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Resistome characterisation from catchment to effluent in a treatment plant without hospital inputs: implications for AMR monitoring

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ABSTRACT

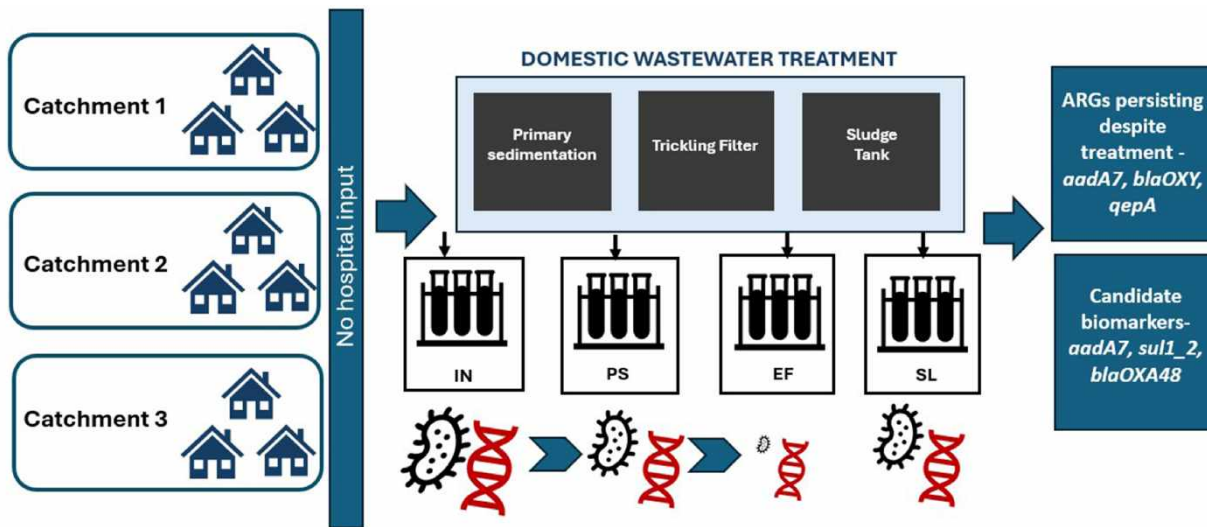
Wastewater surveillance is increasingly used to monitor antimicrobial resistance (AMR) at community scales. This study aimed to (1) assess AMR removal across treatment stages, (2) identify persistent versus responsive antimicrobial resistance genes (ARGs), and (3) characterise resistome profile of a wastewater treatment plant (WWTP) in Northeast England with no hospital inputs. Analyses included physicochemical profiling, microbial culturing, and high-throughput quantitative polymerase chain reaction targeting ARGs. Presumptive extended spectrum β -lactamase (ESBL)-producing *Escherichia coli* were effectively reduced by over two logs in the WWTP, although viable ESBL *E. coli* was still present in effluent. Comparative analyses revealed significant reductions along treatment stages for most ARGs. However, some ARGs, such as *aadA7*, *blaOXY*, and *qepA*, showed no significant reduction, indicating some ARGs persist through treatment, an important consideration for future monitoring programmes. The resistome profiles from three sub-catchments were similar, with only one ARG (*lnuC*) showing significant variation. This suggests that domestic-only catchments without hospital inputs may exhibit relatively uniform ARG profiles, although these results are specific to the WWTP studied and requires further validation. Finally, three ARGs, *aadA7*, *sul1_2*, and *blaOXA48* were identified as potential biomarkers for AMR monitoring in WWTP, based on their abundance, strong correlation patterns, and clinical relevance.

Key words: antimicrobial resistance, biomarker, community resistome, persistent genes, wastewater surveillance, wastewater treatment plant

HIGHLIGHTS

- Baseline resistome profile established for wastewater treatment plant (WWTP) with no hospital input, useful for comparison in hospital-based studies.
- Identified antimicrobial resistance genes (ARGs) persistent through treatment warranting attention and ones removed, indicating antimicrobial resistance (AMR) removal efficiency.
- Strong similarity of AMR profile across community catchments.
- Three ARGs *aadA7*, *sul1_2*, and *blaOXA48* identified as potential biomarkers for tracking resistance patterns in community WWTP.

GRAPHICAL ABSTRACT



INTRODUCTION

The spread of antimicrobial resistance (AMR) is a serious global public health concern. If left unaddressed estimates have suggested, it will cause 10 million deaths per year by 2050 (O' Neill 2016). While AMR spread through clinical settings is well documented (Rusotto *et al.* 2025), the environment is becoming increasingly recognised as a key reservoir and dissemination pathway for AMR (Sassi *et al.* 2025). Wastewater treatment plants (WWTPs) are recognised as both reservoirs of AMR and pathways to the wider environment (Gao *et al.* 2022). These facilities receive wastewater containing antibiotic residues, resistant bacteria and antimicrobial resistance genes (ARGs) from diverse sources, including households, industries, and healthcare settings. While WWTP processes are normally designed to remove organic matter and, as a by-product, some pathogens, they were not designed to remove AMR and are often less efficient at removing ARGs and other AMR determinants (Raza *et al.* 2022). However, WWTPs still serve an important function in reducing AMR release to the environment compared with locations with no treatment (UNEP 2023), emphasising their unique role as both reservoirs and potential control points.

Given that WWTPs serve as a central collection point of community wastewater discharges, wastewater surveillance (WS) at these sites can provide a practical approach for monitoring spatial and temporal AMR distribution patterns. Despite the widespread interest in WS for AMR monitoring, currently, there is no universal strategy for it in WWTPs (Chau *et al.* 2022). Several recent studies have highlighted the importance of sampling at multiple points in the treatment process, including settled sludge, to better comprehend the treatment performance and removal dynamics (Gorecki *et al.* 2024).

While conventional WWTP processes can inactivate many resistant bacteria, ARGs usually persist in the environment with minimal degradation, often detected in effluent and receiving waterways (UNEP 2023). WWTPs are not simply the passive conduits for ARGs, they are usually a series of unique processes, many of which function as active bioreactors where horizontal and vertical gene transfer can occur among microorganisms (Quintela-Baluja *et al.* 2019). This dynamic environment promotes the proliferation of existing ARGs or the emergence of new resistant organisms within the treatment stages. Hence, measuring gene abundance at the inlet and outlet may not fully reflect AMR prevalence or removal efficiency.

An in-depth understanding of AMR patterns in WWTPs is critical to mitigating the resistance spread through these facilities and into the environment. However, the difficulty in standardising AMR surveillance in WWTPs lies in the complexity of sewage composition and the variable performance of treatment processes (Chau 2022). Identifying suitable biomarkers to represent the diverse and dynamic nature of AMR in WWTPs remains an important challenge. Some studies have proposed using bacteria such as *Enterococcus*, *Pseudomonas aeruginosa*, and members of the *Enterobacteriaceae* family, including faecal coliforms, due to their association with ARGs and their ubiquity in wastewater ecosystems (Matheu *et al.* 2017; Hiller *et al.* 2019). Among these, *E. coli* is commonly used as an indicator organism for AMR surveillance and is recommended by the WHO and several national guidelines (Jones *et al.* 2023). Genetic targets have also been suggested,

including *mexE*, *mexF*, *blaCTX-M*, *blaSHV*, *tetC*, *tetX*, *qnrC*, *qnrS*, *sul1*, *sul2* among others (Wang *et al.* 2020). However, the overarching strategies for WS are lacking, including reasonable control markers for monitoring AMR at the catchment scale. While a few studies have started establishing biomarker panels for AMR surveillance in wastewater (Teixeira *et al.* 2023), it has rarely focused specifically on domestic-only catchments without healthcare inputs. This article addresses that gap by characterizing the resistome profile of a small WWTP serving purely community inputs in Northeast England with no significant hospital inputs, to contribute to the understanding of resistome profiles in domestic-only wastewater systems for future studies.

The main aim of this study is to provide a comprehensive analysis of AMR patterns within a small-scale WWTP and its contributing sub-catchments. Specifically, we sought to (1) quantify AMR removal efficiencies by characterizing the reduction of both viable ESBL *E. coli* and a broad spectrum of ARGs across the WWTP’s main treatment stages; (2) identify ARGs that persist through treatment as potential indicators of environmental resilience, and those that are strongly reduced as markers of treatment effectiveness, to inform future AMR monitoring strategies; and (3) assess spatial variation by comparing resistome profiles of three distinct sub-catchments to characterise the resistome of a system without hospital or major industrial inputs.

METHODS

Site selection and sampling design

The study was conducted at a small-scale WWTP located in Northeast England, serving a population equivalent of approximately 30,000. It was selected based on key logistical and scientific requirements: (i) it receives wastewater from three distinct catchment areas entering through physically separate inlets, and (ii) it contains accessible sampling points along key treatment stages. Additionally, there were no hospitals and major industrial sources discharging their wastewaters into the sewer network feeding this WWTP, ensuring that the study primarily examines domestic wastewater.

The three catchments were distinct in influent characteristics (Table 1) in which Inlet 1 (IN1) was almost wholly domestic, whereas Inlet 2 (IN2) was primarily domestic (over 90%), but also had smaller industrial operations (around 35 types), including metal processing facilities, warehouses, a phosphorous production plant, a meat processing industry, and a waste management site. IN1 mostly had waste management companies. Inlet 3 (IN3) had a museum, an important tourist attraction in the area.

The WWTP included screening, grit removal, primary sedimentation (PS), a trickling filter system for secondary treatment, and final sedimentation. Sludge generated was recycled to the influent sump, and treated effluent was discharged into the River Team. Trickling filters are one of the most used secondary treatment methods in small- to mid-sized UK WWTPs and are representative of conventional systems in the region (Marquet *et al.* 2007). Sampling points were selected at the different inlets and along different treatment points at the PS tank, effluent from the secondary sedimentation tank, and the sludge tank. The output directly from the trickling filter was not accessible during the sampling campaign, so it was collected from the secondary sedimentation tank. A schematic of the treatment layout and sampling strategy is shown in Figure 1.

Sampling campaign

The sampling campaign was conducted over 7 weeks between October and December 2022. Samples were collected weekly on Wednesdays between 09:30 and 12:30 (except Week 3, which was sampled on a Thursday). Seven locations were sampled: three influent catchments (IN1, IN2, and IN3), the inlet sump which is the combined influent from three sub-catchments (INS), the tank after PS, the final effluent (EF), and sludge (SL) (Figure 1). Composite samples of a final volume of 2 L were collected by sampling every 15 min over 3 h using either manual collection (IN1–3, INS, SL) or autosamplers (PS, EF).

Table 1 | Details of catchment areas for the three inlets entering the WWTP

Sample	Details of catchments
IN1	661 domestic households, 1 nursing home, and 22 non-household customers
IN2	11,574 domestic households, 9 nursing homes, and 850 non-household customers
IN3	453 households with no nursing homes

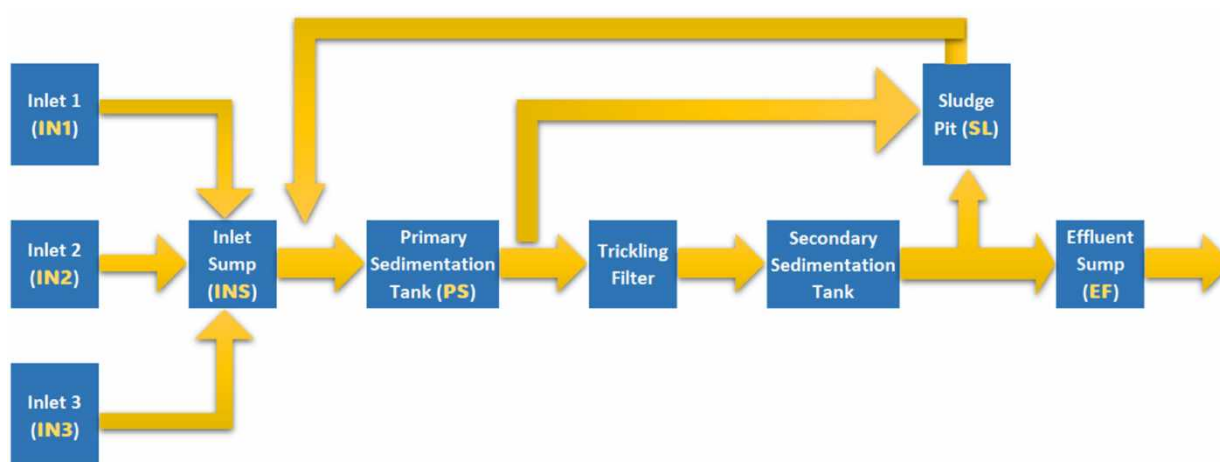


Figure 1 | Schematic diagram of influent, treatment, and discharge flows in the study WWTP. Wastewater from three separate inlets (IN1, IN2, and IN3) mixes in the inlet sump (INS) and proceeds through primary sedimentation (PS), a trickling filter, and secondary sedimentation. Treated effluent is discharged via the effluent sump (EF), while sludge is returned to the inlet sump (SL).

Samples were transported on ice, stored at 4 °C, and processed within 24 h. Sludge samples were collected on five of the seven sampling days.

Physicochemical and microbial analysis

Water quality parameters including temperature, pH, and conductivity were measured *in situ* using a digital portable multi-meter (HQ30D, Hach Lange, US). Once transported to the laboratory, samples were analysed for ammonia, chemical oxygen demand (COD), total nitrogen, and orthophosphate using cuvette test kits (LCK 303, 514, 238 and 348, Hach Lange, US) according to manufacturer instructions. Measurements were performed with a DR6000 Benchtop spectrophotometer.

Escherichia coli and cefotaxime-resistant *E. coli* were cultured using Tryptone Bile X-Glucuronide agar with and without the addition of cefotaxime (4 µg/mL). Cefotaxime resistance at this concentration is widely used as a phenotypic indicator of ESBL production, though it does not exclude other resistance mechanisms such as AmpC β -lactamases. In the absence of confirmatory ESBL testing, cefotaxime-resistant isolates are referred to hereafter as ‘presumptive ESBL *E. coli*’. Ten-fold serial dilutions were plated and incubated at 37 °C for 20 h. Colonies showing typical blue-green coloration were counted and expressed as colony-forming units per millilitre. ESBL-producing *E. coli* was chosen because it is a globally recognized faecal indicator organism and a WHO-associated high-priority, multidrug-resistant pathogen, making it an ideal sentinel for tracking community-level AMR (WHO 2024). While confirmatory testing was not performed in this study, the use of cefotaxime-supplemented media as a screening method is a standard approach in environmental AMR studies where isolate-level confirmation is not practical at scale (Calarco *et al.* 2024).

DNA extraction and qPCR

DNA was extracted using a phenol:chloroform:isoamyl alcohol protocol. Briefly, centrifuged biomass was processed with phenol:chloroform:isoamyl alcohol (25:24:1) using Phase Lock tubes, followed by chloroform cleanup and column-based purification (NucleoSpin® Dx Virus, Macherey-Nagel). DNA was eluted in DNase-free water. All samples were quantified using Qubit and Nanodrop and stored at –20 °C until further use.

Quantitative PCR (qPCR) was performed to measure 16S rRNA (Harms *et al.* 2003) and *Bacteroidetes* marker gene concentrations (Green *et al.* 2014). An additional qPCR was performed for each sample using green fluorescent protein (gfp) as an internal control to assess potential PCR inhibition (Jobling *et al.* 2023). Assays were conducted in 96-well plates using SsoAdvanced Universal Probes Supermix (BioRad, USA) and 10-fold and 100-fold diluted DNA templates. Each 15 µL reaction contained 2 µL DNA, 7.5 µL of Supermix, 0.3 µL each primer (10 µM), 0.15 µL probe (10 µM), 0.975 µL Bovine Serum Albumin (BSA) (6 mg/mL), and qPCR-grade water. Plates were run at 60 °C (16S) and 57 °C (*Bacteroides*, gfp) on a qPCR thermocycler. Threshold cycle (C_q) values and starting quantities were extracted from the qPCR software output. Primer

sequences and additional reagent details are provided in the Supplementary Information. All extracted DNA was within quality ranges, with 260/280 ratios above 1.8 and 260/230 ratios mostly exceeding 2.0, confirming minimal contamination (Lucena-Aguilar *et al.* 2016) and no inhibition was detected.

High-throughput qPCR analysis

High-throughput qPCR (HT-qPCR) was done to quantify a broad set of ARGs and mobile genetic elements (MGEs) using the SmartChip™ Real-Time PCR system (TakaraBio, Japan) by Resistomap Oy (Helsinki, Finland) (Stedtfeld *et al.* 2018). The analysis was conducted in two stages. In stage 1, DNA from four treatment points – INS, PS, EF, and SL – was pooled by combining equal volumes of DNA from weeks 3, 4, 5, 6, and 7. These particular weeks were selected as they included sludge samples, which were absent in weeks 1 and 2. The pooled samples were screened against a panel of 384 ARGs, taxonomic markers, and MGEs to obtain an overview of the resistome in the WWTP.

A targeted 96-gene panel was designed for stage 2-based primarily on relevance to wastewater and public health in North-east England, and inclusion in national or global AMR surveillance priorities (Pruden *et al.* 2021; WHO 2021; Liguori *et al.* 2022). The pooled analysis from stage 1 was then utilized to confirm that this designed panel was appropriate for these specific matrices and to ensure that rarer, geographically-specific genes were captured.

In stage 2, HT-qPCR was performed on individual samples from IN1, IN2, IN3, PS, EF, and SL collected during weeks 3, 5, and 7, chosen based on sludge availability and environmental variability. While the resulting dataset is modest in size, appropriate non-parametric statistical tests (Kruskal–Wallis) and conservative filtering criteria (detection in ≥ 4 of 12 samples) were applied to ensure robust analysis.

Data processing and statistical analysis

HT-qPCR data were processed using a threshold cycle (C_t) cutoff of 27 (Muziasari *et al.* 2017), with genes showing amplification in fewer than two of three replicates discarded to ensure data quality. Relative abundance of each gene was calculated using the $2^{-\Delta C_t}$ method. Using the data from stage 1, the list of 96 ARGs was processed and the sampling weeks were decided. The relative abundance of each gene from stage 2 was then converted to absolute abundance by normalising against *16S rRNA* gene copy numbers obtained via conventional qPCR (Daw Elbait *et al.* 2024). All statistical analyses were conducted using R (version 4.3.2) and Minitab 21.

For the analysis of AMR trends across the treatment stages, influent values were derived by averaging the IN1, IN2, and IN3 values from each sampling week, as HT-qPCR in stage 2 was performed on individual inlet samples to enable catchment-level comparisons (Objective 3) rather than on the combined inlet sump (INS). To validate this approach, the averaged IN1–IN3 relative abundances were compared against the stage 1 INS pooled values: the two datasets were strongly correlated (Spearman $\rho = 0.92$, $p < 0.001$; $n = 80$ genes), confirming that the averaged influent values reasonably approximate the combined influent. These were compared against the corresponding PS, EF, and SL values from the same weeks (3, 5, and 7). To explore the relationships between the results of physicochemical analysis, culturing, and HT-qPCR analysis, Pearson's correlation analysis was conducted between ARG abundance, physicochemical parameters, and culturable *E. coli* concentrations. To identify statistically significant differences in gene abundance between the four treatment stages, a Kruskal–Wallis test was applied to each gene individually. Only genes detected in at least 4 of the 12 samples were retained to minimise the influence of sparsely detected targets. Genes with p -values below 0.05 were considered statistically significant. A similar approach was applied to influent samples, where IN1, IN2, and IN3 were compared across 3 weeks, and only genes with at least four non-zero detections were retained.

Gene abundances that showed statistically significant differences were visualised using dot plots with median values and standard deviation error bars. This format was selected due to its suitability for small datasets and was consistent with common practice in environmental AMR studies. The correlations and other factors were considered to find biomarkers suitable to represent the AMR patterns in wastewater from the WWTP.

RESULTS AND DISCUSSION

Sampling conditions

Weather parameters including ambient temperature and three-day precipitation average were recorded to assess their potential influence on wastewater composition (Met Office 2024). Precipitation ranged from 1.5 to 14 mm across the campaign, but correlation analysis showed no significant relationship between rainfall, temperature, or influent depth. This suggests

that short-term environmental fluctuations (Supplementary Information (SI) Table S1) did not influence the wastewater composition during the sampling period. Considering the three influents, IN1 received intermittent pumped flow, while IN2 and IN3 were continuous. IN2 consistently had the highest flow depth, peaking at 90.3 cm during Week 5, compared with 35.1 and 25.4 cm in IN1 and IN3, respectively. These differences reflect variations in upstream load, likely driven by higher residential and industrial contributions in the IN2 catchment (Table 1). Precise flow rates could not be calculated due to unavailable cross-sectional data; therefore, flow depth was used here as a relative indicator of upstream hydraulic loading. The consistently greater depth at IN2 suggests a higher volumetric flow, likely driven by its larger residential and industrial catchment area.

Physicochemical analysis

Variation in physicochemical characteristics was observed across sampling locations (Table 2). However, compared to typical values in municipal wastewater, COD (200–700 mg/L), ammonia (30–50 mg/L), and phosphate (2–10 mg/L), the physicochemical parameters measured here fell within expected ranges (Burton *et al.* 2013; Guven *et al.* 2019; Zou *et al.* 2019; Tweib 2024). COD values ranged from 127 ± 17 mg/L in IN3 to 613 ± 82 mg/L in IN2, while ammonia ranged from 31 ± 5 to 48 ± 8.3 mg-N/L across influents. Phosphate was also highest in IN2 (5.98 mg/L ± 0.64), compared with IN3 (1.91 mg/L ± 0.76). These trends mirror observed differences in catchment profiles, with IN2 receiving the highest non-domestic inputs. In contrast, pH was consistent across all measurements (7.58 ± 0.22 to 8.25 ± 0.17), indicating minimal acid–base variation throughout the system, with differences falling within the instrument’s accuracy range (± 0.1 pH units).

Ammonia concentrations reduced significantly through the treatment process – from 43.41 ± 18.5 mg/L in the inlet sump (INS) to 3.07 ± 0.99 mg/L in the effluent (one-way analysis of variance, $p < 0.001$). COD similarly decreased from 528 ± 119 mg/L at INS to 66.9 ± 11.5 mg/L at the effluent, with calculated removal efficiencies of 27–77% in the PS tank and 60–74% in the trickling filter. Although differences in removal performance were not statistically significant ($p = 0.29$), these values are typical of trickling filter WWTPs (Aslam *et al.* 2017; Lasaki *et al.* 2023).

Microbial analysis

E. coli concentrations ranged from $2.12 (\pm 0.64) \times 10^4$ to $5.64 (\pm 1.39) \times 10^4$ CFU/mL in influent samples and decreased to $(2.13 \pm 0.89) \times 10^3$ CFU/mL in final effluent, representing approximately a 1-log reduction across the treatment process (Figure 2(a)). As such, *E. coli* remained detectable in both the treated effluent and sludge, which is common in conventional WWTPs, confirming its utility as an indicator of WWTP performance. ESBL *E. coli* was also detected at all sampling points, including the effluent ($(2.03 \pm 1.41) \times 10^1$ CFU/mL), indicating that resistant *E. coli* are discharged into the receiving environment. Although ESBL *E. coli* counts were lower than total *E. coli*, their detection in treated outputs highlights the importance of monitoring both total microbial loads and resistant subpopulations. Across the three influents, IN2 consistently had higher levels of both total and ESBL *E. coli*, consistent with its more diverse catchment.

Table 2 | Average values of physicochemical and microbiological parameters

Parameters	Sampling points						
	IN1	IN2	IN3	INS	PS	EF	SL
pH	8.06 ± 0.18	8.25 ± 0.17	7.84 ± 0.08	8.22 ± 0.05	7.80 ± 0.15	7.78 ± 0.17	7.58 ± 0.22
Conductivity ($\mu\text{S}/\text{cm}$)	675 ± 79.7	693 ± 111	721 ± 139	690 ± 87.9	609 ± 62.4	547 ± 58.9	553 ± 53.5
Phosphate (mg/L)	3.96 ± 1.40	5.98 ± 1.69	1.91 ± 0.76	5.70 ± 1.58	4.06 ± 1.11	2.33 ± 0.53	15.4 ± 4.37
Ammonia (mg/L)	37.8 ± 15.3	42.76 ± 21.9	31.27 ± 14.9	43.41 ± 18.5	28.20 ± 7.44	3.07 ± 0.99	4.24 ± 1.65
COD (mg/L)	269 ± 89.7	613 ± 216	127 ± 45.9	528 ± 119	203 ± 45.7	66.9 ± 11.5	$2,190 \pm 843$
<i>E. coli</i> (CFU/mL)	$(3.41 \pm 1.02) \times 10^4$	$(5.64 \pm 1.39) \times 10^4$	$(2.12 \pm 0.64) \times 10^4$	$(5.15 \pm 1.16) \times 10^4$	$(2.14 \pm 0.78) \times 10^4$	$(2.13 \pm 0.89) \times 10^3$	$(4.85 \pm 1.64) \times 10^4$
ESBL <i>E. coli</i> (CFU/mL)	$(3.40 \pm 1.83) \times 10^2$	$(1.59 \pm 0.91) \times 10^3$	$(7.62 \pm 2.88) \times 10^1$	$(1.65 \pm 0.81) \times 10^3$	$(7.96 \pm 3.54) \times 10^2$	$(2.03 \pm 1.41) \times 10^1$	$(9.86 \pm 3.79) \times 10^2$

Note: Values represent mean $\pm$ standard deviation based on 7 weekly samples.

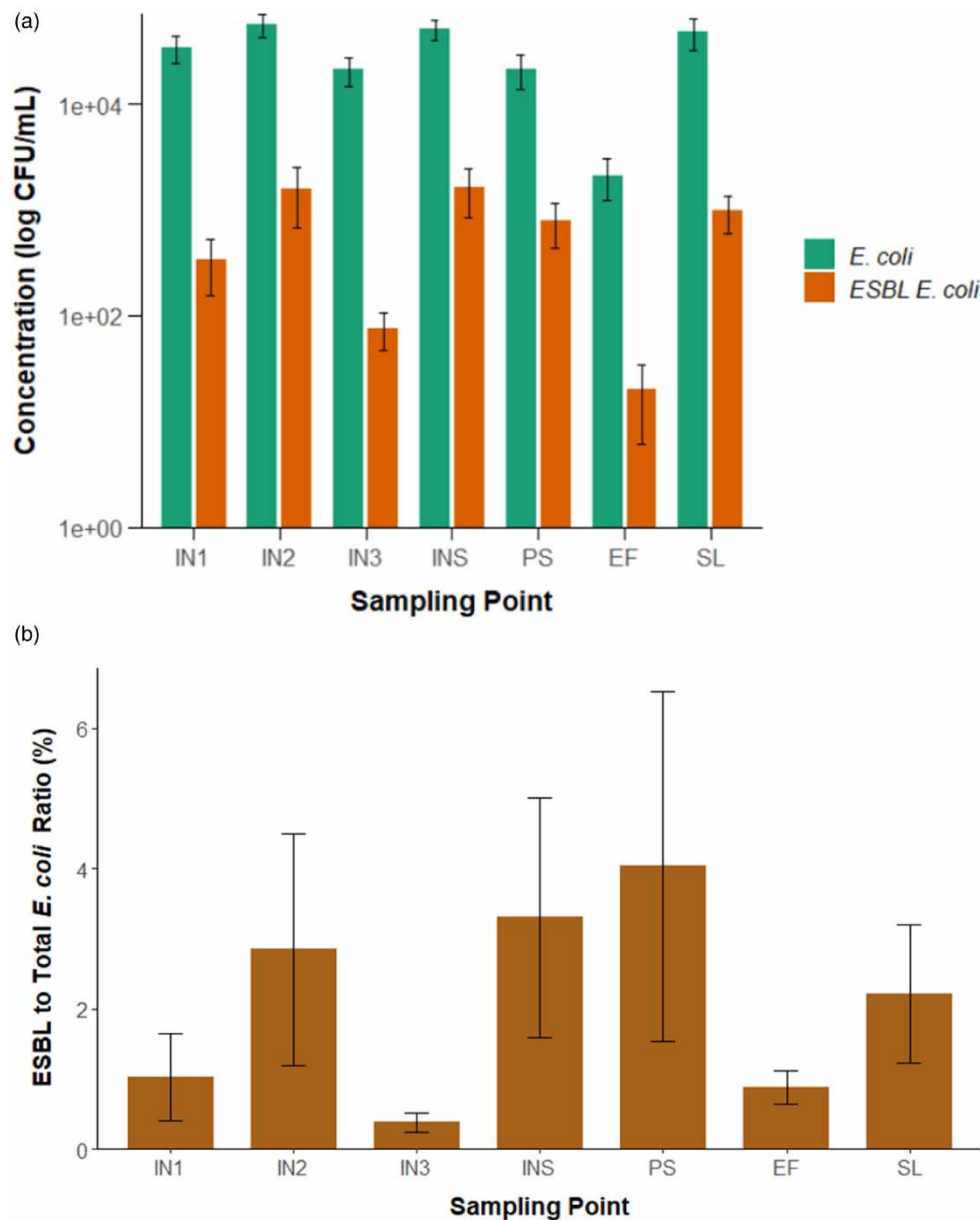


Figure 2 | Concentration and resistance profile of *E. coli* across WWTP sampling points. (a) Log-transformed concentrations of total *E. coli* and ESBL-producing *E. coli* (CFU/mL) across all sampling points. (b) Percentage of resistant *E. coli* relative to total *E. coli* counts, highlighting spatial differences in resistance prevalence across the treatment process.

To better understand the dynamics, the ratio of ESBL *E. coli* to total *E. coli* was calculated for each sampling day and averaged by location. This approach allows comparison of resistance prevalence independent of absolute bacterial load, providing a standardised index that is more useful for evaluating treatment efficacy and comparing across sites. The ratio provides a normalised indicator of resistance prevalence within the culturable *E. coli* population (Figure 2(b)). While absolute *E. coli* concentrations declined along the treatment process, the ESBL *E. coli* to *E. coli* percent ratio slightly increased after PS (3–4%). This transient increase in the ratio suggests that early treatment stages may act as bioreactors, where mixing and high organic loads support short-term enrichment of resistant subpopulations. Overall, the ratio in the effluent was approximately 0.88%, suggesting that although substantial reductions in ESBL *E. coli* occurred (1.65×10^3 CFU/mL in IN to 2.03×10^1 CFU/mL in EF), a consistent subset of resistant organisms is discharged from the system. Notably,

sludge samples also harboured higher levels of ESBL *E. coli*, but those organisms are recirculated in the WWTP system and not discharged.

While reductions in culturable *E. coli* support treatment efficacy, the persistent detection of ESBL producers, particularly in the WWTP effluents, suggests that more study is needed to determine whether further treatment is needed for AMR-specific mitigation. In the following sections, we explore how culturing microbial trends relate to HT-qPCR data to compare methods and assess spatial differences in ARG prevalence, especially between catchments.

High-throughput qPCR

HT-qPCR was conducted in two stages, targeting ARGs, taxonomic genes, and MGEs. In stage 1, DNA from pooled samples, including sub-samples from INS, PS, EF, and SL, were analysed. The panel of 384 genes revealed elevated abundances of genes typically associated with community wastewater, such as *intl1_1*, *aadA7*, and *qepA*. The gene families with the highest abundance included aminoglycoside and macrolide-licosamide streptogramin B (MLSB), with INS showing the highest overall ARG abundance, followed by SL, PS, and EF (Table 3). This initial screening indicated higher ARG prevalence and diversity upstream and within the WWTP, with absolute gene abundances highest in INS and sludge samples.

The preliminary analysis informed the selection of 96 ARGs for stage 2 analysis. The 96-gene array was used to investigate AMR trends from the different sub-catchments and treatment stages, focusing on samples from the three influents (IN1, IN2, and IN3) and treatment stage samples (PS, EF, and SL), collected during weeks 3, 5, and 7. Of the 96 targeted genes, 83 were detected in one or more samples and 13 genes were absent or below detectable levels.

Approximately 81% of the detected genes ($n = 67$) were consistently present across all treatment stages, including the WWTP effluent. The analysis revealed the persistence of ARGs throughout the treatment process, with a reduction in total abundance in the effluent, though the difference was relatively small (a factor of 10). The trickling filter demonstrated moderate reduction in ARG concentrations, with a decrease from 1.66×10^8 copies per mL in the IN to 2.45×10^7 copies per mL in the effluent, supporting other studies highlighting the lower effectiveness of trickling filters in reducing AMR (Lamba & Ahammad 2017; Petrovich *et al.* 2018). As volumetric flow data were unavailable, these represent concentration-based reductions rather than mass-based removal efficiencies.

The absolute abundance of ARGs varied across treatment stages, with MLSB and tetracycline resistance genes showing the highest levels in influent and PS samples (Figure 3). MLSB-associated ARGs exhibited a large reduction in abundance through the treatment phases (from 4.95×10^7 copies per mL in the IN to 3.08×10^6 copies per mL in the effluent); however, overall reduction in concentration of most gene families was not substantial. Trimethoprim-associated ARGs were completely removed during the treatment process from 7.11×10^4 copies per mL in the influent, unlike most other gene families,

Table 3 | Absolute abundances of ARG families and MGEs present in the four pooled samples obtained from HT-qPCR conducted – stage 1

Gene family	Absolute abundances of gene families (gene copies/mL)			
	INS	PS	EF	SL
Aminoglycoside	1.12×10^8	4.13×10^7	2.33×10^7	1.89×10^8
Beta lactam	2.91×10^7	1.04×10^7	4.79×10^6	3.78×10^7
Multi Drug Resistance (MDR)	6.57×10^7	2.60×10^7	1.24×10^7	9.85×10^7
MGE	4.51×10^8	1.66×10^8	3.16×10^7	2.20×10^8
MLSB	1.62×10^8	5.36×10^7	1.35×10^7	9.87×10^7
Other	2.03×10^7	8.68×10^6	2.79×10^6	1.95×10^7
Phenicol	5.30×10^6	2.02×10^6	9.33×10^5	7.19×10^6
Quinolone	2.29×10^7	8.84×10^6	6.77×10^6	5.27×10^7
Sulfonamide	1.12×10^7	4.26×10^6	1.20×10^6	9.16×10^6
Tetracycline	5.94×10^7	2.43×10^7	4.34×10^6	2.82×10^7
Trimethoprim	6.94×10^6	3.20×10^6	8.57×10^5	7.10×10^6
Vancomycin	1.67×10^7	6.78×10^6	3.91×10^6	2.98×10^7

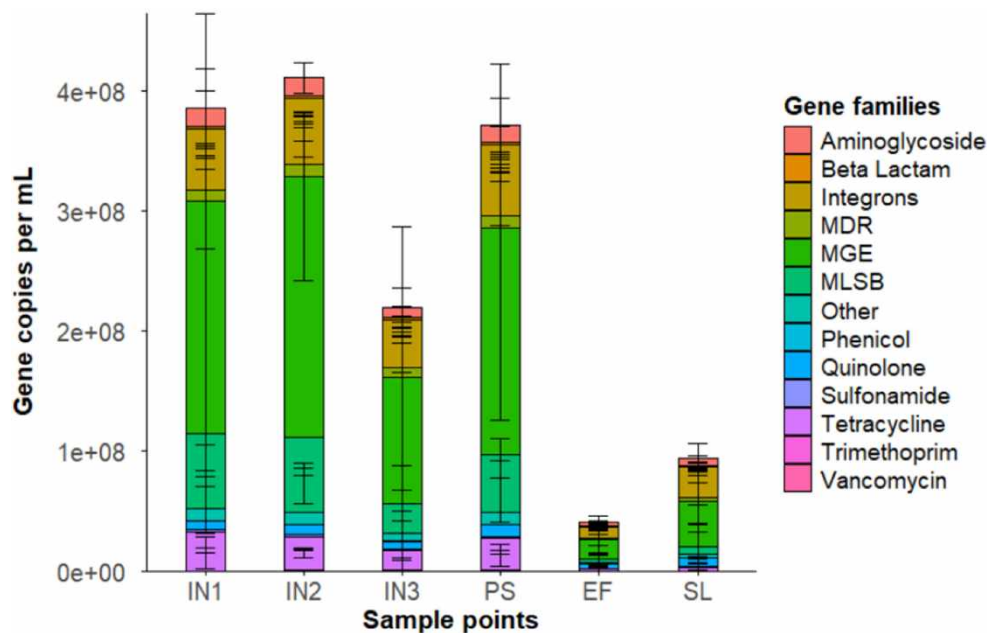


Figure 3 | Stacked bar plot showing distribution of ARG families across treatment stages and influents. The plot illustrates the absolute abundance of ARG families (gene copies per millilitre) at various treatment stages and influents (IN1, IN2, IN3, PS, EF, and SL). The overall trend highlights the limited efficiency of the trickling filter in reducing ARGs, with only a 16.35% reduction in total ARG concentration observed.

which showed minimal reduction. However, this observation may reflect the inclusion of only one trimethoprim-associated gene *dfrA1_1* in the stage 2 array, highlighting the need for further investigation into gene-specific persistence patterns.

Despite the reduction in ARGs from influent to effluent of the 96 genes measured, 67 of these were present in all samples, demonstrating their persistence through the treatment process (Figure 4). Four genes were present only in influent samples and were removed following primary treatment, while seven were present in both IN and PS, but not in EF, indicating

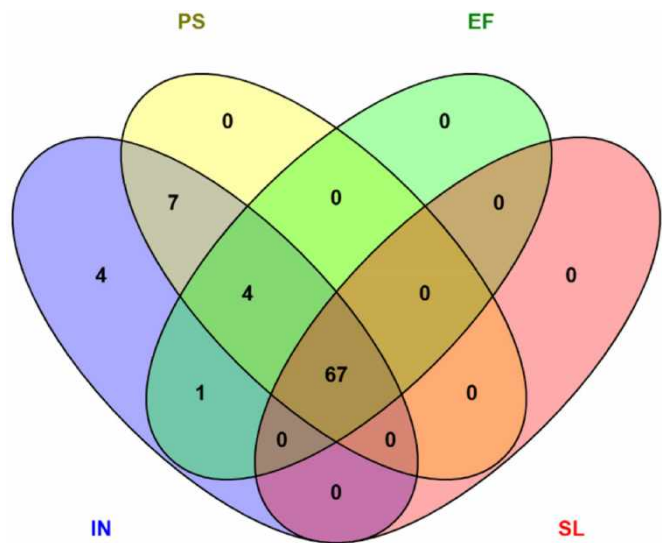


Figure 4 | Venn diagram representing distribution of resistance-related genes (ARGs, MGEs, and taxonomic genes) in different samples. Venn diagram shows that 67 out of 83 genes detected were present in all stages of treatment including effluent, four genes were removed in the primary treatment, and seven genes were removed in secondary treatment.

removal during the trickling filters or secondary sedimentation tank. The β -lactamase gene *blaOXA48* was uniquely detected in the influent and effluent but absent in the PS sample. This suggests that the gene was present at concentrations below the limits of detection of the assay after primary treatment but may have reappeared in the effluent due to increased concentration or enrichment within the downstream processes (Quintela-Baluja *et al.* 2019). Although this could also have been due to stochastic detection near the limit of detection, differences in PCR inhibition across sample matrices, or sampling variability. Nevertheless, such findings further emphasize the challenges of fully eliminating ARGs through conventional WWTP processes.

Correlation analysis between ARGs and wastewater parameters

As ARGs are largely derived from the faecal component of wastewater, their abundance is expected to correlate with conventional wastewater indicators reflecting organic and microbial load. To evaluate whether the conventional physicochemical parameters or ESBL *E. coli* counts reflect the genetic patterns observed in the HT-qPCR analysis, Pearson's correlation coefficients were calculated between absolute ARG abundances and key water quality and microbial indicators (Figure 5 and SI: Table S2). While several ARGs showed moderate to strong correlations ($r > 0.6$) with specific parameters, particularly ammonia, and ESBL *E. coli*, no consistent pattern emerged across all genes or parameters. Ammonia exhibited the strongest average correlation with ARGs, likely reflecting its high concentration in wastewater rather than a direct functional relationship. When averaging across all samples, pH demonstrated the strongest correlation with many ARGs; however, when considering individual samples, pH only correlated strongly with ARGs detected in IN and PS samples, whereas COD displayed the strongest correlation with ARGs in EF and SL samples.

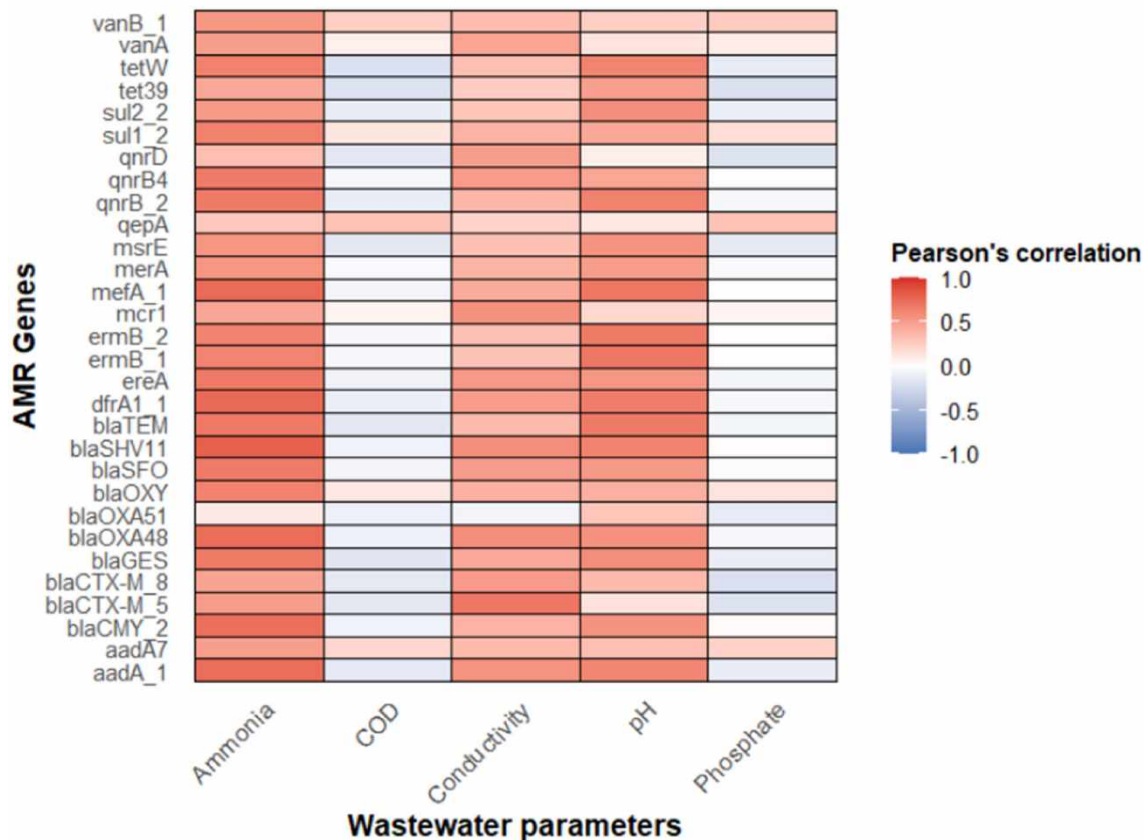


Figure 5 | Heatmap showing correlation between ARGs and wastewater parameters. Pearson's correlation analysis done to quantify the correlation between absolute abundance of ARGs and physicochemical and microbial parameters is illustrated in the heatmap. pH and ammonia showed the highest correlation with the ARGs.

Correlation between ARGs and ESBL *E. coli* was generally weak across all comparisons ($r < 0.4$), suggesting that the measured resistance phenotype does not directly reflect ARG abundances in this system. This disconnect may result from differences in gene carriage among the resistant phenotypes, or a general lack of strong selection pressures for specific ARGs within this system, which is primarily sourced from residential dwellings – i.e. secondary pollutants such as metals, industrial chemicals, and pharmaceuticals, are probably low. Overall, the analysis highlights that neither physicochemical variables nor culture-based ESBL counts strongly correlate with ARG levels in a consistent way within this system.

ARG variation across treatment stages

To identify ARGs that differed significantly across the treatment stages, Kruskal–Wallis (KW) test was applied to all genes from the stage 2 HT-qPCR panel that were detected in at least four samples, to reduce noise from sporadically detected genes. This non-parametric test enabled comparison of gene abundances across different treatment stages while accounting for non-normal data distributions. Two categories of findings emerged from this analysis: genes showing statistically significant differences between stages, and genes which show no significant differences, indicating persistence across treatment.

Twelve ARGs displayed significant variation across the treatment stages ($p < 0.05$), including genes from the tetracycline (*tet39*, *tetW*), MLSB (*ermB_1*, *ermB_2*, *mefA_1*, *msrE*), MDR (*sugE*, *trcB*, *pcoA*), aminoglycoside (*aadA_1*, *strB*), and Other (*uidA*) families, alongside eight MGEs and three taxonomic markers (Figure 6). These genes exhibited measurable changes in abundance across the stages, e.g. the aminoglycoside-associated gene *aadA_1* decreased from 1.54×10^6 copies per mL in IN to 1.24×10^6 copies per mL after PS and then to 8.35×10^4 copies per mL in effluent. The significant reduction of these genes does not confirm effective elimination but may indicate that their abundance is partially reduced by primary or secondary treatment processes. Among the MGEs, significant differences were observed for genes such as *IS26_1*, *Tn3*, *Tn5*, and *tnpA_1*, while taxonomic markers like *Firmicutes*, *Enterococci*, and *Bacteroidetes* also exhibited shifts in distribution (SI: Figures S1 and S2). Together, these findings help identify genes that may serve as dynamic markers of treatment effectiveness under conventional operation.

Among the ARGs that demonstrated significant reduction across the treatment stages, several are associated with clinically relevant organisms. For example, *tetW*, a tetracycline resistance gene, is commonly found in *Clostridium difficile*

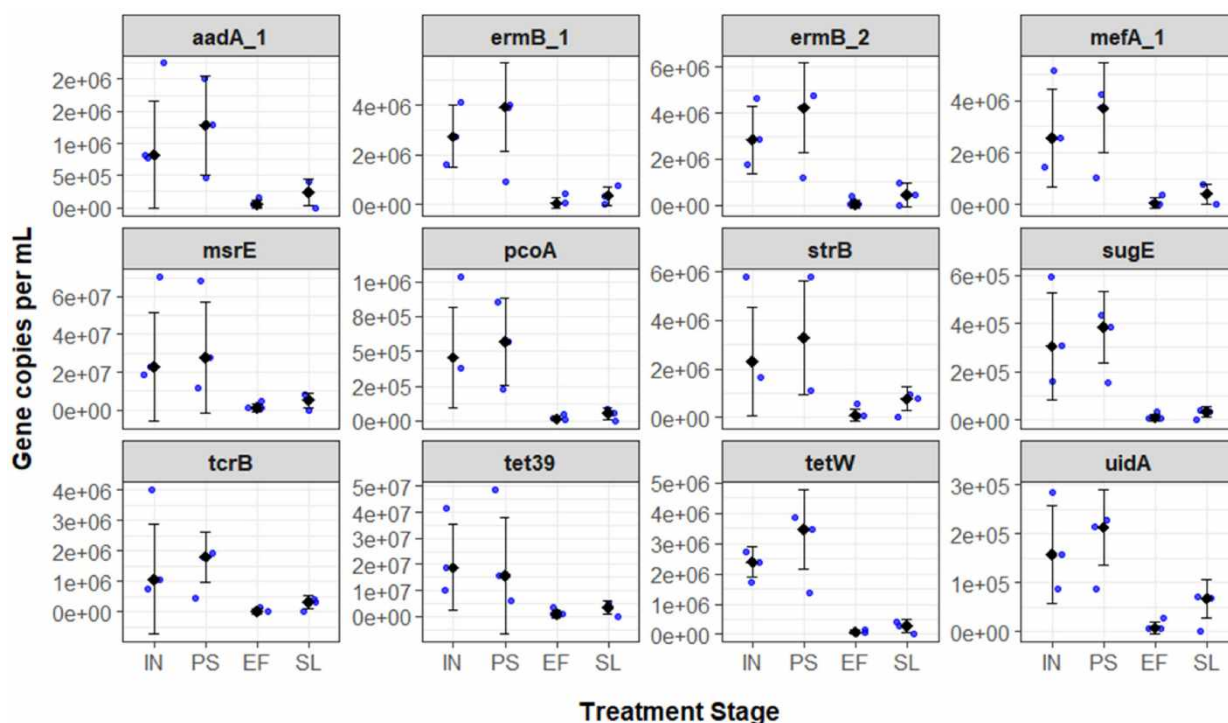


Figure 6 | Dot plots of ARGs with significant differences across treatments. Dot plot shows the significant ARGs as per KW test ($p < 0.05$) across the different treatment stages, which highlight their effective response to the primary and secondary treatment stages.

and *Bacteroides fragilis*, which are part of the gut microbiota and linked to healthcare-associated infections (Scott *et al.*, 2000; Baines & Wilcox 2015). MLSB-associated resistance gene *ermB* occurs in *Streptococcus pneumoniae* and *Enterococcus faecalis*, both of which are important in respiratory and bloodstream infections (Kresken *et al.* 2004; Soares *et al.* 2023). The aminoglycoside resistance gene *aadA1* has been detected in *Salmonella enterica* and *Shigella flexneri*, pathogens of concern in gastrointestinal disease (Havlickova *et al.* 2009; Peng 2024). The MDR-associated gene *sugE* is known to occur in *Escherichia coli* and *Klebsiella oxytoca* (Zou *et al.* 2014; Sajeew *et al.* 2023), while *acrB* has been linked to *Enterococcus faecium* in both animal and human infections (Amachawadi *et al.* 2013). The observed reductions suggest that the WWTP partially limits the environmental release of ARGs associated with high-risk organisms.

While the conventional threshold for statistical significance is $p < 0.05$, our interest was not only limited to identifying genes that significantly change across treatment stages but also in recognising those that remain consistently abundant regardless of treatment. To identify candidate persistent genes, we applied a two-criteria approach: (1) genes were required to maintain abundances within the same order of magnitude across all treatment stages and (2) among these genes, the ones showing the least variation in abundance were identified using a KW p -value threshold of $p > 0.3$ as a filtering criterion. We acknowledge that non-significance in a KW test with small sample sizes does not confirm stability, as it may reflect limited statistical power. However, the genes identified through this approach maintained abundances within the same order of magnitude from influent to effluent, in contrast to genes classified as significantly reduced, which showed reductions of one or more orders of magnitude (e.g., *aadA_1*: from 10^6 gc/mL in IN to 10^4 gc/mL in EF). This difference in magnitude provides additional support for their classification as candidate persistent genes, beyond the statistical filter alone. These identified genes are shown in Figure 7 and included ARGs such as *aadA7*, *blaOXY*, *qepA*, and *vanA* with *qepA* showing the least variation between treatment stages ($p = 0.47$). The lack of significant reduction suggests that these genes were not effectively removed during treatment and were detected at comparable levels in both influent and effluent. Such patterns are concerning, as they may indicate these genes persist throughout the system due to their association with stable host organisms or protective biofilm environments.

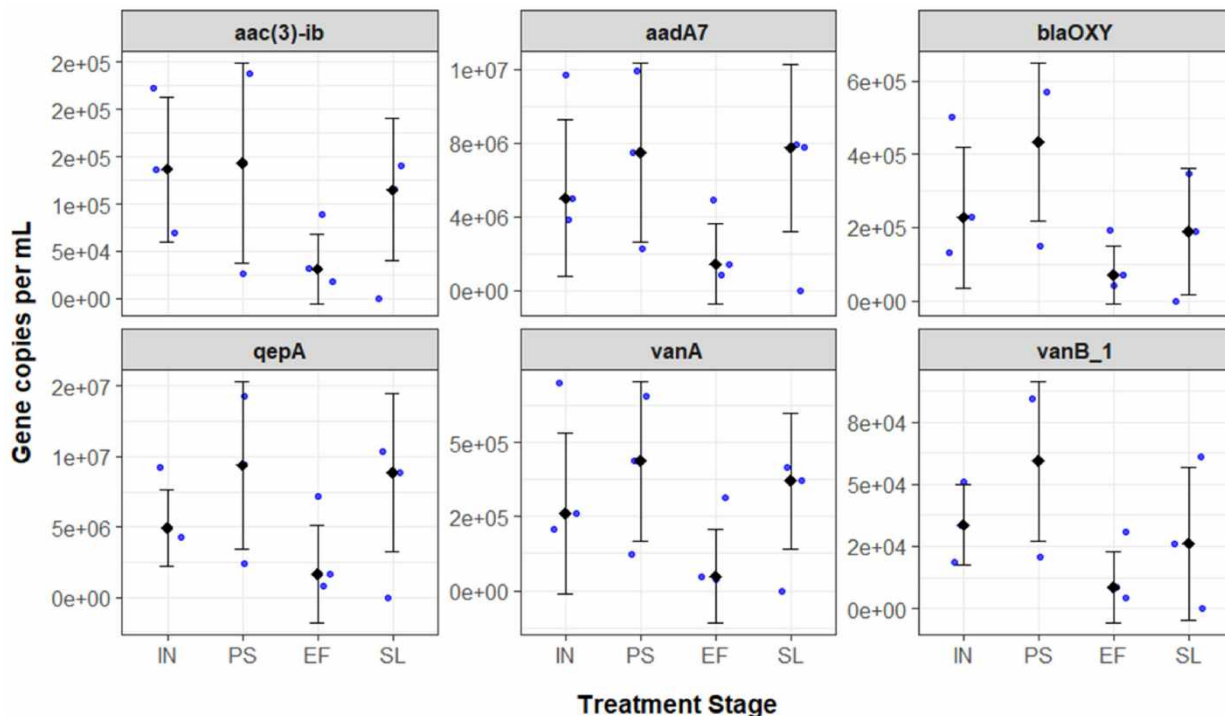


Figure 7 | Dot plots of ARGs with least significant differences across treatment. Dot plot shows the least significant ARGs as per KW test ($p > 0.3$) and consistent detection across the different treatment stages which highlight their persistence through the treatment stages and their potential for Horizontal Gene Transfer and spreading into the environment.

Although culture data were limited to *E. coli*, many of the ARGs identified as poorly removed during treatment, such as *blaOXA*, *qepA*, *vanA*, and *aadA7*, are associated with clinically important organisms beyond *E. coli*. For example, *blaOXA* genes are frequently detected in *Acinetobacter baumannii* and *Klebsiella pneumoniae*, both notable for carbapenem resistance and hospital outbreaks (Hou & Yang 2015; Li *et al.* 2022). *qepA* has been reported in *Shigella spp.* and fluoroquinolone-resistant *E. coli* (Yanat *et al.* 2016; Webb *et al.*, 2019), while *vanA* and *vanB* are commonly found in vancomycin-resistant *Enterococcus faecium* (Hammerum *et al.* 2024).

Their detection in treated wastewater is not unusual and reflects the complexity of ARG dynamics in biological treatment systems. This interpretation aligns with studies that have similarly identified ARGs persisting through wastewater treatments (Majeed *et al.* 2021). The findings from the study conducted in Thailand detected *qepA*, *vanA*, and *vanB* in both influent and effluent of a tropical WWTP, suggesting incomplete removal of these genes through conventional treatment (Srathongneam *et al.* 2024). The presence of the same genes at comparable levels before and after treatment in this study underlines the importance of continued monitoring to better understand the range of resistance genes present and their potential link to upstream microbial communities, rather than indicating any operational shortcoming.

ARGs across influent catchments

The same analysis approach was applied to samples from the three influent catchments. Of the 96 genes tested, only one gene (*lnuC*) showed a statistically significant difference between the catchments ($p < 0.05$) (Figure 8). This suggests a relatively uniform resistome composition across the three catchments during the sampling period and that the AMR profile was similar at geographically close catchments in NE England despite containing different waste input sources, upstream populations, and flow characteristics. *lnuC* was significantly more abundant in catchment 2 (IN2) and is associated not only with *Streptococcus agalactiae* but also with *Clostridium difficile* and *Staphylococcus epidermidis*, indicating a broad spectrum of potential host organisms and highlighting diverse upstream sources of AMR (Imwattana *et al.* 2021; Soni *et al.* 2022).

Although only one gene was statistically distinct, the method highlights a pathway for pinpointing localised AMR sources in larger systems. The shared profile supports the representativeness of combined influent sampling, a valuable insight for surveillance and monitoring strategies in urban settings where residential and industrial inputs are interconnected. It also suggests that catchments like those in this study could be explored as candidate reference sites for future comparisons with catchments receiving hospital or major industrial discharges, though this would require validation across broader

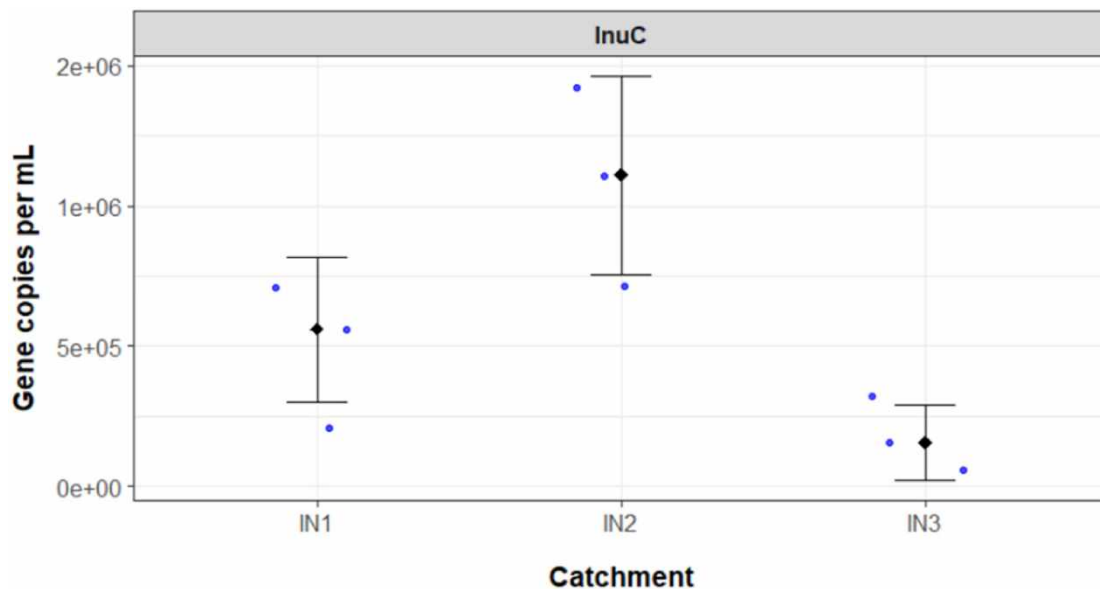


Figure 8 | Dot plots of ARG with least significant differences across influent catchments. Dot plot shows *lnuC* gene, associated with lincosamide resistance, was the only gene found to differ significantly in abundance between the three influent catchments (Kruskal–Wallis test, $p < 0.05$). This spatial variation may reflect localised antimicrobial use or differing microbial inputs within the sewer sub-catchments.

temporal and seasonal scales. While catchment 2 (IN2) had higher flow due to its higher depth, which was driven by its substantially higher upstream contributions (Table 1) and comparable channel width, the overall similarity in absolute ARG abundances across catchments is consistent with the comparable microbial concentrations observed (16S rRNA gc/mL were of the same order of magnitude across all three inlets). This pattern parallels the physicochemical data, where parameters such as ammonia also fell within similar ranges across influents despite differing catchment sizes (Table 2). In domestic catchments within the same region, larger populations generate proportionally greater wastewater volumes, maintaining similar per-volume concentrations. This finding suggests that, in systems without direct medical or pharmaceutical inputs, ARGs may stabilise due to population mixing and continuous flow in the sewer network.

Identification of candidate AMR biomarker

This study explored whether a subset of ARGs could serve as practical indicators of AMR patterns observed across wastewater samples. The aim was not to propose a universal surveillance protocol, but to identify a small set of genes that could summarise broader trends within the current dataset. To identify potential biomarkers for AMR monitoring in this WWTP, we evaluated ARGs using four criteria: (1) average abundance across samples, (2) correlation with other ARGs, (3) unique spatial detection patterns, and (4) clinical relevance. These factors were selected to prioritise genes that are not only representative of total resistance but also detectable, interpretable, and actionable measures in routine monitoring efforts.

Among the 72 genes analysed, aminoglycoside gene *aadA7*, sulfonamide gene *sul1_2*, and beta-lactam gene *blaOXA48* consistently emerged as strong candidates. *aadA7* and *sul1_2* were among the most abundant genes across all 16 samples and exhibited strong positive correlations with the broader resistome. This suggests that changes in their abundance may reliably reflect shifts in the overall AMR profile.

In contrast, *blaOXA48* displayed an irregular detection pattern, being detected in both influent and effluent, but absent from intermediate stages. Even though detections were sporadic and at low abundances, *blaOXA48* was included as a candidate biomarker not on the basis of this spatial pattern alone, but due to its clinical relevance as a carbapenemase gene of critical concern. All three genes are clinically relevant; *aadA7* and *sul1_2* are commonly associated with multidrug-resistant Enterobacteriaceae via Integrons, while *blaOXA48* encodes a carbapenemase of critical concern in hospital-acquired infections.

To further assess representativeness, we performed principal component analysis on gene abundance profiles. *aadA7* and *sul1_2* showed high eigenvector loadings in the first principal component (PC1 > 0.12), which explained over 54% of the variance in the dataset. Although *blaOXA48* had lower loadings (0.08–0.09), it remained higher than most other ARGs, justifying its inclusion based on its distinct spatial pattern. In summary, *aadA7* and *sul1_2* are proposed as generalist biomarkers due to their high prevalence, broad correlation, and association with commonly used antimicrobials. The third gene *blaOXA48*, while not broadly representative, offers targeted insight into beta-lactam resistance behaviour within treatment stages. This combination of generalist and specialist indicators could enable more nuanced AMR surveillance in wastewater systems.

CONCLUSION

This study provides an integrated assessment of AMR trends across similar catchments and the associated WWTP with no hospital input. The study's aims of (1) assessing AMR reduction, (2) identifying persistent versus responsive ARGs, and (3) establishing a domestic reference profile to build evidence base for future wastewater surveillance were fulfilled. Although reductions were observed in expected physicochemical parameters and culturable *E. coli*, some ARGs persisted in the WWTP effluent, emphasising the passage of AMR through conventional treatment processes, particularly in systems employing trickling filters. Correlation analysis did not identify significant connections between physicochemical parameters, microbial counts, and ARG abundance in this WWTP. However, the KW test revealed that selected ARGs showed significant differences across treatment stages (e.g., *tetW*, *ermB_1*, *aadA_1*), which may be useful as practical indicators of reduction in AMR abundance for this WWTP. In contrast, persistent genes such as *qepA*, *aadA7*, and *vanA* warrant attention due to their continued detection in effluent and likely release into receiving waters. The similarity of AMR profiles across the three sub-catchments during the study period, none of which receive hospital discharge, suggests this site warrants further investigation as a potential reference for evaluating systems with stronger anthropogenic influences, such as hospital or industrial inputs. This study also identified three ARGs, *aadA7*, *sul1_2*, and *blaOXA48* as potential biomarkers for

tracking the resistance patterns in wastewater from this WWTP, offering practical markers for future surveillance studies in small-scale WWTPs with no hospital input.

Distinguishing between responsive and persistent ARGs across treatment stages and catchments can offer valuable insights into treatment efficiency and inform future AMR monitoring strategies. These findings also highlight the limitations of a conventional secondary treatment in mitigating AMR releases despite its relatively effective treatment. Finally, deliberately targeting a system with an absence of hospital inputs contributes to the underlying evidence base for domestic-only wastewater, which may be valuable for future comparisons with hospital-influenced catchments. Identifying similar control sites elsewhere in the United Kingdom will be important for strengthening national wastewater surveillance efforts. These findings can support the refinement of AMR monitoring protocols and provide a platform for better identifying higher priority genes for future WS monitoring, particularly in systems with similar treatment technologies or demographic inputs.

Limitations of the study

This study has a few limitations that should be considered while interpreting the findings. Sampling was conducted weekly on Wednesday mornings over 7 weeks (October–December 2022), capturing only autumn/winter conditions. Seasonal variation in antibiotic prescribing, microbial activity, and rainfall-driven dilution may influence ARG profiles. Stage 2 analysis was constrained to 3 weeks (weeks 3, 5, and 7) due to the cost of high-throughput molecular analysis and sludge availability, reducing statistical power; non-parametric tests and conservative filtering were applied to account for this, though the classification of persistent genes should be considered preliminary. Volumetric flow data were unavailable, so reported reductions in ARG abundance are concentration-based and may be influenced by dilution or volume partitioning across treatment stages. Cefotaxime-resistant *E. coli* was used as a phenotypic indicator of ESBL production, but without confirmatory testing, some isolates may carry alternative resistance mechanisms such as AmpC β -lactamases. Finally, the findings are specific to this WWTP and its domestic catchments in Northeast England. Environmental, geographical, and demographic factors may influence ARG profiles in other communities, and validation across different settings and seasons is needed.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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