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Targeting current and future threats: recent methodological trends in environmental antimicrobial resistance research and their relationships to risk assessment

Tucker R. Burch,^{ab} Ryan J. Newton,^c Lee K. Kimbell,^{id}^d Emily Lou LaMartina,^c Kassidy O'Malley,^{id}^d San Marie Thomson,^d Christopher W. Marshall^{id}^e and Patrick J. McNamara^{id}^{*d}

Antimicrobial resistance (AMR) is a growing public health threat. Improved surveillance of AMR's genetic indicators in environmental reservoirs should lead to a more comprehensive understanding of the problem at a global scale, as with SARS-CoV-2 monitoring in sewage. However, the "best" monitoring approach is unclear. Some scientific works have emphasized monitoring for the abundance of already-known antimicrobial resistance genes (ARGs); others have emphasized monitoring for the potential of new ARGs to arise. The goal of this study was to examine which methods were employed by highly-cited papers studying AMR in environmental engineering and agricultural systems, thus providing insight into current and future methodological trends for monitoring ARGs. We searched recent (2018–2020) literature documenting AMR in five environmental matrices: wastewater, surface water, drinking water, stormwater, and livestock manure. We selected the most highly-cited papers across these matrices (89 papers from 17 809 initial results) and categorized them as using targeted methods (e.g., qPCR), non-targeted methods (e.g., shotgun metagenomics), or both. More than 80% of papers employed targeted methods. Only 33% employed non-targeted methods, and the use of targeted versus non-targeted methods varied by environmental matrix. We posit that improving AMR surveillance in environmental reservoirs requires assessing risk, and that different monitoring approaches imply different objectives for risk assessment. Targeted methods are appropriate for quantifying known threats, particularly in environmental matrices where direct human exposure is likely (e.g., drinking water). However, long-term studies employing non-targeted methods are needed to provide an understanding of how frequently new threats (i.e., novel ARGs) arise.

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Water impact

The majority of recent impactful research on environmental reservoirs of antimicrobial resistance has used targeted methods (e.g. qPCR) to quantify known antibiotic resistance genes (ARGs). To advance the field of antimicrobial resistance risk assessment, non-targeted methods (e.g. metagenomics) in longer time-scale studies are needed to determine the frequency by which new ARGs arise.

^a U.S. Department of Agriculture-Agricultural Research Service, Environmentally Integrated Dairy Management Research Unit, 2615 Yellowstone Drive, Marshfield, WI, 54449, USA

^b Laboratory for Infectious Disease and the Environment, 2615 Yellowstone Drive, Marshfield, WI, 54449, USA

^c School of Freshwater Sciences, University of Wisconsin-Milwaukee, 600 E. Greenfield Ave., Milwaukee, WI, 53204, USA

^d Dept of Civil, Construction & Environmental Engineering, Marquette University, 1637 W Wisconsin Ave, Milwaukee WI, 53233, USA.

E-mail: patrick.mcnamara@marquette.edu

^e Dept of Biological Sciences, Marquette University, 1428 W. Clybourn St., Milwaukee WI, 53233, USA

1. Introduction

Antimicrobial resistance (AMR) is a global health threat which may result in a financial burden of approximately 100 trillion USD and over 10 million deaths by 2050.¹ AMR manifests as antimicrobial resistant bacteria (ARB) in clinical infections and is mediated at the genetic level by antimicrobial resistance genes (ARGs). ARGs are naturally occurring,^{2,3} with human activity contributing to their increased abundance and diversity worldwide,^{4,5} such that ARGs (and the ARB that carry them) are regarded as



environmental pollutants.⁶ Thus, both are ubiquitous in the environment and can be transferred among humans, animals, and environmental media (*e.g.*, water, air, and soil). Consequently, antimicrobial resistance requires a global “One Health” response – a response that considers the intersection of human, animal, and environmental health systematically.^{7,8,155}

This response must be guided by public health data. That is, the occurrence of antimicrobial resistant infections in humans ideally should be linked to each of the One Health sectors and, moreover, to the various human activities within those sectors that drive increasing resistance (*e.g.*, antibiotic use in human medicine and livestock agriculture). Understanding these links in detail would inform strategies for mitigating the impact of human activities on antimicrobial resistance. However, this information is largely lacking because the individual relationships of interest represent small effects in a complex real-world system. Studying them empirically would require enormous numbers of human subjects enrolled in observational studies with little to no control over the relevant environmental factors. Thus, the epidemiological studies required to document links between environmental antibiotic resistance and human health are prohibitively large and expensive.

As a result, scientific and regulatory leaders have proposed risk assessment as an alternative.^{7,9–11} Risk assessment represents a predictive modeling approach, and as such, its strengths relative to the empirical measurements of epidemiology are feasibility and flexibility. Risk assessment can be carried out with limited data to assess emerging hazards, so long as its interpretation is tempered by appropriate acknowledgement of those limitations. Risk assessment can be conducted prospectively in order to project the effects of multiple risk mitigation strategies before they are implemented. Risk assessment also allows for synthesis of otherwise disparate information for the purpose of balancing competing risks, and by extension, the competing interests of different One Health sectors.

2. Paradigms for risk assessment of antimicrobial resistance

In our view, two distinct paradigms have emerged with respect to framing risk assessment for antimicrobial resistance. Adopting the language of Zhang *et al.*, the first of these is concerned with estimating risk for *current threats*, *i.e.*, hazards that are already known to exist.¹² The second is concerned with estimating risk for *future threats*, *i.e.*, novel and unknown hazards that have yet to emerge.¹² These represent profoundly different objectives for risk assessment and can be explained by analogy with the current COVID-19 pandemic. In that case, current threats would relate to the risk of infection from a known variant of SARS-CoV-2, whereas future threats would relate to the risk of a new variant emerging. Both types of risk are important

considerations for formulating public health policy, but each requires its own approach to risk assessment.

These two paradigms effectively simplify the wide variety of specific approaches and priorities that have been proposed by others for AMR risk assessment. Moreover, we see close analogs of these two ideas expressed repeatedly in prior works. They are expressed clearly in Finley *et al.*'s distinction between ancient and “new” antibiotic resistance genes,¹³ in Bengtsson-Palme and Larsson's reference to known resistance genes and novel resistance determinants,¹⁴ and in Larsson and Flach's delineation of transmission events *versus* evolutionary events.¹⁵ More generally, we interpret these previous authors' terminology as converging on the same principles underlying Zhang *et al.*'s concepts of current and future AMR threats, such that their framework seems broadly applicable.

We also argue that the dichotomy of current threats *versus* future threats relates to the analytical methods used in quantifying hazards during exposure assessment. With respect to ARGs, exposure to current threats can be estimated readily provided enough empirical data are available to characterize their circulation in a population. More specifically, current threats have known nucleic acid sequences. They can therefore be targeted *via* methods based on polymerase chain reaction (PCR) to estimate their absolute abundance in a given environment, and abundance can be used to index risk. On the other hand, exposure to future threats depends on a very different set of criteria, like the nature of relevant environmental matrices, microbial diversity and density, and the selective pressure(s) involved. Furthermore, the nucleic acid sequences of future threats cannot be targeted because they are as-yet unknown. We therefore propose that the diversity of ARGs in a given environment can be used to index risk for future threats. That is, we assume the more ARGs an individual is exposed to, the higher the risk is that they are exposed to a novel ARG that has yet to emerge on a wider scale.

Finally, we note that diversity can be estimated only using open-ended methods, in which the ARGs present in a given environmental compartment are not necessarily known *a priori* (*e.g.*, shotgun metagenomics). Thus, we argue that the distinction between current and future threats for ARGs also corresponds to an analogous distinction between targeted and non-targeted molecular methods.

The goal of this perspective was to determine how recent and influential studies have characterized ARGs in environmental media. We sought out highly cited papers from 2018–2020 across five different environmental matrices and examined how the use of these methods aligned with the current threat *versus* future threat paradigm for risk assessment. We identified several PCR-based approaches (*e.g.*, qPCR, ddPCR, and HT-qPCR) as examples of targeted analytical methods for detecting current threats, and we identified metagenomics as a non-targeted method for potentially identifying future threats. Specifically, we aimed to 1) identify which methods were used in each study, 2)



identify discrepancies in methods used across environmental matrices, 3) assess how these methods fill knowledge gaps in risk assessment for antimicrobial resistance, and 4) propose research priorities to address risk assessment for antimicrobial resistance more effectively in the future. We focused on drinking water, wastewater, groundwater, stormwater, and livestock manure because these environmental compartments each represent semi-independent points that could be managed to alter the impacts of antimicrobial resistance on the global One Health scale.

3. Literature review approach

3.1. Literature identification

Our goal in identifying relevant literature was to obtain a current “snapshot” of high-impact ARG studies in the environmental field. Thus, we made no attempt to conduct a comprehensive or detailed literature review. Instead, we designed our initial search to be broad, quickly implemented, and easily reproduced. On April 24, 2021 we searched for the terms “(antibiotic OR antimicrobial) resist* gene” on Scopus (Fig. 1). This initial search returned 83 400 documents. These

were restricted further by limiting to the 3 most recent complete years at the time of the search (*i.e.*, 2018–2020) and by limiting to “articles” only (*i.e.*, reviews, letters, conference papers, and other Scopus document types were excluded). These additional restrictions reduced the search to 17 809 documents (Fig. 1).

3.2. Restriction and sub-division by environmental compartment

The literature search was restricted further to include only those documents addressing five environmental compartments of interest, which we refer to colloquially as wastewater, drinking water, stormwater, groundwater, and livestock manure. More formally, these compartments were defined using the Scopus search terms “wastewater AND (effluent OR biosolids)” (1163 documents), “drinking water” (1097 documents), “storm water OR stormwater” (81 documents), “ground water OR groundwater” (526 documents), and “manure AND (NOT anaerobic digest*)” (129 documents), respectively (Fig. 1). These search terms were applied within the group of 17 809 documents referred to in the previous section.



Fig. 1 Methodological approach for conducting literature review.



Our five environmental compartments were selected because they represent potential points of control for managing AMR in the environment. Additionally, consideration of distinct environmental compartments allowed for comparison of research practices in sub-topics of the environmental ARG research field. It is noted these compartments are high level and that subsets within these compartments could be parsed out further. The drinking water studies, for example, included samples from source waters, treatment processes within a drinking water treatment plant, and finished tap water, all of which can have different antimicrobial resistance threats, exposure risks, and interactions with the microbial community. Hospital and industrial effluents feed into municipal wastewater treatment plants and are incorporated under the wastewater umbrella because municipal wastewater treatment plants collect the diverse inputs into municipal sewers.

3.3. Document prioritization and review process

Documents were prioritized within each environmental compartment based on total citations. More specifically, documents were sorted on total citations (as reported in the Scopus database), then reviewed for inclusion in order from most to least citations. Our rationale for this approach was two-fold. First, we sought to include a maximum of 20 documents from each environmental compartment in order to expedite our review process. Second, we assumed that total citations were an appropriate measure of impact across scholarly works. That is, we assumed that the more a given paper has been cited by other works, the more relevant and influential it is for the environmental ARG research field as a whole. We understand this is not strictly the case as articles can be cited for many different reasons, but this approach is reproducible.

The review process was structured in three stages. In the first stage, the full list of documents for each environmental compartment was reviewed independently by two of the authors for inclusion. The review in this stage was based on article titles and abstracts alone, and inclusion criteria were general. Specifically, it was verified that the article did in fact address the compartment of interest (based on whether or not the authors reported collecting samples from that compartment), and it was verified that ARGs were measured as one of the variables in the study (using any analytical approach and/or method). Additionally, reviewers were instructed to include a maximum of 10 articles from 2018, since the extra time in publication for these articles could result in more citations, and we sought to avoid biasing our results towards those older documents. Each of the two independent reviewers for each environmental compartment produced their own list of “top 20” articles for that compartment, which were forwarded on to the lead author (TRB) for the second stage of review.

In the second stage, each pair of lists for each environmental compartment were compared to identify

discrepancies. Discrepancies were resolved by the lead author in consultation with co-authors and based on further review of the articles in question as necessary (e.g., by reviewing materials and methods sections to clarify the nature of sampling and/or analytical approach used). This stage of review produced five consensus lists, one for each environmental compartment. The lists for wastewater, livestock manure, drinking water, and groundwater each contained the target number of 20 documents, while the stormwater list contained only 12 (see Results for additional detail). These five lists were combined into a single list of 89 documents for the final stage of review (three documents were shared between compartments as discussed in section 4.1).

In the final stage, all 89 documents were reviewed in further detail to extract information of interest for the current study. In particular, the methods section of each document was reviewed in detail to identify the analytical method(s) used: polymerase chain reaction (PCR), real-time quantitative PCR (qPCR), high-throughput qPCR (HT-qPCR), droplet digital PCR (ddPCR), and/or metagenomic analysis. Documents reporting the use of PCR, qPCR, HT-qPCR, and/or ddPCR (but not metagenomics) were classified as using “targeted” methods. Documents reporting the use of metagenomics (but not any of the PCR-based approaches) were classified as using a “non-targeted” method. Documents reporting a mix of targeted and non-targeted methods were classified as “both.” Furthermore, *post hoc* assessment of our search results revealed a small number of studies using approaches that we had not initially anticipated and, rather than exclude these studies, we made the following assignments: isolation on selective media followed by PCR screen ($n = 3$; targeted), functional gene arrays ($n = 2$; targeted), and isolation on selective media followed by genomic sequencing and functional annotation ($n = 2$; non-targeted). Review assignments for this stage were randomized with respect to those in the first stage, such that reviewers were likely to screen papers other than those they had already reviewed in the first stage. For a full process overview, see Fig. 1.

4. Results and discussion

4.1. Characteristics of reviewed documents

Overall, we reviewed a total of 89 documents, 11 less than the maximum possible with our inclusion criteria. The discrepancy was due to a shortfall in the stormwater compartment. More specifically, the groundwater,^{16–35} livestock manure,^{36–55} drinking water,^{56–75} and wastewater^{25,57,76–93} compartments each included the target number of 20 documents, while the stormwater ($n = 12$)^{26,94–104} compartment did not have enough documents meeting our criteria (Fig. 1). This finding suggests that stormwater might be under-studied relative to the other four compartments. Additionally, three documents were shared between compartments, one between the groundwater and wastewater compartments,²⁵ one between the groundwater



and stormwater compartments,²⁶ and one between the drinking water and wastewater compartments.⁵⁷

These 89 documents were highly cited. The average citation count per document was 14.0 citations per year (from 2018–2020). Citation counts also varied by environmental compartment, from a minimum of 3.9 average citations per document per year (stormwater) to a maximum of 31.8 average citations per document per year (wastewater). These high citation counts are consistent with our approach to literature identification and review. We intentionally prioritized highly-cited documents in order to expedite our review process while maintaining focus on the most influential works in the field. Furthermore, variation in citation counts across the environmental matrix likely reflects varying publication practices and varying sizes of the research communities in different sub-fields of environmental AMR research. This finding emphasizes the importance of our decision to sub-divide the literature by environmental compartment in order to control for these differences.

4.2. Methods used in reviewed documents

The primary focus of our literature review was on categorizing documents with respect to the methods they employed for analyzing ARGs. Studies reporting use of PCR, qPCR, HT-qPCR, and/or ddPCR were classified as using “targeted” approaches, while studies reporting use of metagenomics were classified as using a “non-targeted” approach (“both” was an additional category and *ad hoc* designations were made for less common analytical methods, see section 3.3). Before this work commenced, we hypothesized that metagenomics would be the predominant method employed across recent impactful papers. Metagenomics is a newer approach and provides more information on the types of genes present, and we assumed that the most recent method would appear in the most recent documents. However, a majority of documents in our final results employed a targeted method. Overall, 85% of documents were classified as using a targeted approach or both targeted and non-targeted approaches together, while only 33% employed metagenomics or metagenomics with a targeted approach (Fig. 2).

Several factors may account for this finding, the first of which relates to utility and feasibility. For example, the PCR-based analytical methods underlying the targeted category are almost certainly more affordable and analytically accessible than metagenomics. Thus, it could be that researchers implicitly (or even explicitly) regard quantification of current threats as more feasible than quantification of future threats. Alternatively, it could be that researchers regard quantification of current threats as more *useful* than quantification of future threats because the current threats are more immediate. For example, at least some ARGs are known because they have already emerged and caused harm in a clinical setting.^{105,106} Thus, these



Fig. 2 AMR methods employed by environmental compartment.

current threats present a higher immediate risk than future threats that have yet to emerge.

4.3. Variation among approaches across environmental matrices

Varying trends in the methods used to characterize AMR in environmental matrices were found (Fig. 2). In each environmental matrix at least half or more of the studies used targeted methods. The distribution of methods for stormwater was notable compared to other compartments, with the combination of non-targeted and both comprising 42% of the methods, the largest percentage of any of the matrices. As an under-studied field of research, a wider breadth of studies with varying methods could indicate that the field of stormwater research has yet to define a consistent technique for AMR characterization, whereas other fields have (*e.g.*, manure and wastewater). Stormwater AMR is also a newer area of research in comparison to manure and wastewater which raises the question: are appropriate methods selected to address a question, or instead, are standard methods used in hopes of finding an answer to a yet-to-be defined question? Livestock manure and wastewater had the highest counts for targeted approaches. This result indicates that the priority of these fields is quantifying what we know and highlighting that quantification is important for surveillance and understanding how treatment systems work. A crucial component of surveillance, however, is identification of new genes for which non-targeted approaches are required.

Drinking water could also be viewed as a growing field in antibiotic resistance research. The metagenomic fraction (*i.e.*, non-targeted plus both) was larger in drinking water (40%) than in wastewater, manure, or groundwater. Metagenomics could enable researchers to determine if disinfection impacts the diversity of AMR in drinking water as well as if selective pressures are being exerted. As discussed in section 4.8, time-series studies that collect samples from the same location



over time could shed light on the role of selective pressures. These questions are also relevant to wastewater and manure where dense biomass and the selective pressures from different pollutants could be generating hotspots for horizontal gene transfer and resistome diversification. Thus, future directions for wastewater and manure should increase focus on metagenomics to answer these crucial questions. Moreover, only one of the 20 wastewater papers sampled biosolids. Wastewater effluent is a hotter topic than wastewater biosolids, but biosolids are also high biomass samples that should be given more attention in the future.

4.4. Implications of using targeted *versus* non-targeted methods for risk assessment

The framework of current and future threats relates directly to several important principles underlying the practice of risk assessment. These include the very definition of risk itself, as well as the general procedure involved in producing risk estimates. By definition, risk is formally identified as the product of two distinct quantities – 1) the probability of exposure to a hazard and 2) the damage caused once exposed.¹⁰⁷ Procedurally, risk assessment consists of four steps: 1) hazard identification, 2) hazard characterization (a. k.a., dose–response assessment), 3) exposure assessment, and 4) risk characterization.^{108,109} Thus, recognizing the terms “threat” and “hazard” as synonyms, the current threat *versus* future threat paradigm relates directly to the nature of hazards for which probabilities and damage are quantified as well as to the foundational steps of any subsequent risk assessments carried out.

In this work, we have argued that current threats and future threats correspond to targeted and non-targeted analytical methods, respectively. The relative use of targeted *versus* non-targeted methods varies across the environmental compartments reviewed here, with targeted methods predominating overall. Both methods have value, but they have different implications for risk assessment. Resolving the question of which method(s) should be used moving forward requires one to consider the nature of the environmental compartment under study. More specifically, we find it important to ask two related questions with respect to risk assessment for AMR in any specific environmental compartment:

1. Is this compartment a source where new ARGs and/or ARB commonly/frequently emerge?
2. Is this compartment an immediate point of human exposure for ARGs and/or ARB?

In other words, we propose that different environmental compartments correspond to different types of hazards. For compartments where the answer to question 1 is “yes”, these would be categorized as future threats. For compartments where the answer to question 2 is “yes”, these would be categorized as current threats. More generally, with questions 1 and 2 answered for a given environmental compartment, appropriate methods can be selected to inform risk

assessment. Admittedly, question 1 is very difficult to answer. Further work is needed to understand the reservoir of resistance genes and their stability over time and among locations within each environmental compartment. This gap in understanding also attests to the need for studying and identifying future threats across compartments. As this knowledge grows and we answer question 1 for each compartment we can better understand how to mitigate risks and stop the spread of novel AMR.

We believe livestock manure and wastewater are more likely sources of new ARGs and/or ARB due to their high genetic diversity, high biomass concentrations, and presence of chemical stressors. Thus, they would represent future threats under the paradigm proposed here, and non-targeted approaches would be the appropriate tool to employ. Research supports that animals can be reservoirs of new, unknown ARGs.¹¹⁰ In contrast, drinking water and groundwater are immediate points of human exposure for ARGs and ARB because large populations consume them on a daily basis.^{111,112} Thus, they would represent current threats, and targeted methods might be prioritized in these compartments to assess risk. Finally, stormwater crosses both categories. It represents an immediate point of human exposure to the extent it impacts recreational water, and it also can be mixed with sanitary sewage, metals, and other pollutants, thereby producing potential for emergence of new ARGs and/or ARB.

4.5. Strengths and limitation of targeted *vs.* non-targeted approaches

There are a few critical differences in the conclusions that can be drawn from taking a targeted quantitative PCR approach *versus* a non-targeted metagenomics approach. Targeted approaches can measure absolute abundances of ARGs and are particularly suited for time-series analyses that can inform about developing risks as ARGs and ARB increase. The limitation to targeted approaches is that they typically identify a narrow range of targets and are designed to match already known resistance genes. In contrast, non-targeted metagenomics approaches require no prior information and can instead detect any gene present as long as the depth of sequencing is adequate^{113,114} and the functional annotations are accurate.^{115,116} That is, qPCR is limited to examination of “current threats”, whereas metagenomics provides the opportunity to identify future threats. Primary limitations to the non-targeted approach include the cost of sequencing as well as available expertise for appropriately analyzing samples. Additionally, non-targeted approaches do not provide absolute quantification as readily as qPCR can provide. Ideally in all compartments, metagenomic sequencing would provide information about the diversity and relative abundances of the ARGs and ARBs and that information could then be used to follow up with targeted approaches to quantify changing absolute abundances.



Metagenomics also allows for a more nuanced hazard identification, *i.e.*, metagenomics can identify resistance genes that currently do exist but have not yet been identified by researchers. Longitudinal metagenomics can help understand the relative abundance of resistance alleles that arise through *de novo* mutation that are not typically detected in qPCR. Examples of this include loss-of-function mutations in negative regulators of efflux pumps that can cause cross-resistance to many antibiotics and the S81L mutation in DNA gyrase conferring resistance to fluoroquinolones.^{117–120} Surveillance with metagenomics methods can detect emerging resistance genes, including selective sweeps of alleles in a population.^{121–123} Other examples of current, yet unidentified risks that can be identified when shotgun sequences are analyzed include ARGs that were repurposed from other functions, newly mobilizable ARGs that were introduced onto unexpected plasmids or phage genomes, epistatically controlled ARGs where the genetic background unlocked new features, and pleiotropic ARGs.^{124–128} Metagenomics provides genomic context to the ARGs. Genome assemblies and annotations can predict if the ARGs are located on a chromosome, plasmid, resistance island, prophage, or other mobile genetic elements (MGEs).^{129–131} Accurate taxonomic assignment of the ARG-harboring microbe can also help inform risks. A chromosomally-encoded ARG from a non-pathogenic environmental isolate should be considered lower risk than an ARG on a MGE in an opportunistic pathogen. All of these nuances can and should influence risk assessments relating to ARGs and possible human exposure from environmental sources.

4.6. The role of genetic elements in characterizing AMR threats

MGEs are DNA regions that specialize in moving within and between genomes. These elements include plasmids, prophage, transposons, integrons, insertion sequences, and integrative conjugative elements. In most environments, MGEs play a crucial role in moving DNA (genes) among cells, and this has resulted in both significant within-species diversity (known as the species pangenome) and gene transfer between microbial species (*i.e.*, horizontal gene transfer). Ultimately, MGEs provide access to genetic determinants that would otherwise be unlikely to arise in a particular microbial lineage and thus accelerate the timeframe in which new combinations of antibiotic resistance can emerge. In other words, MGEs are key components in understanding future threats related to AMR and have been key components in understanding current threats.¹³²

It is known that the widespread use of antibiotics by humans has resulted in the selection of a diverse set of antibiotic resistance determinants that are now routinely carried by a variety of mobile elements.^{132,133} For example, the increase in disease severity from the pathogens

Pseudomonas aeruginosa and *Acinetobacter baumannii* was the result of resistance acquisition by mobile elements.^{132,134,135} Gene transfer among microbes occurs more frequently when microbial densities are highest, which often occurs in engineered systems (*e.g.*, wastewater treatment). It is also thought that antibiotic pollution creates hotspots for the assembly of ever more complex mobile elements with greater capacities for mobilization,¹³² so engineered environments are primary locations for these developments.

It is clear that MGEs play a large role in the fate of ARGs both within and among environments and thus would be key in assessing ARG risks. However, it is not trivial to quantify MGE activity *in situ*, to categorize their relationships with ARGs, or ultimately to place them into a health risk framework. These challenges have so far limited the number of studies examining MGEs in an environmental risk framework.¹³⁶ There is some evidence that the risk associated with different MGEs varies. Prophages seemingly are less likely to carry ARGs than other elements,¹³⁷ and plasmids can be grouped based on their promiscuity,¹³⁸ but more work is needed to make these measures useful.

As with ARGs, methodological considerations challenge assessing MGEs in an environmental context. MGEs are diverse, so it is difficult to pick meaningful targets to quantify when using PCR-based methods. By nature of being mobile, identical MGEs can be found in many different microbes, so with short-read DNA sequencing technologies, it is difficult to identify both the element and its host source. This identification is becoming easier with long-read sequencing technologies. Our results indicate that it is common for studies (63% of included papers) to quantify one or a few MGEs, the most common of which is the integron-integrase gene *int11*. Interestingly, the proportion of papers that quantified MGEs varied quite a bit between matrices. For both drinking water and wastewater categories, 75% of papers investigated MGEs while only 33% of stormwater papers included them. Groundwater (50%) and manure (70%) were in the middle of the environmental compartments. MGEs relate to risk from AMR by serving as a mechanism to spread ARGs to previously non-resistant organisms, and so as MGEs increase in numbers and diversity so too does AMR.¹³⁹ In this context, characterizing the quantity and/or diversity of MGEs is likely to inform on future AMR hazards, but health risk assessment is challenging because MGEs themselves are not directly harmful to human health.

4.7. Quantitative microbial risk assessment (QMRA) of AMR in the environment

In the terminology used here, published risk assessments for antimicrobial resistance to-date have focused largely on current threats. This stems from the use of quantitative microbial risk assessment (QMRA) in their approach, which requires that a specific hazard be identified. Examples



include risk assessments for methicillin-resistant *Staphylococcus aureus*,¹⁴⁰ fluoroquinolone-resistant *Campylobacter jejuni*,^{141,142} ceftiofur-resistant *Salmonella*,¹⁴³ ampicillin-resistant *Enterococcus faecium* infections,¹⁴⁴ quinupristin–dalfopristin resistance in *Enterococcus faecium* infections,^{145,146} macrolide resistance in both *Enterococcus faecium* and *Campylobacter* spp. infections,^{147,148} and fluoroquinolone and multidrug resistance in *Salmonella* infections.¹⁴² Notably, none of these prior QMRAs for AMR focus on the environmental compartments noted as points of exposure in section 4.4 (*i.e.*, drinking water, groundwater), and only one focuses on the role of the environment in general. The vast majority focus on foodborne exposures. Thus, QMRA for AMR in the environment is still in its infancy, even in terms of current threats, and improved hazard identification (*e.g.*, identifying a consensus group of high-priority AMR hazards relative to environmental transmission) could add substantially to the field's development.

Moreover, because the standard QMRA framework requires identification of specific hazards, QMRA itself is not well-suited to risk assessment for future threats. For example, the dose–response characteristics of future threats cannot be studied because the organisms and/or genes involved are, by definition, unknown. They cannot be isolated in a lab and administered in human feeding trials, as in the classic dose–response experiments used for QMRA of susceptible gastrointestinal pathogens.¹⁰⁸ Even for current threats, dose–response models are extremely limited. We know of only two models intended specifically for ARB (for gentamicin-resistant *Escherichia coli* and for methicillin-resistant *Staphylococcus aureus*).^{140,149} In other cases, dose–response models would have to be extrapolated from corresponding susceptible bacteria.

More generally, and returning to the SARS-CoV-2 analogy from section 2, we find it useful to consider the impact that each new variant has had on that pandemic's trajectory. Public health measures intended to mitigate transmission of COVID have been devised and implemented in the near-complete absence of the sort of dose–response information typically used to conduct QMRA. Furthermore, the most relevant risks with respect to SARS-CoV-2 seem to stem from what we would call future threats. Will a new variant be more or less infectious than the one currently circulating? Will vaccines be more or less effective against a new variant? This observation raises an important question with respect to AMR risk assessment. Given the potential importance of future threats to the long-term impacts of AMR, is it crucial to understand the dose–response characteristics of existing “variants” of ARB and/or ARGs? Is this information the limiting factor for risk assessments of environmental AMR?

Regardless of the answer, the genetic recombination of existing ARB and ARGs is likely to be useful. To that point, we argue that understanding the genetic diversity of ARGs in various environments provides an index for potential emergence of new genotypes – the precursor event for future

epidemics (*i.e.*, future threats). The literature search we have presented here suggests that, despite the relatively long-standing establishment of techniques for metagenomics analyses in the environmental field, metagenomic approaches in recent environmental AMR studies appear to be under-represented relative to qPCR and other “targeted” methods. This is particularly true for environments where future threats might be of greatest concern, like livestock manure and municipal wastewater where high biomass concentrations and genetic diversity provide ample opportunity for the emergence of new ARGs that could threaten the usefulness of antibiotics in the future.

More generally, we propose that risk assessment for future threats could be based on a combination of data from targeted and non-targeted methods. Specifically, AMR threats can be characterized in terms of both their sources and exposure routes (*e.g.*, see questions 1 and 2 in section 4.4). Moreover, numerous combinations of sources (*e.g.*, livestock manure, municipal wastewater) and exposure routes (*e.g.*, drinking water, recreational water, person-to-person contact) exist. Systematic One Health risk assessment for AMR could therefore be based on relative ranking of risk for the various source-exposure combinations of interest, with risk in sources indexed by the diversity of ARGs present and risk for exposure routes indexed by the abundance of ARGs present. The risk index for sources could be quantified using non-targeted approaches, assuming ARG diversity is proportional to the rate at which novel ARGs emerge. In contrast, the risk index for exposure routes could be quantified using targeted approaches, assuming that the abundance of ARGs transmitted per person per unit time is proportional to the rate at which a potential future threat would spread once it emerges. This approach is tractable, integrates both types of data, and lends itself to policy and management interventions at both the source and the point of exposure.

4.8. Implications on future research and the importance of time-series research

The value of using targeted *versus* non-targeted methods also relates to timescales and goals of a particular study. If the research goal is to identify the frequency that new ARGs arise in a compartment, then real-time monitoring using non-targeted methods will need to be employed. If the research goal is to assess treatment efficiency of ARGs, at a wastewater treatment plant (WWTP) for example, then employing targeted analysis to quantify influent and effluent ARGs over time would be required. Data processing time is also an important component. If a WWTP wanted to adjust process control parameters based on observed ARG removal, targeted methods such as qPCR can generate data in a manner of hours and be used to impact process operations if desired. Operational parameters could be tweaked and then abundance measured to determine if those changes improved ARG removal.



On the other hand, over a longer timescale of months, it would be important to know if a WWTP serves as a hotspot for the proliferation of antibiotic resistant organisms, which could contribute to the emergence of new ARGs and/or horizontal gene transfer, thereby increasing the diversity of ARB. HGT is dependent on many factors, including cell density, host and recipient phylogeny (*i.e.* cell structures/defenses), MGE diversity and concentration, and the stresses caused by the physical environment. WWTPs and other water infrastructure alter these factors in ways that suggest HGT and selection for new combinations of mobile elements could be elevated when compared to most natural environments, and a few studies have indicated that this is the case.^{150–153} In theory, a more diverse set of MGEs carried in a microbially diverse system would lead to ever-increasing access by individual organisms to a diverse gene pool and greater potential for the development of new resistant microbes of consequence to human health. The mitigating factor to this scenario of ever-increasing ARGs is the fitness cost that is often associated with acquiring and maintaining ARGs and MGEs in the absence of inhibitory concentrations of antibiotics. Thus, it is important to establish a baseline of current ARG/MGE diversity conditions for each treatment plant in order to evaluate its role in the development of new resistant microorganisms. At this time, metagenomics performed through time is the method that could deliver the necessary information. There is currently a dearth of research employing metagenomics over long time-scales within a given compartment. This dearth represents a crucial gap to fill for the advancement of risk assessment on antimicrobial resistance. Furthermore, based on emerging sequencing technologies such as Oxford Nanopore,¹⁵⁴ in the near future long read metagenomic sequencing will become more common. These sequences will eventually be able to be read in near real-time, meaning that within hours of sampling the MGEs, ARGs, and their host organisms could be known. This approach could likely change how risk assessment is done and help fill in gaps described above about identifying current and future threats.

Future work could also look more deeply at specific niches in any of the five compartments surveyed in this paper. For instance, identification of sewers as a hotspot of threats might be managed differently than wastewater treatment effluent hotspots. Sewers could be hotspots because of environmental conditions within a sewer or because of potential hospital or industrial effluents that feed into them. Wastewater treatment effluent as a hotspot could depend on the treatment processes used at a given plant.

In this perspective, we set out to understand which methodologies have been used in recent highly-cited papers addressing AMR in environmental matrices. Based on our results, most studies implicitly focus on current AMR threats. Moving forward, however, it will be critical to refine approaches geared towards identifying the risks associated with as-yet unknown future threats.

Author credit statement

TB: conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, writing – original draft, writing – review and editing. RN: conceptualization, data curation, formal analysis, validation, investigation, writing – original draft, writing – review and editing. LK: investigation, writing – review and editing. EL: data curation, formal analysis, investigation, visualization, writing – review and editing. KO: investigation, writing – review and editing. SMT: investigation, writing – review and editing. CM: writing – review and editing. PM: conceptualization, investigation, methodology, project administration, supervision, validation, writing – original draft, writing – review and editing.

Conflicts of interest

There are no conflicts of interest to declare.

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