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Bacteriophages as Surrogates for the Fate and Transport of Pathogens in Source Water and in Drinking Water Treatment Processes

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1. Introduction

Less than 1% of the world's fresh water accessible for direct human uses is found in lakes, rivers, reservoirs and those underground sources that are shallow enough to be tapped at an affordable cost. Only this amount is regularly renewed by rain and snowfall, and is therefore available on a sustainable basis (Berger, 2003).

More than a billion people have limited access to safe drinking water; over 2 million die each year from water-related diarrhea, which is one of the leading causes of mortality and morbidity in less economically developed countries (UNICEF and WHO, 2009). In more economically developed countries, increasing demands on water resources raise concerns about sustainable provision of safe drinking water. In 2008, supply and protection of water resources was identified as the top strategic priority of North American water professionals (Runge and Mann, 2008). This is not surprising given the rapidly expanding competition for existing water supplies from industrial, agricultural and municipal development, as well as the vital needs to protect human health and ecosystem functions. The challenge of sustaining supply is further exacerbated by changes in water quality and availability as a direct or indirect result of population growth, urban sprawl, climate change, water pollution, increasing occurrence of natural disasters, and terrestrial and aquatic ecosystem disturbance.

Most of the world population depends on groundwater for their supplies. Due to the proximity of groundwater to sources of microbial contamination, the increasing occurrence of extreme climate events and the lack of adequate disinfection, groundwater is responsible for a large percentage of the waterborne outbreaks of disease worldwide (WHO, 2004; 2011). For example, between 1999 and 2000, 72% of drinking water outbreaks of disease were associated with groundwater. Although the number of groundwater-associated disease outbreaks associated in the United States decreased during 2001–02, the proportion of outbreaks associated with groundwater increased to 92% from 87% (Tufenkji and Emelko, 2011). As a result of such outbreaks and the economic implications of waterborne illness, stricter water quality regulations to protect public health have been implemented in many countries. Significant examples of such regulations include the Surface Water Treatment Rules (SWTR -1989a; 2002) and the Ground Water Rule (2006) by the U.S. Environmental

Protection Agency (USEPA); the revised Bathing Water Directive (2006/7/EC) and the Water Framework Directive (2000/60/EC) by the European Union. The pressure generated by such regulations has increased the need to quantitatively understand and describe microbial pathogen transport and survival in various natural and engineered environments, including treatment systems.

Monitoring the fate and transport of all of the various microorganisms that can cause outbreaks of waterborne disease is cost prohibitive; accordingly, representative organisms such as “indicators” of pathogenic contamination or “surrogates” for the transport and survival of pathogens in various environments are sought. While indicators often originate from the same source and act as signals of pathogen presence, surrogates may or may not be derived from the same source as pathogens and are often introduced into natural and engineered environments to pseudoquantitatively assess pathogen fate and transport. Commonly used surrogates for such investigations include several bacteria, aerobic and anaerobic bacterial endospores, numerous bacteriophages, microbe-sized microspheres, chemically inactivated protozoa, and nonpathogenic, fluorescently labeled bacteria and protozoa (Tufenkji and Emelko, 2011). Bacteriophages meet many of the requirements of “ideal” surrogates because they have many characteristics that are similar to those of mammalian viral pathogens (i.e., size, shape, morphology, surface chemistry, isoelectric points, and physiochemistry), are unlikely to replicate in environments such as the subsurface due to a lack of viable hosts and other limiting factors, pose little risk to the health of humans, plants, and animals, and are easier and less expensive to isolate and enumerate relative to enteric viruses (Tufenkji and Emelko, 2011). All of these factors contribute to the utility of bacteriophages as surrogates for microbial pathogen transport and fate in source waters and in drinking water treatment processes.

This chapter focuses on the utility of bacteriophages as surrogates for the fate and transport of microbial pathogens of health concern in source and drinking waters, with particular reference to: (1) indicating the presence of enteric viruses in natural waters, (2) contributing to microbial source tracking, (3) evaluating the effectiveness of water treatment processes such as disinfection and filtration, and (4) elucidating the mechanisms involved in the fate and transport of enteric viruses in natural or engineered filtration media. Present knowledge acquired through laboratory and field approaches is reviewed and further research needs are identified to respond to current and future challenges in this field.

1.1 Major waterborne microbial pathogens of concern

Although water-transmitted microbial pathogens include bacteria, protozoa, helminthes and viruses, the groups of major threat to human health in freshwater supplies are pathogenic protozoa and enteric viruses (Schijven and Hassanizadeh, 2000) (Table 1). The protozoans *Cryptosporidium* and *Giardia* are among the major causal agents of diarrhoeal disease in humans and animals worldwide, and can even potentially shorten the life span of immunocompromised hosts (WHO, 2004). Their resistant forms (cysts or oocysts) are shed in large numbers by infected animals or humans and are ubiquitous in surface water. They are resistant to harsh environmental conditions and to chemical disinfectants at concentrations commonly used in water treatment plants to reduce bacterial contamination (LeChevallier et al., 1991; Rose, 1997; Karanis et al. 2002; Aboytes et al., 2004). Their small size (*Giardia* cysts 8-13 μm and *Cryptosporidium* oocysts 4-6 μm) and infectious dose (as low as a single organism -

Health Canada, 2004), also contribute to waterborne disease transmission. Several studies have revealed little or no correlation between bacterial fecal indicator and protozoan (oo)cyst densities in source surface waters (reviewed by Health Canada, 2004). These observations highlight the need for: (1) routine monitoring of surface waters for protozoan (oo)cysts or for reliable indicators of their presence and infectivity, and (2) implementation of improved drinking water technologies to effectively protect public health.

Group	Pathogen	Disease
Enteric viruses	Poliovirus	Meningitis, paralysis, fever
		Echovirus Meningitis, diarrhea, rash, fever, respiratory disease
	Coxsackievirus A	Meningitis, herpangina, fever, respiratory disease
	Coxsackievirus B	Myocarditis, congenital heart anomalies, pleurodynia, respiratory disease, fever, rash, meningitis
	New enteroviruses (types 68-71)	Meningitis, encephalitis, acute hemorrhagic conjunctivitis, fever, respiratory disease
	Hepatitis A	Hepatitis
	Enterovirus 72	Infectious hepatitis
	Norovirus	Diarrhea, vomiting, fever
	Calicivirus	Gastroenteritis
	Astrovirus	Gastroenteritis
	Reovirus	Not clearly established
	Rotavirus	Diarrhea, vomiting
	Adenoviruses	Respiratory disease, eye infections, gastroenteritis
	Snow mountain agent	Gastroenteritis
	Epidemic non-A non B hepatitis	Hepatitis
Enteric Protozoa	<i>Acanthamoeba</i> spp	Amoebic encephalitis or keratitis
	<i>Entamoeba histolytica</i>	<i>Cryptosporidium parvum</i> amoebic dysentery
		<i>Giardia lamblia</i> Giardiasis (gastrointestinal disease)
	<i>Naegleria fowleri</i>	Amoebic meningoencephalitis
	<i>Toxoplasma gondii</i>	Toxoplasmosis

Table 1. Water-transmitted microbial pathogens of major concern in drinking water (adapted from: Azadpour-Keeley et al., 2003; CDC, 2003).

The collective designation “enteric viruses” includes more than 140 serological types that multiply in the gastrointestinal tract of both humans and animals (AWWA, 2006). Enteric viruses associated with human waterborne illness include noroviruses, hepatitis A virus (HAV), hepatitis E virus (HEV), rotaviruses and enteroviruses (polioviruses,

coxsackieviruses A and B, echoviruses and four ungrouped viruses numbered 68 to 71) (AWWA, 2006). Enteric viruses are widespread in sewage and some have been detected in wastewater, surface water and drinking water (Gerba and Rose 1990; Payment and Franco, 1993; AWWA, 2006). Although they cannot multiply in the environment, they can survive for several months in fresh water and for shorter periods in marine water (Health Canada, 2004).

Enteric viruses are the most likely human pathogens to contaminate groundwater because they are shed in enormous quantities in feces of infected individuals (10^9 to 10^{10} /g) (Melnick and Gerba, 1980) and their extremely small size (20 to 100 nm) allows them to infiltrate soils, eventually reaching aquifers (Borchardt et al., 2003) (Fig. 1). Depending on physicochemical and virus-specific factors (e.g. size and isoelectric point), viruses can move considerable distances in the subsurface environment (Vaughn et al., 1983; Bales et al. 1993) and persist for several months in soils and groundwater (Keswick et al., 1982; Gerba and Bitton, 1984; Yates et al., 1985; Sobsey et al., 1986; Gerba and Rose, 1990; John and Rose, 2005). Enteroviruses also have been shown to be more resistant to disinfection than indicator bacteria (Melnick and Gerba, 1980; Stetler, 1984; IAWPRC, 1991).

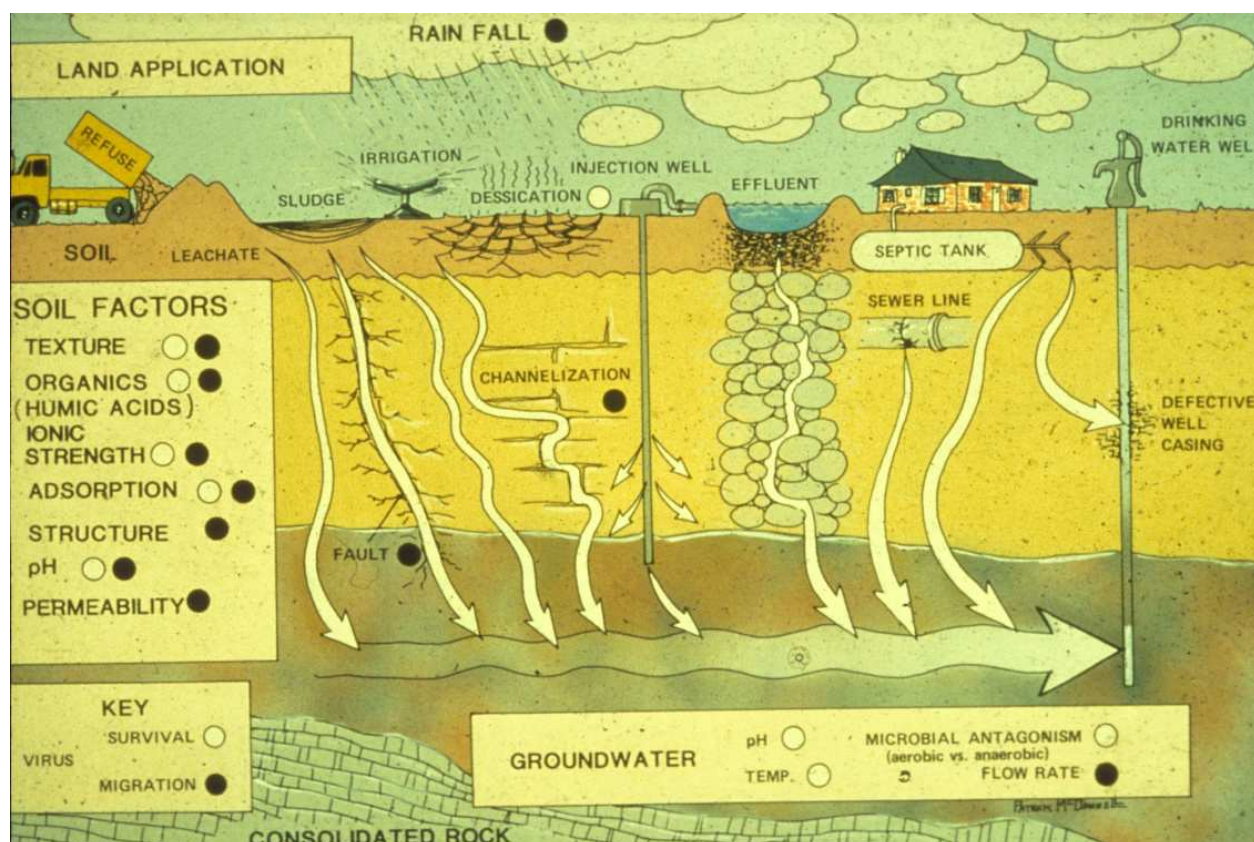


Fig. 1. Migration and survival of viruses and protozoa in the subsurface (adapted from Keswick and Gerba 1980 with permission).

1.2 Source water protection and treatment

In general the multiple-barrier approach to water treatment including watershed or wellhead protection, optimized treatment including disinfection, a well-maintained distribution system, monitoring the effectiveness of treatment, and safe water storage, is the

best approach for reducing the risk of infection to acceptable or non-detectable levels (Health Canada, 2004). Surface and groundwater protection from microbial contamination largely depend on adequate land use policies related to: (1) waste and wastewater management practices, (2) the interaction of contaminated surface water with groundwater supplies (including artificial recharge with treated wastewater) and (3) the effective placement and protection of drinking water wells. Pathogenic protozoa and enteric viruses are considered priority microbial contaminants in drinking water legislation because of the significant role they play in waterborne disease outbreaks and the associated risks to public health, their extended survival in the environment, their considerable resistance to conventional water disinfection processes compared to bacteria, and the often poor or lacking correlation with traditional bacterial water quality indicator numbers.

Commonly used free chlorine concentrations and contact times applied in drinking water treatment are effective in inactivating enteric viruses (Thurston-Enriquez et al. 2003; Health Canada 2004). Ozone is generally considered more efficient against both protozoa and enteric viruses than chlorine or chlorine dioxide (Erickson and Ortega 2006). UV light disinfection, although highly effective for inactivation of protozoa, is not as efficient at inactivating viruses as more traditional chlorine-based disinfection processes (Health Canada, 2004). More recently, the combined performance of UV light and chlorine has been suggested as more effective for reclaimed water disinfection than the use of each process separately (Montemayor et al., 2008).

Effective “green” ways to remove existing and emerging pathogens and produce safe drinking water at lower cost have received much attention in recent years. These include the passage of surface water and/or groundwater through porous media in the subsurface during processes such as riverbank filtration, dune recharge, aquifer storage and recovery, and deep well injection. The need to develop regulations to protect public health coupled with the infeasibility of concentration-based criteria for all known waterborne pathogens has resulted in the evolution of regulatory approaches for water quality and treatment that rely on performance indicators and surrogates and assume specific levels of pathogen reduction through well-operated treatment systems (Tufenkji and Emelko, 2011).

1.3 Global quest for an effective pathogen indicator

Because routine monitoring for pathogens is usually costly and often unrealistic, the use of surrogate parameters (i.e. microbial indicators) to predict the presence of pathogens in water and model their behavior has long been pursued. For decades fecal bacterial indicators (e.g. fecal coliforms and *E.coli*) have been useful to identify fecal contamination to indicate the probable presence of microbial pathogens in water (Payment and Locas, 2011). However, their concentrations rarely correlate well with those of pathogens. Thus, bacterial indicators may signal the probable presence of pathogens in water, but they cannot predict precisely their level of occurrence (Payment and Locas, 2011). They are also not reliable pathogen surrogates because when compared with both virus and protozoa, bacterial indicators are less persistent in the aquatic environment and less resistant to disinfection and removal by other water treatment processes (IAWPRC 1991; Payment and Franco, 1993).

Some enteroviruses have been evaluated for monitoring environmental waters and tracking sources of water pollution (Metcalf, 1978; Goyal, 1983; Payment et al., 1985). However, the limitations associated with their use soon became apparent: (1) they are not constant inhabitants of the intestinal tract and are excreted only by infected individuals and small children, (2) laboratory methods for their detection and quantification are time-consuming, expensive, require high expertise and are restricted to some enteroviruses subgroups, and (3) virion size, surface characteristics and resistance to external agents such as disinfectants vary among subgroups. Some studies have suggested using adenoviruses as an index of human pollution because they have been shown to be more persistent and present in greater numbers than enteroviruses in sewage and fecal contaminated aquatic environments (Pina et al. 1998, Thurston-Enriquez et al. 2003).

When sewage is the source of enteric viruses and protozoa, spores of the anaerobic bacterium *Clostridium perfringens* have been suggested as suitable indicators of the presence and behavior of these pathogens in aquatic environments (Payment and Franco, 1993). Both *Bacillus* spp. aerobic endospores and *Clostridium perfringens* spores have been used as models for the removal of protozoa (oo)cysts and enteric viruses by drinking water treatment processes (Payment and Franco 1993, Rice et al. 1996).

Increasing awareness of the shortcomings of fecal bacteria as indicators of the presence of pathogenic viruses and protozoa in the environment has attracted attention to the potential value of bacteriophages that infect enteric bacteria as indicators and surrogates for evaluating the presence and behavior of human pathogenic viruses in aquatic environments and during water treatment (Noonan and McNabb, 1979; Stetler, 1984; Gerba, 1987; Havelaar, 1987; Havelaar et al., 1993). However, while phage meet many of the requirements as surrogates for enteric viruses and are useful in certain situations, they are not universal indicators, models or surrogates for enteric viruses in water environments because several disadvantages can be associated with their use (further discussed in section 3). For example, enteric viruses have been detected in treated drinking water supplies that yielded negative results for phages, even in presence-absence tests on 500 mL water samples (Ashbolt et al., 2001).

Many years of research gradually elucidated that variations in pathogen input, dilution, retention, and die-off in water environments result in conditions in which relationships/correlations between any pathogen and any indicator may be random, site-specific, and/or time-specific (Grabow, 1996; Payment and Locas, 2011). As a consequence, the present general scientific consensus is that there is no universal indicator of microbial water quality. Each specific situation, set of conditions, and objectives of study require a great deal of judgment to select the best group(s) of pathogen indicator(s) and/or surrogate(s) to be used most effectively (Table 2). Improved molecular detection techniques (e.g. PCR amplification or hybridization) based on host specificity of targeted viral and protozoan pathogens and surrogates in environmental samples may soon enable more reliable source tracking and improved public health surveillance (Scott et al. 2002; Fong and Lipp, 2005). Similarly, in-line microbial and chemical analytical systems installed at critical treatment points may replace microbial indicators and may provide continuous monitoring and reliable data, facilitating decision making. To further assist in process evaluation, efforts also have been made to eliminate ambiguities in the term “microbial indicator”. Several subgroups based on function have been recognized and are now commonly used in the

literature, such as: process indicators or surrogates (useful for demonstrating the efficiency of a process), fecal indicators (that indicate the presence of fecal contamination and imply that pathogens may be present), and index or virus models (indicative of pathogen presence and behavior respectively) (Ashbolt et al. 2001).

Group	Use (publisher use indents)
<i>E. coli</i>	- Indicator of recent fecal pollution and of <u>potential</u> presence of enteric pathogens in water
Enterococci	- Indicators of fecal pollution and indirectly of the <u>potential</u> presence of enteric viruses in groundwater
Somatic coliphages	- Index of sewage contamination - Process indicators - helpful as viral surrogates in evaluating efficiency of drinking water treatment - Some useful as pathogenic viruses models and tracers in transport studies in the subsurface and groundwater
F-RNA phages	- Index of sewage contamination - Index and models of human enteric viruses in contaminated freshwater and shellfish - Process indicators- helpful as viral surrogates in evaluating efficiency of drinking water treatment - Useful in microbial source tracking - Some useful as pathogenic viruses models and tracers in transport studies in the subsurface and groundwater
Phages of <i>B.fragilis</i>	- Indicators of human fecal pollution - Useful in microbial source tracking
<i>C. perfringens</i> spores	- Fecal indicators of both recent and past contamination in surface waters. - Process indicators - helpful as viral and protozoan (oo)cysts surrogates in evaluating drinking water treatment efficiency (e.g. disinfection)

Table 2. Most commonly used pathogen surrogates and their uses (Sources: Havelaar et al. 1993, Health Canada 2004, Payment and Locas 2011)

2. Multifunctionality of bacteriophages

Estimated to be the most widely distributed and diverse entities in the biosphere (McGrath and van Sinderen, 2007), bacterial virus, bacteriophages or phage can be found in all environments populated by bacterial hosts, such as soil, water and animal guts. Their unique characteristics bring several advantages to their use as pathogen surrogates (Table 3). Phages have been successfully used in a variety of environmental applications as follows:

- As **fecal indicators** - the environmental occurrence and persistence of some groups relate to health risks associated with fecal pollution and the potential occurrence of enteric pathogens in aquatic environments (Havelaar, 1987; IAWPCR, 1991; Leclerc et al., 2000; Morinigo et al., 1992; Lucena et al., 2006; Lucena and Jofre, 2010). As a result

<p>Advantages</p> <ul style="list-style-type: none">i. Have no known impact on the environmentii. Are non-toxic and non-pathogenic for humans animals or plantsiii. Have a specific affinity to their bacterial hostiv. Are reasonably similar to mammalian viral pathogens in size, shape, morphology, surface properties, mode of replication and persistence in natural environmentsv. Are colloidal in nature which makes them more adequate virus models then dissolved tracersvi. Are stable over periods of several months under laboratory conditions,vii. Can be detected and enumerated by rapid and inexpensive methods with low detection limits (1 to 2 phage per mL)viii. Can be prepared in large quantities at high concentrationsix. Specific phage groups are similar to specific pathogenic viral groups allowing the use of phage cocktails to simultaneously target several groups of concern. <p>Disadvantages</p> <ul style="list-style-type: none">i. Are excreted by a certain humans and animals all the time while pathogenic viruses are excreted by infected individuals for a short period of time (depending on the epidemiology of viruses, outbreaks of infection, and vaccination). Consequently there is no direct correlation between numbers of phages and viruses excreted by humansii. A wide range of different phage can be detected by methods for somatic coliphagesiii. At least some somatic coliphages may replicate in water environmentsiv. Enteric viruses have been detected in water environments in the absence of coliphagesv. Pathogenic human enteric viruses are excreted almost exclusively by humans, while bacteriophage used in water quality assessment are excreted by humans and animals.vi. The microbiota of the gut, diet and physiological state of animals seems to affect the numbers of coliphages in their fecesvii. The composition and numbers of phages excreted by humans is variable (e.g. patients under antibiotic treatment excrete lower numbers than healthy or non- medicated individuals)viii. As water flows through porous media in the subsurface or engineered filtration processes phage can attach, detach, and re-attach by physico-chemical filtration mechanisms.
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Table 3. Advantages and disadvantages of the use of bacteriophages as viral pathogen surrogates and tracers in aquatic environments (Sources: Havelaar et al., 1993; Ashbolt et al., 2001; Bateman et al., 2006).

phage infecting enteric bacteria are now accepted as useful indicators in water quality control and included in some regulations as required parameters. For example, coliphages are used in the US Water Ground Rule (USEPA, 2006), the drinking water quality regulation for the Canadian Province of Quebec (Anonymous, 2001) and a few USA states regulations regarding required quality for reclaimed water for certain uses (USEPA, 2003).

- In **microbial source tracking** (MST) or identification of fecal contamination sources by genotypic, phenotypic, and chemical methods, phage have proven useful based on their host specificity (Hsu et al. 1995; Hsu et al., 1996; Simpson et al., 2003; Jofre et al., 2011). By identifying problem sources (animal and human) and determining the effect of

implemented remedial solutions MST is of special interest in waters used for recreation (primary and secondary contact), public water supplies, aquifer protection, and protection and propagation of fish, shellfish and wildlife (Simpson et al., 2003).

- As **process indicators** phage groups are often successfully employed as enterovirus surrogates in evaluating the effectiveness of water treatment processes and final product quality. This is the case with filtration and disinfection (Stetler et al., 1984; Payment et al., 1985; Havelaar et al., 1993; Durán et al., 2003; Davies-Colley et al., 2005; Persson et al., 2005; Abbaszadegan et al., 2008).
- As comprehensive pathogenic **virus indices**, phages are not very useful. This is because their numbers seldom seem to correlate to pathogenic viruses numbers in water samples when conventional statistics are applied (Lucena and Jofre, 2010). However, in the future the application of advanced mathematical models to new databases may reduce uncertainty and provide better information about relationships between phage and pathogenic virus numbers (Lucena and Jofre, 2010).
- As viral **models and tracers**, bacteriophages are often used at both field and laboratory scales as biocolloids to estimate the fate and transport of pathogenic viruses in surface and subsurface aquatic environments and through natural and manmade saturated and unsaturated porous media. This use of phage as surrogates for pathogen transport applies to protection of surface and groundwater supplies from microbial contamination, assessment of potential health risk from pathogens in groundwater and design of more efficient treatment systems in removing pathogens from drinking water supplies (Sen, 2011).

3. Main bacteriophage groups used in environmental studies

Three bacteriophage groups, somatic coliphages, male-specific F-RNA phages and *Bacteroides fragilis* phages, have been proposed and are frequently used as surrogates for pathogenic viruses in environmental studies (IAWPRC, 1991; WHO, 2004; Lucena and Jofre, 2010). However, because each group has its pros and cons as a representative of enteric virus presence and behavior in aquatic environments and water treatment processes, no agreement has been reached on which of the three groups best fulfills the index/indicator function.

3.1 Somatic coliphages

Somatic coliphages are the most numerous and most easily detectable phage group in the environment. It is a heterogeneous group whose members infect host cells (*E.coli* and other *Enterobacteriaceae*) by attaching to receptors located in the bacterial cell wall. Their numbers are low in human feces (often $<10 \text{ g}^{-1}$), but abundant in untreated domestic sewage (10^4 to 10^5 particles g^{-1}) and in animal feces (Havelaar et al., 1986).

Somatic coliphages are not usually considered good fecal indicators because some of their hosts are unlikely to be of fecal origin (Hsu et al. 1996), and some of these phage are able to multiply in waters not subjected to fecal pollution (Gerba, 2006). However, some authors argue that the number of somatic phage that replicate in environmental waters is negligible (Jofre, 2009). Moreover, they are not predictive indicators of virus presence or absence in groundwater (Payment and Locas, 2011), though some somatic phage such as T-4, T-7, Φ X174, and PRD-1 have proven useful as viral surrogates of fate and transport in laboratory investigations, pilot trials, and validation testing (WHO, 2004; Lucena and Jofre, 2010).

Phage	Family name	Type	Lipid (%)	pH _{zpc}	Hosts	Phage Size/Shape
T2, T4, T6	<i>Myoviridae</i>	Somatic Linear ds-DNA	0	—	<i>E. coli</i> and other <i>Enterobacteriaceae</i>	Cubic capsid (icosahedral or elongated), long contractile tail, 95 x 65 nm (EM)
T5, λ	<i>Siphoviridae</i>	Somatic Linear ds-DNA	0	—	<i>E. coli</i> and other <i>Enterobacteriaceae</i>	Cubic capsid (icosahedral), long non-contractile tail (150 nm), 54-60 nm (EM)
T3, T7	<i>Podoviridae</i>	Somatic Linear ds-DNA	0	—	<i>E. coli</i> and other <i>Enterobacteriaceae</i>	Cubic capsid (icosahedral), short non-contractile tail, 54-61 nm (EM)
PM2	<i>Corticoviridae</i>	Somatic Linear ds-DNA	13	7.3	<i>Pseudomonas sp.</i> , <i>Pseudoalteromonas sp.</i>	Cubic capsid (icosahedral), with spikes in vertices, no tail, 60 nm (EM)
PRD-1 *	<i>Tectiviridae</i>	Somatic Circular ds-DNA	16	4.5	<i>S. typhimurium</i> and other <i>Enterobacteriaceae</i>	Cubic capsid (icosahedral), no tail, 63 nm (EM) 82 ± 6 nm (DLS)
PR772**	<i>Tectiviridae</i>	F-specific Linear ds-DNA	—	3.8-4.2	<i>E. coli</i> and other <i>Enterobacteriaceae</i>	Cubic capsid (icosahedral), no tail, 63 nm (EM)
MS2, Qβ	<i>Leviviridae</i>	F-specific Linear ss-RNA	0	3.9; 5.2	<i>E. coli</i> and <i>Salmonella sp.</i>	Cubic capsid (icosahedral), no tail, 20-30 nm (EM)
φX174	<i>Microviridae</i>	Somatic Circular ss-DNA	0	6.6	<i>Pseudomonas sp.</i> , <i>Pseudoalteromonas sp.</i>	Cubic capsid (icosahedral), with spikes in vertices, no tail, 27 nm(EM)
SJ2, fd, M13	<i>Inoviridae</i>	F-specific Circular ss-RNA	0	—	<i>E. coli</i> and <i>Salmonella sp.</i>	Filamentous or rod-shaped, 810 x 6 nm (EM)
<i>Bacteroides fragillis</i> phages	<i>Siphoviridae</i>	Linear ds-DNA	0	—	<i>Bacteroides fragillis</i> HSP40	Icosahedral head (60 nm), flexible non-contractile tail, 150 x 8 nm (EM)

EM - electron microscopy analysis (measures physical diameter of dry particles)
DLS - dynamic light scattering analysis (measures hydrodynamic size of particles in a fluid)
pH_{zpc} - zeta potential charge

Table 4. Characteristics of bacteriophages commonly used as pathogenic virus surrogates in environmental studies (adapted from Mesquita et al., 2010).

Bacteriophage PRD-1 (Table 4) in particular has emerged as an important viral model for studying microbial transport through a variety of subsurface environments. Its popularity is due to its similarity to human adenoviruses in size (~62nm) and morphology (icosahedric), its relative stability over a range of temperatures and low degree of attachment in aquifer sediments (Harvey and Ryan, 2004; Ferguson et al., 2007).

3.2 F-RNA bacteriophages

F or male specific RNA bacteriophages are a homogeneous group of phage that attach to fertility fimbriae (F-pili or sex-pili) produced by male bacterial cells (possessing an F-plasmid) in certain stages of their growth cycle. Since the F-plasmid is transferable to a wide range of Gram-negative bacteria, F-specific bacteriophages may have several hosts besides *E.coli* (Havelaar 1987). This group ranks second in abundance in water environments although its persistence in surface waters, mainly in warm climates is low (Chung and Sobsey, 1993; Mocé-Llivina et al., 2005).

F-RNA bacteriophages have been most extensively studied due to their similarity (in size, shape, morphology and physiochemistry) to many pathogenic human enteric viruses, namely enteroviruses, caliciviruses, astroviruses and Hepatitis A and E virus (Jofre et al., 2011) (Table 4). These phages are infrequently detected in human and animal feces (10^3 g⁻¹) or in aquatic environments despite their frequent detection in wastewater (10^3 to 10^4 mL⁻¹) (Havelaar et al., 1986; Gerba, 2006). Further research is needed to clarify if their consistently higher concentrations in sewage relative to feces are the result of direct environmental input or multiplication. If the latter is true, F-RNA bacteriophages may not be acceptable fecal pollution indicators (Havelaar et al., 1990). Jofre et al. (2011) suggested that the environmental multiplication of these phages is unlikely, however, because F-pili production only occurs at temperatures above 25°C and replication does not occur in nutrient-poor environments and requires a minimum host density of 10^4 colony forming units (cfu) per mL.

The presence of F-RNA phage in high numbers in wastewater and their resistance to chlorination contribute to their usefulness as process indicators, indices of sewage pollution, and conservative models of human viruses in water and shellfish (Havelaar et al., 1993; Havelaar, 1993; Love and Sobsey, 2007). They are also promising in microbial source tracking since they can be subdivided in four antigenically distinct serogroups. Because those predominating in humans (groups II and III) differ from those predominating in animals (groups I and IV), it is possible to distinguish between human (higher public health risk) and animal wastes by serotyping or genotyping F-RNA coliphage isolates (Hsu et al., 1995; Hsu et al., 1996; Scott et al., 2002).

F-RNA bacteriophages MS2 and f2 (Table 4) are morphologically similar to enteroviruses and are frequently used to study viral resistance to environmental stressors, disinfection and other treatment processes (Havelaar, 1986, Havelaar et al., 1993; WHO, 2004). These phage have been shown to attach poorly to soil particles and survive relatively well in groundwater (Goyal and Gerba, 1979; Yates et al., 1985; Powelson et al., 1990). As a result, Havelaar (1993) described F-RNA phage as a “worst case” virus model for virus transport in soil. Bacteriophage transport in the subsurface is reviewed in section 5 of this chapter.

Together, somatic and F-specific bacteriophages counts in water samples are usually designated as “total coliphage count”. Some bacterial strains can be used to enumerate both simultaneously (Guzmán et al., 2008). Their enumeration may be a good alternative for determination of viral contamination in poorly contaminated waters such as groundwater and drinking water or in double disinfection water treatments (Lucena and Jofre, 2010).

3.3 Bacteriophages of *Bacteroides fragilis*

Bacteriophages of *Bacteroides fragilis* and other *Bacteroides* species rank third in abundance in natural waters. They have been suggested as potential indicators of human viruses in the environment by Tartera and Jofre (1987). Their host *Bacteroides fragilis* is a strict anaerobic bacterium abundant in human feces. These bacteriophages attach to the host bacteria cell wall and have narrow host range. They occur only in human feces (10^8 g⁻¹) and in environmental samples contaminated with human fecal pollution (Havelaar et al., 1986). Consequently they are useful in microbial source tracking, helping to differentiate human from animal contamination (Ebdon et al. 2007; Lucena and Jofre, 2010). In contrast with other phage they are absent from natural habitats and unable to multiply in the environment (Tartera et al., 1989). They also decay in the environment at a rate similar to that of enteric viruses. The main drawbacks associated with their use as routine fecal indicators, are that: (1) their host is a strict anaerobe requiring complex and tedious cultivation methodology, (2) their numbers in water may be low requiring concentration from large volumes, and (3) different hosts are needed for different geographic areas. Within this group, the most commonly used bacteriophages in environmental and treatment resistance studies are B40-8 and B56-3 (Lucena and Jofre, 2010).

4. Available methodology for bacteriophage detection, enumeration and propagation

Relatively simple and reliable methods for detection, isolation, enumeration and characterization of bacteriophages from natural sources are available in the literature. These include classic culture-based techniques using liquid or solid bacteriological media, as well as more recent physico-chemical, immunological, immunofluorescence, electron microscopy, and molecular methods. However, a lack of methodology standardization and quality control has for decades limited the use of phage data for comparison studies. This situation has improved since the publication of standardized plaque assays and presence/absence methods in the USA and Europe. For somatic coliphages (APHA, EWWA, and WEF, 2005; EPA, 2001a; 2001b), F-specific RNA phages (ISO, 1995; ISO, 2000; EPA, 2001a; 2001b) and bacteriophages infecting *Bacteroides fragilis* (ISO, 2001).

Sobsey et al. (1990) developed a simple, inexpensive and practical procedure for the detection and recovery of F-RNA bacteriophages from low turbidity water using mixed cellulose and acetate filters with 47 mm diameter and 0.45 µm pore size. A slightly modified version of this method has shown excellent performance for recovery of somatic and F-specific phages, and bacteriophages of *Bacteroids fragilis* in up to 1L water samples (Mendez et al., 2004). Rapid bacteriophage detection methods involving enrichment steps followed by latex agglutination or bioluminescence (Love and Sobsey, 2007) and molecular approaches have also been developed and recently reviewed by Jofre et al. (2011).

Specific methods for the production of the large-volume, high-titer purified bacteriophage suspensions that are necessary for many types of environmental fate and transport studies were, until very recently, difficult to find in the refereed literature. Given that system chemistry and other surface-related characteristics of phage particles, may substantially contribute to observations of their environmental fate and transport behavior in many types of porous media filtration systems used for water treatment (Pieper et al., 1997; Harvey and Ryan, 2004; Cheng et al., 2007), it is critical to consider the impacts of the propagation/purification protocol on those factors. In response to this need, a selected sequence of rapid, reliable, and cost-effective procedures to propagate and purify high-titer bacteriophage suspensions has recently been proposed (Mesquita et al., 2010). This methodology emphasizes the most important factors required to ensure maximum bacteriophage yields, minimum change on phage particles surface characteristics, and low dissolved organic carbon (DOC) concentration in the final suspensions.

Many of the methods routinely used to quantify microscopic discrete particles such as bacteriophages are known to yield highly variable results arising from sampling error and variations in analytical recovery (i.e., losses during sample processing and errors in counting); thereby leading to considerable uncertainty in particle concentration or \log_{10} -reduction estimates (Emelko et al., 2008; 2010; Schmidt et al., 2010). For example, sampling error is substantially greater than analytical error when organisms are present in relatively low concentrations; in these cases, improved sampling (i.e., resulting in counts of approximately 10 or more organisms in a sample or, in some cases, several replicates) substantially contributes to reducing uncertainty. In contrast, when organisms are present in higher and homogeneous concentrations, uncertainty in concentration estimates can be reduced by decreasing analytical errors (Emelko et al., 2008; 2010). Emelko et al. (2010) demonstrated that uncertainty in concentration and removal estimates derived from microbial enumeration data can be addressed when these errors are properly considered and quantified. The development and use of such quantitative approaches is an essential component of strategies (e.g., the monitoring of surrogate parameters/pathogens, experimental design, and data analysis) for better evaluating microorganism transport and fate in source and treated drinking waters.

5. Bacteriophages contribution to predicting pathogen transport in filtration porous media

In the last two centuries a large number of field studies have evaluated the transport of bacteriophages in the subsurface (especially through the vadose zone) at different field sites around the world (Rossi, 1994; Collins et al., 2006; Pieper et al., 1997; Bales et al., 1997; Dowd et al., 1998; Rossi et al., 1998; Sinton et al., 1997; Ryan et al., 1999; Auckenthaler et al., 2002; McKay et al., 2000; Schijven and Hassanizadeh, 2000; Schijven, 2001; Harvey and Harms, 2002; Ryan et al., 2002; Harvey and Ryan, 2004; Blanford et al., 2005; Harvey et al., 2007; Ferguson et al., 2007). PRD-1, MS-2 and Φ X174 have also been extensively used at controlled laboratory conditions to elucidate physicochemical effects on virus transport through a variety of porous media (Bales et al., 1991; Bales et al., 1993; Schulze-Makuche et al., 2003; Zhuang and Jin, 2003; Han et al., 2006; Sadeghi et al., 2011).

Based on existing data, major environmental factors affecting enteric viruses and phage survival and transport through soil, porous media and in groundwater have been identified

(Table 5). Due to the complexity of interactive factors controlling survival and transport there is great variability among study outcomes, however. It is, at present, generally accepted that the main processes for viral removal in water filtration through porous media

Factors	Findings
1.	Temperature - a major controlling factor for virus inactivation usually with greater inactivation at temperatures above 20°C. This may be due to more rapid denaturation of viral capsid proteins or potential degradation of extracellular enzymes with increased temperature
2.	Native Microbial activity - Inactivation rates have often been reported to be lower in the absence of groundwater bacteria possibly because bacterial enzymes and protozoa may destroy viral capsid protein. However, other studies have found the opposite to be true.
3.	Moisture content - Different viruses and phage (MS2 and PRD-1) have been reported to have different inactivation rates in groundwater, saturated, unsaturated and dry soils. Migration seems to increase under saturated flow conditions.
4.	Nutrients - addition when native organisms are present seems to determine decreased viral inactivation. Possibly because the nutrients offered protection from inactivation by enzymatic attack or acted as alternate nutrient sources for the native bacteria
5.	Aerobic and anaerobic condition - Anaerobic conditions have been shown to slow down poliovirus and coxsackievirus inactivation. It has been suggested this is potentially an interactive factor with the impact of native microorganisms since low oxygen will minimize negative microbial activity.,
6.	pH - most enteroviruses are stable over a pH range of 3 to 9, survival may be prolonged at near neutral; low pH favors virus attachment and high pH detachment from soil particles
7.	Salt species and concentration - some viruses are protected from inactivation by certain cations; the reverse is also true. Generally increasing the concentration of ionic salts and cation valences enhances virus attachment.
8.	Association with soil and other particles - in many cases viral survival is prolonged by attachment to soil, although the opposite has also been observed. Usually virus transport through the soil is slowed or prevented by association with particles. However, attachment to solid surfaces appears to be virus-type-dependent
9.	Soil properties - effects on survival are probably related to the degree of virus attachment: greater virus migration is usually observed in coarse-textured soils, while there is a high degree of virus retention by the clay fraction of soil.
10.	Virus type - particle-structure may be a deciding factor in attachment/detachment and inactivation by physical, chemical and biological factors.
11.	Organic matter (OM) - may protect virus from inactivation or reversibly retard virus infectivity. Soluble OM seems to compete with virus particles for attachment sites on soil.
12.	Hydraulic conditions - increasing hydraulic loads and flow rates usually increase virus transport.

Table 5. Major factors determining viral survival and transport in the subsurface and in groundwater (adapted from: Azadpour-Keeley et al., 2003; John and Rose, 2005).

are physio-chemical attachment/detachment and inactivation (Keswick and Gerba, 1980; Yates et al., 1987; Bales et al., 1991; 1997; Gitis et al., 2002; Tufenkji and Emelko, 2011). Virus attachment and inactivation depend on the type virus, as well as on the physico-chemical properties of the water and soil or filtration media grain (Schijven and Hassanizadeh, 2000; Tufenkji and Emelko, 2011). Physical and physico-chemical processes such as advection, dispersion, diffusion, and physico-chemical filtration all contribute to attenuation of virus concentrations (Schijven and Hassanizadeh 2000; Tufenkji and Emelko, 2011). Various physico-chemical forces may be involved in the attachment of viruses to soil or filtration media particles including, hydrogen bonding, electrostatic attraction and repulsion, Van der Waals forces and covalent ionic interaction (Murray and Parks; 1980). Straining (i.e. physical blocking of movement) may come into play in some environments as well (Bradford et al., 2006).

The unsaturated or vadose zone (i.e. the layer between the land surface and the groundwater table) where much of the subsurface contamination originates, passes through, or can be eliminated before it contaminates surface and subsurface water resources has gained particular attention in recent years. In unsaturated conditions, additional and more complex mechanisms are involved in pathogen transport such as: variability in ionic strength, pH and water content, particle capture at the water-gas interface, particle capture at the solid-water-gas interface, and preferential flow or retention in the immobilization zone (Sen, 2011). Biological processes such as growth and decay, active attachment or detachment, survival, random mobility and chemotaxis are also believed to strongly affect virus transport in saturated and unsaturated porous media (Sen, 2011). Less information is available regarding the fate of pathogenic protozoa in the vadose zone (Harvey et al., 1995; Harvey et al., 2002; Hancock et al., 1998; Brush et al., 1999; Harter et al., 2000; Darnault et al., 2004; Davies et al., 2005), however, the physico-chemical processes that affect virus fate and transport also apply to protozoan cysts and oocysts during soil transport, albeit to a different extent (Schijven and Hassanizadeh, 2000).

The growing database of information concerning phage attachment, inactivation and transport behavior in porous media has led to their use as viral surrogates in mathematical models used to describe viral transport within physically or geochemically heterogeneous granular media at environmentally-relevant field scales (Rehmann et al., 1999; Schijven and Hassanizadeh, 2000; Schijven et al., 2000; Bhattacharjee et al., 2002; Schijven et al., 2010). As they continue to improve, such models may become useful tools in decision making related to in public health protection because they may ultimately be incorporated into quantitative microbial risk assessment to: (1) assess groundwater vulnerability, especially of highly vulnerable geological settings (i.e. fractured rock aquifers, cross-connecting bore holes, or leaking well cases in sandstone and shale aquifers) in combination with significant sources of contamination (i.e. wastewater treatment plants, septic tanks and animal manure), (2) simulate the transport of viruses from a contamination source at or near the surface to a groundwater abstraction well, and (3) evaluate set back distances from abstraction wells from potential contamination sources for source protection (Schijven et al. 2010).

6. Conclusions and recommendations for future research

Considerable progress has been made in understanding how suitable bacteriophages are as surrogates for pathogenic enteric viruses. As a result, they have become invaluable tools in environmental research and are often successfully used in a variety of applications, namely:

- The use of somatic and F-specific coliphages as indices of water contamination by sewage and as process indicators in the evaluation of drinking water and the efficacy of drinking water treatment processes.
- The use of F-specific bacteriophages as indices and models of human enteric viruses in contaminated water, shellfish and agricultural products and in microbial source tracking.
- The use of particular somatic and F-specific bacteriophages to improve the understanding of the multiple physical, chemical and biological processes affecting biocolloid transport in saturated and unsaturated subsurface environments.
- The use of bacteriophages of *B. fragilis* as indicators of human fecal contamination and in microbial source tracking.

Additional research efforts are needed in the following areas:

- Use of more sensitive and reliable methodologies (i.e. standardized cultural procedures, molecular and other techniques) to minimize the variance between reported and actual numbers of bacteriophages in field and laboratory studies and allow the development of more complete and reliable databases.
- Use of more consistent experimental procedures to reduce variability among researchers' findings. Standardized protocols are required for the preparation (propagation, concentration and purification) of bacteriophages to be used in laboratory and field scale studies, as well the use of phage from well known sources such as the American Type Culture Collection (ATCC) or the Canadian Felix d'Herelle Reference Center for Bacterial Viruses to avoid differences in the viruses themselves.
- Evaluation of the complex interactions of native groundwater organisms with introduced enteric microbes (including enteric bacteriophage) and the environmental factors that influence them.
- Evaluation of the impact of viral structure and surface properties on attachment/detachment and inactivation of virus particles in various environments.
- Improved understanding of the transport and survival of both bacteriophages and pathogenic enteric viruses in surface water and the subsurface is needed; not only at laboratory scale to clarify the generic mechanisms involved, but also at field scale at settings with specific environmental conditions (water matrixes, flow regimes, hydrogeological and filtration media characteristics, etc.) in an attempt to clarify conflicting evidence previously reported on the extent of inactivation and immobilization of viruses by some physico-chemical and biological factors.
- Development of sound databases reflecting the occurrence, persistence and transport of viral particles in natural environments and water treatment systems that can be used to improve mathematical models of microbial fate and transport.
- Development of microbial fate and transport models taking into account the many factors affecting virus fate and transport under various conditions applicable to: improve viral contamination control in specific environments, ensure compliance with current water quality regulations, help in the selection and control of treatment processes and ultimately improve public health protection.
- Further investigation of the usefulness of bacteriophages for source tracking purposes. Taking advantage of the stringent host specificity of some phage groups and the speed, high specificity and sensitivity of molecular detection methods in order to better

characterize sources of contamination in aquatic environments so that appropriate and cost-effective water quality remediation plans can be developed.

In the future, the progress of such applications will reveal the true potential of bacteriophages as viral pathogen surrogates in water and water treatment.

7. References

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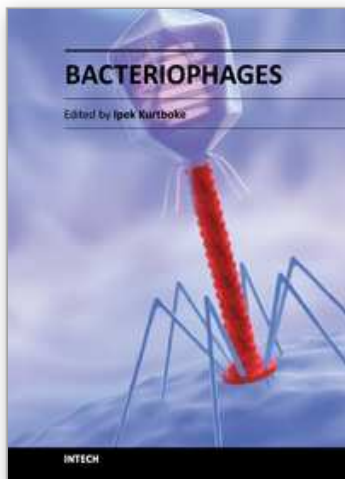
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Bacteriophages have received attention as biological control agents since their discovery and recently their value as tools has been further emphasized in many different fields of microbiology. Particularly, in drug design and development programs, phage and prophage genomics provide the field with new insights.

Bacteriophages reveals information on the organisms ranging from their biology to their applications in agriculture and medicine. Contributors address a variety of topics capturing information on advancing technologies in the field. The book starts with the biology and classification of bacteriophages with subsequent chapters addressing phage infections in industrial processes and their use as therapeutic or biocontrol agents. Microbiologists, biotechnologists, agricultural, biomedical and sanitary engineers will find Bacteriophages invaluable as a solid resource and reference book.

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