# Standardization in Global Environmental Antibiotic Resistance Genes (ARGs) Surveillance

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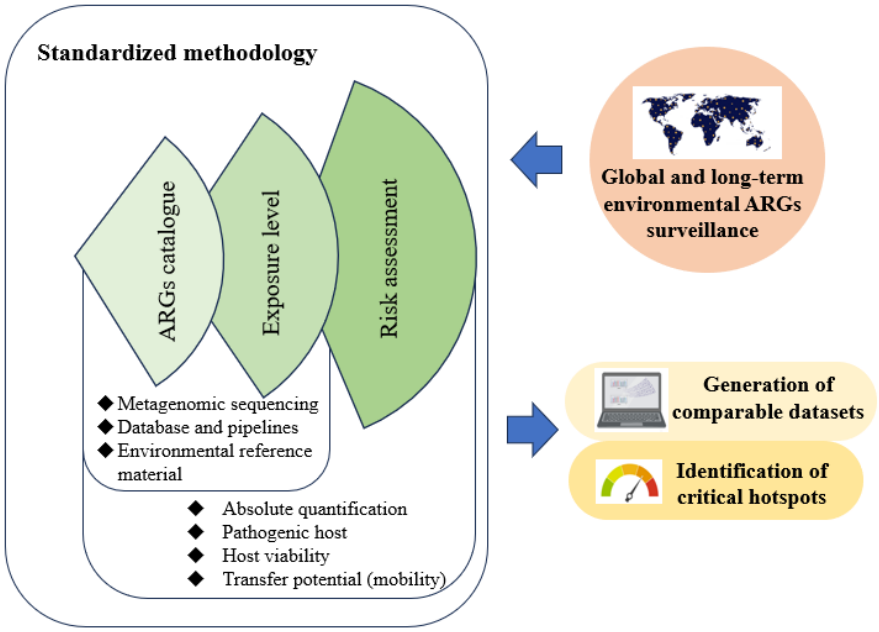
**Abstract**

Antimicrobial resistance (AMR) poses an increasing threat to global health. To deliberate the distribution and transmission of environmental resistance in a wide geographic and longitudinal scope, standardization of the analytical methods is desperately required for the extensive implementation of large-scale environmental antibiotic resistance gene (ARGs) surveillance. In this review, a standardized surveillance method using metagenomic analysis, coupled with proper quantification tools and environmental reference materials as technical benchmarks, was established to facilitate the generation of comparable and informative resistome datasets. As global and long-term ARGs surveillance has recently been performed in various environmental compartments, increasing efforts are also needed for assessing the health risk of ARGs. The development of risk assessment schemes that incorporate factors including transfer potential, host species, viability, and absolute quantification is essential to the regulatory guidelines for high-risk priority ARGs. This review provides guidance to ARGs surveillance regarding the level and the risk of ARG exposure, especially to identify and address critical hotspots.

**Keywords:**

Antimicrobial resistance (AMR), Antibiotic resistance gene (ARGs), Risk assessment, Metagenomics, Standardization, Quantification

## Graphic abstract



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## 1. Introduction

Antimicrobial resistance (AMR) is an increasing threat to global health. In clinical perception, the main risk of antibiotic-resistant genes (ARGs) and antibiotic-resistant bacteria (ARB), especially antibiotic-resistant pathogens, is causing antibiotic treatment failure for humans and animals. Since the first antibiotic-resistance case was well-documented in the 1940s, ARB, especially antimicrobial-resistant pathogens have attracted widespread global attention (De Oliveira et al., 2020; Zignol et al., 2016). It is worth noting that AMR is not only a medical issue but also has environmental dimensions. The concept of "One Health", which recognizes the interconnectedness of the health of humans, animals, and the environment, is now widely accepted within the scientific community. The prevalence of ARGs in diverse environmental habitats could contribute to their spread and transfer, potentially leading to the failure of antibiotic therapy in humans and animals through exposure. For example, the outbreak of *Salmonella enterica* infection resistant to first-line drugs in Pakistan from November 2016 to December 2017 was suspected to be attributed to the contaminated drinking water (Qamar et al., 2018). Additionally, workers from animal farms or live poultry markets were threatened by a higher AMR risk with occupational exposure to the resistome from their working environment (Maciel-Guerra et al., 2022; Wang et al., 2021). These examples clearly warned us of the urgency for the surveillance and mitigation of environmental AMR. The efforts to control the ARGs and ARB at the hotspots in the environment are much cheaper and more effective than in hospitals and farms.

To control and tackle AMR issues and ARGs pollution, a better understanding of the distribution and transmission of ARG in the environment is needed. Previous studies have illustrated the

prevalence of ARGs and/or ARB in various environmental compartments, such as wastewater (Munk et al., 2022), drinking water (Ma et al., 2019), soil (Delgado-Baquerizo et al., 2022), and air (Xie et al., 2022). The degree of human activity was the main driver closely related to the distribution of resistance genes in the environment (Li et al., 2015b). Since AMR is a global issue, international collaborations are desperately needed to monitor the exposure level of ARG pollution although there would be some barriers and difficulties. World Health Organization (WHO) launched The Global Antimicrobial Resistance and Use Surveillance System (GLASS) in 2015, aiming to standardize routine sample collection for clinical purposes for a set of pathogens (WHO, 2020). There have been sporadic global studies for environmental AMR surveillance in the last few years, mainly on wastewater treatment plants (WWTPs) (Munk et al., 2022) and soil samples (Delgado-Baquerizo et al., 2022). However, our knowledge of the global distribution and spread of the environmental antibiotic resistome is still limited. In addition to global geographical monitoring, long-term surveillances at environmental hotspots are also of great importance. For these purposes, more systematic and comparable datasets are needed to identify important pollution sources and compare the level of resistance in different areas. Through this approach, we can gain a complete understanding of the spread and evolution of resistance genes over time and how polluted environments impact the proliferation of antibiotic resistance.

Furthermore, it is critical to assess the risk of ARGs at various exposure levels. Bacteria could spontaneously evolve to be antibiotic-resistant *via* genetic mutations in natural environments. The excessive usage and discharge of antibiotics in the environment has significant ecological toxicity (Sharma et al., 2023) and drivers the AMR development (Dubey et al., 2020). The

massive proliferation of ARB increases the ARGs in the environment by vertical gene transfer under antibiotic selection pressure; and the horizontal gene transfer (HGT), including conjugation, transformation, and transduction, facilitates the spread of genes, which could occur across different environments and along bacterial lineage even linking non-pathogenic bacteria to pathogens (Smillie et al., 2011). Thus, for the risk assessment of ARGs, their HGT potential and association with pathogens are important indexes considering their contribution to AMR development in the environment. However, there is no agreed general framework yet, and a standardized and quantitative approach is needed to address the ARG pollution and its risk.

The global surveillance and risk assessment of AMR at a large spatial-temporal scale requires establishing a standardized methodology and transforming it into a routine activity of environmental-quality monitoring. This review aimed to identify effective approaches for standardizing methodologies for monitoring ARGs in the environment so that frequent and regular global surveillance can be implemented and the risk of ARGs can be assessed in a uniform framework. In this review, we systematically elaborated on the current studies and main knowledge gaps in the monitoring and risk assessment of ARGs in environmental compartments. Constructive suggestions on a standardized surveillance framework based on metagenomic analysis and benchmarking were given. This review also outlined the future research directions on global and long-term surveillance of ARGs and ARBs in the environment, with an emphasis on incorporating absolute quantification and host viability into the risk assessment framework surpassing the previous frameworks.

## 2. Detection and analysis methods of ARGs

The prevalence of AMR at the global scale has been revealed by recent studies from environmental and clinical perspectives (Zhang et al., 2022b; Zignol et al., 2016). A prerequisite for obtaining a global perspective of the environmental antibiotic resistome is to delineate ARGs irrespective of the differences in different geographical regions, the time frame, or the environmental compartment. The convincing results require consistent experimental and analysis methods, including the process of sample collection, pretreatment, DNA extraction, ARGs detection, and bioinformatic analysis (Figure 1). Therefore, it is imperative to standardize the framework to improve the comparability of datasets across worldwide studies.

## 2.1 Comparison of metagenomics and qPCR methods

Quantitative polymerase chain reaction (qPCR) with the advantages of being highly sensitive and low cost, has been broadly applied to evaluating the prevalence of ARGs, especially for highly clinically-relevant ARGs (Keenum et al., 2022; Pruden et al., 2021). Similarly, ddPCR (droplet-digital polymerase chain reaction) is more tolerant to PCR inhibitors, and could detect the concentration of specific ARGs with a lower limit of detection than qPCR (Cao et al., 2015). Both qPCR and ddPCR are based on the PCR technique and fluorescent signals. One of the primary drawbacks of the PCR technique is the requirement for pre-selection of targets, which leads to a limited number of available primers and low throughput. Another challenge is the notorious PCR bias, that is, PCR results could be affected by many factors, including the primer design, reaction volume, instrument, matrix effect, and the expertise of the operator, etc. (Park et al., 2021). This makes it challenging to directly compare the performance of qPCR or ddPCR. For environmental samples, inhibitors from different environmental contaminants could

exacerbate the problem (Kokkoris et al., 2021). Although researchers have attempted to address these issues by normalizing the quantification to the 16S ribosomal RNA (rRNA) gene abundance within the same sample, and using the same equipment and operational conditions (Parnanen et al., 2019), the disparities in the qPCR or ddPCR data from different labs or regions cannot be ignored in the interpretation and comparison of global antibiotic resistome data.

Metagenomic sequencing is a promising method for the detection and quantitation of ARGs with higher feasibility for standardization at a global scale. Without the limitation of primer design on targeted ARGs sequences, metagenomics has higher throughput and broader detection spectrum of ARGs compared to qPCR or ddPCR. Metagenomic data could also help discover novel ARGs if combined with functional screening *via* deep learning approaches (Arango-Argoty et al., 2018). Additionally, the metagenomics results would provide more contextual information, including bacterial compositions, ARG hosts, mobile genetic elements (MGEs), and other genes that provide co-selection opportunities, e.g., gene elements associated with heavy metal resistance (Li et al., 2022; Ma et al., 2016). More importantly, the previous datasets of metagenomics could be retrospectively re-analyzed by more advanced tools in the future when the knowledge of the novel resistance genes, MGEs, or pathogen species is available. Although the current metagenomic method is not comparable to qPCR in terms of detection sensitivity and cost, the advantages mentioned above have made metagenomic sequencing the most promising tool in the ARG surveillance. In summary, qPCR and the metagenomics have their own strengths and complement each other (Table 1). To study the global resistome, a whole collection of ARGs in complex environmental systems in a wide spatio-temporal span, the metagenomic method is much more preferred, especially considering

the comparability.

## 2.2 Database and bioinformatic tools

The comprehensive database is the core of the analysis of ARGs *via* metagenomics methods. Various databases and analysis tools have been designed for the annotation and quantification of ARGs. The Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2022), ResFinder database (Bortolaia et al., 2020), and Structured Antibiotic Resistance Genes (SARG) database (Yin et al., 2018), are some widely used databases for the analysis of ARGs. Understanding the structure and differences of databases is crucial for choosing the appropriate one. ResFinder, the first online bioinformatics tool, has its own ARGs database for the detection of ARGs (ResFinder database) and chromosomal gene mutations for a set of pathogenic microorganisms (PointFinder database). In 2020, a new version was released which included genotype-to-phenotype tables compiled by searching various databases and extensive literature (Bortolaia et al., 2020). CARD is an ontologically structured database that is updated based on an interplay of manual literature curation, computational text mining, and genome analysis (Jia et al., 2017). CARD encompasses large numbers of ontology terms, reference sequences, as well as mutations, accompanied by a bioinformatic tool, Resistance Gene Identifier (RGI) software, to annotate ARG from genomic or metagenomic sequences (Alcock et al., 2022). The newest version v3.2.4 announced in 2022 further expanded the curation to 458 gene families (180 new since v3.0.3 announced in 2020), and introduced the CARD Short Names, a CARD-specific abbreviation for ARG names associated with Antibiotic Resistance Ontology terms, to support machine learning algorithm (Alcock et al., 2022). SARG database was firstly developed

by merging CARD and Antibiotic Resistance Genes Database (ARDB) in a hierarchical structure (type-subtype-reference sequence) with some improvements (Yang et al., 2016), and now it was further expanded by adding more carefully selected and curated ARGs from NCBI-NR database and CARD (Yin et al., 2018). In addition, one more information layer, mechanisms of ARGs, was added for a better understanding of ARGs, and different sub-databases were generated respectively for long reads and short reads (Yin et al., 2022a). The database has been updated to a more comprehensive and accurate version as the sequencing technique and analysis demand developed. Besides, an online platform, ARGs-OAP, was also developed for fast annotation and quantification of ARGs from environmental metagenomic datasets based on SARG, which can be accessed through both a web interface and a command line software. The ARGs-OAP also provided high-throughput screening of potential new resistance genes using Hidden Markov Models (Yin et al., 2018).

ARGs-OAP is recommended in this review as one of the standard quantification pipelines of environmental ARG, because of its comprehensive database, concise interface, and easily interpretable outputs with diverse units. Besides the default SARG database, the newly announced version of ARGs-OAP also supports a customized database (Yin et al., 2022a).

Different popular quantification units are available following the ARGs-OAP pipeline, including copies of ARGs per prokaryote's cell, copies of ARGs per copy of 16S rRNA, 'RPKM' (number of ARGs sequences per kilobase per million sequences), etc. (Yin et al., 2022a). The unit "copy per cell", with the normalization considering sequencing depth, ARG lengths, and the variable numbers of 16S rRNA in different species, is a more preferable way to present the relative abundance of ARGs, since it has the straightforward biological meaning for better

communication and comparison, and could be developed into absolute abundance, which is more useful in comparing the exposure level and finding polluted hotspots (Yin et al., 2022b).

### 2.3 Environmental reference materials (ERM)

Reference materials with known characteristics have crucial roles in sequencing by providing standards for quality control and proficiency testing (Hardwick et al., 2017). The impacts of the common technical variables during the generation of metagenomic datasets, such as extraction kit, skills of operating person, extraction batch, library preparation batch, and even different sequencing platforms, cause difficulty in discerning confident biological signals, especially for longitudinal time-series studies or inter-laboratory comparison. To benchmark these technical variables, an ERM should be introduced into the sample batch at the very beginning of sample treatment to ensure precision and reproducibility. The ability of ERM to reflect the consistent impacts of the undesirable variations to real environment samples is important. Unlike simple commercial mock communities, environmental samples retain high community complexity and more matrix effects, which challenges the selection of proper ERM. Thus, ERM is required to have the characteristics much closer to the real sample to reflect the consistent impacts. For example, in the ARGs surveillance of activated sludge samples, ERM prepared using the well-homogenized activated sludge will be preferred (Yang et al., 2023a). For scientific community-wise standardization in comparative studies of large-scale environmental ARGs surveillances, the prepared ERM can be split into a large number of small portions, and shipped to different labs to serve as benchmarks.

## 3. Global environmental antibiotic resistance surveillance

Antibiotic resistance is frequently found in various environmental compartments such as WWTPs, aquaculture systems, livestock facilities, hospitals, and industrial effluents (Figure 2). These environmental compartments provide both reservoirs and transmission paths for ARB and ARGs (Huijbers et al., 2015). Global and longitudinal studies on ARGs will give more insights into their geographical and temporal trends of abundance and diversity profile. This section summarizes the recent studies on global ARGs and ARB surveillance (Table 2), with a special focus on the water environment, and highlights the requirements to develop a standardized pipeline for a better comparison of large-volume datasets across disparate temporal and/or spatial ranges.

### 3.1 Wastewater treatment plants (WWTPs)

As one of the connection hubs between human and the environment, wastewater treatment plants are important sources of ARGs, especially the activated sludge process which has been widely used for nutrient removal from wastewater in WWTPs (Bengtsson-Palme et al., 2016; Pazda et al., 2019). Sewage receives pollutants from animal and human excreta, industrial and hospital liquid waste, etc., and has unique characteristics of high bacterial diversity, high biomass, intensive cell contacts, and interactions. Besides, antibiotics and other pollutants adsorbed on the surface of activated sludge flocs could be the selective pressures for ARGs (Zhang et al., 2021b). All these factors provide favorable conditions for the horizontal transfer of ARGs. In activated sludge, the enhanced HGT, together with the rapid growth of bacteria will further deteriorate the ARG proliferation. A 9-year longitudinal metagenomic study of the

activated sludge collected from a WWTP of Hong Kong showed that the relative abundance and profiles of ARGs changed significantly every two to three years, and the resistome of activated sludge has higher HGT potentials compared with soil resistome (Yin et al., 2019). In the end, ARB and ARGs in sewage and activated sludge could enter the treated wastewater and be discharged into the environment. For example, a large-scale WWTP (440,000 population equivalents) in Germany with the annual mean discharge of 1.165 $m^3/s$ of the effluent discharged 1.97 E+17 *Escherichia coli* and 1.4 E+13 *Enterococci* cells per day into the receiving river (Jager et al., 2018). The continuous discharge of the large amount of effluent definitely will increase the risk of resistome in the natural aquatic environment (Corno et al., 2019; Schwermer & Uhl, 2021).

Metagenomic analysis of sewage was regarded as an ethically acceptable and economically feasible approach for continuous global surveillance and prediction of AMR (Hendriksen et al., 2019). The influent of a WWTP could be used as the representative sample of resistome in the guts of a human population of thousands of people from the catchment area. Global AMR monitoring in sewage has revealed regional patterns influenced directly by geography in the abundance and diversity of ARGs, while shared ARGs were also identified among different regions (Hendriksen et al., 2019; Munk et al., 2022; Riquelme et al., 2022). Among these studies, the biggest global sewage datasets for ARG surveillance included 757 sewage samples from 243 cities in 101 countries covering a 3-year time series, and the results suggested a more locally tailored solution for fighting AMR (Munk et al., 2022). These studies involve large volume of sample collection and processing in different labs from global wide, thus a standardized and consistent protocol for experiments was crucial to reveal the geographic

hotspots and the socioeconomic factors affecting ARGs transmission events.

### 3.2 Aquatic environments

Aquatic environments, especially surface water such as rivers, lakes, and oceans, are environmental reservoirs of ARGs, receiving WWTP effluent and runoffs affected by anthropogenic activities. Human fecal pollution could enter surface water via discharged effluents through aging or inadequate wastewater infrastructure (Damashek et al., 2022). The discharge of effluent from coastal fishery farms results in an increase of ARGs in the marine environment (Raza et al., 2022). If surface water or reclaimed wastewater is used in agriculture irrigation or fishery farming, it can introduce ARGs into food, leading to human exposure to ARB and ARGs through food consumption (Christou et al., 2019; Giatsis et al., 2015; Liu et al., 2020). In addition, there are significant connections between surface water and groundwater in terms of their ARG profiles, especially stronger during the rainy season (Zhang et al., 2022a). As the source of freshwater for drinking, groundwater contaminated with ARGs and ARBs pose high resistant risk to human (Zainab et al., 2020).

However, in addition to these snapshot studies from different aquatic sectors, there is a lack of surveillance over a large spatiotemporal scale, and the occurrence, proliferation and dissemination of ARGs in aquatic environments have not been completely evaluated yet. Many studies of ARGs in surface water used the methods of qPCR and ddPCR to detect specific types of ARGs and other indicators (Damashek et al., 2022; Raza et al., 2022). As discussed in the second section, these amplification-based methods have low throughput and have big challenges in performing cross-study comparison. Using metagenomic analysis, the antibiotic

297 resistome of a single water body could be directly compared to previous studies from other
298 regions to assess the prevalence of ARGs, as well as the dissemination risk indicated by mobile
299 genetic elements (MGEs) (Chen et al., 2019). Although the different analysis pipelines were
300 applied in different studies, the publicly released datasets could be downloaded and re-analyzed
301 using the same tools. For this purpose, there is an urgent need to adopt a consistent protocol in
302 the sample processing procedures in different batches or labs before the sequencing data is
303 generated, so that the technical variation could be controlled and a more nuanced view of data
304 interpretation could be realized. Additionally, detailed metadata is essential for antibiotic
305 resistance research in aquatic environments, especially socioeconomic factors such as human
306 population density and type of land use, besides traditional metadata of coordinates of sampling
307 sites and environmental factors. It has been reported that along a single river increased antibiotic
308 resistance was observed as human activities increased (Lee et al., 2020). Collecting data on
309 socioeconomic factors in a standardized and quantitative way allows for the systematic
310 evaluation of the impact of human activity on the environmental resistome.
311
312 **3.3 Drinking water**
313 ARGs and ARB remaining in treated drinking water pose health risks to the human gut by direct
314 exposure or indirect transmission among water bacteria to human-related bacteria (Vaz-Moreira
315 et al., 2014). While some disinfection technologies of drinking water treatment could decrease
316 ARGs and ARB, the UV disinfection even showed to enrich the total ARG abundance, for
317 which bacterial community shift may be the key factor (Jia et al., 2020). There is also concern
318 that disinfection may increase the potential for ARG transfer among bacterial genera (Jin et al.,

2020). Therefore, there should be greater concern about monitoring the resistome in drinking water, which is currently not a routine practice. Previous studies have revealed that a broad spectrum of ARGs was found in the tap drinking water over a wide range of countries and regions, with an abundance range of 0.028 to 1.0 copies of ARG per cell (Ma et al., 2017; Ma et al., 2019). ARGs harbored by pathogens make the circumstance worse, as they have the potential to induce resistant bacteria infection, and lead to antibiotic failures and disease outbreaks (Qamar et al., 2018). In disinfected drinking water and tap water, pathogens contributed to 14.7 and 18.5% of the total bacteria community, which is higher than that of raw water before disinfection (Dias et al., 2020). Based on the detection of ARGs and pathogen in drinking water, Ma et al. proposed a strategy to classify drinking water samples into three categories: A- ARG-carrying pathogens detected, B- ARGs and pathogens detected but no ARG-carrying pathogens, and C-only ARGs detected (no pathogen detected) (Ma et al., 2019). This classification system provided a solution to reflect the potential risks for further management of drinking water in terms of AMR. However, it's still hard to assess the risk only based on the relative abundance, as the lower relative abundance of pathogen or ARGs may be the result of dilution by the higher total cell concentration in raw water before disinfection. Absolute quantification of ARB and ARGs is necessary in this context.

### 3.4 Other environmental habitats (soil, air, etc.)

The various environmental sectors are inextricably linked to each other. Although there are unique microbial feature and ARGs profile in each environmental habitat, clear transitions and connections of the bacterial communities existed among air, soil, water and human-associated

habitats (Zhao et al., 2022). Similar to ARGs surveillance in water environments, the methods and principles discussed above could be extended to surveillance for other environmental settings. Soil AMR has also begun to receive global attentions; however, it remains lacking in comprehensive understanding and the prediction for global antibiotic resistome hotspots. One recent study analyzed topsoil ARGs from 1012 sites from 35 countries across all continents using a high-throughput quantitative PCR approach targeting 285 unique ARGs and 10 MGEs, indicating the direct relationship between MGEs and ARGs is far more important than the effects of other environmental factors (Delgado-Baquerizo et al., 2022). However, the conclusions were confined to the occurrence and relative abundance of ARGs and a small number of MGEs by the limitation of throughput of qPCR. Zheng et al. re-analyzed 1088 soil metagenomic data retrieved from public archives to generate a high-resolution quantitative map of ARGs, and revealed the tight association of soil ARGs with anthropogenic factors such as livestock, irrigation, and manure (Zheng et al., 2022). In order to obtain solid conclusions based on data collected from public archives, strict screening criteria must be applied to consistent experimental design and accurate habitat information.

To identify the potential emission sources of ARGs and understand their dissemination in the environment, quantitative source tracking based on metagenomic sequencing and machine-learning classification provided a practical solution (Li et al., 2020). Our previous study developed a source-tracking platform and revealed the contribution of human feces, animal feces, and activated sludge from wastewater treatment plant to antibiotic resistome in sediment samples (Li et al., 2018b). But it's important to note that the identification of sources is largely dependent on the model built by representative datasets, which requires a holistic record of

resistome of diverse putative sources covering a wide geographical and temporal scale.

## 4. Factors to be addressed for risk assessment of ARGs

To define the emergency of controlling ARGs, more emphasis should be put on the risk of ARB and ARGs to human and animal health. With increasing studies on ARGs, it has been realized that risk assessment of ARGs in the environment resistome is more important than simply abundance profiling (Martinez et al., 2015). However, there is still no widely accepted framework for assessing the risk of ARGs at various levels of exposure. Recently, an ARG risk assessment framework was proposed based on three major factors, including whether the ARG is enriched in an anthropogenic environment, its HGT potential (gene mobility), and whether carried by pathogens (Zhang et al., 2021a). This framework proposes a basic risk assessment using metagenomic data, and provides a practical method to identify those high-risk ARGs from the general surveillance profile. Based on this framework, a list of ARGs with high priority to be controlled in the environment was proposed which could be periodically updated with new datasets and knowledge. In several recent studies related to the risk assessment of ARGs, a similar core principle was adopted to identify high-risk ARGs in global samples from various habitats like sewage and animal feces (Qian et al., 2021; Zhang et al., 2022b). Indeed, host pathogenicity, mobility, and prevalence in human-associated environments were widely acknowledged as three essential factors in predicting the human health risks of ARGs; In addition, the viability of ARG-carrying bacteria cells is also taken into account in this review, as live cells contribute more for the transfer of ARGs in the environment than dead cells (Figure 3).

### 4.1 Host tracking

Due to the emergent risk that ARGs harbored by pathogens pose to human health, there has been a focus on tracking the host of these genes. Earlier research (Li et al., 2015b) that examined the potential host of ARGs based on empirical associations between the abundance of ARGs and bacterial species often overestimated the strength of these relationships, as spurious correlations may also result from their synchronous responses towards the same hidden factors (Rice et al., 2020). The rapid development of high-throughput sequencing and bioinformatic tools enable contig and/or genome assembly, which largely facilitates identification of co-occurrences of ARG and host, as well as related MGEs in various environmental compartments (Ma et al., 2016); however, most of the assembled contigs are not long enough, and the number of contigs and MAGs are also limited, resulting in massive loss of low-abundance ARGs (Qiu et al., 2022; Su et al., 2022). For example, in a previous study, only 1200 ARG-carrying contigs over 500 bp were assembled and identified for host tracking, and these contigs only carried 35.8% ARG subtypes found in the short-read based analysis (Qiu et al., 2022). Compared to the methods based on short-read assembly, direct long-read sequencing is a more promising technique for host tracking. As two dominant long-read sequencing platforms, Nanopore sequencing of Oxford Nanopore Technologies (ONT) recorded the longest read length of 4 Mb (https://nanoporetech.com/products/promethion), and single-molecule real-time (SMRT) sequencing of Pacific Biosciences (PacBio) could provide the read length up to 50 kb (Amarasinghe et al., 2020). Unlike the probability-dependent co-occurrence analysis and contigs assembly algorithm, long reads gave the true physical linkage pattern of ARGs, host

and MGEs. Using Nanopore long sequences, a total of 10 resistant pathogens were detected in a WWTP in Hong Kong, most of which carried multiple ARGs (covering 6 types of resistance) (Che et al., 2019). The host tracking also demonstrated a variety of ARB were present both in influents and effluents of WWTP, indicating they were not completely removed and with high potential to disseminate into receiving environments (Che et al., 2019). Further coupled with strains or population screening by selected antibiotics, the long-reads-based method could detect more ARG hosts with low abundance (Che et al., 2022; Peng et al., 2022).

### 4.2 Horizontal gene transfer (HGT)

ARGs could spread through HGT *via* MGEs, including plasmids, transposons, integrative conjugative elements (ICEs), and bacteriophages (phages). In environmental resistome, ARGs co-existed with MGEs, were often regarded as high human risk as they had higher potential to transfer to pathogens. High transfer potential was found in activated sludge biofilm bacteria, which highlights the need for increased awareness and caution when it comes to controlling and assessing the spread of ARGs in WWTPs (Qiu et al., 2018). Direct experimental evidences were provided for the function of conjugative plasmids and ISs in mediating the transfer of ARGs in complex communities of activated sludge (Che et al., 2021). MGE-mediated ARG dissemination was also confirmed between human gut commensals and pathogens, and some broad host range elements were even predicted to have crossed bacterial phyla (Forster et al., 2022). Recently, phages from diverse environments (e.g., pig feces, sewage, marine and freshwater) have been found harboring ARGs and may facilitate the transfer of antibiotic resistance among bacterial hosts in natural environments (Lekunberri et al., 2017; Wang et al.,

2018).

To understand the dissemination and HGT mechanism in the environment, long-read sequencing is an effective method to reveal the connecting patterns of ARGs and associated MGEs. Similar to host tracking, long reads provide more accurate and direct evidence for the physical linkage of ARG and MGE. Using Nanopore and Pacbio sequencing technologies, it has been demonstrated that the transfer potential of different ARG types varied significantly, and those ARGs in lower abundance might have higher risk due to the location on plasmids (Qian et al., 2021). In the three compartments of WWTPs, influent, AS and effluent, plasmid-associated ARGs contributed to a larger proportion of the total resistome than chromosome-carrying ARGs (Che et al., 2019). However, it should be noted that it is challenging for computational methods to accurately determine the host bacteria for plasmids, and the extraction of plasmids from complex environmental samples was also hindered by the bias on smaller plasmid size and contamination of chromosomal DNA (Li et al., 2015a). As promising technologies, Hi-C and meta3C (metagenomic chromosome conformation capture) based on the *in vivo* proximity-ligation have been applied to capture the interaction of plasmid, integron or phage with host chromosomes, although there are still many challenges such as the clustering artifact caused by highly abundant genomes in complex samples (Marbouty et al., 2017; Stalder et al., 2019).

### 4.3 Absolute quantification of ARGs and ARB

The absolute quantification could generate more meaningful biological insights, and facilitate the comparative analysis across different environmental sectors. It's quite challenging for the

comparison using relative quantification of ARGs, which only provides partial observations of dynamics and may cause misleading correlations. Among different absolute quantification methods, a simple one is to calculate the concentration of ARGs using metagenomic data based on total microbial DNA mass under the assumption of a consistent extraction efficiency (Yin et al., 2022b). Another way is to use spike-in cells or internal standard DNAs to correct variations in DNA extraction and sequencing (Wang et al., 2024). Yang et al. developed a method using *Escherichia coli* spike-in cells in known numbers labeled with a marker gene (a *mClover3* fluorescence protein gene), and Nanopore sequencing data was used to investigate the removal of ARGs and pathogens in WWTPs (Yang et al., 2022). In this circumstance, compared to the relative abundance, the absolute concentration is a better indicator for quantitatively measuring the risk associated with treated wastewater.

### 4.4 Viable cells

Live bacteria cells are the viable carriers of genes, and serve as reservoirs which are more significant than dead cells. The culture-based method for the identification of viable cells and their resistance phenotypes served as a golden standard methodology. However, the high abundance and diversity of bacterial communities in environmental samples interfere with the cultivation of ARB of low abundance (Schreiber et al., 2021). Additionally, ARB cells in the viable and not culturable state also escaped from cultivation, although they even have higher antibiotic resistance (Lin et al., 2017). Given these reasons, the sequencing-based method could be an alternative method for environmental surveillance of viable ARB, as demonstrated in a recent study differentiating viable cells and dead cells based on robust metagenomic sequencing

by applying propidium monoazide (PMA) to prevent nonviable DNA amplification (Yang et al., 2023b). Although this method may be compromised by lower sensitivity at this time, it caters to the need to fine-tune ARB/ARG risk assessment.

To conclude, to get an accurate result in global and long-term ARG surveillance, it's essential to establish and follow a standardized framework to uniform the methods, avoid the technical errors introduced in the large-scale sampling campaign, and also make it possible for re-analysis of the datasets in future studies. There are still some challenges in each part of the whole framework, which calls for more careful evaluations and joint efforts by the scientific communities. From the very beginning of the sample collection and treatment, a thorough design is needed and the incorporation of proper ERM in the pretreatment of samples is encouraged. A widely accepted framework for assessing the risk of ARGs is urged to be constructed and applied, in addition to the monitoring of ARG exposure levels. Although we have incorporated four factors in the risk assessment in this review, further improvements in the analysis methods of each factor are still needed for a more precise assessment, especially for the absolute quantification and the host viability.

With all these aspects discussed in this review, it's hopeful to establish the scientific criteria and regulatory standards for priority ARGs with high risk, like the microbial water quality standard for *E. coli* and other pathogens. The final and most important aim is to implement mitigation measures to reduce the exposure level and the risk, especially to take action at the critical environmental hotspots.

## 5. Conclusions

Taking steps to tackle AMR in the environment has become increasingly urgent. In the current stage, in order to understand the development of environmental AMR, it is time to monitor exposure levels and health risks from global and long-term perspectives. The need for international collaborations cannot be overstated, although some obstacles may occur. Thus, ARGs surveillance requires organized-systematic-international-joint efforts using standardized quantitative methods. In this review, metagenomic methods were recommended for quantitatively analyzing ARGs in large-scale sampling campaigns. A standardized and quantitative framework was established for generating comparable and large-volume datasets to address the resistant risk, from sample treatment to bioinformatic analysis (Figure 1). In order to assess health risks for ARGs at different levels of exposure, it was essential to measure the absolute quantification of ARGs and ARB and determine the viability of ARB, as well as consider the role of horizontal gene transfer and the host. This review provides a systematic standardization framework for ARGs and ARB surveillance in different environmental compartments and geographic hotspots.

## Declaration of Competing Interest

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.

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## Figures and tables with captions

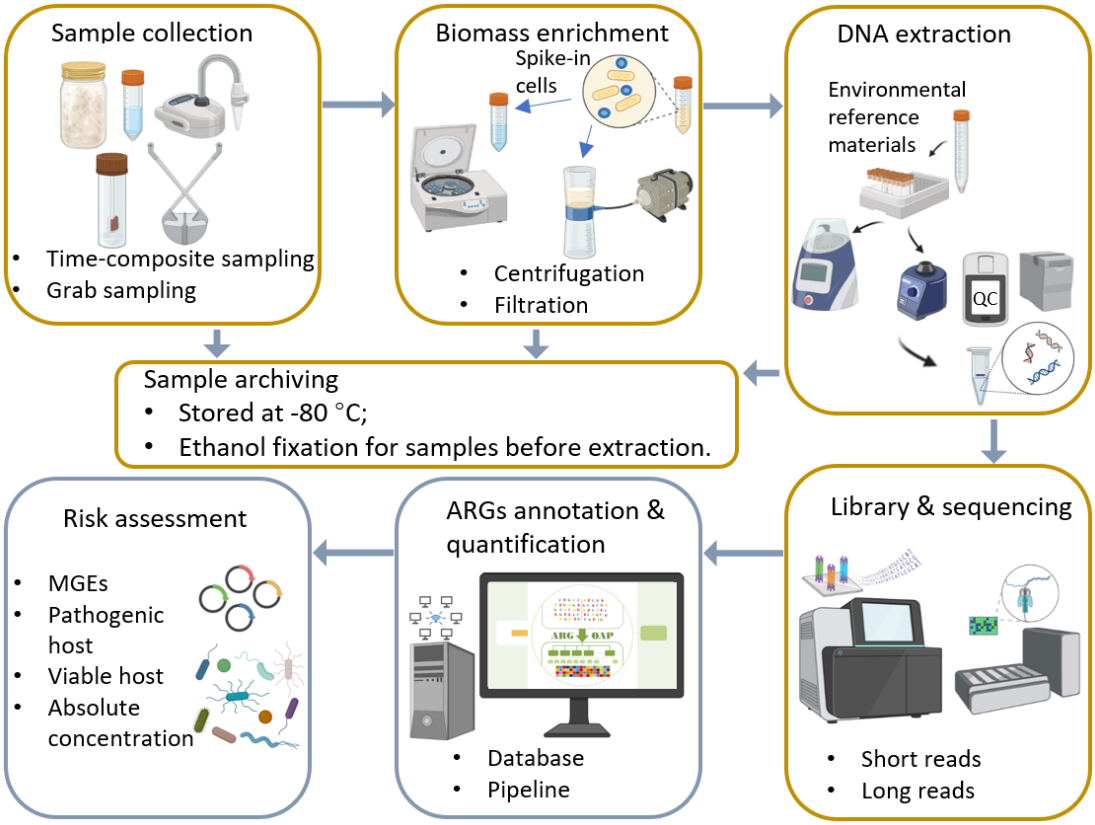



Figure 1 A standard quantification framework for environmental ARGs surveillance by metagenomic analysis, including sample collection, enrichment, archiving, DNA extraction, sequencing, and bioinformatic analysis. Processes in brown boxes would cause irreversible effects to the following steps and the results, while those in blue boxes could be re-treated after data collection.

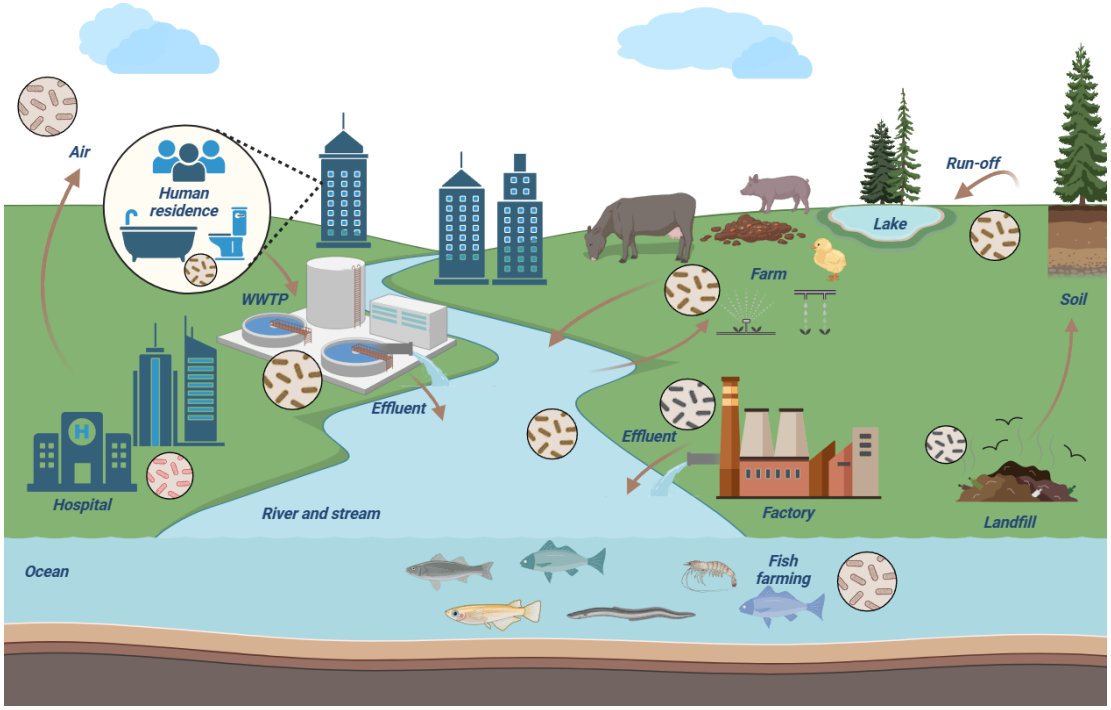
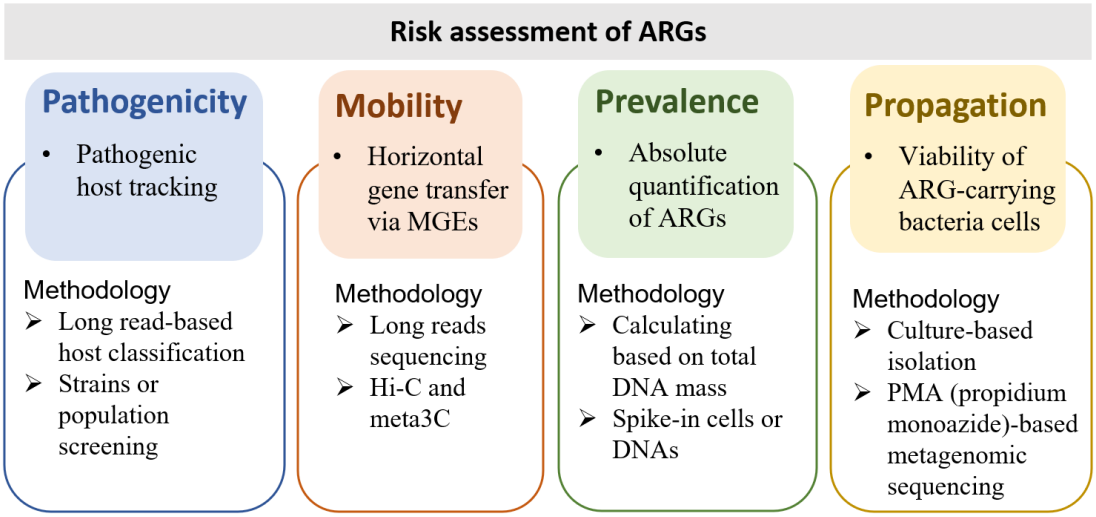



Figure 2 Environmental compartments provide hotspots and routes for antibiotic resistance dissemination.

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857 Figure 3 Factors to be addressed for risk assessment of ARGs, including pathogenicity, mobility,

858 prevalence and propagation.

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Table 1 Comparison of metagenomics and PCR methods for the analysis of ARGs

| Methods | Sensitivity | Cost | Throughput | Prior knowledge of target genes | Contextual information | Other limitations |
|---|---|---|---|---|---|---|
| Metagenomic sequencing | Low | High | High | No | Yes | Complex data processing |
| qPCR | High | Lower | Low | Yes | No | PCR bias |
| ddPCR | Higher | Low | Low | Yes | No | PCR bias |

862 Table 2 Recent studies for global and long-term environmental antibiotic resistance surveillance

| Environmental compartment | Samples and datasets | Geographic or longitudinal scope | ARG analysis methods | Recent study |
|---|---|---|---|---|
| WWTPs | 97 activated sludge samples | Sampling monthly from a Hong Kong WWTP spanning 9 years | Metagenomic sequencing, the pipeline of ARGs-OAP v2.0 with database SARG v2.0, using a unit of ARG copies per cell | (Yin et al., 2019) |
| WWTPs | Domestic sewage collected from 79 sample locations | Covering 7 geographical regions from 74 cities in 60 countries | Metagenomic sequencing, the ResFinder tool, using a unit of fragments per kilobase per million fragments (FPKM) | (Hendriksen et al., 2019) |
| WWTPs | 757 urban sewage samples | Covering 243 cities in 101 countries in a 3-year time series | Metagenomic sequencing, the ResFinder tool | (Munk et al., 2022) |
| Surface water | 992 stream water samples | Sampling seasonally during 5 years from 115 sites across the Upper Oconee watershed (Georgia, USA) | qPCR targeting 6 selected ARGs | (Damashek et al., 2022) |
| Oceans | Re-analyzing 347 metagenomes and 182 metatranscriptomes from marine samples | Covering 181 globally distributed locations in oceans | CARD database (CARD 2022, v3.2.5), using a unit of reads per million per kilobase (RPKM) | (Xu et al., 2023) |
| Drinking water | 98 metagenomes of biofilters from drinking water treatment plants collected from public archives | Covering China, Dutch, Denmark, Switzerland, and the USA | ARG abundance was estimated using scripts in ARGs-OAP v2.0 based on a combined ARG database (ARG sequences mainly from SARG database) | (Liang et al., 2023) |
| Drinking water | Drinking water samples collected from the point of use | Covering 25 cities in mainland China, Hong Kong, Macau, Taiwan, South Africa, Singapore and the USA | Metagenomic sequencing, ARDB database, using Usearch and BLASTX to search ARGs | (Ma et al., 2017) |
| Soil | Composite soil samples (from multiple soil cores) | Covering 1012 sites from 35 countries across all continents | qPCR approach targeting 285 unique ARGs and 10 MGEs | (Delgado-Baquerizo et al., 2022) |
| Soil | 258 soil samples collected from China and 1385 soil metagenomic datasets from NCBI Sequence Read Archive | Soil metagenomes collected from across the globe to generate a global map of soil ARG-carrying pathogens | Metagenomic sequencing, using ARG-OAP v.2.2 with database SARG v2.2 to obtain the annotation of ARG profiles | (Wang et al., 2023) |
| Soil | Re-analyzing 1088 soil metagenomic data retrieved from public archives | Covering 430 locations in the global map | ARG annotation via the pipeline ARGs-OAP 2.3 with SARG reference databases, using a unit of ppm (reads carrying ARGs per million reads) | (Zheng et al., 2022) |
| Air | Ambient total particulate matter samples | Sampling across 19 world cities in 13 countries; and a 10-year longitudinal study in Xi'an, China | qPCR targeting 30 ARG subtypes and 2 MGEs | (Li et al., 2018a) |
| Various environmental habitats | 1723 metagenomes from public archives categorized into 13 habitats, encompassing industrial, urban, agricultural, and natural environments | Covering 83 countries and spanning most continents and oceans | ARGs-OAP (v3.2.2) with SARG v 3.0, quantifying in a unified unit (ARG copy per cell number) | (Yin et al., 2023) |

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