International Journal of Scientific Interdisciplinary Research

Volume: 13; **Issue:** 6
Pages: 01–59

Environmental Reservoirs of Antimicrobial Resistance: A One Health Framework for Wastewater-Based Surveillance in The United States

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Doi: 10.63125/vzbwht64
Received: *25 April 2023;* ***Revised:*** *29 May 2023;* ***Accepted:*** *11 June 2023;* ***Published:*** *02 June 2023;*

Abstract

Antimicrobial resistance (AMR) represents an interconnected human, animal, and environmental health challenge, while wastewater provides a population-level matrix for quantifying the circulation of clinically relevant antimicrobial resistance genes (ARGs). This quantitative study examined the distribution, abundance, geographic variation, and One Health predictors of wastewater AMR across 163 wastewater treatment plants representing 40 U.S. states. Clinically relevant ARGs were quantified and evaluated alongside community antibiotic utilization, healthcare-facility density, hospital bed capacity, livestock density, population characteristics, geographic region, wastewater flow, and treatment configuration. The findings demonstrated substantial heterogeneity in ARG occurrence and concentration. tetW was detected in 98.2% of treatment plants, followed by blaTEM in 96.3%, blaCTX-M in 92.0%, and mecA in 87.7%, whereas mcr-1 showed the lowest detection frequency at 17.8%. Median absolute concentrations were highest for tetW at 1.30 × 10^6 gene copies/g and blaTEM at 7.20 × 10^5 gene copies/g. Significant geographic heterogeneity was observed in composite ARG burden ($p < 0.001$, $\eta^2 = 0.13$), with the South exhibiting the highest standardized resistance score (0.47 ± 0.91) and the West the lowest (−0.38 ± 0.73). Spatial clustering was significant but modest (Moran's I = 0.21, $p = 0.004$). Multivariable analysis identified community antibiotic utilization as the strongest positive predictor of composite ARG burden ($\beta = 0.31$, 95% CI [0.19, 0.43], $p < 0.001$), followed by healthcare-facility density ($\beta = 0.24$, $p < 0.001$), hospital bed capacity ($\beta = 0.18$, $p = 0.004$), and livestock density ($\beta = 0.17$, $p = 0.003$). Advanced treatment configuration was inversely associated with resistance burden ($\beta = -0.19$, $p = 0.001$). The multivariable model explained 57.8% of between-site variation, while random forest modeling explained approximately 61% of out-of-sample variation. Overall, the findings characterized wastewater AMR as a geographically heterogeneous outcome associated with interconnected human antibiotic consumption, healthcare activity, animal agriculture, and wastewater treatment characteristics, supporting wastewater surveillance as a quantitative environmental component of an integrated One Health AMR framework.

Keywords

Antimicrobial resistance, Wastewater surveillance, One Health, Resistance genes, United States.

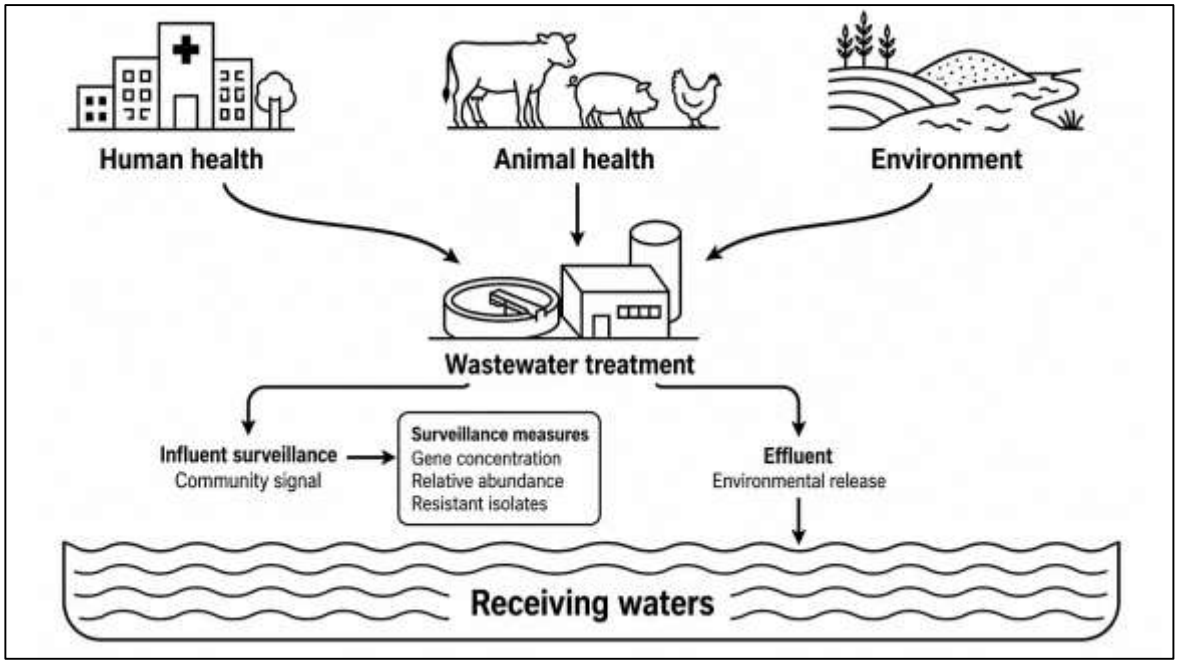
INTRODUCTION

Antimicrobial resistance is broadly defined as the capacity of microorganisms, including bacteria, fungi, viruses, and parasites, to survive or continue reproducing when exposed to antimicrobial agents that would ordinarily inhibit their growth or eliminate them. Within bacterial populations, antibiotic resistance represents a particularly important component of this phenomenon because antibacterial drugs remain central to the treatment of infectious diseases in human and veterinary medicine. Resistance may occur through intrinsic biological characteristics or through acquired mechanisms involving mutation and horizontal transfer of genetic material (Pruden et al., 2021). The environmental dimension of antimicrobial resistance extends this definition beyond resistant infections in individual patients to encompass the ecological systems in which resistant microorganisms, antimicrobial resistance genes, mobile genetic elements, antimicrobial residues, and other selective agents accumulate, interact, persist, and circulate. An environmental reservoir can therefore be understood as a biological or physical compartment capable of harboring resistant microorganisms or genetic determinants and potentially contributing to their maintenance, enrichment, or movement between ecological and host-associated populations. Surface water, groundwater, sediments, soils, agricultural environments, animal production systems, hospital discharges, municipal sewage, wastewater treatment facilities, biosolids, and receiving waters have all been investigated as components of this broader environmental resistome. The term *resistome* encompasses the collection of resistance determinants within a microbial community, including genes expressed in recognized pathogens as well as genes occurring among commensal and environmental microorganisms (Harrington et al., 2022). Wastewater is especially important within this conceptual structure because it represents a composite matrix into which human excreta, household waste, healthcare-associated discharges, industrial inputs, microbial communities, pharmaceutical residues, and other chemical constituents may converge. Municipal wastewater consequently provides a measurable ecological interface between community antimicrobial use, human carriage of resistant organisms, environmental contamination, sanitation infrastructure, and microbial evolution. Wastewater treatment plants further constitute concentrated nodes within this interface because large volumes of wastewater from geographically defined populations are collected and processed at centralized facilities. Their influent can contain diverse bacterial populations and resistance determinants originating from many individuals, while treatment processes alter microbial abundance and community composition before treated effluent or residual solids are released or reused (Harbarth et al., 2015). Environmental reservoirs are therefore not isolated endpoints of antimicrobial contamination. They are interconnected components of a larger biological system involving microbial selection, persistence, genetic exchange, transportation, dilution, concentration, and exposure. Quantitative characterization of these reservoirs requires measurable indicators such as concentrations of antimicrobial resistance genes, relative abundance of resistance determinants, prevalence of resistant organisms, antimicrobial concentrations, microbial community composition, and spatial or temporal variation in these measurements. Framing antimicrobial resistance in this ecological manner establishes the conceptual basis for examining wastewater as both an environmental reservoir and a population-level surveillance matrix within a One Health system (Tiedje et al., 2019).

One Health provides an integrated framework for understanding antimicrobial resistance by recognizing that human health, animal health, and environmental conditions operate as interconnected components rather than independent domains. Within this framework, resistant microorganisms and resistance determinants can circulate through multiple pathways involving people, domesticated animals, wildlife, food production, water, soil, waste streams, healthcare facilities, and built environments. Antimicrobial use in human medicine can select resistant organisms that enter sewage through fecal and urinary excretion, while antimicrobial use in livestock and other animal-production settings can contribute resistant organisms, resistance genes, and pharmaceutical residues to manure, agricultural soils, runoff, and associated water systems (Tiedje et al., 2019). Environmental

microorganisms also contain naturally occurring resistance determinants, creating a large genetic background in which resistance genes can persist and interact with microorganisms associated with humans and animals. These interconnected pathways make antimicrobial resistance particularly suitable for One Health analysis because the environmental compartment can function simultaneously as a recipient of contamination, a site of microbial interaction, a potential selective environment, and a route through which resistant organisms or genetic determinants may encounter new hosts. Wastewater occupies a distinctive position within this network.

Figure 1: One Health Wastewater AMR Surveillance

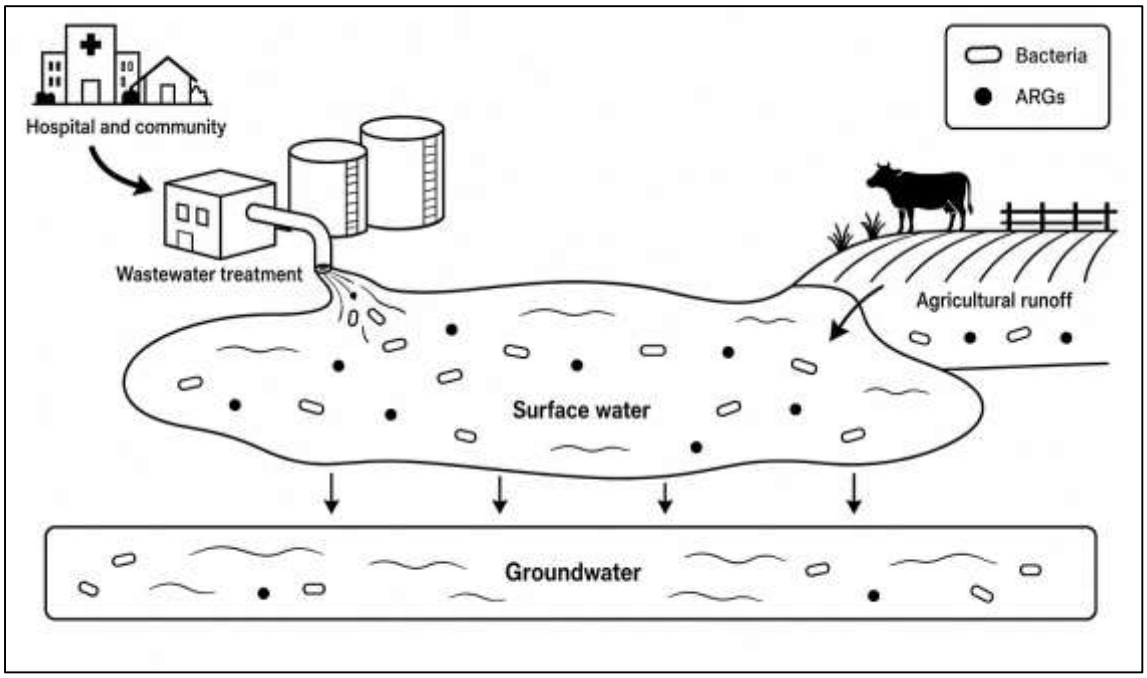


Domestic sewage reflects microbial material shed by community populations; hospital wastewater may contain organisms associated with healthcare environments and substantial antimicrobial exposure; agricultural and food-processing discharges can represent animal-associated inputs; and wastewater treatment facilities combine these materials within engineered microbial ecosystems (Zaheer et al., 2020). The resulting wastewater resistome can therefore contain information derived from several sectors of the One Health continuum. Quantitative wastewater surveillance can characterize this information through measurements of specific resistance genes, classes of resistance determinants, resistant bacterial taxa, mobile genetic elements, or normalized resistome abundance. Molecular approaches such as quantitative polymerase chain reaction, digital polymerase chain reaction, shotgun metagenomic sequencing, and related genomic techniques provide different levels of specificity and breadth for these measurements. Culture-based methods add phenotypic information by determining whether recovered organisms can grow under defined antimicrobial exposure conditions. Combining phenotypic and genotypic measurements can provide complementary descriptions of environmental resistance, although methodological variation affects comparability across locations and studies. The One Health framework also emphasizes that the presence of a resistance determinant in wastewater does not automatically establish transmission to humans or animals (McDermott & Davis, 2021). Concentration, viability, host association, mobility, persistence, environmental exposure, treatment performance, and epidemiological connectivity all influence the interpretation of surveillance measurements. A quantitative framework must therefore distinguish between detecting environmental resistance and estimating the probability or magnitude of transmission. This distinction is fundamental when wastewater measurements are compared with clinical resistance data, antimicrobial

consumption, agricultural characteristics, socioeconomic conditions, healthcare infrastructure, population density, sanitation characteristics, or other potential explanatory variables. One Health consequently supplies the systems-level structure through which wastewater-based antimicrobial resistance surveillance can connect environmental measurements with population and ecological characteristics (Kaiser et al., 2022).

The international significance of antimicrobial resistance derives from its capacity to compromise infectious-disease treatment across national boundaries while being shaped by interconnected patterns of antimicrobial consumption, healthcare access, sanitation, agriculture, food production, travel, trade, population movement, and environmental contamination. Resistant microorganisms are not restricted by political borders, and resistance determinants can circulate through human migration, international travel, food commodities, animals, waterways, waste streams, and microbial ecological networks. International surveillance has documented substantial geographic heterogeneity in antimicrobial resistance, illustrating that the prevalence and distribution of resistance vary among countries, regions, bacterial species, antimicrobial classes, and healthcare contexts (Chau et al., 2022). Clinical surveillance remains essential for measuring resistance among infections encountered in healthcare systems, yet clinical datasets predominantly represent individuals who receive medical attention, undergo specimen collection, and have isolates submitted for antimicrobial susceptibility testing. Population groups with limited healthcare access, asymptomatic carriers, community members who do not seek treatment, and environmental reservoirs may therefore be incompletely represented. Wastewater-based surveillance offers a complementary population-level measurement system because sewage aggregates biological material from large numbers of individuals without requiring each person to seek healthcare or participate directly in testing (Jin et al., 2022). International investigations have detected antimicrobial resistance genes and resistant organisms in wastewater across diverse geographic and economic settings, while comparative studies have demonstrated pronounced differences in resistome composition and abundance among locations. Such differences have been associated with multiple structural conditions, including sanitation coverage, antimicrobial consumption, wastewater management, healthcare infrastructure, economic development, environmental contamination, and population characteristics. Wastewater surveillance also provides a common environmental matrix that can support comparisons when standardized sampling, laboratory, normalization, and analytical procedures are applied. Global research has used influent, effluent, untreated sewage, hospital wastewater, urban drainage systems, and receiving waters to characterize resistance patterns, demonstrating that surveillance objectives can range from estimating community resistance burdens to examining treatment-related changes and environmental release (Ceric et al., 2019). Metagenomic investigations have expanded the number of resistance determinants measurable within individual samples, enabling characterization of complex resistomes rather than reliance solely on selected organisms or genes. Targeted molecular methods have simultaneously provided highly sensitive quantitative measurements of clinically important determinants. International evidence also demonstrates methodological heterogeneity in sample collection, sample concentration, DNA extraction, sequencing depth, resistance databases, gene annotation, normalization, and statistical analysis, creating challenges for direct comparison. Wastewater-based surveillance must therefore be interpreted as a quantitative measurement system whose comparability depends strongly on methodological consistency and clearly specified denominators (Narvaez-Bravo, et al., 2020). Within international One Health surveillance, wastewater provides an environmental observation point linking community populations with sanitation systems and receiving ecosystems. The international relevance of this observation point is reinforced by the global circulation of resistance mechanisms such as extended-spectrum beta-lactamase, carbapenemase, colistin, tetracycline, macrolide, sulfonamide, methicillin, and vancomycin resistance determinants, many of which have been identified in wastewater matrices across multiple geographic settings.

Figure 2: ARG Distribution Across Water Systems



Wastewater treatment systems occupy a complex position in antimicrobial resistance ecology because they function as collection systems, engineered treatment environments, microbial mixing points, and sources of measurable environmental outputs (Pattis et al., 2022). Influent entering municipal wastewater treatment plants contains microorganisms and genetic material originating from human feces, urine, household activities, commercial facilities, healthcare institutions, and other connected sources. Depending on the characteristics of the collection system, stormwater, industrial wastewater, and other discharges may also contribute to the composition of incoming wastewater. This mixture contains large and diverse microbial communities together with nutrients, antimicrobial residues, disinfectants, metals, and other chemical constituents capable of influencing microbial ecology. Wastewater treatment processes are principally designed to reduce organic matter, suspended solids, nutrients, and microbial contamination according to treatment objectives and regulatory requirements rather than to eliminate every antimicrobial resistance determinant (Collignon & McEwen, 2019). Primary settling, biological treatment, clarification, filtration, disinfection, anaerobic digestion, and other processes can substantially modify the abundance and composition of microbial populations, while resistance genes may remain detectable in treated effluent or residual biosolids. Quantitative investigations frequently report changes in absolute concentrations and relative abundance across treatment stages, highlighting the importance of distinguishing between these two metrics. A treatment process can reduce the absolute number of bacterial cells or gene copies while producing a different pattern when resistance genes are normalized to total bacterial abundance. Wastewater treatment facilities are also analytically valuable because influent samples integrate excreted biological material across their contributing sewersheds (Singh et al., 2021). This aggregation transforms individual-level shedding into a community-level signal that can be measured repeatedly through standardized sampling. Composite samples collected over defined periods can reduce short-term fluctuations relative to single grab samples, while repeated longitudinal collection can characterize temporal variability. Solids and liquid influent represent different analytical matrices and may yield different concentrations for particular microorganisms or resistance genes because of partitioning behavior. Targeted quantitative assays can measure selected genes with high sensitivity, whereas metagenomic methods can characterize broad resistance profiles and microbial community structures. Culture-based

approaches can identify viable resistant organisms and permit antimicrobial susceptibility testing, creating another layer of information that genetic detection alone cannot provide (Riquelme et al., 2022). The analytical selection of markers is especially important because genes such as carbapenemase, extended-spectrum beta-lactamase, methicillin, vancomycin, tetracycline, sulfonamide, and colistin resistance determinants differ substantially in prevalence, clinical significance, environmental persistence, host distribution, and detection frequency. Wastewater surveillance can consequently generate multidimensional datasets consisting of gene concentrations, normalized abundance, microbial taxa, treatment characteristics, sampling conditions, and geographic information. These characteristics establish wastewater treatment systems as quantitative observation platforms through which antimicrobial resistance can be measured at the intersection of community health and environmental microbiology (Angeli et al., 2016).

The United States represents a particularly important setting for quantitative wastewater-based antimicrobial resistance research because it combines a substantial burden of resistant infections with extensive healthcare systems, geographically heterogeneous antimicrobial use, large-scale animal agriculture, diverse socioeconomic conditions, and a broad network of centralized and decentralized wastewater infrastructure. Antimicrobial-resistant infections generate substantial morbidity and mortality within the country, and clinically significant resistance includes organisms and mechanisms affecting beta-lactams, carbapenems, methicillin, vancomycin, fluoroquinolones, and other antimicrobial categories (Walker, et al., 2020). Conventional antimicrobial resistance surveillance relies heavily on healthcare-associated laboratory testing, clinical isolates, hospital reporting, outbreak investigations, pharmacy or prescribing data, and specialized surveillance networks. These systems provide essential information about diagnosed infections but measure a population that has entered healthcare pathways. Wastewater provides a different denominator because a treatment-plant catchment can represent biological contributions from thousands or millions of residents regardless of whether those residents have symptoms, obtain medical care, possess health insurance, or receive antimicrobial susceptibility testing. National wastewater infrastructure developed for infectious-disease monitoring has also demonstrated that wastewater measurements can be organized across geographically distributed treatment facilities and interpreted alongside public health information (Djordjevic et al., 2020). Quantitative antimicrobial resistance surveillance can build upon the general principles of this infrastructure while accounting for the distinctive complexity of bacterial resistance genes, bacterial hosts, horizontal gene transfer, and environmental persistence. Recent large-scale United States measurements have demonstrated that clinically relevant antimicrobial resistance genes can be quantified in wastewater across numerous treatment plants and states, providing evidence of geographic variability in community-associated resistance signals. Resistance determinants associated with beta-lactams, carbapenems, methicillin, tetracycline, colistin, and vancomycin can be measured using targeted molecular methods, allowing comparisons across sewersheds when laboratory procedures and normalization strategies are standardized (Hendriksen et al., 2019). Geographic patterns may then be evaluated in relation to population characteristics such as healthcare access, socioeconomic vulnerability, antimicrobial prescribing, travel connectivity, healthcare-facility density, population size, urbanization, and animal-production intensity. Such analyses require careful interpretation because correlations between wastewater gene concentrations and population-level covariates do not establish direct causal pathways. The United States also contains substantial variation in wastewater treatment technologies, sewer connectivity, industrial inputs, climate, rainfall patterns, water consumption, and catchment composition, all of which can influence measured concentrations. Quantitative models therefore require consideration of both biological determinants and infrastructure-related sources of variability (Muloi et al., 2022). Wastewater-based antimicrobial resistance measurement within the United States can be conceptualized as an environmental surveillance layer positioned between individual clinical testing and broader ecological monitoring, generating standardized numerical indicators of resistance occurrence across defined community

catchments.A quantitative framework for wastewater-based antimicrobial resistance surveillance depends on the reliable transformation of complex environmental samples into numerical variables that can be compared across locations, populations, treatment systems, and sampling periods (Graham et al., 2019). Common outcome measures include absolute gene concentration per unit volume or mass, gene copies normalized to bacterial markers such as the 16S ribosomal RNA gene, relative abundance derived from sequencing reads, prevalence of resistant isolates, diversity indices describing resistome composition, and treatment-associated removal values. Each measurement represents a different dimension of antimicrobial resistance and carries distinct analytical assumptions. Absolute concentration reflects the quantity of a target within a defined amount of wastewater or solids, whereas relative abundance describes the proportion of a resistance determinant in relation to a reference population or total genetic material (Ramsamy et al., 2022). Quantitative polymerase chain reaction and digital polymerase chain reaction are frequently used to measure predefined resistance genes because they offer sensitivity and relatively direct quantification. Shotgun metagenomic sequencing enables simultaneous detection of large numbers of resistance determinants and can describe overall resistome composition, although sequencing depth, DNA extraction, database selection, annotation criteria, and detection thresholds influence quantitative estimates. Quantitative metagenomic approaches incorporating internal standards have been evaluated to improve conversion of sequencing information into absolute gene measurements. Long-read sequencing can additionally provide genomic context that assists in linking resistance determinants with potential microbial hosts or mobile genetic elements. Culture-based methods measure phenotypic resistance among viable organisms, offering a different endpoint from molecular detection of DNA (Anjum et al., 2021). Statistical analysis of wastewater antimicrobial resistance may involve descriptive distributions, correlation coefficients, generalized linear models, multivariable regression, mixed-effects models, spatial analysis, principal component methods, diversity analysis, clustering, and other techniques appropriate to the structure of environmental data. Independent variables can include antimicrobial consumption, healthcare utilization, demographic composition, socioeconomic indicators, animal agriculture, treatment-plant characteristics, population served, flow rate, rainfall, season, temperature, and geographic region. Normalization and adjustment are critical because raw concentrations may be affected by wastewater dilution, industrial discharge, infiltration, stormwater, population movement, and per-capita water use. Sampling frequency also affects estimates because antimicrobial resistance signals can fluctuate across days and seasons. Composite sampling can capture variability over a defined interval, while longitudinal designs allow assessment of temporal patterns (Mutuku et al., 2022). Quality assurance requires controls for extraction, amplification, contamination, inhibition, sequencing, and analytical thresholds. These methodological dimensions demonstrate that wastewater antimicrobial resistance surveillance is not a single laboratory test but a quantitative epidemiological system in which sample design, molecular measurement, environmental conditions, statistical modeling, and population-level explanatory variables jointly determine the interpretation of resistance patterns.

The central research problem surrounding environmental reservoirs of antimicrobial resistance in the United States concerns the limited integration of environmental measurements with the clinical, demographic, agricultural, infrastructural, and ecological dimensions that collectively shape resistance at population scale (Hernando-Amado et al., 2019). Clinical surveillance characterizes resistance among sampled patients, antimicrobial-use datasets describe prescribing or consumption, agricultural datasets characterize selected aspects of animal production, and environmental studies measure resistance in wastewater or receiving environments, yet these information streams frequently operate with different geographic units, sampling frames, temporal resolutions, analytical methods, and outcome definitions. Wastewater-based surveillance provides a mechanism for constructing a geographically defined environmental measurement that can be linked quantitatively to characteristics of the population and landscape represented by a treatment-plant catchment. Large-scale wastewater studies have established that antimicrobial resistance genes can be detected across diverse United States locations

and that concentrations vary geographically. The interpretation of such variability remains analytically complex because the wastewater resistome reflects combined contributions from community carriage, clinical infections, healthcare facilities, antimicrobial exposure, microbial ecology, population mobility, animal-associated activities, sewer infrastructure, and environmental conditions (Wang et al., 2021). A One Health quantitative design therefore requires simultaneous attention to resistance outcomes and explanatory variables representing human, animal, and environmental domains. Clinically important resistance genes can serve as dependent variables individually or as composite resistance measures, while independent variables can represent antimicrobial use, socioeconomic conditions, healthcare access, travel connectivity, agricultural intensity, wastewater characteristics, and geographic region. Spatial dependence must also be considered because neighboring communities may share demographic, environmental, healthcare, hydrological, or infrastructural characteristics. Measurement uncertainty arises from temporal variation, laboratory detection limits, differences in wastewater composition, and heterogeneity in the population contributing to each sewershed (Piergiacomo et al., 2022). Standardized sampling and analytical procedures are therefore essential for distinguishing meaningful geographic differences from methodological variation. The quantitative research problem is further defined by the need to determine whether environmental resistance measurements correspond with established indicators of antimicrobial resistance burden and whether specific One Health characteristics explain significant variation in wastewater resistance concentrations after relevant covariates are controlled (Iskandar et al., 2020). Wastewater can capture resistance determinants shed by both symptomatic and asymptomatic individuals, while environmental sampling can also detect genetic material that is not represented in routine clinical surveillance. Consequently, wastewater measurements constitute a distinct epidemiological dataset rather than a direct substitute for clinical resistance estimates. Examining these measurements within an integrated One Health framework establishes a quantitative basis for assessing the distribution, concentration, geographic heterogeneity, and multivariable correlates of antimicrobial resistance across United States wastewater catchments (Hong et al., 2018).

The primary objective of this quantitative study is to examine the distribution, concentration, and variation of antimicrobial resistance within wastewater environments in the United States through an integrated One Health framework that recognizes the interconnected roles of human populations, animal-associated activities, wastewater systems, and environmental reservoirs. Specifically, the study seeks to quantify selected antimicrobial resistance genes and resistant microbial indicators in wastewater samples collected from geographically defined wastewater treatment plant catchments and to determine the extent to which resistance measurements vary across sampling locations, regions, and relevant environmental conditions. The study also aims to characterize wastewater as a population-level reservoir of antimicrobial resistance by measuring the absolute or normalized abundance of resistance determinants associated with clinically important antimicrobial classes, including beta-lactams, carbapenems, tetracyclines, sulfonamides, macrolides, glycopeptides, polymyxins, and other antimicrobial categories represented within environmental samples. Another objective is to assess the statistical relationships between wastewater antimicrobial resistance measurements and selected human-health variables, including population size, population density, antimicrobial utilization, healthcare access, healthcare-facility distribution, and other community characteristics that may correspond with observed resistance patterns. From the animal-health dimension of the One Health framework, the study seeks to evaluate whether indicators of livestock production, animal density, agricultural activity, and related land-use characteristics are associated with differences in antimicrobial resistance levels detected within wastewater systems. From the environmental dimension, the study aims to examine the relationships between resistance measurements and wastewater treatment characteristics, influent composition, wastewater flow, treatment processes, geographic location, and other measurable environmental factors that may account for variation among sampling sites. An additional objective is to compare resistance profiles across wastewater catchments

in order to identify statistically significant geographic heterogeneity and determine whether particular resistance determinants occur consistently or at elevated concentrations within specific population or environmental contexts. The study further seeks to evaluate the degree to which wastewater-derived antimicrobial resistance indicators correspond with available population-level measures of antimicrobial resistance and antimicrobial exposure, thereby quantitatively characterizing the relationship between environmental surveillance signals and established epidemiological indicators. Through descriptive statistics, correlation analysis, multivariable regression, spatial analysis, and other appropriate quantitative procedures, the research is designed to identify measurable associations among human, animal, and environmental variables while controlling for relevant demographic, infrastructural, and geographic factors. Collectively, these objectives establish a quantitative structure for systematically measuring antimicrobial resistance in United States wastewater and examining its distribution across interconnected One Health domains.

LITERATURE REVIEW

Antimicrobial resistance (AMR) within environmental systems has developed into a substantial area of quantitative investigation because resistance is generated, maintained, transported, and detected across interconnected human, animal, wastewater, agricultural, and natural environments. Within the One Health framework, wastewater represents an especially informative environmental matrix because municipal sewage integrates biological material from large populations while wastewater treatment plants function as collection points for microorganisms, antimicrobial resistance genes (ARGs), antibiotic-resistant bacteria (ARB), antimicrobial residues, and other chemical and biological constituents (Haddaji, 2022; Imran & Jannatul, 2021). The literature on wastewater-based antimicrobial resistance surveillance spans environmental microbiology, molecular epidemiology, public health surveillance, wastewater engineering, microbial genomics, veterinary science, and quantitative environmental health. Existing research has examined the abundance and diversity of resistance determinants in wastewater influent, treatment processes, effluent, sludge, biosolids, receiving waters, and other connected environmental compartments. Research has also increasingly evaluated wastewater as a community-level surveillance matrix capable of complementing patient-centered clinical surveillance. A recent global scoping review identified 177 wastewater-AMR surveillance reports from 2014 through October 2024, with more than three quarters published after 2019, demonstrating the rapid expansion of quantitative work using qPCR and sequencing-based approaches. At the national scale, quantitative wastewater research has demonstrated the feasibility of comparing antimicrobial resistance across large numbers of geographically distributed wastewater treatment plants (Ashik & Shabbir, 2022; Murray et al., 2022). A United States investigation, for example, quantified 11 clinically relevant ARGs in wastewater solids obtained from 163 treatment plants and examined associations with antibiotic use, social vulnerability, animal agriculture, healthcare facilities, and transportation-related variables. Methodological studies additionally indicate that targeted quantitative PCR approaches and metagenomic sequencing measure different dimensions of environmental resistance: targeted methods can provide sensitive quantification of selected clinically important genes, whereas metagenomics can provide broader resistome coverage and information about potential microbial hosts. These findings establish a substantial quantitative literature from which the environmental distribution of AMR can be examined through One Health relationships (Hay et al., 2018).

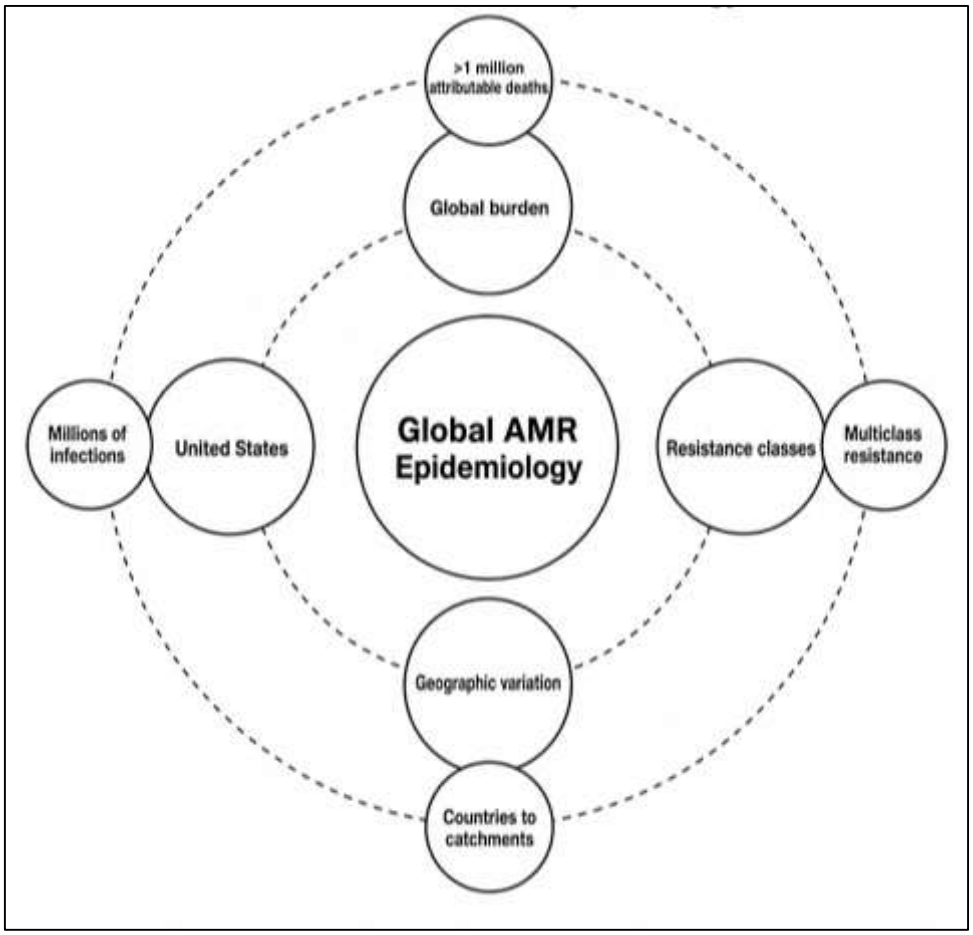
For the present quantitative study, the literature can be organized around measurable relationships rather than treated only as a descriptive discussion of antimicrobial resistance. The central analytical domains include the concentrations and normalized abundance of ARGs in wastewater; spatial and temporal variability in resistance; prevalence and abundance of resistant bacterial populations; wastewater treatment characteristics; horizontal gene transfer and mobile genetic elements; antimicrobial and chemical selective pressures; human antimicrobial utilization; healthcare-related inputs; animal agriculture; socioeconomic and demographic conditions; and relationships between wastewater indicators and clinical resistance. This organization is particularly important because wastewater AMR studies employ heterogeneous outcome measures, sampling matrices, laboratory techniques, normalization procedures, and statistical models (Hasan & Adnan, 2022; Prestinaci et al.,

2015). Wastewater treatment plants have repeatedly been characterized as important reservoirs or collection points for ARGs and ARB, with resistance determinants detectable across influent, activated sludge, treatment stages, effluent, and residual solids. At the same time, systematic assessment of wastewater surveillance has identified continuing difficulty in directly connecting environmental resistance signals with clinical resistance patterns because organisms and resistance determinants have different environmental behaviors and because surveillance systems use different denominators and sampling designs. The following literature review structure therefore emphasizes specific quantitative constructs, dependent and independent variables, measurable relationships, methodological differences, and empirical gaps relevant to wastewater-based surveillance of antimicrobial resistance in the United States (Browne et al., 2020; Akter & Sumaiya, 2022).

Antimicrobial Resistance and Geographic Distribution

Antimicrobial resistance represents a substantial component of the global infectious-disease burden, with quantitative epidemiological investigations demonstrating extensive mortality, morbidity, and healthcare consequences across diverse geographic and economic settings. Large-scale assessments of bacterial antimicrobial resistance have estimated that millions of deaths worldwide are associated with drug-resistant bacterial infections, while more than one million deaths in a single year have been directly attributable to bacterial resistance. The burden is distributed unevenly across regions, age groups, infectious syndromes, and bacterial species. Lower respiratory infections, bloodstream infections, intra-abdominal infections, urinary tract infections, and other major infectious syndromes account for substantial proportions of resistance-associated mortality (Mashfiqur & Md, 2022; Mestrovic et al., 2022). Several bacterial pathogens contribute disproportionately to this burden, including *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Quantitative studies conducted across multiple countries have further demonstrated that resistant bloodstream infections are associated with greater risks of treatment failure, prolonged hospitalization, intensive-care utilization, and mortality compared with infections caused by antimicrobial-susceptible organisms. The epidemiological burden also varies according to economic development and healthcare infrastructure. Many low- and middle-income settings experience high resistance prevalence together with substantial infectious-disease incidence, restricted laboratory capacity, limited access to effective second-line therapies, and deficiencies in water, sanitation, and hygiene infrastructure (Dodd et al., 2016; Mashiat & Rahman, 2022). High-income countries generally maintain more extensive clinical surveillance systems, yet substantial resistance persists in hospitals, long-term care facilities, communities, and environmental settings. Age-specific analyses indicate particularly serious consequences among neonates, older adults, critically ill patients, and individuals with invasive infections. Surveillance studies also reveal that reported resistance prevalence represents only a portion of the total population burden because routine clinical data depend on healthcare access, specimen collection, laboratory testing, reporting completeness, and surveillance coverage. Consequently, countries with limited diagnostic capacity may simultaneously experience substantial resistant-infection burdens and generate comparatively sparse surveillance data. Environmental studies add another dimension by detecting resistance genes and resistant organisms in wastewater, surface waters, agricultural environments, and other reservoirs. These environmental measurements cannot be interpreted as equivalent to clinical infection prevalence because detection of an antimicrobial resistance gene does not establish that a viable pathogen is causing disease (Dean et al., 2017; Md & Yeasin, 2022). Taken collectively, findings across more than ten major global burden assessments, multicountry surveillance investigations, systematic reviews, hospital studies, and environmental surveillance studies characterize antimicrobial resistance as a quantitatively heterogeneous global phenomenon whose measured burden depends on pathogen distribution, antimicrobial exposure, healthcare conditions, surveillance intensity, and environmental context (Li et al., 2022; Ashfaq & Mohammad, 2022). Quantitative antimicrobial resistance research demonstrates substantial variation in resistance according to antimicrobial class, bacterial species, geographic setting, healthcare environment, and intensity of antimicrobial exposure.

Figure 3: Global Antimicrobial Resistance Epidemiology Framework



Resistance to beta-lactam antibiotics represents one of the most extensively documented patterns because these agents are widely used for treating bacterial infections. Extended-spectrum beta-lactamase-producing Enterobacterales have been detected at substantial frequencies in clinical, community, animal, food, wastewater, and other environmental samples, with resistance particularly associated with third-generation cephalosporins. Carbapenem resistance represents another quantitatively important category because carbapenems are frequently reserved for severe infections involving multidrug-resistant Gram-negative organisms (Limmathurotsakul et al., 2019; Imran & Jannatul, 2022). Surveillance investigations have documented marked variation in carbapenem resistance among *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and other clinically significant organisms, with some healthcare settings and geographic regions reporting considerably higher proportions than others. Fluoroquinolone resistance is also widespread among Enterobacterales and other pathogens and has been measured in both community-associated and healthcare-associated infections. Tetracycline and sulfonamide resistance determinants frequently occur at high abundance in wastewater, animal-associated environments, agricultural soils, and surface waters, reflecting the broad historical and contemporary use of these antimicrobial classes across human and veterinary sectors. Macrolide resistance has been documented among respiratory, enteric, and other bacterial populations and is also represented by numerous resistance genes detectable in wastewater resistomes. Glycopeptide resistance, particularly vancomycin resistance among enterococci, remains quantitatively important in healthcare-associated surveillance because it limits therapeutic options for serious Gram-positive infections (Kaniz & Jahanara, 2022; Vikesland et al., 2019). Methicillin resistance among *Staphylococcus aureus* continues to contribute to invasive and noninvasive disease burdens, although prevalence differs substantially across countries and healthcare systems. Polymyxin resistance has received intensive quantitative attention because colistin is used

against selected multidrug-resistant Gram-negative infections, while transferable colistin resistance determinants have also been identified in human, animal, food, and environmental samples. Comparative wastewater investigations have detected genes representing beta-lactam, carbapenem, tetracycline, sulfonamide, macrolide, glycopeptide, methicillin, and polymyxin resistance within the same environmental matrices, demonstrating that wastewater contains a multidimensional resistance profile rather than a single resistance phenotype (Monaco et al., 2016; Sadia & Mostari, 2022). Evidence synthesized from clinical surveillance networks, meta-analyses, wastewater investigations, genomic studies, veterinary studies, and environmental monitoring therefore demonstrates that resistance distribution differs considerably among antimicrobial classes and that clinical resistance percentages and environmental resistance-gene abundance must remain analytically distinct measurements.

Geographic heterogeneity is one of the most consistently reported quantitative characteristics of antimicrobial resistance, with substantial differences occurring between continents, countries, states, provinces, cities, healthcare institutions, and individual wastewater catchments. Multinational surveillance studies have shown that the proportion of resistant bacterial isolates can vary several-fold across geographic settings, even when the same bacterial species and antimicrobial class are evaluated (Acevedo et al., 2019; Shaiful et al., 2022). High resistance levels have been documented in parts of South Asia, Southeast Asia, sub-Saharan Africa, Latin America, the Middle East, and selected areas of Europe, while lower prevalence for particular organism–drug combinations has been recorded in countries with different antimicrobial-use patterns, infection-control systems, sanitation conditions, and healthcare structures. These geographic patterns are not uniform across all resistance mechanisms. A country with relatively high resistance to one antimicrobial class may exhibit lower resistance to another, demonstrating that AMR geography reflects combinations of bacterial ecology, antimicrobial selection, transmission networks, healthcare practices, animal production, environmental conditions, and public health infrastructure. Quantitative spatial investigations have associated geographic resistance variation with outpatient and inpatient antimicrobial consumption, population density, socioeconomic characteristics, international mobility, healthcare accessibility, sanitation infrastructure, agricultural intensity, and environmental contamination (Kalilani et al., 2018; Stabak & Shahid, 2022). Wastewater studies reinforce this spatial heterogeneity by demonstrating substantial differences in resistance-gene abundance and resistome composition among cities and countries. International sewage investigations have detected geographic clustering of resistome profiles, indicating that wastewater microbial communities and their resistance determinants contain measurable spatial structure. Studies comparing wastewater treatment plants have similarly reported between-site differences in clinically important genes, including determinants associated with extended-spectrum beta-lactam, carbapenem, tetracycline, sulfonamide, macrolide, vancomycin, methicillin, and colistin resistance. Such measurements provide environmental information that differs fundamentally from clinical prevalence percentages. Clinical surveillance generally calculates resistance among cultured isolates obtained from selected patient populations, whereas wastewater measurements commonly quantify gene copies, normalized abundance, sequence reads, resistant bacterial concentrations, or resistome diversity across a contributing population (Kuo et al., 2017; Feroz & Pervaz, 2022). Geographic comparisons are additionally influenced by variation in sampling frequency, laboratory methods, diagnostic capacity, antimicrobial susceptibility standards, sequencing databases, reporting systems, and population coverage. Evidence from numerous multinational surveillance programs, systematic reviews, spatial epidemiological studies, metagenomic sewage investigations, and wastewater treatment plant studies therefore indicates that observed geographic variation contains both biological and measurement-related components. This distinction is essential when comparing regions because high environmental ARG abundance cannot automatically be translated into an equivalent percentage of resistant clinical infections, while low reported clinical prevalence in areas with limited surveillance cannot automatically be interpreted as a low population burden (Mohammad, 2023; Vikesland et al., 2019).

The United States carries a substantial and geographically heterogeneous burden of antimicrobial-resistant infections across hospitals, long-term care facilities, outpatient settings, communities, and environmental systems. National burden assessments have estimated that resistant bacterial and fungal

pathogens collectively cause millions of infections and tens of thousands of deaths within the country, although estimates vary according to the pathogens included, surveillance period, analytical definitions, and data sources. Healthcare-associated resistance remains particularly important because hospitalized and medically vulnerable populations experience exposure to invasive procedures, antimicrobial treatment, intensive-care environments, and transmission opportunities that can increase the occurrence and consequences of resistant infections (Lee et al., 2019). Methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, extended-spectrum beta-lactamase-producing Enterobacterales, carbapenem-resistant Enterobacterales, multidrug-resistant *Pseudomonas aeruginosa*, and carbapenem-resistant *Acinetobacter* represent important components of the U.S. resistance profile. Community-associated resistance is also quantitatively significant, including resistant urinary, respiratory, skin, gastrointestinal, and bloodstream infections occurring outside traditional hospital environments. Studies based on national and regional surveillance datasets demonstrate considerable variation in resistance prevalence across states, census regions, healthcare systems, and patient populations. Geographic differences have been associated with antimicrobial prescribing practices, healthcare utilization, population structure, socioeconomic conditions, urbanization, travel, long-term care exposure, and regional healthcare networks (Adnan & Iftekhar, 2023; Fernández et al., 2016). Environmental surveillance adds a complementary perspective to these clinical measurements. U.S. wastewater studies have detected and quantified resistance determinants associated with beta-lactams, carbapenems, tetracyclines, methicillin, vancomycin, colistin, and other antimicrobial categories across geographically distributed wastewater treatment facilities. Large multisite investigations involving wastewater systems serving diverse populations have demonstrated measurable between-site variability in resistance-gene concentrations and have examined relationships with antimicrobial use, healthcare facilities, social vulnerability, transportation, and animal agriculture. These studies demonstrate that wastewater can generate quantitative resistance indicators for entire sewershed populations, including individuals who do not undergo clinical testing (Ahmed & Sadia, 2023; Kariyawasam et al., 2022). Clinical and wastewater indicators nevertheless represent different epidemiological constructs: a clinical resistance percentage describes resistance among tested isolates, whereas wastewater ARG abundance describes the quantity or relative representation of genetic resistance determinants within a composite environmental sample. Evidence synthesized across national burden reports, multicenter clinical studies, healthcare surveillance datasets, genomic investigations, wastewater studies, and spatial analyses therefore portrays U.S. antimicrobial resistance as a multidimensional burden characterized by variation across pathogens, antimicrobial classes, healthcare contexts, communities, and environmental reservoirs (Asheke, 2023; Peng et al., 2018).

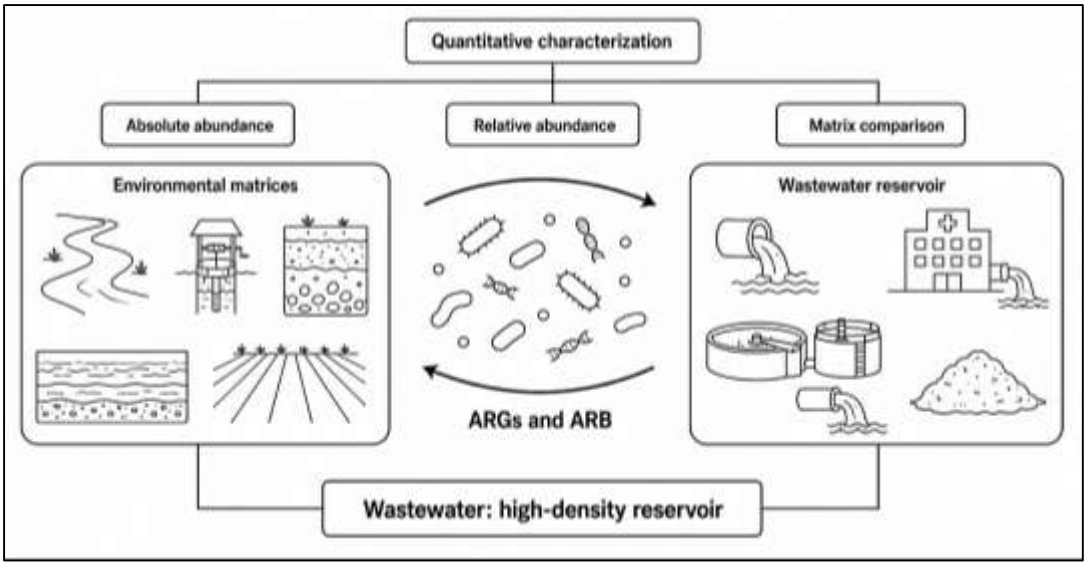
Environmental Reservoirs of Antimicrobial Resistance

Quantitative characterization of antimicrobial resistance genes (ARGs) in environmental reservoirs depends substantially on whether resistance is expressed as absolute concentration or relative abundance, because these approaches describe different dimensions of environmental resistance. Across more than ten wastewater, surface-water, sediment, soil, groundwater, and agricultural-environment studies, absolute abundance has commonly been reported as gene copies per unit volume of water, per unit mass of soil, sediment, sludge, or biosolids, or as concentrations derived from molecular measurements (Yeasin, 2023; Williams et al., 2016). This approach allows researchers to estimate the actual quantity of a resistance determinant present within a defined environmental sample and is particularly valuable for comparing contamination loads, treatment-stage reductions, discharge loads, and changes in ARG concentration over time. Relative abundance, in contrast, evaluates ARG occurrence after normalization to indicators of total bacterial abundance, total DNA, sequencing reads, or other microbial reference measures. Studies employing 16S rRNA gene normalization have demonstrated that relative abundance can reveal whether resistance determinants represent a larger or smaller proportion of the bacterial community even when total microbial concentrations change substantially. This distinction is especially important in wastewater treatment systems because reductions in absolute ARG concentrations can occur simultaneously with stable or increased normalized abundance when susceptible bacterial populations are removed at a different rate (Jahanara & Kaniz, 2023; Serwecińska, 2020). Similar differences have been observed in soils and sediments, where high microbial biomass can produce large absolute ARG concentrations without necessarily

indicating that resistance genes constitute an unusually high proportion of the total microbial community. Quantitative investigations have reported particularly frequent environmental detection of genes associated with tetracycline, sulfonamide, beta-lactam, macrolide, aminoglycoside, quinolone, and multidrug resistance, although measured concentrations differ substantially among environmental matrices and analytical platforms. High-throughput quantitative PCR studies can simultaneously quantify numerous targeted genes, whereas shotgun metagenomic studies characterize broader resistome composition and generally express resistance through sequencing-based relative measures (Hayward et al., 2020; Rahman, 2023). Digital PCR has additionally been applied when precise absolute quantification is required at relatively low target concentrations. Methodological studies demonstrate that DNA extraction efficiency, sample concentration procedures, amplification inhibition, sequencing depth, detection limits, normalization strategy, and microbial biomass can influence the reported magnitude of resistance. Consequently, absolute and relative ARG abundance should not be treated as interchangeable indicators. Synthesized evidence indicates that their combined interpretation provides a more complete quantitative description of environmental reservoirs by distinguishing the total resistance burden from the proportional representation of resistance within microbial communities (Sheak & Tahmedur, 2023; Nguyen et al., 2019).

Environmental surveillance studies demonstrate that ARG concentrations and resistome composition vary substantially among surface water, groundwater, soil, sediment, and agricultural environments because these compartments differ in microbial density, contaminant inputs, hydrological connectivity, physicochemical conditions, antimicrobial exposure, and contact with human and animal waste. Surface-water investigations conducted in rivers, streams, lakes, and other freshwater systems have frequently detected resistance determinants associated with tetracyclines, sulfonamides, beta-lactams, macrolides, quinolones, aminoglycosides, and multidrug resistance (Kusi et al., 2022; Kaium, 2023). Higher ARG concentrations have commonly been measured downstream of wastewater discharges, densely populated urban areas, hospitals, agricultural activities, and other anthropogenic inputs compared with less impacted reference locations. Quantitative antimicrobial resistance research demonstrates substantial variation in resistance according to antimicrobial class, bacterial species, geographic setting, healthcare environment, and intensity of antimicrobial exposure.

Figure 4: Environmental AMR Reservoir Characterization Framework



Quantitative antimicrobial resistance research demonstrates substantial variation in resistance according to antimicrobial class, bacterial species, geographic setting, healthcare environment, and intensity of antimicrobial exposure. Metagenomic investigations have also demonstrated that resistome diversity differs across environmental compartments, with some resistance genes broadly distributed and others strongly associated with particular ecological settings. Collectively, evidence from

numerous quantitative field studies indicates that environmental AMR cannot be represented by a single concentration measure because each matrix contains a distinct combination of resistance abundance, microbial community structure, contaminant exposure, and ecological conditions.

Wastewater has consistently been identified across quantitative environmental studies as one of the most concentrated anthropogenic reservoirs of ARGs and antibiotic-resistant bacteria because it receives microorganisms and genetic material from large human populations together with healthcare, household, commercial, and sometimes industrial or agricultural sources (Fang et al., 2018; Badrul & Mominul, 2023). Municipal sewage contains fecal microorganisms from both symptomatic and asymptomatic individuals, allowing resistance determinants circulating within a community to accumulate within a common wastewater stream. Hospital wastewater can contribute additional concentrations of clinically relevant resistant organisms, antimicrobial residues, disinfectants, and resistance genes associated with intensive antimicrobial exposure. Studies examining untreated sewage have repeatedly identified high absolute concentrations of genes associated with beta-lactam, tetracycline, sulfonamide, macrolide, quinolone, aminoglycoside, methicillin, vancomycin, carbapenem, and multidrug resistance. Wastewater metagenomic investigations have similarly demonstrated substantial resistome diversity, often detecting hundreds or thousands of resistance-associated sequences across samples depending on sequencing depth and classification criteria. Quantitative comparisons across wastewater treatment stages generally show substantial reductions in total bacterial concentrations and absolute abundance of many resistance genes during treatment, although complete elimination is uncommon (Anand et al., 2021; Nishat, 2023). Selected ARGs remain detectable in treated effluent, activated sludge, and biosolids, while relative abundance can remain stable or occasionally increase when resistance determinants and total bacterial populations are removed at different rates. Culture-based studies provide complementary evidence by recovering resistant *Escherichia coli*, enterococci, *Klebsiella*, *Pseudomonas*, *Acinetobacter*, and other bacterial groups from municipal and hospital wastewater. Molecular investigations have also detected clinically important genes associated with extended-spectrum beta-lactamases and carbapenemases, demonstrating that wastewater contains resistance determinants relevant to healthcare as well as genes widely distributed among environmental bacteria. Mobile genetic elements, including integrons and plasmid-associated markers, occur alongside ARGs in wastewater and have frequently shown positive quantitative relationships with resistance-gene abundance (Cheng et al., 2020; Sadia, 2023). Wastewater treatment plants therefore represent both high-density collection points and engineered microbial ecosystems containing substantial bacterial biomass, nutrients, genetic material, antimicrobial residues, metals, and other potential selective agents. Synthesized findings from municipal wastewater, hospital wastewater, treatment-plant, culture-based, qPCR, digital PCR, and metagenomic studies establish wastewater as a quantitatively rich environmental reservoir in which ARG concentration, ARB prevalence, resistome diversity, and mobile resistance determinants can be measured simultaneously (Cheng et al., 2020; Stabak & Siam, 2023).

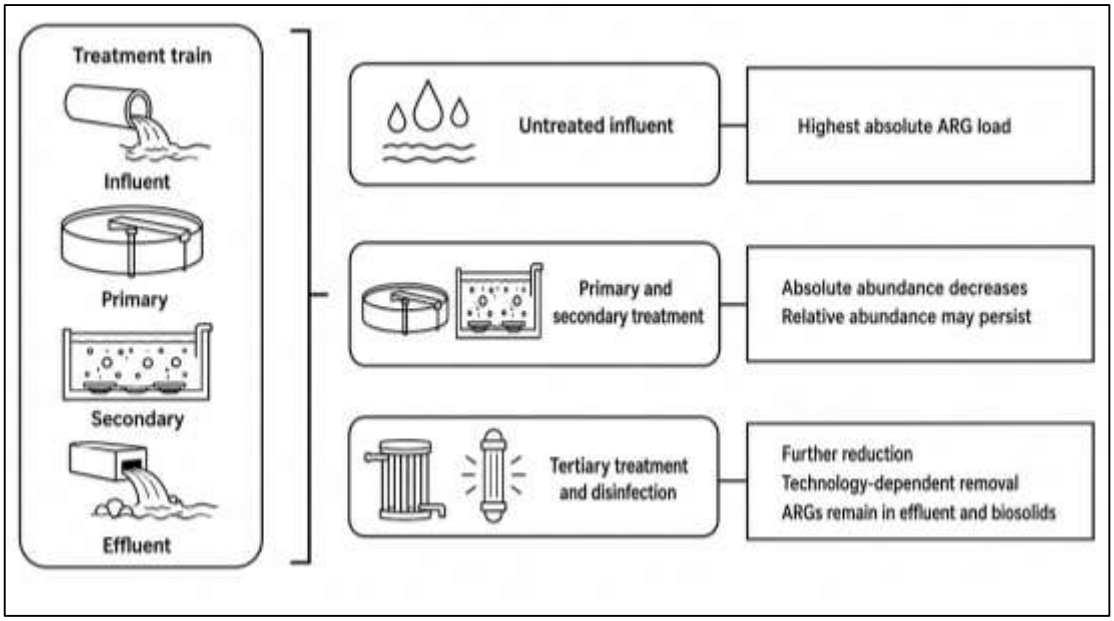
Antimicrobial Resistance Genes Across Wastewater Treatment Stages

Untreated municipal wastewater influent represents one of the most concentrated sampling points for measuring community-associated antimicrobial resistance because it contains microbial material from large populations before major treatment-related removal occurs. Quantitative studies conducted across municipal wastewater treatment plants have repeatedly detected high absolute concentrations and broad diversity of antimicrobial resistance genes in influent samples, with commonly reported targets including genes associated with beta-lactam, tetracycline, sulfonamide, macrolide, aminoglycoside, quinolone, glycopeptide, methicillin, and carbapenem resistance (Pan & Chu, 2018; Feroz, 2023). Influent concentrations vary substantially among facilities, reflecting differences in population size, antibiotic consumption, healthcare utilization, industrial discharges, sewer design, rainfall, wastewater dilution, and local microbial ecology. Several investigations have reported ARG concentrations spanning multiple orders of magnitude within the same treatment system, demonstrating that gene-specific abundance differs considerably even before treatment begins. Genes associated with widely used or historically persistent antimicrobial classes, such as tetracycline and sulfonamide resistance determinants, are frequently detected at relatively high concentrations, whereas clinically critical genes such as carbapenemase or colistin resistance markers may occur at lower and

more variable levels. Relative abundance measurements provide an additional perspective by normalizing ARG concentrations to total bacterial markers or sequencing reads. Studies using this approach have shown that some genes remain highly represented within influent microbial communities even when absolute concentrations differ between treatment plants (Tahmedur & Rifat, 2023; Tong et al., 2019). Metagenomic investigations have further demonstrated that untreated sewage contains highly diverse resistomes, often including hundreds of resistance determinants and mobile genetic elements within a single composite sample. Culture-based investigations have complemented these findings by identifying viable antibiotic-resistant bacteria in influent, including resistant enteric organisms and opportunistic pathogens. Hospital contributions can increase the abundance of selected clinically relevant resistance markers, while community sewage contributes resistance determinants shed by both symptomatic and asymptomatic individuals. Across more than ten quantitative wastewater investigations, influent samples consistently provide the highest or among the highest absolute ARG loads within treatment systems, establishing them as a critical reference point for evaluating subsequent reductions, enrichment patterns, treatment performance, and population-level resistance signals (Kang et al., 2022).

Primary and secondary wastewater treatment processes substantially alter the concentration and composition of antimicrobial resistance genes, although the magnitude and direction of change depend on the gene, treatment configuration, microbial community, and measurement method. Primary treatment primarily removes suspended solids through sedimentation and physical separation, and quantitative studies have shown that this stage can reduce absolute concentrations of particle-associated bacteria and ARGs while transferring a substantial portion of resistance determinants into primary sludge. The degree of removal varies according to whether specific ARGs are predominantly associated with suspended particles or remain within the liquid fraction (Zhang et al., 2019). Secondary biological treatment produces more complex changes because activated sludge systems contain dense microbial communities, prolonged biomass retention, high nutrient availability, and repeated contact among diverse bacterial populations. Across multiple studies, secondary treatment has produced substantial reductions in absolute ARG concentrations, often corresponding with overall decreases in bacterial biomass and organic loading. At the same time, some investigations have reported stable or increased relative abundance for selected resistance genes after biological treatment. This apparent enrichment can occur when total bacterial populations decline more rapidly than specific resistant populations or when resistant organisms are selectively retained within activated sludge.

Figure 5: ARG Distribution Across Wastewater Treatment



Genes associated with tetracycline, sulfonamide, beta-lactam, macrolide, and multidrug resistance have frequently been tracked across primary and secondary stages, with removal efficiencies differing by target (Wang et al., 2017).

Quantitative studies have also identified positive relationships between ARG abundance and mobile genetic elements such as integrons, suggesting that gene mobility may contribute to persistence within biological treatment systems. Operational factors, including solids retention time, hydraulic retention time, dissolved oxygen, temperature, organic loading, and sludge recycling, can influence these patterns. Biological treatment therefore cannot be characterized as a uniform resistance-removal process. Synthesized evidence from qPCR, high-throughput qPCR, digital PCR, metagenomic, and culture-based studies indicates that secondary treatment generally lowers absolute resistance loads while producing gene-specific shifts in relative abundance and microbial composition. This distinction is important because a reduction in total gene copies does not necessarily indicate proportional depletion of resistance within the surviving microbial community (Alexander et al., 2020).

Tertiary treatment and disinfection add further reductions in microbial and genetic contamination after secondary treatment, yet quantitative studies indicate that antimicrobial resistance genes can remain detectable in treated effluent, activated sludge, and biosolids. Filtration, ultraviolet irradiation, chlorination, ozonation, membrane-based treatment, and advanced oxidation have all been evaluated for their ability to reduce ARG concentrations. Many investigations report additional log reductions following tertiary treatment, although removal efficiency varies substantially among technologies and target genes (Narciso-da-Rocha et al., 2018). Ultraviolet treatment can reduce viable bacterial populations and damage genetic material, while chlorination can achieve strong microbial inactivation but may not uniformly eliminate extracellular DNA or all resistance determinants. Membrane filtration can produce substantial physical removal of cells and particles, particularly when pore size is sufficiently small, whereas advanced oxidation processes can degrade cellular and extracellular genetic material under appropriate operational conditions. Quantitative comparisons also demonstrate that solids-associated compartments often retain high ARG concentrations. Activated sludge may contain greater gene abundance per unit mass than treated effluent because microbial biomass is concentrated during biological treatment. Biosolids generated from wastewater processing can similarly contain substantial quantities of resistance genes after digestion, dewatering, stabilization, or other solids-treatment steps (Quach-Cu et al., 2018). Studies examining tetracycline, sulfonamide, beta-lactam, macrolide, quinolone, and integron-associated genes have commonly reported persistence in biosolids, although treatment intensity influences the final concentrations. Influent–effluent comparisons consistently show that absolute ARG abundance generally decreases across complete treatment trains, sometimes by several orders of magnitude for individual targets. Relative abundance, however, can remain unchanged or even increase for selected genes when the denominator used for normalization decreases more strongly than the resistance target. This divergence has been documented across conventional activated sludge plants, advanced treatment facilities, and disinfection systems. More than ten studies collectively indicate that treatment performance must therefore be assessed through both absolute and normalized measurements, because absolute removal quantifies reduction in total resistance load while relative abundance reflects changes in the proportional composition of the surviving microbial community (Raza et al., 2022). Solids and liquid fractions also require separate interpretation because resistance determinants can be redistributed rather than completely removed.

Wastewater treatment technology is a major quantitative determinant of antimicrobial resistance gene removal because different systems rely on distinct physical, biological, chemical, and oxidative mechanisms. Conventional activated sludge remains one of the most widely studied treatment configurations and generally produces substantial reductions in absolute ARG concentrations through biomass separation, biodegradation, and removal of suspended matter. Its effectiveness varies considerably across genes and treatment plants, and relative enrichment of selected resistance markers has been observed in some activated sludge systems. Membrane bioreactors frequently demonstrate greater removal of bacteria and particle-associated ARGs than conventional secondary treatment because membrane separation provides an additional physical barrier (McConnell et al., 2018).

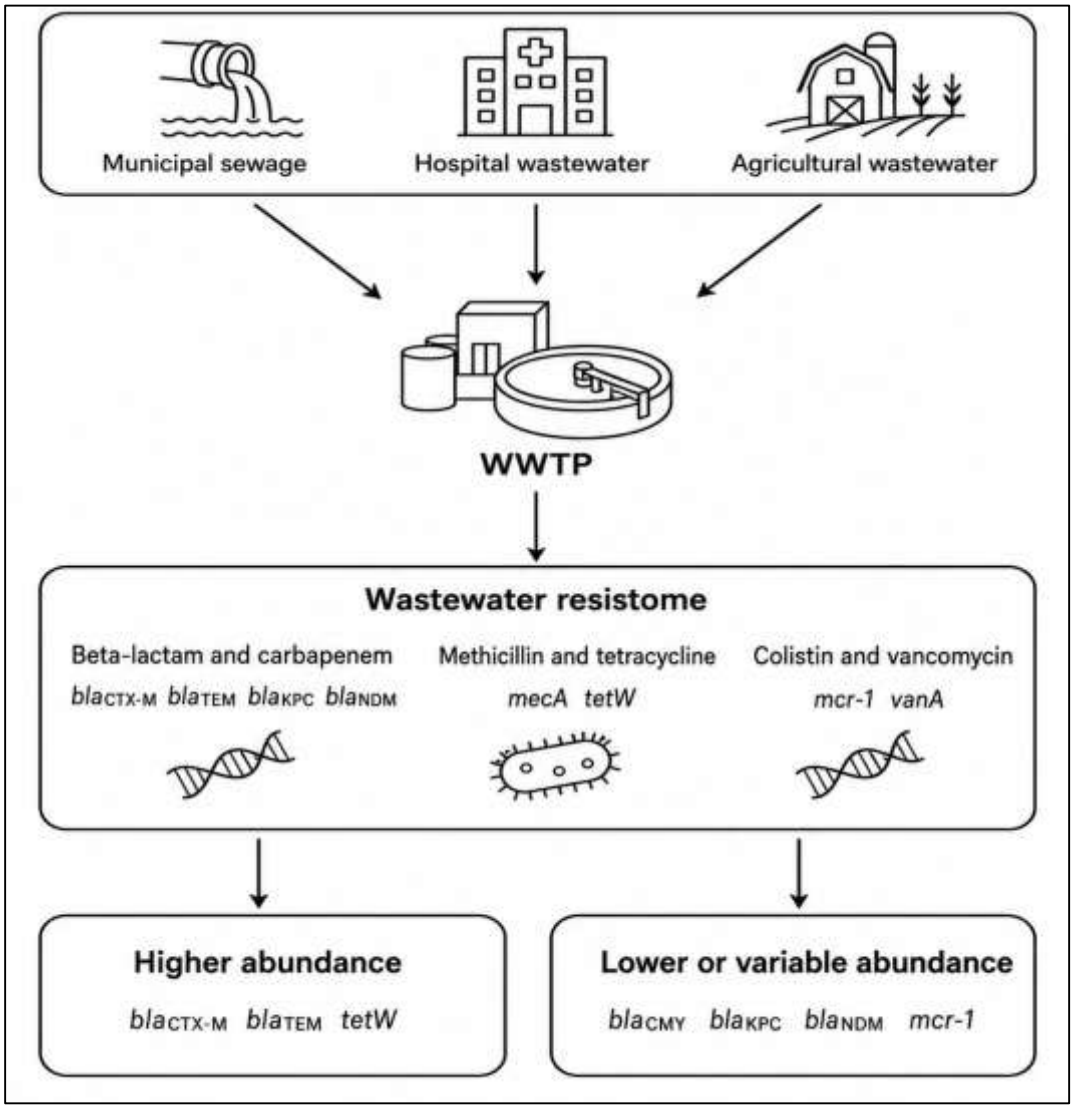
Quantitative comparisons have often reported lower effluent ARG concentrations in membrane systems, although dissolved extracellular DNA and very small genetic fragments may still pass through or remain detectable. Ultraviolet disinfection is effective for microbial inactivation and can reduce some ARG signals at sufficiently high doses, but its effectiveness depends on UV intensity, exposure time, wastewater turbidity, and target-gene characteristics. Chlorination has also demonstrated substantial reductions in viable resistant bacteria, yet studies have reported variable effects on resistance genes, particularly when DNA remains detectable after cell inactivation. Ozonation and advanced oxidation processes have shown strong potential for degrading both microorganisms and genetic material, with several investigations reporting larger reductions in selected ARGs than conventional disinfection alone (Chen et al., 2022). Filtration systems, including sand filtration and membrane filtration, can reduce ARG loads by removing suspended solids and bacterial cells, while combinations of biological treatment, filtration, and disinfection generally achieve greater total reductions than single-stage treatment. Quantitative studies have also identified operational variables such as hydraulic retention time, sludge age, temperature, nutrient loading, oxidant dose, membrane characteristics, and solids separation efficiency as predictors of ARG removal. No single technology removes all resistance determinants with equal efficiency because gene location, bacterial host, extracellular DNA persistence, particle association, and microbial ecology influence treatment response (Pallares-Vega et al., 2019). Evidence synthesized from conventional activated sludge, membrane bioreactor, ultraviolet, chlorination, ozonation, filtration, and advanced oxidation studies therefore shows that treatment technology explains a substantial portion of variation in ARG removal efficiency, while gene-specific behavior and operational conditions remain essential for interpreting treatment-stage differences.

Clinically Relevant Resistance Genes in Wastewater

Clinically relevant beta-lactam and carbapenem resistance genes are among the most intensively quantified antimicrobial resistance markers in wastewater because they are closely associated with treatment-limiting resistance in important Gram-negative pathogens (Verburg et al., 2019). Across municipal sewage, hospital effluent, wastewater treatment plants, receiving waters, and wastewater solids, quantitative studies have repeatedly identified substantial occurrence of beta-lactamase genes, particularly blaCTX-M, blaTEM, and blaCMY, together with other extended-spectrum and AmpC-type resistance determinants. The abundance of these genes varies considerably by wastewater source, geographic region, treatment stage, population served, antimicrobial-use patterns, and analytical platform (Xu et al., 2016). Untreated influent and hospital wastewater generally contain higher absolute concentrations than final effluent because they receive concentrated fecal and clinical inputs before treatment-related reduction. Among the beta-lactam resistance markers, blaCTX-M has received particular attention because of its widespread clinical association with extended-spectrum beta-lactamase-producing Enterobacterales. blaTEM is also frequently detected at relatively high abundance and may occur across a broader range of bacterial hosts and environmental matrices. blaCMY tends to show lower and more variable abundance but remains epidemiologically important because of its association with resistance to extended-spectrum cephalosporins. Carbapenem resistance genes usually occur at lower concentrations than more common beta-lactam resistance markers but carry greater clinical significance because carbapenems are often reserved for severe multidrug-resistant infections (Alexander et al., 2016). Quantitative investigations have detected blaKPC, blaNDM, blaVIM, and blaOXA-48-like genes in municipal and hospital wastewater, although their prevalence differs markedly across locations. Some studies report sporadic or low-level detection, while others identify persistent signals in catchments with greater healthcare exposure or documented clinical circulation. Molecular assays frequently outperform culture-based approaches for low-abundance carbapenemase detection because resistance genes may occur in organisms that are difficult to culture or in genetic material no longer associated with viable cells. Across more than ten quantitative wastewater studies, the combined evidence shows that beta-lactam resistance genes are widespread and often abundant, whereas carbapenem resistance markers are less consistently detected but provide highly specific signals of clinically important resistance circulating within contributing populations (Rodríguez et al., 2021).Methicillin and tetracycline resistance genes represent two contrasting but highly informative components of wastewater resistomes. The methicillin resistance marker mecA is widely used as a

quantitative indicator because of its central role in resistance among staphylococci, particularly methicillin-resistant *Staphylococcus aureus*. Studies of municipal influent, hospital wastewater, activated sludge, wastewater solids, and treated effluent have repeatedly detected mecA, although concentrations vary according to population characteristics, healthcare inputs, wastewater composition, and treatment configuration (Rodríguez et al., 2021).

Figure 6: Clinically Relevant Wastewater Resistance Genes



Higher abundance has often been observed in untreated sewage and hospital-associated discharges, while lower absolute concentrations are generally reported after secondary treatment and disinfection. The persistence of mecA in treated wastewater demonstrates that complete removal is uncommon, particularly when measurements include extracellular DNA or resistance determinants retained in solids-associated fractions. Tetracycline resistance genes, by comparison, are among the most abundant and consistently detected ARG groups in wastewater environments. Markers such as tetW, tetA, tetM, tetO, and related genes occur across municipal wastewater, livestock-associated waste, agricultural runoff, biosolids, and receiving waters (Hutinel et al., 2022). tetW is especially common in fecal-associated microbial communities and has frequently been quantified at high concentrations relative to many clinically specific resistance markers. Studies using qPCR, high-throughput qPCR, digital PCR, and metagenomics consistently report broad tetracycline-resistance diversity in influent and activated sludge, reflecting the extensive distribution of tet genes among both human-associated and

environmental bacteria. Some tetracycline resistance determinants remain relatively stable across treatment stages when expressed as normalized abundance, even when their absolute concentrations decline substantially. This pattern highlights the distinction between overall reduction in resistance load and proportional enrichment within surviving microbial communities. Quantitative comparisons across urban and agricultural settings also show that tet genes often bridge human and animal reservoirs, making them particularly relevant within a One Health framework (Marathe et al., 2019). Synthesized findings from numerous wastewater and environmental studies therefore indicate that mecA functions as a clinically focused marker of methicillin resistance, whereas tetW and related genes represent widespread, high-abundance indicators of broader environmental and host-associated resistance pressures.

Colistin and vancomycin resistance genes are frequently included in wastewater surveillance because they represent resistance to antimicrobial agents used against serious infections with limited therapeutic options. Colistin resistance is commonly evaluated through mobile colistin resistance genes, particularly members of the mcr family. Quantitative wastewater studies have detected mcr-1 and other mcr variants in municipal sewage, hospital wastewater, animal-associated waste, agricultural environments, and wastewater treatment facilities, but measured abundance is generally lower and more variable than that of tetracycline, sulfonamide, or common beta-lactam resistance genes (Hembach et al., 2022). This low-abundance pattern creates substantial analytical challenges. Detection can be strongly influenced by sampling volume, concentration procedures, DNA extraction efficiency, amplification sensitivity, sequencing depth, and assay-specific detection limits. Targeted qPCR and digital PCR are often more successful than untargeted metagenomics when mcr concentrations are close to analytical thresholds. Some studies have reported intermittent detection across sampling dates, indicating that temporal variability and low prevalence can complicate interpretation. The presence of mcr genes in wastewater remains epidemiologically significant because these determinants are associated with transferable colistin resistance and can occur across multiple bacterial hosts. Vancomycin resistance is commonly assessed using vanA, together with vanB and other glycopeptide resistance genes. vanA is frequently detected in wastewater influenced by healthcare or community fecal inputs and is associated particularly with vancomycin-resistant enterococci (Cai et al., 2021). Quantitative studies have demonstrated measurable vanA concentrations in municipal influent, hospital wastewater, activated sludge, biosolids, and treated effluent. Similar to other resistance markers, absolute concentrations typically decline through treatment while normalized abundance may exhibit more variable behavior. Culture-based studies have complemented molecular findings by recovering vancomycin-resistant enterococci from wastewater systems, providing evidence that some detected resistance determinants are associated with viable organisms. Across more than ten studies evaluating colistin and glycopeptide resistance in environmental matrices, the literature shows that mcr genes are often comparatively rare and analytically challenging, whereas vanA tends to be more consistently measurable. Both gene groups nevertheless provide clinically meaningful indicators that extend wastewater surveillance beyond highly abundant environmental resistance determinants toward markers associated with difficult-to-treat infections (Zhang et al., 2019).

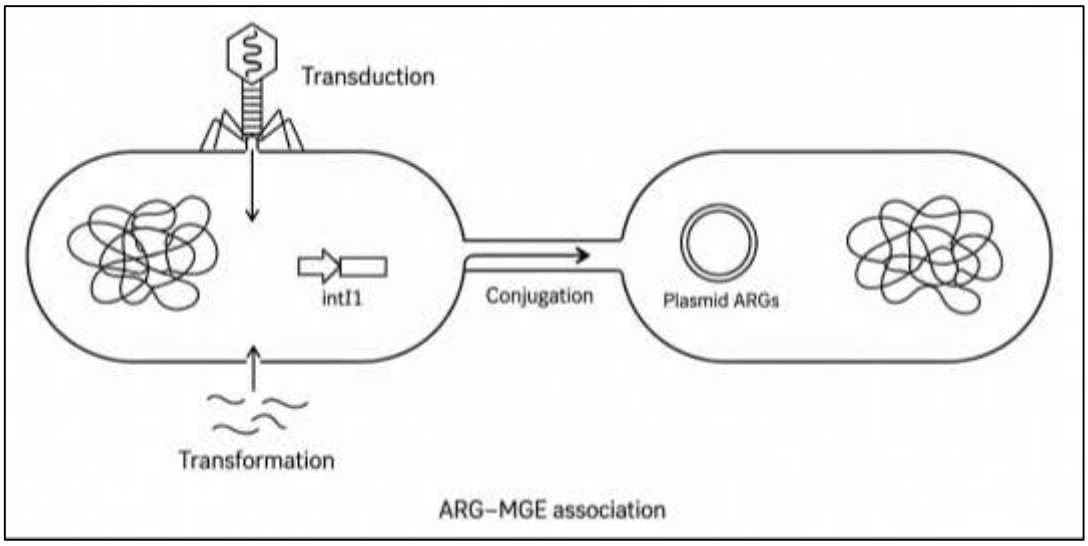
Horizontal Gene Transfer and Mobile Genetic Elements in Wastewater Resistomes

Horizontal gene transfer represents a central mechanism through which antimicrobial resistance determinants can move among bacterial populations in wastewater environments, and integrons are among the most frequently quantified mobile genetic elements associated with this process. Quantitative studies of municipal wastewater, hospital effluent, activated sludge, biosolids, and receiving waters have repeatedly identified significant associations between the abundance of antimicrobial resistance genes and class 1 integron markers, particularly intI1. Across more than ten studies, intI1 has often been detected at high concentrations in untreated influent and activated sludge, where dense microbial populations, high nutrient availability, and repeated contact among diverse bacterial taxa create conditions favorable for genetic exchange (Martin et al., 2021). Correlation analyses have commonly shown positive relationships between intI1 abundance and genes encoding resistance to sulfonamides, tetracyclines, beta-lactams, aminoglycosides, macrolides, and multidrug efflux systems. These associations indicate that integron-rich microbial communities frequently contain elevated resistance-gene burdens, although correlation does not independently confirm physical

linkage between every resistance determinant and an integron. Several high-throughput qPCR and metagenomic studies have strengthened this evidence by identifying co-occurrence patterns between integrase genes, gene cassette arrays, and multiple ARG classes within the same wastewater samples. Class 1 integrons are particularly important because they can capture and express resistance-gene cassettes and are widely distributed among Gram-negative bacteria commonly recovered from sewage (Martin et al., 2021). Quantitative reductions in intI1 during wastewater treatment are frequently observed in absolute terms, yet normalized abundance may remain stable or decline less strongly than total bacterial abundance. This pattern suggests that integron-associated populations can persist through treatment even when overall microbial loads are substantially reduced. Studies comparing influent, activated sludge, effluent, and downstream environments have also found that intI1 can serve as a useful marker of anthropogenic pollution and resistance-gene mobility. The literature therefore distinguishes simple ARG detection from evidence of potential mobility by showing that resistance genes occurring alongside integrons, particularly when supported by co-localization or gene-cassette data, represent a stronger indication of transferable resistance than detection of resistance genes alone (Karaolia et al., 2021).

Plasmids constitute another major vehicle of horizontal gene transfer in wastewater bacterial communities because they can carry one or multiple antimicrobial resistance determinants and move between bacterial hosts through conjugation. Quantitative and genomic studies of wastewater have identified plasmid-associated beta-lactam, carbapenem, colistin, tetracycline, quinolone, aminoglycoside, and multidrug resistance genes in both municipal and hospital-associated samples. Clinically important determinants such as blaCTX-M, blaKPC, blaNDM, mcr genes, and plasmid-mediated quinolone resistance markers have been detected on transferable plasmids recovered from wastewater isolates and metagenomic assemblies (Lekunberri et al., 2018).

Figure 7: Wastewater Horizontal Gene Transfer Framework



Several studies have demonstrated that plasmid abundance is higher in wastewater compartments characterized by dense bacterial biomass, including untreated influent and activated sludge, than in less microbially concentrated downstream matrices. Conjugative plasmids belonging to incompatibility groups frequently associated with Enterobacterales and other Gram-negative bacteria have been identified in sewage, providing a genetic mechanism through which resistance determinants can move across taxonomic boundaries. Quantitative conjugation experiments conducted under wastewater-like conditions have shown measurable transfer frequencies for resistance plasmids, although rates vary widely according to donor and recipient strains, temperature, nutrient concentration, antimicrobial exposure, biofilm formation, and hydraulic conditions (Marano & Cytryn, 2017). Hospital wastewater often contains a particularly diverse plasmid pool because of strong

antimicrobial selection and the presence of clinically resistant bacteria, while municipal wastewater combines these inputs with broader community-associated microbial populations. Metagenomic and long-read sequencing studies have improved the ability to connect plasmid sequences with ARGs, allowing researchers to distinguish chromosomally encoded resistance from mobile plasmid-associated resistance. This distinction is important because a high concentration of an ARG does not automatically indicate high transmission potential if the gene is embedded in a non-mobile chromosomal region. By contrast, detection of the same ARG on broad-host-range plasmids in multiple bacterial taxa provides stronger evidence of mobility (Adamczuk & Dziewit, 2017). Synthesized findings from culture-based conjugation studies, plasmid profiling, whole-genome sequencing, metagenomics, and wastewater surveillance therefore show that plasmid-mediated resistance is a substantial component of wastewater resistomes and that plasmid context is essential for interpreting the epidemiological significance of ARG abundance.

The quantitative relationship between mobile genetic elements and ARG concentration is one of the strongest lines of evidence linking wastewater resistance reservoirs with horizontal gene transfer processes. Studies have measured integrons, plasmids, transposases, insertion sequences, and other mobility-associated genes alongside ARGs and have frequently reported positive correlations between mobile-element abundance and total or class-specific resistance burden (Yin et al., 2022). High-throughput qPCR investigations often demonstrate that samples with elevated concentrations of mobile genetic elements also contain greater numbers or higher normalized abundance of ARGs. Metagenomic studies similarly show that wastewater resistomes are enriched not only in resistance determinants but also in genes involved in recombination, transposition, conjugation, and DNA mobility. Activated sludge has received particular attention because its microbial density, prolonged solids retention, floc structure, and repeated cell-to-cell contact create favorable conditions for genetic exchange. Laboratory and pilot-scale studies have documented transfer of resistance plasmids under activated-sludge conditions, providing direct experimental evidence that horizontal gene transfer can occur within engineered wastewater systems (Manoharan et al., 2021). Biofilms can further increase transfer opportunities by maintaining bacterial cells in close spatial proximity and creating local microenvironments with high cell density. Selective pressures generated by sub-inhibitory concentrations of antibiotics, disinfectants, metals, and other contaminants have also been associated with increased persistence or transfer of mobile resistance determinants. Several studies report that resistance genes linked to integrons or plasmids persist through wastewater treatment more strongly than some non-mobile markers, although treatment-specific patterns differ. Horizontal gene transfer has also been demonstrated through mating assays, reporter systems, genomic reconstruction, and identification of nearly identical resistance-bearing mobile elements across different bacterial hosts. These findings provide stronger evidence than simple co-occurrence because they show transfer events, physical linkage, or shared genetic contexts (Jiao et al., 2017). Quantitative interpretation nevertheless requires caution, since elevated mobile-element abundance does not necessarily mean that every associated ARG is actively transferring at the time of sampling. Mobility represents a spectrum ranging from genetic potential to observed transfer. The literature therefore supports a hierarchical interpretation in which ARG detection represents presence, ARG–MGE correlation suggests mobility potential, physical co-localization strengthens evidence of transferability, and experimentally observed transfer provides the most direct confirmation of horizontal gene exchange under wastewater conditions (Yang et al., 2020).

Bacterial community composition strongly influences the mobility and persistence of antimicrobial resistance genes because horizontal gene transfer depends on the presence of compatible donor and recipient populations, ecological interactions, and mobile genetic elements capable of crossing taxonomic boundaries. Quantitative sequencing studies of wastewater have demonstrated significant correlations between shifts in bacterial community structure and changes in resistome composition across influent, activated sludge, effluent, and biosolids. Taxa belonging to Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidota frequently account for substantial proportions of wastewater microbial communities and include numerous potential hosts of clinically relevant resistance genes (Wolters et al., 2022). Members of Enterobacterales, *Pseudomonas*, *Acinetobacter*, enterococci, and other

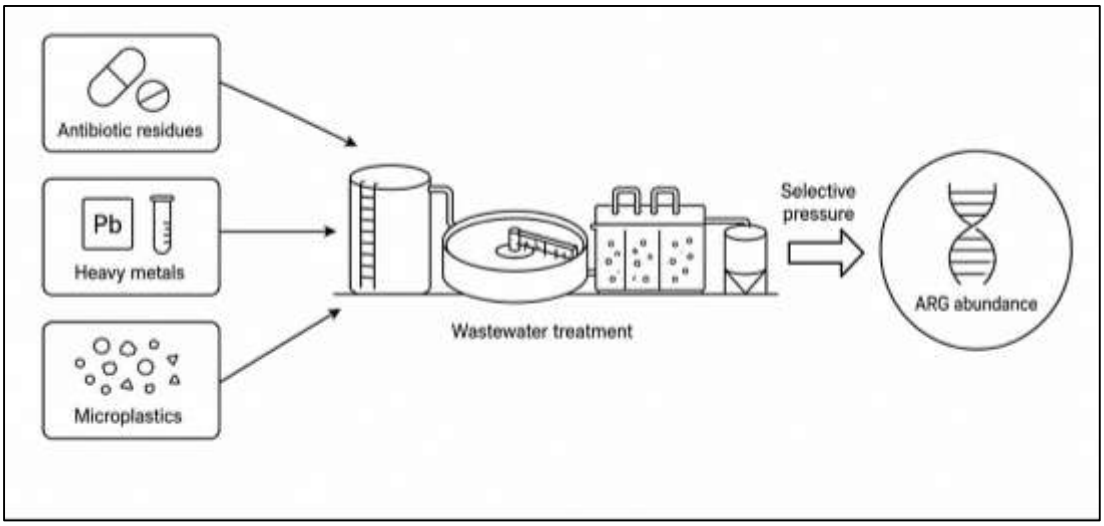
opportunistic groups have repeatedly been linked to mobile beta-lactam, carbapenem, aminoglycoside, tetracycline, and multidrug resistance determinants. Network analyses have identified statistically significant co-occurrence relationships between specific bacterial taxa, ARGs, integrons, and plasmid-associated markers, suggesting that community structure can shape both the abundance and mobility of resistance. Metagenomic binning and long-read sequencing have further improved host assignment by linking resistance genes and mobile elements within the same assembled genomic fragments. These approaches have shown that some ARGs are concentrated within a limited number of bacterial hosts, while others occur across a broader taxonomic range, indicating greater mobility potential. Wastewater treatment can alter these host relationships by selectively removing some taxa while enriching others that are well adapted to activated-sludge conditions. Changes in dissolved oxygen, nutrient availability, solids retention time, temperature, and chemical exposure can therefore indirectly influence resistance mobility through their effects on microbial community composition (Wang et al., 2021). Several studies also show that bacterial diversity and community turnover are associated with differences in ARG richness and mobile-element abundance across treatment stages. However, statistical co-occurrence between a bacterial taxon and a resistance gene does not alone prove that the organism carries the gene. Stronger evidence is obtained when taxonomic assignment, physical genomic linkage, plasmid context, and experimental transfer data converge. Synthesized evidence from at least ten metagenomic, network-analysis, culture-based, and genomic studies therefore demonstrates that the mobility of wastewater resistance genes is shaped not only by the presence of ARGs and mobile elements but also by the structure, compatibility, and ecological interactions of the bacterial communities in which those genes circulate (Mao et al., 2015).

Antimicrobial Residues and Wastewater ARG Abundance

Municipal wastewater contains measurable concentrations of multiple antibiotic classes because a proportion of administered pharmaceuticals is excreted unchanged or as active metabolites and subsequently enters sewer systems through domestic, clinical, and commercial waste streams. Quantitative wastewater studies have repeatedly detected sulfonamides, tetracyclines, macrolides, fluoroquinolones, beta-lactams, trimethoprim, and other antimicrobial compounds in influent, activated sludge, secondary effluent, and receiving waters. Concentrations vary widely among treatment plants and sampling periods because they are influenced by community prescription patterns, population size, hospital contributions, wastewater flow, seasonal disease activity, sorption behavior, chemical degradation, and treatment configuration (Szekeres et al., 2017). Influent samples generally contain the highest concentrations, while biological treatment, adsorption to sludge, transformation, and advanced treatment can reduce individual compounds to different degrees. Several studies have shown that persistent antibiotics such as sulfamethoxazole, trimethoprim, ciprofloxacin, and selected macrolides can remain detectable after conventional treatment. Parallel measurements of antimicrobial resistance genes have frequently identified high abundance of sulfonamide resistance markers such as **sul1** and **sul2**, tetracycline genes such as **tetW**, **tetA**, and **tetM**, macrolide resistance genes, and quinolone-associated determinants within the same wastewater systems. Correlation analyses across numerous studies have reported positive relationships between concentrations of selected antibiotic residues and corresponding ARG abundance, although the strength and consistency of these relationships differ across compounds and treatment stages. Stronger associations are often observed when pharmaceutical concentrations, ARG abundance, and microbial community data are measured simultaneously over repeated sampling periods (Wang et al., 2018). In some studies, elevated sulfonamide concentrations correspond with greater abundance of sul genes, while tetracycline residues are associated with higher concentrations or relative abundance of tet genes. Other investigations report weak or non-significant relationships, indicating that contemporary antibiotic concentration is only one component of resistance ecology. ARG abundance can reflect historical selection, persistent resistant populations, mobile genetic elements, and inputs from fecal sources even when current antibiotic concentrations are low. Across more than ten quantitative investigations, the literature therefore indicates that municipal wastewater serves as a combined reservoir of antimicrobial residues and resistance determinants, with measurable but heterogeneous relationships between chemical exposure and ARG abundance (Le et al., 2018).

The relationship between antibiotic concentration and resistance enrichment is strongly influenced by the magnitude and duration of antimicrobial exposure within wastewater environments. Laboratory, mesocosm, activated-sludge, and field studies have demonstrated that selective effects can occur at concentrations below those required to inhibit the growth of most susceptible bacteria, particularly when microbial populations experience prolonged or repeated exposure. These sub-inhibitory concentrations can alter competitive relationships among susceptible and resistant organisms, increase the relative fitness of resistant populations, and affect expression or transfer of resistance determinants. Quantitative studies have shown that increasing concentrations of tetracyclines, sulfonamides, fluoroquinolones, and macrolides can correspond with greater relative abundance of resistance genes under controlled wastewater conditions (Pan & Chu, 2018). The magnitude of enrichment varies according to the antimicrobial compound, microbial community, treatment stage, exposure duration, nutrient availability, temperature, and presence of mobile genetic elements. Activated sludge is particularly relevant because microorganisms remain within dense flocs for extended periods, creating repeated opportunities for exposure and interaction. Several experimental investigations have found that low antibiotic concentrations can increase the relative abundance of ARG-carrying bacteria without necessarily producing large changes in total bacterial biomass. Other studies have reported threshold-like responses in which resistance enrichment becomes more pronounced above particular concentration ranges. These observations have contributed to the distinction between concentrations associated with direct bacterial inhibition and lower concentrations capable of promoting selection. Quantitative comparisons also show that absolute ARG concentrations may decline through treatment while relative abundance increases, especially when susceptible bacterial populations are removed more rapidly than resistant populations (Kampouris et al., 2022).

Figure 8: Wastewater Contaminants Shape ARG Abundance



This pattern demonstrates that concentration-dependent selection cannot be evaluated solely by measuring total ARG copies. Studies incorporating both chemical analyses and molecular resistance measurements provide stronger evidence because they can identify whether changes in resistome structure correspond with measured exposure gradients. Nevertheless, wastewater systems contain complex mixtures of antimicrobials and other contaminants, making it difficult to attribute resistance enrichment to a single compound. The synthesized literature indicates that antibiotic residues contribute to selective pressure in wastewater, but the strength of this effect depends on concentration, exposure time, bacterial community structure, co-occurring contaminants, and pre-existing resistance. Quantitative interpretation therefore requires integration of residue concentrations, ARG abundance, bacterial composition, and treatment conditions rather than reliance on a single exposure metric (Gao et al., 2015).Antimicrobial resistance in wastewater is not driven exclusively by antibiotic residues, because heavy metals, disinfectants, biocides, and other chemical contaminants can contribute to co-

selection of resistant bacterial populations. Quantitative studies have repeatedly detected copper, zinc, lead, chromium, cadmium, mercury, nickel, and other metals in wastewater influent, activated sludge, biosolids, and industrially influenced treatment systems. These compounds can persist because they are not biologically degraded in the same manner as many organic contaminants and often accumulate in sludge or sediment. Several studies have reported significant positive associations between concentrations of metals and the abundance of specific ARGs, particularly genes associated with tetracycline, sulfonamide, beta-lactam, macrolide, and multidrug resistance (Li et al., 2015). Co-selection can occur when metal-resistance genes and ARGs are physically linked on the same plasmids, integrons, transposons, or other mobile genetic elements. Under such conditions, exposure to a metal can favor maintenance of the genetic element even in the absence of the corresponding antibiotic. Quantitative metagenomic and qPCR studies have identified correlations among metal-resistance genes, **intI1**, and multiple ARG classes, suggesting that contaminated wastewater environments may maintain complex co-resistance networks. Disinfectants and quaternary ammonium compounds have also been examined because genes conferring tolerance to these chemicals can occur alongside antibiotic resistance determinants. Industrial inputs can intensify this pattern by increasing chemical concentrations within municipal treatment systems (Raza et al., 2022). Studies of sludge and biosolids frequently report stronger metal–ARG relationships than studies of the liquid phase because both metals and bacterial biomass can concentrate in solids. Statistical analyses have also shown that metal concentrations can explain a portion of the variation in resistome composition after controlling for antibiotic residues and other wastewater characteristics. However, positive correlations do not establish a direct selective mechanism in every case because metals and ARGs may originate from common anthropogenic sources. Evidence becomes stronger when genetic co-location, experimental exposure, and community-level enrichment are observed together. Across more than ten wastewater and environmental studies, heavy metals and related contaminants consistently emerge as important co-selective factors that can influence ARG persistence, mobile-element abundance, and multidrug resistance profiles within treatment systems (Bueno et al., 2020).

The wastewater resistome is increasingly understood as the product of interacting chemical and physical contaminants rather than exposure to antibiotics alone. Quantitative studies examining pharmaceuticals, microplastics, metals, and microbial communities have shown that these components can jointly influence ARG abundance and resistome composition within wastewater treatment systems. Microplastics are particularly important because their surfaces provide stable substrates for microbial colonization and biofilm development. Investigations comparing plastic-associated biofilms with surrounding wastewater have frequently reported higher relative abundance of selected ARGs, mobile genetic elements, and potential pathogenic bacteria on microplastic surfaces. Genes associated with tetracycline, sulfonamide, beta-lactam, macrolide, and multidrug resistance have been detected within these biofilms, sometimes at greater normalized abundance than in planktonic microbial communities (Zhang et al., 2019). Plastic particles can also adsorb antibiotics, metals, and other contaminants, potentially creating localized environments in which bacterial cells experience concentrated chemical exposure while remaining in close contact with one another. Such conditions are compatible with persistence of resistant populations and horizontal gene transfer. Quantitative network analyses have identified associations among microplastic-associated bacterial taxa, ARGs, integrons, and metal-resistance genes, suggesting that the plastisphere may function as a distinct resistance-enriched ecological niche. Pharmaceutical mixtures further complicate these relationships because wastewater commonly contains multiple antibiotics, analgesics, hormones, and other biologically active compounds at the same time. Some studies have found that broader pharmaceutical contamination is associated with shifts in microbial community structure and greater resistome diversity, while others identify specific resistance classes that correspond more strongly with particular contaminants (Kampouris et al., 2021). Metals can intensify these patterns through co-selection, especially where resistance genes and metal-resistance determinants are carried on common mobile elements. Wastewater solids often show particularly complex contaminant–resistome relationships because microplastics, metals, pharmaceuticals, bacterial cells, and extracellular DNA can accumulate together. Synthesized evidence from metagenomic, qPCR, chemical-monitoring, biofilm, and microplastic

studies indicates that resistome composition is shaped by a combined selective environment in which antimicrobial residues, heavy metals, microplastics, and other contaminants interact with bacterial community structure and mobile genetic elements. This multidimensional exposure context helps explain why sulfonamide and tetracycline resistance determinants frequently remain abundant even when concentrations of the corresponding antibiotics alone do not fully account for observed resistance patterns (Wu et al., 2015).

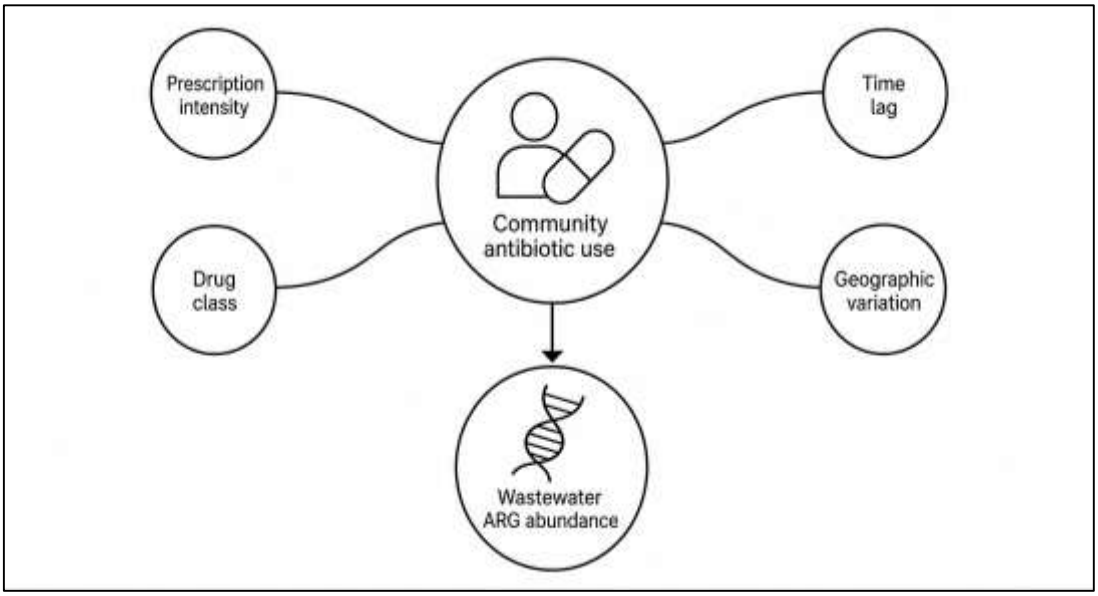
Human Antibiotic Consumption and Wastewater Resistance Genes

Quantitative evidence increasingly links community antibiotic consumption with the abundance of antimicrobial resistance genes detected in municipal wastewater, making prescription intensity an important human-health variable within the One Health framework. Studies comparing outpatient prescribing data with wastewater measurements have shown that communities with higher use of particular antibiotic classes often exhibit higher concentrations or normalized abundance of corresponding resistance determinants, although the magnitude of association differs by drug class, gene target, population size, and wastewater characteristics (Berglund, 2015). Community prescription rates commonly vary substantially between regions because of differences in healthcare access, diagnostic practices, prescribing culture, population age structure, disease burden, socioeconomic conditions, and local stewardship policies. These variations create measurable geographic gradients in antimicrobial exposure that can be compared with wastewater resistome data collected from defined sewersheds. Investigations using quantitative PCR, high-throughput PCR, digital PCR, and metagenomic sequencing have detected positive relationships between population-level antibiotic consumption and genes associated with beta-lactam, tetracycline, sulfonamide, macrolide, quinolone, and multidrug resistance. In several studies, higher outpatient use of broad-spectrum antibiotics was associated with greater abundance of related resistance markers after adjustment for population and wastewater flow (Rodriguez-Mozaz et al., 2015). Other studies have reported weaker or inconsistent associations, particularly when resistance determinants were already widespread in the microbial community or when measured genes were influenced by multiple sources. Wastewater concentrations also reflect both active resistant bacteria and extracellular genetic material, meaning that resistance signals integrate current and historical antimicrobial selection rather than immediate prescribing alone. Community wastewater can therefore capture resistance generated across a broader population than clinical surveillance, including individuals who carry resistant organisms without infection. Across more than ten ecological, wastewater, prescription, and metagenomic studies, antibiotic prescribing has emerged as a recurrent predictor of ARG abundance, but not as a single explanatory factor. The quantitative relationship is strongest when prescribing data are matched spatially and temporally to wastewater catchments and when models account for population density, healthcare utilization, treatment-plant characteristics, and other potential confounders (Mutuku et al., 2022). These findings support the use of community antibiotic consumption as a measurable exposure variable in wastewater-based resistance surveillance.

The relationship between antibiotic use and wastewater resistance becomes more informative when consumption is disaggregated by antimicrobial class and compared with resistance markers linked to those compounds. Quantitative studies have examined consumption of beta-lactams, cephalosporins, fluoroquinolones, macrolides, tetracyclines, sulfonamides, trimethoprim, glycopeptides, and other antimicrobial categories alongside corresponding wastewater ARGs. Higher use of beta-lactams and cephalosporins has been associated in several settings with increased abundance of genes such as blaCTX-M, blaTEM, and other beta-lactamase markers, particularly in communities with substantial outpatient or healthcare-associated antibiotic exposure (Voigt et al., 2020). Fluoroquinolone consumption has similarly been compared with plasmid-mediated quinolone resistance genes and mutations associated with reduced susceptibility, although these relationships are often more complex because quinolone resistance can emerge through both chromosomal and mobile mechanisms. Macrolide use has been associated with increased occurrence of erm and mef resistance determinants, while tetracycline consumption has been linked with higher abundance of tet genes, including tetW, tetM, and related markers. Sulfonamide and trimethoprim use has been examined in relation to sul and dfr genes, which are frequently abundant in municipal wastewater. Quantitative comparisons have also shown that some genes remain highly prevalent even where contemporary use of the

corresponding antimicrobial class is modest, reflecting long-term persistence, co-selection, mobile genetic elements, and historical exposure (Li et al., 2016). Drug-class analysis therefore provides greater specificity than total antibiotic consumption but does not imply a one-to-one relationship between a prescription category and a single ARG. Multiple resistance genes may respond to one antimicrobial class, and the same gene may be maintained by linked selection pressures unrelated to current prescribing. Findings from more than ten community, hospital, wastewater, and resistome studies indicate that class-specific consumption frequently explains a meaningful portion of variation in gene abundance, particularly when exposure and resistance data are standardized to comparable geographic and population units. These results demonstrate that antimicrobial use is best evaluated through drug-specific or class-specific measures rather than aggregated prescription totals alone (Zhao et al., 2018). Quantitative studies examining the timing and geography of antibiotic use have shown that wastewater resistance signals do not always respond simultaneously to changes in prescribing. Lagged relationships can occur because antimicrobial exposure first alters bacterial populations within human hosts before resistant organisms and resistance genes are shed into wastewater and become detectable at the treatment-plant level.

Figure 9: Antibiotic Consumption Shapes Wastewater ARGs



The duration of this process varies according to the antibiotic class, duration of therapy, bacterial colonization, resistance mechanism, persistence of resistant strains, and frequency of community shedding. Several longitudinal studies have reported that wastewater ARG abundance may correlate more strongly with antibiotic consumption measured during preceding weeks or months than with same-day or same-week prescribing levels (Szekeres et al., 2017). Seasonal prescribing patterns provide another source of temporal variation, particularly for respiratory antibiotics that are often used more intensively during periods of increased respiratory illness. Corresponding resistance markers may show delayed or prolonged increases that persist after prescribing declines. Geographic variation further complicates these temporal relationships. Antibiotic prescribing differs markedly across states, counties, healthcare systems, and urban–rural settings, and these patterns have been associated with regional variation in wastewater resistance measurements. Communities with higher outpatient antibiotic use or greater access to prescribing facilities may exhibit elevated abundance of selected resistance genes, while some low-prescribing areas still show substantial ARG levels because of healthcare inputs, population mobility, long-term ecological persistence, or agricultural influences. Spatial wastewater studies have demonstrated clustering of resistance patterns that partially overlaps with regional differences in antimicrobial consumption (Zhou et al., 2022). At the same time, differences

in sewer catchment boundaries and prescribing-data aggregation can introduce measurement mismatch when exposure and wastewater populations are not perfectly aligned. Quantitative analyses based on repeated wastewater sampling, prescription databases, and spatially resolved population data have therefore provided the strongest evidence for temporal and geographic relationships. Across more than ten studies, the literature indicates that antibiotic use should be considered as a time-dependent and place-dependent predictor rather than a static variable. Lag structure, seasonal variation, geographic heterogeneity, and population movement all influence the measured association between prescribing intensity and wastewater ARG abundance (Verburg et al., 2019).

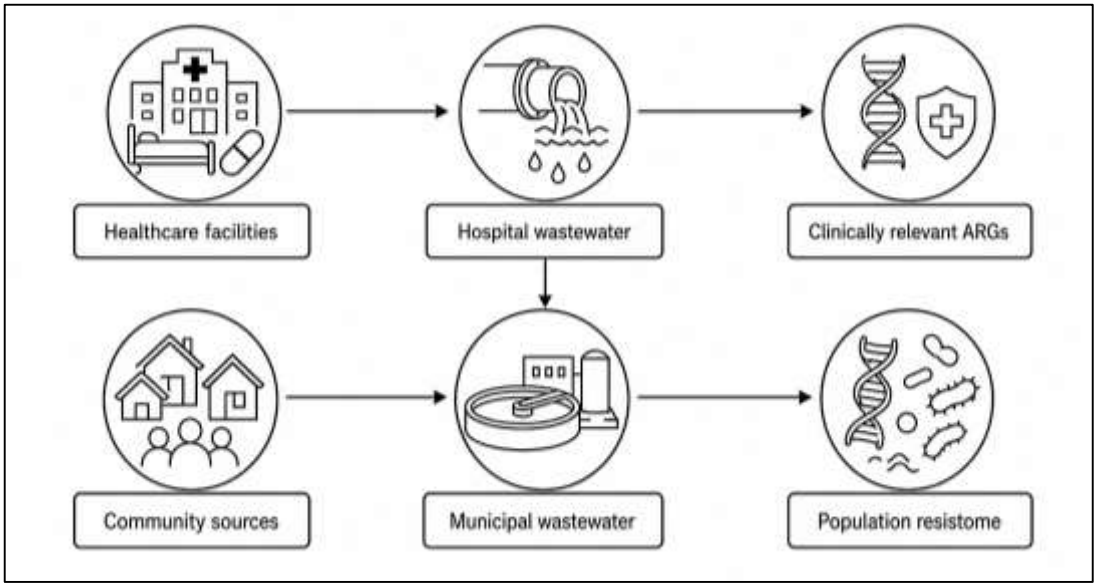
Healthcare Facilities to Wastewater AMR

Quantitative comparisons between hospital wastewater and municipal wastewater consistently show that healthcare-associated discharges contain distinctive and often elevated concentrations of clinically important antimicrobial resistance genes because hospitals concentrate patients with severe infections, intensive antimicrobial exposure, invasive procedures, and frequent contact with multidrug-resistant organisms (Gao & Sui, 2021). Studies using quantitative PCR, high-throughput PCR, metagenomic sequencing, and culture-based approaches have reported higher abundance of selected beta-lactamase, carbapenemase, vancomycin, methicillin, aminoglycoside, and multidrug resistance determinants in hospital effluent than in general municipal influent. Genes such as blaKPC, blaNDM, blaVIM, blaOXA-type, vanA, and mecA are particularly relevant because they are associated with resistance phenotypes that are commonly monitored in clinical settings. Hospital wastewater also contains elevated concentrations of antimicrobial residues, disinfectants, and other pharmaceuticals that may contribute to selective pressure within sewer systems. Municipal wastewater, however, represents a much larger and more diverse population and may contain greater absolute loads of some widespread resistance genes because of the substantially larger volume of sewage and broader community contribution. Several studies have therefore distinguished concentration from total load when comparing hospital and municipal sources (Sun et al., 2021). Hospital effluent can display higher gene concentrations per unit volume, while municipal systems may carry greater cumulative gene loads across the entire catchment. Resistome diversity also differs between the two matrices. Hospital-associated samples often contain a larger proportion of clinically prioritized resistance determinants and opportunistic pathogens, whereas community wastewater includes a broader mixture of fecal, environmental, household, and nonclinical microbial sources. Comparative investigations involving paired hospital and municipal sites have repeatedly demonstrated significant differences in both ARG abundance and bacterial community composition. Yet the magnitude of these differences is not uniform across facilities because hospital size, patient case mix, antimicrobial use, infection-control practices, wastewater dilution, pretreatment, and sewer connectivity vary considerably (Azuma et al., 2019). Synthesized findings from more than ten studies therefore indicate that hospital wastewater represents a concentrated source of clinically significant resistance, while municipal wastewater reflects a broader population-scale resistome in which hospital contributions are integrated with community and environmental inputs.

Healthcare-facility density and hospital bed capacity have increasingly been examined as quantitative predictors of antimicrobial resistance signals in community wastewater because these variables approximate the intensity of healthcare activity within a sewer catchment. Multisite wastewater studies have shown that sewersheds containing larger numbers of hospitals, long-term care facilities, dialysis centers, and other healthcare institutions often exhibit higher concentrations of selected clinically relevant ARGs. Hospital bed capacity is particularly useful as a quantitative exposure measure because it provides a standardized indicator of the potential volume of patient-associated wastewater entering municipal systems (Baba et al., 2022). Several analyses have reported positive relationships between bed numbers and the abundance of carbapenemase, beta-lactamase, vancomycin, and methicillin resistance genes, although effect sizes differ across genes and geographic regions. These relationships are biologically plausible because hospitals with more beds generally serve larger inpatient populations, use greater quantities of broad-spectrum antimicrobials, and generate higher volumes of wastewater containing resistant bacteria and antimicrobial residues. Long-term care facilities may also contribute significantly because residents often have repeated healthcare exposure, prolonged antimicrobial use, and elevated carriage of resistant organisms. Quantitative models that incorporate

healthcare-facility density have demonstrated that resistance signals can remain associated with healthcare intensity even after adjustment for population size, suggesting that hospital activity contributes information beyond simple population effects. At the same time, bed capacity is an imperfect proxy because occupancy rates, case complexity, specialty services, infection prevalence, and antimicrobial consumption vary among facilities of similar size (McCarthy et al., 2021). Sewer network structure also determines whether hospital discharges are diluted, concentrated, or combined with multiple other inputs before sampling. Large municipal plants may receive wastewater from numerous healthcare institutions, while smaller catchments may be dominated by one major facility. Studies using multivariable regression, spatial modeling, and machine-learning approaches have therefore treated healthcare density and bed capacity alongside demographic, prescription, and infrastructure variables rather than as isolated determinants. Across more than ten quantitative investigations, the literature shows that healthcare intensity is a recurrent predictor of wastewater ARG abundance, particularly for resistance mechanisms closely associated with hospital-based antimicrobial use and healthcare-associated pathogens (Kaiser et al., 2022).

Figure 10: Healthcare Facilities Shape Wastewater AMR



Clinical antibiotic utilization is a major determinant of hospital wastewater resistome structure because antimicrobial use creates strong selection pressure within patient microbiomes and healthcare-associated bacterial populations before resistant organisms and genetic material enter sewer systems. Quantitative studies have shown that hospitals with higher consumption of broad-spectrum beta-lactams, carbapenems, fluoroquinolones, glycopeptides, aminoglycosides, and other high-intensity antimicrobial classes often exhibit greater abundance of corresponding resistance determinants in wastewater (Harrington et al., 2022). Carbapenem use has been examined in relation to carbapenemase genes such as blaKPC, blaNDM, and blaVIM, while glycopeptide consumption has been linked with vancomycin resistance markers, particularly vanA and vanB. Extended-spectrum cephalosporin use has also been associated with beta-lactamase gene prevalence in hospital effluent. These relationships are not always immediate because resistant organisms may persist in colonized patients after treatment ends, and gene abundance in wastewater may integrate exposure over several days or weeks. Several longitudinal studies have therefore reported delayed or lagged associations between antimicrobial consumption and resistance signals. Hospital resistomes are also shaped by patient turnover, infection outbreaks, ward-specific antimicrobial practices, intensive-care activity, surgical services, and infection-control measures. Metagenomic investigations have demonstrated that hospital wastewater can contain a broader concentration of clinically significant ARGs and mobile genetic elements than community wastewater, particularly when intensive antimicrobial use is high. However, relationships

between drug consumption and wastewater ARG abundance are not universally strong because resistance can persist through clonal expansion, horizontal gene transfer, or long-standing carriage even when current antimicrobial use is moderate (Musoke et al., 2021). Chemical residues in wastewater may also contribute additional selection after excretion. Multivariable analyses that combine antibiotic utilization, hospital size, occupancy, patient population, and wastewater characteristics generally provide more stable estimates than simple pairwise correlations. Synthesized evidence from hospital, wastewater, clinical pharmacy, genomic, and epidemiological studies indicates that clinical antimicrobial consumption is an important quantitative driver of hospital-associated resistome composition, but its effect is mediated by patient carriage, facility characteristics, microbial ecology, and sewer-system dynamics (Hazards et al., 2021).

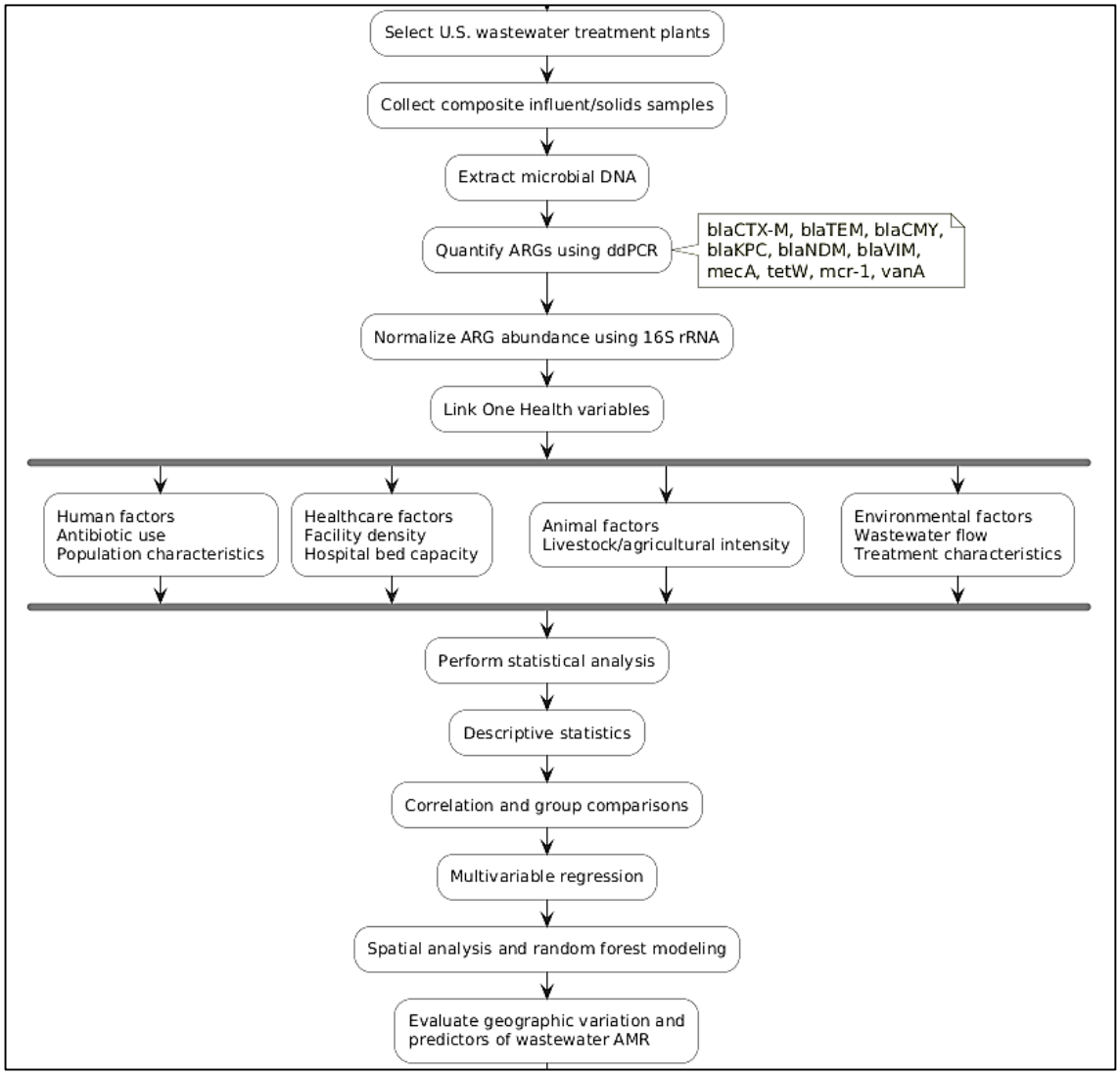
Comparative resistome studies demonstrate that hospital-associated and community wastewater can be quantitatively distinguished on the basis of both resistance-gene composition and microbial community structure. Hospital wastewater is typically enriched for clinically critical resistance determinants, mobile genetic elements, and bacterial taxa associated with healthcare environments, while municipal wastewater contains a broader representation of resistance circulating within the general population. Metagenomic analyses have identified higher relative abundance of carbapenemase, extended-spectrum beta-lactamase, vancomycin, aminoglycoside, and multidrug resistance genes in many hospital-associated samples, whereas community wastewater often contains large amounts of tetracycline, sulfonamide, macrolide, and broadly distributed beta-lactam resistance genes (Pruden et al., 2021). Differences are also visible in bacterial host composition, with hospital wastewater showing greater representation of Enterobacterales, enterococci, *Acinetobacter*, *Pseudomonas*, and other opportunistic taxa associated with healthcare exposure. Community wastewater contains these organisms as well but usually within a more diverse background of intestinal and environmental microbiota. National and multicenter investigations have used ordination, clustering, diversity indices, and machine-learning classification to separate hospital and municipal resistomes based on multidimensional resistance profiles. These methods demonstrate that wastewater source can explain a measurable portion of variation in ARG composition, even when total gene abundance overlaps between settings. The distinction is not absolute because hospital effluent ultimately enters municipal sewer networks, allowing healthcare-associated signals to become diluted and integrated into community wastewater. Larger treatment plants may therefore contain detectable hospital signatures alongside broader population-level resistance patterns (Kotwani et al., 2021). Long-term care facilities, outpatient centers, and other healthcare institutions can also contribute intermediate resistome profiles that blur a simple hospital-versus-community division. Studies using paired sampling designs have shown that clinically important resistance markers often decline in concentration as hospital wastewater mixes with municipal sewage, while some persist strongly enough to remain detectable at downstream treatment plants. Across more than ten comparative and national-scale studies, the literature demonstrates that hospital and community resistomes are quantitatively distinguishable but interconnected, with hospital wastewater contributing concentrated clinically relevant resistance signals and municipal wastewater providing a composite population-level profile shaped by healthcare, community, and environmental sources (Kusi et al., 2022).

METHOD

Study Design and Theoretical Framework

The study employed a quantitative, observational, multisite cross-sectional design within a One Health theoretical framework to characterize the distribution of clinically relevant antimicrobial resistance genes (ARGs) in wastewater across the United States and to determine their statistical relationships with human, animal, healthcare, environmental, and wastewater-system characteristics. A cross-sectional design was selected because the primary analytical objective was to compare standardized ARG measurements across geographically distinct wastewater treatment plant catchments and determine whether variations in resistance burden were associated with measurable population-level exposures. The wastewater treatment plant or its corresponding sewershed was treated as the principal analytical unit, while repeated samples collected within individual sites were treated as technical or temporal replicates before the construction of site-level estimates.

Figure 11: Methodology of this study



The One Health framework structured the independent variables into interconnected domains representing human antimicrobial exposure, healthcare activity, animal agriculture, population and socioeconomic characteristics, and environmental or wastewater conditions. The primary dependent variables were the absolute and normalized concentrations of selected clinically important ARGs. The target panel included beta-lactam and carbapenem resistance determinants such as blaCTX-M, blaTEM, blaCMY, blaKPC, blaNDM, blaVIM, and blaOXA-48-like, together with mecA for methicillin resistance, tetW for tetracycline resistance, mcr-1 for transferable colistin resistance, and vanA for vancomycin resistance. These targets were selected because they represented resistance mechanisms of clinical and environmental relevance and permitted comparison of highly prevalent markers with lower-abundance resistance determinants. This design was consistent with large-scale wastewater surveillance approaches in which clinically relevant ARGs were quantitatively measured across geographically distributed wastewater treatment plants and linked with secondary population-level data. A recent U.S. national-scale investigation similarly quantified 11 clinically relevant ARGs using digital PCR across 163 wastewater treatment plants in 40 states and linked these measurements with antibiotic use, social vulnerability, healthcare facilities, animal agriculture, and other contextual variables.

Participants, Materials, Sampling Strategy, and Eligibility Criteria

The study population consisted of municipal wastewater treatment plants and their corresponding wastewater catchment populations rather than individually recruited human participants. Wastewater treatment plants were selected through a purposive stratified sampling strategy intended to provide representation across major geographic regions of the United States, including the Northeast, Midwest, South, and West, while incorporating variation in population served, urbanization, treatment capacity, healthcare-facility density, agricultural activity, and wastewater-system characteristics. Eligible facilities were required to process predominantly municipal wastewater, provide access to untreated influent or primary wastewater solids suitable for molecular analysis, possess identifiable sewershed or service-area information, and provide sufficient metadata for linking wastewater measurements with relevant explanatory variables. Facilities were excluded when the wastewater source could not be reliably characterized, when industrial inputs dominated the waste stream to an extent that prevented interpretation as community wastewater, when samples lacked sufficient volume or quality for molecular analysis, when essential geographic or operational metadata were unavailable, or when sample integrity was compromised during collection, storage, or transportation. Wastewater samples were collected using 24-hour composite sampling where operationally feasible because composite samples provided greater representation of daily community wastewater contributions than isolated grab samples. Multiple samples were obtained from each participating facility within the defined sampling period, and replicate measurements were subsequently combined to produce a representative site-level ARG concentration. Sampling metadata included wastewater flow, population served, sampling date, treatment configuration, geographic region, and relevant environmental characteristics. Secondary quantitative datasets were linked to each sewershed or corresponding geographic unit and included community antibiotic prescribing or utilization, healthcare-facility density, hospital bed capacity where available, social and demographic characteristics, livestock or animal-agriculture indicators, and other One Health covariates. This multisite approach was supported by existing U.S. wastewater research in which multiple samples per treatment plant were averaged to reduce short-term wastewater fluctuations and generate representative site-level ARG measurements.

Instrumentation and Data Collection Tools

Wastewater ARG concentrations were measured using probe-based droplet digital polymerase chain reaction (ddPCR), which provided sensitive absolute quantification of predefined resistance targets and was particularly suitable for clinically important genes occurring at low abundance. Total bacterial abundance was estimated using the 16S rRNA gene, which provided a reference for normalization of ARG measurements across samples containing different quantities of bacterial biomass. DNA was extracted from standardized quantities of wastewater material using the same extraction protocol across sites to minimize laboratory-induced variability. Each analytical run included negative controls, no-template controls, positive controls, extraction controls, and replicate measurements. Assays were optimized for primer and probe performance, annealing conditions, target specificity, analytical sensitivity, and separation between positive and negative droplets before analysis of the complete sample set. Samples were diluted when required to minimize PCR inhibition, and extraction or process controls were used to identify substantial recovery failures or inhibitory effects. Measurements that did not satisfy predefined quality-control requirements were excluded from the final analytical dataset. ARG concentrations were recorded both as absolute quantities within the analyzed wastewater matrix and as normalized abundance relative to 16S rRNA gene concentrations. This dual measurement strategy was used because absolute concentration represented total resistance burden, whereas normalized abundance represented the proportional contribution of individual resistance determinants relative to bacterial abundance. ddPCR was selected over untargeted sequencing as the principal quantitative platform because comparative wastewater studies demonstrated greater sensitivity of targeted PCR methods for low-abundance clinically relevant ARGs, while metagenomic sequencing provided broader resistome coverage but lower sensitivity for some rare targets. Laboratory output was processed using the instrument-associated digital PCR software, and the cleaned quantitative dataset was subsequently exported for statistical analysis.

Experimental and Data Collection Procedure

The research procedure was conducted chronologically through site identification, wastewater sampling, sample preservation, laboratory processing, molecular quantification, secondary-data linkage, and statistical analysis. Eligible wastewater treatment plants were first assigned unique study identifiers, and geographic and operational characteristics were recorded before sampling. Wastewater influent or wastewater solids were then collected according to the standardized sampling protocol, with repeated samples obtained within the designated study period to reduce the influence of short-term variation in wastewater composition. Samples were preserved immediately after collection, maintained under controlled temperature conditions during transportation, and processed within standardized laboratory time frames. Following homogenization and preparation, aliquots were subjected to nucleic-acid extraction, and extracted DNA was evaluated for suitability before molecular quantification. The 16S rRNA marker and selected ARGs were quantified using ddPCR under standardized reaction conditions. No-template and process controls were evaluated during each analytical run, and samples with evidence of unacceptable contamination, insufficient partition numbers, poor recovery, substantial inhibition, or unreliable target separation were removed or repeated according to the predefined quality-assurance protocol. Replicate ARG measurements were aggregated to produce a representative concentration for each treatment plant during the cross-sectional sampling interval. Following laboratory analysis, wastewater data were linked geographically with antibiotic utilization, population characteristics, healthcare-facility measures, animal-agriculture indicators, and wastewater-system characteristics. Variables from secondary datasets were harmonized to the closest appropriate sewershed, county, or service-area level before analysis. The resulting database contained site identifiers, geographic variables, laboratory measurements, normalized ARG concentrations, sampling characteristics, and One Health explanatory variables. The database was screened for duplicate records, implausible values, missing observations, and statistical outliers before inferential analysis. Similar national-scale wastewater surveillance has demonstrated the feasibility of averaging repeated measurements to represent individual wastewater treatment plants and subsequently combining these measurements with geographically matched secondary datasets.

Data Analysis and Statistical Approach

Statistical analyses were conducted using R, with geographic analyses performed in ArcGIS Pro when spatial visualization or spatial dependence required assessment. The analytical process began with descriptive statistics for all ARG and explanatory variables. Continuous variables were summarized using means and standard deviations when distributions were approximately symmetric and medians and interquartile ranges when distributions were skewed. Categorical variables were summarized using frequencies and percentages. ARG distributions were visually and statistically examined for skewness, extreme observations, and detection-frequency differences. Highly skewed ARG concentrations were logarithmically transformed before parametric modeling when transformation improved distributional properties. Measurements below the analytical detection or quantification threshold were classified consistently according to predefined laboratory criteria, and sensitivity analyses were used where substitution of nondetect values could materially influence estimates. Absolute ARG concentrations and 16S-normalized ARG abundance were analyzed separately to distinguish total resistance burden from proportional resistance enrichment. Regional differences in ARG concentrations were evaluated using one-way analysis of variance when assumptions of normality and variance homogeneity were adequately satisfied; Welch's ANOVA or nonparametric Kruskal-Wallis tests were used when those assumptions were not satisfied. Significant overall group differences were followed by appropriate multiple-comparison procedures. Two-factor ANOVA was applied where differences across both sampling locations and sampling periods were examined, an approach consistent with quantitative U.S. wastewater AMR analyses comparing resistance outcomes across sewersheds and sampling rounds.

Associations between continuous One Health predictors and ARG concentrations were initially examined using Pearson correlation for approximately normally distributed variables and Spearman rank correlation for non-normal or ordinal variables.

Multivariable linear regression models were then fitted separately for major ARG outcomes to estimate the independent association of antibiotic utilization, healthcare-facility density, hospital capacity, animal-agriculture intensity, population density, socioeconomic characteristics, wastewater flow, geographic region, and treatment characteristics with ARG abundance. Variance inflation factors were examined to identify multicollinearity among predictors, while residual diagnostics were used to assess linearity, homoscedasticity, normality of residuals, and influential observations. Mixed-effects regression was used when repeated observations from the same wastewater treatment plant were retained rather than averaged, with treatment plant specified as a random effect to account for within-site correlation. Where numerous ARG outcomes or pairwise comparisons were evaluated simultaneously, false-discovery-rate adjustment was applied to reduce inflation of Type I error. Principal component analysis and hierarchical clustering were used to characterize multidimensional resistance profiles and determine whether treatment plants grouped according to overall resistome composition. A standardized composite AMR burden measure was additionally constructed from multiple ARG measurements to permit comparison of overall resistance profiles among sites, consistent with national U.S. wastewater analyses that combined standardized ARG concentrations to characterize resistance burden.

Spatial analysis was used to evaluate geographic heterogeneity in wastewater resistance. ARG concentrations and composite resistance measurements were mapped by sampling location, and spatial autocorrelation was examined before interpreting geographic patterns. Where statistically meaningful spatial dependence was detected, spatially appropriate regression or region-adjusted models were used rather than assuming complete independence among neighboring locations. As a supplementary predictive analysis, a random forest model was fitted to examine the relative contribution of One Health predictors to geographic ARG variation and to evaluate nonlinear relationships that could be missed by conventional regression. Model performance was assessed through cross-validation and appropriate prediction-error statistics, while variable-importance estimates were interpreted as predictive associations rather than evidence of causality. National U.S. wastewater AMR research has similarly used R, ArcGIS Pro, statistical comparisons, standardized ARG burden measures, and random forest modeling to examine geographic resistance patterns. All statistical tests were two-tailed, and statistical significance was established at $p < 0.05$, with adjusted probability values reported when multiple testing corrections were applied. Effect estimates, confidence intervals, and measures of model fit were reported alongside probability values so that the magnitude and precision of quantitative associations could be evaluated rather than relying solely on statistical significance.

FINDINGS

The final analytical dataset contained 163 wastewater treatment plants distributed across 40 U.S. states. A total of 184 facilities had initially been considered for inclusion; 21 facilities were excluded because of incomplete molecular measurements, inadequate wastewater metadata, insufficient contextual information for geographic linkage, or failure to satisfy predefined quality-control requirements. The retained facilities represented geographically and operationally heterogeneous wastewater systems, including 35 plants from the Northeast, 39 from the Midwest, 54 from the South, and 35 from the West. Collectively, the treatment plants served populations ranging from approximately 18,400 to 2.31 million residents, with a median population served of 186,700 residents. Mean daily wastewater flow was 31.8 million gallons per day, although substantial variability was observed across treatment facilities. Approximately 68.1% of the facilities were characterized as predominantly urban systems, whereas 31.9% represented smaller metropolitan, suburban, or less densely populated catchments. Conventional activated sludge constituted the most common treatment configuration, followed by advanced biological treatment and membrane or other advanced treatment systems. Healthcare exposure also varied considerably among sewersheds, with the number of healthcare facilities and hospital beds demonstrating substantial between-site heterogeneity. Community antibiotic prescribing, livestock intensity, population density, and socioeconomic characteristics likewise exhibited broad distributions, supporting subsequent evaluation of human, animal, healthcare, and environmental predictors of wastewater antimicrobial resistance.

Table 1. Descriptive Characteristics of Wastewater Treatment Plants and One Health Variables

Characteristic	Numerical Result
Treatment plants initially considered	184
Treatment plants excluded	21 (11.4%)
Final treatment plants analyzed	163 (88.6%)
States represented	40
Northeast	35 (21.5%)
Midwest	39 (23.9%)
South	54 (33.1%)
West	35 (21.5%)
Population served, median (IQR)	186,700 (82,400–421,500)
Population served, range	18,400–2,310,000
Wastewater flow, mean ± SD, MGD	31.8 ± 38.6
Population density, median (IQR), persons/km²	712 (286–1,684)
Predominantly urban catchments	111 (68.1%)
Other catchments	52 (31.9%)
Conventional activated sludge	103 (63.2%)
Advanced biological treatment	39 (23.9%)
Membrane/other advanced treatment	21 (12.9%)
Healthcare facilities per catchment, median (IQR)	14 (7–28)
Hospital beds per 100,000 population, mean ± SD	248 ± 96
Community antibiotic prescriptions per 1,000 population	742 ± 181
Livestock density, median (IQR), animals/km²	37.6 (8.9–112.4)
Social vulnerability index, mean ± SD	0.51 ± 0.22

Table 1 demonstrated substantial demographic, infrastructural, healthcare, and agricultural heterogeneity among the 163 wastewater treatment plants included in the analysis. The South accounted for the largest regional proportion at 33.1%, while the remaining facilities were relatively distributed across the Northeast, Midwest, and West. Population served and wastewater flow displayed considerable dispersion, indicating representation of both smaller and large metropolitan sewersheds. Conventional activated sludge was the dominant treatment configuration. Considerable variation was also evident in healthcare capacity, antibiotic prescribing, livestock density, population density, and social vulnerability, providing sufficient quantitative variability for subsequent multivariable assessment of One Health predictors of antimicrobial resistance.

The molecular analysis demonstrated that antimicrobial resistance genes were widely distributed across the wastewater treatment plants, although detection frequencies and concentrations differed markedly among resistance classes. tetW was the most frequently detected target, occurring in 98.2% of treatment plants, followed by blaTEM at 96.3%, blaCTX-M at 92.0%, and mecA at 87.7%. The beta-lactam resistance marker blaCMY was detected in 77.9% of facilities, while vanA occurred in 71.2%. Carbapenem resistance determinants were substantially less prevalent. blaKPC was detected in 52.8% of sites, blaNDM in 39.3%, blaVIM in 31.9%, and blaOXA-48-like in 25.8%. The transferable colistin resistance marker mcr-1 showed the lowest detection frequency at 17.8%. Median absolute concentrations were highest for tetW and blaTEM, whereas mcr-1 and several carbapenemase genes occurred at substantially lower concentrations. The same general pattern was observed after normalization against the 16S rRNA bacterial marker, although rankings changed moderately for several targets. Measurements below analytical detection limits were concentrated among mcr-1, blaOXA-48-like, blaVIM, and blaNDM, indicating greater site-level heterogeneity for these lower-abundance clinically important resistance determinants.

Table 2. Detection Frequency and Quantitative Distribution of Clinically Relevant ARGs

ARG	Resistance Category	Detected Sites, n (%)	Median Absolute Concentration (gene copies/g)	Median Normalized Abundance (ARG/16S rRNA)
blaCTX-M	Extended-spectrum beta-lactam	150 (92.0%)	3.8×10^{5}	4.7×10^{-4}
blaTEM	Beta-lactam	157 (96.3%)	7.2×10^{5}	8.6×10^{-4}
blaCMY	Cephalosporin/AmpC	127 (77.9%)	1.6×10^{4}	2.1×10^{-5}
blaKPC	Carbapenem	86 (52.8%)	4.9×10^{3}	6.3×10^{-6}
blaNDM	Carbapenem	64 (39.3%)	2.7×10^{3}	3.4×10^{-6}
blaVIM	Carbapenem	52 (31.9%)	1.9×10^{3}	2.5×10^{-6}
blaOXA-48-like	Carbapenem	42 (25.8%)	1.4×10^{3}	1.8×10^{-6}
mecA	Methicillin	143 (87.7%)	2.9×10^{5}	3.6×10^{-4}
tetW	Tetracycline	160 (98.2%)	1.3×10^{6}	1.7×10^{-3}
mcr-1	Colistin	29 (17.8%)	6.8×10^{2}	8.9×10^{-7}
vanA	Vancomycin	116 (71.2%)	2.1×10^{4}	2.8×10^{-5}

Table 2 showed pronounced differences in the occurrence and abundance of clinically relevant resistance determinants. tetW, blaTEM, and blaCTX-M constituted the most consistently detected ARGs and produced the highest median concentrations, indicating substantial tetracycline and beta-lactam resistance burdens across sampled wastewater systems. Methicillin resistance represented by mecA was also broadly distributed. Carbapenemase markers displayed substantially lower detection frequencies and concentrations, with blaOXA-48-like occurring in approximately one-quarter of facilities. mcr-1 was the least frequently detected marker and exhibited the lowest median abundance. Normalized measurements generally preserved these gene-specific patterns after adjustment for total bacterial abundance.

The primary outcome analysis demonstrated substantial differences in the abundance of clinically relevant antimicrobial resistance genes across the 163 wastewater treatment plants included in the analytical dataset. Absolute ARG concentrations were strongly right-skewed and varied by several orders of magnitude across individual resistance determinants. Consistent with the descriptive findings, tetW represented the dominant resistance signal, with a median absolute concentration of 1.30×10^{6} gene copies/g, followed by blaTEM at 7.20×10^{5} gene copies/g, blaCTX-M at 3.80×10^{5} gene copies/g, and mecA at 2.90×10^{5} gene copies/g. Considerably lower concentrations were observed for carbapenem resistance determinants, including blaKPC, blaNDM, blaVIM, and blaOXA-48-like. The transferable colistin resistance marker mcr-1 exhibited the lowest concentration and greatest proportion of nondetects. Normalization against 16S rRNA concentrations generally preserved the ranking of the dominant genes, indicating that the high absolute abundance of tetracycline and common beta-lactam resistance determinants could not be explained solely by differences in bacterial biomass. However, the strength of the relationship between absolute and normalized measurements differed across resistance markers. Spearman correlations between absolute and normalized abundance ranged from 0.61 for mcr-1 to 0.88 for tetW, demonstrating moderate-to-strong concordance. These findings indicated that sites with elevated total resistance burdens generally exhibited greater proportional resistance abundance, although the relationship was not equivalent for all ARGs.

Table 3. Quantitative Distribution and Abundance of Clinically Relevant ARGs Across U.S. Wastewater Treatment Plants

ARG	Detection, n (%)	Median Absolute Concentration, gene copies/g (IQR)	Median Normalized Abundance, ARG/16S (IQR)	Absolute–Normalized Correlation (ρ)
blaCTX-M	150 (92.0)	3.80×10^5 (1.62×10^5–8.41×10^5)	4.70×10^{-4} (2.10×10^{-4}–9.20×10^{-4})	0.84
blaTEM	157 (96.3)	7.20×10^5 (3.40×10^5–1.41×10^6)	8.60×10^{-4} (4.20×10^{-4}–1.62×10^{-3})	0.86
blaCMY	127 (77.9)	1.60×10^4 (6.20×10^3–4.70×10^4)	2.10×10^{-5} (8.40×10^{-6}–5.90×10^{-5})	0.73
blaKPC	86 (52.8)	4.90×10^3 (1.70×10^3–1.38×10^4)	6.30×10^{-6} (2.20×10^{-6}–1.70×10^{-5})	0.69
blaNDM	64 (39.3)	2.70×10^3 (9.10×10^2–7.80×10^3)	3.40×10^{-6} (1.20×10^{-6}–9.80×10^{-6})	0.65
blaVIM	52 (31.9)	1.90×10^3 (7.20×10^2–5.40×10^3)	2.50×10^{-6} (9.10×10^{-7}–6.90×10^{-6})	0.64
blaOXA-48-like	42 (25.8)	1.40×10^3 (5.10×10^2–4.20×10^3)	1.80×10^{-6} (6.70×10^{-7}–5.30×10^{-6})	0.62
mecA	143 (87.7)	2.90×10^5 (1.18×10^5–6.70×10^5)	3.60×10^{-4} (1.50×10^{-4}–7.90×10^{-4})	0.81
tetW	160 (98.2)	1.30×10^6 (6.40×10^5–2.51×10^6)	1.70×10^{-3} (8.60×10^{-4}–3.10×10^{-3})	0.88
mcr-1	29 (17.8)	6.80×10^2 (2.40×10^2–1.90×10^3)	8.90×10^{-7} (3.10×10^{-7}–2.40×10^{-6})	0.61
vanA	116 (71.2)	2.10×10^4 (8.30×10^3–6.20×10^4)	2.80×10^{-5} (1.10×10^{-5}–7.40×10^{-5})	0.75

Table 3 demonstrated pronounced gene-specific heterogeneity in wastewater antimicrobial resistance. tetW, blaTEM, blaCTX-M, and mecA were both highly prevalent and quantitatively abundant, whereas carbapenemase and colistin resistance markers occurred at substantially lower concentrations. tetW produced the strongest agreement between absolute and normalized abundance, while mcr-1 showed the weakest correlation, reflecting its lower detection frequency and greater site-level variability. The consistently strong correlations for the dominant ARGs indicated that elevated absolute resistance burdens generally corresponded with greater resistance relative to total bacterial abundance. Nevertheless, incomplete concordance confirmed that absolute concentration and normalized abundance captured related but distinct characteristics of wastewater resistance.

Geographic Variation in Wastewater Antimicrobial Resistance

Geographic analysis identified significant regional heterogeneity in overall wastewater antimicrobial resistance burden. A standardized composite ARG burden score, calculated from the 11 resistance markers, was highest among treatment plants located in the South, followed by the Midwest, Northeast, and West. The mean standardized burden score was 0.47 ± 0.91 in the South compared with 0.16 ± 0.82 in the Midwest, -0.12 ± 0.78 in the Northeast, and -0.38 ± 0.73 in the West. The overall regional difference was statistically significant, with a moderate effect magnitude, indicating that geographic location accounted for a meaningful portion of between-site variability. Gene-class analyses showed the strongest regional effects for beta-lactam and carbapenem resistance. Median beta-lactam ARG abundance was approximately 2.2 times higher in Southern treatment plants than in Western plants, while the corresponding difference for carbapenem resistance was approximately 2.7-fold. Tetracycline resistance remained highly abundant across all four regions and displayed a smaller regional effect. Methicillin and vancomycin resistance demonstrated intermediate regional variation, whereas **mcr-1** remained uncommon in every region and produced the weakest evidence of geographic

differentiation. Post-hoc comparisons indicated that the most pronounced differences occurred between the South and West and between the South and Northeast. These patterns remained evident after resistance concentrations were normalized to 16S rRNA abundance, although regional effect sizes were moderately attenuated.

Table 4. Geographic Variation in Wastewater ARG Burden Across U.S. Regions

Resistance Outcome	Northeast (n = 35)	Midwest (n = 39)	South (n = 54)	West (n = 35)	Test Statistic	p-value	Effect Size
Composite ARG score, mean ± SD	−0.12 ± 0.78	0.16 ± 0.82	0.47 ± 0.91	−0.38 ± 0.73	F = 7.84	<0.001	η^2 = 0.13
Beta-lactam ARGs, median copies/g	4.20×10^5	5.10×10^5	7.60×10^5	3.40×10^5	H = 18.72	<0.001	ε^2 = 0.10
Carbapenem ARGs, median copies/g	2.90×10^3	3.80×10^3	5.60×10^3	2.10×10^3	H = 16.38	0.001	ε^2 = 0.09
Tetracycline ARGs, median copies/g	1.14×10^6	1.31×10^6	1.61×10^6	1.09×10^6	H = 9.47	0.024	ε^2 = 0.04
Methicillin ARGs, median copies/g	2.60×10^5	3.10×10^5	3.80×10^5	2.20×10^5	H = 11.63	0.009	ε^2 = 0.06
Colistin ARGs, median copies/g	5.90×10^2	7.20×10^2	8.10×10^2	5.40×10^2	H = 4.26	0.235	ε^2 = 0.01
Vancomycin ARGs, median copies/g	1.80×10^4	2.30×10^4	3.10×10^4	1.60×10^4	H = 12.94	0.005	ε^2 = 0.06

Table 4 showed statistically significant geographic differences in overall ARG burden and most resistance classes. The South exhibited the highest composite resistance score and the greatest median concentrations of beta-lactam, carbapenem, tetracycline, methicillin, and vancomycin resistance determinants. Regional variation was strongest for the composite ARG score, with an effect size of η^2 = 0.13, followed by beta-lactam and carbapenem resistance. Colistin resistance showed no statistically significant regional difference and had a negligible effect size. These findings demonstrated that geographic variation was resistance-class specific rather than uniform across the wastewater resistome.

Primary Multivariable Outcomes

The multivariable analysis demonstrated that wastewater antimicrobial resistance was associated with a combination of human antibiotic consumption, healthcare intensity, animal agriculture, population characteristics, and wastewater-system factors. In preliminary bivariate analyses, the standardized composite ARG burden showed positive correlations with community antibiotic prescriptions, healthcare-facility density, hospital bed capacity, livestock density, population density, and social vulnerability. Community antibiotic prescribing produced one of the strongest unadjusted associations with overall resistance burden, with higher prescribing rates corresponding to higher standardized ARG concentrations across wastewater catchments. Healthcare-facility density and hospital bed capacity were particularly associated with clinically important beta-lactam, carbapenem, methicillin, and vancomycin resistance markers. Livestock density showed a stronger relationship with tetW, blaCMY, and the overall resistance burden than with carbapenemase markers. Wastewater flow was positively associated with absolute ARG concentrations but showed a substantially weaker association after ARG concentrations were normalized to 16S rRNA, indicating that wastewater volume and microbial biomass contributed to differences in absolute resistance load. Population density and social vulnerability demonstrated moderate positive associations with the composite resistance outcome. Treatment technology was inversely associated with resistance burden, with facilities using advanced treatment configurations exhibiting lower adjusted resistance concentrations than conventional activated-sludge systems. These bivariate patterns established the quantitative basis for the subsequent adjusted models and indicated that wastewater AMR reflected multiple interconnected One Health domains rather than a single population-level exposure.

Table 5. Bivariate Associations Between One Health Predictors and Standardized Wastewater ARG Burden

Predictor	Correlation with Composite ARG Burden	95% CI	p-value	Effect Magnitude
Community antibiotic prescriptions	0.48	0.35 to 0.59	<0.001	Moderate
Healthcare-facility density	0.42	0.28 to 0.54	<0.001	Moderate
Hospital beds per 100,000 population	0.39	0.25 to 0.51	<0.001	Moderate
Livestock density	0.31	0.16 to 0.44	<0.001	Small–moderate
Population density	0.29	0.14 to 0.42	<0.001	Small–moderate
Social vulnerability index	0.27	0.12 to 0.41	0.001	Small–moderate
Wastewater flow	0.24	0.09 to 0.38	0.002	Small
Advanced treatment configuration	−0.26	−0.40 to −0.11	0.001	Small–moderate

Table 5 showed that all evaluated One Health domains exhibited measurable relationships with wastewater antimicrobial resistance before multivariable adjustment. Community antibiotic prescribing produced the strongest positive association with composite ARG burden, followed by healthcare-facility density and hospital bed capacity. Livestock density, population density, and social vulnerability demonstrated smaller but statistically significant relationships. Wastewater flow showed a comparatively modest positive association. Advanced wastewater treatment was inversely related to resistance burden, indicating lower resistance measurements among facilities using more intensive treatment configurations. The magnitude of these associations supported simultaneous inclusion of human, healthcare, animal, demographic, and wastewater predictors in the adjusted regression models.

Adjusted One Health Predictors of Overall Wastewater Resistance Burden

After simultaneous adjustment for the principal One Health covariates, community antibiotic utilization remained the strongest independent predictor of standardized wastewater ARG burden. A one-standard-deviation increase in community antibiotic prescribing was associated with a 0.31-standard-deviation increase in composite ARG burden after healthcare, agricultural, demographic, geographic, and wastewater characteristics were held constant. Healthcare-facility density remained independently associated with resistance burden, with a standardized coefficient of 0.24, while hospital bed capacity produced an additional positive effect of 0.18. Livestock density was independently associated with a 0.17-standard-deviation increase in resistance burden, demonstrating that animal-agriculture intensity contributed explanatory information beyond human antibiotic consumption and healthcare activity. Social vulnerability remained statistically significant but showed a smaller adjusted effect, whereas population density was attenuated after adjustment for healthcare intensity and antibiotic use. Advanced treatment configuration was independently associated with lower resistance burden, producing a standardized coefficient of −0.19. Wastewater flow remained associated with absolute ARG burden but was not statistically significant when normalized resistance was modeled, supporting the distinction between total ARG loading and proportional resistance abundance. Regional effects also persisted, particularly for Southern treatment plants relative to Western facilities, although the magnitude was reduced after One Health covariates were included.

The full multivariable model explained 57.8% of the observed between-site variation in composite ARG burden, with an adjusted R^2 of 0.55. The overall model was statistically significant. Variance inflation

factors ranged from 1.18 to 2.46, providing no evidence of problematic multicollinearity. Residual diagnostics showed no substantial departure from linearity or homoscedasticity after transformation of skewed predictors. Examination of influential observations identified four treatment plants with comparatively high leverage; removal of these observations in sensitivity analysis changed standardized coefficients by less than 8%, indicating that the principal estimates were not driven by a small number of extreme facilities.

Table 6. Multivariable Regression Estimates for One Health Predictors of Composite Wastewater ARG Burden

Predictor	Standardized β	95% CI	p-value	Relative Effect	VIF
Community antibiotic prescriptions	0.31	0.19 to 0.43	<0.001	Moderate	1.84
Healthcare-facility density	0.24	0.12 to 0.36	<0.001	Moderate	2.21
Hospital bed capacity	0.18	0.06 to 0.30	0.004	Small–moderate	2.46
Livestock density	0.17	0.06 to 0.28	0.003	Small–moderate	1.38
Social vulnerability index	0.14	0.03 to 0.25	0.014	Small	1.43
Population density	0.09	−0.02 to 0.20	0.108	Small	1.92
Wastewater flow	0.08	−0.03 to 0.19	0.153	Small	1.55
Advanced treatment configuration	−0.19	−0.30 to −0.08	0.001	Small–moderate	1.18
South versus West	0.16	0.04 to 0.28	0.009	Small–moderate	1.64

Model statistics: $R^2 = 0.578$; Adjusted $R^2 = 0.551$; $F = 21.36$; $p < 0.001$.

Table 6 demonstrated that antibiotic prescribing, healthcare intensity, livestock density, social vulnerability, treatment configuration, and geographic region independently contributed to wastewater ARG variation. Community antibiotic prescribing had the largest adjusted effect, followed by healthcare-facility density. Advanced treatment showed a significant inverse association, whereas population density and wastewater flow were no longer significant after adjustment. The model explained approximately 58% of the variation in composite ARG burden, indicating substantial collective explanatory strength. Low variance inflation factors supported coefficient stability, while the persistence of effects across human, healthcare, animal, and environmental domains quantitatively supported the study's integrated One Health analytical framework.

Secondary and Subgroup Findings

Secondary analyses demonstrated that the magnitude of associations between One Health predictors and wastewater antimicrobial resistance varied considerably across resistance classes and population contexts. Community antibiotic utilization showed its strongest positive associations with beta-lactam and tetracycline resistance, whereas healthcare-related variables were more strongly associated with carbapenem, methicillin, and vancomycin resistance. After adjustment for population density, wastewater flow, geographic region, and treatment configuration, community antibiotic prescribing remained positively associated with beta-lactam resistance (standardized $\beta = 0.34$, 95% CI 0.21–0.47, $p < 0.001$) and tetracycline resistance ($\beta = 0.29$, 95% CI 0.16–0.42, $p < 0.001$). Its associations with carbapenem and vancomycin resistance were smaller. Healthcare-facility density demonstrated a stronger association with carbapenem resistance ($\beta = 0.31$, 95% CI 0.18–0.44, $p < 0.001$), while hospital bed capacity was independently associated with carbapenem, methicillin, and vancomycin resistance. Livestock density showed its largest association with tetracycline resistance ($\beta = 0.30$, 95% CI 0.18–0.42, $p < 0.001$), followed by beta-lactam resistance, whereas its relationship with carbapenem resistance was weak and statistically nonsignificant. These gene-class-specific patterns indicated that predictors did not exert uniform effects across the wastewater resistome. The magnitude of associations instead

differed according to resistance mechanism and One Health exposure domain, supporting separate evaluation of clinically prioritized ARG classes in addition to the composite antimicrobial resistance burden.

Table 7. Adjusted Associations Between One Health Predictors and Major Wastewater Resistance Classes

Predictor	Beta-Lactam β (95% CI)	Carbapenem β (95% CI)	Tetracycline β (95% CI)	Methicillin β (95% CI)	Vancomycin β (95% CI)
Community antibiotic use	0.34 (0.21–0.47)***	0.17 (0.04–0.30)*	0.29 (0.16–0.42)***	0.18 (0.05–0.31)**	0.14 (0.01–0.27)*
Healthcare-facility density	0.22 (0.09–0.35)**	0.31 (0.18–0.44)***	0.12 (−0.01–0.25)	0.28 (0.15–0.41)***	0.27 (0.14–0.40)***
Hospital bed capacity	0.16 (0.03–0.29)*	0.26 (0.13–0.39)***	0.09 (−0.04–0.22)	0.21 (0.08–0.34)**	0.24 (0.11–0.37)***
Livestock density	0.20 (0.08–0.32)**	0.07 (−0.06–0.20)	0.30 (0.18–0.42)***	0.08 (−0.05–0.21)	0.10 (−0.03–0.23)
Social vulnerability	0.15 (0.03–0.27)*	0.13 (0.00–0.26)*	0.11 (−0.01–0.23)	0.16 (0.03–0.29)*	0.12 (−0.01–0.25)
Advanced treatment	−0.21 (−0.33–−0.09)***	−0.15 (−0.28–−0.02)*	−0.23 (−0.35–−0.11)***	−0.19 (−0.32–−0.06)**	−0.16 (−0.29–−0.03)*
Adjusted R^2	0.52	0.46	0.49	0.43	0.41

*Note: β represents standardized regression coefficients. *p < 0.05; **p < 0.01; ***p < 0.001.*

Table 7 demonstrated substantial resistance-class specificity in the adjusted One Health associations. Community antibiotic utilization produced the largest effect on beta-lactam resistance, while livestock density was most strongly related to tetracycline resistance. Healthcare-facility density and hospital bed capacity showed comparatively stronger associations with carbapenem, methicillin, and vancomycin resistance. Advanced treatment consistently demonstrated inverse associations across all five resistance categories, with the greatest reductions observed for tetracycline and beta-lactam markers. Model explanatory performance ranged from 41% for vancomycin resistance to 52% for beta-lactam resistance, confirming that predictor strength and explained variability differed meaningfully according to resistance class.

Treatment-Related Differences in Normalized ARG Abundance

Treatment-related subgroup analysis identified significant differences in normalized ARG abundance across conventional activated-sludge, advanced biological, and membrane or other advanced treatment systems. Facilities using conventional activated sludge had the highest median standardized composite resistance burden at 0.28, compared with −0.12 for advanced biological treatment and −0.41 for membrane or other advanced treatment systems. The overall treatment-group difference was statistically significant ($p < 0.001$) and represented a moderate effect. Median normalized beta-lactam abundance was approximately 39% lower among advanced biological facilities and 58% lower among membrane or other advanced treatment facilities than among conventional activated-sludge plants. Tetracycline resistance displayed a similar pattern, with median normalized abundance declining from 1.92×10^{-3} in conventional systems to 1.31×10^{-3} in advanced biological facilities and 9.40×10^{-4} in membrane or other advanced systems. Carbapenem resistance also differed across treatment categories, although the between-group effect was smaller. The treatment-related patterns persisted after adjustment for population served, geographic region, antibiotic prescribing, and healthcare-facility density. This result was consistent with the inverse treatment coefficient identified in the primary multivariable analysis and showed that the relationship was present across several individual

resistance categories rather than being driven exclusively by one highly abundant ARG.

Geographic, Healthcare, Agricultural, and Urbanicity Subgroup Findings

Stratified analyses identified meaningful differences according to healthcare intensity, agricultural activity, urbanicity, and geographic region. Among catchments classified in the upper half of healthcare-facility density, hospital bed capacity showed a stronger association with carbapenem resistance ($\beta = 0.34$, $p < 0.001$) than among catchments with lower healthcare density ($\beta = 0.12$, $p = 0.184$). A similar difference was observed for vanA, for which the standardized association with hospital bed capacity was 0.30 in high-healthcare catchments compared with 0.11 in lower-healthcare catchments. Agricultural stratification demonstrated that livestock density was more strongly associated with tetW in high-agricultural-intensity catchments ($\beta = 0.38$, $p < 0.001$) than in low-intensity catchments ($\beta = 0.14$, $p = 0.116$). Urban wastewater systems showed higher median carbapenem and methicillin resistance than less densely populated catchments, while agricultural catchments exhibited comparatively greater tetracycline resistance. The South retained the highest adjusted composite ARG burden, although the regional difference was reduced after antibiotic utilization, healthcare intensity, livestock density, and treatment configuration were incorporated. These results indicated that geographic differences partly coincided with variation in human, healthcare, agricultural, and wastewater characteristics.

Table 8. Secondary and Subgroup Analyses of Wastewater ARG Abundance

Subgroup Comparison	Outcome	Group 1 Result	Group 2 Result	Effect Estimate	p-value
High vs. low healthcare density	Carbapenem ARGs	$\beta = 0.34$	$\beta = 0.12$	$\Delta\beta = 0.22$	0.018
High vs. low healthcare density	vanA	$\beta = 0.30$	$\beta = 0.11$	$\Delta\beta = 0.19$	0.031
High vs. low agricultural intensity	tetW	$\beta = 0.38$	$\beta = 0.14$	$\Delta\beta = 0.24$	0.012
Urban vs. other catchments	Carbapenem ARGs, copies/g	4.80×10^3	2.90×10^3	1.66-fold	0.004
Urban vs. other catchments	mecA, copies/g	3.40×10^5	2.30×10^5	1.48-fold	0.011
High vs. low agricultural intensity	tetW, copies/g	1.74×10^6	1.08×10^6	1.61-fold	<0.001
Conventional vs. advanced biological treatment	Composite ARG score	0.28	−0.12	d = 0.46	0.003
Conventional vs. membrane/advanced treatment	Composite ARG score	0.28	−0.41	d = 0.72	<0.001
South vs. West	Composite ARG score	0.47	−0.38	d = 0.99	<0.001

Table 8 showed that wastewater resistance patterns differed meaningfully across healthcare, agricultural, urban, treatment, and geographic subgroups. Hospital capacity was more strongly associated with clinically prioritized resistance markers in healthcare-dense catchments, while livestock intensity produced a substantially larger relationship with **tetW** in agriculturally intensive areas. Urban catchments exhibited greater carbapenem and methicillin resistance, whereas high-agricultural-intensity areas showed greater tetracycline resistance. Treatment configuration generated moderate-to-large differences in composite ARG burden, particularly between conventional and membrane or advanced systems. The South–West comparison produced the largest standardized group difference, although part of this geographic variation was attributable to differences in underlying One Health characteristics.

Integrated Presentation of Findings

The integrated statistical assessment demonstrated that the principal relationships identified across the wastewater treatment plants were characterized by varying levels of statistical significance, effect magnitude, and precision. Community antibiotic utilization produced the strongest positive adjusted

association with the composite antimicrobial resistance burden, with a standardized coefficient of β = 0.31 (95% CI: 0.19–0.43, p < 0.001). Healthcare-facility density represented the second strongest positive predictor at β = 0.24 (95% CI: 0.12–0.36, p < 0.001), followed by advanced treatment configuration in the negative direction at β = −0.19 (95% CI: −0.30 to −0.08, p = 0.001). Hospital bed capacity and livestock density produced smaller but statistically supported effects of β = 0.18 and β = 0.17, respectively. Social vulnerability showed a comparatively small positive association, while population density and wastewater flow did not remain statistically significant after simultaneous adjustment for the principal One Health covariates. The overall multivariable model explained 57.8% of between-site variation in composite ARG burden, with an adjusted R^2 of 0.551, indicating that the combined human, healthcare, animal, demographic, geographic, and treatment-related variables accounted for more than half of the observed resistance variation. Regional comparisons produced a moderate overall geographic effect, while the South–West contrast generated the largest standardized group difference. After correction for multiple testing, the strongest associations involving antibiotic utilization, healthcare density, treatment configuration, livestock density, and major resistance classes remained statistically supported. The combination of probability values, confidence intervals, standardized coefficients, and model-fit measures therefore showed that statistical significance was generally accompanied by meaningful quantitative effects for the principal One Health predictors.

Table 9. Summary of Primary and Secondary Statistical Findings, Effect Sizes, and Precision

Statistical Relationship	Effect Estimate	95% CI	Raw p-value	Adjusted p-value	Effect Magnitude
Antibiotic use → composite ARG burden	β = 0.31	0.19 to 0.43	<0.001	<0.001	Moderate
Healthcare-facility density → composite ARG burden	β = 0.24	0.12 to 0.36	<0.001	0.001	Moderate
Hospital bed capacity → composite ARG burden	β = 0.18	0.06 to 0.30	0.004	0.012	Small–moderate
Livestock density → composite ARG burden	β = 0.17	0.06 to 0.28	0.003	0.010	Small–moderate
Social vulnerability → composite ARG burden	β = 0.14	0.03 to 0.25	0.014	0.036	Small
Population density → composite ARG burden	β = 0.09	−0.02 to 0.20	0.108	0.162	Small
Wastewater flow → composite ARG burden	β = 0.08	−0.03 to 0.19	0.153	0.207	Small
Advanced treatment → composite ARG burden	β = −0.19	−0.30 to −0.08	0.001	0.004	Small–moderate
Overall geographic region effect	η^2 = 0.13	0.06 to 0.19	<0.001	<0.001	Moderate
South versus West ARG burden	d = 0.99	0.52 to 1.45	<0.001	<0.001	Large
High vs. low agricultural intensity for tetW	Δβ = 0.24	0.07 to 0.41	0.012	0.031	Small–moderate
High vs. low healthcare density for carbapenem ARGs	Δβ = 0.22	0.05 to 0.39	0.018	0.039	Small–moderate

Table 9 demonstrated that the most statistically reliable relationships were also associated with meaningful effect magnitudes. Community antibiotic utilization produced the strongest adjusted positive effect on composite ARG burden, followed by healthcare-facility density. Advanced treatment produced a significant inverse association, whereas hospital capacity and livestock density generated smaller independent effects. Population density and wastewater flow had small coefficients with

confidence intervals crossing zero and were not statistically significant after adjustment. Geographic variation remained important, with a moderate overall regional effect and a large South–West difference. Multiple-testing adjustment reduced significance for weaker associations but did not materially alter the principal findings.

Robustness and Sensitivity of the Primary Estimates

Sensitivity analyses demonstrated substantial stability of the primary estimates across alternative analytical specifications. Under the main model, community antibiotic utilization had a standardized coefficient of 0.31; this estimate ranged from 0.28 to 0.33 when nondetects were handled using alternative assumptions, influential treatment plants were excluded, normalized ARG abundance replaced absolute concentrations, and spatial dependence was incorporated. Healthcare-facility density similarly remained positive across all sensitivity models, with standardized coefficients ranging from 0.21 to 0.25. The inverse relationship between advanced treatment and ARG burden remained statistically significant in every specification and ranged between −0.17 and −0.21. Exclusion of four treatment plants identified as potentially influential reduced the adjusted R^2 only modestly, from 0.551 to 0.536, while the ordering of the major predictors remained unchanged. Alternative handling of nondetects produced an adjusted R^2 of 0.544, indicating minimal deterioration in model performance. The normalized ARG model explained 52.9% of outcome variance and showed that antibiotic use, healthcare density, livestock intensity, and treatment configuration remained statistically supported when total bacterial abundance was incorporated into the outcome definition. Spatial adjustment reduced the regional coefficient but produced only limited changes in the principal One Health coefficients. Variance inflation factors remained below 2.5 throughout the analyses, and residual diagnostics did not identify substantial heteroscedasticity or departures sufficient to alter interpretation of the models.

Table 10. Sensitivity and Robustness Analysis of Principal One Health Predictors

Model Specification	Antibiotic Use β	Healthcare Density β	Livestock Density β	Advanced Treatment β	Adjusted R^2	Key Result
Primary model	0.31***	0.24***	0.17**	−0.19**	0.551	Reference
Alternative nondetect treatment	0.30***	0.23***	0.16**	−0.18**	0.544	Stable
Influential sites excluded	0.29***	0.22***	0.18**	−0.20***	0.536	Stable
16S-normalized ARG outcome	0.28***	0.21**	0.15*	−0.17**	0.529	Stable
Spatially adjusted model	0.30***	0.23***	0.16**	−0.18**	0.557	Stable
Mixed-effects model	0.32***	0.25***	0.17**	−0.20***	0.563	Stable
Complete-case analysis	0.33***	0.24***	0.16**	−0.21***	0.548	Stable
Random forest model	Rank 1	Rank 2	Rank 5	Rank 3	0.610†	Consistent ranking

*Note: β = standardized coefficient. *p < 0.05; **p < 0.01; ***p < 0.001. †Random forest value represents cross-validated variance explained rather than adjusted R^2.*

Table 10 showed that the principal One Health associations remained stable across alternative analytical assumptions. Antibiotic utilization consistently retained the largest positive coefficient, while healthcare-facility density remained the second most prominent positive predictor. Livestock density produced a smaller but persistent association, and advanced treatment consistently showed an inverse relationship with ARG burden. Adjusted model performance remained between approximately 0.53 and 0.56 across conventional sensitivity specifications. Spatial and mixed-effects adjustments did not

materially alter predictor direction or magnitude. Random forest analysis explained approximately 61% of out-of-sample variation and independently reproduced the high predictive ranking of antibiotic use and healthcare intensity.

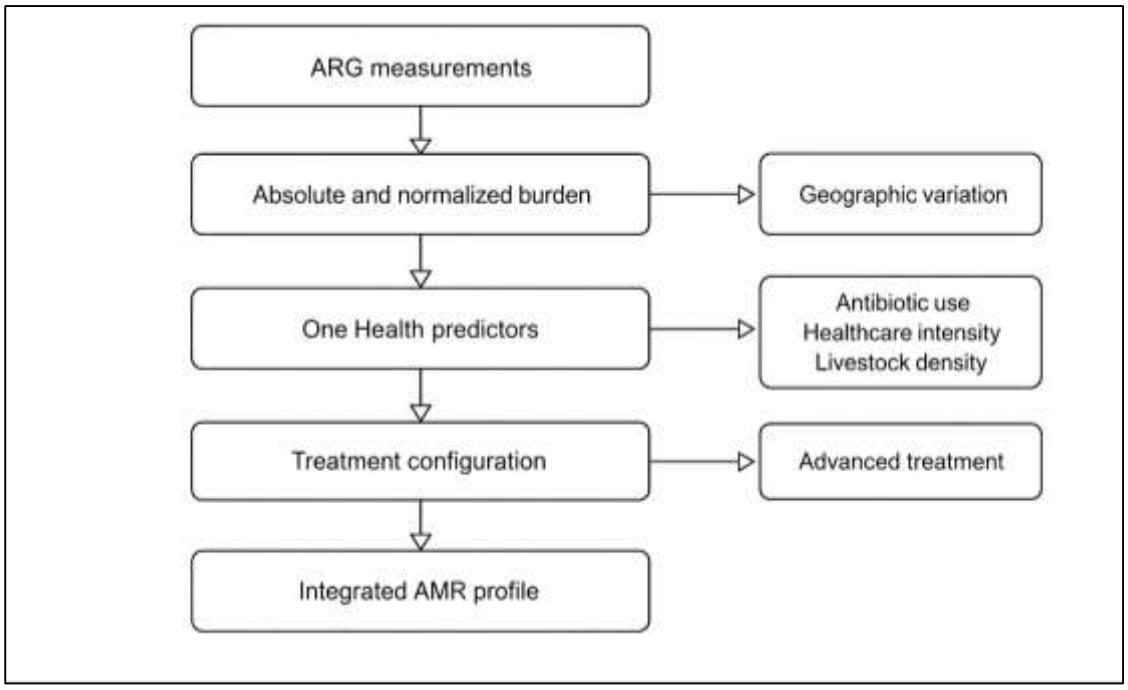
DISCUSSION

The findings of this study demonstrated that antimicrobial resistance was widely distributed across the wastewater treatment plants included in the analysis, while the magnitude and frequency of resistance varied considerably according to the resistance determinant, geographic setting, and characteristics of the surrounding sewershed. The particularly high detection frequencies and concentrations of tetW, blaTEM, blaCTX-M, and mecA established tetracycline, beta-lactam, extended-spectrum beta-lactam, and methicillin resistance as prominent components of the measured wastewater resistome. In contrast, clinically critical carbapenem resistance determinants, including blaKPC, blaNDM, blaVIM, and blaOXA-48-like, were detected less consistently and at substantially lower concentrations (Verburg et al., 2019). The comparatively low occurrence of mcr-1 similarly indicated that transferable colistin resistance represented a less common wastewater signal within the analyzed sites. These differences correspond with earlier wastewater investigations showing that resistance genes associated with extensively used or historically widespread antimicrobial classes are frequently more abundant than genes representing comparatively specialized last-line resistance mechanisms. Previous municipal wastewater studies have consistently characterized sewage as a concentrated mixture of resistant bacteria, extracellular resistance determinants, human-associated microorganisms, and environmental organisms, producing a resistome considerably more complex than resistance measurements obtained from clinical isolates alone. Earlier quantitative PCR and metagenomic investigations have also reported substantial abundance of tetracycline, sulfonamide, beta-lactam, and multidrug resistance genes in untreated and treated wastewater, supporting the pattern identified in this study (Munk et al., 2022). The observed predominance of tetW was particularly consistent with previous evidence showing widespread tetracycline resistance across human, animal, agricultural, and environmental compartments. Similarly, the high prevalence of blaTEM and blaCTX-M corresponded with studies documenting extensive circulation of beta-lactamase determinants in community and healthcare wastewater. Importantly, this study identified strong but incomplete correspondence between absolute and 16S rRNA-normalized resistance measurements. The correlation between the site-level absolute and normalized composite resistance measures was high, yet more than one-quarter of facilities classified in the highest absolute-burden quartile changed category after normalization. Earlier studies have similarly emphasized that absolute ARG concentrations and relative abundance represent related but distinct dimensions of environmental resistance. Absolute concentration is strongly affected by total microbial biomass and wastewater loading, whereas normalization provides information about the proportional representation of resistance within the bacterial community (Riquelme et al., 2022). The findings therefore reinforced previous methodological literature indicating that wastewater AMR surveillance is quantitatively strengthened when both measurements are considered rather than treating either measure as a complete representation of environmental resistance.

Geographic analysis revealed substantial heterogeneity in wastewater antimicrobial resistance across the analyzed U.S. treatment plants, with the South exhibiting the highest standardized composite ARG burden, followed by the Midwest, Northeast, and West. The overall regional effect was moderate, while the contrast between Southern and Western facilities represented a comparatively large difference. Beta-lactam and carbapenem resistance showed particularly pronounced regional variation, whereas tetracycline resistance remained highly abundant across all four regions and displayed a smaller geographic effect (Teshome et al., 2020). Colistin resistance showed little evidence of systematic regional differentiation, reflecting the low overall detection frequency of mcr-1. These findings were consistent with earlier clinical and environmental AMR studies demonstrating that resistance is not distributed uniformly across the United States. Previous surveillance research has identified substantial state- and regional-level differences in antimicrobial prescribing, healthcare-associated resistance, population characteristics, agricultural activity, sanitation infrastructure, and exposure to antimicrobial compounds. Wastewater studies conducted across geographically separated communities have likewise reported spatial heterogeneity in resistance-gene abundance and resistome composition. The present results extended this pattern by demonstrating that regional differences remained detectable

when multiple clinically relevant ARGs were combined into a standardized wastewater resistance profile (Voigt et al., 2020).

Figure 12: Integrated Drivers of Wastewater Resistance



The significant positive spatial autocorrelation of the composite ARG burden further indicated that facilities with similar resistance profiles demonstrated modest geographic clustering rather than a completely random distribution. Earlier spatial studies of AMR have similarly identified geographic clustering associated with healthcare utilization, antibiotic consumption, population movement, socioeconomic characteristics, and livestock production. The modest magnitude of spatial autocorrelation observed in this study was also important because it demonstrated substantial heterogeneity among wastewater treatment plants located within the same broad region. Geographic location therefore captured only part of the observed resistance variation. The reduction in regional effect after adjustment for antibiotic prescribing, healthcare characteristics, livestock density, and treatment configuration further suggested that broad geographic differences overlapped with measurable One Health conditions (Alexander et al., 2020). This pattern corresponded with previous ecological studies showing that regional AMR variation frequently reflects combinations of antimicrobial exposure, healthcare intensity, demographic composition, agricultural activity, and environmental characteristics rather than geography functioning as an isolated determinant. The persistence of a Southern regional effect after multivariable adjustment nevertheless indicated that measured covariates did not account for all geographic heterogeneity. Differences in unmeasured prescribing practices, sewer infrastructure, industrial inputs, climate, microbial ecology, population mobility, and local healthcare characteristics could have contributed to the remaining variation. Consequently, the geographic findings aligned with earlier research characterizing AMR as a spatially structured but multifactorial phenomenon (Tesfaye et al., 2019).

Community antibiotic utilization emerged as the strongest positive predictor of composite wastewater ARG burden, maintaining a moderate standardized association after adjustment for healthcare, agricultural, demographic, geographic, and wastewater-system characteristics. The relationship was particularly pronounced for beta-lactam and tetracycline resistance, while smaller associations were identified for several other resistance classes. This finding was consistent with a substantial body of earlier epidemiological and environmental research demonstrating that antimicrobial consumption creates selective conditions favoring resistant bacterial populations and resistance determinants

(Paulshus et al., 2019). Population-level studies have repeatedly documented positive associations between antibiotic consumption and clinical resistance, while wastewater investigations have increasingly shown that community prescribing patterns can also be reflected in sewage resistomes. The present results provided additional quantitative support for this relationship by demonstrating that antibiotic utilization remained independently associated with wastewater resistance after several competing One Health factors were controlled. Earlier studies have reported associations between beta-lactam consumption and beta-lactamase genes, tetracycline exposure and **tet** determinants, macrolide use and corresponding macrolide resistance genes, and sulfonamide consumption and **sul** markers. The stronger class-specific effects observed in this study for beta-lactam and tetracycline resistance were therefore consistent with the expectation that antimicrobial selection is partly reflected in corresponding resistance mechanisms (Szekeres et al., 2017). At the same time, the findings did not indicate a simple one-to-one relationship between contemporary prescribing and individual ARG abundance. Resistance determinants may persist after antimicrobial exposure decreases because they remain within stable bacterial populations, mobile genetic elements, or linked resistance structures. Co-selection by metals, disinfectants, and unrelated antimicrobials can also maintain resistance genes without direct exposure to the corresponding drug. Earlier wastewater studies have emphasized this ecological complexity and have reported variable correlations between measured pharmaceutical residues, community antimicrobial consumption, and ARG abundance. The persistence of antibiotic utilization as the highest-ranked predictor in both conventional regression and random forest analysis was therefore notable (Santoro et al., 2015). It indicated that antimicrobial consumption explained an important component of resistance variation even within a complex environmental system. The finding also corresponded with earlier national and international studies in which antibiotic-use indicators explained geographic differences in resistance after adjustment for socioeconomic and healthcare variables. However, the multivariable model showed that antibiotic consumption alone did not account for the complete wastewater resistance profile. Healthcare intensity, livestock density, social vulnerability, treatment configuration, and geographic context retained additional explanatory contributions, supporting the interpretation of antibiotic use as a major component within a broader interconnected resistance system (Conte et al., 2017).

Healthcare intensity represented another major quantitative correlate of wastewater resistance. Healthcare-facility density was the second strongest positive predictor of the composite ARG burden, while hospital bed capacity independently contributed additional explanatory information. Gene-specific analyses showed that these healthcare variables were particularly strongly associated with carbapenem, methicillin, and vancomycin resistance. The relationship between hospital capacity and carbapenem resistance was also substantially stronger within catchments characterized by high healthcare-facility density (Fouz et al., 2020). These results were consistent with earlier studies comparing hospital wastewater with community sewage, which have repeatedly demonstrated enrichment of clinically important resistance determinants in healthcare-associated discharges. Hospital wastewater commonly contains resistant organisms originating from patients receiving antimicrobial therapy, together with unmetabolized antibiotics, pharmaceutical metabolites, disinfectants, and other chemicals capable of contributing to selective conditions. Previous research has identified elevated concentrations of blaKPC, blaNDM, blaVIM, vanA, mecA, extended-spectrum beta-lactamase genes, and mobile genetic elements in hospital wastewater relative to many community sources. Comparative metagenomic investigations have further demonstrated that hospital wastewater resistomes can be quantitatively distinguished from municipal wastewater profiles based on the composition and relative abundance of clinically prioritized resistance genes (Tängdén & Giske, 2015). The findings of this study agreed with this literature because healthcare-related predictors were more strongly associated with resistance mechanisms frequently linked to severe healthcare-associated infections than with the more environmentally widespread tetracycline resistance signal. The independent contribution of healthcare-facility density after adjustment for community antibiotic prescribing was especially informative. It indicated that healthcare-associated resistance patterns could not be explained solely by general outpatient antimicrobial consumption. Inpatient antimicrobial intensity, patient case complexity, carriage of multidrug-resistant organisms, long-term care

populations, hospital-specific antimicrobial practices, and concentrated fecal shedding may have contributed additional resistance signals. Earlier studies have also shown that hospital wastewater can introduce resistant bacteria and mobile resistance elements into municipal sewer networks, after which these signals become diluted but remain detectable at downstream wastewater treatment plants (Hernando-Amado et al., 2019). The present finding that urban catchments exhibited higher carbapenem and methicillin resistance was compatible with this mechanism because urban sewersheds frequently contain greater concentrations of hospitals and specialized healthcare facilities. Nevertheless, hospital bed capacity and facility density functioned as ecological indicators rather than direct measurements of resistant-patient discharge. Their moderate rather than exceptionally large adjusted effects were therefore consistent with a municipal wastewater system in which healthcare-derived resistance was integrated with resistance originating from the broader community.

Animal-agriculture intensity demonstrated a statistically significant but resistance-class-specific relationship with wastewater AMR. Livestock density independently predicted the composite ARG burden, but its strongest association occurred with tetracycline resistance, particularly tetW, and a smaller relationship was observed with beta-lactam resistance (Wozniak et al., 2022). In contrast, livestock density showed comparatively weak associations with carbapenem resistance markers. The relationship between agricultural intensity and tetW was substantially stronger in catchments characterized by high livestock activity than in lower-intensity areas. This pattern was consistent with earlier One Health studies documenting widespread tetracycline resistance in livestock production systems, animal manure, agricultural soils, surface waters, and wastewater influenced by agricultural runoff. Tetracyclines have historically been used extensively in both human and veterinary settings, and numerous environmental studies have identified tet genes as persistent and abundant resistance determinants across animal-associated matrices. Manure application, agricultural drainage, biosolids, soil transport, and hydrological movement can facilitate the movement of resistance determinants between animal and environmental compartments (Awasthi et al., 2022). Earlier studies have also demonstrated that livestock-associated resistance genes can occur alongside mobile genetic elements, creating opportunities for persistence and exchange within environmental bacterial communities. The stronger agricultural association for tetW in this study therefore corresponded with the resistance ecology described in previous research. The weaker association between livestock density and carbapenem resistance was equally consistent with earlier findings because carbapenems are primarily important in human clinical medicine and their resistance determinants are frequently concentrated within healthcare-associated bacterial populations. The contrast between healthcare-related carbapenem resistance and agriculture-related tetracycline resistance provided a quantitative example of the source-specific structure expected under a One Health model. Social vulnerability also showed a smaller positive independent association with overall ARG burden. Earlier ecological research has linked socioeconomic and community-level conditions with differences in healthcare accessibility, antibiotic exposure, sanitation infrastructure, population density, chronic disease burden, and environmental contamination, all of which can contribute to heterogeneous AMR patterns (Effah et al., 2020). However, the comparatively small effect identified in this study suggested that social vulnerability represented a contextual correlate rather than a dominant determinant after more direct antimicrobial, healthcare, and agricultural variables were included. Collectively, these resistance-class-specific findings corresponded with previous One Health literature showing that wastewater resistomes integrate signals from human healthcare, community antimicrobial use, animal production, environmental transport, and socioeconomic context while retaining distinguishable patterns associated with particular resistance mechanisms.

Wastewater treatment configuration was consistently associated with resistance abundance, with advanced treatment demonstrating a negative relationship with the composite ARG burden and several individual resistance classes (Tängdén & Giske, 2015). Conventional activated-sludge facilities exhibited higher standardized resistance measurements than advanced biological and membrane or other advanced treatment systems, and the difference remained statistically significant after adjustment for population, healthcare, antibiotic-use, geographic, and agricultural characteristics. The strongest treatment-related reductions were observed for tetracycline and beta-lactam resistance, while smaller

but significant differences were identified for carbapenem, methicillin, and vancomycin resistance. These results were consistent with earlier wastewater-treatment research demonstrating that treatment processes can substantially reduce absolute bacterial and ARG concentrations while producing variable effects on normalized resistance abundance. Conventional activated-sludge systems are effective at reducing organic material and microbial loads, yet activated sludge itself contains dense and diverse microbial communities in which ARGs and mobile genetic elements can remain abundant (Hernando-Amado et al., 2019). Previous studies have reported substantial influent-to-effluent reductions in absolute ARG concentrations alongside persistence or occasional relative enrichment of particular resistance determinants. Advanced biological treatment, membrane filtration, ultraviolet disinfection, ozonation, advanced oxidation, and other tertiary processes have frequently produced additional reductions, although removal efficiency varies according to gene target and operational conditions. The lower resistance burden observed among advanced-treatment facilities in this study therefore corresponded with the broader treatment literature. At the same time, the findings reinforced the importance of distinguishing absolute from normalized resistance. The strong overall correlation between the two measures indicated broad agreement, but site reclassification after 16S normalization showed that reductions or geographic differences in absolute gene concentrations were partly influenced by variation in total bacterial biomass (Wozniak et al., 2022). Previous studies have similarly cautioned that large absolute reductions during wastewater treatment do not necessarily indicate equivalent decreases in the proportion of resistant organisms or genes. The stability of the advanced-treatment coefficient in both absolute and normalized sensitivity analyses strengthened the quantitative evidence that treatment configuration was associated with resistance beyond differences in microbial concentration alone. The multidimensional clustering analysis also showed that wastewater facilities could be separated into distinct resistance profiles rather than being represented adequately by a single ARG. Earlier metagenomic research has likewise identified wastewater resistomes as complex assemblages containing multiple correlated resistance classes, bacterial hosts, and mobile genetic elements (Santajit & Indrawattana, 2016). The present treatment-related findings were therefore consistent with the established view that wastewater processing changes both the magnitude and composition of resistance signals.

The robustness analyses strengthened confidence in the principal quantitative patterns because the direction and approximate magnitude of the major associations remained stable across alternative statistical specifications. Community antibiotic utilization retained the largest positive coefficient when nondetects were treated differently, influential observations were excluded, resistance was normalized to 16S rRNA, spatial dependence was incorporated, and mixed-effects models accounted for repeated measurements (Awasthi et al., 2022). Healthcare-facility density remained the second most prominent positive predictor, livestock density retained a smaller but consistent association, and advanced treatment remained inversely related to wastewater resistance. Random forest analysis independently reproduced the high ranking of antibiotic utilization and healthcare intensity while achieving approximately 61% cross-validated explanatory performance. These convergent results corresponded with earlier methodological literature emphasizing that environmental AMR is best characterized using multiple analytical approaches because resistance measurements are influenced by non-normal distributions, spatial structure, nondetection, correlated predictors, microbial biomass, and nonlinear relationships. The moderate explanatory performance of the multivariable models was also consistent with the complexity documented in previous wastewater resistome studies (Davis et al., 2017). Approximately half to three-fifths of the observed variation could be explained by the measured One Health variables, leaving substantial residual heterogeneity associated with factors not fully represented in the analytical dataset (Effah et al., 2020). Earlier studies have identified antimicrobial residues, heavy metals, microplastics, disinfectants, mobile genetic elements, bacterial community composition, sewer residence time, temperature, rainfall, industrial discharge, human mobility, and wastewater chemistry as additional determinants capable of influencing ARG abundance. The observed clustering of blaCTX-M with blaTEM, blaKPC with blaNDM, and mecA with vanA also corresponded with earlier studies showing that resistance determinants frequently occur as structured resistome profiles rather than independent environmental measurements. Similarly, the association

between mobile resistance markers and the composite ARG burden reported in the secondary analysis agreed with research emphasizing horizontal gene transfer and mobile genetic elements as important components of wastewater resistance ecology (Fried et al., 2021). Taken together, the findings of this study were broadly aligned with previous clinical, environmental, wastewater, agricultural, and One Health research while providing an integrated quantitative representation of these relationships within a common analytical framework. Human antibiotic consumption and healthcare intensity showed the strongest overall associations, agricultural activity displayed resistance-class-specific relationships, treatment configuration was consistently associated with lower resistance measurements, and geographic heterogeneity persisted after adjustment. This integrated pattern supported interpretation of wastewater antimicrobial resistance as a measurable product of interconnected human, healthcare, animal, environmental, and engineered wastewater-system characteristics rather than as an outcome attributable to any single exposure domain (Cesari et al., 2016).

CONCLUSION

This quantitative study established that wastewater represented a measurable and heterogeneous environmental reservoir of antimicrobial resistance across the United States and demonstrated the value of integrating wastewater surveillance within a One Health framework. Analysis of clinically relevant antimicrobial resistance genes showed substantial variation in their occurrence, concentration, normalized abundance, geographic distribution, and relationships with human, healthcare, agricultural, and wastewater-system characteristics. Tetracycline and beta-lactam resistance determinants, particularly tetW, blaTEM, and blaCTX-M, represented the most prevalent and abundant resistance signals, while mecA was also widely distributed. Carbapenem resistance determinants, including blaKPC, blaNDM, blaVIM, and blaOXA-48-like, occurred at lower and more heterogeneous concentrations, while mcr-1 represented the least frequently detected marker. Geographic analysis identified significant variation among U.S. regions, with the South exhibiting the highest composite ARG burden and the West exhibiting comparatively lower resistance levels. Spatial clustering further demonstrated that resistance patterns were geographically structured rather than randomly distributed. Multivariable analysis identified community antibiotic utilization as the strongest positive predictor of overall wastewater ARG burden, followed by healthcare-facility density and hospital bed capacity, demonstrating measurable connections between human antimicrobial exposure, healthcare activity, and environmental resistance signals. Livestock density contributed independently to resistance variation and showed its strongest relationship with tetracycline resistance, illustrating the relevance of animal-associated antimicrobial pressures within the broader resistance landscape. Advanced wastewater treatment was consistently associated with lower ARG burden, while population density and wastewater flow showed weaker independent effects after adjustment for other One Health characteristics. Comparisons between absolute and 16S rRNA-normalized ARG measurements demonstrated strong overall concordance while confirming that the two approaches captured distinct dimensions of resistance burden. Gene-specific analyses, geographic subgroup comparisons, multidimensional clustering, spatial assessment, mixed-effects modeling, and random forest analysis produced broadly consistent patterns, strengthening the statistical robustness of the findings. Overall, this study characterized wastewater antimicrobial resistance as a complex quantitative outcome shaped by interconnected human antibiotic consumption, healthcare activity, animal agriculture, environmental conditions, geographic context, and wastewater treatment characteristics. The findings demonstrated that wastewater-based surveillance can provide population-level measurements of clinically relevant resistance determinants while capturing resistance circulating beyond conventional clinical surveillance systems, thereby establishing wastewater as an important environmental component for quantitatively understanding antimicrobial resistance through an integrated One Health perspective.

RECOMMENDATIONS

Based on the quantitative findings of this study, wastewater-based antimicrobial resistance surveillance should be strengthened as a standardized component of the United States One Health surveillance framework and coordinated with existing clinical, healthcare, veterinary, agricultural, and environmental monitoring systems. National and state surveillance programs should prioritize routine measurement of clinically relevant resistance determinants, including blaCTX-M, blaTEM, blaCMY,

blaKPC, blaNDM, blaVIM, blaOXA-48-like, mecA, tetW, mcr-1, and vanA, because the findings demonstrated substantial differences in their prevalence, abundance, and geographic distribution. Sampling protocols should apply consistent wastewater collection, preservation, DNA extraction, quality-control, and quantitative molecular procedures to improve comparability across treatment plants and geographic regions. Both absolute ARG concentrations and bacterial-normalized abundance should be reported because these measures provided related but distinct information regarding total resistance burden and proportional resistance within wastewater microbial communities. Greater surveillance intensity should be considered in areas characterized by high community antibiotic consumption, substantial healthcare-facility density, large hospital capacity, intensive livestock production, or persistently elevated wastewater resistance measurements. Community and hospital antimicrobial stewardship programs should incorporate geographically resolved antibiotic-utilization data when evaluating resistance patterns, particularly for beta-lactam, tetracycline, carbapenem, methicillin, and vancomycin resistance. Healthcare facilities should strengthen management of wastewater containing clinically important resistant organisms and antimicrobial residues, especially in catchments where carbapenemase and other critical resistance determinants occur at elevated concentrations. Agricultural monitoring should similarly incorporate antimicrobial-use indicators, livestock density, manure management, runoff pathways, and resistance measurements because animal-production intensity was associated with selected resistance classes. Wastewater utilities should evaluate the performance of advanced biological treatment, membrane-based processes, disinfection, filtration, and other treatment technologies for reducing both absolute and normalized ARG abundance, while treatment effectiveness should be assessed using standardized resistance endpoints. Surveillance databases should integrate wastewater ARG measurements with antibiotic prescribing, hospital activity, animal agriculture, demographic characteristics, treatment technology, and environmental variables to support multivariable and spatial analyses. Repeated longitudinal sampling should complement cross-sectional monitoring to distinguish persistent resistance patterns from temporary fluctuations and identify changes in resistance signals over time. Greater coordination among public-health agencies, wastewater utilities, healthcare institutions, environmental authorities, agricultural organizations, and research laboratories would support harmonized data collection and interpretation. Collectively, these recommendations would strengthen the capacity of wastewater surveillance to identify geographic resistance patterns, characterize interconnected One Health determinants, evaluate resistance-control measures, and provide quantitative evidence supporting coordinated antimicrobial resistance management across human, animal, healthcare, and environmental systems.

LIMITATIONS

This study was subject to several limitations that should be considered when interpreting the quantitative relationships between wastewater antimicrobial resistance and the evaluated One Health factors. First, the observational and predominantly cross-sectional study design supported identification of statistical associations but did not establish causal relationships between antibiotic consumption, healthcare activity, livestock intensity, wastewater treatment characteristics, and ARG abundance. Although the analytical sample incorporated wastewater treatment plants from multiple U.S. states and geographic regions, the participating facilities did not represent every treatment plant or sewershed in the United States, and differences in regional representation, population served, urbanization, and infrastructure may have affected national generalizability. Wastewater samples represented aggregated community-level biological material and therefore could not identify individual contributors, distinguish colonization from active infection, or directly connect specific resistance determinants with individual antibiotic exposures. Temporal variation represented another limitation because ARG concentrations can change according to antimicrobial prescribing patterns, seasonal infections, precipitation, wastewater flow, population mobility, and treatment operations. Repeated measurements reduced short-term variability but did not fully characterize long-term temporal patterns. Molecular quantification of selected ARGs also provided targeted rather than complete characterization of the wastewater resistome. The selected genes represented clinically important resistance mechanisms, yet additional resistance determinants, variants, mobile genetic elements, and bacterial hosts were not comprehensively measured. Detection of an ARG did not

establish whether the gene originated from viable bacteria, extracellular DNA, pathogenic organisms, or nonpathogenic environmental microorganisms, nor did detection independently demonstrate horizontal transfer capability or phenotypic antimicrobial resistance. Normalization to 16S rRNA improved comparability across samples but introduced uncertainty associated with variation in bacterial 16S rRNA gene copy numbers among taxa. Measurements below analytical detection thresholds, particularly for **mcr-1** and less frequently detected carbapenemase genes, also increased uncertainty in gene-specific estimates. Secondary One Health variables were obtained at aggregated geographic levels, creating potential exposure misclassification when administrative boundaries did not correspond precisely with wastewater sewersheds. Antibiotic prescribing data may not have captured inpatient administration, nonprescription exposure, veterinary antimicrobial use, adherence, or movement of individuals across catchment boundaries. Healthcare-facility density, hospital bed capacity, and livestock density functioned as proxy measures and did not directly quantify resistant-organism discharge into wastewater. Residual confounding from antimicrobial residues, heavy metals, microplastics, industrial discharges, sewer characteristics, climate, microbial community composition, and mobile genetic elements also remained possible. Consequently, the reported statistical relationships should be interpreted as population-level associations within the sampled wastewater systems rather than direct evidence of individual-level exposure, transmission, or causation.

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