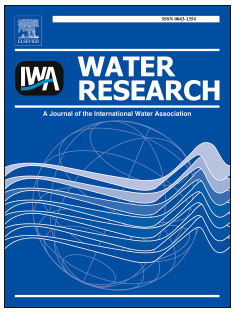
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Groundwater resources as a global reservoir for antimicrobial-resistant bacteria

Luisa Andrade, Madeleine Kelly, Paul Hynds, John Weatherill, Anna Majury, Jean O'Dwyer

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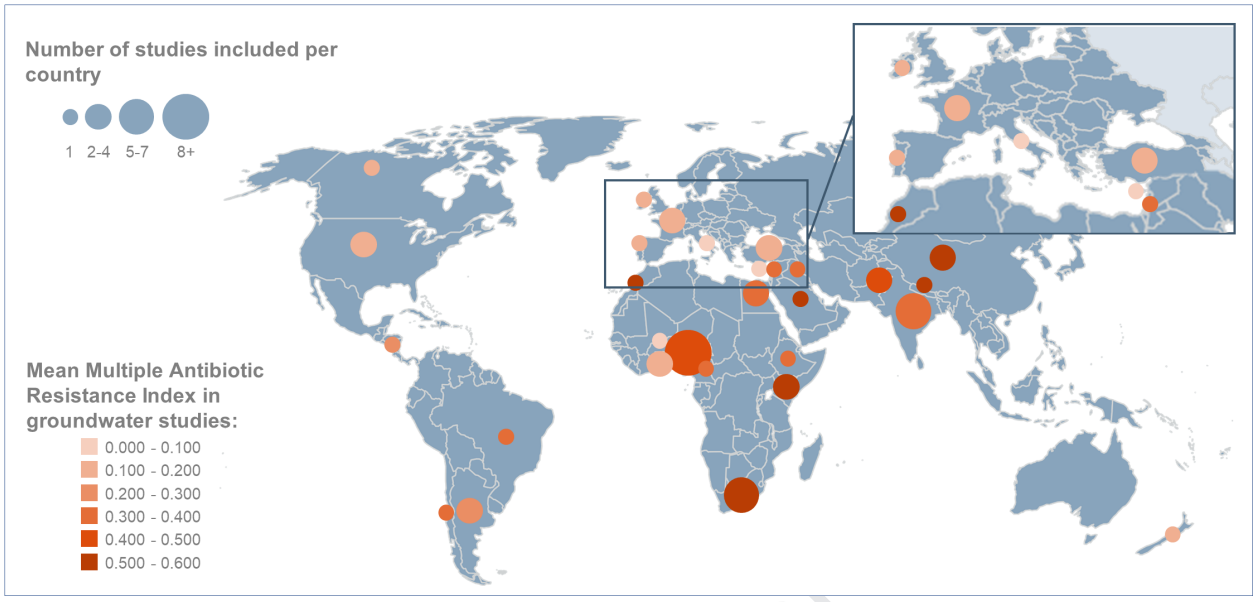
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Title: Groundwater resources as a global reservoir for antimicrobial-resistant bacteria

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Abstract

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 understanding the sources and pathways for antimicrobial-resistant bacteria (ARB) is critical. The current study presents the first systematic review and pooled analysis of ARB occurrence in global groundwater supplies, which are used as primary drinking water sources by 2.2 billion people 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 aggregated groundwater isolates were resistant to ≥ 1 and ≥ 3 antimicrobials, respectively. Where bacteria were present, ARB were identified in 76.9% ± 33.7 of individual wells and springs. Our 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 their occurrence.

Keywords: Groundwater; Antimicrobial Resistance; Drinking water; Environment and Health; Risk Factors.

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1. Introduction

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

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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 well substantiated within the scientific literature. A recent review by Murphy et al. (2017) 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 gastrointestinal infection potentially attributable to groundwater consumption per year. Thus, the potential implications of groundwater-borne ARB pose a significant threat to public health, allied with an already high global burden of infection. A recent study in the Republic of Ireland found that 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 groundwater supplies (O'Dwyer et al., 2017). As such, the ubiquity of contaminant sources, in concurrence with the presence of bacteria and sub-therapeutic antimicrobial concentrations, suggests that vulnerable groundwater systems may be a significant and frequently overlooked reservoir for ARB (Wellington et al., 2013). Indeed, research carried out with 878 Canadian individuals has shown that consumers of *E. coli* contaminated groundwater are 1.26 times more likely to be colonised by antimicrobial resistant *E. coli* than non-consumers (Coleman et al., 2012). Nonetheless, there is presently no consensus regarding the role of groundwater in the global dissemination of ARB. The extent of this threat is further complicated by the nuances of groundwater contamination mechanisms, which are typically determined and/or driven by numerous environmental and source-specific risk factors (e.g. source design, location and

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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.

2. Methods

2.1 Literature identification

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

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Table 1: Search terms employed in the current study

Element	Description	Search terms				
Population	All non-saline groundwater	Groundwater, Ground Water, Aquifer, Subsoil, Subsurface, Borehole, Bore Well, Bored Well, Dug Well, Well Water, Water Well				
Outcome(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*, *Enterococci*, *Campylobacter*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Shigella*, *Klebsiella*, *Enterobacter*, *Enterobacteriaceae*

2.2 Study selection

Studies uncovered during the identification phase (i.e. database and supplementary searches) were independently screened by two authors using explicit eligibility criteria (Table 2) via title and abstract assessment. Articles without an available full text were excluded, with eligibility disagreements resolved via provisional inclusion. All "provisionally included" and manually identified studies had full-texts manually (i.e., no computer-assisted techniques employed) and independently assessed (eligibility assessment) using the presented criteria (Table 2). Disagreements at this stage were resolved via author panel consensus.

Excluded studies were those that: i) reviewed previously published studies, ii) incorporated controlled elements in their study design (i.e., spiking or laboratory-based (micro-, meso-) groundwater environments), iii) examined thermal/hot springs, iv) did not study water samples derived from groundwater sources (e.g., surface and marine water), v) combined resistance data from multiple environmental media, vi) did not examine ≥ 10 groundwater samples (including articles where sampling number was not reported), vii) did not analyse antimicrobial resistance/susceptibility in bacterial isolates from groundwater (including studies where bacteria

were not found), viii) did not report number of isolates tested, ix) did not report bacterial resistance (or susceptibility) to each antimicrobial tested against (Figure 1).

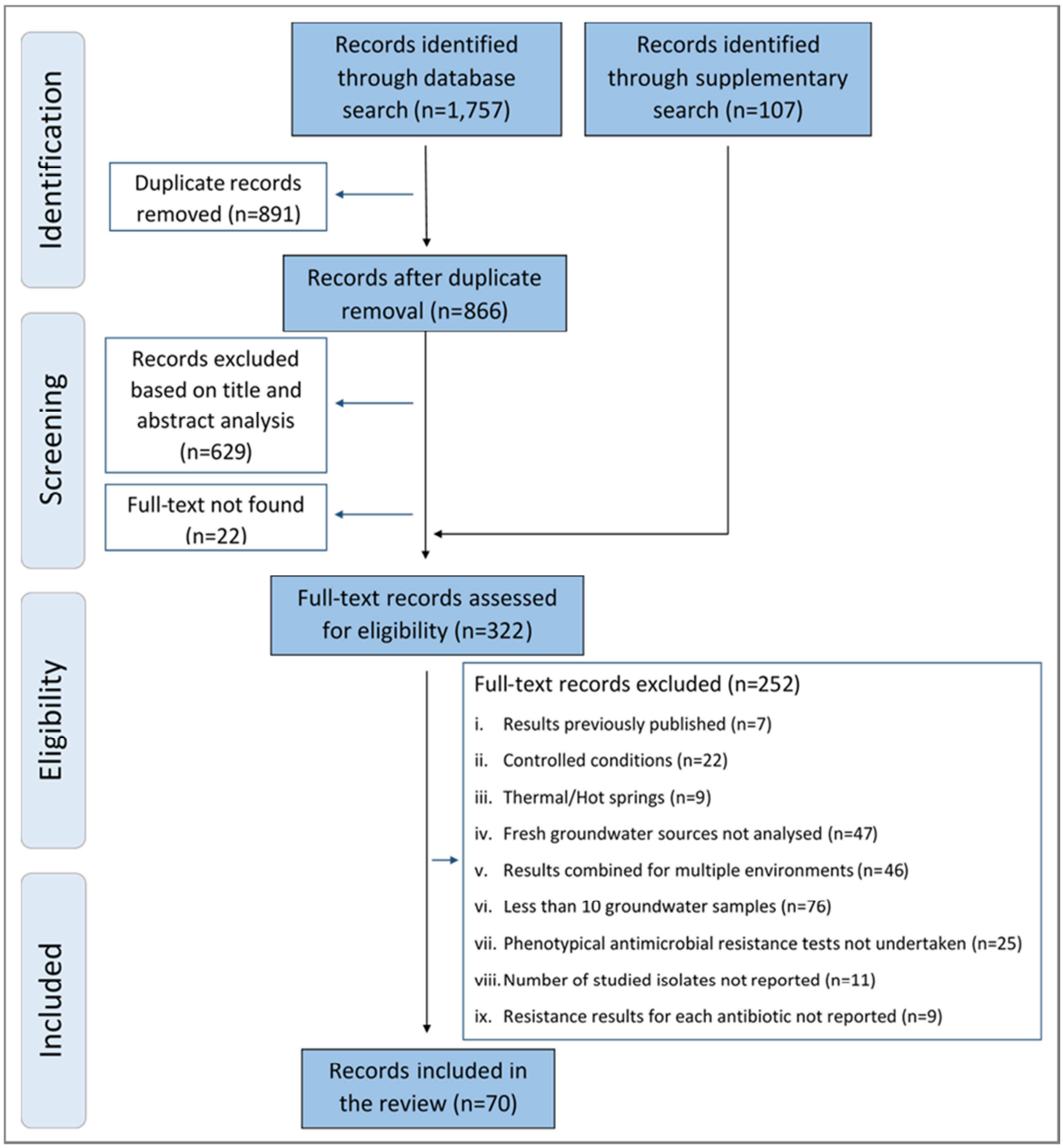
Table 2: Eligibility (inclusion/exclusion) criteria employed

Inclusion Criteria	Exclusion Criteria
Study type: All peer-reviewed articles excluding reviews	**Study type:** Academic reviews; grey literature
Language: English	**Language:** non-English
Population: Naturally occurring groundwater environments; groundwater sources (i.e. wells and springs)	**Population:** Artificial groundwater media (i.e. lab-based); pre-packaged water; Thermal/Hot springs; surface water bodies; maritime aquatic environments; wastewater treatment plants; soil; saline, brackish, or soil water.
Exposure: Pre-existing environmental exposures (i.e. prior to study)	**Exposure:** Any controlled exposure (i.e. spiking)
Event/Outcome: Antimicrobial-resistant (or susceptible) bacteria found in groundwater resources	**Event/Outcome:** Absence of bacterial contamination in groundwater; absence of antimicrobial resistance profiling of groundwater isolates
Study design: Analysis of ≥ 10 groundwater samples; results including percentages of bacterial isolates resistant/susceptible to each antimicrobial tested against.	**Study design:** 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:** -

2.3 Study Inclusion

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

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

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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.

2.5 Data extraction

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

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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 re-assessed. 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).

Where key variables were unclear or not explicitly documented, article authors were contacted for clarification and/or articles were analysed for identifiable characteristics, and thus 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 (≥ 100), as previously defined by Bain et al. (2014), with a maximum threshold established whereby data from one study comprising > 5,000 samples were not extracted to prevent geographical and/or analytical bias (Coleman et al., 2013); however, data reported from a smaller sample (n=657) in this study were included in analyses. As such, the maximum sample number from a single included study was 939 (Akoachere et al., 2013).

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).

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

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

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).

$$MAR\ index = \frac{y}{n \times x} \qquad \text{[Equation. 1]}$$

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 cross-study 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

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

2.7 Data analyses

Three dependent variables were calculated and used to quantify presence of ARB and/or Multidrug Resistant Bacteria (MRB) in groundwater and represent the main findings from each included study (i.e. studies were the analytic unit throughout analyses), namely: i) occurrence rates of antimicrobial resistance (≥ 1 antimicrobial) amongst all groundwater isolates tested; ii) occurrence rates of multidrug resistance (≥ 3 antimicrobials) amongst all groundwater isolates tested; and iii) calculated study-specific MAR indices.

Dependent variables were not normally distributed and could not be normalized using standard (transformation) techniques, thus non-parametric Mann-Whitney U and Kruskal-Wallis tests were employed to identify categorical associations between dependant and independent variables (i.e. the extracted characteristics outlined in Table 3). Where significance was found ($p < 0.05$) and the independent variable described three or more levels of measurement, Dunn's (non-parametric) 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.

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

Categories		Sub-categories	Non-parametric test Sub
Environmental			
1.	Economic classification [a]	Low; Lower middle; Upper middle; High income	Kruskal-Wallis
2.	Climate [b]	Tropical; Arid; Cold; Temperate	Kruskal-Wallis
3.	Sampling period [c]	Wet; Dry	Mann-Whitney U
4.	Settlement type [d]	Urban; Rural	Mann-Whitney U
5.	Waste source adjacent to study site [e]	Human waste; Animal waste	Mann-Whitney U
Source-specific			
6.	Source type	Well; Spring	Mann-Whitney U
7.	Well type	Hand-dug; Bored	Mann-Whitney U
Study-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

[a] economic classification according to the World Bank (2018); [b] climate classified according to Peel et al. (2007); [c] wet and dry as defined in Peel et al. (2007); [d] urban settlements include urban, sub-urban or peri-urban regions; [e] 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 analyses. A
259 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
263 as four of the five climatic zones were represented. Overall, 51.4% (n=36) of identified studies were
264 undertaken in lower-middle income countries, followed by countries classified by high (21.4%; n=15)
265 and upper-middle (21.4%; n=15) incomes. There were two primary sampling regimes identified, with
266 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

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was exclusively undertaken during wet and dry seasons in 41.4% (n=24) and 32.7% (n=19) of studies, respectively; however, just 10.3% of studies (n=6) explicitly reported this information.

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

292 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).

294 ***Table 4: Summary of principal characteristics extracted, with study (n=70) and groundwater sample (n=8,741) number***
295 ***associated 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 [a]					
Low income	3 (4.3)	149 (1.7)	**Primary settlement type** [e]		
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 [b]					
Tropical	25 (35.7)	3,506 (40.1)	**Waste adjacent to GW sites** [f]		
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 [c]			**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 [g]	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)
Sample size [d]			**Human consumption**		
Small (< 30)	13 (18.6)	-	Yes	57 (81.4)	7,036 (80.5)
Medium (30 - 99)	34 (48.6)	-	No	3 (4.3)	299 (3.4)
Large (≥ 100)	23 (32.8)	-	Not reported	10 (14.3)	1,406 (16.1)
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)
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)			

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

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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.

3.3. Antimicrobial-resistant bacteria in global groundwater

Where reported (n=20 studies; 28.6%), ARB were identified in 31.4% ± 32.6 of studied groundwater sources and in 76.9% ± 33.7 of sources where bacteria were present. Additionally, 80.2% ± 29.0 and 57.2% ± 36.8 of pooled groundwater isolates (3,456 and 1,403 isolates, respectively) were resistant to ≥ 1 (ARB; n=55; 78.6%) and ≥ 3 antimicrobials (MRB; n=32; 45.7%), respectively.

As shown (Figure 2), consistently high occurrence rates of antimicrobial resistance were found 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 high rates of multidrug resistance (96.6% ± 5.8). Moreover, *Pseudomonas* spp. (0.544 ± 0.237) were 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 1st generation Cephalosporins (0.594 ± 0.325), Glycopeptides (0.549 ± 0.378), 2nd generation Cephalosporins (0.529 ± 0.316) and Sulphonamides (0.517 ± 0.315) were associated with the highest mean MAR Indices. Conversely, lowest mean values were associated with Carbapenems (0.040 ± 0.078) and 4th generation Cephalosporins (0.150 ± 0.236).

A calculated mean MAR index of 0.352 ± 0.207 represents the level of ARB presence in global groundwater during the total review period (i.e. between 1976 and 2018; Figure 3), and of 0.359 ± 0.207 from 2010 to 2018 (not shown). Just one study exhibited a calculated MAR index of 0.000 (zero) (i.e. Traoré et al., 2015). MAR index values were highest in Kenya ($\bar{x}$ ± SD= 0.549 ± 0.208),

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

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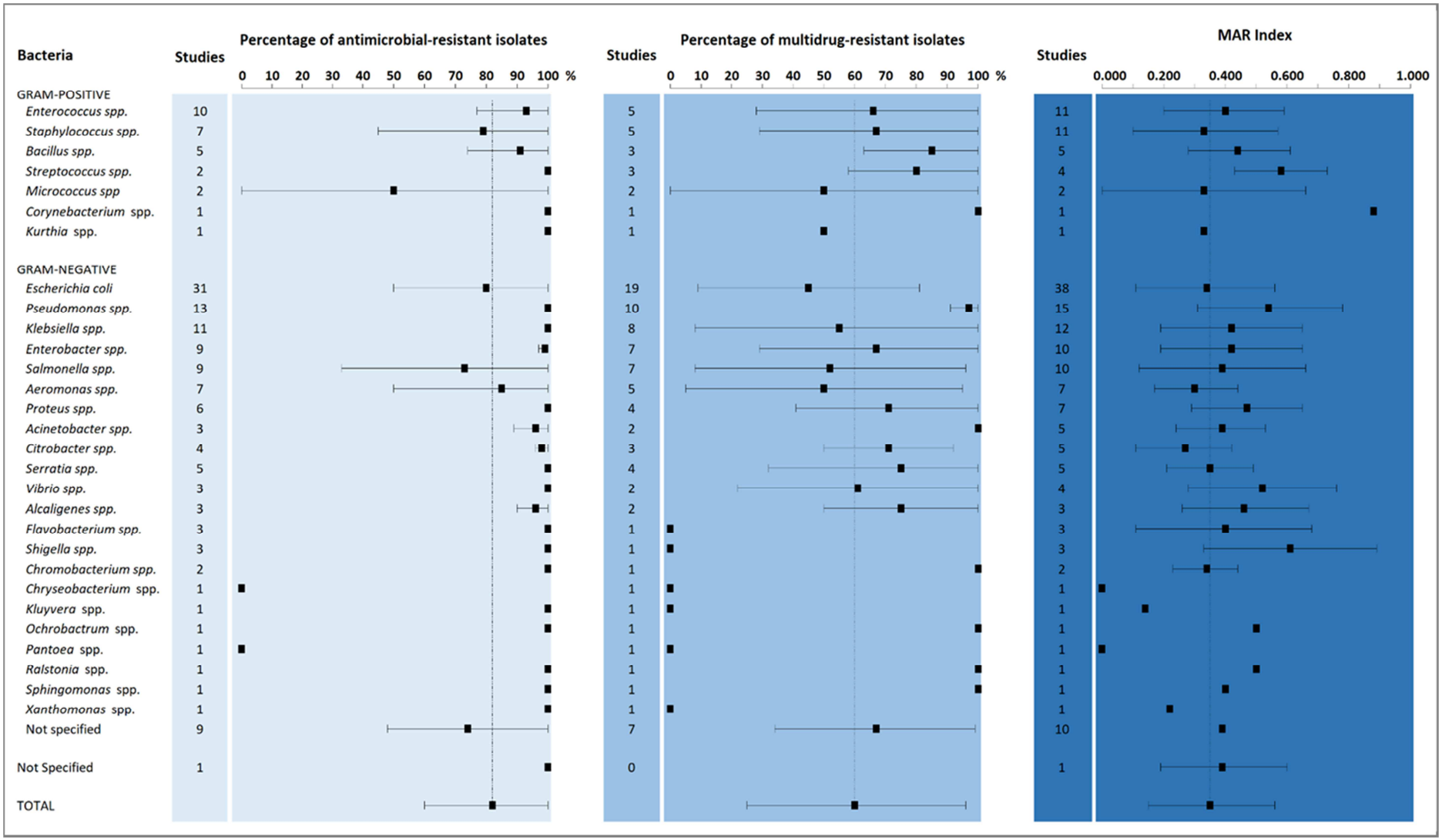


Figure 2: Forest plot of i) antimicrobial resistance, ii) multidrug resistance and iii) calculated multiple antimicrobial resistance (MAR) Indices in groundwater isolates from each genus examined

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

Bacteria	Aminoglycosides[1]	Carbapenems[2]	Cephalosporins				Fluoroquinolones[7]	Glycopeptides[8]	Lincosamides[9]	Macrolides[10]	Penicillins[11]	Sulfonamides[12]	Tetracyclines[13]	TOTAL
			1st gen[3]	2nd gen[4]	3rd gen[5]	4th gen[6]								
	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)[c #]	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)[a]	0.599 ± 0.284 (5)	0.421 ± 0.333 (15)	0.375 ± 0.391 (19)[a]	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)[a]*	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)[a]*	0.586 ± 0.268 (3)	0.428 ± 0.384 (5)	0.261 ± 0.360 (7)[a]*	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 ± 0.000 (1)[a]	0.798 ± 0.236 (3)	0.484 ± 0.358 (5)	0.276 ± 0.246 (6)[a]	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 ± 0.000 (1)[a]	0.902 ± 0.000 (1)	1.000 ± 0.000 (1)	0.338 ± 0.413 (7)[a]	-	0.134 ± 0.246 (9)[b]	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)	-[a]	0.555 ± 0.016 (2)	0.524 ± 0.278 (4)	0.494 ± 0.271 (4)[a]	-	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 ± 0.000 (1)[a]*	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 ± 0.000 (1)[a]	0.536 ± 0.036 (2)	0.667 ± 0.333 (2)	0.111 ± 0.157 (3)[a]	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[§]	**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

[1] Amikacin, Gentamicin, Kanamycin, Neomycin, Netilmicin, Paromomycin, Streptomycin and/or Tobramycin; 61 studies

[2] Biapenem, Ertapenem, Imipenem and/or Meropenem; 16 studies

[3] Cefadroxil, Cefalexin, Cefazolin, Cefradine and/or Cephaloridine; 14 studies

[4] Cefaclor, Cephalothin, Cefamandole, Cefoxitin and/or Cefuroxime; 26 studies

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

[6] Cefepime; 8 studies

[7] Ciprofloxacin, Enrofloxacin, Levofloxacin, Moxifloxacin, Nalidixic acid, Nitrofurantoin, Norfloxacin, Ofloxacin, Pefloxacin and/or Sparfloxacin; 57 studies

[8] Teicoplanin and/or Vancomycin; 15 studies
[9] Clindamycin and/or Lincomycin; 7 studies
[10] Erythromycin and/or Roxithromycin; 21 studies
[11] Amoxicillin, Amoxicillin-clavulanic acid, Ampicillin, Ampicillin-sulbactam, Ampiclox, Carbenicillin, Cloxacillin, Mecillinam, Methicillin, Oxacillin, Piperacillin, Piperacillin-tazobactam, Ticarcillin and/or Ticarcillin-clavulanic acid; 66 studies
[12] Sulfamethoxazole, Sulfanilamide, Sulphafurazole and/or Sulfabenzamide-sulfacetamide-sulfathiazole (i.e. "Triple Sulfa"); 18 studies
[13] Chlortetracycline, Doxycycline, Oxytetracycline and/or Tetracycline; 50 studies
[a] Priority 1 (i.e. critical) in the global priority list of antimicrobial-resistant bacteria[7]
[b] Priority 2 (i.e. high) in the global priority list of antimicrobial-resistant bacteria[7]
[c] Priority 3 (i.e. medium) in the global priority list of antimicrobial-resistant bacteria[7]
* 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[7]
# The only *Streptococcus* species included in the global medium priority list of antimicrobial-resistant bacteria is *Streptococcus pneumoniae*[7]
§ 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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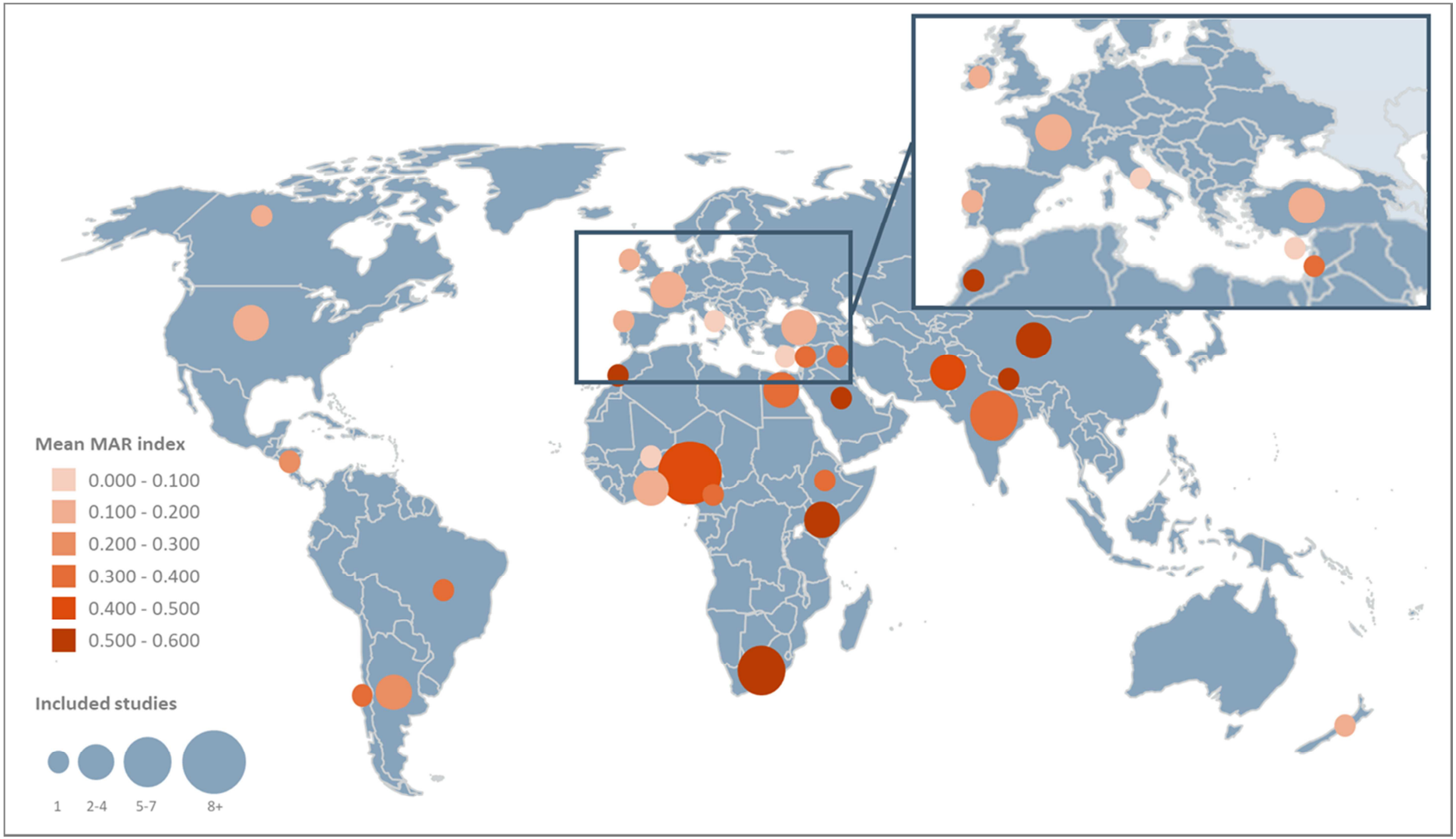


Figure 3: Mean Multiple Antimicrobial Resistance (MAR) Indices in global groundwater from 1976-2018 (n = 70) in each included country

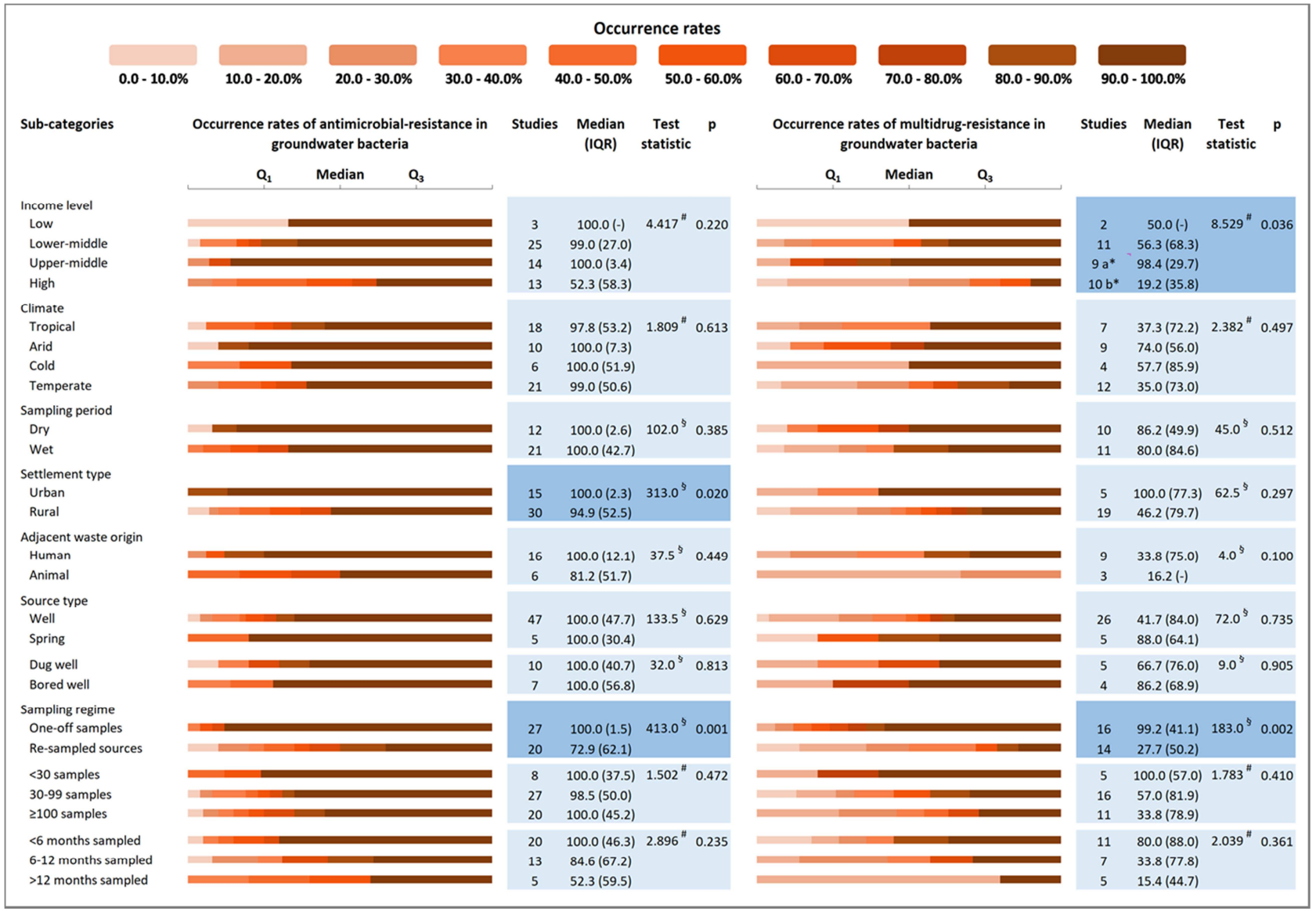
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3.4. Potential drivers of antimicrobial-resistant bacteria in groundwater

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).

MAR indices were significantly lower in groundwater from high-income countries when compared to upper-middle (p=0.018) and lower-middle (p=0.002) income countries (Figure 5), and 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 settlements (p=0.001) and when waste sources adjacent to groundwater supplies were predominantly of human (e.g. septic tanks and wastewater treatment plants) as opposed to animal (e.g. animal grazing and manure spreading) origin (p=0.005). No significant differences were found when comparing MAR index distributions with sources type (i.e. wells versus springs) or source construction (i.e. hand-dug versus bored wells). One-off sampling regimes (n=37) yielded significantly higher MAR indices in groundwater when compared to regimes where re-sampling was employed (n=23) (p=0.003).

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Sub-categories	Occurrence rates of antimicrobial-resistance in groundwater bacteria (Q_1 Median Q_3)	Studies	Median (IQR)	Test statistic	p	Occurrence rates of multidrug-resistance in groundwater bacteria (Q_1 Median Q_3)	Studies	Median (IQR)	Test statistic	p
Income level										
Low		3	100.0 (-)	4.417 #	0.220		2	50.0 (-)	8.529 #	0.036
Lower-middle		25	99.0 (27.0)				11	56.3 (68.3)		
Upper-middle		14	100.0 (3.4)				9 a*	98.4 (29.7)		
High		13	52.3 (58.3)				10 b*	19.2 (35.8)		
Climate										
Tropical		18	97.8 (53.2)	1.809 #	0.613		7	37.3 (72.2)	2.382 #	0.497
Arid		10	100.0 (7.3)				9	74.0 (56.0)		
Cold		6	100.0 (51.9)				4	57.7 (85.9)		
Temperate		21	99.0 (50.6)				12	35.0 (73.0)		
Sampling period										
Dry		12	100.0 (2.6)	102.0 §	0.385		10	86.2 (49.9)	45.0 §	0.512
Wet		21	100.0 (42.7)				11	80.0 (84.6)		
Settlement type										
Urban		15	100.0 (2.3)	313.0 §	0.020		5	100.0 (77.3)	62.5 §	0.297
Rural		30	94.9 (52.5)				19	46.2 (79.7)		
Adjacent waste origin										
Human		16	100.0 (12.1)	37.5 §	0.449		9	33.8 (75.0)	4.0 §	0.100
Animal		6	81.2 (51.7)				3	16.2 (-)		
Source type										
Well		47	100.0 (47.7)	133.5 §	0.629		26	41.7 (84.0)	72.0 §	0.735
Spring		5	100.0 (30.4)				5	88.0 (64.1)		
Dug well		10	100.0 (40.7)	32.0 §	0.813		5	66.7 (76.0)	9.0 §	0.905
Bored well		7	100.0 (56.8)				4	86.2 (68.9)		
Sampling regime										
One-off samples		27	100.0 (1.5)	413.0 §	0.001		16	99.2 (41.1)	183.0 §	0.002
Re-sampled sources		20	72.9 (62.1)				14	27.7 (50.2)		
<30 samples		8	100.0 (37.5)	1.502 #	0.472		5	100.0 (57.0)	1.783 #	0.410
30-99 samples		27	98.5 (50.0)				16	57.0 (81.9)		
≥100 samples		20	100.0 (45.2)				11	33.8 (78.9)		
<6 months sampled		20	100.0 (46.3)	2.896 #	0.235		11	80.0 (88.0)	2.039 #	0.361
6-12 months sampled		13	84.6 (67.2)				7	33.8 (77.8)		
>12 months sampled		5	52.3 (59.5)				5	15.4 (44.7)		

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 = 25th percentile; Q_3 = 75th percentile; IQR = Interquartile range = $Q_3 - Q_1$; p ≥ 0.1; p < 0.05; # Kruskal-Wallis test; § 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).

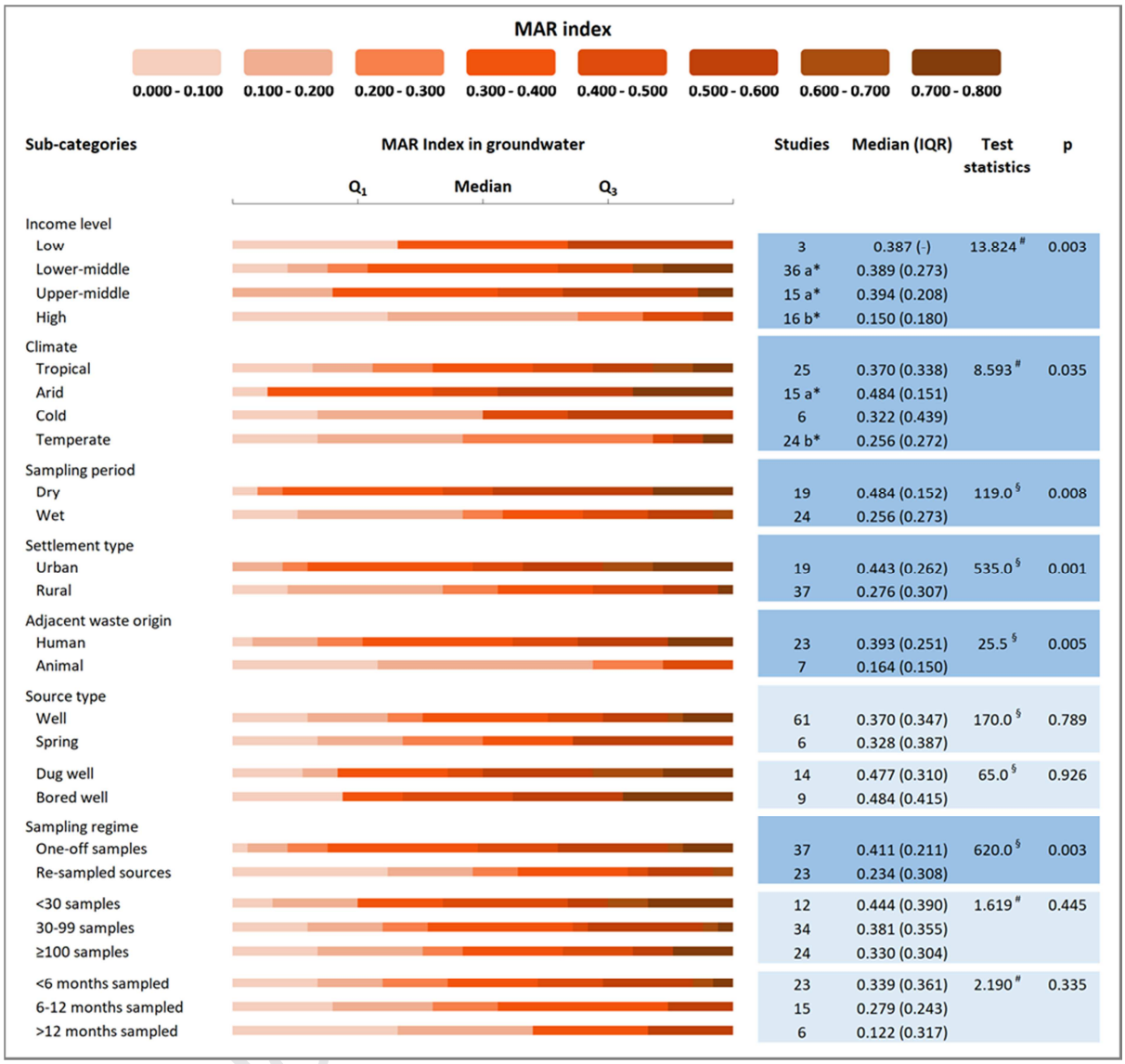


Figure 5: Summary of descriptive and non-parametric test results for the calculated Multiple Antimicrobial Resistance (MAR) indices in groundwater (iii; n=70). Q_1 = 25th percentile; Q3 = 75th percentile; IQR = Interquartile range = $Q_3 - Q_1$; $p \geq 0.05$; $p < 0.05$; # Kruskal-Wallis test; § Mann-Whitney 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).

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4. Discussion

Antimicrobial resistance is a widely recognised global public health threat with growing evidence of its spread beyond clinical settings now available within the scientific literature (Bradford and Harvey, 2017; Larsson et al., 2018; Opatowski et al., 2019). Most recently, the role of the natural aquatic environment in the dissemination of ARB has gained interest (Suzuki et al., 2017; Sanderson et al., 2018), with groundwater resources being particularly relevant given the reliance of 2.2 billion people around the world on this source of drinking water (Murphy et al., 2017). Moreover, due to the widely held belief that groundwater is an intrinsically 'clean' water source, many sources lack treatment, in spite of well document incidences of faecal contamination as a result of agricultural and wastewater sources (Schets et al., 2005; Hynds et al., 2014; Murphy et al., 2017). As such, the risks posed via consumption of contaminated groundwater are significant, with a recent United Nations (UN) report referring to discharge of contaminated wastes to the environment and inadequate access to clean water as key drivers of antimicrobial resistance (IACG, 2019). However, despite the importance of groundwater for human consumption and its potential role in the global resistome, to date no comprehensive synthesis of previous studies of ARB in the subsurface has been undertaken. The current study sought to address this gap in the scientific literature.

Occurrence rates of ARB amongst groundwater-derived isolates were consistently high across the seventy included studies, which were undertaken in geographic, climatologic and socioeconomically diverse regions (Figure 2; Table 4). In the pooled analysis, studies where isolates were not obtained were excluded and hence our findings are relevant to groundwater sources where bacteria are present, which, where reported, accounted for 31.4% ± 32.6 of studied groundwater sources. Results show that four fifths (80.2% ± 29.0) of aggregated groundwater isolates were resistant to one or more antimicrobial agents, with ARB identified in 76.9% ± 33.7 of individual groundwater sources where bacteria were present. These findings seem to suggest that in the minority of groundwater sources were bacteria were present they were often resistant to at

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

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

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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.

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 varying climates, anthropogenic practices, hydrogeological settings, and groundwater source types. This represents a common limitation within blended groundwater and microbiological studies; due 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 included studies explicitly reported meteorological, hydrogeological, and detailed source-specific data, respectively. Accordingly, in combination with the inherently high degree of variability in reporting identified across identified studies, more robust quantitative examination (i.e., meta-regression) and (multi-)collinearity diagnostics could not be undertaken as part of the current study, which may be partially accountable (due to the multiple instances of missing data) for the lack of statistical significance between some external factors and ARB/MRB occurrence in groundwater (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 undertaken, as studies could not be weighted using any meaningful outcome measure. As such, retrospective pooled analyses with the analytical units (i.e. individual study findings) weighted equally was employed to identify significant trends in this research field (Figure 4 and Figure 5). Result suggest that studies undertaken in high income countries reported significantly lower MAR indices compared to lower and upper middle income countries ($p = 0.003$), with similar results found for MRB occurrence (i.e. lower MRB rates reported in groundwater from high income countries; $p = 0.036$). This is likely due to lower antimicrobial diversity, lack of sanitation, poor hygiene practices

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

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

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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 tetracycline compounds, in particular, are restricted to fast preferential and macropore flow or require facilitation of co-transport with mobile colloids, such as dissolved organic matter (Thiele-Bruhn, 2003). Nevertheless, an increase in the global occurrence of ARB and MRB in groundwater 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 geographic location.

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

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

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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% ± 33.4 and MRB = 38.9 ±
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 antimicrobial resistance can be spread, and highlight the need for further research looking at
537 the occurrence of ARB and MRB in the subsurface environment.

538

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

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quantify the risks associated with groundwater consumption and antimicrobial resistance. Thus, a key recommendation of this research is the requirement for the scientific community to refine their approach to groundwater- and health-related research. Specifically, it is imperative that future studies employ:

- hydrogeological, meteorological, climatic and detailed source-specific data measurement and 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 ingress mechanisms of antimicrobial resistant bacteria in groundwater systems.

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 resistance and anthropogenic climate change, consolidation of robust research approaches and systematic design of sampling programmes to help answer pressing global questions is not only warranted, but necessary. Regardless, findings of this study offer valuable insights into the extent and significance of groundwater as a potential source of ARB, and provides guidance for future research to guide policy development, action plans and remediation efforts/technologies to safeguard public health into the future.

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Author contributions

L.A. and M.K. performed the initial review of literature. All authors (L.A., M.K., P.H., J.W., A.M. and J.OD.) determined final inclusion. Statistical analyses were carried out by L.A. in consultation with J.OD. and P.H. All authors were involved in the writing of the manuscript, with J.OD., P.H. and J.W. 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

☒ 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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