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Persistence of emerging viral fecal indicators in large-scale freshwater mesocosms

Justin Greaves, Daniel Stone, Zhenyu Wu, Kyle Bibby*

Department of Civil and Environmental Engineering and Earth Sciences, University of Notre Dame, IN, 46556, USA

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ABSTRACT

Fecal indicator bacteria (FIB) are typically used to monitor microbial water quality but are poor representatives of viruses due to different environmental fate. Viral fecal indicators have been proposed as alternatives to FIB; however, data evaluating the persistence of emerging viral fecal indicators under realistic environmental conditions is necessary to evaluate their potential application. In this study, we examined the persistence of five viral fecal indicators, including crAssphage and pepper mild mottle virus (PMMoV), and three bacterial fecal indicators (*E. coli*, enterococci and HF183/BacR287) in large-scale experimental ponds and freshwater mesocosms. Observed inactivation rate constants were highly variable and ranged from a minimum of $-0.09\ d^{-1}$ for PMMoV to a maximum of $-3.5\ d^{-1}$ for HF183/BacR287 in uncovered mesocosms. Overall, viral fecal indicators had slower inactivation than bacterial fecal indicators and PMMoV was inactivated more slowly than all other targets. These results demonstrate that bacterial fecal indicators inadequately represent viral fate following aging of sewage contaminated water due to differential persistence, and that currently used fecal indicator monitoring targets demonstrate highly variable persistence that should be considered during water quality monitoring and risk assessment.

Introduction

Globally, at least 80% of wastewater enters the environment untreated (UNWWAP, 2017), and approximately 40% of the United States' waterways do not meet the Clean Water Act criteria, primarily due to low microbial water quality (Wade et al., 2006). Sewage contaminated waters are responsible for more than two million deaths per year globally (Betancourt et al., 2014). Microbial water quality monitoring, therefore, is necessary to protect human health; however, sewage contaminated waters may contain a large diversity of pathogens, including bacteria, viruses, and protozoa. To overcome challenges associated with monitoring all pathogens, fecal indicator bacteria (FIB) such as *E. coli* and enterococci are typically used as indicators of fecal pollution in water.

FIB have significant limitations despite their widespread application. Studies have also shown that FIB have differing persistence than viral pathogens in water (Hjelmsø et al., 2017). Viruses are predicted to be the greatest source of infectious risk due to exposure from sewage-contaminated waters (Boehm et al., 2015; Crank et al., 2019). Viral indicators have been proposed to better represent viruses in sewage contaminated water than FIB (Bibby et al., 2019; Hjelmsø et al., 2017). Indicator organisms do not necessarily have to be nonpathogenic; they can be any microorganism (pathogenic or nonpathogenic) that is present in sewage. Previous studies have investigated human pathogenic viruses such as polyomaviruses (HPyV) and adenoviruses (AdV) as possible viral indicators (Ahmed et al., 2014; Liang et al., 2015); however, the low concentrations in sewage and varying prevalence by region have limited the suitability of HPyV and AdV as viral indicators (Harwood et al., 2013).

Viral indicators that are both abundant and highly sewage-associated have been recently proposed, including crAssphage and pepper mild mottle virus (PMMoV) (Stachler et al., 2017). CrAssphage is a double stranded DNA virus discovered though the cross-assembly of unknown sequences of human fecal metagenomes (Dutilh et al., 2014). PMMoV is a single stranded RNA Tobamovirus discovered in 1983 to infect peppers (Wetter et al., 1984). Both are highly abundant and widely present in wastewater globally but are measured using molecular assays (Dutilh

* Corresponding author.
E-mail address: KBibby@ND.edu (K. Bibby).

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et al., 2014; Hamza et al., 2011).

Prior fecal indicator persistence studies have focused on smaller experimental scales ranging between laboratory microcosms to small-scale experimental mesocosms (less than 100 L) (Ahmed et al., 2019). Experimental scale may influence fecal indicator marker persistence through multiple mechanisms, including altered biological activity and diversity and sunlight exposure. Additional investigations are necessary to explore emerging fecal indicator inactivation characteristics at scales larger than previously done, including emerging viral fecal indicators. In addition, the environmental persistence of both crAssphage and PMMoV has been relatively understudied and they have yet to be comparatively studied in the same system.

The goal of the current study is to examine the inactivation rate constants of emerging viral fecal pollution indicators under representative surface water conditions and compare observed inactivation rate constants with other microbial indicators of human fecal pollution. In this study, three culturable indicators along with five molecular based indicators were examined. Molecular indicators in this study do not represent microorganism viability; however, molecular measures would be directly applied in environmental monitoring purposes (Crank et al., 2019). A large-scale pond study and a smaller scale mesocosm study were used to examine fecal indicator persistence. Wastewater was added to either the test ponds or mesocosms as a representative source of fecal pollution, and the concentrations of each fecal indicator were monitored. The inactivation characteristics for each fecal pollution indicator will help inform water quality monitoring efforts, the development of viral fecal pollution indicators, and risk models of exposure to sewage contamination.

Materials and methods

Pond study

Experiments were completed between July 31st and August 28th, 2018 at the Notre Dame linked experimental ecosystem Facility (ND-LEEF). ND-LEEF is an outdoor research facility located in St. Patrick's County Park in South Bend, Indiana. All experimental procedures were approved by facility management and county authorities. The facility has two constructed ponds with a volume of 925 m^3 and a maximum depth of 2 m lined with sediment and plants. It also has a waterproof concrete liner under the sediment to prevent seepage into groundwater. The pond was filled with groundwater two years prior to the start of experiment then allowed to remain exposed to the environment. Prior to the start of this study, all pumped water supplying the ponds were turned off to allow for a closed system. Wastewater primary influent samples were collected from a local wastewater treatment plant and released at different points along each pond to reach a dilution of 0.01%. Previous studies have shown this level of contamination to be close to real world scenarios (Malla et al., 2019; Nguyen et al., 2016). This level of contamination also helps us understand the benefit of highly abundant fecal indicators such as PMMoV and crAssphage. Sodium bromide was also released into each pond to a final concentration of 0.5 mg/L as a conservative dilution tracer. One-liter samples were collected approximately 0.3 m below the water surface from two locations on each pond at 2 h (to allow natural mixing in the pond), 1, 2, 3, 4, 5, 6, 8, 10, 14, and 28 days after wastewater release using sterile 1-L bottles. Samples were processed for molecular measurements, water quality characteristics, and culturable testing within 8 h of sampling.

Mesocosm study

Mesocosm experiments were conducted from July 31st, 2018 to August 28th, 2018 at the ND-LEEF site. Four 300 L tanks were filled with 200 L of pond water. Two of the four tanks were covered with high density polyethylene cloth (shade cloth) to block sunlight. UV irradiance was measured using the UVA/B light meter under and above the shade cloth once before the start of experiment. UV was measured daily for the first eight days using a UVA/B meter (Sper Scientific, AZ, USA). Wastewater samples were collected from a local wastewater treatment plant and spiked into each tank at a 1% dilution. Sodium bromide was released into each tank to a final concentration of 0.5 mg/L as a conservative dilution tracer. One-liter samples were collected at 2 h (to allow for initial mixing), and 1, 2, 3, 4, 5, 6, 8, 10, 14, and 28 days after wastewater release from each tank using sterile 1-L bottles. Samples were then processed within 24 h of collection.

Physiochemical testing

Conductivity and turbidity were measured in all samples including initial pond samples prior to wastewater release. Water and air temperature were measured in the initial and final pond and mesocosm samples while pH was measured only in the initial pond samples. Bromide concentration in each sample was measured using a Thermo ICS-5000 ion chromatography system (Thermo Fisher, MA, USA). UVA and UVB irradiance was measured using a UVA/B light meter (Sper Scientific, AZ, USA) during the pond and mesocosm experiments.

Culturable indicator testing

200 mL of each sample was used for enterococci, *E. coli*, and somatic coliphage measurements. Enterococci was measured using the USEPA method 1600, *E. coli* was measured using the USEPA method 1603, and somatic coliphage was measured using USEPA method 1602. Negative controls (sterile water) were run with all samples and no control indicated contamination.

Microbial concentration and DNA/RNA extraction

Samples were concentrated for molecular analyses as previously described (Stachler et al., 2018). Briefly, 500 mL of each sample was adjusted to a pH of 3.5 with HCl and then filtered through an electronegative 0.45 μm mixed cellulose ester filter (Pall) (Staley et al., 2012). Filters were then transferred to preloaded bead tubes (Qiagen) and stored at −20 °C for DNA/RNA extraction. The QIAGEN DNeasy PowerSoil kit was used to extract DNA from all membrane filters for the pond study following manufacturer instructions. The QIAGEN AllPrep PowerViral DNA/RNA kit was used for simultaneous DNA and RNA extraction from membrane filters for the mesocosm study following manufacturer instructions.

Molecular analysis

Molecular indicators were measured using ddPCR and previously published assays for crAssphage (CPQ56), HF183/BacR287, HPyV, AdV, and PMMoV. CrAssphage, PMMoV and HF183/BacR287 were quantified for the pond study. Assays in this study have shown comparable or improved detection and quantification as qPCR when adapted to ddPCR (Cao et al., 2015; Hayden et al., 2013; Rački et al., 2014; Stachler et al., 2019). Primers, probes, and cycling conditions for each assay are displayed in Table S1. For DNA targets, reaction mix in each well was made up of 10 μL of ddPCR supermix for probes (Bio-Rad, CA, USA), 1 μL of primer probe mix, 4 μL of

DNase-free water and 5 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. Samples were run in duplicates for the first three days of samples to calculate regression line between duplicates (Fig. S1). The limit of detection was calculated for each reaction well and results are displayed in Table S2.

For the RNA target evaluated (PMMoV), the reaction mix in each droplet cartridge well was made up of 5 μL of One-step RT-ddPCR advanced kit supermix (Bio-Rad, 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. Final concentration of primers and probes were 900 nM and 250 nM respectively. Samples were run in technical duplicates for the first three days of each experimental sampling period and single ddPCR runs for subsequent sample timepoints. The limit of detection was calculated for each reaction well and results are displayed in Table S2 ddPCR performance metrics are summarized in Table S3.

Data analysis

Inactivation curves from both the pond and mesocosm study were fit to a first-order decay model ($N_t = N_0 e^{k_{obs} t}$) where $k_{obs}(d^{-1})$ can be calculated as the slope of the regression line $\ln(N_t / N_0)$ versus time (Silverman et al., 2013). For crAssphage and PMMoV, a pseudo-first order inactivation rate constant, $k_{uv}(m^2\ J^{-1})$, can be calculated. The depth averaged UV fluence can be calculated by first calculating the average light irradiance at total depth z using the equation employed by Silverman et al. (Maraccini et al., 2016; Silverman et al., 2013).

$$I_{\lambda z} = I_{\lambda,0} \frac{(1 - 10^{-\alpha_\lambda z})}{2.303 \alpha_\lambda \times z} = I_{\lambda,0} \times S_\lambda$$

Where $I_{\lambda,0}$ is the wavelength-specific irradiance incident on the water surface (W/m2), α_λ is the decadic absorbance of the water matrix, z is the path length and. S_λ is the light screening factor. Due to the UV meter measurements being a cumulative value for the wavelength range from 280 to 400 nm, the equation above was modified by summing S_λ to the following:

$$I_z = I_0 \times \sum_{280}^{400} S_\lambda$$

Where I_z is the average light irradiance at depth z. The depth averaged UV fluence (J/m^2) can then be calculated by the following:

$$F_z = I_z \times t$$

A pseudo-first order inactivation rate constant, $k_{uv}(m^2\ J^{-1})$, can then be calculated as the slope of the line $\ln(N_t/N_0)$ versus the depth averaged UV fluence.

All inactivation rate constants were calculated using GraphPad PRISM 7.0d (La Jolla California, USA) fixing the y-intercept to zero. Statistical correlation between pond and mesocosm pseudo-first order inactivation rate constants were performed using two-way ANOVA followed by Fisher's LSD multiple comparisons test. Statistical correlation between microbial inactivation rate constants in the mesocosm study was performed using multiple linear regression and two-way ANOVA followed by a Fisher's LSD multiple comparisons test. There were two mesocosms per treatment (uncovered or covered). Each inactivation value from each treatment was used as a variable in the two-way ANOVA analysis and each inactivation curve from each treatment was used as replicates in the multiple linear regression to compare between treatments and among microbes. k_{obs} values from the mesocosm experiment were used in the comparison between treatments due to the similar setups, water depths and matrices.

Systematic review

We performed a short systematic review using search methods described in previously published meta-analyses identifying a total of 64 unique inactivation rate constants across the seven different microbes tested in this study (Table S4) (Boehm et al., 2018, 2019). Briefly, Web of Science core collection and PubMed were searched in February 2019. The search items used were "(X) AND (water OR seawater OR freshwater) AND (persistence OR decay OR inactivation)" where X represents one of the seven indicators used in this study. Details of the review process are provided in the SI.

Results

Pond study

Pond persistence experiments were completed in two experimental closed system 925 m^3 ponds from July 31st to August 28th, 2018. System was assumed to be well-mixed. Wastewater samples were collected from a local wastewater treatment plant and added into each pond with wastewater at a 0.01% total concentration. Samples were collected approximately 0.3 m below the water surface daily at two separate locations in each pond at each sampling time point for water quality characterization and microbial quantification. CrAssphage, PMMoV and HF183/BacR287 were measured in the ponds because of their high concentrations in wastewater. The culturable microbes *E. coli,* coliphage and enterococci were tested and not detectable in the pond after 2 h. AdV and HPyV were not measured in the ponds since they would not be detectable in the ponds based on pond sewage dilution and calculated detection limit.

Sample conductivity and turbidity averaged 0.32 mS/cm and 0.08 NTU, respectively. Mean water temperature in the ponds was 24.7 °C. The pH of initial samples before wastewater release was 8.6. No culturable indicators were detected in the initial pond samples before or following wastewater release. In the initial wastewater samples, crAssphage, PMMoV and HF183/BacR287 had concentrations of 1.03×10^9, 1.38×10^8 and 4.26×10^8 genome copies/L, respectively. CrAssphage and PMMoV were detectable for five and eight days, respectively, in both ponds (Fig. 1). HF183/BacR287 was only detectable for the 2-h and one-day time points, so inactivation rate constants were not calculated. Molecular fecal indicator target concentrations following wastewater release were normalized to a conservative bromide tracer concentration by simply dividing by the concentration of bromide in each sample to account for dilution. k_{obs} and k_{UV} were then calculated based on these normalized values. The mean k_{obs} values for PMMoV and crAssphage were $-0.41\ d^{-1}$ and $-0.98\ d^{-1}$, respectively (Table 1). The mean k_{UV} values for PMMoV and crAssphage were $-0.009\ m^2\ J^{-1}$ and $-0.023\ m^2\ J^{-1}$, respectively (Table 2).

Mesocosm study

Mesocosm experiments were conducted from July 31st, 2018 to August 28th, 2018. Four 300 L tanks were filled with 200 L of pond water, and two of the four tanks were covered with shade cloth which blocks 96.7% of UVA + UVB. Due to the high reduction in incident light by the shade cloth, the covered mesocosms were assumed to be in little to no sunlight throughout the experiment. Wastewater samples were collected from a local wastewater treatment plant and added into each mesocosm at a 1% total

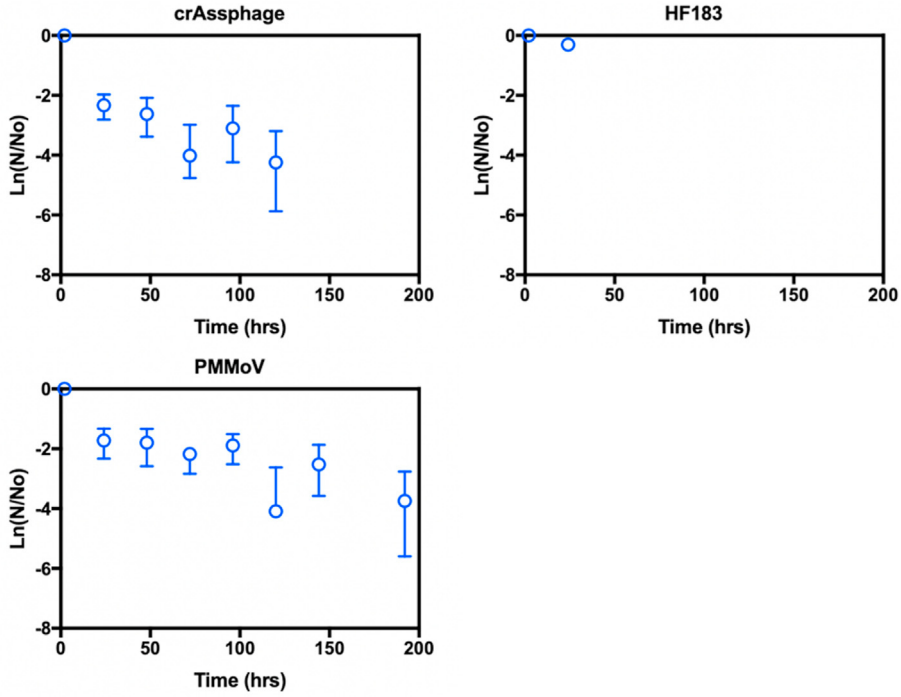

Fig. 1. Inactivation of crAssphage, PMMoV and HF183/BacR287 in the pond experiment. Each data point represents the average of duplicate pond experiments. Error bars represent average ddPCR 95% Poisson-based confidence intervals to calculate the concentration between each sample. The error bars may not be visible at some data points because they are smaller than the data point or the value is zero. All values were corrected for dilution using bromide concentration. Detection limits for all conditions are included in Table S2.

Table 1
Inactivation rate constants for crAssphage and PMMoV for the two different ponds.

Marker	k_{obs} (d^{-1})	SD	95% CI	R^2
Crassphage	−1.20	0.18	−1.74 to −0.67	0.78
HF183/BacR287			N/A	
PMMoV	−0.57	0.10	−0.31 to −0.01	0.53

Table 2
UV and Depth normalized inactivation rate constants (k_{UV}) for crAssphage and PMMoV for the ponds and mesocosms.

Marker	Sample	k_{UV} (m^2 J^{-1})	SD	95% CI	R^2
Crassphage	Pond	−0.10	0.02	−0.18 to −0.03	0.65
	Mesocosm	−0.09	0.01	−0.12 to −0.08	0.88
PMMoV	Pond	−0.05	0.01	−0.07 to 0.02	0.49
	Mesocosm	−0.03	0.00	−0.04 to −0.01	0.66

concentration. A single sample was collected daily from each tank for water quality characterization and microbial quantification.

Water conductivity and turbidity averaged 0.34 mS/cm and 0.16 NTU, respectively. The mean water temperature in the covered tanks was 24.2 °C and in the uncovered tanks was 26.2 °C. Daily peak UV intensity varied from 3000 to 9900 mW/cm^2 (Fig. S2).

No fecal indicators measured in this experiment were detected in mesocosms prior to wastewater release. In the initial wastewater sample, somatic coliphage, *E. coli*, and enterococci concentrations were 3.82×10^6 PFU/L, 3.20×10^6 CFU/L, and 9.67×10^6 CFU/L, respectively. CrAssphage and HF183/BacR287 concentrations were 1.93×10^8 and 4.83×10^8 genome copies/L, respectively. AdV, HPyV, and PMMoV concentrations were 9.98×10^5, 1.57×10^6, and 2.22×10^7 genome copies/L, respectively.

Culturable target inactivation in the mesocosm experiments is shown in Fig. 2 and inactivation rate constants are shown in Table 3. Fecal pollution indicator concentrations were corrected for dilution (e.g., rain) following initial release using the bromide tracer. Enterococci and somatic coliphage were detectable for 2 h (first sampling time point) and two days, respectively, in the uncovered mesocosms and both were detectable for five days in the covered mesocosms. *E. coli* was detectable for two days in the covered mesocosm and 2 h in the uncovered mesocosms. Inactivation rate constants for enterococci in the uncovered mesocosm and *E. coli* in both the covered and uncovered mesocosms were not calculated as insufficient data was available in the quantifiable range. Enterococci and somatic coliphage had mean k_{obs} values of −1.20 d^{-1} and -0.62 d^{-1}, respectively, in the covered mesocosm, while somatic coliphage had a mean k_{obs} value of −1.23 d^{-1} in the uncovered mesocosm.

Molecular target inactivation for mesocosm experiments is shown in Fig. 3 and inactivation rate constants are shown in Table 3. CrAssphage was detectable for 10 days in the covered mesocosms and eight days in the uncovered mesocosms. CrAssphage had mean k_{obs} values of −0.74 d^{-1} and -1.01 d^{-1} in the covered and uncovered mesocosms, respectively, and had a mean k_{UV} value of −0.006 m^2 J^{-1} in the uncovered mesocosm (Table 2). HF183/BacR287 was detectable for five days in the covered mesocosms and three days in the uncovered mesocosms. HF183/BacR287 had mean k_{obs} values of −1.65 d^{-1} and -3.58 d^{-1} in the covered and uncovered mesocosms, respectively. AdV was detectable for four and three days in the covered and uncovered mesocosms, respectively, while HPyV was detectable for three days in both mesocosms. AdV had mean k_{obs} values of −0.90 d^{-1} and -2.02 d^{-1} in the covered and uncovered mesocosms, respectively. HPyV had mean k_{obs} values of −0.54 d^{-1} and -1.16 d^{-1} in the covered and uncovered mesocosms, respectively. PMMoV was detectable for at least 28 days (end of experiment) for both uncovered and covered mesocosms. PMMoV had mean k_{obs} values of −0.20 d^{-1} and -0.09 d^{-1} in the covered and uncovered mesocosms, respectively. PMMoV also

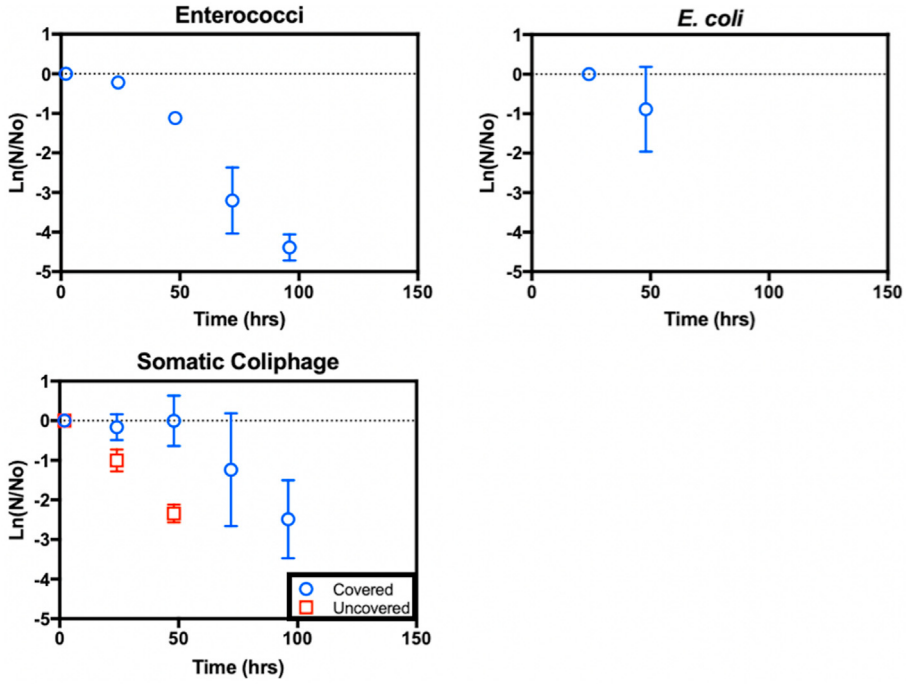

Fig. 2. Inactivation of culturable indicators enterococci, *E. coli*, and somatic coliphage in mesocosm experiments. Detection limits for all conditions are included in Table S2. Each data point represents the average of duplicates. Error bars represent the calculated 95% confidence interval for each sample.

Table 3
Mean k_{obs} values for all culturable and molecular indicators measured in the mesocosm study.

Marker	Sample Type	k_{obs} (d^{-1})	SD	95% CI	R^2
HF183	Covered	−1.53	0.16	−1.94 to −1.12	0.79
	Uncovered	−3.76	0.45	−5.71 to −1.81	0.91
ENT	Covered	−0.99	0.12	−1.31 to −0.60	0.89
	Uncovered			N/A	
E. coli	Covered			N/A	
	Uncovered			N/A	
AdV	Covered	−0.62	0.26	−1.60 to 0.37	0.59
	Uncovered	−1.59	0.46	−3.57 to 0.39	0.76
Somatic Co**liphage**	Covered	−0.46	0.12	−0.78 to −0.14	0.88
	Uncovered	−1.14	0.06	−1.39 to −0.88	0.99
CrAssphage	Covered	−0.78	0.09	−0.99 to −0.56	0.73
	Uncovered	−1.15	0.11	−1.41 to −0.89	0.78
HPyV	Covered	−0.44	0.17	−1.18 to 0.30	0.62
	Uncovered	−1.05	0.13	−1.61 to −0.49	0.90
PMMoV	Covered	−0.27	0.07	−0.43 to −0.12	0.47
	Uncovered	−0.13	0.03	−0.20 to 0.06	0.33

had a mean k_{UV} value of $-0.002\ m^2\ J^{-1}$ in the uncovered mesocosm. k_{UV} values were only calculated for crAssphage and PMMoV because they are the only microbes quantifiable after one day in the pond experiments.

Statistical correlation

Pond and uncovered mesocosm k_{UV} values were compared using a two-way ANOVA followed by a Fischer's LSD multiple comparisons test to evaluate potential differences due to experimental setup. In the pond study, PMMoV k_{UV} values were statistically smaller than PMMoV uncovered mesocosm k_{UV} values, whereas crAssphage k_{UV} values were not statistically different from crAssphage uncovered mesocosm k_{UV} values ($p = 0.1$ for crAssphage and $p = 0.04$ for PMMoV). All fecal indicator duplicate k_{obs} values for the uncovered mesocosm study were compared using two-way ANOVA followed by Fisher's LSD multiple comparisons test (Fig. S3). All fecal pollution indicators were also compared using Multiple linear regression's correlation coefficient to evaluate correlation (Fig. S4).

ANOVA results comparing fecal indicator k_{obs} values are presented in Fig. S3. ANOVA results showed that HPyV, crAssphage, and somatic coliphage k_{obs} values were not significantly different in the uncovered mesocosms ($p > 0.05$); however, all other comparisons between microbial k_{obs} values in the uncovered mesocosm had significant differences ($p < 0.05$). Multiple linear regression results of the uncovered mesocosms showed HPyV, crAssphage and somatic coliphage were significantly correlated ($p < 0.05$) with each other and AdV (Fig. S4). Multiple linear regression results also showed PMMoV and HF183 to be significantly different from each other and the other viral indicators in the uncovered mesocosms (Fig. S4).

In the covered mesocosms, ANOVA did not identify significant differences between crAssphage, HPyV, AdV and somatic coliphage k_{obs} values and enterococci and HF183/BacR287 k_{obs} values. PMMoV was statistically different from AdV, crAssphage, HF183/BacR287, and enterococci but not statistically different from somatic coliphage and HPyV. Multiple linear regression results showed all viral fecal indicators had significant correlations ($p < 0.05$) with each other in the covered mesocosms except for comparison between crAssphage and PMMoV (Fig. S4). HF183 was significantly ($p < 0.05$) correlated with enterococci in the covered mesocosms (Fig. S4).

Discussion

Literature comparison of inactivation rate constants for fecal pollution indicators

Fecal pollution indicator inactivation rate constants in water are essential to inform risk interpretation associated with fecal pollution monitoring in surface water. In this study, we investigated the inactivation of seven fecal pollution indicators, including the

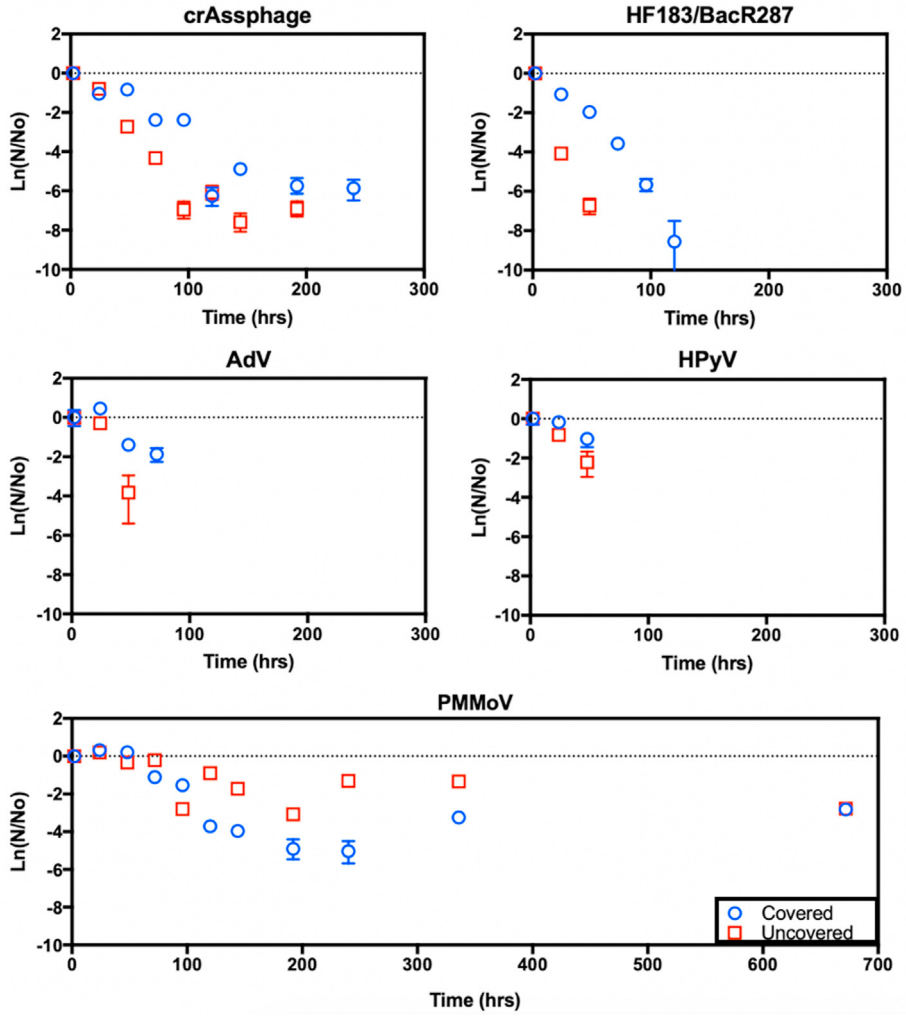

Fig. 3. Inactivation of molecular indicators crAssphage, HF183/BacR287, AdV, HPyV and PMMoV in mesocosm experiments. Each data point represents the average of duplicates. Error bars represent average ddPCR 95% confidence level between duplicates. Blue represents the covered mesocosm while red represents the uncovered mesocosm. Detection limits for all conditions are included in Table S2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

emerging viral indicators crAssphage and PMMoV. In order to provide enhanced context for our study results, we performed a short systematic review to identify literature value inactivation rate constants across the seven different microbes tested then compared them to the k_{obs} values determined in this study. This systematic review will help us to understand research gaps and recognize best practices associated with performing inactivation experiments (Boehm et al., 2019).

We were able to briefly compare HF183/BacR287 and uncovered enterococci values with previous literature, but we were unable to develop literature comparisons for *E. coli* in either condition or enterococci in the uncovered mesocosms due to insufficient data to calculate inactivation rate constants in our study. Enterococci k_{obs} values for the covered condition in the current study were smaller than five out of the six inactivation rate constants from previous studies in the absence of sunlight (Fig. 4) (ranging from −0.130 to −2.4 d^{-1}) (Ahmed et al., 2014; Anderson et al., 2005; Craig et al., 2019; Eichmiller et al., 2014; Jeanneau et al., 2012; Kirs et al., 2016). HF183/BacR287 k_{obs} values in the uncovered mesocosm were smaller than all 16 literature value inactivation rate constants whereas in the covered mesocosm, mean k_{obs} values were smaller than seven out of the nine literature value inactivation rate constants (Fig. 4) (ranging from −0.03 to −2.55 d^{-1}) (Ahmed et al., 2014, 2019; Bae and Wuertz, 2015; Balleste et al., 2018, 2019; Dick et al., 2010; Eichmiller et al., 2014; Gilpin et al., 2013; Green et al., 2011; He et al., 2016; Jeanneau et al., 2012; Kirs et al., 2016; Liang et al., 2012; Walters and Field, 2009).

Two previous studies that reported inactivation rate constants for PMMoV in the absence of sunlight were identified. The current study PMMoV k_{obs} values in the absence of sunlight was smaller than these two inactivation rate constants (−0.05 d^{-1} and -0.053 d^{-1}) (Hamza, 2011; Rachmadi et al., 2016). Literature values on crAssphage inactivation in the absence of sunlight were also not available to develop literature comparisons with crAssphage in the covered condition. In the presence of sunlight, however, crAssphage had mean k_{obs} values that were smaller than all three inactivation rate constants reported in previous studies (Ahmed et al., 2019; Balleste et al., 2019). We were able to compare all other viral inactivation rate constants with previous studies done in both the absence and presence of sunlight. Somatic coliphage, AdV and HPyV had k_{obs} values that were slightly smaller (not significant) than the mean value from the literature review (Fig. 4) (Ahmed et al., 2014, 2019; Bae and Wuertz, 2015; Balleste et al., 2019; Chendorain et al., 1998; Craig et al., 2019; Elmahdy et al., 2018; Ibrahim et al., 2019; Kirs et al., 2016; Prevost et al., 2016; Wu et al., 2016).

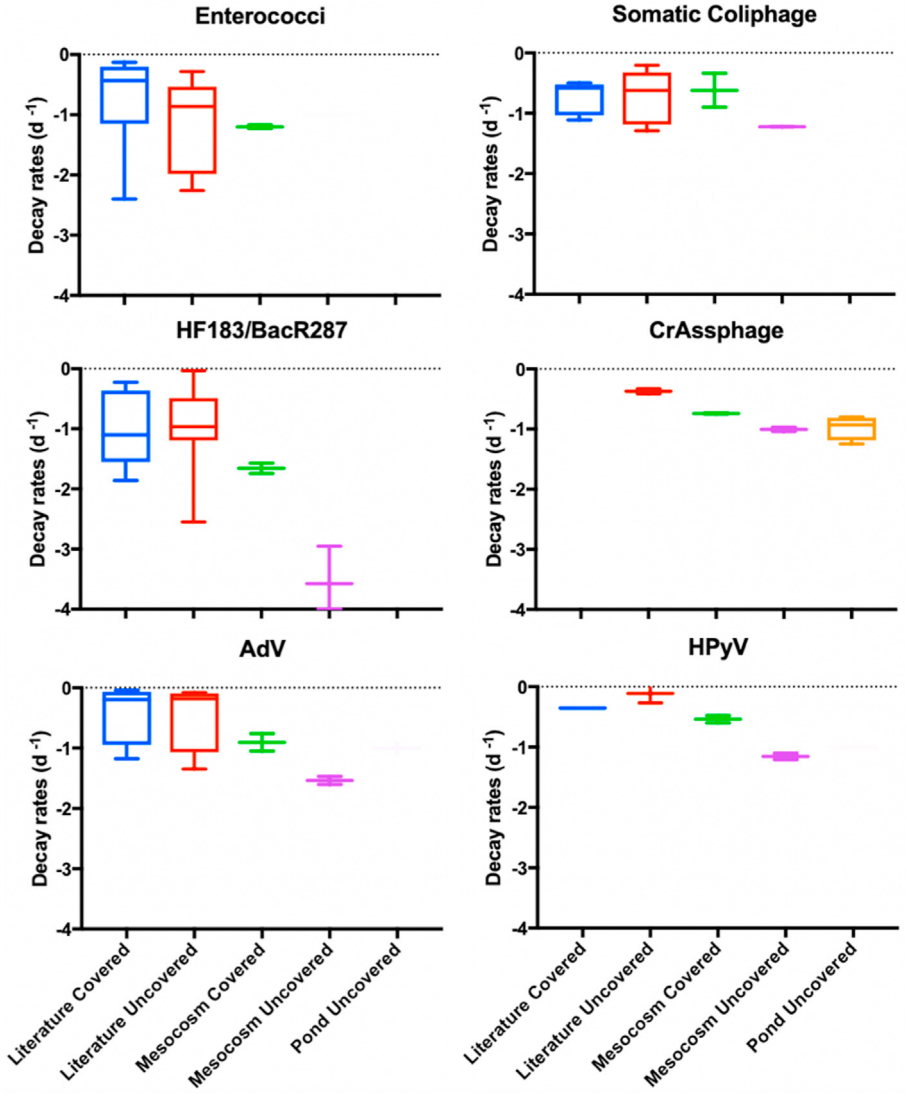

Fig. 4. k_{obs} values from this study compared to inactivation rate constants from previous study for enterococci, somatic coliphage, HF183/BacR287, crAssphage, HPyV, and AdV. Previous study values contain inactivation rate constants from freshwater conditions only. The edge of the boxes on the figure represent the 25th and 75th percentile while the middle line represents the median. The whiskers represent the lowest and highest data point. In cases where there are only three studies, only the middle line and whiskers are present. The dotted line represents the zero mark and initial level at the 2-h time point.

When we compared k_{UV} values between pond and mesocosm study we determined pond crAssphage k_{UV} values were slightly smaller (not statistically) than uncovered mesocosm crAssphage k_{UV} values ($p = 0.1$) and that PMMoV pond k_{UV} values were significantly smaller than the mesocosm values. The faster inactivation in the current pond study compared to the mesocosm study may be due to multiple factors that can appear with scaling up that can affect microbial inactivation such as microbiota (Sagarin et al., 2016). While biological activity was not directly tested at the different scales, scaling up may introduce changes in the microbial community diversity that may not have been present or possible at the lower scales. This could also explain why decay in our study was faster than previously observed. The model systems in our study were significantly larger and more exposed to the environmental factors than other model systems. We used ponds with a volume of 925,000 L and mesocosms with a volume of 300 L, whereas previous study approaches have used dialysis bags, microcosms and smaller mesocosms (10–20L). These results may demonstrate that it is important to not overextend inactivation rate constants from smaller scale studies to larger scale applications.

The larger inactivation rate constants in the presence of sunlight compared to the absence of sunlight shows that sunlight has an effect on indicator persistence. Previous studies, however, do not account for or measure UV fluence in their experiments hence this could also be a reason for the differences between this study and previous literature. Other physical characteristics such as pH, temperature and salinity could play a role in the differences between this study and previous literature comparison; however, most studies did not report values for all physical characteristics hence we cannot determine the role these parameters play on the differential inactivation rate constants. It is important for future studies to report all relevant conditions. Additional research is also needed to understand how these different physical factors affect the inactivation of fecal indicators under large-scale conditions.

Comparison between viral and bacterial persistence

A significant motivation for developing a viral fecal pollution

indicator is the inadequacy of bacterial fecal pollution indicators to represent viral fate. In this study, the bacterial fecal indicators enterococci, *E. coli*, and HF183/BacR287 decayed faster in the uncovered mesocosms compared to viral indicators, suggesting that bacterial fecal indicators were more susceptible to inactivation stressors than the viral fecal indicators. Prior studies have observed similar relationships between viral and bacterial fecal indicators, attributed to a number of factors (Ahmed et al., 2019). One factor is the smaller size of viruses that may adsorb to larger particles, which can shield the virus from UV radiation (Rao et al., 1984). Viruses such as phages (e.g., crAssphage, somatic coliphage) can also be shielded from UV when present in a bacterial host or if host cell debris is adsorbed onto the virus. Inactivation of viral fecal indicators were also slower than the inactivation of bacterial fecal indicators in the covered mesocosm. One previously suggested reason for this is that bacteria are more susceptible to predation than viruses due to the presence of eukaryotes such as protozoa and bacteriophages in surface waters (Chen and Williams, 2012). Further research into the mechanisms of fecal indicator inactivation is still necessary to interpret the differences between viral and bacterial indicators. In addition, these results demonstrate the differential fate of bacterial and viral indicators and highlight the necessity of viral indicators to accurately demonstration viral fate in the environment.

Extended persistence of PMMoV

Inactivation of PMMoV was significantly slower than all other microbial targets tested in this experiment. The PMMoV k_{obs} values between the covered and uncovered mesocosm were also not significantly different (p-value = 0.7186). A potential contributing factor to the extended persistence of PMMoV is its single stranded RNA genome. Previous studies have shown DNA is more photoreactive than RNA, hence making RNA genomes more UV resistant (Kundu et al., 2004; Qiao and Wigginton, 2016). However, a recent study by Qiao et al. suggested that ssRNA viruses such as norovirus have equal resistance to UV degradation as dsDNA viruses such as AdV, HPyV, somatic coliphage, and crAssphage (Qiao et al., 2018). Additional research is needed to parse out the differences between these two different types of viruses in more realistic conditions. The extended persistence of PMMoV could also be due to its long rod-shaped protein capsid (approximate length of 312 nm) which could influence inactivation (Hamza, 2011; Kitajima et al., 2018). These results demonstrate that the extended persistence of PMMoV should be considered during viral indicator evaluation and when including viral indicators into quantitative microbial risk assessment.

We used a ratio approach to examine the differential persistence of crAssphage and PMMoV (Fig. 5). The predicted crAssphage/PMMoV ratio varied over orders of magnitude within a week of sewage release, highlighting the significantly differential persistence of crAssphage and PMMoV in aged sewage. This analysis also highlights the limitations associated with using only one fecal indicator to assess risk. These results also highlight the value of using multiple fecal indicators to assess microbial water quality in a toolbox approach to account for differential fate and inactivation.

Study Limitations

The primary limitation of the current study is the consideration of a single set of water quality parameters and weather conditions, as differing water quality and weather conditions would provide a more complete picture of fecal indicator persistence. In addition, both crAssphage and PMMoV have only been applied as molecular indicators to date; future research should evaluate how these molecular identifications correlate with viable human pathogens. Another limitation of the current study is that the majority of the targets used are molecular targets hence there is uncertainty around whether organisms are viable. Future research should investigate ways to assess viability of molecular targets through the development of culturable assays or the use of propidium monoazide.

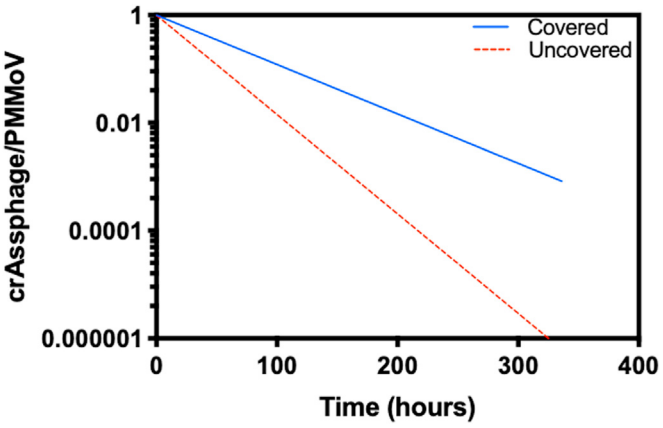

Fig. 5. The ratio of crAssphage concentration normalized by PMMoV concentration over time for covered and uncovered mesocosms. Values calculated from mean first order k_{obs} values determined in the mesocosm study.

Another limitation of our study is the inability to demonstrate mechanisms driving the significant differences between the pond and mesocosm study. Additional research is needed to reveal how scaling up experiments affects decay. The calculations used in this study assumed ponds and mesocosms to be well-mixed, but this was not directly assessed. The final limitation of our study is that visible light was not measured throughout the experiment. Visible light has been shown to contribute to the inactivation of microorganisms though the reaction with colored dissolved organic matter (Traving et al., 2017). Further research should investigate the effects of visible light on these viral fecal indicators and compare them to human pathogens.

Conclusion

- Bacterial indicators HF183/BacR287, *E. coli*, and enterococci inactivated more rapidly than viral indicators crAssphage, HPyV, AdV, somatic coliphage and PMMoV, demonstrating the importance of including viral indicators in the microbial water quality toolkit.
- PMMoV, an ssRNA virus, has extended persistence compared to other viral indicators in water. This suggests that the extended persistence should be taken into account during risk and exposure modeling.
- All targets except PMMoV decayed faster in the presence of sunlight than in the absence of sunlight, emphasizing sunlight as a primary driver of fecal indicator persistence in surface waters.
- Ultimately, the relative inactivation observed between FIB and the promising viral indicators used in this study will help develop each fecal pollution indicator for use in microbial water quality assessment improving risk of infection analysis for fecal-contaminated waters.

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 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). Universities and non-profit researchers interested in using this technology must obtain a research license from the USEPA. 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wroa.2020.100067.

References

Ahmed, W., Gyawali, P., Sidhu, J.P., Toze, S., 2014. Relative inactivation of faecal indicator bacteria and sewage markers in freshwater and seawater microcosms. Lett. Appl. Microbiol. 59 (3), 348–354.
Ahmed, W., Zhang, Q., Kozak, S., Beale, D., Gyawali, P., Sadowsky, M.J., Simpson, S., 2019. Comparative decay of sewage-associated marker genes in beach water and sediment in a subtropical region. Water Res. 149, 511–521.
Anderson, K.L., Whitlock, J.E., Harwood, V.J., 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. Appl. Environ. Microbiol. 71 (6), 3041–3048.
Bae, S., Wuertz, S., 2015. Decay of host-associated Bacteroidales cells and DNA in continuous-flow freshwater and seawater microcosms of identical experimental design and temperature as measured by PMA-qPCR and qPCR. Water Res. 70, 205–213.
Balleste, E., Garcia-Aljaro, C., Blanch, A.R., 2018. Assessment of the decay rates of microbial source tracking molecular markers and faecal indicator bacteria from different sources. J. Appl. Microbiol. 125 (6), 1938–1949.
Balleste, E., Pascual-Benito, M., Martin-Diaz, J., Blanch, A.R., Lucena, F., Muniesa, M., Jofre, J., Garcia-Aljaro, C., 2019. Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments. Water Res. 155 (15), 233–244.
Betancourt, W.Q., Duarte, D.C., Vasquez, R.C., Gurian, P.L., 2014. Cryptosporidium and Giardia in tropical recreational marine waters contaminated with domestic sewage: estimation of bathing-associated disease risks. Mar. Pollut. Bull. 85 (1), 268–273.
Bibby, K., Crank, K., Greaves, J., Li, X., Wu, Z., Hamza, I.A., Stachler, E., 2019. Metagenomics and the development of viral water quality tools. NPJ Clean Water 2 (1), 1–13.
Boehm, A., Soller, J.A., Shanks, O.C., 2015. Human-associated fecal quantitative polymerase chain reaction measurements and simulated risk of gastrointestinal illness in recreational waters contaminated with raw sewage. Environ. Sci. Technol. Lett. 2 (10), 270–275.
Boehm, A.B., Graham, K.E., Jennings, W.C., 2018. Can we swim yet? Systematic review, meta-analysis, and risk assessment of aging sewage in surface waters. Environ. Sci. Technol. 52 (17), 9634–9645.
Boehm, A.B., Silverman, A.I., Schriewer, A., Goodwin, K., 2019. Systematic review and meta-analysis of decay rates of waterborne mammalian viruses and coliphages in surface waters. Water Res. 164, 114898.
Cao, Y., Raith, M.R., Griffith, J.F., 2015. Droplet digital PCR for simultaneous quantification of general and human-associated fecal indicators for water quality assessment. Water Res. 70 (1), 337–349.
Chen, H., Williams, H.N., 2012. In: Azam, F. (Ed.), mBio.
Chendorain, M., Yates, M., Villegas, F., 1998. The fate and transport of viruses through surface water constructed wetlands. Am. Soc. Agron. 27 (6), 1451–1458.
Craig, D.L., 2019. Comparison of decay rates of faecal indicator organisms in recreational coastal water and sediment. Duncan.Craig@flinders.edu.au, Department of Environmental Health, F.U., GPO Box 2100, Adelaide 5001, Australia (E-mail:,), Fallowfield, H.J., Duncan.Craig@flinders.edu.au, Department of Environmental Health, F.U., GPO Box 2100, Adelaide 5001, Australia (E-mail:,), Cromar, N.J., Duncan.Craig@flinders.edu.au, Department of Environmental Health, F.U., GPO Box 2100, Adelaide 5001, Australia (E-mail: and). Water Supply 2 (3), 131–138.
Crank, K., Petersen, S., Bibby, K., 2019. Quantitative microbial risk assessment of swimming in sewage impacted waters using CrAssphage and pepper mild mottle virus in a customizable model. Environ. Sci. Technol. Lett. 6 (10), 571–577.
Dick, L.K., Stelzer, E.A., Bertke, E.E., Fong, D.L., Stoeckel, D.M., 2010. Relative decay of Bacteroidales microbial source tracking markers and cultivated Escherichia coli in freshwater microcosms. Appl. Environ. Microbiol. 76 (10), 3255–3262.
Dutilh, B.E., Cassman, N., McNair, K., Sanchez, S.E., Silva, G.G.Z., Boling, L., Barr, J.J., Speth, D.R., Seguritan, V., Aziz, R.K., 2014. A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. Nat. Commun. 5.
Eichmiller, J.J., Borchert, A.J., Sadowsky, M.J., Hicks, R.E., 2014. Decay of genetic markers for fecal bacterial indicators and pathogens in sand from Lake Superior. Water Res. 59, 99–111.
Elmahdy, M.E.I., Magri, M.E., Garcia, L.A., Fongaro, G., Barardi, C.R.M., 2018. Microcosm environment models for studying the stability of adenovirus and murine norovirus in water and sediment. Int. J. Hyg Environ. Health 221 (4), 734–741.
Gilpin, B.J., Devane, M., Robson, B., Nourozi, F., Scholes, P., Lin, S., Wood, D.R., Sinton, L.W., 2013. Sunlight inactivation of human polymerase chain reaction markers and cultured fecal indicators in river and saline waters. Water Environ. Res. 85 (8), 743–750.
Green, H.C., Shanks, O.C., Sivaganesan, M., Haugland, R.A., Field, K.G., 2011. Differential decay of human faecal Bacteroides in marine and freshwater. Environ. Microbiol. 13 (12), 3235–3249.
Hamza, I., 2011. Evaluation of pepper mild mottle virus, human picobirnavirus and Torque teno virus as indicators of fecal contamination in river water. Water Res. 45 (3), 1358–1368.
Hamza, I.A., Jurzik, L., Uberla, K., Wilhelm, M., 2011. Evaluation of pepper mild mottle virus, human picobirnavirus and Torque teno virus as indicators of fecal contamination in river water. Water Res. 45 (3), 1358–1368.
Harwood, V.J., Boehm, A.B., Sassoubre, L.M., Vijayavel, K., Stewart, J.R., Fong, T.T., Caprais, M.P., Converse, R.R., Diston, D., Ebdon, J., Fuhrman, J.A., Gourmelon, M., Gentry-Shields, J., Griffith, J.F., Kashian, D.R., Noble, R.T., Taylor, H., Wicki, M., 2013. Performance of viruses and bacteriophages for fecal source determination in a multi-laboratory, comparative study. Water Res. 47 (18), 6929–6943.
Hayden, R.T., Gu, Z., Ingersoll, J., Abdul-Ali, D., Shi, L., Pounds, S., Caliendo, A.M., 2013. Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus. J. Clin. Microbiol. 51 (2), 540–546.
He, X., Liu, P., Zheng, G., Chen, H., Shi, W., Cui, Y., Ren, H., Zhang, X.X., 2016. Evaluation of five microbial and four mitochondrial DNA markers for tracking human and pig fecal pollution in freshwater. Sci. Rep. 6 (35311).
Hjelmsø, M.H., Hellmér, M., Fernandez-Cassi, X., Timoneda, N., Lukjancenko, O., Seidel, M., Elsässer, D., Aarestrup, F.M., Löfström, C., Bofill-Mas, S., Abril, J.F., Girones, R., Schultz, A.C., 2017. Evaluation of methods for the concentration and extraction of viruses from sewage in the context of metagenomic sequencing. PloS One 12 (1).
Ibrahim, E.M.E., El-Liethy, M.A., Abia, A.L.K., Hemdan, B.A., Shaheen, M.N., 2019. Survival of E. coli O157:H7, Salmonella Typhimurium, HAdV2 and MNV-1 in river water under dark conditions and varying storage temperatures. Sci. Total Environ. 648, 1297–1304.
Jeanneau, L., Solecki, O., Wéry, N., Jardé, E., Gourmelon, M., Communal, P.-Y., Jadas-Hécart, A., Caprais, M.-P., Gruau, G., Pourcher, A.-M., 2012. Relative decay of fecal indicator bacteria and human-associated markers: a microcosm study simulating wastewater input into seawater and freshwater. Environ. Sci. Technol. 46 (4), 2375–2382.
Kirs, M., Caffaro-Filho, R.A., Wong, M., Harwood, V.J., Moravcik, P., Fujioka, R.S., 2016. Human-associated Bacteroides spp. and human polyomaviruses as microbial source tracking markers in Hawaii. Appl. Environ. Microbiol. 82 (22), 6757–6767.
Kitajima, M., Sassi, H.P., Torrey, J.R., 2018. Pepper mild mottle virus as a water quality indicator. NPJ Clean Water 1 (1), 19.
Kundu, L.M., Linne, U., Marahiel, M., Carell, T., 2004. RNA is more UV resistant than DNA: the formation of UV-induced DNA lesions is strongly sequence and conformation dependent. Chemistry 10 (22), 5697–5705.
Liang, L., Goh, S.G., Vergara, G.G., Fang, H.M., Rezaeinejad, S., Chang, S.Y., Bayen, S., Lee, W.A., Sobsey, M.D., Rose, J.B., Gin, K.Y., 2015. Alternative fecal indicators and their empirical relationships with enteric viruses, Salmonella enterica, and Pseudomonas aeruginosa in surface waters of a tropical urban catchment. Appl. Environ. Microbiol. 81 (3), 850–860.
Liang, Z., He, Z., Zhou, X., Powell, C.A., Yang, Y., Roberts, M.G., Stoffella, P.J., 2012. High diversity and differential persistence of fecal Bacteroidales population spiked into freshwater microcosm. Water Res. 46 (1), 247–257.
Malla, Bikash, et al., 2019. Evaluation of human-and animal-specific viral markers and application of crassphage, pepper mild mottle virus, and tobacco mosaic virus as potential fecal pollution markers to river water in Japan. Food Environ. Virol. 11 (4), 446–452.
Maraccini, P., Mattioli, M., Sassoubre, L., 2016. Solar inactivation of enterococci and Escherichia coli in natural waters: effects of water absorbance and depth. Environ. Sci. Technol. 50 (10), 5068–5076.
NguyenThi Mai, Huong, et al., 2016. Seasonal variability of faecal indicator bacteria numbers and die-off rates in the Red River basin, North Viet Nam. Sci. Rep. 6, 21644.
Prevost, B., Goulet, M., Lucas, F.S., Joyeux, M., Moulin, L., Wurtzer, S., 2016. Viral persistence in surface and drinking water: suitability of PCR pre-treatment with intercalating dyes. Water Res. 91, 68–76.
Qiao, Z., Wigginton, K.R., 2016. Direct and indirect photochemical reactions in viral

RNA measured with RT-qPCR and mass spectrometry. Environ. Sci. Technol. 50 (24), 13371–13379.
Qiao, Z., Ye, Y., Chang, P.H., Thirunarayanan, D., Wigginton, K.R., 2018. Nucleic acid photolysis by UV254 and the impact of virus encapsidation. Environ. Sci. Technol. 52 (18), 10408–10415.
Rachmadi, A.T., Kitajima, M., Pepper, I.L., Gerba, C.P., 2016. Enteric and indicator virus removal by surface flow wetlands. Sci. Total Environ. 542 (Pt A), 976–982.
Rao, V.C., Seidel, K.M., Goyal, S.M., Metcalf, T.G., Melnick, J.L., 1984. Isolation of enteroviruses from water, suspended solids, and sediments from Galveston Bay: survival of poliovirus and rotavirus adsorbed to sediments. Appl. Environ. Microbiol. 48 (2), 404–409.
Rački, N., Dreo, T., Gutierrez-Aguirre, I., Blejec, A., Ravnikar, M., 2014. Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples. Plant Methods 10 (1), 42.
Sagarin, R.D.A., Blanchette, John Carol A., Brusca, Richard C., Chorover, Jon, Cole, Julia E., Micheli, Fiorenza, Munguia-Vega, Adrian, Rochman, Chelsea M., Bonine, Kevin, van Haren, Joost, Troch, Peter A., 2016. Between control and complexity: opportunities and challenges for marine mesocosms. Front. Ecol. Environ. 14 (7), 389–396.
Silverman, A.I., Peterson, B.M., Boehm, A.B., McNeill, K., Nelson, K.L., 2013. Sunlight inactivation of human viruses and bacteriophages in coastal waters containing natural photosensitizers. Environ. Sci. Technol. 47 (4), 1870–1878.
Stachler, E., Akyon, B., Carvalho, N.A.d., Ference, C., Bibby, K., 2018. Correlation of crAssphage qPCR markers with culturable and molecular indicators of human fecal pollution in an impacted urban watershed. Environ. Sci. Technol. 53 (13), 7505–7512.
Stachler, E., Crank, K., Bibby, K., 2019. Co-occurrence of crAssphage with antibiotic resistance genes in an impacted urban watershed. Environ. Sci. Technol. 6 (4), 216–221.
Stachler, E., Kelty, C., Sivaganesan, M., Li, X., Bibby, K., Shanks, O.C., 2017. Quantitative CrAssphage PCR assays for human fecal pollution measurement. Environ. Sci. Technol. 51 (16), 9146–9154.
Staley, C., Gordon, K.V., Schoen, M.E., Harwood, V.J., 2012. Performance of two quantitative PCR methods for microbial source tracking of human sewage and implications for microbial risk assessment in recreational waters. Appl. Environ. Microbiol. 78 (20), 7317–7326.
Traving, S.J., Rowe, O., Jakobsen, N.M., Sørensen, H., Dinasquet, J., Stedmon, C.A., Andersson, A., Riemann, L., 2017. The effect of increased loads of dissolved organic matter on estuarine microbial community composition and function. Front. Microbiol. 8 (351).
UNWWAP, 2017. In: Connor, R. (Ed.), Wastewater: the Untapped Resource. UNESCO, Paris, p. 198.
Wade, T.J., Calderon, R.L., Sams, E., Beach, M., Brenner, K.P., Williams, A.H., Dufour, A.P., 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspect. 24–28.
Walters, S.P., Field, K.G., 2009. Survival and persistence of human and ruminant-specific faecal Bacteroidales in freshwater microcosms. Environ. Microbiol. 11 (6), 1410–1421.
Wetter, C., Conti, M., Altschuh, D., Tabillion, R., Van Regenmortel, M.H.V., 1984. Pepper mild mottle virus, a tobamovirus infecting pepper cultivars in Sicily. Phytopathology 74 (4), 405–410.
Wu, J., Cao, Y., Young, B., Yuen, Y., Jiang, S., Melendez, D., Griffith, J.F., Stewart, J.R., 2016. Decay of coliphages in sewage-contaminated freshwater: uncertainty and seasonal effects. Environ. Sci. Technol. 50 (21), 11593–11601.