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PII: S0166-0934(22)00193-8

DOI: <https://doi.org/10.1016/j.jviromet.2022.114645>

Reference: VIRMET114645

To appear in: *Journal of Virological Methods*

Received date: 13 October 2022

Accepted date: 31 October 2022

Please cite this article as: Brian R. McMinn, Asja Korajkic, Adin C. Pemberton, Julie Kelleher, Warish Ahmed, Eric N. Villegas and Kevin Oshima, Assessment of Two Volumetrically Different Concentration Approaches to Improve Sensitivities for SARS-CoV-2 Detection During Wastewater Monitoring, *Journal of Virological Methods*, (2022)
doi:<https://doi.org/10.1016/j.jviromet.2022.114645>

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Assessment of Two Volumetrically Different Concentration Approaches to Improve Sensitivities for SARS-CoV-2 Detection During Wastewater Monitoring

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Abstract

Wastewater monitoring for severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), the virus responsible for the global coronavirus disease 2019 (COVID-19) pandemic, has highlighted the need for methodologies capable of assessing viral prevalence during periods of low population infection. To address this need, two volumetrically different, methodologically similar concentration approaches were compared for their abilities to detect viral nucleic acid and infectious SARS-CoV-2 signal from primary influent samples. For Method 1, 2L of SARS-CoV-2 seeded wastewater was evaluated using a dead-end hollow fiber ultrafilter (D-HFUF) for primary concentration, followed by the CP Select™ for secondary concentration. For Method 2, 100mL of SARS-CoV-2 seeded wastewater was evaluated using the CP Select™ procedure. Following D-HFUF concentration (Method 1), significantly lower levels of infectious SARS-CoV-2 were lost (*P* value range: 0.0398 to 0.0027) compared to viral gene copy (GC) levels detected by the US Centers for Disease Control (CDC) N1 and N2 reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) assays. Subsamples at different steps in the concentration process were also taken to better characterize the losses of SARS-CoV-2 during the concentration process. During the centrifugation step (prior to CP Select™ concentration), significantly higher losses (*P* value range: 0.0003 to <0.0001) occurred for SARS-CoV-2 GC levels compared to infectious virus for Method 1, while between the methods, significantly higher infectious viral losses were observed for Method 2 (*P* = 0.0002). When analyzing overall recovery of endogenous SARS-CoV-2 in wastewater samples, application of Method 1 improved assay sensitivities (*P*= <0.0001) compared with Method 2; this was especially evident during periods of lower COVID-19 case rates within the sewershed. This study describes a method which can successfully concentrate infectious SARS-CoV-2 and viral RNA from wastewater. Moreover, we demonstrated that large volume wastewater concentration provides additional sensitivity needed to improve SARS-CoV-2 detection, especially during low levels of community disease prevalence.

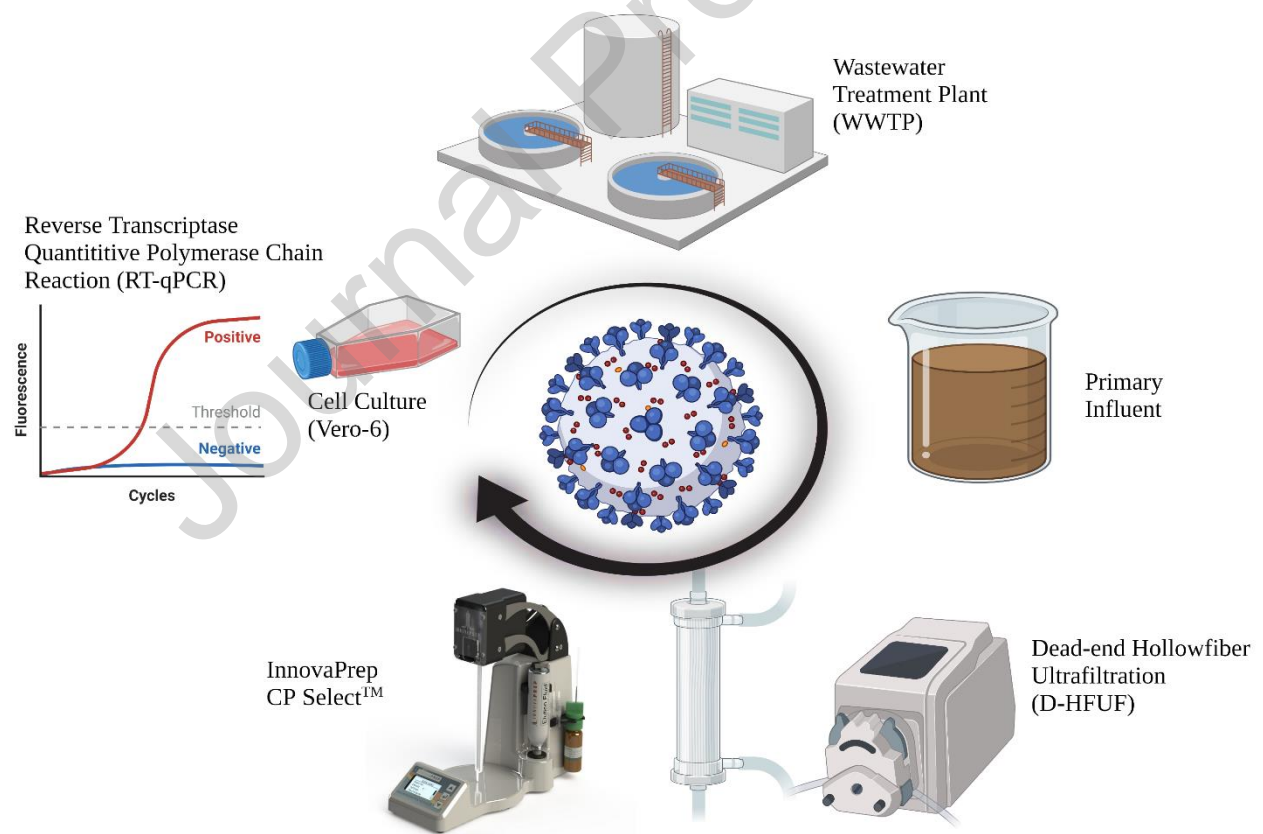
Keywords

Wastewater, SARS-CoV-2, Environmental Sampling, qPCR, Large Volume, Ultrafiltration

Funding Source

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. All work presented in this manuscript was funded by the United States Environmental Protection Agency.

Graphical Abstract



1. Introduction

SARS-CoV-2, the viral agent responsible for the current COVID-19 global pandemic, has spread to every country of the world and is responsible for 6.5 million deaths worldwide, one million in the United States alone as of August of 2022 (Oxford, 2021). While significant attention has been given to establishing human infection rates of SARS-CoV-2 through individual testing of mostly symptomatic patients, only more recently has a focus been placed on population-level monitoring via wastewater. Monitoring of wastewater, recently described as wastewater-based epidemiology (WBE) as a possible population screening tool has gained attention (Ahmed et al., 2020a; Ai et al., 2021). The advantage of wastewater screening is that both symptomatic and asymptomatic individuals (shedding virus particles through their stool) are represented within sanitary waste collection systems (Farkas et al., 2020). Wastewater analysis could act as a non-invasive way to assess community level infection rates to provide more timely and accurate assessments of viral presence and spread.

Estimating levels of circulating SARS-CoV-2 through wastewater analysis has inherent limitations with current virus detection methodologies, particularly with regards to analytical volumes processed (Ahmed et al., 2021b; Ai et al., 2021; Cuevas-Ferrando et al., 2021; Gonzalez et al., 2020). Current methodologies used to concentrate and detect SARS-CoV-2 have, by necessity, been developed quickly to analyze wastewater samples ranging from 50-250 mL, while avoiding the time constraints and difficulties associated with large volume wastewater concentration approaches (Ahmed et al., 2021b; Ai et al., 2021; Randazzo et al., 2020). While small volume wastewater samples can be informative during high community infection rates, these volumes can limit the ability to use wastewater to estimate circulating levels of virus, especially during early stages of disease outbreaks. In addition, pathogenic viruses are often found in wastewater at low densities necessitating the concentration of larger volumes of

wastewater (1-100L) to achieve detectable virus levels (Korajkic et al., 2022). As this pandemic evolves or future pandemics occur, the ability to detect low levels of circulating viruses and estimate infection rates within communities, especially during early stages of an outbreak, is important and improvements in method sensitivity will be critical to detect early onset of disease outbreaks for more effective implementation of appropriate remediation to minimize and thwart disease spread.

An easy to use, quick, field-deployable concentration method that has the flexibility to effectively monitor emerging pathogens and/or pandemic related microorganisms (viruses, bacteria, protozoa, etc.) is needed. Ideally, the method will be capable of large volume processing allowing increased sensitivity, while being amenable to both molecular and culture-based analyses to better monitor or address future pandemic related microorganisms. Technologies such as hollow-fiber ultrafilters (HFUF), repurposed medical dialysis filters, have been utilized for this application and have shown effectiveness in recovery of diverse microorganisms of interest from a variety of liquid matrices (Cuevas-Ferrando et al., 2021; Liu et al., 2012; McMinn et al., 2017; Morales-Morales et al., 2003). Upon sample concentration, washing of captured microorganisms from the ultrafilter surfaces is performed using a mild surfactant solution which allows for recovery of both viable and non-viable microorganisms present (Hill et al., 2005; Korajkic et al., 2021). Methods capable of relaying infectious virus status are critical to understanding the risk to public health, establishing the fate and transport of viral pathogens like SARS-CoV-2 (and viral variants) through wastewater, and determining viral persistence. Once developed, these methodologies could play a major role in efforts to better inform public health officials in the United States and abroad for addressing the COVID-19

pandemic, while also providing necessary tools to address future pandemics (Kitajima et al., 2020).

In this study, we evaluated and compared two methods, 1) a large volume method (D-HFUF) capable of analyzing large volumes (2L) of wastewater, and 2) a small volume (100mL) method (CP Select) also using HFUF technology. For the first series of experiments, laboratory cultured stocks of SARS-CoV-2 were seeded into autoclaved primary influent to assess the ability of the D-HFUF and the CP Select for recovery of both infectious SARS-CoV-2 and gene copies levels of SARS-CoV-2 RNA from wastewater. We documented viral losses through each concentration process to aid in identifying areas where these concentration methodologies could be improved. For the second series of experiments, we applied the methods (D-HFUF and CP Select) to concentrate endogenous levels of SARS-CoV-2 in wastewater samples to determine if larger volume analysis results in improved assay sensitivity.

2. Materials and Methods

2.1 SARS-CoV-2 Stock Preparation

Vero-6 cells (Cat. #ATCC CRL 1586, American Type Culture Collection, Manassas, VA) were grown to confluency in 75cm², filter-capped flasks (ThermoFisher Scientific, Waltham, MA) and incubated at 37°C; 5% CO₂. Each 75cm² flask contained 30mL of cell maintenance media; Dulbecco's Modified Eagles Medium (DMEM), with high glucose with L-glutamine (ThermoFisher Scientific); along with 10% fetal bovine serum (FBS) (ThermoFisher Scientific). Prior to infection, used maintenance media was removed and cell monolayers were washed with 10mL of Dulbecco's Phosphate Buffered Saline (DPBS) (ThermoFisher Scientific). To acquire

high levels of infectious SARS-CoV-2 (Washington State strain, USA/WA1/2020), the virus was propagated as described below. Briefly, 1mL of frozen SARS-CoV-2 (10^5 plaque forming units/100mL (PFU/100mL) were thawed and diluted in 20 mL of sterile maintenance media. Three milliliter volumes of SARS-CoV-2 viral dilutions were added to 4-75cm² flasks of 100% confluent Vero-6 monolayers and allowed to incubate for 1 h at 37°C; 5% CO₂. Thirty milliliters of fresh maintenance media were added, and flasks were incubated for an additional 72 h at 37°C, 5% CO₂. Following the 72 h incubation, flasks were subjected to two freeze/thaw cycles at -80°C and at room temperature, respectively. Cell debris was pelleted by centrifugation at $4,500 \times g$ for 10 minutes. The resulting supernatant was collected and syringe filtered through a 0.22µm filter (ThermoFisher Scientific) prior to aliquoting into 1mL volumes in cryovials. Viral titers were assessed from filtered supernatant using both RT-qPCR and plaque assay as described below. Viral titers of 1×10^6 infectious particles/mL were aliquoted and used for seeding experiments in this study. Virus seeding stocks were stored at -80°C until use.

2.2 Infectious SARS-CoV-2 Detection

Levels of infectious SARS-CoV-2 were measured according to Mendoza et al., 2020 (Mendoza et al., 2020). Briefly, 6-well plates (Corning, Corning, NY) were seeded with 4×10^5 Vero-6 cells per well and incubated at 37°C at 5% CO₂ for 3 days for 90% confluent cell monolayer formation. Once confluent, the maintenance media was removed, and cell monolayers were washed with 1mL of DPBS. Following the wash step, the monolayers were inoculated with 100µl SARS-CoV-2 seeding suspension or wastewater concentrate, or 1:10 dilutions of SARS-CoV-2 seeding suspensions, with all virus inoculums run in triplicate. Following addition of virus inoculum, plates were allowed to incubate for 1 h at 37°C; 5% CO₂. During the 1-h incubation, plates were gently rocked by hand every 15 min to facilitate viral

attachment/adsorption. Following the 1-h infection, wells were overlaid with a liquid overlay media (LOM), consisting of a 3mL of a 1:1 mixture of 3% carboxymethylcellulose and overlay diluent (2× Minimum Essential Media [MEM] (500mL), 10% FBS, 10mL of 200mM L-glutamine, 7.5mL of 100× sodium bicarbonate, and 10mL of 100× nonessential amino acids (ThermoFisher Scientific) were added to each plate well and allowed to incubate for 72 h at 37°C; 5% CO₂. Following the 72 h incubation, the liquid overlay was removed, and cell monolayers were washed with 1mL of DPBS. Cell monolayers were then fixed by adding 1mL of 4% paraformaldehyde (ThermoFisher Scientific) and incubated for 1 h at room temperature. Cell monolayers were then stained with 0.5% crystal violet solution (1g crystal violet dissolved in 20mL absolute ethanol and 80mL sterile DI water; ThermoFisher Scientific) and incubated for 15 min at room temperature. Crystal violet was then removed from cells through three washes with 1mL of DI water and allowed to dry. The resulting viral plaques were enumerated and reported as PFU/100mL.

2.3 Wastewater Collection and Treatment

Using a sterile 10L carboy, primary wastewater influent samples (following primary settling and screening) were collected (from August through October) from an urban wastewater treatment (WWTP) facility located within the metropolitan sewerage district of Cincinnati, Ohio. This facility processes between 55-96 million gallons (MGD) of waste/day, serving a population of 237,000 residents (<https://www.msdc.org/>). Wastewater samples were transported immediately (on ice) to the US Environmental Protection Agency (USEPA) laboratories located in Cincinnati, Ohio for processing. Upon arrival at the laboratory, wastewater samples were either autoclaved for 60 min at 121°C to inactivate any SARS-CoV-2 present (SARS-CoV-2 seeding and recovery experimentation) or were left untreated and transferred to a Biosafety

Level-3 (BSL-3) laboratory for assessing levels of endogenous SARS-CoV-2. In a previous study, sample pre-treatment (autoclaving) manipulation did not alter the recovery of enveloped viruses compared to un-autoclaved wastewater (Ahmed et al., 2020b). All wastewater samples were stored overnight at 4°C prior to experimentation the following day. No SARS-CoV-2 gene copies or infectious particles) were detected in the autoclaved wastewater samples.

2.4 Concentration Method 1

Autoclaved wastewater aliquots (2L) were measured using a graduated cylinder and transferred into a sterile 2.5L Erlenmeyer flask containing a stir bar. To each 2L wastewater aliquot, 1 mL of SARS-CoV-2 seed (1×10^6 infectious particles/mL) was added and allowed to mix for 3 min prior to concentration. Dead-end hollow-fiber ultrafiltration (D-HFUF) concentration was used to concentrate 2L volumes of wastewater using 15S Asahi Kasei Rexeed ultrafilters (Dial Medical Supply, Chester Springs, PA) and a peristaltic pump (Masterflex L/S Easy Load, Cole Parmer, Vernon Hills, IL) set at 300 rpm (1.5L/min). Following sample filtration, filters were eluted following procedures detailed in McMinn et al., 2022 (McMinn et al., 2021).

Filter eluates were centrifuged ($12,000 \times g$ for 30 min) to remove particulates prior to secondary concentration. For secondary sample concentration, the Concentrating Pipette Select™ (CP Select™) (InnovaPrep, Drexel, MO) was used to reduce the approximate 100mL volumes of primary filter eluate or primary influent to volumes more applicable to molecular analyses (eluate volume ranged between 670-932 μ l). Using InnovaPrep's CP Select™ Wastewater Application settings, primary filter eluates were passed through the concentrating pipette tip (CPT) ultrafilter (InnovaPrep) and upon sample filtration ultrafilter tips were eluted (2

times) using a hand-driven syringe elution procedure previously described (McMinn et al., 2021).

2.5 Concentration Method 2

For seeding and recovery experimentation, a 2L sample of autoclaved primary influent was seeded with 1mL of SARS-CoV-2 and was allowed to mix for 3 min at room temperature prior to aliquoting 100mL volumes for further processing. For experiments targeting endogenous SARS-CoV-2, 100mL volumes of primary influent were analyzed directly. Prior to CP Select concentration, all wastewater samples were centrifuged at $12,000 \times g$ for 30 min to remove particulates. All samples were then processed as described above using the InnovaPrep CP Select™.

2.6 Determining SARS-CoV-2 Loss During Concentration Procedures

Throughout both primary and secondary concentration, subsamples were collected (400µl for culture and 200µl for nucleic acid extraction) to determine SARS-CoV-2 losses at each step of the concentration procedure. For Method 1, subsamples were taken: 1) from the 2L of autoclaved wastewater following the addition of SARS-CoV-2 seed and mixing for 3 min, 2) from the filter eluate (post D-HFUF concentration), and 3) following centrifugation of the eluate but prior to CP Select™ secondary concentration. For Method 2, subsamples were taken: 1) from the 100mL autoclaved wastewater following the addition of SARS-CoV-2 seed and mixing for 3 minutes, and 2) following centrifugation of the 100mL virus seeded wastewater. Both Method 1 and Method 2 were evaluated for their ability to concentrate endogenous SARS-CoV-2 from unamended primary influent samples collected as described above. Percent of virus losses were calculated using the following formula: $SC/S \times 100 = PR$ here: SC = SARS-CoV-2 concentrations in sample filtrate/eluate, S = SARS-CoV-2 concentrations in seeded material from

prior concentration step (i.e., levels of SARS-CoV-2 in seeded sewage prior to D-HFUF concentration), PR = percent recovery. The percent loss (L) was calculated as follows; $L = 100 - PR$ SARS-CoV-2 genomic copies or infectious virus.

2.7 Endogenous SARS-CoV-2 Detection

Primary influent wastewater samples were collected and were immediately transferred to a BSL-3 facility (without autoclaving) and processed using the methods described above (Method 1 and Method 2). Endogenous levels of SARS-CoV-2 resulting from the two methods were quantified and then compared to COVID-19 case numbers (corresponding to the sample collection dates) for the sewershed, retrieved from Ohio Department of Health (ODH) COVID-19 Dashboard which displayed confirmed case counts by symptom onset date (Ohio).

2.8 Nucleic Acid Extractions and RT-qPCR

Viral RNA was extracted from 200 μ l portions of each seed, subsample, and sample concentrate using a Qiagen All Prep PowerViral Kit (Qiagen, Valencia, CA) according to manufacturer's instructions, resulting in 100 μ l extract volumes. All sample extracts were analyzed in triplicate unless otherwise noted. One extraction blank (EB), including all reagents but no nucleic acid RNA template, was included with each extraction batch. Purified RNA extracts were stored at -80°C no longer than 30 days. SARS-CoV-2 RNA was quantified using a one-step quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) using the U.S. Center for Disease Control and Prevention N1 and N2 primers sets (Control, 2021). N1 forward primer (2019-nCoV_N1-F) GAC CCC AAA ATC AGC GAA AT, TaqMan probe (2019-nCoV_N1-P) FAM-/ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1, and reverse primer

(2019-nCoV_N1-R) TCT GGT TAC TGC CAG TTG AAT CTG, and N2 forward primer (2019-nCoV_N2-F) TTA CAA ACA TTG GCC GCA AA, TaqMan probe (2019-nCoV_N2-P) FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1, and reverse primer (2019-nCoV_N2-R) GCG CGA CAT TCC GAA GAA, were used to detect SARS-CoV-2 RNA. Viral RNA present was amplified using the RNA UltraSense™ One-Step Quantitative RT-PCR System (Applied Biosystems, Foster City, CA) following manufacturer's instructions. Each reaction consisted of; 1.25µl enzyme mix, 5µL of 5× reaction mix, 2.5µL BSA fraction V (Sigma Aldrich), 0.05µL ROX, 3.0µL RT-PCR master mix (1µM primer and 80nM probe), 2µL template, and 11.2µL nuclease-free water, for a total reaction volume of 25µL. One step RT-qPCR reaction conditions were as follows: 50.0°C for 15 min, 95.0°C for 2 min, 3 cycles at 95.0°C for 15 s followed by 60.0°C for 30 s. All sample RT-qPCR TaqMan assays were performed in triplicate using a QuantStudio3 real-time qPCR system with threshold manually set to 0.03 (Applied Biosystems). Potential inhibition was assessed by comparing cycle thresholds (Ct) of undiluted and samples diluted 10-fold in sterile AE buffer (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0), which is expected to delay the detection of the target by 3.3 Ct, as $2^{3.3} = 10$, assuming 100% assay amplification efficiency. Data acceptance thresholds of ± 1.0 Ct were selected based on the expectation that a Ct difference between an uninhibited sample and a 10-fold diluted sample be no more than one Ct if assuming 0.5 cycle natural variability (pipetting errors, nucleic acid losses through adhering to equipment surfaces, low level sample inhibition, etc.) between qPCR sample replicates (Cao et al., 2012). Six no template controls (NTCs) were included with each instrument run.

2.9 Standard curve preparation

Armored RNA Quant® SARS-CoV-2 (Asuragen, Austin, TX) (1×10^{11} copies/mL) was extracted as described above and used as a reference RNA standard. The resulting RNA extract was

quantified by RT-qPCR using the most probable number (MPN) approach (McMinn, Korajkic, and Grimm, 2016). Briefly, utilizing a ten-fold decimal dilution series starting with the undiluted extract (five replicates each) GC present were quantified for each dilution series with dilutions spanning 10 to 10^6 copies per $2\mu\text{l}$. All dilutions were prepared using sterile AE buffer for use as calibration standards. A master standard curve was created based on five individual standard curves for each assay (N1 and N2 RT-qPCR assays), resulting slope and y-intercepts were averaged between the five replicate standard curves and this data was then used for quantifying GC of SARS-CoV-2 present in samples during the study. Average slope, R^2 , y-intercept, and amplification values were -3.32 ± 0.05 , 0.99 ± 0.00 , 36.39 ± 0.72 and 1.03 ± 0.00 for N1 and -3.23 ± 0.25 , 0.98 ± 0.01 , 35.19 ± 1.09 and 1.03 ± 0.00 for N2, respectively. N1 and N2 assay lower limit of quantification (LLOQ) was identified as an average of all 10 copy standards and equaled 34.279 and 33.519, Ct respectively, during this analysis. A total of 48 individual negative control reactions (combination of EBs and NTCs) were below the LLOQ, indicating absence of extraneous RNA contamination.

2.10 Data statistical analyses

Percent recovery data were \log_{10} arcsine square root transformed prior to statistical analyses. Paired t-tests and one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests (GraphPad Prism 8.3.1, GraphPad Software, La Jolla, CA, USA) were used to determine effectiveness of each step of the concentration procedure. Levels of infectious SARS-CoV-2 virus were compared to the N1 and N2 RT-qPCR signals. Performance of Method 1 was then compared to Method 2 ($\alpha = 0.05$ for all tests).

3. Results

3.1 Sample Inhibition Analysis

To assess the presence of inhibitory substances, all subsamples and sample concentrates were screened for inhibition of the RT-qPCR reactions. Deviations between undiluted and 10-fold diluted samples ranged from -0.52 to 0.65 Ct for the N1 qPCR primer set and from -0.11 to 0.57 Ct for the N2 RT-qPCR primer set. No notable evidence of sample inhibition (difference of ± 1.0 Ct between undiluted and diluted sample) was observed for any subsample or sample concentrate analyzed using the N1 and N2 RT-qPCR assays.

3.2 Infectious Viral Particles vs. Viral RT-qPCR GC

To compare SARS-CoV-2 infectious particles with the GC signal (N1 and N2 qPCR), paired samples of SARS-CoV-2 seeding mixture, sub-samples, and sample concentrates were analyzed. Irrespective of seeding mixture, subsample, or sample concentrate measured between culture and RT-qPCR, the N1 and N2 gene copies detected by RT-qPCR were nearly 4- \log_{10} or higher in concentration (average viral PFU/mL vs N1 and N2 gene copies/mL). Overall average N1 GC signals throughout the study were $3.72 \pm 0.38 \log_{10}$ greater than SARS-CoV-2 infectious signals, while the N2 RT-qPCR signal averaged $3.69 \pm 0.35 \log_{10}$ greater. The largest differences in signal concentrations occurred during the final elution step (following CP Select™ concentration) where RT-qPCR assay signal concentrations averaged $4.15 \pm 0.64 \log_{10}$ and $4.16 \pm 0.47 \log_{10}$ higher than the infectious signal for the US CDC N1 and N2 assays, respectively. Overall, concentrations for both N1 and N2 assays (regardless of concentration step analyzed) were found to be significantly higher than infectious SARS-CoV-2 concentrations ($P \leq 0.0001$) assessed through experimentation, while there were no statistically significant differences between the two RT-qPCR assays.

3.3 SARS-CoV-2 Loss at the Primary Concentration Step of Method 1

For Method 1, 1mL of SARS-CoV-2 seed was inoculated and mixed into 2L of autoclaved primary treated wastewater and concentrated using D-HFUF. SARS-CoV-2 losses were assessed following addition of the virus seed to the treated wastewater prior to D-HFUF concentration. Initial losses of the SARS-CoV-2 virus seeds prior to D-HFUF filtration were $81.1\pm 6.4\%$ for culture and, $73.4\pm 18.5\%$ and $65.6\pm 13.6\%$ for the N1 and N2 RT-qPCR assays, respectively. Losses of SARS-CoV-2 virus seeds following D-HFUF concentration averaged $82.2\pm 10.9\%$, $50.7\pm 9.6\%$, and $47.7\pm 7.5\%$ for culturable SARS-CoV-2, N1, and N2 RT-qPCR assays, respectively. Overall, significantly higher losses were observed for culture-based SARS-CoV-2 (P value range: 0.0398 to 0.0027), as opposed to either N1 or N2 RT-qPCR assays through D-HFUF concentration.

3.4 SARS-CoV-2 Loss During Centrifugation

Following D-HFUF concentration for Method 1 and prior to direct analysis of seeded wastewater for Method 2, filter eluates and seeded wastewater samples were centrifuged to remove particulate matter prior to the InnovaPrep CP Select™ concentration step (InnovaPrep, 2021). Assessment of SARS-CoV-2 losses resulting from centrifugation of samples were measured from subsamples collected prior to and post centrifugation (as described in the Materials and Method section). Infectious SARS-CoV-2 losses during centrifugation for filter eluates (Method 1), ranged from 0-13% (Fig 1). The observed losses from the same filter eluates analyzed using the N1 and N2 RT-qPCR assays ranged from 30-35% and 32-47%, respectively which were significantly higher compared with infectious virus losses (P value range: 0.0003 to

<0.0001). For seeded wastewater samples (100mL) that were processed using Method 2, infectious SARS-CoV-2 losses during centrifugation ranged from 16-84%, while observed losses using the N1 or N2 RT-qPCR assays ranged from 23-63% and 18-67%, respectively. There were no significant differences in centrifugation losses of infectious virus compared to GC levels of virus signal observed during Method 2. Infectious SARS-CoV-2 was lost at significantly higher levels ($P = 0.0002$) during centrifugation in Method 2 compared to Method 1 (Figure 1). No other significant differences in losses were observed between the two methods and analytical techniques used during centrifugation.

3.5 SARS-CoV-2 Loss During CP Select™ Concentration

SARS-CoV-2 viral losses were assessed during CP Select™ concentration under two different conditions (Fig 1). Losses were either assessed from Method 1 filter eluates (post centrifugation) or were determined from seeded wastewater samples for Method 2 (post centrifugation). For Method 1 filter eluates, losses through CP Select™ concentration for infectious SARS-CoV-2 averaged $8.8 \pm 0.8\%$, while N1 and N2 RT-qPCR signal losses averaged $30.8 \pm 0.7\%$ and $37.8 \pm 6.8\%$, respectively. There were no statistically significant differences in losses occurring between infectious SARS-CoV-2 and SARS-CoV-2 GC signal. For Method 2, infectious SARS-CoV-2 virus signal was lost at an average of $67.6 \pm 13.4\%$, while losses for the N1 and N2 assays averaged $42.3 \pm 8\%$ and $46.0 \pm 6.1\%$, respectively. There were no statistically significant differences in losses occurring between infectious SARS-CoV-2 and for either molecular marker during this stage of concentration.

3.6 Overall Recovery of SARS-CoV-2 Through Method 1 and Method 2 Concentration

Final cumulative methodological recoveries of infectious virus, GC N1 and N2 signals for SARS-CoV-2 seeded primary influent during Method 1 averaged 15%, 23%, and 21%, respectively. For Method 2, overall final methodological recoveries of infectious SARS-CoV-2 averaged 18%, while GC levels were recovered at an average of 27% for N1 and 32% for N2. Additionally, the overall concentration factor (beginning sample volume through final RNA extract volume) for Method 1 was 10000:1, while for Method 2 it was 1000:1.

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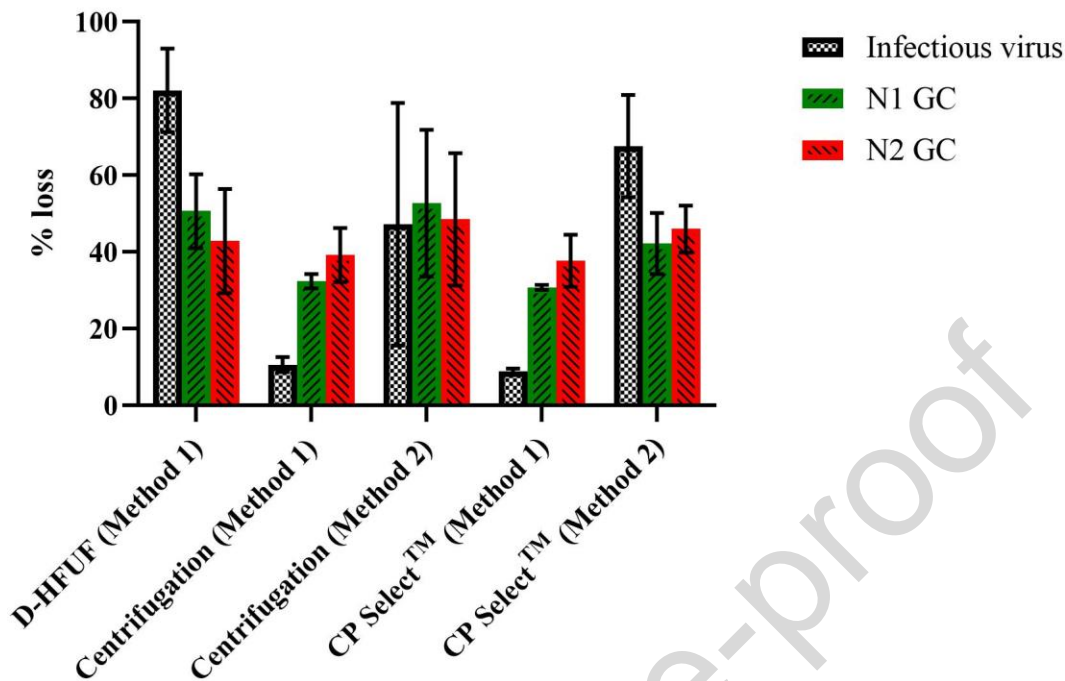


Figure 1. SARS-CoV-2 Infectious and GC Signal Losses During Method 1 and Method 2 Concentration

3.7 Endogenous SARS-CoV-2 Detection

To compare the performance of Method 1 (D-HFUF and CP Select™) to that of Method 2 (CP Select™) in a real-world wastewater surveillance effort (targeting endogenous levels of SARS-CoV-2), unamended wastewater samples were concentrated using each method and viral concentrations recovered were then compared. Wastewater sampling occurred during two different timepoints to evaluate each concentration method for their ability to detect fluctuating levels of endogenous SARS-CoV-2. For the first sampling timepoint, where community infection rates were the most prevalent (18.9 confirmed COVID-19 cases within sewershed), Method 1 detected $3.29 \pm 0.08 \log_{10}$ and $2.90 \pm 0.04 \log_{10}$ GC/100mL of SARS-CoV-2 using the N1 and N2 RT-qPCR assays, respectively (Table 1). During the same sampling timepoint, Method 2 detected $2.29 \pm 0.08 \log_{10}$ and $2.20 \pm 0.11 \log_{10}$ GC/100mL for N1 and N2 RT-qPCR

signals, respectively which were significantly lower ($P < 0.0001$) than concentrations determined using Method 1. During the second sampling event, during which time community infection rates were lower (12.1 confirmed COVID-19 cases within sewershed), Method 1 detected 2.94 ± 0.08 and $2.64 \pm 0.14 \log_{10}$ GC/100mL for N1 and N2 RT-qPCR signals, respectively. By contrast, either no N1 and N2 RT-qPCR signals were detected or the concentrations fell below assay LLOQ when Method 2 was used. Infectious endogenous SARS-CoV-2 viruses were also not detected (analytical volumes equal to 500mL for Method 1 and 50mL for Method 2) in any of the unamended wastewater samples tested.

Table 1. Endogenous SARS-CoV-2 Levels in Primary Influent

| Sample Time Point | Infection Rate ^a | Method | Target | Replicate | Concentration ^b | Average±Standard Deviation |
|-------------------|-----------------------------|--------|------------|-----------|----------------------------|----------------------------|
| 1 | 18.9 | 1 | N1 | 1 | 3.33 | 3.29±0.08 |
| | | | | 2 | 3.20 | |
| | | | | 3 | 3.33 | |
| | | 2 | N2 | 1 | 2.93 | 2.90±0.04 |
| | | | | 2 | 2.86 | |
| | | | | 3 | 2.92 | |
| | | 1 | N1 | 1 | 2.24 | 2.29±0.08 |
| | | | | 2 | 2.38 | |
| | | | | 3 | 2.26 | |
| 2 | N2 | 1 | 2.27 | 2.20±0.11 | | |
| | | 2 | 2.07 | | | |
| | | 3 | 2.25 | | | |
| 2 | 12.1 | 1 | N1 | 1 | 2.85 | 2.94±0.08 |
| | | | | 2 | 3.01 | |
| | | | | 3 | 2.95 | |
| | | 2 | N2 | 1 | 2.74 | 2.64±0.14 |
| | | | | 2 | 2.71 | |
| | | | | 3 | 2.48 | |
| | | 1 | N1 | 1 | Below LLOQ ^c | ND ^d |
| | | | | 2 | Below LLOQ | |
| | | | | 3 | Below LLOQ | |
| 2 | N2 | 1 | Below LLOQ | ND | | |
| | | 2 | Below LLOQ | | | |
| | | 3 | Below LLOQ | | | |

^a Confirmed COVID-19 cases within sewershed

^b Concentration reported as log₁₀ GC per 100mL

^c Lower limit of quantification (LLOQ)

^d Non-detect (ND) due to unquantifiable levels of RT-qPCR product

4. Discussion

Wastewater surveillance has shown to be a useful means to track SARS-CoV-2 RNA prevalence within a given community. However, as reported in a recent review article, methodological improvements are needed to minimize false-positive and false-negative errors to improve detection of low-level SARS-CoV-2 signal in wastewater (Ahmed et al., 2022).

Specifically, the establishment of targeted volume criteria for wastewater monitoring to provide

uniform data collection requirements is needed to ease information amalgamation between laboratories (Ahmed et al., 2020a; Gonzalez et al., 2020). To date, many existing studies monitoring wastewater for SARS-CoV-2 have used small grab samples to link wastewater virus concentrations to community level incidences of disease (Ahmed et al., 2020a; Ahmed et al., 2020d; Ahmed et al., 2021b; Randazzo et al., 2020; Venugopal et al., 2020). In some cases, virus concentrations were drawn from data at or nearing the detection limit of analytical techniques (i.e., RT-qPCR, droplet digital PCR [ddPCR]), limiting the predictive capabilities of the approach when community infection rates are low during early phases of the outbreak (Bivins et al., 2021; Ciesielski et al., 2021). To date, improved methodologies capable of providing increased sensitivities for SARS-CoV-2 detection, like analyzing larger volumes of wastewater, are limited, and still needed. Increased sample volume could improve assay sensitivities to more accurately estimate active infection rates (both symptomatic and asymptomatic), especially during early phases of disease outbreaks within a community (Gerrity et al., 2021; Kitajima et al., 2020). This is especially true for wastewater monitoring efforts in cases where viral variants and sub-variants (e.g., delta and omicron) are emerging, a critical timepoint where implementing mitigation efforts will be most effective. However, careful considerations such as choosing the appropriate method, and appropriate target volume assessments must be considered prior to sample analysis. Additionally, a concentration method must be validated to establish method performance since sensitivities to concentration processes can vary for each target pathogen.

In this study we evaluated two methods that have the potential to provide increased detection sensitivities that could be beneficial for monitoring disease incidence, especially during early phases of an outbreak. Prior to experimentation, virus stock titers revealed a nearly $4 \log_{10}$

difference in concentration of infectious SARS-CoV-2 compared with that of the N1 or N2 RT-qPCR molecular signals, a trend that continued during all steps of the method comparison experimentation. Since RT-qPCR can detect both infectious and non-infectious virions, these results are not surprising and have been observed elsewhere (Berg et al., 2021; La Scola et al., 2020; Mak et al., 2020). One of the potential explanations for the 4- \log_{10} difference in concentration between infectious SARS-CoV-2 and N1/N2 gene targets is the ability of the N1 and N2 primer sets to detect SARS-CoV-2 genomic and sub-genomic RNA (sgRNA) found in infectious and non-infectious particles (Lu et al., 2020). These RT-qPCR assays amplify two regions of the nucleocapsid gene, which are part of the viral genome that generates the most abundant sub-genomic RNA species during coronavirus replication and represents all the expressed sgRNAs by the virus (Kim et al., 2020), thus resulting in much higher sensitivities. Sender et al. also reported much higher molecular signals detected when RT-qPCR targets genomic and sub-genomic RNA as compared to theoretical total virus particles present in host cells (Sender et al., 2021).

Since wastewater is not typically used to track respiratory enveloped viruses and their interactions within and sensitivities to this matrix are not well established, we investigated virus losses upon addition of SARS-CoV-2 to autoclaved primary influent. We observed considerable SARS-CoV-2 seed losses (ranging from 66-81%) for both infectious and molecular signal. Our results support the notion that viral adsorption to wastewater particulates is likely occurring, as indicated by the initial reduction of culturable and N1/N2 RT-qPCR signals. This finding highlights important considerations when extrapolating SARS-CoV-2 wastewater-based monitoring data with active patient infection rates, where virus signal can be severely

compromised within minutes of exposure to wastewater matrices (Berg et al., 2021; Noorimotlagh et al., 2021; Tiwari et al., 2021).

Size exclusion technologies are well established concentration methodologies that have the added advantage of more readily retaining microbial target infectious status as opposed to other similarly purposed filtration methodologies (Ahmed et al., 2020c; McMinn et al., 2016; Nemudryi et al., 2020; Tiwari et al., 2021; Torii, Furumai, and Katayama, 2021). Additionally, size exclusion filtration, in a dead-end configuration, requires minimal expertise to setup and run, readily lending itself to field deployment where processing upwards of 100L of environmental water at rates of 1L/min can be achieved (Haramoto et al., 2018; McMinn et al., 2017; McMinn et al., 2021; McMinn et al., 2018). A critical step in establishing performance metrics of an effective concentration methodology is identifying sensitivities for a given viral target. This is certainly the case with SARS-CoV-2 (enveloped respiratory virus) since this virus is structurally different from enteric viral strains (non-enveloped) for which the majority of viral concentration methods were developed (Francy et al., 2013). During Method 1, significant loss of SARS-CoV-2 infectious signal was observed compared to that of the N1 and N2 RT-qPCR signals during D-HFUF concentration. Infectious virus signal sensitivities during this processing step could be due to virions being irreversibly bound to particulates within the sample, damaged during filtration/elution processes, or sensitivity to the mild surfactant used to wash filter surfaces (Katz et al., 2018; Liu et al., 2012; Morales-Morales et al., 2003). Both the D-HFUF and CP select™ have been demonstrated to be effective concentration methods for SARS-CoV-2 and its surrogates from wastewater for detection using molecular methods (Ahmed et al., 2021a; McMinn et al., 2021). Unfortunately, as the proprietary foam-based elution options supplied by InnovaPrep are not designed for recovery of infectious virus; modifications to the elution

procedure using a syringe-driven elution with the D-HFUF elution solution were developed (McMinn et al., 2021). This elution solution has been previously used to successfully recover both infectious and molecular viral targets (coliphage, beta-coronavirus OC43, enteric viruses, viral microbial source tracking (MST) markers), in a variety of matrices (Korajkic et al., 2022; Korajkic et al., 2021; McMinn et al., 2021), but its use to successfully isolate infectious SARS-CoV-2 (or other enveloped viruses) has not been demonstrated to date. In this study, infectious SARS-CoV-2 was successfully recovered using this modified elution procedure in conjunction with the D-HFUF elution solution, suggesting that this method can be used to isolate infectious viral particles, although further method refinement is needed.

Prior to CP Select™ concentration for both Method 1 and Method 2, particulate removal using centrifugation is recommended in InnovaPrep's CP Select™ Wastewater Analysis Protocol to avoid filter fouling (InnovaPrep, 2021). However, viral losses determined through removal of particulates during centrifugation were observed and significantly impacted the SARS-CoV-2 RT-qPCR signals when compared to that of infectious signal, but only following D-HFUF concentration (Method 1). Reduced viral loss in filter eluates (Method 1) could be attributed to concentrates being suspended in an elution solution, which by design, limit virus attachment to particulates and filter surfaces through use of mild surfactants (Hill et al., 2005). Wastewater monitoring efforts have identified that significant levels of SARS-CoV-2 are recovered from particulates separated during sample processing, highlighting the importance of processing both liquid and solid fractions of wastewater to assure optimal SARS-CoV-2 signal detection (Casanova et al., 2009; Farkas et al., 2018; Graham et al., 2021; Mackowiak et al., 2018). There are numerous options available for processing viral nucleic acids from solids, but unfortunately, obtaining infectious virus signal from solids still proves difficult since processes used to release

attached viruses can inadvertently result in virus inactivation (Farkas et al., 2017; Mackowiak et al., 2018). Nevertheless, the methods used in the current study were successful in recovery of seeded infectious SARS-CoV-2 virus from wastewater. Optimization of an effective SARS-CoV-2 wastewater monitoring method will need to address issues with processing particulates within samples, as they can be a significant source of the overall viral signal as well as effectively recovering infectious viruses from this matrix. Fortunately, a solids analysis (processing of retained solids post centrifugation) could be easily incorporated into each of the two concentration methods described herein.

To assess the performance of the two concentration methods under real-world wastewater surveillance scenarios, we processed unamended primary influent samples to quantify levels of endogenous SARS-CoV-2. During the first sampling event coinciding with the higher incidence of COVID-19 positive cases in the sewershed, Method 1 detected significantly higher concentrations of both N1 and N2 RT-qPCR signals, compared to Method 2. During the second sampling event with lower confirmed COVID-19 positive cases, N1 and N2 RT-qPCR signals were also detected using Method 1, but they were below the assay LLOQ for Method 2. Concentration patterns of SARS-CoV-2 detected in the tested wastewater samples paralleled the numbers of confirmed COVID-19 cases during each of the two timepoints sampled. However, extrapolation of these findings for public health relevance is difficult, as many wastewater surveillance efforts to date have attributed fluctuations in wastewater signal to patient shedding patterns, wastewater flow, or the absence of mild or asymptomatic carriers in reported public health data (Ahmed et al., 2021b; He et al., 2020; Li et al., 2021; Pan et al., 2020; Randazzo et al., 2020). While additional field studies are needed to generate similar comparative data, our results suggest sample volume could be a critical component for obtaining endogenous virus

signal during wastewater surveillance and for enhancing our ability to assess low community levels of infection more accurately.

Similar to a few existing examples in the literature (Rimoldi et al., 2020; Westhaus et al., 2021), no endogenous infectious SARS-CoV-2 was detected in the wastewater samples collected for this study. These results indicate that SARS-CoV-2 is either quickly inactivated in wastewater, irreversibly adheres to wastewater particulates, or as recent studies have suggested, infected individuals do not excrete detectable infectious virus in their stool (2001, 2001; Ahmed et al., 2020b; Albert et al., 2021; Carducci et al., 2020; Cerrada-Romero et al., 2022; Tiwari et al., 2021). Taken together, these findings suggest the need to further understand the feasibility and importance of monitoring infectious SARS-CoV-2 in environmental matrices (e.g., wastewater).

5. Conclusion

Useful insights on SARS-CoV-2 presence in populations have been collected from current wastewater monitoring efforts, but inherent limitations associated with small-volume sample analysis make it difficult to obtain data pertinent for assessing disease burden within a given community. Implementing a method capable of processing larger wastewater volumes has the potential to concentrate virus signal 20-fold over small volume methodologies. Large volume methods will be key in characterizing viral stability in environmental systems, assessing the risks of secondary exposure through contaminated waters, and ensuring current wastewater treatment processes are adequate for viral removal/inactivation. It is hoped that efforts such as those described herein will lead to the eventual identification and establishment of standardized wastewater methods allowing for more meaningful data comparisons generated from multiple geographically dispersed sewersheds. Large volume methods could enhance the generation of data during instances of low community infection (e.g., corresponding to early onset of the

disease outbreak), aiding in better estimating viral prevalence, tracking viral diversity, and recognizing geographic hotspots for implementation of disease abatement efforts.

Acknowledgments

We would like to thank Dr. Suman Pradhan of the University of Cincinnati for providing SARS-CoV-2 virus stocks, and Mr. David Feldhake of Pegasus Technical Services for providing contractual cell culture support.

Disclaimer

The United States Environmental Protection Agency through its Office of Research and Development funded and managed the research described here. It has been subjected to the Agency's administrative review and approved for publication. The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

References

- 2001, I.S. 2001. Water Quality-Detection and Enumeration of Bacteriophages-Part 4: Enumeration of bacteriophages infecting *Bacteriodes fragilis* University of Brighton.
- Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tschärke, B., Verhagen, R., Smith, W.J.M., Zaugg, J., Dierens, L., Hugenholz, P., Thomas, K.V. and Mueller, J.F., 2020a. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community. *Sci Total Environ* 728, 138764.
- Ahmed, W., Bertsch, P.M., Bibby, K., Haramoto, E., Hewitt, J., Huygens, F., Gyawali, P., Korajkic, A., Riddell, S., Sherchan, S.P., Simpson, S.L., Sirikanachana, K., Symonds, E.M., Verhagen, R., Vasani, S.S., Kitajima, M. and Bivins, A., 2020b. Decay of SARS-CoV-2 and surrogate murine hepatitis virus RNA in untreated wastewater to inform application in wastewater-based epidemiology. *Environ Res* 191, 110092.
- Ahmed, W., Bertsch, P.M., Bivins, A., Bibby, K., Farkas, K., Gathercole, A., Haramoto, E., Gyawali, P., Korajkic, A., McMinn, B.R., Mueller, J.F., Simpson, S.L., Smith, W.J.M., Symonds, E.M., Thomas, K.V., Verhagen, R. and Kitajima, M., 2020c. Comparison of

- virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. *Sci Total Environ* 739, 139960.
- Ahmed, W., Bivins, A., Bertsch, P.M., Bibby, K., Choi, P.M., Farkas, K., Gyawali, P., Hamilton, K.A., Haramoto, E., Kitajima, M., Simpson, S.L., Tandukar, S., Thomas, K. and Mueller, J.F., 2020d. Surveillance of SARS-CoV-2 RNA in wastewater: Methods optimisation and quality control are crucial for generating reliable public health information. *Curr Opin Environ Sci Health*.
- Ahmed, W., Bivins, A., Simpson, S.L., Smith, W.J.M., Metcalfe, S., McMinn, B., Symonds, E.M. and Korajkic, A., 2021a. Comparative analysis of rapid concentration methods for the recovery of SARS-CoV-2 and quantification of human enteric viruses and a sewage-associated marker gene in untreated wastewater. *Sci Total Environ* 799, 149386.
- Ahmed, W., Simpson, S.L., Bertsch, P.M., Bibby, K., Bivins, A., Blackall, L.L., Bofill-Mas, S., Bosch, A., Brandao, J., Choi, P.M., Ciesielski, M., Donner, E., D'Souza, N., Farnleitner, A.H., Gerrity, D., Gonzalez, R., Griffith, J.F., Gyawali, P., Haas, C.N., Hamilton, K.A., Hapuarachchi, H.C., Harwood, V.J., Haque, R., Jackson, G., Khan, S.J., Khan, W., Kitajima, M., Korajkic, A., La Rosa, G., Layton, B.A., Lipp, E., McLellan, S.L., McMinn, B., Medema, G., Metcalfe, S., Meijer, W.G., Mueller, J.F., Murphy, H., Naughton, C.C., Noble, R.T., Payyappat, S., Petterson, S., Pitkanen, T., Rajal, V.B., Reyneke, B., Roman, F.A., Jr., Rose, J.B., Rusinol, M., Sadowsky, M.J., Sala-Comorera, L., Setoh, Y.X., Sherchan, S.P., Sirikanchana, K., Smith, W., Steele, J.A., Sabburg, R., Symonds, E.M., Thai, P., Thomas, K.V., Tynan, J., Toze, S., Thompson, J., Whiteley, A.S., Wong, J.C.C., Sano, D., Wuertz, S., Xagorarakis, I., Zhang, Q., Zimmer-Faust, A.G. and Shanks, O.C., 2022. Minimizing errors in RT-PCR detection and quantification of SARS-CoV-2 RNA for wastewater surveillance. *Sci Total Environ* 805, 149877.
- Ahmed, W., Tschärke, B., Bertsch, P.M., Bibby, K., Bivins, A., Choi, P., Clarke, L., Dwyer, J., Edson, J., Nguyen, T.M.H., O'Brien, J.W., Simpson, S.L., Sherman, P., Thomas, K.V., Verhagen, R., Zaugg, J. and Mueller, J.F., 2021b. SARS-CoV-2 RNA monitoring in wastewater as a potential early warning system for COVID-19 transmission in the community: A temporal case study. *Sci Total Environ* 761, 144216.
- Ai, Y., Davis, A., Jones, D., Lemeshow, S., Tu, H., He, F., Ru, P., Pan, X., Bohrerova, Z. and Lee, J., 2021. Wastewater SARS-CoV-2 monitoring as a community-level COVID-19 trend tracker and variants in Ohio, United States. *Sci Total Environ* 801, 149757.
- Albert, S., Ruiz, A., Peman, J., Salavert, M. and Domingo-Calap, P., 2021. Lack of evidence for infectious SARS-CoV-2 in feces and sewage. *Eur J Clin Microbiol Infect Dis* 40, 2665-2667.
- Berg, M.G., Zhen, W., Lucic, D., Degli-Angeli, E.J., Anderson, M., Forberg, K., Olivo, A., Sheikh, F., Toolsie, D., Greninger, A.L., Cloherty, G.A., Coombs, R.W. and Berry, G.J., 2021. Development of the RealTime SARS-CoV-2 quantitative Laboratory Developed Test and correlation with viral culture as a measure of infectivity. *J Clin Virol* 143, 104945.
- Bivins, A., Kaya, D., Bibby, K., Simpson, S.L., Bustin, S.A., Shanks, O.C. and Ahmed, W., 2021. Variability in RT-qPCR assay parameters indicates unreliable SARS-CoV-2 RNA quantification for wastewater surveillance. *Water Res* 203, 117516.
- Cao, Y., Griffith, J.F., Dorevitch, S. and Weisberg, S.B., 2012. Effectiveness of qPCR permutations, internal controls and dilution as means for minimizing the impact of

- inhibition while measuring *Enterococcus* in environmental waters. *J Appl Microbiol* 113, 66-75.
- Carducci, A., Federigi, I., Liu, D., Thompson, J.R. and Verani, M., 2020. Making Waves: Coronavirus detection, presence and persistence in the water environment: State of the art and knowledge needs for public health. *Water Res* 179, 115907.
- Casanova, L., Rutala, W.A., Weber, D.J. and Sobsey, M.D., 2009. Survival of surrogate coronaviruses in water. *Water Res* 43, 1893-1898.
- Cerrada-Romero, C., Berastegui-Cabrera, J., Camacho-Martinez, P., Goikoetxea-Aguirre, J., Perez-Palacios, P., Santibanez, S., Jose Blanco-Vidal, M., Valiente, A., Alba, J., Rodriguez-Alvarez, R., Pascual, A., Oteo, J.A., Miguel Cisneros, J., Pachon, J., Casas-Flecha, I., Cordero, E., Pozo, F. and Sanchez-Cespedes, J., 2022. Excretion and viability of SARS-CoV-2 in feces and its association with the clinical outcome of COVID-19. *Sci Rep* 12, 7397.
- Ciesielski, M., Blackwood, D., Clerkin, T., Gonzalez, R., Thompson, H., Larson, A. and Noble, R., 2021. Assessing sensitivity and reproducibility of RT-ddPCR and RT-qPCR for the quantification of SARS-CoV-2 in wastewater. *J Virol Methods* 297, 114230.
- Control, C.f.D. 2021. 2019-Novel Coronavirus (2019-nCoV) Real-time RT-PCR Primers and Probes. In: National Center for Immunization and Respiratory Diseases (NCIRD), D.o.V.D. (Ed).
- Cuevas-Ferrando, E., Perez-Cataluna, A., Allende, A., Guix, S., Randazzo, W. and Sanchez, G., 2021. Recovering coronavirus from large volumes of water. *Sci Total Environ* 762, 143101.
- Farkas, K., Cooper, D.M., McDonald, J.E., Malham, S.K., de Rougemont, A. and Jones, D.L., 2018. Seasonal and spatial dynamics of enteric viruses in wastewater and in riverine and estuarine receiving waters. *Sci Total Environ* 634, 1174-1183.
- Farkas, K., Hassard, F., McDonald, J.E., Malham, S.K. and Jones, D.L., 2017. Evaluation of Molecular Methods for the Detection and Quantification of Pathogen-Derived Nucleic Acids in Sediment. *Front Microbiol* 8, 53.
- Farkas, K., Hillary, L.S., Malham, S.K., McDonald, J.E. and Jones, D.L., 2020. Wastewater and public health: the potential of wastewater surveillance for monitoring COVID-19. *Curr Opin Environ Sci Health* 17, 14-20.
- Francy, D.S., Stelzer, E.A., Brady, A.M., Huitger, C., Bushon, R.N., Ip, H.S., Ware, M.W., Villegas, E.N., Gallardo, V. and Lindquist, H.D., 2013. Comparison of filters for concentrating microbial indicators and pathogens in lake water samples. *Appl Environ Microbiol* 79, 1342-1352.
- Gerrity, D., Papp, K., Stoker, M., Sims, A. and Frehner, W., 2021. Early-pandemic wastewater surveillance of SARS-CoV-2 in Southern Nevada: Methodology, occurrence, and incidence/prevalence considerations. *Water Res X* 10, 100086.
- Gonzalez, R., Curtis, K., Bivins, A., Bibby, K., Weir, M.H., Yetka, K., Thompson, H., Keeling, D., Mitchell, J. and Gonzalez, D., 2020. COVID-19 surveillance in Southeastern Virginia using wastewater-based epidemiology. *Water Res* 186, 116296.
- Graham, K.E., Loeb, S.K., Wolfe, M.K., Catoe, D., Sinnott-Armstrong, N., Kim, S., Yamahara, K.M., Sassoubre, L.M., Mendoza Grijalva, L.M., Roldan-Hernandez, L., Langenfeld, K., Wigginton, K.R. and Boehm, A.B., 2021. SARS-CoV-2 RNA in Wastewater Settled Solids Is Associated with COVID-19 Cases in a Large Urban Sewershed. *Environ Sci Technol* 55, 488-498.

- Haramoto, E., Kitajima, M., Hata, A., Torrey, J.R., Masago, Y., Sano, D. and Katayama, H., 2018. A review on recent progress in the detection methods and prevalence of human enteric viruses in water. *Water Res* 135, 168-186.
- He, X., Lau, E.H.Y., Wu, P., Deng, X., Wang, J., Hao, X., Lau, Y.C., Wong, J.Y., Guan, Y., Tan, X., Mo, X., Chen, Y., Liao, B., Chen, W., Hu, F., Zhang, Q., Zhong, M., Wu, Y., Zhao, L., Zhang, F., Cowling, B.J., Li, F. and Leung, G.M., 2020. Temporal dynamics in viral shedding and transmissibility of COVID-19. *Nat Med* 26, 672-675.
- Hill, V.R., Polaczyk, A.L., Hahn, D., Narayanan, J., Cromeans, T.L., Roberts, J.M. and Amburgey, J.E., 2005. Development of a rapid method for simultaneous recovery of diverse microbes in drinking water by ultrafiltration with sodium polyphosphate and surfactants. *Appl Environ Microbiol* 71, 6878-84.
- InnovaPrep. 2021. Concentrating Sars-CoV-2 from Raw and Primary Wastewater Using the InnovaPrep® Concentrating Pipette™ (Revision C).
- Katz, A., Pena, S., Alimova, A., Gottlieb, P., Xu, M. and Block, K.A., 2018. Heteroaggregation of an enveloped bacteriophage with colloidal sediments and effect on virus viability. *Sci Total Environ* 637-638, 104-111.
- Kim, D., Lee, J.Y., Yang, J.S., Kim, J.W., Kim, V.N. and Chang, H., 2020. The Architecture of SARS-CoV-2 Transcriptome. *Cell* 181, 914-921 e10.
- Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E. and Rose, J.B., 2020. SARS-CoV-2 in wastewater: State of the knowledge and research needs. *Sci Total Environ* 739, 139076.
- Korajkic, A., Kelleher, J., Shanks, O.C., Herrmann, M.P. and McMinn, B.R., 2022. Effectiveness of two wastewater disinfection strategies for the removal of fecal indicator bacteria, bacteriophage, and enteric viral pathogens concentrated using dead-end hollow fiber ultrafiltration (D-HFUF). *Sci Total Environ* 831, 154861.
- Korajkic, A., McMinn, B.R., Herrmann, M.P., Pemberton, A.C., Kelleher, J., Oshima, K. and Villegas, E.N., 2021. Performance evaluation of a dead-end hollow fiber ultrafiltration method for enumeration of somatic and F+ coliphage from recreational waters. *J Virol Methods* 296, 114245.
- La Scola, B., Le Bideau, M., Andreani, J., Hoang, V.T., Grimaldier, C., Colson, P., Gautret, P. and Raoult, D., 2020. Viral RNA load as determined by cell culture as a management tool for discharge of SARS-CoV-2 patients from infectious disease wards. *Eur J Clin Microbiol Infect Dis* 39, 1059-1061.
- Li, X., Zhang, S., Shi, J., Luby, S.P. and Jiang, G., 2021. Uncertainties in estimating SARS-CoV-2 prevalence by wastewater-based epidemiology. *Chem Eng J* 415, 129039.
- Liu, P., Hill, V.R., Hahn, D., Johnson, T.B., Pan, Y., Jothikumar, N. and Moe, C.L., 2012. Hollow-fiber ultrafiltration for simultaneous recovery of viruses, bacteria and parasites from reclaimed water. *J Microbiol Methods* 88, 155-61.
- Lu, X., Wang, L., Sakthivel, S.K., Whitaker, B., Murray, J., Kamili, S., Lynch, B., Malapati, L., Burke, S.A., Harcourt, J., Tamin, A., Thornburg, N.J., Villanueva, J.M. and Lindstrom, S., 2020. US CDC Real-Time Reverse Transcription PCR Panel for Detection of Severe Acute Respiratory Syndrome Coronavirus 2. *Emerg Infect Dis* 26.
- Mackowiak, M., Leifels, M., Hamza, I.A., Jurzik, L. and Wingender, J., 2018. Distribution of *Escherichia coli*, coliphages and enteric viruses in water, epilithic biofilms and sediments of an urban river in Germany. *Sci Total Environ* 626, 650-659.

- Mak, G.C., Cheng, P.K., Lau, S.S., Wong, K.K., Lau, C.S., Lam, E.T., Chan, R.C. and Tsang, D.N., 2020. Evaluation of rapid antigen test for detection of SARS-CoV-2 virus. *J Clin Virol* 129, 104500.
- McMinn, B.R., Huff, E.M., Rhodes, E.R. and Korajkic, A., 2017. Concentration and quantification of somatic and F+ coliphages from recreational waters. *J Virol Methods* 249, 58-65.
- McMinn, B.R., Korajkic, A. and Grimm, A.C., 2016. Optimization and evaluation of a method to detect adenoviruses in river water. *J Virol Methods* 231, 8-13.
- McMinn, B.R., Korajkic, A., Kelleher, J., Herrmann, M.P., Pemberton, A.C., Ahmed, W., Villegas, E.N. and Oshima, K., 2021. Development of a large volume concentration method for recovery of coronavirus from wastewater. *Sci Total Environ* 774, 145727.
- McMinn, B.R., Rhodes, E.R., Huff, E.M., Wanjugi, P., Ware, M.M., Nappier, S.P., Cyterski, M., Shanks, O.C., Oshima, K. and Korajkic, A., 2018. Comparison of somatic and F+ coliphage enumeration methods with large volume surface water samples. *J Virol Methods* 261, 63-66.
- Mendoza, E.J., Manguiat, K., Wood, H. and Drebot, M., 2020. Two Detailed Plaque Assay Protocols for the Quantification of Infectious SARS-CoV-2. *Curr Protoc Microbiol* 57, ecpmc105.
- Morales-Morales, H.A., Vidal, G., Olszewski, J., Rock, C.M., Dasgupta, D., Oshima, K.H. and Smith, G.B., 2003. Optimization of a reusable hollow-fiber ultrafilter for simultaneous concentration of enteric bacteria, protozoa, and viruses from water. *Appl Environ Microbiol* 69, 4098-4102.
- Nemudryi, A., Nemudraia, A., Wiegand, T., Surya, K., Buyukyoruk, M., Cicha, C., Vanderwood, K.K., Wilkinson, R. and Wiedenheft, B., 2020. Temporal Detection and Phylogenetic Assessment of SARS-CoV-2 in Municipal Wastewater. *Cell Rep Med* 1, 100098.
- Noorimotlagh, Z., Mirzaee, S.A., Jaafarzadeh, N., Maleki, M., Kalvandi, G. and Karami, C., 2021. A systematic review of emerging human coronavirus (SARS-CoV-2) outbreak: focus on disinfection methods, environmental survival, and control and prevention strategies. *Environ Sci Pollut Res Int* 28, 1-15.
- Ohio, S.o. COVID-19 dashborad: Ohio Coronavirus Wastewater Monitoring Network
Oxford, U.o. 2021. Coronavirus (COVID-19) Deaths. In: Data, O.W.I. (Ed).
- Pan, Y., Zhang, D., Yang, P., Poon, L.L.M. and Wang, Q., 2020. Viral load of SARS-CoV-2 in clinical samples. *Lancet Infect Dis* 20, 411-412.
- Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simon, P., Allende, A. and Sanchez, G., 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. *Water Res* 181, 115942.
- Rimoldi, S.G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D., Maresca, M., Longobardi, C., Mancon, A., Romeri, F., Pagani, C., Cappelli, F., Roscioli, C., Moja, L., Gismondo, M.R. and Salerno, F., 2020. Presence and infectivity of SARS-CoV-2 virus in wastewaters and rivers. *Sci Total Environ* 744, 140911.
- Sender, R., Bar-On, Y.M., Gleizer, S., Bernshtein, B., Flamholz, A., Phillips, R. and Milo, R., 2021. The total number and mass of SARS-CoV-2 virions. *Proc Natl Acad Sci U S A* 118.

- Tiwari, A., Phan, N., Tandukar, S., Ashoori, R., Thakali, O., Mousazadesh, M., Dehghani, M.H. and Sherchan, S.P., 2021. Persistence and occurrence of SARS-CoV-2 in water and wastewater environments: a review of the current literature. *Environ Sci Pollut Res Int*.
- Torii, S., Furumai, H. and Katayama, H., 2021. Applicability of polyethylene glycol precipitation followed by acid guanidinium thiocyanate-phenol-chloroform extraction for the detection of SARS-CoV-2 RNA from municipal wastewater. *Sci Total Environ* 756, 143067.
- Venugopal, A., Ganesan, H., Sudalaimuthu Raja, S.S., Govindasamy, V., Arunachalam, M., Narayanasamy, A., Sivaprakash, P., Rahman, P., Gopalakrishnan, A.V., Siama, Z. and Vellingiri, B., 2020. Novel wastewater surveillance strategy for early detection of coronavirus disease 2019 hotspots. *Curr Opin Environ Sci Health* 17, 8-13.
- Westhaus, S., Weber, F.A., Schiwy, S., Linnemann, V., Brinkmann, M., Widera, M., Greve, C., Janke, A., Hollert, H., Wintgens, T. and Ciesek, S., 2021. Detection of SARS-CoV-2 in raw and treated wastewater in Germany - Suitability for COVID-19 surveillance and potential transmission risks. *Sci Total Environ* 751, 141750.

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.

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

Highlights

- A large volume method (2L) was evaluated against a small volume method (100mL).
- Total virus was 4 log₁₀ higher compared to infectious virus for SARS-CoV-2 seed.
- Significant levels of infectious SARS-CoV-2 was lost ($P \leq 0.0398$) during D-HFUF.
- Method 1 outperformed Method 2 ($P = <0.0001$) for endogenous SARS-CoV-2 detection.
- To better forecast disease spread, large volume (2L) concentration is beneficial.