Bacteriophages as faecal indicators in environmental systems: Historical perspectives and recent uses

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Abstract
The relationship between various indicator organisms and the presence of enteric viruses in treated drinking water, surface water and groundwater has been the focus of numerous investigations in the recent years. Faecal indicators have been found to be useful for pointing out the potential presence of enteric viruses in various water sources. Commonly used indicators include bacteria (e.g. *E. coli*, enterococci and *Clostridium perfringens* spores), and bacteriophages. The most appropriate indicator is one that can provide information about the presence of viruses in groundwater or surface water sources or the removal/inactivation of viruses by conventional water treatment processes. Bacteriophages can be recovered and detected by many different techniques, and numerous promising approaches are still in the developmental stage. It should be noted, however, that meaningful and universally accepted guidelines for the recovery and detection of bacteriophages in water environments are still underway.

Keywords: somatic coliphages, F-specific RNA phages, *Bacteroides fragilis* phages, enteric viruses, contamination, waterborne diseases

Introduction

The microbial contamination of water often leads to large outbreaks of waterborne diseases. Almost half of the waterborne disease outbreaks reported every year are associated with groundwaters contaminated with microorganisms originating from animal feeding operations, decentralized wastewater treatment systems (e.g. septic tanks), leaking sewage pipes, treated sewage sludges (biosolids), and artificial aquifer recharge with treated wastewater effluents [1,2]. During the course of typical wastewater treatment operations, most of the microorganisms are removed. However, some pathogens are often resistant to chlorination [3] and may still be present in effluents, which can contaminate recreational waters and drinking water supplies [4].

Among all classes of waterborne pathogens, viruses can cause a wide range of diseases and symptoms. A large number of epidemics caused by viruses have been reported in the literature [5]. Hepatitis A virus, caliciviruses, adenoviruses, rotavirus, and enteroviruses have the greatest effect on public health. Numerous studies have documented the presence of enteroviruses in raw and treated drinking water [6], wastewater [7], and sludge [8], as well as the presence of enteroviruses and adenoviruses in seawater [9].

Rotaviruses have been detected in sewage [10], river water [11], groundwater [1], and drinking water [12].

Viruses are of submicron size [13] and they can penetrate easier tight subsurface formations than larger size microorganisms such as protozoa [14]. Consequently, it is quite important to monitor the levels of viruses in groundwater and aquatic systems. Virus monitoring in environmental systems is often accomplished with the aid of indicator bacteria (faecal coliforms, *Escherichia coli*, and faecal streptococci), which are employed as sole indicators of faecal pollution.

It has been reported in the literature that viruses present in various aquatic systems frequently survive longer than indicator faecal bacteria [9,4]. Moreover, there is evidence that some viruses may be more resistant to rough environmental conditions, and water treatment processes, than coliform organisms [15]. Consequently, the unreliability of bacterial model mcroorganisms [4] led to the search for alternatives. Several bacteriophage groups, such as somatic coliphages, F+ specific (male-specific), RNA bacteriophages [16], and *Bacteroides fragilis* bacteriophages [17] have been identified as promising indicator candidates for faecal contamination and viral presence.

This work looks at the history and examines some of the faecal indicators used to assess the microbiological quality of water, highlighting the current limitations and also possible future developments.The potential of using bacteriophages as models for the fate of viruses in natural waters and water treatment systems is also examined. Special attention is given to the somatic coliphages, F-specific RNA phages and phages of *Bacteroides fragilis*.

Bacteriophages as indicators

Bacteriophages are increasingly employed in various environmental applications as indicator organisms of human pathogenic viruses because they possess all the elements of true viruses and permit easy, fast and inexpensive isolation [18,19]. Three types of bacteriophages, namely: somatic coliphages, F-specific RNA phages, and Bacteroides fragilis phages have been employed in environmental water samples as specific indicators of human enterovirus contamination. For all of these bacteriophage groups the International Standardization Office (ISO) has recommended appropriate procedures for their detection in water. Furthermore, numerous studies suggest that these bacteriophages are reliable indicators [16,20,21]. Table 1 presents a comprehensive compilation of studies where bacteriophages have successfully been employed as indicators.

Table 1: Compilation of studies where bacteriophages have been used as indicators

Categories of bacteriophages (correlation with pathogens)	**Indicators of:**	**Bacteriophages frequently used as indicators**	**Sources (counts)**
Somatic coliphages (Yes/No)			
[55,59]	Faecal contamination [21,37,44, 46]	T2, T4, T6 (T even phages), T-odd, λ, T5, T7, T3, ΦX174, S13, PRD4 [23,25,28,40]	domestic sewage (10^3-10^4pfu/ml) [29,31,35,38]
			storm-water-runnoff (10^0-10^3pfu/100ml) [27]
			greywater sediment samples (10^4-10^6 pfu/g) [54]
			primary sludge (10^5-10^8 pfu/100g) [45,47,48,52]
			activated sludge (10^5-10^7 pfu/100g) [45,47]
			thickened sludge (primary+activated sludge) (10^4-10^7 pfu/100g) [41]
			raw municipal sewage (10^3 to 10^6pfu/ml) [50]
			faeces of man, cattle, pigs, chickens and other animals (10^0-10^6 pfu/g) [30,51,53]
Male spesific F+ phages (Yes) [44,55]			
	Sewage contamination		
	[21,34,53]		storm-water-runnoff (10^0-10^2pfu/100ml) [27]
Serogroup I	Animal Faecal contamination	MS2, f2, R17,JP501	greywater sediment samples (10^8-10^9 pfu/g) [54]
Serogroup II	Human Faecal contamination	GA,DS,TH1,BZ13, KU1,JP34	primary sludge (10^3-10^8 pfu/100g) [45,47,48,52]
Serogroup III	Human Faecal contamination	Qβ, VK, ST, TW17	activated sludge (10^2-10^5 pfu/100g) [45, 47]
Serogroup IV	Animal Faecal contamination [26,32,36,53]	SP, FI, TW19, TW26, MX1, ID2 [2,22,23,25,40-42,58,61]	thickened sludge (primary+activated sludge) (10^2-10^3 pfu/100g) [41] raw sewage (10^2 to 10^5pfu/ml) [45,50,57]
			faeces of man, cattle, pigs, chickens and other animals-(10^0-10^5 pfu/g) [51]
B. fragilis phages (Yes)			
[17,55]	Human Faecal contamination [15,17,24,33, 39,43,56,60]	phages using *Bacteroides fragilis* strain HSP38 phages using *Bacteroides fragilis* strain RYC20 [49]	primary sludge (10^2-10^5 pfu/100g) [45,47]
			activated sludge (10^3 pfu/100g) [45,47]
			thickened sludge (primary+activated sludge) (10^3 pfu/100g) [41]
			faeces of man, cattle, pigs, chickens and other animals (10^0-10^2 pfu/g) [51]

Somatic coliphages

Somatic coliphages are bacteriophages that attach directly to the lipopolysaccharide of E. coli and certain closely related members of the bacterial family Enterobacteriacea [62,63]. Somatic coliphages are proven to be accurate faecal indicators. Sewage usually harbours high numbers of somatic coliphages. They have also been detected in storm-water-runnoff [27], greywater (e.g. wastewater from bath shower, kitchen, and laundry but without input from toilets) samples [54], and animal-rearing operations [64], as well as in slaughterhouses [51], hospital wastewater [53] and bioaerosols around wastewater treatment plants [65]. Somatic coliphages have been found to generally outnumber F-RNA phages in wastewater and raw water sources [66].

Somatic coliphages present some shortcomings, one of them being their heterogeneity (high adaptability to environmental conditions), whereas both F-specific RNA [67] and B. fragilis [15] bacteriophages are much more homogeneous. Somatic coliphages are classified into four groups with significant genetic differences, namely: Myoviridae [DNA], Siphoviridae [DNA], Podoviridae [DNA], and Microviridae [DNA]. Also, F-specific RNA phages are subdivided into two main groups: Leviviridae [RNA], and Inoviridae [DNA]. Not all groups of bacteriophages exhibit the same behavior in aquatic and environmental systems. For water quality applications, homogeneous phages are recommended. Important drawbacks of somatic coliphages are their replication potential in estuarine water [68] and the presence of autochtonous (native inhabitants or indigenous) bacteriophages in unpolluted water [69]. However, Muniesa and Jofre [70] indicated that the unique combination of the presence of somatic coliphages, host bacterium densities, and bacterial physiological conditions needed for phage replication are rarely expected to be found in natural water environments. Moreover, in water samples collected far from a pollution source or in water samples taken subsequently to the process of chemical disinfection, predominant bacteriophages may be different from phages detected in sewage and in freshly polluted waters [71].

Somatic coliphages are detectable by relatively simple, inexpensive, and rapid plaque assays [39]. Somatic coliphages attach to the bacterial cell wall and under optimal conditions may lyse the host cell within a 20-30 minutes period. They produce plaques of widely different size and morphology. The methodology to detect somatic coliphages is very simple and results may be obtained within 4-6 hours.

Male-specific bacteriophages

Male-specific (F^+) bacteriophages are coliphages that infect *E. coli* via the bacterial sex-pilus, the genes for which are located on the F-plasmid, which is produced only at temperatures near 37°C or higher [35]. The F^+ coliphages can be RNA-containing (FRNA phages) or DNA-containing (FDNA phages) coliphages. [72] reported that site specific factors, which are not yet understood influence the reliability of coliphages as indicator organisms. Hence, until these factors have been thoroughly investigated, the use of a F-specific phage as an indicator should be carefully examined for the specific conditions of each particular site or bench scale experiment.

FRNA coliphages are classified into four serological types: serogroups I, II, III, IV. Faeces and in particular human faeces do not appear to be an important source of F-specific bacteriophages [21,30,53,67]. However, animal and human faeces contain different serotypes of RNA coliphages, suggesting that these phages can be used to predict the source of faecal pollution [73,74]. Members of F-specific RNA coliphages are highly associated with faecal contamination from different sources and/or domestic sewage [73]. Therefore, the presence of F-specific phage in water often designates the existence of sewage pollution.

The physical structure, composition, and morphology of F-RNA coliphages and also their failure to multiply in water environments, closely resemble those of many human enteric viruses [75]. Many experiments confirmed that the resistance of F-RNA coliphages to unfavourable conditions (presence of various chemicals, heat treatment, chlorination) and disinfection processes resembles or exceeds that of most human enteric viruses [21]. Furthermore, it has been reported in the literature that F-RNA phages are resistant to various chemicals [44], heat treatment [76], sunlight [77], ultraviolet light [78], chlorination [79], and typical water treatment processes [63].

Detection of F-RNA coliphages by plaque assays is not as simple as for the case of somatic coliphages. The reason is that the F fimbriae with receptor sites for the phages are produced only by host bacteria in the logarithmic growth phase (the logarithm of the population density rises linearly with time). This implies that the preparation of host cultures for plaque assays has to be timed carefully in order to have the host bacteria in the logarithmic growth phase. Even then, the plaques are relatively small and mottled because many bacteria in the plaque area may not be lysed. Successful plaque assay procedures for F-RNA coliphages have been formulated. However, it is most important that the instructions of these procedures are followed closely [80].

B. fragilis phages

Bacteroides fragilis is an obligate anaerobic bacterium (it can only survive in the absence of molecular oxygen) found in high concentrations in human faeces. Hence, the presence of phages that infect these bacteria is considered to be indicative of human faecal contamination. The genus *Bacteroides* is found in the human gastrointestinal tract in large numbers (more than 10^9-10^{10}/g faeces), compared to coliform bacteria, which range from 10^6 to 10^8/g faeces [81]. It should be noted that the difficulty to recover *B. fragilis* phages from waters with low levels of faecal pollution limits their use as faecal indicators.

Sun et al. [82] suggested that *B. fragilis* phages may be a better indicator for water bacteriology than classical bacteriological indicators used in water treatment. Also *B. fragilis* phages are more resistant to inactivation by chlorine than other micro-organism models such as poliovirus type 1 and *E. coli* [83]. Compared to coliphages and enteric viruses, *B. fragilis* phages proved to be relatively resistant to unfavourable conditions, at least in certain water environments [84].

Plaque assays for *B. fragilis* phages are more complicated, expensive, labour-intensive and time-consuming than those for somatic and F-RNA coliphages. Rather

complex growth media supplemented with antibiotics are required, and plates have to be incubated under strictly anaerobic conditions. Details on plaque assays for *B. fragilis* phages have been reported by Tartera et al. [60] and ISO [85]. A molecular procedure based on the polymerase chain reaction (PCR) technique may be more sensitive for the detection of *B. fragilis* HSP40 phages than plaque assays [56].

Conclusions

Water faecal contamination is an undesired reality encountered in many countries. To prevent major outbreaks of infectious disease caused by pathogenic microorganisms the scientific community has searched for various indicators that could be used to alert their presence. Among the possible indicators, bacteriophages are receiving increasing attention because of the concern with waterborne viral diseases. Bacteriophages have been studied worldwide as faecal indicators because of the ease of their detection and their morphological similarity to human viruses. In addition, detection of human viruses is still a highly skilled and costly process. However, low concentrations of all types of bacteriophages in groundwater limit their power to predict the presence of enteric viruses. There is little concordance in the literature regarding phage detection methods, thus making comparisons extremely difficult. Different authors have used different hosts, phage concentration methods, and end-point determinations. Also, markedly different volumes of sample have been employed. In addition, bacteriophage concentration methods are not reproducible. Moreover, there is no consensus on the best bacterial host strain, and there is a lack of consistent recovery of bacteriophages from individual faecal specimens. Nonetheles, bacteriophages have shown good potential application as indicators in certain situations, but their use is premature at this time, and a number of critical issues must be addressed in order for them to meet minimum regulatory requirements.

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