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Intraday variability of indicator and pathogenic viruses in 1-h and 24-h composite wastewater samples: Implications for wastewater-based epidemiology

Warish Ahmed [a,*], Aaron Bivins [b], Paul M. Bertsch [a], Kyle Bibby [b], Pradip Gyawali [c], Samendra P. Sherchan [d], Stuart L. Simpson [e], Kevin V. Thomas [f], Rory Verhagen [f], Masaaki Kitajima [g], Jochen F. Mueller [f], Asja Korajkic [h]

[a] CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, QLD, 4102, Australia
[b] Department of Civil & Environmental Engineering & Earth Sciences, 156 Fitzpatrick Hall, University of Notre Dame, Notre Dame, IN, 46656, USA
[c] Institute of Environmental Science and Research Ltd (ESR), Porirua, 5240, New Zealand
[d] Department of Environmental Health Sciences, Tulane University, 1440 Canal Street, New Orleans, LA, 70112, USA
[e] CSIRO Land and Water, Lucas Heights, NSW, 2234, Australia
[f] Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 20 Cornwall Street, Woolloongabba, QLD, 4103, Australia
[g] Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North West 8, Kita-ku, Sapporo, Hokkaido, 060-0032, Japan
[h] United States Environmental Protection Agency, Office of Research and Development, 26W Martin Luther King Jr. Drive, Cincinnati, OH, 45268, USA

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ABSTRACT

We monitored the density of fecal indicator viruses crAssphage and pepper mild mottle virus (PMMoV) and human pathogen adenovirus (HAdV) in influent from a wastewater treatment plant in Brisbane, Australia in 1-h and 24-h composite samples. Over three days of sampling, the mean concentration of crAssphage gene copies (GC)/mL in 24-h composite samples did not differ significantly ($p = 0.72$), while for PMMoV GC/mL (p value range: 0.0002–0.0321) and HAdV GC/mL (p value range: 0.72–0.92), significant differences in concentrations, were observed on one day of sampling compared to the other two. For all three viruses the variation observed in 1-h composite samples was greater than the variation observed in 24-h composite samples. For crAssphage, in 54.1% of 1-h composite samples, the concentration was less than that observed in 24-h composite samples; whereas for PMMoV and HAdV the concentration was less in 79.2% and 70.9% of 1-h composites, respectively, compared to the relevant 24-h composite. Similarly, the concentration of crAssphage DNA in 1-h compared to 24-h composite samples did not differ ($p = 0.11$) while the concentrations of PMMoV ($p < 0.0001$) and HAdV ($p < 0.0001$) in 1-h composites were significantly different from 24-h composites. These results suggest that 24-h composite samples offer increased analytical sensitivity and decreased variability compared to 1-h composite samples when monitoring wastewater, especially for pathogenic viruses with low infection rate within a community. Thus, for wastewater-based epidemiology applications, 24-h composite samples are less likely to produce false negative results and erroneous public health guidance.

1. Introduction

Several groups of non-enveloped enteric viruses, such as human adenovirus (HAdV), astrovirus (AtVs), enterovirus (EV), hepatitis A (HAV) and E (HEV) viruses, norovirus (NoV), rotavirus (RoV), aichi virus (AiV), sapovirus (SaV) and torqueteno virus (TTV) are commonly found in wastewater (Xagoraraki and O'Brien, 2020). These viruses are waterborne and known to cause gastroenteritis, respiratory disease, common cold, meningitis, liver disaese, nausea, vomiting and fever in infected individuals (Frankhauser et al., 2002; Clark and McKendrick, 2004; Ganesh and Lin, 2013). Enteric virus transmission occus mainly through fecal-oral route via human feces (Kotwal et al., 2014). Enveloped viruses such as, influenza A (H1N1) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have also been reported to be present in wastewater (Lago et al., 2003; Heijnen and Medema, 2009; Ahmed et al., 2020a, 2020b, 2020c; Haramoto et al., 2020; La Rosa et al., 2020a; Medema et al., 2020; Randazoo et al., 2020a; Sherchan et al., 2020). Unlike non-enveloped viruses, less is known about this mode of transmission for enveloped viruses. However, consistent detection of SARS-CoV-2 RNA in feces, anal swabs (Yeo et al., 2020), and untreated wastewater combined with the binding affinity of SARS-CoV-2 to ACE2 receptors, which are abundant in small intestine epithelial cells, suggest that fecal-oral transmission is plausible for this virus (Mohapatra et al., 2020).

Human viruses present in municipal wastewater originate from human feces with rare examples (e.g., polyomaviruses) that may also be

* Corresponding author. Ecosciences Precinct, 41 Boggo Road, Dutton Park, 4102, Queensland, Australia.
E-mail address: Warish.Ahmed@csiro.au (W. Ahmed)

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excreted in urine (Xagoraraki and O'Brien, 2020). Therefore, the concentration of human viruses in wastewater is expected to depend on patterns of human defecation and urination. Intraday fluctuations in virus concentration in municipal wastewater are also expected to differ depending on a number of factors, such as the size of both the service population and catchment, the virus type, the type of sewer sytem, groundwater intrusion, and climatic conditions. Monitoring of human pathogenic and indicator viruses in municipal wastewater has been used to assess the micobiological quality of wastewater treatment plant (WWTP) influent and to monitor viral diseases in a community, known as wastewater-based epidemiology (WBE) (Bivins et al., 2020). WBE is a powerful tool which can be used to detect not only pathogens significant to public health but also chemicals or illicit drugs at the community level by monitoring wastewater (Choi et al., 2018; Sims and Kasprzyk-Hordern et al., 2020; Devault and Karolak, 2020).

WBE has been attracting much attention as a complementary tool to monitor the degree of viral disease prevalence in a community, including coronavirus disease 2019 (COVID-19). WBE can provide information on the prevalence of the viruses in the community, spatial and temporal trends on the circulation of viruses, and screening of asymptomatic individuals. WBE could be a valuable tool for resource-limited regions where clincal testings are limited or not available. Complementing clinical testing, WBE can be used as an early warning tool or to detect hot spots for better management of on-going and upcoming pandemics (La Rosa et al., 2020b; Medema et al., 2020; Thompson et al., 2020).

The application of WBE can be greatly impacted by temporal fluctuations of viral loads in influent wastewater at a wastewater treatment plant (WWTP), however, the temporal dynamics of viruses in wastewater are underreported. A recent review paper has identified several sources of uncertainty in WBE, such as sampling approaches (i.e., grab vs. composite), lack of knowledge on the persistence of viral nucleic acid in wastewater, low recovery efficiency of virus concentration methods, and which molecular assays provide the most sensitive detection and enable accurate quantification at low levels (Kitajima et al., 2020).

Currently, inadequate comparison and reporting of the efficacy of wastewater sampling and virus concentration methods for the detection of enteric viruses and enveloped viruses from wastewater impedes WBE applications. Sampling strategies are particularly important, since poorly designed strategies can introduce biases (i.e., false negatives) in data interpretation. Several studies have used grab sampling approaches for monitoring of enteric and enveloped viruses in wastewater (Ahmed et al., 2020a, 2020b, 2020c; Haramoto et al., 2020; Tandukar et al., 2020; McCall et al., 2020; Gyawali and Hewitt, 2018). These methods collect an untreated wastewater sample at a selected sampling site at a single moment in time. However, grab sampling methods may be inadequate for detection of low levels or diurnal variations of enteric or enveloped viruses in untreated wastewater. Composite samples collected using volume, flow or time-based sampling modes pooling the wastewater into a single sample provide the basis for representative sample collection over extended periods of time (Ort et al., 2010). For enteric virus concentrations or other enveloped viruses such as SARS-CoV-2 in wastewater, little information is available on which of these sampling approaches provide the more appropriate means to collect a representative sample.

The aim of this study was to investigate the intraday variabity of two indicator viruses, namely crAssphage and pepper mild mottle virus (PMMoV) and a pathogenic virus (i.e., human adenovirus (HAdV)) with varying levels in an urban wastewater treatment plant (WWTP) in Brisbane. The two indicator viruses selected were chosen because, unlike some other viral indicators (e.g., bacteriophages) both crAssphage and PMMoV are human-associated (Ahmed et al., 2019), owing to the consumption of vegetables infected with PMMoV and crAssphage as a commensal member of the human gut microbiota, and subsequently, both are prevalent in untreated wastewater across wide geographic regions, and exhibit temporal stability. Both have been recently used to normalize SARS-CoV-2 RNA in wastewater (Medema et al., 2020b). Conversely, HAdV, is a human pathogen capable of infecting both the respiratory and gastrointestinal tracts (Lynch et al., 2011). The prevalence of HAdV in wastewater is expected to be much lower than crAssphage and PMMoV due to the lower prevalence of infection-associated shedding. By considering viruses that are both more and less prevalent among the population and subsequently in wastewater, the observations of the current study can inform the design of appropriate wastewater sampling strategies for sensitive and reliable monitoring of pathogenic viruses in wastewater for WBE or other applications such as recycled water safety.

2. Materials and methods

2.1. Wastewater sampling

Untreated wastewater samples were collected from the influent of an urban WWTP in Brisbane, Australia, using a conventional autosampler ISCO 3700 (Teledyne ISCO, Inc., Lincoln, NE, USA). The autosampler base contained ice to keep the samples cool during sample collection. The autosampler was programmed to collect a sample every 15 min over a 24-h period that were composited to provide 24 1-h composite over three separate days (June 22, 2020, June 25, 2020, and July 01, 2020), yielding a total of 72 1-h composite samples. In addition, equal volume aliquots from each of the 24 1-h composite samples were pooled to create a one 24-h composite sample for each of the three days yielding a total of three 24-h composite samples for three days. The sewer network is comprosed of reticulated, branched and trunk main sewers. No precipitation occurred in the WWTP catchment during wastewater sampling. The hydraulic treatment capacity of the WWTP is approximately 170 ML/day and average dry weather flow is 140 ML/day. Approximately 17% of wastewater was estimated to be from commercial and industrial sources including waste from landfills, hospitals and an airport. The maximum residence time is estimated to be 11–12 h, and there are 83 pump stations in the catchment. Samples were transported on ice to the laboratory, stored at 4 °C and processed within 5 days. While five days is a longer holding time compared to customary ~24 h, a recent study reported that the SARS-CoV-2 and murine hepatitis virus (MHV) RNA in untreated wastewater at 4 °C can persist for > 30 days, with minimal to no decay observed within the first 5 days (Ahmed et al., 2020a, 2020b, 2020c) suggesting no adverse effects from the extended holding times.

2.2. Viral nucleic acid extraction

RNeasy Mini and DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) were used to directly extract nucleic acid from 140 μL to 200 μL of untreated wastewater, without any pre-treatment or concentration, respectively. A QIAcube Connect platform (Qiagen) was used to eluate 60 μL of RNA and 100 μL of DNA from lysates. All nucleic acid samples were stored at −80 °C and subjected to qPCR and RT-qPCR analyses within 1–3 days after nucleic acid extraction to avoid losses associated with storing, as well as freezing and thawing of nucleic acid samples.

2.3. Determination of PCR inhibition

An experiment was conducted to determine the presence of PCR inhibition in nucleic acid extracted from wastewater samples using a Sketa22 and MHV real-time PCR assay (Besselsen et al., 2002; Haugland et al., 2005). Known gene copy (GC) numbers of *Oncorhynchus keta* DNA (2×10^2 GC/reaction) and MHV RNA (2×10^4 GC/reaction) were added in the qPCR and RT-qPCR reactions (without sample)

and the quantification cycle (Cq) values obtained acted as reference points. The same amounts of *O. keta* and MHV were also added into qPCR and RT-qPCR reactions in the presence of wastewater nucleic acid samples. If the Cq value of a wastewater nucleic acid sample increases (i.e., two Cq values), the sample was considered to have PCR inhibitors (Staley et al., 2012).

2.4. qPCR and RT-qPCR assay

Previously published qPCR and RT-qPCR assays were used for the analysis of the crAssphage (CPQ_056 assay) (Stachler et al., 2017), HAdV (Heim et al., 2003) and PMMoV (Rosario et al., 2009; Haramoto et al., 2013). The primers and probes for these qPCR assays are shown in Supplementary Table ST1 along with qPCR cycling parameters. For all qPCR assays, synthetic DNA (4 μg) in plasmid cloning vectors or gBlocks gene fragments were purchased from the Integrated DNA Technologies (Coralville, IA, USA). qPCR standards were prepared from the synthetic DNA, ranging from 10^6 to 1 GC/μL of DNA. CrAssphage and HAdV qPCR amplifications were performed in 20 μL reaction mixtures using SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, Richmond, CA, USA). CrAssphage qPCR mixtures contained 10 μL of Supermix, 1000 nM of forward and reverse primer, 100 nM probe and 3 μL of template DNA. HAdV qPCR mixture contained 10 μL of Supermix, 200 nM of forward and reverse primer, 200 nM probe and 3 μL of template DNA. The qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories). All qPCR reactions were performed in triplicate. PMMoV RT-qPCR mixtures contained 10 μL of qScript XLT 1-Step RT-qPCR ToughMix (QuantaBio, Beverly, MA, USA), 200 nM of forward and reverse primer, 80 nM probe and 5 μL of template RNA. For each qPCR run, a series of standard (3×10^6 to 3 GC/reaction) and no template controls ($n = 3$) were included. The qPCR assay limit of detection (ALOD) values were determined from Cq values of the three separate standard curves run for each assay. qPCR ALOD values were defined as the number of copies that could be detected and quantified in 2/3 qPCR assays (Senkbeil et al., 2019). The sample limit of detection (SLOD) was calculated by dividing the ALOD by the RNA template volume (i.e., 3 μL for crAssphage and HAdV and 5 μL for PMMoV) added to the qPCR/RT-qPCR well and then multiplying this number by the total volume of RNA extracted from each sample (100 μL for crAssphage and HAdV and 60 μL for PMMoV) to yield SLOD.

2.5. Quality control

A reagent blank and an extraction blank were included for each batch of nucleic acid extraction to ensure no carryover contamination occurred during extraction. No carryover contamination was observed in reagent blank samples. To minimize potential qPCR and RT-qPCR contamination, nucleic acid extraction and PCR setup were performed in separate laboratories.

2.6. Data analysis

GraphPad Prism 8.3.1 (GraphPad Software, La Jolla, CA, USA) was used to conduct one-way analysis of variance (ANOVA) with Tukey's multiple comparison test to evaluate differences in concentrations of each individual virus across different sampling days, and to compare concentrations of three viruses tested in 1-h and 24-h composite samples. The same software was also used to conduct unpaired *t*-test to test the difference in concentration of each virus in the 1-h composite samples versus 24-h composite samples ($\alpha = 0.05$ for both tests).

3. Results

3.1. PCR inhibition, performance characteristics and limit of detection

Mean Cq values for *O. keta* in sterile water ranged from 31.6 to 31.9, while these values ranged from 31.6 to 32.1 for the 1-h composite samples and 31.2 to 32.0 for 24-h composite samples (Supplementary Table ST2). The difference in mean Cq value for *O. keta* in the 1-h and 24-h composite samples collected in different days compared to sterile water samples were −0.10 to 0.70 and −0.40 to 0.40, respectively. Similarly, mean Cq values for MHV in sterile water ranged from 24.5 to 24.9, while these values ranged from 24.5 to 24.9 for the 1-h composite samples and 24.5 to 28.5 for the 24-h composite samples (Supplementary Table ST2). The differences in mean Cq values for MHV in 1-h and 24-h composite samples collected on different days compared to sterile water samples ranged from −0.50 to 0.10 and −0.40 to 0.30, respectively. Based on the Cq values, all DNA and RNA samples were considered to be free from PCR inhibition and were used for downstream qPCR and RT-qPCR analysis. The amplification efficiencies of crAssphage, HAdV and PMMoV assays were within the prescribed range (96–104%) of MIQE guidelines (Bustin et al., 2009). The correlation coefficient (R^2) values for all assays were between 0.96 and 0.99. The slope of the standard curves and Y-intercept values are shown in Supplementary Table ST3. The ALOD values were 2, 1, and 3 GC/μL of nucleic acid for crAssphage, HAdV and PMMoV, respectively. The SLOD values for crAssphage and HAdV were 66.6 and 33.3 GC/200 μL of untreated wastewater sample. Similarly, the SLOD value for PMMoV was 36 GC/200 μL of untreated wastewater sample.

3.2. Concentration of viruses in untreated wastewater

Concentrations of crAssphage, HAdV and PMMoV in the 1-h and 24-h time-based composite samples are shown in Fig. 1. Among the three viruses analysed, the mean concentrations of crAssphage, HAdV and PMMoV in the 1-h composite samples ranged from 5.94 to 5.96, 3.54 to 3.95 and 4.90 to 5.28 $\log_{10}$ GC/mL, respectively, over three sampling dates (Table 1). One-way ANOVA indicated that the pooled mean concentration (5.95 ± 0.13 $\log_{10}$ GC/mL) of crAssphage in the 1-h composite samples over the entire sampling campaign was significantly ($p < 0.0001$) greater than HAdV (3.69 ± 0.43 $\log_{10}$ GC/mL) and PMMoV (5.08 ± 0.34 $\log_{10}$ GC/mL). The mean concentration of PMMoV was also significantly greater ($p < 0.0001$) than HAdV.

Similarly, the mean concentration (5.92 ± 0.08 $\log_{10}$ GC/mL) of crAssphage in the 24-h composite samples collected over three sampling dates was significantly (*p* value range: 0.0021 - <0.0001) greater than HAdV (3.93 ± 0.18 $\log_{10}$ GC/mL) and PMMoV (5.33 ± 0.006 $\log_{10}$ GC/mL). The mean concentration of PMMoV was also significantly ($p < 0.001$) greater than HAdV. We also compared the concentration of all three viruses among three sampling days. The mean concentration of crAssphage in the 24-h composite sample collected on June 22, 2020 did not differ significantly from June 25, 2020 and July 01, 2020 (*p* value range: 0.72–0.92). The mean concentration of HAdV in the 24-h composite sample on June 22, 2020 differed significantly (*p* value range: 0.002–0.006) from June 25, 2020 and July 01, 2020. However, the mean concentration of HAdV on June 25, 2020 did not differ significantly ($p = 0.9536$) from July 01, 2020. The mean concentration of PMMoV in the 24-h composite sample on June 22, 2020 differed significantly (*p* value range: 0.0002–0.03) from June 25, 2020 and July 01, 2020, but there was no statistically significant difference between June 25, 2020 and July 01, 2020 ($p = 0.1980$).

We also compared the variation in concentration of all three viruses in the 24-h composite samples with the concentration in the 1-h composite samples. The standard deviations (SDs) of crAssphage in the 1-h

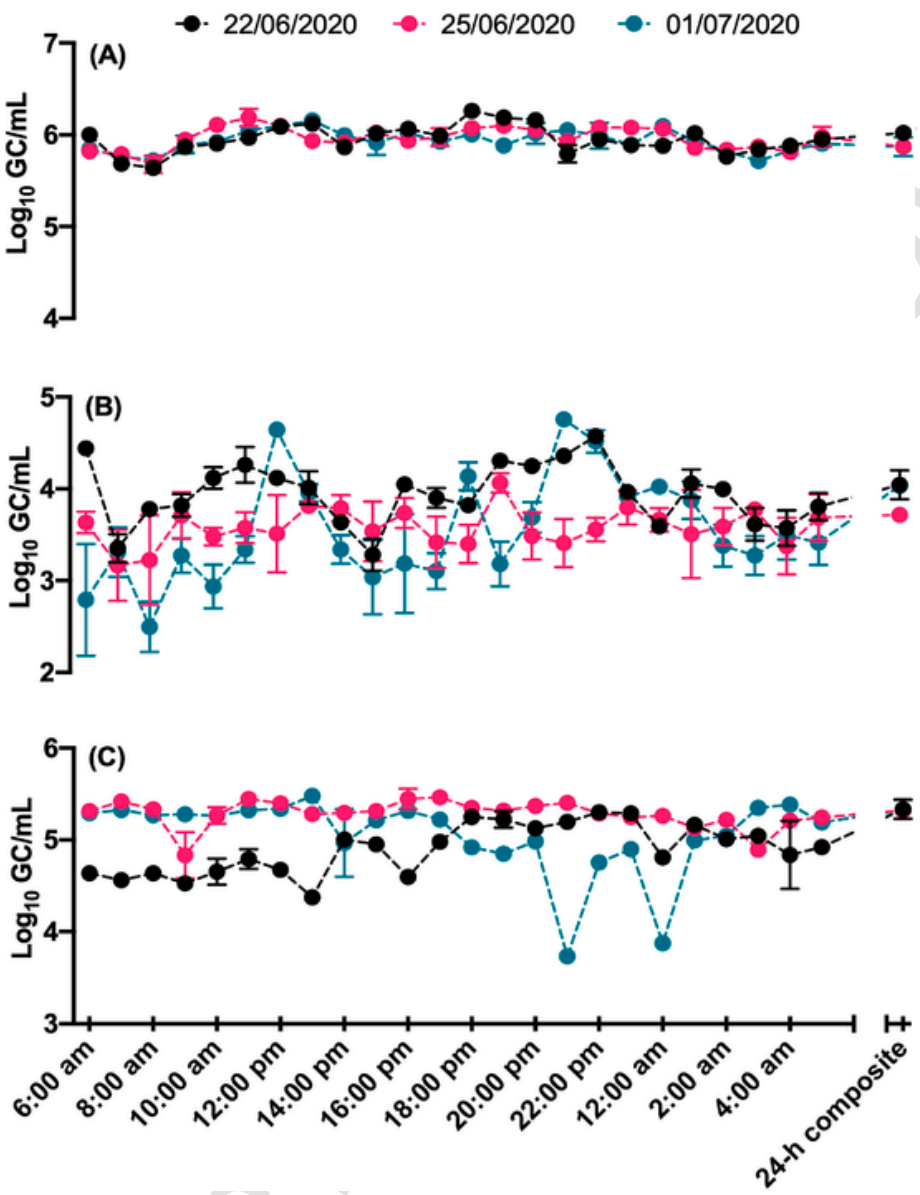

Fig. 1. Concentrations (log_{10} GC/mL) of (A) crAssphage, (B) HAdV, and (C) PMMoV in the 1-h and 24-h time-based composite samples.

composite samples ranged from 0.11 to 0.15 log_{10} GC/mL over the three sampling dates, whereas the SD for 24-h time-based composite samples was 0.08 log_{10} GC/mL over three sampling dates. A greater variation was observed for HAdV in the 1-h composite samples, with SDs ranging from 0.20 to 0.57 log_{10} GC/mL over three sampling dates compared to 24-h time-based composite samples (0.18 log_{10} GC/mL) over three sampling dates. A greater variation was also observed for PMMoV in the 1-h composite samples, with SDs ranging from 0.15 to 0.43 log_{10} GC/mL over three sampling dates compared to 24-h time-based composite samples (0.006 log_{10} GC/mL) over three sampling dates. The co-efficient of variation (CV) values for crAssphage over three days were small ranging from 1.97 to 2.57%. However, CV values for HAdV and PMMoV were much greater than crAssphage, ranging from 5.65 to 16.3% and 2.90–8.63%, respectively.

We determined the percentage increase or decrease in concentration by determining the difference in the concentration of viruses between 1-h and 24-h composite samples and then divideded the increase by the original number and multiply the value by 100. Overall, the concentration of crAssphage was greater in 24-h time-based composite samples compared to 39/72 (54.1%) of the 1-h composite wastewater samples (Fig. 2). Similarly, concentrations of HAdV and PMMoV were greater in 24-h composite samples compared to 51/72 (70.8%) and 57/72 (79.2%) 1-h composite wastewater samples (Fig. 2), but not all comparisons were statistically significant. Specifically, direct comparison of crAssphage concentrations between the 1-h composite samples and 24-h time-based composite samples indicated no statistically significant difference ($p = 0.1082$), while concentrations in the latter sample type were significantly higher for both HAdV and PMMoV ($p < 0.0001$ for both).

4. Discussion

Achieving a representative sample is vital for the successful application of WBE as a sensitive early warning system or to monitor trends of virus circulation in the community. Poorly designed sampling procedures may introduce biases and contribute to false-negative results (WRF, 2020). False negative results (i.e., virus infections present in the community but the wastewater analytical method fails to yield a signal) could have economic and legal consequences in wastewater sur-

Table 1
Mean (log_{10} GC/mL), range and 95% CI levels of crAssphage, HAdV and PMMoV in the 1-h composite untreated wastewater samples collected on three separate days.

Concentrations (log_{10} GC/mL)	Sampling dates			
	22/06/2020 [a]	25/06/2020 [a]	01/07/2020 [a]	22/06-2020 – 01/07/2020 [b]
CrAssphage				
Mean	5.95	5.96	5.94	5.95
SD	0.15	0.12	0.11	0.13
Min	5.64	5.68	5.72	5.64
Max	6.26	6.19	6.16	6.26
Lower 95% CI	5.89	5.91	5.89	5.92
Upper 95% CI	6.01	6.02	5.98	5.98
CV (%)	2.57	2.12	1.97	2.22
HAdV				
Mean	3.95	3.58	3.54	3.69
SD	0.33	0.20	0.57	0.43
Min	3.28	3.17	2.50	2.50
Max	4.57	4.06	4.76	4.76
Lower 95% CI	3.80	3.49	3.30	3.59
Upper 95% CI	4.09	3.66	3.79	3.79
CV (%)	8.46	5.65	16.3	11.9
PMMoV				
Mean	4.90	5.28	5.05	5.08
SD	0.27	0.15	0.43	0.34
Min	4.37	4.83	3.73	3.73
Max	5.30	5.46	5.48	5.48
Lower 95% CI	4.78	5.22	4.87	5.00
Upper 95% CI	5.01	5.35	5.23	5.16
CV (%)	5.55	2.90	8.53	6.73

[a] 1-h composite for each day.
[b] 1-h composite for three days.

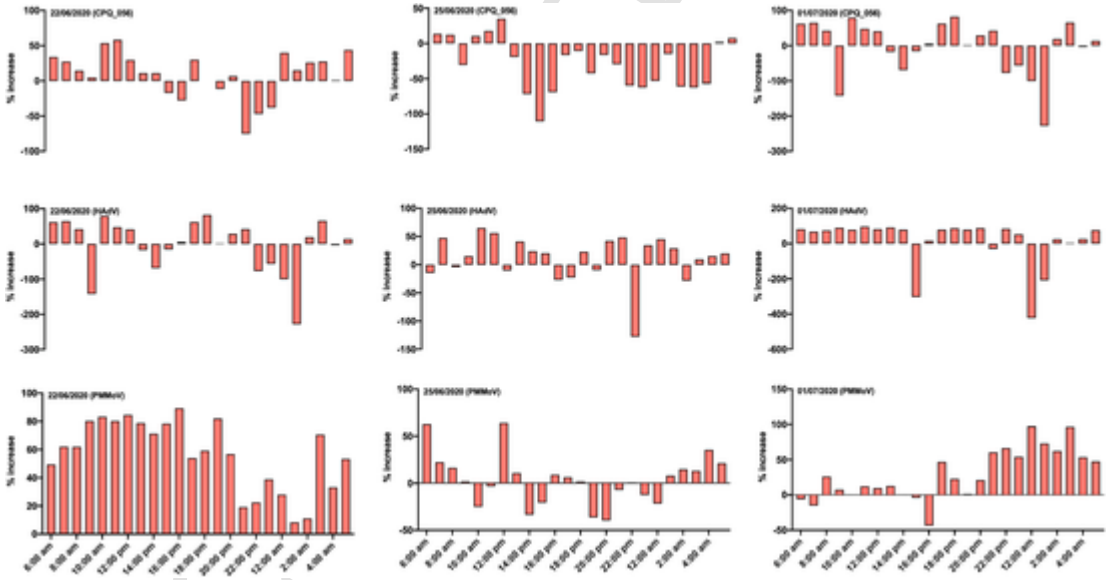

Fig. 2. Percentage increase of crAssphage, HAdV and PMMoV in 24-h composite samples compared to the 1-h composite samples.

veillance. The concentration of viruses, especially pathogenic viruses, in untreated wastewater is expected to vary throughout the day depending on a number of factors, including the infection prevalence in the community, the frequency and timing of shedding into the sewer, concentrations in the body fluids entering the wastewater stream, variations in flow due to water introduced from non-sewer sources (e.g., washing systems), and the travel time of wastewater from households to the sampling point (O'Reilly et al., 2020; Polo et al., 2020). The concentration of pathogenic viruses is also typically much lower compared to indicator viruses such as PMMoV and crAssphage (Symonds et al., 2018) necessitating collection of larger sample volumes or creation of a composite sample. The sampling strategies for virus detection in wastewater range from composite samples collected over a 24-h period (La Rosa et al., 2020b; Westhaus et al., 2020) to single grab samples collected during the peak flow (Polo et al., 2020), while other researchers collect grab wastewater samples in the morning (Randazzo et al., 2020b), and all are based on relatively small volumes (typically 50–250 mL). From the available data on the concentrations of enteric viruses and SARS-CoV-2 RNA in wastewater, it is not clear how widely the signal may vary throughout the day or whether different sampling strategies affect the outcome.

In this study, we determined the concentrations of crAssphage, HAdV and PMMoV in samples collected as 1-h composites and 24-h composite samples prepared from mixing 24 1-h samples collected from

a large urban WWTP over three sampling days in Southeast Queensland, Australia. Even though our study was conducted at a large WWTP (serving > 500,000 people), recent studies indicating no difference in concentrations of various indicators (including crAssphage and other less abundant viral indicators such as human polyomavirus) between urban and rural WWTP suggest that our findings could be extended to a wide range of sewerage systems (Korajkic et al., 2020; Mayer et al., 2018). While it would have been ideal to determine the concentrations of SARS-CoV-2 RNA in wastewater samples directly compare their loadings and variability with enteric viruses; however, the prevalence of COVID-19 in the catchment and concentrations of SARS-CoV-2 have been low resulting in many non-detects or below the limit of quantification (Ahmed et al., 2020a, 2020b, 2020c). As a result, we selected two indicator viruses and a pathogenic virus as potentially similar or greater to SARS-CoV-2 loadings in wastewater samples, had the prevalence of SARS-CoV-2 been higher in the community.

In this study, we did not perform virus concentration instead viruses were directly extracted from a small volume of wastewater. This is because the selected viruses are highly abundant in untreated wastewater throughout the world. The concentration of crAssphage, HAdV and PMMoV in wastewater would have been lower (due to loss during concentration process and variabiity of recovery) if a virus concentration method used in this study. However, this does not affect the overall results of this study.

Over the course of three sampling days, concentration of HAdV and PMMoV in 1-h composite samples varied, albeit not in the same manner (e.g., HAdV concentrations were the highest during the first sampling day, while PMMoV concentrations were the highest during the second day). On the other hand, crAssphage concentrations were similar across the sampling days. Recent report suggested using PMMoV concentrations in the wastewater as an internal reference to calibrate SARS-CoV-2 concentrations across the samples (Wu et al., 2020). While this is a promising approach that can help normalize viral targets typically present in low concentrations, such as SARS-CoV-2, caution should be exercised as concentrations of potential normalization viruses (e.g., PMMoV) may not co-vary with the desired viral pathogen target on a daily basis. Future research efforts need to examine intraday variability of SARS-CoV-2 and prospective viral calibrators to determine feasibility of this approach.

Among the three viruses analysed, the concentrations of crAssphage in the 1-h and 24-h composite samples were up to three orders of magnitude greater than HAdV and PMMoV. This is consistent with previous findings where we showed crAssphage concentration was much greater than other indicator and pathogenic viruses in wastewater in Southeast Queensland, Australia and USA. The high concentration of crAssphage in wastewater makes it a more sensitive marker gene for tracking faecal pollution in environmental waters (Hughes et al., 2016; Ahmed et al., 2019; Korajkic et al., 2020). The intraday variability of crAssphage and PMMoV concentrations were much lower than HAdV. This is because these indicator viruses are typically shed by both healthy and infected people, whereas, pathogenic viruses are only shed by those who are infected. However, the CV of PMMoV concetrations were considerably higher than the CV of crAssphage concentrations. While both indicator viruses are human-associated, crAssphage is a bacteriophage likely infecting *Bacteroides intestinalis* a commensal inhabitant of human gastrointestinal tract (Shkoporov et al., 2018) while PMMoV is a plant virus infecting peppers belonging to *Capsicum* spp. (Fauquet et al., 2005). Therefore, the concentrations of PMMoV in any sewerage system are directly dependent on dietary intake of infected pepper plants by the contributing populations which is likely to be variable throughout the day resulting in the elevated CVs. Therefore, measurements of pathogenic viruses such as HAdV or other enteric viruses such as norovirus or enterovirus, typically present in lower concentrations, are expected to be more variable throughout the day. Of note, HAdV concentrations measured in this study are comparable to those reported by others (Ahmed et al., 2019; Hamza et al., 2019; Elmahdy et al., 2020) suggesting that the greater intraday variability of HAdV compared to indicator viruses present in higher concentrations is likely not limited to this study. Assuming these viruses are indicative of the variability in SARS-CoV-2, the results indicate that collecting a grab sample at a pre-determined time may be inferior to a 24-h composite sample when seeking to achieve sensitive detection of low levels of enteric viruses and SARS-CoV-2 RNA in wastewater and avoid false negatives.

A recent study recommended the collection of grab sample during the peak hour of flow may be a good approach for surveillance of SARS-CoV-2 in wastewater (Polo et al., 2020). However, the travel time of wastewater from households to the WWTP in some catchments can typically range from between 2 and 12 h or greater in some cases, and therefore, it may be difficult to determine when is the best time to collect a grab sample (Castigliono et al., 2013). Furthermore, it is unclear if collecting a sample during the predicted peak hour of toilet usage will capture the strongest signal or result in a more diluted signal (Michael-Kordatou et al., 2020; WRF, 2020). Another consideration are the combined sewerage systems where wastewater is considerably diluted during the heavy rainfalls or periods of snow fall and snow melt. For example, during combined sewer overflow (CSO) events, the contribution of rainwater to wastewater can range from 5 to 40% (Passerat et al., 2011), whereas the seasonal dilution factor during the snowmelt period can be as high ~6900 (Madoux-Humery et al., 2013). These circumstances present unusual case scenarios that merit further consideration when selecting the best sampling approach for a given wastewater system.

In this study, the concentrations of all three viruses were greater in 54.1–79.2% in 24-h composite samples when compared with 1-h composite samples, suggesting that composite samples may be preferable over a sample taken at a pre-determined time when the concentration of the target virus (es) is expected to be low due to low shedding in the community. The longer the composite sampling durations (i.e., 24-h or 72-h), the more representative the sample will be, however, shorter durations such as 2-h, 4-h and 6-h sampling over 24-h and 72-h period may also be equally representative when the travel time of wastewater from households to the WWTP is shorter. It is important to note that we had observed significant differences in concentrations between the 1-h and 24-h composite samples for PMMoV and HAdV, suggesting that the two sample types are not interchangeable within the study or across the studies.

One drawback of composite sampling is that setting up auto samplers can be expensive and the collection system needs to provide cool storage conditions (refrigeration or filled with ice) to maintain the integrity of the sample. However, recent studies suggested longer persistence of SARS-CoV-2 RNA in water and wastewater even at 15–20 °C. (Ahmed et al., 2020a, 2020b, 2020c; Bivins et al., 2020). It should be noted that, results obtained in this study were based on wastewater samples from a large urban WWTP with specific catchment characteristics, and different results may be observed for wastewater systems with significantly different catchment characteristics. The results presented in this study also reflect a scenario over a short duration (i.e., three days) and do not include seasonal and weather variations. Therefore, it would be useful to undertake similar studies for a range of different catchments, seasons and under variable weather (dry vs. wet) and climatic conditions (sub-tropical vs. temperate). Further studies should focus on comparing time-based vs. flow-based composite sampling and how that influences the results for wastewater surveillance of the SARS-CoV-2 signal. Also, the appropriate volume of composite samples that should be analysed to avoid false-negative detection should be

considered carefully especially when the prevalence of the target virus is low.

Representative wastewater sampling along with effective concentration and sensitive detection methods are needed for successful application of WBE at the community level. Our results based on two indicator viruses (PMMoV and crAssphage) and a pathogenic virus (HAdV) indicate that there is intra- and inter-day variability of concentrations, which is especially noticeable with targets present in lower concentrations (e.g., HAdV). Therefore, in this study, time-based 24-h composite sample typically yielded greater concentrations of viruses especially HAdV and PMMoV compared to the 1-h composite samples. Extrapolating our findings to SARS-CoV-2 RNA in wastewater, the 24-h composite samples are likely to be superior to single grab samples or the 1-h composite samples, and would facilitate more sensitive detection of the virus. However, this assertion will need to be further examined.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Credit author statement

Warish Ahmed - Formal analysis. Aaron Bivins - Formal analysis. Paul M. Bertsch - Study design. Kyle Bibby - Writing. Pradip Gyawali - Formal analysis. Samendra P. Sherchan – Formal analysis. Stuart L. Simpson - Writing and design. Kevin V. Thomas - Formal analysis. Rory Verhagen - Sampling. Masaaki Kitajima - Writing. Jochen F. Mueller - Study design and Sampling. Asja Korajkic- Formal analysis

Disclaimers

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