GLOBAL WATER PATHOGEN PROJECT

PART TWO. INDICATORS AND MICROBIAL SOURCE TRACKING MARKERS

GENERAL AND HOST- ASSOCIATED BACTERIOPHAGE INDICATORS OF FAECAL POLLUTION

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Summary

Different groups of bacteriophages have been comprehensively tested as indicators of faecal pollution and as markers for microbial source tracking (MST), as surrogate indicator for viral pathogens (index), and as potential indicators of public health risk. Because of their composition and structure, which are similar to other viruses, their outcome after remaining in water bodies and after water treatments mimics that of human and animal viruses much better than any other group of indicators. In this subchapter, bacteriophages will be organized in three *working groups* rather than in *taxons*, mostly because the current methods for their detection and quantification are adapted to these working groups. The three working groups of bacteriophages are taxonomically complex and diverse.

The groups that have been extensively studied are somatic coliphages, F-specific RNA bacteriophages, also named sexual coliphages though the two terms do not mean exactly the same, and bacteriophages infecting selected strains of *Bacteroides*. Somatic coliphages are bacteriophages infecting *E. coli* through the cell wall, F-specific RNA bacteriophages infect *E. coli* through the sexual pili encoded by the F plasmid and phages infecting *Bacteroides* strains are supposed to infect also through the cell wall.

Somatic coliphages and F-specific RNA bacteriophages can be used as indicators of general faecal contamination, whereas some selected host strains of *Bacteroides* can be used for MST since they preferably detect phages present in a given faecal source, as for example human waste water and excreta. As well, the proportions of genotypes of F-specific RNA phages in a given sample can be used for MST.

Feasible and cost effective protocols standardized by the International Standardization Organization and the United States Environmental Protection Agency for the detection of infectious bacteriophages belonging to the three groups are available. Molecular methods for some phages are also available, but realistic molecular methods detecting all the phages contained in an entire group do not exist. In addition, practical and quite feasible bacteriophage concentration methods for up to one litre of sample are on hand.

The literature shows many data on the concentrations of the three groups of bacteriophages detected by standardized methods in point sources of faecal pollution from different areas of the world. The most abundant group are somatic coliphages that roughly exceed the numbers of RNA bacteriophages by a logarithm in all types of faecal sources and by 1.5 to 2 logarithms the numbers of phages infecting *Bacteroides* when these are enumerated with a strain appropriated to the source of faecal contamination. In all sources, *E. coli* uses to outnumber somatic coliphages by less than one logarithm.

Data on phages' persistence in different types of waters

and resistance to waste water treatments are available, and allow outlining bacteriophages belonging to the three groups of phages from moderate to high persistence. Concentrations of the three groups of bacteriophages in contaminated aquatic and non-aquatic systems are also available and will be provided and discussed. The relative proportions between the numbers of *E. coli* and the three groups of bacteriophages change after tertiary treatments and after prolonged persistence in the environment, to the extent that the phages, mostly somatic coliphages outnumber *E. coli*.

In contrast, sound data on the meaning of enumerating bacteriophages to infer either the occurrence of intestinal pathogens, especially viruses, or the health risks for humans are scarce and frequently incomplete. The rather rare information available will be reported and discussed.

Bacteriophages as indicators of general and host-associated faecal pollution

1.0 General Characteristics of Bacteriophages (Phages)

Bacteriophages or viruses that infect bacteria are extremely abundant in nature, probably the most abundant life form on Earth. They outnumber bacteria in most studied habitats (Weinbauer, 2004), including human and animal -associated microbial communities (Letarov and Kulikov, 2009).

Phages which infect faecal bacteria are important virus indicators and viral surrogates for wastewater treatment efficacy. They are very useful in pollution assessment and control but like all phage carry on very specific activities in nature including in sewage and wastewater environments.

Phage infect and lyse bacteria thus they contribute to bacterial mortality, releasing organic compounds. They have an important impact on the cycling of organic matter in the biosphere (Suttle, 1994). On the other hand, they control microbial diversity by selecting for some types of bacteria that are resistant to their attack. As well, many phages can mobilize genetic material among different host bacteria in a process known as transduction. By transduction, genetic material can be introduced into a bacterium by a phage that has previously replicated in another bacterium, in which it packaged random DNA fragments (generalized transduction) or the DNA adjacent to the prophage attachment site (specialized transduction).

Phages can only replicate in metabolizing host cells. Once within a host bacterium, phages replicate by one of two ways, the lytic and the lysogenic cycles. In the lytic cycle, phages immediately after infection multiply, rapidly disrupting their host cells from within and then releasing the phage progeny that varies regularly between 10 and 1000 depending on the phage and the physiological status of the host cell. This process can be as short as 25-30 minutes in fast growing host bacteria. Phages that only

follow the lytic cycle are known as virulent phages. In contrast, temperate, or lysogenic, phages can follow the lysogenic cycle, in which the genome of the temperate phage remains in the host bacteria, replicating along with it. At this stage, the phage is known as a prophage, which can be induced to follow the lytic cycle. Induction occurs either spontaneously or when stimulated by natural or anthropogenic inductors.

Bacteriophages can only replicate inside susceptible host bacteria. A given phage can only infect certain bacteria to the point that different strains of the same species differ in their susceptibility to phage attack (Muniesa et al., 2003; Thingstad et al., 2014). Receptor molecules on the surface of the bacteria mainly determine the host-specificity of phages. Phage receptors have been described in different parts of bacteria (capsule, cell wall, flagella and pili). Phages attached to the receptors located in the cell wall are typically known as somatic phages. Most of the known phages infect a limited number of strains.

In their extracellular phase, a phage consists of a

genome of either RNA or DNA surrounded by a protein coat named capsid. These particles are known as virions. Many phages also contain additional structures such as tails and spikes. Much less frequently, they contain lipids. In addition, they display a range of nucleic acid structures consisting of either double stranded (ds) or single stranded (ss) RNA or DNA, but never both. Phages may readily be grouped on the basis of a few gross characteristics including host range, morphology, nucleic acid, strategies of infection, morphogenesis, phylogeny, serology, sensitivity to physical and chemical agents, and dependence on properties of hosts and environment. The present classification adopted by the International Committee on Taxonomy of Viruses is mostly based in phage morphology and characteristics of the nucleic acid (Fauquet and Fargette, 2005). Phages of particular interest in water quality assessment are included into the seven families whose characteristics are summarized in Figure 1.

Figure 1. Most common morphological types in somatic coliphages and F-specific phages. Bar 50 nm.

Owing to their simple structure and composition, virions persist quite successfully in the environment and are moderately resistant to natural and anthropogenic stressors (Grabow, 2001). It is likely that phages infecting bacteria indigenous to a given habitat are less persistent than their bacterial host (Ogunseitan et al., 1990), but also that phages survive better than host bacteria in habitats where they are aliens (Grabow, 2001). This will be the case of phages infecting enteric bacteria once outside the gut.

Typically, infectious phages are detected by their effects, mostly lysis, on the host bacteria that they infect. The most important factor in defining a method for the detection of a given phage or group of phages is the bacterial host strain. Easy methods to detect their presence or absence in a given volume of sample are available.

Phages are counted by direct quantitative plaque assays (Adams, 1959) (Figure 2). The plaque assay provides the results in plaque forming units (PFU). A plaque forming unit is an entity, usually a single virion but also e.g. a clump of virions that originates a single plaque. PFU are also denominated for example in the ISO standards, plaque forming particles (pfp). The presence of phages in a given volume of sample can also be determined by the qualitative presence-absence enrichment test (Adams, 1959) (Figure 3). Enrichment of multiple tube serial dilutions allows estimating numbers of phages by “quantal” methods, as for example the most probable number procedure.

Figure 2. Bacteriophage enumeration by double agar layer method

Figure 3. Detection of bacteriophage by the presence/absence method

Phages are used as faecal indicators to mimic enteric viruses better than any other group of indicators, and show moderate resistance and persistence in the water environment and through wastewater treatment (Armon and Kott, 1996; Grabow, 2001; IAWPRC Study group on health related water microbiology, 1991; Jofre, 2007). This

is one of the reasons why different groups of phages infecting enteric bacteria have been proposed as either faecal or viral indicators. Phages used as indicators for faecal pollution and microbial source tracking, indicators as surrogates for pathogens (index) and indicators of public health risk have been assembled according to the detection

methods. Coliphage are those viruses that infect the bacteria *E. coli* and somatic coliphages (IAWPRC Study group on health related water microbiology, 1991; Kott et al., 1974), F-specific RNA phages (Havelaar et al., 1990; IAWPRC Study group on health related water microbiology, 1991), and phages infecting *Bacteroides* (IAWPRC Study group on health related water microbiology, 1991; Tartera and Jofre, 1987) are those extensively studied regarding their potential as indicators of various aspects of water quality control. Reviews with a wide coverage of this potential application of phages as indexes, indicators or markers exist (Armon and Kott, 1996; Grabow, 2001; IAWPRC Study group on health related water microbiology, 1991; Jofre, 2007).

Other than the fact that they mimic various aspects of virus behaviour, the appeal of phages as indicators lies in the availability of feasible, fast and cost effective detection methods, and their abundance in wastewaters of human and animal origin. Moreover, samples can be kept at 4°C for at least 48 hours without any significant change in the numbers of infectious phages (Mendez et al., 2002); and small volume samples can be kept for months at -20°C or -80°C after the addition of 10% v/v glycerol (Mooijman et al., 2005). Reference suspensions of phages needed for quality assurance are easily prepared and conserved (Mendez et al., 2002; Mooijman et al., 2005). Finally, phenomena such as *stress*, *injury* or *reactivation* that frequently lead to misinterpretation of environmental data on bacterial indicators are not applicable to phages.

1.1 Somatic Coliphages

The term somatic coliphages defines the phages infecting *Escherichia coli* through the cell wall. Most known somatic coliphages found in municipal wastewater belong to the *Myoviridae*, *Siphoviridae*, *Podoviridae* and *Microviridae* families (Muniesa et al., 1999; Rajala-Mustonen and Heinonen-Tanski, 1994). Somatic coliphages most frequently used as model organisms are ϕ X174, PDR1, T2 and T7.

Somatic coliphages attach to the bacterial cell wall and may lyse the host cell in 25-30 min producing between 100 and 1000 phages per infected cell depending on both the phage and the physiological state of the cell. They produce plaques (Figure 2) of widely different size and morphology.

Host strains of somatic coliphages include *Escherichia coli* and related species such as *Shigella* spp and *Klebsiella* spp. Some of these may occur in pristine waters, so exceptionally somatic coliphages can find hosts in these environments. Several sorts of studies have shown many factors limit somatic coliphage replication in water environments. 1) The rather narrow host range of phages infecting the host strains used in the standardized methods (Muniesa et al., 2003). 2) The high densities of host bacteria and phages needed to ensure phage replication (Muniesa and Jofre, 2004; Wiggins and Alexander, 1985). 3) The presence in water of numerous background bacterial flora and particulate material that interferes with coliphage replication (Muniesa and Jofre, 2004; Wiggins and Alexander, 1985). 4) The metabolic activity of the host

needed for phage replication is too low in the water environment (Cornax et al., 1991). 5) The envelope stress response that affects *E. coli* when released into the environment that could trigger responses diminishing phage infection (Perez-Rodriguez et al., 2011; Raivio, 2011). Moreover, the contribution of lysogenic induction to the presence of free coliphages is not noticeable. Hernandez-Delgado and Toranzos (Hernández-Delgado and Toranzos, 1995) have shown that neither sewage isolates nor laboratory phage strains replicated in pristine river water in a tropical area. These and other data reviewed (Jofre, 2009) strongly suggest that the contribution of somatic coliphages replicated outside the gut to the numbers of somatic coliphages detected in water environments is negligible.

Different host strains of *E. coli* as well as different assay media count different numbers and types of somatic coliphages (Havelaar et al., 1990; Muniesa et al., 1999). Consequently, from now on in this chapter preferential attention is given to data obtained with the standardized methods indicated.

1.2 F-specific and F-specific RNA Bacteriophages

F-specific bacteriophages, also termed sexual coliphages or male-specific phages infect bacteria through the sex pili, which are coded by the F plasmid first detected in *E. coli* K12. F-specific RNA phages, a subgroup of F-specific phages consist of a simple capsid of cubic symmetry of 21-30 nm in diameter and contain a single-stranded RNA as the genome. The F-specific RNA phages group (*Leviviridae*) contains two genera (*Levivirus* and *Allolevirus*) and three minor unclassified groups (Fauquet and Fargette, 2005). *Levivirus* contains subgroups I and II, whereas *Allolevirus* contains subgroups III and IV. These four groups coincide with the serotypes first described by Furuse (Furuse, 1987). Posterior genomic characterization has allowed establishing that genogroups match with the serotypes (Beekwilder et al., 1996; Hsu et al., 1995), at least for practical purposes. Other F-specific phages are the rod-shaped DNA phages of the family *Inoviridae* (Fauquet and Fargette, 2005).

F-specific RNA phages used as model are MS2 and f2 belonging to genotype I, GA to genotype II, Q β to genotype III and F1 to genotype IV. Sero and genogrouping of RNA F-specific phages has been used to differentiate subgroups in waters receiving faecal wastes, and the study of subgroups distribution may contribute to source tracking of faecal pollution. This is because groups I and IV are the predominant in waters contaminated with animal faecal residues whereas groups II and III are mostly associated with human pollution (Jofre et al., 2011). Long and collaborators have also studied whether typing of F-specific DNA phages could be useful for microbial source tracking (MST), but results seem to indicate that they are not (Long et al., 2005).

The F plasmid is transferable to a wide range of Gram-negative bacteria and so F-specific phages may have several hosts. Yet, it is thought that their main natural host is *E. coli*. The sexual pili are not synthesized below 32°C. In

spite of the temperature conditioned production of sexual pili, necessary for phage replication, conflicting reports on replication of F-specific phages in wastewater and groundwater exist (Havelaar and Pot-Hogbeem, 1988). Nevertheless, Woody and Cliver reviewed conditions affecting F-specific RNA phage replication other than pili formation in the host bacteria, and concluded that replication outside the gut cannot be excluded but also that it is very improbable (Woody and Cliver, 1997). Therefore, the influence of replication outside the gut in the numbers of these phages in the environment can be considered as negligible.

The infectious process of F-specific RNA phages is inhibited by the presence of RNase in the assay medium, which can be used to distinguish between the F-specific RNA phages and the other phages than can infect the host bacteria. These are the rod-shaped F-specific DNA phages of the family *Inoviridae*, which also infect the host cell through the sex pili as well as the somatic phages that can infect the bacterial host used.

1.3 Bacteriophages Infecting *Bacteroides*

Bacteriophages infecting strains of several species of *Bacteroides* have been detected in faeces and wastewater contaminated with faecal wastes (Jofre et al., 2014). Replication of phages infecting *Bacteroides* outside of the gut of warm-blooded animals is even more unlikely than in the case of the coliphages referred to in previous sections. This is due to the additional stringent requirements of the host strain regarding anaerobiosis and nutrients that are unlikely to occur and coincide in natural water environments (Tartera and Jofre, 1987).

Phages infecting different *Bacteroides* species

described so far are all tailed and the vast majority of them have the morphology corresponding to the family *Siphoviridae* (McLaughlin and Rose, 2006; Queralt et al., 2003; Tartera and Jofre, 1987). As well, the genome sequence of the few *Bacteroides* infecting phages studied corresponds to that of *Siphoviridae* (Hawkins et al., 2008; Ogilvie et al., 2013).

Phages infecting *Bacteroides* seem to infect the host through the cell wall and therefore are somatic phages (Puig et al., 2001) yet most phages infecting *Bacteroides* have a quite narrow host range (Cooper et al., 1984; Kory and Booth, 1986; Tartera and Jofre, 1987).

In addition to the narrow host range of phages, strains of *Bacteroides* spp. differ in their ability to recover phages in faecal material, wastewaters and wastewater contaminated waters (Payán et al., 2005; Puig et al., 1999). *Bacteroides* strains differ also in their capability to detect phages in the faeces of different animal species, including humans, and hence in their ability to determine the origin of faecal contamination in a given sample. Some strains of *B. fragilis*, such as RYC2056 and VPI3625, detect phages both in human and non-human faecal wastes (Blanch et al., 2006; Kator and Rhodes, 1992; Puig et al., 1999). Others are able to discern the faecal source. Those detecting higher numbers of phages are reported in Table 1. However, this source specificity is not absolute and though seldom, host strains reported in Table 1 detect very low numbers of phages in the non-corresponding sources. The method proposed by Payán et al. (2005) allows isolating hosts appropriate for a given host and a given geographical area with reasonable success. The total cost associated with each attempt to isolate new strains did not exceed 1,000 euros including consumables and labour (approximately 1 month).

Table 1. Host strains of *Bacteroides* discerning faecal sources and country of isolation

Area	Host strain	Sample Type	Source of origin	Percent Positive	Concentration Average (range) PFU/100 mL at source ^a	Reference
Colombia	<i>B. fragilis</i> HB13	Municipal wastewater	Human	100%	1E+04 to 1E+05	Payan et al., 2005
Colombia	<i>B. fragilis</i> CA8	Municipal wastewater	Human	100%	1E+02 to 1E+04	Venegas et al., 2015 ^b
Spain	<i>B. fragilis</i> HSP40	Clinical sample	Human	100%	1E+03 to 1E+04	Tartera and Jofre, 1987
Spain	<i>B. thetaiotaomicron</i> GA17	Municipal wastewater	Human	100%	1E+04 to 1E+5.5	Payan et al., 2005
Spain	<i>B. fragilis</i> PG76	Swine slaughterhouse	Pig	100%	1E+05 to 1E+06	Gómez-Doñate et al., 2011 ^b
Spain	<i>B. fragilis</i> PG1226	Swine slaughterhouse	Pigs	100%	1E+05 to 1E+06	Gómez-Doñate et al., 2011 ^b
Spain	<i>B. thetaiotaomicron</i> CW18	Cattle slaughterhouse	Cows	70%	1E+03 to 1E+04	Gómez-Doñate et al., 2011 ^b

Area	Host strain	Sample Type	Source of origin	Percent Positive	Concentration Average (range) PFU/100 mL at source ^a	Reference
Spain	<i>B. fragilis</i> PL122	Poultry slaughterhouse	Poultry	82%	1E+03 to 1E+04	Gómez-Doñate et al., 2011 ^b
Switzerland	<i>B. fragilis</i> ARABA 84	Municipal wastewater	Human	100%	1E+03 to 1E+04	Wicki et al., 2011 ^b
Switzerland	<i>B. fragilis</i> ARABA KBA60	Slaughterhouse	Cattle and horses	67%	1E+02	Wicki et al., 2011 ^b
United Kingdom (UK)	<i>B. fragilis</i> GB124	Municipal wastewater	Human	100%	1E+04 to 1E+5.5	Payan et al., 2005

^aMunicipal wastewater or slaughterhouse (specific) wastewater; ^bIsolated according to the method described by Payán et al. (2005)

Strains RYC2056, GA17, GB124 and PG2166 were selected as those giving higher and more consistent counts in the matching wastewaters and are those that will be further discussed.

2.0 Detection and Concentration Methods

Generally phage are assayed without concentration directly with their respective host as plaque assay or in a

presence absence format.

2.1 Somatic Coliphages

Methods for the detection and enumeration of somatic coliphages have been standardized by ISO 10705-2 (Anonymous, 2000), USEPA 1601 and 1602 (EPA, 2001a; 2001b) and Standard Methods (Rice, 2012), (Table 2).

Table 2. Standardized methods for the detection of somatic coliphages

Target indicator	Host strain	Name of assay	Type of assay (results)	Expression of results	Time for results	Control and Reference materials	Standard method	Reference
Somatic coliphages ^{a,b,c}	<i>E. coli</i> WG5	Plaque assay (double agar layer- DAL)	Quantitative	Plaque forming units (PFU)/ ^a given volume	6 hours	Quality control included in the standard method. Not commercial reference materials, but easy to prepare	ISO 10705-2	Anonymous, 2000
Somatic coliphages	<i>E. coli</i> WG5	Presence/absence. Spot test after enrichment	Qualitative	Presence or absence in a given volume. Can be adapted to the MPN	24 hours	Quality control included in the standard method. Not commercial reference materials, but easy to prepare	ISO 10705-2	Anonymous, 2000
Somatic coliphages	<i>E. coli</i> CN13	Plaque assay (single agar layer-SAL)	Quantitative	Plaque forming units (PFU) per a given volume	6 hours	Not commercial reference materials, but easy to prepare	USEPA METHOD 1602	U.S. EPA, 2001b
Somatic coliphages	<i>E. coli</i> CN13	Presence/absence	Qualitative	Presence or absence in a given volume. Can be adapted to the MPN	24 hours	Not commercial reference materials, but easy to prepare	USEPA METHOD 1601	U.S. EPA, 2001a

Target indicator	Host strain	Name of assay	Type of assay (results)	Expression of results	Time for results	Control and Reference materials	Standard method	Reference
Somatic coliphages	<i>E. coli</i> C	Plaque assay (double agar layer- DAL)	Quantitative	Plaque forming units (PFU) per a given volume	6 hours	Not commercial reference materials, but easy to prepare	Standard Method 9224B	Green, 2000

^aDetection principle is replication of phages in host strain; ^bPre-treatment not needed for water; extraction from solids; ^cVolume tested adjustable

Other than some details on media and assay conditions, the three methods use *E. coli* C as host strain. These are either *E. coli* ATCC 13706 (Rice, 2012) or their nalidixic acid resistant clones *E. coli* CN13 (ATCC 700609) used in the USEPA 1601 and 1602 methods, or *E. coli* CN, more frequently referred as WG5, (ATCC 700078) used in the ISO 10705-2 method. Hosts resistant to nalidixic acid were introduced to minimize the growth of background bacteria that frequently interferes in the correct visualization of plaques in the plaque assay test. This allows quantifying phages in samples avoiding filtration of the sample to eliminate the background bacteria. Of course, filtration through 0.22 µm non-protein binding membrane filters such as those of polyvinylidene fluoride or polyether sulfone can also be applied to eliminate background bacteria present in the sample. In contrast, membrane filters of mixed cellulose and acetate membranes will adsorb and consequently retain phages. Strains ATCC13706, CN13 and WG5 detect similar numbers of phages in water matrixes when using the same method (Grabow et al., 1993; Guzmán et al., 2008). Other, non *C. E. coli* host strains count lesser numbers of somatic coliphages (Grabow et al., 1993; Havelaar and Hogeboom, 1983; Stetler, 1984). One of these strains used frequently (Rose et al., 2004) is *E. coli* C3000 (ATCC 15597) that is an Hfr strain derived from K12, which is used to count simultaneously somatic coliphages and F-specific phages. Strain *E. coli* CB390 was tailored to detect the same numbers of somatic coliphages as WG5 and as many F-specific phages as host strains used in standardized procedures (Guzmán et al., 2008) and it detects similar number of F-specific phages but more somatic coliphages than C3000.

ISO-10705-2 (Anonymous, 2000) includes both the double agar layer (DAL) plaque assay method for the quantification of PFU (Figure 2) and the presence/absence test (Figure 3) than can also be adapted to a more probable number format. A simplified version of these methods can be found in Havelaar and Hogeboom (Havelaar and Hogeboom, 1983). Mooijman et al. (2005) proved that the implementation of the ISO standard method is feasible in routine microbiology laboratories without previous experience in phages.

USEPA Method 1601 (U.S. EPA, 2001a) deals with the presence absence method and EPA method 1602 (U.S. EPA, 2001b) with the single agar layer (SAL) plaque assay. A

simplified version of these methods can be read in USEPA (U.S. EPA, 2001c). USEPA (U.S. EPA, 2003a, 2003b) has carried out interlaboratory validation tests of methods 1601 and 1602. A double agar layer (DAL) plaque assay method with strain C as the host is described in the 22nd edition of Standard Methods (Rice, 2012).

Results using these plaque assay methods can be obtained in 6 hours, though the habitual period of assay is 18 hours.

Detection and enumeration of somatic coliphages by the standardized methods is quite cost effective. The cost in material, media, reagents and labour is similar to the one for detection of faecal coliforms or *E. coli*. It can be done in routine microbiology laboratories. Additionally, the ISO procedure includes optional steps for laboratories with limited equipment.

Additionally to the host strains there are minor differences in the media between the standardized procedures. USEPA and ISO methods count similar numbers of somatic coliphages (Guzmán et al., 2008). As well, the present Standard Methods method is supposed to provide similar counts than ISO and USEPA. In contrast, the Standard Methods procedure described in previous editions has been reported to perform poorly (Green, 2000).

Regarding fast methods, no procedures based in serologic detection or PCR have been described that are applicable to the detection of the group somatic coliphages in waters. The diversity of phages included in this group makes this approach complex. In contrast, FAST PHAGE, which is applicable to the methods based on presence/absence of USEPA and ISO, has been described by Salter and Durbin (Salter and Durbin, 2012). This method is based on a rapid extracellular beta-galactosidase enzyme release and detection during the coliphage induced lysis.

Volumes of sample tested as described in the methods can be scaled up keeping the proportions of the mixtures of medium, host suspension and sample and using different size Petri dishes accordingly.

2.2 F-specific and F-specific RNA Bacteriophages

Host strains to detect sexual coliphages must produce sexual pili encoded by the F-plasmid. Hfr *E. coli* strains

such as C3000 (ATCC 15597) were firstly used for this purpose, but these strains also detect high numbers of somatic coliphages. Later, strains *Escherichia coli* HS(*E.coli* Famp, ATCC 700891)(Debartolomeis and Cabelli, 1991)and *Salmonella entericaserovar typhimurium* (most frequently reported as *Salmonella typhimurium*) WG49 (NCTC 12484)(Havelaar and Hogeboom, 1984)were tailored to detect mainly F-specific phages, though both still detect a very small proportion of somatic phages infecting either *E. coli* or *S. typhimurium*. Strains HS and WG49 were selected as host strains in the standardized methods mentioned later on. The phages detected by these strains are counted as sexual or F-specific coliphages. The number of F-specific RNA phages is the difference between the numbers of phages counted in the absence and in the presence of RNase in the assay medium. More than 90-95 % of the phages detected in sewage by strains HS and WG49 are F-specific RNA phages (Debartolomeis and Cabelli, 1991; Havelaar and Hogeboom, 1984), but this percentage may be lower in receiving waters and treated

wastewaters. Counts achieved by strains HS and WG49 are similar (Chung et al., 1996; Grabow et al., 1993). Because of the genetic markers (resistance to ampicillin and capacity to use lactose), the stability (and re-selection) of strain WG49 is easier to check than that of strain HS that has no explicit genetic markers.

Standardized methods for the detection and enumeration of both F-specific and F-specific RNA phages exist (Table 3). The ISO-10705-1 (Anonymous, 1995)standard method for the detection and enumeration of phages endorse *Salmonella typhimurium* WG49 as host strain and include a step with RNase in the assay medium. Consequently, it detects both F-specific and F-specific RNA phages. The ISO standard includes both the double layer plaque assay method and the presence-absence method. A simplified version can be found in Havelaar and Hogeboom (1984)and Standard Methods (Rice, 2012). Mooijman et al. (2005) proved that the implementation of the ISO standard method is feasible in routine microbiology laboratories without previous experience in phages.

Table 3. Methods for the detection of F-specific phages , F-specific RNA phages and subgroups of F-specific RNA phages

Target indicator	Host strain	Name of assay (type of results)	Detection principle	Pre-treatment	Expression of results	Time for results	Control and reference material	Standard method	Reference
F-specific and F-specific RNA phages ^{a,b,c}	<i>S. typhimurium</i> WG49	Plaque assay (double agar layer-DAL) (quantitative)	Replication of phages in host strain	Not needed for water; extraction from solids	Plaque forming units (PFU) per a given volume	18 hours	Quality control included in the standard method. Not commercial reference materials, but easy to prepare	ISO10705-1	Anonymous, 1995
F-specific phages	<i>S. typhimurium</i> WG49	Presence/absence (spot test after enrichment) (qualitative)	Replication of phages in host strain	Not needed for water; extraction from solids	Presence/absence in a given volume. Can be adapted to the MPN	24 hours	Quality control included in the standard method. Not commercial reference materials, but easy to prepare	ISO10705-1	Anonymous, 1995
F-specific phages	<i>E. coli</i> HS (Famp)	Plaque assay (single agar layer-SAL) (quantitative)	Replication of phages in host strain	Not needed for water; extraction from solids	Plaque forming units (PFU) per a given volume	18 hours	Quality control included in the standard method. Not commercial reference materials, but easy to prepare	USEPA1602	U.S. EPA, 2001b

Target indicator	Host strain	Name of assay (type of results)	Detection principle	Pre-treatment	Expression of results	Time for results	Control and reference material	Standard method	Reference
F-specific phages	<i>E. coli</i> HS (Famp)	Presence/ absence (spot test after enrichment) (qualitative)	Replication of phages in host strain	Not needed for water; extraction from solids	Plaque forming units (PFU) per a given volume	24 hours	Quality control included in the standard method. Not commercial reference materials, but easy to prepare	USEPA 1601	U.S. EPA, 2001a
F-specific RNA phages subgroups (genotyping)	<i>S. typhimurium</i> WG49 or <i>E. coli</i> HS (Famp)	Plaque (obtained by either DAL or SAL) hybridization (quantitative)	Replication of phages in host strain followed by plaque hybridization by specific probes for each subgroup	Not needed for water; extraction from solids	Percentages of plaques hybridizing with each probe	48 hours	No reference material available. But control with model phages are easy to prepare	No standard method available	(Anonymous, 1995; U.S. EPA, 2001b) for plaque assay and (Schaper and Jofre, 2000) for plaque hybridization
F-specific RNA phages subgroups (genotyping)	<i>S. typhimurium</i> WG49 or <i>E. coli</i> HS (Famp)	Plaque (obtained by either DAL or SAL) genotyping by RT-PCR (quantitative)	Replication of phages in host strain followed by qRT-PCR of phages in the plaques	Not needed for water; extraction from solids	Percentages of plaques that react to pairs of primers corresponding to each subgroup	26-28 hours	No reference material available. But control with model phages are easy to prepare	No standard method available	(Anonymous, 1995; U.S. EPA, 2001b) for plaque assay and (Kirs and Smith, 2007; Ogorzaly and Gantzer, 2006) for qRT-PCR
F-specific RNA phages subgroups (serotyping)	<i>S. typhimurium</i> WG49 or <i>E. coli</i> HS (Famp)	Culture latex agglutination and typing. Combines a two-step enrichment process (presence/absence) and latex agglutination serotyping (quantitative or qualitative)	Replication of phages in host strain followed by agglutination with specific antibodies	Not needed for water; extraction from solids	Percentages of plaques agglutinating with each antibody	5-24 hours	No reference material available. But control with model phages are easy to prepare	No standard method available	(Anonymous, 1995; U.S. EPA, 2001b) and for enrichment; (Love and Sobsey, 2007)
F-specific RNA phages subgroups (genotyping)	Not needed	Multiplex qRT-PCR (qualitative)	Amplification of fragments of genome present in the sample	Concentration from water; extraction from solids	Genome copies in the sample	3-4 hours	No reference material available. But control with model phages are easy to prepare	No standard method available	Wolf et al., 2010

^a Detection principle is replication of phages in host strain; ^b Pretreatment not needed for water; extraction from solids; ^c Volume tested adjustable

EPA Method 1601 (U.S. EPA, 2001a) standard deals with the presence-absence method and EPA method 1602 (U.S. EPA, 2001b) with the single layer plaque assay (SAL) for the detection of F-specific phages. These methods use *E. coli* Famp as host strain. A simplified version can be found in USEPA (U.S. EPA, 2001c) and Standard Methods (Rice, 2012). USEPA (U.S. EPA, 2003a, 2003b) has carried out interlaboratory validation tests on methods 1601 and 1602.

Results using both plaque assay methods can be

obtained in 18 hours.

The cost in material, media and reagents and labour for the detection of F-specific coliphages is similar to that for the detection of somatic coliphages. The cost for the detection of F-specific RNA phages is 10-15 % higher because of the need of RNase and the double number of plates. It can be done in routine microbiology laboratories.

Using any one of the methods, volumes tested as

described in the basic standardized methods can be scaled up keeping the proportions of the mixtures of assay media and sample.

Molecular methods are available. However, they are for a specific phage or for a subgroup, not for the full group. The sum of genome copies (GC) obtained by RT-qPCR (Reverse transcription-quantitative polymerase chain reaction) of the different subgroups accounts for all F-specific RNA phages. These methods are referred to later on in the section on F-specific RNA phages for microbial source tracking section.

Different procedures for detecting F-RNA phage subgroups are available, some based on serological recognition and others based on nucleic acid sequence recognition (Table 3).

Among the first group of methods, there are the "neutralization" method first described (Furuse, 1987) and the culture latex agglutination and typing test (Love and Sobsey, 2007). The latex agglutination method is fast and can be applied *in situ*, though it may require pre-enrichment. However, nowadays accessibility of specific antisera is less practical than the availability of nucleic acid probes and primers.

Methods based either on plaque hybridization with specific probes and those based on RT-PCR seem more realistic. Nowadays RT-qPCR is commonly used.

Plaque hybridization is applied on plaques grown by either the ISO-10705-1 or the USEPA 1602 standards (Beekwilder et al., 1996; Hsu et al., 1995; Schaper and Jofre, 2000). This method implies the transfer of the plaques to four different N⁺ hybond membranes and posterior hybridization of each membrane with a probe specific for each one of the 4 subgroups. It has the advantage that many plaques can be studied at once.

Reverse transcription-polymerase chain reaction (RT-PCR) assays have been developed for MS2, the prototypical F-RNA phage of subgroup I (O'Connell et al., 2006), the different genogroups (Ogorzaly and Gantzer, 2006) and multiplex for all subgroups (Friedman et al., 2009; Kirs and Smith, 2007; Wolf et al., 2010). RT-PCR can be applied either to phages recovered from plaques obtained by the plaque assay method or directly (RT-qPCR) to determine the number of genome copies (GC) present in a given sample. The first approach allows apportioning the subgroups of the infectious phages, whereas the second does not provide insight in the infectiousness of the phages, but results can be obtained within a few hours.

The actual concentrations of phages belonging to each one of the subgroups in a given sample can be estimated by applying the percentages corresponding to each group to the concentration of F-specific RNA phages.

In contrast, direct RT-qPCR can provide directly the real numbers of GC. The numbers of GC usually exceed those of infectious phages determined by plaque assay. This difference is actually observed in faeces, and even varies from sample to sample (Hartard et al., 2015). In raw human

and animal wastewaters, the GC numbers exceed the values of infectious phages by between 1.5 and 2.6 log₁₀ units. However, this is not always the case, probably because of poor efficiency of the RT qPCR applied on certain types of samples or because of aggregation-disaggregation of phage particles. Thus, (Hata et al., 2013) detected more PFUs than GC in the influent of a wastewater treatment plant, but detected more GC in the effluent. Additionally, the difference in GC and PFUs will likely increase after inactivation in water environments and after water treatment, since it is well known that phage GC signals are more persistent in nature and more resistant to treatments than the plaque assay measuring infectious viruses.

2.3 Bacteriophages Infecting *Bacteroides*

The method used for the *Bacteroides* phages, namely ISO-10705-4 (Anonymous, 2001), includes both the double agar layer (DAL) plaque assay method for the quantification of PFU and the presence/absence test. A simplified version of these methods can be read in Araujo et al. (2001). These methods are similar to those described for coliphages, the only difference being that *Bacteroides* has to be grown under anaerobic conditions and that incubation times are longer. However, anaerobic jars and sachets are appropriate for cultures in Petri dishes and screw capped tubes completely filled with culture medium are enough for liquid cultures. Manipulation can be done on the open bench. The ISO methods are applicable to all the host strains referred to earlier. Mooijman et al. (2005) proved that the implementation of the ISO standard method is feasible in routine microbiology laboratories without previous experience with phages.

The cost in material, media, reagents and labour for the detection of phages infecting *Bacteroides* is similar to that for detection of somatic coliphages with an additional 10-15 % for anaerobic conditions. It can be done in routine microbiology laboratories. Additionally, the ISO procedure includes optional steps for laboratories with limited equipment.

2.4 Methods for Concentration

The presence/absence method allows testing of relatively large volumes (up to one litre) (Grabow, 2001), however concentration may be required either because greater volumes need to be tested or because quantification is required. Most methods described for concentrating animal viruses are not adequate for concentrating phages (Grabow, 2001). For volumes ranging from 10 to 1000 mL, two methods are recommended. For water with low turbidity, Sobsey et al. (1990) developed a simple, inexpensive and practical procedure for the recovery and detection of F-specific phages using mixed cellulose and acetate membrane filters with a diameter of 47 mm and a pore size of 0.45 µm after addition of salts and pH adjustment. This method was slightly modified by Mendez et al. (2004b) showing an excellent performance for up to 1 litre of sample for concentrating somatic coliphages, F-specific RNA phages and phages infecting *Bacteroides*. For samples with high turbidity, flocculation with magnesium hydroxide (Schulze and Lenk, 1983) is practicable for the

three groups of phages (Contreras-Coll et al., 2002). Phage can also be concentrated by ultrafiltration as mentioned for other viruses.

3.1 Faeces

3.1.1 Somatic coliphages

Somatic coliphages have been isolated in variable percentages of human and animal stool samples (Table 4).

3.0 Data on Occurrence in the Environment

Table 4. Presence and concentrations of somatic coliphages in faeces

Area	Host	Number of species ^a	Method	Extraction procedure	Amount of sample ^b	Detection limit	Percent Positive (Range) ^c [numbers of samples] ^d	Concentration Average (range) ^e PFU/g	Reference
France	Human	1	ISO-DAL	Suspension in peptone saline	1 g	1/g	68% [193]	4.3E+01 (<1 to 7.3E+05)	Gantzer et al., 2002
Holland	Human	1	ISO-like DAL	Suspension in peptone saline	1 g	10/g	80% [10]	6.1E+04	Havelaar et al., 1986
Holland	Animal	7	ISO-like DAL	Suspension in peptone saline	1 g	10/g	100% (100 to 100) [70]	4.1E+04 to 2.2E+07	Havelaar et al., 1986
Korea	Human	1	USEPA-SAL	Suspension in phosphate buffered saline (PBS)	5 g	1/g	91% [11]	3.0E+02 (<1 to 3.8E+04)	Lee et al., 2009
Korea	Animal	4	USEPA-SAL	Suspension in PBS	5 g	1/g	39.7% (25 to 63) [29]	1.0E+01 to 1.4E+03 (<1 to 1.0E+04)	Lee et al., 2009
South Africa	Human	1	ISO- P/A	Direct to enrichment	1 g	1/g	54% [90]	Not reported	Grabow et al., 1993
South Africa	Animal	16	ISO- P/A	Direct to enrichment	1 g	1/g	38% (38 to 100) [155]	Not reported	Grabow et al., 1993
Spain	Human	1	ISO-DAL	Suspension in PBS	1 g	1/g	49% [100]	1.0E+05 (< 1 to 1.7E+07)	Martinez-Castillo et al., 2013
Spain	Animal (Cattle)	1	ISO-DAL	Suspension in PBS	1 g	1/g	100% [28]	8.7E+06 (6.0E+04 to 3.1E+07)	Colomer-Lluch et al., 2011
Switzerland	Human	1	ISO-DAL	Suspension in peptone saline	50 g	10/g	73% [55]	2.5E+02 (<10 to 6.8E+04)	Diston and Wicki, 2015
Switzerland	Animal	7	ISO-DAL	Suspension in peptone saline	50 gb1	10/g	82% (82 to 100) [46]	1.0E+01 to 2.0E+07 (<10 to 1.0E+08)	Diston and Wicki, 2015
USA	Animal	12	USEPA-SAL	Suspension in water	1 g	1/g	Isolation in 6/2 species [260]	<1 to 6.0E+04	McMinn et al., 2014

^a Number of species tested; ^b From a single stool, ^{b1} composite samples; ^c Range of positive samples in the different species; ^d Sum of samples corresponding to the different species tested; ^e Range of average values corresponding to the different species

Reported percentages of positive human specimens range from 54 to 91%, whereas those of animal samples range from <1 to 100 %. The variability is such that some publications report the isolation of somatic coliphages in all animal species tested and in 100% of the samples tested (Havelaar et al., 1986) whereas other authors failed to isolate somatic coliphages from any sample of 50 % of species analysed (McMinn et al., 2014). As well, the concentrations reported are very variable and range from <1 to 7.3×10^5 /gram in humans and from <1 to 10^8 in animals. Animal species studied (Table 4) include farm, domestic and wild species more likely contributing to faecal contamination of the environment.

These percentages and concentrations are very likely an underestimation. Such supposition is grounded on the facts that stool is a very complex matrix and no methods have been settled for defining the amounts of sample to be analysed or the need of applying extraction procedures. Regarding the amount of sample to be tested, if, as expected, coliphages replicate as lytic phages in the gut following the *kill the winner model* of phage bacteria population dynamics (Rodriguez-Valera et al., 2009), the number of phages will probably vary in form of waves of abundance in the gut content moving towards the cecum. This model involves a phage-bacteria population dynamics in which an increase in a host population (the winner) is followed by an increase in the rate at which that winner is

killed by phages with the corresponding increase in the phage population. In fact, in measuring phages infecting *Bacteroides*, Tartera and Jofre (Tartera and Jofre, 1987) tested different stool specimens of the same individual and detected values ranging from <1 to $>2.4 \times 10^8$ infectious phages/gram; very likely composite samples will provide a better view of the presence and concentrations of phages in the stool samples. Additionally, Jones and Johns (Jones and Johns, 2009) reported for F-specific phages that increasing the amount of sample increased the percentage of samples from which phages were isolated as well as extraction and polyethylene glycol precipitation improved the detection.

3.1.2 F-specific and F-specific RNA bacteriophages

F-specific RNA phages have been isolated in variable percentages of human and animal stool samples (Table 5). The same consideration as for somatic coliphages regarding the great variability of results reported apply for F-specific RNA phages. Reported percentages of positive human specimens range from 6 to 73%, whereas those of animal samples of different species range from 0 to 100 %. Also, the concentrations reported are very variable and range from <1 to 1×10^4 PFU/gram in humans and from <1 to $>1.2 \times 10^6$ in animals. Animal species studied (Table 5) include farm, domestic and wild species that most likely contribute to faecal contamination of the water environment.

Table 5. Presence and concentrations of F-specific and F-specific RNA phages in faeces

Area	Host	Number of species ^a	Method	Extraction procedure	Amount of sample ^b	Detection limit	Percent Positive (Range) ^c [number of samples] ^d	Concentration Average (range) ^e PFU/g	Reference
Holland	Human	1	ISO-DAL	Suspension in peptone saline	1g	10/g	10% [10]	<1	Havelaar et al., 1986
Holland	Animal	7	ISO-DAL	Suspension in peptone saline	1g	10/g	0% (0 to 100) [70]	<10 to >1.2E+06d	Havelaar et al., 1986
Korea	Human	1	USEPA-SAL	Suspension in PBS	5 g	1/g	73% [11]	5 (<1 to 2.8E+02)	Lee et al., 2009
Korea	Animal	4	USEPA-SAL	Suspension in PBS	5 g	1/g	25% (25 to 100) [29]	1 to 5 (<1 to 2.0E+02)	Lee et al., 2009
South Africa	Human	1	ISO- P/A	Direct to enrichment	1g	1/g	26% [90]	-	Grabow et al., 1993
South Africa	Animal	16	ISO- P/A	Direct to enrichment	1g	1/g	18.4% (20 to 84) [155]	-	Grabow et al., 1993
USA	Human	1	USEPA-SAL	Suspension in tryptone broth	1g	1/g	8% [13]	< 1	Calci et al., 1998
USA	Animal	12	USEPA-SAL	Suspension in tryptone broth	1 g	1/g	0% (0 to 25) [1081]	1.5 to 1.1E+04 ^d	Calci et al., 1998

^a Number of species tested; ^b from a single stool sample; ^cRange of positive samples in the different species; ^dSum of samples corresponding to the different species tested; ^e Range of average values corresponding to the different species

The comments regarding the potential underestimation of the values made for somatic coliphages are applicable to F-specific RNA phages.

Several reports on the distribution of subgroups in human and animal faeces are available. All reports indicate that subgroups II and III are generally associated with human faecal material, whereas subgroups I and IV make up the majority in animal faecal wastes. This distribution is observed whatever the method used. Nevertheless, this association is not always 100 % exact. Thus, Havelaar et al. (1990) have detected subgroup II in pig faeces; Schaper et al. (Schaper et al., 2002b) subgroup II in faeces of pigs, cattle and poultry; Hsu et al. (1995) subgroup II in pig faeces; Hartard et al. (2015) subgroup II in faeces of ducks and geese; and Cole and Sobsey (2003) subgroups II and III in faeces of cows.

3.1.3 Bacteriophages infecting *Bacteroides*

Phages infecting different strains of *Bacteroides* have been isolated in variable percentages of human and animal stool samples (Table 6). In this case, the host strain introduces the greater factor of variability since as indicated earlier *Bacteroides* strains differ in their capability to detect phages in different faecal sources. *Bacteroides fragilis* RYC2056, has been reported to recover phages from 28% of human stool specimens, although it also recovers phages from animal faeces (Puig et al., 1999), with the maximum incidence, 30%, in pigs. *Bacteroides fragilis* HSP40 phages have been isolated from 10-13 % of human stool samples, but never from animal faeces, with values ranging from >1 to 1.2×10^4 PFU/gram (Gantzer et al., 2002; Grabow et al., 1995; Tartera and Jofre, 1987). GB-124 has been reported in 4% of human samples and never in animal samples (Diston and Wicki, 2015; McMinn et al., 2014).

Table 6. Presence and concentrations of phages infecting different host strains of *Bacteroides* in faeces

Area	Host	Number of species ^a	Detection Method	Extraction procedure	Amount of sample ^b	Detection limit	Percent Positive (Range) ^d [number of samples] ^c	Concentration Average (range) ^d PFU/g	Reference
France	Human	1	ISO PFU (RYC2056)	Suspension in peptone saline	1 g	1/g	11.0% [193]	7.0E+01 (<1 to 0.2E+04)	Gantzer et al., 2002
Spain	Animal	5	ISO P/A (RYC2056)	Suspension in peptone saline	1g ^{b1}	1/g	0 to 31.0% [45]	-	Puig et al., 1999
Switzerland	Human	1	ISO PFU (GB124)	Suspension in peptone saline	50 g	5/g	4.0% [55]	5.5E+01 (<5 to 1.0E+02)	Diston and Wicki, 2015
Switzerland	Animal	7	ISO PFU (GB124)	Suspension in peptone saline	50 g	5/g	0.0% [46]	<5	Diston and Wicki, 2015
USA	Animal	12	ISO PFU (GB124)	Water	1g	1/g	0.0% [260]	<1	McMinn et al., 2014

^a Number of species tested; ^b Single stool, ^{b1} Composite sample; ^c Sum of samples corresponding to the different species tested; ^d Range of positive samples in the different species

3.2 Raw Wastewater

3.2.1 Somatic coliphages

Somatic coliphages are the most abundant indicator phages in raw municipal and hospital wastewater. Their

concentrations are usually less than one order of magnitude lower than those of faecal coliforms (or *E. coli*) wherever they have been counted (Table 7). Bacterial indicators and somatic coliphage concentrations in raw municipal wastewaters are comparable regardless of the geographical location and the income level of the country.

Table 7. Concentrations of somatic coliphages in untreated sewage from municipal wastewater (human faecal source)

Area	Host strain	Sample type/ Matrices	Detection Method	Number of samples	Percent Positive	Concentration Average (range) PFU/100 mL	Reference
Argentina	<i>E. coli</i> WG5	Raw sewage	ISO 10705-2	36	100%	6.0E+05 (1.0E+05 to 5.0E+06)	Lucena et al., 2004
Canada	<i>E. coli</i> C	Raw sewage	APHA previous 22nd edition	10	100%	2.9E+05 (1.0E+05 to 7.0E+05)	Zhang and Farahbakhsh, 2007
China	<i>E. coli</i> WG5	Raw sewage	ISO 10705-2	18	100%	2.0 E+06 (1.0 E+05 to 4.9 E+06)	Fu et al., 2010
Colombia	<i>E. coli</i> WG5	Raw sewage	ISO 10705-2	36	100%	6.0E+05 (1.3E+03 to 1.0E+07)	Lucena et al., 2004
European Countries (Spain, Holland, France, UK, Germany, Greece, Italy, Finland, Austria Ireland)	<i>E. coli</i> WG5	Raw sewage	ISO 10705-2	29	100%	Median 8.5E+06 (4.0E+04 to 3.0 E +07)	Contreras-Coll et al., 2002
European Countries (France, Spain, Sweden, UK)	<i>E. coli</i> WG5	Municipal sewage and hospital wastewater	ISO 10705-2	110	100%	1.0E+06 (1.0E+04 to 4.5E+07)	Blanch et al., 2006
Netherlands	<i>E. coli</i> WG5	Raw sewage	ISO 10705-2	5	100%	5.0E+05 (2.9E+05 to 7.3E+05)	Lodder and de Roda Husman, 2005
South Africa	<i>E. coli</i> WG5	Settled municipal wastewater	ISO 10705-2	24	100%	1.6E+06 (4.0E+05 to 7.1E+06)	Grabow et al., 1993
Tunisia	<i>E. coli</i> WG5	Raw sewage	ISO 10705-2	15	100%	Median 1.0E+06 (1.0E+05 to 1.5E +07)	Yahya et al., 2015

In Argentina, Lucena et al. (2003) studied samples of twenty-eight septic tanks and reported the detection of somatic coliphages in all samples tested with values ranging from 10^6 to 10^7 PFU/100 mL, which are similar to those reported for sewage in the area.

The reported values of somatic coliphages range from 10^4 - 10^5 PFU/100 mL in manures to 4×10^8 in abattoir wastewaters (Table 8). The proportion of these counts with counts of faecal coliforms and/or *E. coli* are similar to those found in municipal wastewater.

Table 8. Concentrations of somatic coliphages in untreated point sources of animal pollution

Area	Host strain	Sample type/ Matrices	Detection Method	Faecal Source	Number of samples	Percent Positive	Concentration Average (range) PFU/100 mL	Reference
Colombia (South America)	<i>E. coli</i> WG5	Slaughterhouse wastewater effluents	ISO 10705-2	Animal	5	100%	(4.0E+06 to 5.0E+07)	Venegas et al., 2015
Europe (France, UK, Spain, Sweden and Cyprus)	<i>E. coli</i> WG5	Slaughterhouse wastewater effluents and slurries	ISO 10705-2	Animal :Pigs, cows and poultry	57 wastewater + 59 slurries	100%	5.0E+06 (1.2E+02 to 4.0E+09)	Blanch et al., 2006
Holland	<i>E. coli</i> WG5	Slaughterhouse wastewater effluents	ISO 10705-2	Animal: Pigs and calves	19	100%	4.5E+06	Havelaar et al., 1986
New Zealand	<i>E. coli</i> NZRM 2718	Meat processing lagoon	APHA previous 22nd edition	Animal	25	100%	8.8E+03 (8.0E+02 to 6.3E+04)	Donnison and Ross, 1995
South Africa	<i>E. coli</i> WG5	Slaughterhouse process water	ISO 10705-2	Animal	12	100%	2.8E+06 (2.5E+04 to 3.3E+04)	Grabow et al., 1993
Tunisia (North Africa)	<i>E. coli</i> WG5	Slaughterhouse wastewater effluents	ISO 10705-2	Animal	6	100%	2.0E+06 (1.0E+04 to 1.7E+07)	Yahya et al., 2015
USA	<i>E. coli</i> CN13	Swine wastewater lagoon	USEPA 1602	Animal Swine	10	100%	2.5E+07	Hill and Sobsey, 1998

Importantly enough, at source of pollution, somatic coliphages do not show seasonal differences (Muniesa et al., 2012; Zhang and Farahbakhsh, 2007).

3.2.2 F-specific and F-specific RNA bacteriophages

F-specific phages and F-specific RNA phages are the second most abundant indicator phages in raw municipal and hospital wastewater with most frequently reported

values ranging from 10^4 to 10^6 PFU/100 mL. These values are usually between one and two orders of magnitude lower than the numbers of faecal coliforms (or *E. coli*) and somatic coliphages (Table 9). The numbers of F-specific and F-specific RNA phages in wastewaters are comparable wherever they have been determined regardless of the geographical location and the income (level of development) of the country (region/area). Numbers of F-specific phages in sewage do not show seasonal differences (Haramoto et al., 2015; Zhang and Farahbakhsh, 2007).

Table 9. Concentrations of F-specific and F-specific RNA phages in raw municipal sewage (Faecal Source Human)

Area	Host strains	Detection Method	Number of samples	Percent Positive	Concentration ^{a,b} Average (range) PFU/100 mL	Reference
Argentina	<i>S. typhimurium</i> WG49	ISO 10705-1	36	100%	7.0E+04 ^b (1.5E+03 to 1.2E+06)	Lucena et al., 2003
Canada	<i>E. coli</i> HS(pFamp)	Double agar layer method	10	100%	1.8E+05 ^a (5.0E+04 to 5.0E+05)	Zhang and Farahbakhsh, 2007
Colombia	<i>S. typhimurium</i> WG49	ISO 10705-1	38	100%	2.0E+05 ^b (1.0E+04 to 2.0E+06)	Lucena et al., 2003

Area	Host strains	Detection Method	Number of samples	Percent Positive	Concentration ^{a,b} Average (range) PFU/100 mL	Reference
European Countries (Spain, Holland, France, UK, Germany, Greece, Italy, Finland, Austria, Ireland)	<i>S. typhimurium</i> WG49	ISO 10705-1	29	100%	6.5E+05 ^b (1.0E+04 to 1.0E+06)	Contreras-Coll et al., 2002
European Countries (France, Spain, Sweden, UK)	<i>S. typhimurium</i> WG49	ISO 10705-1	110	100%	2.0 E+05 ^{b,d} (5.0E+01 to 9.0E+06)	Blanch et al., 2006
Japan	<i>S. typhimurium</i> WG49	ISO 10705-1	24	100%	6.0E+05 ^a	Hata et al., 2013
Netherlands	<i>S. typhimurium</i> WG49	ISO 10705-1	5	100%	3.2E+05 ^a (6.7E+04 to 8.4E+06)	Lodder and de Roda Husman, 2005
South Africa	<i>S. typhimurium</i> WG49	ISO 10705-1	24	100%	3.8E+05 ^{a,c} (7.0E+04 to 1.2E+06)	Grabow et al., 1993
Tunisia	<i>S. typhimurium</i> WG49	ISO 10705-1	15	100%	1.0E+05 ^b (1.0E+03 to 1.7E+06)	Yahya et al., 2015
USA	<i>E. coli</i> HS(pFamp)	USEPA1602	14	100%	5.2E+05 ^a (8.5E+04 to 8.7E+05)	Calci et al., 1998

^aF-specific phages; ^bF-specific RNA phages ^craw sewage after settling; ^dincluded hospital wastewater

Lucena et al. (2003) enumerated F-specific RNA phages by the ISO methods of twenty-eight septic tank samples in Argentina and reported the detection of F-specific RNA phages in all samples tested with values ranging from 10⁵ to 10⁶ PFU/100 mL, which are similar to those reported for sewage in the area. Calci et al. (1998) using the USEPA methods detected F-specific phages in 10 of 17 samples of

septic tank samples with values ranging from <10 to 10 PFU/100 mL.

Also, F-specific and F-specific RNA phages are detected in substantial concentrations in abattoir wastewater and animal slurries and manures. The reported values range from 10⁴PFU/100 mL in animal waste slurries to 2x10⁸ in abattoir wastewaters (Table 10).

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Table 10. Concentrations of F-specific and F-specific RNA phages in point sources of animal faecal pollution

Area	Host strain	Sample type/ Matrices	Detection Method	Number of samples	Percent Positive	Concentration ^{a,b} Average (range) PFU/ 100 mL	Reference
European Countries (Spain, Holland, France, UK, Germany, Greece, Italy, Finland, Austria, Ireland)	<i>S. typhimurium</i> WG49	Slaughterhouse wastewater effluents and slurries	ISO 10705-1	100	100%	6.0E+04 b (5.0E+01 to 2.6E+08)	Blanch et al., 2006
Holland	<i>S. typhimurium</i> WG49	Slaughterhouse wastewater	ISO 10705-1	19	100%	3.5E+06 ^{a,c}	Havelaar et al., 1990
New Zealand	<i>S. typhimurium</i> WG49	Meat processing Lagoon	ISO 10705-1	14	100%	4.8E+03 ^b (1.0E+03 to 5.1E+04)	Donnison and Ross, 1995
South Africa	<i>S. typhimurium</i> WG49	Slaughterhouse wastewater	ISO 10705-1	24	100%	1.6E+05 ^a (1.5E+03 to 4.9E+05)	Grabow et al., 1993
USA	<i>E. coli</i> HS (pFamp)	Raw swine wastewater	USEPA 1602	10	100%	6.4E+04 ^a	Hill and Sobsey, 1998

^a F-specific phages; ^b F-Specific RNA phages; ^c identified as pigs and calves

The subgroup percentages detected in raw wastewaters are reported in Table 11. As in the case of faeces, this distribution is not absolute. Most of the data reported in

table 11 refer to phages enumerated by either the USEPA or the ISO methods and subgrouping of the phages in plaques. Wolf et al. (2010) and Hata et al. (2013) detected GC by multiplex RT-q-PCR and RT-q-PCR.

Table 11. F-specific RNA bacteriophages subgroups distribution in different point sources of faecal contamination

Area	Sample type	Method	F-specific RNA phages (PFU/100 mL)	% Subgroups ^a				Reference
				I	II	III	IV	
Holland	Hospital raw wastewater	Plaques + serology	1.0E+06 to 1.0 E +07	1V	42	U	PM	Havelaar et al., 1990
Holland	Wastewater raw abattoir	Plaques + serology	1.0E +05	77	0	6	16	Havelaar et al., 1990
Japan	Municipal raw wastewater	Plaques + qRT-PCR	~1.6E+05	1	41	20	3	Haramoto et al., 2015
Japan	Secondary effluent (municipal)	Plaques + qRT-PCR	~3.0E+02	73	13	8	0	Haramoto et al., 2015
Japan	Municipal raw wastewater	RT-qPCR (GC)	~4.0E+05	10	50	40	-	Hata et al., 2013
Japan	Secondary effluent (municipal)	RT-qPCR (GC)	~1.0E+04	>70	-	<10	-	Hata et al., 2013
South Africa	Hospital raw wastewater	Plaques + plaque hybridization	1.0E+05	6	42	52	0	Schaper and Jofre, 2000
Spain	Municipal raw wastewater	Plaques + plaque hybridization	1.0E+05 to 1.0E+06	2.2	61.5	34.7	1.6	Schaper and Jofre, 2000
Spain	Secondary effluent (municipal)	Plaques + plaque hybridization	1.0E+04	54.3	43.3	1.7	0.7	Schaper and Jofre, 2000
USA	Municipal raw wastewater	Replication of phages in host strain followed by agglutination with specific antibodies (CLAT)	~5.0E+04	23	10	57	0	Brion et al., 2002
USA	Municipal raw wastewater	CLAT	~3.0E+05	3.8	53.8	12.9	3.8	Cole et al., 2003

^a Values indicate the percentage of samples where the subgroup was detected. If the percentages do not sum 100, it means that there were untyped plaques.

3.2.3 Bacteriophages infecting *Bacteroides*

In this case, the numbers detected depend on the host strains used since these differ in detection regarding the source of faecal contamination and there are also some geographical differences.

Concentration of phages detected by the non-discerning strain RYC2056 are relatively consistent everywhere around the world both in human and animal wastewaters with most frequent values ranging from 10^4 to 10^5 PFUs/100 mL. (Table 12).

Table 12. Concentrations of phages in raw sewage detected by *Bacteroides fragilis* strain RYC2056

Area	Sample type/ Matrices	Faecal Source / Host indicated	Number of samples	Percent positive	Concentration ^a Average (range) PFU/100 mL	Reference
Argentina, Colombia, France and Spain.	Municipal sewage	Human	104	100%	6.0E+04	Lucena et al., 2003
Colombia	Municipal sewage	Human	5	100%	1.2E+04	Venegas et al., 2015
Several European Countries (France, Spain, Sweden, UK)	Municipal and hospital sewage	Human	108	100%	1.0E+04 (5.0E+01 to 3.6E+05)	Blanch et al., 2006
Several European Countries (France, Spain, Sweden, UK)	Abattoir wastewater and slurries	Animal	110	100%	3.1E+03 (5.0E+01 to 1.0E+06)	Blanch et al., 2006
Thailand	Municipal sewage	Human	71	100%	(1.4E+02 to 1.4E+05)	Sirikanchana et al., 2014
Tunisia	Municipal sewage	Human	15	100%	Median E+04 (1.0E+02 to 1.7E+05)	Yahya et al., 2015
Tunisia	Abattoir wastewater	Animal	9	100%	Median 2.5E+04 (1.0E+02 to 3.0E+05)	Yahya et al., 2015

^a Method for all studies ISO 107 05-4 with host strain *B. fragilis* RYC2056

Lucena et al. (2003) detected strain RYC2056 phages in 77 % of samples from twenty-eight septic tank samples in Argentina with counts ranging from 10^2 to 10^3 PFU/100 mL. The lower numbers of *Bacteroides* strain RYC2056 phages compared to coliphages in the source of contamination, and the anaerobic conditions required for their cultivation, tend to discourage the use of RYC2056 phages as general

indicator, at least in temperate climates, despite attractive features.

Table 13 shows counts of phages in wastewater of human origin obtained by using *Bacteroides* host strain GA and GB124 which yielded the highest counts in assays on hosts specific for human *Bacteroides* phages.

Table 13. Concentrations of bacteriophages infecting *Bacteroides* host strains discerning the origin of contamination in raw wastewater

Area	Host strain	Sample type/ Matrices	Faecal Source/ Host indicated	Number of samples	Percent positive	Concentration Average (range) PFU/100 mL	Reference
Colombia	<i>B. thetaiotaomicron</i> GA17	Municipal wastewater	Human	5	100%	3.2E+04 (1.0E+04 to 5.9E+05)	Venegas et al., 2015
Colombia	<i>B. thetaiotaomicron</i> GA17	Abattoir wastewater	Animal	4	0%	<100 all samples	Venegas et al., 2015
European Countries (France, Spain, Sweden, UK)	<i>B. thetaiotaomicron</i> GA17	Municipal wastewater	Human	73	100%	1.5E+04 (5.0E+01 to 7.0E+05)	Blanch et al., 2006
European Countries (France, Spain, Sweden, UK)	<i>B. thetaiotaomicron</i> GA17	Abattoir wastewater and slurries	Animal	71	7%	5.5E+01 (<5.0E+01 to 1.0E+03) Only 7% of samples >50	Blanch et al., 2006
Spain	<i>B. thetaiotaomicron</i> GA17	Municipal wastewater	Human	43	100%	5.9E+04 (4.0E+03 to 3.5E+05)	Muniesa et al., 2012
Spain	<i>B. thetaiotaomicron</i> GA17	Abattoir wastewater	Animal	125	3.2%	(2.0E+01 to 2.0E+03) Only 3.2% of samples >50	Gómez-Doñate et al., 2011
Thailand	<i>B. thetaiotaomicron</i> GA17	Municipal wastewater	Human	71	0%	<19	Sirikanchana et al., 2014
Tunisia	<i>B. thetaiotaomicron</i> GA17	Municipal wastewater	Human	15	100%	1.0E+04 (1.0E+03 to 1.7 E+05)	Yahya et al., 2015
Tunisia	<i>B. thetaiotaomicron</i> GA17	Abattoir wastewater	Animal	6	16%	(<1 to 1.0E+03) Only 16% of samples >100	Yahya et al., 2015
UK	<i>B. fragilis</i> GB124	Animal slurries	Animal	30	0%	<100	Ebdon et al., 2007
USA	<i>B. fragilis</i> GB124	Primary sewage effluent	Human	5	100%	(2.0E+01 to 3.8E+02)	McMinn et al., 2014

Detection of phages by strains GA17, GB124 show a certain geographic discontinuity, which was previously appreciated for strain HSP40 (Kator and Rhodes, 1992; McLaughlin and Rose, 2006; Sirikanchana et al., 2014). In Southern Europe, Tunisia and Colombia, most phage concentrations detected by GA17 ranged between 5×10^4 and 5×10^5 PFUs/100 mL. McMinn et al. (2014) have reported values of phages detected by strain GB214 in primary sewage effluents studied in several states of USA with average values in some states well below 10^3 to some states with average values nearby 10^4 /100 mL.

As it happens with somatic and F-specific coliphages, the presence of phages infecting strains RYC2056 and GA17 in sewage shows no seasonality (Muniesa et al., 2012).

3.3 Treated Wastewater

Detailed information on phages as treatment indicators

as well as on removal during treatment will be given in *Using indicators to assess microbial treatment and disinfection efficacy* within this section and in chapters within the technology section. Here, data on phages found in different kinds of wastewater treatment effluents categorized as secondary are provided.

3.3.1 Somatic coliphages

Primary sedimentation, up flow anaerobic sludge blanket processes, flocculation-aided sedimentation, activated sludge digestion, activated sludge digestion plus precipitation and trickling filters remove bacterial indicators and somatic coliphages in numbers ranging from 0.3 to 3.0 log₁₀. Differences in the elimination of faecal indicator bacteria and somatic coliphages are minor and vary slightly from treatment to treatment. Therefore, the values of somatic coliphages in secondary effluents range mostly from 10^3 to 10^5 PFU/100 mL (Table 14).

Table 14. Concentrations of somatic coliphages in secondary activated sludge effluents (faecal source humans)

Area	Host strain	Detection Method	Number of samples	Percent positive	Concentration Average (range) PFU/100 mL	Reference
Canada	<i>E. coli</i> C	APHA previous 22nd edition	-	100%	(2.0E+02 to 1.0E+03)	Zhang and Farahbakhsh, 2007
China	<i>E. coli</i> WG5	ISO 10705-2	14	100%	9.4E+03 (1.0E+03 to 4.9E+04)	Fu et al., 2010
Netherlands	<i>E. coli</i> WG5	ISO 10705-2	5	100%	2.2E+04 (2.5E+04 to 1.1E+05)	Lodder and de Roda Husman, 2005
South Africa	<i>E. coli</i> WG5	ISO 10705-2	12	100%	2.9E+03 (7.0E+02 to 6.8E+04)	Grabow et al., 1993
Spain	<i>E. coli</i> WG5	ISO 10705-2	48	100%	1.6E+05	Costán-Longares et al., 2008
Spain	<i>E. coli</i> WG5	ISO 10705-2	8	100%	7.8E+05 (4.5E+02 to 2.3E+07)	Gomila et al., 2008
Tunisia	<i>E. coli</i> WG5	ISO 10705-2	15	100%	Median 6.0E+04 (1.2E+04 to 7.2E+04)	Yahya et al., 2015

Another point source is the effluents of waste stabilization ponds (or oxidation ponds, or lagoons) of very varied configuration and extent of treatment. Therefore, the effluents released after lagooning are quite diverse regarding the concentration of indicators (Alcalde et al., 2003; Campos et al., 2002; Gomila et al., 2008; Lucena et al., 2004). Moreover, counts of indicators in the effluents can vary between seasons in climates with marked seasonal variation.

DeBorde et al. (1998) studied the septic effluents of a high school in the USA and detected somatic coliphages in all 43 samples tested with values between 10^4 and 10^5 PFU/100 mL, though in this case the host strains used were not those recommended in the standardized methods.

3.3.2 F-specific and F-specific RNA bacteriophages

Primary sedimentation, up flow anaerobic sludge blanket processes, flocculation-aided sedimentation, activated sludge digestion, activated sludge digestion plus precipitation and trickling filters remove bacterial indicators, somatic coliphages, F-specific and F-specific RNA phages in numbers ranging from 0.3 to $3.0 \log_{10}$. The reported values of F-specific and F-specific RNA phages in secondary effluents range mostly from 10^2 to 10^4 PFU/100 mL (Table 15). The relative proportions of the F-specific and F-specific RNA phages with other indicators remain similar to those in untreated wastewater.

Table 15. Concentrations of F-specific and F-specific RNA phages in activated sludge secondary effluents (Faecal sources human)

Area	Indicator/Marker Name	Host strain	Detection Method	Numbers of samples	Percent positive	Concentration Average (range) PFU/100 mL	Reference
Canada	F-specific phages	<i>S. typhimurium</i> WG49	ISO 10705-1	10	100%	(5.0E+02 to 1.0E+03)	Zhang and Farahbakhsh, 2007
Japan	F-specific phages	<i>S. typhimurium</i> WG49	ISO 10705-1	24	100%	1.0E+04	Hata et al., 2013
Netherlands	F-specific phages	<i>S. typhimurium</i> WG49	ISO 10705-1	5	100%	4.8E+04 (5.3E+03 to 1.2E+05)	Lodder and de Roda Husman, 2005
South Africa	F specific phages	<i>S. typhimurium</i> WG49	ISO 10705-1	12	100%	1.6E+03 (1.0E+02 to 3.6E+03)	Grabow et al., 1993
Spain	F-specific RNA phages	<i>S. typhimurium</i> WG49	ISO 10705-1	8	84.2%	6.8E+04 (<100 to 1.5E+05)	Gomila et al., 2008
Spain	F-specific RNA phages	<i>S. typhimurium</i> WG49	ISO 10705-1	48	100%	1.2E+04	Costán-Longares et al., 2008
Tunisia	F-specific RNA phages	<i>S. typhimurium</i> WG49	ISO 10705	15	100%	Median 1.0E+04	Yahya et al., 2015
USA	F-specific phages	<i>E. coli</i> HS(pFamp)RR	USEPA 1602	14	79%	3.0E+04 (<100 to 2.1E+05)	Calci et al., 1998
USA (Hawaii)	F-specific RNA phages	<i>S. typhimurium</i> WG49	ISO 10705-1	4	NR	8.5E+03	Luther and Fujioka, 2004
NR - Not Reported							

As in the case of somatic coliphages, the concentrations of F-specific phages detected in effluents released by lagooning are quite variable. In climates with marked seasonal variation the numbers of pathogens and indicators in the effluents may vary between summer and winter in the same treatment facility (Alcalde et al., 2003; Gomila et al., 2008; Hill and Sobsey, 1998; Lucena et al., 2004).

DeBorde et al. (1998) studied the septic effluents of a high school (350 persons) in the USA and detected F-specific phages in all 43 samples tested with values ranging from 10^4 to 10^5 /100 mL, though this number might be an overestimation since the host strain used in this study, C3000, detects also somatic coliphages.

In secondary effluents of municipal activated sludge plants, there is a very significant increase in the percentage of subgroup I and the practical disappearance of subgroup IV (Table 11). This has been observed after either subgrouping of plaques or by direct determination of GC copies.

3.3.3 Bacteriophages infecting *Bacteroides*

Numbers of *Bacteroides* phages in secondary effluents range mostly from 5×10 to 10^4 PFU/100 mL (Table 16). The relative proportions of the *Bacteroides* phages with other indicators remain similar to those in untreated wastewater.

Table 16. Concentrations of bacteriophages infecting *Bacteroides* host strains discerning the origin of contamination in activated sludge secondary effluents (faecal source human)

Area	Host strain	Number of samples	Percent positive	Concentration ^a Average (range) PFU/100 mL	Reference
Spain	<i>B. thetaiotaomicron</i> GA17	33	100%	Median 3.0×10^3 (50 to 7×10^4)	Muniesa et al., 2012
Spain	<i>B. thetaiotaomicron</i> GA17	73	75.3%	4.1×10^3 (<50 to 3.6×10^5)	Gomila et al., 2008
Tunisia	<i>B. thetaiotaomicron</i> GA17	6b	0%	<100	Yahya et al., 2015
Tunisia	<i>B. thetaiotaomicron</i> GA17	15	100%	Median 2.9×10^2 (21 to 7.0×10^3)	Yahya et al., 2015
UK and Denmark	<i>B. fragilis</i> GB124	110	100%	8.5×10^3 (1.0×10^2 to 5.0×10^4)	Ebdon et al., 2007

^a Detection method ISO10705-4; ^b Slaughterhouse

As in the case of somatic coliphages, the numbers of *Bacteroides* phages in lagoon effluents are quite variable. In climates with marked seasonal variation the quality of the effluents depends on the season (Campos et al., 2002; Gomila et al., 2008; Lucena et al., 2004).

3.4 Untreated Sludge

3.4.1 Somatic coliphages

The numbers of reports about concentrations of somatic coliphages in untreated sludge (primary sludge, raw sludge (mix of primary and activated), activated sludge and

thickened sludge) are relatively scarce as compared to reports on phages in treated sludge. Most of the reported results are summarized in Table 17. Somatic coliphages were detected in all samples tested. Though concentrations in the different reports are difficult to compare since the concentrations reported in the scientific literature are referred to different measures such as dry mass, wet mass and volumes, and since different extraction procedures have been used, their concentrations are quite high in all reports. The highest concentrations are reported in primary sludge and the lowest in activated sludge. Numbers above 10^7 PFU/gram of dry weight have been reported for primary and raw sludge. Their counts in sludge remain in proportion to those of bacterial faecal indicators and other phages in raw wastewater.

Table 17. Concentrations of somatic coliphages in untreated sludge from a municipal wastewater treatment plant (faecal source human)

Area	Host strain	Detection Method	Number of samples	Percent positive	Concentration ^a Average (range)	Reference
Canada	<i>E. coli</i> C	APHA previous 22nd edition	10	100%	1.7E+06/100 g of wet matter	Chauret et al., 1999
Colombia	<i>E. coli</i> WG5	ISO10705-2	12	100%	1.5E+07/100 g of dry matter	Campos et al., 2013
Greece	<i>E. coli</i> WG5	ISO10705-2	40	100%	1.0E+07/100 g of wet matter	Mandilara et al., 2006
Spain	<i>E. coli</i> WG5	ISO10705-2	6	100%	3.5E+09/100 g of dry matter	Guzmán et al., 2007
Spain	<i>E. coli</i> WG5	ISO10705-2	10	100%	1.1E+07/100 g of wet matter	Lasobras et al., 1999
Spain	<i>E. coli</i> WG5	ISO10705-2	10	100%	1.1E+06/100 g of wet matter	Lasobras et al., 1999
Tunisia	<i>E. coli</i> WG5	ISO10705-2	15	100%	Median 1.0E+06/100 mL	Yahya et al., 2015
Tunisia	<i>E. coli</i> WG5	ISO10705-2	6	100%	Median 1.0E+06/100 mL	Yahya et al., 2015

^a PFU per mass or volume as indicated in the cells

3.4.2 F-specific and F-specific RNA bacteriophages

The same general comments done for somatic coliphages in untreated sludge mentioned earlier apply for F-specific RNA phages. Most of the reported results regarding untreated sludge are summarized in Table 18. F-specific RNA phages were detected in all samples tested.

As in raw and treated wastewater they rank second in abundance among the indicator phages. The highest numbers were reported in primary sludge and the lowest in activated sludge. Concentrations well over 10⁶PFU/gram of dry weight have been reported for primary and raw sludge. Their concentration in sludge and proportion to faecal bacteria and other phages remain similar to that in raw wastewater.

Table 18. Concentrations of F-specific and F-specific RNA phages in untreated sludge from municipal wastewater treatment plant(faecal source human)

Area	Indicator/Marker Name	Target Organism /Gene	Number of samples	Percentage positives	Concentration ^a Average (range)	Reference
Greece	F-specific phages	<i>S. typhimurium</i> WG49	40	100%	1.0E+06 to 1.0E+07/100 g of wet matter	Mandilara et al., 2006
Spain	F-specific RNA phages	<i>S. typhimurium</i> WG49	6	100%	1.2E+09/100 g of dry matter	Guzmán et al., 2007
Spain	F-specific RNA phages	<i>S. typhimurium</i> WG49	10	100%	4.4 E+06/100 g of wet matter	Lasobras et al., 1999
Spain	F-specific RNA phages	<i>E. coli</i> HS(pFamp)RR	10	100%	1.6 E+05/100 g of wet matter	Lasobras et al., 1999
Tunisia	F-specific RNA phages	<i>S. typhimurium</i> WG49	15	100%	Median 5E+04/100 mL	Yahya et al., 2015

^a PFU per mass or volume as indicated in the cells, Detection method ISO 10705-1

Phages in plaques obtained from municipal untreated sludge gave the following distribution: 56% of serogroup I, 38% of group II and 6% had a mixed population. This pattern resembles that of secondary effluents more closely than that of raw wastewater (Nappier et al., 2006).

3.4.3 Bacteriophages infecting *Bacteroides*

The same general comments made for somatic coliphages in non-treated sludges apply to *Bacteroides* phages. Most of the reported results regarding non-treated

sludge are summarized in Table 19. As in raw and treated wastewater they rank third in abundance among the indicator phages. *Bacteroides* phages were detected in all samples tested when the matching (faecal source and geographic area) host strain was used. Highest counts were reported in primary sludge and the lowest in activated sludge. Concentrations well over 10^4 PFU/gram of dry weight have been reported for primary and raw sludge. Their numbers in sludge and proportion to faecal bacterial and other phages remained similar to that in raw wastewater.

Table 19. Concentrations of bacteriophages infecting *Bacteroides* host strains discerning the origin of contamination in untreated sludge from municipal wastewater treatment plants (faecal source human)

Area	Number of samples	Percentage positive	Concentration ^a Average (range) PFU/100 mL liquid sludge	Reference
Spain	12	100%	Median 5.0E+05 (2.0E+05 to 7.0 E+05)	Muniesa et al., 2012
Tunisia	15	100%	Median 4.0 E+03 (5.0 E+02 to 7.0 E+03)	Yahya et al., 2015

^aDetection method ISO10705-4 with *B. thetaiotaomicron* GA17 host

4.0 Persistence

Persistence of pathogens and indicators in water environments depends on both external factors related to water condition and in the microbe itself. Each microbe behaves differently. In the case of viruses and phages even simple mutations can change the resistance to treatment and persistence in the environment (Wigginton and Kohn, 2012). Because of this, model experiments with viruses and phages adapted to grow in laboratory culture should be viewed cautiously.

Most available data on phage persistence determined in model experiments refer to laboratory grown model phages and will be reviewed in more detail in the persistence section.

4.1 General Indicator Bacteriophages

Coliphages are present in raw sewage in numbers high enough to carry out model inactivation experiments after diluting sewage in fresh, brackish or marine water. In contrast, concentrations of phages infecting *Bacteroides* in raw sewage are not high enough to perform in situ experiments like those reported for coliphages and consequently phages grown in the laboratory have to be inoculated into the mixture. The short discussion in this chapter is predominantly on coliphages naturally occurring in sewage and laboratory grown phages infecting *Bacteroides* and correspond to experiments in which the persistence of the three groups of phages was studied at once. The results show that indicator phages persist in water environments for longer than *E. coli* does (Table 20).

Table 20. Persistence of the different groups of bacteriophages.

Assay characteristics	Habitat description	Physical features	Additional experimental conditions	T90 in hours <i>E. coli</i> /FC	T90 in hours Somatic coliphages	T90 in hours F-specific RNA phages	T90 in hours <i>Bacteroides</i> phages	References
"In situ" inactivation in a 560- 600 mm depth (300 L) open-top chambers (A)	River water	Winter (12°C) and dark	3% v/v sewage and river water (C)	100	2303	>2300	NR	Sinton et al., 2002
A	River water	Winter (12°C) and sunlight	C	6.9	15.9	19.1	NR	Sinton et al., 2002
A	River water	Summer (16°C) and sunlight	C	3.3	8.3	12.5	NR	Sinton et al., 2002
A	Sea water	Winter (12°C) and dark	2% v/v sewage and sea water(D)	63	102	156	NR	Sinton et al., 1999
A	Sea water	Winter (12°C) and sunlight	D	7.7	51.3	20.3	NR	Sinton et al., 1999
A	Sea water	Summer (16°C) and dark	D	49	52	52	257 ^a	Sinton et al., 1999
A	Sea water	Summer (16°C) and sunlight	D	1.7	7	4.8	4.3 ^a	Sinton et al., 1999
Sample in sealed dialysis tube placed "in situ" at a 20-25 cm depth placed (B)	River water	Summer (20-25°C) and sunlight	2% v/v sewage and river water	64	118	62	>118 ^a	Durán et al., 2002
B	River water	Winter (6-10°C) and sunlight	2% v/v sewage and river water	233	385	323	>385 ^a	Durán et al., 2002
B	Sea water	Summer (>25°C)	2% v/v sewage and sea water	9.8	53	14	95 ^a	Mocé-Llivina et al., 2003
Wetland sediments (naturally occurring)	Wetland sediments	NR	NR	27	370	NR	NR	Stenström and Carlander, 2001
NR - Not Reported								
^a Laboratory grown phages were spiked in the mixture								

Indirect information regarding comparative persistence is also provided by the changes of the ratios between the numbers of conventional bacterial indicators and phages in water environments with aged pollution. Different studies report a decrease of the ratio in both river and marine waters when compared to the ratios of sewage (Contreras-Coll et al., 2002; Ibarluzea et al., 2007; Lucena et al., 2003; Mocé-Llivina et al., 2005; Skrabber et al., 2002). This diminution confirms the observation of the model

experiments.

As a whole, it can be concluded that persistence of the three groups of indicator phages is intermediate and as reviewed by Verbyla and Mihelcic (Verbyla and Mihelcic, 2014) their persistence is in the range of that of human viruses.

We think that it is worth mentioning that the great

majority of data regarding the presence and persistence of F-specific RNA phages as a group in water have been obtained in cold and temperate climates. But, evidences exist indicating that in environments with temperatures higher than 25°C F-specific RNA phages persist shorter than the other phages and bacterial indicators (Agulló-Barceló et al., 2013; Alcalde et al., 2003; Cole et al., 2003; Durán et al., 2002; Guzmán et al., 2007; Mandilara et al., 2006; Mocé-Llivina et al., 2005). Geographic areas with surface sea and fresh water temperatures over 25°C either all year round (between 40° latitude North and 40° latitude South) or during the warm seasons (many areas of Europe and USA) are quite extensive. Then, the persistence of F-specific and F-specific RNA phages needs some extra verification before rating their persistence and recommending their use in warm regions.

4.2 Host Specific Indicator Bacteriophages

Many experiments regarding persistence of F-specific RNA subgroups in the water environment indicate a significant difference in the persistence among the subgroups. Indeed, persistence studies with phage isolates belonging to the different subgroups (Cole et al., 2003; Schaper et al., 2002a) and with naturally occurring phages (Hartard et al., 2015; Muniesa et al., 2009) indicate differential persistence between subgroups. In all cases subgroup I being the more persistent followed by subgroups II, III and IV in this order. These diverse survival rates of subgroups of F-specific RNA phages to environmental stressors constitute a limitation to their suitability as trackers of the origin of faecal pollution. This fact is aggravated by a similar behaviour in advanced wastewater treatments. All together these features lead to a marked preponderance of subgroup I in surface waters (Brion et al., 2002; Cole et al., 2003; Stewart-Pullaro et al., 2006).

All the results obtained in the in situ experiments point out that phages infecting different host strains of *Bacteroides*, including those that allow tracking faecal sources, are more persistent than *E. coli* and are in the range of persistence of somatic coliphages. Regarding the ratios between numbers of different indicators, Muniesa et al. (2012) reported that the numerical ratios between somatic coliphages/phages infecting *Bacteroides* strains in wastewaters are maintained in river and marine water. This fact points out that the persistence of phages infecting *Bacteroides* is similar to that of somatic coliphages. Having this in mind, Muniesa et al. (2012) have suggested that the numerical ratios between the concentrations of somatic coliphages and other phages detected by strains able to identify host associated faecal contaminant are a good marker for microbial source tracking.

5.0 Short Review on the Application of Phage as Indicators

5.1 General Quality Indicators

Coliphages have been used in academia for many years as both faecal and viral indicators. The high concentrations found in raw wastewaters and in many other matrixes

contaminated with faecal remains, the easy, fast and cost effective methods, the persistence in the water environment and the resistance to treatments (see chapters dealing with sanitation, disinfection and persistence), which resemble those of viruses, make the indicator phages good surrogate indicator candidates for various set-ups.

Additionally, regulatory authorities in different areas of the world are beginning to consider phages as indicators of water quality and for validating and verifying water treatment processes.

5.1.1 Quality indicators of recreational waters

A substantial amount of data about the presence and levels of indicator phages, mostly coliphages, in all type of surface waters and in very different climatic areas is available (Burbano-Rosero et al., 2011; Contreras-Coll et al., 2002; Ebdon et al., 2007; Haramoto et al., 2005; Ibarluzea et al., 2007; Jiang et al., 2007; Lucena et al., 2003; Rezaeinejad et al., 2014; Taylor et al., 2001). The general trend is that the three groups of phages keep the same proportions as in raw wastewater, but they have reduced the concentration gap with either *E. coli* or faecal coliform bacteria. This shift in concentrations is quite prominent in samples with values of either *E. coli* or faecal coliforms below 10^2 - 10^3 CFU/100 mL (Contreras-Coll et al., 2002; Ibarluzea et al., 2007; Lucena et al., 2003; Skrabber et al., 2002). This is probably due to the greater persistence of phages. The potential application of coliphages in quality control of bathing and recreational surface waters is currently being studied by USA regulatory authorities (U.S. EPA, 2015), though at present, neither concentrations nor which coliphages should be considered have been defined.

5.1.2 Indicators for the efficiency of reclamation processes and the quality of reclaimed water

Many of the tertiary treatment processes applied for water reclamation have as the major aim the removal and inactivation of pathogens. The most frequently used processes include filtration and disinfection by chemicals or UV radiation or a combination. The tertiary effluents generally contain substantially reduced numbers of pathogens and indicators, but the proportions between different indicators found in raw wastewater and secondary effluents changed after treatment.

A substantial amount of information is available on the removal of somatic coliphages and F-specific coliphages by tertiary treatment processes and on the numbers of surviving phages in treated effluents (Costán-Longares et al., 2008; den Blanken, 1985; Gomila et al., 2008; Luther and Fujioka, 2004; Mandilara et al., 2006; Montemayor et al., 2008; Nieuwstad et al., 1988; Rose et al., 2004; Rubiano et al., 2012; Soriano et al., 2011; Stiegel et al., 2013; Zhang and Farahbakhsh, 2007).

Removal of both groups of phages varies for different treatment processes and counts of surviving phages may range between 0.5 and $>4.0 \log_{10}$ units. Typically counts of somatic and F-specific coliphages in many tertiary effluents

(reclaimed waters) are higher than those of faecal indicator bacteria, or at least, the difference is much smaller than in secondary effluents. According to the only available report on *Bacteroides* phages, their behaviour is similar to that of coliphages.

Because of these kinds of results indicating that the resistance of phages to many treatment processes (see chapters on sanitation and disinfection) resembles that of viruses, coliphages have been included in some regulations regarding water reclamation. Among these are the states of Queensland, Australia (Queensland Government, 2005) and North Carolina, USA (North Carolina Administration, 2011). Both regulations specify a given reduction in numbers of coliphages by the treatment processes concerned as well as the maximum number of coliphages permitted in the reclaimed water.

5.1.3 Quality indicators of groundwater

The presence of indicator phages in groundwater has very frequently been reported as presence in 1 litre after concentration and in most studies the results are reported as presence/absence (Abbaszadegan et al., 2003; Borchardt et al., 2004; Jung et al., 2011; Locas et al., 2007; Lucena et al., 2006).

In summary, somatic coliphages were detected in 8.7% (Payment and Locas, 2011) to 22.4% (Lucena et al., 2006) of groundwater samples, and F-specific phages in 1% (Borchardt et al., 2004) to 18.3% of samples (Lucena et al., 2006). The only study including phages of *Bacteroides* reports 1.7% (Lucena et al., 2006). Thus again somatic coliphages are the most abundant ones, followed by F-specific coliphages and *Bacteroides* phages. In two of these studies, partial data on aquifers with no faecal contamination and aquifers heavily contaminated are reported, and the values of positive isolations of phages clearly match with the expected contamination. Thus, for somatic coliphages values range from 0% to 62 % (Lucena et al., 2006) and from 0% to 25 % (Locas et al., 2007).

In an analysis of 100 mL groundwater samples, Lucena et al. (2006) reported the presence of *E. coli* in 18.8%, somatic coliphages in 22.4% and F-specific RNA phages in 18.1% of samples, and in a similar analysis Lee et al. (2011) reported the same indicators to be present in 15%, 12% and 7.5% of samples.

The USEPA rule on groundwater considers coliphages as alternative indicators for quality assessment of groundwater (U.S. EPA, 2006)

5.1.4 Quality indicators of drinking water

This refers to disinfected water only. Since indicator phages are more resistant to treatment processes than bacterial indicators (except spores of *Clostridium*) it can be expected that coliphages and *Bacteroides* phages are detected in samples of drinking water treated to eliminate the bacterial faecal indicators. This was confirmed by the detection of coliphages in a meaningful percentage of drinking water samples with free residual chlorine and no,

or very low, numbers of bacterial indicators in Canada, Egypt and Peru (El-Abagy et al., 1988; Palmateer et al., 1990; Ratto et al., 1989). Méndez et al. (2004a) detected neither *E. coli* nor faecal coliforms in 427 samples of 100 mL of a metropolitan water network containing >0.1 mg/L of chlorine, but detected somatic coliphages, F-specific RNA phages and *Bacteroides* phages in 8.8, 10 and 4.5 % of 1000 mL samples, respectively. Likewise, Armon et al. (1997) detected faecal coliforms, somatic coliphages, F-specific coliphages and *Bacteroides* phages in 0.7, 5.6, 7.1 and 5 %, respectively, of 1536 100 mL-samples of disinfected water in Israel. Therefore, coliphages arise as attractive indicators for disinfected drinking water that will provide some more protection than the traditional bacterial indicators. We are not aware of any drinking water quality regulation that considers phages as indicators.

5.1.5 Quality indicators for treated sludge

In many countries, regulations require the treatment of sludge for certain destinations as for example in agriculture. As seen in a previous section, coliphages are quite abundant in untreated sludge. The most frequent treatments of sludge are thermophilic digestions, pasteurization, lime stabilization and composting. Anaerobic mesophilic digestion shows a poor hygienization of all the indicators including phages, with values of somatic coliphages as high as 4.4×10^6 PFUs/gram (dry weight) of digested sludge (Guzmán et al., 2007). Thermophilic digestions, pasteurization and composting achieving very significant reductions in the number of bacterial indicators showed in-between reductions of F-specific RNA and very moderate reduction in the numbers of somatic coliphages (Astals et al., 2012; Guzmán et al., 2007). Also, storage in quick lime is much more efficient in reducing the numbers of faecal coliforms than those of somatic coliphages (Campos et al., 2002).

Therefore, phage indicators, particularly somatic coliphages, seem to be a serious candidate to be considered as an indicator of hygienization in sludge management. Western Australia (Western Australian Government, 2012) and Colombia (Republica de Colombia, 2014) have introduced coliphages in the regulations about the quality of biosolids (treated sludge) to be applied in agriculture.

5.2 Other Potential Uses

In order not to unreasonably enlarge this section it is only mentioned that among other applications related to water quality, phages are also considered as potential indicators for the microbiological quality of shellfish, as well as markers for the transport of microorganisms in soil, and to validate filters.

5.2.1 Indicators for microbial source tracking

Among the subgroups of F-specific RNA phages, subgroup I seems to predominate in surface waters. This is difficult to interpret because subgroup I also appear to be the most persistent. In contrast, the few existing data (Ebdon et al., 2007; Muniesa et al., 2012) seem to indicate

that phages infecting *Bacteroides* and their ratio to somatic coliphages seem a good option for MST of surface waters where *E. coli* concentrations are equal to or greater than 10^3 CFUs/100 mL.

Lee et al. (2011) found that 85% of 60 F-RNA phages isolated from groundwater being subgroup I. No sound data about phages infecting strains GA17 and GB124 in groundwater are available in order to evaluate their potential use for MST in groundwater.

Information on the presence and values of coliphages in solid matrixes is relatively abundant, mostly in untreated and treated sludge, in contrast it is scarce about phages infecting *Bacteroides* and non-existing for F-specific RNA phage subgroups. The few data available seem to indicate that the ratio between the numbers of somatic coliphages and phages infecting *Bacteroides* will remain in these matrixes similar to the ratio in sewage (Guzmán et al., 2007; Lasobras et al., 1999; Yahya et al., 2015).

5.2.2 Target enumeration to infer the occurrence of intestinal pathogens

Additionally to their worth as indicators of faecal contamination the indicator phages have been viewed as potential surrogates of human viruses. Consequently, the possible relationship between presence and levels of indicator phages and human viruses in waters has been studied, though with disparate results. However, these studies are quite varied with respect to the assessed parameters and the methods used. Phages have usually been evaluated by plaque assay by standardized (or equivalent methods) in small volumes, and without a concentration procedure. Human viruses have been evaluated after concentration of great volumes and posterior detection with very different methods, such as plaque assay on cell culture and different PCR approaches. Most of these counts, mostly in matrixes where the numbers of phages and viruses are low and mostly those of viruses have a great amount of uncertainty that can explain part of the heterogeneity of the results.

Some studies have failed to show any relationship in fresh surface waters (Hot et al., 2003; Jiang et al., 2007; Viau et al., 2011; Westrell et al., 2006), marine surface water (Boehm et al., 2009; Jiang et al., 2007) and groundwater (Abbaszadegan et al., 2003; Borchardt et al., 2004; Payment and Locas, 2011).

Others have found relationships between human viruses and phage. Jiang et al. (2001) found that the presence of human adenovirus detected by nested-PCR was significantly associated with F-specific coliphages in marine surface waters impacted by urban run-off.

Ballester et al. (2005) described that the presence of astroviruses and adenoviruses was significantly associated with the presence of both somatic and F-specific coliphages in coastal water impacted by WWTP. The presence of rotavirus and enterovirus was only linked to the presence of F-specific phages. Human viruses were detected by ICC-nPCR and ICC-RT-nPCR.

Love et al. (2014) described that the presence of F-specific coliphages was associated with the presence of genome copies of adenoviruses but not noroviruses in coastal waters.

Rezaeinejad et al. (2014) found that in urbanized catchments of fresh water in tropical Singapore F-specific PFU numbers were associated to counts of genome copies of noroviruses but not with GC of adenoviruses, astroviruses and rotaviruses.

Mocé-Llivina et al. (2005) described a significant association between the presence of enteroviruses enumerated on cell culture and somatic coliphages in marine waters.

Skraber et al. (2004) revealed that in a French river the numbers of samples positive for infectious enterovirus as well as genome copies of enteroviruses and of noroviruses increased with increasing densities of somatic coliphages.

Even if results are far away from indicating a clear correlation between densities of indicator coliphages and human viruses in waters, there is evidence that somatic and F-specific coliphages are more strongly associated with pathogenic viruses than the traditional bacterial indicators.

5.2.3 Relation to risk

Several epidemiological studies conducted to evaluate the relationships between the presence of indicator phages in surface waters and swimming illnesses have been performed with disparate outcomes. However, overall the epidemiological evidence suggests a likely relationship between coliphages and human health.

Von Schirnding et al. (1992) conducted a prospective cohort study at 2 South African marine beaches. The numbers of coliphages detected were fairly low and no statistically significant relation was found between sickness and numbers of somatic coliphages.

In studies at freshwater lakes in Germany, Wiedenmann et al. (2006) found a significantly increased risk of gastroenteritis for persons bathing in water with concentrations of somatic coliphages greater than 10 PFU/100 mL versus non-bathing fellows.

In an epidemiological study to evaluate water quality and health effects for waters at a marine beach in Florida (Abdelzaher et al., 2011), the detection of somatic coliphages overlapped with the highest illness days. However, no significant correlation between health outcomes and somatic coliphages was observed.

Lee et al. (1997) found in Great Britain a statistically significant association between risk of gastrointestinal illness and density of F-specific RNA coliphages.

Van Asperen et al. (1998) did not find exposure-response association between F-specific RNA phages and gastrointestinal illness in a study performed in Holland.

In a study on swimmers at marine beaches in

California, Colford et al. (2007) found skin rash and diarrhoea, but no other health symptoms, to correlate with the presence of F-specific phages but not with that of somatic coliphages.

In a comparison of swimmers to non-swimmers at marine beaches in Alabama, Mississippi, and Rhode Island, a significantly higher risk of gastrointestinal illness has been reported for days when coliphages were present (Wade et al., 2010). No epidemiological studies seem to be on record regarding a correlation of phages in drinking water to related disease.

An overlapping between a jaundice outbreak and a high incidence of somatic coliphages in potable water occurred in a municipality of West Bengala, India in 2014. Somatic coliphages ranging from 40 to 250 PFU/100 mL were detected in 16 out of 20 samples during the outbreak detected in December 2013, whereas *E. coli* was detected in only 2 of the samples. A few weeks after the outbreak, somatic coliphages were only detected in four out of twenty samples with positive values ranging from 10 to 50 PFU/100 mL, and *E. coli* in none (Mookerjee et al., 2014).

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