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Decay of SARS-CoV-2 and surrogate murine hepatitis virus RNA in untreated wastewater to inform application in wastewater-based epidemiology

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ABSTRACT

Wastewater-based epidemiology (WBE) demonstrates potential for COVID-19 community transmission monitoring; however, data on the stability of SARS-CoV-2 RNA in wastewater are needed to interpret WBE results. The decay rates of RNA from SARS-CoV-2 and a potential surrogate, murine hepatitis virus (MHV), were investigated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in untreated wastewater, autoclaved wastewater, and dechlorinated tap water stored at 4, 15, 25, and 37 °C. Temperature, followed by matrix type, most greatly influenced SARS-CoV-2 RNA first-order decay rates (k). The average $T_{90}$ (time required for 1-log reduction) of SARS-CoV-2 RNA ranged from 8.04 to 27.8 days in untreated wastewater, 5.71 to 43.2 days in autoclaved wastewater, and 9.40 to 58.6 days in tap water. The average $T_{90}$ for RNA of MHV at 4 to 37 °C ranged from 7.44 to 56.6 days in untreated wastewater, 5.58–43.1 days in autoclaved wastewater, and 10.9 to 43.9 days in tap water. There was no statistically significant difference between RNA decay of SARS-CoV-2 and MHV; thus, MHV is suggested as a suitable persistence surrogate. Decay rate constants for all temperatures were comparable across all matrices for both viral RNAs, except in untreated wastewater for SARS-CoV-2, which showed less sensitivity to elevated temperatures. Therefore, SARS-CoV-2 RNA is likely to persist long enough in untreated wastewater to permit reliable detection for WBE application.

1. Introduction

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an enveloped positive-sense RNA virus belonging to the genus Betacoronavirus in the family Coronaviridae (Coronaviridae Study Group of the International

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Variable controlling microbial decay and can impact the persistence of vi
atitude of SARS-CoV-2 RNA in untreated wastewater following exposure by an infected individual (Hart and Halden, 2020). Quantifying the decay rate of SARS-CoV-2 RNA is important for WBE because decay in the RNA signal between the time of excretion in feces and the time of sampling from the wastewater collection system may lead to systematic bias in subsequent estimates relevant to public health. Additionally, wastewater sample storage and handling conditions prior to analysis could also create bias in viral RNA quantification.

Considering the rigorous biosafety requirements necessary for working with infectious SARS-CoV-2, surrogate viruses, such as murine hepatitis virus (MHV), are useful for assessing method performance and for quality assurance and control during monitoring campaigns (Ahmed et al., 2020b). Surrogate viruses are also useful for assessing decay characteristics under various environmental conditions (Casanova et al., 2009; Ye et al., 2016; De Carvalho et al., 2017). MHV belongs to the same genus as SARS-CoV-2 and is structurally and morphologically similar (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020). However, direct comparisons of MHV and SARS-CoV-2 decay characteristics have not been made. This information is critical to assess the utility of MHV to be used as a persistence surrogate for SARS-CoV-2 in wastewater and other matrices.

Temperature has been identified as a significant environmental variable controlling microbial decay and can impact the persistence of viruses in environmental systems. Increased decay rates are typically observed at temperatures >20 °C, compared to those <15 °C (Korajkic et al., 2019; Muirhead et al., 2020). However, while prolonged viral persistence at lower temperatures has been observed for non-enveloped viruses, it is unclear if enveloped viruses, including SARS-CoV-2, persist in a similar manner. For example, infectious viruses and/or the nucleic acids of somatic and F+ coliphages (Lee and Sobsey, 2011; Sokolova et al., 2012), as well as coxsackievirus B5, echovirus 6 and noroviruses all persisted longer at lower temperatures (Lo et al., 1976; Wait and Sobsey, 2001; Skraber et al., 2007; Ngaosa et al., 2008). The existing data suggest that SARS-CoV-2 surrogates, such as MHV and transmissible gastroenteritis virus can remain infectious for up to seven days when seeded in pasteurized settled sewage, up to 22 days in deionised water at 25 °C, and 4 weeks or longer in these matrices at lower temperatures (<4 °C) (Casanova et al., 2009). Similarly, feline infectious peritonitis virus (FIPV) and human coronavirus (CoV) 229E were found to also remain infectious significantly longer in tap water at 4 °C compared to 23 °C (Gundy et al., 2009). For SARS-CoV, the etiologic agent of the SARS epidemic that occurred in 2003, it was found to remain infectious for 14 days in wastewater and 17 days in feces and urine at 4 °C, but less than 2 days in hospital wastewater, domestic sewage and tap water at 20 °C (Wang et al., 2005). Thus, while data suggest that the stability of enveloped viruses is negatively affected by elevated temperatures, observations for SARS-CoV-2 RNA have not yet been published and may significantly impact interpretation of WBE surveillance data.

Biotic interactions, such as predation by bacterivorous protozoa, are a known cause of bacterial decay in many different environments, including wastewater (Korajkic et al., 2019). Considerably less is known about the direct (ingestion) or indirect (adsorption) effect of protozoa on viruses. Some controlled, laboratory feeding studies demonstrated uptake of various bacteriophages (Pinheiro et al., 2007; Deng et al., 2014) and adenvirus by predatory protozoa (Scheid and Schwarzenberger, 2012). Others have observed more rapid decay of bacteriophages (Wanjugu et al., 2016; Booncharoen et al., 2018), adenviruses (Rigotto et al., 2001; McMinn et al., 2020), poliovirus (Rachmadi et al., 2016) and echovirus 11 (Olive et al., 2020) when seeded in unprocessed water than in filtered and/or autoclaved ambient water, suggesting that microbial communities may influence viral decay rates. Compared to temperature, even less is known about the effect of protozoan predation on enveloped viruses such as SARS-CoV-2.

Much of what is currently surmised concerning decay of SARS-CoV-2 in various aquatic matrices is derived from studies of other human CoV or viral surrogates. Gundy et al. (2009) determined the survival of human CoV 229E and FIPV in water and wastewater using plaque assay or median tissue culture infectious dose (TCID50) techniques. The survival of both human CoV 229E and FIPV showed similar patterns which was highly dependent on water temperature, level of organic matter, and biological activity. In tap water, the T99 (99% or 2-log10 reduction) of both human CoV 229E and FIPV were lower at 23 °C (7–9 days) than 4 °C (87 days). The inactivation rates of these viruses were greater in filtered tap water compared to unfiltered tap water at 23 °C, suggesting increased protection and survival in the presence of organic matter and suspended solids. CoVs were inactivated rapidly in wastewater, with T99 values of 3 days (Gundy et al., 2009). Another study compared the decay of enveloped viruses (i.e. MHV and 46 bacteriophage) to non-enveloped viruses (i.e., MS2 and T3 bacteriophages) and found that enveloped viruses generally decayed faster in both pasteurized and untreated wastewater. However, the effect of sample pasteurization, and by extension the presence of microbial communities, could not be determined because experiments were terminated before 90% of the enveloped viruses were inactivated (Ye et al., 2016).

There are critical gaps in our understanding of the persistence of enveloped viruses, and in particular, SARS-CoV-2. The objective of this study was to compare the decay characteristics of SARS-CoV-2 and MHV RNA using reverse transcription quantitative PCR (RT-qPCR), with attention to the effect of: (i) the water matrix (untreated wastewater, autoclaved wastewater and dechlorinated tap water); (ii) environmentally relevant temperatures (4, 15, 25, and 37 °C); and, (iii) wastewater microbiota (comparison of untreated wastewater and autoclaved wastewater). To achieve this, microcosms containing each of the three matrices were seeded with SARS-CoV-2 (gamma-irradiated) and MHV, and incubated at the four temperatures in the dark and sampled over the course of 33 days.

2. Materials and methods

2.1. Sources of SARS-CoV-2 and MHV

A working stock of SARS-CoV-2 hCoV-19/Australia/VIC01/2020 isolated by the Victorian Infectious Diseases Reference Laboratory (VIDRL) was provided by the Australian Centre for Disease Preparedness (ACDP) after gamma irradiation (Cobalt-60 using an MDS Nordion Irradiator) with 50 kGy or 5 Mrad dose. Gamma irradiation was necessary to eliminate the risk of infection during handling of SARS-CoV-2 in a biological safety level 2 (BSL-2) laboratory. The MHV stock was obtained from 20 fecal samples of naturally infected mice collected from a snake farm. The presence of MHV in the mouse fecal samples was determined using an RT-qPCR assay (further details provided below). Each MHV-positive fecal sample (approximately 250 mg) was suspended into 1 mL of phosphate buffered saline (1 × PBS) and then the samples were pooled together to produce a homogeneous fecal slurry. The slurry was centrifuged at 1000 × g for 10 min. The pellet was discarded, and the supernatant was stored at −80 °C for 3 days. This supernatant, containing MHV, is referred to as the MHV suspension. RNA was extracted from 15-μL aliquots of gamma-irradiated SARS-CoV-2 and the MHV suspension using the QiAamp Viral RNA Mini Kit (Qiagen,
Valencia, CA, USA) according to the manufacturer’s instructions. The concentrations of gamma-irradiated SARS-CoV-2 and MHV seeding stocks were determined using the SARS-CoV-2 CDC N1 and MHV RT-qPCR assays, respectively (see below for detailed methodologies).

2.2. Wastewater sample preparation

A sample of untreated wastewater (2 L) was collected from a wastewater treatment plant (WWTP) in Brisbane, Australia and transported to the laboratory on ice. The WWTP receives domestic waste water from approximately 325,000 people, and also includes industrial and hospital wastewaters. While stormwater also enters the WWTP, the study catchment did not receive any precipitation during the 24 h period prior to the wastewater sample collection. The wastewater sample was ported to the laboratory on ice. The WWTP receives domestic waste water from approximately 325,000 people, and also includes industrial and hospital wastewaters. While stormwater also enters the WWTP, the study catchment did not receive any precipitation during the 24 h period prior to the wastewater sample collection. The wastewater sample was stored at 4 °C for 24 h before setting up the microcosm experiment. The collected wastewater sample was RT-qPCR negative for both SARS-CoV-2 and MHV as determined according to the methods described elsewhere (Ahmed et al., 2020a, 2020b).

2.3. Microcosm set up, treatments, and sampling

The microcosms were established in 15-mL sterile conical tubes. Each microcosm contained 4.99 mL of one of the three water matrices: (i) untreated wastewater (indigenous microbiota present), (ii) autoclaved (121 °C for 30 min under 15 psi of pressure) wastewater (indigenous microbiota inactivated), or (iii) dechlorinated tap water. All microcosm waters were seeded with 5×10^6 (indigenous microbiota inactivated), or (iii) dechlorinated tap water. All microcosm waters were seeded with 5×10^6 copies of SARS-CoV-2 genome (e.g., divided by 2) (Bustin et al., 2009).

2.4. Viral RNA extraction

Viral RNA was extracted from each replicate microcosm subsample (140 μL aliquot). Briefly, a final volume of 60 μL of RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). All RNA samples were stored at −80 °C and subjected to RT-qPCR analysis within one day of RNA extraction.

2.5. RT-qPCR analyses

RT-qPCR assays with hydrolysis probes were used to quantify the RNA of SARS-CoV-2 and MHV at each time point from each microcosm (Besselen et al., 2020a; US CDC, 2020). The primers and probe sequences, and qPCR cycling parameters, are shown in Table 1. All RT-qPCR assays were performed in 25-μL reaction mixtures using the QIAGEN OneStep RT-PCR Kit (Qiagen). CDC N1 RT-qPCR mixtures contained 5 μL of RT-PCR buffer (5×), 2019-nCoV Kit (500 nM of forward primer, 500 nM of reverse primer and 125 nM of probe) (Catalogue No. 10006606), 50 ng/μL of bovine serum albumin, 1 μL of RT-PCR Enzyme Mix, 1 μL of dNTP Mix and 3 μL of template RNA. The MHV RT-qPCR mixture contained 5 μL of RT-PCR buffer (5×), 300 nM of forward primer, 300 nM of reverse primer, 400 nM of probe, 1 μL of RT-PCR Enzyme Mix, 1 μL of dNTP Mix, and 3 μL of template RNA. The RT-qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories). All RT-qPCR reactions were performed in triplicate. For each RT-qPCR run, a series of three positive (SARS-CoV-2 hCoV-19/Australia/VIC01/2020) and no template (RNase-free water) controls were included.

Baseline thresholds (75 for SARS-CoV-2 and 150 for MHV) were selected manually for all RT-qPCR data generated in this study. For RT-qPCR assays, 2019-nCoV_N plasmid control (Catalogue No. 10006625) and a double-stranded DNA gene fragment containing 108 bp MHV assay amplicon, were purchased from Integrated DNA Technologies (Coralville, IA, USA) and used to generate the standard curves. Both CDC N1 and MHV standard dilutions ranged from 1 × 10^3 to 1 copy/μL. RT-qPCR data were generated using master standard curves reported in our recent studies (Ahmed et al., 2020a, 2020b). For each assay in this study, the assay limit of detection (ALOD), defined as the minimum copy number with a 95% probability of detection, was determined as previously described (Verbyla et al., 2016). The mean SARS-CoV-2 and MHV copy numbers were corrected for the difference between the double-stranded standard curve material and the single-stranded virus genome (e.g., divided by 2).

2.6. Effects of matrix on RT-qPCR amplification

The effects of untreated and autoclaved wastewater samples on RT-qPCR amplifications of SARS-CoV-2 and MHV were determined by comparing the Cq values obtained for wastewater samples to the tap water samples (i.e., reference point). A sample was considered to have PCR inhibitors (i.e., matrix interference) when Cq value was >2 compared to the reference Cq from tap water. The variation in Cq values or matrix effects on SARS-CoV-2 and MHV quantification was calculated using an equation described elsewhere (Livak and Schmittgen, 2001).

2.7. Quality control

To minimize qPCR contamination, RNA extraction and RT-qPCR analysis were performed in separate laboratories. A reagent blank was also included during nucleic acid extraction to detect contamination during extraction. All method and reagent blanks were negative for SARS-CoV-2 and MHV RNA.

2.8. Decay rate and T90 calculations, and statistical analysis

Only SARS-CoV-2 and MHV RNA concentrations (GC/mL) greater than the ALOD for each treatment (water types and various temperatures) were used to calculate the decay rate. Observed RNA concentrations were linearized using the natural log (ln)-transformation of the normalized concentrations as shown in equation (1) (Chick, 1998). These values and their associated time points (i.e., days) were used to calculate the first-order decay rate constants in units per day by linear regression using GraphPad Prism Version 8.3.1 (GraphPad Software, La Jolla, CA, USA). The runs test was used to evaluate the appropriateness of the linear

| Table 1 | Primers and probes and RT-qPCR cycling parameters used in this study. | Cycling parameters | Reference |
|---------|------------------------------------------------------------------|-------------------|-----------|
| SARS-CoV-2 CDC N1 | F-GAC CCC AAA ATC AGG GAA AT | 50 °C for 10 min for RT; 95 °C for 5 min and 45 cycles of 95 °C for 10 s, 55 °C for 30 s. | US CDC (2020) |
| MHV | F-GGA AC TCT TGT TGG GCA TTA TAC T | 50 °C for 10 min for RT; 95 °C for 5 min and 45 cycles of 95 °C for 15 s, 60 °C for 1 min. | Besselen et al. (2002) |
model and fit was assessed by \( r^2 \) and root mean square error (RMSE). \( r^2 \) assesses the proportion of the variance explained by the independent variable given the selected model, while the RMSE assesses fit by measuring the distance of the observed measures from the fitted line.

\[
\ln\left( \frac{C}{C_0} \right) = -kt
\]

(1)

The first-order decay rate constant and the associated 95% confidence interval were estimated by linear regression, where \( C \) and \( C_0 \) are the concentrations of GC in the microcosms at time \( t \) and time 0, respectively, and \( k \) is the decay rate constant. The time required to achieve a 90% (one log) reduction (\( T_{90} \)) was calculated using equation (2).

\[
T_{90} = -\frac{\ln(0.1)}{k}
\]

(2)

After mean \( k \) values were estimated at each temperature for each matrix, the values were \( \log_{10} \) transformed and linear regression was used to characterize the relationship between temperature and first-order decay rate constant within each matrix. The fit of the regression was assessed by means of \( r^2 \) values and RMSE as previously described. GraphPad Prism was also used to perform two-way ANOVA with Tukey’s multiple comparison tests to evaluate the effect of treatment factors (matrix type and temperature) as well as paired \( t \)-test to compare the decay of SARS-CoV-2 and MHV RNA (\( \alpha = 0.05 \) for both tests).

3. Results

3.1. RT-qPCR assays performance characteristics, assay limit of detection (ALOD) and matrix inhibition

The master standard curves used in this study were within the prescribed range of Minimum Information for Publication of Quantitative (MIQE) real-time PCR guidelines (Bustin et al., 2009), and the parameters have been reported in our recent studies (Ahmed et al., 2020b, 2020c). The ALOD (i.e., lowest copy number detectable) was 3 copies/reaction for both the CDC N1 and MHV assays. The mean Cq value for untreated wastewater and autoclaved wastewater did not increase more than two Cq values from the tap water reference point at day 0, indicating that the wastewater matrices had negligible inhibitory effects on RT-qPCR quantification of SARS-CoV-2 and MHV RNA (Table 2) (Dalman et al., 2012).

3.2. Estimated first-order decay rates

The temperature of the microcosms was monitored throughout the study with temperature data loggers (HOBO) and remained stable throughout the duration of the experiment (mean and standard deviations of 4.0 ± 0.2 °C, 15.0 ± 0.1 °C, and 25.0 ± 0.2 °C and 37.0 ± 0.2 °C). The mean concentrations of SARS-CoV-2 and MHV RNA in the studied microcosms at day 0 ranged from 6.03 ± 0.20 and 5.84 ± 0.13 log_{10} GC/mL, respectively. The declining concentrations of SARS-CoV-2 and MHV RNA in wastewater and tap water microcosms over 33 days at 4, 15, 25 and 37 °C are shown in Fig. 1. For SARS-CoV-2 RNA in untreated wastewater, mean first-order decay rate constants (\( k \)) ranged from 0.084/day at 4 °C to 0.286/day at 37 °C with \( r^2 \) values from 0.71 to 0.87 (Table 3). In autoclaved wastewater, first order decay rates ranged from 0.054/day at 4 °C to 0.405/day at 37 °C (\( r^2 \) values of 0.85–0.95) and were less than the constants for untreated wastewater at all temperatures except 37 °C. For MHV RNA, decay rate constants in untreated wastewater (0.042/day at 4 °C to 0.311/day at 37 °C) were less than those observed for autoclaved wastewater (0.053/day at 4 °C to 0.412/day at 37 °C). However, decay rate constants in untreated wastewater at the mid-temperatures (0.082/day at 15 °C to 0.135/day at 25 °C) were greater than those observed for autoclaved wastewater (0.068/day at 15 °C to 0.132/day at 25 °C). Estimated first-order decay rate constants for tap water are also summarized in Table 3. Considering all temperature treatments, the average SARS-CoV-2 RNA \( T_{90} \) (time required for 1 log_{10} reduction) ranged from 8.04 to 27.8 days in untreated wastewater, 5.71 to 43.2 days in autoclaved wastewater, and 9.40 to 58.6 days in tap water. The averaged \( T_{90} \) for MHV RNA at 4 to 37 °C ranged from 7.44 to 56.6 in untreated wastewater, 5.58 to 43.1 in autoclaved wastewater, and 10.9 to 43.9 days in tap water.

Regardless of matrix, there was no statistically significant difference between decay characteristics of SARS-CoV-2 and MHV (\( p \) value range: 0.239–0.391). For both viruses, temperature was the most significant environmental variable contributing to the observed variation in the dataset (>80%), followed by matrix (~5%) (Table 4). Interaction between variables, while significant, was observed infrequently (<10%), suggesting the effect of temperature is rarely dependent on the matrix (Table 4). Irrespective of the virus type and matrix (Table 4), there was no statistically significant difference in decay at lower temperatures (4 vs 15 °C), but the decay at higher temperatures (25 and 37 °C) was significantly faster compared to the lower temperatures. When comparing decay between matrices at the same temperatures, there was no significant difference for MHV at 4 °C, but SARS-CoV-2 RNA decayed faster in untreated wastewater compared to the tap water (\( p = 0.0136 \)). A similar outcome was observed for both viruses at 15 °C (\( p \) value range: 0.0113 - <0.0001), but not 25 °C (Table 4). Lastly, at 37 °C, RNA from both viruses decayed significantly faster in autoclaved wastewater compared to both untreated wastewater and tap water (\( p < 0.0001 \)) and in untreated wastewater compared to tap water (\( p \) value range: 0.0264 - <0.0001) (Table 4).

3.3. Temperature and first-order decay rates

Log_{10} transformed mean decay rates for both SARS-CoV-2 and MHV RNA within each matrix at temperatures observed in the study are shown in Fig. 2. Equations describing the linear regression fit for each virus and matrix are summarized in Table 5. The fit of each linear model was reasonable with \( r^2 \) values ranging from 0.953 to 0.696 and root
Fig. 1. Mean decay curves of SARS-CoV-2 and MHV RNA over time (days) in untreated wastewater, autoclaved wastewater and tap water microcosms. The measurements were linearized premised on first-order decay, in which the natural log (ln)-transformed measured concentration at each time point was divided by the concentration at time zero. In some instances, the error bars (SD) are too small to illustrate.
| Matrix            | Temperatures (°C) | SARS-CoV-2 k (mean ± SD) [95% CI slope] | SARS-CoV-2 $r^2$ | RMSE | Runs test | $T_{90}$ days (mean ± SD) |  | MHV k (mean ± SD) [95% CI slope] | MHV $r^2$ | RMSE | Runs test | $T_{90}$ days (mean ± SD) |
|-------------------|-------------------|----------------------------------------|-----------------|------|-----------|------------------------|---|--------------------------|-----------|------|-----------|------------------------|
| Untreated wastewater | 4                 | 0.084 ± 0.013 [0.103 to 0.064]         | 0.79            | 0.37 | NS p = 0.33 | 27.8 ± 4.45 [22.4 to 50.1] |  | 0.042 ± 0.009 [0.058 to 0.026] | 0.80      | 0.30 | NS p = 0.10 | 56.6 ± 14.2 [39.7 to 88.6] |
|                   | 15                | 0.114 ± 0.012 [0.144 to 0.083]         | 0.71            | 0.59 | NS p = 0.33 | 20.4 ± 2.13 [16.0 to 27.7] |  | 0.082 ± 0.011 [0.094 to 0.069] | 0.95      | 0.24 | NS p = 0.13 | 28.5 ± 4.43 [24.5 to 33.4] |
|                   | 25                | 0.183 ± 0.008 [0.219 to 0.149]         | 0.87            | 0.67 | NS p = 0.33 | 12.6 ± 0.59 [10.5 to 15.5] |  | 0.135 ± 0.019 [0.158 to 0.111] | 0.94      | 0.45 | NS p = 0.10 | 17.3 ± 2.46 [14.6 to 20.7] |
|                   | 37                | 0.286 ± 0.008 [0.370 to 0.202]         | 0.74            | 1.10 | NS p = 0.20 | 8.04 ± 0.23 [6.22 to 11.4] |  | 0.311 ± 0.026 [0.371 to 0.250] | 0.90      | 0.79 | NS p = 0.40 | 7.44 ± 0.61 [6.20 to 9.21] |
|                    |                   |                                        |                 |      |            |                       |   |                                        |           |      |            |                       |
| Autoclaved wastewater | 4                 | 0.054 ± 0.007 [0.077 to 0.030]         | 0.95            | 0.14 | NS p = 0.90 | 43.2 ± 5.95 [29.9 to 76.7] |  | 0.053 ± 0.005 [0.062 to 0.046] | 0.95      | 0.14 | NS p = 0.40 | 43.1 ± 4.02 [37.1 to 50.1] |
|                   | 15                | 0.077 ± 0.006 [0.094 to 0.060]         | 0.85            | 0.32 | NS p = 0.30 | 29.9 ± 2.39 [24.5 to 38.4] |  | 0.068 ± 0.004 [0.091 to 0.044] | 0.82      | 0.44 | NS p = 0.33 | 33.9 ± 1.97 [25.3 to 52.3] |
|                   | 25                | 0.171 ± 0.011 [0.196 to 0.146]         | 0.93            | 0.48 | NS p = 0.33 | 13.5 ± 0.85 [11.8 to 15.8] |  | 0.132 ± 0.019 [0.153 to 0.110] | 0.94      | 0.41 | NS p = 0.30 | 17.6 ± 2.46 [15.1 to 20.9] |
|                   | 37                | 0.405 ± 0.035 [0.477 to 0.333]         | 0.94            | 0.59 | NS p = 0.33 | 5.71 ± 0.50 [4.82 to 6.91] |  | 0.412 ± 0.018 [0.539 to 0.291] | 0.84      | 1.00 | NS p = 0.50 | 5.58 ± 0.25 [4.27 to 7.91] |
| Tap water          | 4                 | 0.039 ± 0.006 [0.049 to 0.030]         | 0.83            | 0.17 | NS p = 0.33 | 58.6 ± 8.68 [47.0 to 76.7] |  | 0.052 ± 0.005 [0.060 to 0.044] | 0.94      | 0.15 | NS p = 0.40 | 43.9 ± 4.17 [38.4 to 52.3] |
|                   | 15                | 0.045 ± 0.004 [0.062 to 0.027]         | 0.28            | 0.33 | NS p = 0.13 | 51.2 ± 4.54 [37.1 to 85.3] |  | 0.033 ± 0.006 [0.037 to 0.028] | 0.96      | 0.07 | NS p = 0.70 | 71.2 ± 12.3 [62.2 to 82.2] |
|                   | 25                | 0.151 ± 0.001 [0.187 to 0.116]         | 0.78            | 0.68 | NS p = 0.13 | 15.2 ± 0.45 [12.3 to 19.9] |  | 0.124 ± 0.006 [0.160 to 0.087] | 0.86      | 0.69 | NS p = 0.13 | 18.6 ± 0.98 [14.4 to 26.5] |
|                   | 37                | 0.245 ± 0.001 [0.289 to 0.200]         | 0.88            | 0.86 | NS p = 0.33 | 9.40 ± 0.03 [7.96 to 11.5] |  | 0.212 ± 0.013 [0.238 to −0.186] | 0.96      | 0.50 | NS p = 0.40 | 10.9 ± 0.68 [9.67 to 12.4] |

NS: Non significant deviation from the model; SD: standard deviation.
mean square error (RMSE) values from 0.051 to 0.178. As expected, the log_{10} transformed first-order decay constant (k) per day, for SARS-CoV-2 and MHV RNA in untreated wastewater, autoclaved wastewater, and tap water. The linear equations, r^2, and RMSE values reported are from linear regressions of the log_{10}-transformed mean k values observed at four temperatures in each matrix during the study.

4. Discussion

WBE is gaining momentum as a potentially valuable management tool in efforts to detect and respond with appropriate public health interventions to keep COVID-19 infection prevalence within a manageable threshold for health systems. Many research teams have reported the application of this tool to detect SARS-CoV-2 RNA in wastewater, with the intent to monitor the prevalence of COVID-19 and provide early warning detection of infection resurgence of the virus in communities. There is a need to understand the persistence of SARS-CoV-2 and its nucleic acid in water and wastewater environments to properly interpret SARS-CoV-2 RNA measurements from wastewater collection systems (Kitajima et al., 2020; La Rosa et al., 2020b; Silverman and Boehm, 2020).

The decay of SARS-CoV-2 and MHV RNA in wastewater was measured at
four environmentally relevant temperatures covering cold, temperate, sub-tropical, and tropical latitudes using RT-qPCR. In addition to untreated wastewater, autoclaved wastewater (i.e., reduced biological activity) and dechlorinated tap water (free of enzymatic activity) were included to determine the impacts of variable matrices on the persistence of SARS-CoV-2 and MHV RNA. High concentrations of gamma-irradiated SARS-CoV-2 and MHV were seeded into these three wastewater matrices so that at least 2 to 3 log reductions could be monitored over the 33-day monitoring period. Virus concentration was not performed; instead, the RNA of both viruses was directly extracted from the test samples at multiple time points over the course of the study. Importantly, RT-PCR inhibition for either the SARS-CoV-2 or MHV assays was not observed during the study. This is probably because RNA was directly extracted from a small volume (140 μL) of wastewater samples. A recent study investigating the decay of enveloped Zika virus RNA in wastewater using RT-qPCR also reported the absence of PCR inhibition in diluted and undiluted wastewater samples (Mairhead et al., 2020). However, when virus is concentrated from a large volume of wastewater (>100 mL) collected from different WWTPs, inhibitors may affect RT-qPCR amplifications (Schrader et al., 2012). MHV particles were also seeded because the impacts of gamma-irradiation on the integrity of SARS-CoV-2 particles were unknown and gamma-irradiated SARS-CoV-2 will seldom ever be excreted by infected individuals. The inclusion of MHV also enabled comparison of the decay patterns between gamma-irradiated SARS-CoV-2 (human) and non-gamma-irradiated MHV (animal) coronaviruses.

In this study, MHV and SARS-CoV-2 RNA showed comparable persistence. RNA from both viruses in untreated wastewater and autoclaved wastewater persisted for 18 to 25 days at 37 °C. This is particularly important for WBE as there is limited knowledge on the persistence of SARS-CoV-2 RNA in wastewater. Our results suggest that temperatures as high as 37 °C are not likely to affect the detection of SARS-CoV-2 RNA at hydraulic retention times typical of wastewater collection systems, i.e. <24 h transit time to a sample collection point (i.e., WWTP influent). Further, the results indicate that samples should not be retained at these higher temperatures; rather they should be stored and transported to the location for analyses in a chilled state (e.g. refrigerated). This may be especially important for WBE in tropical and warmer sub-tropical environments. Fortunately, the prolonged persistence of both SARS-CoV-2 and MHV RNA at 4 and 15 °C suggests that wastewater samples can be stored at 4 °C without significant degradation for a considerable length of time. This is particularly important for laboratories where resources and throughput are limited, and sample storage is unavoidable.

The first-order decay rate constant for each matrix increased with increasing temperature for both SARS-CoV-2 and MHV RNA. Across all matrices included in the study, temperature was the most significant driver of decay followed by matrix type. Nevertheless, the decay rate of SARS-CoV-2 RNA in untreated wastewater was less sensitive to increases in temperature than MHV and SARS-CoV-2 RNA in other matrices. A significant difference in first-order decay rate constants between MHV and SARS-CoV-2 was only identified in untreated wastewater, which indicates that gamma radiation may possibly influence decay rates in biologically active matrices. The viral state (i.e., infectivity) of SARS-CoV-2 in feces is currently uncertain; while the presence of infectious SARS-CoV-2 in feces has previously been reported (Xiao et al., 2020), findings from other studies indicate the majority of virus is inactivated prior to excretion (Wöllel et al., 2020). As a compliment to the current study, it would be ideal to use intact SARS-CoV-2 as the seeding material, however, this could not be done due to high biological activity level (BSL-3) requirements for working with infectious SARS-CoV-2. Therefore, gamma-irradiated SARS-CoV-2 was used in our study. Gamma irradiation is known to preserve the integrity of viral morphology and protein structure (Elliot et al., 1982), and does not affect PCR results at excessive radiation up to 5 Mrads used in this study (Lowy, 2005; Leung et al., 2020). Future research is needed to better understand the SARS-CoV-2 RNA decay using gamma-irradiated versus intact SARS-CoV-2.

Despite the inactivation of indigenous microbiota, the observed decay for MHV RNA was slightly faster in autoclaved wastewater than untreated wastewater at 4 and 37 °C. For SARS-CoV-2 RNA, decay at 37 °C was also faster in autoclaved wastewater than untreated wastewater. It is possible that RNases, which are not inactivated by autoclaving (Green and Sambrook, 2019), were released into solution by cell lysis and that these enzymes contributed to increased RNA decay compared to untreated wastewater. It is also possible that the RNA decay rates of both viruses were increased due to other changes in the physicochemical characteristics of the wastewater resulting from autoclaving (Jones and Prasad, 1968).

The findings from the current study suggest that RNA from SARS-CoV-2 and its surrogate MHV decay slowly in both wastewater and dechlorinated tap water samples held at common environmental temperatures under controlled laboratory conditions. Since the conditions in this study may not be representative of the environmental conditions (i.e., diurnal temperature variation) in all wastewater collection systems, future decay studies that employ in situ diffusion chambers or dialysis bags are needed to confirm the decay rates identified in this study (Boehm et al., 2019). The presence of biofilms in the sewerage system may also increase the persistence of viruses (Skraber et al., 2007). Additionally, owing to methodological constraints, SARS-CoV-2 and MHV were seeded at RNA concentrations of approximately 6-log_{10} GC/mL. In wastewater collection systems, much lower concentrations are likely, and observed decay parameters might differ. Thus, additional studies are needed to explore how biofilms and lower concentrations may affect the SARS-CoV-2 RNA decay in wastewater systems (Choi et al., 2020).

Nevertheless, the current study demonstrates that the SARS-CoV-2 RNA signal is unlikely to degrade significantly at the time scales and temperatures typical of most wastewater collection systems, nor decay if stored at 4 °C after collection. The similar decay of MHV RNA identifies it as a suitable persistence surrogate for SARS-CoV-2. Future studies will need to identify if the RNA of MHV is concentrated with efficiencies similar to SARS-CoV-2. Also, while not imperative for WBE, an increased understanding of SARS-CoV-2 infectivity in feces and wastewater collection systems would enhance our understanding about possible transmission routes. The slow decay of SARS-CoV-2 RNA supports its detection and quantification as a viable surveillance tool to monitor the presence of COVID-19 in the community.

### Disclaimers

The views expressed in this article are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. The U.S. Environmental Protection Agency through the Office of Research and Development provided technical direction but did not collect, generate, evaluate, or use the environmental data described herein.

### Author Statement

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### 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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