Microbiome

RESEARCH Open Access

Beyond borders: plasmids drive a shared antibiotic resistome in European urban water systems

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Abstract

Background Urban wastewater systems (UWSs) act as reservoirs and conduits for the dissemination of antibiotic resistance genes (ARGs), with plasmids playing a central role in their spread. Despite their significance, the diversity and persistence of plasmids in UWSs remain underexplored.

Results This study applies a multi-omics approach, including metagenomic and direct plasmidome sequencing, high-throughput qPCR array, and whole genome sequencing of plasmid isolates, to comprehensively profile the microbial plasmidome and resistome on 78 samples across UWSs in Denmark, Spain, and the UK. We successfully uncovered an extensive plasmid and ARG diversity that could not be fully captured by a single method, especially identified 78,574 plasmids, including 20,925 plasmids previously unreported. We also observed that plasmids carried a disproportionate share of clinically relevant ARGs, particularly beta-lactamase resistance genes; most importantly, they were preferentially located on transmissible plasmids. Furtherly, plasmids harbor ARG can enhance their persistence in wastewater ecosystems, especially harboring multiple types of ARGs. Moreover, *Bacteroides* emerged as a unique persistent ARG reservoir not only for harboring and disseminating diverse resistance genes especially in residential-relevant areas, but also emerged as a major driver of antimicrobial resistance dynamics across different wastewater treatment processes.

Conclusions Overall, this work provides the first attempt at a holistic description of the UWSs' resistome, its structure, dynamics, and mobility and significantly expands the current knowledge.

Keywords Urban water systems, Resistome, Plasmid, Antibiotic resistance genes, Bacteroidota, Metagenomics

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Introduction

Antibiotic resistance has become a growing public health concern, exacerbated by the overuse and misuse of antibiotics in recent years. The emergence, evolution, and dissemination of antibiotic resistance genes (ARGs) beyond clinical settings and into the environment have been increasingly recognized [1, 2]. Recent research has increasingly focused on the proliferation of ARGs in urban wastewater systems (UWSs) [3–5]. These systems represent a unique interaction between human-associated and environmental bacteria. UWSs were originally designed to collect and transport human

waste from residential areas and eventually treat it and introduce its effluent into natural watercourses such as creeks, beaches, and the sea [6, 7]. Meanwhile, hospitals and residential areas continuously discharge a vast array of ARGs into UWSs. As a result, these systems are both a reservoir and a conduit for the spread of ARGs in the environment [8, 9] and increase the risk of ARGs being transferred to pathogenic bacteria, further aggravating the public health crisis. As such, examining the composition and abundance of ARGs in urban wastewater can provide crucial insights into the mechanisms driving antibiotic resistance in microbial communities.

Antibiotic-resistant bacteria harboring plasmids contribute significantly to the overall UWS resistome [10]. Genera such as *Escherichia*, *Klebsiella*, and *Pseudomonas* are frequently identified as key players in the context of plasmid-borne ARGs, especially for carrying multiple resistance genes [11–13]. Nevertheless, a study showed that *qnrS*, a quinolone ARG, is significantly associated with Aeromonas [14], and another study suggested that IS*994*, an insertion sequence mobile genetic element (MGE), is only found to be related to *Renibacterium salmoninaru* [15]. These findings imply that some ARGs or MGEs can frequently be detected in a single host species, suggesting that the spread of these genes may also largely depend on the persistence of the host bacterium, rather than the resistance selection effect. A comprehensive understanding of the specific connections between ARGs and their bacterial hosts in UWSs remains underexplored. Horizontal gene transfer (HGT) plays a critical role in disseminating ARGs across bacterial populations, significantly accelerating the spread of resistance [15, 16]. Through HGT mechanisms such as conjugation, transduction, and transformation, ARGs can move between diverse bacterial species, allowing bacteria initially susceptible to antibiotics to acquire resistance rapidly [17, 18]. This process is facilitated by MGEs like plasmids, transposons, and integrons, which act as vectors carrying ARGs across bacterial populations [19–21]. Studies indicate that ARGs are commonly associated with MGEs, enabling ARGs to spread rapidly among different bacterial species [21]. For instance, plasmids, small, and circular DNA molecules can carry multiple resistance genes and facilitate their horizontal transfer between bacteria, enhancing the overall resilience of microbial populations and playing a vital role in disseminating ARGs within UWSs [18, 22, 23]. This capacity for multidrug resistance is especially concerning, as it increases the risk of ARGs spreading to clinically relevant bacteria, including opportunistic and *bona fide* human pathogens, thereby posing a significant challenge to public health.

Research methods for studying plasmid and resistome in urban wastewater have evolved significantly, incorporating experimental-based methods and high-throughput sequencing technologies. These methodologies allow for comprehensive profiling of the resistome and plasmidome, providing insights into the relationships between microbial communities and their associated resistance genes. For instance, PCR-based methods that rely on current and robust databases of both plasmids and genes of relevance are effectively utilized to detect plasmids and their resistome genes [15, 24]. High-throughput sequencing, such as metagenomic and direct plasmid sequencing, enables the identification of novel ARGs and plasmids, facilitating a better understanding of the resistome's structure and dynamics [15, 23, 25]. Additionally, bioinformatics tools play a crucial role in analyzing the large datasets generated from these technologies, enabling researchers to explore the genetic context of ARGs and their transfer mechanisms. RGI [26] and Plaspline [27] facilitate the detection and association of plasmid-borne ARGs using short-read shotgun metagenomics. However, fully assembling larger conjugative plasmids, which often carry multiple ARGs, remains notoriously difficult [28]. Therefore, it is essential to employ a combination of methods to comprehensively reveal the resistome's structure and dynamics in UWSs. Addressing these challenges is critical for improving the reliability and comparability of research findings, ultimately contributing to effective management strategies for antibiotic resistance in urban wastewater.

In this study, we collected a combined 78 samples from four sites along the UWSs in three medium-sized cities in Denmark (DK), Spain (SP), and the UK in winter and summer. We implemented a multi-omics strategy that combined three complementary sequencing approaches: direct plasmidomic sequencing, metagenomic sequencing, and whole-genome sequencing of plasmid isolates. This integrated approach provided comprehensive coverage of both the plasmidome (total plasmid content) and resistome (total ARG content) within UWSs. We enhanced our analysis by incorporating microbiome profiles and high-throughput qPCR data collected during the same sampling campaign. This methodological framework enabled us to examine the composition, behavior, and transmission mechanisms of plasmids and ARGs in UWSs, as well as their relationships with environmental variables, treatment processes, and microbial populations. By elucidating the mechanisms behind ARG dissemination in wastewater environments, this research provides critical insights into how plasmids contribute to the spread of antibiotic resistance.

Methods

Sampling and collection

Samples used in this study were collected previously, as described in Yu et al. [22]. Comparable UWSs from medium-sized cities (>250 k inhabitants) in Denmark, Spain, and the UK located in Odense, Santiago de Compostela, and Durham County, respectively, were used in this study. In each country, hospital sewer (HS), residential sewer (RS), the mixture of HS and RS in the influent water (MS), and the biological treatment basins (BTP) within the treatment plant were sampled. Specifically, activated sludge basins were applied to the BTP facilities used in the wastewater treatment plants (WWTPs) in DK and SP, while a biofilter was employed in the UK. Sampling campaigns at the four sites were performed in the winter and summer of 2018 using automatic samplers to obtain 24-h flow proportional samples, either directly in DK and the UK or after mixing hourly samples according to flow information in SP. Two sewer lines were sampled for HS in DK (sewer lines of two different wards of the same hospital), while a single HS line was sampled in the other two countries. Three replicates per site and season were collected on three consecutive days without rain events. In total ($n=78$), 24 samples from the UK, 24 samples from SP, and 30 samples from DK were obtained. Biological samples were immediately stored in coolers at 4 °C and transported back to the lab for further processing. Samples from sewer waters (HS, RS & MS) were concentrated tenfold by centrifugation before freezing at −80 °C. All samples were then sent within the week of the last sampling day on dry ice to a single location (Section for Microbiology, Copenhagen, Denmark) for DNA extraction.

Metagenomic sequencing

Environmental DNA from all 78 samples was extracted and subjected to shotgun metagenomic sequencing. Briefly, genomic DNA from 500 µL of each sample was extracted using the NucleoSpin Soil kit (Macherey–Nagel, Düren, Germany). Sequencing libraries ($n=78$ samples; 3 replicates per location) were built with the Kapa Hyper Prep kit for Illumina (KAPA Biosystems, Wilmington, MA, USA) and sequenced by Admera-Health using 150 bp paired-end mode on a NovaSeq instrument (Illumina) generating 2.3×10^9 reads (approx. 690 Gbp) in total and a mean of 3.1×10^7 reads (approx. 9 Gbp) per sample.

16S-rRNA gene sequencing

Detailed 16S rRNA gene sequencing information was recorded in our previous study [24]. Bacterial and archaeal community compositions were assessed by high-throughput amplicon sequencing. A fragment spanning the hypervariable regions V3−V4 of the 16S rRNA gene was amplified from each DNA sample by an initial PCR step using the primers Uni341F (5′-CCTAYGGGRBGCASCAG-3′) and Uni806R (5′-GGACTACNNGGGTATCTAAT-3′). Amplicon library sequencing was performed on an Illumina MiSeq platform using Reagents Kit v3 [2×300 cycles] in paired-end mode.

High-throughput qPCR array

High-throughput qPCR array data were obtained in our previous study [24]. Briefly, a set of 120 primer pairs primarily targeting β-lactamases genes and MGEs using high-throughput qPCR (SmartChip, Takara Bio, Japan). SYBR green qPCR assays were performed for selected genes of interest: bla_{VIM}, bla_{NDM}, bla_{KPC}, and $bla_{\text{CTX-M}}$ for qPCR quantification using the BioRad CFX C1000 system (BioRad, Hercules, CA, USA). These four genes have clinical relevance and could be detected in high abundance in hospital sewers and all parts of the three countries' UWSs [23].

Conjugative plasmid isolate sequencing

Conjugative plasmids were isolated from the regional hospital (HS) and residential sewers (RS) in three countries using mating assays. Donor samples were thawed on ice, homogenized with sterile metal beads, filtered (10 µm), and cell density was measured by FACS (SYBR green staining). Samples were diluted to 3.78×10^6 cells/100 µL in TE buffer with 0.9% NaCl. The recipient strain, *E. coli* MG1655 tagged with mCherry and kanamycin resistance (50 µg/mL), was cultured to 3.78×10^7 cells/mL. Solid surface filter mating was performed at donor to recipient ratios of 1:5 and 1:10 (final density 30,000 cells/mm2). Mixtures (100 µL) were spotted onto sterile 0.22 µm membrane filters on LB agar with cycloheximide (10 µg/mL) and incubated at 25 °C for 24 h. Recipient cells were first sorted by FACS based on size and mCherry fluorescence to enrich for the mCherry-tagged population. The sorted cells were then plated on LB agar containing kanamycin and ampicillin to select for true transconjugants. Only colonies resistant to both antibiotics and exhibiting red fluorescence were confirmed as transconjugants. Red fluorescent colonies were confirmed as transconjugants and stored at −80 °C. Transfer efficiency was calculated as transconjugants per million recipient cells. Single transconjugants were cultured in selective LB broth for plasmid extraction using the Plasmid Mini AX kit. Libraries from 96 plasmids (grouped in 8 sets) were prepared with the SQK-RBK004 kit (Oxford Nanopore Technologies), barcoded, pooled, purified, quantified by Qubit, and sequenced on Flongle flow cells.

Plasmidome sequencing

The direct plasmidome approach was recorded in our previous study [22]. Briefly, UWS samples were pretreated by filtration, vortex, and sonication and resuspended in TE buffer. Afterwards, a pre-lysis cocktail of cell-wall degrading enzymes (lysozyme, mutanolysin, and lysostaphin) was used to facilitate the lysis of Gram-positive bacteria. Alkaline lysis was applied to remove chromosomal DNA, followed by Plasmid-Safe™ ATP-Dependent DNase (Lucigen, UK) digestion. Plasmid-Safe DNase will digest any fragments of dsDNA with open 3′ or 5′ termini, hence removing fragmented chromosomal DNA. The purified plasmid DNA was then quality-checked, libraries prepared, and sequenced on an Illumina NextSeq platform (Illumina, San Diego, CA, USA) with a v2.5 sequencing kit (Illumina).

Plasmid catalogs generation, annotation, classification,phylogenetic tree construction, and abundance profiling

Two shotgun datasets, metagenomic and plasmidomic, were generated after sequencing. Then, an in-house bioinformatic pipeline, *Plaspline* (version 1.4) [22, 27], for plasmid analysis was implemented. These two datasets were restructured, and plasmid genomes were isolated through quality control, assembly, restructuring, and verifying circular plasmid contigs (regarded as putative plasmids from then on), generating a circular plasmid catalog (> 90% identity and > 90% coverage). In addition, linear plasmid contigs were identified, and a linear plasmid genome catalog was generated (> 85% identity and > 80% coverage). These steps were processed for metagenomic and plasmidomic datasets separately. Next, we merged the metagenomic and plasmidomic circular plasmid catalogs and the linear plasmid genome catalog, individually, and the merged linear plasmid catalog was blasted to the merged circular plasmid catalog to remove redundancy (> 85% identity and > 80% coverage) and generated the final wastewater plasmid catalog. The slightly lower thresholds for linear plasmids reflect their generally lower structural completeness and annotation confidence. This relaxation facilitates the merging of potentially spurious sequences with true plasmids, thereby minimizing the retention of false positives in the final catalog.

Later, *Plaspline* uses *MOB-recon* and *MOB-typer* modules from the *MOB-suite* (version 2.0.1) [29] software to classify plasmids. These modules classified putative plasmids from metagenome contigs and searched for replication gene (*rep*), mobilization protein (*mob* relaxases), mate-pair formation (MPF), and transfer origin (*oriT*). To reduce the skewed abundance estimation generated by merging reads from different sequencing technologies, only metagenomic reads were used to calculate plasmid abundance. *Bwa* (version 0.7.17) [30, 31], *Samtools* (version 1.9) [31], and *Msamtools* (version 0.9) were recruited in *Plaspline*. The abundance was calculated and normalized based on fragments per kilobase of sequence per million reads, while the contig/genomes were filtered if their coverage was less than 55%.

A plasmid phylogenetic tree was constructed using the plasmid replicase marker gene. MAFFT (version 7.475) [32] was used to perform multiple alignments of plasmid replicase protein sequences. The multiple alignment results were then fed into IQ-TREE (version 2.0.3) [33] to reconstruct a phylogeny of plasmids, with the parameter "-MFP" to determine the best-fit model of the SH-like approximate Likelihood ratio test using 1000 bootstrap replicates. The final phylogenetic tree was visualized and annotated using iTOL (version 4) [34]. Antibiotic-resistant plasmid tree (carrying ARGs or phenotypes on antibiotic resistance) is constructed based on their abundance used by "scipy.cluster.hierarchy" with "euclidean" distance.

UWS microbial and plasmid gene catalog generation, annotation, and abundance calculation

To create the total UWS microbial and the plasmid gene catalogs, we first filtered contigs by size and used the ones longer than 2 Kb for the downstream analysis. Next, we utilized *prodigal* (version 2.6.3) to identify open reading frames (ORFs) from the assembled contigs (for plasmid sequences, this was done with plasmid contigs). Subsequently, we employed *mmseqs2* (version 12.113e3) [35] to eliminate redundancy among the ORFs based on nucleic acid sequence similarity, thereby generating the gene catalog. This step used the "easy-cluster" mode with "–cov-mode 2" setting thresholds greater than 85% identity and 80% coverage. Then, ARGs were annotated using the CARD Resistance Gene Identifier (RGI, version 5.2.1) [26]. Later, the total UWS microbial gene abundance was calculated by mapping metagenomic reads. For this analysis, we utilised *Bwa* (version 0.7.17) [30], *Samtools* (version 1.9) [31], and *Msamtools* (version 0.9). The abundance was determined and normalized based on fragments mapped per kilobase of sequence per million reads. Genes with coverage < 80% were discarded. For plasmid gene abundance, we calculated a gene's abundance as the total abundance of all plasmid sequences that included that gene within the sample.

Notably, some plasmids were assembled exclusively from the plasmidomic dataset without corresponding metagenomic read mapping. To estimate the abundance of these plasmids, we employed a machine learning model for prediction. Plasmids detected by both metagenomic and plasmidomic reads were used as the training dataset, where the plasmidomic-read-based abundance served as

the input features (x), and the metagenomic-read-based abundance as the output label (y). For plasmids lacking metagenomic read mapping, their plasmidomic-read-based abundance formed the prediction input (x). To improve prediction accuracy, additional features including the total number of reads per sample and plasmid length were incorporated during model training. To develop a more effective predictive model, we compared *LinearRegression*, *Lasso*, *Ridge*, *ElasticNet*, *BayesianRidge*, *DecisionTreeRegressor*, *MLPRegressor*, *RandomForestRegressor*, *XGBRegressor*, and *LGBMRegressor* learning models using the training and predicting datasets and optimizing for the best parameters. Model evaluation was conducted using mean absolute error (MAE) and mean squared error (MSE) as metrics to assess prediction accuracy. These parameters were manually adjusted to enhance the performance of each model. All processes were executed using the Python package *sklearn* (version 1.0.2; https://scikit-learn.org/).

Plasmid host annotation and construction of a potential plasmid transfer network

For each plasmid reconstructed from UWSs, we inferred the most probable host based on sequence similarity with plasmids from PLSDB (version v.2023_11_03_v2) [36], which in most cases includes information on the host from which the plasmid was isolated. We estimated genomic similarity by calculating the Jaccard Index (JI) based on shared 21-bp *k*-mers, with a minimum JI threshold of 0.3, as described in Acman et al. [37] JI values were calculated using Bindash (version 1.0) [38, 39]. All matching hosts were recorded if a plasmid matched with more than one host. Finally, each bacterial host was annotated with information on its linked plasmids in PLSDB (NCBI taxa). To validate the results using PLSDB-based host annotation, we also performed host annotation by assessing sequence similarity between binned and unbinned plasmids derived from *Vamb* (version 3.0.9) [38] results.

Based on the number and identity of plasmids associated with different hosts, we constructed a network of putative horizontal plasmid transmission events. Specifically, a plasmid transfer event was presumed to have occurred if plasmids with the same contig were found in two or more bacterial hosts.

Taxonomic annotation and calculation of abundance

Metagenome-assembled genomes (MAGs) were binned and taxonomically annotated using *Vamb* (version 3.0.9) [38] and GTDB-Tk (version 2.4.0) [40]. Species abundance calculation and taxonomic profiling of reads were carried out using *MetaPhlAn* (version 3.0) [41] at the species level.

Statistical analysis

All statistical analyses were performed using Python (version 3.12.4) packages "scipy (version 1.13.1)", "*statsmodels* (version 0.14.2)", and "*skbio* (version 0.6.2)". Moreover, the alpha and beta diversity of plasmid and bacteria were calculated by "skbio.diversity.alpha_diversity" and "skbio.diversity.beta_diversity" functions. Moreover, principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity was applied to evidence the different patterns of plasmid ARGs found in the different UWS compartments. Wilcoxon rank-sum tests were executed with "skbio.stats.ranksums", ordinary least squares (OLS) regression, and linear regression tests were performed with "statsmodels.api.OLS" and "scipy.stats.linregress". Permutational multivariate analysis of variance (PERMANOVA) and testing were conducted using "skbio.stats.ordination.pcoa" and "skbio.stats.distance.permanova" (permutations = 999). The p-values were adjusted using "statsmodels.stats.multitest.fdrcorrection." The phylogenetic signal (Pagels' λ) was computed using the R package "phytools" (version 2.0–3).

Results

Plasmidomic and metagenomic sequencing significantly improves the detection of plasmids and ARGs

To thoroughly investigate plasmids and ARGs present in UWSs, we leveraged sampling in three European countries, spanning their respective UWSs. We employed a combination of methodologies, including direct plasmidomic sequencing, metagenomic sequencing, and whole-genome sequencing of plasmid isolates (Fig. 1a). Through these efforts, we successfully assembled a comprehensive catalog of UWSs' plasmidome and resistome, with 78,574 different plasmids, of which 20,925 were never reported in the current database (PLSDB, version v.2023_11_03_v2). Moreover, these plasmids belonged to 670 rep-groups (classified by identical plasmid backbone genes), the most prevalent one was IncP, rep_cluster_10, and rep_cluster_2238 (Supplementary Fig. 1e).

Our analyses revealed differences between metagenomic and plasmidomic sequencing in plasmid detection and characterisation, particularly concerning their size. Metagenomic sequencing tended to reconstruct larger plasmids, whereas plasmidomic sequencing was more effective at detecting smaller plasmids (Fig. 1b). Moreover, plasmids identified by the two methods showed significant differences. Only 1015 of the 3992 complete plasmid genomes detected via plasmidomic sequencing were also identified by metagenomic sequencing (Fig. 1b). Notably, a substantial number of plasmids (11,227 in total), including both complete circular plasmids and partially assembled plasmids, were exclusively detected through plasmidomic sequencing (Fig. 1a).

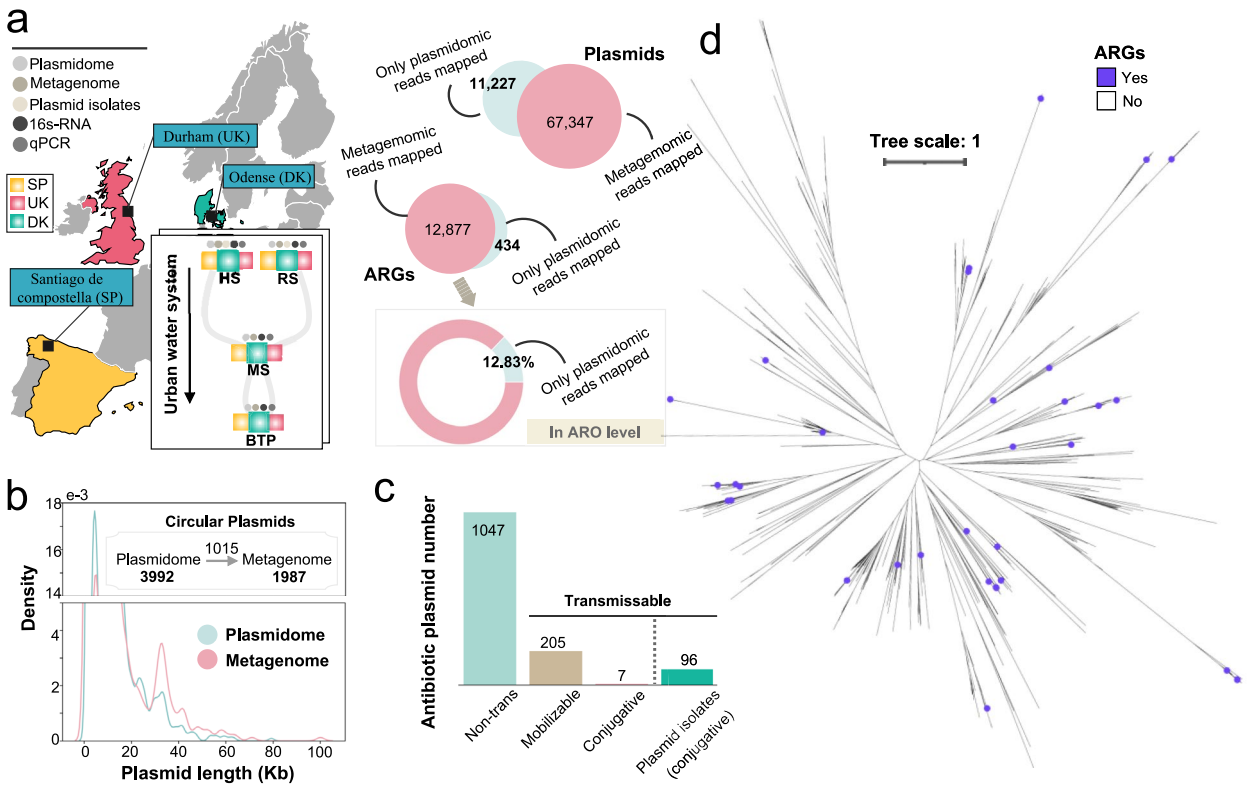

Fig. 1 Assembling the pan-European plasmidome and its associated resistome. **a** Sampling and sewer and wastewater flow schematic. A flow diagram illustrating the movement from hospital sewer (HS) and residential sewer (RS) to the mixture sewer (MS), and then from MS to wastewater sampled in the biological treatment basins (BTP). The Venn diagrams compare plasmids and ARGs detected from metagenomic and plasmidomic sequences. **b** The length distribution of the circular plasmids recovered from metagenomic and plasmidomic sequencing is shown. **c** Bar plot represents different types of plasmids that harbor ARGs. **d** The phylogenetic tree of plasmids based on the plasmid replicase gene is displayed, and the antibiotic resistance plasmids are marked

These findings underscore the complementary nature of these two methods. Specifically, when we narrowed our focus to complete circular plasmid genomes, our analysis showed that conjugative plasmids typically exceeded 15 Kb in size, mobilizable plasmids ranged between 5 and 10 Kb, and non-transmissible plasmids were generally less than 5 Kb (Supplementary Fig. 1c). This observation aligns perfectly with prior research indicating a correlation between plasmid size and mobility [34].

Finally, we compiled an urban water ARG catalog comprising 12,877 genes. Intriguingly, 434 ARGs were exclusively detected through direct plasmid sequencing, yet they accounted for approximately 12.83% of the total ARG pool at the Antibiotic Resistance Ontology (ARO) level (Fig. 1a). This suggests that by integrating plasmidome sequencing, we identified a subset of ARGs that otherwise would have been overlooked by metagenomic sequencing alone. This successful augmentation of the detection of the UWS resistome underscores the critical significance of combining these two methodologies.

Detection of antibiotic resistance-encoding plasmids in UWSs

To investigate the role of plasmids in disseminating ARGs throughout, we constructed a comprehensive catalog of antibiotic resistance plasmids (ARPs) from initially combined plasmidomic and metagenomic sequencing data. This integration resulted in a larger collection of ARPs, encompassing 163 out of 670 rep-group plasmids that contained ARGs (Fig. 1d). Notably, we detected only seven conjugative and 205 mobilizable ARPs (Fig. 1c).

Additionally, to expand the UWS's ARPs catalog, we exogenously isolated and sequenced 96 conjugative plasmids, which confer phenotypic resistance to antibiotics. However, only 32 of these plasmids were also identified through plasmidomic or metagenomic sequencing data, and only with a low coverage (0.3) indicating relatively low abundance of these exogenous isolated plasmids in the UWS (Supplementary Fig. 1 d). This finding underscores the challenges of comprehensively detecting conjugative ARPs in the UWSs using current omics methodologies.

Plasmids harbor more beta-lactamase genes

To further investigate the resistome dynamics, we analyzed the composition of total ARGs (tARGs) and plasmid-encoded ARGs (pARGs) along UWSs. A total of 1155 tARGs at the ARO level, distributed across 172 ARG families and 55 drug classes (Fig. 2a), were identified, 19% of which were encoded on plasmids. This proportion increased to 40% at the gene family level and 80% at the drug class level (Fig. 2a).

We observed distinct differences between the compositions of pARGs and tARGs. As no significant seasonal variation was detected (Richness, Wilcoxon rank-sum test, $P_{tARGs} = 0.178$, $P_{pARGs} = 0.236$), samples from different seasons were merged for subsequent analyses. Notably, efflux pump resistance genes were the predominant ARGs in the total UWSs gene pool as expected, while the most prevalent pARGs were beta-lactamase genes (Fig. 2b). Additionally, we found that also *aph*, *mph* (aminoglycoside and macrolide phosphotransferases, respectively), and *sul* (sulfonamide resistance) genes had a higher relative abundance in pARGs compared to tARGs (Fig. 2b).

Interestingly, within the tARG pool, specific resistance genes, such as efflux pumps, *ef-tu*, *arr*, and *grc*, exhibited an increasing trend along the urban water flow. In contrast, other ARGs, such as beta-lactamases and *ant*, showed a decreasing trend (Fig. 2b). However, we observed a contrasting trend for these ARGs once they were located on plasmids. For example, despite a significant reduction in the relative abundance of total beta-lactamase resistance genes at BTP, plasmid-encoded beta-lactamase genes remained abundantly present at BTP across all three countries (Fig. 2b).

We also noticed that the composition of ARGs varied between different countries. The composition at HS in Denmark differed from that in Spain and the UK, while the composition at BTP in the UK differed from that in Spain and Denmark (Fig. 2b). Additionally, we observed that the ARG richness in BTP in the UK was significantly higher than in the other two countries (Supplementary Fig. 2a). This could be attributed to the different

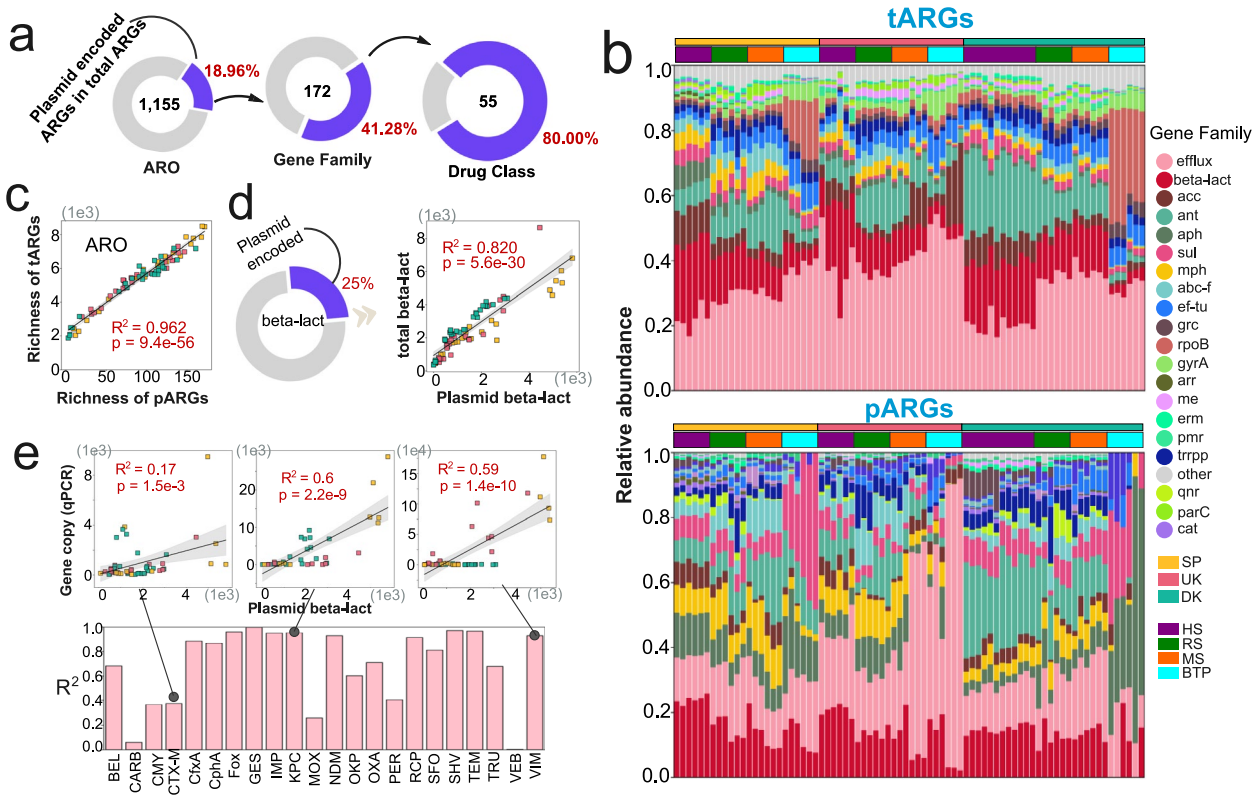

Fig. 2 Resistome composition of European urban sewer systems. **a** The comparison of tARGs (total ARGs) and pARGs (ARGs encoded by plasmids) is shown. **b** The composition of tARGs and pARGs among different countries and sewers is shown. **c** The correlation between the richness of pARGs and tARGs is displayed. **d** The contribution of plasmid-encoded beta-lactamase resistance genes to total beta-lactamase resistance genes is shown. **e** Bar plot displaying the correlation coefficient (R^2) between tARGs and pARGs for each beta-lactamase resistance gene, based on gene relative abundance measured in fragments per kilobase million and normalized by 16S rRNA gene abundance. The dot plot represents the correlation between plasmid-encoded beta-lactamase resistance genes and gene copy numbers obtained from qPCR analysis

wastewater treatment processes employed in the UK BTP, indicating that the treatment process can significantly impact the downstream antibiotic resistome.

To further explore differences in ARGs across countries, we compared the abundance of each ARG across different sewers. We observed that Spain had the highest ARG abundance in nearly all samples, followed by Denmark, which had higher levels than the UK (Supplementary Fig. 2b). Notably, we observed that the primary differences in ARG composition between pARGs and tARGs were beta-lactamase resistance genes. Nevertheless, the variation in beta-lactamase resistance genes on plasmids across the different countries was less pronounced compared to tARGs (Supplementary Fig. 2b). We detect a high abundance of plasmid-encoded beta-lactamase resistance genes in nearly all samples (Fig. 2b), underscoring the significance of plasmids as vectors for beta-lactamase resistance genes across European countries.

Plasmids disproportionally contribute to tARGs

Due to the unique role of plasmids in encoding ARGs, we speculate that plasmids may have a strong impact on the total ARG pool along the UWSs. We observed a positive correlation between the abundance of pARGs and tARGs, particularly for beta-lactamase genes (Fig. 2c and d, Supplementary Fig. 2c; ordinary least squares). Specifically, we found that plasmid-encoded beta-lactamase genes accounted for 25% of the total beta-lactamase gene diversity on the UWSs (Fig. 2d).

Furthermore, when analyzing individual beta-lactamase genes, we found that nearly all plasmid-encoded beta-lactamase genes exhibited a positive correlation with their total abundance in the UWSs (Fig. 2e; ordinary least squares). The correlation coefficient exceeded 0.3 for most genes and approached 1 for genes such as bla_{GES} and bla_{SHV} (Fig. 2e; ordinary least squares), further emphasizing the significant contribution of plasmids to the total beta-lactamase gene pool in UWSs.

For validation, we randomly selected four beta-lactamase genes with moderate correlation coefficients and performed qPCR to assess their copy numbers. We observed a positive correlation between their copy numbers and their abundance when encoded on plasmids (Fig. 2e), reinforcing the significant role of plasmids in promoting beta-lactamase genes in UWSs.

The presence of ARGs enhances the prevalence of plasmids

The unique role of pARGs in UWSs raises questions about the dynamics of ARPs in these systems. To further explore ARPs, we examined their distribution and found that a large group was shared across countries. Spain had the highest number of ARPs compared to other countries (Fig. 3a; Supplementary Fig. 3a). Notably, many of these ARPs encoding efflux pump and beta-lactamase genes exhibited higher prevalence and mobility (Fig. 3a). Approximately 23% of ARPs contained multiple ARGs, with one of them harboring up to 10 ARGs that all belong to the glycopeptide resistance gene cluster (*gly*). Meanwhile, the majority carried two ARGs (Fig. 3a). Notably, beta-lactamase, macrolide phosphotransferase (*mph*), *msr*-type ABC-F protein (*abc-f*), *aph*, *ant*, sulfonamide resistant gene (*sul*), and *gly* were the primary ARGs carried by multiple ARPs. In particular, *gly* was predominant among ARGs with multiple annotations (Fig. 3a). Furthermore, we found that many ARPs persisted throughout the UWS, especially in the UK, where half of the ARPs detected at MS were also observed downstream in BTP (Fig. 3b). Intriguingly, some ARPs were highly abundant at BTP in the UK, comprising over 60% of the total ARGs, whereas in SP and DK, this proportion was below 40% (Fig. 3c). Interestingly, we observed that these plasmids representing over 60% ARPs at BTP in the UK are significantly larger plasmid sizes than ARPs whose proportion was below 40% at BTP in SP and DK (Fig. 3c; Wilcoxon rank-sum test; $P=0.0012$). This indicates that wastewater treatment processes can significantly select ARPs that leading to different dissemination risks of ARGs since conjugative and mobilizable plasmids are usually larger than non-mobilizable ones.

The detection of ARPs carrying multiple ARGs across UWSs prompts the question of whether carrying ARGs can augment plasmid prevalence. To address this, we compared the prevalence of different types of plasmids across all samples. We found that plasmids carrying ARGs had a significantly higher prevalence compared to those without ARGs (Wilcoxon rank-sum test; $P_{\text{single ARG–non-ARG}}=2.26\text{e}-11$; $P_{\text{multiple ARG–non-ARG}}=3.48\text{e}-14$). Specifically, plasmids carrying multiple ARGs exhibited even higher prevalence than those carrying a single ARG (Fig. 3d; Wilcoxon rank-sum test; $P_{\text{single ARG–multiple ARG}}=5.87\text{e}-14$), suggesting that the presence of ARGs can indeed enhance plasmid prevalence, particularly with respect to the number of ARGs carried by the plasmids.

Interestingly, we identified a subset of ARPs carrying multiple ARGs with an exceptionally high prevalence, detected in over 50% of samples. Their prevalence was significantly higher than that of other multiple ARG carriers (less than 40%) (Wilcoxon rank-sum test; $P=1.32\text{e}{-35}$). Moreover, nearly 72% of these highly prevalent multiple ARG carriers were still present at BTP, whereas only 5% of the other multiple ARG carriers were detected at BTP. Additionally, we found that most of the highly prevalent multiple ARG carriers harbored at least two different types of ARGs, with OXA beta-lactamase being the most common,

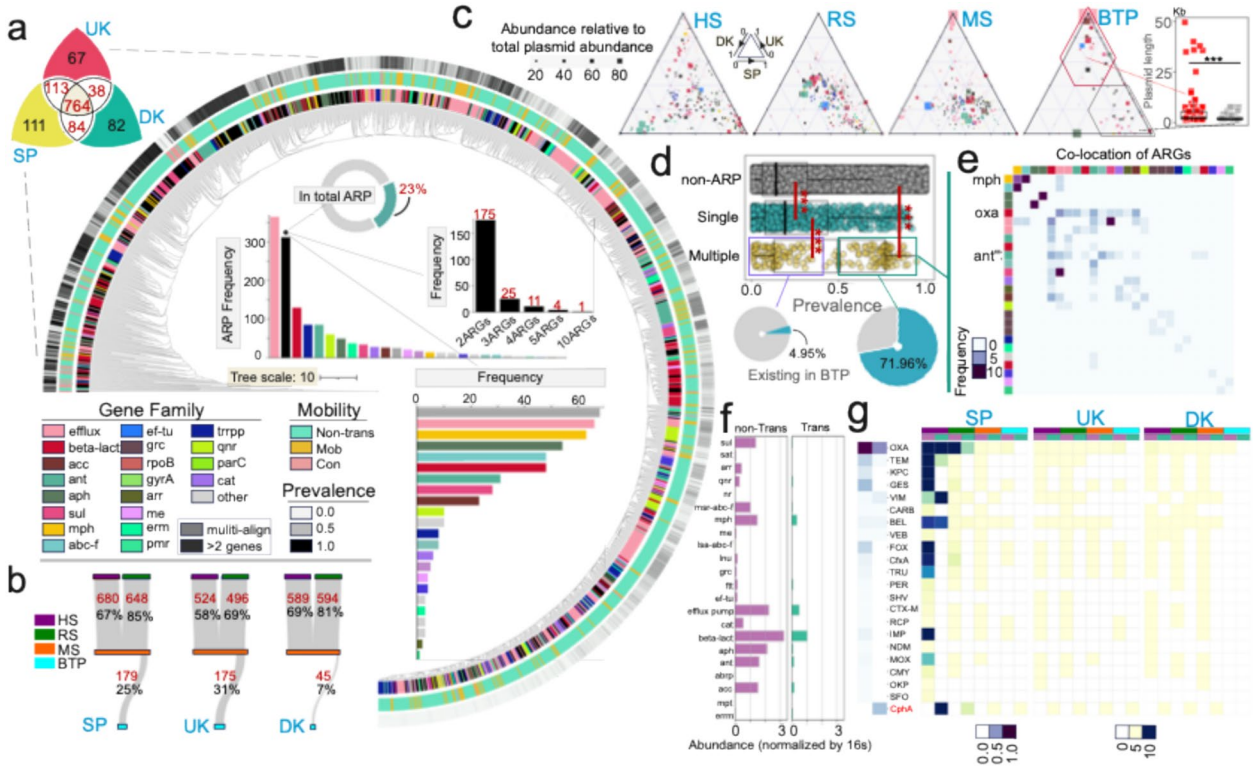

Fig. 3 Spatial ecology structures the urban sewage plasmidome and associated resistome. **a** Venn diagram showing shared and unique antibiotic plasmids at UWSs across different countries. The tree of antibiotic resistance plasmids (ARP) is constructed according to their abundance. The innermost layer annotates the ARG types (at the gene family level) harbored by the plasmids, the next layer represents plasmid mobility, and the outer layer indicates plasmid prevalence. **b** Trajectories of different ARPs along the UWSs in each country. **c** The comparison of different ARPs (gene family level) in the same sewer among different countries is displayed. The boxplot shows the length of plasmids that are abundant at BTP among different countries. **d** Prevalence of plasmids grouped by the number of ARGs they harbor. The pie chart shows the prevalence of plasmids carrying multiple ARGs at BTP. **e** The co-location of ARGs harbored by higher prevalence plasmids is displayed. **f** Total abundance of ARGs on transmissible (green) and non-transmissible plasmids (purple) is shown normalizing by 16S-rRNA gene abundance. **g** The abundance (fpkm) of each beta-lactamase gene at different sewers in each country and different types of plasmids is shown

frequently coexisting with most other types of ARGs (Fig. 3e). In contrast, most of the lower prevalence multiple ARG carriers predominantly harbored the same type of ARGs (Supplementary Fig. 3c), indicating that the prevalence of plasmids increases proportionally with the number of distinct types of ARGs they harbor.

To further verify this, we re-grouped multiple ARG carriers based on whether the ARGs conferred resistance to the same antibiotic. We observed that the prevalence of ARPs carrying multiple ARGs resistant to two types of antibiotics was significantly higher than those carrying multiple ARGs but resistant to the same antibiotic (Wilcoxon rank-sum test; $P = 0.019$). This suggests that the prevalence of ARPs increases with the diversity of ARG types they encode.

More ARGs with high clinical relevance were found on transmissible plasmids

Mobile ARGs pose a higher risk of dissemination. Here, we investigated and compared the abundance of ARGs carried by different plasmid types according to their mobility. Most plasmid-borne ARGs were found to be non-transmissible (Fig. 3f). However, ARGs with high clinical relevance, such as beta-lactamases, *qnr* and *mph*, were most abundant in transmissible plasmids (Fig. 3f). Therefore, we conducted a more detailed analysis of all beta-lactamase genes (Fig. 3g). Results revealed that bla_{TEM}, bla_{VIM}, bla_{BEL}, and bla_{OXA} were ubiquitously present on plasmids across all sewer compartments in all three countries (Fig. 3g). Additionally, bla_{CfxA}, bla_{FOX}, bla_{GES}, bla_{IMP}, bla_{KPC}, bla_{OXA}, and bla_{CphA} were exclusively abundant in the Spanish HS and RS samples,

indicating a higher prevalence of clinically relevant ARGs, even in RS, in Spain. Interestingly, most of these ARGs were exclusively carried by non-transmissible plasmids, while bla_{CphA} was solely located on transmissible plasmids (Fig. 3g). This suggests a preference for different ARGs for different types of plasmids, with these clinically relevant ARGs potentially posing a higher risk of dissemination.

Antibiotic resistance plasmids have a broader host range

To get a complete picture of plasmids and their potential bacterial hosts, we compared the non-circularized and circularized (complete) plasmid contigs obtained here with published plasmids (in PLSDB) whose bacterial hosts are known. Using this approach, we were able to construct networks depicting putative plasmid-bacteria associations of 19.32% plasmids in this study (Supplementary Fig. 4a). Most of these connections were between plasmids and members of *Enterococcus*, *Bacteroides*, *Acinetobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Salmonella* (Fig. 4a; Supplementary Fig. 4a). Importantly, we identified *Acinetobacter*, *Escherichia*, *Klebsiella*, and *Salmonella* as the primary bacterial hosts of ARPs in UWSs (Fig. 4a; Supplementary Fig. 4a). Next, based on the network linking plasmids and their potential bacterial hosts, we defined potential plasmid

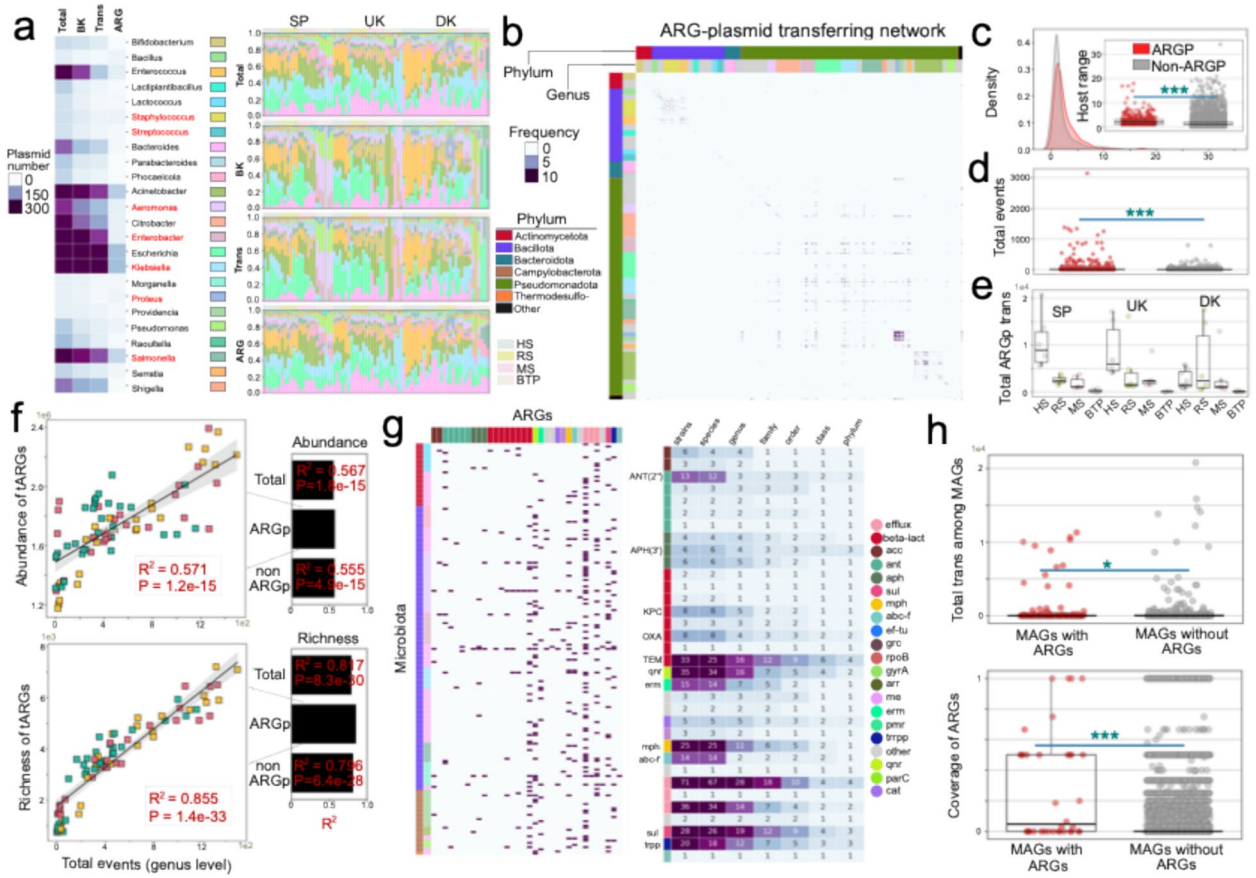

Fig. 4 Identifying plasmids' hosts and potential transfer networks. **a** The heatmap depicts the richness of plasmids in different bacterial host genera. "Total" indicates the richness of all plasmids; "Backbone," "Trans," and "ARG" indicate plasmids with backbone genes, genes that are conjugative and mobilizable, and ARG genes, respectively. The bar plot shows the total number of plasmids in each bacterial host phylum. **b** Networks of potential plasmid transfer (pPT) events between species were constructed for all countries and sewers. The outermost strip represents phylum-level host assignments, while the inner strip indicates whether the events involve ARPs. **c** The host range of AR- and non-ARPs at the level of individual plasmids and plasmid groups (which share the same replication genes) is shown. **d** The total pPT events of each bacterial host were compared based on whether ARPs (ARP) were involved. "Red" represents events involving ARPs, while "gray" represents events without ARPs. **e** The pPT events at each sewer are shown. **f** The association between total pPT events and the richness and abundance of tARGs was calculated. **g** The network between the microbiota and each ARG that is harbored by host-annotated plasmids is shown. **h** The shared ARGs between two MAGs were calculated. "pMAGs" represents the shared ARGs between two MAGs that harbor the same ARP, while "non-pMAGs" represents the shared ARGs between two MAGs without shared ARPs

transfer (pPT) events as instances where the same plasmid was found in different bacterial hosts. The majority of pPT events occurred within phyla (Fig. 4b; Supplementary Fig. 4b). As expected, the biggest proportion of pPT events was observed within a single phylum. However, significant pPT events also took place across different phyla, e.g., Actinomycetota with Pseudomonadota, and Bacteroidota with Bacillota (Fig. 4b; Supplementary Fig. 4b).

We next examined the host range of plasmids. Most plasmids were Limited to a single bacterial host, although some exhibited a broader host range. When analyzed at the contig level, most plasmids were found in no more than five different bacterial hosts, with a maximum of 17 hosts. At the rep-group level, most plasmids had a maximum of 10 hosts, with the maximum extending to 33 hosts (Fig. 4c). Notably, we found that ARPs had a significantly broader host range than non-ARPs, both at the contig and rep-group levels (Fig. 4c; Wilcoxon rank-sum test; Pcontig-level = 0.003; Prep-group-level = 0.004), emphasizing the crucial role of plasmids in the dissemination of ARGs within the UWSs.

Interestingly, the frequency of pPT events involving ARPs among different taxa was higher than those involving plasmids not encoding ARGs (Fig. 4d; Wilcoxon rank-sum test, $P = 6.59e-06$). This further indicates that plasmids play a crucial role in the dissemination of ARGs between different taxa. In particular, we observed a clear north-to-south gradient in the prevalence of clinically relevant pPT across Europe, with the highest number occurring in Spain (Fig. 4e; Wilcoxon rank-sum test). This corresponds to the trend of clinically relevant ARGs, indicating that plasmid-borne resistance may contribute to this trend.

To better understand plasmids' roles in disseminating ARGs in UWSs, we finally focused on each ARG that could potentially be transferred by plasmids among different microbiotas. As expected, we found that the efflux pump category had the highest transferring frequency, with the broadest host range and the highest number of transfer events (Fig. 4g). Importantly, we found that *qnr*, *mph*, *sul*, and beta-lactamase ARGs were the most frequently detected after the efflux pump category. Notably, bla_{TEM} had the broadest host range, which was transferable via plasmids across four different phyla (Fig. 4g). This suggests that these clinically relevant ARGs were more frequently disseminated among microbiota through plasmid-mediated transfer, highlighting the crucial role of plasmids in spreading clinically relevant ARGs in UWSs.

Plasmid transfer promotes the diversity of UWS ARGs

To further investigate the role of pPT events on the spread of ARGs in the UWSs, we conducted a correlation analysis between the diversity of tARGs and pPT events, finding a significant correlation between the richness and abundance of tARGs and pPT events in the UWSs (Fig. 4f; ordinary least squares). This indicates that plasmids may play a crucial role in the diversity and abundance of ARGs within the UWS, especially for ARP-mediated pPT events, which displayed the highest correlation coefficient (Fig. 4f).

To further confirm that plasmids contribute to ARG transfer among microbiota, we focused on the plasmids and ARGs within each MAG. We improved host annotation methods by calculating genomic similarity between plasmids in each MAG and the rest of the plasmids in this study that were not binned. We then analyzed only the MAGs containing plasmids and ARGs to construct the pPT network and events. We found that MAGs involved in plasmid transfer shared more identical ARGs at the ARO level (Fig. 4h; Wilcoxon rank-sum test; $P = 0.01$). Specifically, the coverage, defined as the number of identical ARGs divided by the total number of ARGs between two plasmid-linked MAGs, was significantly higher (Fig. 4h; Wilcoxon rank-sum test; $P = 4.6e-6$). This further supports the notion that plasmids play a significant role in spreading ARGs among microbiota in the UWSs.

Plasmid-carrying taxa exhibit higher ARG diversity and host-specific variability

Next, we examined the correlation between ARGs and microbiota composition in UWSs. Our analysis revealed a negative correlation between overall microbiota diversity (richness) and the abundance of both total ARGs (tARGs) and plasmid-borne ARGs (pARGs) (Fig. 5a). However, when analyzing individual ARGs, we observed a divergence in correlation patterns: the diversity (richness) of tARGs exhibited an overall positive correlation with microbiota diversity. In contrast, pARG diversity showed a negative correlation with microbiota richness (Supplementary Fig. 5a). These results suggest that pARGs are associated with a more specialized subset of the UWS microbiota, whereas tARGs are more broadly distributed among diverse bacterial communities.

Further analysis identified 377 out of 721 metagenome-assembled genomes (MAGs) across 25 phyla carrying ARGs (Fig. 5b). Among these, Pseudomonadota, Bacteroidota, Bacillota_A, and Actinomycetota harbored the most diverse ARG repertoires. Notably, in these dominant phyla, over half of the MAGs contained ARGs (Supplementary Fig. 5b). Additionally, the random distribution of ARG-containing MAGs in the phylogenetic tree (Fig. 5b) suggests that ARGs are not restricted to specific lineages but are instead widely distributed across diverse taxa.

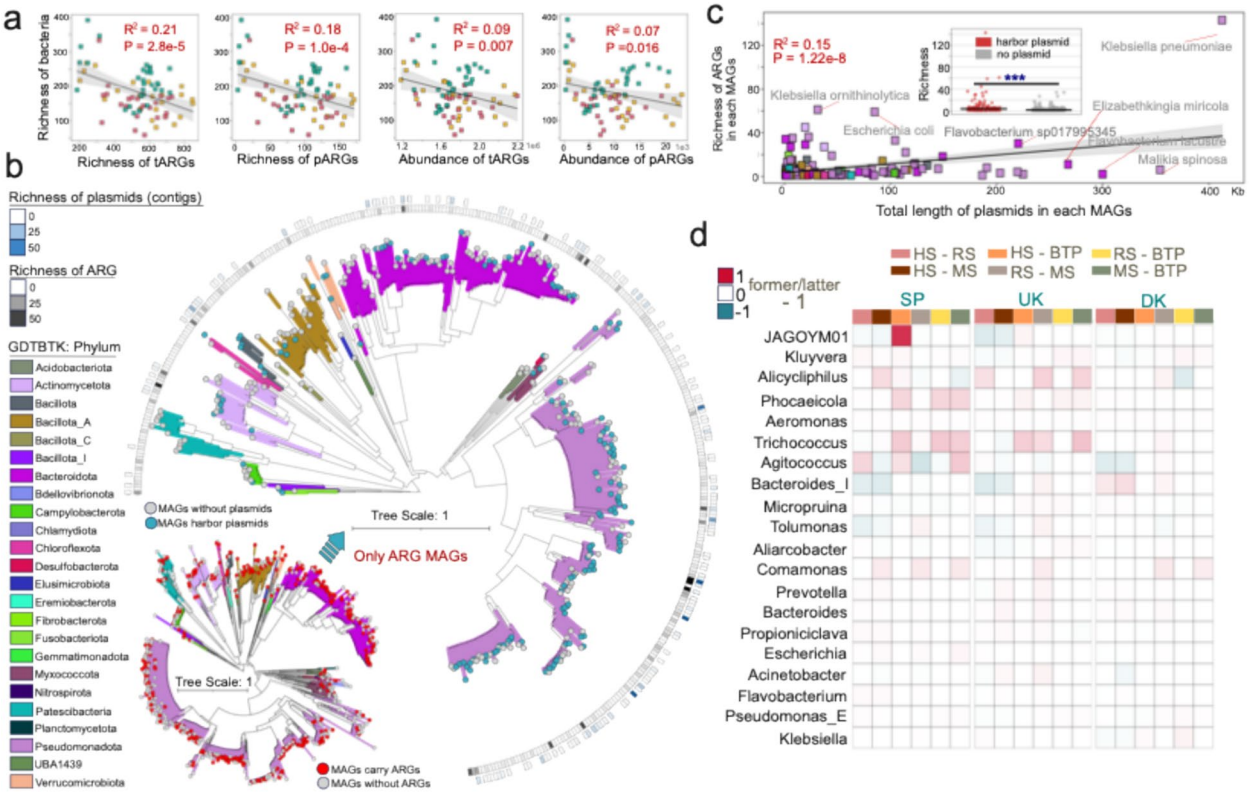

Fig. 5 Phylogenetic and geographical patterns of ARG distribution in metagenome-assembled genomes from wastewater environments. **a** The correlation between the richness and abundance of bacteria and each pARG and tARG is displayed. **b** Phylogenetic tree of MAGs annotated using GTDB-Tk. The smaller tree (without layers) represents all MAGs generated in this study that have GTDB-Tk annotations. Nodes are marked to indicate whether the MAG harbors ARGs (red dots), and branches are color-coded by phylum. The outer-layered tree includes only MAGs containing ARGs. Nodes are colored to show whether the MAGs harbor plasmids (blue), with branches also color-coded by phylum. **c** The correlation between total plasmid length and the richness of ARGs in each MAG is shown, along with a box plot representing the richness of ARGs between MAGs that harbor plasmids and MAGs that do not harbor plasmids. **d** The differential of the richness of ARGs detected from the top 20 genera (genus level) is displayed across different sewers and countries

Our analyses revealed that 257 out of the 377 AR-MAGs harbor plasmids (Fig. 5b) and that the plasmid-containing MAGs exhibit a greater diversity of ARGs (Fig. 5c; Wilcoxon rank-sum test; $P = 3.7e-09$). This suggests that plasmids significantly promote the presence of ARGs in specific taxa. As expected, the total length of plasmids in each AR-MAG is positively correlated with the richness of ARGs within that MAG (Fig. 5c; linear regression test; $P = 1.22e-08$; $R^2 = 0.15$) and supports the fact that some specific taxa preferentially encode ARGs on plasmids rather than on chromosomes. Among these, Pseudomonadota and Bacteroidota are the dominant phyla harboring plasmids and ARGs, particularly *Klebsiella pneumoniae*, which carries the largest sum of detected plasmid sequences and the highest diversity of ARGs. However, we also identified bacteria within these two phyla that possess a large amount of plasmid sequences but exhibit low diversity of ARGs, e.g., *Malikia spinosa*, *Elizabethkingia miricola*, *Flavobacterium lacustre*, and *Flavobacterium* sp. 017995345, while *Klebsiella ornithinolytica* and *Escherichia coli* have relatively less plasmid sequences but high ARG diversity, suggesting that within phyla the density of ARG on plasmids can vary (Fig. 5c).

Bacteroides as persistent ARG carriers throughout UWSs

ARG diversity and abundance varied across UWS microbiota and geographic regions, with the highest diversity observed at HS in most cases. However, *Bacteroides_I*, *Tolumonas*, *Agitococcus*, and *JAGOYM01* exhibited greater ARG diversity at RS and persisted at MS, demonstrating their role as consistent ARG carriers throughout the UWS, especially at residential-relevant areas (Fig. 5b). *Klebsiella* and *Pseudomonas_E* were the dominant ARG-carrying genera across all regions, particularly at BTP (Supplementary Fig. 5c). However, *Bacteroides_I* remained a key reservoir of ARGs along the entire UWS

continuum, from RS to MS, suggesting a sustained role in ARG persistence and mobility.

ARG abundance also displayed regional variation in BTP samples: Spain and Denmark exhibited higher ARG contributions from Acidobacteriota, Actinomycetota, and Myxococcota, whereas the UK had greater ARG abundance from Bacillota, Bacteroidota, and Campylobacterota. These differences suggest that the biofiltration-based wastewater treatment in the UK site may select for distinct ARG-hosting microbiota compared to the activated sludge systems used in the sites in Spain and Denmark.

Overall, these findings highlight that *Bacteroides* not only serve as major ARG reservoirs but also persist throughout the entire UWS pipeline, reinforcing their role in ARG dissemination across different wastewater compartments and geographic regions.

Discussion

In this study, we have undertaken a comprehensive investigation of the urban water plasmidome and resistome by employing multi-omics approaches, including direct plasmidomic sequencing, metagenomic sequencing, and whole-genome sequencing of plasmid isolates. The investigation spanned UWSs across three European countries, providing an unprecedented insight into the diversity and complexity of plasmids and ARGs in these environments.

Our finding highlights the need to integrate multiple sequencing approaches to capture the full diversity of plasmids. To better capture the full diversity of plasmids in urban water systems, we integrated metagenomic, plasmidomic, and isolate-based sequencing approaches. Metagenomic sequencing was more effective at reconstructing larger plasmids, while plasmidomic sequencing favored smaller plasmids (Fig. 1d), consistent with prior observations [42]. Although this combination provided a broader view of the plasmidome (Fig. 1b), the detection of conjugative and mobilizable plasmids—especially those carrying ARGs—remained limited (Fig. 1c). Therefore, we included an isolate-based approach that was intended to expand the known pool of conjugative ARPs, complementing the limited findings from sequencing. However, many of the phenotypically resistant plasmids isolated from wastewater treatment samples did not contain ARGs detectable by current databases, suggesting a gap between resistance phenotypes and genotypic annotations. Besides, most of the plasmid isolates were not detected by plasmidomic and metagenomic sequencing (Supplementary Fig. 1 d); this underscores current limitations in sequencing methods [42] and databases [29], such as the low abundance of certain conjugative plasmids—which makes them difficult to detect reliably—and the high sequence similarity among plasmid backbones and mobile genetic elements, which hampers accurate assembly and classification, which highlights the need to further explore the urban water plasmidome [43, 44].

Our previous study showed that plasmids harbor diverse ARGs in UWSs, but the proportion of plasmid-borne ARG was not estimated [22]. In the present study, we show that genes conferring resistance to over 80% of all drug classes could be found on plasmids (Fig. 2a). We found that plasmid-encoded resistance genes were very different from total resistance genes (Fig. 2d). The diversity of plasmid resistance genes (pARGs) strongly correlates with total resistance genes (tARGs), showing that plasmids always carry a significant portion of all resistance genes (Fig. 2c and d). MAGs harboring plasmids exhibited significantly higher ARG diversity than those without plasmids (Fig. 5b and 5c), confirming that plasmids are major contributors to ARG diversity in UWSs. This finding not only expands upon previous studies highlighting plasmids' role in ARG dissemination [13, 15, 45] but also brings a new perspective on plasmid-encoded ARGs in UWSs. Notably, one crucial finding is that UWS plasmids encoded more clinically relevant resistance genes (Fig. 2b), and these ARGs have higher mobility (Fig. 3f) and are most frequently transferred between microbiota in UWSs (Fig. 3g). Specifically, some beta-lactamase genes are exclusively located on transmissible plasmids (Fig. 3g). These findings support the notion of a complex interaction between plasmids and their cargo, which could influence both the dissemination dynamics of these genes and the persistence of plasmids in microbial communities [19].

Another significant finding is that carrying ARGs can improve the persistence of plasmids. Our comparative analysis confirmed that plasmids harboring ARGs have significantly higher prevalences than non-ARG-carrying plasmids. Specifically, plasmids with multiple ARGs exhibited even greater prevalence, particularly those carrying distinct types of resistance genes (Fig. 3d). Recent studies have proved that various factors can impact plasmid persistence, such as fitness effects [46], plasmid transfer and interactions [47, 48]. Our findings suggest that specific plasmid traits can enhance their persistence, likely because these traits confer fitness benefits to their microbial hosts [49] and particularly when multiple benefits are provided, such as antibiotic resistance towards different compounds [50]. This suggests that the presence of multiple ARGs enhances plasmid persistence and dissemination within UWS environments. Indeed, we did observe that ARPs had a broader host range (Fig. 4c) and higher pPT events (Fig. 4d), highlighting the crucial role of plasmids in carrying and spreading ARGs,

underscoring the increasing urgency of developing and implementing strategies to limit plasmid abundance to control the spread of these critical genes.

One of the most striking findings was that the plasmidome and resistome were highly similar across all three countries (Denmark, Spain, and the UK). Despite differences in local antibiotic use, microbial composition, and wastewater treatment technologies, we found a large fraction of plasmid-encoded ARGs present in all regions (Fig. 3a, Supplementary Fig. 3a). This suggests that UWSs are not isolated genetic reservoirs but are interconnected through environmental dissemination pathways. Notably, highly transmissible plasmids carrying ARGs, particularly beta-lactamases, were detected in every country and across multiple UWS compartments (Fig. 3g). The persistence of these resistance determinants in treated wastewater further highlights their high mobility and potential for dissemination into natural water systems. Moreover, our analysis of potential plasmid transfer networks (pPT events) revealed a consistent north-to-south gradient, with Spain exhibiting the highest frequency of clinically relevant pPT (Fig. 4e). This gradient aligns with previous studies indicating that the abundance of clinically relevant ARGs is often higher in southern European regions [22]. Notably, this study only included three cities from three European countries, which imposes a limitation on the geographical scope. Future studies should incorporate more countries across Europe to provide a more comprehensive perspective. The widespread geographical overlap of plasmid-encoded ARGs underscores the critical need for international monitoring and mitigation strategies. Given that ARG-carrying plasmids can persist in both treated and untreated wastewater, there is a significant risk of their dissemination into human-associated and environmental bacterial populations, reinforcing the urgency of global surveillance efforts.

Previous studies have shown that antibiotics have specific effects on the gut microbiome and cause specific microbial perturbation [51], and the gut resistome composition is intimately connected to the taxonomic structure of the gut microbiome [52]. In our study, we found that the UWS resistome, both tARGs and pARGs, showed negative correlations to microbiota diversity and abundance (Fig. 5a), which is possibly due to the UWS resistome and plasmidome are also determined by specific taxa. Indeed, our further analyses confirmed that not all MAGs carry ARGs and/or harbor plasmids (Fig. 5b). *Klebsiella* and *Pseudomonas_E* were the dominant genera carrying ARGs in UWSs, in accordance with previous studies [19, 53]. However, an unexpected result was the significant role of Bacteroidota, particularly *Bacteroides_I*, in the resistome and plasmidome of UWSs. Our earlier research indicated that this phylum contains many plasmids in the infant gut microbiome and plays a crucial role in plasmid transfer [27]. In the present study, we found that the resistome of *Bacteroides_I* dominated the north-to-south gradient of clinically relevant antibiotic resistomes (Fig. 5d) and was notably affected by various wastewater treatment processes (Supplementary Fig. 2a). Furthermore, bacteria in this phylum exhibited varying resistome abundances across different regions (Supplementary Fig. 5c). These findings suggest that *Bacteroides_I* also plays a significant role in shaping the resistome and plasmidome of UWSs. However, most ARGs in existing databases are not derived from this taxon [36], indicating that its contribution has been significantly underestimated.

Our findings highlight the public health and environmental risks posed by UWSs as reservoirs for ARG persistence and dissemination. Many of the identified plasmids carry clinically relevant resistance genes, and the presence of transmissible plasmids in BTP samples (Figs. 3c, f, and g and 4g) suggests that current treatment processes, particularly biofiltration, may not effectively remove high-risk resistance determinants from hospital effluents. To address these risks, wastewater monitoring should be enhanced to track high-risk plasmids and ARGs, particularly in downstream environments. Treatment processes must be optimized to improve the removal of transmissible ARG-carrying plasmids, and international collaboration is essential for detecting and mitigating the spread of globally shared resistance determinants. Future research should assess the impact of wastewater-derived ARGs on human health, particularly their potential uptake by pathogens. Investigating targeted filtration, advanced disinfection, and microbial community engineering may provide new strategies to limit plasmid-mediated resistance dissemination. These findings reinforce the need to view wastewater treatment as a critical point of intervention in controlling antibiotic resistance.

Conclusion

This study provides a comprehensive analysis of plasmids and ARGs in UWSs across Denmark, Spain, and the UK, revealing previously unrecognized diversity of plasmids and their central role in ARG dissemination. Integrating plasmidomic, metagenomic, and whole-genome sequencing, we show that UWS plasmids encode the most clinically relevant ARGs, particularly beta-lactamases, and enhance ARG persistence and mobility. Despite regional differences, a large fraction of plasmid-encoded ARGs is shared across countries, highlighting UWSs as interconnected reservoirs of resistance. Plasmids carrying multiple ARGs were more prevalent and broadly distributed, reinforcing their role in HGT.

Bacteroidota emerged as a key ARG reservoir persisting throughout UWS compartments. Bacteroidota's ARG diversity was shaped by treatment processes, with activated sludge retaining more plasmid-encoded ARGs than biofiltration, suggesting Bacteroidota's overlooked role in wastewater resistome dynamics. Wastewater treatment selectively influenced plasmid retention, but transmissible plasmids persisted in treated wastewater, indicating current treatment methods do not eliminate high-risk ARGs. These findings underscore the need for optimized treatment strategies and enhanced plasmid surveillance to limit plasmid-mediated resistance spread and inform future intervention efforts.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-025-02253-0.

Supplementary Material I.

Acknowledgements
This research was funded by a Joint Programming Initiative-Antimicrobial Resistance grant (JPIAMR; DARWIN project #7044-00004B) to BFS and Novo Nordisk Foundation Data Science Collaborative Research Programme 2020 under grant agreement NNF 200C0062223 (Machine learning methods for data-driven discovery of antibiotic resistance plasmid dissemination and evolution) to SJS. We also want to thank Dr. Sabela Balboa Mendez, Dr. Marcos Quintela Baluja, and all the sewers' systems operators involved in the sampling campaign.

Authors' contributions
WLH processed the bioinformatic and statistical analysis and produced the incorporated figures and wrote the first draft of the manuscript; BFS, AD, JN and SJS designed the sampling campaigns and the overall experimental work; ZW, ZFY, AKO performed experimental work; ZFY, JSM, BFS, AD, JN and SJS gave suggestions for the bioinformatic analysis; all the co-authors reviewed and offered suggestions for this manuscript.

Funding
Open access funding provided by Copenhagen University This study was supported by the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR), grant #7044-00004B (DARWIN project) awarded to B.F.S., and by the Novo Nordisk Foundation Data Science Collaborative Research Programme 2020, grant NNF200C0062223 awarded to S.J.S. for the project "Machine learning methods for data-driven discovery of antibiotic resistance plasmid dissemination and evolution."This work was partially supported by DeiC National HPC (g.a. DeiC-KU-L2-55) through the University of Copenhagen's IT Datalab. Novo Nordisk Foundation Data Science Collaborative Research Programme 2020,NNF 200C0062223,Joint Programming Initiative-Antimicrobial Resistance grant,DARWIN project #7044-00004B

Data availability
Shotgun metagenomic and plasmidome sequencing data have been deposited in the European Nucleotide Archive (ENA) under BioProject accession PRJEB85938. The curated, non-redundant plasmid catalog is publicly available on Zenodo under https://doi.org/10.5281/zenodo.13143815.

Declarations

Ethics approval and consent to participate
Not applicable. This study does not involve human participants, animal experiments, or the use of personally identifiable data.

Consent for publication
All authors have read and approved the final version of the manuscript and consent to its publication.

Competing interests
The authors declare no competing interests.

Received: 2 June 2025 Accepted: 4 September 2025
Published online: 22 January 2026

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