



Insights into the circular

the cryptic plasmidome and its derived antibiotic resistome in the urban water systems

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Full length article

Insights into the circular: The cryptic plasmidome and its derived antibiotic resistome in the urban water systems

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ABSTRACT

Plasmids have been a concern in the dissemination and evolution of antibiotic resistance in the environment. In this study, we investigated the total pool of plasmids (plasmidome) and its derived antibiotic resistance genes (ARGs) in different compartments of urban water systems (UWSs) in three European countries representing different antibiotic usage regimes. We applied a direct plasmidome approach using wet-lab methods to enrich circular DNA in the samples, followed by shotgun sequencing and *in silico* contig circularisation. We identified 9538 novel sequences in a total of 10,942 recovered circular plasmids. Of these, 66 were identified as conjugative, 1896 mobilisable and 8970 non-mobilisable plasmids. The UWSs' plasmidome was dominated by small plasmids (≤ 10 Kbp) representing a broad diversity of mobility (MOB) types and incompatibility (Inc) groups. A shared collection of plasmids from different countries was detected in all treatment compartments, and plasmids could be source-tracked in the UWSs. More than half of the ARGs-encoding plasmids carried mobility genes for mobilisation/conjugation. The richness and abundance of ARGs-encoding plasmids generally decreased with the flow, while we observed that non-mobilisable ARGs-harbouring plasmids maintained their abundance in the Spanish wastewater treatment plant. Overall, our work unravels that the UWS plasmidome is dominated by cryptic (*i.e.*, non-mobilisable, non-typeable and previously unknown) plasmids. Considering that some of these plasmids carried ARGs, were prevalent across three countries and could persist throughout the UWSs compartments, these results should alarm and call for attention.

1. Introduction

In the last decades, horizontal gene transfer (HGT) has gained more and more awareness (Stokes and Gillings, 2011). Reasons behind this are HGT not only potentiates the cargo genes such as antibiotic resistance genes (ARGs) spread among similar and distinct microbes in various environments but even accelerates the occurrence of several multidrug-resistant (MDR) organisms in different ecological niches (Sun et al., 2019). Currently, plasmids are of particular concern as they provide an efficient and rapid transmission capacity (Pinilla-Redondo et al., 2018).

Moreover, other MGEs such as insertion sequences, integrons and gene cassettes as well as transposable elements can constitute the accessory regions of the plasmids (Partridge et al., 2018). Specifically, conjugative plasmids are of particular interest since they possess several comprehensive genetic characteristics, such as mobility (MOB) genes [including an origin of transfer (*oriT*), a relaxase, a type IV coupling protein (T4CP)] and mating pair formation genes (*i.e.*, type IV secretion system, T4SS) (Smillie et al., 2010). These features enhance these well-reported plasmids maintenance and mobility within bacterial populations by conjugation. However, plasmid fitness costs to the hosts under different

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environmental conditions vary remarkably (Pinilla-Redondo et al., 2018), and plasmid persistence remains mysterious (Carroll and Wong, 2018). The total plasmid populations in a specific niche (microbiome or environment) are known as plasmidome. We and others have previously used the term mobilome (Jorgensen et al., 2014a), but given that mobilome can cause ambiguity for referring to all the MGEs, we use the term plasmidome in the following text.

Various plasmidome approaches have been made in diverse environments, such as cow rumen (Brown Kav et al., 2020), rat cecum (Jorgensen et al., 2014b), human gut (Suzuki et al., 2019; Willmann et al., 2019), soil (Luo et al., 2016), sediments (Mootapally et al., 2021), surface water (Perez et al., 2020), groundwater (Kothari et al., 2019), and extensively in wastewater (Ferreira et al., 2019; Li et al., 2015; Li et al., 2012; Zhang et al., 2011). However, the methodologies in previous studies were compromised in terms of biased targets and lack of needed bioinformatic tools. Conventionally, environmental plasmidome studies were performed under strong selective pressures that alter plasmid dynamics and bias towards phylogenetic evolution of plasmids compared to their indigenous habitats (Hulter et al., 2017). Traditional cultural-dependent methods (antibiotic screening for resistance plasmids and *in situ* matings with fluorescent reporters) and cultural-independent techniques [exogenous plasmid isolation, PCR-based replicon typing (PBRT) and relaxase/MOB typing, epicPCR] targets a relatively small part of the entire plasmidome (Rios Miguel et al., 2020; Saak et al., 2020). Sequencing-based methods such as shotgun metagenomics and whole-genome sequencing and single-cell genomics facilitate an unbiased characterization of known and novel plasmids in a sample. However, large quantities of sequencing-yield reads are discarded due to chromosomal contamination in nontargeted sequencing approaches, which tends to be not cost-effective. Accordingly, there is no consensus on a universal methodology for plasmidome study.

Most Europeans reside in urban areas (>70 %), and urban water systems (UWSs) play a crucial role in today's city infrastructure and the water cycle (Kaushal et al., 2015). Nevertheless, with the intensive use of antibiotics for domestic and hospital purposes, wastewater treatment plants (WWTPs) have been considered hotspots of antibiotic resistance (AR) and potential reservoirs for ARGs evolution and dissemination to the environment (Singh et al., 2019; Yin et al., 2019). This is mainly due to the actual goal of WWTPs, which was exclusively to remove the majority of solids, organic matter and nutrients in wastewaters and ensure sanitary effluent discharging to the downstream water bodies, yet not intended to reduce ARGs (Pruden, 2014). Superabundant studies have been made on detecting antibiotics, ARGs, antibiotic-resistant bacteria and ARGs harbouring plasmids (AR plasmids) through PCRs or selective screenings in wastewaters (Cacace et al., 2019; Novo et al., 2013; Osińska et al., 2020; Pallares-Vega et al., 2019; Paulshus et al., 2019; Yuan et al., 2020). Meanwhile, researchers have been intrigued by the possible association between microbiome and antibiotic resistome in the WWTPs by using high-throughput qPCR analysis (Buelow et al., 2020; Quintela-Baluja et al., 2019; Yu et al., 2020), or combined 16S profiling and metagenomics (Che et al., 2019; Ekwanzala et al., 2020; Guo et al., 2017; Jia et al., 2017; Ju et al., 2019; Liu et al., 2019; Ng et al., 2019; Yin et al., 2019). Nonetheless, the dynamics of the plasmidome and the plasmid-mediated mobile antibiotic resistome in the UWSs are poorly understood. On the other hand, different countries have specific antibiotic consumption practices, which have been documented as an explanation for distinctive human gut resistomes (Forslund et al., 2013). Considering the discharge of human excrements to the sewers, the wastewater antibiotic resistome can be influenced by the regional antibiotic usage (Yin et al., 2019; Yu et al., 2020). Herein, we proposed investigating the UWS plasmidome in a dynamic perspective concerning different compartments during the UWS processing. Denmark, Spain and the United Kingdom, located in northern, southern and western Europe with country-specific antibiotic use practices, were chosen in this study (Fig. S1). Sewage and wastewater samples were collected from the sewer catchments to the WWTP biological treatment

process basin (BTP) during summer and winter in 2018, and a direct plasmid DNA metagenomic sequencing strategy skipping the widely used transposon-aided capture (TRACA) and multiple displacement amplification (MDA) was applied. This was in light of methodology studies that suggested direct sequencing of plasmid DNA samples uncovered more larger-sized plasmids (Alanin et al., 2021; Dean et al., 2001). Moreover, we introduced a plasmid purification using an exonuclease plasmid-safe digestion to shear and remove chromosomal DNA before building sequencing libraries (Browne et al., 2019).

To our best understanding, we present the full UWS plasmidome for the first time, shedding light on how the plasmids and plasmid derived mobile antibiotic resistome migrate in different compartments in the UWSs.

2. Materials and methods

2.1. Subjects and sample pre-treatment

Comparable UWSs from Denmark, Spain and the United Kingdom located in Odense, Santiago de Compostela and Durham County, respectively, were recruited in this study (Fig. S2). At each UWS, hospital sewer (HS), residential sewer (RS), mixed sewer (MS, *i.e.*, the mixture of HS and RS, and was regarded as the WWTP influent), and treated wastewater after the BTP in the WWTP were sampled (Fig. 1A). Specifically, for the BTP facilities used in the WWTPs, biological treatment basins were applied in Denmark and SP, while biofilter was employed in the United Kingdom. Sampling campaigns at the four sites were performed in the winter and summer of 2018 using ISCO automatic samplers for 24-hour flow (50 mL per 5 min) in Denmark and the United Kingdom, while 24-hour-time proportional samples in SP (mixing hourly samples according to flow information). Two sewer lines were operated and sampled for HS in Denmark (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 United Kingdom, 24 samples from Spain, and 30 samples from Denmark were obtained. Detailed sampling information was recorded in our previous study (Li et al., 2021).

All samples were initially cooled with ice on-site, then 100 mL of each sample was spun down by a centrifuge (Eppendorf, Hamburg, Germany) at 10,000 g for 8 min at 4 °C in the laboratory. The supernatants were removed, while the pellets were resuspended in 20 % glycerol stock to reach a final volume of 10 mL for storage at -80 °C.

2.2. The direct plasmidome approach

The direct plasmidome approach applied here refers to our collaborators (Alanin et al., 2021; Jorgensen et al., 2014b), and was further modified in the lab (Fig. S3). Briefly, samples were pretreated by filtration, vortex and sonication and resuspended in TE buffer. Afterwards, an enzymatic lysis (lysozyme, mutanolysin, and lysostaphin) was performed to break the Gram-positive cell walls. Then alkaline lysis and plasmid-safe DNase digestion were performed to obtain fine plasmid DNA. Quality-checked DNA was proceeded to the library preparation and finally sequenced on an Illumina NextSeq platform with a v2.5 sequencing kit (Illumina, San Diego, CA, USA). The detailed methodology was introduced in Text S1.

2.3. Bioinformatic analysis

2.3.1. Processing the raw data

An in-house bioinformatic pipeline *Plaspline* (version: 1.1, <https://github.com/Wanli-HE/Plaspline>) for plasmidome analysis was implemented in this study. 150 bp paired-end raw reads from the 78 samples were processed through quality control, assembly for circular contigs (regarded as putative plasmids from then on), identification and

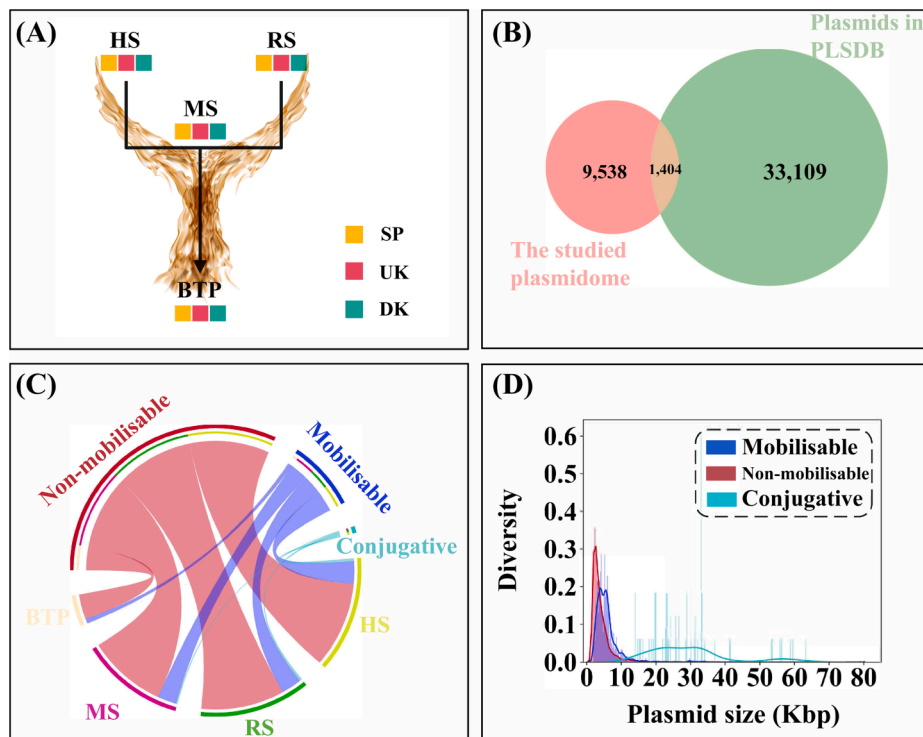


Fig. 1. General information of the UWS plasmidome. (A) Schematic of the sewer and wastewater flow (from HS and RS to MS, then from MS to BTP). (B) Number of plasmids recovered in this study compared to PLSDB database v2021_06_23. (C) Distribution of plasmids in different treatment compartments in the UWSs. (D) Distribution of plasmid size from each mobility group. HS: hospital sewer; RS: residential sewer; MS: mixed sewer, i.e., the mixture of HS and RS; BTP: WWTP biological treatment process basin. In Fig. 1B, the intersection in the Venn diagrams represents the hit plasmids of the present plasmidome with PLSDB.

removal of redundant circular plasmids, calculations of plasmid abundance based on reads mapping (normalization by 16S-rRNA gene copy per sample thereafter), and plasmid annotation and classification. Briefly, *de novo* metagenome assembly was performed using *metaSPAdes* version 3.14.1 (Nurk et al., 2017) with trimmed and filtered sequence reads as the input (default parameters were applied, *k-mer*: 21,33,55). Contigs longer than 1 Kbp were selected for the downstream analysis. Circular contigs were then reconstructed by *metaSPAdes* version 3.14.1 (Nurk et al., 2017) using the “-plasmid” option38 and *SCAPP* version v0.1.4 (Pellow et al., 2021) with max *k-mer* 55. Spurious plasmids (false positives, i.e., non-plasmid circular elements) were removed from the dataset using *plasmidVerify* (version: May 1, 2020, <https://github.com/ablab/plasmidVerify>), which classified contigs as plasmid or non-plasmid based on their gene contents. A non-redundant catalog of circular plasmid genomes was constructed using *MMseqs* (version: 12.113e3, <https://github.com/soedinglab/MMseqs2>) with sequence identity >90 % and coverage >95 % (“-cov-mode” 0). Specifically, we consider the presence of a plasmid in one sample when >80 % of the plasmid sequence was covered by at least one read. And this was applied in the plasmid source tracking analysis.

2.3.2. PLSDB similarity search

All the retrieved plasmid sequences were submitted to PLSDB (database version: 2021_06_23; tool version: 0.4.1–386-gd7e4b70b05) and searched by blast+ (version: 2.12.0 +) to evaluate plasmid similarity to the input (identity ≥ 0.8 and coverage ≥ 0.7) (Camacho et al., 2009). Plasmid sequences without hit were considered novel plasmids.

2.3.3. Correlation analysis with bacterial taxa

16S rRNA sequencing for the same sampling campaign was performed (Li et al., 2021), and the data was recruited for the correlation analysis of plasmid richness and microbial richness by using the R package “*ggtrendline*” (version: 1.0.1, <https://github.com/PhDMei>

wp/ggtrendline). Details for the 16S rRNA sequencing can be found in Text S2.

2.3.4. Statistical analysis

Richness index of the plasmidome was estimated by the “*skbio.diversity*” function (version: 0.4.2, <https://scikit-bio.org/docs/0.4.2/diversity.html>) in Python (Van Rossum and Drake, 2009). Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity was applied to evidence the different patterns of plasmid communities’ through found in the different UWS compartments. actors influencing the plasmidome composition in samples such as: countries of origin, season, and UWS compartments were compared by significance tests (Wilcoxon rank-sum test), and plasmidome similarities (ANOSIM analysis) were observed by using python packages “*skbio*” (version: 0.5.7, <https://scikit-bio.org/>).

3. Results

3.1. UWS plasmidome in a nutshell

After the direct plasmid DNA metagenome sequencing, the raw data were implemented in the circular module of *Plaspline* and passed through the plasmid verification and clustering. Collectively, we recovered 10,942 non-redundant plasmids: 66 conjugative, 1906 mobilisable and 8970 non-mobilisable plasmids. Based on the PLSDB similarity search, we discovered 9,538 novel plasmids (approximately 87.2 % of the present plasmidome), which expands our knowledge of the UWS plasmidome (Fig. 1B). Non-mobilisable plasmids accounted for the most part in every compartment in the UWSs, followed by mobilisable and conjugative plasmids (Fig. 1C). Meanwhile, we observed a similar pattern with the plasmid database (Smillie et al., 2010) of the association between plasmid size and mobility in Fig. 1D. We illustrated the mean peak rankings of the plasmid length: conjugative plasmids >

mobilisable plasmids > non-mobilisable plasmids (only one peak was detected in this study).

Relative abundances of known MOB types (MOB_Q, MOB_V, MOB_F and MOB_P) of plasmids showed a declining trend along with the flow, yet the relative abundances of MOB_unknown steadily grew during the UWS processing (Fig. S4A). On the other hand, the plasmid Inc groups could be arguably divided into four groups based on their fluctuational relative abundance (Fig. S4B). ‘Inc Group 1’ plasmids including several rep_cluster_xxxx showed higher relative abundance in the BTPs. ‘xxxx’ here stands for the miscellaneous numbering of the rep_clusters, which is due to the fact that contemporary *in silico* analysis hasn’t incorporated these clusters with the canonical established replicon classification system. ‘Inc Group 2’ including IncQ1, IncQ2, IncI1, IncFII possessed the most diverse Inc categories, and their relative abundances mostly showed a decreasing propensity with the UWS processing. ‘Inc Group 3’ containing multiple rep_cluster_xxxx were persistent during the UWS processing. ‘Inc Group 4’ such as IncW showed superior relative abundance in the sewer lines, however, were likely to be removed in the BTPs.

Meanwhile, we perceived a general decreasing trend in plasmid richness with the flow regardless of country (Fig. 2A). There was

consistently retained high plasmid richness in the sewers (HS, RS and MS) in Spain with plasmid richness in the Spanish RS being significantly ($p < 0.01$) higher than in the Danish and British RS. Overall, Denmark held the lowest plasmid richness throughout the UWS processing. It is noteworthy that the British BTP remained significantly ($p < 0.01$) higher plasmid richness compared to the other two countries. Basically, the plasmids in HS and RS were dissimilar except in Spain, MS was shaped by RS, and BTP plasmids were remarkably different from the sewers (Fig. 2B). Interestingly, the plasmid populations in the three countries appeared totally different in the separated compartments (Fig. 2C).

3.2. The common and persistent plasmids in the UWSs

Given the background of the globalization of ARGs, we assumed plasmids were also internationally spread and ‘shared’ by different countries. In this study, we discovered a shared pool of plasmids in every treatment compartment (Fig. 3A). The ‘biggest share’ was found in HS ($n = 2000$, 24.5%), followed by MS ($n = 1857$, 25.3%), RS ($n = 1776$, 22.8%) and BTP ($n = 67$, 3.0%). Spain and the United Kingdom consistently shared the most plasmids in HS, MS and BTP, while Spain and Denmark shared more plasmids in RS. Notably, Spain possessed the

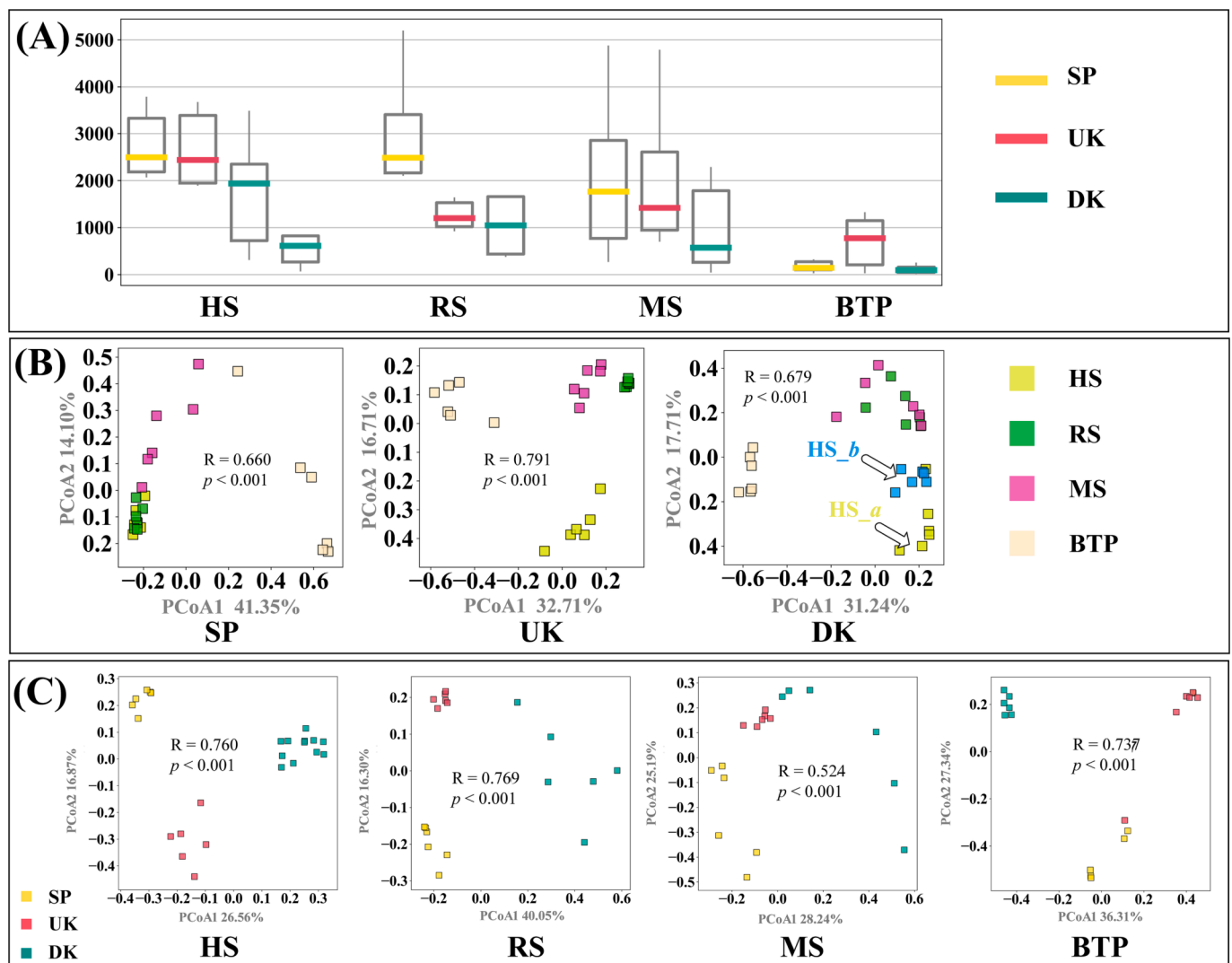


Fig. 2. Plasmid diversity and distribution in the UWSs. (A) The dynamic plasmid richness along with the UWS processing. (B) PCoA analysis based on BrayCurtis distances for the UWS plasmidome from different UWS compartments in each country. (C) PCoA analysis based on BrayCurtis distances for the UWS plasmidome from different countries in each UWS compartment. HS: hospital sewer; RS: residential sewer; MS: mixed sewer, i.e., the mixture of HS and RS; BTP: WWTP biological treatment process basin. HS_a and HS_b were the two sewer lines operated in Denmark.

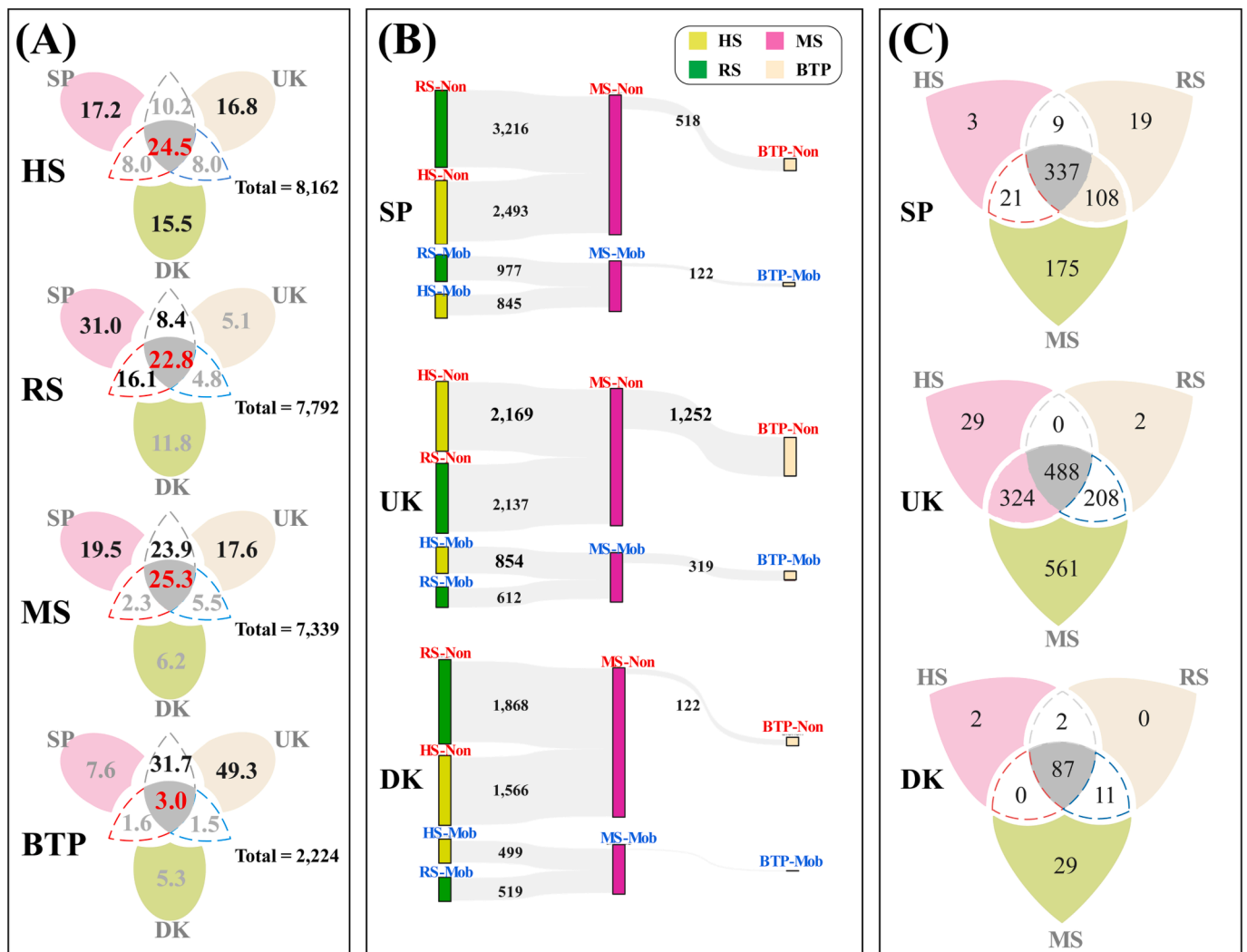


Fig. 3. The plasmid persistence in the UWSs. (A) Common plasmids shared by different countries in each UWS compartment. (B) Plasmid trajectories in the UWSs. (C) Source distributions of the source-trackable BTP plasmids in each country. HS: hospital sewer; RS: residential sewer; MS: mixed sewer, i.e., the mixture of HS and RS; BTP: WWTP biological treatment process basin. Numbers inside of the Venn diagram of Fig. 3A represent the percentage of the total number of shared plasmids. The numbers in Fig. 3B and the numbers inside the Venn diagram of Fig. 3C represent the absolute plasmid numbers. In Fig. 3B, the suffix 'Mob' and 'Non' after 'HS', 'RS', 'MS' and 'BTP' mean 'plasmids with mobility' and 'plasmids without mobility', respectively.

highest number of country-distinct plasmids in all the sewers, while the United Kingdom owned the most distinct plasmids in BTP (Fig. 3A).

Based on the knowledge of the richness and abundance of most plasmids declined with the UWS processing (Figs. 2A and S4B), we wondered about the fate of a specific group (mobility) of plasmids. Therefore, we detected the plasmidome trajectory, i.e., the plasmid populations flowing from an upstream treatment stage to a downstream treatment stage in the UWSs. The plasmidome trajectories of different mobility groups from HS and RS to MS, and from MS to BTP were studied (Fig. 3B). RS predominantly contributed to the plasmid flow to MS in Spain and Denmark, however, HS played a bigger role in the United Kingdom for transporting plasmids to MS. And the majority (>75 %) of the plasmid flows were composed of non-mobilisable plasmids in the trajectories of HS to MS and RS to MS. Considering the striking plasmid richness in the British BTP (Fig. 2A), we were curious about where these plasmids came from and what could be the reasoning. The plasmidome trajectory showed these British BTP plasmids were dominantly occupied by non-mobilisable plasmids. Nevertheless, these BTP plasmids mainly originated from either RS or HS, or alternatively, the indigenous MS plasmid populations remained a question. Thereby, a source-tracking analysis was performed for the BTP plasmids. We found that 671, 131

and 1,612 BTP plasmids were trackable to the upstream sewer compartments in Spain, Denmark and the United Kingdom, respectively (Fig. 3C). 337, 87 and 488 plasmids were universally propagated in the sewer catchments in Spain, Denmark and the United Kingdom, respectively, and finally flowed to the BTP in each country. Intriguingly, MS was the key BTP plasmids contributor regardless of country (Fig. 3C).

In Fig. 3A, the intersection in the Venn diagrams represents the commonly shared plasmids between/among countries. In Fig. 3C, the intersection in the Venn diagrams represents the common plasmids found in both/all sewers that also showed occurrence in BTP.

3.3. The UWS plasmidome associated antibiotic resistance

3.3.1. AR plasmids

Non-mobilisable plasmids took a large part (82.1 %) in the present UWS plasmidome, while in the identified 216 AR plasmids, we observed a high ratio (52.3 %) of plasmids with mobility (Fig. 4A). Plasmid trajectory analysis reveals the persistence features of AR plasmids in the UWSs (Fig. 4B). The sewer sources (HS and RS) both contributed considerably to the end of sewer (MS) in the three countries, however, AR plasmids could hardly flow to the BTP in Spain and Denmark. In light

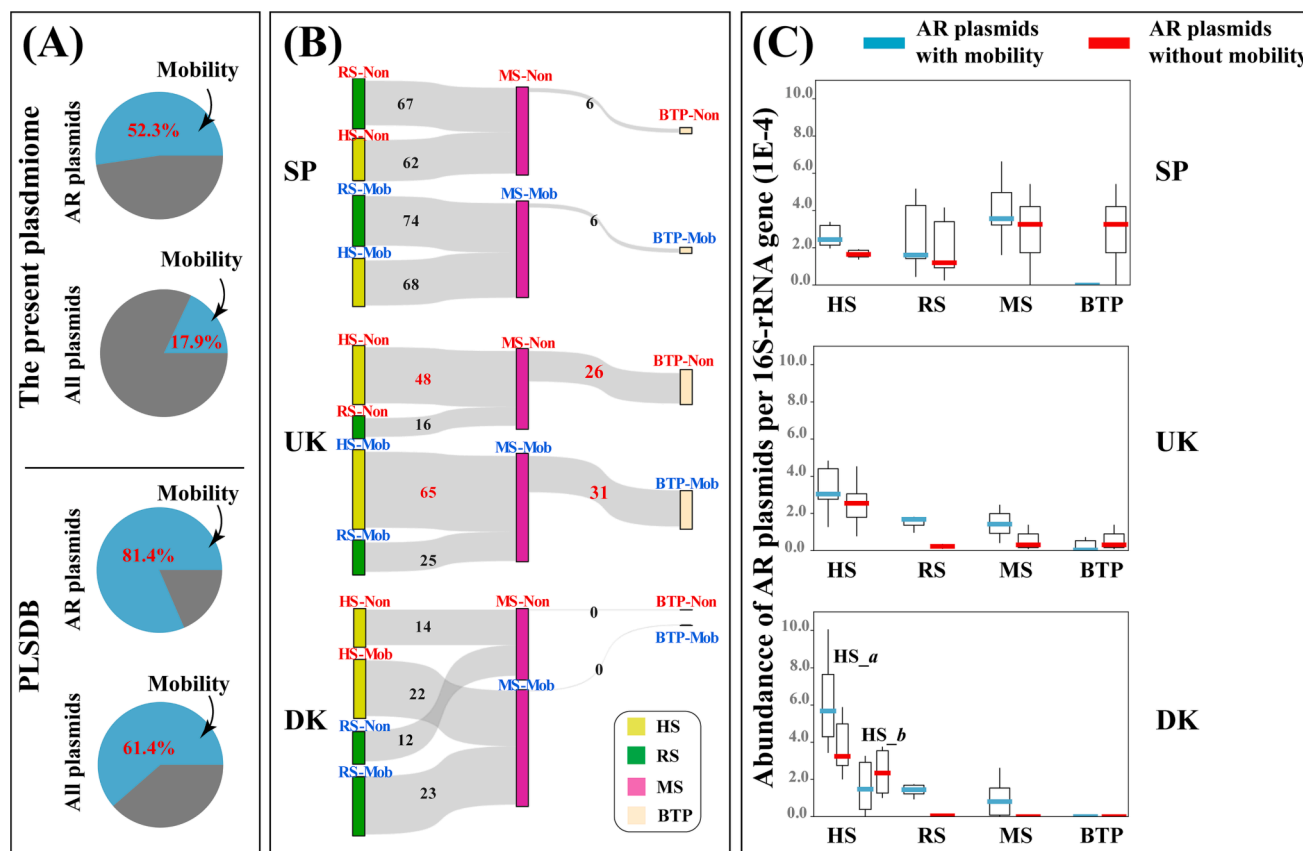


Fig. 4. The general pattern of the AR plasmids in the UWSs. (A). Mobility of the UWS plasmidome with comparison to the PLSDDB database. (B) AR plasmid trajectories in the UWSs. (C) The dynamic AR plasmid abundances along with the UWS processing. HS: hospital sewer; RS: residential sewer; MS: mixed sewer, i.e., the mixture of HS and RS; BTP: WWTP biological treatment process basin. In Fig. 4B, the suffix ‘Mob’ and ‘Non’ after ‘HS’, ‘RS’, ‘MS’ and ‘BTP’ mean ‘AR plasmids with mobility’ and ‘AR plasmids without mobility’, respectively.

of the tremendous plasmid populations transported from MS to BTP in the United Kingdom (Fig. 3B), we also detected remarkable AR plasmids that were delivered to the British BTP.

By recruiting the microbiome dataset of the same sampling campaign (Li et al., 2021), the plasmidome abundance was normalized per 16S-rRNA (Fig. 4C). We illustrated the mobilisable AR plasmids abundance generally dropped along with the flow in all countries. In Denmark and the United Kingdom, non-mobilisable AR plasmids consistently kept at a low level of abundance in RS, MS and BTP though it was comparable in HS with Spain. In contrast, the AR plasmid abundance was promoted in the Spanish MS from the sewer sources (HS and RS), and the BTP retained a similar level of abundance with MS in Spain.

3.3.2. Plasmid-borne ARGs

Plasmid-borne ARGs were dragged and analyzed to explore the plasmidome derived resistome. Basically, ARGs in RS and MS were similar in every country, and HS seemed also closely connected to other sewers in Spain (Fig. 5A). A seeming country coding pattern of ARGs was discovered in the sewer compartments, and it appeared the ARG groups from different countries were more distinct in the raw sewers (HS and RS) than in MS (Fig. 5B). We then examined the abundance of a specific family of the plasmid-borne ARGs (Fig. 5C). In Denmark and the United Kingdom, RS always harboured the lowest abundance of ARGs except for *tet*, *lnu* and *qnr* genes, whilst RS carried various and prosperous ARGs in Spain. Generally, Spain harboured the most diverse and abundant ARGs in the sewer catchments. United Kingdom showed unique high ARG abundances of *bla*, *mph*, *ant* and *abc-f* families in BTP. Denmark steadily exhibited low (or under detection threshold) ARG abundances except for *tet*, *beta-lac* and *erm*. Particularly, *qnr* genes were exclusively

detected in the Danish raw sewers (HS and RS).

4. Discussion

4.1. The state-of-art plasmidome methodology

With the present plasmidome methodology and bioinformatic analysis, we didn't detect any significant changes in the abundance of plasmid per sample (Wilcoxon rank-sum test, $p = 0.12$) from the two sampling seasons, therefore we combined them as replicates. The plasmid functional transfer system generally requires at least 2 Kbp length for mobilisation and ca. 15 Kbp for conjugation (Smillie et al., 2010). Plasmids with larger sizes are normally of more interest considering HGT possibilities (Alanin et al., 2021; Kothari et al., 2019; Norman et al., 2014), and capacity for harbouring various cargo genes such as ARGs. Smillie et al. elaborated the association between plasmid size and mobility with detection of the distribution of different plasmids in the database according to plasmid size (Smillie et al., 2010). Following their findings, we displayed the UWS plasmidome exhibiting a similar pattern (Fig. 1D), which somehow supports the feasibility and reliability of this work.

Aiming to improve the detection of larger-sized circular elements (>10 Kbp), we applied the direct plasmid DNA metagenome sequencing approach, excluding TRACA and MDA (Alanin et al., 2021; Dean et al., 2001). The biggest plasmid contig we captured was 79 Kbp, which harboured many hypothetical protein coding sequences (Fig. S5), suggesting the cryptic nature of the UWS plasmidome. Nonetheless, we harvested considerable small-sized (≤ 10 Kbp) plasmid contigs (Fig. 1D), which was probably because the method preferred capturing and

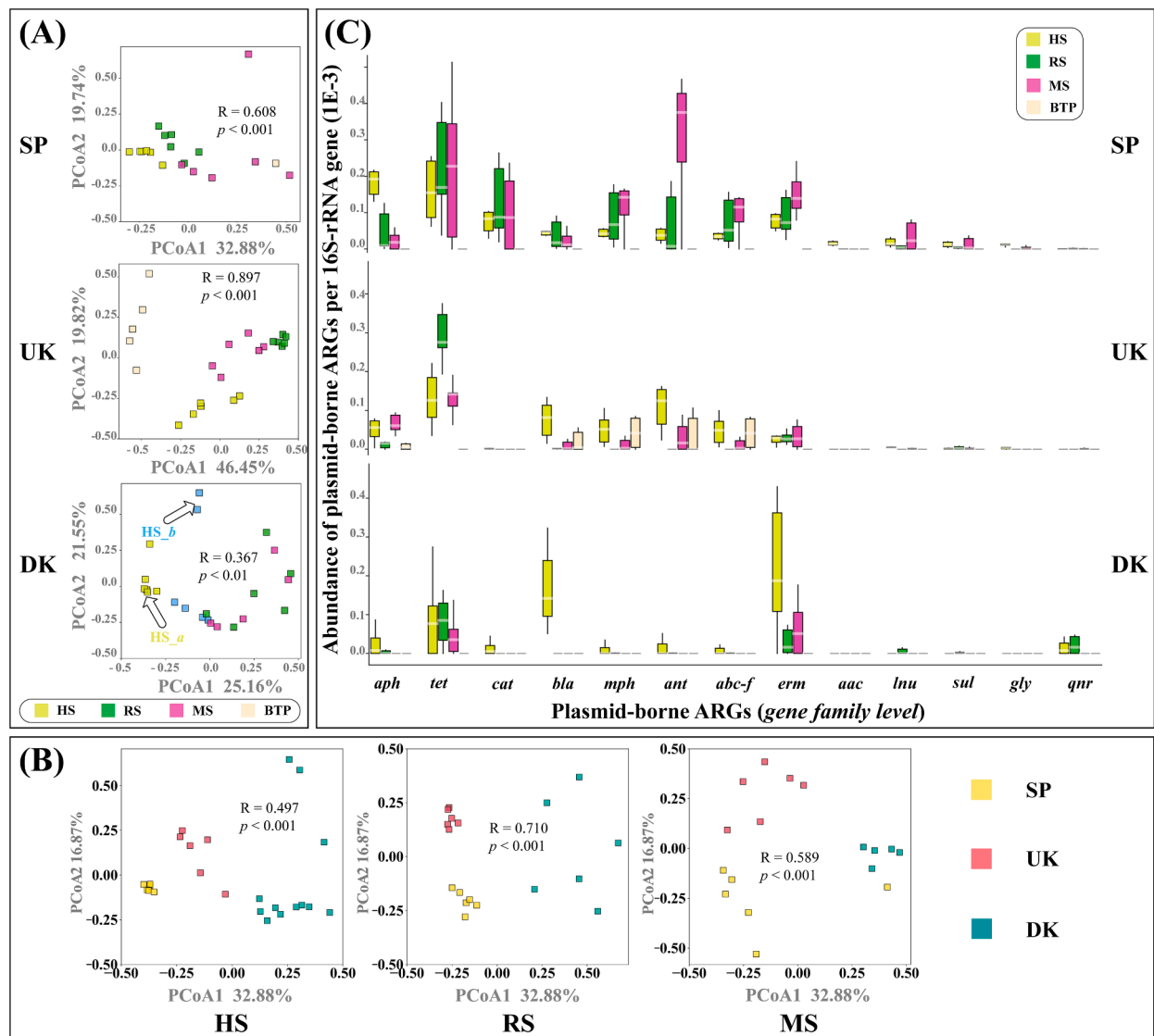


Fig. 5. Distributions and abundances of plasmid derived ARGs in the UWSs. (A) PCoA analysis based on BrayCurtis distances for the plasmid derived ARGs from different UWS compartments in each country. (B) PCoA analysis based on BrayCurtis distances for the plasmid derived ARGs from different countries in each UWS compartment. (C) The dynamic AR plasmid abundances along with the UWS processing. HS: hospital sewer; RS: residential sewer; MS: mixed sewer, *i.e.*, the mixture of HS and RS; BTP: WWTP biological treatment process basin. In Fig. 5A, the yellow and blue squares represent the two hospital sewer lines operated in Denmark. ARG family abbreviations: aph (aminoglycoside phosphotransferase), tet (tetracycline-resistant ribosomal protection protein), cat (chloramphenicol acetyltransferase), bla (beta-lactamase), mph (macrolide phosphotransferase), ant (aminoglycoside nucleotidyltransferase), abc-f (miscellaneous abc-f subfamily ATP-binding cassette ribosomal protection proteins), erm (erm 23 s ribosomal RNA methyltransferase), aac (aminoglycoside acetyltransferase), lnu (lincosamide nucleotidyltransferase), sul (sulfonamide resistant sul), gly (glycopeptide resistance gene cluster), qnr (quinolone resistance protein). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assembling small contigs while was difficult to cover complete long contigs (>10 Kbp). Meanwhile, this may also be caused by the fact that the target UWS plasmidome was substantially rich in smaller genetic elements. One groundwater plasmidome study showed the isolation of extremely large plasmids (>1 Mbp) using MDA with a deep sequencing strategy (Kothari et al., 2019). Consequently, one can argue that not recovering large-sized plasmids is due to sequence depth (sufficient informative reads for the assembly) and sequencing method (long-reads-sequencing-based plasmidome metagenomics such as Oxford Nanopore and PacBio single-molecule real-time can help improve assemblies of large plasmids since they inherently preserve the genomic context). This urges a necessity to perform deeper sequencing and improve the contemporary sequencing approaches (e.g., combining reads from long-reads- and short-reads-based sequencing) for the sake of a higher resolution for the large-sized fraction of the plasmidome.

4.2. Uncovering the cryptic UWS plasmidome

Though plasmids can be classified in various ways, the genetic-traits-based grouping methods such as MOB typing and replicon typing are universally applied (Orlek et al., 2017). We showed that the UWS plasmidome was basically a blend of plasmids with MOB_unknown (undivided MOB) types (Fig. S4A). This suggests the nature of UWS plasmidome, and a recent report indicated that the contemporary plasmid database contained large quantities of MOB_{less} plasmids (Coluzzi et al., 2022). Apart from the MOB typing, traditional plasmid Inc grouping, defined by conjugation experiment data, is also used from time to time given that it is the most consistent typing method with the plasmid mobility scheme (Partridge et al., 2018). With the expansion of the whole genome sequencing data, the Inc group database tethering with replicon information gets rapidly developed, and many *in silico*

typing tools are publicly accessible (Carattoli et al., 2014; Orlek et al., 2017; Partridge et al., 2018; Robertson and Nash, 2018). More than 28 Inc groups have been recognised so far, and many contain subgroups (Shintani et al., 2015). Our study uncovered that plasmids belonging to the miscellaneous rep_clusters were the most prevalent groups in the UWSs (Fig. S4B), which had not been reported by conventional PBRT or metagenome studies in the wastewater environment (Kothari et al., 2019; Lira et al., 2020; Tymensen et al., 2019). Taken together, our work showed the UWS plasmidome was dominated by these cryptic plasmids, which were non-mobilisable, not typeable under the current MOB and Inc typing scheme and previously unknown (*i.e.*, novel) plasmids.

In addition, we perceived that the UWS plasmidome was essentially a collection of non-mobilisable plasmids, whereas PLSDb showed a prevailing pattern of plasmids with mobility (mobilisable/conjugative) in the database (Fig. 4A). This can be attributed to the fact that mobilisable and conjugative plasmids typically carry more functional traits or specific features than non-mobilisable plasmids, and they take critical roles in HGT for spreading ARGs. These conspicuous characteristics lead to massive “mobilisable plasmids” and “conjugative plasmids” oriented projects, and thus, their sequences constituted a major part of the PLSDb database.

4.3. The dynamic UWS plasmidome

As a critical step in urban water management, WWTPs have been of particular concern in environmental studies for years (Kaushal et al., 2015; Pruden, 2014). However, there's a lack of examining the plasmidome dynamics along with the UWS processing due to method biases (targeting only some MGE marker genes) or incomplete sampling construction (Lira et al., 2020; Ng et al., 2019; Pallares-Vega et al., 2019; Stalder et al., 2019; Yadav and Kapley, 2019; Yu et al., 2020). This study targeted the sewer catchments (HS, RS and MS) and the BTPs in the WWTPs. It is arguably true that the samples from the front- and post-BTPs (e.g., the primary settler, the secondary settler, and the tertiary filter) and the upstream and downstream rivers were also worth surveys to obtain a full-frame image of the fate of UWS plasmidome. Unfortunately, we met difficulties building the plasmidome libraries for sequencing these sites. Within this sampling scope, MS was sampled after the convergence of the raw sewer lines and was supposed to be the convergence of HS and RS. Consequently, it is reasonable to speculate that MS contained the highest plasmid richness and abundance. However, this was not the common feature in the present study (Fig. 2A and S5B). The plasmid richness in MS mainly maintained the level of RS, while lower than HS. With regard to the plasmid abundance, it was hard to conclude the general pattern for this sophisticated plasmidome, whilst we observed increased plasmid abundance in MS compared to HS and RS in a few “Inc Group 2” plasmids with miscellaneous rep_cluster_xxxx replicons. Apart from this, we discovered that all the sewer lines (HS, RS and MS) carried significantly ($p < 0.01$) higher richness of plasmids than BTPs (Fig. 2A). This could be explained by the dramatic environmental changes for the plasmid hosts flowing from the sewer lines to the WWTPs, while plasmid maintenance is closely related to its host (Carroll and Wong, 2018), and BTPs can remove several microbes (Barancheshme and Munir, 2017; Bouki et al., 2013; Hiller et al., 2019; Kumar and Pal, 2018; Sharma et al., 2016). We evidenced this with a remarkable correlation ($p < 0.0001$) of plasmid richness and microbiota richness (Fig. S7). Strikingly, the British BTP retained significantly ($p < 0.01$) higher plasmid richness than the other two countries, which was probably owing to the different BTP facilities employed in the United Kingdom (biofilter) compared to Denmark and Spain (biological treatment basin).

HS and RS can be considered as separated niches given that distinct selection pressure is exerted in the corresponding environment. And they usually possess different microbial community compositions and plasmid profiles considering the different sewer physicochemical properties (Buelow et al., 2020; Yuan et al., 2020). Herein, we showed the

distinct HS and RS plasmid populations in Denmark and the United Kingdom (Fig. 2B). Nonetheless, we discovered a close relationship between the plasmid populations in the Spanish HS and RS, which could be attributed to the extensive domestic use of antibiotics in Spain (Fig. S1), and indeed the Spanish HS plasmidome appeared distinct compared to Denmark and the United Kingdom (Fig. 2C). Interestingly, we figured out that the MS plasmidome was closely related to that in RS regardless of country. This indicates the MS plasmid communities might be notably shaped by the injection of RS plasmids with the flow.

On the other hand, we illustrated a clear routine of ‘vanishment’ for the HS-distinct plasmids, whereas RS-distinct and MS-distinct plasmids were not totally removed in the BTP (Fig. S6A). Nonetheless, the HS-distinct plasmids should not be overlooked as hospital wastewaters were detected as pools of plasmids with mobility (Wang et al., 2018). In view of the mobility features carried by these HS-distinct plasmids, it is reasonable to speculate that the harboured ARGs might be already widely disseminated in the sewer microbiome (Buelow et al., 2020; Szekeres et al., 2017; Wang et al., 2018).

4.4. The common UWS plasmidome across different European countries

Recently, a network analysis has revealed the shared plasmid clusters of gut microbiomes from distinct human groups (Conteville and Vicente, 2022). This implies that plasmids could be spread and shared among niches despite spatial-temporal differences. In this study, we showed the constantly evolving plasmid community structure along with the UWS processing as well as the documentation of different UWSs in different European countries. We revealed that plasmids from different countries were shared in every UWS stage (Fig. 3A). And we noticed a ‘bigger share’ in the sewers than in the BTPs, which was probably due to the distinct treatment settings implemented in the United Kingdom. Many ARGs are associated with plasmids (Munk et al., 2022), and predecessors reported country-specific antibiotic resistance profiles in seven different European WWTPs (Pärnänen et al., 2019). Irrespectively, our observation of the common pool of the three European countries’ wastewater-derived plasmids alarms the bell since their emergence implied they probably have already been widespread in the urban water cycles in the whole of Europe and would potentially acquire ARGs from those AR plasmids through HGT and create cross-national resistance issues (Frost et al., 2005; Rodríguez-Beltrán et al., 2021).

4.5. The persistent plasmidome in the UWSs

Plasmid persistence is constantly a hot topic in the field and is frequently reported to infer its potential mechanism in diverse environments (Carroll and Wong, 2018). In the present UWS plasmidome, we investigated the emergence and prevalence of plasmid persistence using a plasmidome trajectory model (Fig. 3B). We observed that the persistent plasmidome was mainly composed of non-mobilisable plasmids. As known, plasmids can lower their mobility and become less dependent on mobilisation and conjugation to be maintained in the bacterial communities (Alonso-Del Valle et al., 2021). Meanwhile, compensatory mutations can happen to alleviate plasmid fitness costs to hosts (San Millan, 2018). In view of the variations of selection pressure in different compartments of the UWSs, we assumed the plasmid groups with mobility gradually lost competitive advantages with the flow, resulting in non-mobilisable plasmids outcompeting mobilisable and conjugative plasmids. Alternatively, another explanation can be the overwhelming number of non-mobilisable plasmids in the raw sewers, and the reduction during sewer transportation and WWTP processing to them was minor. This calls for further bioinformatic work and sampling campaigns looking into extensive sets of plasmid sequences to reveal the persistent plasmidome genomic contexts.

4.6. Wastewater plasmid source tracking

Considering the blossom of persistent plasmids in the UWSs, we were aware of their occurrence in the BTPs. BTPs are of particular importance because they constitute a potential mobile genetic and antibiotic resistance reservoir to the WWTP effluent and downstream rivers (Cacace et al., 2019; Makowska et al., 2016; Osińska et al., 2020). Accordingly, tracking the origin of the BTP plasmids would help us understand its composition (intrinsic vs nomadic) and the gateway to prevent specific groups of plasmids from flowing to the WWTPs. In the present study, a multitude of BTP plasmids was trackable to the front treatment compartments (Fig. 3C). And the majority of these trackable BTP plasmids were from a common pool contributed by the three sewer lines. Further, we noticed that the trackable BTP plasmids were dominantly contributed by MS. This seems plausible when considering MS was the confluence of HS and RS, as well as the direct inflow to the WWTP. Retrospectively, Dottorini et al. showed that activated sludge community composition was strongly reflected by the corresponding influent wastewater microbial composition in 11 Danish WWTPs (Dottorini et al., 2021). This supports our assumption as the fate of plasmid is strongly associated with its host (Carroll and Wong, 2018).

We imagined these ubiquitous plasmids were common goods shipped from different origins (HS, RS) to the transit hub (MS) and finally deposited in the retention centre (BTP). Meanwhile, they resembled goodies and were preserved at each site. Given that the BTP plasmids were essentially (>70 %) occupied by these nomadic plasmids, we anticipated they might offer their hosts beneficial traits to be retained in the UWSs and thus became prevalent. Alternatively, these plasmids might fortuitously taxi on those durable microbes which survived along the sewer pipelines till the BTPs. Another hypothesis can be that the backbones of these persistent plasmids were not changed while some genetic contexts alternation/rearrangement/movement happened (compensatory mutation), for instance, accessory genes (e.g. mobility genes, ARGs) could be discarded to lower plasmid fitness costs to the hosts (Pinilla-Redondo et al., 2018; Rodríguez-Beltrán et al., 2021). In other words, plasmids can flourishingly adapt the cargo genes for specific environments, and the variability of plasmid fitness effects promotes plasmid persistence in bacterial communities (Alonso-Del Valle et al., 2021; Yu et al., 2022). Our findings of such ubiquitous plasmids in the UWSs paved the way toward understanding the biological and ecological facts of the BTP plasmids. Further studies on these plasmids are necessary to explore their genetic repertoire, which could be pathogen-related and poses health risks to the downstream rivers.

4.7. Fate of the AR plasmids in the UWSs

WWTPs are well-characterized hotspots of antibiotic resistance though they arguably remove antibiotics, ARGs and antibiotic-resistant bacteria to some extent (Michael et al., 2013; Rizzo et al., 2013). A pan-European survey of the UWSs shows the WWTP effluent were responsible for the antibiotic resistome enrichment in the receiving water bodies (Cacace et al., 2019). This suggests the potential health risks posed by WWTPs and the significance of monitoring the antibiotic resistance issues along with the UWS processing to track the crucial barrier (compartment) for prevention.

In general, we found a dissimilar pattern of AR plasmids in RS and HS, and a close relationship between AR plasmids in RS and MS (Fig. S8A). These could be due to the distinct nature of different sewers, and the confluence impacts from the upstreams, respectively. RS derives from the urban residential area and is consistently a key source of antibiotics, ARGs and antibiotic-resistant bacteria flowing to the WWTPs (Pallares-Vega et al., 2019; Quintela-Baluja et al., 2019; Yu et al., 2020). HS originates in hospitals, and it appears plausible that HS harbours higher ARG abundance than RS, given that a more intense amount of antibiotics are used in the clinical environment (Buelow et al., 2020). However, Buelow et al. reported that HS could be neglected compared to

RS as not contributing significantly to the quantity and diversity of ARGs in the UWSs (Buelow et al., 2018). This contradiction typically emerges in case studies and can be explained by differences in antibiotic usage, local wastewater effluent load, hydrological conditions, sampling strategies, etc. The message from these studies is that HS and RS are both essential contributors to the wastewater antibiotic resistome in the UWSs. However, this point has been buried in numerous studies (Kim et al., 2019; Novo et al., 2013; Pallares-Vega et al., 2019; Quintela-Baluja et al., 2019; Yu et al., 2020).

Antibiotic resistance issues in different countries vary considering differing antibiotic usage and practises (Huijbers et al., 2020; Pärnänen et al., 2019; Rodríguez-Mozaz et al., 2020). Similarly, we were able to cluster the AR plasmid populations by country in each sewer catchment (Fig. S8B). Specifically, Spanish and British AR plasmid populations tended to be similar yet dissimilar to those in Denmark. Regarding the dynamics of the AR plasmids, we discovered:

- (1) In Denmark, HS harboured a relatively high AR plasmid abundance compared to RS, and flowed comparable numbers of AR plasmids with RS to MS (Fig. 4B and C). However, all AR plasmids in Denmark were eliminated in the BTP, which could be associated with the strict discharge regulations in the local environment (Chhetri et al., 2022). And the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme reported lower use of the main antimicrobial classes compared to the other two countries (<https://www.danmap.org/reports> and Fig. S1), possibly contributing to the general lower AR issue in Denmark.
- (2) In Spain, we discovered similar numbers of AR plasmids with and without mobility flowed from sewer sources to MS and then to BTP (Fig. 4B). Plasmids can act as selfish parasites, while plasmid-encoded ARG traits, in turn, promote the stability, maintenance and transmission of themselves (Ghaly and Gillings, 2022). And AR plasmids with mobility have continuously been headlines in wastewater studies (Uluseker et al., 2021). A remarkable number of AR plasmids in the Spanish UWS plasmidome carried mobility genes, whereas they seemingly got removed in the BTP in view of abundance (Fig. 4C). And a group of stubborn non-mobilisable AR plasmids in the Spanish BTP was found to retain a similar plasmid abundance with MS. This could be due to the fact that Spain consumes high amounts of antibiotics in the community and hospital sectors (Fig. S1) and higher concentrations or relative abundances of antibiotics, antibiotic-resistant bacteria and ARGs were detected in the Spanish wastewaters than in other western and northern European countries (Huijbers et al., 2020; Pärnänen et al., 2019; Rodríguez-Mozaz et al., 2020). Therefore, the high selection pressure in the Spanish BTP favours plasmids encoding ARGs, and these non-mobilisable AR plasmids were preserved for the sake of beneficial traits for survival and lower fitness costs compared to mobilisable/conjugative plasmids.
- (3) In the United Kingdom, AR plasmid relative abundances decreased with the UWS processing, but intriguingly, a larger number of AR plasmids flowed from MS to BTP than in the other two countries (Fig. 4B and C). This could be due to the higher hospital antibiotic consumption in the UK compared to the other two countries (Fig. S1), and hospital wastewater (i.e., HS) can potentially exacerbated the AR issue in the downstream environment (Perry et al., 2021). These HS-borne AR plasmids persisted in the sewer line to the BTP though their abundances have been reduced remarkably (Figs. 4B, C and S6A).

Above all, the non-mobilisable AR plasmids remained abundant along with the UWS processing in Spain. These AR plasmids were not strictly settled in fixed niches but could be disseminated to other environments with the WWTP processing, while their prevalence and dominance depend on the trade-off of plasmid fitness costs and benefits

to the hosts and abiotic factors (e.g., selective pressure), which demands further investigations to understand their genetic evolution and possible infection to naive communities though they were without mobility (Coluzzi et al., 2022).

On the other hand, we discovered 111 AR plasmids universally distributed in UWSs across the three countries (Fig. S9), featuring resistance to aminoglycoside, beta-lactam, fluoroquinolone, fosfomicin, lincosamide, macrolide, phenicol, rifamycin, sulfonamide, tetracycline, and metals (lead, cadmium, zinc, mercury and copper). 60 of these common AR plasmids were mobilisable, and some of which carried MDR (Fig. S10A, C, and D). This suggests that plasmid-borne ARGs could readily be widespread in these countries. Munk et al. also observed that common ARGs were sometimes found on plasmids in sewage sampled in 101 countries (Munk et al., 2022).

4.8. The plasmidome derived antibiotic resistome

Plasmids play a key role in bacterial ecology and evolution. Moreover, the synteny features of the ARG loci on the plasmid contigs bring the possibility of ARG horizontally transferring to pathogens (Frost et al., 2005; Rodríguez-Beltrán et al., 2021). Our high-throughput qPCR-based resistome network study for the same sampling campaign demonstrated a significant ($p < 0.05$) correlation among the extended-spectrum beta-lactamase and carbapenemase genes and MGEs marker genes (Li et al., 2021). And researchers showed that the evolution of resistome was mainly associated with the interaction of microbial communities and MGEs (Buelow et al., 2020; Ju et al., 2019).

In the present work, we inspected the antibiotic resistome in this circular plasmidome dataset and discovered similar country and treatment compartment patterns with the AR plasmids (Fig. 5A and B). Sewer-distinct ARGs were all eliminated after the UWS processing (Fig. S6B). Aminoglycoside, beta-lactam, macrolide, chloramphenicol, and tetracycline resistance genes were ubiquitous in the sewer lines while they were almost removed in the BTP except in the United Kingdom (Fig. 5C). These ARG types have been also frequently reported in previous wastewater studies (Guo et al., 2017; Pallares-Vega et al., 2019; Quintela-Baluja et al., 2019; Yin et al., 2019), which reflects the plasmid derived resistome contributes to the total resistome. Generally, Spanish RS harboured relatively higher abundant ARGs than the other two countries, which was probably due to the excessive community usage of antibiotics (Fig. S1). And the occurrence of ARGs belonging to *bla* (including *bla*_{OXA}, *bla*_{SCO} and *bla*_{TEM} variants), *mph*, *ant* and *abc-f* in the British BTP suggests the incapability of reducing these ARGs using biofilters. ERM genes showed surprisingly high abundance in the Danish HS, which would be associated with local clinical practices. Generally, the wastewater ARG abundance mirrored the pattern of antibiotic use, environmental temperature and WWTP size (Pärnänen et al., 2019). Our results implied that the treatment facilities also played an important role in shaping the highly mobile resistome in the UWSs.

UWSs are seemingly central conduits of AR to and from pathogens and environmental strains (Pruden, 2014). In light of the importance of surveillance on ARGs with high mobility (Yin et al., 2021), we unravelled that mobilisable/conjugative AR plasmids were persistent in the studied UWS processes (Fig. 4B). These mobile AR determinants threaten the downstream UWS compartments and WWTP downstream rivers considering their potential horizontal transmission and becoming prevalent and ubiquitous in diverse environmental settings thereafter (Dolejska and Papagiannitsis, 2018), eventually posing potential risks to human health. On the other hand, the ARG and ARG-carrying-bacteria profiles often change over time with the wastewater flow from the raw sewer to the WWTP (Auguet et al., 2017; Dai et al., 2022; Ekwanzala et al., 2020). Our results showed that different UWS compartments in different countries exhibited diverse resistome patterns (Fig. 5). This indicates that the plasmidome resistome carriage and maintenance in the studied UWS environment were variable and the resistome flow in different countries varied. Overall, the emergence of common and

persistent plasmid harbouring ARGs during the UWS processing implies the prevalence and propagation of plasmid-mediated ARGs in the UWS environment, which emphasises the urgency to implement regular surveillance and risk management that is appropriate for the different regions and treatment stages.

Collectively, our study deciphered the cryptic UWS plasmidome which was dominated by non-mobilisable, non-typeable and previously unknown plasmids, and the UWS plasmidome derived antibiotic resistome was shaped by geographic-regional and treatment-sectional variations. Out of research priority, we exclusively focused on the circular plasmidome in this study. However, inspecting the dataset on the linear contigs would also potentially unearth their encoded ARGs and provide a full resistome view of the plasmidome.

CRedit authorship contribution statement

Zhuofeng Yu: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Wanli He:** Conceptualization, Software, Formal analysis, Visualization, Writing – review & editing. **Franziska Klincke:** Conceptualization, Software, Writing – review & editing. **Jonas Stenløkke Madsen:** Conceptualization, Data curation, Writing – review & editing. **Witold Kot:** Methodology, Writing – review & editing. **Lars Hestbjerg Hansen:** Methodology, Writing – review & editing. **Marcos Quintela-Baluja:** Resources, Writing – review & editing. **Sabela Balboa:** Resources, Writing – review & editing. **Arnaud Dechesne:** Resources, Project administration, Writing – review & editing. **Barth Smets:** Conceptualization, Funding acquisition, Writing – review & editing. **Joseph Nesme:** Conceptualization, Data curation, Supervision, Funding acquisition, Methodology, Project administration. **Søren Johannes Sørensen:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hereby, the authors declare no other competing financial interests for the manuscript ENVINT-D-23-03040-R1: [Insights into the circular: the cryptic plasmidome and its derived antibiotic resistome in the urban water systems].

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

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