145 (1.7)
7 - 12 months	15 (21.4)	3,009 (34.4)	6-10	35 (50.0)	4,660 (53.3)
> 12 months	6 (8.6)	1,907 (21.8)	11–15	20 (28.6)	2,589 (29.6)
Not reported	26 (37.1)	2,314 (26.5)	16 - 20	13 (18.6)	1,347 (15.4)
·	· · /	, , ,		ζ, γ	, , ,
Sampling regime			Type of bacteria		
One-off sampling	37 (52.9)	2,920 (33.4)	Gram-positive	8 (11.4)	461 (5.3)
Re-sampled sources	23 (32.9)	4,405 (50.4)	Gram-negative	49 (70.0)	6,412 (73.4)
Not reported	10 (14.3)	1,416 (16.2)	Both	12 (17.1)	1,793 (20.5)
			Not specified	1 (1.4)	75 (0.9)
Validity score					
Low (≤ 5)	5 (7.1)	245 (2.8)			
Medium (6 – 8)	19 (27.1)	2,377 (27.2)			
High (≥ 9)	46 (65.7)	6,119 (70.0)			

<sup>a</sup> Countries economically classified according to the Work Bank (2018); <sup>b</sup> Climate classification according to Peel et al. (2007); <sup>c</sup> as defined in Peel et al. (2007); <sup>d</sup> Sample size classified following approach in Bain et al. (2014); <sup>e</sup> Urban settlements include urban, sub-urban and/or peri-urban regions; <sup>f</sup> Human waste encompasses septic tanks and wastewater treatment plants, and animal waste encompasses animal grazing fields, agricultural fields where manure is spread, etc.; <sup>g</sup> "Mixed" treatment before use encompasses studies in which treated and untreated supplies were combined during analysis

#### 296 **3.2.** Critical appraisal of study validity

Study validity scores obtained varied greatly among included studies, spanning from 5 to 13,
however most studies were considered of medium to high quality (i.e. ≥6; 92.8%; n=65). The
frequency of studies in each quality category are summarized in Table 3 and complete analysis can
be found in Supplementary Materials.

## 301 **3.3.** Antimicrobial-resistant bacteria in global groundwater

302 Where reported (n=20 studies; 28.6%), ARB were identified in 31.4% ± 32.6 of studied

303 groundwater sources and in 76.9% ± 33.7 of sources where bacteria were present. Additionally,

304 80.2% ± 29.0 and 57.2% ± 36.8 of pooled groundwater isolates (3,456 and 1,403 isolates,

respectively) were resistant to  $\geq$  1 (ARB; n=55; 78.6%) and  $\geq$  3 antimicrobials (MRB; n=32; 45.7%),

306 respectively.

As shown (Figure 2), consistently high occurrence rates of antimicrobial resistance were found 307 308 among *Pseudomonas* (99.9% ± 0.2), *Klebsiella* (99.8% ± 0.6) and *Enterobacter* spp. (99.2% ± 2.2), as well as others which were reported in fewer studies (i.e. <7), with Pseudomonas spp. also exhibiting 309 310 high rates of multidrug resistance (96.6% ± 5.8). Moreover, *Pseudomonas* spp. (0.544 ± 0.237) were 311 associated with some of the highest calculated MAR indices, across the 15 studies that examined the genus (Figure 2). MAR indices calculated within antimicrobial classes (Table 5) indicate that 1<sup>st</sup> 312 generation Cephalosporins (0.594  $\pm$  0.325), Glycopeptides (0.549  $\pm$  0.378), 2<sup>nd</sup> generation 313 314 Cephalosporins ( $0.529 \pm 0.316$ ) and Sulphonamides ( $0.517 \pm 0.315$ ) were associated with the highest 315 mean MAR Indices. Conversely, lowest mean values were associated with Carbapenems (0.040 ± 0.078) and  $4^{\text{th}}$  generation Cephalosporins (0.150 ± 0.236). 316 317 A calculated mean MAR index of  $0.352 \pm 0.207$  represents the level of ARB presence in global

318 groundwater during the total review period (i.e. between 1976 and 2018; Figure 3), and of 0.359  $\pm$ 

319 0.207 from 2010 to 2018 (not shown). Just one study exhibited a calculated MAR index of 0.000

320 (zero) (i.e. Traoré et al., 2015). MAR index values were highest in Kenya ( $x \pm SD = 0.549 \pm 0.208$ ),

- 321 Nepal (0.545), China (0.537 ± 0.041), Morocco (0.528), Saudi Arabia (0.516) and South Africa (0.513 ±
- 322 0.122). At a continental level, MAR indices were highest in Africa (0.423  $\pm$  0.202) and Asia (0.370  $\pm$
- 323 0.201), followed by Central and South America (0.281 ± 0.098), Oceania (0.173 ± 0.000), Europe
- 324  $(0.138 \pm 0.083)$  and North America  $(0.110 \pm 0.045)$ .

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327 Figure 2: Forest plot of i) antimicrobial resistance, ii) multidrug resistance and iii) calculated multiple antimicrobial resistance (MAR) Indices in groundwater isolates from each genus

328 examined

#### 329 Table 5: Summary statistics of MAR indices calculated within antimicrobial classes and bacterial species

Bacteria	Aminoglycosides <sup>1</sup>	Carbapenems <sup>2</sup>	-	Cephal	losporins		Fluoroquinolones <sup>7</sup>	Glycopeptides <sup>8</sup>	Lincosamides <sup>9</sup>	Macrolides <sup>10</sup>	Penicillins <sup>11</sup>	Sulfonamides <sup>12</sup>	Tetracyclines 13	TOTAL
			1st gen <sup>3</sup>	2nd gen <sup>4</sup>	3rd gen ⁵	4th gen <sup>6</sup>								
	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)	mean ± SD (n)
Gram-positive														
Enterococcus spp.	0.344 ± 0.268 (7)	-	0.333 ± 0.333 (2)	0.923 ± 0.000 (1)	0.615 ± 0.440 (3)	-	0.188 ± 0.190 (6)	0.433 ± 0.350 (7)	0.692 ± 0.000 (1)	0.466 ± 0.245 (8)	0.518 ± 0.302 (10)	0.600 ± 0.400 (2)	0.417 ± 0.262 (9)	0.396 ± 0.198 (11)
Staphylococcus spp.	0.250 ± 0.314 (10)	-	0.451 ± 0.399 (3)	0.380 ± 0.302 (3)	0.283 ± 0.349 (7)	-	0.270 ± 0.390 (7)	0.285 ± 0.184 (3)	0.424 ± 0.348 (5)	0.208 ± 0.273 (4)	0.350 ± 0.255 (10)	0.629 ± 0.000 (1)	0.293 ± 0.194 (7)	0.334 ± 0.235 (11)
Bacillus spp.	0.243 ± 0.388 (5)	-	0.286 ± 0.286 (2)	0.583 ± 0.083 (2)	0.322 ± 0.349 (4)	-	0.333 ± 0.471 (3)	-	0.889 ± 0.079 (3)	0.545 ± 0.455 (2)	0.554 ± 0.246 (5)	0.000 ± 0.000 (1)	0.071 ± 0.071 (2)	0.443 ± 0.163 (5)
Streptococcus spp.	0.544 ± 0.390 (3)	-	-	0.683 ± 0.111 (2)	0.646 ± 0.256 (3)	-	0.545 ± 0.386 (3)	0.606 ± 0.000 (1)	0.714 ± 0.000 (1)	0.412 ± 0.412 (2)	0.561 ± 0.174 (4) <sup>c#</sup>	0.861 ± 0.000 (1)	0.585 ± 0.068 (3)	0.581 ± 0.146 (4)
Micrococcus spp.	0.406 ± 0.406 (2)	-	-	0.469 ± 0.000 (1)	0.328 ± 0.328 (2)	-	0.422 ± 0.422 (2)	-	0.813 ± 0.000 (1)	0.000 ± 0.000 (1)	0.286 ± 0.286 (2)	-	0.000 ± 0.000 (1)	0.332 ± 0.332 (2)
Gram-negative														
Escherichia coli	0.270 ± 0.302 (35)	0.051 ± 0.107 (7) <sup>a</sup>	0.599 ± 0.284 (5)	0.421 ± 0.333 (15)	$0.375 \pm 0.391$ (19) <sup>a</sup>	0.036 ± 0.055 (4)	0.212 ± 0.226 (34)	0.486 ± 0.367 (3)	-	0.402 ± 0.288 (4)	0.486 ± 0.335 (36)	0.394 ± 0.281 (8)	0.428 ± 0.277 (28)	0.339 ± 0.226 (38)
Pseudomonas spp.	0.322 ± 0.371 (15)	0.151 ± 0.157 (4) <sup>a</sup> *	0.950 ± 0.000 (1)	0.666 ± 0.356 (6)	0.478 ± 0.380 (10)	0.185 ± 0.185 (2)	0.332 ± 0.371 (13)	0.194 ± 0.000 (1)	-	0.750 ± 0.204 (3)	0.614 ± 0.298 (15)	0.780 ± 0.137 (4)	0.643 ± 0.342 (10)	0.544 ± 0.237 (15)
Klebsiella spp.	0.307 ± 0.321 (12)	0.000 ± 0.000 (1) <sup>a</sup> *	0.586 ± 0.268 (3)	0.428 ± 0.384 (5)	0.261 ± 0.360 (7) <sup>a</sup> *	0.000 ± 0.000 (1)	0.260 ± 0.358 (11)	0.080 ± 0.080 (2)	-	0.750 ± 0.250 (2)	0.666 ± 0.229 (12)	0.956 ± 0.000 (1)	0.442 ± 0.285 (8)	0.423 ± 0.231 (12)
Enterobacter spp.	0.329 ± 0.406 (10)	$0.000 \pm 0.000$ (1) <sup>a</sup>	0.798 ± 0.236 (3)	0.484 ± 0.358 (5)	0.276 ± 0.246 (6) <sup>a</sup>	0.000 ± 0.000 (1)	0.272 ± 0.367 (9)	-	-	0.542 ± 0.208 (2)	0.591 ± 0.212 (10)	0.857 ± 0.000 (1)	0.404 ± 0.342 (6)	0.424 ± 0.229 (10)
Salmonella spp.	0.300 ± 0.353 (9)	$0.000 \pm 0.000$ (1) <sup>a</sup>	0.902 ± 0.000 (1)	1.000 ± 0.000 (1)	0.338 ± 0.413 (7) <sup>a</sup>	-	$0.134 \pm 0.246$ (9) <sup>b</sup>	1.000 ± 0.000 (2)	-	0.802 ± 0.217 (3)	0.577 ± 0.410 (10)	0.427 ± 0.357 (3)	0.308 ± 0.372 (8)	0.391 ± 0.271 (10)
Aeromonas spp.	0.011 ± 0.018 (7)	0.000 ± 0.000 (2)	0.586 ± 0.309 (3)	0.813 ± 0.000 (1)	0.159 ± 0.268 (5)	0.000 ± 0.000 (1)	0.000 ± 0.000 (6)	1.000 ± 0.000 (1)	1.000 ± 0.000 (1)	0.778 ± 0.314 (3)	0.730 ± 0.331 (7)	1.000 ± 0.000 (1)	0.100 ± 0.200 (5)	0.304 ± 0.135 (7)
Proteus spp.	0.384 ± 0.432 (7)	- <sup>a</sup>	0.555 ± 0.016 (2)	0.524 ± 0.278 (4)	0.494 ± 0.271 (4) <sup>a</sup>	-	0.366 ± 0.317 (6)	-	-	0.500 ± 0.000 (1)	0.524 ± 0.274 (7)	1.000 ± 0.000 (1)	0.683 ± 0.331 (4)	0.471 ± 0.182 (7)
Acinetobacter spp.	0.117 ± 0.194 (5)	$0.000 \pm 0.000$ (1) <sup>a</sup> *	0.467 ± 0.033 (2)	0.708 ± 0.257 (3)	0.594 ± 0.427 (4)	0.000 ± 0.000 (1)	0.263 ± 0.327 (4)	-	0.750 ± 0.000 (1)	1.000 ± 0.000 (1)	0.563 ± 0.343 (5)	0.833 ± 0.000 (1)	0.000 ± 0.000 (2)	0.388 ± 0.151 (5)
Citrobacter spp.	0.000 ± 0.000 (5)	0.000 ± 0.000 (1)	0.180 ± 0.055 (2)	0.563 ± 0.438 (2)	0.250 ± 0.433 (4)	0.000 ± 0.000 (1)	0.000 ± 0.000 (4)	-	-	0.625 ± 0.000 (1)	0.505 ± 0.346 (5)	0.588 ± 0.000 (1)	0.123 ± 0.102 (3)	0.269 ± 0.155 (5)
Serratia spp.	0.200 ± 0.400 (5)	$0.333 \pm 0.000$ (1) <sup>a</sup>	0.536 ± 0.036 (2)	0.667 ± 0.333 (2)	0.111 ± 0.157 (3) <sup>a</sup>	0.000 ± 0.000 (1)	0.167 ± 0.289 (4)	-	-	0.250 ± 0.250 (2)	0.652 ± 0.129 (5)	0.429 ± 0.000 (1)	0.000 ± 0.000 (3)	0.351 ± 0.138 (5)
Vibrio spp.	0.359 ± 0.178 (4)	-	-	0.415 ± 0.000 (1)	0.360 ± 0.454 (3)	-	0.000 ± 0.000 (2)	1.000 ± 0.000 (1)	-	0.590 ± 0.410 (2)	0.820 ± 0.312 (4)	0.875 ± 0.125 (2)	0.491 ± 0.425 (4)	0.520 ± 0.237 (4)
Alcaligenes spp.	0.389 ± 0.437 (3)	-	0.750 ± 0.000 (1)	0.500 ± 0.000 (1)	0.250 ± 0.250 (2)	-	0.375 ± 0.375 (2)	-	-	0.000 ± 0.000 (1)	0.757 ± 0.236 (3)	0.417 ± 0.000 (1)	0.250 ± 0.250 (2)	0.461 ± 0.205 (3)
Flavobacterium spp.	0.250 ± 0.204 (3)	-	0.500 ± 0.000 (1)	0.500 ± 0.000 (1)	0.500 ± 0.500 (2)	-	0.250 ± 0.250 (2)	-	0.750 ± 0.000 (1)	0.000 ± 0.000 (1)	0.611 ± 0.283 (3)	0.333 ± 0.000 (1)	0.000 ± 0.000 (2)	0.397 ± 0.282 (3)
Shigella spp.	0.567 ± 0.419 (3)	-	-	0.833 ± 0.000 (1)	0.458 ± 0.458 (2)	-	0.375 ± 0.375 (2)	-	-	1.000 ± 0.000 (1)	0.617 ± 0.238 (3)	1.000 ± 0.000 (1)	0.500 ± 0.500 (2)	0.607 ± 0.273 (3)
Chromobacterium spp.	0.000 ± 0.000 (2)	1.000 ± 0.000 (1)	0.667 ± 0.000 (1)	-	0.500 ± 0.000 (1)	0.000 ± 0.000 (1)	0.000 ± 0.000 (1)	-	-	-	0.750 ± 0.250 (2)	0.000 ± 0.000 (1)	0.000 ± 0.000 (1)	0.339 ± 0.106 (2)
TOTAL <sup>§</sup>	0.253 ± 0.274 (61)	0.040 ± 0.078 (16)	0.594 ± 0.325 (14)	0.529 ± 0.316 (26)	0.326 ± 0.291 (38)	0.150 ± 0.236 (8)	0.178 ± 0.199 (57)	0.549 ± 0.342 (15)	0.493 ± 0.325 (7)	0.493 ± 0.325 (21)	0.492 ± 0.313 (66)	0.517 ± 0.315 (18)	0.393 ± 0.285 (50)	0.352 ± 0.207 (70)

MAR Index = Multiple antimicrobial resistance index = aggregate antimicrobial resistance score of all isolates tested / (number of isolates tested x number of antimicrobials tested against) (Krumperman, 1983). = mean MAR index ≤0.200; = mean MAR index from 0.201 to 0.400 ; = mean MAR index from 0.401 to 0.600; = mean MAR index from 0.601 to 0.800, and = mean MAR index from 0.801 to 1.000

SD = Standard deviation

n = number of studies

<sup>1</sup>Amikacin, Gentamicin, Kanamycin, Neomycin, Netilmicin, Paromomycin, Streptomycin and/or Tobramycin; 61 studies

<sup>2</sup> Biapenem. Ertapenem. Imipenem and/or Meropenem: 16 studies

<sup>3</sup>Cefadroxil, Cefalexin, Cefazolin, Cefradine and/or Cephaloridine; 14 studies

<sup>4</sup> Cefaclor, Cephalothin, Cefamandole, Cefoxitin and/or Cefuroxime; 26 studies

<sup>5</sup> Cefixime, Cefixime, Cefoperazone, Cefoperazone-sulbactam, Cefotaxime, Cefotaxime-clavulanic Acid, Cefpodoxime, Cefsulodin, Ceftazidime, Ceftiofur and/or Ceftriaxone; 28 studies

<sup>6</sup>Cefepime; 8 studies

<sup>7</sup> Ciprofloxacin, Enrofloxacin, Levofloxacin, Moxifloxacin, Nalidixic acid, Nitrofurantoin, Norfloxacin, Ofloxacin, Pefloxacin and/or Sparfloxacin; 57 studies

<sup>8</sup> Teicoplanin and/or Vancomycin; 15 studies

<sup>9</sup> Clindamvcin and/or Lincomycin; 7 studies

<sup>10</sup> Erythromycin and/or Roxithromycin; 21 studies

<sup>11</sup>Amoxicillin, Amoxicillin-clavulanic acid, Ampicillin, Ampicillin-sulbactam, Ampiclox, Carbenicillin, Cloxacillin, Mecillinam, Methicillin, Oxacillin, Piperacillin-tazobactam, Ticarcillin and/or Ticarcillin-clavulanic acid; 66 studies <sup>12</sup> Sulfamethoxazole, Sulfanilamide, Sulphafurazole and/or Sulfabenzamide-sulfacetamide-sulfathiazole (i.e. "Triple Sulfa"): 18 studies

<sup>13</sup> Chlortetracycline, Doxycycline, Oxytetracycline and/or Tetracycline; 50 studies

<sup>a</sup> Priority 1 (i.e. critical) in the global priority list of antimicrobial-resistant bacteria<sup>7</sup>

<sup>b</sup> Priority 2 (i.e. high) in the global priority list of antimicrobial-resistant bacteria<sup>7</sup>

<sup>c</sup> Priority 3 (i.e. medium) in the global priority list of antimicrobial-resistant bacteria<sup>7</sup>

\* The only Pseudomonas, Klebsiella and Acinetobacter species included in the global critical priority list of antimicrobial-resistant bacteria are Pseudomonas aeruginosa, Klebsiella pneumonia and Acinetobacter baumannii, respectively<sup>7</sup> <sup>#</sup> The only Streptococcus species included in the global medium priority list of antimicrobial-resistant bacteria is Streptococcus pneumoniae<sup>7</sup>

<sup>5</sup> The total calculated MAR Indices were obtained per study, amalgamating results for all bacteria tested (included ones of unspecified genus) and using Equation 1. All studies were weighted equally

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ις Det. Ing Equation 1. All stu.





334 Figure 3: Mean Multiple Antimicrobial Resistance (MAR) Indices in global groundwater from 1976-2018 (n = 70) in each included country

#### 335 **3.4.** Potential drivers of antimicrobial-resistant bacteria in groundwater

As shown (Figure 4), occurrence rates of antimicrobial resistance in groundwater bacteria were significantly higher in urban settlements (p=0.020), with rates of MRB in groundwater from high income countries significantly lower than those from upper-middle income countries (p=0.035). Studies that employed one-off sampling regimes yielded significantly higher percentages of both antimicrobial and multidrug resistance in groundwater bacteria when compared to regimes where re-sampling was employed (p=0.001 and p=0.002, respectively).

342 MAR indices were significantly lower in groundwater from high-income countries when 343 compared to upper-middle (p=0.018) and lower-middle (p=0.002) income countries (Figure 5), and 344 significantly higher in arid versus temperate regions (p=0.021) and in samples collected during dry versus wet seasons (p=0.014). Significantly higher MAR indices were also observed in urban 345 346 settlements (p=0.001) and when waste sources adjacent to groundwater supplies were 347 predominantly of human (e.g. septic tanks and wastewater treatment plants) as opposed to animal 348 (e.g. animal grazing and manure spreading) origin (p=0.005). No significant differences were found 349 when comparing MAR index distributions with sources type (i.e. wells versus springs) or source 350 construction (i.e. hand-dug versus bored wells). One-off sampling regimes (n=37) yielded 351 significantly higher MAR indices in groundwater when compared to regimes where re-sampling was 352 employed (n=23) (p=0.003).

		Occurrence rates		
0.0 - 10.0%	6 10.0 - 20.0% 20.0 - <b>30.0% 30.0 - 4</b>	0.0% 40.0 - 50.0% 50.0 - 60.0%	60.0 - 70.0% 70.0 - 80.0% 80.0 - 90.0%	6 90.0 - 100.0%
Sub-categories	Occurrence rates of antimicrobial-resistance in groundwater bacteria O. Median O.	Studies Median Test p (IQR) statistic	Occurrence rates of multidrug-resistance in groundwater bacteria O. Median O.	Studies Median Test p (IQR) statistic
Income level Low Lower-middle Upper-middle High		3       100.0 (-)       4.417 #       0.220         25       99.0 (27.0)         14       100.0 (3.4)         13       52.3 (58.3)		2 50.0 (-) 8.529 <sup>#</sup> 0.036 11 56.3 (68.3) 9 a* 98.4 (29.7) 10 b* 19.2 (35.8)
Climate Tropical Arid Cold Temperate		18       97.8 (53.2)       1.809 <sup>#</sup> 0.613         10       100.0 (7.3)       6       100.0 (51.9)         21       99.0 (50.6)       99.0 (50.6)		7       37.3 (72.2)       2.382 #       0.497         9       74.0 (56.0)         4       57.7 (85.9)         12       35.0 (73.0)
Sampling period Dry Wet		12 100.0 (2.6) 102.0 <sup>§</sup> 0.385 21 100.0 (42.7)		10 86.2 (49.9) 45.0 <sup>§</sup> 0.512 11 80.0 (84.6)
Settlement type Urban Rural		15 100.0 (2.3) 313.0 <sup>§</sup> 0.020 30 94.9 (52.5)		5 100.0 (77.3) 62.5 <sup>§</sup> 0.297 19 46.2 (79.7)
Adjacent waste origin Human Animal		16 100.0 (12.1) 37.5 <sup>§</sup> 0.449 6 81.2 (51.7)		9 33.8 (75.0) 4.0 <sup>§</sup> 0.100 3 16.2 (-)
Source type Well Spring		47 100.0 (47.7) 133.5 <sup>§</sup> 0.629 5 100.0 (30.4)		26 41.7 (84.0) 72.0 <sup>§</sup> 0.735 5 88.0 (64.1)
Dug well Bored well		10 100.0 (40.7) 32.0 <sup>§</sup> 0.813 7 100.0 (56.8)		5 66.7 (76.0) 9.0 <sup>§</sup> 0.905 4 86.2 (68.9)
Sampling regime One-off samples Re-sampled sources <30 samples 30-99 samples		27 100.0 (1.5) 413.0 <sup>§</sup> 0.001 20 72.9 (62.1) 8 100.0 (37.5) 1.502 <sup>#</sup> 0.472 27 98.5 (50.0)		16         99.2 (41.1)         183.0 <sup>§</sup> 0.002           14         27.7 (50.2)         5         100.0 (57.0)         1.783 <sup>#</sup> 0.410           16         57.0 (81.9)         9         100.0 (57.0)         1.783 <sup>#</sup> 0.410
≥100 samples <6 months sampled 6-12 months sampled >12 months sampled		20         100.0 (45.2)           20         100.0 (46.3)         2.896 <sup>#</sup> 0.235           13         84.6 (67.2)         5         52.3 (59.5)		11         33.8 (78.9)           11         80.0 (88.0)         2.039 <sup>#</sup> 7         33.8 (77.8)           5         15.4 (44.7)

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Figure 4: Summary of descriptive and non-parametric test results for occurrence rates of i) antimicrobial (n=55) and ii) multidrug resistance (n=32) amongst groundwater isolates tested.  $Q_1 = 25^{th}$  percentile;  $Q_3 = 75^{th}$  percentile; IQR = Interquartile range =  $Q_3 - Q_1$ ;  $p \ge 0.1$ ; p < 0.05; # Kruskal-Wallis test; <sup>§</sup> Mann-Whitney U test; \* Dunn's pairwise tests (each similar letter denotes a subset of each category whose distribution do not differ significantly from each other at the p < 0.05 level and different letters indicate statistically significant differences at the p < 0.05 level).





358

359 Figure 5: Summary of descriptive and non-parametric test results for the calculated Multiple Antimicrobial Resistance

360 (MAR) indices in groundwater (iii; n=70).  $Q_1 = 25^{th}$  percentile; Q3 = 75<sup>th</sup> percentile; IQR = Interquartile range =  $Q_3 - Q_1$ ;

361 p ≥ 0.05; p < 0.05; Kruskal-Wallis test; <sup>§</sup> Mann-Whitney U test; \* Dunn's pairwise tests (each similar letter denotes a

362 subset of each category whose distribution do not differ significantly from each other at the p < 0.05 level and different 363 letters indicate statistically significant differences at the p < 0.05 level).

#### 365 4. Discussion

366 Antimicrobial resistance is a widely recognised global public health threat with growing 367 evidence of its spread beyond clinical settings now available within the scientific literature (Bradford 368 and Harvey, 2017; Larsson et al., 2018; Opatowski et al., 2019). Most recently, the role of the natural 369 aquatic environment in the dissemination of ARB has gained interest (Suzuki et al., 2017; Sanderson 370 et al., 2018), with groundwater resources being particularly relevant given the reliance of 2.2 billion 371 people around the world on this source of drinking water (Murphy et al., 2017). Moreover, due to 372 the widely held belief that groundwater is an intrinsically 'clean' water source, many sources lack 373 treatment, in spite of well document incidences of faecal contamination as a result of agricultural 374 and wastewater sources (Schets et al., 2005; Hynds et al., 2014; Murphy et al., 2017). As such, the 375 risks posed via consumption of contaminated groundwater are significant, with a recent United 376 Nations (UN) report referring to discharge of contaminated wastes to the environment and 377 inadequate access to clean water as key drivers of antimicrobial resistance (IACG, 2019). However, 378 despite the importance of groundwater for human consumption and its potential role in the global 379 resistome, to date no comprehensive synthesis of previous studies of ARB in the subsurface has 380 been undertaken. The current study sought to address this gap in the scientific literature. 381 Occurrence rates of ARB amongst groundwater-derived isolates were consistently high across the seventy included studies, which were undertaken in geographic, climatologic and 382 383 socioeconomically diverse regions (Figure 2; Table 4). In the pooled analysis, studies where isolates 384 were not obtained were excluded and hence our findings are relevant to groundwater sources 385 where bacteria are present, which, where reported, accounted for 31.4% ± 32.6 of studied 386 groundwater sources. Results show that four fifths (80.2% ± 29.0) of aggregated groundwater 387 isolates were resistant to one or more antimicrobial agents, with ARB identified in 76.9% ± 33.7 of 388 individual groundwater sources where bacteria were present. These findings seem to suggest that in 389 the minority of groundwater sources were bacteria were present they were often resistant to at

390 least one antimicrobial. Just one study, undertaken in Burkina Faso, reported no ARB in examined 391 groundwater sources (Traoré et al., 2015). The high mean MAR index values calculated across 392 differing regions (Figure 3), when considered in concurrence with high occurrence rates of MRB 393 (57.2% ± 36.8), further highlights the presence of multidrug resistance as a particular issue within 394 groundwater sources. Compounding this, myriad ARB listed on the World Health Organisation 395 priority list as 'critical' (WHO, 2017) were specifically noted in across included studies (Table 5); a 396 high proportion of which were used for human consumption (e.g. Ribeiro et al., 2014; Maran et al., 397 2016; O'Dwyer et al., 2017). Accordingly, pooled results point to groundwater as an environment 398 characterised by the presence of ARB and MRB, and highlight its potential role in the spread of antimicrobial resistance both within the environment and directly to humans via consumption of, 399 400 frequently untreated, drinking water (Coleman et al., 2012).

401 Similarly, the abovementioned concurrently high incidence of ARB and MRB, and elevated 402 pooled mean MAR index (0.352 ± 0.207), suggests the presence of significant selective pressures 403 within groundwater systems, potentially resulting from extended residence times along subsurface 404 pathways with exposure to sub-therapeutic concentrations of antimicrobial residues. Continuous 405 release of human and veterinary antimicrobials to the natural environment has been shown to 406 facilitate their ingress to both surface and groundwater sources (Van Schaik, 2015); however, 407 occurrence rates of antimicrobial resistance in surface water bacteria found in middle- and high-408 income countries (O'Flaherty and Cummins, 2017) are lower than those encountered in the current 409 study. Unlike surface water, extended residence times associated with subsurface systems may lead 410 to prolonged bacterial exposure to antimicrobial residues (at low concentrations), resistance genes 411 and ARB within a relatively confined and oftentimes buffered (e.g. UV, pH and temperature) 412 environment (Van Schaik, 2015; Williams-Nguyen et al., 2016). Compounding this, bacterial isolates 413 may present high rates of resistance prior to subsurface ingress, with a recent study reporting that 414 tetracycline-resistant *E. coli* strains were more mobile than susceptible strains in saturated porous 415 media (Walczak et al., 2011), and thus can be characterised by higher rates of transport in the

subsurface. However, there is limited research exploring this hypothesis in diverse soil and aquifer
types. Indeed, further research is required which combines microbial source tracking, advanced
hydrogeological modelling, antimicrobial susceptibility testing and pharmaceutical residue
concentrations to facilitate a greater understanding of the intricacies of microbial transport and
resistance acquisition by these microbes in the subsurface.

421 More generally, pooled analyses suggest a paucity of research specifically addressing the mechanisms mediating the occurrence of ARB and MRB in groundwater, particularly with respect to 422 423 varying climates, anthropogenic practices, hydrogeological settings, and groundwater source types. 424 This represents a common limitation within blended groundwater and microbiological studies; due 425 to their multidisciplinary nature, important source-pathway-receptor information are oftentimes omitted, overlooked or unreported (Hynds et al., 2014). For example, just 8.6%, 17.1% and 18.6% of 426 427 included studies explicitly reported meteorological, hydrogeological, and detailed source-specific 428 data, respectively. Accordingly, in combination with the inherently high degree of variability in 429 reporting identified across identified studies, more robust quantitative examination (i.e., meta-430 regression) and (multi-)collinearity diagnostics could not be undertaken as part of the current study, 431 which may be partially accountable (due to the multiple instances of missing data) for the lack of 432 statistical significance between some external factors and ARB/MRB occurrence in groundwater 433 (Figure 4). Moreover, owing to the nature of the research reporting within identified studies, it was not possible to calculate "treatment" effects (effect sizes), thus a meta-analysis could not be 434 435 undertaken, as studies could not be weighted using any meaningful outcome measure. As such, 436 retrospective pooled analyses with the analytical units (i.e. individual study findings) weighted 437 equally was employed to identify significant trends in this research field (Figure 4 and Figure 5). 438 Result suggest that studies undertaken in high income countries reported significantly lower MAR 439 indices compared to lower and upper middle income countries (p = 0.003), with similar results found 440 for MRB occurrence (i.e. lower MRB rates reported in groundwater from high income countries; p = 441 0.036). This is likely due to lower antimicrobial diversity, lack of sanitation, poor hygiene practices

442	and reduced antimicrobial stewardship in areas characterised by lower mean incomes (Morgan et
443	al., 2011; Ayukekbong et al., 2017); however, the asymmetry in study numbers from each area (i.e.
444	just 22.9% in high income countries; Table 4) likely impacted study findings.

445 Climatologically, pooled analysis indicates that groundwater samples collected during dry 446 periods yielded significantly higher MAR indices (p=0.008) than during wet periods, directly 447 contrasting previous findings in surface water environments (Sanderson et al., 2018). While 448 precipitation is roundly acknowledged as a primary driver of groundwater contamination (Hynds et 449 al., 2012; Andrade et al., 2018), in the context of antimicrobial resistance, the evidence is less 450 compelling. For example, drier periods may enable higher concentration of antimicrobial residues 451 within the subsurface (Dhar et al., 2008), thus leading to increased bacterial exposure to sub-452 therapeutic levels of antimicrobial residues. Moreover, as temperature is directly correlated with 453 bacterial proliferation, even marginal increases in subsurface temperatures may increase bacterial 454 loading in-situ (John and Rose, 2005). However, it is important to note that in the absence of specific 455 hydrogeological information, interpreting the impact of seasonality on ARB and MRB occurrence is 456 largely speculative in this instance, further reiterating the need for a more "holistic" approach to 457 multidisciplinary groundwater research. Socio-geographically, groundwater sources located in 458 categorically urban (as opposed to rural) regions were associated with significantly higher MAR 459 indices (p=0.001; Figure 5). Similarly (and likely collinear with this previous finding), supplies 460 predominantly adjacent to human waste sources (e.g. septic tanks and wastewater treatment 461 plants) were characterised by a significantly higher (p=0.005) mean MAR index than those adjacent 462 to animal waste sources (e.g. animal grazing and manure sprecan eiading). This is possibly driven by 463 the antimicrobial residual concentration differentials between urban and rural areas. For example, 464 while antimicrobial concentrations in manure are typically orders of magnitude higher than those 465 encountered in wastewaters (Arikan et al., 2009; Sabri et al., 2018), the spatiotemporal exposure is 466 inconsistent, due to the diffuse nature of the source. Conversely, wastewater treatment 467 infrastructures typically result in point source contamination mechanisms, providing a consistent

468 source of low-dose antimicrobial residues. Moreover, transmission (infiltration) of common veterinary antimicrobials in the subsurface may be confined and thus spatially limited. Transport of 469 470 tetracycline compounds, in particular, are restricted to fast preferential and macropore flow or 471 require facilitation of co-transport with mobile colloids, such as dissolved organic matter (Thiele-472 Bruhn, 2003). Nevertheless, an increase in the global occurrence of ARB and MRB in groundwater 473 sources is expected due to current and future population growth, urbanisation, lack of sanitation, inappropriate wastewater treatment, and the misuse and over-use of antimicrobials, irrespective of 474 475 geographic location.

476 Focusing on the identification of factors driving ARB and MRB occurrence, significant research 477 challenges also exist due to inherent complexities associated with the genetic acquisition of antimicrobial resistance, in addition to the multifaceted hydrogeological mechanisms governing 478 479 subsurface contamination. For example, while MAR indices calculated within Carbapenems, which 480 are generally used as "last line" drugs (Van Boeckel et al., 2014), were found to be significantly lower 481 than other classes, several similarly prescribed antimicrobials, such as Glycopeptides (Van Boeckel et 482 al., 2014), were associated with relatively high mean MAR index values (Table 4). Accordingly, 483 antimicrobial usage itself may not be a primary driver of MRB or ARB in groundwater, despite 484 remaining an undeniably important factor in the broader antimicrobial resistance crisis. This is in line 485 with findings from Collignon et al (2018) that antimicrobial consumption was not significantly 486 associated with the global indices for antimicrobial resistance.

At the genus level, there was significant variance observed in the rates of encountered
antimicrobial resistance (Figure 2; Table 5). Ubiquitous members of the soil microbiome *Pseudomonas* spp., *Klebsiella* spp. and *Enterobacter* spp. exhibited some of the highest rates of
antimicrobial resistance at 99.9% ± 0.2, 99.8% ± 0.6 and 99.2% ± 2.2, respectively. These findings are
particularly significant as both Pseudomonas and Klebsiella spp. are considered to be ubiquitously
"soil-resident" bacteria, and as such, may characterise the local and/or source specific resistome

493 more accurately than faecal indicator and/or (opportunistic) pathogens, which are often absent. 494 However, it should be noted that a proportion of this resistance is attributable to 'intrinsic 495 resistance', and thus, should not be interpreted as a wholly anthropogenic phenomenon in all cases. 496 For example, Pseudomonas aeruginosa is intrinsically resistant to many antimicrobial agents 497 accredited to the low permeability of its outer membrane (Livermore, 1984), the expression of 498 various efflux pumps with wide substrate specificity (Livermore, 2001) and the naturally occurring 499 chromosomal AmpC blactamase (Nordmann & Guibert, 1998). A more compelling assessment of 500 acquired resistance focuses on E. coli, which was the most frequently examined bacterial species 501 across the studies, with pooled isolates exhibiting below average, but considerably high, rates of 502 resistance to  $\geq$  1 (79.8 ± 30.1) and  $\geq$  3 (45.4 ± 36.0) antimicrobial agents. Mechanisms explaining 503 between-species variation could not be determined in the current study, as it may reflect locally 504 specific circumstances. However, drawing from previous field and modelling work (Hynds et al., 505 2012), findings may suggest higher rates of ARB ingress and resistance acquisition taking place in the 506 subsurface via "traditional" recharge, with lower rates of antimicrobial resistance associated with 507 rapid entry (i.e. runoff at wellhead and/or preferential flow) into groundwater sources. As such, it is 508 recommended that future studies perform antimicrobial susceptibility assays, where possible, using 509 both faecal and soil-resident bacteria to accurately establish the extent of resistance within 510 microbial communities in groundwater, and their most likely ingress mechanisms. Moreover, as 511 molecular methods of antimicrobial resistance characterisation (specific gene targeting) increase in 512 popularity, it is important that isolate-specific phenotypic (i.e. culture-based) research be 513 undertaken to permit a greater understanding of the specific public health significance of 514 environmental exposures to ARB."

As with any literature findings-based study, it is important to acknowledge that data used for analyses are limited to previous reports; and thus, the potential for reporting bias exists and should be highlighted (i.e. studies targeting susceptible areas or groundwater sources where ARB contamination is suspected). Over half (52.9%) of identified studies included in this review based

519	their findings on "one-off" (i.e. non-temporal) groundwater sampling regimes, and were associated
520	with significantly higher MAR indices (p=0.003). Moreover, a widespread lack of explicit
521	methodology for achieving sample representativeness was identified during validity appraisal
522	(Supplementary Materials 1). As such, the occurrence rates of ARB and MRB in global groundwater
523	garnered from this review may represent an overestimate, with true values likely more accurately
524	reflected via means calculated from temporal studies only (i.e. ARB = 65.5% $\pm$ 33.4 and MRB = 38.9 $\pm$
525	32.0). However, the issue of temporal and spatial representativeness still represents a significant
526	concern. Baseline sampling and analytical procedures should be developed and routinely employed
527	via unbiased and robust spatiotemporal studies, following consistent methodologies, in diverse
528	(hydro)geological and climatic settings.
529	The current study is the first to integrate findings from international literature regarding the
530	occurrence of ARB and MRB in groundwater sources. While this research is undoubtedly topical,
531	identified studies highlight a long-standing issue, with the earliest relevant research available within
532	the scientific literature (Cooke, 1976) reporting antimicrobial resistance in 48.9% of 321 total and
533	faecal coliform isolates (species unspecified) from groundwater sources in New Zealand. As such,
534	and as groundwater remains an important source of potable water for a considerable proportion of
535	the global population, the results presented show that groundwater is a significant environment
536	where antimicropial resistance can be spread, and highlight the need for further research looking at
	where antimerobian esistance can be spread, and ingringht the need for further research looking at

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## 539 **5.** Conclusion

540 Study results highlight groundwater as a noteworthy source of global ARB and MRB, and the 541 pressing need for more representative studies (i.e. "baseline" work) on this topic. Additionally, lack 542 of more robust methodologies and of key relevant data were identified as persistent issues among 543 published work, making it difficult to ascertain a comprehensive global perspective, which is vital to

544	quantify the risks associated with groundwater consumption and antimicrobial resistance. Thus, a
545	key recommendation of this research is the requirement for the scientific community to refine their
546	approach to groundwater- and health-related research. Specifically, it is imperative that future
547	studies employ:
548	hydrogeological, meteorological, climatic and detailed source-specific data measurement and
549	reporting;

- temporal, rather than one-off, sampling methodologies;
- spatial distribution (as an attempt to achieve representativeness); and
- assessment of both faecal and soil-resident bacteria to establish the presence, origin and
- 553 ingress mechanisms of antimicrobial resistant bacteria in groundwater systems.
- 554 The importance of efforts to understand and prevent the spread of antimicrobial resistance cannot be overstated. In an era characterised by significant global challenges, including antimicrobial 555 556 resistance and anthropogenic climate change, consolidation of robust research approaches and 557 systematic design of sampling programmes to help answer pressing global questions is not only 558 warranted, but necessary. Regardless, findings of this study offer valuable insights into the extent 559 and significance of groundwater as a potential source of ARB, and provides guidance for future 560 research to guide policy development, action plans and remediation efforts/technologies to 561 safeguard public health into the future.

562

#### 563 Acknowledgements

The current study was made possible thanks to the support and funding provided by the Irish Centre for Research in Applied Geosciences (iCRAG) and Geological Survey of Ireland (GSI) under the remit of their Environmental Geosciences Postgraduate Programme.

## 568 *Author contributions*

- 569 L.A. and M.K. performed the initial review of literature. All authors (L.A., M.K., P.H., J.W., A.M. and
- 570 J.OD.) determined final inclusion. Statistical analyses were carried out by L.A. in consultation with
- 571 J.OD. and P.H. All authors were involved in the writing of the manuscript, with J.OD., P.H. and J.W.
- 572 providing final approval for submission.
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## Highlights

- Global analysis of antimicrobial-resistant bacteria in groundwater sources •
- Where reported, 31.4% ± 32.6 of studied sources harboured resistant bacteria
- In total, 80.2% ± 29.0 of aggregated isolates were antimicrobial-resistant •
- Overall, 57% ± 36.8 of isolates were resistant to ≥3 antimicrobials •
- Results highlight groundwater is a noteworthy source of antimicrobial resistance •

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#### **Declaration of interests**

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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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