Journal of Virological Methods 320 (2023) 114790

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocols

Comparing solid-based concentration methods for rapid and efficient recovery of SARS-CoV-2 for wastewater surveillance

Doris Yoong Wen Di [a], Bo Li [a], Min Ki Jeon [a], Tao Yan [a,b,*]

[a] Department of Civil and Environmental Engineering, University of Hawaii at Manoa, Honolulu, HI 96822, USA
[b] Water Resources Research Center, University of Hawaii at Manoa, Honolulu, HI 96822, USA

ARTICLE INFO

Keywords:
SARS-CoV-2
Wastewater
Solid fraction
Viral concentration
Precipitation
Flocculation

ABSTRACT

As wastewater-based surveillance of SARS-CoV-2 attracts interest globally, there is a need to evaluate and identify rapid and efficient methods for concentrating enveloped viruses in wastewater. When comparing five precipitation/flocculation-based concentration methods (including aluminum hydroxide adsorption-precipitation, AHAP; zinc acetate precipitation, ZAP; skimmed milk flocculation, SMF; $FeCl_3$ precipitation, FCP; and direct centrifugation, DC), AHAP was found to be the most efficient method in terms of seeded BCoV recovery (50.2 %). Based on the BCoV recovery efficiency and turnaround time, the AHAP and DC methods were selected and tested on five additional wastewater samples containing both seeded BCoV and indigenous wastewater SARS-CoV-2 RNA. The BCoV recovery (DC: average = 30.1 %, s_x = 14.7 %; AHAP: average = 33.0 %, s_x = 14.2 %) and SARS-CoV-2 based on the N2 gene assay (DC: average = 3.6×10^3 gene copies or GC/mL, $s_x = 1.9 \times 10^3$ GC/mL; AHAP: average=3.0×10^3 GC/mL, $s_x = 2.0 \times 10^3$ GC/mL) of both methods were not significantly different in solid fraction ($p = 0.89$). This study showed significant higher BCoV recovery and SARS-CoV-2 viral RNA in wastewater solid fraction ($p = 0.006$) than liquid fraction. Our result suggests that the solid fraction of wastewater samples is more suitable for recovering enveloped viruses from wastewater, and the DC and AHAP methods equally provide suitably rapid, cost-effective, and significantly higher recovery of SARS-CoV-2 viral RNA in wastewater samples.

1. Introduction

The coronavirus disease 2019 (COVID-19) global pandemic is caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus (Wu et al., 2020b), which is found primarily at the respiratory tract of infected individuals. Since fecal shredding of SARS-CoV-2 viral RNA has also been observed in significant percentage of infection (Gupta et al., 2020; Wu et al., 2020c), detection of SARS-CoV-2 RNA in municipal wastewater, which collects fecal wastes in addition to some other human bodily wastes, was reported (Kitajima et al., 2020; Peccia et al., 2020). Subsequently, detection and quantification of SARS-CoV-2 RNA in raw wastewater have been reported globally, including in Australia (Ahmed et al., 2020a), Brazil (Prado et al., 2020), Chile (Ampuero et al., 2020), China (Zhang et al., 2020), France (Wurtzer et al., 2020), Italy (La Rosa et al., 2020; Rimoldi et al., 2020), Israel (Or et al., 2020), Japan (Haramoto et al., 2020), Netherlands (Medema et al., 2020), Spain (Randazzo et al., 2020), Turkey (Kocamemi et al., 2020a), and USA (Gonzalez et al., 2020; Li et al., 2021; Nemudryi et al., 2020; Sherchan et al., 2020; Wu et al., 2020a). Several studies have also shown that the concentration of viral RNA in wastewater correlated with community prevalence of COVID-19 clinical cases (Ahmed et al., 2021; Li et al., 2021; Medema et al., 2020; Peccia et al., 2020; Randazzo et al., 2020; Stadler et al., 2020).

Since fecal wastes from SARS-CoV-2 infected individuals undergo significant dilutions upon entering the municipal wastewater collection systems, efficient wastewater viral concentration is needed for effective wastewater-based surveillance of COVID-19. Various methods have been used to concentrate and recover SARS-CoV-2 from wastewater, including ultrafiltration (Sherchan et al., 2020), polyethylene glycol (PEG) precipitation (Wu et al., 2020a), ultracentrifugation (Ampuero et al., 2020; Jafferali et al., 2021), and filtration with an electronegative membrane (Ahmed et al., 2020a). Several recent studies have compared recovery efficiencies of various concentration methods with enveloped surrogate viruses seeded in wastewater. For example, Ahmed et al.

* Corresponding author at: University of Hawaii at Manoa, Department of Civil and Environmental Engineering, 2540 Dole Street, 383 Holmes Hall, Honolulu, HI 96822, USA.
E-mail address: taoyan@hawaii.edu (T. Yan).

https://doi.org/10.1016/j.jviromet.2023.114790
Received 1 May 2023; Received in revised form 26 July 2023; Accepted 5 August 2023
Available online 7 August 2023
0166-0934/© 2023 Elsevier B.V. All rights reserved.

(2020) showed that an electronegative membrane filtration method exhibited the best recovery (65.7 %) of seeded murine hepatitis virus (MHV) when compared with ultrafiltration (28.0–56.0 %), polyethylene glycol (PEG) precipitation (44.0 %), and centrifugation (33.5 %) (Ahmed et al., 2020b); LaTurner et al. (2021) reported the best recovery of seeded bovine coronavirus by direct centrifugation (3.84 %) when compared with electronegative membrane filtration methods (0.57–0.96 %), PEG precipitation (0.08 %), and ultrafiltration (0.36 %) (LaTurner et al., 2021); and Philo et al. (2021) reported the best recovery of seeded human coronavirus OC43 (6.5–9.1 %) by a skimmed milk flocculation method when compared with bag-mediated filtration system (BFMS) (0.04–0.7 %), PEG precipitation (3.2 %), and ultrafiltration (1.0%) (Philo et al., 2021). The varying results reported by these studies might be caused by the different wastewater samples used, and also the different treatment on the solid and liquid fractions the wastewater matrix (e.g. Ahmed et. al. (2020a, 2020b) and LaTurner et. al. (2021) primarily analyzed the liquid fraction and discarded the solid fraction of wastewater samples).

Although most prior studies focused on the liquid fraction of wastewater for SARS-CoV-2 viral RNA concentration (Ahmed et al., 2020a; Ahmed et al., 2020b; Kocamemi et al., 2020a; La Rosa et al., 2020; LaTurner et al., 2021; Medema et al., 2020; Or et al., 2020; Rimoldi et al., 2020; Zhang et al., 2020), several studies have recently reported high concentrations of SARS-CoV-2 RNA in wastewater sludge (Balboa et al., 2021; Kocamemi et al., 2020b; Peccia et al., 2020), and that SARS-CoV-2 RNA gene copies predominantly resides in the solid fraction of the wastewater samples (Graham et al., 2021; Li et al., 2021). Therefore, methods that can capture SARS-CoV-2 viral gene copies in both the solid and liquid fractions of wastewater samples would potentially provide optimal concentration and recovery of SARS-CoV-2 viral RNA for wastewater-based surveillance. Additional considerations include wastewater processing throughput volumes and turnaround time (TAT). The capability of processing large wastewater volumes is essential for wastewater sentinel surveillance when community COVID-19 disease burden is low, which is likely the scenario for most human communities in the post-vaccination era. Short TAT would on one hand preserve target RNA integrity during sample process, while on the other hand enable quick surveillance results and facilitate timely decision making.

Therefore, this study compared the performance of five different methods that are based on the wastewater solid fractions and utilize precipitation, adsorption, and/or coagulation and flocculation mechanisms to further concentrate SARS-CoV-2 viral RNA from the liquid fraction of wastewater. The methods were first evaluated based on their recovery from wastewater samples of seeded bovine coronavirus (BCoV) as the surrogate enveloped virus. The SARS-CoV-2 RNA indigenous of the wastewater samples were recovered by the different methods and quantified based on the N1, and N2 and E gene two-step RT-qPCR assays. Other process parameters, including total RNA extracted, total TAT, and process costs, were also considered. The results presented in this study will allow researchers to select a rapid, efficient, and cost-effective concentration method for domestic wastewater for designing and implementing wastewater-based surveillance of SARS-CoV-2 in human communities.

2. Materials and methods

2.1. Wastewater sampling and BCoV seeding

Flow-weighted daily composite raw wastewater samples were collected on 8/31/2020 (WW4) from the Sand Island wastewater treatment plants (treating ca. 58 % of total daily wastewater flow) in the City and County of Honolulu (Hawaii, USA). This wastewater sample was used to compare the five precipitation/flocculation-based concentration methods for the recovery of seeded BCoV. The sampling time coincided with a COVID-19 outbreak in the community, with a 7-day average new case number of 303 on 8/31/2020. The wastewater samples were collected in sterile plastic containers and stored at −80 °C before processing. Frozen wastewater samples were first fully thawed and thoroughly mixed before viral concentration procedures.

In addition, five wastewater samples (WW1: 8/28/2020; WW2: 8/29/2020; WW3: 8/30/2020; WW5: 9/1/2020; and WW6: 9/8/2020) collected from the same wastewater treatment plant were subsequently used to compare the BCoV recovery rate and SARS-CoV-2 RNA concentration between direct centrifugation (DC) and aluminum hydroxide adsorption-precipitation (AHAP) methods, i.e. without and with precipitation/flocculation treatments. The DC method was tested here as a reference baseline because of its simplicity and fast TAT. The concentration of SARS-CoV-2 RNA in the wastewater samples (both solid and liquid fractions) were previously determined by PEG precipitation and shown in Table S1. (Li et al., 2021).

BCoV (Zoetis; Kalamazoo, MI, USA) was used as the enveloped virus surrogate, and was seeded into the wastewater at a final concentration of 5.3×10^3 BCoV GC/mL (WW4) and 4.0×10^5 BCoV GC/mL (WW1, WW2, WW3, WW5, and WW6). The seeded wastewater was fully mixed by stirring at 4 °C for 1 h. The RNA extracted from the wastewater before seeding the BCoV were quantified for BCoV using qPCR in triplicate, which confirmed the absence of BCoV RNA.

2.2. Wastewater viral concentration methods

The fully mixed wastewater sample was divided into 20 mL aliquots (in triplicates) and subjected to five different concentration methods (Methods A-E; Fig. S1), which are described in detail below. In general, the wastewater sample was amended with or without chemical coagulants and flocculants and then incubated for a varying amount of time depending on the methods. The samples were then centrifuged at 38,400 × *g* for 30 mins at 4 °C in a high-speed centrifuge Avanti J-E (Beckman Coulter; Brea, CA, USA) to separate suspended solids (referred to as solid fractions) from the wastewater supernatant. Solid fractions were subjected to direct viral RNA extraction as described below. The liquid supernatants were collected and subjected to PEG precipitation to determine the amount of viruses remaining after treatment. The PEG precipitation followed the procedure described by Hjelmso et al. (Hjelmso et al., 2017). Briefly, 80 g/L of PEG 8000 (VWR; PA, USA) and 17.5 g/L of NaCl (VWR; PA, USA) were added to the supernatant, agitated overnight (100 rpm) at 4 °C, and centrifuged at 38,400 × *g* for 30 mins at 4 °C. After carefully decanting the supernatant, the viral pellet at the bottom of the centrifuge bottle was thoroughly resuspended in 500 μL of phosphate buffer saline (PBS) and referred to as the liquid fractions of the wastewater samples.

2.2.1. (A) Direct centrifugation (DC)

The DC process followed the procedure described by Li et al. (Li et al., 2021). This is the baseline process without amendment of any chemical coagulants and flocculants to enhance viral partition from wastewater liquid to solids, also acts as a viral concentration process control. The BCoV-seeded wastewater samples were subjected to direct solid and liquid separation by centrifugation, and the liquid supernatants were further treated with PEG precipitation.

2.2.2. (B) Zinc acetate precipitation (ZAP)

The ZAP process followed the procedure described by Sokol et al. (Sokol et al., 1968). One part of 1 M zinc acetate solution (J.T. Baker Chemical Co.; Phillipsburg, NJ, USA) at pH 5.0 was added to 50 parts of wastewater samples. The samples were allowed to stand for 20 mins at 4 °C without shaking. The samples were then swirled to suspend floc before centrifugation to pellet suspended solids, and the liquid supernatants were further treated with PEG precipitation.

2.2.3. (C) Aluminum hydroxide adsorption-precipitation (AHAP)

The AHAP process followed the procedure described by Randazzo

et al. (Randazzo et al., 2020). Briefly, wastewater pH was first adjusted to 6.0 using 1 N of HCl (VWR; PA, USA), 1:100 of 0.9 N aluminum chloride ($AlCl_3$) solution (Thermo Fisher Scientific; Waltham, MA, USA) was added into wastewater samples, and pH was readjusted to 6.0 with 1 N NaOH (VWR; PA, USA). The wastewater samples were mixed at 150 rpm for 15 mins at 4 °C before 1:20 of 3 % beef extract solution (pH 7.4) was added. The samples were then agitated at 150 rpm for 10 mins at 4 °C, and then centrifuged to pellet suspended solids. The liquid supernatants were further treated with the PEG precipitation.

2.2.4. (D) Skimmed milk flocculation (SMF)

The SMF process followed the procedure described by Calgua et al. (Calgua et al., 2008). Wastewater pH was first adjusted to 3.5 using 1 N HCl. Pre-flocculated skim milk solutions (Criterion Hardy Diagnostics; Santa Maria, CA, USA) were added into the pH-adjusted wastewater samples at 1:100 ratio. The samples were then mixed (100 rpm) overnight at 4 °C, and then centrifuged to pellet suspended solids. The liquid supernatants after treatment were further processed using the PEG precipitation.

2.2.5. (E) $FeCl_3$ precipitation (FCP)

The FCP process followed the procedure described by John et al. (John et al., 2011). After 1 mg/L of $FeCl_3$ (final concentration) (Thermo Fisher Scientific; Waltham, MA, USA) was added into the wastewater samples, the samples were shaken vigorously for 1 min and repeated 3 times and allowed to settle for 1 h at 4 °C before adding 1:1000 of 0.1 M EDTA-0.2 M $MgCl_2$-0.2 M ascorbate buffer into the $FeCl_3$-treated samples. The samples were shaken vigorously for 30 s, agitated overnight (100 rpm) at 4 °C, and were centrifuged to pellet suspended solids. The liquid supernatants from centrifugation were further processed using PEG precipitation.

2.3. Viral RNA extraction and two-step reverse transcription quantitative PCR (RT-qPCR)

The solid fractions (wet weight, ranging from 15 to 312 mg) and the liquid fractions (500 μL) were subjected to viral RNA extraction and eluted into a final 30 μL volume of RNA products by using QIAamp® Viral RNA Mini Kit (Qiagen; CA, USA). Carrier RNA supplied in the RNA extraction kit was not added in the RNA extraction process. RNA concentrations were measured by using Qubit™ RNA BR Assay Kit with a Qubit 4 Fluorometer (Invitrogen; Carlsbad, CA, USA). Before further analysis, the RNA samples were diluted and normalized to 10 ng/μL final concentrations to minimize potential matrix effects (inhibition and/or competition) on RT-qPCR (Graham et al., 2021). Reverse transcription (RT) was performed to obtain complementary DNA (cDNA) by using random hexamers (Promega; Madison, WI, USA) and SuperScript® IV Reverse Transcriptase (Thermo Fisher Scientific; Waltham, MA, USA) according to manufacturers' instructions. Briefly, 1 μL of RNA templates (10 ng), 0.5 mM dNTP, 2.5 μM random hexamers, and nuclease free water were added to a volume of 13 μL. This RNA-primer mix were heated at 65 °C for 5 mins using a GeneAmp® PCR System 9700 (Applied Biosystem; Beverly, MA, USA) followed by incubation on ice for at least 1 min. A mixture of 1 × SSIV buffer, 5 mM DTT, 2 U/μL of RNase inhibitor (Promega; Madison, WI, USA), and 200 U/μL of SuperScript® IV Reverse Transcriptase in a total volume of 7 μL were added to the ice-cooled RNA-primer mix. The combined reaction mixture was then incubated sequentially at 23 °C for 10 mins, 55 °C for 10 mins, and 80 °C for 10 mins. The cDNA products from the RT reactions were then stored at −20 °C for 24–48 h before being used as DNA template for subsequent real-time PCR (qPCR) quantification in the various assays.

2.4. qPCR assays

qPCR assays for BCoV, SARS-CoV-2 E gene, N gene (N1 and N2) were performed as previously described (Li et al., 2021). Details of the primers and probes used were summarized in Table S2 and S3. Each RNA sample for each qPCR assay was performed in duplicate in an ABI 7300 qPCR System (Applied Biosystem; Beverly, MA, USA). The primers and probes were obtained from Integrated DNA Technologies (IDT) (Coralville, IA, USA) and Biosearch Technologies (Novato, CA, USA). Each qPCR reaction mixture had a final volume of 20 μL and comprised of 1 × GoTaq® Probe qPCR Master Mix (Promega; Madison, MI, USA), 0.15 μM of forward and reverse primers each (SARS-CoV-2 E, N1, and N2 genes, and BCoV), 0.05 μM of probe (SARS-CoV-2 E, N1, and N2 genes, and BCoV), 1–5 μL of template cDNA, and molecular grade nuclease-free water. The qPCR thermal cycling conditions started with DNA polymerase activation and initial denaturing at 95 °C (2 mins for SARS-CoV-2 N1 and N2 genes, BCoV; and 3 mins for SARS-CoV-2 E gene) and followed by 45 thermo cycles of denaturation and annealing/extension. Each thermo cycle included a denaturation step at 95 °C (3 s for SARS-CoV-2 N1 and N2 genes; 15 s for SARS-CoV-2 E gene and BCoV), and an annealing and extension step (at 55 °C and 30 s for SARS-CoV-2 N1 and N2 genes; 56 °C and 28 s for BCoV; and 58 °C and 30 s for SARS-CoV-2 E gene).

SARS-CoV-2 positive control templates (E, N1, and N2 gene fragments) were reverse transcribed and qPCR amplified from the genomic RNA of a SARS-CoV-2 strain (Isolate USA_WA1/2020; BEI Resources, Manassas, VA, USA). BCoV positive control template was generated from bovine coronavirus (BCoV) vaccine (Zoetis; Kalamazoo, MI, USA). RNA extraction (from SARS-CoV-2 and BCoV), cDNA synthesis by RT, and qPCR amplification of the target genes followed the methods described above. The RT-qPCR amplicon sizes were confirmed by 1.5 % agarose gel electrophoresis and illustration by an UVP GelStudio (Analytik Jena; Upland, CA, USA). Target DNA amplicons were excised and extracted using a QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA), and quantified using Qubit™ 1 × dsDNA HS Assay Kit with a Qubit 4 Fluorometer (Invitrogen; Carlsbad, CA, USA). The RT-qPCR standard curves were generated using ten-fold serial dilutions of the target template DNAs (10^1 to 10^6 copies per reaction). The amplification efficiencies were 98.0 % with an R^2 value of 1.00 for the E gene assay (slope = −3.37; y intercept = 39.67), 91.1 % with an R^2 value of 0.98 for the N1 assay (slope = −3.56; y intercept = 39.85), 91.2 % with an R^2 value of 0.99 for the N2 assay (slope = −3.55; y intercept = 39.89), 101 % with an R^2 value of 0.99 for the BCoV assay (slope = −3.30; y intercept = 38.90).

2.5. Quality assurance and data analysis

Undiluted and ten-fold diluted of RNA (from BCoV and wastewater spiked with BCoV, in both solid and liquid fractions) was tested to check for potential RT-qPCR inhibition and/or competition (Graham et al., 2021). Data showed that dilutions at the 10 ng/μL and 1 ng/μL levels used for RT-qPCR exhibited good recovery of BCoV, but the latter showed higher variations amongst replicates. Hence, the RNA concentrations in the samples in this study were all diluted to 10 ng/μL in RT-qPCR to minimize potential inhibition and/or competition.

Each batch of RT-qPCR reactions for each gene assay contained at least one positive control and three non-template controls (NTCs). The results were accepted only when the positive control yield anticipated C_t values based on established calibration curves and all NTCs yield negative results (i.e., $C_t > 40$). For each sample and target gene combination, duplicate RT-qPCR reactions were performed, and the arithmetic mean C_t values and the standard deviation (s_x) were used for subsequent analysis. Dry weight of solid fractions was calculated by determining their water content by measuring the weight difference of aliquots before and after oven drying at 120 °C overnight.

The recovery efficiency of the seeded BCoV by the concentration methods was calculated based upon the GC quantified per GC seeded as follow:

$$\text{Recovery Efficiency } (\%) = \frac{\text{Total BCoV viral RNA GC recovered}}{\text{Total BCoV viral RNA GC seeded}} \times 100$$

The mean and standard deviation of recovery efficiency for each concentration methods were calculated and used to plot graphs. One-way analysis of variance (ANOVA) was used to determine if significant differences in BCoV recovery exist among the concentration methods tested and SARS-CoV-2 E, N1, and N2 gene quantities. Least significant difference (LSD) test was used for post-hoc evaluation ($p = 0.05$) in SPSS ver 16.0 (IBM; Armonk, NY, USA). Graphs were generated using the Seaborn Python package in Jupyter notebook.

3. Results

3.1. Total RNA eluted by different concentration methods

The total amount of solids concentrated and RNA extracted from wastewater showed significant differences amongst the five treatment processes (Fig. S2). The AHAP method showed the highest average concentration solid of 2.91 mg/mL, which is 3.5, 3.3, 8.8, and 4.9-fold significantly higher ($p < 0.001$) than the solid recovered by Methods DC, ZAP, SMF, and FCP, respectively (Fig. S2-A). Although the high solid mass recovered may also be contributed by the coagulants and flocculants used in the methods, total RNA eluted from Method AHAP was also significantly higher ($p < 0.001$) than those by the other methods (Fig. S2-B). The eluted total RNA concentration by Method AHAP was 0.711 μg/mL (s_x =0.165, n = 3), which is 4.7, 4.4, 2.5, and 3.1-fold higher than the total RNA recovered by DC (0.153 μg/mL), ZAP (0.163 μg/mL), SMF (0.280 μg/mL), and FCP (0.230 μg/mL), respectively. Overall, the amount of total RNA recovered from the liquid fractions after wastewater solid removal by the different methods (DC: 0.043 μg/mL ZAP: 0.012 μg/mL; AHAP: 0.014 μg/mL; SMF: 0.016 μg/mL; FCP: 0.048 μg/mL) was significantly lower ($p < 0.001$) than those recovered from the solid fractions.

3.2. Recovery of seeded BCoV by different concentration methods

Recovery efficiencies of enveloped viruses by the different solid concentration methods were determined based on recovery of the seeded BCoV. The total BCoV recovered in solid fractions ranged from 11.7 % to 50.2 % of the total seeded BCoV gene copies, while only was 0.1–9.8 % recovered in the liquid fractions after treatment by the solid concentration methods (Fig. 1). The recovery of BCoV in solid fractions was significantly higher ($p = 0.011$) than in liquid fractions. In the solid fractions, AHAP showed the highest recovery rate of BCoV (50.2 %), followed by FCP (35.7 %), DC (34.3 %), SMF (18.1 %) ZAP (11.7 %). BCoV recovery in the solid fractions from the AHAP method was significantly higher than the ZAP method ($p = 0.023$) and the SMF method ($p = 0.049$), while no significant difference was observed between the AHAP method and DC ($p = 0.29$) and FCP ($p = 0.34$) due to large variations as indicated by the standard deviation of triplicate experiments. Unlike the high percentage of recovery in the solid fractions, the recovery of BCoV in the liquid fractions by PEG precipitation after solid removal by the AHAP treatment was only (0.38 %). This was higher than the recovery by ZAP (0.1 %) and lower than the recovery in SMF (1.57 %) but without statistical significance (ZAP: $p = 0.87$; SMF, $p = 0.53$), and was significantly lower than the recovery in liquid fractions by DC (9.8 %, $p < 0.001$) and FCP (7.9 %, $p = 0.002$) methods. This indicates that the AHAP method concentrated and recovered significant higher portion of the seeded BCoV viral gene copies into the solid fractions, and left a low residual BCoV gene copies in the supernatant after centrifugation.

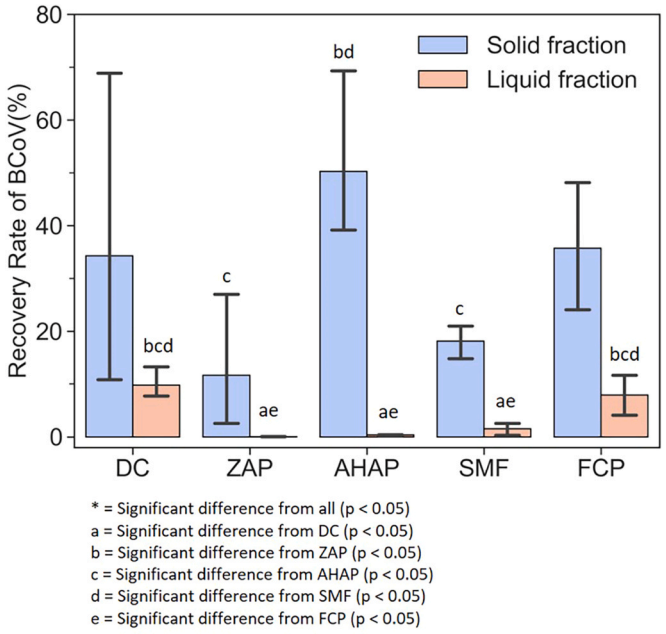

Fig. 1. Percentage of seeded BCoV recovered from the solid fraction by the five different concentration methods and the percentages remaining in the liquid fraction after treatment.

3.3. SARS-CoV-2 quantification

The performance of the five methods in concentrating SARS-CoV-2 viral RNA from the wastewater samples was evaluated by using the N1, N2, and E gene assays (Fig. 2). The highest N1 gene level (Fig. 2-A) was detected in the solid fractions generated by the AHAP method (3.3×10^4 GC/mL), which was significantly higher than the N1 gene level detected in the solid fractions from all other methods: SMF (1.3×10^4 GC/mL; $p = 0.019$), FCP (1.1×10^4 GC/mL; $p = 0.011$), ZAP (4.6×10^3 GC/mL; $p = 0.003$), and DC (2.1×10^3 GC/mL; $p = 0.001$). Similarly, the highest N2 gene levels (Fig. 2-B) were also detected in the solid fractions from the AHAP method (3.8×10^3 GC/mL), which are significantly higher than those detected in the solid fractions from all other methods: SMF (1.5×10^3 GC/mL; $p = 0.038$), FCP (1.2×10^3 GC/mL; $p = 0.049$), DC (1.1×10^3 GC/mL; $p = 0.025$), and ZAP (5.4×10^2 GC/mL; $p = 0.019$). The E gene assay (Fig. 2-C) showed that the highest concentration was detected in the solid fractions from the SMF method (1.3×10^4 GC/mL), following by AHAP (5.6×10^3 GC/mL), ZAP (3.8×10^3 GC/mL), FCP (1.3×10^3 GC/mL), and DC (7.6×10^2 GC/mL). However, the triplicate samples by the SMF method showed a large variation in the E gene quantification, which resulted in no statistically significant difference in the results between SMF and AHAP ($p = 0.187$).

The SARS-CoV-2 RNA level detected in the liquid fractions from all wastewater concentration methods were always lower than that detected in the corresponding solid fractions. For example, the solid fractions from the AHAP method always detected higher levels of SARS-CoV-2 RNA than the corresponding liquid fractions: 33.9-fold (s_x =26.2-fold) based on the N1 assay ($p = 0.05$), 94.1-fold (s_x =274.8-fold) based on the N2 assay ($p = 0.12$), and 40.9-fold (s_x =12.8-fold) based on the E gene assay ($p = 0.05$).

3.4. Method comparison between DC and AHAP

Based on the higher BCoV recovery and shortest turnaround time determined previously (Fig. 1 and Fig. S1), the recovery of BCoV between DC and AHAP methods were compared by using six additional wastewater samples. The range of total amount of solid concentrated by AHAP method (2.91 – 5.69 mg/mL) were found to be significantly

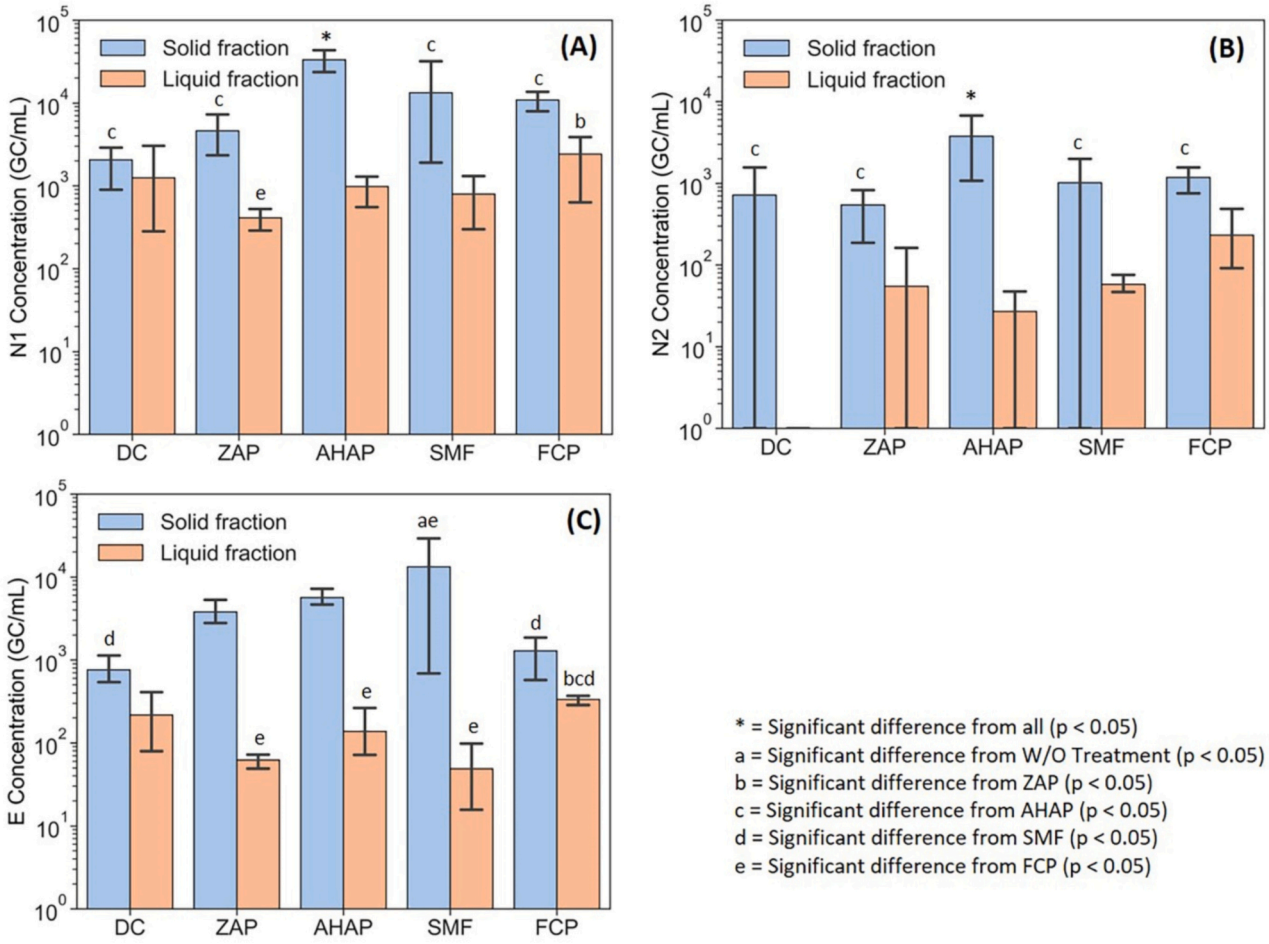

Fig. 2. Comparison of SARS-CoV-2 RNA concentration in the solid fraction of wastewater samples generated by the five different concentration methods and that remaining in the liquid fraction after treatment based on the N1 assay (A), N2 assay (B), and E gene assay (C).

higher ($p < 0.001$) than DC method (0.84 – 1.20 mg/mL) as shown in Fig. S3-A. However, the total RNA eluted from AHAP method (0.140 – 0.711 μg/mL) were not significantly different ($p = 0.282$) from DC method (0.150 – 0.220 μg/mL) (Fig. S3-B). Wastewater sample WW4 showed the highest RNA eluted was also determined to have the highest SARS-CoV-2 RNA as determined previously by Li et. al. (Li et al., 2021) (Table S1).

The overall BCoV recovery in the wastewater solid fraction was found to be significantly higher than in the liquid fraction ($p = 0.006$) (Fig. 3). In wastewater solid fraction, the range of BCoV recovery by DC method was 34.0–85.0 % and by AHAP method was 0.1–77.5 % (Fig. 3-A). On the other hand, in wastewater liquid fraction, the range of BCoV recovery by DC method was 0.03–9.8 % while by AHAP method was 0.4–7.1 % (Fig. 3-B). The recovery of BCoV by DC was found to be not significantly different from AHAP in both solid fraction ($p = 0.886$) and liquid fraction ($p = 0.790$).

Similarly, both DC and AHAP methods resulted in significantly higher SARS-CoV-2 RNA (as indicated by N2 gene copies) in wastewater solid fraction than liquid fraction ($p < 0.001$) as shown in Figs. 4 and S4. The SARS-CoV-2 N2 assay was used here due to its robust performance in our hands. The average SARS-CoV-2 N2 gene concentrated using DC method in wastewater solid fraction (3.6×10^3 GC/mL, $s_x = 1.9 \times 10^3$ GC/mL) was 22.8-fold (s_x = 13.8-fold) higher than liquid fraction (1.6×10^2 GC/mL, $s_x = 1.4 \times 10^2$ GC/mL). Furthermore, the average SARS-CoV-2 N2 gene concentrated using AHAP method in wastewater solid fraction (3.0×10^3 GC/mL, $s_x = 2.0 \times 10^3$ GC/mL) was 15.0-fold (s_x =34.5-fold) higher than liquid fraction (2.0×10^2 GC/mL, $s_x = 5.7 \times 10^1$ GC/mL).

4. Discussion

This study compared five wastewater concentration methods to recover the enveloped viral RNA, including seeded BCoV and indigenous SARS-CoV-2 RNA, rapidly and efficiently in municipal wastewater. This is important because wastewater surveillance of SARS-CoV-2 RNA has been shown to provide a useful tool in tracking the transmission of COVID-19 in human communities, as studies have shown that SARS-CoV-2 RNA in wastewater is associated with COVID-19 cases in the community from which wastewater are collected (Ahmed et al., 2020a; Graham et al., 2021; Li et al., 2021; Peccia et al., 2020). Considering the significant dilution and complexity of the wastewater matrix itself, it is crucial to have rapid and efficient concentration method in order to assess the actual SARS-CoV-2 viral load in the wastewater of communities. Understanding and accurate measurements of SARS-CoV-2 in wastewater would allow public health officials to act or develop appropriate mitigation strategies needed by the community in time.

Given the emerging evidences of dominant presence of enveloped viruses in the wastewater solid fraction (Graham et al., 2021; Li et al., 2021; Parra-Guardado et al., 2022; Ye et al., 2016), a wastewater concentration strategy that focuses on wastewater solids while recovering additional viral biomass from the liquid fraction is expected to achieve optimal recovery. In this study, the average recovery rate of the seeded BCoV by the DC method (i.e. without additional chemical assistance by adsorption, coagulation and flocculation) in the solid fraction (34.3 %) were already much higher than in the liquid fraction (9.8 %), indicating that when the enveloped viral surrogate was introduced into the wastewater, the viral particles quickly adsorbed onto wastewater solid

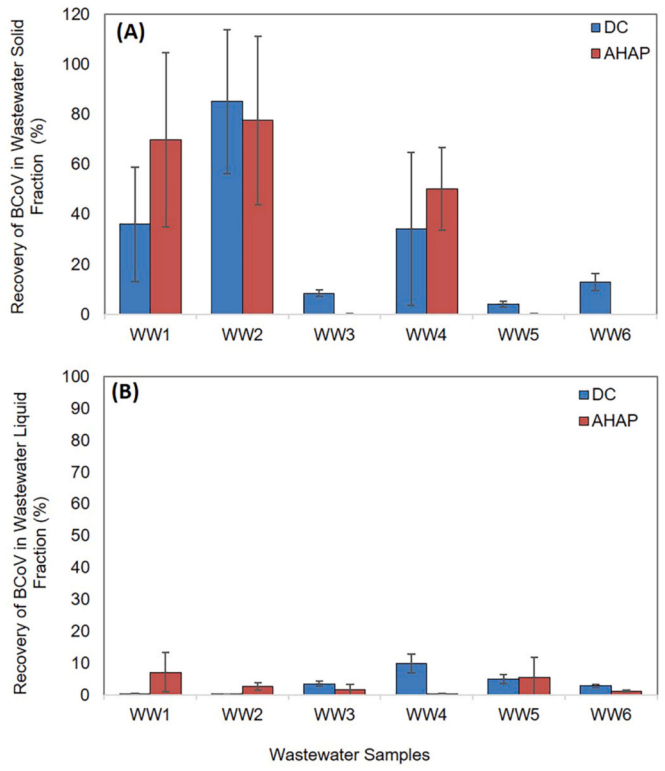

Fig. 3. Percentage of seeded BCoV recovered from the solid fraction of six wastewater samples by direct centrifugation (DC) and aluminum hydroxide adsorption-precipitation (AHAP) methods and the percentages remaining in the liquid fraction after treatment.

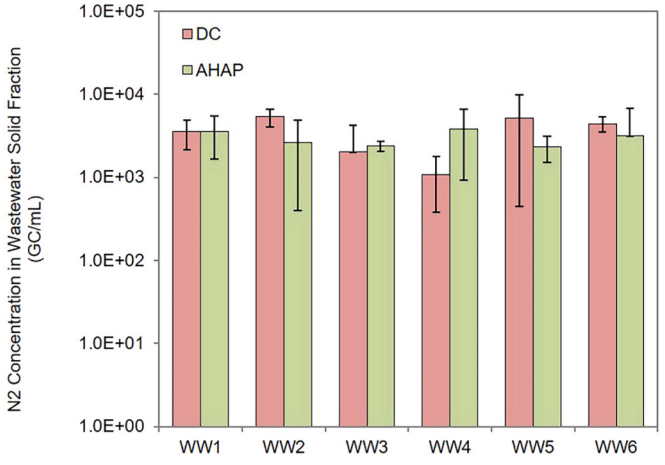

Fig. 4. Comparison of SARS-CoV-2 N2 gene concentration in the solid fraction of six wastewater samples generated by direct centrifugation (DC) and aluminum hydroxide adsorption-precipitation (AHAP) methods.

surfaces. This may be explained by the solid-liquid partition behaviors of enveloped viruses in wastewater; Ye et al. (2016) reported partition coefficients between solid and liquid fractions of wastewater to be 1500 mL/g and 1200 mL/g for seeded enveloped MHV and *Pseudomonas* Phi6 virus, respectively (Ye et al., 2016). Several previous studies showed that SARS-CoV-2 RNA exist predominantly in the solid fractions of wastewater. For example, Li et al. (2021) showed that wastewater solids generated by direct centrifugation contained 90.5 % ($s_x =$ 8.1 %) of N1 gene, 92.5 % ($s_x =$ 14.1 %) of N2 gene, and 82.5 % ($s_x =$ 19.9 %) of E gene of the total mass distribution of SARS-CoV-2 RNA (Li et al., 2021), and Graham et al. (2021) also showed that measurement ratios of N1 and N2 genes in settled primary sludge were between 320- and 3100-times higher concentrations than influent on a per mass basis (Graham et al., 2021). These observations may be primarily caused by natural embedment of the enteric virus in fecal solids (Hejkal et al., 1981; Wellings et al., 1976), although preferential partition of viral particles from liquid to solid may have also contributed to the observations.

Since majority of the enveloped viruses in wastewater already resides in the solid fraction (Graham et al., 2021; Li et al., 2021; Parra-Guardado et al., 2022; Ye et al., 2016), additional enhancement of their partition from the liquid phase to the solid phase could enable a wastewater solid-based concentration method for rapid, low cost, and optimal recovery. Previous studies have shown that the adsorption of nonenveloped enteric viruses onto solid particles were controlled primarily by electrostatic interactions and hydrophobic interactions (Armanious et al., 2016; Lytle and Routson, 1995), and partitioning behaviors of enveloped and nonenveloped viruses affect the recovery rate of these viruses from wastewater (Ye et al., 2016). Since limited information is available regarding surface properties of SARS-CoV-2 virus, including its isoelectric point, surface hydrophobicity, and their changes in response to environmental conditions such as pH or metal addition, exact physical and chemical mechanisms to enhance SARS-CoV-2 virus partition from wastewater liquid to solid remain unclear. However, the observed recovery of BCoV by the AHAP method suggests that multiple factors, including coagulation and adsorption, could enhance this process. Aluminum chloride is a widely-used chemical coagulant, which enhances the aggregation of colloidal particles in wastewater through charge neutralization of negatively charged colloids and incorporation of particles in an amorphous hydroxide precipitate (Duan and Gregory, 2003). This is support by the observation of AHAP method generating the largest amount of solid from the wastewater samples (Fig. S2-A and Fig. S3-A). Some previous studies also noted that addition of aluminum ions can enhance viral adsorption to solid substrates (Lukasik et al., 2000).

In this study, when compared between DC and AHAP methods in additional wastewater samples, the BCoV and SARS-CoV-2 quantifications were not significantly different. Study done by Giron-Guzman *et. al.* showed that direct capture system produces better SARS-CoV-2 detection in wastewater samples compared to aluminum-based adsorption-precipitation method depending on the RT-qPCR target region (Girón-Guzmán et al., 2023). However, inhibition challenges also occur in the wastewater solid fractions. Study done by Parra-Guardado *et. al.* demonstrated that although enhanced concentration/extraction protocol increased RNA extracts and RT-qPCR detection sensitivity, it also produced false-negative results or inaccurate quantification due to the RNA extracts where it was susceptible to RT-qPCR amplification inhibition compared to direct extraction method (Parra-Guardado et al., 2022). Study done by Yu et. al. showed that the addition of enhancement increased the virus releasing steps in aluminum hydroxide precipitation concentration method when compare to beef extract elution (Yu et al., 2022), which was used in this study. Complete dissolution of the aluminum hydroxide precipitates will enhance the sufficient release of the trapped viruses, however, extreme pH conditions when dissolving aluminum hydroxide may have strong impact on virus viability and structural stability which lead to a decrease in PCR detection efficiency (Yu et al., 2022).

The highest recovery performance of the seeded BCoV by the AHAP method was further supported by the high recovery performance of SARS-CoV-2 viral RNA in the wastewater samples. BCoV was chosen as the surrogate virus to measure the recovery efficiency because it is a mammal coronavirus with presumed similar surface properties as SARS-CoV-2, easier availability, and less stringent biosafety requirements. The utility of seeded BCoV as a reliable indicator of recovery was also observed in a previous comparative study where the recovery of BCoV was found to reflect the magnitudes of recovery of N1 and N2 in the same order of methods used (LaTurner et al., 2021). Other studies have

used other enveloped viruses for either process control or for method evaluation, including MHV (Ahmed et al., 2020b), human coronavirus (HCoV) 229E (Rosa et al., 2021), human coronavirus OC43 (Philo et al., 2021), *Pseudonmonas* phage Phi6 (Sherchan et al., 2020), porcine coronavirus (PEDV) (Randazzo et al., 2020), transmissible gastroenteritis virus (TGEV) (Mlejnkova et al., 2020), bovine respiratory syncytial virus (BRSV) (Gonzalez et al., 2020), and BCoV (Gonzalez et al., 2020; Graham et al., 2021). Further investigation is needed to determine the recovery of different enveloped virus in wastewater due to the partition efficiency to wastewater solids.

Like the other methods tested, the AHAP method was also developed and tested primarily for non-enveloped viruses, and has been shown to be a reliable and efficient virus concentration method in wastewater (Rice et al., 2012). Aluminum hydroxide, a strong adsorbent and coagulant formed during hydrolysis-precipitation reactions, allows viral particle to be destabilized and aggregated from fine particulate matter into larger particulates by the intermediate polymers (Matsui et al., 2003). AHAP method has been used to concentrate viruses in sewage, achieving 1000-fold concentration of poliovirus and echovirus from wastewater when the wastewater samples collected was during a low virus concentration time of the year (Wallis and Melnick, 1967). Recovery ratios of type 1 poliovirus and enterovirus from sewage-contaminated water by using AHAP were 50 % and 40 %, respectively (Fattal et al., 1977). A study compared the recovery of human enteric viruses in influent and effluent wastewater and showed slightly higher norovirus genogroup I (NoV GI), rotavirus (RV), and astrovirus (HAstV) in AHAP method than ultracentrifugation (Randazzo et al., 2019). Hepatitis E virus (HEV) recovered by using AHAP ranged from 7.0 % to 20.5 % compared to ultracentrifugation of 8.0–16.8 % (Cuevas-Ferrando et al., 2019). PEDV used as surrogate to SARS-CoV-2 were recovered at ranges of 11 ± 3.5 % (influent) and 3.3 ± 1.6 % (effluent) by using AHAP (Randazzo et al., 2020).

When compared with the other methods in terms of processing time, costs, processing volume, and method complexity, the AHAP method also performed the best (Table 1). Since aluminum salts in water hydrolyze rapidly (Matsui et al., 2003), the total processing time to separate wastewater solids with liquids takes approximately 1 h. ZAP uses similar processing time, but exhibited significantly lower virus recovery. SMF was developed to concentrate nonenveloped virus by direct binding of the viral particle to the organic flocculants under acidic conditions, with a recommended 10 h of stirring to obtain a maximum adsorption of viruses (Calgua et al., 2008). However, studies have shown that acidification of sample to pH 4 yielded the lowest recoveries (Ahmed et al., 2020b), probably because acidification affect virus integrity and infectivity (Abdelzaher et al., 2008). FCP was also developed to increase virus recovery, preferably with overnight agitating (John et al., 2011). However, longer incubation and mixing of wastewater samples with flocculants and coagulants did not seem to increase the recoveries of enveloped virus from wastewater. Another benefit of precipitation, coagulation, and flocculation-based viral concentration methods is equipment availability and their suitability for processing large volumes of wastewater samples, which is often needed to increase the detection of certain pathogenic viruses because the concentration of target viruses in the wastewater often experience significant dilutions. Methods that rely upon ultrafiltration and ultracentrifugation may have practical limitations such as sample volume throughput and/or requirement of high-cost equipment.

Table 1
Procedural and theoretical comparison of the precipitation-, coagulation-, and flocculation-based virus concentration methods evaluated in this study.

Method Characteristics	Concentration Methods			
	ZAP	AHAP	SMF	FCP
Advantages				
1. Rapid (1 h processing time to obtain solid and liquid subsamples)	X	X		
2. Relatively inexpensive supplies and equipment	X	X	X	X
3. Large volume of wastewater can be processed (up to 1 L and more)	X	X	X	X
4. Simple and easy steps	X	X	X	X
5. The cost per sample is low	X ($0.12/g)	X ($0.06/g)	X ($0.14/g)	X ($0.41/g)
Disadvantages				
1. Time consuming (more than 16 h to obtain solid and liquid subsamples)			X	X
2. pH adjustment required		X		
3. Pellet formed is relatively low and hard to collect	X		X	X
Chemical Property				
pH during the processed (begin to end)	6.54 ± 0.05 to 6.25 ± 0.03	6.0 ± 0.0 to 6.75 ± 0.09	3.48 ± 0.21 to 4.32 ± 0.24	7.48 ± 0.03 to 7.07 ± 0.05

5. Conclusions

This study concluded that both DC and AHAP methods are equally rapid, efficient, high throughput and low-cost enveloped virus concentration methods for the municipal wastewater, including the detection of SARS-CoV-2. The high-speed centrifugation, rapid coagulants formation and high precipitants density yielded higher RNA concentration from wastewater samples and higher enveloped virus recovery (both BCoV and SARS-CoV-2), in the solid fraction of the wastewater samples. The high efficiency, short TAT, low cost, and equipment affordability would make both the DC and AHAP methods a useful tool in detecting SARS-CoV-2 RNA in municipal wastewater, which can play an important role in screening and monitoring community outbreak of COVID-19. These methods may also be beneficial to wastewater-based surveillance of other viruses of concern.

Credit authorship contribution statement

Doris Yoong Wen Di: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Bo Li:** Data curation, Formal analysis, Investigation, Software. **Min Ki Jeon:** Data curation, Formal analysis, Investigation, Software. **Tao Yan:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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.

Data Availability

Data will be made available on request.

Acknowledgements

This material is based upon work supported by the National Science Foundation under Grant No. (CBET-2027059). The authors would like to thank the Department of Environmental Service at City and County of Honolulu for wastewater sample collection, and Dr. Mark Borchardt and Susan Spencer (Agricultural Research Service, USDA) for providing the bovine coronavirus vaccine.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2023.114790.

References

Abdelzaher, A.M., Solo-Gabriele, H.M., Wright, M.E., Palmer, C.J., 2008. Sequential concentration of bacteria and viruses from marine waters using a dual membrane system. J. Environ. Qual. 37 (4), 1648–1655.

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., Verhagen, R., Smith, W.J.M., Zaugg, J., Dierens, L., Hugenholtz, P., Thomas, K.V., 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., 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., Kitajima, M., 2020b. 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., Tscharke, 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., Mueller, J.F., 2021. 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.

Ampuero, M., Valenzuela, S., Valiente-Echeverria, F., Soto-Rifo, R., Barriga, G.P., Chnaiderman, J., Rojas, C., Guajardo-Leiva, S., Díez, B., Gaggero, A., 2020. medRxiv: the preprint server for health sciences. SARS-CoV-2 Detect. Sew. Santiago Chile-Prelim. Results.

Armanious, A., Aeppli, M., Jacak, R., Refardt, D., Sigstam, T., Kohn, T., Sander, M., 2016. Viruses at solid–water interfaces: a systematic assessment of interactions driving adsorption. Environ. Sci. Technol. 50 (2), 732–743.

Balboa, S., Mauricio-Iglesias, M., Rodriguez, S., Martínez-Lamas, L., Vasallo, F.J., Regueiro, B., Lema, J.M., 2021. The fate of SARS-COV-2 in WWTPS points out the sludge line as a suitable spot for detection of COVID-19. Sci. Total Environ. 772, 145268.

Calgua, B., Mengewein, A., Grunert, A., Bofill-Mas, S., Clemente-Casares, P., Hundesa, A., Wyn-Jones, A.P., Lopez-Pila, J.M., Girones, R., 2008. Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. J. Virol. Methods 153 (2), 79–83.

Cuevas-Ferrando, E., Randazzo, W., Perez-Cataluna, A., Sanchez, G., 2019. HEV occurrence in waste and drinking water treatment plants. Front. Microbiol. 10, 2937.

Duan, J., Gregory, J., 2003. Coagulation by hydrolysing metal salts. Adv. Colloid Interface Sci. 100, 475–502.

Fattal, B., Katzenelson, E., Hostovsky, T., Shuval, H.I., 1977. Comparison of adsorption-elution methods for concentration and detection of viruses in water. Water Res. 11, 955–958.

Girón-Guzmán, I., Díaz-Reolid, A., Cuevas-Ferrando, E., Falcó, I., Cano-Jiménez, P., Comas, I., Pérez-Cataluña, A., Sánchez, G., 2023. Evaluation of two different concentration methods for surveillance of human viruses in sewage and their effects on SARS-CoV-2 sequencing. Sci. Total Environ. 862, 160914.

Gonzalez, R., Curtis, K., Bivins, A., Bibby, K., Weir, M.H., Yetka, K., Thompson, H., Keeling, D., Mitchell, J., 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., 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 (1), 488–498.

Gupta, S., Parker, J., Smits, S., Underwood, J., Dolwani, S., 2020. Persistent viral shedding of SARS-CoV-2 in faeces - a rapid review. Colorectal Dis.: Off. J. Assoc. Coloproctology Gt. Br. Irel. 22 (6), 611–620.

Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci. Total Environ. 737, 140405.

Hejkal, T.W., Wellings, F.M., Lewis, A.L., LaRock, P.A., 1981. Distribution of viruses associated with particles in waste water. Appl. Environ. Microbiol. 41 (3), 628–634.

Hjelmso, M.H., Hellmer, M., Fernandez-Cassi, X., Timoneda, N., Lukjancenko, O., Seidel, M., Elsasser, D., Aarestrup, F.M., Lofstrom, C., Bofill-Mas, S., Abril, J.F., Girones, R., Schultz, A.C., 2017. Evaluation of methods for the concentration and extraction of viruses from sewage in the context of metagenomic sequencing. PloS One 12 (1), e0170199.

Jafferali, M.H., Khatami, K., Atasoy, M., Birgersson, M., Williams, C., Cetecioglu, Z., 2021. Benchmarking virus concentration methods for quantification of SARS-CoV-2 in raw wastewater. Sci. Total Environ. 755, 142939.

John, S.G., Mendez, C.B., Deng, L., Poulos, B., Kauffman, A.K., Kern, S., Brum, J., Polz, M. F., Boyle, E.A., Sullivan, M.B., 2011. A simple and efficient method for concentration of ocean viruses by chemical flocculation. Environ. Microbiol. Rep. 3 (2), 195–202.

Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E., Rose, J.B., 2020. SARS-CoV-2 in wastewater: state of the knowledge and research needs. Sci. Total Environ. 739, 139076.

Kocamemi, B.A., Kurt, H., Hacioglu, S., Yarali, C., Saatci, A.M. and Pakdemirli, B. 2020a. First Data-Set on SARS-CoV-2 Detection for Istanbul Wastewater in Turkey. medRxiv: the preprint server for health sciences.

Kocamemi, B.A., Kurt, H., Sait, A., Sarac, F., Saatci, A.M. and Pakdemirli, B. 2020b. SARS-CoV-2 detection in Istanbul wastewater treatment plant sludges. medRxiv: the preprint server for health sciences.

La Rosa, G., Iaconelli, M., Mancini, P., Ferraro, G.B., Veneri, C., Bonadonna, L., Lucentini, L., Suffredini, E., 2020. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Sci. Total Environ. 736, 139652.

LaTurner, Z.W., Zong, D.M., Kalvapalle, P., Gamas, K.R., Terwilliger, A., Crosby, T., Ali, P., Avadhanula, V., Santos, H.H., Weesner, K., Hopkins, L., Piedra, P.A., Maresso, A.W., Stadler, L.B., 2021. Evaluating recovery, cost, and throughput of different concentration methods for SARS-CoV-2 wastewater-based epidemiology. Water Res. 197, 117043.

Li, B., Di, D.Y.W., Saingam, P., Jeon, M.K., Yan, T., 2021. Fine-scale temporal dynamics of SARS-CoV-2 RNA abundance in wastewater during A COVID-19 lockdown. Water Res. 197, 117093.

Lukasik, J., Scott, T.M., Andryshak, D., Farrah, S.R., 2000. Influence of salts on virus adsorption to microporous filters. Appl. Environ. Microbiol. 66 (7), 2914–2920.

Lytle, C.D., Routson, L.B., 1995. Minimized virus binding for tests of barrier materials. Appl. Environ. Microbiol. 61 (2), 643–649.

Matsui, Y., Matsushita, T., Sakuma, S., Gojo, T., Mamiya, T., Suzuoki, H., Inoue, T., 2003. Virus inactivation in aluminum and polyaluminum coagulation. Environ. Sci. Technol. 37 (22), 5175–5180.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. Environ. Sci. Technol. Lett. 7 (7), 511–516.

Mlejnkova, H., Sovova, K., Vasickova, P., Ocenaskova, V., Jasikova, L., Juranova, E., 2020. Preliminary study of sars-Cov-2 occurrence in wastewater in the Czech Republic. Int. J. Environ. Res. Public Health 17 (15).

Nemudryi, A., Nemudraia, A., Wiegand, T., Surya, K., Buyukyoruk, M., Cicha, C., Vanderwood, K.K., Wilkinson, R., Wiedenheft, B., 2020. Temporal detection and phylogenetic assessment of SARS-CoV-2 in municipal wastewater. Cell Rep. Med. 1 (6), 100098.

Or, I.B., Yaniv, K., Shagan, M., Ozer, E., Erster, O., Mendelson, E., Mannasse, B., Shirazi, R., Kramarsky-Winter, E., Nir, O., 2020. Regressing SARS-CoV-2 sewage measurements onto COVID-19 burden in the population: a proof-of-concept for quantitative environmental surveillance. medRxiv: the preprint server for health sciences.

Parra-Guardado, A., Sweeney, C., Hayes, E., Trueman, B., Huang, Y., Jamieson, R., Rand, J., Gagnon, G., Stoddart, A., 2022. Development of a rapid pre-concentration protocol and a magnetic beads-based RNA extraction method for SARS-CoV-2 detection in raw municipal wastewater. Environ. Sci.: Water Res. Technol. 8 (1), 47–61.

Peccia, J., Zulli, A., Brackney, D.E., Grubaugh, N.D., Kaplan, E.H., Casanovas-Massana, A., Ko, A.I., Malik, A.A., Wang, D., Wang, M., Warren, J.L., Weinberger, D. M., Arnold, W., Omer, S.B., 2020. Measurement of SARS-CoV-2 RNA in wastewater tracks community infection dynamics. Nat. Biotechnol. 38 (10), 1164–1167.

Philo, S.E., Keim, E.K., Swanstrom, R., Ong, A.Q.W., Burnor, E.A., Kossik, A.L., Harrison, J.C., Demeke, B.A., Zhou, N.A., Beck, N.K., Shirai, J.H., Meschke, J.S., 2021. A comparison of SARS-CoV-2 wastewater concentration methods for environmental surveillance. Sci. Total Environ. 760, 144215.

Prado, T., Fumian, T.M., Mannarino, C.F., Maranhão, A.G., Siqueira, M.M., Miagostovich, M.P., 2020. Preliminary results of SARS-CoV-2 detection in sewerage system in Niterói municipality, Rio de Janeiro, Brazil. Mem. do Inst. Oswaldo Cruz 115.

Randazzo, W., Piqueras, J., Evtoski, Z., Sastre, G., Sancho, R., Gonzalez, C., Sanchez, G., 2019. Interlaboratory comparative study to detect potentially infectious human enteric viruses in influent and effluent waters. Food Environ. Virol. 11 (4), 350–363.

Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simon, P., Allende, A., Sanchez, G., 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. Water Res. 181, 115942.

Rice, E.W., Bridgewater, L. and Association, A.P.H. (2012) Standard methods for the examination of water and wastewater, American public health association Washington, DC.

Rimoldi, S.G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D., Maresca, M., Longobardi, C., Mancon, A., Romeri, F., 2020. Presence and infectivity of SARS-CoV-2 virus in wastewaters and rivers. Sci. Total Environ. 744, 140911.

Rosa, G.L., Mancini, P., Ferraro, G.B., Veneri, C., Iaconelli, M., Bonadonna, L., Lucentini, L., Suffredini, E., 2021. SARS-CoV-2 has been circulating in northern Italy since December 2019: Evidence from environmental monitoring. Sci. Teh. Total Environ. 750, 141711.

Sherchan, S.P., Shahin, S., Ward, L.M., Tandukar, S., Aw, T.G., Schmitz, B., Ahmed, W., Kitajima, M., 2020. First detection of SARS-CoV-2 RNA in wastewater in North America: a study in Louisiana, USA. Sci. Total Environ. 743, 140621.

Sokol, F., Kuwert, E., Wiktor, T.J., Hummeler, K., Koprowski, H., 1968. Purification of rabies virus grown in tissue culture. J. Virol. 2 (8), 836–849.

Stadler, L.B., Ensor, K., Clark, J.R., Kalvapalle, P., LaTurner, Z.W., Mojica, L., Terwilliger, A.L., Zhuo, Y., Ali, P. and Avadhanula, V. 2020. Wastewater Analysis of SARS-CoV-2 as a Predictive Metric of Positivity Rate for a Major Metropolis. medRxiv: the preprint server for health sciences.

Wallis, C., Melnick, J.L., 1967. Concentration of viruses on aluminum and calcium salts. Am. J. Epidemiol. 85 (3), 459–468.

Wellings, F.M., Lewis, A.L., Mountain, C.W., 1976. Demonstration of solids-associated virus in wastewater and sludge. Appl. Environ. Microbiol. 31 (3), 354–358.

Wu, F., Zhang, J., Xiao, A., Gu, X., Lee, W.L., Armas, F., Kauffman, K., Hanage, W., Matus, M., Ghaeli, N., Endo, N., Duvallet, C., Poyet, M., Moniz, K., Washburne, A.D., Erickson, T.B., Chai, P.R., Thompson, J., Alm, E.J., 2020a. SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases. mSystems 5 (4).

Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian, J.H., Pei, Y.Y., Yuan, M.L., Zhang, Y.L., Dai, F.H., Liu, Y., Wang, Q.M., Zheng, J.J., Xu, L., Holmes, E.C., Zhang, Y.Z., 2020b. A new coronavirus associated with human respiratory disease in China. Nature 579 (7798), 265–269.

Wu, Y., Guo, C., Tang, L., Hong, Z., Zhou, J., Dong, X., Yin, H., Xiao, Q., Tang, Y., Qu, X., Kuang, L., Fang, X., Mishra, N., Lu, J., Shan, H., Jiang, G., Huang, X., 2020c. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. The lancet. Gastroenterol. Hepatol. 5 (5), 434–435.

Wurtzer, S., Marechal, V., Mouchel, J.M., Maday, Y., Teyssou, R., Richard, E., Almayrac, J.L., Moulin, L., 2020. Evaluation of lockdown effect on SARS-CoV-2 dynamics through viral genome quantification in waste water, Greater Paris, France, 5 March to 23 April 2020. Eur. Surveill.: Bull. Eur. sur Les. Mal. Transm. = Eur. Commun. Dis. Bull. 25 (50).

Ye, Y., Ellenberg, R.M., Graham, K.E., Wigginton, K.R., 2016. Survivability, partitioning, and recovery of enveloped viruses in untreated municipal wastewater. Environ. Sci. Technol. 50 (10), 5077–5085.

Yu, L., Tian, Z., Joshi, D.R., Yuan, L., Tuladhar, R., Zhang, Y., Yang, M., 2022. Detection of SARS-CoV-2 and other viruses in wastewater. Optim. Autom. Alum. hydroxide Adsorpt. Method Virus Conc. ACS EsT. Water 2 (11), 2175–2184.

Zhang, D., Ling, H., Huang, X., Li, J., Li, W., Yi, C., Zhang, T., Jiang, Y., He, Y., Deng, S., Zhang, X., Wang, X., Liu, Y., Li, G., Qu, J., 2020. Potential spreading risks and disinfection challenges of medical wastewater by the presence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) viral RNA in septic tanks of Fangcang Hospital. Sci. Total Environ. 741, 140445.