

Wastewater-Based Antimicrobial Resistance Surveillance: A Systematic Review of Metagenomic and Complementary Molecular Evidence from Wastewater and Wastewater-Impacted Environments

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Abstract:

Introduction: Wastewater and wastewater-impacted environments are increasingly used to monitor antimicrobial resistance genes (ARGs) and characterize resistance determinants relevant to environmental AMR surveillance. This systematic review aimed to synthesize evidence on ARG prevalence, diversity and persistence, with particular attention to the analytical resolution of shotgun metagenomics, complementary molecular evidence, methodological quality, environmental drivers and public-health interpretation.

Methods: PubMed and Scopus were searched for eligible studies published from January 2018 to July 2025, with Google Scholar used as a supplementary screening source. The initial search was done on July 31, 2025. The final database comprised 652 PubMed/Scopus records, and the first 20 Google Scholar result pages (approximately 200 records) were screened without being counted as a stable PRISMA record set. The study included 15 studies while JBI design-specific appraisal was supplemented by a metagenomic methodological appraisal for the 13 included studies that used shotgun metagenomics. The review was not prospectively registered and no protocol was prepared. Moreover, the scope amendment made during revision is acknowledged as a review limitation.

Results: Clinically relevant ARGs, including *blaCTX-M*, *sul1*, *tetM*, *erm*, and *mcr-1*, were detected across global, national, and local wastewater-associated environments. Mobile genetic elements were frequently reported in association with ARGs, indicating potential mobility of resistance determinants. However, this does not necessarily mean the ARGs were horizontally acquired or transmitted. Differences in sampling methods, analytical procedures, ARG databases, normalisation methods, and reporting procedures hindered direct comparisons between studies. Resistome profiles were found to be different depending on the environmental and operational conditions, such as seasonal changes and input of hospital wastewater, but the effect these factors have needs to be further confirmed based on standardised and long-term investigation.

Conclusion: Wastewater metagenomics is a promising complementary One Health surveillance approach for characterizing environmental AMR patterns, but its clinical and public-health utility requires further validation. Standardized sampling, sequencing, normalization and reporting, together with longitudinal clinical correlation and exposure-based interpretation, are needed before wastewater resistome measurements can be used routinely for actionable public-health decisions.

Keywords: Antimicrobial resistance, wastewater metagenomics, resistome, one health, public health, environmental safety, antimicrobial resistance genes.

1. INTRODUCTION

Inflation Antimicrobial Resistance (AMR) is a property of microorganisms that allows them to survive the action of

antimicrobial drugs. The mechanisms that can decrease the effectiveness of an antimicrobial drug are encoded by genes known as Antimicrobial Resistance Genes (ARGs) at the genetic level [1]. The potential mobility of ARGs can be

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enhanced by the gene transfer capacity of Mobile Genetic Elements (MGEs) like plasmids, integrons and transposons, which can enable the transfer of ARGs among microbial populations [2]. Wastewater contains diverse microbial communities with ARGs and MGEs, making it an important matrix for detecting and characterizing resistance determinants. The identification of the presence of the gene, however, does not confirm that it is expressed, the viability of the host organism, genomic linkage, horizontal transfer, environmental exposure or transmission to humans or animals. A comprehensive global analysis in 2019 found bacterial AMR as directly responsible for 1.27 million deaths while associated with an additional 4.95 million deaths globally [3].

Furthermore, the burden of bacterial AMR is distributed unevenly across geographical regions, globally, only 56% of domestic wastewater was safely treated in 2024 leading to a continuous discharge of inadequately treated effluents into the receiving water bodies. These effluents are environmental reservoirs for the dissemination of Antimicrobial-Resistant Bacteria (ARB) and antimicrobial resistance genes (ARGs), promoting horizontal gene transfer and contributing to the global propagation of antimicrobial resistance [4]. These figures justify an urban-wastewater focus and motivate a metagenomics-anchored evidence synthesis that can resolve ARG diversity, context, and mobility with decision-relevant specificity.

Shotgun metagenomics, sequencing all DNA in a sample, enables culture-independent profiling of ARG diversity and their genomic contexts [5]. Metagenomic surveys of urban sewage have mapped strong geographic structure in the resistome at city and global scales [6]. A more comprehensive study by Munk *et al.* [7] studied 757 samples of urban sewage from 243 cities in 101 countries, again showing significant geographical differences in the antimicrobial resistance profiles. The geographic scope differs between large-scale wastewater resistome studies. Munk *et al.* [7] also reported a broader global analysis involving urban sewage from 101 countries. These studies should therefore be interpreted according to their individual sampling frameworks. Clinically relevant markers such as mobilised colistin resistance (*mcr*) genes for colistin and carbapenemases such as *bla*_{NDM}/*bla*_{OXA}, as well as heavy-metal-linked proxies like integron integrase 1 (*intI1*), are consistently detectable in urban influent and, treated effluents from municipal Waste Water Treatment Plants (WWTPs) following secondary or tertiary treatment including Conventional Activated Sludge (CAS) or Membrane Bioreactor (MBR) with chlorination, UV radiation or ozonation [8].

Prior syntheses leave actionable gaps for public health and environmental safety. Scope mixing is common: reviews often combine phenotypic assays, targeted qPCR/amplicon data, and metagenomics, making it difficult to distinguish the unique contribution of metagenomics in urban municipal systems [9, 10]. Risk translation is underdeveloped: while concordance between wastewater and clinical AMR has been discussed, frameworks converting metagenomic signals into exposure-relevant indicators are still nascent [10]. For example, Ko *et al.* [11] critiques the limited integration of metagenomic data into

actionable public health metrics, showing a gap in translating environmental data to real-world applications.

Methodological heterogeneity remains a barrier, as variations in database choices, read vs. assembly-based inference, and normalisation methods hinder cross-study comparability. Rumi *et al.* [12] identify how inconsistent database use leads to varying ARG identifications, resulting in data bias. Tools like MetaCompare/2.0 for resistome risk ranking are inconsistently applied, limiting their utility. Martínez-Quintela *et al.* [13] found fragmented process-stage quantification, where diverse technologies yield differing results on ARG removal. Furthermore, Papp *et al.* [14] reported sludge and biosolids are vital for ARG persistence studies and remain underexplored, thereby hindering comprehensive environmental risk assessment. A focused systematic review of metagenomic insights into urban wastewater resistomes is warranted. A more focused synthesis is required to identify the extent to which a wastewater shotgun metagenomics approach differs from other molecular methods employed in wastewater AMR surveillance. Previous reviews have mostly combined different approaches, while the present review focused on the metagenomic evidence and consulted selected non-metagenomic studies primarily for methodological comparison and contextual support.

Although shotgun metagenomics has been increasingly applied to study the occurrence of antimicrobial resistance in urban wastewater, available data are currently quite limited in the degree of comparability between different studies, given the use of different sampling strategies, sequencing depths, reference databases, and normalization procedures [15, 16]. Most investigations are descriptive with little effort made to validate wastewater ARG profiles against clinical resistance data, nor are there thresholds for public-health action based on exposure [10, 11]. In addition, the association of ARG–mobile genetic elements are not well resolved, and the assessment is not complete over the entire treatment steps, which leaves gaps in understanding of transmission risks [16]. A focused synthesis is thus needed to normalise metagenomic evidence, assess ARG persistence in wastewater treatment systems and to determine clinically relevant indicators for One Health surveillance and policy action.

This review's contribution is a focused synthesis of evidence relating to wastewater AMR, with a particular focus on the analytical resolution of shotgun metagenomics. Past reviews have provided a summary of wastewater-associated antimicrobial resistance through various molecular, microbiological, and epidemiological methods; however, variations in analytical resolution, sequencing strategies, and environmental matrices hinder direct comparisons to previous studies. This systematic review focuses primarily on shotgun-metagenomic evidence while incorporating selected complementary molecular studies to provide broader context for wastewater-associated AMR surveillance. The review examines ARG diversity, analytical pipelines, treatment-stage patterns, ARG–mobile genetic element associations, and the extent to which genomic approaches can resolve resistance determinants within wastewater and wastewater-impacted environments.

2. METHODOLOGY

2.1. Research Design

This systematic review synthesized evidence on Antimicrobial Resistance Genes (ARGs) in wastewater and wastewater-impacted environments. The review was metagenomics-anchored: shotgun metagenomic studies formed the principal evidence base, while targeted qPCR and culture-based studies were retained as complementary molecular evidence when they met the stated wastewater-associated eligibility criteria. Eligible matrices comprised municipal and hospital wastewater, wastewater-treatment influent and effluent, sludge/biosolids, and receiving aquatic environments demonstrably affected by wastewater discharge. Because complementary molecular methods and wastewater-impacted receiving environments were added during manuscript revision, this post hoc amendment is reported explicitly and considered when interpreting the breadth and comparability of the synthesis. Study identification, screening and reporting followed PRISMA 2020 [17].

2.2. Literature Search Strategy

A systematic literature search was conducted using PubMed and Scopus, with Google Scholar used as a supplementary dynamic screening source. The initial search was done on July 31, 2025, which is the final search date. The final PubMed and Scopus retrieval counts reported in the PRISMA accounting are the counts through July 31, 2025.

Google Scholar was not treated as an equivalent bibliographic database because it does not provide a stable exportable result set. The first 20 result pages (approximately 200 results) were manually screened; the interface estimate of approximately 1,170 results was not entered into the PRISMA identification total. No additional unique eligible study was added from the Google Scholar manual screening. PubMed and Scopus records were exported to Zotero for deduplication, with automated duplicate identification followed by manual checking. The retained search strings and documented counts are presented in Supplementary Appendix A.

2.3. Eligibility Criteria

Studies were eligible if they reported primary data on ARGs or antimicrobial-resistant organisms in wastewater or a receiving environmental matrix explicitly linked to wastewater discharge. Eligible matrices were municipal wastewater, hospital wastewater, WWTP influent/effluent, sludge/biosolids, and wastewater-impacted canals, rivers or surface waters. Eligible analytical approaches included shotgun metagenomics and complementary targeted molecular or culture-based AMR characterization. Reviews, editorials, conference abstracts, studies without a defined wastewater/wastewater-impact context, studies without relevant AMR outcomes, and reports with insufficient methodological information for interpretation were excluded.

2.4. Data Screening

The final PubMed and Scopus searches identified 652 records (PubMed $n = 105$; Scopus $n = 547$). Twenty-two

duplicate records were removed before screening, leaving 630 records for title and abstract screening. The Google Scholar interface estimate was not included in these identification totals; approximately 200 Google Scholar results (first 20 pages) were screened as a supplementary source (Supplementary Appendix A).

Of the 630 records screened, 586 were excluded at title/abstract stage. Forty-four reports were sought for retrieval; two could not be retrieved, leaving 42 full-text reports assessed for eligibility. Twenty-seven reports were excluded (Supplementary Appendix B). These include the following: studies outside the wastewater/wastewater-impact scope ($n = 7$), not relevant to AMR outcome ($n = 10$), those that were review/editorial/commentary ($n = 5$), or contained insufficient methodological information ($n = 5$). Fifteen studies were included in the systematic review and narrative synthesis. The additional eligible study was identified in the update on July 31, 2025 and was included in these counts, as indicated in Fig. (1).

Two reviewers independently screened the title and abstract and, based on the predetermined inclusion criteria, determined whether the articles met the inclusion criteria. Reports were then evaluated in full text at the same time for potentially eligible reports. At both meetings, differences were resolved by discussion and consensus. Records from the bibliographic databases were merged before screening, and duplicate records were eliminated before the assessment of the titles and abstracts. The reasoning for including or excluding articles at the screening stage was recorded to help with PRISMA reporting purposes.

The scope of this review was refined during the revision process to include wastewater and wastewater-impacted environments, including municipal and hospital wastewater, wastewater treatment plant influents and effluents, sludge/biosolids, and receiving environments affected by wastewater discharge. This amendment was introduced to better capture the environmental pathways through which antimicrobial resistance genes (ARGs) occur and persist.

2.5. Data Extraction and Analysis

Data from the 15 included studies were extracted independently by two reviewers using a structured template. Extracted variables included author/year, study aim, setting/location, number and type of sites where reported, environmental matrix, analytical method, treatment stage or comparison, principal ARG findings, and study/appraisal limitations. Discrepancies were resolved by discussion and consensus. Because the included studies differed in sampling design, sequencing depth, ARG databases, normalization, matrix and analytical platform, formal meta-analysis was not appropriate. A narrative synthesis was organized around ARG diversity and prevalence, genomic context/potential mobility, treatment-stage persistence or removal, and environmental/public-health interpretation. No formal sensitivity analysis, reporting-bias analysis, or GRADE-style certainty assessment was undertaken.

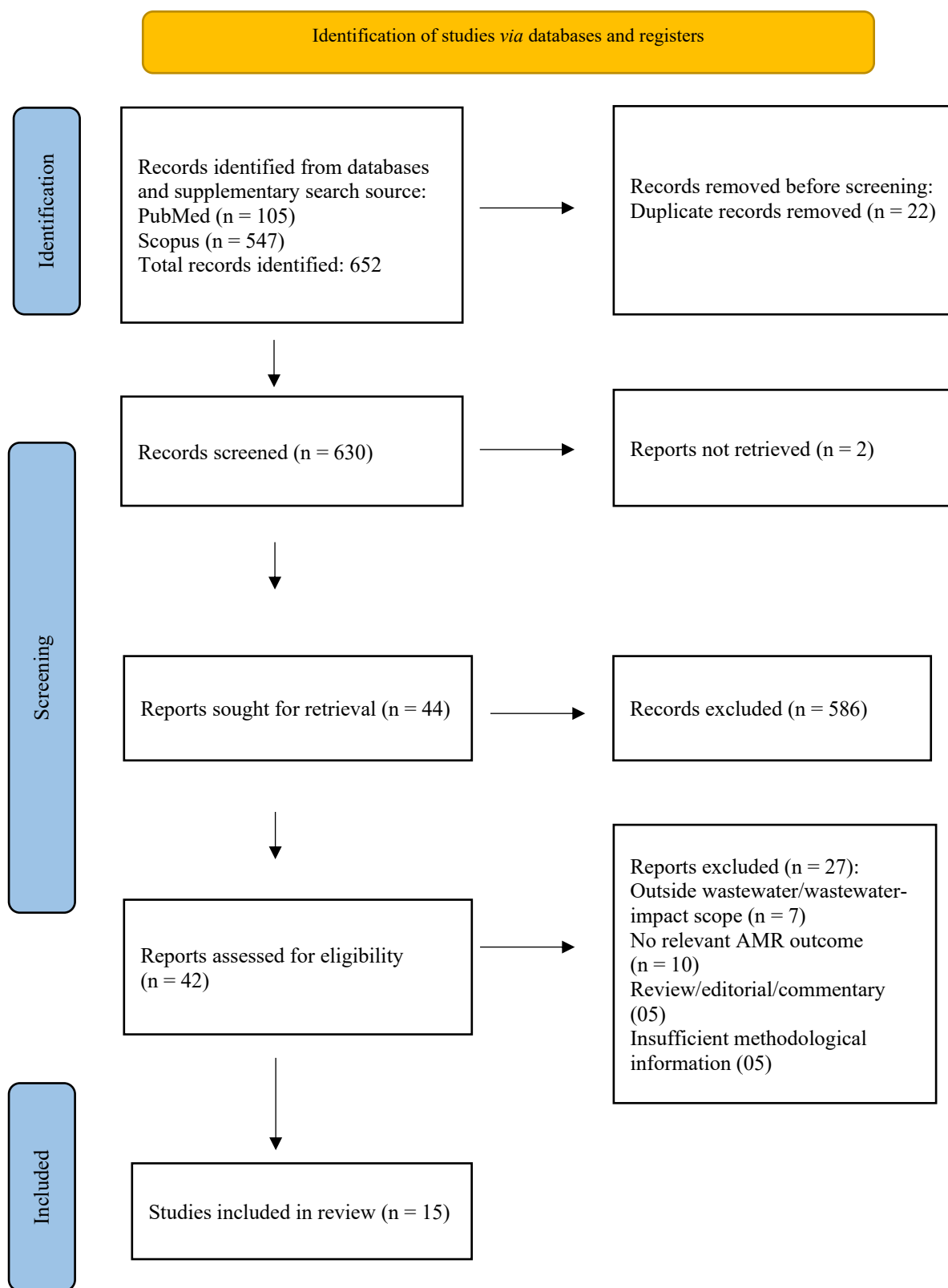


Fig. (1). PRISMA flow diagram.

2.6. Quality Assessment

JBIC critical appraisal tools were selected according to the way each included study was reported. Studies structured as cross-sectional comparisons of settings, sources or analytical approaches were appraised with the JBIC Analytical Cross-Sectional Studies Checklist [18], whereas descriptive occurrence/prevalence surveys were appraised with the JBIC Prevalence Studies Checklist [19, 20]. Ten studies were assigned to the cross-sectional checklist and five to the prevalence checklist, with each included study appearing once. Responses were coded as Yes, No, Unclear or Not applicable according to whether the published report clearly satisfied the item, failed it, provided insufficient information, or made the item inapplicable. The JBIC tools were used to characterize reporting and methodological limitations, not to validate the review's scientific conclusions (Supplementary Appendix C).

A methodological appraisal of studies using metagenomic approaches was performed in addition to the general appraisal of the observational-study tools, because there are some technical sources of bias which are not fully captured by the general tools used for observational study appraisal. The appraisal took into account sampling representativeness and replication, sampling and concentration, principle of DNA extraction, depth of sequencing and QC procedures, negative controls, antimicrobial-resistance gene database and versions, identification thresholds, read-based *versus* assembly-based analyses, abundance normalisation, host assignment, linkage of mobile genetic elements, and availability of sequencing data and analytical code, as indicated in Supplementary Appendix D, E.

2.7. Ethical Consideration

Ethical standards were observed in line with international and institutional guidelines relevant to the study designs included in this review. This review did not involve direct experimentation with humans or animals. Ethical approval was not required and informed consent was not obtained since this was a secondary analysis of previously made studies. However, every effort was made to ensure that there was strict reporting, transparency and that guidance in conducting and reporting reviews was followed.

3. RESULTS

3.1. ARG Diversity and Prevalence Across Wastewater Matrices

The 15 included studies comprised predominantly shotgun-metagenomic investigations together with selected complementary molecular studies. The study by Hendriksen *et al.* [21] showed a link between national antibiotic use prevalence, sanitation infrastructure and prevalence of ARGs, highlighting the role of sociodemographic factors. Similarly, Becsei *et al.* [22] noted differences among five cities in Europe, where the outcomes of bioburden testing were affected by *Pseudomonas* blooms that inhibited the detection of some ARGs. This highlights that the resistome may suffer from microbial community composition bias.

Hospital-wastewater studies reported higher ARG abundance or diversity than municipal/community wastewater in some settings, including clinically relevant *bla*NDM-1, *bla*OXA and *mcr* variants (Table 1). These observations identify settings with elevated resistance determinants but do not by themselves demonstrate selection, amplification, dissemination or transmission. Jantharadej *et al.* [23] reported 611 ARG subtypes in Bangkok canal-water samples, including abundant β -lactam resistance determinants and pathogenic genera such as *Arcobacter* and *Burkholderia*. The canal findings are consistent with wastewater influence on the receiving environment, but do not establish direct transmission pathways.

Interestingly, culture-based and targeted qPCR approaches, such as those conducted by Tsvetanova and Boshnakov [24] and Magnano San Lio *et al.* [25] detected fewer ARGs but a higher relative abundance of clinical relevance resistance genes including *erm*(B) or *bla*OXA. This illustrates the trade-off between detection breadth and quantitative sensitivity, indicating that methodological approaches directly influence the diversity and composition of the resistome observed. Although these studies have all demonstrated the ubiquitous presence of ARGs across wastewater matrices, the diversity and abundance reported is systematic, depending on sampling design, sequencing depth and analytical methods (Supplementary Appendix D, E).

3.2. ARG Co-occurrence, Genomic Context, and Potential Mobility

The included studies provided different levels of evidence regarding the genomic context and potential mobility of ARGs. Detection of ARGs in metagenomic reads confirmed the presence of ARGs in a sample. In contrast, taxonomic co-occurrence only confirmed the presence of ARGs and a specific microbial group within a microbial community. However, these levels of evidence should not be interpreted as equivalent to demonstrated transmission, because genomic association or potential mobility does not confirm successful horizontal gene transfer, survival of resistant organisms, or human exposure. More evidence was available for ARGs localized to assembled contigs, metagenome-assembled genomes, plasmids, integrons, or other mobile genetic elements. Genomic linkage does not necessarily imply horizontal gene transfer, but can imply potential for mobility. Likewise, the presence of ARGs in taxa that contain opportunistic pathogens does not mean that the resistance gene was on a viable pathogenic strain unless a host assignment was done with high enough genomic specificity. The integron (*intI1*) was enriched in the influent samples and often found to be associated with the carbapenemase genes such as *bla*NDM and colistin resistance genes such as *mcr-1* as reported by Sekizuka *et al.* [26]. In the same way, plasmid associated ARGs were also identified in *E. coli*, *Klebsiella* and *Acinetobacter* by Prieto Riquelme *et al.* [27] indicating that the distribution of resistomes is related to the bacterial host and the potential for genetic mobility.

Variability across time was observed in some investigations. In Rotterdam, the microbial community of the sewer changed significantly across the seasons [26]. In Copenhagen, the microbial community was different.

However, measurements of selected ARGs showed seasonal variation in effluents from the Copenhagen WWTP [26], with some resistance determinants being more abundant in summer than winter effluents. While longitudinal surveillance of selected ARGs was provided in three Sicilian wastewater-treatment plants by Magnano San Lio *et al.* [25], the authors did not provide supporting analysis to conclude that the plants experienced seasonal changes due to prescription. A comparison of genomic findings across studies revealed significant variability in ARG types and prevalence due to differences in sampling context, sequencing depth, and analysis methods. For example, Hendriksen *et al.* [21] detected a wide range of ARGs globally, while Silvester *et al.* [28] found a higher prevalence of carbapenem resistance in hospital wastewater (Table 2). Studies with higher sequencing depth captured rare ARGs, while those using 16S rRNA sequencing missed broader resistome diversity [15, 25]. These genomic discrepancies emphasize the need for standardized methodologies to enable more consistent and comparable findings across studies, highlighting the critical role of hospital effluents in the environmental occurrence or dissemination potential of AMR determinants. The evidence was therefore interpreted hierarchically as follows: ARG detection in unassembled reads; ARG–taxon co-occurrence; ARG localization within assembled contigs or metagenome-assembled genomes; ARG linkage to plasmids, integrons or other mobile elements; and, at the highest level, direct evidence of environmental or human transmission of viable phenotypically resistant organisms. Most included studies provided evidence within the first four levels of this hierarchy; however, none demonstrated direct environmental or human transmission of viable resistant organisms.

Acosta *et al.* [29] showed that meropenem enrichment increased detection of rare carbapenemases, demonstrating that analytical sensitivity can affect whether low-abundance ARGs are detected. Low abundance alone should not be interpreted as disproportionate public-health risk without information on host, viability, mobility, exposure and clinical relevance. Becsei *et al.* [22] and Jantharadej *et al.* [23] also reported associations between ARG signals and microbial taxa. Co-occurrence or association with MGEs can indicate possible genomic context or mobility potential, but does not demonstrate horizontal gene transfer or subsequent transmission to humans or animals.

3.3. Stage-Wise ARG Removal or Persistence

The changes in the indicators of antimicrobial resistance in each of the stages of the wastewater-treatment process were different depending on both the treatment process and the method of measurement. Reported outcomes were absolute abundance, relative abundance, detection frequency, prevalence of resistant organisms, and log reduction, and were not directly interchangeable and were interpreted separately. If an overall biomass of bacteria is decreased during treatment, a decrease in absolute ARG concentration can occur with an increase in relative abundance.

Tsvetanova and Boshnakov [24] reported decreases in culturable counts of AMR bacterial species in treated effluents in all cases, except for Enterobacteriaceae and Enterococcus, which were detected. Sekizuka *et al.* [26] observed about a 1-log reduction in some signals associated with the ARGs from the influent to effluent. Majeed *et al.* [30] reported a total ARG abundance approximately 50% less in secondary effluent than in influent, and that approximately 90% of the ARG types found in secondary effluent also appeared in the influent. The findings suggest that the ARG diversity continues to exist despite a decline in some abundance measures. Quantitative comparison of the performance of the different treatments was not appropriate due to differences in the data analysis platform employed, microbial denominators, and sequencing depths used in the included studies. Treatment results were therefore evaluated and compared within each study, based on the results reported in each and not across technologies by incompatible measures.

3.4. Metagenomic Methodological Appraisal

The metagenomic appraisal revealed differences in the reported technical and analytical parameters among metagenomic studies (Table 2). Some discrepancies were noted regarding the reporting of sampling replication, sequencing depth, quality control procedures, ARG database selection, normalisation methods, assembly-based analyses, host assignment, and the availability of sequencing data/analytical code [28, 29, 31]. These differences are sources of methodological heterogeneity and should be taken into account when comparing metagenomic findings from studies.

Table 1. Comparison of major antibiotic resistance genes (ARGs) reported across wastewater studies.

Study	Wastewater Matrix	Major ARGs Reported	Key Finding
Hendriksen <i>et al.</i> 2019 [21]	Urban wastewater (60 countries)	β -lactamases, macrolide, tetracycline	High global ARG diversity (557 ARGs); prevalence linked to antibiotic use and sanitation.
Becsei <i>et al.</i> 2024 [22]	Municipal wastewater	β -lactam, macrolide ARGs	ARG profiles varied among cities; microbial composition influenced detection.
Jantharadej <i>et al.</i> 2022 [23]	Canal water affected by sewage	β -lactam ARGs	Surface water reflected upstream sewage resistome.
Tsvetanova and Boshnakov, 2025 [24]	Wastewater	<i>erm(B)</i> , <i>blaOXA</i>	Targeted methods detected fewer ARGs but quantified clinically relevant genes effectively.

Table 2. Metagenomic methodological appraisal of included metagenomic studies.

Study	Sampling Representativeness	Replication	Sample Processing/ DNA Extraction	Sequencing Depth & QC	ARG Database/Version	Detection Threshold	Read/Assembly Approach	Normalization Method	Host Assignment	ARG–MGE Linkage	Raw Data Availability
Davis <i>et al.</i> 2025 [15]	Yes	Yes	Yes	Yes	Yes (CARD)	Yes	Read-based metagenomics	Yes (16S normalization/ qPCR supported)	Unclear	Unclear	Yes
Knight <i>et al.</i> 2024 [16]	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics	Yes	Unclear	Unclear	Yes
Hendriksen <i>et al.</i> 2019 [21]	Yes	Yes	Yes	Yes	Yes (ResFinder/CARD-based annotation)	Yes	Read-based metagenomics	Yes	Unclear	Unclear	Yes
Becsei <i>et al.</i> 2024 [22]	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics + MAG analysis	Yes	Yes	Yes	Yes
Jantharadej <i>et al.</i> 2022 [23]	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics + 16S	Yes	Unclear	Unclear	Yes
Sekizuka <i>et al.</i> 2022 [26]	Yes	Yes	Yes	Yes	Yes (MEGARes/CARD)	Yes	Shotgun metagenomics	Yes	Yes	Yes	Yes
Prieto Riquelme <i>et al.</i> 2022 [27]	Yes	Yes	Yes	Yes	Yes (CARD)	Yes	Shotgun metagenomics	Yes (16S normalized abundance)	Yes	Yes	Yes
Silvester <i>et al.</i> 2025 [28]	Yes	Yes	Yes	Yes	Yes (MEGARes)	Yes	Shotgun metagenomics	Yes	Unclear	Unclear	Yes
Acosta <i>et al.</i> 2025 [29]	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics ± enrichment	Yes	Unclear	Unclear	Yes
Rout <i>et al.</i> 2023 [31]	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics	Unclear	Unclear	Unclear	Yes
Fresia <i>et al.</i> 2019 [35]	Yes	Yes	Yes	Yes	Yes (CARD)	Yes	Read-based metagenomics	Yes	Unclear	Unclear	Yes

Assessment categories: Yes = criterion clearly reported and satisfied; No = criterion reported but not satisfied; Unclear = insufficient information to determine; Not reported = information unavailable in the publication; Not applicable = criterion not relevant to the study design.

Table 3. Summary of methods and outcomes of the 15 included studies.

Author/Year	Aim of the Study	Methodology	Treatment / Intervention	Findings of the Study	Key Appraisal Observations
Davis <i>et al.</i> 2025 [15]	Benchmarking quantitative metagenomics vs ddPCR	Shotgun metagenomics (94 Gb), meta-sequin spike-ins, ddPCR validation	U.S. WWTP (influent, activated sludge, effluent)	ARG quantification feasible; LoD ~1 copy/μL; depth critical (~100 Gb)	Quantitative benchmarking and spike-in calibration were reported; host/MGE linkage remained limited.
Knight <i>et al.</i> 2024 [16]	National wastewater AMR surveillance	Shotgun metagenomics vs HT-qPCR	47 WWTPs in Wales + hospital effluents	Metagenomics detected 491 ARGs; HT-qPCR showed greater sensitivity for low-abundance targeted genes	Broad multi-site coverage with metagenomics/HT-qPCR comparison; host/MGE linkage was not consistently resolved.
Hendriksen <i>et al.</i> 2019 [21]	Global surveillance of ARGs in untreated sewage	Shotgun metagenomics, Illumina HiSeq, ResFinder	Untreated sewage from 79 sampling sites representing 74 cities in 60 countries	557 ARGs identified; β-lactamases, macrolide, tetracycline dominant; carbapenemases detected; resistome profiles mirrored clinical AMR patterns	Clear sampling framework and sequencing workflow; host assignment and ARG–MGE linkage were not consistently resolved.
Becsei <i>et al.</i> 2024 [22]	Temporal surveillance across 5 European cities	Shotgun metagenomics, MAG reconstruction	Untreated sewage, time-series	2,332 MAGs assembled; <i>Pseudomonas</i> blooms masked ARG detection; strong city-level differences	Replication and MAG analysis were reported; microbial blooms may affect detection and interpretation.
Jantharadej <i>et al.</i> 2022 [23]	Resistome in urban transport canal	Shotgun metagenomics, 16S	10 canal sites (Bangkok)	15 ARG types, 611 subtypes; β-lactams are the most abundant; pathogen hotspots (<i>Arcobacter</i> , <i>Burkholderia</i>)	Shotgun metagenomics plus 16S; surface-water matrix limits direct inference about wastewater-treatment performance.
Tsvetanova & Boshnakov, 2025 [24]	ARGs in Bulgarian WWTP	Culture + PCR (<i>Enterobacteriaceae</i> , <i>Enterococcus</i>)	Medium-sized WWTP (influent vs effluent)	Resistant bacteria persisted/increased; 8.8% effluent <i>Enterobacteriaceae</i> ESB ⁺ ; Enterococci dominant	Culture/PCR study; metagenomic-specific appraisal was not applicable; JBI prevalence appraisal was used.
Magnano San Lio <i>et al.</i> 2025 [25]	ARG surveillance in Sicily WWTPs	qPCR for key ARGs	3 WWTP influents (tertiary vs secondary)	<i>blaOXA</i> , <i>blaTEM</i> , <i>blaCTX-M</i> , erm(B) prevalent; erm(B) highest	Targeted qPCR study; metagenomic-specific appraisal was not applicable; JBI cross-sectional appraisal was used.
Sekizuka <i>et al.</i> 2022 [26]	ARG surveillance in Japan WWTPs	Shotgun metagenomics, MEGARes/CARD	16 WWTPs (influent, effluent, river)	408 ARGs; <i>blaCTX-M</i> , <i>blaTEM</i> , mcr-1 in influents; ~1 log reduction post-treatment	Sequencing, normalization and genomic-context reporting were comparatively detailed.

Prieto Riquelme <i>et al.</i> 2022 [27]	Develop and demonstrate a standardized metagenomics-based approach for internationally comparable wastewater AMR surveillance	Shotgun metagenomics; CARD-based ARG annotation; 16S rRNA-normalized relative abundance	14 influent sewage samples from 12 WWTPs across six international locations	Resistome composition differed geographically; Asian sewage generally showed higher relative ARG abundance than European/US samples.	Harmonized sampling and genomic-context analysis were reported; sample numbers per site were limited.
Silvester <i>et al.</i> 2025 [28]	Hospital vs community wastewater	Shotgun metagenomics, MEGARes v3, AMR++	3 hospitals & 3 linked WWTPs (Wales)	Hospital effluents showed greater ARG relative abundance and diversity than community WWTP samples; ARG removal varied across WWTPs and was incomplete.	Multi-site metagenomics were reported; site variability and incomplete host/MGE resolution remained.
Acosta <i>et al.</i> 2025 [29]	Enrichment-based detection of rare ARGs	Shotgun metagenomics ± culture enrichment	Raw and culture-enriched wastewater from two tertiary-care hospitals and two nearby urban neighbourhoods	1,225 ARGs; enrichment (esp. meropenem) improved detection of carbapenemases; hospitals had higher ARG loads	Enrichment increased rare-ARG detection but can alter community composition and relative abundance.
Majeed <i>et al.</i> , 2021 [30]	ARGs across WWTP stages	Shotgun metagenomics (MetaStorm, CARD)	U.S. WWTP (influent → effluent)	Total ARG relative abundance decreased by approximately 50%; about 90% of ARG types detected in secondary effluent were also detected in influent.	Transparent metagenomic workflow; relative rather than absolute quantification limited treatment interpretation.
Rout <i>et al.</i> 2023 [31]	Characterization of bacterial diversity, ARGs and virulence factors in River Ganga sediments	Shotgun metagenomic analysis	Ganga River sediments, India	ARGs and virulence factors showed spatial variation across river sediments; Proteobacteria predominated	Shotgun metagenomics of river sediment; contextual environmental evidence only, with incomplete host/MGE linkage.
Talat <i>et al.</i> 2023 [34]	Resistome in Indian hospital wastewater	Shotgun metagenomics (Illumina)	6 hospitals (urban, rural)	183 ARGs; <i>bla</i> NDM-1, <i>bla</i> OXA, <i>mcr</i> -3, <i>mcr</i> -5 detected; plasmid-mediated ARGs frequent	Shotgun metagenomics reported clinically relevant ARGs and plasmid-associated signals; no direct transmission evidence.
Fresia <i>et al.</i> 2019 [35]	Resistomes in sewage vs coastal beaches	Shotgun metagenomics, CARD	Montevideo sewage outfalls & beach waters	Sewage carried >90% of ARG classes vs 40% in beaches; overlapping with clinical clones.	Sampling and sequencing methods were reported; some sampling limitations and incomplete host/MGE resolution remained.

3.5. Implications for Environmental and Public Health Risk

Repeated detection of clinically relevant ARGs in wastewater and wastewater-impacted waters supports their use for environmental hazard identification and surveillance. Detection does not establish gene expression, viable pathogenic hosts, horizontal transfer, direct exposure or transmission. Likewise, co-occurrence of ARGs with microbial taxa or MGEs in the same sample should not be interpreted as genomic colocalization without contig-, plasmid- or MAG-level evidence. Knight *et al.* [16] and Hendriksen *et al.* [21] illustrate population-level surveillance applications, while ARGs such as *bla*NDM-1 and *mcr* variants detected in hospital and municipal wastewater indicate clinically relevant resistance determinants in environmental matrices [23, 28], as indicated in Table 3. Rout *et al.* [31] provides contextual evidence from Ganga River sediments rather than evidence of wastewater-treatment efficacy.

Across the included studies, ARG detection patterns varied with sampling context, treatment stage, microbial ecology, sequencing depth, database coverage and analytical method [32, 33]. Recurrent detection of β -lactamase, sulfonamide and aminoglycoside resistance determinants supports environmental surveillance, but does not establish dissemination or quantitative public-health risk [34, 35]. The quality appraisals identified both strengths and limitations, including variation in sampling design, sequencing depth, normalization, host assignment and reporting thresholds. These methodological limitations should be considered when interpreting consistency and generalisability across studies.

4. DISCUSSION

4.1. Principal Findings

A key difference throughout the body of evidence is that wastewater metagenomics shows the presence, relative abundance, and diversity of ARGs, and at times, the context of the ARGs in the genome. Read-based detection cannot tell if the resistance genes are expressed or if the microbial host is alive. Taxonomic co-occurrence can be used to infer ecological association but not necessarily host assignment, while contig-, MAG-, plasmid-, or integron-level linkage can be used to infer genomic context and potential mobility. Even these approaches do not demonstrate actual horizontal transfer, environmental exposure, or transmission to humans. Wastewater ARG detection, therefore, should be considered a measure of possible hazard and surveillance value, not necessarily as public health importance. Accordingly, the synthesis presented here describes recurring patterns across heterogeneous studies rather than estimating a common effect size across wastewater settings.

This review is not intended to prove that ARGs are present in wastewater, which is already proven, but to contrast the different ways in which shotgun-metagenomic approaches can characterize resistome diversity, quantify treatment stage change, elucidate ARG hosts and mobile genetic context, and provide cross-study surveillance. The review distinguishes

metagenomic evidence from complementary targeted methods. It identifies key considerations where sequencing depth, annotation databases, normalization, and assembly strategy have an impact on interpretation and comparability of wastewater AMR results.

The most substantial contributions to the evidence base derive from global-scale investigations. Hendriksen *et al.* [21] analysed sewage from 79 sites in 60 countries, identifying substantial geographic variability in ARG abundance correlated with sanitation infrastructure and socio-economic indices. Similarly, Prieto Riquelme *et al.* [27] employed harmonized sampling to show cross-national differences in resistome composition. These studies exemplify the scalability of metagenomic wastewater surveillance and align with international frameworks such as the World Health Organisation's Global Antimicrobial Resistance and Use Surveillance System (GLASS), which has begun developing environmental modules [32]. The United Nations Environment Programme's 2023 AMR update also emphasized wastewater as a priority surveillance domain within a One Health strategy [33]. Both Hendriksen *et al.* [21] and Prieto Riquelme *et al.* [27] did not directly connect resistome data with patient-level clinical phenotypes, thus restricting their direct translational value.

4.2. Diversity and Distribution of ARGs

Regional and national studies added context on spatial, temporal and source-related variation. Hospital effluents were reported to have higher ARG abundance or diversity than community wastewater in some included studies, including Talat *et al.* [34]. Seasonal associations were reported in some datasets, but these should be interpreted cautiously unless supported by repeated longitudinal sampling and appropriate analysis. Environmental conditions such as rainfall, runoff and temperature may influence wastewater-associated resistome patterns, but detection patterns alone do not demonstrate transmission. Wastewater surveillance may therefore contribute complementary population-level information within a One Health framework, while proposed early-warning or regulatory applications still require validated thresholds, exposure assessment and clinical correlation [33].

Innovation was evident at the methodological level in a study reported by Davis *et al.* [15], where shotgun metagenomics was benchmarked against droplet digital PCR, using spike-in standards to quantify detection limits, and concluded that metagenomic data remain semi-quantitative unless calibrated. This finding aligns with broader evaluations that sequencing-based resistome profiling requires internal standards to avoid bias [31]. Authors compare high-throughput qPCR with metagenomics, revealing strong convergence for standard ARGs but divergence for rare ones, a discrepancy also reported in other comparative platforms [25]. Findings further demonstrated that culture enrichment improved detection sensitivity for low-abundance ARGs, though it distorted community structure and relative abundances. Together, these studies underline the methodological fragmentation of the field, an issue also highlighted by the OECD. It has called for

harmonised pipelines and inter-laboratory calibration before wastewater AMR data can be used for policymaking.

Smaller-scale studies provided contextual evidence from receiving environments. Rout *et al.* [31] examined Ganga River sediments and reported spatial variation in ARGs and virulence factors; these findings do not measure wastewater-treatment efficacy. Fresia *et al.* [35] reported ARGs in Montevideo sewage and coastal waters, supporting environmental co-occurrence across connected settings but not demonstrating direct transmission, treatment-stage removal, or human exposure. Such receiving-environment studies should therefore be interpreted as contextual environmental evidence rather than as direct measures of WWTP performance or quantitative public-health risk.

Across the included studies, recurrent ARG classes and clinically relevant determinants were detected in wastewater-associated matrices. Their repeated detection supports the value of environmental surveillance, but does not establish expression, viability, host pathogenicity, horizontal transfer, or human exposure. Studies of the mobilome may identify genomic contexts compatible with potential mobility [36], but potential mobility should not be described as demonstrated dissemination without direct linkage evidence. Reproducibility is additionally affected by sequencing depth, annotation databases, primer sets, and normalization choices.

4.3. Influence of Wastewater-Treatment Stages

Wastewater surveillance may provide complementary information on population-level resistance patterns and environmental AMR dynamics, although its relationship with clinical AMR outcomes requires further validation. Several global initiatives, including the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) [37], have advocated the importance of developing standardized environmental AMR surveillance strategies. Despite this, many governments consider monitoring wastewater an experimental practice. Feasibility of wastewater-based AMR surveillance has been explored in different settings; however, evidence from low-resource environments remains limited and requires further validation [38, 39]. However, broader implementation requires evidence linking wastewater resistome measurements with clinical AMR indicators, together with validated thresholds and standardized interpretation frameworks.

4.4. Strengths and Limitations

This review has notable strengths. It systematically synthesized 15 studies across global, national, and local scales, applying JBI cross-sectional and prevalence tools for structured appraisal. By incorporating hospital-specific and community-level data and methodological comparisons, the review captures a broad spectrum of the resistome landscape. However, limitations are equally important. Studies included are very variable in design, sequencing depth, and analysis pipelines and cannot be meta-analyzed, forcing narrative integration. Geographic representation is still biased towards Europe and Asia, and Africa and Latin America are still under-represented. Furthermore, none of the studies directly linked

wastewater ARG profiles with clinical AMR outcomes, limiting translational potential.

Methodological weaknesses further constrain interpretation. Short-read sequencing cannot always resolve plasmid contexts or gene hosts, reducing insight into ARG mobility. Variation in annotation thresholds and bioinformatics pipelines undermines cross-study comparability. Few studies accounted for hydraulic or sewer-network variables such as dilution or retention time, which may confound apparent ARG concentrations. These issues echo cautions raised in methodological reviews of metagenomic AMR surveillance [40]. A limitation of this review is the lack of a predetermined protocol and prospective registration. The eligibility criteria were applied uniformly at the screening phase; however, as the scope was revised to incorporate complementary molecular evidence and wastewater-impacted environmental matrices, there was potential for post-hoc methodological decisions to be made.

There are also limitations to the quality appraisal. JBI tools offer a framework for the design and reporting of an observational study, but they are not specifically aimed at environmental metagenomic studies. This may lead them to miss aspects of technical bias such as sequencing depth, extraction method, bioinformatic thresholds, normalisation, negative controls, and genomic host assignment. The supplementary metagenomic-specific appraisal was therefore used, not as an alternative, but to complement the JBI appraisal. The review scope was broadened during revision to include complementary molecular approaches and wastewater-impacted environmental matrices alongside shotgun-metagenomic wastewater studies. This enhances the representation of information from wastewater-associated AMR data, but added methodological variation and restrained the ability to make direct comparisons across studies based on different platforms. This review has a limitation in that there was no prospective, registered protocol. Eligibility criteria were applied uniformly during screening, but the scope was refined during the revisions, which could have led to potential post hoc methodological decisions.

Future work should prioritize standardized metagenomic protocols, including transparent sequencing-depth thresholds, annotation pipelines and quantitative calibration [37, 39]. Integration with clinical surveillance requires prospective correlation with antibiograms, validated thresholds and exposure-based interpretation. Implementation in low- and middle-income settings also depends on broader health-system and resource considerations [40]. Portable sequencing and near-real-time analysis are promising methodological developments, but wastewater metagenomics should currently be characterized as a potential complementary surveillance approach requiring further validation rather than as an established predictor of clinical AMR or individual risk.

CONCLUSION

This systematic review indicates that wastewater and wastewater-impacted environments provide useful matrices for

detecting and characterizing antimicrobial resistance genes (ARGs). The 15 studies incorporated (mostly shotgun-metagenomic studies with strategic complementary molecular evidence) found clinically relevant ARGs in a wide range of wastewater-related environments. These data confirm the potential for identifying environmental hazards and surveillance at the population level, but do not determine expression, viability, horizontal transfer, exposure, clinical transmission, or individual risk. Wastewater metagenomics is thus a promising complementary One Health surveillance method, and not a validated clinical or public-health predictor.

There is considerable sampling- through-to- sequencing, bioinformatics pipeline, ARG database heterogeneity, normalization, and reporting heterogeneity that drives cross-study consideration. Longitudinal validation of wastewater resistome needs further standardization, clinical correlation and exposure-based risk assessment before it can be utilized routinely as an actionable part of public-health monitoring.

LIST OF ABBREVIATIONS

AMR	=	Antimicrobial Resistance
ARB	=	Antimicrobial-Resistant Bacteria
ARGs	=	Antimicrobial Resistance Genes
CAS	=	Conventional Activated Sludge
GLASS	=	Global Antimicrobial Resistance and Use Surveillance System
IntI1	=	Integron Integrase 1
JB	=	Joanna Briggs Institute
MBR	=	Membrane Bioreactor
MCR	=	Mobilised Colistin Resistance
MGEs	=	Mobile Genetic Elements
UMWW	=	Urban Municipal Wastewater
WWTPs	=	Wastewater Treatment Plants

APPENDICES

Supplementary Appendix A. Database-Specific Search Documentation.

Source	Final Search Date	Retained Query/Approach	Documented Fields/Limits	Records	PRISMA Handling
PubMed	July 31, 2025	((("wastewater"[Title/Abstract] OR "waste water"[Title/Abstract] OR sewage [Title/Abstract] OR WWTP[Title/Abstract]) AND (urban [Title/Abstract] OR municipal [Title/Abstract] OR city [Title/Abstract]) AND (metagenom*[Title/Abstract] OR "shotgun sequencing"[Title/Abstract] OR "whole genome shotgun"[Title/Abstract]) AND ("antimicrobial resistance"[Title/Abstract] OR ARG[Title/Abstract] OR resistome[Title/Abstract] OR "resistance gene"[Title/Abstract]))	Title/Abstract field tags are preserved in the retained syntax. Publication window reported in the manuscript: Jan 2018–Jul 2025. No additional undocumented filter is inferred.	105	Included in PRISMA identification total.

AUTHOR'S CONTRIBUTION

The author was responsible for study conceptualization, methodology design, data compilation, analysis and interpretation, and manuscript writing.

REPORTING GUIDELINES

This study was conducted in accordance with PRISMA guidelines.

AVAILABILITY OF DATA AND MATERIALS

The Supplementary Appendices submitted with this review contains the search documentation, screening-decision accounting, complete data-extraction table, study-level JBI appraisal, and study-level metagenomic methodological appraisal. No new primary sequencing dataset or analytical code was generated by this review. The supplied screening records did not preserve a reliable citation-level mapping for every full-text exclusion; this limitation is disclosed in the Supplementary File rather than reconstructing unsupported bibliographic entries.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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Declared none.

DECLARATION OF AI

Author declares usage of Artificial Intelligence (AI) tools for language editing and manuscript refinement. They approve the final manuscript and take responsibility for the published content.

Scopus	July 31, 2025	("wastewater" OR "waste water" OR sewage OR WWTP) AND (urban OR municipal OR city) AND ("metagenom" OR "shotgun sequencing" OR "whole-genome shotgun") AND ("antimicrobial resistance" OR ARG OR resistome OR "resistance gene")	The retained file preserves this core Boolean syntax and the final count, but not a reliable machine-readable record of all interface field wrappers/filters.	547	Included in PRISMA identification total.
Google Scholar	July 31, 2025	("wastewater" OR "waste water" OR sewage OR WWTP) AND (urban OR municipal OR city) AND ("metagenom" OR "shotgun sequencing" OR "whole-genome shotgun") AND ("antimicrobial resistance" OR ARG OR resistome OR "resistance gene") AND (integron OR intI1 OR plasmid OR transposon OR "mobile genetic element")	Supplementary dynamic screening source. First 20 pages manually screened. Interface estimate (~1,170) was not used as a stable record count.	~200 screened	Not included in PRISMA identification total; no additional unique eligible study added from this manual screen.

Supplementary Appendix B. Full-Text Screening Decision Accounting.

Decision ID	Report on Identifier Status	Specific Exclusion Reason
FT01	Citation-level identifier not recoverable from supplied screening records	Outside wastewater/wastewater-impact scope
FT02	Citation-level identifier not recoverable from supplied screening records	Outside wastewater/wastewater-impact scope
FT03	Citation-level identifier not recoverable from supplied screening records	Outside wastewater/wastewater-impact scope
FT04	Citation-level identifier not recoverable from supplied screening records	Outside wastewater/wastewater-impact scope
FT05	Citation-level identifier not recoverable from supplied screening records	Outside wastewater/wastewater-impact scope
FT06	Citation-level identifier not recoverable from supplied screening records	Outside wastewater/wastewater-impact scope
FT07	Citation-level identifier not recoverable from supplied screening records	Outside wastewater/wastewater-impact scope
FT08	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT09	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT10	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT11	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT12	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT13	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT14	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT15	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT16	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT17	Citation-level identifier not recoverable from supplied screening records	No relevant AMR outcome
FT18	Citation-level identifier not recoverable from supplied screening records	Review/editorial/commentary
FT19	Citation-level identifier not recoverable from supplied screening records	Review/editorial/commentary
FT20	Citation-level identifier not recoverable from supplied screening records	Review/editorial/commentary
FT21	Citation-level identifier not recoverable from supplied screening records	Review/editorial/commentary
FT22	Citation-level identifier not recoverable from supplied screening records	Review/editorial/commentary
FT23	Citation-level identifier not recoverable from supplied screening records	Insufficient methodological information for interpretation
FT24	Citation-level identifier not recoverable from supplied screening records	Insufficient methodological information for interpretation
FT25	Citation-level identifier not recoverable from supplied screening records	Insufficient methodological information for interpretation
FT26	Citation-level identifier not recoverable from supplied screening records	Insufficient methodological information for interpretation
FT27	Citation-level identifier not recoverable from supplied screening records	Insufficient methodological information for interpretation

Supplementary Appendix C. Study-Level JBI Appraisal.

Checklist assignment was based on reported study design: cross-sectional comparison studies used the JBI Analytical Cross-Sectional Studies Checklist, while descriptive occurrence/prevalence surveys used the JBI Prevalence Studies Checklist. Each of the 15 included studies appears once. Yes = clearly satisfied; No = not satisfied; Unclear = insufficient reporting; N/A = not applicable.

JBI Question	Jantharadej <i>et al.</i> , 2022	Magnano San Lio <i>et al.</i> , 2025	Talat <i>et al.</i> , 2023	Acosta <i>et al.</i> , 2025	Majeed <i>et al.</i> , 2021	Sekizuka <i>et al.</i> , 2022	Silvester <i>et al.</i> , 2025	Becsei <i>et al.</i> , 2024	Davis <i>et al.</i> , 2025	Knight <i>et al.</i> , 2024
1. Were the criteria for inclusion in the sample clearly defined?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2. Were the study subjects and the setting described in detail?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
3. Was the exposure measured in a valid and reliable way?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
4. Were objective, standard criteria used for measurement of the condition?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
5. Were confounding factors identified?	Unclear	Yes	Unclear	Yes	Yes	Yes	Yes	Unclear	Yes	Yes
6. Were strategies to deal with confounding factors stated?	No	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes
7. Were the outcomes measured in a valid and reliable way?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
8. Was an appropriate statistical analysis used?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

JBI Prevalence Study Checklist.

JBI Question	Rout <i>et al.</i> , 2023	Fresia <i>et al.</i> , 2019	Hendriksen <i>et al.</i> , 2019	Riquelme <i>et al.</i> , 2022	Tsvetanova & Boshnakov, 2025
1. Was the sample frame appropriate to address the target population?	Yes	Yes	Yes	Yes	Yes
2. Were study participants sampled in an appropriate way?	Yes	Yes	Yes	Yes	Yes
3. Was the sample size adequate?	Unclear	Unclear	Yes	Unclear	Unclear
4. Were the study subjects and the setting described in detail?	Yes	Yes	Yes	Yes	Yes
5. Was the data analysis conducted with sufficient coverage of the identified sample?	Yes	Yes	Yes	Yes	Yes
6. Were valid methods used for the identification of the condition?	Yes	Yes	Yes	Yes	Yes
7. Was the condition measured in a standard, reliable way for all participants?	Yes	Yes	Yes	Yes	Yes
8. Was there an appropriate statistical analysis?	Yes	Yes	Yes	Yes	Yes
9. Was the response rate adequate, and if not, was the low response rate managed appropriately?	N/A	N/A	N/A	N/A	N/A

Supplementary Appendix D. Metagenomic Methodological Appraisal of Included Studies.

This appraisal applies only to the 13 included studies that used shotgun metagenomics. The two complementary non-metagenomic studies (Tsvetanova & Boshnakov [24]; Magnano San Lio *et al.* [25]) are covered by the JBI appraisal but are not included in this metagenomic-specific table.

Study	Sampling Representativeness	Replication	Sample Processing /DNA Extraction	Sequencing Depth & QC	ARG Database/Version	Detection Threshold	Read/Assembly Approach	Normalization Method	Host Assignment	ARG-MGE Linkage	Raw Data Availability
Hendriksen <i>et al.</i> (2019)	Yes	Yes	Yes	Yes	Yes (ResFinder/CARD-based annotation)	Yes	Read-based metagenomics	Yes	Unclear	Unclear	Yes
Davis <i>et al.</i> (2025)	Yes	Yes	Yes	Yes	Yes (CARD)	Yes	Read-based metagenomics	Yes (16S normalization /qPCR supported)	Unclear	Unclear	Yes

Fresia et al. (2019)	Yes	Yes	Yes	Yes	Yes (CARD)	Yes	Read-based metagenomics	Yes	Unclear	Unclear	Yes
Knight et al. (2024)	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics + qPCR comparison	Yes	Unclear	Unclear	Yes
Majeed et al. (2021)	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics	Yes	Unclear	Unclear	Yes
Prieto Riquelme et al. (2022)	Yes	Yes	Yes	Yes	Yes (CARD)	Yes	Shotgun metagenomics	Yes (16S normalized abundance)	Yes	Yes	Yes
Sekizuka et al. (2022)	Yes	Yes	Yes	Yes	Yes (MEGARes/CARD)	Yes	Shotgun metagenomics	Yes	Yes	Yes	Yes
Silvester et al. (2025)	Yes	Yes	Yes	Yes	Yes (MEGARes)	Yes	Shotgun metagenomics	Yes	Unclear	Unclear	Yes
Acosta et al. (2025)	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics ± enrichment	Yes	Unclear	Unclear	Yes
Becsei et al. (2024)	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics + MAG analysis	Yes	Yes	Yes	Yes
Rout et al. (2023)	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics	Unclear	Unclear	Unclear	Yes
Talat et al. (2023)	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics	Yes	Yes	Yes	Yes
Jantharadej et al. (2022)	Yes	Yes	Yes	Yes	Yes	Yes	Shotgun metagenomics + 16S	Yes	Unclear	Unclear	Yes

Assessment categories: Yes = clearly reported/satisfied; No = reported but not satisfied/not performed; Unclear = insufficient information; Not reported = information unavailable in the publication; N/A = not applicable.

Supplementary Appendix E. Complete Data-Extraction Table.

The table below reproduces the study-level extraction fields retained in the review and separates study findings from appraisal observations. Values are reported as documented in the manuscript/source extraction; no missing bibliographic or screening information has been invented.

Author/Year	Aim of the Study	Methodology	Treatment / Intervention	Findings of the Study	Key Appraisal Observations
Davis <i>et al.</i> [15]	Benchmarking quantitative metagenomics vs ddPCR	Shotgun metagenomics (94 Gb), meta-sequin spike-ins, ddPCR validation	U.S. WWTP (influent, activated sludge, effluent)	ARG quantification feasible; LoD ~1 copy/μL; depth critical (~100 Gb)	Quantitative benchmarking and spike-in calibration were reported; host/MGE linkage remained limited.
Knight <i>et al.</i> [16]	National wastewater AMR surveillance	Shotgun metagenomics vs HT-qPCR	47 WWTPs in Wales + hospital effluents	Metagenomics detected 491 ARGs; HT-qPCR showed greater sensitivity for low-abundance targeted genes	Broad multi-site coverage with metagenomics/HT-qPCR comparison; host/MGE linkage was not consistently resolved.
Hendriksen <i>et al.</i> [21]	Global surveillance of ARGs in untreated sewage	Shotgun metagenomics, Illumina HiSeq, ResFinder	Untreated sewage from 79 sampling sites representing 74 cities in 60 countries	557 ARGs identified; β-lactamases, macrolide, tetracycline dominant; carbapenemases detected; resistome profiles mirrored clinical AMR patterns	Clear sampling framework and sequencing workflow; host assignment and ARG-MGE linkage were not consistently resolved.
Becsei <i>et al.</i> [22]	Temporal surveillance across 5 European cities	Shotgun metagenomics, MAG reconstruction	Untreated sewage, time-series	2,332 MAGs assembled; Pseudomonas blooms masked ARG detection; strong city-level differences	Replication and MAG analysis were reported; microbial blooms may affect detection and interpretation.
Jantharadej <i>et al.</i> [23]	Resistome in urban transport canal	Shotgun metagenomics, 16S	10 canal sites (Bangkok)	15 ARG types, 611 subtypes; β-lactams are the most abundant; pathogen hotspots (Arcobacter, Burkholderia)	Shotgun metagenomics plus 16S; surface-water matrix limits direct inference about wastewater-treatment performance.
Tsvetanova & Boshnakov [24]	ARGs in Bulgarian WWTP	Culture + PCR (Enterobacteriaceae, Enterococcus)	Medium-sized WWTP (influent vs effluent)	Resistant bacteria persisted/increased; 8.8% effluent Enterobacteriaceae ESBL+; Enterococci dominant	Culture/PCR study; metagenomic-specific appraisal was not applicable; JBI prevalence appraisal was used.
Fresia <i>et al.</i> [35]	Resistomes in sewage vs coastal beaches	Shotgun metagenomics, CARD	Montevideo sewage outfalls & beach waters	Sewage carried >90% ARG classes vs 40% in beaches; overlap with clinical clones.	Sampling and sequencing methods were reported; some sampling limitations and incomplete host/MGE resolution remained.

Magnano San Lio et al. [25]	ARG surveillance in Sicily WWTPs	qPCR for key ARGs	3 WWTP influents (tertiary vs secondary)	<i>blaOXA</i> , <i>blaTEM</i> , <i>blaCTX-M</i> , <i>erm(B)</i> prevalent; <i>erm(B)</i> highest	Targeted qPCR study; metagenomic-specific appraisal was not applicable; JBI cross-sectional appraisal was used.
Sekizuka et al. [26]	ARG surveillance in Japan WWTPs	Shotgun metagenomics, MEGAREs/CARD	16 WWTPs (influent, effluent, river)	408 ARGs; <i>blaCTX-M</i> , <i>blaTEM</i> , <i>mcr-1</i> in influents; ~1 log reduction post-treatment	Sequencing, normalization and genomic-context reporting were comparatively detailed.
Prieto Riquelme et al. [27]	Develop and demonstrate a standardized metagenomics-based approach for internationally comparable wastewater AMR surveillance	Shotgun metagenomics; CARD-based ARG annotation; 16S rRNA-normalized relative abundance	14 influent sewage samples from 12 WWTPs across six international locations	Resistome composition differed geographically; Asian sewage generally showed higher relative ARG abundance than European/US samples.	Harmonized sampling and genomic-context analysis were reported; sample numbers per site were limited.
Silvester et al. [28]	Hospital vs community wastewater	Shotgun metagenomics, MEGAREs v3, AMR++	3 hospitals & 3 linked WWTPs (Wales)	Hospital effluents showed greater ARG relative abundance and diversity than community WWTP samples; ARG removal varied across WWTPs and was incomplete.	Multi-site metagenomics was reported; site variability and incomplete host/MGE resolution remained.
Acosta et al. [29]	Enrichment-based detection of rare ARGs	Shotgun metagenomics ± culture enrichment	Raw and culture-enriched wastewater from two tertiary-care hospitals and two nearby urban neighbourhoods	1,225 ARGs; enrichment (esp. meropenem) improved detection of carbapenemases; hospitals had higher ARG loads	Enrichment increased rare-ARG detection but can alter community composition and relative abundance.
Majeed et al., [30]	ARGs across WWTP stages	Shotgun metagenomics (MetaStorm, CARD)	U.S. WWTP (influent → effluent)	Total ARG relative abundance decreased by approximately 50%; about 90% of ARG types detected in secondary effluent were also detected in influent.	Transparent metagenomic workflow; relative rather than absolute quantification limited treatment interpretation.
Rout et al. [31]	Characterization of bacterial diversity, ARGs and virulence factors in River Ganga sediments	Shotgun metagenomic analysis	Ganga River sediments, India	ARGs and virulence factors showed spatial variation across river sediments; Proteobacteria predominated	Shotgun metagenomics of river sediment; contextual environmental evidence only, with incomplete host/MGE linkage.
Talat et al. [34]	Resistome in Indian hospital wastewater	Shotgun metagenomics (Illumina)	6 hospitals (urban, rural)	183 ARGs; <i>blaNDM-1</i> , <i>blaOXA</i> , <i>mcr-3</i> , <i>mcr-5</i> detected; plasmid-mediated ARGs frequent	Shotgun metagenomics reported clinically relevant ARGs and plasmid-associated signals; no direct transmission evidence.

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