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Temporal and spatial variation of third-generation cephalosporin-resistant and ESBL-producing *Escherichia coli* in a Swedish urban water system

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ABSTRACT

Third-generation cephalosporin-resistant *E. coli* (3GCR-Ec), including extended-spectrum beta-lactamase *E. coli* (ESBL-Ec), have been detected in environmental water, and are likely discharged by wastewater treatment plants (WWTPs). This study examined the occurrence of 3GCR- and ESBL-Ec in the Swedish urban river Fyrisån, Uppsala, from March to November 2023. Water samples were collected every two months from four sites: upstream of the WWTP (upWWTP), near the WWTP effluent (WWTP), downstream of the WWTP (dWWTP), and Lake Mälaren. Samples were analyzed for 3GCR-Ec and ESBL-Ec using culture- and sequence-based methods. A total of 40 3GCR-Ec isolates were subjected to antibiotic sensitivity testing via microtiter plates and whole-genome sequencing (WGS), followed by in-silico analysis. Significantly higher concentrations of 3GCR-Ec and ESBL-Ec were found at the WWTP compared to Lake Mälaren ($P<0.05$). When comparing WWTP and upWWTP, significantly higher concentrations of 3GCR-Ec ($P<0.05$) and borderline significantly higher concentrations of ESBL-Ec ($P = 0.0556$) were detected at WWTP. All tested *E. coli* isolates ($n = 40$) were multidrug-resistant (MDR), with no resistance to imipenem or meropenem. The virulence- and MDR-associated sequence types ST69, ST10, ST1193, and ST1722 were present at multiple sampling points, while ST131 was found only downstream of the WWTP in March and November. Nearly identical strains were detected at WWTP and dWWTP, indicating WWTP as the origin and persistence of resistant strains in the river. Antibiotic-resistance genes (ARGs) were detected in all isolates; 65 % of isolates carried an ESBL gene, and 43 % carried $bla_{CTX\text{-}M\text{-}15}$, common in clinically relevant strains. By tracking resistance dynamics over space and time using WGS, this study highlights the role of WWTPs in the dissemination of antimicrobial resistance in aquatic environments and stresses the need for enhanced surveillance and improved sewage treatment.

1. Introduction

Antimicrobial resistance (AMR) is a critical global health issue with severe consequences. In 2022, an estimated 1.27 million deaths were attributed directly to AMR globally, with an additional 4.95 million deaths in 2019 involving AMR as a contributing factor. This number is predicted to increase to 8.22 million deaths, where AMR is a contributing factor, by 2050 [38,39]. This stems from multiple factors, including overuse of antibiotics in medical and agricultural settings and inadequate treatment of wastewater from anthropogenic and industrial sources. A One Health approach, integrating human, animal, and environmental health, has been widely advocated to address the multifaceted nature of AMR [1].

At present, the most commonly used cephalosporins in clinical settings belong to the third generation of this class. They are an integral part of managing severe infections and play an essential role in managing complications from advanced medical procedures, such as organ transplantation and immune-modulating therapies. Extended-spectrum beta-lactamases (ESBL) represent an antibiotic resistance mechanism occurring in bacteria globally, conferring resistance to the beta-lactam class of antibiotics, including cephalosporins [52]. The rise of ESBL-producing bacteria, including ESBL-producing *E. coli* (ESBL-Ec), has become a pressing healthcare concern. In 2021, resistant *E. coli* was among the three leading pathogens in resistance-related mortality globally, making up approximately 14 % of all deaths attributable to AMR in individuals over the age of five [39]. Among ESBLs, enzymes with potent hydrolytic activity against cefotaxime (*CTX*), such as *CTX-M-15*, have become dominant worldwide [6,57]. The rapid and massive spread of *CTX-M* enzymes is changing the ESBL epidemiology, leading to increased use of carbapenem-class antibiotics and highlighting the need for effective strategies to monitor current and emerging trends in the spread of these enzymes [6].

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The spread of antibiotic-resistant bacteria (ARB) from anthropogenic sources to nearby ecosystems is well documented [13,50]. Hospital and municipal wastewater effluents are contributors to the dissemination of ESBL-Ec [14]. For example, Davidova-Gerzova et al. [14] demonstrated the spread of MDR *E. coli* from such wastewater into rivers in the Czech Republic, illustrating the transmission pathways of resistant strains into surface waters. However, data from countries with low AMR prevalence, such as Sweden, remain limited. A recent systematic review from the Nordic countries further showed that carbapenemase- and ESBL-producing *E. coli* and *Klebsiella* spp. are commonly detected in both clinical samples and wastewater, highlighting the need for wastewater-based surveillance for early detection of ARB emergence [50]. The World Health Organization has proposed ESBL-Ec concentrations in environmental samples as a global surveillance indicator in a project called "Global Tricycle Surveillance", which specifically measures the presence and abundance of ESBL-Ec across human, food sources, and environmental sectors (World Health [54]).

The use of antibiotics in society eventually leads to dissemination of ARB and antibiotic resistance genes (ARGs) in the environment [26]. Antibiotics and their active metabolites are frequently discharged into wastewater at sub-minimally inhibitory concentrations (MICs), creating a selective environment that favors bacteria harboring ARGs [58]. In this environment, horizontal gene transfer (HGT) facilitates the spread of resistance genes to previously susceptible bacteria [11].

While many studies have shown the presence of ESBL-producing bacteria in urban surface waters globally [13,14,19], temporal and spatial variations, especially with regard to genetic relatedness of isolates, persistence of specific clones, and distribution of ARGs, remain poorly understood. This is particularly relevant in high-income countries, where the prevalence of AMR is low, such as Sweden [49]. The extent to which wastewater treatment plants (WWTPs) contribute to the selection and dissemination of resistance in such settings remains unclear. A recent comprehensive systematic review on ARB and ARG in WWTPs [29] suggests that positive selection of ARB during the wastewater treatment process contributes to the abundance of ARB in WWTP effluents. This selection is likely driven by sub-inhibitory concentrations of antimicrobial compounds present in wastewater from public and hospital sources, highlighting the need to understand the dissemination and persistence of bacteria released from WWTPs into surface waters. Studies evaluating the effectiveness of ESBL-Ec removal in WWTPs report varying results, with reductions in ESBL-Ec concentrations ranging from 51 % to 99.9 % after treatment [3,28].

Data show a linear relationship between the proportion of antibiotic-resistant *E. coli* found in wastewater treatment plants and isolates from respective nearby clinics in European countries [22]. Specifically, the study by Huijbers et al. [22] found that, across ten European countries, resistant *E. coli* in wastewater mirrored the geographic trends observed in clinical isolates, with strong correlations for aminopenicillins and fluoroquinolones. However, the extent to which treated effluents contribute to AMR dissemination further downstream, and how this varies over time, remains unexplored. According to a recent meta-analysis on wastewater surveillance strategies in Nordic countries, recipient surface waters receiving wastewater from WWTPs were reported to have an increased prevalence of ARB, ARGs, and mobile genetic elements [50]. A review article on ESBL-producing Enterobacteriaceae in surface waters highlighted the need for further research on the fate of ARB in surface waters and called for a "human health risk assessment to evaluate the impact of their presence" [13]. Considering the global scale of the AMR challenge, the severity of its consequences if left unaddressed, and the current uncertainty regarding the extent to which WWTPs contribute to the dissemination of ARB and ARGs over time, there is a critical need for studies that provide detailed insights into this subject.

To address this, our study's hypothesis was that WWTPs contribute to the dissemination and persistence of 3GCR- and ESBL-Ec, and their associated ARGs, in urban aquatic environments over space and time. In this study, we investigated the presence and abundance of 3GCR-Ec and ESBL-Ec in surface waters receiving effluent from a municipal WWTP in Sweden over a nine-month period. To the best of our knowledge, this is one of the few studies that goes beyond CFU quantification or gene detection in a Swedish water system by exploring the genetic relatedness of isolates, the presence of clinically relevant clones, and the distribution of ARGs over space and time. By combining WGS with temporal and spatial sampling, our study provides novel insights into how WWTPs may act as both reservoirs and points of release for MDR *E. coli* clones and ARGs. By further integrating genotypic and phenotypic resistance profiles, we provide novel insights into the environmental persistence and dissemination of specific clones and ARGs, highlighting the role of WWTPs in AMR environmental dissemination and the potential public health impact.

2. Materials and methods

2.1. Study design and sampling

This study was conducted in Uppsala, the fourth-largest city in Sweden, located 71 km north of the capital, Stockholm. As of 2023, Uppsala has an estimated population of approximately 245,329 ([55], n.d.). Water sampling was carried out at five time points (March, May, July, September, and November in 2023) in the Fyrisån river (59°47′12″N 17°39′20″E), which flows through Uppsala. Samples were collected from four designated points, always starting at the lake (Mälaren), followed by the sampling point downstream of the WWTP (dWWTP), the sampling point next to the WWTP (WWTP), and finally upstream of the WWTP (upWWTP) (Table S1). The sampling point next to the WWTP is located near the effluent discharge of Uppsala's municipal WWTP, which employs mechanical, biological and chemical treatment processes before discharging effluents into the river. Sampling was conducted around the same time of the day, between 9:00 am and 11:00 am. The distances between the collection points ranged between 1.7 to 4.1 kilometers, with precise coordinates detailed in Table S1. At each location, three biological replicate grab samples of 250 ml river water were collected using autoclaved, sterile glass bottles, amounting to 60 samples overall (Table S1). The bottles were submerged approximately 50 cm from the water surface to avoid surface contamination. Collected samples were immediately stored at 4 – 6 °C in a cooler box with ice blocks and processed for microbiological analysis within 24 h.

2.2. Microbiology

3GCR-Ec were isolated from environmental water samples by filtering 100 ml of undiluted water through 0.45 µm nitrocellulose filters (Cytiva, Japan). The filters were directly placed onto selective chromogenic agar plates (CHROMagar™ C3GR, Paris, France) containing a third-generation cephalosporin, and incubated overnight at 37 °C. Presumptive *E. coli* colonies, appearing as pink colonies on the chromogenic plates, were sub-cultured overnight at 37 °C for species confirmation. From each sampling location, four to six pink colonies were randomly selected for species confirmation using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) at the Department of Clinical Microbiology, Uppsala University Hospital (BRUKER). The proportion of colonies identified as *E. coli* by MALDI-TOF was used to estimate the total number of 3GCR-Ec on the plate (e.g., if 3 out of 6 pink colonies were *E. coli* by MALDI-TOF, a total of 50 pink colonies on the plate was estimated as 25 colonies of 3GCR-Ec). The confirmed *E. coli* colonies were preserved in 15 % glycerol-containing LB (Luria-Bertani) broth (BD Difco™) and stored at -80 °C for subsequent analysis. The concentration of 3GCR-Ec at each sampling time/point was calculated as the average concentration of the three biological replicates (in CFU/100ml).

2.3. Antimicrobial susceptibility test

A total of 40 3GCR-Ec isolates were selected to determine their minimum inhibitory concentrations (MICs) against 21 antibiotics using Sensititre™ Gram Negative GNX3F AST plates (TREK Diagnostic system Ltd., West Sussex, UK), following the manufacturer's instructions. Briefly, after overnight incubation on LB at 37 °C, 2-3 single colonies were dissolved into 5 ml of 0.9 % sterile normal saline. The optical density (OD) of the suspension was adjusted to 0.5 McFarland standard using a Sensititre™ Nephelometer (Thermo Fisher Scientific, US). Subsequently, 50 µl of the adjusted inoculum was transferred into 10 ml of Muller-Hinton broth (NutriSelect® Plus, Sigma-Aldrich), and 50 µl of the working inoculum was dispensed into each well of the GNX3F plate (Thermo Fisher Scientific, US). The plates were incubated overnight at 37 °C. The MIC breakpoint results were interpreted following the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (European Committee on Antimicrobial Susceptibility Testing [[17]ST], 2025).

2.4. DNA extraction and sequencing

In this study, we modified the boiling method described by Bollet et al. for DNA extraction [7]. Briefly, 4 to 6 bacterial colonies were suspended in 200 µl of distilled water in 1.5 ml microfuge tubes and microwaved at 650 W for 4 min. The suspensions were centrifuged at 13,330 rpm for 2 min using a MICRO STAR 17 centrifuge (VWR, Belgium), and the supernatant containing genomic DNA was transferred into new sterile 1.5 ml microfuge tubes. The quality of the extracted DNA was assessed for protein contamination based on the A_{260}/A_{280} ratio using a Nanodrop (Thermo Scientific NanoDrop 1000 UV-VIS Spectrophotometer, USA). DNA concentration was quantified at A_{260} using Qubit 2.0 (Thermo Fisher Scientific, USA). The DNA samples were sent to Novogene for whole genome sequencing (WGS), performed using the Illumina platform if the concentration of genomic DNA was above 100 ng/µl and the A_{260}/A_{280} ratio was 1.8-2.0.

2.5. Genomic assembly and multi-locus sequence typing

Isolates were labeled based on their sampling location (A = upWWTP, B = WWTP, C = dWWTP, and D = Lake Mälaren), replicate number, and the sampling date (DDMMYY) (Table S2). De novo assembly of Illumina raw reads was performed using the EnteroBase database tools [59]. The QAssembly pipeline v3.61 employed Sickle v1.33 [24] for quality- and adapter-trimming and for removing bases with quality scores below 10 [59]. Trimmed reads were assembled using SPAdes v3.9.1, with k-mer sizes of 21, 33, 55, and 77 [42]. Reads were aligned back to the assemblies to improve consensus base call accuracy using BWA v0.7.12-r1039 [27]. Quality control of the assemblies was performed using established criteria for the *Escherichia* genus, including genome size (3.7–6.4 Mbp), N50 values greater than 20kb, number of contigs ≤ 800, proportion of scaffolding placeholders (N's) below 3 %, and at least 70 % of contigs assigned as *E. coli* using Kraken v0.10.5-beta [53]. A second verification of genome quality was performed with CheckM v1.1.3 [40], ensuring completeness above 99 % and contamination below 1 % (Table S2). The assembled genomes were further analyzed to assign their MLST using the NServ genotype integrated into the EnteroBase database [59,60].

2.6. Pangenome and phylogenetic analysis

Genomes were annotated using prokka v1.14.6 [45] and annotations, including the well-annotated reference *E. coli* strain K-12 (NC_000913.3), were used as input for PIRATE v1.0.5 [4], as described before [37]. Briefly, gene orthologs were classified as gene families after classification at various MCL thresholds using default parameters. A core gene-by-gene multiple sequence alignment was generated using MAFFT, consisting of core genes present in >95 % of all isolates. A phylogenetic tree was reconstructed based on a core gene-by-gene alignment, using the maximum-likelihood algorithm implemented in RAxML v8.2.11 [48], with GTRGAMMA as a substitution model. The degree of genetic similarity between isolates was investigated by calculating the average nucleotide identity (ANI) of all 40 genomes using FastANI v1.33 [23].

2.7. Identification of putative antibiotic resistance determinants

All *E. coli* genome assemblies were screened for the presence of ARGs using ResFinder v4.6.0 [10,12]. A locus match was defined as >90 % sequence identity over >70 % of the gene sequence length. A gene presence/absence matrix was generated to include information for each gene.

2.8. Estimation of ESBL-Ec concentrations

Based on the genetic profiles of the selected 3GCR-Ec isolates, most strains were identified to carry ESBL genes (26/40, Table S3). To compare the distribution of ESBL-Ec spatially and temporally, we estimated the concentration of ESBL-Ec at each sampling location and time point. This estimation was based on the assumption that the proportion of ESBL-Ec among the total 3GCR-Ec remains relatively constant over time at the same sampling location, as it should receive a similar influx of *E. coli* types. Thus, the proportion ESBL-Ec/3GCR-Ec was calculated for sequenced 3GCR-Ec isolates from each sampling site. For each water sample, the 3GCR-Ec concentration was then multiplied by the ESBL-Ec/3GCR-Ec proportion of the corresponding sampling location to obtain the estimated ESBL-Ec concentration.

2.9. Statistical analysis

The Shapiro-Wilk normality test was implemented using the RStats package from R Commander v4.4.1 [20,43] to assess the distributions of the 3GCR-Ec and ESBL-Ec concentrations (measured as CFU/100ml) for each sampling location. Concentrations of 3GCR-Ec were normally distributed at WWTP ($P = 0.2496$) and dWWTP ($P = 0.9854$), while they were not at upWWTP ($P = 0.003232$) and Mälaren ($P = 0.000131$). Similarly, for ESBL-Ec concentrations, they were normally distributed at WWTP ($P = 0.2517$) and dWWTP ($P = 0.9842$), but not at upWWTP ($P = 0.003556$) or Mälaren ($P = 0.000131$). Normally distributed concentrations for 3GCR-Ec and ESBL-Ec were compared using the Student's *t*-test, while the Mann-Whitney test was used to compare non-normally distributed groups. These tests were performed using Prism v10.4.0 for Windows 10 (GraphPad Software, Boston, Massachusetts, USA). Differences were considered statistically significant at $P < 0.05$ and indicated with an asterisk ($^* = P < 0.05$). Unless otherwise stated, summary statistics are presented as medians, followed by interquartile range (IQR) in parentheses.

3. Results

3.1. Temporal and spatial variation of 3GCR- and ESBL-Ec in Fyrisån river

Water sampling for 3GCR-Ec was conducted at four locations along the Fyrisån river in Uppsala, Sweden, during March, May, July, September, and November 2023 (Fig. 1A). The concentrations of 3GCR-Ec and estimated ESBL-Ec varied over time (Table S4). In July, far lower concentrations than any other time point were recorded for all locations except for Mälaren (e.g., a 15-fold lower 3GCR-Ec concentration when comparing the average of all locations, except Mälaren, in July to the second-lowest month, September, Fig. 1B, Table S4). Lake Mälaren maintained low concentrations throughout the sampling period (Fig. 1B and D).

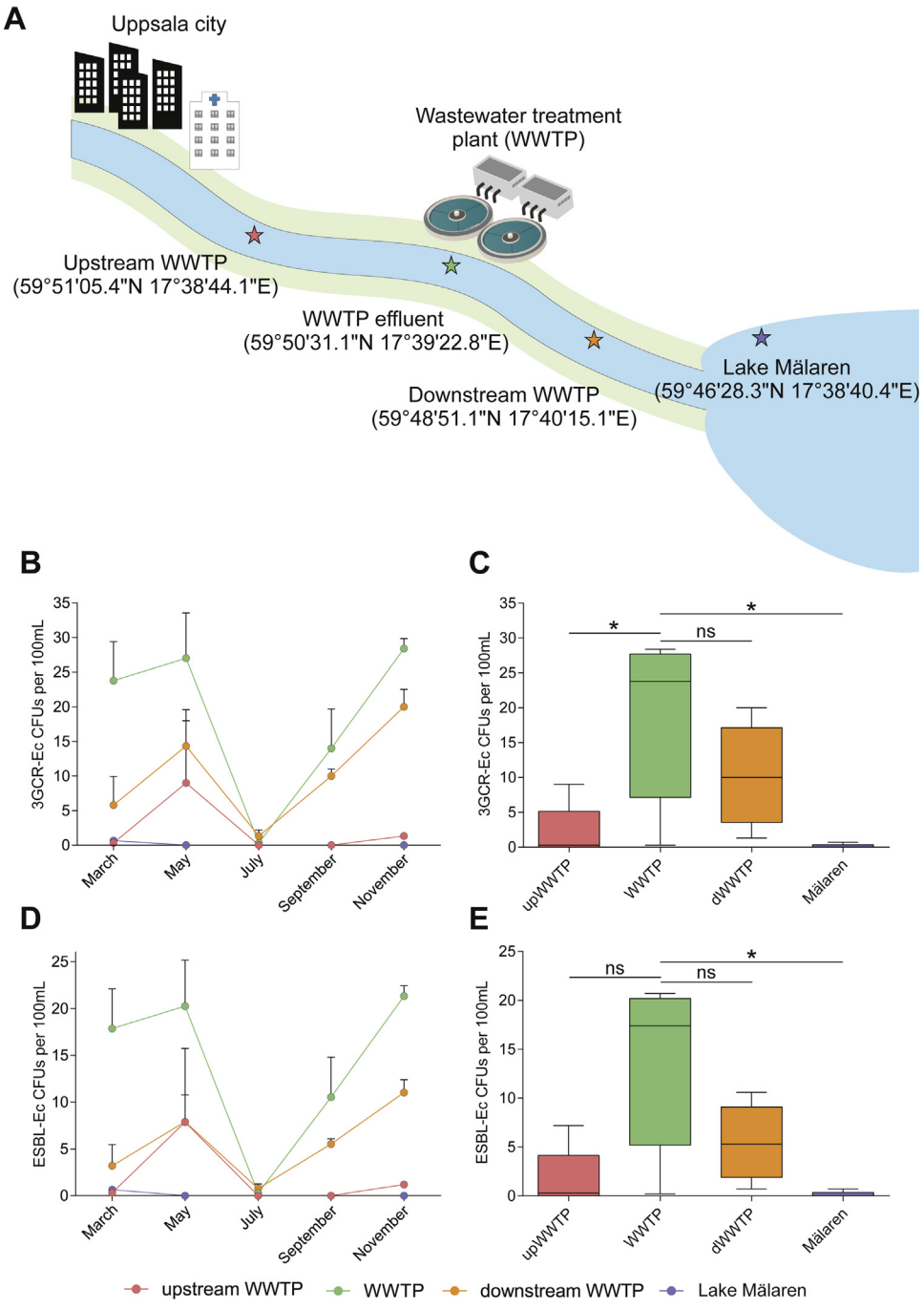

Fig. 1. (**A**) Schematic map of the study area in Uppsala, Sweden, showing the sampling locations along the Fyrisån river: upstream of WWTP (upWWTP, red star), near the WWTP effluent (WWTP, green star), downstream of the WWTP (dWWTP, orange star), and the recipient Mälaren lake (Mälaren, blue star). The exact geographical coordinates are indicated at each sampling site. (**B**) Temporal variation in 3GCR-Ec concentrations in CFU/100mL across sampling points (red = upWWTP, green = WWTP, orange = dWWTP, blue = Mälaren). Error bars represent the standard error of the mean (SEM). (**C**) Box plots showing 3GCR-Ec concentrations in CFU/100mL at each sampling site: upWWTP (red), WWTP (green), dWWTP (orange), and Mälaren (blue). Pairwise comparisons of means (Mann-Whitney U tests) are shown with significance ($P < 0.05$) indicated by an asterisk and non-significance by 'ns'. The center line represents the median value, while the upper and lower bounds of the boxes correspond to the first and third quartiles. (**D**) Temporal variation in ESBL-Ec concentrations in CFU/100mL across sampling points. Error bars represent SEM. (**E**) Box plots showing ESBL-Ec concentrations in CFU/100mL at each sampling site: upWWTP (red), WWTP (green), dWWTP (orange), and Mälaren (blue). Pairwise comparisons of means (Mann-Whitney U tests) are shown with significance ($P < 0.05$) indicated by an asterisk and non-significance by 'ns'. The center line represents the median value, while the upper and lower bounds of the boxes correspond to the first and third quartiles.

The concentrations of 3GCR-Ec at WWTP (median 23.80, IQR: 7.15 - 27.70) were significantly higher than upWWTP (median: 0.30, IQR: 0.00 - 5.150; Mann-Whitney U test, $P = 0.0397$) (Fig. 1C). Similarly, concentrations of 3GCR-Ec at WWTP were significantly higher than at Mälaren (median: 0.0, IQR: 0.0 - 0.35; Mann-Whitney U test, $P = 0.0159$) (Fig. 1C). However, there was no statistically significant difference between the median of WWTP and dWWTP (median 10.00; IQR: 3.55-17.15; Student t-test, $P = 0.2165$) (Fig 1C).

For ESBL-Ec, estimated concentrations at WWTP (median 17.36, IQR: 5.20 - 20.2) were significantly higher than Mälaren (median: 0.0, IQR: 0.00 - 0.3333); Mann-Whitney U test, $P = 0.0159$) (Fig. 1E). A borderline significant difference was observed between WWTP and upWWTP (median: 0.27, IQR: 0.0 - 4.13; Mann-Whitney U test, $P = 0.0556$), while there was no significant difference between WWTP and dWWTP (median: 5.3, IQR: 1.890- 9.098; Student's *t*-test, $P = 0.1021$) (Fig. 1E).

3.2. *High rates of in vitro multidrug resistance among* E. coli *isolates in Fyrisån river*

A total of 40 3GCR-Ec isolates, validated as *E. coli* with MALDI-TOF, were further analyzed for their in vitro antibiotic resistance profile. These were sampled from upWWTP ($n = 5$), WWTP ($n = 15$), dWWTP ($n = 19$), and Lake Mälaren ($n = 1$). The isolates were assessed for resistance to twenty-one antibiotics (Table S5), using the EUCAST criteria. All tested isolates exhibited multidrug resistance (resistance to at least three different antibiotic classes, as defined by [33] (Table S5). The highest resistance rate was observed for ticarcillin/clavulanic acid (38/40; 95 %), followed by cefotaxime (36/40; 90 %) (Table S5). Resistance to tobramycin, ampicillin/sulbactam (33/40; 83 %), aztreonam, and ceftazidime, was also common (32/40; 80 %) (Table S5). In contrast, all isolates were susceptible to imipenem and meropenem (Table S5).

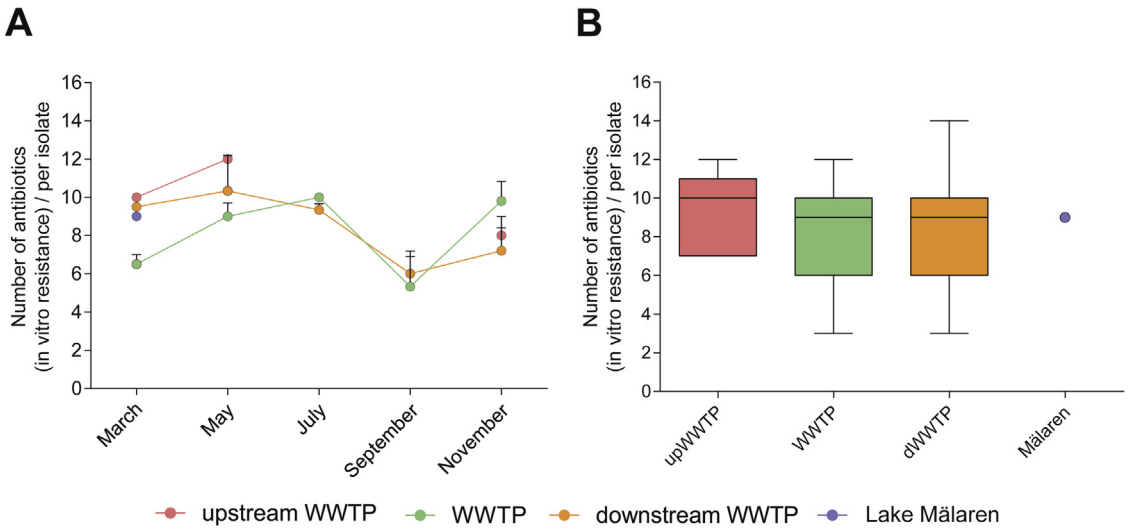

Fig. 2. (**A**) Temporal variation in the number of antibiotics each 3GCR-Ec isolate is resistant to (in vitro resistance based on AST) across sampling points (red = upWWTP, green = WWTP, orange = dWWTP, blue = Mälaren). Error bars represent the SEM. **(B)** Box plots showing the number of antibiotics to which each isolate is resistant to at each sampling site. The center line represents the median value, while the upper and lower bounds of the boxes correspond to the first and third quartiles.

The number of antibiotics to which isolates were resistant remained fairly consistent over time and across locations (Fig. 2A and B). The highest average resistance was observed at upWWTP in May (12 antibiotics), while the lowest was recorded at WWTP in September (5 antibiotics) (Fig. 2A).

3.3. Genetically similar 3GCR-Ec strains detected across multiple locations and time points

To understand the genetic diversity among 3GCR-Ec along the Fyrisån river, a maximum-likelihood (ML) phylogenetic tree was reconstructed based on the gene-by-gene sequence alignment of 3,359 genes present in >95 % of isolates. The 40 3GCR-Ec isolates were assigned to 28 multilocus sequence types (STs) (Fig. 3A, Table S2). Out of these, seven STs were detected at multiple times points, and eight STs were detected at multiple locations along the Fyrisån river (Fig. 3B and C). Three ST69 isolates were obtained from upWWTP in November (1/5; 20 %), WWTP in March (1/15; 6.7 %), and dWWTP in July (1/19; 5.3 %), while four ST10 isolates were obtained from WWTP in March (1/15; 6.7 %) and dWWTP in September (1/19; 5.3 %) and November (2/19; 10.5 %), respectively (Fig. 3B and C). Two ST1193 isolates were obtained from WWTP in November (1/15; 6.7 %) and dWWTP in May (1/19; 5.3 %), while two isolates belonging to ST1695 were detected at upWWTP in March (1/5; 20 %) and at WWTP in September (1/15; 6.7 %), respectively (Fig. 3B and C). Similarly, three ST1722 isolates were obtained from WWTP in May (1/15; 6.7 %), and dWWTP in May (1/19; 5.3 %) and November (1/19; 5.3 %), respectively (Fig. 3A and C). Two ST88 isolates were obtained from the WWTP (1/15; 6.7 %) and dWWTP (1/19; 5.3 %) in July, while two ST38 isolates were obtained from the WWTP in May (1/15; 6.7 %) and November (1/15; 6.7 %) (Fig. 3A and C). Lastly, two ST131 isolates were obtained from dWWTP in March (1/19; 5.3 %) and November (1/19; 5.3 %), respectively (Fig. 3A and C).

To assess the level of genetic similarity among isolates of the same STs and identify potential cases where the same strain is isolated from different time points and locations, we calculated the pairwise average nucleotide identity (ANI) for shared genes across all genome pairs. High ANI values (>99.96 %) were observed in two ST88 genomes (id: B2_2_100723 and id: C2_2_100723) and two ST1722 genomes (id: B3_SP_310523 and 5_C3_SP_1_310523) (Table S6). The ST88 and ST1722 isolates were obtained from WWTP and dWWTP in July and May, respectively (Fig. 3A and C). These findings suggest that certain strains may persist in this environment.

3.4. Multiple and diverse antibiotic resistance genes detected in 3GCR-Ec isolates

To obtain a comprehensive in silico resistance profile, we screened all genomes for the presence of AMR genetic determinants using ResFinder [10]. We identified a total of 50 ARGs, conferring predicted resistance to beta-lactams, sulfonamides, trimethoprim, aminoglycosides, macrolides, amphenicol, tetracyclines, and fluoroquinolones (Table S3). The number of ARGs detected in each isolate varied over time and space, with the highest number of ARGs (median numbers or actual numbers if only one isolate present) detected in isolates at WWTP and dWWTP in July (13 ARGs), and the lowest at Lake Mälaren in March (3 ARGs) (Fig. 4A). No statistically significant differences were found in the ARG numbers per isolate per location (Fig. 4B).

ESBL-associated genes from the bla$_{CTX\text{-}M}$ group were highly prevalent, detected in 63 % (25/40) of all isolates across all sampling months (Fig. 4C, Table S3). The most common of those genes, bla$_{CTX\text{-}M\text{-}15}$, was detected in 43 % (17/40) of all isolates and found at all locations and in every sampling month except for July. Its distribution was as follows: (2/6) in March, (3/8) in May, (6/9) in September, (6/13) in November, and (2/5) at upWWTP, (8/15) at WWTP, (6/19) at dWWTP, and (1/1) at Mälaren (Fig. 4C, Table S3). The *sul1* gene, conferring sulfonamide resistance, was also detected in 53 % of the isolates (21/40) (Fig. 4C, Table S3). Moreover, the gene *aac(6')-Ib-cr*, which confers predicted combined resistance to aminoglycosides and quinolones [46], was detected in 5 % (2/40) of the isolates (Fig. 4C, Table S3). One isolate, obtained from dWWTP in July, carried the colistin resistance gene *mcr-9* as well as the ESBL-encoding gene bla$_{SHV\text{-}12}$ (Fig. 4C, Table S3). Phenotypically, this isolate was resistant to polymyxin B but sensitive to colistin, in line with previous findings that this gene does not always confer phenotypic colistin resistance [8,51].

4. Discussion

Our results demonstrate a significantly higher concentration of 3GCR-Ec comparing the sampling point just by the WWTP outlet and the point upstream of the WWTP, and a borderline significant higher

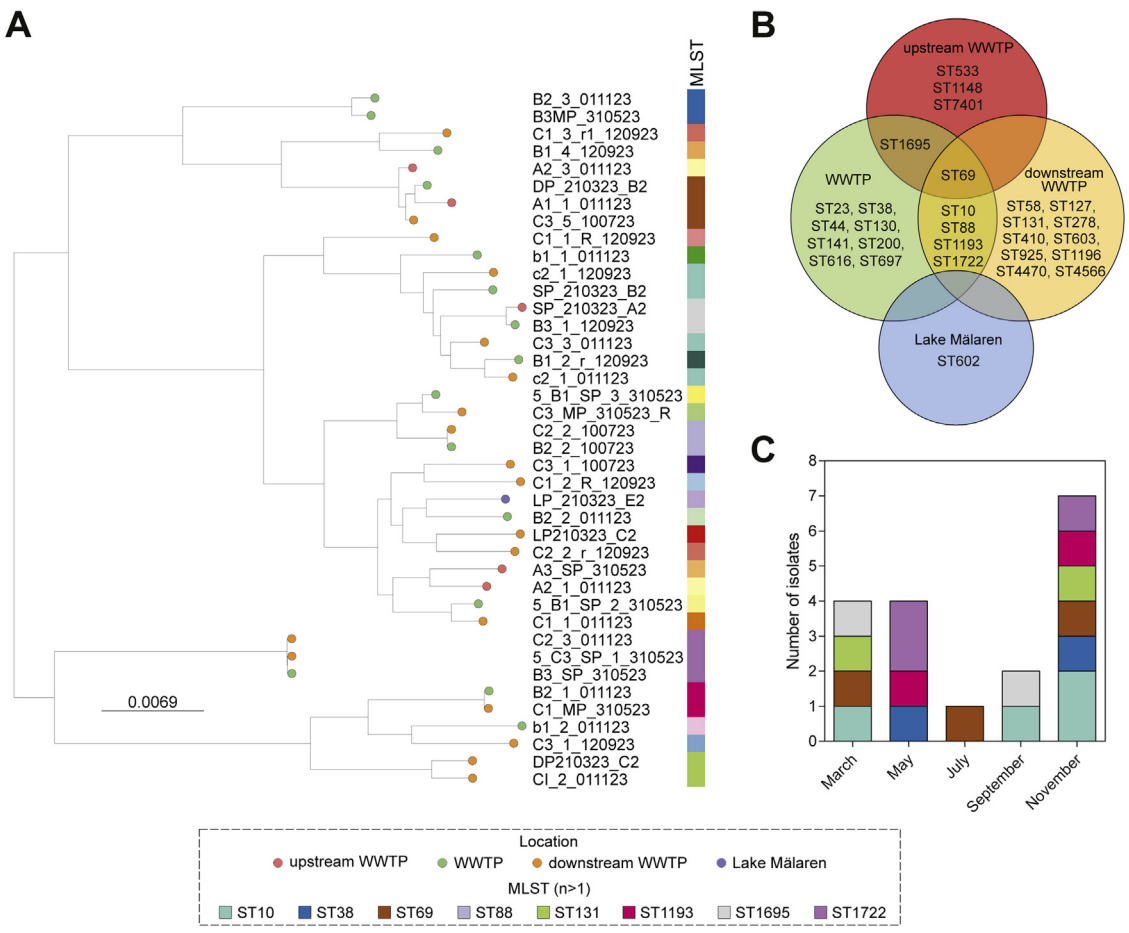

Fig. 3. **(A)** Phylogeny showing the population structure of the 40 3GCR-Ec strains reconstructed a maximum-likelihood algorithm (RAxML) from a core alignment (*n*=3,359 genes shared by at least 95 % of all the isolates). The origin of each isolate is shown in the nodes (red = upWWTP, green = WWTP, orange = dWWTP, blue = Mälaren). The leaf names correspond to sample metadata information including the sample location (A= upWWTP, B= WWTP, C= dWWTP or D= Mälaren), number of the biological replicate from which the strain was isolated (A1, A2, or A3) and sampling date (DDMMYY). Interactive visualization is available on Microreact https://microreact.org/project/skYFEqgScJvssPkSrZJysc-fyrisan-project. (**B**) Venn diagram showing the distribution of all sequence types (STs) detected across sampling locations, and which STs were found in multiple locations. (**C**) Bar plot showing the distribution of the STs detected across different sampling months. The number of isolates attributed to each ST is illustrated on the *y*-axis while the different STs are shown on the *x*-axis with different colors.

concentration of ESBL-Ec. This strongly indicates the WWTP as a contributing factor to the contamination of river water with 3GCR-Ec and indicating contamination of ESBL-Ec. Previous studies on the efficiency of ESBL-Ec removal in WWTP treatment processes show varying results, from a 51 % decrease in detection rate between influent and effluent in a Chinese WWTP [28], to >99,9 % in a Japanese WWTP [3]. It is thus likely to assume that the WWTP in our setting removes a majority of ESBL-Ec, but our results still indicate that pathogenic strains of ESBL-Ec from the WWTP are being disseminated into receiving waters used for recreation by the population. One previous Swedish study showed an increase of ARGs in river water downstream from a WWTP in a similar setting, even though no ARB concentrations were measured [5]. An increase in ARGs in surface waters has been attributed primarily to fecal contamination of the receiving waters, rather than natural selection [25]. Our results corroborate these findings, as we demonstrate that the WWTP outlets likely contaminate surface waters with viable antibiotic-resistant bacteria.

The concentration of 3GCR-Ec and ESBL-Ec seemed to decrease along the course of the river; both 3GCR-Ec and ESBL-Ec concentrations were significantly lower in the receiving lake than at the WWTP outlet. This gives us some insight into how the concentrations of ARBs are affected in receiving larger water bodies. Possible contributing factors to the low ESBL-Ec concentration in lake water include dilution effects [31] and unfavorable survival and/or replication conditions for the bacteria in river and lake water (including no or low selective pressure from antibiotic residues) [34]. The clear difference at the sampling point where the river has entered the large volume of water in the lake suggests that dilution effects are important.

We found no significant difference in 3GCR-Ec or ESBL-Ec concentrations between site WWTP and dWWTP, 3,1 km downstream in the river, suggesting that 3GCR-Ec and ESBL-Ec concentrations are relatively stable in a substantial part of the river system downstream of the main source of contamination. This may be due to resistant bacteria from the WWTP being maintained in the river, and/or influx of new resistant bacteria; in this regard, it is interesting to note the presence of a bird sanctuary between WWTP and dWWTP. Previous studies have demonstrated that the occurrence of ESBL-Ec is approximately three times higher in wild gulls compared to community carriers in Sweden [2], suggesting that the bird sanctuary, located upstream of the dWWTP sampling point, could be a potential source influencing the observed patterns in our results. One study has examined concentrations in a similar setting along a Japanese river and received similar results [56], although this study did not measure concentrations in the water body receiving the river water.

There was a difference in 3GCR-Ec and ESBL-Ec concentrations between months, with sampling in July and, to a lesser degree, September yielding lower concentrations compared to March, May, and November. Notably, the observed peak in May, followed by a sharp drop in July

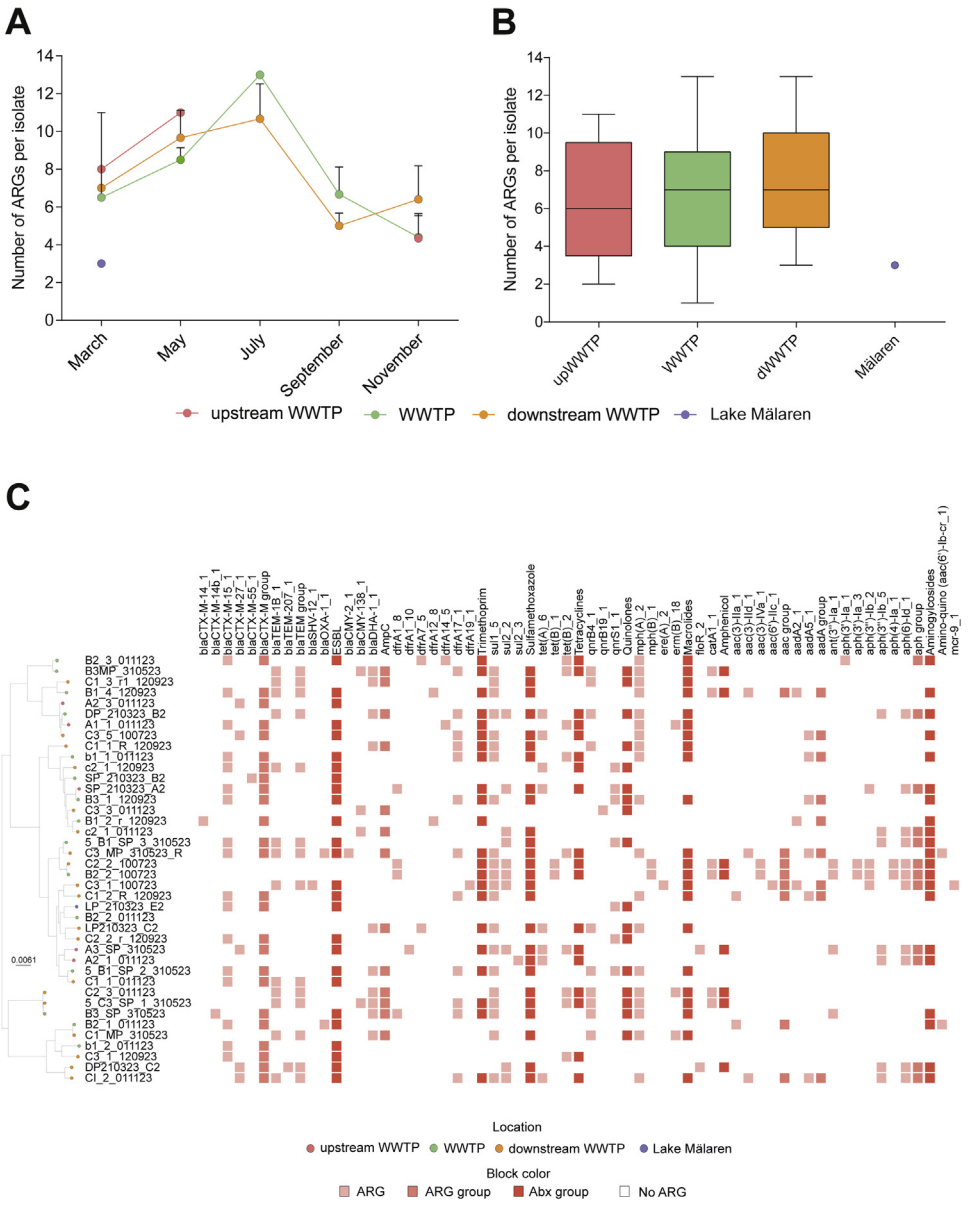

Fig. 4. (A). Temporal variation in the number of ARGs detected in each isolate per month for the four sampling points (red = upWWTP, green = WWTP, orange = dWWTP, blue = Mälaren). Error bars represent the SEM. **(B)** Box plot showing the number of ARGs per isolate per location. The center line represents the median value, while the upper and lower bounds of the boxes correspond to the first and third quartiles. **(C).** Presence of ARGs in the 40 3GCR-Ec isolates. The same phylogenetic tree was used with the nodes colored by location (red = upWWTP, green = WWTP, orange = dWWTP, blue = Mälaren). The ARGs identified by ResFinder and groups of antibiotic classes and genes they confer resistance to were colored in different shades of red. The multidrug efflux pump *mdf(A)* gene that was present in all isolates is not shown. Interactive visualization is available on Microreact: https://microreact.org/project/skYFEqgScJvssPkSrZJysc-fyrisan-project.

and a subsequent increase in November, suggests a potential seasonal cycle, although more sampling is needed to confirm this pattern. This could potentially be due to differences in weather and rainfall, where higher flows in the river, with the same number of bacteria disseminated into the water, could result in lower concentrations. Previous studies on seasonal differences in ESBL-Ec abundance showed a clear increase in abundance during the winter months compared to summer [28,44]. Our results are limited by a small sample size and demonstrate no clear temporal pattern in 3GCR-Ec concentration, although lower concentrations were found during summer/early autumn months, consistent with previous findings [28,44].

We found an extensive resistance to beta-lactam antibiotics among the 40 tested 3GCR-Ec isolates, which is not surprising given that they were grown on selective plates containing third-generation cephalosporin. However, resistance to the aminoglycoside antibiotic tobramycin was also common, and all isolates were MDR, demonstrating extensive resistance to other antibiotic classes and indicating a treatment challenge should those strains infect and cause disease in humans. Each isolate was resistant to between 3 and 14 of the 21 tested antibiotics, with no clear trend across sampling times or locations, suggesting that the phenotypic resistance among 3GCR-Ec in the Fyrisån river is relatively stable. The single isolate from the receiving lake had one of the lowest ARG counts (3/50), suggesting a potentially lower resistance burden compared to the river, although this needs to be interpreted with caution due to the limited sample size. Additionally, our data provide no evidence of carbapenem resistance, either phenotypically or genotypically, consistent with the fact that carbapenem resistance is still rare in Sweden [49].

Among the STs detected, several are known to be associated with human infections and multidrug resistance, including ST10, ST38, ST69, ST131, and ST1193 [35,41]. Additionally, ST10, ST38, ST69, and ST88 are commonly found in livestock and companion animals [15,35]. This suggests the contribution of both human and animal sources to the presence of ESBL-Ec in the river and receiving lake, highlighting the importance of a One Health approach in understanding AMR transmission in the environment.

Our phylogenetic analyses revealed instances of genetically similar isolates being found at upWWTP, WWTP and dWWTP, with some sampled from different locations up to six months apart. For example, very high ANI values (indicating nearly identical strains) were observed in ST88 and ST1722 isolates, detected at WWTP and dWWTP in July and May, respectively, indicating that resistant *E.coli* strains likely originating from the WWTP can persist in the river environment. Additionally, ST1695, a rarely described ST known to carry carbapenemase genes

[30], was detected both upWWTP and WWTP, while ST69, an ST frequently found in human clinical isolates [16], was detected at upWWTP, WWTP, and dWWTP in different months. This suggests the dissemination of human pathogenic clones from sources upstream of the WWTP. While the exact source of contamination is unknown and beyond the scope of this study, it is unlikely that these bacteria are native to the river. The Fyrisån river has three main tributaries, each passing through smaller communities and villages, and the river itself is bordered by large agricultural areas, all of which could potentially contribute to the bacterial dissemination. These findings, together with the observation of shared resistance gene profiles across sampling sites, suggest that both clonal dissemination and horizontal gene transfer may contribute to the spread and persistence of resistance, even where bacterial concentrations differ.

The collected *E. coli* harbored various beta-lactamase genes, with $bla_{CTX\text{-}M\text{-}15}$ present in a substantial proportion of samples (17/40, 42 %) across all sampling locations. This supports previous reports identifying this beta-lactamase gene as one of the most prevalent ESBL genes in the world [6,21]. Even though $bla_{CTX\text{-}M\text{-}15}$ has been associated with human carriage, it has also been found among gulls in Sweden [2], companion animals in multiple European countries [18], and among livestock [36]. As all of these are possible sources of contamination in the river, we cannot draw the conclusion that humans constitute the only origin of this gene.

Among the beta-lactamase genes found, we identified three AmpC genes known to relocate to plasmids and become plasmid-borne (pAmpC). These genes were found in both WWTP and dWWTP, with the majority (69 %) detected at dWWTP. This could suggest a livestock-associated source, as pAmpC genes have been primarily linked to farmed animals, particularly poultry, rather than humans in Sweden [9]. Notably, a tributary running through an agrarian landscape joins the river just 1.8 km upstream from the dWWTP sampling point, potentially contributing to the presence of pAmpC genes. One isolate carried the *mcr-9* gene, a recently identified allele of the mobile colistin resistance (*mcr*) gene family, which has shown inconsistent associations with colistin resistance [47,51]. In our study, this isolate was resistant to polymyxin B but not to colistin, and also harbored $bla_{SHV\text{-}12}$, an ESBL gene linked to cefiderocol resistance [32]. Both colistin and cefiderocol are antibiotic agents used as last-line treatment in severe infections caused by MDR bacteria. Continued monitoring of the presence of ESBL, pAmpC and *mcr* genes remains important to understand their epidemiology and emergence.

5. Major implications and future perspectives

This study demonstrates that WWTPs play a critical role in the dissemination of MDR *E. coli* and various ARGs in urban water systems, even in high-income settings with advanced wastewater treatment processes and relatively low AMR prevalence. The detection of nearly identical clones and shared resistance genes across sampling sites and months suggests the persistence and spread of AMR in aquatic environments, highlighting the importance of integrating genomic surveillance into routine monitoring programs. Our findings emphasize the need for improved wastewater treatment technologies and policy measures, aimed at reducing the release of resistant bacteria and genes into natural water bodies. Future studies should prioritize higher temporal resolution sampling to capture short-term fluctuations, and explore additional environmental reservoirs, including agricultural animals and wildlife, as well as transmission pathways, to inform risk assessment and mitigation strategies.

6. Conclusion

Our study demonstrates that a WWTP in a high-income country, with comparatively low AMR levels, can contribute to the contamination of surface river water with antibiotic-resistant *E. coli*. The resistant isolates harbored common ESBL-genes, like $bla_{CTX\text{-}M}$, and bla_{SHV}, which are known to spread via plasmids through horizontal gene transfer. Many of the STs identified in the study are commonly found among humans, further indicating human fecal contamination, likely originating from the WWTP effluent. Contamination of waterways in close proximity to humans could ultimately lead to a higher prevalence of ESBL carriage in the population, and eventually increase the number of infections with ESBL-producing *E. coli*. While the prevalence of ESBL-Ec was low in the receiving lake, likely due to dilution, further research is needed to better understand the impact of the contaminated river on downstream waters. We recommend increased surveillance of ESBL-producing *Enterobcteriaceae* in WWTP effluents and waterways, and further exploration of wastewater treatment strategies that more effectively remove ARB before discharge into the environment.

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Data availability

Raw illumina reads have been deposited to the GenBank database in the National Center for Biotechnology Information (NCBI) under the BioProject accession PRJNA1229472 (https://www.ncbi.nlm.nih.gov). SRA accession numbers and BioSample accessions for each genome are provided in Table S2. The full set of assemblies has been deposited at the public data repository FigShare under https://doi.org/10.6084/m9.figshare.28505435.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ebraheem D. Elmarghani: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **Robert Spörndly:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Evangelos Mourkas:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Josef D. Järhult:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nexres.2025.100436.

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