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Cross-assembly phage and pepper mild mottle virus as viral water quality monitoring tools—potential, research gaps, and way forward

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Abstract

Microbial water quality is currently assessed by fecal indicator bacteria that are poor representatives of viral pathogens in the environment. Viruses are predicted to account for the majority of infectious risk from exposure to sewage contaminated water. Previously developed viral indicators suffer from a lack of human specificity, low concentrations in sewage, or both. In this commentary review, we discuss recent advances in developing cross-assembly phage (crAssphage) and pepper mild mottle virus (PMMoV) as viral water quality indicators. CrAssphage and PMMoV are abundant in and highly associated with human sewage, correlate with viral pathogens in sewage contaminated environments and globally present. Future work is necessary to describe crAssphage and PMMoV fate in the environment, local variation in abundance and genetic makeup, and relationship between molecular detections and pathogen viability, among other areas. These developments will allow the integration of crAssphage and PMMoV into quantitative microbial risk assessment and water quality regulation.

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Introduction

Direct measurements of waterborne pathogens are currently impractical for routine monitoring of drinking and recreational water. Instead, fecal indicator bacteria are monitored as proxies for fecal-oral pathogens. While fecal indicator bacteria are widely used for routine water quality monitoring, the fate and transport of fecal indicator bacteria do not adequately mimic viral pathogens. The results of this discrepancy are outbreaks of waterborne disease associated with viruses despite fecal indicator bacteria being absent or in compliance with regulations [25,36,55,60]. Both epidemiology [27] and risk assessments [15,20,52,69] indicate that viruses are responsible for a significant portion of waterborne disease. Viral fecal pollution indicators would have a wide variety of potential applications, including recreational and irrigation water quality monitoring, as well as process monitoring for wastewater treatment and water reuse applications.

Prior targets have been developed as viral water quality indicators. Bacteriophages, particularly somatic coliphages and F-specific RNA phages infecting *E. coli* and phages infecting *Bacteroides fragilis*, have been tested as viral water quality indicators [12,28,38,40]. Both coliphages and phages infecting *Bacteroides* are not specific to a sole-source wastewater stream and are inconsistently associated with human viruses in water [16,17,39,49,68,84] and waterborne disease [1,19,41,47,81,82]. In addition to bacteriophages, human enteric viruses including adenoviruses [2,86], polyomaviruses [34,54], enteroviruses [56], and noroviruses [86] have also been examined as more specific fecal pollution indicators and microbial source tracking markers [4]. While human viruses are more specific indicators of waterborne disease risks than nonpathogenic fecal indicators, in general they are less abundant in wastewater streams which makes their detection in contaminated environments challenging.

Currently, a viral pollution indicator that is both abundant in and specific to wastewater is needed. Here, we focus on pepper mild mottle virus (PMMoV) and crossassembly phage (crAssphage) as recently developed viral fecal pollution indicators. PMMoV is a plant pathogen that is abundant in human feces [89], likely from the consumption of infected pepper products, such as hot sauce. PMMoV was subsequently proposed as a viral fecal pollution indicator [62]. Reviews covering the development and application of PMMoV as a viral water quality monitoring tool have recently been published [43,74,77]. CrAssphage is a highly abundant human gut bacteriophage [21]. CrAssphage has been confirmed as a *Bacteroides* bacteriophage by culture [66] and identified as a prototypical member of an entirely new viral family [88]. While crAssphage as a viral water quality monitoring tool is a fairly recent development, a recent review paper briefly covered its development and use [14].

Some selected similarities and differences between crAssphage and PMMoV are compared in Table 1. In this commentary, we highlight recent findings applying crAssphage and PMMoV as viral water quality tools, as well as research needs to support their continued development as we move toward their application in water quality regulation.

CrAssphage and PMMoV fate in the environment

The principal driver for using viral water quality markers such as PMMoV and crAssphage is both their improved ability to detect sewage contamination because of their high enrichment in sewage and that their fate (i.e. transport and persistence) in the environment more closely mimics pathogenic viruses than bacterial indicators. Here, we discuss previous work on the comparative fate of crAssphage and PMMoV with both water quality indicators and pathogens. Representative environmental crAssphage concentrations from these studies are shown in Table 2; PMMoV concentrations in environmental water samples have recently been reviewed [43,74].

In general, crAssphage correlates well with existing bacterial fecal indicators immediately following sewage contamination events. CrAssphage has similar concentrations to the bacterial fecal indicator target HF183 in untreated and treated sewage [44,70]. CrAssphage also correlates well with HF183, *E. coli*, and enterococci in sewage and surface water samples before and after rain events but is present at higher concentrations [5,6,13,70]. CrAssphage also co-occurs with antibiotic resistance genes in sewage-impacted environments [71]. In recently contaminated environments, crAssphage co-occurs with human viruses such as adenovirus, polyomavirus, and norovirus in sewage but has concentrations up to five orders of magnitude higher than these viruses [23,70]. Previous studies have shown correlation between crAssphage and other viral targets and pathogens throughout the wastewater treatment process [23,51]. A recent study found that crAssphage statistically correlated with adenovirus, polyomavirus, and somatic coliphage during activated sludge wastewater treatment process [87]. The similar behavior between crAssphage and other sewage-associated viral pathogens in sewage-contaminated environments suggests it may be a reliable indicator of human fecal pollution; however, additional work, especially mechanistic work demonstrating crAssphage fate, is necessary to confirm these observations.

Similar to crAssphage, PMMoV correlates well with existing bacterial fecal indicators following fresh sewage contamination events, including HF183 in sewage and sewage-impacted surface waters [3,73] and *E. coli* and enterococci in sewage-impacted waters [64]. PMMoV also co-occurs with antibiotic resistance genes in sewage

Table 1

Comparison between crAssphage and PMMoV.

	CrAssphage	PMMoV
Genome	dsDNA ~90 kbp	ssRNA ~6.3 kb
Shape	Isometric with tail	Elongated rods
Size	Diameter ~75 nm Tail ~36 nm	Diameter ~18 nm Length ~300 nm
Host organism	Bacteria (*Bacteroides* spp.)	Plants (Peppers)
Sewage concentration[a]	7–9.5 log GC/L	~6–10.3 log GC/L
Culturable?	Single isolate cultured in strain of *Bacteroides intestinalis*	Culturable in pepper plants; difficult to quantify via culture

[a] Range from Ref. [20].

Table 2

Observed crAssphage concentrations ranges. All values measured by molecular methods (qPCR or dPCR). Concentrations are reported as described in the original study with the exception of unit conversion.

Environment	Location	Concentration Range (Log10 copies/100 mL)	% Positive	Reference
Wastewater				
Untreated	Thailand	4.23–6.19	100	[44]
Untreated	United Kingdom	5.3–9.3	100	[23]
Untreated	United States	7.23 ± 0.36	100	[87]
Untreated	Spain	7.4–8.9	100	[26]
Untreated	Japan	9.98–11.03	100	[51]
Treated	Thailand	3.78–4.89	100	[44]
Treated	Spain	5.34 ± 0.59	100	[13]
Treated	United Kingdom	7–8	100	[23]
Treated	United States	4.35 ± 0.80	100	[87]
Treated	Japan	8.54–10.00	100	[51]
Environmental waters				
Stream	United States	4.02–6.04	100	[70]
River	Spain	5.42 ± 0.60	100	[13]
River	United Kingdom	3.5–7.5	94	[23]
Variable	Nepal	3.1 ± 0.9	62	[50]
Stream	Spain	3.46 ± 1.29	100	[13]
Urban lake	Australia	ND-2.44 (dry weather) 2.61–5.33 (wet weather)	10 (dry weather) 95 (wet weather)	[5]
Urban lake	Australia	2.06 ± 0.04 (dry weather) 3.53 ± 0.89 (wet weather)	31 (dry weather) 89 (wet weather)	[6]
Storm drain	United States	2.60–3.65 (dry weather) 2.62–3.91 (wet weather)	41.6 (dry weather) 66.6 (wet weather)	[3]

ND-non-detect; PCR, polymerase chain reaction.

and sewage-impacted environments [46]. PMMoV occurred more frequently and at higher concentrations than adenovirus, polyomavirus, and norovirus in sewage-impacted surface waters [37,51,53,74,78]. Additionally, PMMoV correlated with and had higher concentrations than enterovirus, Aichi virus, and polyomavirus in sewage contaminated waters [50]. PMMoV reduction levels (0.7—0.9 $\log_{10}$ reduction) during wastewater treatment were also less than other viruses, suggesting resistance to wastewater treatment [42,65]. This implies PMMoV may be more applicable as a conservative indicator of fecal pollution.

Both crAssphage and PMMoV have longer environmental persistence than bacterial indicators [8,31]. CrAssphage persistence correlates with other viral pathogens such as adenovirus, polyomavirus, and coliphage [8,13], whereas PMMoV decay is slower than other viral indicators, including adenovirus, polyomavirus, and torque teno virus [31]. The difference in crAssphage and PMMoV persistence is likely driven by differences in their genomes (dsDNA versus ssRNA, respectively); however, additional work is necessary to examine the comparative decay of crAssphage, PMMoV, and viral pathogens in diverse aquatic environments. The enhanced persistence of PMMoV should be more fully described to determine the suitability of PMMoV application to detect an aged sewage release.

Transport of viral fecal indicators in the environment has remained relatively understudied because of the inherent difficulty and variability of performing transport experiments in the environment. Previous studies have examined viral transport on the small-scale laboratory-controlled level and have shown viral capsid structure to play a crucial role [10,11]. Few studies, however, have examined fecal indicator transport at larger scales and even fewer have mechanistically examined the transport of novel fecal indicators [10,24]. Additional research is necessary to evaluate the difference in transport between crAssphage, PMMoV, and viral pathogens.

Geographic variability of CrAssphage and PMMoV

Fecal pollution indicators have previously demonstrated differential performance across geographical areas [35,83]. This variability is likely driven at least in part by geographic variations in diet (PMMoV), human gut microbiomes (crAssphage), or both. The potential for differential performance by viral fecal pollution indicators makes it necessary to determine variability in geographic performance during viral indicator assay development, selection, and evaluation [14,80].

CrAssphage has been detected in wastewater globally. Edwards et al. sequenced crAssphage PCR products

from wastewater from 70 different locations in 23 countries across five continents demonstrating global occurrences [22]. We note that this study did not provide quantitative detections. CrAssphage has also been quantified in wastewater in the United States [72], Spain [26], Nepal [50], Japan [51], Thailand [44], and the United Kingdom [23]. Finally, given the recent description of crAssphage as a fecal pollution indicator, we expect additional reports of crAssphage occurrence in wastewater to be forthcoming.

PMMoV was detected globally in wastewater as well, including in Australia [37], Bolivia [75], Costa Rica [79], Germany [31], Japan [48], Nepal [67], South Korea [32], the United States of America [42,62,65], New Zealand [29], and Vietnam [46]. PMMoV has also been detected in groundwater [63], recreational water (e.g. Ref. [3]), and in constructed wetlands treating wastewater [61].

While global presence suggests the high potential of both crAssphage and PMMoV, one possible challenge is local variability in target abundance in sewage and specificity to sewage. For example, assessments of crAssphage specificity in the US and Europe have shown strong human association [26,72]; however, a recent assessment in Kathmandu, Nepal, suggested much lower specificity with detections in multiple animal fecal samples [50]. Local target variations are likely because of variability in diet and human gut microbiomes, including both target abundance and strain variability. CrAssphage diversity has recently been shown to be locally clustered within countries, cities, and individuals with thousands of strains of crAssphage spread around the world from human feces-associated environments [22]. Similarly, country-wide PMMoV variation has been observed in Japanese drinking water [33].

Metagenomic methods could be used to examine crAssphage and PMMoV diversity [14]; however, this approach is currently severely limited by bias from sampling locations. As an example of sample bias, in a recent meta-analysis of the human gut metagenomes, 88.35% of samples were from North America and Europe [9]. Greater metagenome geographic sample diversity is needed to inform the development and application of viral fecal pollution indicators. Working with sewage for this application has considerable advantages, even potentially generating complete genomes of novel uncultivated viruses with fewer restrictions than working with samples from individual patients [85].

CrAssphage and PMMoV application in quantitative microbial risk assessment

Novel indicator viruses such as crAssphage and PMMoV have significant potential advantages in risk-based analyses of human contact with wastewater-impacted environments (Figure 1). Specific advantages include improved representation of pathogenic viruses and high concentrations in wastewater which improves our ability to detect wastewater contamination in the environment and quantify subsequent human health risk. Quantitative Microbial Risk Assessment (QMRA) is a tool that enables quantifying human health risk and the associated uncertainty for decision making. Directly measuring infectious pathogens is the most accurate way to estimate risk; however, measuring all pathogens in an environment may not be feasible, either because of low concentration and prevalence or the diversity of potential pathogen targets. One approach to address this is to use a ratio to estimate pathogen concentrations based on indicator concentrations. This method assumes contamination from a single source (typically wastewater) and identical fate and transport of both the indicator and pathogens. This method is based on the current World Health Organization recommendation to convert between FIB concentrations and viral pathogen concentrations [57,76]. Recent QMRAs have applied a variety of viral indicators including crAssphage, PMMoV, and adenovirus to represent viral pathogens using a ratio approach [18,20,45]. Crank et al. used this approach with crAssphage and PMMoV in a QMRA that showed the potential to reduce the current USEPA Recreational Water Criteria (based on fecal indicator bacteria detection limits) of approximately 32 illnesses per 1000 swimmers to approximately 1 illness per 1000 swimmers [20].

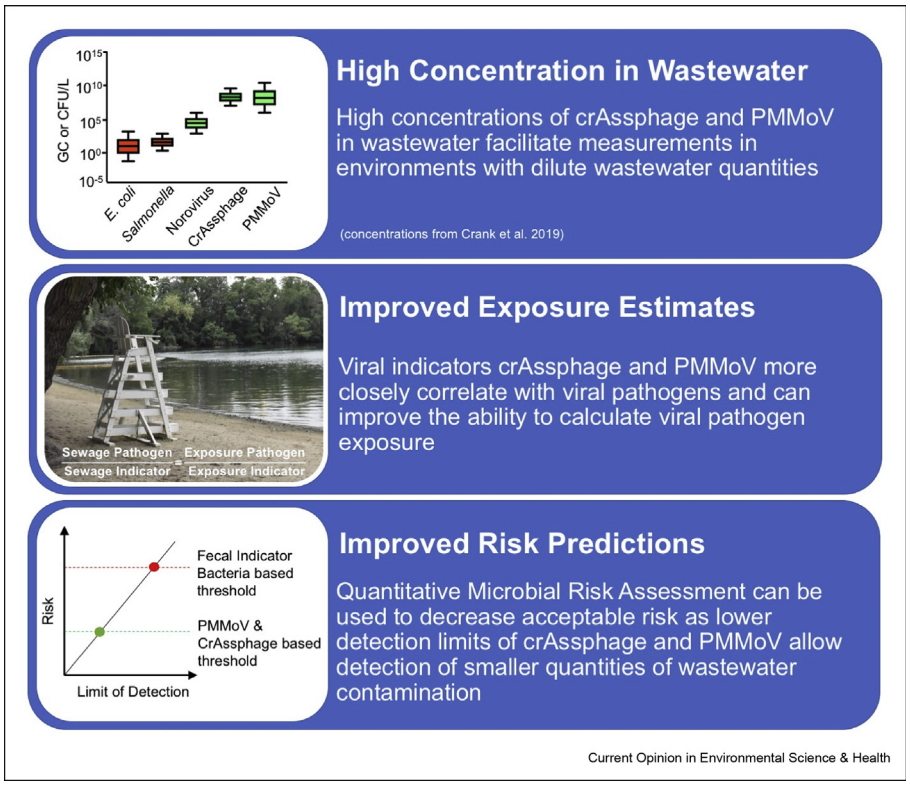
A key limitation to this approach is the assumed ratio of indicator to pathogen, which is generally representative only of a fresh sewage contamination event. Improved research on the fate and transport of crAssphage and PMMoV in relation to viral pathogens, as well as the local occurrence of crAssphage and PMMoV will enable modeling of more complex scenarios and improve the utility of QMRA for this approach. The WHO acknowledges that a risk-based approach is vital to understanding and preventing human disease, and as viral diseases account for the majority of gastrointestinal illnesses, improved methods for quantification and risk characterization are needed to move forward [58,59].

Research needs

Multiple critical research needs are necessary to move crAssphage and PMMoV from research to regulatory use.

Detection of both crAssphage and PMMoV relies on molecular methods, as they are not currently culturable from environmental samples. Both qPCR and ddPCR methods have been applied to detect crAssphage and PMMoV; a recent review paper discussed the relative advantages of each approach [30]. Molecular detections have unknown correlation with infectious viral

Figure 1



Advantages of crAssphage and PMMoV in regulatory and quantitative microbial risk assessment applications.

pathogens [30]. QMRAs have corrected for this using a live/dead fraction or assuming that all organisms are infectious; however, more research is necessary for modeling the infectious risk based on concentration values from molecular methods alone [7,20,45], or alternatively, methods to culture crAssphage or PMMoV.

Another potential challenge is sample processing. A major reason why crAssphage and PMMoV have been proposed as novel indicators is their abundance in wastewater, but sample concentration is typically still necessary, even in high biomass samples. Thus far, all viral concentration methods have been largely tested *ad hoc*. Further comparison of viral concentration techniques is required to optimize molecular methods and to quantify concentration efficiency to connect detected concentrations with regulatory limits and QMRA.

There is also a need to connect the detection of crAssphage and PMMoV with the fate of viral pathogens in the environment to support their application in QMRA. Vital research needs that must be addressed are the differential fate and transport of crAssphage and PMMoV compared with the pathogens they represent and improved models to represent crAssphage, PMMoV, and viral pathogen decay. While observations to date support the co-occurrence of crAssphage and PMMoV with viral pathogens, most studies have been conducted in locations with known sewage impacts. Future studies should include more pristine sites to support crAssphage and PMMoV application in those settings.

Finally, there is a need for local validation and verification of crAssphage and PMMoV to confirm their local utility despite their global prevalence. Analysis of samples taken at exposure sites do not allow highly accurate pathogen exposure risk without further characterization of the source of fecal pollution. The continued global evaluation of crAssphage, PMMoV, and other viral targets will further support their use in regulatory applications and QMRA. In addition to local assay validation, it would be beneficial to conduct multilaboratory assay validation for widely used crAssphage and PMMoV assays.

Conclusions

The high abundance in sewage and human association of crAssphage and PMMoV supports their continued development as viral water quality monitoring tools. Multiple research needs, including improved description of fate in the environment of crAssphage, PMMoV, and viral pathogens; geographic variability of the abundance and genetic makeup of crAssphage and PMMoV;

and improved connection between molecular crAssphage and PMMoV detections and viral pathogen infectious risk will support the use of crAssphage and PMMoV in QMRA and regulatory applications.

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Conflict of interest statement

K.B. is a co-inventor on a US patent application entitled "Cross-Assembly Phage DNA Sequences, Primers and Probes for PCR-based Identification of Human Fecal Pollution Sources" (Application Number: 62/386,532). United States universities and non-profit researchers interested in using this technology must obtain a research license from the US EPA. To apply for a research license, please request additional information from ftta@epa.gov. The authors declare no other conflict of interest.

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