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Quantification and fate of plasmid-specific bacteriophages in wastewater: Beyond the F-coliphages

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\textbf{A R T I C L E   I N F O}

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- Virus
- Conjugation
- F-specific bacteriophages
- Antimicrobial resistance
- Water treatment

\textbf{A B S T R A C T}

Plasmid-specific bacteriophages specifically infect bacteria carrying conjugal plasmids. While wastewater has been used as isolation source for such phages, to date, only the distribution and ecology of RNA phages specific to the F plasmid have been described, because they serve as a water quality indicator. Yet, several other plasmid classes have higher clinical and ecological relevance, and the distribution, fate, and ecology of the phages that target them remain uncharacterized. We aimed to (i) provide an experimental platform to quantify the abundance of plasmid-specific phages applicable to several different conjugal plasmid classes, (ii) describe the distribution of such phages in wastewater systems, and (iii) relate their abundance to plasmid abundance and to municipal wastewater treatment processes. We introduced four model conjugal plasmids, belonging to incompatibility groups IncP-1, IncN, IncHI1, or IncF into an avirulent Salmonella enterica strain, for which somatic phages are at low abundance in wastewater. These strains were used in double layer agar assays with waters from contrasting sources. Plasmid-specific phages were common in wastewater but rare in river water. Hospital wastewater contained significantly more IncP-1, but fewer IncF- and IncN-specific phages than domestic wastewater. This pattern did not match that of plasmid abundance estimated by Inc group targeting high-throughput quantitative PCR. The comparison between influent and effluent of wastewater treatment plants revealed a reduction in phage concentration by ca. 2 log, without significant contribution of primary settling. Overall, the ubiquity of these phages hints at their importance for plasmid ecology, and can provide opportunities in water quality monitoring and in ecological management of mobile resistance genes.

1. Introduction

Bacteriophages, viruses infecting bacteria, are ubiquitous in the environment and there is an increasing awareness of their role in the ecology and evolution of bacterial communities. They are especially abundant and diverse in wastewater (Olsen \textit{et al.}, 2020) and are even considered as novel tools to control unwanted microbes in environmental engineering applications (Mathieu \textit{et al.}, 2019).

Plasmid-specific bacteriophages infect bacteria carrying conjugal plasmids, typically using the conjugal pilus as receptor (Penttinen \textit{et al.}, 2021). They are also known as plasmid-dependent or male-specific. Unlike somatic phages, the host range of these phages is not directly constrained by the bacterium’s chromosomal content, but by the type of plasmid(s) it carries. Indeed, plasmids of different incompatibility groups differ in the type of transfer apparatus they encode; therefore, plasmid-specific phages have some specificity towards one or a few plasmid classes (Frost, 1993).

RNA phages targeting the conjugative machinery of the F plasmid, which belongs to the IncF plasmid incompatibility group, have been studied for several decades in water systems and used as water quality indicator. Indeed, RNA F-coliphages are thought to be associated to fecal contamination and to have a fate in the environment similar to that of other RNA viruses of public health relevance, such as the human enteric viruses (IAWPRC Study Group on Health Related Water Microbiology, 1991). The main hot spots of F-coliphages are the feces of humans and other warm-blooded animals, with different dominant F-coliphage subgroups in different animals (Coles \textit{et al.}, 2003). Consequently, much effort has been dedicated to the description of the distribution of these phages in the environment. F-coliphages are clearly more abundant in wastewater than in ambient water: on average, \(8 \times 10^5\) PFU/L vs less than 10 PFU/L according to a meta-analysis (Nappier \textit{et al.}, 2019). These averages cover a large inter-sample variability, with significant...

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geographical and seasonal trends (Nappier et al., 2019). However, little correction for somatic phage abundance.

Summary of phage concentration as a function of sample type and plasmid carriage of the target strain. We report results of DLA counts from plates without RNAse after Table 1.

To the best of our knowledge, the only available abundance data is for IncH2-specific phages, present at $10^6$ to $10^9$ PFU/L in sewage samples collected in Ireland (Nuttal et al., 1987). The presence of phages specific to other plasmid classes has been reported in diverse wastewater sources, since it is the main source for isolation (Bradley et al., 1982, 1981; Coetzee et al., 1982; Mahler et al., 1991; Olsen et al., 1974), but quantitative information has been lacking.

Filling this gap is important because this incomplete knowledge impedes our understanding of the ecology of plasmids and their interactions with phages. It is especially relevant because plasmids occurring in wastewater can contribute to the dissemination of antimicrobial resistance in the environment and because plasmid-specific phages have the potential to reduce the load of plasmid-borne antimicrobial resistance, as suggested in laboratory experiments ( Cairns et al., 2018; Colom et al., 2019; Jalsavuori et al., 2011; Ojala et al., 2016, 2013; Penttinen et al., 2021). However, whereas a standard enumeration method has long been available for the F-specific phages (Anonymous, 2001) to which several improvements have been made e.g. (Blanch et al., 2020), none exist for other plasmid groups. Therefore, in this work, we created a set of strains to quantify phages able to lyse bacteria carrying plasmids belonging to the IncP-1, IncHI, and IncN classes, which are often implicated in the dissemination of antimicrobial resistance (Rozwandowicz et al., 2018). Leveraging this set of strains, we next described phage abundance in contrasting water samples and explored their fate in municipal wastewater treatment plants. Specifically, we hypothesized that hospital wastewater may carry a load of plasmid-specific phages differing from that of domestic wastewater because the former is often enriched in mobile antimicrobial resistance genes (Hassoun-Kheir et al., 2020; Li et al., 2021; Manoharan et al., 2021).

2. Materials and methods

2.1. Generation of bacterial strains harboring conjugative plasmids for bacteriophage enumeration

 Cultures of avirulent Salmonella enterica serovar Typhimurium MHM112 ATCC® BAA2828 ( de Moraes et al., 2016) harboring one of the four conjugative plasmids, pKJK5::gfp (IncP-1, (Kümper et al., 2015)), drR27 (IncHI, derepressed version of R27 (Lawley et al., 2003)), pKM101 (IncN, (Langer et al., 1981)), and R1adr19 (IncFII, derepressed version of R1, (Clerget et al., 1981)) were used as targets for phage enumeration (see Table S1 for the list of strains used here). We used derepressed plasmids or plasmids with high transfer ability to facilitate phage detection and S. enterica was selected as plasmid host because sewage contains few somatic phages against S. enterica strains compared to other Enterobacteriaceae (Havelaar et al., 1985; Havelaar and Hogeboom, 1984). The strains were generated by surface mating assays using Escherichia coli strains as plasmid donors: strains MG1655, DH5α, BW27783, and CHS33, harboring pKJK5::gfp, drR27, pKM101, and R1adr19, respectively. Due to the lack of selectable markers in S. enterica MHM112, the plasmids were initially transferred to a fastidious intermediate donor strain, E. coli ST18, a ΔhemA deletion mutant requiring 5-aminolevulinic acid (ALA) for growth (Thoma and Schober, 2009) and with resistance to streptomycin and trimethoprim.

The initial donor and intermediate recipient strains were grown overnight in Luria-Bertani (LB) media with appropriate antibiotics and ALA at 37 °C while shaking. These cultures were centrifuged for 10 min at 6720 × g and resuspended in 0.9% NaCl solution. The donor and the intermediate recipient strains were mixed in a 1:1 ratio and plated on LB plates supplemented with 50 µg/mL ALA. Cultures of donors or recipients alone were used as negative controls. After overnight incubation at 25 °C for drR27 and at 37 °C for pKJK5, pKM101, and R1adr19, the bacteria were collected with an inoculation loop and suspended in 0.9% NaCl solution. Dilutions were plated on LB plates supplemented with 50 µg/mL ALA and 50 µg/mL streptomycin along with either 10 or 20 µg/mL tetracycline (to select for the intermediate recipient strain harboring drR27 or pKJK5, respectively) or 100 µg/mL ampicillin (pKM101 and R1adr19). A colony from each of these plates was then used in a subsequent surface mating with S. enterica MHM112 as described above. To obtain MHM112 with each of the plasmids, the mating mixtures were plated on LB plates supplemented with appropriate antibiotics to select for the plasmids, but without ALA to eliminate the intermediate strains. Additionally, to confirm the success of the surface mating assay, the putative transconjugants were plated on Xylose Lysine Deoxycholate (XLD) plates for visual inspection as S. enterica and E. coli colonies have distinct morphotypes on this media.

2.2. Enumeration of bacteriophages with DLA assay

To enumerate bacteriophages in environmental samples, we adopted the classical double layer agar (DLA) assay (Clokie and Kropinski, 2009). The environmental water samples were centrifuged for 17 min at 4820 × g. The supernatant was filtered into a sterile container through a 25 mm Whatman™ glass fiber membrane (GF/F) syringe filter with a pore size of 0.7 µm to reduce the bacterial load while retaining the viral fraction in the filtrate. S. enterica MHM112 carrying one of the plasmids or without any plasmid (somatic control) were grown overnight (18–20 h) in LB at 37 °C while shaking. These target strains were diluted in LB to an OD600 of 0.5 and 1 ml of this suspension was mixed with 100 µL or 1 ml of the filtered water sample and with 3 ml soft agar medium (0.7% agar in LB broth) supplemented with CaCl2 and glycerol at a final concentration of 5 mM and 5%, respectively (Santos et al., 2009) in sterile glass tubes prewarmed at 48–50 °C. The mixture was then poured on top of duplicate LB plates with appropriate antibiotics to select for the plasmids. To specifically enumerate DNA-bacteriophages and infer abundance of RNA-bacteriophages by subtraction from total counts, duplicate plates were prepared with soft agar supplemented with RNAse A at 40 µg/mL, following the ISO 10705–1 protocol (Anonymous, 2001). All plates were incubated overnight at 25 °C before counting the plaques to estimate phage concentration (as PFU/mL, after multiplying by the concentration factor for the low biomass samples where necessary, see below). We report plasmid-specific phage concentrations after subtracting the number of S. enterica somatic phages we detected.
Table 2

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<th>Location</th>
<th>Type</th>
<th>Date</th>
<th>TSS g/L</th>
<th>VSS g/L</th>
<th>PFU/mL</th>
<th>pKJK5 DNA</th>
<th>pKJK5 RNA</th>
<th>drR27 DNA</th>
<th>drR27 RNA</th>
<th>pKM101 DNA</th>
<th>pKM101 RNA</th>
<th>r1drd19 DNA</th>
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<td>0</td>
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</tr>
<tr>
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<td>influent WWTP</td>
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<td>0.194</td>
<td>4.25</td>
<td>0</td>
<td>0</td>
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</table>

2.3. Collection and processing of water samples

Twenty-nine water samples (freshwater or wastewater from different sources and at different degrees of treatment) were collected in Southern Sweden and Eastern Denmark, using either grab samples (river samples, some of the wastewater samples) or 24 h flow-proportional sampling (samples from municipal wastewater treatment plants). The three river samples were from Usserød Å, a peri-urban stream north of Copenhagen (DK). They cover different degrees of human influence: the stream origin at a lake (station 1), a station mostly influenced by agricultural activity (station 6), and a station mostly influenced by the urban environment (about 500 m downstream of a small municipal WWTP, station 7). More details can be found elsewhere (Lemaire, 2021). Grab samples (2 L) of untreated wastewater were collected from three hospitals within 100 km of Copenhagen (cities of Slagelse, Herlev, and Hillerød; with 367, 938, and 520 beds, respectively).

The fate of plasmid-specific phages was studied in three municipal wastewater treatment plants in southern Sweden. They treat water of the cities of Malmo, Visby, and Gothenburg (Ryaverket: 10⁶ PE, Visby: 5 × 10⁶ PE, Sjölunda: 3 × 10⁵ PE). Samples were collected twice within a single week of the Fall 2020 at the influent, after primary clarification (gravity settling), and at the effluent, using 24 h flow-proportional sampling. The treatment train in these plants included four moving bed bioreactors (MBBRs) and a secondary clarification step (Visby); activated sludge and secondary filtration, two MBBRs and tertiary filtration with disk filters (Ryaverket) (Wang et al., 2020); activated sludge and secondary filtration, trickling filter, MBBR, and flotation (Sjölunda).

For samples with low suspended matter (river samples and WWTP effluents), we concentrated 7 to 9 L of the samples to about 250 mL prior to enumeration. We used hollow fiber filtration as described in (McMinn et al., 2017), which reports a recovery between 20 and 80% for F-co-liphages. The samples were kept refrigerated at 4 °C until processing. Samples collected in Denmark were processed within 48 h, while samples collected in Sweden were processed within 10 days of collection.

Total and volatile suspended solids (TSS and VSS) were measured prior to any concentration and according to standard methods (American Public Health Association, 2014).

2.4. Molecular analysis

2.4.1. DNA extraction

DNA extraction was carried out using the NucleoSpin soil kit (Macherey-Nagel, Düren, Germany) following the user manual and using SL2 lysis buffer and without enhancing buffer SX. The starting material was 0.5 mL of biomass concentrated by centrifugation and resuspended in saline solution (0.9% NaCl) supplemented with glycerol (10% v/v final concentration): the wastewater samples were concentrated 10 fold, and the low biomass samples 100 fold (combining the hollow fiber filtration described in 2.3 and centrifugation). The low biomass samples were the river samples and the WWTP effluents. The concentrated biomass and extracted DNA were kept at –80 °C for up to 6 months before extraction or before qPCR. The DNA concentration was measured with Qubit® Fluorometer using the HS dsDNA kit (Invitrogen, Maryland, MD, USA). An extraction blank (Milli-Q water) was included in each batch of DNA extraction.

2.4.2. High-Throughput qPCR

A selection of 120 β-lactamases genes and genes associated to gene mobility (transposases, integrases, IS elements) were quantified using high-throughput qPCR (HT-qPCR) as described previously (Li et al., 2021). The quantification was performed by Resistomap (Helsinki, Finland) on a SmartChip™ Real-Time PCR Cycler (Takara Bio, San Jose, CA) for 40 cycles with 100 nL reaction comprised 1 × SmartChip™ Green Gene Expression Master Mix (TakaraBio), nuclease-free PCR-grade water, 300 nM of each primer and a template
concentration of 2 ng/µL. This technology and microchip design were shown to compare favorably to conventional qPCR assays run with standard curves (Stedtfeld et al., 2018). For each assay, dissociation melt curve analysis was conducted at the end of the reaction to confirm that the observed melting behavior was consistent with expectations and we checked the absence of amplification for the DNA extraction blanks that served as negative control. No problem was detected with both types of quality control. Of particular interest are the primer sets targeting plasmids, as introduced in the ARG primer set 2.0 (Stedtfeld et al., 2018), which target IncN (oriT, rep, korA), IncP-1 (oriT, trfa), IncF (FIC), and IncHI2 (smr0018) plasmids. We filtered out data for genes and samples where two or more of the HT-qPCR three technical replicates had a Cycle Threshold (CT) >28 as previously suggested (Stedtfeld et al., 2018). In addition, we checked that at least one of the technical replicates did not suffer from sub-optimal efficiency of amplification (< 1.8), compared to the ideal value of 2. We converted the copy number of the plasmid-related genes to relative abundance by dividing them by the copy number of the 16S rRNA gene from the same qPCR assay, to make our plasmid quantification robust against sample-specific partial PCR inhibition or variable DNA extraction recovery. This plasmid abundance relative to bacterial abundance could then be compared to phage concentration relative to (i.e., divided by) the VSS concentration, used as a proxy of bacterial concentration, and always measured on samples not subjected to concentration step.

2.5. Statistical methods

Data analysis was conducted in R version 4.1.0. We used statistical methods robust against non-normality of the data (e.g., median, median absolute deviation, Kruskal-Wallis rank sum test to test for concentration differences, and Spearman correlation test). Significance threshold was set at \( p < 0.05 \). Lastly, the Vegan package (Oksanen et al., 2021) was used to generate Principal Coordinate Analysis ordination plots of the phage compositions across samples.

3. Results and discussion

3.1. Effectiveness of the set of strains for environmental enumeration of plasmid-specific phages

Across the tested water samples, somatic phages forming plaques against the plasmid-free Salmonella MHM112 were rare (median abundance: 0.02 and 31 PFU/mL in river and wastewater samples, respectively, Table 1) which allows the quantification of plasmid-specific phages with a low detection limit. This confirms \( S.\ enterica \) as an appropriate host for plasmid-specific phage enumeration in the environment, as previously proposed (Havelaar et al., 1985; Havelaar and Hogeboom, 1984). The detection limit was even lower for RNA-phages, as we did not find evidence of RNAse-sensitive somatic phages against MHM112 in eight out of the eleven wastewater samples surveyed. We verified that the vast majority of phages purified from the plaques of the
plasmid-bearing strains displayed a lack of infectivity against the plasmid-free strain but could infect other hosts carrying plasmids from the same or related plasmid incompatibility groups.

3.2. Distribution of plasmid-specific phages

There are clear differences between wastewater and river samples, with the latter characterized by low phage abundance (Table 1). In river samples, the abundance of phages targeting drR27 or R1drd19 was always below detection limit of the DLA assay and below 10 PFU/mL for the other plasmids. pKJK5-specific phages were clearly above detection limit in the two river samples with highest turbidity and pKM101 only in the most turbid sample (Table S2). The river samples were collected during a summer rainstorm which transitorily mobilized solids, possibly including viral particles or viral particles attached to solids (Fauvel et al., 2017), hence the possible link between suspended solid and viral load for these samples. An incomplete phage recovery during the concentration of the river samples might have contributed to, but cannot fully explain, the large difference between river and wastewater samples (the latter being processed without concentration).

The wide distribution of pKJK5-specific phages observed in the river samples was confirmed in the wastewater samples, where such phages were always present at concentrations larger than 45 PFU/mL (Table 2). Phages targeting drR27 and pKM101 were detected in most wastewater samples with our DLA assay (8 out of 12 samples, 9 out of 11 samples), while those targeting the IncFII plasmid R1drd19 were often either below detection limit (4 out of 10 samples) or at low abundance (median when detected: 41 PFU/mL). This low abundance contrasts with the high F-coliphage abundance typically reported in wastewater (Havelaar et al., 1985; Nappier et al., 2019). For the limited number of samples for which we compared the performance of our test strain with the one classically used for F-coliphage enumeration (ATCC700891,
both strains yielded similarly low phage counts, suggesting that the wastewater samples we tested were characterized by a low concentration of these phages.

For wastewater samples, neither the concentration of individual phage types nor that of all plasmid-specific phages combined significantly correlated with the suspended solids concentration (VSS or TSS, Spearman’s $\rho < 0.42$ and $p > 0.17$), suggesting that biomass concentration is not a good predictor of plasmid-specific phage distribution in raw wastewater samples. However, we found that hospital wastewater and the influent of municipal WWTP presented several compositional differences (Table 1). The hospital samples had higher concentrations of pKJK5-specific phages (median $10^{3.4}$ vs $126$ PFU/mL, Kruskal-Wallis rank sum test $p = 0.02$). In contrast, the samples from the WWTP influents tended to contain more pKM101 and R1drd19-specific phages (Table 1 and Fig. 1, $p = 0.06$).

We compared phage abundance with that of plasmid-associated genes, as measured with the ARG HT-qPCR array, after normalization by the VSS concentration and by 16S rRNA gene copy number, respectively (Fig. 2). All HT-qPCR assays and samples passed quality control and all plasmid types were detected in at least five samples. Genes associated with IncP (oriT and trfA) were detected in all samples, while the ones associated with IncHI2, IncN, or IncF were below detection limit in the river samples (Fig. 2). The detection of the IncF-specific gene was restricted to the WWTP influent samples and always with low relative abundance (median: $1.5 \times 10^{-5}$ copies per 16S rRNA gene copy).

We observed slightly different patterns of coexistence between phages and their target depending on the plasmid class. We rarely observed coexistence of high abundances of the phages and their targets. Instead, typically, when one was abundant, the other was not. This was most clearly the case for the IncN plasmids and pKM101-specific phages, as well as for the IncHI2 plasmids and the drR27-specific phages (Fig. 2). This pattern is consistent with a high phage efficiency in controlling the density of their preys, sometimes termed the ‘killing the winner’ hypothesis, as previously observed in water treatment systems (Shapiro et al., 2009). However, in absence of time series of the abundance data, we cannot confirm that such scenario has been taking place. For IncP-1 plasmids and their phages, the pattern was more complex. In this case, the relationship between phage and plasmid abundance depended on the type of sample. We noted a positive correlation for both the hospital samples and WWTP influent samples, but with very different slopes (Pearson’s product-moment correlation: 0.97, $p = 0.05$ and 0.85, $p = 0.07$; respectively). This effort at relating abundance of plasmids and phages that target them is complicated by the fact that the specificity of any plasmid primer set is unlikely to perfectly match phage specificity. Indeed, plasmid-specific phages can often infect carriers of plasmid(s) of several incompatibility groups. For example, PRD1-like phages target bacteria carrying plasmids from the IncP1, W, or N groups (Bamford et al., 1995). In contrast, the primers we used target single groups or even subgroups of plasmids (e.g., smr0018 only IncHI2, when the host range of IncH-specific phages typically spans the HI1, HI2 and HI11 groups (Coetzee et al., 1985)).

### 3.3. Types of phage genomes

For a subset of samples, we compared the number of plaques on the DLA plates in presence and absence of RNAse (Table 2). This revealed that drR27-specific RNA-phages are more abundant than their DNA counterparts, while the opposite was true for pKM101 (Wilcoxon paired test: $p = 0.06$ and $p = 0.008$, respectively). These dominance patterns

Fig. 3. Profile of phage abundance across three Swedish wastewater treatment plants. In each plant, phage abundance was measured in the influent, effluent, and after primary settling. For each location, the whiskers represent the span of the abundances observed for two non-consecutive days over a week in the fall of 2020 while the middle point indicates the average. The two horizontal dashed lines illustrate the detection limits for the two first sampling points (unconcentrated samples, in brown) and the effluent (concentrated samples, in blue), respectively. Data points on the detection limit lines indicate an absence of plaque for these samples.
are consistent with the fact that all IncH-specific phages isolates described to date are RNA phages (Coetzee et al., 1985; Nuttal et al., 1987), while all IncN-specific phages known to date are DNA phages (Bamford et al., 1995; Bradley et al., 1981; Khatoon et al., 1972). For pKJK5 and R1drd19, in contrast, we did not identify a clear dominance pattern, which is indicative of higher overall diversity of phages targeting these plasmid groups.

3.4. Fate of plasmid-specific phages through municipal wastewater treatment plants

Across the phage types and the WWTPs, we observed that primary clarification did not reduce phage concentration in the water phase (Fig. 3). This limited effect of settling, attributable to the small size of the viral particles, is consistent with previous research (Lewis and Metcalf, 1988). However, the rest of the treatment trains strongly reduced phage concentrations, which dropped by ca. 2 log in the effluent compared to the influent. We note that these removal estimates might be biased by the incomplete recovery of phages by the concentration procedure used for the plant effluent samples. This recovery was estimated to 20–80% (McMinn et al., 2017). The overall plant-wide removal is similar, albeit a bit lower, than observed in Ryaverket for all viruses, as measured by metagenomic sequencing in a separate sampling effort (3 to 4 log, (Wang et al., 2020)). Our experimental design does not allow identification of the process unit(s) most responsible for phage removal. Activated sludge, where viruses can become associated with microbial biomass and be removed from the liquid stream has been reported to constitute the most significant barrier against viruses (IAWPRC Study biomass and be removed from the liquid stream has been reported to constitute the most significant barrier against viruses (IAWPRC Study Group on Health Related Water Microbiology, 1991; Lewis and Metcalf, 1988; Tanji et al., 2002). However, we do not observe a strong difference in removal between the two plants that include this process unit (Sjöundna and Ryaverket) and the one which does not (Visby). It is thus likely that the biofilms in MBBRs capture phage virions in a similar fashion as activated sludge flocs do.

4. Conclusions

This first effort at describing the distribution of plasmid-specific phages beyond the F-coliphages generated the following insights:

- A set of Salmonella enterica serovar Typhimurium strains was constructed and used to detect and quantify phages targeting four important plasmid classes. They open new opportunities in water quality monitoring and for understanding the fate and ecology of conjugal plasmids and the resistance genes they may carry.
- Plasmid-specific phages are more abundant in wastewater than ambient water, suggesting that gut microbiomes are the main sources of these phages. Phages specific to IncP-1 plasmids are especially consistently found in wastewater samples and at elevated concentration in hospital wastewater. They could serve as sensitive fecal indicator, complementing the established F-coliphage indicators.
- The fate of all types of plasmid-specific phages conforms municipal wastewater treatment plants, and especially secondary treatment, as effective barriers against environmental dissemination of viruses of fecal origin.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Supplementary materials

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References

