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Bacteriophages in sewage: abundance, roles, and applications

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One sentence summary: This paper reviews the abundance, roles, and applications of bacteriophages from sewage.
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Abstract

The raw sewage that flows through sewage systems contains a complex microbial community whose main source is the human gut microbiome, with bacteriophages being as abundant as bacteria or even more so. Phages that infect common strains of the human gut bacteriome and transient bacterial pathogens have been isolated in raw sewage, as have other phages corresponding to non-sewage inputs. Although human gut phages do not seem to replicate during their transit through the sewers, they predominate at the entrance of wastewater treatment plants, inside which the dominant populations of bacteria and phages undergo a swift change. The sheer abundance of phages in the sewage virome prompts several questions, some of which are addressed in this review. There is growing concern about their potential role in the horizontal transfer of genes, including those related with bacterial pathogenicity and antibiotic resistance. On the other hand, some phages that infect human gut bacteria are being used as indicators of fecal/viral water pollution and as source tracking markers and have been introduced in water quality legislation. Other potential applications of enteric phages to control bacterial pathogens in sewage or undesirable bacteria that impede the efficacy of wastewater treatments, including biofilm formation on membranes, are still being researched.

Keywords: bacteriophages, sewage, somatic phages, microbial source tracking, transduction, faecal indicators

Introduction

Bacteriophages, viruses that infect bacteria, are commonly acknowledged to have played an important role in the development of molecular biology science, and their properties are being increasingly harnessed in various biotechnological applications.

Viral ecology studies in diverse environments indicate that phages are highly ubiquitous and essential elements of natural microbial systems (Weinbauer 2004), where they are agents of bacterial mortality (Fuhrman and Schwalbach 2003), nutrient regeneration (Weitz *et al.* 2015), and horizontal gene transfer (Canchaya *et al.* 2003). Therefore, they are key drivers of bacterial abundance, activity and community composition in natural ecosystems, including animal guts (Rodriguez-Valera *et al.* 2009, Mills *et al.* 2013, Minot *et al.* 2013).

The key role played by phages in shaping the bacterial microbiota of the human gut ecosystem has been extensively researched (Mills *et al.* 2013, Scanlan 2017, Guerin and Hill 2020). Recent studies show that the gene flux generated by phages is not restricted to a single bacterial species or genus, but that they create gene flow networks across phylogenetically distinct bacteria (Camarillo-Guerrero *et al.* 2021). Phages dominate the viral fraction of human gut microbiota (Letarov and Kulikov 2009, Reyes *et al.* 2012), and up to 10^{12} virus-like particles (VLPs) per ml have been reported in human faeces (Hoyles *et al.* 2014). Regarding diversity, more than 142 000 non-redundant viral genomes, mostly those of phages, have been detected in the human gut based on data in the Gut Phage Database (Camarillo-Guerrero *et al.* 2021). Among all this variety, the worldwide predominance of crAssphage and crAss-like phages has emerged (Dutilh *et al.* 2014, Edwards *et al.* 2019). CrAss-like phages are associated with the *Bacteroidetes*, which is the most abundant bacterial phylum in the human gut microbiome.

The aim of this review is to picture the gut phages occurrence in sewage and they role as vectors of horizontal gene transfer, to show their potential application as faecal indicators, microbial source trackers or to be applied in internal processes of a WWTP.

Bacteriophages in raw sewage

The raw sewage circulating in sewage systems harbours a complex microbial community, largely of human origin. Faecal bacteria do not replicate in raw sewage, with the exception of a few genera of the phylum *Proteobacteria*, which are infrequent in the gut (García-Aljaro *et al.* 2019). Even when an autochthonous population derived from non-sewage inputs proliferates in the sewers, the bacterial and phage profile of sewage before it reaches a wastewater treatment plant (WWTP) continues to be shaped mainly by the human gut microbiome.

As in faeces, phages are an important component of the microbial content of sewage, as demonstrated by various methods. Total viral particles can be determined by electron microscopy,

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epifluorescence microscopy or flow cytometry, while a metagenomics approach sheds light on the composition and diversity of the sewage phageome, also providing information about host bacterial species, including pathogenic strains.

Values in the literature for VLPs in the sewage entering WWTPs vary considerably, which is likely due to inter-site (Gulino *et al.* 2020) or intra-site variability (Brown *et al.* 2019) and the variable efficiency of methods used for VLP detection (Wu and Liu 2009). Nevertheless, most reported concentrations of VLPs (corresponding mainly to phages) in sewage systems range from 10^8 to 10^9 per ml (Wu and Liu 2009, Tamaki *et al.* 2012), which is much higher than in any other water environment studied so far. These values are similar to those reported for faeces (Hoyles *et al.* 2014) considering that daily each person defecates 150–200 g and contributes an average of 200 l of water to urban sewage.

Phages infecting strains of diverse species of cultivable gut bacteria have been reported in raw sewage, including *Escherichia coli* (Jebri *et al.* 2017), *Shigella* (Muniesa *et al.* 2003), *Klebsiella* (Muniesa *et al.* 2003, Wangkahad *et al.* 2015), *Enterobacter* (Wangkahad *et al.* 2015), *Salmonella* (Carey-Smith *et al.* 2006), *Pseudomonas* (Essoh *et al.* 2015), *Aeromonas* (Wangkahad *et al.* 2015), *Staphylococcus* (Synnott *et al.* 2009), *Enterococcus* (Bonilla *et al.* 2010, Vijayavel *et al.* 2014) and *Bacteroides* (Jebri *et al.* 2017). However, relatively few studies have quantified infectious phages, except those infecting *E. coli*, commonly known as coliphages, with reported values from 4.0 to 5.0 $\log_{10}$ units per ml (Jebri *et al.* 2017). Other cultivable phages are those infecting the most abundant bacteria in the gut: the phylum *Bacteroidetes*. For example phage infecting *Bacteroides tehaiotaomicron*, show values ranging from 2.0 to 3.0 $\log_{10}$ units per ml (Jebri *et al.* 2017).

Non-cultivable phages in sewage have also been studied by electron microscopy and metagenomics, the results revealing a huge diversity. Electron microscopy images show a wide morphological range, with *Myoviridae*, *Syphoviridae*, *Pedovoridae*, and *Microviridae* being the principle forms (Wu and Liu 2009, Brown *et al.* 2019). Metagenomics studies report a great variability in the metagenomic composition of raw sewage samples, with a high percentage of sequences not identified in databases, as well as a predominance of DNA phages (Cantalupo *et al.* 2011, Tamaki *et al.* 2012, Gulino *et al.* 2020). The collection of the existing metagenomic data of viromes from sewage and joining analysis efforts will provide more comprehensive insight into sewage virome profiles. However, as the human microbiota is the main source of the raw sewage microbial population (García-Aljaro *et al.* 2019), it can be assumed that most phages in sewage are found in the human gut. Indeed, the most abundant phage in human faeces, crAssphage, was identified in wastewater in all the 14 WWTPs tested in New York, showing a far greater ubiquity than any other identified phage (Gulino *et al.* 2020). Moreover, using a real time qPCR approach, Ballesté *et al.* (2021) reported about 10^6 and 10^7 crAssphage genomic copies per ml of municipal wastewater, ten thousand times more than the highest number of phages detected so far by culture of *Bacteroides* host strains (Payan *et al.* 2005). Besides, in the study of WWTPs in New York, a low percentage of samples were found to contain phages infecting bacteria involved in carbon and sulphur cycling, which can grow in biofilms formed in the sewers (Gulino *et al.* 2020).

The number of phage particles in activated sludge liquor is significantly higher than in raw sewage (Ewert and Paynter 1980, Wu and Liu 2009, Brown *et al.* 2019), indicating that phage replication occurs in the reactors, which is the most common biological secondary treatment of wastewater in high-income countries. Differences in the microbiota of raw sewage and activated sludge determined by metagenomics (Ye and Zhang 2013, Caiv 2014, Liu *et al.* 2017) indicate a shift in the composition of microbial communities in the effluents versus influents. As most phages are associated with specific host taxa, and the evolution of bacterial and viral communities is therefore closely linked (Fuhrman and Schwalbach 2003), the phageome of activated sludge also differs from that of raw sewage (Otawa *et al.* 2007, Parsley *et al.* 2010, Tamaki *et al.* 2012), with variations in abundance and diversity over time (Brown *et al.* 2019). Clear phage-host associations have been reported in activated sludge plants during bulking episodes (Liu *et al.* 2017).

As emphasised by this brief overview, phages are present in raw sewage and WWTPs in great abundance and variety. However, their study is still in its infancy and many questions remain to be answered, such as whether this density of phages has any significance for public health and if they have any useful applications.

Phage populations in the gut have been reported to differ substantially between healthy and diseased cohorts (Mills *et al.* 2013, Norman *et al.* 2015, Manriquev2017), a finding that has generated considerable interest in how phages shape our gut microbiome. Although great numbers of VLPs, mostly phages, have been detected in potable, well and reclaimed water, their metagenomics do not coincide with those found in the human gut or sewage (Rosario *et al.* 2009). Therefore, the potential role of gut phages that can reach humans through the consumption of faecally polluted water remains to be elucidated and will not be discussed here further.

In contrast, there is growing evidence that phages can act as vehicles of gene transfer, including those encoding toxins and antibiotic resistance, and in this manner they can have an undesirable impact on gut microbiota, including pathogens. This subject, one of increasing concern, is covered in the review.

Regarding potential applications of phages, we will look at their use as indicators of faecal/viral contamination and as faecal source markers, as well as their potential use in wastewater treatment processes.

Bacteriophage-mediated gene transfer in sewage

There is mounting evidence that phages in sewage may play a significant role in gene transfer as mobile genetic elements (MGEs). These are segments of DNA that encode enzymes and other proteins involved in the movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility) (Frost *et al.* 2005). MGEs act as vectors of horizontal gene transfer, which is the main evolutionary mechanism of prokaryotes (Darmon and Leach 2014, Vos *et al.* 2015). The set of MGEs within a cell, known as the mobilome (Siefert 2009), includes plasmids, transposons, integrons, genomic islands, conjugative integration elements, gene transfer agents, and phages.

The ubiquity, abundance, persistence, and versatility of phages makes them ideal vehicles for the transfer of genes between bacteria, even between those of different taxa or from different biomes. Phages found in sewage also show similar persistence, as discussed in other sections (Muniesa *et al.* 1999, Allué-Guardia *et al.* 2012, Calero-Cáceres and Muniesa 2016). Unlike other horizontal gene transfer mechanisms, transduction does not require contact between the donor and the recipient cell.

Phages infecting intestinal bacteria are released by defecation, either as free phages or some lysogens after the induction of prophages located within the bacterial chromosomes, remaining free in extra-intestinal environments (Muniesa *et al.* 2011). Consequently, phages of faecal origin encoding genes related with virulence can be found in raw sewage or animal wastewater, including those encoding the *E. coli* Shiga toxin type 1 (Dumke *et al.* 2006, Grau-Leal *et al.* 2015), Shiga toxin type 2 (Muniesa and Jofre 1998,

Tanji *et al.* 2002, Muniesa *et al.* 2004a, Dumke *et al.* 2006, Imamovic *et al.* 2010), new Shiga toxin 2 subtypes (García-Aljaro *et al.* 2006) and the cytolethal distending toxin (Allué-Guardia *et al.* 2011).

More recently, interest has grown in phages that mobilize antibiotic resistance genes (ARGs), far less studied than ARG transfer mediated by plasmids and transposons. Early studies detected β-lactamases in phages isolated from sewage (Muniesa *et al.* 2004b) and their abundance was subsequently quantified in sewage (Colomer-Lluch *et al.* 2011a, 2014, Marti *et al.* 2014), animal wastewater (Colomer-Lluch *et al.* 2011b) and sludges produced in WWTPs (Calero-Cáceres *et al.* 2014). Other groups of ARGs, such as those encoding resistances to sulfonamides (*sul1*) (Calero-Cáceres *et al.* 2014), quinolones (*qnrA*, *qnrS*) (Colomer-Lluch *et al.* 2014) and tetracycline (*tetW*) (Anand *et al.* 2016) have also been abundantly detected in phages, whereas those carrying methicillin resistance genes (*mecA*) (Colomer-Lluch *et al.* 2011a,b) and aminoglycoside resistance genes (*armA*) (Colomer-Lluch *et al.* 2014) are less common. Metagenomic analysis of the viral fraction isolated from sewage samples allows a more general overview of ARG diversity in sewage viromes (Subirats *et al.* 2016, Lekunberri *et al.* 2017).

The abundance of phages encoding virulence genes and ARGs increases the chances of their transduction to the resident bacteria in the sewage environment. Moreover, some bacterial populations in WWTPs, although not those of faecal origin, are metabolically active during the treatment process, a necessary condition for transduction to occur. Transduction was traditionally considered a rare event, thought to happen about once every 10^7–10^9 phage infections. Yet other studies (Evans *et al.* 2010, Kenzaka *et al.* 2010), and some recently described mechanisms of transduction (Chen *et al.* 2018), suggest a higher frequency, possibly several orders of magnitude greater. In this scenario, gene transfer by transduction in sewage environments could be taking place every second at an exceptionally high rate (Muniesa *et al.* 2013).

Transduction in a wastewater environment has been observed in *Proteobacteria, Bacteroidetes, Actinomycetales*, and *Firmicutes* (Del Casale *et al.* 2011a,b), but not in other classes of bacteria (e.g., *Deltaproteobacteria*, *Nitrospira*, *Planctomycetes*), indicating that not all bacterial groups are equally involved in gene transfer in a wastewater environment. Phages from wastewater (Gunathilaka *et al.* 2017) and biosolids (Ross *et al.* 2015) have shown the potential to transduce ARGs to the bacterial population, although the extent of ARG transduction events within the WWTP remains to be elucidated.

In environments with high concentrations of phages and their bacterial hosts, such as the gut and sewage, genetic material can be exchanged in other ways. Indeed, the constant lysis and turnover of bacterial populations during the natural life cycle of a lytic phage may be underestimated as a mechanism for the liberation of DNA, including plasmids and antibiotic resistance determinants. Subsequent genetic exchange may involve mechanisms such as transformation and delivery by outer membrane vesicles (Fulsundar *et al.* 2014, Keen *et al.* 2017, Crippen *et al.* 2020), which would be important among unrelated bacteria in environments that receive inputs from several sources, such as sewage.

Bacteriophages as indicators of viral/fecal pollution

Phages were proposed as indicators of faecal contamination as early as the 1940s, when they were studied by Guelin and Collaborators (1948) in the marine waters of the French Atlantic coasts (Guelin 1948). Using clearly defined methodologies, they established a correlation between phages and the proximity of faecal discharge of urban origin, considering the dilution effect associated with tide kinetics.

In the following decades, numerous studies enumerated and evaluated the diversity of phages infecting enteric bacteria, mainly *E. coli*, in wastewater and faeces (Dhillon *et al.* 1970, Ayres 1977). The relationship of these phages with bacterial indicators (Kott *et al.* 1974, Bell 1976) and enteric viruses in water (Simková and Cervenka 1981, Stetler 1984) began to be investigated. During this period, studies were initiated on the resistance of phages infecting enteric bacteria to different water treatments (Friberg and Hammarström 1956, Weber-Schutt 1966, Joyce and Weiser 1967, Vaughn and Ryther 1974), and data on their environmental persistence in different ecosystems were provided (Kott *et al.* 1974, Gerba and Schaiberger 1975). However, until the late 1980s, information on the ecology of these phages remained limited, which led to unsubstantiated assumptions about phage behaviour. For example, it was claimed that somatic coliphages can replicate in the water environment and consequently cannot accurately reflect faecal microbial contamination (Vaughn and Metcalf 1975, Seeley and Primrose 1980, Grabow *et al.* 1981). As reviewed by Jofre (2009), it was subsequently demonstrated that such replication is highly unlikely and if it were to occur, the effect on the concentration of somatic coliphages in the environment would be negligible (Jofre 2009).

As research in the field progressed, four groups of phages found in sewage attracted attention as potential determinants of microbial water quality: somatic coliphages, which infect *E. coli* through the cell wall, F-specific coliphages, which infect *E. coli* through the sex pili, phages infecting *Bacteroides* and those infecting enterococci (IAWPRC 1991, Bonilla *et al.* 2010). The proportions in which these proposed viral indicators of faecal pollution occur in sewage has been extensively studied (Contreras-Coll *et al.* 2002, Rose *et al.* 2004, Blanch *et al.* 2006), and it has been consistently documented that urban wastewater contains more somatic than F-specific coliphages or phages infecting *Bacteroides* or enterococci (Jebri *et al.* 2017, Nappier *et al.* 2019, Jofre *et al.* 2021).

As phages that infect enteric bacteria are always present in wastewater, they can be considered as indicators of faecal contamination. Moreover, as phages are affected by inactivation processes in a similar way to human or animal viruses, the presence of phages of faecal origin in the treated water can generally predict human faecal viruses. A correlation between human viruses and phages has been demonstrated in samples of surface water, groundwater, sediments and shellfish (Jofre *et al.* 1989, Havelaar *et al.* 1993, Brion *et al.* 2005), although other studies report a lack of clear association (Haramoto *et al.* 2005, Lodder *et al.* 2010, Rezaeinejad *et al.* 2014). In any case, coliphages are more strongly associated with pathogenic viruses than traditional bacterial markers and even more so than other groups of human viruses (Wu *et al.* 2011, Ballesté *et al.* 2021). Although coliphages are not index microorganisms for specific virus groups, their presence indicates faecal contamination, potentially including pathogenic faecal human viruses (Jofre *et al.* 2016).

Now established in regulations for water quality management, coliphages meet the operational requirements to be selected as microbial indicators (World Health Organization 2017): they are not a pathogenic microorganism; they are universally present in human and animal faeces in high numbers; under natural conditions they do not multiply in water; they are more numerous than faecal pathogens; they persist, are inactivated and respond to disinfection treatments in a similar way to faecal pathogens;

and can be easily detected by simple and inexpensive culture methods.

Before an indicator can be included in national or international regulations, standardized protocols need to be established that can be applied in different laboratories to provide robust and comparable results (IAWPRC 1991). In the case of phage indicators, it was not until the late 1990s that methodology began to be standardized for the enumeration of somatic and F-specific coliphages and phages infecting *Bacteroides* spp.

Standardized methods were developed, almost simultaneously, by the International Standardization Organization (ISO) and the United States Environment Protection Agency (the US EPA) for detecting somatic coliphages (International Standardization Organization 2000, US EPA 2001a,b) and F-specific coliphages (International Standardization Organization 1995, US EPA 2001a,b). A standardized protocol for phages infecting enterococci is not yet available, but an ISO method exists for phages infecting *Bacteroides*, which uses *B. fragilis* RYC2056 (ATCC 700786) as a host strain (International Standardization Organization 2001). This method can also be applied with phages associated with other *Bacteroides* spp. that effectively discriminate between sources of faecal contamination in water and have been used for microbial source tracking (MST) studies, as described in the following section.

The standardized ISO and EPA protocols for somatic and F-specific coliphage analysis, which are very similar and produce comparable results, have been widely applied in the last decades. They outline a qualitative procedure (a presence/absence spot test) that can be adapted to quantitative approaches based on a most probable number test or the enumeration of plaque forming units (PFUs) on host bacteria layers. Additionally, the ISO has defined a validation procedure for methods that concentrate phages from water when volumes higher than 10 mL need to be analysed (International Standardization Organization 2003).

The US EPA standardized protocols for somatic and F-specific coliphage detection also include two different methods applicable to both types of phages. Method 1601 is a quantitative approach based on single agar layer (SAL) assays for PFU enumeration (US EPA 2001a), whereas the qualitative method 1602 uses presence/absence assays (US EPA 2001b). The US EPA has recently revised and adapted these methods for application in recreational water and wastewater, using ultrafiltration and SAL assays (US EPA 2018a), and for the analysis of secondary wastewater, also using SAL procedures (US EPA 2018b).

Although ISO and the US EPA methods can be easily implemented in routine microbiology laboratories without experience in phages, they can be cumbersome in laboratories with limited equipment and insufficiently trained staff. Although the protocols from both organizations are similar, they use different host strains for the various groups of targeted phages. Among the few comparative studies carried out, some authors report that methods with the DAL technique provide slightly higher values than those using SAL assays (Mooijman *et al.* 2001).

As indicated by the ISO and the US EPA standardized protocols, origin traceability of the host strains and positive control coliphages is important, as is strain quality control and maintenance, so that the genotypic and phenotypic properties that characterize the stipulated strains are not lost. For example, both ISO and the US EPA methods use nalidixic acid-resistant variants to minimize the growth of accompanying microbiota in polluted water samples, which frequently interferes with visualization of plaques. Otherwise, a previous step of filtration through membrane filters of 0.22 μm pore diameter made of materials that do not adsorb proteins is advised.

In the case of F-specific coliphage enumeration, in both the ISO and the US EPA standardized methods, host strains must express the sexual pili, encoded in the F plasmid or F-derived plasmids and are not synthesized below 32ºC. In addition, markers are used in these host strains to improve their selection and stability: the capacity to degrade lactose in *Salmonella enterica* WG49 in the ISO method and ampicillin resistance in the *E. coli* HS/Famp strain in the US EPA methods.

Despite efforts to standardize protocols, a small percentage of divergent results is reported for phage total numbers and the proportions of phage groups. It cannot be ruled out that some of the notable variations in wastewater analysis outcomes between studies in different laboratories around the world could be related to insufficient quality control of the host strains and how long they are maintained (Wu *et al.* 2011, Nappier *et al.* 2019).

Of the aforementioned four types of enteric phages, somatic coliphages are the most frequently used as viral indicators of water quality, as they have the highest concentrations in wastewater and are simple to analyse. However, there is still some debate regarding which type of coliphage is the most suitable for application as a faecal/viral indicator. As F-specific coliphages are usually more resistant to ultraviolet light than somatic coliphages, they are used to monitor and validate ultraviolet-disinfection water treatments (Leclerc *et al.* 2000, Montemayor *et al.* 2008). However, F-specific coliphages have lower persistence in surface waters, especially in warmer climates, and are less resistant to inactivating heat or high pH treatments (Jofre 2007). It was therefore proposed to detect both types simultaneously, and standardized methods based on modified host strains were devised for total coliphage enumeration in a single analysis. Initially, the *E. coli* strain C3000 (ATCC 15597), extensively used in conjugation studies of the 1960s, was employed, but it was observed that this modified strain detects lower amounts of somatic coliphages than the standardized strains. The problem was subsequently overcome by the engineering of *E. coli* strain CB390 (CECT9198) (Guzmán *et al.* 2008), which can match the results of established methods that enumerate F-specific and somatic coliphages separately (Guzmán *et al.* 2008, Bailey *et al.* 2017).

Consequently, coliphages have been used to assess the quality of drinking water, wastewater sanitation, water reuse treatments, and hygienization of sludge, biosolids and sediments, and the safety of some foods (mainly shellfish). Since the beginning of the millennium, mainly somatic, but also F-specific coliphages, have been progressively incorporated in national and international regulations and guidelines, including those of the WHO, for the management of microbiological quality (Table 1).

Despite the availability of feasible and cost-effective presence/absence and quantitative (PFU) methods standardized by regulatory agencies, the routine analysis of coliphages as viral indicators will require faster and more user-friendly methods. Different analytical procedures have been proposed, including those that can be adapted to 100 mL of water, thus avoiding the need to concentrate phages from smaller volumes (Blanch *et al.* 2020).

Bacteriophages as microbial source tracking markers

Faecal indicators have been used to determine water quality for over a century, and now they are also being employed to determine the origin of the pollution. Microbial source tracking (MST) (Malakoff 2002) is based on the detection of particular

Table 1. List of national and International regulations and guidelines including coliphages as viral indicators for different water matrices, biosolids, sludge, and food (mainly shellfish).

Year	Country	Regulation sector	Description
1989	USA	Integrity membranes and UV	USEPA. 1989. Drinking Water; national Primary Drinking Water Regulations; Filtration; Disinfection; Turbidity, *Giardia lamblia*; Viruses; Legionella; and Heterotrophic Bacteria; Final Rule. 40CFR Parts 141 and 142. Federal register 54: 27486-27541. Washington D.C.
1999	UK	Integrity membranes and UV	DWI. 1999. The Water Supply (Water Quality) (Amendment) Regulations 1999: Cryptosporidium in Water Supplies. Department for Environment, Food and Rural Affairs. Statutory Instruments No. 1524. United Kingdom Legislation (available at http://united-kingdom-legislation.vlex.co.uk/vid/water-supply-quality-amendment-28393731)
2001	Canada (Quebec)	Drinking Water	Loi sur la qualité de l'environnement : règlement sur la qualité de l'eau potable c. Q.-2, r. 18.1.1. Gazette Officielle du Québec 24, 3561. Government of Quebec, Montreal, Quebec, Canada
2005	Australia (Queensland)	Reclaimed water	Queensland Government. 2005. Queensland Water Recycling Guidelines. Queensland Environmental Protection Agency. Brisbane. Australia.
2006	Australia (Northern Territory)	Reclaimed water	DRAFT Northern Territory Interim Guidelines for Management of Recycled Water Schemes 2006
2006	Australia (Northern Territory)	Reclaimed water	Australian Guidelines for Water Recycling: Managing Health and Environmental Risks (Phase 1), 2006
2006	Australia (Western Australia)	Reclaimed water	Australian Guidelines for Water Recycling: Managing Health and Environmental Risks (Phase 1) 2006
2006	USA	Groundwater	40 CFR Parts 9, 141, and 142 National Primary Drinking Water Regulations: Ground Water Rule; Final Rule.
2001	USA	Integrity membranes and UV	USEPA. 2001. Low-pressure membrane filtration for pathogen removal: application, implementation and regulatory issues. EPA 815-C-01-001. Environmental Protection Agency. Washington D.C.
2007	Australia (New South Wales)	Recycled Water	Interim NSW Guidelines for Management of Private Recycled Water Schemes, 2007
2008	Australia	Recreational Water	Guidelines for Managing Risks in Recreational Water (Emerging interest)
2008	Canada	Recreational Water	Guidelines for Canadian Recreational Water Quality (Third edition 2012)
2009	Australia (Western Australia)	Recycled Water	Draft Government of Western Australia Department of Health. Draft Guidelines for the Use of Recycled Water in Western Australia. Initial External Consultation
2009	USA	Molluscan shellfish	Guide for the control of Molluscan Shellfish. National shellfish Sanitation program. 2017 Revised
2011	Australia	Drinking Water	National Water Quality Management Strategy. Australian Drinking Water Guidelines 6 (version 3.2 updated February 2016)
2011	Canada	Drinking Water	Guidelines for Canadian Drinking Water Quality: Guideline Technical Document - Enteric Viruses
2011	USA (North Caroline)	Reclaimed water	North Carolina Environmental Quality. North Carolina Adm. Code 15A NCAC 2U Reclaimed Water; North Carolina Department of Environment and Natural Resources. Regulatory Review of North Caroline.
2012	Australia (Western Australia)	Biosolids	Western Australian guidelines for biosolids management. Department of Environment and Conservation
2012	India	Drinking water	Indian Standard. ISO 10500: 2012. Drinking Water – Specification. Bureau of Indian Standards. Manak Bhavan, 9 Bahadur Shah Zafar Marg, New Delhi.
2014	Colombia	Biosolids	Decreto Número 1287 por el cual se establecen criterios para el uso de los biosólidos generados en plantas de tratamiento de aguas residuales municipales,
2014	France	Reclaimed water	Arrêté du 25 juin 2014 modifiant l'arrêté du 2 août 2010 relatif à l'utilisation d'eaux issues du traitement d'épuration des eaux résiduaires urbaines pour l'irrigation de cultures ou d'espaces verts.
2017	WHO	Drinking Water	Guidelines for Drinking - water Quality. Fourth Edition Incorporating the First Addendum. 7 810 Microbial Microbial aspects.
2017	WHO	Drinking Water	Potable reuse. Guidance for producing safety drinking water.
2018	USA	Recreational water	Review of coliphages as possible indicators of fecal contamination for ambient water quality. EPA 820-R-15-098
2020	Europe	Reclaimed Water	Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 on minimum requirements for water reuse. OJ L 177, 5.6.2020, p. 32–55
2020	Europe	Drinking Water	DIRECTIVE (EU) 2020/2184 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2020 on the quality of water intended for human consumption. OJ L 435, 23.12.2020, p. 1–62
2021	France	Sludge	Arrêté du 20 avril 2021 modifiant l'arrêté du 30 avril 2020 précisant les modalités d'épandage des boues issues du traitement des eaux usées urbaines pendant la période de covid-19. Journal Oficial de la République Française, Texte 4 sur 188. 27 Mai 2021.

host-associated bacteria, mainly *Bacteroidetes* and *Bifidobacterium* (Bernhard and Field 2000, Shanks *et al.* 2008, Mieszkin *et al.* 2009, Gomez-Donate *et al.* 2012, Green *et al.* 2014), or viruses, either phages or host pathogens, as discussed below. By identifying the potential source of faecal pollution, these markers allow a better management of water and the application of effective restoration measures.

F-specific RNA phages, a fraction of F-specific coliphages, were the first phage candidates suggested as a potential MST tool. These viruses belong to the family *Fiersviridae* (Francki *et al.* 1991) and are divided into two genera, *Emesvirus* and *Qubevirus* (Friedman *et al.* 2009, Callanan *et al.* 2021), each of which are classified in two serotypes or genogroups: genogroups I and II for *Emesvirus* and III and IV for *Qubevirus* (Hsu *et al.* 1995, Beekwilder *et al.* 1996). The proportions of these genogroups in faecal pollution can serve to discriminate between human and warm-blooded animal inputs, given that groups II and III predominate in the former, and groups I and IV in the latter (Furuse 1987, Havelaar *et al.* 1990, Hsu *et al.* 1995, Schaper *et al.* 2002).

F-specific RNA phages can be detected either by culture, as explained in the previous section, or by molecular methods such as real-time quantitative polymerase chain reaction (RT-qPCR). After obtaining plaques by culture, the genotypes can be distinguished by plaque hybridization using specific probes (Schaper *et al.* 2002), or analysing a phage suspension from the plaques by RT-qPCR (Ogorzaly and Gantzer 2006, Wolf *et al.* 2008). RT-qPCR can also be carried out directly on the viral fraction of a sample (Wolf *et al.* 2008).

However, different phage groups display variable resistance to environmental stressors and inactivation treatments such as disinfection. Several studies on both laboratory and environmental phages have shown that phages belonging to genogroups I and II (Levivirus) are more resistant than those of III and IV (Allolevivirus) (Schaper *et al.* 2002, Long and Sobsey 2004, Muniesa *et al.* 2009, Haramoto *et al.* 2015). Consequently, the variability in genogroup proportions after natural or human-driven inactivation impairs their suitability for MST.

Even before or parallel to the growing use of host-specific *Bacteroidetes* as MST markers, phages infecting *Bacteroides* strains isolated from humans or animals were also being promoted as pollution source trackers (Tartera *et al.* 1989, Payan *et al.* 2005, Gómez-Doñate *et al.* 2011). As the sensitivity of *Bacteroides* strains used to detect phages varies considerably between regions, a suitable strain needs to be isolated from each area (Payan *et al.* 2005). As a result, several *Bacteroides* strains have been isolated for use in MST in different countries (e.g., Spain, Colombia, UK), successfully discriminating between human and animal (mainly cattle, swine, poultry, and horse) faecal pollution (Tartera and Jofre 1987, Ebdon *et al.* 2007, Gómez-Doñate *et al.* 2011, Venegas *et al.* 2015). Numbers of *Bacteroides* phages infecting *B. thetaiotaomicron* GA17 strain range from 10^3-10^5 PFU/100 mL in municipal sewage to 50 to 10^4 PFU/100 ml in secondary effluents, being rarely detected in wastewaters from animal slaughterhouses (Muniesa *et al.* 2012, Venegas *et al.* 2015, Yahya *et al.* 2015). On the other hand, numbers of *Bacteroides* phages of animal origin range between 10^3 and 10^4 PFU/100 ml for cattle and poultry (phages infecting *B. thetaiotaomicron* CW18 and *B. fragilis* PL122, respectively) and 10^4 to 10^5 PFU/100 ml for swine (phages infecting *B. fragilis* PG76 and *B. fragilis* PG1226) (Gomez-Donate *et al.* 2012).

The great majority of phages infecting *B. fragilis* detected in sewage belong to the *Siphoviridae* family and have flexible tails and dsDNA (Tartera and Jofre 1987, Lasobras *et al.* 1997, Ogilvie *et al.* 2012); for example, phage B40-8, which infects *B. fragilis* strain GA17, and phage FB124-14, which infects *B. fragilis* strain GB-124, isolated in Spain and the UK, respectively. These phages have a very narrow host range and infect through receptors in the cell wall (Ogilvie *et al.* 2012, Jofre *et al.* 2014), which situates them in the group of somatic phages. As phages infecting *Bacteroides* have similar persistence to somatic coliphages, the ratio of both phage types (i.e., host-specific and a general indicator) was proposed as a good marker for MST (Muniesa *et al.* 2012).

Recent studies have identified a new group of phages infecting *Bacteroides*, known as crAssphage, which metagenomic studies have revealed to be the most abundant phage in sewage (Dutilh *et al.* 2014). This phage family has a worldwide distribution, having been found in sewage in all the continents except Antarctica, and has therefore been proposed as a human-associated molecular marker for MST (Stachler and Bibby 2014, García-Aljaro *et al.* 2017, Cinek *et al.* 2018). Four crAssphage have been isolated so far, all with *Podoviridae* morphology, although only two of them have been partially characterized, ΦcrAss001 (Shkoporov *et al.* 2018) and ΦcrAss002 (Guerin *et al.* 2021), whose hosts are *B. intestinalis* and *B. xylanisolvens*, respectively. The other two phages were isolated in *B. thetaiotaomicron* (Hryckowian *et al.* 2020). Moreover, the replication cycles of these phages are still mostly unknown. As culture methods are difficult to be implemented, to date only molecular techniques have been developed for crAssphage detection and quantification. The worldwide use of qPCR-based analysis has yielded average contents of between 10^4 and 10^9 GC/100 ml (García-Aljaro *et al.* 2017, Stachler *et al.* 2017, Ahmed *et al.* 2018a, Farkas *et al.* 2019, Kongprajug *et al.* 2019, Malla *et al.* 2019, Crank *et al.* 2020, Wu *et al.* 2020) and a high correlation with other bacterial and viral MST markers has been observed (Ahmed *et al.* 2018b, Ballesté *et al.* 2019, Edwards *et al.* 2019). These values are higher than those of other known human viruses, such as adenoviruses, noroviruses or polyomaviruses (Bofill-Mas *et al.* 2006, Ballesté *et al.* 2021), or bacterial MST markers, such as human *Bifidobacteria* (Gomez-Donate *et al.* 2012) or human *Bacteroidetes* (Green *et al.* 2014).

Finally, host-specific phages infecting enterococci have been postulated as another alternative MST indicator (Bonilla *et al.* 2010), although no standardized method for their detection is available. Several bacterial hosts have been isolated from human, cattle and pigs, which showed high specificity and abundances between 10^2–10^4 PFU 100 ml^{-1}[11] in animal runoff or sewage in England (Purnell *et al.* 2011) and Thailand (Wangkahad *et al.* 2017).

Prospective applications of bacteriophages in wastewater treatment processes

Phage-mediated bacterial mortality may improve the performance of wastewater treatments by controlling the abundance of harmful bacteria. Laboratory studies have tested phage efficacy against pathogenic bacteria, as well as filamentous bacteria causing bulking and foaming in activated sludge and membrane-fouling bacteria, among others. A few examples are given here.

The application of phages infectious for specific strains of pathogenic bacteria is envisaged as an additional tool to the range of treatments already used by WWTPs to reduce pathogen numbers in sewage (Curtis 2003). Laboratory tests have shown that communities of *Salmonella* (Turki *et al.* 2012) and *E. coli* (Beheshti Maal *et al.* 2015) are negatively affected by specific phage treatment.

Activated sludge treatment plants quite frequently suffer from the presence of filamentous bacteria that cause bulking and foaming on the surfaces of aeration reactors. A clear phage-host association has been reported in activated sludge plants during these episodes (Liu *et al.* 2015). Therefore, the addition of specific

lytic phages should reduce the numbers of problematic bacteria. In laboratory-scale experiments, isolated phages that infect foam-causing bacteria have prevented foam stabilization (Petrovski *et al.* 2011, Liu *et al.* 2015).

Membrane-based treatments are increasingly being used in WWTPs, but their operation can be impaired by the formation of bacterial biofilms, which results in a loss of flow rate (Wu *et al.* 2017). Addressing this issue, treatments with lytic phages assayed in the laboratory have shown promising results in inhibiting bacterial fouling of membranes (Goldman *et al.* 2009, Bhattacharjee *et al.* 2015, Ayyaru *et al.* 2018).

However, although promising results have been obtained on a laboratory level, the application of phages in WWTPs in the real world is more complex, due to a number of shortcomings (Ji *et al.* 2020). These include narrow host specificity, the difficulty of isolating and producing suitable phages, the emergence of resistant hosts, non-specific adsorption, phage decay, and last, but not least, as already discussed, the potential role of phages in gene transfer. This approach to controlling undesirable bacteria in wastewater treatments is, however, still in its infancy and considerable research remains to be done to render the procedures applicable in real-life settings. The progress made in phage therapy for bacterial infections in human medicine and husbandry (Gordillo Altamirano and Barr 2019, Kortright *et al.* 2019) may help to resolve some of the present limitations.

Conclusions

The aim of this review is to show the importance of bacteriophages in sewage and uncover their potential applications. Their great abundance and diversity in sewage mirrors the diversity observed in faeces and for the moment there is no information whether the water way has any role in shaping the gut virome and hence the gut microbiome. However, currently there are already some applications of gut phages. Thus, some of them like somatic coliphages and F-RNA phages are being used as faecal indicators together with bacterial indicators like *E. coli* and *Enterococci*. Since they have a similar persistence and environmental behaviour than animal viruses, they can act as a better proxy for them, allowing a better prediction of viral presence in water. In fact, some regulatory agencies have already included them in their regulations regarding water quality. For this reason, their use as microbial source trackers to determine the source of a pollution is also increasing, specially after the discovery of crAssphage as the most abundant bacteriophage in human faeces. Before its detection, just culture techniques were used to detect *Bacteroides* phages and molecular techniques as qPCR have also been developed to detect human and animal specific F-RNA phages. Also, the use of lytic phages in WWTPs can control the concentration of pathogenic bacteria or those impairing the treatment like bacteria causing bulking. However, currently these applications are still in laboratory stages. On the other hand, bacteriophages role in gene transfer as mobile genetic elements between bacteria like virulence related genes or antibiotic resistance genes has to be considered. In fact, sewage includes abundant autochthonous bacterial populations growing in sewage which could, by transduction, interchange these genes with bacteria of the human gut, including pathogens.

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