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Comparative fate of CrAssphage with culturable and molecular fecal pollution indicators during activated sludge wastewater treatment

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ABSTRACT

Wastewater treatment plants are typically monitored using fecal indicator bacteria to ensure adequate microbial water quality of the treated effluent. Fecal indicator bacteria exhibit poor correlation with virus fate in the environment, including during wastewater treatment. Viral-based microbial source tracking methods have the potential to overcome this limitation. The recently discovered human gut bacteriophage crAssphage is a promising viral human fecal indicator. In this current study, primary influent, primary effluent, secondary effluent, and final effluent of a conventional activated sludge wastewater treatment plant were analyzed for a suite of fecal indicators to evaluate the suitability of crAssphage as a wastewater process indicator for virus removal. CrAssphage was the most abundant fecal indicator measured through the wastewater treatment process. Culturable and molecular bacterial fecal pollution indicators showed higher removal than viral fecal pollution indicators, including crAssphage, confirming the necessity of a viral-specific fecal monitoring target. CrAssphage was strongly correlated with adenovirus and polyomavirus molecular indicators through the wastewater treatment process. Literature comparison demonstrated site-specific removal of molecular fecal indicators during wastewater treatment highlighting the need for local performance validation. The high abundance of crAssphage and correlation with pathogenic viruses suggests the potential suitability of crAssphage as a viral fecal pollution process indicator during wastewater treatment.

1. Introduction

The World Health Organization estimates that 1.8 billion people globally consumed fecal-contaminated water in 2014 (WHO/UNICEF Joint Water Supply and Sanitation Monitoring Programme, 2014), and that 9.1% of the global burden of disease and 6.3% of all deaths are due to unsafe water, inadequate sanitation, and poor hygiene (Pruss-Ustun and WHO, 2008). Wastewater disposal, either prior to or following treatment, is a primary source of fecal pathogens in the water environment. Wastewater may contain a diverse array of pathogens, including viruses, bacteria and protozoa.

Pathogens in wastewater are traditionally monitored using fecal indicator bacteria (FIB), such as *E. coli* and enterococci. Despite their widespread implementation, FIB have well-known limitations, including inadequately capturing viral risk to human health (Schmitz et al., 2016). Relevant to the current study, FIB do not correlate well with enteric viruses through wastewater treatment (Carducci et al., 2009; Okoh et al., 2010; Sinclair et al., 2009). Notably, viral pathogens are predicted to pose the greatest infection risk following human exposure in receiving water (Boehm et al., 2015; Crank et al., 2019). A viral fecal pollution indicator to understand viral fate and release during wastewater treatment is needed to improve monitoring efforts to protect public health.

Microbial source tracking (MST) methods based on host-specific assays for water monitoring have the potential to differentiate fecal contamination sources and better capture human health risk. Sewage-associated indicators are particularly significant because human fecal contamination poses the greatest human health risk (Soller et al., 2010, 2014). A leading MST method is the HF183/BacR287 assay, developed from the 16S rRNA gene of *Bacteroides dorei* and closely related taxa (Ahmed et al., 2016; Bernhard and Field, 2000; Green et al., 2014). This assay has been widely used due to its high prevalence and concentration in sewage for nearly two decades (Mayer et al., 2018). The HF183/BacR287 assay is considered one of the best performing sewage-associated source tracking assays and consistently performs well in multi-laboratory validation studies (Boehm et al., 2013), though HF183/BacR287 was found weakly correlated with viral indicators after secondary wastewater treatment (Hughes et al., 2017).

Previously proposed viral fecal pollution indicators include somatic coliphage, human adenovirus (HAdV), and human polyomavirus

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(HPyV), among others. Somatic coliphage infects and replicates in coliform bacteria and is found in elevated levels in fecal material of both humans and animals (Ng et al., 1993). Previous associations have been demonstrated between somatic coliphage and both infectious enteroviruses and enterovirus genomes in treated wastewater samples (Gantzer et al., 1998; Pina et al., 1998). HAdV and HPyV are common human viral pathogens with DNA genomes. Both the molecular HAdV assay and the molecular HPyV assay have been widely suggested as a viral fecal pollution indicators (Albinana-Gimenez et al., 2006; Bauer et al., 2011). One significant limitation for these assays is that HAdV and HPyV are typically detected at concentrations orders of magnitude lower than the HF183/BacR287 assay in wastewater (Stachler et al., 2018), which may be a challenge when the detection of contamination level is low in environmental water samples.

Cross-assembly phage (crAssphage) is a recently developed viral fecal pollution indicator. CrAssphage was discovered by metagenomic data mining and was reported to be the most abundant virus in human gut (Dutilh et al., 2014) and was later shown to be globally distributed (Edwards et al., 2019). Further metagenomic evaluation demonstrated that crAssphage is closely associated with human fecal waste, and was suggested for human fecal source identification (Stachler and Bibby, 2014). Recently, it has been shown that 'crAssphage' is the prototypical member of a family of crass-like phages (Yutin et al., 2018); for the purposes of this paper, 'crAssphage' refers to the prototypical crAssphage (i.e., crAssphage *sensu stricto*). Subsequently, molecular microbial source tracking assays have recently been published (Cinek et al., 2018; García-Aljaro et al., 2017; Stachler et al., 2017). Molecular crAssphage assays have recently been successfully deployed in environmental waters with demonstrated correlation with microbial source tracking markers, pathogens, and antibiotic resistance genes (Ahmed et al., 2018a,b; Ballesté et al., 2019; Kongprajug et al., 2019; Stachler et al., 2018, 2019). CrAssphage was previously reported to have a higher persistence than molecular bacterial MST markers (Ballesté et al., 2019). CrAssphage has also been suggested as a marker of human health risk in sewage impacted waters (Bibby et al., 2019; Crank et al., 2019). Few studies have yet conducted a process-based comparison of crAssphage performance as a human fecal indicator through wastewater treatment, although recent studies have evaluated crAssphage removal during wastewater treatment (Farkas et al., 2019; Malla et al., 2018).

The overarching goals of the current study were to evaluate crAssphage removal during activated sludge wastewater treatment as well as the suitability of crAssphage as a viral process indicator during wastewater treatment. Samples were taken from the primary influent, primary effluent, secondary effluent, and final effluent of a conventional activated sludge wastewater treatment plant. The removal and co-occurrence of the evaluated fecal indicators were statistically compared through the wastewater treatment process. In addition, observed removals were compared with literature values to provide context for study evaluations. The current study demonstrates the fate of crAssphage and other fecal indicators to evaluate crAssphage's suitability as a viral removal process indicator during activated sludge wastewater treatment.

2. Material and methods

2.1. Study site and sampling

Samples were collected from an anonymous conventional activated sludge wastewater treatment plant (WWTP) in Indiana, USA. Wastewater treatment process details and permitted monthly effluent limits are provided in the Supplemental Information; no process upset occurred, and no samples exceeded permitted effluent limits during the sampling period. Samples were collected at approximately 9:00 AM (EDT) on 11 days from June 2018 to September 2018. The samples were collected from primary influent (n = 11), primary effluent (n = 11), secondary effluent (n = 8), and final effluent (n = 11) as indicated in Fig. S1. All samples were stored in one-liter sterile containers, transported on ice and returned to the laboratory for analysis within one hour of collection. The temperature, flow rate, and dissolved oxygen (D.O.) information for each sampling date were provided by the WWTP as the average of daily measured values. While the seasonality of crAssphage in wastewater is currently unknown, previous studies demonstrated no clear seasonal trend for other viral molecular indicators including HAdV (Hamza et al., 2019; Schmitz et al., 2016) and HPyV (Hamza and Hamza, 2018; Prado et al., 2019).

2.2. Chemical parameter characterization

Sample pH was measured using a B10P Benchtop pH Meter (VWR International, Radmor, PA). Turbidity was measured using a 2100P Portable Turbidimeter (HACH, Loveland, CO). Conductivity was measured using HACH PocketPro (HACH, Loveland, CO). All parameters were tested in triplicate.

2.3. Enumeration of culturable indicators

E. coli were measured by membrane filtration and cultured on Difco modified mTEC agar (BD, Franklin Lakes, NJ) following EPA Method 1603 (USEPA, 2002). Enterococci were measured by membrane filtration and culturing on Difco mEI agar (BD, Franklin Lakes, NJ) following EPA Method 1600 (USEPA, 1997). Somatic coliphages were enumerated via a single-agar layer plaque assay procedure according to EPA Method 1602 (USEPA, 2001).

2.4. ddPCR assays

Wastewater samples were concentrated for subsequent DNA extraction immediately upon receipt at the lab as described previously (Ahmed et al., 2015; Stachler et al., 2018). Previous recovery estimates using this method for HAdV and HPyV using this method ranged from 31% to 78% (Ahmed et al., 2015). Briefly, 100 mL of each sample was pH adjusted to pH 3.5 by adding hydrochloric acid and filtered through a 47 mm diameter 0.45 μm GN-6 Metricel mixed cellulose ester filter membrane (Pall, Port Washington, NY) (Ahmed et al., 2015; McQuaig et al., 2009). DNA for all samples was extracted immediately after sample concentration using a DNeasy PowerSoil Kit (Qiagen, Valencia, CA), following the manufacturer's protocol. Extracted DNA was stored at − 20 °C prior to analysis. Droplet digital polymerase chain reaction (ddPCR) assay primers and probes are reported in Table 1. TaqMan hydrolysis probe assays targeting crAssphage (CPQ56) (Stachler et al., 2017), the HF183/BacR287 molecular indicator region (Green et al., 2014), human adenovirus (HAdV) (Heim et al., 2003), and human polyomavirus (HPyV) (McQuaig et al., 2009) were performed as described previously (Stachler et al., 2019). Additional assay details are included in the Supporting Information, including ddPCR summary statistics (Table S1).

Technical assay reproducibility for CPQ56, HF183/BacR287, HAdV and HPyV was assessed by running duplicate for most samples (86.36% (38 out of 41), 65.91% (29 out of 41), 72.73% (32 out of 41) and 75.00% (33 out of 41) samples, respectively). Results demonstrated high technical reproducibility (Fig. S2) with R^2 = 0.999.

2.5. Controls

Negative process controls for mTEC and mEI plates were performed each sampling day with sterile buffer. Negative process controls for the somatic coliphage procedure were performed each sampling day with DI water. Filter control DNA extractions were performed by placing a sterile filter in a bead mill tube, and extraction blanks were performed and tested by ddPCR for all assays. One no template control (NTC) assay was performed for each ddPCR cartridge with molecular grade water.

Table 1
Assay primers and probes.

Assay	*Primer or probe*	*Sequence 5′ - 3′*	*Reference*
CPQ56	056F1	CAGAAGTACAAACTCCTAAAAAACGTAGAG	Stachler et al. (2017)
	056R1	GATGACCAATAAACAAGCCATTAGC	
	056P1	(FAM)-AATAACGATTTACGTGATGTAAC-(MGB)	
HF183/BacR287	HF183	ATCATGAGTTCACATGTCCG	Green et al. (2014)
	BacR287	CTTCCTCTCAGAACCCCTATCC	
	BacP234MGB	(FAM)-CTAATGGAACGCATCCC-(MGB)	
HAdV	AQ1	GCCACGGTGGGGTTTCTAAACTT	Heim et al. (2003)
	AQ2	GCCCCAGTGGTCTTACATGCACATC	
	Adeno, AP	(56-FAM)-TGCACCAGACCCGGGCTCAGGTACTCCGA-(3BHQ_1)	
HPyV	SM2	AGTCTTTAGGGTCTTCTACCTTT	McQuaig et al. (2009)
	P6	GGTGCCAACCTATGGAACAG	
	KGJ3	(FAM)-TCATCACTGGCAAACAT-(MGBNFQ)	

All NTCs and process controls were negative throughout the course of the study.

2.6. Statistical analyses

Concentrations of fecal pollution indicators through each process step were normalized as the ratios over crAssphage concentrations to evaluate differential fate. Pearson correlation coefficients (r) were calculated on the means of log-transformed data in RStudio 1.1.463 (RStudio, Inc., Boston, MA) between all indicators used in the study (i.e., both culturable and ddPCR concentrations) using two-tailed 95% confidence intervals. Correlation coefficients are characterized by the following scale (Evans, 1996) for comparison purposes: 0.40–0.59 (weak correlation), 0.60–0.79 (moderate correlation), 0.80–1.0 (strong correlation). Graphs from the current study were drawn in RStudio 1.1.463 using averages of data sets with R 3.6.0 (packages used: tidyverse, readxl, ggpubr, scales, gridExtra, Hmisc, reshape2).

3. Results and discussion

3.1. Wastewater treatment plant and sample characteristics

Samples were collected at an anonymous conventional activated sludge wastewater treatment plant at 11 timepoints from June 2018 to September 2018. Primary influent flowrates ranged from 0.39 m^3/s to 0.73 m^3/s and temperatures on sampling days ranged from 16.11 to 26.67 °C. D.O., flowrate, temperature, and dissolved oxygen information on sampling days are shown in Table S2. Turbidity and conductivity measurements are shown in Figs. S3 and S4, respectively. No process upset occurred during the sampling period.

3.2. Culturable fecal indicator quantification

Culturable *E. coli*, enterococci, and somatic coliphage concentrations through each process step are presented in Fig. 1. *E. coli* concentrations averaged 5.74 $\log_{10}$ CFU/100 mL in primary influent and 5.40 $\log_{10}$ CFU/100 mL in primary effluent, and all samples were below detection limit (20 CFU/100 mL) in secondary and final effluent. Enterococci concentrations averaged 5.94 $\log_{10}$ CFU/100 mL in primary influent and 5.73 $\log_{10}$ CFU/100 mL in primary effluent, and all samples were below detection limit (20 CFU/100 mL) in secondary and final effluent. Somatic coliphage concentrations averaged 5.45 $\log_{10}$ PFU/100 mL in primary influent and 5.42 $\log_{10}$ PFU/100 mL in primary effluent. Seven out of eight secondary effluent samples were in the quantifiable range (5 PFU/100 mL detection limit) with an average 3.47 $\log_{10}$ PFU/100 mL concentration. One out of twelve final effluent samples were quantifiable with a concentration of 1.18 $\log_{10}$ PFU/100 mL.

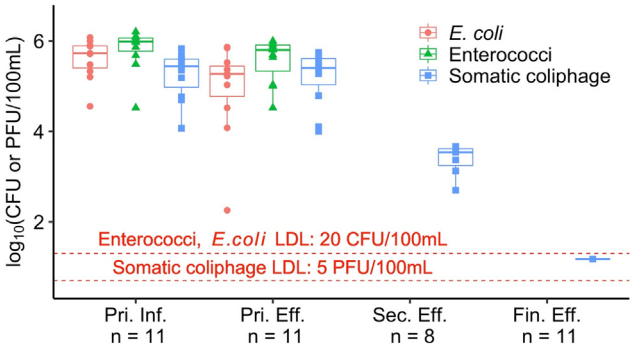

Fig. 1. Culturable *E. coli*, enterococci, and somatic coliphage abundances throughout each process step. X-axis shows sample source (Pri. Inf.: primary influent, Pri. Eff.: primary effluent, Sec. Eff.: secondary effluent, Fin. Eff.: final effluent) and sample number count; y-axis shows concentration in $\log_{10}$ colony forming unit (CFU) per 100 mL for *E. coli* and enterococci or $\log_{10}$ plaque forming unit (PFU) per 100 mL for somatic coliphage. The lower detection limit (LDL) for each assay is shown by the dashed red line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Molecular fecal indicator quantification

The concentrations of molecular fecal source tracking indicators crAssphage (measured as CPQ56), HF183/BacR287, human enteric adenovirus (HAdV), and human polyomavirus (HPyV) through each process step are presented in Fig. 2. CrAssphage concentrations

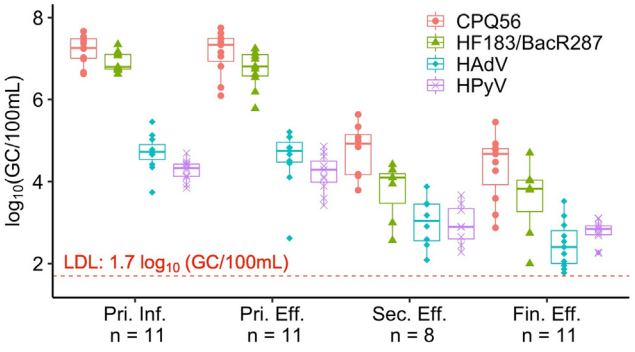

Fig. 2. Molecular crAssphage (CPQ56), HF183/BacR287, HAdV, and HPyV abundance throughout each process step. X-axis shows sample source (Pri. Inf.: primary influent, Pri. Eff.: primary effluent, Sec. Eff.: secondary effluent, Fin. Eff.: final effluent) and sample number count; y-axis shows concentration in $\log_{10}$ GC/100 mL. Lower detection limit was 1.70 $\log_{10}$ (GC/100 mL). The lower detection limit (LDL) for each assay is shown by the dashed red line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

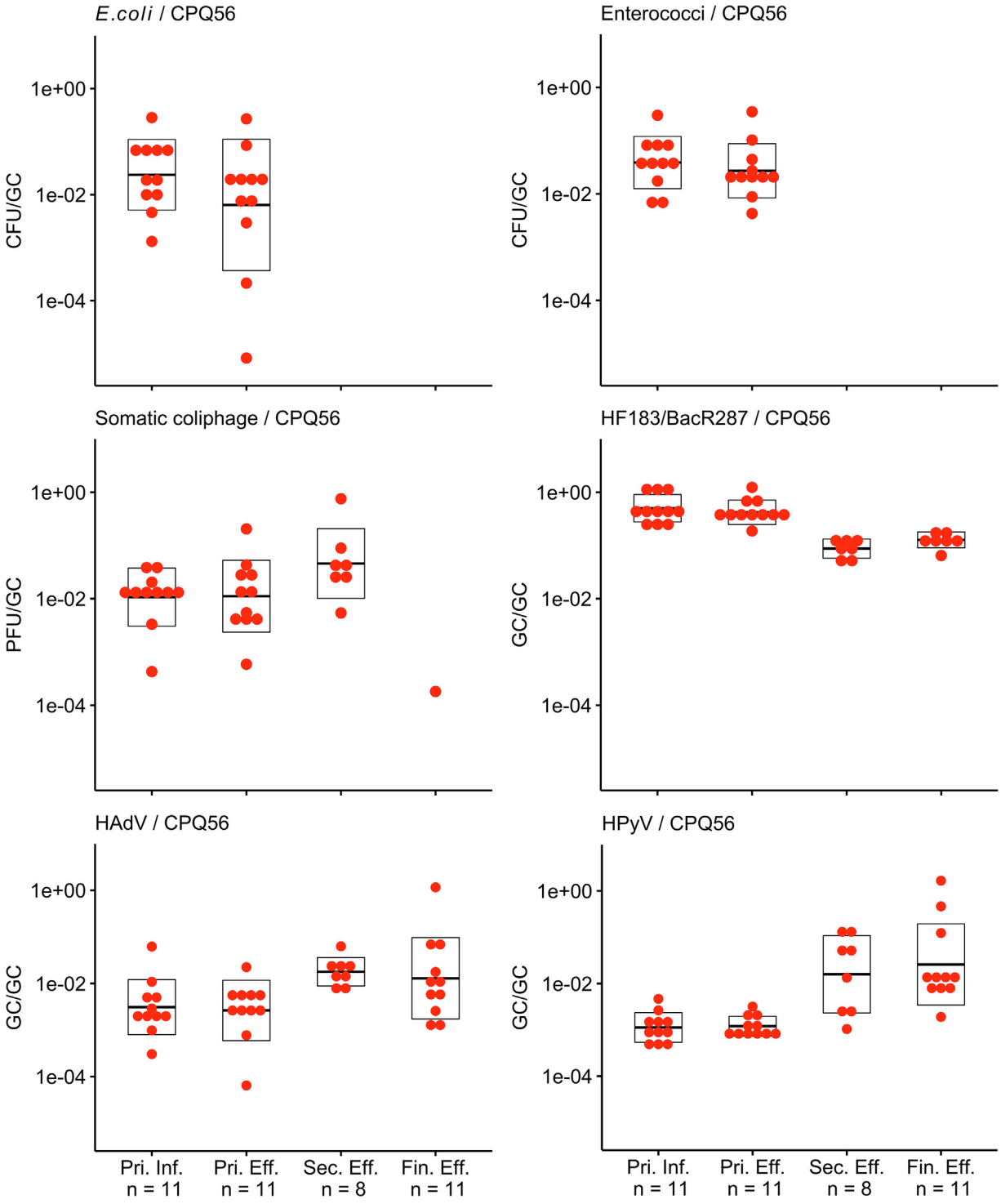

Fig. 3. Ratios of culturable *E. coli*, enterococci and somatic coliphage, and molecular HF183/BacR287, HAdV and HPyV over crAssphage (CPQ56). X-axis shows sample source and sampling count; y-axis shows ratio in CFU/GC, PFU/GC, and GC/GC. Boxplots show mean and standard deviation.

averaged 7.23 $\log_{10}$ GC/100 mL in primary influent, 7.14 $\log_{10}$ GC/100 mL in primary effluent, 4.75 $\log_{10}$ GC/100 mL in secondary effluent, and 4.35 $\log_{10}$ GC/100 mL in final effluent. No sample was below the detection limit (1.70 $\log_{10}$ GC/100 mL). HF183/BacR287 concentrations averaged 6.92 $\log_{10}$ GC/100 mL in primary influent, 6.76 $\log_{10}$ GC/100 mL in primary effluent, 3.77 $\log_{10}$ GC/100 mL in secondary effluent, and 3.59 $\log_{10}$ GC/100 mL in final effluent. One out of eight secondary effluent samples and four out of eleven final effluent samples were below the detection limit (1.70 $\log_{10}$ GC/100 mL). HAdV concentrations averaged 4.71 $\log_{10}$ GC/100 mL in primary influent, 4.56 $\log_{10}$ GC/100 mL in primary effluent, 3.00 $\log_{10}$ GC/100 mL in secondary effluent, and 2.47 $\log_{10}$ GC/100 mL in final effluent. No sample was below the detection limit (1.70 $\log_{10}$ GC/100 mL). HPyV concentrations averaged 4.28 $\log_{10}$ GC/100 mL in primary influent, 4.21 $\log_{10}$ GC/100 mL in primary effluent, 2.95 $\log_{10}$ GC/100 mL in secondary effluent, and 2.77 $\log_{10}$ GC/100 mL in final effluent. No sample was below the detection limit (1.70 $\log_{10}$ GC/100 mL).

3.4. Ratio of fecal pollution indicators and CrAssphage

Fig. 3 demonstrates ratios of concentrations of fecal pollution indicators through each process step normalized over crAssphage concentrations. These ratios were employed as a measure of differential fate of crAssphage and other fecal pollution indicators through the wastewater treatment process. *E. coli* and enterococci were normalized to CFU/GC, somatic coliphage as PFU/GC, and HF183/BacR287, HAdV and HPyV as GC/GC. Only samples where both targets were within the quantifiable range were included. All samples were below detection limit (20 CFU/100 mL) for both *E. coli* and enterococci in secondary and final effluent. Employing the *E. coli* and enterococci lower detection limit over crAssphage concentrations would result in average ratios of 3.55×10^{-4} CFU/GC and 8.99×10^{-4} CFU/GC for both targets in the secondary and final effluent, respectively; these values represent significant decreases over the ratio observed in both primary influent and primary effluent. CrAssphage was the most abundant target evaluated in 190 of 192 pairs where both targets were quantifiable.

3.5. Co-occurrence analysis

Pearson correlation coefficients (r) were calculated between each pair of culturable indicators and molecular indicators (Fig. 4). A large portion of indicators were correlated to each other, with 71% (15 out of 21) of comparisons indicating statistically significant correlation ($p < 0.05$). All molecular indicators, both bacterial and viral, showed a strong correlation with each other. Among these molecular indicators, crAssphage and HF183/BacR287 showed the strongest correlation ($r = 0.99$); however, we note that this data only includes samples where both targets were in the quantifiable range. CrAssphage, HF183/BacR287, and HPyV showed no or weak correlation with *E. coli* and enterococci. For culturable indicators, *E. coli* and enterococci did not show a significant correlation with each other, though they both showed weak correlation with somatic coliphage. Somatic coliphage showed moderate correlation with HAdV and HPyV, and a strong correlation with crAssphage and HF183/BacR287.

3.6. Differential fate of fecal pollution indicators during wastewater treatment

The average $\log_{10}$ removal through the treatment process varied with a maximum $\log_{10}$ removal of greater than 4.64 for enterococci and a minimum $\log_{10}$ removal of 1.51 for HPyV. Given that our overarching goal was to evaluate the suitability of crAssphage as a viral fecal

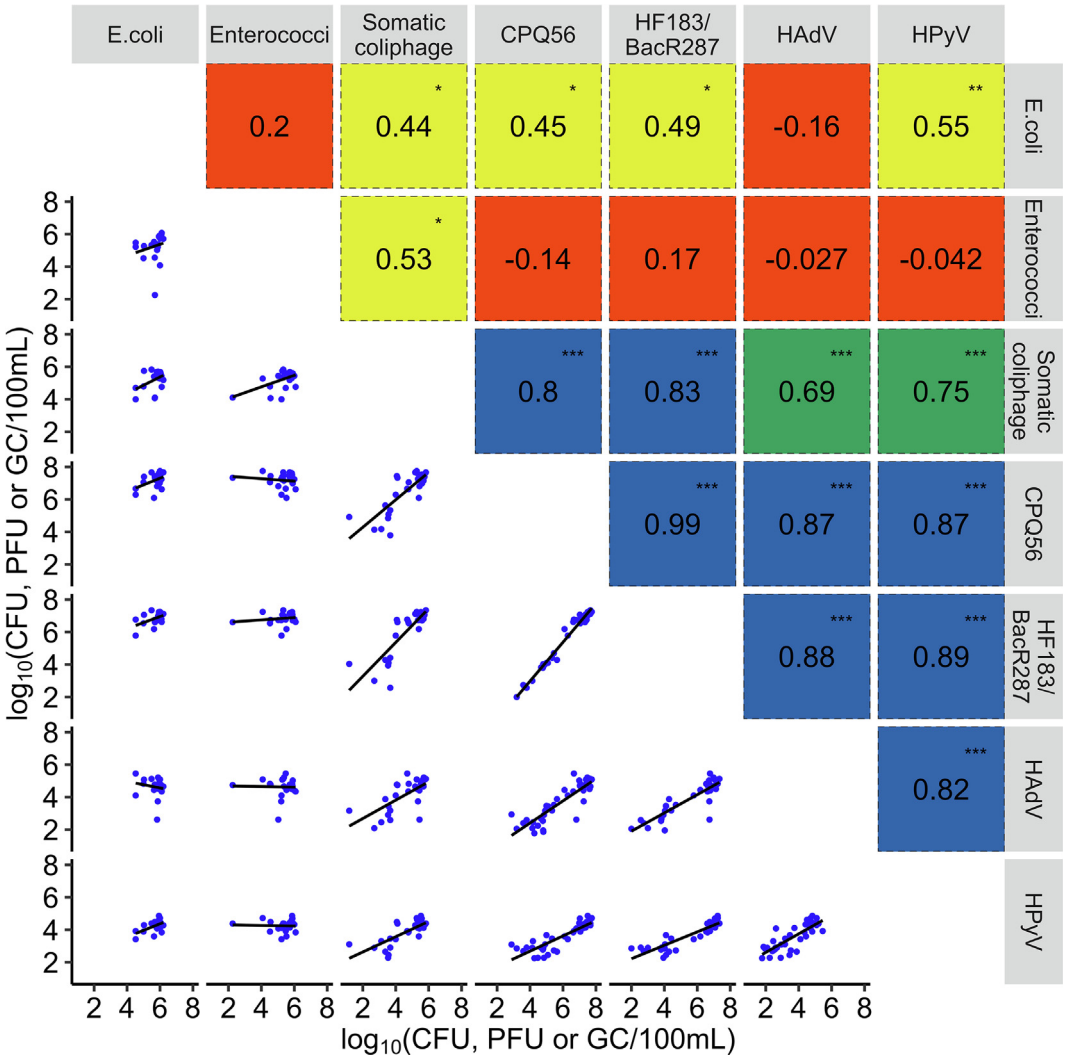

Fig. 4. Pearson correlation coefficient (r) matrix for fecal pollution indicators. Lower left shows data points for each paired data set with a linear regression line. Upper right shows Pearson correlation coefficient for each paired data set with the color of the box indicating its r value. Orange for 0.40 and below (no obvious correlation), yellow for 0.40–0.59 (weak correlation), green for 0.60–0.79 (moderate correlation), and blue for 0.80–1.0 (strong correlation). Asterisks show P-value for each pair data set (no asterisk: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$).

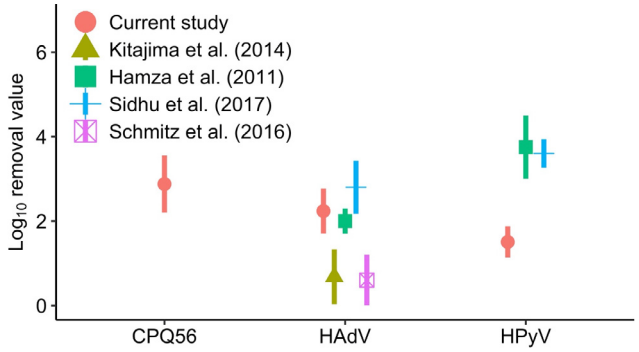

Fig. 5. Log_{10} removal values from the current and literature studies. Crossbar chart indicates means and standard deviations of log_{10} removal values collected. X-axis shows indicators name. Y-axis shows log_{10} removal values from primary influent to final effluent.

pollution process indicator during wastewater treatment, we first normalized all fecal pollution indicators by the measured crAssphage concentrations (as CPQ56). The ratios of measured fecal pollution indicators over CPQ56 are shown in Fig. 3. We then conducted a co-occurrence analysis results based on Pearson's correlation coefficients (r) between each pair of culturable and molecular indicators (Fig. 4).

The culturable bacterial fecal indicators *E. coli* and enterococci had a different fate from molecular indicators, including crAssphage. Notably, *E. coli* and enterococci were not detected in secondary and final effluents. Even in samples in the detectable range, *E. coli* and enterococci correlated poorly with crAssphage and other indicators including somatic coliphage, HF183/BacR287, HAdV, and HPyV (Fig. 4). The high removal of culturable bacterial fecal indicators through the wastewater treatment process agrees with previous study comparing culturable bacterial fecal indicators with HF183/BacR287, HAdV, and HPyV molecular fecal indicators (McQuaig et al., 2009).

Somatic coliphages, a culturable viral fecal pollution indicator, exhibited a strong or moderate correlation with all molecular indicators (Fig. 4). This differs from a previously reported weak or no correlation between somatic coliphage and molecular human fecal indicators including HAdV, astrovirus, rotavirus, and norovirus GI and GII in surface water (Hot et al., 2003) and the previously reported poor correlation of somatic coliphage with crAssphage and HPyV in combined sewage overflow (CSO) events (Stachler et al., 2018). Prior to disinfection, somatic coliphage presented a similar fate as crAssphage. The ratio of somatic coliphage and crAssphage from primary influent, primary effluent, and secondary effluent did not exhibit a statistically significant fluctuation (Wilcox test, $P > 0.05$). Somatic coliphage was detected in the majority of secondary effluent samples (seven out of eight) but not detected in the majority of final effluent samples (10 out of 11), whereas crAssphage was detected in all samples. We note that since somatic coliphages were quantified using plaque assay whereas crAssphage was measured with ddPCR, the plaque negative samples may still contain non-viable somatic coliphage RNA. This may also indicate differing disinfection characteristics and abundances between crAssphage and somatic coliphage.

The molecular bacterial fecal indicator HF183/BacR287 was strongly correlated with crAssphage, HAdV, and HPyV in the current study (Fig. 4) but a larger relative portion of HF183/BacR287 was removed than crAssphage, HAdV, and HPyV in secondary and final effluent. This differs from previously reported weak or moderate correlation coefficients between HF183/BacR287 and crAssphage and HPyV in a CSO-impacted stream, which would likely be more variable than wastewater treatment processes (Stachler et al., 2018). The strong correlation between HF183/BacR287 and HAdV and HPyV agrees with previously reported results before and after primary wastewater treatment (Hughes et al., 2017). The ratios of HF183/BacR287 and crAssphage statistically significantly decreased from primary effluent to secondary effluent and final effluent (Wilcox test, both primary effluent versus secondary effluent, primary effluent versus final effluent $P < 0.05$), indicating that HF183/BacR287 was removed in a larger relative removal portion than crAssphage through secondary treatment and chlorination/dechlorination. Notably, one out of eight secondary effluent samples and four out of 11 final effluent samples were below the detection limit for HF183/BacR287, whereas crAssphage, HAdV, and HPyV were detected in all samples (Fig. 2).

HAdV and HPyV were strongly correlated with crAssphage through the wastewater treatment process (Fig. 4) but were removed with a smaller relative portion than crAssphage. The ratios of HAdV and HPyV and crAssphage showed a statistically significant increase during secondary treatment (Wilcox test, $P < 0.05$), which indicates a smaller relative removal for both viruses than crAssphage; however, the strong correlation of crAssphage with HAdV and HPyV through wastewater treatment suggests a similar removal mechanism (Omura et al., 1989).

3.7. Literature comparison of crAssphage, HAdV, and HPyV fate during wastewater treatment

Log_{10} removal values for viral molecular indicators from the current study were compared with previously reported values to contextualize our observations (Fig. 5). Studies were chosen that targeted the same molecular viral targets and were conducted in conventional activated sludge urban municipal wastewater treatment plants with chlorination/dechlorination before final discharge. Hamza et al. (2011) (Hamza et al., 2011) tested HAdV and HPyV removal through a German WWTP with 12 paired samples. Sidhu et al. (2017) (Sidhu et al., 2017) evaluated HAdV (38 paired samples) and HPyV (39 paired samples) removal through an Australian WWTP. Kitajima et al. (2014) (Kitajima et al., 2014) studied HAdV (12 paired samples) removal through an US WWTP. Schmitz et al. (2016) (Schmitz et al., 2016) observed HAdV (12 paired samples) removal through an US WWTP. Log_{10} removal values were compared with a two-sample *t*-test. We note that other relevant studies (e.g., (Farkas et al., 2019; Hamza and Hamza, 2018; Hamza et al., 2019; Hata et al., 2013; Prado et al., 2019)) were not included due to process or assay differences that do not allow direct comparison.

Log_{10} removal values of viral molecular fecal indicators through wastewater treatment process showed inter-study variation, likely caused by variability in the processes and influent composition. The log_{10} removal value of HAdV from the current study (2.24 ± 0.53) was statistically indistinguishable to observations from Hamza et al. (2.00 ± 0.30) ($P = 0.20$), lower than Sidhu et al. (2.80 ± 0.63) ($P < 0.01$) and higher than Kitajima et al. (0.68 ± 0.65) ($P < 0.01$) and Schmitz et al. (0.60 ± 0.60) ($P < 0.01$). Average influent concentrations of HAdV for Hamza et al., Sidhu et al., Kitajima et al. and Schmitz et al. studies were all higher than in the current study (0.84, 1.86, 0.14 and 0.09 log_{10} GC/100 mL higher, respectively). The log_{10} removal value of HPyV was 1.51 ± 0.37, was statistically significantly lower than both Ahmed et al. (3.75 ± 0.75) ($P < 0.01$) and Sidhu et al. (3.60 ± 0.34) ($P < 0.01$). The influent concentrations of HPyV for the Hamza et al. and Sidhu et al. studies were 2.01 and 2.83 log_{10} GC/100 mL higher than the current study, respectively.

The log_{10} removal value of crAssphage from the current study was 2.88 ± 0.68, higher than observed removals for both HAdV and HPyV ($P < 0.01$). While crAssphage had a higher starting concentration in primary influent (Fig. 2), the difference in removal is likely specific to crAssphage because it was removed in larger relative proportion than HAdV or HPyV during secondary treatment and chlorination/dechlorination. A recent evaluation of crAssphageshowed a 2.22 log_{10} removal value through activated sludge wastewater treatment plant (Kongprajug et al., 2019), similar to our observations of 2.88 ± 0.68 log_{10} removal; however, method and process variation limit more direct study comparison. Further studies should examine different processes and influent compositions to better characterize the variability of crAssphage removal through wastewater treatment. Ultimately, these

results highlight process-specific behaviors and the necessity for local indicator performance verification.

3.8. Suitability of crAssphage as a fecal pollution wastewater process indicator

CrAssphage has multiple characteristics that would be advantageous for a viral process indicator during wastewater treatment. It had the highest abundance among the fecal indicators used in the current study (Fig. 2), facilitating its detection. CrAssphage shows a high specificity to human fecal pollution (Stachler et al., 2017); however, we note that molecular crAssphage markers have previously been identified in animal sources (Malla et al., 2019). Finally, strong co-occurrence and similar fate between crAssphage, HAdV, and HPyV through the wastewater treatment process suggests similar removal mechanisms between crAssphage and human DNA viral pathogens.

CrAssphage also has potential limitations as a process indicator during wastewater treatment. CrAssphage is currently not easily culturable from environmental samples, although a crAss-like phage has previously been cultured (Guerin et al., 2018) and the ability to culture environmental crAss-like phages may exist in the future. The inability to culture crAssphage necessitates the application of molecular methods, which have an unknown correlation with microbial viability. Also, as a dsDNA virus, crAssphage may be limited as a representative of RNA viruses (Bibby et al., 2019), which were not evaluated in the current study. Finally, there is not yet an epidemiological link between crAssphage occurrence and human health impacts; however, recent work based using quantitative microbial risk assessment suggests that crAssphage may be useful for this application (Crank et al., 2019).

3.9. Study implications

The purpose of the current study was to evaluate crAssphage removal through activated sludge wastewater treatment and to compare the fate of crAssphage with other fecal pollution indicators. CrAssphage was the most abundant fecal indicator measured throughout the study, facilitating its detection. Molecular viral indicators, including crAssphage, demonstrated lower removals than bacterial indicators, highlighting the need for a process indicator for viral pathogen removal during wastewater treatment. CrAssphage was strongly correlated with the DNA human viral pathogens HAdV and HPyV through the wastewater treatment process. A comparison of study results with literature values demonstrates site-specific variability suggesting the necessity to locally verify wastewater process indicator performance (Stoeckel and Harwood, 2007). Ultimately, this study suggests the strong potential to use crAssphage as a viral fecal pollution process indicator during wastewater treatment.

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CRediT authorship contribution statement

Zhenyu Wu: Data curation, Formal analysis, Investigation, Writing - review & editing. **Justin Greaves:** Investigation, Writing - review & editing. **Lillian Arp:** Investigation, Writing - review & editing. **Daniel Stone:** Investigation, Writing - review & editing. **Kyle Bibby:** Conceptualization, Data curation, Formal analysis, Investigation, Writing - review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: 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.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105452.

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