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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

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HIGHLIGHTS

- Concentration methods varied by microbial target.
- Concentrating pipette yielded better recovery for SARS-CoV-2 than adsorption-extraction method.
- SARS-CoV-2 RNA recovery was greater for samples with higher titer seeds.
- Laboratories must empirically validate methods for water matrix and microbial target.

GRAPHICAL ABSTRACT

ABSTRACT

To support public-health-related disease surveillance and monitoring, it is crucial to concentrate both enveloped and non-enveloped viruses from domestic wastewater. To date, most concentration methods were developed for non-enveloped viruses, and limited studies have directly compared the recovery efficiency of both types of viruses. In this study, the effectiveness of two different concentration methods (Concentrating pipette (CP) method and an adsorption-extraction (AE) method amended with MgCl\(_2\)) were evaluated for untreated wastewater matrices using three different viruses (SARS-CoV-2 (seeded), human adenovirus 40/41 (HAdV 40/41), and enterovirus (EV)) and a wastewater-associated bacterial marker gene targeting Lachnospiraceae (Lachno3). For SARS-CoV-2, the estimated mean recovery efficiencies were significantly greater by as much as 5.46 times, using the CP method than the AE method amended with MgCl\(_2\). SARS-CoV-2 RNA recovery was greater for samples with higher titer seeds regardless of the method, and the estimated mean recovery efficiencies using the CP method were 25.1 ± 11% across ten WWTPs when wastewater samples were seeded with 5 × 10\(^4\) gene copies (GC) of SARS-CoV-2. Meanwhile, the AE method yielded significantly greater concentrations of indigenous HAdV 40/41 and Lachno3 from wastewater compared to the CP method. Finally, no significant differences in indigenous
1. Introduction

Wastewater has been shown to be an effective means to monitor populations for infectious microorganisms, as these agents are often shed in the feces of infected individuals (Wigginton et al., 2015; Zhou et al., 2017). In March 2020, the World Health Organization (WHO) classified coronavirus disease 2019 (COVID-19) as a global pandemic, requiring unprecedented actions to surveil viral spread in populations worldwide. Although the virus responsible for COVID-19, severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), is primarily transmitted through respiratory droplets (Morawska and Cao, 2020; Yu et al., 2020), measurements of SARS-CoV-2 RNA in the feces of infected individuals showed evidence of viral shedding (Zhang et al., 2020; Wölfel et al., 2020). Researchers worldwide (https://arcg.is/1aummW) have begun screening untreated wastewater samples for the presence of SARS-CoV-2 RNA to track the disease’s progression in communities through wastewater surveillance.

Due to the rapid onset of COVID-19 and quickly following wastewater surveillance response, methodologies deployed for SARS-CoV-2 RNA concentration from wastewater were largely techniques developed and optimized for non-enveloped enteric viruses. The methods included polyethylene glycol precipitation (PEG) (Mull and Hill, 2012; Gyawali et al., 2019; Wu et al., 2020), different adsorption-extraction (AE) procedures (Symonds et al., 2014; Ahmed et al., 2015; Ahmed et al., 2020a,b), utilization of various centrifugal devices (Symonds et al., 2009; Ahmed et al., 2015), and ultracentrifugation (Fumian et al., 2010; Ye et al., 2016). These concentration methods have exhibited variable efficiencies with percent recoveries generally ~25% (Gerrity et al., 2021; Gonzalez et al., 2020; La Rosa et al., 2021; Jafferali et al., 2021; Randazzo et al., 2020; Wang et al., 2005; Ye et al., 2016).

Recently, a method that uses an automated concentrating pipette (CP) (Concentrating pipette Select instrument) to eluate viruses using elution fluid showed promising results as it recovered ~48% (McMinn et al., 2021) of OC43 Betacoronavirus (American Society for Testing and Materials, 2020). A potential advantage of this CP method is that it relies on size-exclusion to concentrate microorganisms. Thus, an initial volume of 0.1–1 L can be concentrated to an eluate volume of ≤1 mL. This makes it potentially applicable to concentrate various microbial targets from water and wastewater for the sensitive detection of clinically significant microorganisms using molecular detection techniques (e.g., [RT]-qPCR, ddPCR).

Several groups of non-enveloped enteric viruses, including human adenovirus (HAdV) and enterovirus (EV), are frequently found in wastewater (Xagorarakis and O’Brien, 2020; Hughes et al., 2017) and are etiological agents of gastroenteritis, respiratory disease, meningitis, and common cold (Clark and McKendrick, 2004; Ganesh and Lin, 2013). Unlike SARS-CoV-2, these viruses are waterborne, mainly transmitted via fecal–oral route (Kotwal and Cannon, 2014), and have been subjects of interest for the development of most virus concentration methods for water/wastewater to date. However, simultaneous comparisons of a given method regarding its ability to recover both enveloped, and non-enveloped viruses are rare, and the few extant reports offer conflicting conclusions.

In this study, we simultaneously evaluated the effectiveness of two concentration methods (AE supplemented with the MgCl2 and CP methods) for the recovery of gamma-irradiated SARS-CoV-2 seeded in wastewater. We also quantified indigenous human enteric viruses (i.e., HAdV 40/41 and EV) with clinical significance and different morphologies, and a novel wastewater-associated marker gene Lachnospiraceae 3 (Lacho3). Lachnospiraceae are commensal inhabitants of the human GI tract, and the Lacho3 marker gene is highly abundant in untreated wastewater (McLellan et al., 2018). Such marker gene could be used to normalize pathogen quantities in wastewater samples. Furthermore, unlike the AE method, the CP method requires a centrifugation step (i.e., to separate wastewater solids from the liquid) before concentration. Because earlier studies indicated that enveloped viruses, including SARS-CoV-2, may be bound to particulate matter in the wastewater (Ye et al., 2016; Ahmed et al., 2020a,b), we have also analyzed the resulting pellets from the centrifugation step that is required for the CP method for all four targets measured in this study. This was done to account for the loss of microbial targets through centrifugation step. The findings of this study will enable researchers better identify the optimal method for recovery and quantification of viral and bacterial targets from untreated wastewater for monitoring efforts as well as more routine surveillance practices.

2. Materials and methods

2.1. Sources of SARS-CoV-2

Gamma-irradiated SARS-CoV-2 hCoV-19/Australia/VIC01/2020 was provided by the Australian Centre for Disease Preparedness (ACDP). CSIRO. Gamma irradiation was necessary to mitigate the risk of infection associated with handling SARS-CoV-2 in a biosafety containment level 2 (BC2) laboratory where this study was conducted. Upon receiving the gamma-irradiated virus stock, the tubes were stored at −80 °C for eight weeks. The concentration (2.92 × 106 ± 3.62 × 105 GC/μL) of the SARS-CoV-2 stock was determined directly using the CDC N1 RT-qPCR assay as described in the following section.

2.2. Wastewater sample preparation

Two composite untreated wastewater samples (500 mL) were collected from two metropolitan wastewater treatment plants (WWTP A and B) in early 2021. Wastewater samples were kept at 4 °C for 48 h. The pH of the wastewater samples collected from WWTP A and B were 7.23 ± 0.20 and 7.65 ± 0.15, respectively. Wastewater samples collected from WWTP B (TSS = 825 mg/L) were more turbid than WWTP A (TSS = 703 mg/L). The selected WWTPs treat mainly domestic wastewater from approximately ~15,000 (WWTP A) and ~45,000 (WWTP B) people. The catchments where the WWTPs are located did not receive any precipitation 24-h before the wastewater sampling. Before seeding with gamma-irradiated SARS-CoV-2, aliquots (3 × 50 mL) of each wastewater sample were screened for the presence of SARS-CoV-2 RNA. This was done to confirm that background levels (if any) of SARS-CoV-2 did not affect the recovery estimates. SARS-CoV-2 virus stock was serially diluted in phosphate buffer saline (PBS). 10-fold (2.72 × 105 ± 4.91 × 104 GC) and 100-fold (3.25 × 104 ± 1.96 × 103 GC) diluted SARS-CoV-2 were added to each 50-mL aliquot of untreated wastewater samples.

2.3. Virus concentration

Viruses were concentrated from gamma-irradiated SARS-CoV-2 seeded wastewater samples using two virus concentration methods (Fig. 1). The AE method is commonly used to concentrate enteric viruses from water/wastewater (Symonds et al., 2014; Ahmed et al., 2015; Ahmed et al., 2020b). The AE method began with the addition of...
MgCl₂ to the sample to achieve a final concentration of 25 mM MgCl₂. The wastewater sample subsequently passed through 0.45-μm pore-size, 47-mm diameter electron-negative HA membranes (HAWPO4700; Merck Millipore Ltd., Sydney, Australia) via a magnetic filter funnel (Pall Corporation) and filter flask (Merck Millipore Ltd.) (Ahmed et al., 2020a,b). The membrane was immediately inserted into a 5-mL-bead-beating tube for RNA extraction (described below).

The CP method utilized an automated, rapid concentrator instrument (InnovaPrep, Drexel, MO, USA) for concentrating bacteria, protozoa, and viruses from water matrices simultaneously. The CP method began with the centrifugation of each wastewater sample at 4000 g for 30 min using a Beckman Coulter Avanti J-15R Centrifuge at 4 °C. Supernatant was then concentrated using an unirradiated 0.05 μm PS hollow fibre filter CP tip (Cat. No. CC08004-200) using the CP. Custom wastewater application settings provided by the manufacturer were used (valve open ms: 700; pulse: 1; Foam factor: 10; valve closed ms: 3 s; flow start: 3 s; flow end: 10 s; flow min start: 40 s; ext delay: 3 s; pump: 25% and ext pump delay: 1 s). Upon concentration, the tip was eluted twice using CP elution fluid (HC08001) containing 0.075% Tween 20 and 25 mM Tris (Cat. No. HC08001) and is the supernatant sample lysate from the bead-beating tube was further processed following the manufacturer’s recommendations to obtain a final elution volume of 60 μL RNA was extracted directly from pellets using RNeasy Power Microbiome Kit (Cat. No. 26000–50) (Qiagen). Glass beads in the bead-beating tubes were replaced with garnet beads. Summarily, 650 μL of buffer PM1 and 6.5 μL of β-Mercaptoethanol (Sigma-Aldrich) were added into each bead-beating tube. Bead-beating tubes were homogenized using a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, FR) at conditions 3 × 15 s at 10,000 rpm at a 10 s interval. Tubes were further centrifuged at 13,000 g for 1 min to pellet the filter debris and beads. Ly-sate from the bead-beating tube was further processed following the manufacturer’s recommendations to obtain a final elution volume of 100 μL of nucleic acid. DNase I solution was omitted from the protocol to isolate both RNA and DNA.

For the CP method, nucleic acid was extracted from 140 μL of the CP eluate using QIAamp Viral RNA Mini Kit (Cat. No. 52905) (Qiagen) with a minor modification. A buffer AVE volume of 100 μL was used to elute nucleic acid instead of 60 μL. RNA was extracted directly from pellets using RNeasy Power Microbiome Kit (Cat. No. 26000–50) (Qiagen). Glass beads in the bead-beating tubes were replaced with garnet beads. Summarily, 650 μL of buffer PM1 and 6.5 μL of β-Mercaptoethanol (Sigma-Aldrich) were added into each bead-beating tube. Bead-beating tubes were homogenized using a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, FR) at conditions 3 × 15 s at 10,000 rpm at a 10 s interval. Tubes were further centrifuged at 13,000 g for 1 min to pellet the filter debris and beads. Ly-sate from the bead-beating tube was further processed following the manufacturer’s recommendations to obtain a final elution volume of 100 μL of nucleic acid. DNase I solution was omitted from the protocol to isolate both RNA and DNA. All nucleic acid samples were stored at −20 °C and subjected to RT-qPCR and qPCR analysis within 48 h of extraction to avoid losses associated with storing as well as freezing and thawing of nucleic acid preparations.

2.5. Inhibition assessment

Before RNA extraction, known quantities (1.5 × 10⁴ GC) of murine hepatitis virus (MHV) were seeded into each lysate and pellet as a molecular process control. The quantity of MHV suspension was also added to distilled water (same volume of each lysate and pellet) and subjected to nucleic acid extraction. The reference Cq values obtained for MHV-seeded distilled water (for all methods) were compared with the Cq values of the MHV seeded into each wastewater lysate and pellet to obtain information on potential RT-PCR inhibition. If the Cq value of the RNA sample was >2 compared to the reference Cq value for distilled water, the sample was considered inhibited (Ahmed et al., 2020b). The presence of PCR inhibition in nucleic acid samples extracted from wastewater was assessed using an MHV RT-PCR assay (Besselsen et al., 2002). All samples were analyzed alongside three no-template controls.

2.6. RT-qPCR and qPCR analyses

Previously published RT-PCR, RT-qPCR, and qPCR assays were used for MHV, SARS-CoV-2, EV, HAdV 40/41, and Lachno3 detection and
quantification in extracted wastewater concentrate samples (Besselsen et al., 2002; Ko et al., 2005; Cashdollar et al., 2013; Feng et al., 2018; CDC, 2020). For the MHV, EV, HAdV 40/41, and Lachno3 assays, gBlocks gene fragments were purchased from Integrated DNA Technologies (Integrated DNA Technology Coralville, IA, USA). Gamma-irradiated SARS-CoV-2, as previously described, was used as an RT-qPCR standard for the SARS-CoV-2 CDC N1 assay. CDC N1, EV, HAdV 40/41, and Lachno3 standard dilutions ranged from 1 × 10^6 to 1 copy/µL. Primer and probe sequences and cycling conditions are shown in Table 1. MHV, CDC N1, and EVs RT-qPCR analyses were performed in 20 µL reaction mixtures using TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystem, California, USA). MHV RT-PCR mixture contained 5 µL of Supermix, 300 nM of forward primer, 300 nM of reverse primer and 400 nM of probe, and 5 µL of template RNA. CDC N1 RT-qPCR mixture contained 5 µL of Supermix, 2019-nCoV Kit (500 nM of forward primer, 500 nM of reverse primer and 125 nM of probe) (Catalogue No. 10006060), and 5 µL of template RNA. EVs RT-qPCR mixture contained 5 µL of Supermix, 300 nM of forward primer, 900 nM of reverse primer and 125 nM of probe, and 5 µL of template RNA. HAdVs 40/41 and Lachno3 qPCR assays were performed in 20 µL reaction mixtures using 2 × QuantiNova Probe PCR Master Mix (Qiagen). HAdV 40/41 qPCR mixture contained 5 µL of Master Mix, 400 nM of forward primer, 400 nM of reverse primer and 100 nM of probe, and 3 µL of template nucleic acid. Lachno3 qPCR mixture contained 5 µL of Master Mix, 1000 nM of forward primer, 1000 nM of reverse primer and 100 nM of probe, and 3 µL of nucleic acid. The RT-qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Richmond, CA, USA) using manual settings for threshold and baseline. The estimated copy numbers for EV in each wastewater concentrate sample were corrected for the difference between the double-stranded standard curve (i.e., gBlocks gene fragment) material and the single-stranded EV (e.g., divided by 2).

2.7. Recovery efficiency variability of SARS-CoV-2 within and across WWTPs

Since the CP method demonstrated a superior recovery of SARS-CoV-2 RNA compared to the AE method during the experiments described above, further evaluation of the CP method was undertaken by analyzing 30 archived wastewater samples collected from 10 additional WWTPs over three sampling events (WWTPs C-L). Approximately, 5.10 × 10^4 GC gamma-irradiated SARS-CoV-2 viruses were seeded into each wastewater sample. Before seeding, an aliquot (i.e., 50 mL) of each wastewater sample was screened for SARS-CoV-2 RNA. This was done that background levels (if any) of SARS-CoV-2 did not affect the recovery estimates. Virus concentration and RT-qPCR analysis was performed as described above.

2.8. SARS-CoV-2 recovery efficiency

The SARS-CoV-2 recovery efficiency of each replicate for each concentration method was calculated based on the copies quantified per by RT-qPCR as follows:

\[
\text{Recovery Efficiency (\%)} = \frac{\text{Total viral RNA gene copies recovered in concentrated wastewater}}{\text{Total viral RNA gene copies seeded in wastewater}} \times 100
\]

The mean and standard deviation (SD) for each concentration method was calculated.

2.9. Quality control

To minimize RT-qPCR and qPCR contamination, nucleic acid extraction and RT-qPCR set up were performed in separate laboratories. A method negative control was included for each concentration method. A reagent negative control was also included during nucleic acid extraction to account for any contamination during extraction. All negative controls were negative for the analyzed targets.

2.10. Statistical analysis

Concentration (EV, HAdV 40/41, and Lachno3) and percent recovery (SARS-CoV-2) estimates were normalized using log_{10} and arcsine square root transformations, respectively, before statistical analyses. One-way analyses of variance (ANOVA) using GraphPad Prism Version 8.3.1 (GraphPad Software, La Jolla, CA, USA) software were used to assess whether percent recovery efficiencies of SARS-CoV-2 obtained through AE and CP methods, including the pellet, significantly differed. ANOVA was also used to test whether the recovery of SARS-CoV-2 RNA among WWTPs (WWTPs C-L) differed significantly and to compare concentrations of EV, HAdV 40/41, and Lachno3 using AE and CP methods, and in pellets. Finally, unpaired t-tests were used to compare percent recovery estimates of SARS-CoV-2 between WWTPs A-B and WWTPs C-L. Statistical significance was defined as p < 0.05.

Table 1: Primers, probes and cycling parameters used in this study.

| Targets | Gene | Oligonucleotide sequences (5′ - 3′) | Primers and probes (nM) | Cycling parameters | References |
|---------|------|----------------------------------|-------------------------|-------------------|-----------|
| MHV     | M    | F: CGA ACT TCT CTT TCG GCA TTA TAC T R: ACC ACA AGA TTA TCA TTT TCA CAA CAT A P: FAM-AQA TGC TAC GCG TCG TCT AAC CGA ACT GT-BHQ | 300 | 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 | Besselsen et al., 2002 |
| SARS-CoV-2 | N | F: GACCCCCAAAATCAGCGCAAT R: TCAGTTACTGCACTGAATCTG P: FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ | 500 | 50 °C for 10 min for RT; 95 °C for 5 min and 45 cycles of 95 °C for 15 s, 60 °C for 30 s | US CDC, 2020 |
| EVs     | 5′ LTR | F: CCT CCG GCC CCT GAA TG R: ACC GGA TGG CCA ATC CAA P: FAM-CGG AAC GCA CTA CTT TGG GTG TCC GT-TAMRA | 125 | 50 °C for 10 min, 95 °C for 5 min, 45 cycles of 95 °C for 1 min, 60 °C for 30 s | Cashdollar et al., 2013 |
| HAdV 40/41 | Fibre | gene | F: AAC TTT TCT TCT TAA TAG ACC CC R: AGG GGG CTA GAA AAC AAA A P: CTG ACA GGC GCA CTC TCT GC | 100 | 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C 30 s | Ko et al., 2005 |
| Lachno3 | 16S | rRNA | F: CAA CGG GAA GAA TAC TCT ACC A A: CCC AGA GTG CCC ACC TTA AAT P: FAM-CTC TGA GGC GTC TTT AAT CCG A-TAMRA | 100 | 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 63 °C for 45 s | Feng et al., 2018 |
3. Results

3.1. RT-qPCR and qPCR assay performance

The RT-qPCR standard curves for CDC N1 (SARS-CoV-2), EV, HAdV 40/41, and Lachno3 had a dynamic linear range of quantification from $5 \times 10^0$ to 5 gene copies (GC)/reaction ($1 \times 10^0$ to 1 GC/µL). The slopes of the standard curves were $-3.437$ (CDC N1), $-3.502$ (EVs), $-3.399$ (HAdV 40/41), and $-3.367$ (Lachno3) (Table 2). The amplification efficiencies were 95.4, 96.9, and 98.2% for CDC N1, EVs, HAdV 40/41, and Lachno3, respectively. The correlation coefficient ($r^2$) ranged from 0.996 to 0.999 for all assays. The assay limit of detection was 1.8, 2.3, 2.4 and 1.5 GC/µL for CDC N1, EV, HAdV 40/41, and Lachno3, respectively. All wastewater RNA samples were within the 2-Cq values of the reference Cq value; thus, no RT-qPCR inhibition was identified (Table 3).

3.2. Gamma-irradiated SARS-CoV-2 concentrations and recovery in untreated wastewater

Wastewater samples used for seeding SARS-CoV-2 did not contain detectable background SARS-CoV-2 as determined by the CDC N1 RT-qPCR assay. The concentrations of SARS-CoV-2 CDC N1 in 10-fold and 100-fold diluted SARS-CoV-2 were $2.72 \times 10^5 \pm 4.91 \times 10^5$ and $3.25 \times 10^4 \pm 1.96 \times 10^4$ GC and were used to determine the recovery efficiency of the two concentration methods examined.

The estimated mean recovery efficiencies using CP method of gamma-irradiated SARS-CoV-2 in WWTP A and B were $55.5 \pm 11.8$ and $65 \pm 23.6\%$, respectively when $2.72 \times 10^5$ GC SARS-CoV-2 was seeded (Table 4). Similarly, mean recovery efficiencies of gamma-irradiated SARS-CoV-2 in WWTP A and B were $39.4 \pm 4.18$ and $29.4 \pm 9.66\%$ when $3.25 \times 10^4$ GC SARS-CoV-2 was seeded. The estimated mean recovery efficiencies using the AE method were 2.10 to 5.46 times lower than the CP method and ranged from 7.21 $\pm$ 1.85 to 26.4 $\pm$ 4.73%. For the high and low titers, the AE method showed similar patterns to that of the CP method; greater recovery was achieved for high titer seed compared to a slightly lower recovery for the lower titer seeded in the samples. Irrespective of the seed titer, the estimated SARS-CoV-2 recoveries obtained by the CP method were significantly greater ($p < 0.05$) than the AE method. Recovery efficiency of SARS-CoV-2 RNA from pellets were much lower than both the AE and CP methods and ranged from 3.79 to 8.21%.

3.3. Concentrations of enteric viruses and Lachno3 marker in wastewater samples collected from WWTP-A and -B

AE and CP methods were also evaluated for their relative consistency in quantifying EV, HAdV 40/41, and Lachno3 in untreated wastewater samples collected from WWTP A and B (Table 5). The concentrations of both Lachno3 and HAdV 40/41 were $8.83 \pm 0.18 \log_{10}$ and $6.44 \pm 0.16 \log_{10}$ GC/L, respectively using the AE method. The CP method yielded the highest concentrations of EV ($5.24 \pm 0.47 \log_{10}$ GC/L) compared to the AE method ($4.27 \pm 0.62 \log_{10}$ GC/L) and the resultant pellets from the CP method ($3.53 \pm 0.34 \log_{10}$ GC/pellet). The concentrations of both HAdV 40/41 ($5.04 \pm 0.23 \log_{10}$ GC/pellet) and Lachno3 ($7.34 \pm 0.65 \log_{10}$ GC/pellet) in the pellets were greater than CP method. Comparisons of relative consistency in quantifying EV concentrations indicated there was no statistically significant difference between the AE and CP methods ($p > 0.05$), suggesting that either the AE or CP method was capable of consistently quantifying EV from untreated wastewater samples. The AE method significantly yielded more HAdV 40/41 and Lachno3 (p < 0.05) compared to the CP method indicating that the CP method may be less sensitive in quantifying these two analytes from the untreated wastewater. The concentrations of Lachno3 in pellets were significantly greater (p < 0.05) than the CP method.

3.4. Variations in SARS-CoV-2 recovery efficiency within and among WWTPs using the CP method

Seeding archived wastewater samples (WWTP C-L) with $5.10 \times 10^4$ GC gamma-irradiated SARS-CoV-2 resulted in estimated mean recovery efficiencies of 25.1 $\pm$ 11% using the CP method across 10 WWTPs (Fig. 2), which was significantly lower ($p < 0.05$) compared to mean recovery efficiencies obtained for WWTP A and B. The percentage recovery variations within WWTPs over the three sampling events ranged from 11.4 to 34.8% (WWTP J) and 7.0 to 14.7% (WWTP L). However, comparison of SARS-CoV-2 recovery estimates among WWTPs (WWTP C-L; three samples/WWTP) indicated no statistically significant difference ($p > 0.05$).

4. Discussion

In this study, the mean recovery efficiency of gamma-irradiated SARS-CoV-2 using the AE method ranged from 7 and 26% and coefficient of variation between 19 and 29% in untreated wastewater samples from two WWTPs, which is greater than the recoveries reported in other studies (Feng et al., 2021; Gonzalez et al., 2020). Importantly, the MHV recovery estimated in our previous study (Ahmed et al., 2020b), which was greater than those observed for AE method in this study, was only for the concentration step, whereas, in this study, it includes the efficiency of both the concentration and extraction steps. Comparison of recovery efficiencies between studies are difficult, since recovery rates will vary depending on the surrogate, PCR assays, standard curves, and characteristics of the wastewater samples used (Kantor et al., 2021). Nonetheless, within-study comparisons between methods can provide useful information. When compared to AE, the CP method recovery for SARS-CoV-2 was significantly greater, with mean recoveries between 29 and 65%, consistent with a previous study reporting 48% recovery for OC43, a SARS-CoV-2 surrogate (McMinn et al., 2021).

Table 2

| RT-qPCR/qPCR performance characteristics. | Efficiency (%) | Linearity | Slope | Y-intercept | ALD (GC/µL) of nucleic acid |
|------------------------------------------|---------------|----------|-------|-------------|------------------------------|
| CDC N1                                   | 95.4          | 0.996    | −3.437| 38.639      | 1.8                          |
| EVs                                      | 93.0          | 0.996    | −3.502| 41.254      | 2.3                          |
| HAdV 40/41                               | 96.9          | 0.996    | −3.399| 40.062      | 2.6                          |
| Lachno3                                  | 98.2          | 0.999    | −3.367| 39.502      | 1.5                          |

Table 3

| Sample ID | Mean Cq values |
|-----------|----------------|
|           | AE method | CP method | Pellet* |
| WWTP A-1  | 26.14       | 26.09       | 26.06       |
| WWTP A-2  | 26.04       | 26.10       | 26.10       |
| WWTP B-1  | 26.08       | 26.11       | 26.01       |
| WWTP B-2  | 26.08       | 26.07       | 26.04       |
| WWTP C    | 26.15       | 25.93       | 26.14       |
| WWTP D    | 26.08       | 26.02       | 26.17       |
| WWTP E    | 26.11       | 26.08       | 25.93       |
| WWTP F    | 25.94       | 26.08       | 26.14       |
| WWTP G    | 26.05       | 26.07       | 25.88       |
| WWTP H    | 26.07       | 26.03       | 25.74       |
| WWTP I    | 26.12       | 26.02       | 26.00       |
| WWTP J    | 25.00       | 26.01       | 25.95       |
| WWTP K    | 26.00       | 26.06       | 25.99       |
| WWTP L    | 26.11       | 26.06       | 26.02       |
| Reference point | 26.14 |

AE method: adsorption-extraction. CP method: concentrating pipette.

* Resultant pellet from the CP method.
However, the CP method also exhibited greater within-sample SARS-CoV-2 recovery variation, with coefficient of variation between 10 and 37% for wastewater samples in this study.

Even when similar methods are used, recovery efficiencies for various SARS-CoV-2 surrogates are highly variable between laboratories and WWTPs (Pecson et al., 2021; Chik et al., 2021). For example, the AE method had 67% mean recovery efficiency for MHV seeded into wastewater in Australia (Ahmed et al., 2020b) and 5.5 to 6% recovery efficiencies for BCoV and BRSV seeded in wastewater in Virginia, USA (Gonzalez et al., 2020). SARS-CoV-2 recoveries as low as 0% from wastewater were reported for versions of the AE method that relied on centrifugation of sample prior to filtration and application of different bead-beating instrument (Sapula et al., 2021). Similarly, low recovery efficiencies, using the AE method, were reported when smaller 2-mL bead-beating tubes were used in the lysis step of the extraction protocol (RNeasy PowerMicrobiome Kit) compared to the 5-mL-bead beating tubes used in this study (Jafferli et al., 2021; Bivins et al., 2021). Differences in extraction kits, reverse-transcription, and quantification methods or deviation or modification of optimized methods can also cause recovery efficiencies to vary among within and among laboratories.

Such large variation in recovery could represent a significant limitation for the use of RT-qPCR data in quantitative models or trend analysis (Feng et al., 2021; Bivins et al., 2021). This variation could be driven by physicochemical characteristics of the wastewater being sampled such as pH, temperature, salinity, or suspended solids (Mohanpatra et al., 2021). Importantly, the CP method mean recovery efficiency for seeded SARS-CoV-2 was not different among 10 different WWTPs. Less variation in recovery between WWTPs could strengthen longitudinal quantitative and correlative analyses within and between locations, thus giving the CP method an advantage over the AE method. On the other hand, when wastewater surveillance is intended for early detection of incident COVID-19 in communities without local transmission, maximizing the recovery efficiency would improve the likelihood of detecting low/trace levels of SARS-CoV-2 RNA in wastewater. Despite lower recovery at decreased seeding titer, the CP method demonstrated greater mean recovery for SARS-CoV-2 at both seeding levels than the AE method. Thus, the CP method may afford more sensitive detection of SARS-CoV-2 RNA in wastewater when the concentration of SARS-CoV-2 is moderate to high (i.e., 4–6 \( \text{log}_{10} \) GC/100 mL). Detection sensitivity of the CP method can be further improved by extracting nucleic acid from eluate and pellets separately by combined analysis if required. Both methods are rapid; however, CP requires additional time if the wastewater sample is not centrifuged before ultrafiltration. In this study, only 50 mL of wastewater was processed, with no observed inhibition, but concentration of larger volumes of wastewater to improve sensitivity could induce PCR inhibition. Another limitation of the CP method is the effective sample volume (ESV = 1.31 to 1.74 mL/sample), which is lower than the AE method (15 mL/sample); therefore, this method may not be sensitive enough for trace detections and may require further optimization.

| WWTPs and samples | Mean ± SD recovery of SARS-CoV-2 RNA |
|-------------------|------------------------------------|
| A method          | CP method                          | Pellet* |
| EVs               |                                   |        |
| WWTP A-1          | 4.81 ± 0.11                        | 5.73 ± 0.10 | 3.80 ± 0.20 |
| WWTP A-2          | 4.80 ± 0.12                        | 5.57 ± 0.08 | 3.86 ± 0.11 |
| WWTP B-1          | 3.87 ± 0.52                        | 4.88 ± 0.88 | 3.25 ± 0.17 |
| WWTP B-2          | 3.63 ± 0.22                        | 4.80 ± 0.17 | 3.24 ± 0.24 |
| WWTP A-3          | 6.47 ± 0.17                        | 4.89 ± 0.09 | 5.11 ± 0.10 |
| WWTP A-2          | 6.66 ± 0.14                        | 4.60 ± 0.08 | 5.33 ± 0.06 |
| WWTP B-1          | 6.33 ± 0.18                        | 3.38 ± 0.23 | 4.89 ± 0.07 |
| WWTP B-2          | 6.32 ± 0.07                        | 3.44 ± 0.12 | 4.83 ± 0.26 |
| Lachno3           |                                   |        |
| WWTP A-1          | 8.67 ± 0.08                        | 5.33 ± 0.10 | 6.73 ± 0.14 |
| WWTP A-2          | 8.68 ± 0.07                        | 5.29 ± 0.17 | 6.86 ± 0.08 |
| WWTP B-1          | 9.00 ± 0.11                        | 3.53 ± 0.54 | 8.04 ± 0.06 |
| WWTP B-2          | 8.97 ± 0.11                        | 4.00 ± 0.19 | 7.76 ± 0.35 |

### Table 4

Recovery (mean ± SD) of SARS-CoV-2 RNA from ten wastewater treatment plants (WWTPs) using a concentrating pipette (CP method). Each WWTP was sampled three times.
determine the starting titer of the seeding material (i.e., gamma-irradiated SARS-CoV-2). While this may limit the interpretation of the absolute recovery, it does not preclude the comparisons between the AE and CP methods.

The greater recoveries observed by the CP method for the enveloped virus (i.e., SARS-CoV-2) were inconsistent with the relative quantities observed for bacteria and non-enveloped viruses. For Lachnospiraceae, spore-forming gram-negative bacteria, the greatest concentration was measured using the AE method, followed by the pellets, and the CP method with the lowest amount. This order was consistent for HAdV 40/41, a non-enveloped virus with a double-stranded DNA genome, with the AE method demonstrating the highest concentration, followed by the pellet, and the CP method. However, for EV, a non-enveloped virus with a single-stranded RNA genome, the CP method yielded the highest concentration, followed by the AE method, with the pellet last. These observations suggest that the most efficient recovery method may not be ‘one size fits all’ and is likely dependent on the morphology and physiology of the microorganisms of interest. This agrees with recovery experiments performed using both non-enveloped and enveloped viruses, which indicate inconsistent trends in recovery between the two morphologies (Haramoto et al., 2009; Randazzo et al., 2020). While this study was not designed to determine the mechanistic basis for these differences, in lieu of such understanding, an optimal concentration method should be determined empirically for each relevant combination of water matrix and microbial target. Differences in recovery efficiencies between microbial agents could become an important source of bias during normalization of quantitative SARS-CoV-2 data, such as has been done using pepper mild mottle virus, could potentially introduce bias into quantitative analyses (Feng et al., 2021; Bivins et al., 2021).

In this study, the CP method achieved greater mean recovery of SARS-CoV-2 than the AE method when SARS-CoV-2 were seeded into wastewater, but this increased recovery was accompanied by increased variability as well. While the CP method seems to be advantageous over AE, the Concentrating Pipette Select™ is a specialized piece of laboratory equipment with associated consumables that are far more expensive and less readily available than the materials and equipment required for the AE method. The improved performance of the CP method was inconsistent across microbial targets or virus morphology, thus reinforcing that technological sophistication does not always guarantee optimal performance. Laboratories performing wastewater surveillance must empirically validate methods for the pertinent application or use case. Despite their limitations, whole process controls, including surrogate coronaviruses, are critical for establishing optimal protocols and ensuring correct data interpretation for wastewater surveillance of pathogens or other microbial targets. Finally, since there is no concentration method that can effectively recover all pathogens from wastewater, it will be important to optimize the concentration method that most effectively meets the needs and priorities of the surveillance and/or monitoring program.

Disclaimers
The research presented was not performed or funded by USEPA and was not subject to USEPA’s quality system requirements. 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.

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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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