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Bacteriophages from faecal contamination are an important reservoir for AMR in aquatic environments

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ABSTRACT

Bacteriophages have been shown to play an important role in harbouring and propagating antibiotic resistance genes (ARGs). Faecal matter contains high levels of phages, suggesting that faecal contamination of water bodies may lead to increased antimicrobial resistance (AMR) levels due to increased phage loading in aquatic environments. In this study, we assessed whether faecal pollution of three rivers (Rivers Liffey, Tolka, and Dodder) was responsible for increased levels of ARGs in phage particles using established phage-faecal markers, focusing on four ARGs (bla_{TEM}, *tet*(O), *qnr*S, and *sul*1). We observed all four ARGs in phage fractions in all three rivers, with ARGs more frequently observed in agricultural and urban sampling sites compared to their source. These findings highlight the role of faecal pollution in environmental AMR and the impact of agricultural and urban activities on water quality. Furthermore, our results suggest the importance of including phages as indicators when assessing environmental AMR, as they serve as significant reservoirs of resistance genes in aquatic environments. This study provides important insights into the role of faecal pollution and phages in the prevalence of AMR in the environment and the need for their inclusion in future studies to provide a comprehensive understanding of environmental AMR.

1. Introduction

Antimicrobial resistance (AMR) poses a global threat to public health (Ferri et al., 2017; Prestinaci et al., 2015). A global assessment of the effect of AMR in 2019 estimated that 1.27 million deaths were directly attributable to bacterial AMR (Collaborators, 2022). In the absence of mitigating actions, this number is expected to increase to 10 million by 2050 (O'Neill, 2016). There are many reasons why AMR develops and spreads. These include the overuse and misuse of antimicrobial drugs, incomplete prescribed antimicrobial treatments, and poor sanitation practices (File Jr et al., 2014; Velazquez-Meza et al., 2022). As a result, there is an urgent need for better stewardship of existing antimicrobials, improved sanitation, and adequate infection prevention control to reduce the impact of AMR.

It is becoming increasingly clear that the environment plays an important role in spreading AMR, as resistant microbial populations can survive and persist in natural environments such as soil and water (Musoke et al., 2021). The One Health approach, which recognises the interconnection of human, animal, and environmental health, is essential to addressing this issue. Effective management practices and policies are needed to reduce the spread of AMR in the environment, including promoting the responsible use of antimicrobial agents in human and animal settings and the need for surveillance and monitoring of environmental reservoirs of AMR. Policies that limit the discharge of antimicrobial agents into the environment and the development of sustainable alternatives to antimicrobials can also help mitigate the environmental contribution to AMR. Thus, environmental research provides crucial opportunities to mitigate transmission patterns among humans, animals, and their shared environments (Eisenberg et al., 2007; Kagan et al., 2002).

To date, most studies that assess AMR in the environment have focused on bacteria (Larsson and Flach, 2022; Singer et al., 2016). Given the impact of drug-resistant bacteria, the emphasis on bacteria is understandable. Bacterial populations are known to exchange resistance genes through the processes of conjugation and transformation (Sun et al., 2019; von Wintersdorff et al., 2016). However, recent research has

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shown that bacteriophages also play a crucial role in harbouring and propagating ARGs, particularly via lateral transduction, a novel mechanism discovered in recent years (Chen et al., 2018). Furthermore, phages are also valuable tools for microbial source tracking, exemplified by the identification of the crAssphage through metagenomic analysis of human faecal samples (Gómez-Doñate et al., 2011; Kapoor et al., 2021; Mocé-Llivina et al., 2005; Shkoporov et al., 2018). The crAssphage specifically infects *Bacteroides intestinalis* and represents the most abundant bacteriophage family in the human gut. The abundance and distribution of gut microbiota can vary across different geographical regions; however, the crAssphage sequence has been found in various geographic locations, with minimal impact of diet, age, or illness affecting its presence (Edwards et al., 2019; Honap et al., 2020). This indicated a near omnipresence in the global human population and has crAssphage recognised as a valuable marker for tracking human-associated sources of pollution (Ballesté et al., 2021; García-Aljaro et al., 2017; Sala-Comorera et al., 2021b; Stachler et al., 2017).

If faecal pollution is found to contribute to phage loading, it is plausible that phages may harbour ARGs making them an important reservoir for resistance genes in aquatic environments. Therefore, phages serve as both a vehicle for propagating resistance in the environment and, due to their abundance in the gut microbiome, a reliable indicator of faecal pollution (Colomer-Lluch et al., 2011; Gómez-Doñate et al., 2011; Sala-Comorera et al., 2021b; Toribio-Avedillo et al., 2021). Aquatic pollution is a complex issue, and the quality of water is known to be influenced by geographical pressures, including land use (Li et al., 2023; Nolan et al., 2023). Anthropogenic, agricultural or industrial activities that occur proximal to aquatic environments can contribute to environmental pollution, and indeed the importance of these environments for the provision of a range of water-based activities makes aquatic systems particularly vulnerable (Harrison et al., 2019; Li et al., 2023; Reynolds et al., 2021).

Current studies that analyse environmental resistance typically focus on culture-specific microbes and molecular analyses of bacterial resistance genes. Consequently, these studies may significantly underestimate the levels of AMR in ecological niches. To further understand this issue, we present a study investigating the role of phages in environmental AMR. The stability of phages is a cause for concern because they have the potential to introduce ARGs into the aquatic microbiota. Moreover, phage particles can accumulate in our food sources, leading to further transmission of AMR. Indeed, phages carrying ARGs have been found in retail food such as fresh-cut vegetables, meat and dairy products (Blanco-Picazo et al., 2022; Blanco-Picazo et al., 2023; Gómez-Gómez et al., 2019; Larrañaga et al., 2018).

Specifically, this study sought to identify whether faecal pollution was responsible for increased levels of ARGs in phages using established phage-faecal markers in three rivers, from their source to outfall in Dublin Bay, on the east coast of Ireland. The chosen ARGs in our study confer resistance to widely consumed classes of antimicrobials used in Ireland, such as ciprofloxacin, sulphonamides, tetracyclines, and beta-lactams. Our results demonstrate that faecal pollution leads to phage loading, coinciding with a higher number of phage particles carrying ARGs This finding underscores the importance of phages as reservoirs of resistance genes in aquatic environments and highlights the need for additional indicators when assessing resistance.

2. Methods

2.1. Study area and water collection

The Rivers Dodder, Tolka and Liffey are three rivers which converge in Dublin and discharge into Dublin Bay (Fig. 1). The River Dodder in its upland catchments flows through reasonably rural agricultural lands before entering the urban areas of Dublin City and discharging to Dublin Bay. The main stream length of the River Dodder is 26 km, and it drains a catchment area of *c.*120 km^2. The River Liffey, with a length of 125 km and a catchment area of approximately 1250 km^2, drains peat and bogland in its headwater catchments before flowing through agricultural areas in County Kildare, suburban areas of west Dublin and the urban area of Dublin city, before its outfall to Dublin Bay. The River Tolka, with a catchment area of approximately 148 km^2 and a main stream length of approximately 22 km, rises in productive agricultural lands in County Meath before flowing through suburban and urban areas of Dublin to its outfall on the north-western fringe of Dublin Bay.

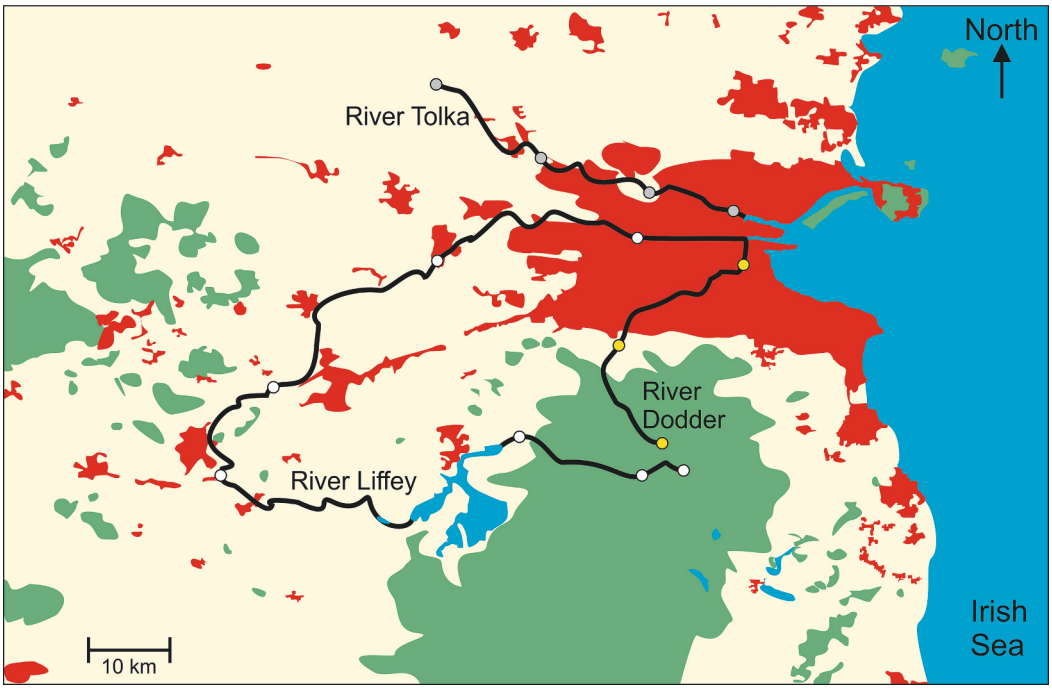

Fig. 1. Sampling sites of rivers. Fourteen sampling points across the Rivers Tolka, Dodder and Liffey. The colour seen in the map matches the EPA CORINE (Co-ORdinated INformation on the Environment). Red (urban), yellow (agricultural) and green (bogland) can be seen in the illustrated map.

Water samples were collected every two to three weeks at fourteen locations within the catchments of the Rivers Liffey, Dodder and Tolka over a one-year period from May 2019–May 2020 (Fig. 1; Supplementary Table 2; $n = 231$). Samples were collected from the source of each river and in downstream agricultural and urban locations to reflect accurate changes in faecal pollution and ARG concentrations. Seven samples were taken from the River Liffey, one from the source located in the bog land of the Wicklow mountains, four in downstream sampling points located in agricultural areas and two more downstream in urban areas located in Dublin City. Three samples were taken from the River Dodder, one from the source located in the Wicklow mountains, one located downstream in agricultural areas and one more located in the urban area of Dublin City. Four samples were taken from the River Tolka, two were taken from agricultural areas, of which its source is located, and two more from downstream urban sampling points. The EPA CORINE (Co-ORdinated INformation on the Environment) was used to assign land use to a sampling point. Duplicate one-litre samples were collected in autoclaved water bottles from each sampling location, stored at 4 °C and processed within 24 h.

2.2. Enumeration of faecal indicator organisms

The enumeration of somatic coliphages (SOMCPH) was carried out using the ISO 10705-2 double agar layer method (ISO, 2000). Volumes >10 ml were concentrated by the addition of $MgCl_2$ to a final concentration of 0.05 M and subsequent filtration through a 0.22 μm mixed cellulose ester membrane filter (Merck Millipore). Filters were added to a flask containing 5 ml of 1 % (w/v) beef extract (Neogen), 3 % (v/v) Tween 80 (MP Biomedicals) and 0.5 M NaCl. Phage elution was accomplished using an ultrasound bath for 4 min (ISO, 2003; Méndez et al., 2004).

2.3. Extraction of DNA

Water (100 ml) was filtered through 0.22 μm mixed cellulose ester membrane filters (Merck Millipore). The filters were subsequently placed in 500 μl of GITC buffer (5 M guanidine thiocyanate, 100 mM EDTA [pH 8] and 0.5 % sarkosyl) and stored at −20 °C. DNA was extracted using a previously described modification of the DNeasy Blood and Tissue kit protocol (Qiagen, Germany) (Gourmelon et al., 2007).

2.4. DNA extraction from phages

DNA of phages present in water was extracted using the protocol described by Colomer-Lluch et al. (2011). Briefly, water samples (100 ml) were passed through 0.22 μm low protein binding polyethersulfone filters. The filtrate was concentrated 200-fold using 100 kDa Amicon Ultra-15 Centrifugal Filter units (Millipore). Chloroform extraction was carried out on concentrated samples with a 1:1 (v/v) ratio followed by a DNAse treatment (100 U/ml) and incubated at 37 °C for 70 min. DNAse was then inactivated at 80 °C for 10 min. To ensure the complete removal of free DNA, an aliquot of 10 μl was collected and subjected to PCR using 16S rDNA primers, as well as ARG specific primers prior to digestion of the phage capsids. Digestion of the phage capsids was carried out using proteinase K (0.2 mg/ml) for 60 min at 56 °C, followed by a phenol/chloroform extraction and ethanol precipitation. The resulting DNA was dissolved into 20 μl water.

2.5. AMR gene & crAssphage marker quantification

The bla_{TEM}, *qnr*S, *tet*(O) and *sul*1 ARG (Supplementary Table 2) markers were quantified on the Roche Lightcycler 96 platform. SYBR Green 1 (Roche Diagnostics, Switzerland) fluorochrome was used, and all qPCR cycle conditions included a 10 min pre-incubation at 95 °C for 10 min and a subsequent melt curve analysis. Targeted primers and qPCR amplification conditions were used to amplify DNA targets, and ARGs were expressed as gene copies per 100 ml (gc/100 ml). Linearised standards between 10 and 10^6 gene copies were included in each run to quantify target gene levels in each sample. Positive (plasmid standard) and negative no-template (PCR grade water) controls were included. The absence of non-package DNA was verified by non-PCR amplification of ARG targets and 16S rRNA gene from DNA phage extraction controls. Only negative samples for both the ARG target and the 16S rRNA gene was used for further analysis. The amplification efficiency of the assays was calculated using the equation (Rutledge and Côté, 2003).

$$E = 10\left(\frac{1}{slope}\right) - 1$$

The efficiency of all the assays was between 90 and 110 %. All qPCR cycle conditions included a 10 min incubation at 95 °C and a melt curve analysis. The limit of detection (LOD) was defined as the lowest concentration of DNA that could be detected in at least 95 % of replicates. The limit of quantification (LOQ) was determined as the lowest concentration of DNA quantified within 0.5 standard deviations of the $\log_{10}$ concentration (Blanchard et al., 2012) (Supplementary Table 2).

The crAss_2 marker was quantified using a FAM-labelled TaqMan probe (García-Aljaro et al., 2017). Undiluted and 10-fold diluted samples were analysed in a reaction mixture (20 μl) containing the appropriate primers (Supplementary Table 1) and either the FastStart Essential DNA Probes Master or FastStart Essential DNA Green Master (Roche Diagnostics, Switzerland).

2.6. Data analysis

Univariate analysis was carried out using GraphPad Prism 10 software. A significance cut-off of $p \leq 0.05$ was used for all analyses. Bivariate relationships were investigated using qPCR results for each target ARGs and the human-associated phages source tracking marker-crAssphage and somatic coliphages.

3. Results

3.1. Phage markers show increasing levels of faecal pollution as rivers flow through agricultural and urban environments

The crAssphage marker (to identify human faecal contamination) was detected at all sampling locations in the Rivers Liffey and Dodder. At the source of the River Liffey, the crAssphage marker was observed in 18 % of the samples and ranged over one order of magnitude (Figs. 2(f) and 3(c); $n = 15$), while at the source of the River Dodder, the crAssphage marker was observed in 6 % of the samples and ranged over one order of magnitude (Figs. 2(e) and 3(b); n = 15). In both rivers, the lowest levels of the crAssphage marker were observed at the source compared to sampling locations further downstream. At downstream locations within agricultural lands of the River Liffey, an increase in human faecal pollution was observed, with 41 % of the samples being above the detection limit and in which the crAssphage marker ranged over four orders of magnitude ($n = 68$). At sampling locations within the agricultural land use of the River Dodder catchment, an increase in human faecal pollution was observed compared to observations at the river's source location, with 64 % of the samples being above the detection limit and in which the crAssphage marker ranged by over one order of magnitude ($n = 17$). In both rivers, the highest prevalence of crAssphage marker was observed in downstream urban sampling locations. At these locations in the River Liffey, the crAssphage marker was observed in 81 % of the samples and ranged over two orders of magnitude ($n = 33$). At urban sampling locations in the River Dodder, the crAssphage marker was observed in 91 % of the samples and ranged over two orders of magnitude ($n = 16$).

Of note, the source of the River Tolka is in the agricultural area of Dunshaughlin, Co. Meath (Fig. 1). The human-associated crAssphage

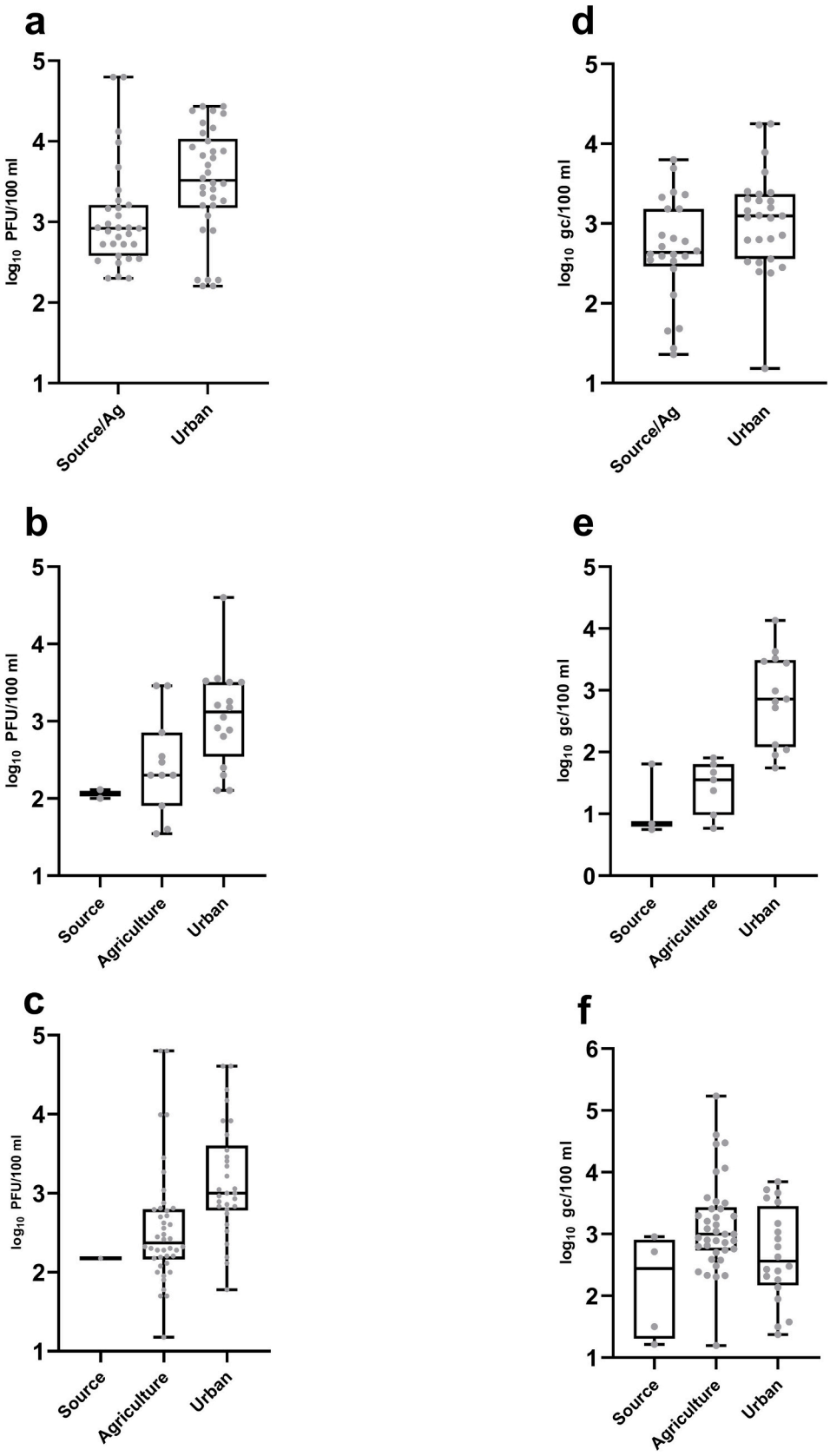

Fig. 2. Somatic coliphages and crAssphage in rivers. (a)(b)(c) Depicts somatic coliphages from the Rivers Tolka, Dodder, and Liffey. (d)(e)(f) Show crAssphage levels from the Rivers Tolka, Dodder, and Liffey. The levels are shown in their designated land use, source, and agricultural and urban land uses (EPA Corine land use). Samples were taken over one year ($n = 231$), and the box plot only represents positive samples and depicts all points and median values, upper and lower quartile percentile values.

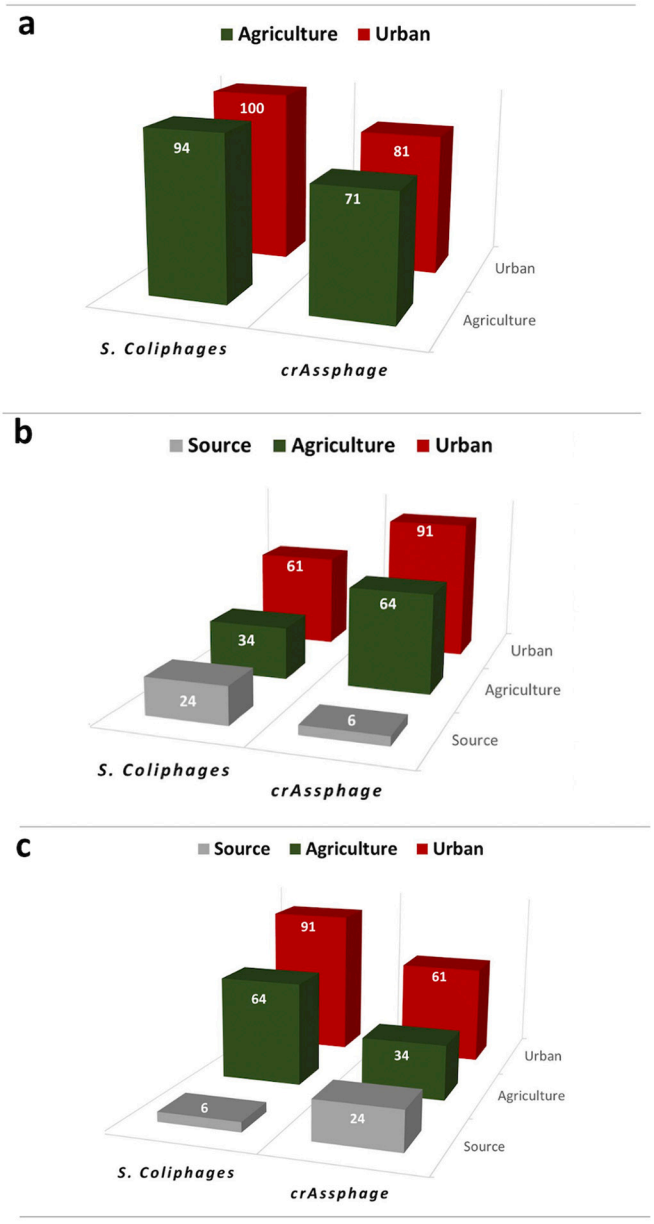

Fig. 3. Percentage of positive detection for crAssphage and somatic coliphages in rivers. Rivers Tolka (a), Dodder (b) and the Liffey (c) clustered based on designated land uses.

marker was positive for 71 % of samples at this location and ranged over two orders of magnitude (Figs. 2(d) and 3(a); $n = 34$). The increasing prevalence of the crAssphage marker in the downstream was again observed in the River Tolka, albeit to a considerably lesser extent, with 81 % of the samples in downstream urban areas being positive and ranging over one order of magnitude ($n = 33$).

Somatic coliphages were also detected at all sampling points in the Rivers Liffey and Dodder (Figs. 2 and 3). At the River Liffey source, somatic coliphages were detected in 14 % of the samples but varied by less than an order of magnitude (Figs. 2(c) and 3(c); $n = 15$). At the source of the River Dodder, somatic coliphages were observed in 24 % of the samples, and similar to the River Liffey, levels varied by less than an order of magnitude (Figs. 2(b) and 3(b); n = 15; n = 15). Increases in somatic coliphages downstream of the source locations in both the Liffey and Dodder rivers suggests an increase in faecal pollution from agricultural inputs. For the River Liffey, somatic coliphages at the sampling locations dominated by land use of agriculture were observed in 69 % of the samples and varied by over three orders of magnitude ($n = 68$). In the River Dodder, somatic coliphages were observed in 64 % of the samples and ranged by over one order of magnitude ($n = 17$). The highest prevalence of the somatic coliphages marker were observed further downstream in urban sampling points in both rivers. In the River Liffey, somatic coliphages were observed in 81 % of the samples and ranged over two orders of magnitude ($n = 33$). In the River Dodder, somatic coliphages were observed in 91 % of the samples and ranged over two orders of magnitude ($n = 16$).

In the River Tolka, somatic coliphages were observed at all sampling points. At the source of the River Tolka, somatic coliphages were observed in 94 % of the samples and ranged over two orders of magnitude (Figs. 2(a) and 3(a); $n = 34$). The highest prevalence of the somatic coliphages marker was found further downstream in urban sampling points, where somatic coliphages were observed in 100 % of the samples and ranged over two orders of magnitude ($n = 33$).

3.2. ARGs carrying phages are most prevalent in agricultural and urban areas

All four ARGs were detected in phage fractions derived from the Rivers Liffey, Dodder and Tolka (Figs. 4 and 5). The River Liffey and Dodder profiles were similar, as both had the lowest levels of ARGs in phage fractions at their sources. At the source of the River Liffey, 14.3 % of the samples were positive for ARGs in phage fractions, and all four ARGs were within an order of magnitude (Figs. 4(c) and 5(c); $n = 15$). In comparison, 3 % of samples were positive at the source of the River Dodder and varied by less than one order of magnitude (Figs. 4(b) and 5(b); n = 15). In downstream agricultural sampling points, an increase in the prevalence of ARGs in phages was observed. At agricultural sampling points in the River Liffey, 36.5 % of the phage fraction samples contained ARGs and ranged over three orders of magnitude ($n = 68$). In agricultural sampling points in the River Dodder, 28.5 % of the phage fraction samples contained ARGs and ranged over three orders of magnitude ($n = 17$). In downstream urban sampling points on both rivers, similar profiles emerge. In urban sampling points in the River Liffey, 52 % of the phage samples contained ARGs ($n = 33$). Similarly, at urban sampling points in the River Dodder, 42.3 % of the phage samples were positive for ARGs.

In contrast to both the Rivers Liffey and Dodder, the source of the River Tolka is in an agricultural area (Fig. 1). Here, 62.5 % of the phage samples contained ARGs and ranged over two orders of magnitude (Figs. 4(a) and 5(a); n = 17). In downstream urban sampling points in the River Tolka, a slight decrease was evident where 57.3 % of the phage samples were positive for ARGs (n = 33).

3.3. Faecal pollution is associated with increased levels of ARGs in phage fractions

All three of the studied rivers showed varying levels of significant correlation between two faecal-associated phage markers and ARGs in phage fractions (Table 1). Somatic coliphages correlated with all four resistance genes in phages (p-value < 0.005), while the human-associated crAssphage marker correlated with both the bla_{TEM} (p-value < 0.005) and *qnr*S (p-value < 0.05) resistance genes. The River Liffey exhibited the highest correlation between the phage-faecal-associated marker and antimicrobial resistance genes found in phages. After the River Liffey, the River Dodder showed a correlation between the studied markers, as somatic coliphages correlated with both the *tet* (O) (p-value < 0.001) and *qnr*S (p-value < 0.05) ARGs. The River Tolka exhibited no correlation between the phage faecal-associated markers and the resistance genes (Table 1).

4. Discussion

This study investigated rivers that are constantly being influenced

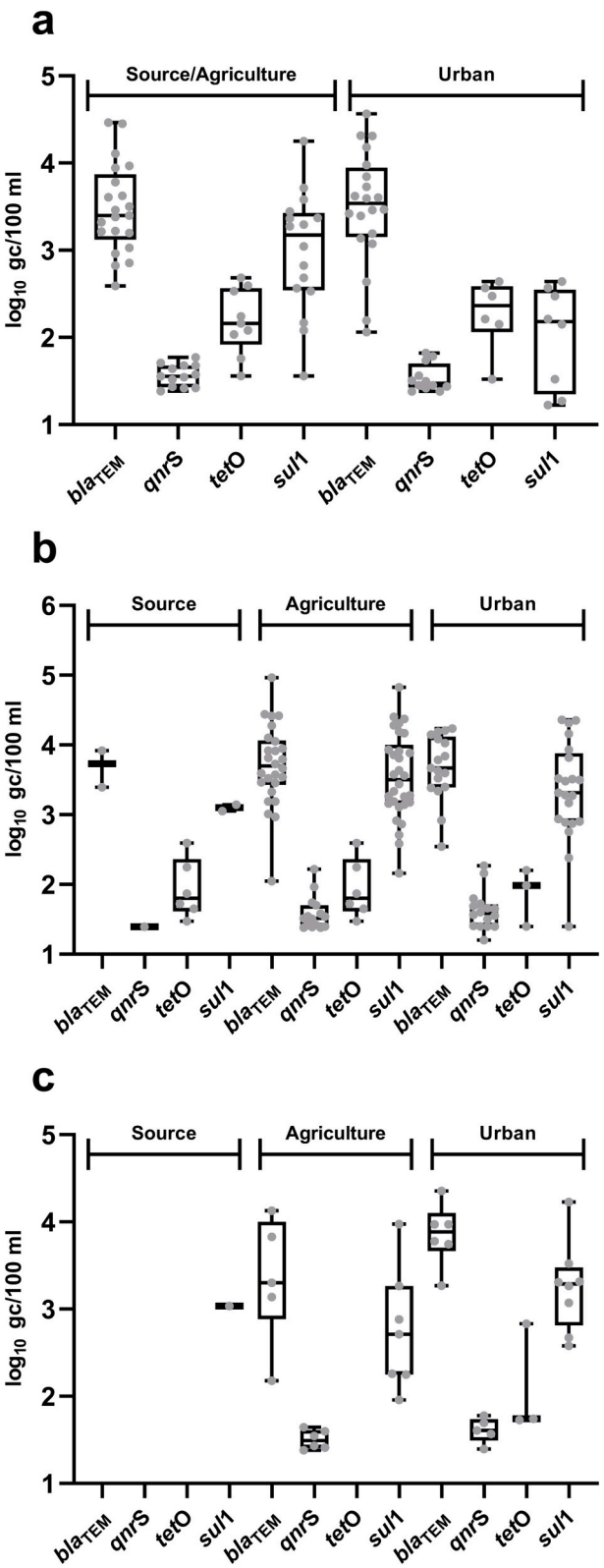

Fig. 4. The concentration of ARGs in phage fractions. Rivers (a) Tolka, (b) Dodder, and (c) Liffey are shown. The concentration of four antimicrobial resistance genes is shown from each river, bla_{TEM}, *qnr*S, *tet*(O) and *sul*1. Sampling points were arranged based on their designated land use, source, and agricultural and urban land uses (EPA Corine land use). Samples were taken over one year (n = 231), and the box plot only represents positive samples and depicts all points and median values, upper and lower quartile percentile values.

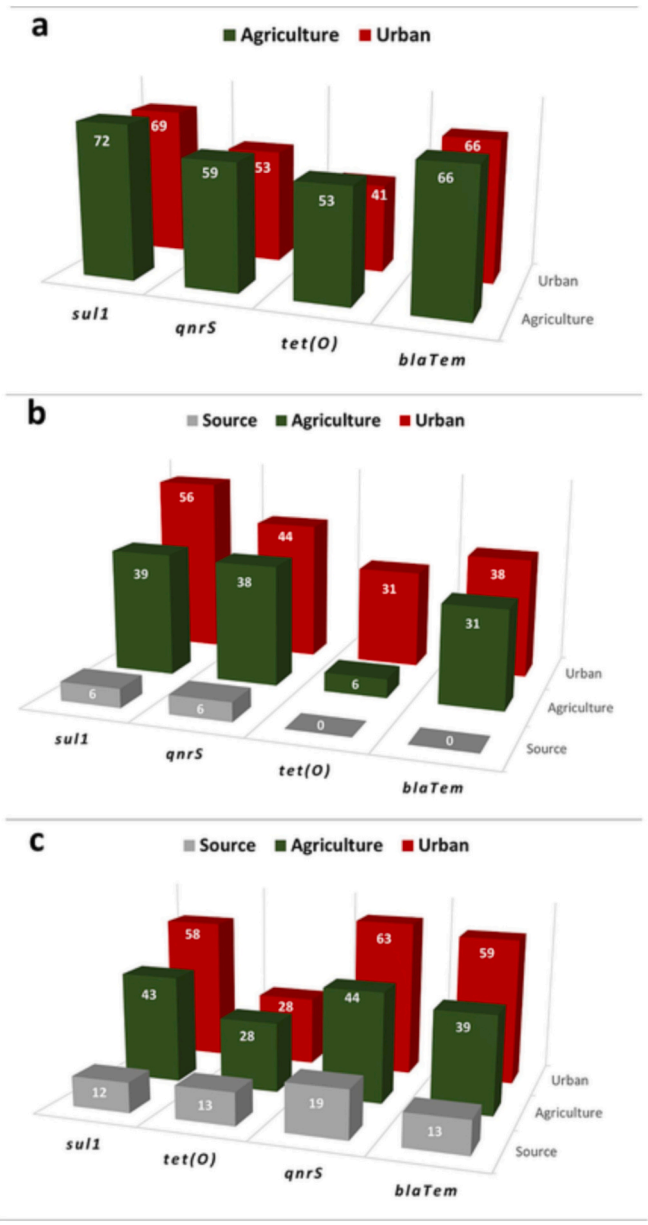

Fig. 5. Percentage of positives of ARGs in phage fractions in rivers. Rivers Tolka (a), Dodder (b) and Liffey (c) are shown and clustered based on their designated land use. Sampling points were assigned based on their designated land use, source, and agricultural and urban land uses (EPA Corine land use), *n* = 231.

Table 1
Correlations between phage faecal markers and ARGs in phage DNA fractions.

River	Phages marker	bla_{TEM}	*sul*1	*tet*(O)	*qnr*S
All rivers	S. Coliphage	0.205***	0.254***	0.224***	0.203***
	crAssphage	0.218***	0.089	0.106	0.149*
Liffey	S. Coliphage	0.189*	0.305***	0.180	0.330***
	crAssphage	0.172*	0.013**	−0.001	0.165*
Tolka	S. Coliphage	−0.117	−0.087	−0.425	0.087
	crAssphage	0.049	−0.006	−0.096	0.082
Dodder	S. Coliphage	0.413	0.315	0.793***	0.501*
	crAssphage	0.099	0.291	0.506	0.239

* $p \leq 0.05$.
** $p \leq 0.005$.
*** $p \leq 0.001$.

and shaped by nearby agricultural and urban activities, which may negatively impact their water quality. The main objective of this study was to determine whether the presence of faecal pollution is associated with the loading of phages and if this loading affects the occurrence and concentration of ARGs in phage particles. Consequently, given the stability of phages in aquatic environments, they may be a significant reservoir for AMR in aquatic environments.

Our study observed that some ARGs in phage particles correlated with faecal pollution using phage-faecal-associated markers. Our results also highlight changes in the concentration and presence of ARGs in phage particles, with similarities between all three rivers. In general, the presence of ARGs observed in phage particles increased in the downstream river directions, peaking at the most downstream sampling locations, a pattern that reflects the path of the watercourses through agricultural and urban land uses. These results suggest that faecal pollution can lead to increased levels of AMR-carrying phage particles in these systems. Thus, faecal pollution is contributing not only to greater levels of AMR in the environment, but increases the persistence of AMR, as studies on the decay rates of faecal indicator organisms have observed phages to persist significantly longer than other faecal indicator bacteria in different aquatic environments (Herrig et al., 2019; Korajkic et al., 2019). Thus, highlighting the need to consider phages when discussing environmental AMR.

The levels of ARGs in the phage fractions of this study reached over five orders of magnitude, and resistance genes were detected in nearly two-thirds of the samples. Filtration methods are frequently used to concentrate water samples prior to DNA extraction and subsequent analysis (Hinlo et al., 2017). Bacteriophages pass through commonly used filters e.g., 0.22 μm membranes, and are therefore not included in DNA extraction. Targeted bacteriophage concentration and extraction methods are therefore necessary to avoid that phage levels are underestimated. Applying filtration methods, the levels and prevalence of ARGs reported in this study, up to 50 %, could go unreported if the focus remains solely on ARGs in bacteria in the environment. Thus, AMR in other aquatic environments is likely to be underrepresented, especially if these environments are contaminated with faecal pollution. The levels observed in this study were similar to previously reported faecal-impacted bathing waters (Sala-Comorera et al., 2021a). Interestingly, bla_{TEM} was most observed in anthropogenically impacted areas. In some cases, the concentration observed in this study was in the same order of magnitude as WWTP influent (Rodriguez-Mozaz et al., 2015). Penicillins are the most prescribed outpatient antimicrobials in Ireland (DOH, 2018). The resistance gene bla_{TEM} works by hydrolysing penicillins and narrow spectrum β-lactams. However, they are not effective against newer generation cephalosporins. Consequently, the high dependence on penicillins may contribute to increased bla_{TEM} resistance genes in faecal-polluted waters.

The levels and presence of faecal contamination and ARGs in phages differ significantly between the source of each river, and through agricultural and urban areas. These results highlight the impact of external pressures, such as farming practices and urban pollution, on phage loading. This finding is supported by previous studies (Buelow et al., 2021; Czekalski et al., 2015; Reynolds et al., 2020; Samreen et al., 2021). The changes in the type and concentration of AMR can provide valuable information, serving as microcosms of potential prediction of geographical resistance, similar to wastewater-based epidemiology (Reynolds et al., 2022; Sims and Kasprzyk-Hordern, 2020).

The results indicate that phage loading coincides with faecal pollution and may pose a health risk to recreational users of these waters or downstream watershed bodies (Doménech-Sánchez et al., 2008). It is becoming clear that phages can transfer DNA from the environment to human and animal body-associated microbiomes, for example through ingestion of faecally-polluted water containing ARG-harbouring phages (Muniesa et al., 2013). In addition, ARG-harbouring bacteriophages may enter the food chain through consumption of shellfish harvested from faecally contaminated water (Blanco-Picazo et al., 2023). It is worth noting that the acquisition of ARGs via bacteriophages may be overlooked, as most literature on antimicrobial resistance focuses on bacteria alone. Our study suggests that additional indicators, besides faecal indicator bacteria, could provide valuable information on AMR in aquatic environments.

5. Conclusions

This study demonstrates that faecal contamination of waterbodies is associated with increased phage levels and with increased levels of phage associated ARG. These findings highlight the important role of bacteriophage particles as potential reservoirs of antimicrobial resistance genes in the environment, which would be overlooked if assessment of environmental ARG focuses solely on bacteria. Phage particles harbouring ARG genes may enter human and animal microbiomes through ingestion of contaminated water or food, e.g., shellfish. This study therefore highlights the need to include phages in assessing water quality to provide a comprehensive understanding of AMR in aquatic environments and mitigate potential public health risks.

CRediT authorship contribution statement

Tristan M. Nolan: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Laura Sala-Comorera:** Validation. **Liam J. Reynolds:** Validation. **Niamh A. Martin:** Investigation. **Jayne H. Stephens:** Investigation. **Gregory M.P. O'Hare:** Writing – review & editing. **John J. O'Sullivan:** Writing – review & editing. **Wim G. Meijer:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165490.

References

Ballesté, E., Blanch, A.R., Mendez, J., Sala-Comorera, L., Maunula, L., Monteiro, S., Farnleitner, A.H., Tiehm, A., Jofre, J., García-Aljaro, C., 2021. Bacteriophages are good estimators of human viruses present in water. Front. Microbiol. 12, 619495.

Blanchard, P., Regnault, J., Schurr, F., Dubois, E., Ribière, M., 2012. Intra-laboratory validation of chronic bee paralysis virus quantitation using an accredited standardised real-time quantitative RT-PCR method. J. Virol. Methods 180 (1–2), 26–31.

Blanco-Picazo, P., Gómez-Gómez, C., Morales-Cortes, S., Muniesa, M., Rodríguez-Rubio, L., 2022. Antibiotic resistance in the viral fraction of dairy products and a nut-based milk. Int. J. Food Microbiol. 367, 109590.

Blanco-Picazo, P., Morales-Cortes, S., Ramos-Barbero, M.D., García-Aljaro, C., Rodríguez-Rubio, L., Muniesa, M., 2023. Dominance of phage particles carrying antibiotic resistance genes in the viromes of retail food sources. ISME J. 17 (2), 195–203.

Buelow, E., Ploy, M.C., Dagot, C., 2021. Role of pollution on the selection of antibiotic resistance and bacterial pathogens in the environment. Curr. Opin. Microbiol. 64, 117–124.
Chen, J., Quiles-Puchalt, N., Chiang, Y.N., Bacigalupe, R., Fillol-Salom, A., Chee, M.S.J., Fitzgerald, J.R., Penadés, J.R., 2018. Genome hypermobility by lateral transduction. Science 362 (6411), 207–212.
Collaborators, A.R., 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet 399 (10325), 629–655.
Colomer-Lluch, M., Jofre, J., Muniesa, M., 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. PLoS One 6 (3), e17549.
Czekalski, N., Sigdel, R., Birtel, J., Matthews, B., Bürgmann, H., 2015. Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes. Environ. Int. 81, 45–55.
DOH, 2018. Ireland's First One Health Report on Antimicrobial Use and Antimicrobial Resistance.
Doménech-Sánchez, A., Olea, F., Berrocal, C.I., 2008. Infections related to recreational waters. Enferm. Infecc. Microbiol. Clin. 26 (Suppl. 13), 32–37.
Edwards, R.A., Vega, A.A., Norman, H.M., Ohaeri, M., Levi, K., Dinsdale, E.A., Cinek, O., Aziz, R.K., McNair, K., Barr, J.J., Bibby, K., Brouns, S.J.J., Cazares, A., de Jonge, P. A., Desnues, C., Díaz Muñoz, S.L., Fineran, P.C., Kurilshikov, A., Lavigne, R., Mazankova, K., McCarthy, D.T., Nobrega, F.L., Reyes Muñoz, A., Tapia, G., Trefault, N., Tyakht, A.V., Vinuesa, P., Wagemans, J., Zhernakova, A., Aarestrup, F. M., Ahmadov, G., Alassaf, A., Anton, J., Asangba, A., Billings, E.K., Cantu, V.A., Carlton, J.M., Cazares, D., Cho, G.S., Condeff, T., Cortés, P., Cranfield, M., Cuevas, D. A., De la Iglesia, R., Decewicz, P., Doane, M.P., Dominy, N.J., Dziewit, L., Elwasila, B.M., Eren, A.M., Franz, C., Fu, J., Garcia-Aljaro, C., Ghedin, E., Gulino, K. M., Haggerty, J.M., Head, S.R., Hendriksen, R.S., Hill, C., Hyöty, H., Ilina, E.N., Irwin, M.T., Jeffries, T.C., Jofre, J., Junge, R.E., Kelley, S.T., Khan Mirzaei, M., Kowalewski, M., Kumaresan, D., Leigh, S.R., Lipson, D., Lisitsyna, E.S., Llagostera, M., Maritz, J.M., Marr, L.C., McCann, A., Molshanski-Mor, S., Monteiro, S., Moreira-Grez, B., Morris, M., Mugisha, L., Muniesa, M., Neve, H., Nguyen, N.P., Nigro, O.D., Nilsson, A.S., O'Connell, T., Odeh, R., Oliver, A., Piuri, M., Prussin Ii, A.J., Qimron, U., Quan, Z.X., Rainetova, P., Ramírez-Rojas, A., Raya, R., Reasor, K., Rice, G.A.O., Rossi, A., Santos, R., Shimashita, J., Stachler, E.N., Stene, L.C., Strain, R., Stumpf, R., Torres, P.J., Twaddle, A., Ugochi Ibekwe, M., Villagra, N., Wandro, S., White, B., Whiteley, A., Whiteson, K.L., Wijmenga, C., Zambrano, M.M., Zschach, H., Dutilh, B.E., 2019. Global phylogeography and ancient evolution of the widespread human gut virus crAssphage. Nat. Microbiol. 4 (10), 1727–1736.
Eisenberg, J.N., Desai, M.A., Levy, K., Bates, S.J., Liang, S., Naumoff, K., Scott, J.C., 2007. Environmental determinants of infectious disease: a framework for tracking causal links and guiding public health research. Environ. Health Perspect. 115 (8), 1216–1223.
Ferri, M., Ranucci, E., Romagnoli, P., Giaccone, V., 2017. Antimicrobial resistance: A global emerging threat to public health systems. Crit. Rev. Food Sci. Nutr. 57 (13), 2857–2876.
File Jr., T.M., Srinivasan, A., Bartlett, J.G., 2014. Antimicrobial stewardship: importance for patient and public health. Clin. Infect. Dis. 59 (suppl_3), S93–S96.
García-Aljaro, C., Ballesté, E., Muniesa, M., Jofre, J., 2017. Determination of crAssphage in water samples and applicability for tracking human faecal pollution. Microb. Biotechnol. 10 (6), 1775–1780.
Gómez-Doñate, M., Payán, A., Cortés, I., Blanch, A.R., Lucena, F., Jofre, J., Muniesa, M., 2011. Isolation of bacteriophage host strains of Bacteroides species suitable for tracking sources of animal faecal pollution in water. Environ. Microbiol. 13 (6), 1622–1631.
Gómez-Gómez, C., Blanco-Picazo, P., Brown-Jaque, M., Quirós, P., Rodríguez-Rubio, L., Cerdà-Cuellar, M., Muniesa, M., 2019. Infectious phage particles packaging antibiotic resistance genes found in meat products and chicken feces. Sci. Rep. 9 (1), 13281.
Gourmelon, M., Caprais, M.P., Ségura, R., Le Mennec, C., Lozach, S., Piriou, J.Y., Rincé, A., 2007. Evaluation of two library-independent microbial source tracking methods to identify sources of fecal contamination in French estuaries. Appl. Environ. Microbiol. 73 (15), 4857–4866.
Harrison, S., McAree, C., Mulville, W., Sullivan, T., 2019. The problem of agricultural 'diffuse' pollution: getting to the point. Sci. Total Environ. 677, 700–717.
Herrig, I., Seis, W., Fischer, H., Regnery, J., Manz, W., Reifferscheid, G., Böer, S., 2019. Prediction of fecal indicator organism concentrations in rivers: the shifting role of environmental factors under varying flow conditions. Environ. Sci. Eur. 31 (1), 59.
Hinlo, R., Gleeson, D., Lintermans, M., Furlan, E., 2017. Methods to maximise recovery of environmental DNA from water samples. PLoS One 12 (6), e0179251.
Honap, T.P., Sankaranarayanan, K., Schnorr, S.L., Ozga, A.T., Warinner, C., Lewis, C.M., 2020. Biogeographic study of human gut-associated crAssphage suggests impacts from industrialization and recent expansion. PLoS One 15 (1), e0226930.
ISO, 2000. ISO 10705-2: Water Quality. Detection and Enumeration of Bacteriophages–Part 2: Enumeration of Somatic Coliphages. International Organisation for Standardisation.
ISO, 2003. ISO 10705-3: 2003 Water Quality-Validation of Methods for Concentration of Bacteriophages From Water.
Kagan, L.J., Aiello, A.E., Larson, E., 2002. The role of the home environment in the transmission of infectious diseases. J. Community Health 27 (4), 247–267.
Kapoor, R., Ebdon, J., Wadhwa, A., Chowdhury, G., Wang, Y., Raj, S.J., Siesel, C., Durry, S.E., Mairinger, W., Mukhopadhyay, A.K., Kanungo, S., Dutta, S., Moe, C.L., 2021. Evaluation of low-cost phage-based microbial source tracking tools for elucidating human fecal contamination pathways in Kolkata, India. Front. Microbiol. 12, 673604.
Korajkic, A., Wanjugi, P., Brooks, L., Cao, Y., Harwood, V.J., 2019. Persistence and decay of fecal microbiota in aquatic habitats. Microbiol. Mol. Biol. Rev. 83 (4).
Larrañaga, O., Brown-Jaque, M., Quirós, P., Gómez-Gómez, C., Blanch, A.R., Rodríguez-Rubio, L., Muniesa, M., 2018. Phage particles harboring antibiotic resistance genes in fresh-cut vegetables and agricultural soil. Environ. Int. 115, 133–141.
Larsson, D.G.J., Flach, C.-F., 2022. Antibiotic resistance in the environment. Nat. Rev. Microbiol. 20 (5), 257–269.
Li, H., Zhao, B., Wang, D., Zhang, K., Tan, X., Zhang, Q., 2023. Effect of multiple spatial scale characterization of land use on water quality. Environ. Sci. Pollut. 30, 7106–7120.
Méndez, J., Audicana, A., Isern, A., Llaneza, J., Moreno, B., Tarancón, M.L., Jofre, J., Lucena, F., 2004. Standardised evaluation of the performance of a simple membrane filtration-elution method to concentrate bacteriophages from drinking water. J. Virol. Methods 117 (1), 19–25.
Mocé-Llivina, L., Lucena, F., Jofre, J., 2005. Enteroviruses and bacteriophages in bathing waters. Appl. Environ. Microbiol. 71 (11), 6838–6844.
Muniesa, M., Colomer-Lluch, M., Jofre, J., 2013. Potential impact of environmental bacteriophages in spreading antibiotic resistance genes. Future Microbiol. 8 (6), 739–751.
Musoke, D., Namata, C., Lubega, G.B., Niyongabo, F., Gonza, J., Chidziwisano, K., Nalinya, S., Nuwematsiko, R., Morse, T., 2021. The role of Environmental Health in preventing antimicrobial resistance in low- and middle-income countries. Environ. Health Prev. Med. 26 (1), 100.
Nolan, T.M., Reynolds, L.J., Sala-Comorera, L., Martin, N.A., Stephens, J.H., O'Hare, G. M.P., O'Sullivan, J.J., Meijer, W.G., 2023. Land use as a critical determinant of faecal and antimicrobial resistance gene pollution in riverine systems. Sci. Total Environ. 162052.
O'Neill, J., 2016. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations.
Prestinaci, F., Pezzotti, P., Pantosti, A., 2015. Antimicrobial resistance: a global multifaceted phenomenon. Pathog. Glob. Health 109 (7), 309–318.
Reynolds, L.J., Sala-Comorera, L., Martin, N.A., Nolan, T.M., Stephens, J.H., Gitto, A., O'Hare, G.M.P., O'Sullivan, J.J., Meijer, W.G., 2020. Correlation between antimicrobial resistance and faecal contamination in small urban streams and bathing waters. Sci. Total Environ. 739, 140242.
Reynolds, L.J., Martin, N.A., Sala-Comorera, L., Callanan, K., Doyle, P., O'Leary, C., Buggy, P., Nolan, T.M., O'Hare, G.M.P., O'Sullivan, J.J., Meijer, W.G., 2021. Identifying sources of faecal contamination in a small urban stream catchment: a multiparametric approach. Front. Microbiol. 12, 661954.
Reynolds, L.J., Gonzalez, G., Sala-Comorera, L., Martin, N.A., Byrne, A., Fennema, S., Holohan, N., Kuntamukkula, S.R., Sarwar, N., Nolan, T.M., Stephens, J.H., Whitty, M., Bennett, C., Luu, Q., Morley, U., Yandle, Z., Dean, J., Joyce, E., O'Sullivan, J.J., Cuddihy, J.M., McIntyre, A.M., Robinson, E.P., Dahly, D., Fletcher, N.F., Carr, M., De Gascun, C., Meijer, W.G., 2022. SARS-CoV-2 variant trends in Ireland: wastewater-based epidemiology and clinical surveillance. Sci. Total Environ. 838 (Pt 2), 155828.
Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242.
Rutledge, R.G., Côté, C., 2003. Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res. 31, e93.
Sala-Comorera, L., Nolan, T.M., Reynolds, L.J., Venkatesh, A., Cheung, L., Martin, N.A., Stephens, J.H., Gitto, A., O'Hare, G.M.P., O'Sullivan, J.J., Meijer, W.G., 2021a. Bacterial and bacteriophage antibiotic resistance in marine bathing waters in relation to rivers and urban streams. Front. Microbiol. 12, 718234.
Sala-Comorera, L., Reynolds, L.J., Martin, N.A., Pascual-Benito, M., Stephens, J.H., Nolan, T.M., Gitto, A., O'Hare, G.M., O'Sullivan, J.J., García-Aljaro, C., 2021b. crAssphage as a human molecular marker to evaluate temporal and spatial variability in faecal contamination of urban marine bathing waters. Sci. Total Environ. 789, 147828.
Samreen, Ahmad, I., Malak, H.A., Abulreesh, H.H., 2021. Environmental antimicrobial resistance and its drivers: a potential threat to public health. J. Glob. Antimicrob. Resist. 27, 101–111.
Shkoporov, A.N., Khokhlova, E.V., Fitzgerald, C.B., Stockdale, S.R., Draper, L.A., Ross, R. P., Hill, C., 2018. ΦCrAss001 represents the most abundant bacteriophage family in the human gut and infects Bacteroides intestinalis. Nat. Commun. 9 (1), 4781.
Sims, N., Kasprzyk-Hordern, B., 2020. Future perspectives of wastewater-based epidemiology: monitoring infectious disease spread and resistance to the community level. Environ. Int. 139, 105689.
Singer, A.C., Shaw, H., Rhodes, V., Hart, A., 2016. Review of antimicrobial resistance in the environment and its relevance to environmental regulators. Front. Microbiol. 7, 1728.
Stachler, E., Kelty, C., Sivaganesan, M., Li, X., Bibby, K., Shanks, O.C., 2017. Quantitative CrAssphage PCR assays for human fecal pollution measurement. Environ. Sci. Technol. 51 (16), 9146–9154.
Sun, D., Jeannot, K., Xiao, Y., Knapp, C.W., 2019. Editorial: horizontal gene transfer mediated bacterial antibiotic resistance. Front. Microbiol. 10, 1933.
Toribio-Avedillo, D., Blanch, A.R., Muniesa, M., Rodríguez-Rubio, L., 2021. Bacteriophages as fecal pollution indicators. Viruses 13 (6).
Velazquez-Meza, M.E., Galarde-López, M., Carrillo-Quiróz, B., Alpuche-Aranda, C.M., 2022. Antimicrobial resistance: one health approach. Vet. World 15 (3), 743–749.
von Wintersdorff, C.J., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S., van Alphen, L.B., Savelkoul, P.H., Wolffs, P.F., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7, 173.