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Particle association and size fraction of molecular viral fecal pollution indicators in wastewater†

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Fecal indicator bacteria currently used for water quality monitoring inadequately represent viral fate in water systems, motivating the development of viral fecal pollution indicators. Molecular viral fecal pollution indicators such as crAssphage and pepper mild mottle virus (PMMoV) have emerged as leading viral fecal pollution indicator candidates due to ease and speed of measurement and target specificity. Elucidating the fate of molecular viral fecal indicators in water systems is necessary to facilitate their development, broader adoption, and ultimately their association with infectious risk. A significant mechanism controlling the behavior of viral indicators in environmental waters is association with particles, as this would dictate removal *via* settling and transport characteristics. In this study, we investigated the particle associations of six molecular fecal pollution targets (crAssphage, PMMoV, adenovirus, human polyomavirus, norovirus, HF183/BacR287) in wastewater using a cascade filtration approach. Four different filters were employed representing large settleable particles (180 μm), larger (20 μm) and smaller suspended particles (0.45 μm), and non-settleable particles (0.03 μm). All molecular targets were detected on all particle size fractions; however, all targets had their highest concentrations on the 0.45 μm (percent contribution ranging from 40% to 80.5%) and 20 μm (percent contribution ranging from 3.9% to 39.4%) filters. The association of viral fecal pollution targets with suspended particles suggests that particle association will dictate transport in environmental waters and that sample concentration approaches based upon particle collection will be effective for these targets.

Water impact

The majority of fecal viral indicators in wastewater are attached to settleable particles. Particle associations have significant impacts on viral fecal indicator transport through environmental waters. Incorporating particle association through transport modeling or suitable fecal indicator selection is essential to accurately measuring environmental fecal contamination.

Introduction

Sewage contamination in freshwater water resources is a global problem affecting more than two billion people.[1] Illnesses from fecal contaminated waters are a leading cause of child deaths globally,[2] and exposure to sewage contaminated recreational water results in $2.2–3.7 billion in annual economic loss in the US alone.[3] Viruses are predicted to account for the majority of illnesses caused by sewage contaminated water under direct exposure scenarios.[4,5] Hence, viral fecal indicators have been proposed to improve water quality monitoring upon commonly used bacterial indicators.

Non-particle associated viral indicators and free DNA or RNA that may be detected using molecular methods typically would not settle in water systems unless attached to a larger particle. Indicator attachment to larger particles would also dictate removal efficiency in wastewater treatment techniques using sedimentation. In addition, particle association could also make indicators more resistant to sunlight and other inactivation processes, thereby increasing their persistence through shielding.[6] Fecal indicator attachment to particles depends on the water matrix, surface charge, shape, and size of the virus/molecular target and particle characteristics.[7] Differing viral indicators have differing attachment mechanisms, which dictates variable particle associations profiles. Previous studies have examined the particle association of a subset of sewage associated RNA viruses (*e.g.*, PMMoV and norovirus genogroup I and II[8]) using a

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sequential filtration approach.[8,9] Symonds *et al.* found PMMoV, rotavirus, and norovirus in sewage had low associations with particles above 180 μm and higher associations with particles below 180 μm or free flowing.[9] Similar trends were seen by da Silva *et al.*, which showed Norovirus GII had higher concentrations on the 0.45 μm filter than on the 180 μm filter.[8] Currently, there is a critical knowledge gap regarding the particle association of a number of other promising viral indicators, such as crAssphage. Viral association with particles in smaller size ranges is also understudied and is important for understanding effective size of virus which could be used to model transport of virus in streams.

Multiple promising viral indicators, such as adenovirus (AdV), human polyomavirus (HPyV), crAssphage, and PMMoV, have been proposed as indicators of fecal pollution. AdV and HPyV are human pathogenic viruses that cause a wide range of illnesses but their low concentrations in sewage and varying prevalence by region have limited their broad suitability.[10] CrAssphage and PMMoV non-pathogenic viruses (a bacteriophage and plant virus, respectively) that have been proposed as fecal indicators due to their high abundance in and specificity to wastewater.[11,12] CrAssphage and PMMoV also have greater similarity in decay and codetection with viral pathogens than FIB.[11–15] Both crAssphage's and PMMoV's application as fecal indicators has been thoroughly reviewed elsewhere.[16] However, further studies on the characteristics and mechanisms of the fate and transport of crAssphage and PMMoV are needed to further develop them as suitable viral indicators of fecal pollution.

Beyond viral indicators, further measurements of the bacterial target, HF183/BacR287 (HF183), and the viral pathogen, norovirus (NoV), can provide important particle association profile comparisons between viral pathogenic and bacterial targets. HF183 is a bacterial target applied as a PCR assay developed in 2004 from the HF183 *Bacteroides* 16S rRNA genetic marker.[17] It is highly abundant in wastewater and wastewater impacted environments.[12,18] NoV is a single stranded RNA viral pathogen that causes acute gastroenteritis in children and has since been detected in wastewater samples.[19] Due to the low concentrations of NoV in wastewater, indicators with higher concentrations are usually measured instead to understand fecal contamination in sewage impacted waters.

All indicators and microbial targets explored here are generally applied using molecular detection methods, (*i.e.*, based on DNA/RNA detection), which provides benefits such as target specificity and speed of measurement. However, molecular-based measurements do not provide information on viability due to capturing both intact viruses and free flowing extracellular DNA/RNA. Free flowing viral DNA/RNA and intact viruses have different sizes and exterior composition which could affect environmental persistence. Both free flowing viral DNA/RNA and intact viruses have the ability to interact with larger particles[20,21] and, as previously mentioned, the attachment of viruses to larger particles in wastewater can be a major factor controlling both decay and transport in sewage impacted waters.[8,9] Therefore, there is a critical need to determine the effective size that viral particle/DNA/RNA association creates for each molecular indicator.

In the current study we examined the particle association of molecular fecal indicators PMMoV, crAssphage, AdV, HPyV, HF183/BacR287 (HF183), and NoV GII in wastewater. We used a cascade filtration format through four different filter sizes to differentiate between operationally defined sizes for settleable particles (>180 μm), suspended particles (20–180 μm and 0.45–20 μm), and non-settleable particles (>0.03 μm). The particle association profiles of these targets will help to inform both fecal indicator transport mechanisms and appropriate target concentration approaches to facilitate target detection.

Methods

Experimental setup

Wastewater samples were collected from the primary influent of a regional St. Joseph County, IN, USA wastewater treatment plant monthly from September to December 2019. Samples were then filtered through cascade filtration format as described previously by da Silva *et al.* (Fig. S1†).[8] Briefly, 50 mL of each sample was sequentially filtered through 180 μm nylon net filter (Millipore), 20 μm nylon net filter (Millipore), and 0.45 μm polyethersulfone membrane filter (Sterlitech). The final filtrate from the 0.45 μm filter was then filtered through a 0.03 μm polyethersulfone membrane filter (Sterlitech). Sample volumes were optimized prior to experimentation to maintain constant filter flow rates and minimize plugging. Filter sorption of 'free' viruses or particles was not directly assessed, *i.e.*, particle sizes were operationally defined by collection on the appropriate filter. Flow rates were also not directly maintained or measured but the volumes used (50 mL) were previously tested on wastewater samples so to ensure efficient filtration and concentration of fecal targets with no filter clogging. The four filters and 400 μL of the initial primary influent sample (single samples were done on each day) were stored at −80 °C in bead tubes for DNA/RNA extractions. The QIAGEN AllPrep PowerViral DNA/RNA kit was used for simultaneous DNA and RNA extraction from samples and membrane filters in this study following manufacturer instructions. For direct extraction of initial wastewater sample, 400 μL of wastewater was placed in DNA/RNA kit tube and extraction was done following manufacturer instructions as with membrane samples.

Physiochemical testing

Conductivity, turbidity, total suspended solids, and pH were measured in all wastewater samples. The air and water temperature at the wastewater treatment plant was also recorded. Sewage particle size distribution above 5 μm was measured using the Fluid Imaging Technologies FlowCam

(Tokyo, Japan) and sewage particle size distribution below 10 μm was measured using Brookhaven BIC NanoBrook Omni (Holtsville, NY).

Molecular analyses

Molecular targets were measured using droplet digital polymerase chain reaction (ddPCR) and previously published assays for crAssphage, HF183, PMMoV, AdV, HPyV and NoV GII.[9,15,22] Assays in this study have shown comparable or improved detection and quantification when adapted to ddPCR.[23–26] Primers, probes, and cycling conditions for each assay are displayed in Table S1.† For DNA markers, reaction mix in each well was made up of 10 μL of ddPCR supermix for probes (BioRad, CA, USA), 1 μL of primer probe mix, 6 μL of DNase-free water and 3 μL of DNA sample to a total volume of 20 μL per well. The final concentration of primers and probes were 900 nM and 250 nM, respectively. Thermocycling conditions for each assay are described in Table S1.†

For the RNA markers (PMMoV and NoV GII), the reaction mix in each droplet cartridge well was made up of 5 μL of One-step RT-ddPCR advanced kit supermix (BioRad, CA, USA), 2 μL of reverse transcriptase, 1 μL of 300 mM DTT, 6 μL of RNase free water, and 5 μL of RNA sample to a total volume of 20 μL per well. The final concentration of primers and probes were 900 nM and 250 nM, respectively. Thermocycling conditions for each assay are described in Table S1.† The limit of detection was determined for each assay to be 1.70 $\log_{10}$ genome copies/reaction based on a previous study.[27]

Statistical analysis

The concentration on each filter was compared between the different filters for all fecal pollution targets using two-way ANOVA followed by Fischer's LSD multiple comparisons test with a cut-off p-value of 0.05. Each experimental day was used as a replicate variable in the statistical tests.

Results

Sample characteristics

Sample characteristics and other physical details are summarized in the ESI.† Sewage particle size distribution results are shown in Fig. 1. To calculate percent contribution, particles in each size group on the FlowCam (5–20 μm, 20–180 μm, and >180 μm) and BIC Nanobrook Omni (0.03–0.45 μm and 0.45–10 μm) were normalized by the total number of particles detected by each instrument, respectively. Overall, the BIC NanoBrook Omni (range 0.3 nm to 10 μm) and FlowCam (range 5 μm to 6000 μm) results indicated that the majority of the particles were between 0.45 and 20 μm (Fig. 1). More specifically, on the FlowCam, 77.6% of particles were smaller than 20 μm, 22.0% between 20 and 180 μm, and 0.30% above 180 μm.

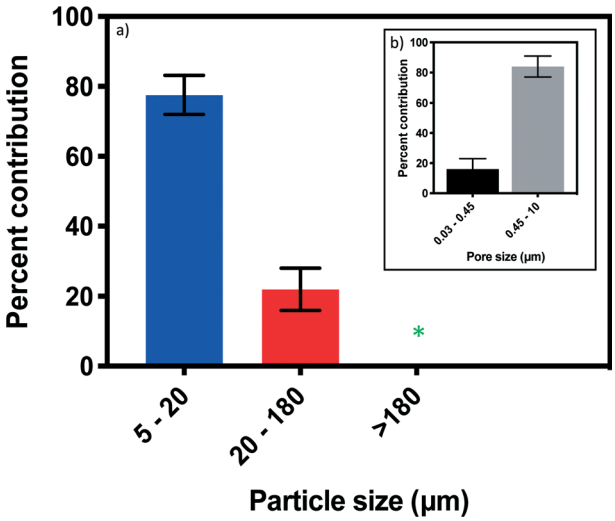

Fig. 1 Sewage particle size distribution for particles larger than 5 μm as measured by the FlowCam (a). Data was grouped into 5–20 μm, 20–180 μm, and >180 μm particle size groups. Number of particles in each size group was divided by total number of particles in all size groups then multiplied by 100 to get percent contribution for FlowCam measurements. Inlet figure (b) shows sewage particle size fraction below 10 μm as measured by the BIC Nanobrook Omni. Data was grouped into 0.03–0.45 μm and 0.45–10 μm particle size groups. Number of particles in each size group was divided by total number of particles in all size groups then multiplied by 100 to get percent contribution for Nanobrook measurements. The scattering technique used by the NanoBrook allows it to only pick up the three highest size distribution peaks. Each point represents the average of five sampling points throughout the experiment. Data from all replicates are presented in Tables S1 and S2.†

The BIC NanoBrook Omni employs a technique that can only pick up the three highest peaks; hence, analysis with the BIC NanoBrook Omni showed 83.9% of particles (the three highest peaks) between 0.45 and 10 μm.

Target capture efficiency

To assess efficiency of target capture by the filters, the total target concentration on the four filters were normalized by the target concentration in the direct extraction of the initial wastewater sample. AdV, HPyV, and NoV GII were below detection limits in the direct extraction, consistent with concentrations observed by filtration experiments. The capture efficiencies were calculated for each day of the experiment and displayed in Fig. 2. PMMoV, crAssphage, and HF183 had average filtration capture efficiencies of 92.5%, 129.6%, and 116.4%, respectively, when compared to direct extraction. The values observed over 100% may be due to variability in target concentration within the wastewater matrix coupled with a small extraction volume or other related factors. These data confirm that the experiments were effective in measuring the targets and that a significant fraction was not missed in the cascade filtration experimental design.

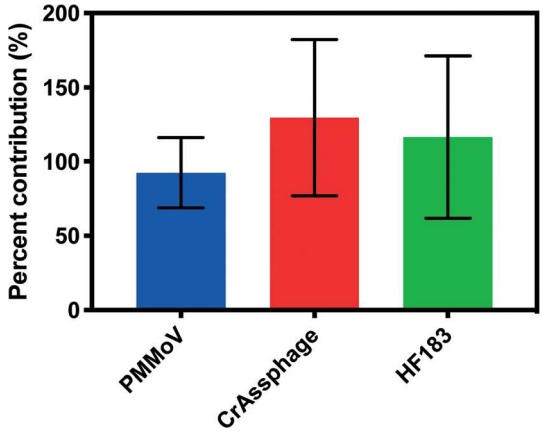

Fig. 2 Capture efficiency of viral fecal targets crAssphage, PMMoV, and bacterial target HF183. Percent contribution was calculated by dividing the total concentration on all the filters by the concentration found in the direct extraction of the initial wastewater sample then multiplying by 100. Detection limits for all conditions are included in the ESI.† Error bars represent the standard deviation for each sample. The five replicate experiments were averaged to achieve graphs displayed above.

Target size distribution

The size distribution of molecular targets as assessed by cascade filtration is shown in Fig. 3. The relative contribution of each size fraction was calculated by dividing the concentration on each filter by the sum of the concentrations on all the filters for that sample. All viral molecular targets had the highest concentrations on the 0.45 μm filter, followed by the 20 μm filter. PMMoV, crAssphage, NoV GII, and AdV had their third highest concentrations on the 180 μm filter, followed by their lowest concentrations on the 0.03 μm filter. HPyV had its third highest concentrations on the 0.03 μm filter followed by their lowest concentrations on the 180 μm filter. HF183 had its highest concentration on the 0.45 μm filter, followed by the 180, 20, and 0.03 μm filter.

Statistical analysis

For individual molecular targets, the concentrations on each filter were compared only within each target type using a one-way ANOVA followed by a Fischer's LSD multiple comparison. ANOVA results showed significantly higher concentrations on the 0.45 μm filter than on the other three filters for crAssphage, HPyV, NoV GII, and HF183. Concentrations on the other three filters (0.03, 20 and 180 μm) were not significantly different for crAssphage, HPyV, NoV GII, and HF183. Also, when comparing within the size distribution of PMMoV and AdV, the concentrations on the 180 μm and 0.45 μm filters and the 180 μm and 0.03 μm filters were not statistically different. The capture efficiency between PMMoV, crAssphage, and HF183 was not statistically different as determined using a one-way ANOVA followed by a Fischer's LSD multiple comparisons test.

Discussion

Impact of particle association for viral fecal indicator fate

Our study showed that viral targets were primarily associated with particles greater than 0.45 μm, which has implications for how they persist and transport in the environment. Typically, viral particles are in the 30–300 nm range and free DNA or RNA may be even smaller. However, all viral targets tested in this study had their highest concentrations on the 0.45 μm filter, implying that their functional hydrodynamic diameter would be larger than the free viral particles. As larger particles tend to settle faster, differential transport is expected with differences in sizes. To put our data in context, settling velocities for each filter pore size (180, 20, 0.45 and 0.03 μm) were calculated using Stoke's equation (details in ESI†). The results showed that the particles captured on the 180 μm filter represent particles that will settle during primary sedimentation in wastewater treatment plants (settling velocity >4.03 cm min^{-1}). Particles captured on the 0.45 and 20 μm filters represent particles that may settle depending on the mixture within the system (settling velocities $>3.5 \times 10^{-5}$ and >0.05 cm min^{-1} for 0.45 and 20 μm, respectively). Particles trapped on the 0.03 μm filter represent non-settling particles in water (settling velocity $>1.1 \times 10^{-7}$ cm min^{-1}). Therefore, these fecal targets could have vast differences in transport in flowing water than targets that are not particle associated. These results also show sedimentation processes in a wastewater treatment plant are likely not the primary removal mechanism for the fecal targets tested in this study as targets were mainly associated with particles smaller than 180 μm (settleable particles). This has also been shown in previous studies where concentration of fecal targets remains high after primary sedimentation.[27,28]

The highest total number of particles in the samples was observed between 0.45 and 20 μm, similar to the viral target size fraction with the highest concentrations on the 0.45 μm filter. Given challenges in accurately quantifying the number and composition of particles across these size ranges, we did not attempt to normalize by particle number. Viral targets may be associating with the most numerous particles in sewage; hence, viral target transport would mainly depend on the type and abundance of particles. Furthermore, if we extend this assumption to environmental matrices where smaller size particles (in the range 0.03–0.45 μm) have a higher abundance relative to larger particles (in the range 0.45–180 μm), viral targets could instead associate highly with smaller size particles. However, additional research investigating particle association in environmental matrices is necessary to prove this assumption.

The viral targets HPyV and PMMoV had statistically similar concentrations on both the 20 and 0.45 μm filters whereas the other fecal targets had significantly lower concentrations on the 20 μm filter than the 0.45 μm filter. HPyV and PMMoV's higher percentage contribution on these particles between 20 and 180 μm could then lead to different

settling times resulting in differing transport and persistence patterns between these viral targets (HPyV and PMMoV) and the other fecal targets (crAssphage, AdV, HF183, and NoV) tested in this study. Differences between observations for these two viral groups could be due to a number of reasons not tested in this study, such as genome structure or capsid

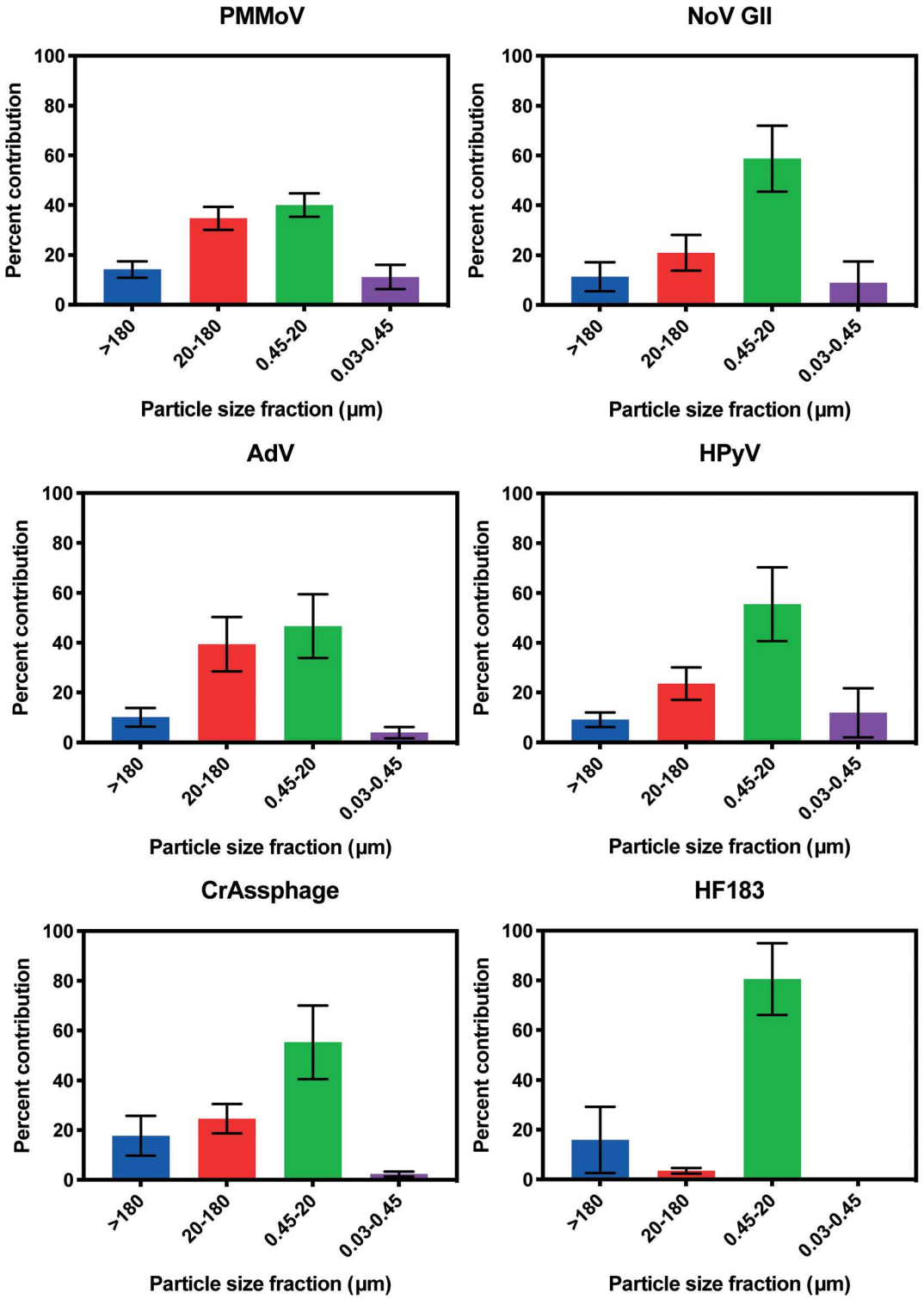

Fig. 3 Particle association of viral fecal targets crAssphage, PMMoV, AdV, NoV GII and HPyV and bacterial target HF183. Percent contribution was calculated by dividing the concentration on each filter by the total concentration on all the filters. Detection limits for all conditions are included in the ESI.† Error bars represent the standard deviation for each sample. The five replicate experiments were averaged to achieve graphs displayed above.

protein differences. The sole bacterial target, HF183, also had its highest concentrations on the 0.45 μm filter. Concentrations on all other filter sizes were significantly lower. In contrast, this suggests that HF183 may be unattached and not associating with particles that are larger than it or that HF183 free DNA is mainly associated with particles between 0.45 and 20 μm.

Literature comparison

Few prior studies have characterized fecal viral particle association in environmental waters. Previous analyses have used a variety of approaches, such as the cascade filtration format used in this study and the continuous flow centrifugation of filtration used in a study done in 1981 by Hejkal *et al.*[21] The study done in 1981 contrasted from our study as it showed viruses to be predominantly on finer particles smaller than 0.3 μm.[21] Study done by Hejkal *et al.* used culturable methods to enumerate viruses whereas our study used molecular methods; this difference in methods may cause differential particle association patterns. Similar to our study, the two previous studies that utilized the cascade filtration format and molecular methods found viruses on a wide range of particles, including particles above 0.3 μm.[8,9] A previous particle association study by Symonds *et al.*[9] found PMMoV may be a suitable surrogate for enteric viruses in particle association studies due to the similar particle association trends in their results but in our study, the addition of the 20 μm filter shows major particle association differences between PMMoV and NoV GII. With previous studies[15,29,30] showing PMMoV having extended persistence compared to other viral indicators and the current study showing differences in particle association between PMMoV and NoV GII, this could ultimately impact the suitability of PMMoV to be a surrogate for enteric viruses. The similar particle associations between crAssphage and the other targets, such as NoV GII, suggests crAssphage may have more potential as a suitable surrogate for NoV GII, AdV, and other enteric viruses; however, further research into their fate and transport mechanisms is still required to conclusively develop a suitable surrogate.

Study limitations

A limitation of the current study is the examination of samples from one wastewater sampling location. Wastewater from varying locations across the globe have different water characteristics that could affect viral particle associations. Water characteristics such as pH and salinity have been shown to affect the attachment of viruses to particles. The quantity of organic matter and number of particles could also lead to differing concentrations. Furthermore, our study only examined a single type of water matrix, *i.e.*, wastewater. In different water matrices with lower levels of contamination, the conclusions from this study may not hold as trends may vary. Further research outside the scope of our study should incorporate wastewater and wastewater contaminated environmental water samples from multiple locations.

Another limitation of the current study is that all the fecal targets used in this study are molecular targets. Though molecular targets provide fast measurement and the ability to detect a wide diversity of targets, they are unable to assess viability due to the capture of both intact viruses and extracellular DNA in its measurement. Both extracellular DNA/RNA and intact viruses can associate with particles through different mechanisms, hence, molecular targets could be overestimating/underestimating viable particle association. Importantly, the majority of proposed fecal indicators, including those evaluated here, are being developed as molecular indicators; thus, they are measured here as they would be used in water quality monitoring applications.

An additional limitation of the current study is the use of different filtration membrane material between larger and smaller pore size filters. The differential filtration material could result in different extraction efficiencies which could impact comparison of fecal targets. Furthermore, our study did not examine extraction efficiencies across the four filters and the direct extraction of the initial wastewater sample; however, comparison of target quantities from direct extractions with the sum from filter extractions suggest that extraction efficiencies did not drive observed trends across particle sizes.

Conclusions

This study showed that the fecal targets evaluated are primarily present on settleable particles in wastewater. These particle interactions change the effective size of our fecal targets in wastewater, changing transport characteristics in the environment. Implications from these findings include the choice of viral concentration approaches, fecal target fate during wastewater treatment and in the environment. Our study also showed differences in particle association profiles between different viral targets. Future work evaluating the particle association of fecal targets in varying environments will be essential for future development of molecular tools for water quality assessment in sewage contaminated waters.

Conflicts of interest

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 **https://ftta@epa.gov**. The authors declare no other conflict of interest.

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