

Review

Coliphages as Model Organisms in the Characterization and Management of Water Resources

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Abstract: Two groups of bacteriophages that infect *Escherichia coli*, somatic and F-specific coliphages, have been used in academia as both fecal and viral indicators for many years. Regulatory authorities in different parts of the world are beginning to consider coliphages as indicators of water quality in a range of settings. However, issues such as their potential replication in natural water environments, the cumbersome detection and enumeration methods, a lack of definition concerning which of the two groups should be included in future regulations, and the lack of a clear correlation between coliphages and human viruses and health risks in different water settings remain controversial. This review attempts to shed some light on these contentious issues. The conclusions are that: 1) supposing that they can replicate in some natural water settings, the contribution of coliphages replicated outside the gut will not affect the numbers contributed by fecal pollution and detected by strains recommended for standardized methods; 2) there are easy, fast, and cost-effective methods that can be used in routine laboratories after a little training; 3) perhaps the best option is to determine both groups in a single step; and 4) the low correlation of coliphages with human viruses and health risks is no worse than the correlation between different human viruses.

Keywords: bacteriophage; somatic coliphage; F-specific phages; water; indicator

1. Introduction

Bacteriophages or phages are viruses that infect bacteria. They consist of at least a nucleic acid molecule, the genome, surrounded by a protein coat called capsid. Many phages also contain additional structures such as tails and spikes. Much less frequently, they contain lipids. The sizes of phages are in the range of viral dimensions. These features imply that in terms of composition, structure, morphology, and capsid size, phages share many properties with animal, and hence human, viruses. In fact, there is an elevated degree of consensus that the persistence in the environment and resistance to treatment of bacteriophages resemble those of viruses. Bacteriophages only can replicate inside susceptible and metabolizing host bacteria. A given bacteriophage can only infect certain bacteria and therefore they are considered as host specific. The degree of host specificity is wide-ranging and depends on the bacteriophage. 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 the most common, normally known as somatic phages.

Two groups of bacteriophages that infect *E. coli*, somatic and F-specific coliphages, have been used as both fecal and viral indicators in academia for many years [1–4]. The high concentration of coliphages found in raw wastewater and many other matrices contaminated with fecal remains,

the easy, fast, and cost-effective detection and enumeration methods, their persistence in the water environment, and their resistance to treatment used for viruses make indicator bacteriophages good surrogate indicators for a range of applications. Additional benefits are that samples can be kept at 4 °C for at least 48 hours without any significant change in the numbers of infectious bacteriophages [5] and that small volume samples can keep their phage densities for months when stored at −20 °C or −80 °C after the addition of 10% *v/v* glycerol [6]. Finally, phenomena such as “stress,” “injury,” and “reactivation,” which frequently lead to the misinterpretation of environmental data on bacterial indicators, do not, as far as it is known, apply to bacteriophages.

Furthermore, regulatory authorities in different parts of the world are beginning to consider coliphages as indicators of water quality. Examples of regulatory guidelines that involve bacteriophages include those concerning water reclamation [7,8], groundwater [9], and biosolids used in agriculture [10,11]. In addition, U.S. regulatory authorities [12] are currently studying the possibility of using coliphages in the quality control of surface water used for bathing and recreation.

However, the use of coliphages as indicators in water quality control is not free from controversy. In this review, we will attempt to address the contentious issues.

2. Coliphages

Coliphages are bacteriophages that infect *E. coli*. In fact, the name is very vague, since it can refer to phages that infect any strain of *E. coli*, and the susceptibility of host strains to different sets of bacteriophages is known to vary. From the point of view of using phages as indicators in the water environment, there is an unofficial consensus that the term applies to phages that infect certain host strains. This will be addressed in the Methods section below. Again, in the field of water quality, the term “coliphage” refers to two groups: somatic coliphages and F-specific coliphages.

Somatic coliphages are a heterogeneous group of bacteriophages that infect *Escherichia coli* through the cell wall (Figure 1). Most known somatic coliphages detected by the host strains recommended in the standardized methods and found in municipal wastewater belong to the *Myoviridae*, *Siphoviridae*, *Podoviridae*, and *Microviridae* families [13,14].

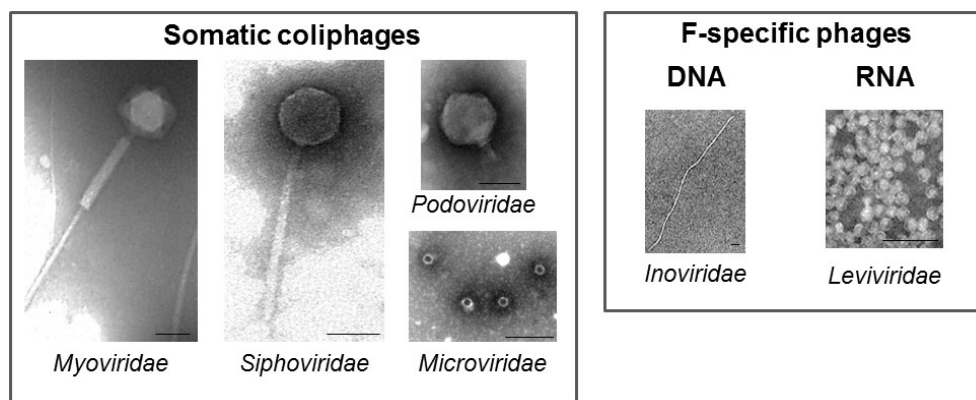


Figure 1. The most common morphological types in somatic coliphages and F-specific phages. Bar 50 nm.

F-specific bacteriophages, also known as sexual coliphages or male-specific bacteriophages, infect bacteria through the sex pili, which are coded by the F plasmid that was first detected in *E. coli* K-12. F-specific RNA bacteriophages, a subgroup of F-specific bacteriophages, consist of a simple capsid with cubic symmetry and a diameter of between 21 nm and 30 nm. They contain a single-stranded RNA molecule as the genome. The F-specific RNA bacteriophage group (*Leviviridae* family) contains two genera (*Levivirus* and *Allolevirus*) and three minor unclassified groups [15]. The genus *Levivirus* contains subgroups I and II, whereas the genus *Allolevirus* contains subgroups III and IV. These four groups

coincide with the serotypes first described by Furuse in 1987 [16]. Subsequent genomic characterization has established that genogroups match the serotypes [17,18], at least for practical purposes. Other F-specific bacteriophages are the rod-shaped DNA bacteriophages of the *Inoviridae* family [15].

As described in further detail below, both coliphage groups are found in significant numbers in raw municipal and animal wastewater as well as animal slurries and manure around the world. It is also worth noting that neither somatic nor F-specific phages show seasonal variation in either pollution sources or surface waters [19–23].

3. Replication of Coliphages Outside the Gut

Host strains of somatic coliphages include *E. coli* and related species, primarily *Shigella* spp. and *Klebsiella* spp. Some *Klebsiella* species may live outside the gut, albeit not in large quantities [24]. *E. coli* has also been reported to replicate in certain water environments [25]. Therefore, somatic coliphages can occasionally find hosts in these environments, including *E. coli* that has replicated in certain settings. Furthermore, somatic coliphages have been reported to replicate in *E. coli* under environmental conditions [26,27]. Some researchers argue that the potential replication of somatic coliphages outside the gut invalidates them as indicators.

It cannot be ruled out that a particular coliphage found in a non-gut sample has not replicated outside the gut and that coliphages replicate in water environments. However, there are numerous reasons to believe that, if they do replicate in water environments, the numbers of coliphages originating from replication outside the gut and detected by the host strains recommended by the standardized methods will not constitute a detectable increase in the numbers of somatic coliphages originating from the fecal pollution measured.

A small number of scientific publications support the statement that somatic coliphages replicate in water environments [26–29]. A meticulous review of the scientific basis of this assumption led us to question it. Vaughn and Metcalf (1975) first reported phage replication in estuarine waters at densities of 1 CFU of *E. coli* host cells and 100 PFU of somatic coliphages per mL [26]. These densities are much lower than those required for replication to occur, according to theoretical models and experimental findings that have emerged since then and are reviewed in detail below. Moreover, with such low numbers, the uncertainty of the enumeration method is enough to explain the increase in the numbers of coliphages reported by the authors. Borrego *et al.* reported replication in seawater after seeding it with selected coliphages and *E. coli* as the host [29]. Replication failed at 18 °C. In contrast, replication occurred at 36 °C, but under experimental conditions with densities of *E. coli* cells and phages that were much higher than the values that naturally occur in contaminated seawater samples. In addition, these experiments were performed with *E. coli* host cells in physiological conditions that were much more appropriate for phage replication than those expected in a water environment. Seeley and Primrose supported their hypothesis of environmental coliphage replication with indirect evidence by isolating phages replicating at low temperatures from river water, which the authors considered to be an indication of replication in the environment [27]. A third study [28] reported that somatic coliphage numbers increased in both sand and biologically active carbon filters in a water reclamation plant. One potential reason proposed to explain the increase in the average coliphage counts in these treatment units was replication. However, such phage replication was never confirmed, and the increase in average numbers may have been due to accumulation and breakthrough at times.

By contrast, a number of the studies addressed below reveal many factors that reduce the probability of somatic coliphage replication in water environments. The joint replication threshold densities of non-replicating *E. coli* and their phages expressed in log₁₀ units account for at least 7.0 log₁₀ units per mL [30,31]. This means that combinations of threshold densities of phages and host cells of 4 log₁₀ units *vs.* 3 log₁₀ units, 3 log₁₀ units *vs.* 4 log₁₀ units, 5 log₁₀ units *vs.* 2 log₁₀ units or *vice versa, etc.*, would be required to allow host–phage encounters. Additionally, less than 3% of naturally occurring hosts (*E. coli*) have been reported to support the replication of phages that infect the host strains used in the standardized methods [32]. This low percentage reduces the densities

of the hosts available for replication in nature and makes it very unlikely that water environments containing the joint replication threshold density of $7.0 \log_{10}$ units per mL would be found. The presence in water of numerous background bacterial flora and particulate materials that interfere with coliphage replication further increases the value of the joint replication threshold densities [30,31]. The metabolic activity of the host needed for phage replication is too low in the water environment to support phage replication [33]. The envelope stress response that affects *E. coli* when it is released into the water environment triggers responses that have been reported to reduce phage infection [34,35]. The contribution of lysogenic induction to the presence of free coliphages detectable by the standard methods is not noticeable [36]. Moreover, Hernandez-Delgado and Toranzos showed that neither sewage isolates nor laboratory phage strains replicate in pristine river water in a tropical area [37]. These and other data reviewed [38] strongly suggest that the contribution of somatic coliphages replicated outside human and animal guts to the numbers of somatic coliphages detected by host strains recommended by the standardized methods is negligible when analyzing water, food, or biosolid samples. The paradigm of environmental coliphage replication as an impediment to the application of coliphages as fecal indicators in water should therefore be revisited.

With regard to F-specific bacteriophages, in spite of the temperature-conditioned ($32\text{ }^{\circ}\text{C}$) production of sexual pili necessary for phage replication, there are conflicting reports on the replication of F-specific bacteriophages in wastewater and groundwater [39]. Nevertheless, Woody and Cliver reviewed the conditions that affect F-specific RNA phage replication in the host bacteria, other than pili formation [40]. They concluded that replication outside the gut cannot be ruled out, but that it is highly improbable and that the influence of replication outside the gut on the numbers of these phages in the water environment can be considered as insignificant compared to those contributed by feces.

4. Are the Methods Cumbersome?

Some people argue that the methods for detecting bacteriophages are cumbersome, but there are many reasons to disagree with this viewpoint. As with all bacteriophages, coliphages can only replicate in metabolizing host cells. Infectious bacteriophages are typically detected by the effects they have on the host bacteria they infect, especially lysis. The most important factor in defining a method for the detection of a given phage or group of bacteriophages is the bacterial host strain. Phages are enumerated by direct quantitative plaque assays [41] (Figure 2); the plaque assay provides the results in plaque-forming units (PFUs). A plaque-forming unit is an entity, usually a single virion, but it may also be a clump of virions that gives rise to a single plaque of lysis in a monolayer of the host strain. PFUs are also known as plaque-forming particles (PFPs) in the ISO standards. The presence of phages in a given volume of sample can also be detected by the qualitative presence/absence enrichment test [41] (Figure 3). The enrichment of multiple tube serial dilutions allows the numbers of phages to be estimated using “quantal” methods such as the most probable number procedure.

Standardized methods are available for both somatic and F-specific coliphages. Mooijman and collaborators [6] and USEPA [42,43] have carried out interlaboratory validation tests of the ISO and USEPA methods described below. In addition, Mooijman *et al.* showed that the implementation of the ISO standard method is feasible in routine microbiology laboratories without any prior experience of bacteriophage handling [6].

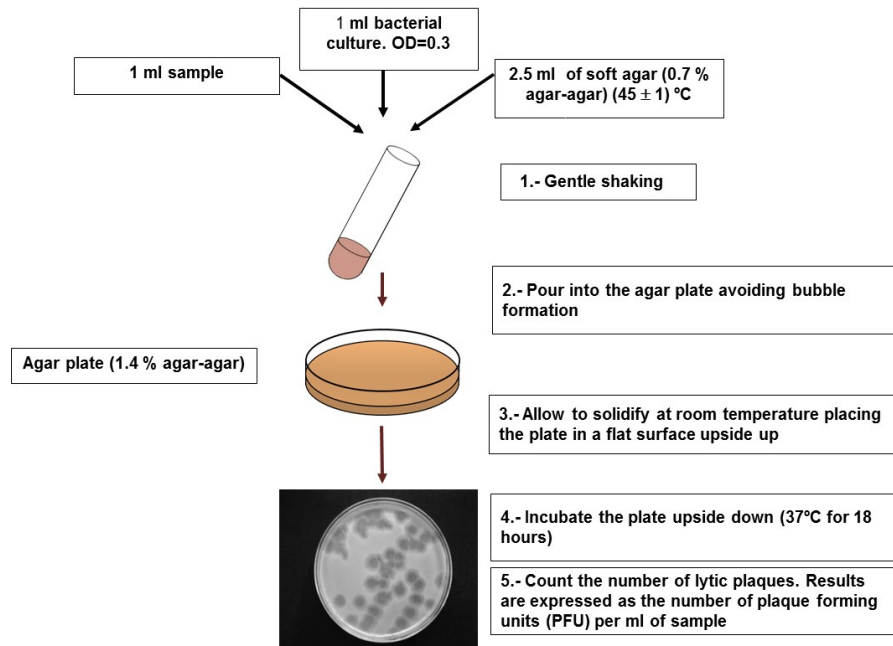


Figure 2. Double agar layer method for phage enumeration.

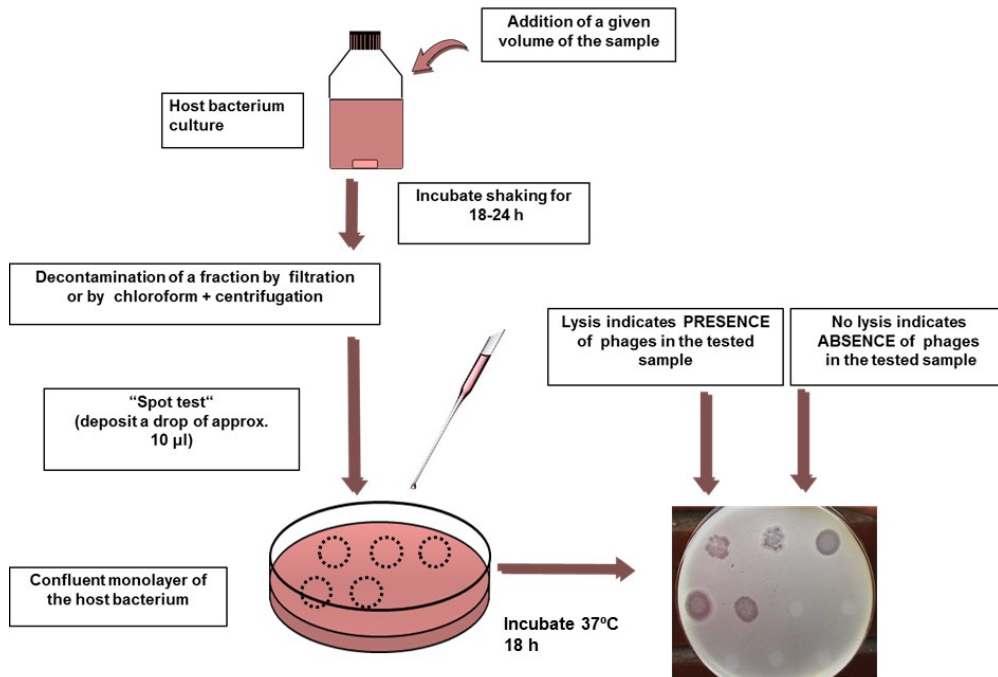


Figure 3. Detection of bacteriophages by the Spot Test method.

4.1. Somatic Coliphages

ISO [44], USEPA [45,46], and Standard Methods [47] have standardized procedures for the detection of somatic coliphages. The three methods use *E. coli* C (ATCC 13706) as the host strain and differ only in minor details relating to the media and assay conditions. The *E. coli* used is either *E. coli* ATCC 13706 (Standard Methods procedure) or its nalidixic acid-resistant clones *E. coli* CN13 (ATCC 700609), used in the USEPA 1601 and 1602 methods [45,46]; or *E. coli* CN13, more frequently referred to as WG5 (ATCC 700078), used in the ISO 10705-2 method [44]. Hosts resistant to nalidixic acid were introduced to minimize the growth of background bacteria that frequently interfere with the

correct visualization of plaques in the plaque assay test. Strains CN13 and WG5 detect similar phage numbers in water matrices when the standardized method is used [48,49]. In addition, the present Standard Methods procedure theoretically provides similar counts to ISO and USEPA. By contrast, the Standard Methods procedure described in previous editions has been reported to perform poorly [50]. ISO-10705-2 [44] includes both the double agar layer (DAL) plaque assay method for the quantification of PFU (Figure 2) and the presence/absence test (Figure 3). A simplified version of these methods can be found at Havelaar and Hogeboom [51]. The USEPA Method 1601 [45] uses the presence/absence procedure and EPA Method 1602 [46] uses the single agar layer (SAL) plaque assay. A simplified version of these methods can be consulted on the USEPA website [52]. Results of quantification by PFU can be obtained within six hours.

4.2. F-Specific Coliphages

Host strains used to detect sexual coliphages must produce sexual pili encoded by the F-plasmid. Strains expressing pili and detecting minimal amounts of somatic coliphages have been tailored. These are strains *E. coli* HS (*E. coli* Famp, ATCC 700891) [53] and *Salmonella enterica* serovar Typhimurium (most frequently reported as *Salmonella typhimurium*) WG49 (NCTC 12484) [54]. The phages detected by these strains are counted as F-specific coliphages or male-specific coliphages or sexual coliphages. The number of F-specific RNA bacteriophages is the difference between the numbers of phages counted in the absence and in the presence of RNase in the assay medium, since this enzyme interferes with the infection of F-specific RNA phages. The counts achieved with strains HS and WG49 are similar [48,55,56]. Because of the genetic markers (resistance to ampicillin and capacity to use lactose), the stability (and re-selection) of strain WG49 is easier to verify than that of strain HS, which has no explicit genetic markers. However, strain HS seems more stable than strain WG49. The ISO-10705-1 standard method [57] for the detection and enumeration of F-specific bacteriophages includes a step with RNase in the assay medium. Consequently, it detects both F-specific and ARN F-specific bacteriophages. The ISO standard includes both the double layer plaque assay method and the presence/absence method. A simplified version can be found at Havelaar and Pot Hogeboom [54] and Standard Methods [47]. The EPA Method 1601 standard procedure [45] uses the presence/absence method and EPA method 1602 [46] uses the single-layer plaque assay (SAL) for the detection of F-specific bacteriophages that use *E. coli* Famp as the host strain. A simplified version can be found at USEPA [52] and Standard Methods [47]. Results using both plaque assay methods can be obtained within 18 hours.

4.3. Molecular and Fast Methods

No procedures based on serologic detection or polymerase chain reaction (PCR) that are applicable to the detection of the full somatic coliphage group in water have been described. The diversity of the bacteriophages in this group makes this approach complex, though not impossible.

For F-specific coliphages, both serological [58] and PCR-based methods are available to quantify F-specific RNA phages [59–62] and F-specific DNA phages [63]. However, these methods are used for a specific phage or subgroup, not for the full group of F-specific bacteriophages. The sum of values obtained for the different subgroups accounts for all F-specific bacteriophages. These methods can be applied directly to the sample, in which case they do not reveal the infectivity status of the detected genomes or genome fragments, or after the enrichment of multiple tube serial dilutions through the standardized methods, which allows phage numbers to be estimated by “quantal” methods such as the most probable number procedure.

Methods based on phage-induced host lysis through the detection of a molecule released by the lysed cell are also available and provide results for infectious bacteriophages. These methods are relatively fast because of the short time needed for a replication cycle and subsequent replication cycles, which lead to a high number of lysed cells in just a few hours in *E. coli*. Thus, Izjeran *et al.* described a method based on a rapid extracellular β -galactosidase enzyme release and detection

during coliphage-induced lysis [64]. Later, a method based on the detection of somatic coliphages through a bioluminescence assay that measured the phage-mediated release of adenylate kinase and detected adenosine 5'-triphosphate was described [65]. In order to obtain results within a day, these methods were improved and adapted to include the enrichment of multiple tube serial dilutions based on the presence/absence test in the USEPA and ISO standards by Salter and Durbin and Salter *et al.*, respectively [66,67]. Ongoing procedures to generate tailored host strains that can detect phage-induced cell lysis would reduce the time required to obtain results to less than four hours, even at low concentrations of somatic coliphages (Muniesa, personal communication). It should be relatively easy to adapt these methods based on the detection of molecules released by host lysis into the medium to user-friendly commercial kits.

4.4. Concentration Methods

Though the presence/absence method allows relatively large volumes to be tested (up to 1 L is quite feasible), concentration may sometimes be required, either because greater volumes need to be tested or because quantification is required. Most methods described for concentrating animal viruses are not suitable for concentrating bacteriophages [3], although ongoing projects may help change this situation. For volumes ranging from 10 to 1000 mL, two methods are recommended. For water samples with low turbidity, 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 was developed [68]. This method was modified slightly [69] and performed extremely well when concentrating somatic coliphages and F-specific RNA phages from samples of up to one liter. For samples with high turbidity, flocculation with magnesium hydroxide [70] is suitable for the two bacteriophage groups [71].

With respect to somatic coliphages, we cannot imagine many scenarios in which it would be necessary to concentrate them from water samples of more than one liter. The ratios of culturable viruses to somatic coliphages (mainly with reference to enteroviruses detected by cell culture) are 10:10⁴ or 10:10⁵ in the scenarios for which data on both are available. The claim is true for raw sewage, secondary and tertiary effluents [72–77], surface waters [73,78–80], and sludge [81,82]. The same ratio is true for infectious rotaviruses detected by cell culture followed by qRT-PCR [83]. No comparative data are available for other infectious viruses *vs.* coliphages in the same sample. However, some data that compared culturable viruses in the same water samples indicate that adenoviruses and enteroviruses [84,85] and rotavirus and enteroviruses [83,86] are found in concentrations of the same order of magnitude, and therefore the same ratio *vs.* somatic coliphages will be applicable. Moreover, the concentration of volumes greater than 1000 L is very seldom recommended and feasible, because higher volumes increase the uncertainty of the concentration methods [87], and if volumes greater than 1000 L are desired, several aliquots of 1000 L are recommended [88].

4.5. Additional Methodological Issues

4.5.1. Scale-Up

For all these standardized methods, the volumes of the samples tested, as described in the Methods section, can be scaled up by maintaining the proportions of the medium mixtures, host suspension, and sample, and, for the agar layer methods, by using larger Petri dishes.

4.5.2. Reference Material

Reference suspensions of model bacteriophages [6] and naturally occurring somatic and F-specific coliphages needed for quality assurance are easily prepared and conserved [5].

4.5.3. Costs

Detection and enumeration of somatic coliphages and F-specific phages by the standardized methods is relatively cost-effective. The costs of the material, media, reagents, and labor are similar to those required for the detection of fecal coliforms or *E. coli*. The cost of detecting F-specific RNA bacteriophages is 10–15% higher because of the need for RNase and double the number of plates. All these methods can be applied in routine microbiology laboratories without the need for extra equipment.

5. Which Group Should Be Used? Somatic vs. F-Specific Coliphages

There is some debate regarding which of the two groups of phages should be addressed by the regulations or whether both should be included. Information about the relative abundance of both phage groups and their relative persistence and resistance would help clarify the issue. Although there is a great deal of literature on these issues, this section only considers reports in which both phage groups have been analyzed by the standardized methods in the same samples.

5.1. Abundance in Pollution Sources

The primary origin of coliphages found in the water environment is human and animal feces. If we consider the percentage of human and animal individuals excreting phages and the amounts of phages excreted, the inputs of somatic coliphages [89–91] in the water environment directly from feces are greater than those of F-specific coliphages [90–93].

Coliphages reach water for human use by either point or diffuse pollution. Point pollution is contributed mostly by raw or treated human and animal (*i.e.*, from abattoirs) wastewater, whereas diffuse pollution is mostly contributed by septic tank overflow, sewer leakage, and the spread of solid waste such as raw and treated sewage sludge, slurry, and manure, and the feces of pets, farmed animals, and wild animals. Other than feces, the main contributors, *i.e.*, raw wastewater, secondary effluents, sludge, and slurry, will be reviewed below as polluting sources, in which somatic coliphages clearly outnumber F-specific phages when both groups of phages are counted using the standardized methods (USEPA or ISO) in the same samples, with a few exceptions.

Thus, somatic coliphages are detected in raw municipal wastewater throughout the world in 100% of the samples tested and values range from 5×10^6 – 10^7 PFUs per 100 mL, with those of F-specific phages ranging from 10^4 to 10^6 PFUs per 100 mL [20,48,71,94,95]. With respect to community septic tanks, somatic and F-specific coliphages were detected in all samples tested, with values ranging from 10^6 to 10^7 PFU per 100 mL and 10^5 to 10^6 PFU per 100 mL, respectively [94].

Regarding animal contamination sources, the reported somatic coliphage values range from 10^4 – 10^5 PFU per 100 mL in slurry and manure to 4×10^8 in abattoir wastewater, whereas the reported values for F-specific bacteriophages range from 10^4 PFU per 100 mL in animal waste slurries to 2×10^8 in abattoir wastewaters [48,96,97].

Primary sedimentation, flocculation-aided sedimentation, activated sludge digestion, activated sludge digestion followed by precipitation, and trickling filters remove somatic coliphages and F-specific bacteriophages in numbers ranging from 50% to 99.9%. The most frequent somatic coliphage values in secondary effluents therefore normally range from 10^3 to 10^5 PFU per 100 mL and those of F-specific and RNA F-specific phages from 10^2 to 5×10^4 PFU per 100 mL [20,48,74,76,95,98]. The relative proportions of somatic coliphages and F-specific and RNA F-specific bacteriophages are similar to those found in untreated wastewater.

The concentrations of somatic and F-specific phages detected in effluents released by lagooning are relatively variable depending on the extent of the treatment. In climates with considerable seasonal variation, the quality of the effluents in terms of pathogens and indicators numbers may vary from summer to winter in the same treatment facility [74,96,99,100]. In any case, the relative proportion of somatic coliphages to F-specific RNA phages remains the same or increases during warm periods.

The same trend, *i.e.*, somatic coliphages outnumbering F-specific phages, is true for primary and raw sludge. Concentrations above 10^7 PFU for somatic coliphages and well over 10^6 PFU for F-specific bacteriophages per gram of dry weight of primary and raw sludge have been reported [81,101].

5.2. Persistence in Water Environments and Resistance to Treatment

Numerous data are available on persistence experiments conducted with laboratory-grown model somatic coliphages (ϕ X174, PDR1, T2, and T7) and F-specific coliphages (f2, MS2, Q β). Nevertheless, we will limit the discussion to naturally occurring coliphages. Concentrations of these phages in raw sewage are high enough to carry out model inactivation experiments after diluting sewage in fresh, brackish, or marine water.

Sinton *et al.* performed *in situ* inactivation experiments with sewage and/or waste stabilization pond effluent mixed with river or seawater in a proportion of 10% and placed at a depth of 560 mm (300 L) in open-top chambers [102,103]. Experiments were performed in winter (approx. 12 °C) and summer (approx. 16 °C), and in the dark and with sunlight. The calculated T_{90} for somatic coliphages ranged from seven hours in seawater, summer, and sunlight to 2303 hours in river water, winter, and darkness; the calculated T_{90} for F-specific RNA bacteriophages ranged from 4.8 hours in seawater, summer, and sunlight to >2303 hours in river water, winter, and darkness.

Durán *et al.* and Mocé-Llivina *et al.* mixed raw municipal wastewater at a proportion of 2% with either river or seawater and placed the mixture in a dialysis tube with a cutoff of 14,000 Daltons that was sealed and placed at a depth of 20 cm to 25 cm in the same site where the river and seawater were collected [104,105]. These sites were in the shadow for part of the day. With respect to somatic coliphages, T_{90} ranged from 53 hours in seawater and summer (>25 °C) to 385 hours in winter (6–10 °C) and river water, whereas F-specific RNA T_{90} ranged from 14 hours in seawater and summer to 323 hours in river water and winter.

Studies of the abatement of both phage groups after releasing manure, slurry, and sludge into soil are scarce. Following the land application of liquid pig manure, Gessel *et al.* found that the numbers of somatic coliphages after application were always higher than the values of F-specific phages, and that the proportion of somatic coliphages to F-specific phages increased moderately with time [106].

At this point in the review, it is worth mentioning that the great majority of data regarding the persistence and presence of F-specific RNA bacteriophages in receiving waters have been obtained in cold and temperate climates. However, some evidence indicates that in areas or periods with surface water temperatures higher than 25 °C the picture might be different. Thus, in the experiments performed in the summer [104,105], the T_{90} of *E. coli* and F-specific RNA bacteriophages were close; this situation was not observed for either somatic coliphages or phages infecting *Bacteroides* that show more persistence than *E. coli*. Also, with respect to oxidation ponds, which can be used for *in situ* inactivation experiments, it was reported that, in a series of stabilization ponds in the dry and warm desert of Judea (Israel), F-specific bacteriophages were removed more efficiently than fecal coliforms and somatic coliphages, primarily during the summer [100]. Moreover, F-specific RNA bacteriophages occurring in secondary effluents are significantly more sensitive to temperatures ranging from 25 °C to 40 °C than *E. coli* and somatic coliphages [107]. Cole *et al.* and Rahman *et al.* determined the numerical ratios between F-DNA phages and F-RNA phages in surface waters and observed that in summer F-specific RNA phages were in the minority, whereas they accounted for more than 90% during the other seasons [108,109]. The abatement of F-specific RNA phages in sludge mesophilic (30–35 °C) anaerobic digestion is faster (approximately two \log_{10} units *versus* one \log_{10} unit) than that of somatic coliphages or *Bacteroides* phages [81,101]. This acceleration in the inactivation kinetics of F-specific bacteriophages with increasing temperatures is also observed in experiments with laboratory-grown bacteriophages. Thus, McLaughlin and Rose described very high decay rates for MS2, an F-specific bacteriophage, in seawater at 30 °C [110], and Allwood *et al.* revealed that the decay rate of MS2 at 37 °C is even greater than that of *E. coli* [111]. All these results suggest that the persistence of F-specific RNA phages might be rated as low in waters with temperatures greater than 25 °C. Since geographic

areas with surface seawater and fresh water temperatures exceeding 25 °C either all year round (between 40° latitude north and 40° latitude south) or during the warm seasons (many areas of Europe and the USA) are fairly extensive, the persistence of F-specific and RNA F-specific bacteriophages requires additional verification before their persistence in warm areas can be rated.

5.3. Resistance to Treatment

It has been mentioned previously that all indicators in general and somatic coliphages and F-specific bacteriophages are eliminated in a similar way in primary and secondary wastewater treatment. However, the situation changes frequently when additional treatments such as filtration through depth filters (sand, anthracite, *etc.*) or membrane filtration and either chemical or physical disinfection are used.

There is a great deal of literature on the effects of these treatments in certain specific somatic (ϕ X174, PDR1, T2, and T7) and F-specific bacteriophages (ϕ 2, MS2, Q β). However, as indicated in a previous section, both groups are heterogeneous and what happens to a particular bacteriophage may not reflect what happens to the whole group. The significant amounts of somatic and F-specific coliphages in some matrices (secondary effluents, some contaminated rivers, raw sludge, *etc.*) make it possible to reveal the effect of the treatments on both groups of bacteriophages as a whole. A certain amount of information is available from pilot or full-scale tertiary treatments used for water reclamation, sludge treatment, and filtration through soil. As discussed below, the two groups respond differently depending on the treatment.

Depth filtration (sand, anthracite, *etc.*), precipitation/flocculation plus filtration [96,112–114], and even membrane filtration reactors [20,114–116] remove somatic and specific coliphages found in secondary effluent in equal measures. Consequently, the proportions of the two groups of bacteriophages are maintained after filtration and somatic coliphages normally outnumber F-specific bacteriophages in the permeated waters.

Chlorination causes a greater effect on somatic coliphages than on F-specific phages, but this difference is not great enough to change the relative proportions, and somatic coliphages outnumber F-specific phages in most chlorinated secondary effluents [76,113,117,118].

With respect to radiation, UV irradiation has a significantly greater effect on somatic coliphages than on F-specific phages, and after strong UV treatment F-specific numbers surpass those of somatic coliphages in many settings [76,117].

Photo-oxidation (H₂O₂, TiO₂) used to be a highly effective treatment for bacteriophages, as described for F-specific bacteriophages, but when photo-oxidizing a secondary effluent, Agulló *et al.* detected significantly greater elimination of F-specific bacteriophages and thus concluded that effluents harbor greater numbers of somatic coliphages [107].

Most sludge treatments required prior to its release in soil are based on heat. Major F-specific phage inactivation is already observed in mesophilic digestion (30–35 °C) and all treatments with higher temperatures increase the difference [81,101,119], so the great majority of biosolids released into the environment contain higher numbers of somatic coliphages than F-specific phages.

Regarding the effect of soil filtration on the abatement of both phage groups, studies that compare both groups are once again in short supply. Sinton *et al.* reported that F-specific RNA phages showed greater attenuation than somatic coliphages in an alluvial gravel aquifer [120].

5.4. Abundance of Bacteriophages in Surface Water and Groundwater

Due to the discharge of raw or treated contaminants and their inactivation once in nature, the gap between the numbers of somatic and F-specific phages in receiving surface water and groundwater is equal to or greater than the gap at the source, with a few exceptions.

Reports concerning concentrations of both phage groups measured by either the USEPA or ISO methods in fresh and marine surface waters indicate that the numbers of somatic coliphages are greater than the numbers of F-specific phages (Table 1).

Most reports on phages in groundwater only account for their presence or absence in a given volume. A few examples of the values found are reported in Table 2. Somatic coliphages predominate overall, but in this case somatic coliphages prevail in some aquifers and F-specific phages in others. This is very likely to depend on the characteristics of the soil, since both phage groups have been reported to adsorb differently to different minerals [121].

Table 1. Number of bacteriophages (PFU / 100mL) in surface water. * Average per site.

Samples	Methods	Number of Samples	Geographical Location	Somatic Coliphages (% +)	F-Specific (% +)	RNA F-Specific (% +)	Reference
River water	ISO	392	Spain, France, Colombia, Argentina	6.2×10^3 (?)		5×10^2 (?)	[94]
Freshwater reservoir	USEPA	65	Singapore	2.2×10^2 (98)	1.1×10^2 (98)		[23]
Sea water	ISO	806 for somatic and 427 for RNA F-specific phages	Spain	32.8 (72.6)		8 (25.5)	[122]
Fresh and sea water	ISO	139	9 European countries, 13 sampling sites	1.7×10^2 (92)		12 (50)	[71]
Fresh and sea water	USEPA	12	California	$2.0\text{--}3.3 \times 10^2$ (100)	$<0.02\text{--}30$ (25)		[123]
River water	USEPA	120	California	$6.0\text{--}10^3$ (?)	$5.0\text{--}1.1 \times 10^2$ (?)		[124]
River water	ISO	96	France	1.7×10^3 (100)	2.0×10^2 (92)		[125]
Sea water	ISO	20	Spain	$<10\text{--}1.2 \times 10^4$ (95)		0–84 (15)	[104]
River water	ISO	75 (10 sites)	The Netherlands	$*8.8\text{--}4.3 \times 10^2$ (100)	$0.04\text{--}93.6$ (?)		[78]
Sea water	USEPA	436	California	3.1–4.9 (median)	0.3 (median)		[126]

Table 2. Bacteriophages in groundwater (% of positive samples).

Sampling site	Method	Number of Samples	Geographical Location	Somatic Coliphages	F-Specific Phages	RNA F-Specific Phages	Reference
Wells of varied characteristics	USEPA	160	Canada	8.7	1.8		[127]
Variety of wells and springs	ISO	197	Argentina, Colombia, France, Spain	41.7		28.8	[128]
Variety of wells	USEPA	39	Korea	12.5	7.5		[129]
Wells and springs	ISO	125	Spain	53.6		36.0	[130]
Variety of wells	F+ ISO, somatic coliphages C3000	444	USA	10.8	9.5		[131]

5.5. General Conclusions for Practical Uses

The general trend is that somatic coliphages outnumber F-specific coliphages. However, there are some situations, such as reclaimed water, in which UV plays the main role in inactivation [117], clayey sediments [121], and groundwater from certain aquifers [128], in which F-specific phages have been observed to predominate.

Therefore, detecting both bacteriophage groups simultaneously would appear to be a good option. Strain *E. coli* C3000 (ATCC 15597) has been used extensively for this purpose, mainly in the USA, but it detects lower numbers of somatic coliphages than strains CN13 and WG5. More recently, Guzmán *et al.* tailored a strain [49], CB390, which detects both phage groups, and reported numbers in Spain similar to the sum of those detected by WG5 or CN13 and those detected by strains WG49 or F (amp) HS. Recently, this strain has been validated in the USA [132] and has proved to be just as efficient as in Spain.

6. Relationship of Coliphages to the Occurrence of Human Viruses and to Health Risk

6.1. Relationship to Human Viruses

In addition to their value as indicators of fecal contamination, indicator bacteriophages have been viewed as potential surrogates of human viruses. Consequently, the possible relationship between the presence and levels of indicator phages and human viruses in water has been studied, though with disparate results.

Thus, many studies have failed to show any correlation in fresh surface waters [133–137], marine surface water [136–138], and groundwater [131,139]. In contrast, other studies have shown some correlation between human viruses and F-specific phages [23,123,126,140,141] and between human viruses and somatic coliphages in surface waters [104,140,142], between enteroviruses and somatic coliphages in sludge [82], and between human viruses and both somatic and F-specific phages in surface water [78]. Thus, even if the results far from indicate a clear correlation between indicator coliphage densities and human viruses in water, there is evidence that somatic and F-specific coliphages are more strongly associated with pathogenic viruses than the traditional bacterial indicators and even a particular pathogenic virus. Accordingly, low rates of co-occurrence or an absence of correlation among different human viruses have been reported in different settings. In groundwater these failures have been identified for enteroviruses, rotaviruses, hepatitis-A viruses, and noroviruses [131,139]; noroviruses, enteroviruses, rotaviruses, adenoviruses, and hepatitis-A viruses [129]; enteroviruses and noroviruses [127]; and enteroviruses, adenoviruses, and reoviruses [143]. In surface waters, a lack of co-occurrence or correlation has been reported for adenoviruses and enteroviruses [136]; enteroviruses and adenoviruses [142]; enteroviruses and adenoviruses [124]; adenoviruses and noroviruses [144]; and enteroviruses and polyomaviruses [145]. In contrast, some studies on surface waters have shown correlations among different pathogenic viruses, such as astroviruses, noroviruses, rotaviruses, and adenoviruses [23]; enteroviruses, reoviruses, noroviruses, and rotaviruses [78]; and adenoviruses and noroviruses [141]. However, these three studies also report correlations between human viruses and coliphages.

Potential theories have been proposed to explain these unequal results. One is that somatic and F-specific coliphages are released into the environment by humans and animals, whereas human viruses are not. For this purpose, the use of bacteriophages found mostly in humans, such as those detected by some specific strains of *Bacteroides* or some genogroups (II and III) of F-specific phages [146], would probably offer more congruent results. Other reasons are strictly methodological, since these studies vary somewhat in terms of the parameters assessed and the concentration and detection procedures used. Bacteriophages have usually been evaluated by plaque assay using standardized (or equivalent) methods in small volumes, and without a concentration procedure. Human viruses have been evaluated after the concentration of great volumes and the subsequent detection using very different methods, such as plaque assay on cell culture and different PCR approaches. Most of these counts, which have mostly been in matrices where the numbers of phages and viruses are low, are subject to a great amount of uncertainty, which partly explains the considerable heterogeneity of the results. Moreover, in our opinion, the statistical methods used to establish associations are not always suited to the characteristics of the data, which are frequently characterized by predominance in non-detected values, with detection limits for the different parameters analyzed not always equivalent.

6.2. Relationship to Health Risk

With respect to the relationship to health risk, seven epidemiological studies conducted to evaluate the relationships between the presence of indicator bacteriophages in surface waters and swimming illnesses have been carried out with disparate outcomes. Three of them only studied F-specific phages, and while Lee *et al.* and Wade *et al.* found some correlation [147,148], Van Asperen *et al.* did not [149]. Wiedenmann *et al.* only studied somatic coliphages and found some correlation [150]. In the studies where both phages were tested, the results are incongruent, since Von Schirnding *et al.* failed to

show any correlation with either somatic or F-specific phages [151], Colford *et al.* found a correlation with F-specific phages [152], and Abdelzaher *et al.* found a correlation with somatic coliphages [145]. No epidemiological studies to correlate phages and disease in drinking water have been performed. However, some overlap between a jaundice outbreak and a high incidence of somatic coliphages in drinking water in a municipality of West Bengala, India was reported in 2014 [153].

Epidemiological studies on the relationship between the presence of human viruses and disease associated with water are scarce and those available fail to show any association between pathogenic viruses in surface waters and infections associated with bathing [145,152].

7. Conclusions

The joint threshold concentrations of the bacterial hosts required to guarantee phage replication and the known concentrations of coliphages detected by the host strains of standardized methods limit the sites where these numbers are reached. Moreover, some basic aspects of phage biology indicate that the replication of coliphages outside the gut is highly inefficient. Therefore, coliphage replication in the water environment cannot be ruled out, but if it does occur the resulting numbers will be lower than those contributed by fecal waste.

There are easy, rapid, and cost-effective standardized methods for detecting and enumerating coliphages. Implementing these methods without the need for extra equipment in routine laboratories is feasible after a little training. It should be relatively easy to adapt recent developments to very fast methods (three to four hours) based on the detection of molecules released into the culture medium by phage-induced host lysis to user-friendly commercial kits.

In fecal pollution sources and most water environments with varying degrees of pollution, numbers of somatic coliphages exceed those of F-specific coliphages. Persistence in the environment is similar, with the exception of warm water, where the survival of F-specific RNA phages is relatively low. With respect to resistance, this depends on the treatment; thus, while F-specific phages survive better after chlorination and UV irradiation used in tertiary treatments, somatic coliphages are more resistant to the thermal treatments used in sludge hygienization. With a few exceptions, the difference in resistance is not enough to make F-specific phages more abundant than somatic coliphages. The general trend is that somatic coliphages outnumber F-specific coliphages in a wide range of samples. However, there are some situations, such as reclaimed water, in which UV plays the main role in inactivation, clayey sediments, and groundwater from certain aquifers, in which F-specific phages have been observed to predominate. Therefore, detecting both bacteriophage groups simultaneously would appear to be a good option. For this purpose, strain CB 390 of *E. coli*, which detects both groups in numbers similar to those detected by somatic and F-specific standardized methods, is available.

Possible correlations between the presence and levels of coliphages and human viruses in water have been studied, though with disparate results. However, the correlations between the presence and levels of different human viruses are no higher.

The epidemiological evidence of the correlation between coliphage concentrations in water and health risks suggests a likely relationship, although no sound statistical correlations have been found. The same statement is valid for human viruses.

As a general conclusion, coliphages that can be detected by easy, rapid, and cost-effective methods are likely to be a better indicator of viruses in fecal contamination than the current FIB (*i.e.*, enterococci and *E. coli*).

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