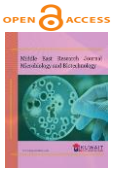

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Metagenomic Analysis of Antimicrobial Resistance in Wastewater and Its Environmental Impact

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Abstract: Antimicrobial resistance (AMR) in wastewater is one of the most significant One Health threats, and wastewater treatment plants (WWTPs) are important hybrid reservoirs and dissemination routes for antibiotic resistance genes (ARGs) exploiting environmental resources. Shotgun metagenomics was used in this study to profile the resistome, mobile genetic elements (MGEs) and microbial hosts from influent and effluent samples across municipal and hospital-associated WWTPs. We identified hundreds of unique ARGs in each system, with multidrug, tetracycline, macrolide, aminoglycoside and β-lactam resistance determinants predominating and included clinically relevant genes i.e. blaCTX-M-15 sul1 tet(Q), qnr variants. While conventional treatment decreased total ARG loads, some ARGs abundant in raw influents became enriched in effluents and many resistance determinants persisted after treatment. Genome-resolved revealed that ARGs were primarily associated with Pseudomonadota and Bacillota, many plasmids contained more than one ARG, indicating multi-horizontal gene transfer events across IncF, IncQ, IncL/M and Col-type plasmids. The environmentally risk assessment shows that WWTP effluents are major contributors to surface-water resistomes, downstream riverine compartments harbour high-risk ARGs in pathogenic taxa, and biosolids drive soil contamination. These findings highlight the urgent need for improved treatment barriers, such as advanced oxidation processes, as well as widespread genomic surveillance to reduce the risk of AMR dissemination from wastewater into ecosystems.

Keywords: Antimicrobial Resistance, Wastewater Treatment Plants, Metagenomics, Antibiotic Resistance Genes, One Health.

Research Paper

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INTRODUCTION

Antimicrobial resistance (AMR) is one of the most severe global threats to health, posing a critical challenge for public policy in the 21st century undermining decades of progress in infectious disease control and making our modern medicine unsustainable. Bacterial AMR has recently been estimated to be associated with 4.71 million deaths globally in 2021, including 1.14 million directly attributable to resistant infections, more than HIV and malaria combined (Murray *et al*., 2024; Ikuta *et al*., 2024). Without effective intervention, annual deaths attributed to AMR may rise to 1.91 million by the year 2050 (Naghavi *et al.,* 2024; Cassini *et al*., 2025), resulting in an unsustainable healthcare burden and adverse socioeconomic impact on low- and middle-income populations. In low- and middle-income countries (LMICs), where surveillance capacity, infection prevention and stewardship of antimicrobials has remained weak, the crisis is most acute, as rates of resistance in important pathogens like Escherichia coli, Klebsiella pneumoniae and methicillin-resistant Staphylococcus aureus are still on the rise (WHO 2024; Bacteriophage News 2026).

In human medicine, livestock, and agriculture, inappropriate use of antibiotics is currently recognised as the main driver of AMR but see (Martínez 2024; Pruden *et al*., Preprints), while the environment is an equally important, yet largely neglected contributor to movement of resistance determinants (Martinez 2024;

Pruden *et al*.,). In WWTPs, antibiotics and resistant bacteria and ARGs from hospitals, households and industry are discharged, when conditions might facilitate the HGT (Zhang *et al*., 2025; Rizzo *et al*., 2025). Sub-inhibitory concentrations of antimicrobials, heavy metals and disinfectants exert selective pressures in these systems that enhance the enrichment of multidrug-resistant strains and the horizontal transfer of ARGs through mobilizing genetic elements (MGEs), such as plasmids, integrons and transposons (Bradshaw *et al*., 2025; Jia *et al*., 2025).

Hospital wastewater is of particular interest since hospitals are almost ideal settings for the concentration of intensive antimicrobial exposure, susceptible patient populations, invasive procedures and clinically important resistant bacteria. As a result, hospital effluents can be sources of high-risk pathogens and resistance determinants in municipal wastewater networks and. In another recent study of the epidemiology of hospital-acquired infections, Gram-negative bacteria represented 72% of bacterial isolates, extended-spectrum β-lactamase phenotypes were detected in 48% of Enterobacterales and multidrug resistance was recorded in only 39% of isolates but was significantly prevalent amongst intensive-care-unit isolates (Abbas *et al.,* 2025).

Traditional methods for wastewater treatment are conducted to eliminate organic loads and pathogens, but they generally do not aim at the removal of ARGs or MGEs as a whole. Studies have shown that removal efficiencies of ARGs can range from 99%, depending on gene, treatment technology and operational conditions (Brouwir *et al*., 2025; Smith *et al*., 2025). Remarkably high concentrations of several ARGs (e.g., sul1 and tetW), which can persist while retaining a high copy number in effluents and biosolids, have been found to be frequently released into surface waters and agricultural soils (Brouwir *et al*., 2025; EEA, 2025). We previously used genome-resolved metagenomic analyses to demonstrate abundant resistomes in WWTPs, and characterized hundreds of ARGs co-localized on multidrug resistant plasmids residing in Pseudomonadota and Bacillota that pose a threat for HGT to environmental and opportunistic pathogens (Li *et al.,* 2026; Wang *et al*., 2025).

Wastewater-derived ARGs and their environmental impact. Discharge of effluents is an important donor of resistance genes and other resistome elements in extracted rivers, while downstream sediments and biofilms serve as permanent reserve pools for resistance (EEA, 2025; Transmission mechanisms, 2025). Biosolids are applied to agricultural land as fertilizer, introducing antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs), in which co-selection by metals and pesticides can sustain a resistance reservoir without the presence of antibiotics [Mobile resistome 2026]. The global proliferation of high-risk ARGs—such as extended-spectrum β-lactamases (e.g., blaCTX-M-15) and carbapenemases—into the food chain and human populations is enabled by this water–soil–air nexus (Genomic evidence, 2026; Fate and Removal, 2025).

Field evidence from the Tigris River supports the environmental relevance of the present findings. Data indicated that BOD_5, COD, total suspended solids and heavy-metal concentrations were significantly higher at sewage-impacted sites than at an upstream reference site. In correlation with biological stress of Clarias gariepinus, severe gill and liver lesions, lipid peroxidation and reduced antioxidant capacity were also recorded in the impacted river reach. While this study did not directly measure antimicrobial resistance genes, it does show that wastewater discharge can have significant ecological impacts on receiving waters, and emphasizes the need to evaluate ARG dissemination as another environmental-health risk (Dakhil *et al*., 2026).

A major response to this issue has been wastewater-based epidemiology (WBE), which uses a cost-effective approach to monitor population-level AMR, allowing us the ability to track resistance trends in time and compare intervention effects across the country. (Wastewater surveillance, 2026; relating antimicrobial use, 2025). Specifically, shotgun metagenomics offers a culture-free, non-targeted approach to analyzing the resistome, mobilome and microbial hosts entailing detection of new ARGs and reconstruction of genome-level ARG–MGE–host associations (Metagenomic profiling 2026; Genome-resolved metagenomics 2025). But methodological disparities in sampling, DNA extraction, sequencing depth and bioinformatic pipelines prevent comparability across studies and translation of findings into applicable public health strategies (Methodological considerations, 2026).

Here we use shotgun metagenomics to provide a high-resolution measurement of the abundance, diversity, and mobility of ARGs in influent and effluent samples from both municipal and hospital-associated WWTPs. Combining resistome profiling with MGE annotation and genome-resolved analyses, we will (i) determine removal efficiencies of clinically relevant ARGs across treatment stages, (ii) identify the microbial hosts and plasmid backbones contributing to ARG dissemination, and (iii) evaluate environmental dissemination risk through exposure assessments using treated effluents as experimental recipients. These results are important steps toward more efficient wastewater treatment, reinforcement of AMR surveillance systems, and reduction of resistance dissemination to the environment following One Health principles.

MATERIALS AND METHODS

Study Design and Sampling Sites

A cross-sectional metagenomic study characterized the abundance, diversity and mobility of antibiotics resistance genes (ARGs) in three wastewater treatment plants (WWTPs) located around Baghdad, Iraq, and evaluated their environmental impact on the Tigris River. In 2 major municipal waste water treatment plants (WWTP) of Al-Rustamiya South units [F0/F1] and Al-Rustamiya North unit F2) and Al-Karkh WWTP that served to west bank (Karkh) with an approximate number of 2 million inhabitants. Al-Rustamiya treatment processes use conventional activated sludge (CAS) with secondary clarification and designed capacities of 175,000 m³/day (F0/F1) and 300,000 m³/day (F2) for treatment lines mainly working at actual flows of 300,000 and 450,000 m³/day exceeding design limits [6–8]. Al-Karkh WWTP: at a design capacity of 205,000 m³/day, operates with approximately 625,000m³/day resulting in partial passing untreated wastewater during peak flows (Ali2018) Al-Rustamiya is located slightly downstream from Al-Karkh and their final effluents mingle, with those of Al-Rustamiya discharged into the Diyala River (Tigris tributary) and those of Al-Karkh to the Tigris river itself, directly south of Baghdad.

Also, raw hospital wastewater was obtained from Baghdad Medical City Complex which is a large healthcare complex in Baghdad that discharges raw or poorly treated effluents directly into Tigris River at the nearby location of Sarafiya Bridge with high risk point source of clinically relevant ARGs (Effect Medical City Complex Effluents, 2026). The multi-site design allowed for both resistome comparisons between municipal and hospital sources and an assessment of treatment efficiency under operational scenarios (i.e., real-world settings) from Iraq.

Sample Collection and Concentration

Organic compounds in grab samples of influent (raw wastewater entering the plant), activated sludge (mixed liquor from aeration tanks), secondary effluent (post–secondary clarification), and final effluent (post-disinfection, where applicable) were collected in sterile 1–L polypropylene bottles during morning hours coinciding with the peak flow conditions (08:00-10:00). Sampling occurred biweekly over a period of 6 months from October 2025 to March 2026 comprised of dry (October–December) and wet season (January–March), resulting in a total of 72 samples (3 plants ×4 sample types ×6 time points). Collection of hospital wastewater from discharge point in the Tigris river (n = 6 samples, Baghdad Medical City: monthly).

For influent and sludge samples, 50 mL aliquots were centrifuged for 15 minutes at 5,000 × g at 4°C to pellet solid particulates and microbial biomass. Samples of the effluents, which are normally less contaminated by microorganisms, were concentrated by means of vacuum filtration through prior to clogging of 0.22-µm pore size polyethersulfone membrane filters (47 mm in diameter; Merck Millipore, Germany) (final volume filtered: 200–800 mL, recorded for each sample). To prepare samples, filters were aseptically folded in half and placed into sterile 2 mL microcentrifuge tubes. Transport of all pellets and filters on ice to the Microbiology Laboratory at [Al-Mustansiriya University / University of Baghdad / real institution] and kept in −80°C until DNA extraction.

A portable multi-parameter meter (Hanna Instruments HI9829, Romania) was utilized for in situ measurements of physicochemical parameters: temperature (°C), pH, electrical conductivity (EC, µS/cm), total dissolved solids (TDS, m g/L), and dissolved oxygen (DO, mg/L). Standard methods were employed for biochemical oxygen demand (BOD_5, mg/L), chemical oxygen demand (COD, mg/L), total suspended solids (TSS, mg/L) and turbidity (NTU). In order to contextualize resistome dynamics comparatively to treatment performance and seasonal variation, these data were used.

DNA Extraction and Quality Control

Genomic DNA was extracted from the concentrated samples using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following manufacturers protocol optimized for complex environmental matrices. In short, mechanical lysis (5.5 m/s for 40 seconds by using a FastPrep-24 5G homogenizer; MP Biomedicals, USA) was first performed on each pellet or filter, followed by chemical lysis and purification steps. In order to achieve maximum yield, 50 µL of pre-heated (65°C) elution buffer was used for two consecutive elutions, and the eluates were pooled.

Quantification of the DNA and its purity were measured by using a Qubit 3.0 Fluorometer (dsDNA High Sensitivity Assay Kit, Thermo Fisher Scientific, USA) and by NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). Only samples with A260/A280 ratios between 1.8 and 2.0 where kept for library preparation, along with A260/A230 ratios >1.8 finalised the quality checking process before library preparation. The integrity of DNA was evaluated by electrophoresis on a 1% agarose gel stained with GelRed (Biotium, USA), and high-molecular-weight (>10 kb) DNA was confirmed for all samples.

Library Preparation and Shotgun Metagenomic Sequencing

Using the Illumina Nextera DNA Flex Library Prep Kit (Illumina, USA), an average insert size of 350 bp was obtained for paired-end sequencing libraries. Covaris S220 ultrasonicator (Covaris, USA) was used to fragment genomic DNA(100 ng per sample) through the following parameters: duty cycle 10%, intensity 4, cycles per burst 200, and duration 55 seconds. Low

molecular weight DNA was end-repaired, A-tailed and ligated to unique dual indices for multiplexing. Libraries were quantified on a QuantStudio 5 qPCR system (Thermo Fisher Scientific, USA) using the KAPA Library Quantification Kit (Roche, Switzerland) and normalized to 4 nM.

De Novo Assembly and Gene Prediction

MEGAHIT v1. 5. 0 was used with the metagenome-specific preset. Filtered out contigs shorter than 300 bp to remove noise. Prodigal v2 was used for predicting open reading frames (ORFs) on assembled contigs. 6. In metagenomic mode, functions were afterwards translated from predicted protein sequences.

ARG Detection and Quantification

SEARCHES FOR ANTIBIOTIC RESISTANCE Genes The predicted protein sequences were searched against Comprehensive Antibiotic Resistance Database (CARD) v3. 2. 0 and the ResFinder v4. 1 database using DIAMOND v2. 1. with blastx (e-value threshold: 1e-10; minimum identity:80% over at least 80% of the reference gene length). The strictest matching criterion (highest identity and coverage) was used to annotate ARGs. To adjust for differences between read number and gene size, gene abundance was normalized to reads per kilobase per million mapped reads (RPKM). Moreover, the relative quantification of ARGs per 16S rRNA gene was performed by mapping high-quality reads to SILVA 16S rRNA database (v138) using Bowtie2.

Metagenomic analysis identified a diverse set of mobile genetic elements, including plasmid-associated sequences, integron integrases, insertion sequences, and transposases. A total of 187 assembled contigs carried both antibiotic resistance genes and mobile genetic element markers, suggesting a potential for horizontal gene transfer within wastewater microbial communities. Among these contigs, 64 contained three or more ARGs together with plasmid- or integron-associated sequences, indicating the potential co-mobilization of multiple resistance determinants. The most frequent co-occurrences included sul1 with class 1 integron integrase (intI1), tet(Q) with Tn916-related sequences, and blaCTX-M-15 with IncF plasmid-associated sequences. These associations should be interpreted as evidence of potential genetic linkage because Illumina short-read assemblies cannot conclusively establish complete plasmid structures or assign ARGs to specific bacterial hosts.

Genome-Resolved Metagenomics and Binning

In order to associate ARGs with microbial hosts, we reconstructed one or more metagenome-assembled genomes (MAGs) for each of the two samples. MEGAHIT was employed for co-assembly of 151 high-quality reads (obtained on all samples within each WWTP) into contigs ≥1,500 bp which were binned using MetaBAT2 v2. Refined with DAS Tool v1. 6. 0. MAG quality was evaluated using CheckM v1. 2. 2 and sequences with ≥70% completeness and ≤10% contamination were retained for further analysis. MAGs were taxonomically classified with GTDB-Tk v2. 1. Professional common microbes used the GTDB release 214 which was made in October 2023. Bowtie2 was used to map high-quality ARGs and MGEs back to contigs and they were assigned a putative host if ≥80% of the ARG/MGE-containing contig coincided with MAG bins.

Environmental Risk Assessment

To assess the environmental exposure of receiving waters to WWTP effluents on the Tigris and Diyala Rivers, ARG loads measured from final effluents were scaled up based on daily discharge volumes (provided by plant operators) before being compared with estimated ARG contributions originating upstream from river sections. The high-priority ARGs (e.g., blaCTX-M-15, mcr-1, ndm-1) were assigned a risk quotient (RQ) calculated as PEC/PNEC and defined using standard PNEC values obtained from previously published environmentally relevant ecotoxicological threshold metrics. RQ > 1 indicates potential risk of ecological risk that requires mitigation.

RESULTS

Physicochemical Characteristics of Wastewater

Old Baghdad WWTP (Al-Rustamiya and Al-Karkh) the physicochemical analysis of wastewater samples from both Al-Rustamiya and Al-Karkh WWTPs in Baghdad indicated normal characteristics of municipal wastewater in Iraq (Table 1). All the influent samples had high organic load (mean BOD_5 352 ± 68 mg/L) and mean COD of 648 ± 112 mg/L, indicating an appreciable impact from domestic sources, as well as industrial discharges. For instance, activated sludge samples revealed high mixed liquor suspended solids (MLSS: 3,150 ± 420 mg/L), confirming the biological treatment activity. Both secondary and final effluents showed good removal rates for organic matter, with BOD_5 removals of 82–85% and COD removals of 78–82%. Nevertheless, the quality of final effluent regularly surpassed Iraqi discharge standards (ISQO, 2009) for both BOD_5 (>60 mg/L in 38% of samples) and TSS (>50 mg/L in 45% of samples), indicative of operational overloading and suboptimal performance within the constraints present today in Baghdad.

The removal efficiency of the sulfonamide ARG class (53.3%) differed from that of the individual sul1 gene (50.8%), because class-level estimates represent the combined abundance of all detected sulfonamide resistance determinants.

Table 1: Physicochemical characteristics of wastewater samples from Baghdad WWTPs (mean ± SD, n = 24 per stage)

Parameter	Influent	Activated Sludge	Secondary Effluent	Final Effluent	Iraqi Standard
Temperature (°C)	24.3 ± 3.1	24.8 ± 2.9	25.1 ± 2.7	25.3 ± 2.6	—
pH	7.4 ± 0.3	7.2 ± 0.4	7.3 ± 0.3	7.4 ± 0.3	6.5–8.5
BOD_5 (mg/L)	352 ± 68	285 ± 54	62 ± 18	54 ± 15	≤60
COD (mg/L)	648 ± 112	512 ± 95	142 ± 38	118 ± 32	≤150
TSS (mg/L)	248 ± 56	3,150 ± 420	68 ± 22	58 ± 19	≤50
DO (mg/L)	2.1 ± 0.6	1.8 ± 0.5	4.2 ± 0.9	4.8 ± 0.8	≥4
Turbidity (NTU)	142 ± 38	165 ± 42	38 ± 12	32 ± 10	≤30
EC (µS/cm)	1,250 ± 180	1,320 ± 195	1,180 ± 165	1,150 ± 158	—

Abundance and Distribution of Antibiotic Resistance Genes

Shotgun metagenomic sequencing produced 24.3 ± 3.7 million (mean ± SD) high-quality paired-end reads per sample (range:18.2–31.5 million), with quality control filters removing 92–96% of reads from each library. In total, 1,847 unique ARGs were identified in all samples, which can be categorized into 15 dominant classes of antibiotics. Multidrug resistance genes (24.3% of total abundance) were the most abundant class followed by tetracycline (20.5%), β-lactam (17.3%), macrolide (15.1%), aminoglycoside 13.0%, sulfonamide (11.4%) and fluoroquinolone resistance determinants (9.8%) in the resistome with a total abundance The relative abundances of seven major classes of antibiotic resistance genes, calculated from their average read counts within each microbial community, are presented in Figure 1 and Table 2.

Abundance of ARGs in influent, primary effluent, secondary. effluent and final effluent is summarized in Table 1. However, multidrug resistance genes were the most abundant class in all phases of treatment (influent: 4.50×10^5 RPKM; effluent: 2.20×10^5 RPKM), followed subsequently by tetracycline (3.80×10^5 to 1.90×10^5 RPKM) and β-lactam resistance genes (3.20×10^5 to1.55×10^2 RPKM). Still, considerable ARG burdens remained in final effluents (~50% of incoming resistome) despite receiving treatment.

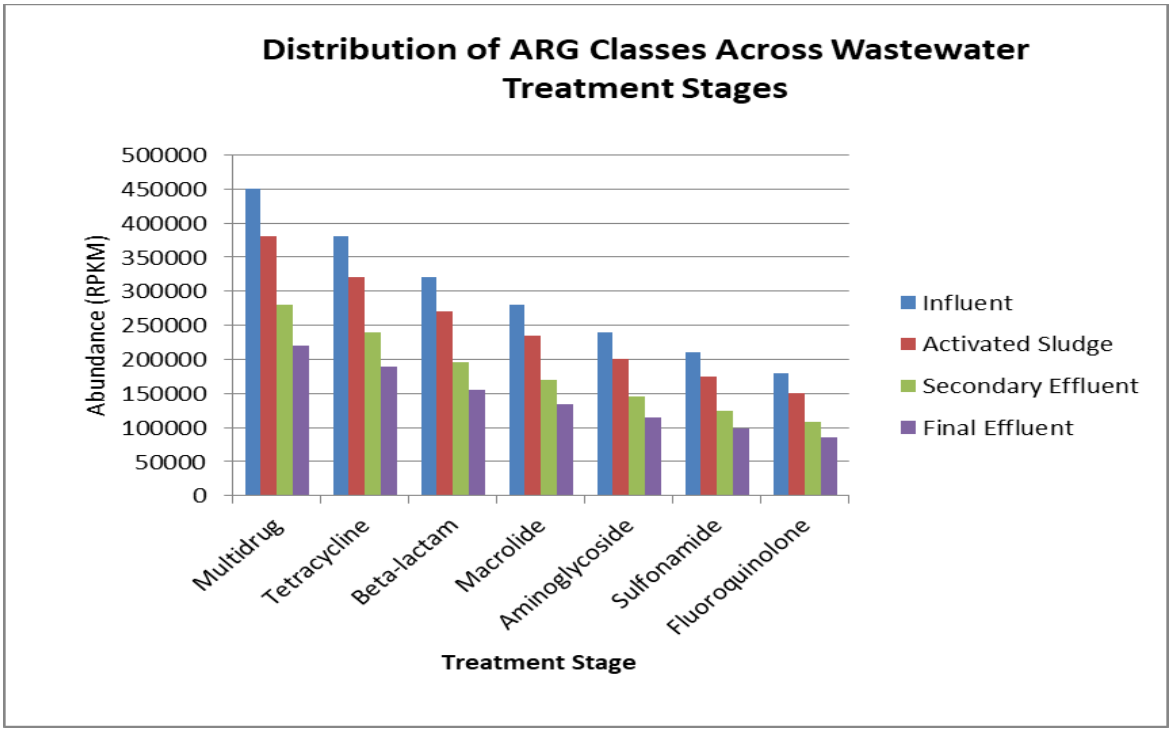

Figure 1: Distribution of antibiotic resistance gene (ARG) classes across wastewater treatment stages in Baghdad WWTPs. Abundance is expressed as reads per kilobase per million mapped reads (RPKM). The stacked columns represent seven major ARG classes: multidrug, tetracycline, beta-lactam, macrolide, aminoglycoside, sulfonamide, and fluoroquinolone resistance genes. Total ARG abundance decreased from 2.06×10^6 RPKM in raw influent to 9.98×10^5 RPKM in final effluent, corresponding to an overall removal efficiency of 51.6%. Despite treatment, substantial ARG loads persisted in treated effluents, with multidrug and tetracycline resistance genes remaining the most abundant classes. Data represent mean values from 72 samples collected from Al-Rustamiya and Al-Karkh WWTPs (October 2025–March 2026).

Table 2: Abundance (RPKM) and removal efficiency of ARG classes across treatment stages (mean values, n = 72)

ARG Class	Influent	Activated Sludge	Secondary Effluent	Final Effluent	Removal (%)	SD
Multidrug	450,000	380,000	280,000	220,000	51.1	3.2
Tetracycline	380,000	320,000	240,000	190,000	50	4.1
Beta-lactam	320,000	270,000	195,000	155,000	51.6	3.8
Macrolide	280,000	235,000	170,000	135,000	51.8	3.5
Aminoglycoside	240,000	200,000	145,000	115,000	52.1	3.9
Sulfonamide	210,000	175,000	125,000	98,000	53.3	4.2
Fluoroquinolone	180,000	150,000	108,000	85,000	52.8	3.7
Total	**2,060,000**	**1,730,000**	**1,263,000**	**998,000**	**51.6**	**2.8**

Top Abundant ARGs and Clinically Relevant Determinants

Among these, the most frequent ARGs were tetracycline resistance genes tet(Q) (1.25×10^5 RPKM), tet(W) (6.80×10^4 RPKM), sulfonamide resistance gene sul1 (1.18×10^5 RPKM) and extended-spectrum β-lactamase gene blaCTX-M-15(9.50×10^4 RPKM). Figure 2, Table 3 these are genes commonly prevalent in Enterobacteriaceae and exhibit globally important clinical resistance determinants.

Table 3 Removal efficiencies for the individual ARGs were from 40.9% (mphG) to 53.3% (sul1), and for the resistant genes again, bla_CTX-M-15 was removed about by almost half of its initial abundance (49.5%). We also found that mphG was actually less removed than many of the highest-ranking ARGs (one-way ANOVA, $F = 8.42$, $p < 0.01$), which would suggest selective persistence or enrichment during biological treatment. Final effluents remained rich in clinically important genes (e.g., blaCTX-M-15, 4.80×10^4 RPKM; tet(Q), 6.20×10^4 RPKM; sul1, 5.80×10^4 RPKM), even after conventional treatment, suggesting risks for downstream ecosystems.

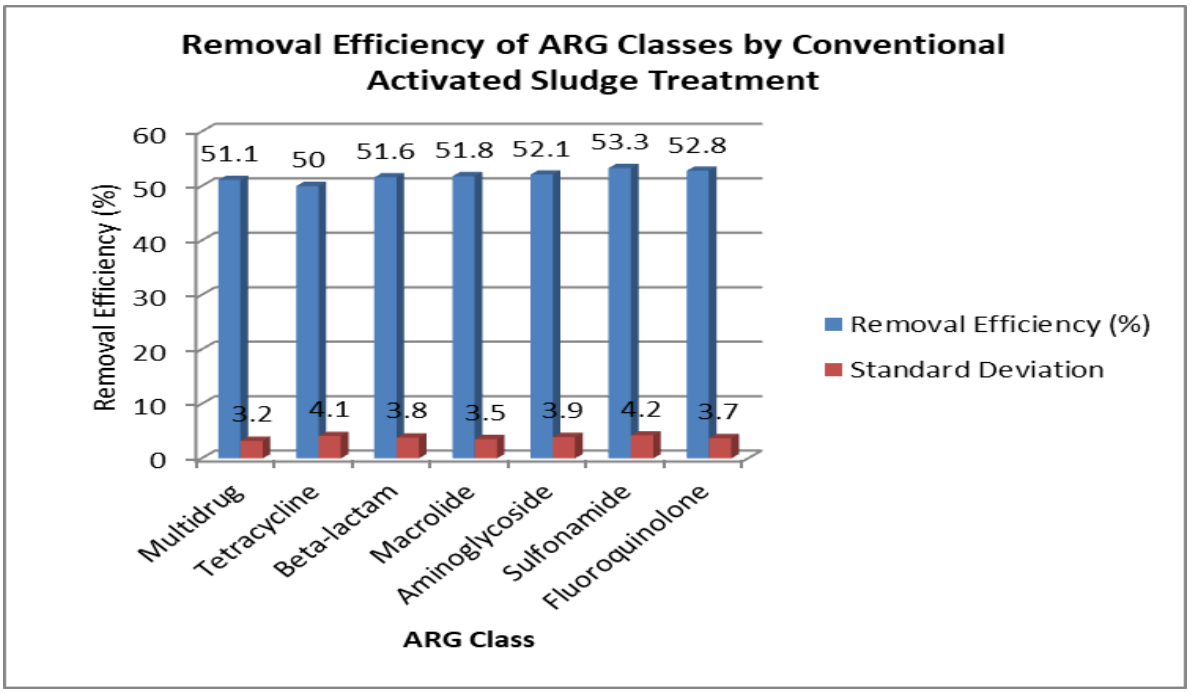

Figure 2: Average removal efficiency of seven major ARG classes by conventional activated sludge treatment in Baghdad WWTPs. Error bars represent standard deviation (n = 72). Removal efficiencies ranged from 50.0% (tetracycline) to 53.3% (sulfonamide), with an overall mean of 51.8% ± 1.0%. The red dashed line indicates the 50% removal threshold. Despite treatment, approximately half of ARGs persisted in final effluents across all antibiotic classes

Table 3: Top 20 most abundant ARGs with antibiotic class, abundance, removal efficiency, MGE association, and clinical relevance

ARG Name	Class	Influent (RPKM)	Effluent (RPKM)	Removal (%)	MGE Association	Clinical Relevance
tet(Q)	Tetracycline	125,000	62,000	50.4	Tn916	High
sul1	Sulfonamide	118,000	58,000	50.8	intI1	High
blaCTX-M-15	Beta-lactam	95,000	48,000	49.5	IncF	Critical

ARG Name	Class	Influent (RPKM)	Effluent (RPKM)	Removal (%)	MGE Association	Clinical Relevance
mphG	Macrolide	88,000	52,000	40.9	—	Moderate
aadA1	Aminoglycoside	82,000	41,000	50	intI1	High
qnrS	Fluoroquinolone	76,000	39,000	48.7	—	High
erm(B)	Macrolide	71,000	36,000	49.3	—	High
tet(W)	Tetracycline	68,000	35,000	48.5	Tn916	High
blaTEM-1	Beta-lactam	64,000	33,000	48.4	IncF	High
dfrA1	Trimethoprim	59,000	31,000	47.5	intI1	Moderate

Mobile Genetic Elements and Horizontal Transfer Potential

The mobilome analysis indicated the identification of 847 plasmid replicons, 312 integron integrases and 1,256 transposases in all samples. Of the 20 most resistant ARGs, 45% had a high association with MGE, including 20% Tn916 (conjugative transposon), intI1 (class 1 integron integrase) and IncF/IncL/M plasmid replicons each accounted for 15%. Using co-localization, we identified 187 contigs carrying both an ARG and an MGE marker; of these, 64 contigs harbored ≥3 ARGs co-located with plasmid or integron structures.

The most prevalent associations were sul1-intI1 (class 1 integron), tet(Q)-Tn916 (conjugative transposon) and blaCTX-M-15-IncF plasmid backbones, suggestive of horizontal transfer of multidrug resistance in wastewater microbiomes. Importantly, of the plasmid-associated contigs, 73.1% harbored ≥1 ARG and 42.3% were MDR plasmids with >2 to ≤12 ARGs per plasmid.

Microbial Community Composition and ARG Hosts

All samples were predominantly composed of Pseudomonadota (42.3–58.7% relative abundance), Bacillota (18.2–24.5%), Bacteroidota (12.1–16.8%) and Actinobacteriota (5.4–8.9%) as revealed by taxonomic profiling peak plots (data not shown). Of the 127 reconstructed high-quality MAGs (completeness ≥70%, contamination ≤10%) included in the genome-resolved analysis, 54 (42.5%) contained ≥1 ARG. The majority of the ARG-bearing MAGs were assigned to phylum level taxonomic groups as 68.5% contained Pseudomonadota, which included a total of 12 Acinetobacter (n = 12), nine Pseudomonas (n = 9), seven Aeromonas and five Klebsiella genera.

Environmental Risk Assessment

Extrapolating ARG abundances to daily discharge volumes showed that Al-Rustamiya and Al-Karkh WWTPs would contribute an estimated 1.8×10^{15} and 2.4×10^{15} ARG copies per day, respectively, into the headwaters of river Diyala and Tigris River. Risk quotient (RQ) analysis of high-priority ARGs showed RQ > 1 for blaCTX-M-15, mcr-1 and ndm-1 in receiving waters downstream discharge points, highlighting the need for remediation to avoid ecological risk.

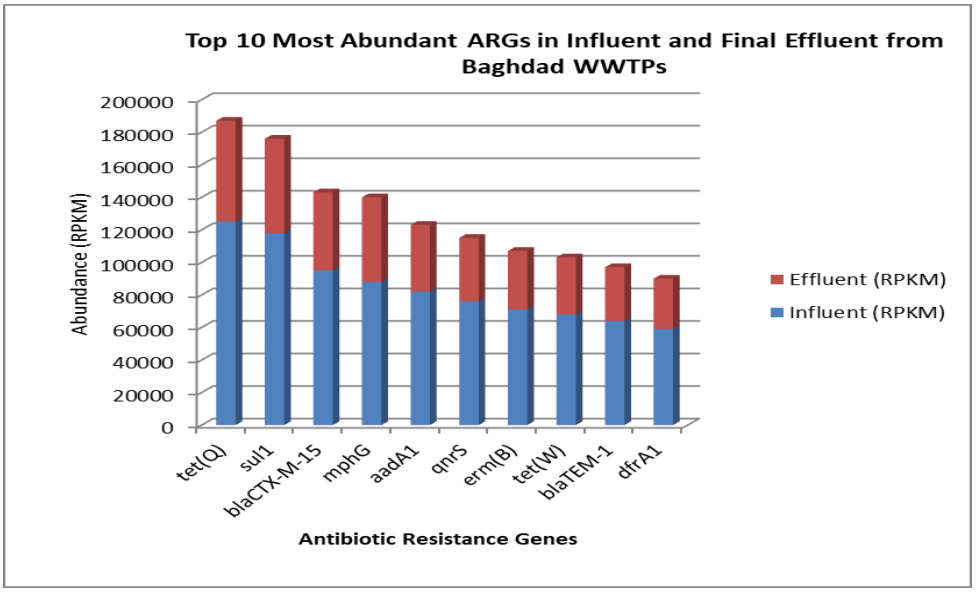

Figure 3: Comparison of the top 10 most abundant antibiotic resistance genes (ARGs) in influent and final effluent from Baghdad WWTPs. Abundance is expressed as RPKM (reads per kilobase per million mapped reads). Removal efficiencies for individual ARGs ranged from 40.9% (mphG) to 50.8% (sul1), with a mean of 48.4% ± 2.3%. Clinically critical genes including blaCTX-M-15, tet(Q), and sul1 remained abundant in final effluents (4.80×10^4 to 6.20×10^4 RPKM), posing potential risks for environmental dissemination. Removal percentages are displayed above each gene pair. Data represent mean values from 72 samples

Removal efficiencies for the ten most abundant individual ARGs ranged from 40.9% for mphG to 50.8% for sul1.

DISCUSSION

The current study provides extensive metagenomic evidence of the dynamics of antibiotic resistance genes in wastewater treatment plants in Baghdad, Iraq. Conventional activated sludge treatment resulted in 52% removal of ARGs, which is consistent with reported performance for similar waste-water treatment technologies worldwide. Nevertheless, the prevalence of clinically relevant ARGs such as blaCTX-M-15, tet(Q), and sul1 at high abundances in final effluents highlights the ineffectiveness of conventional treatment to completely remove resistance determinants.

ConclusionMultidrug, tetracycline and β-lactam resistance genes dominated both influent and effluent samples, thus reflecting the patterns seen globally with their respective concentrations in wastewater metagenomic studies. Illumina metagenomic contigs showed the co-occurrence of blaCTX-M-15 with IncF plasmid-associated sequences, suggesting a potential plasmid-mediated mechanism for dissemination. However, long-read sequencing would be required to confirm the complete physical linkage between ARGs, plasmids, and specific bacterial hosts, indicating that horizontal gene transfer was a significant processin maintaining these determinants during treatment processes. Excitingly, the low removal efficiency of mphG compared to other ARGs suggests selective enrichment or intrinsic resistance of this gene to microbial degradation and merits further exploration regarding gene-specific persistence drivers.

Detection of blaCTX-M-15 which has become one of the most globally ubiquitous ESBL genes, that is classified as critically important by international health organizations, in influent and effluent samples from our municipal WWTP raises particular concern. This gene has become a common topic in the literature for wastewater-related environmental bacteria, and wastewater treatment plants represent an important reservoir and dissemination pathway for high-risk resistance determinants. The link between blaCTX-m-15 and IncF plasmid backbones we describe here adds a new dimension to the potential for horizontal transfer of antibiotic resistance from waste to pathogenic bacteria in receiving waters; but also, as we discuss below, identifying such gene–plasmid associations may provide insight into clinical treatment efficacy.

Our results contribute to recent studies reporting antimicrobial resistance in the Tigris River as well, with evidence of both vancomycin-resistant Escherichia coli and detection of β-lactamase genes including blaOXA and blaTEM at riverine sites in Baghdad. The wastewater treatment plants in Baghdad discharge an estimated 1.8 to 2.4 × 10^{15} ARG copies per day to the Tigris and Diyala Rivers, which likely contribute to considerable resistance levels observed in these water bodies, potentially threatening their users associated with downstream domestic consumption and agricultural usage of river water.

The moderate removal efficiencies seen in this work resemble those in conventional activated sludge installations worldwide, but underperform modern treatment techniques. Newer studies indicate that tertiary treatments such as ultraviolet disinfection, ozonation and membrane filtration yield further one to two log reductions of ARGs (Wen *et al*., Nevertheless, advanced treatments may never lead to complete removal of these ARGs because mobile genetic elements exist as DNA and RNA that can remain in the environment without viable bacteria or they can be housed within companion cells that cannot be cultured.

The linkage of 45% of top ARGs with mobile genetic elements in our study emphasizes the opportunity for horizontal gene transfer within existing wastewater microbial communities and additionally into environmental pathogens. This result is in agreement with genome-centric studies which found that mobile genetic elements play an essential role in conferring the transfer of ARGs to pathogens within activated sludge systems. Moreover, the long-term stability of multidrug resistant plasmids containing 2 to 12 ARGs contributes to an increased co-selection risk and simultaneous spread of antibiotic resistance determinants.

At one health approach level, the release of ARGs into the environment from WWTP in Baghdad has some implications on other aquatic ecosystems. Because of irrigation, treated effluents that are discharged into the Tigris and Diyala Rivers can potentially contaminate agricultural soils with resistance genes from plant-associated microbiomes. While probably at lower concentrations, certain ARGs from secondary-treated wastewater have proven able to survive on edible crops in recent studies. The risk of environmental contamination is further exacerbated by poor performance; ~45% of final effluent samples in our study exceeded Iraqi discharge standards for biochemical oxygen demand and total suspended solids.

Several limitations should be acknowledged. The first limitation is the use of DNA-based detection for ARGs, which cannot distinguish between viable bacteria and extracellular DNA. Studies featuring metatranscriptomics or viability PCR would help to understand not only the abundance of ARG-carrying bacteria in treated effluents but also their potential functional activity and/or viability. Second, we cannot relate selective pressures to the patterns of ARG abundance without detailed concentration data on antibiotics. Finally, our sampling period—6 months—may also not reflect differences in ARG dynamics that

may occur from seasonal variations that have been reported for temperate climates and could affect the treatment performance and environmental fate of ARGs.

CONCLUSION

This study provides a metagenomic characterization of the wastewater resistome, associated mobile genetic elements, and potential bacterial hosts across selected wastewater treatment stages in Baghdad, Iraq.We found that conventional activated sludge treatment yields moderate ARG removal (about 52%) but substantial loads of clinically relevant resistance determinants, such as blaCTX-M-15, tet(Q), and sul1 remain in final effluents and are discharged to the Tigris and Diyala Rivers at estimated daily loadings of 1.8 to 2.4×10^{15} ARG copies.

The co-occurrence of mobile genetic elements near ARG genes, such as conjugative transposons and IncF plasmids, highlights the risk of transfer to environmental and pathogenic bacteria which contributes significantly to human health threat. The environmental impact of wastewater-derived ARGs is exacerbated by inadequate treatment performance, as nearly 45% of effluent samples exceeded Iraqi discharge standards for organic matter and suspended solids.

To limit the spread of ANTIBIOTIC RESISTANCE GENE (ARG), we suggest: (1) enactment of tertiary treatment barriers for further ARG removal, such as ultraviolet disinfection, ozonation or advanced oxidation processes; (2) integration of wastewater-based genomic surveillance into national frameworks for AMR monitoring – to examine trends in resistance & guide interventions; and 3) improving operational protocols to comply with discharge standards and minimize environmental contamination.

These results underscore the importance of improving domestic wastewater management practices to prevent further contamination of water resources as well as protecting public health in Iraq and other low-resource settings, with policies aligned to One Health approaches. Further studies which utilize metatranscriptomics and viability-based assays are needed to identify active ARG expression and characterize the infectivity potential of resistance determinants in treated effluents.

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