Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Short Communication

Development of an efficient wastewater testing protocol for high-throughput country-wide SARS-CoV-2 monitoring

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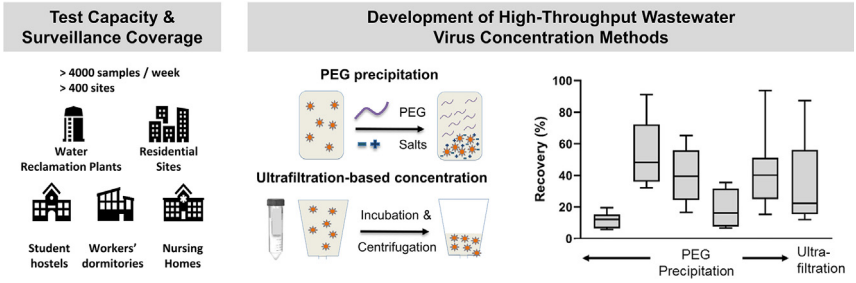
HIGHLIGHTS

- Polyethylene glycol precipitation yielded high SARS-CoV-2 recovery from wastewater.
- Ultrafiltration yielded comparable recovery and require shorter processes.
- Ultrafiltration facilitates high-throughput SARS-CoV-2 wastewater testing.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based surveillance has been widely used as a non-intrusive tool to monitor population-level transmission of COVID-19. Although various approaches are available to concentrate viruses from wastewater samples, scalable methods remain limited. Here, we sought to identify and evaluate SARS-CoV-2 virus concentration protocols for high-throughput wastewater testing. A total of twelve protocols for polyethylene glycol (PEG) precipitation and four protocols for ultrafiltration-based approaches were evaluated across two phases. The first phase entailed an initial evaluation using a small sample set, while the second phase further evaluated five protocols using wastewater samples of varying SARS-CoV-2 concentrations. Permutations in the pre-concentration, virus concentration and RNA extraction steps were evaluated. Among PEG-based methods, SARS-CoV-2 virus recovery was optimal with 1) the removal of debris prior to processing, 2) 2 h to 24 h incubation with 8% PEG at 4 °C, 3) 4000 xg or 14,000 xg centrifugation, and 4) a column-based RNA extraction method, yielding virus recovery of 42.4–52.5%. Similarly, the optimal protocol for ultrafiltration included 1) the removal of debris prior to processing, 2) ultrafiltration, and 3) a column-based RNA extraction method, yielding a recovery of 38.2%. This study also revealed that SARS-CoV-2 RNA recovery for samples with higher virus concentration were less sensitive to changes in the PEG method, but permutations in the PEG protocol

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could significantly impact virus yields when wastewater samples with lower SARS-CoV-2 RNA were used. Although both PEG precipitation and ultrafiltration methods resulted in similar SARS-CoV-2 RNA recoveries, the former method is more cost-effective while the latter method provided operational efficiency as it required a shorter turn-around-time (PEG precipitation, 9–23 h; Ultrafiltration, 5 h). The decision on which method to adopt will thus depend on the use-case for wastewater testing, and the need for cost-effectiveness, sensitivity, operational feasibility and scalability.

1. Introduction

The Coronavirus disease 2019 (COVID-19) has continued to spread worldwide, with close to 400 million infections and more than 5 million deaths recorded as of 29 January 2022. Despite the implementation of control measures globally, disease spread has not been successfully controlled (Gandhi et al., 2020). Surveillance has largely relied on testing of individuals, including testing of symptomatic persons, or screening persons in high-risk groups for early case-identification (Viswanathan et al., 2020). However, these approaches depend on health-seeking behaviours and testing regimes and may not offer objective assessment of disease transmission and burden.

Wastewater-based surveillance has emerged as a useful, non-intrusive tool for monitoring of SARS-CoV-2 community transmission, providing objective information on COVID-19 spread that can be used to complement individual case detection (Daughton, 2020; Thompson et al., 2020). Studies in Singapore (Wong et al., 2021), Netherlands (Medema et al., 2020b) and Italy (La Rosa et al., 2020), among others, have demonstrated the utility of wastewater surveillance to detect SARS-CoV-2 RNA in wastewater prior to the detection of cases. The changes in virus titre over time may also closely represent the dynamics of outbreak in the communities (Gonzalez et al., 2020; Peccia et al., 2020; Randazzo et al., 2020;Wu et al., 2020), highlighting that wastewater surveillance could be a cost-effective way to facilitate the implementation of targeted control measures or to provide situational assessment of COVID-19 spread (Medema et al., 2020a; Thompson et al., 2020).

In Singapore, the COVID-19 situation has evolved over time. A zero-COVID-19 strategy was initially adopted, where swift action was taken to identify and isolate cases when clusters were reported in the community (Lee et al., 2020) or in vulnerable or high-risk groups (Koo et al., 2022; Tan et al., 2021). Subsequently, the implementation of a successful vaccination programme facilitated the transition towards endemicity. After achieving a vaccination rate of 80% in September 2021, a phased approach was taken to relax clinical testing regimes, quarantine requirements and border restrictions (https://www.moh.gov.sg/news-highlights/details/stabilising-our-covid-19-situation-and-protecting-our-overall-healthcare-capacity_24September2021).

Wastewater surveillance for SARS-CoV-2 had been implemented across the country since April 2020 to serve as a surveillance indicator which was independent of the evolving testing regime. Surveillance objectives shifted from prompting for early case detection in the earlier zero-COVID-19 phase to providing situational assessment for targeted follow-up in the transition to endemicity. Surveillance sites include high density living premises such as workers' dormitories and student hostels, and residential apartment blocks where COVID-19 transmission is suspected (Wong et al., 2021). Popular community hubs and wide-area regional nodes (e.g. water reclamation plants) were also surveyed to provide situational assessment (https://www.straitstimes.com/singapore/wastewater-surveillance-sites-for-covid-19-to-double-by-2022-from-current-200-nea). As of January 2022, more than 400 sites have been monitored with approximately 4000 tests done per week.

To support the scale of wastewater testing in Singapore, we sought to identify an efficient SARS-CoV-2 virus concentration protocol for high-throughput testing. Various methodologies including polyethylene glycol (PEG) precipitation (Ahmed et al., 2020c; Torii et al., 2022), filtration using electronegative membranes (Ahmed et al., 2020a; Sherchan et al., 2020), ultrafiltration (Balboa et al., 2021), and ultracentrifugation (Wurtzer et al., 2021) have been used for virus concentration from wastewater samples (Kitajima et al., 2020). Based on potential scalability, automation, and adherence to high containment biosafety principles, we focused on evaluating and optimising the PEG precipitation and ultrafiltration methods. Different parameters required in the pre-concentration, virus concentration and RNA extraction process were evaluated to establish the protocols for both PEG and ultrafiltration methods. A two-phase approach was taken, where 13 methods comprising permutations in test parameters were screened in the initial phase. Test parameters and methods which yielded higher recovery were then further evaluated in the second phase.

Unlike other evaluation studies where a surrogate virus was used (Ahmed et al., 2020b; Kaya et al., 2022; Pecson et al., 2021; Torii et al., 2022), this study utilised raw SARS-CoV-2 positive wastewater samples from various sites which were processed in a high containment laboratory. This approach allowed for the direct calculation of recovery from the methods by comparing the total number of RNA copies in the raw wastewater before concentration and in the final concentrate. Our study identified operational parameters in the virus concentration process that led to higher SARS-CoV-2 RNA recovery for both PEG precipitation and ultrafiltration methods, providing an informative guide on the use of these approaches for laboratories conducting wastewater testing.

2. Methods

2.1. First phase of evaluation

2.1.1. SARS-CoV-2 positive wastewater sample

The positive sample used for the first phase of evaluation was obtained from a manhole that served a workers' dormitory with ongoing transmission. Hourly composite samples were collected for 24 h using the ISCO 3700 Full-Size Portable Sampler (Teledyne Isco Inc., USA), on 22-23 April 2020. Each hourly composite was comprised of four 200 mL samples drawn from the manhole every 15 min. The samples were transported to the laboratory at 4–8 °C. In the laboratory, nine samples which tested positive for SARS-CoV-2 RNA using a PEG precipitation-based method (PEG-H, Table 1) were pooled to create a reference wastewater sample for evaluation of the methods.

2.1.2. PEG precipitation and ultrafiltration concentration methods

PEG precipitation and ultrafiltration was conducted using nine and four modified protocols respectively, with different permutations of test parameters in the pre-concentration, concentration and RNA extraction steps (Table 1 and Table 2). Each protocol used 45 mL of SARS-CoV-2 positive wastewater sample and tests were conducted in duplicate. To compare the effect of the modified steps, we assigned one of the methods to be the base method (PEG-A, Table 1 and ULT-A, Table 2).

Both base methods begin with the removal of debris from the wastewater sample through centrifugation at 4000 $\times g$ for 30 min. For the base method for PEG precipitation (PEG-A), the supernatant was incubated with 8% PEG (Merck Group, Germany) and 0.3 M NaCl (First BASE Laboratories Sdn Bhd, Malaysia) overnight (approx. 16 h) at 4 °C on an orbital shaker at 200 rpm. Subsequently, virus precipitates were collected through centrifugation of the supernatant-PEG mixture at 4000 $\times g$ for 3 h and RNA was extracted using a modified TRIzol-QIAGEN extraction described below. Other methods involved the following modifications from the base method – inclusion of a filtering step using a 0.22 μm polyethersulfone (PES) membrane filter (Corning, New York, USA) after debris removal (PEG-B), varying either the PEG concentration (PEG-C, 20% PEG and PEG-D, 50% PEG), PEG

Table 1
First phase of evaluation - summary of nine polyethylene glycol (PEG) precipitation methods.

Parameter evaluated or metric	Method								
	PEG-A	PEG-B	PEG-C	PEG-D	PEG-E	PEG-F	PEG-G	PEG-H	PEG-I
Pre-concentration centrifugation									
Speed (×*g*)	4000	4000	4000	4000	4000	4000	4000	4000	4000
Duration (h)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pre-concentration filtration	No	Yes	No	No	No	No	Yes	No	Yes
PEG concentration (% w/v)	8	8	20	50	8	8	8	8	8
PEG incubation time (h)	~16[a]	~16	~16	~16	2	~16	~16	~16	~16
PEG precipitation centrifugation									
Speed (×*g*)	4000	4000	4000	4000	4000	14,000	14,000	4000	14,000
Duration (h)	3	3	3	3	3	1.5	1.5	3	1.5
RNA extraction method	TRIzol-QIAGEN	TRIzol-QIAGEN	TRIzol-QIAGEN	TRIzol- QIAGEN	TRIzol-QIAGEN	TRIzol-QIAGEN	TRIzol-QIAGEN	QIAGEN	QIAGEN

[a] Overnight ~16 h.

incubation time (PEG-E, 2 h), or centrifugation speed (PEG-F, 14,000 ×*g* for 1.5 h) for virus precipitation, and varying the RNA extraction method (PEG-H, QIAGEN) (Table 1). A combination of the above-mentioned methods was also evaluated (PEG-G, PEG-I) (Table 1).

For the ultrafiltration base method (ULT-A), 15 mL of the centrifugation supernatant was passed through an Amicon Ultra-15 (molecular weight cut-off 30 kDa) centrifugal ultrafiltration filter unit (Merck Group, Germany) at 2000 ×*g* for 5 min. This step was repeated two additional times using the same ultrafiltration filter unit to process the total sample volume of 45 mL. RNA was extracted from the virus concentrate using the QIAmp Viral RNA Mini kit (QIAGEN Group, Germany). Variation of this base method modified (ULT-B) or eliminated (ULT-C) the centrifugation step to remove debris or modified the RNA extraction method (ULT-D) (Table 2).

For comparability, all methods for PEG concentration and ultrafiltration were conducted in parallel on the same day. To account for sample degradation during storage and to compare the recovery of various methods, SARS-CoV-2 RNA concentration of the raw wastewater sample was determined on the same day of processing through direct RNA extraction of the wastewater sample using QIAGEN method and the molecular assays described below.

2.2. RNA extraction

RNA was extracted using two methods. “QIAGEN”, was carried out using QIAmp Viral RNA Mini kit (QIAGEN, Hilden, Germany). Virus precipitates from the supernatant-PEG mixture were resuspended in 500 μL of phosphate buffer saline (PBS) from which 140 μL was used for RNA extraction. For the ultrafiltration methods, the total retentate volume of approximately 140 μL was used. QIAGEN RNA extraction was conducted following the manufacturer's protocol with a final RNA elution volume of 60 μL.

“TRIzol-QIAGEN”, included TRIzol Reagent as the lysis agent before proceeding to RNA extraction of the aqueous TRIzol lysate using the QIAmp Viral RNA Mini kit (QIAGEN, Hilden, Germany). Briefly, 1 mL of TRIzol was added to the pellet or 140 μL of retentate and processed according to the manufacturer's protocol until the aqueous phase containing RNA was obtained. The aqueous phase was directly added to the QIAamp Mini column. After centrifugation and disposal of the flow through, the column was washed with Buffer AW1 and Buffer AW2 according to the manufacturer's protocol before a final elution with 60 μL Buffer AVE.

Table 2
First phase of evaluation - summary of four ultrafiltration precipitation methods.

Parameter evaluated or metric	Method			
	ULT-A	ULT-B	ULT-C	ULT-D
Pre-concentration centrifugation				
Speed (×*g*)	4000	14,000	No centrifugation	4000
Duration (h)	0.5	0.5		0.5
Ultrafiltration centrifugation				
Speed (×*g*)	2000	2000	2000	2000
Duration (h)	0.12	0.12	0.12	0.12
RNA Extraction	QIAGEN	QIAGEN	QIAGEN	TRIzol-QIAGEN

2.3. Molecular testing

SARS-CoV-2 RNA was detected using a single-plex, real-time quantitative polymerase chain reaction (RT-qPCR) protocol using oligonucleotides and probe described elsewhere (Niu et al., 2020). The single-step SARS-CoV-2 assay mixture contained 1X Luna Universal Probe One-Step Reaction Mix (NEB, USA), 1X Luna WarmStart RT Enzyme Mix, 0.5 μM of each primer, 0.25 μM of the probe and 2.5 μL of the template in a final reaction volume of 20 μL. The amplification protocol included a 10 min reverse transcription step at 55 °C, followed by initial denaturation for 1 min at 95 °C and 45 cycles of denaturation for 10 s at 95 °C and extension for 30 s at 60 °C. The copy number of SARS-CoV-2 RNA was estimated based on a standard curve generated by using triplicates of 10-fold serial dilutions of a known copy number of SARS-CoV-2 synthetic RNA control (Twist BioScience, San Francisco, CA, USA). The dilution series ranged from 10^6 to 10^1 copies per reaction. The threshold of positive detection was set at <40 quantitative cycles (Cq). The limit of detection of the assay, determined based on Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Huggett, 2020), was 20 copies per reaction (Supplemental materials, Fig. S1).

As an additional recovery indicator, the pepper mild mottle virus (PMMoV) faecal marker (Gu et al., 2018) was included in the evaluation. PMMoV RNA was detected using a single-plex RT-qPCR protocol, using oligonucleotides and a probe described elsewhere (Symonds et al., 2019; Zhang et al., 2005). The single-step PMMoV assay mixture and thermal profile were same as that of SARS-CoV-2, except that primers and probe were used at a final concentration of 0.9 μM and 0.4 μM. The copy number of PMMoV RNA was estimated based on a standard curve generated using 10-fold serial dilutions of a known copy number of PMMoV synthetic control (Integrated DNA Technologies, Singapore). The dilution series ranged from 1.5^7–1.5^2 copies per reaction. The threshold of positive detection was set at Cq < 40. The limit of detection determined as per MIQE guidelines was 150 PMMoV RNA copies per reaction (Supplemental materials, Fig. S1).

2.4. Recovery calculation

Recovery of SARS-CoV-2 RNA was calculated as follows:

$$\text{Recovery}\ (\%) = \frac{\text{Number of copies per μL of RNA} * \text{A} * \left(\frac{\text{B}}{\text{C}}\text{mL}\right)}{\text{Number of copies per μL of RNA from raw sewage} * \text{D} * \left(\frac{\text{E}}{\text{F}}\text{mL}\right)} \times 100$$

Where, A = volume of RNA extract obtained from the PEG and Ultrafiltration samples; B = total volume of virus concentrate obtained from the virus concentration process; C = volume of virus concentrate used in the RNA

extraction step; D = volume of RNA extract obtained from raw sewage; E = total raw sewage volume used in virus concentration (45 mL); F = volume of virus concentrate used in the RNA extraction step.

2.5. Second phase of evaluation

2.5.1. SARS-CoV-2 positive wastewater samples

A total of six unique SARS-CoV-2 positive samples from surveillance sites across Singapore were used in the second phase of evaluation. The samples were collected as described in the first phase of evaluation and were pasteurized at 60 °C for 1 h in a water bath before use. Among these samples, three were of lower concentration for SARS-CoV-2 when raw wastewater was extracted without using any concentration methods (Cq: 38–39, <750 copies/mL sewage) and three had higher SARS-CoV-2 concentration (Cq: 34–36; >750 copies/mL sewage) (henceforth denoted as low and high concentration samples). The Cq range for low concentration samples was determined based on the limit of detection of the RT-qPCR assay.

2.5.2. PEG precipitation and ultrafiltration concentration methods

After the first phase of evaluation, five PEG and one ultrafiltration methods were identified for the second phase of evaluation using six SARS-CoV-2 positive samples described above (Table 3). PEG-H, the best PEG method from the first phase, was chosen as the basis for further evaluation. Methods which modified PEG-H in various steps were included: PEG-A, with a different RNA extraction step using TRIzol-QIAGEN RNA extraction; PEG-HX, with a sample filtration step through a 0.22 μm PES membrane filter (Corning, New York, USA) after debris removal; PEG-EX, with a shorter PEG incubation duration of 2 h; and PEG-IX, with a higher centrifugation speed for virus precipitation at 14,000 ×*g* for 1.5 h (Table 3). As the ultrafiltration methods had similar recoveries (Table 2), the ultrafiltration base method (ULT-A) was used to compare with PEG methods evaluated in the second phase of evaluation (Table 3).

2.6. Statistical analysis

The average number of copies and recovery efficiency with range of concentrations were determined. For the second phase, Analysis of Variance (ANOVA) (Supplemental materials, Table S2, S4, S6) and subsequent Tukey's honestly significant difference (HSD) test was done to compare different virus concentration methods using wastewater samples with high and low SARS-CoV-2 concentration. For PMMoV, Tukey HSD was conducted for all samples combined. *P*-values less than 0.05 were considered significant. All statistical tests were performed using R software version 3.6.3. Figures were generated using Graphpad Prism software version 9.3.1.

3. Results

3.1. First phase of evaluation

3.1.1. PEG precipitation recovery

The base method, PEG-A, which included debris removal, 16 h overnight incubation with 8% PEG, centrifugation at 4000 ×*g* for 3 h and TRIzol-QIAGEN RNA extraction yielded a SARS-CoV-2 RNA recovery of 35.7% (Table 4). PEG-B, with an additional filtration step prior to PEG precipitation, decreased SARS-CoV-2 RNA yield from 35.7% to 6.4% (Table 4). An increase in PEG concentration from 8% to 20% in PEG-C gave a similar yield of 44.7% compared to PEG-A. However, further increase of PEG concentration to 50% in PEG-D significantly lowered the yield to 9.3% (Table 4).

A shorter incubation time of 2 h for PEG precipitation in PEG-E resulted in decreased SARS-CoV-2 RNA recovery of 26.8% when compared with PEG-A (Table 4). The use of a higher centrifuge speed for PEG-F showed that centrifugation of virus precipitates at 14000 ×*g* for 1.5 h gave similar yields to 4000×*g* for 3 h (PEG-F: 33.1% vs PEG-A: 35.7%, respectively) (Table 4). This result was further substantiated by comparing PEG-G to PEG-B (PEG-G: 6.9% vs PEG-B: 6.4%, respectively), both of which had different centrifugation speeds but included pre-concentration filtration (Table 4).

PEG-H, which utilizes QIAGEN instead of TRIzol-QIAGEN for RNA extraction, yielded the highest SARS-CoV-2 RNA recovery of 59.5% among all PEG methods evaluated in the first phase. PEG-I which was similar to PEG-H but included additional filtration step and higher centrifugation speeds yielded a recovery of 57.7% (Table 4).

These findings suggested that increasing PEG concentration, varying centrifugation speeds and using TRIzol-QIAGEN for RNA extraction did not improve SARS-CoV-2 RNA recovery. Discordant results were observed with the introduction of a pre-filtration step. Although PEG-I yielded good recovery, methods PEG-B and PEG-G had significantly lower yields. A slight decline in yield was observed when the PEG incubation time was decreased from 16 h to 2 h.

Results from the first phase of evaluation formed the basis for the second phase of evaluation where selected parameters were further evaluated using a total of six samples with low (n = 3) and high (n = 3) SARS-CoV-2 concentrations for more robust comparisons. Among PEG precipitation methods, the best performing PEG-H method which utilised QIAGEN RNA extraction was evaluated. Methods with modifications in sample pre-filtration (PEG-HX), PEG incubation duration (PEG-EX), PEG centrifugation speed (PEG-IX) and RNA extraction (PEG-B) were also compared.

3.1.2. Ultrafiltration recovery

SARS-CoV-2 RNA recovery for all four ultrafiltration methods were not significantly different, with only slight differences in the recovery values

Table 3
Wastewater virus concentration methods for the second phase of evaluation.

Parameter evaluated or metric	Method					
	PEG-A	PEG-H	PEG-EX	PEG-HX	PEG-IX	ULT A
Pre-concentration centrifugation						
Speed (×g)	4000	4000	4000	4000	4000	4000
Duration (h)	0.5	0.5	0.5	0.5	0.5	0.5
Pre-concentration filtration	No	No	No	Yes	No	No
PEG concentration (% w/v)	8	8	8	8	8	NA
PEG incubation time (h)	~16[a]	~16	2	~16	~16	NA
PEG precipitation centrifugation						
Speed (×g)	4000	4000	4000	4000	14,000	NA
Duration (h)	3	3	3	3	1.5	NA
Ultrafiltration centrifugation						
Speed (×g)	NA	NA	NA	NA	NA	2000
Duration (h)	NA	NA	NA	NA	NA	0.12
RNA extraction method	TRIzol- QIAGEN	QIAGEN	QIAGEN	QIAGEN	QIAGEN	QIAGEN

[a] Overnight ~16 h.

Table 4
Comparison of recovery efficiencies for SARS-CoV-2 and PMMOV using nine polyethylene glycol (PEG) precipitation methods.

Parameter evaluated or metric	Method								
	PEG-A	PEG-B	PEG-C	PEG-D	PEG-E	PEG-F	PEG-G	PEG-H	PEG-I
Mean SARS-CoV-2 concentration as $\log_{10}$ RNA copies (range)[a]	5.30 (5.18, 5.42)	4.55 (4.46, 4.64)	5.40 (5.33, 5.47)	4.72 (3.78, 5.66)	5.18 (5.01, 5.35)	5.27 (5.18, 5.36)	4.59 (4.52, 4.66)	5.52 (5.44, 5.60)	5.51 (5.30, 5.72)
Mean percent recovery of SARS-CoV-2 RNA copies (range)[a]	35.7 (25.9, 45.5)	6.4 (5.1, 7.7)	44.7 (37.6, 51.8)	9.3 (18.3, 0.2)	26.8 (16.7, 36.9)	33.1 (26.2, 40.0)	6.9 (5.8, 8.0)	59.5 (49.1, 59.9)	57.7 (31.3, 84.1)
Mean PMMoV concentration as $\log_{10}$ RNA copies (range)[a]	6.92 (7.03, 6.76)	5.88 (5.93, 5.83)	6.93 (7.03, 6.81)	6.32 (6.43, 6.17)	6.59 (6.75, 6.34)	6.72 (6.96, 6.17)	6.35 (6.38, 6.32)	7.15 (7.17, 7.12)	7.20 (7.33, 7.00)
Mean percent recovery of PMMoV RNA copies (range)[a]	25.0 (17.4, 32.6)	2.3 (2.0, 2.5)	25.7 (19.4, 31.9)	6.3 (4.4, 8.2)	11.7 (6.5, 16.8)	15.8 (4.4, 27.2)	6.8 (6.3, 7.2)	42.1 (39.8, 44.3)	47.5 (30.0, 65.0)

[a] n = 2.

(Table 5). Method ULT-A which included a debris removal preconcentration step and QIAGEN RNA extraction yielded SARS-CoV-2 RNA recovery of 14.5%. RNA recovery reduced slightly when the centrifugation speed for debris removal in method ULT-B (10.0%) (Table 5). Consistent with findings for PEG-based methods, the modified TRIzol-QIAGEN RNA extraction method also had a lower recovery of 10.2% (Table 5). Similar results were also observed for PMMOV with ULT-C having the highest yield, followed by ULT-A and ULT-B methods. As these recovery values were not significantly different, the base method ULT-A was used in the second phase of evaluation.

3.2. Second phase of evaluation

3.2.1. Wastewater samples with high SARS-CoV-2 concentration

When wastewater samples with high SARS-CoV-2 concentration were used, methods PEG-EX (52.5%), PEG-H (43.2%) and PEG-IX (42.4%), yielded high and comparable recoveries (Fig. 1, Tables S1 and S3). These findings suggest that the recovery efficiency for wastewater samples with high SARS-CoV-2 concentration were less likely to be influenced by changes in PEG incubation time (PEG-EX) or PEG centrifugation speeds (PEG-IX). Consistent with the results from the first phase, significantly lower SARS-CoV-2 RNA recovery was obtained when the TRIzol-QIAGEN RNA extraction method was used (PEG-A, 11.5%) or when an additional step of filtration after initial centrifugation was included (PEG-HX, 19.5%) (Fig. 1, Table S3). Although the SARS-CoV-2 recovery for PEG-A was significantly lower than PEG-EX, PEG-H and PEG-IX, PEG-HX was only significantly lower than PEG-EX and comparable to PEG-H and PEG-IX. The ultrafiltration method, ULT-A, yielded a recovery of 38.2% and was comparable to all five PEG methods (Fig. 1, Table S3).

3.2.2. Wastewater samples with low SARS-CoV-2 concentration

In general, lower SARS-CoV-2 RNA recoveries (4.0%–19.1%) were obtained when wastewater samples with low SARS-CoV-2 concentration were tested (Fig. 2, Table S1). Among methods evaluated, ULT-A and PEG-HX yielded higher and comparable SARS-CoV-2 RNA recoveries of 19.1% and 16.2%, respectively (Fig. 2, Table S1). Of note, although method PEG-HX (16.2%) with additional filtration step had a slightly higher recovery compared to PEG-H (8.4%), the difference was not statistically significant (Table S5). Significant differences were observed between ULT-A and all methods except PEG-HX, and PEG-HX with PEG-A, PEG-EX and PEG-IX (Table S5).

3.2.3. Pepper mild mottle virus recovery

Among methods evaluated in the second phase, the recovery of PMMoV from wastewater samples differed slightly from SARS-CoV-2. Three better-performing methods, namely, ULT-A (57.6%), PEG-HX (44.4%), and PEG-H (44.2%) had comparable PMMoV RNA recoveries which were not statistically significant from each other (Fig. 3). Among these three methods, ULT-A had statistically significant higher yield than PEG-A, PEG-EX and PEG-IX, while PEG-H and PEG-HX had significantly higher yields than PEG-A only (Table S7).

4. Discussion

Despite the advancement and implementation of wastewater surveillance for SARS-CoV-2 since the beginning of the COVID-19 pandemic, there are still uncertainties surrounding the methodology used for virus concentration (Ahmed et al., 2020d; Pino et al., 2021). This study investigated the effectiveness of two viral concentration methods, namely PEG precipitation and ultrafiltration, for the detection of SARS-CoV-2 in wastewater. Key steps in the virus concentration process for each approach were evaluated with an aim to identify parameters which could improve the recovery of SARS-CoV-2 from wastewater samples and to develop a protocol for large-scale wastewater testing. A range of wastewater samples from different sites and with varying SARS-CoV-2 concentrations were used to

Table 5
Comparison of recovery efficiencies for SARS-CoV-2 and PMMOV using four ultrafiltration methods.

Parameter evaluated or metric	Method			
	ULT-A	ULT-B	ULT-C	ULT-D
Mean of $\log_{10}$ of SARS-CoV-2 RNA copies (range)[a]	4.97 (4.77, 5.17)	4.81 (4.78, 4.84)	5.10 (5.09, 5.11)	4.82 (4.68, 4.96)
Mean recovery of SARS-CoV-2 RNA copies (%, range)[a]	14.5 (8.3, 20.7)	10.0 (9.2, 10.8)	19.5 (19.2, 19.8)	10.2 (7.0, 13.4)
Mean $\log_{10}$ of PMMoV RNA copies (range)[a]	7.18 (6.68, 7.68)	7.19 (5.94, 8.44)	7.28 (6.38, 8.18)	6.87 (6.38, 7.36)
Mean recovery of PMMoV RNA copies (%, range)[a]	29.1 (25.8, 32.4)	29.5 (29.4, 29.6)	36.7 (36.1, 37.3)	14.3 (12.6, 16.0)

[a] n = 2.

provide invaluable information of the performance characteristics of these methods.

The PEG precipitation method consisted of four steps: 1) pre-concentration; 2) PEG addition and incubation; 3) PEG precipitation; 4) RNA extraction. Based on the series of modifications for the PEG precipitation methods, we examined the effects of each step on SARS-CoV-2 recovery. Result from the first and the second phases of evaluation reveal that several PEG precipitation methods could be effective in the concentration of SARS-CoV-2 from wastewater samples with both low and high SARS-CoV-2 concentration. SARS-CoV-2 RNA recovery for wastewater samples with higher virus concentration were less sensitive to changes in the PEG precipitation method, with three of five methods yielding similar recoveries of 42.4–52.5%. In contrast, permutations in the PEG protocol resulted in significantly different yields when wastewater samples with lower SARS-CoV-2 RNA was used, and with lower SARS-CoV-2 recoveries of 8.4–16.2% for the better-performing methods. These findings were generally consistent with the results reported by Pino et al. (Pino et al., 2021), where uncertainties in virus recovery were observed at lower virus concentrations. In that study, the PEG precipitation protocol evaluated also yielded higher recoveries for wastewater samples with high and medium virus concentration (22.8–30%) when compared with the recovery for samples with low virus concentration (9.6%) (Pino et al., 2021). Similar recoveries for PEG precipitation methods of 7.4–59.5% were also reported in other studies (Barril et al., 2021; Falman et al., 2019; Flood et al., 2021).

Initial centrifugation to remove debris in wastewater was sufficient in yielding optimal SARS-CoV-2 recovery for samples with high virus concentration but an additional filtration step seemed to yield slightly higher SARS-CoV-2 recovery for samples with low virus concentration, although not statistically significant. This step removed finer particles which could inhibit the subsequent PCR process (Ahmed et al., 2021; Gallardo-Escárate et al., 2020) and may have improved virus recovery for samples nearer the limit of detection.

Comparable viral yields were obtained using a PEG concentration of either 8% or 20%, suggesting that a PEG concentration of 8% is both cost effective and sufficient for virus exclusion and subsequent precipitation. Increasing PEG concentration to 50% however significantly lowered SARS-CoV-2 recovery in our settings. This could be due to the higher viscosity of the supernatant-PEG solution where removal of the viscous supernatant in the final step may have also removed virus precipitates. Although an increase in PEG concentration may theoretically improve SARS-CoV-2 recovery due to the higher hydrophobicity provided for virus precipitation (Khan et al., 2021), our study revealed that the use of 8–10% PEG concentration described in most studies would be sufficient for virus concentration from wastewater samples (Bar-Or et al., 2021; Kaya et al., 2022; Kevill et al., 2022).

Similar to observations for sample pre-filtration, a shorter incubation duration for PEG precipitation did not affect SARS-CoV-2 recovery for wastewater samples with high SARS-CoV-2 concentration, but a lower yield was recorded when samples with low SARS-CoV-2 concentration were used. In some studies, shortening the incubation period for PEG precipitation did not impact virus recovery (Trujillo et al., 2021) although others have found that longer incubation periods could improve recovery (Flood et al., 2021). The differences observed in these studies could possibly be explained by our findings, where longer incubation periods had no impact on virus recovery for samples with higher virus concentration but may improve recoveries for samples with lower virus concentrations.

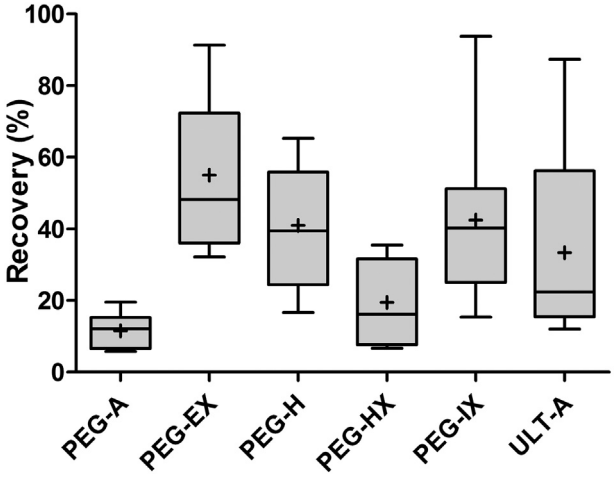

Fig. 1. Second phase of evaluation – SARS-CoV-2 RNA recovery (%) using three wastewater samples with high SARS-CoV-2 concentration (n = 3). PEG-EX, PEG-H and PEG-IX yielded higher recoveries of 42.4–52.5%. The ultrafiltration method, ULT-A, yielded comparable recovery to all five PEG methods. The boxplots represent lower and upper quartile data with median value, with whiskers reflecting the 5%–95% percentile. The 'plus' symbol (+) indicates the average SARS-CoV-2 RNA recovery. Samples were tested in triplicate for each method evaluated.

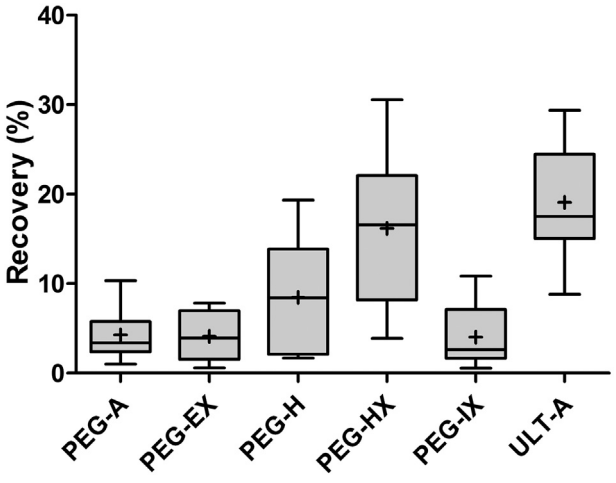

Fig. 2. Second phase of evaluation – SARS-CoV-2 RNA recovery (%) using three wastewater samples with low SARS-CoV-2 concentration (n = 3). ULT-A and PEG-HX yielded higher recoveries of 19.1% and 16.2%, respectively The boxplots represent lower and upper quartile data with median value, with whiskers reflecting the 5%–95% percentile. The 'plus' symbol (+) indicates the average SARS-CoV-2 RNA recovery. Samples were tested in triplicate for each method evaluated.

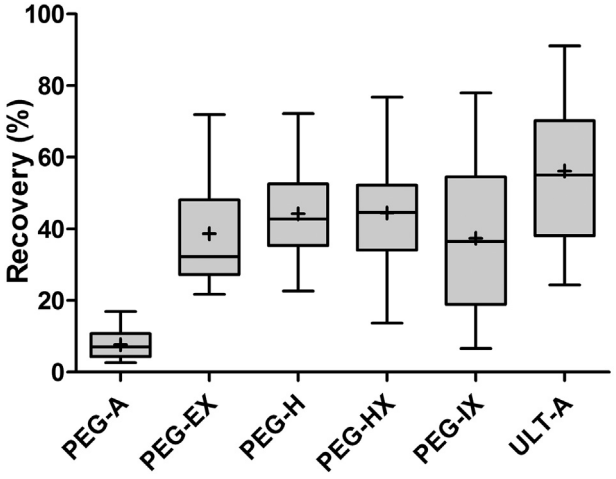

Fig. 3. Second phase of evaluation – PMMoV RNA recovery (%) using all six wastewater samples (n = 6). ULT-A, PEG-HX and PEG-H yielded the highest recoveries of 44.2–57.6%. Among PEG based methods, only PEG-A had significantly lower yield. The boxplots represent lower and upper quartile data with median value, with whiskers reflecting the 5%–95% percentile. Outliers are indicated by points. The 'plus' symbol (+) indicates the average PMMoV RNA recovery. Samples were tested in triplicate for each method evaluated.

The use of different centrifugation speeds for virus precipitation at 4000 ×g or 14,000 ×g consistently yielded similar virus recoveries throughout both phases. This finding is important as centrifugation above 10,000 ×g is part of most published PEG protocols (Ahmed et al., 2020b; Kaya et al., 2022; Kumar et al., 2020; Wu et al., 2020), but is not feasible for large-scale centrifugation in many laboratories without high-speed centrifuges. The use of lower speed centrifuges, which are available in most laboratories will make wastewater testing more accessible. These centrifuges also typically accommodate high number of samples. Additionally, low centrifugal speeds can be achieved with swing rotors, which accumulate the virus precipitate into a visible pellet at the bottom of tubes, facilitating ease of sample recovery. This contrasts with higher centrifugal speeds that are only achieved by fixed angle centrifuge rotors, which cause the precipitate to collect along the vertical axis of the sample tube, rendering it less visible and harder to reconstitute, potentially leading to loss of virus precipitates and lower yields.

The TRIzol step prior to QIAGEN purification of RNA was included to remove inhibitors and over concerns of insufficient viral lysis by the AVL buffer used in the QIAGEN kit (Ngo et al., 2017). The method with TRIzol consistently yielded lowest recovery rates than all other methods in both phases of evaluation, suggested the redundancy of TRIzol when QIAGEN RNA extraction is used. Torri et al. (Torii et al., 2022), which utilised a similar TRIzol-QIAGEN RNA extraction method in the concentration of surrogate viruses from wastewater, also reported lower virus recovery compared to previously reported yields. Our findings thus highlights the importance of the RNA extraction step in wastewater virus concentration and corroborates other studies where RNA extraction via a column based method was effective in yielding optimal virus recoveries (Dumke et al., 2021; Flood et al., 2021).

In the first phase of evaluation, SARS-CoV-2 RNA recovery efficiencies for all four ultrafiltration methods were not significantly different, and the base method with an initial centrifugation at 4000 ×g, ultrafiltration and RNA extraction using QIAGEN RNA extraction was used for subsequent evaluation. Subsequently, when more samples were tested in the second phase, the ultrafiltration method produced comparable recovery rates to better-performing PEG protocols for wastewater samples with higher SARS-CoV-2 concentration and had the highest yield for samples with lower SARS-CoV-2 concentration.

In conclusion, our study showed that the yield of PEG precipitation protocols remains acceptable with the basics of removal of debris, 2–16 h incubation with 8% PEG at 4 °C, 4000 ×g or 14,000 ×g centrifugation and use of the QIAGEN method for RNA purification. Additionally, a relatively simple ultrafiltration method with initial removal of debris, concentration and RNA extraction performed similarly or better than PEG methods. Among the better-performing PEG precipitation and ultrafiltration methods, similar recoveries were obtained for either enveloped SARS-CoV-2 or non-enveloped PMMoV. Although the focus of this study was to identify methods which were suitable for SARS-CoV-2 concentration from wastewater, the findings suggest that these methods could also be used for the concentration of other viruses.

4.1. Considerations for an optimal wastewater testing protocol

The selection of a suitable wastewater testing protocol is highly dependent on the objectives and resources available for a wastewater surveillance programme. PEG precipitation methods yielded similar SARS-CoV-2 RNA recovery compared to ultrafiltration methods, but the latter offered ease of processing and shorter sample processing duration. Although the turn-around-time for PEG precipitation methods were typically 4 h (PEG-E) to 18 h (all other PEG methods) longer than for ultrafiltration methods, PEG precipitation methods may be a suitable choice for wastewater testing in resource-limited settings, especially since single-use ultrafiltration devices may cost around USD $10–15 per device (LaTurner et al., 2021; Trujillo et al., 2021). In addition, PEG-precipitation methods are less likely to face global supply chain shortages associated with ultrafiltration devices.

In Singapore, both PEG precipitation and ultrafiltration methods have been employed in the country-wide wastewater surveillance programme. In scenarios where wastewater surveillance is conducted at wide-area regional nodes to assess the spread of COVID-19 (https://www.straitstimes.com/singapore/health/wastewater-surveillance-enables-wide-area-monitoring), surveillance may not be as time-sensitive and PEG precipitationis an protocols could serve as a sensitive and cost-effective test method. On the other hand, if wastewater surveillance is carried out at specific sites and results informed operational decisions such as prompting for active case-finding or situational monitoring (https://www.channelnewsasia.com/singapore/nea-wastewater-testing-more-locations-covid-19-transmission-1984336), ultrafiltration methods, which offer a shorter turnaround time may be preferred.

4.2. Study limitations

SARS-CoV-2 virus-spiked wastewater samples were not used in this evaluation, which could have limited the concentration of the virus used in this evaluation to those detected in raw sewage wastewater. However, as cell-culture virus isolates are different from viral fragments found in raw sewage wastewater, the use of a known positive real-world sample in our study could provide a more representative sample matrix for evaluation, especially when various sample processing steps were evaluated.

The total number of virus copies in the denominator of the recovery calculation was based on direct RNA extraction and testing of wastewater samples which may limit the analyses to samples with sufficient SARS-CoV-2 virus concentration. Nevertheless, this approach was adopted to avoid comparison with a reference virus concentration method which may also introduce biases in the analyses. Direct testing of wastewater samples also reduces potential virus loss through steps in the wastewater concentration process (Kantor et al., 2021). Taken together, although the study was limited to samples with sufficient SARS-CoV-2 virus concentration, the recovery efficiencies reported in our study were likely to be more reflective of the actual test conditions.

This study was also limited to methods that could be employed on a large operational scale, and other methods such as electronegative membrane filtration (Ahmed et al., 2020b), ultracentrifugation (Wurtzer et al., 2021), or methods requiring large sample volumes were not evaluated as they were impracticable for high-throughput testing.

Some studies have shown the association of SARS-CoV-2 to sludge (Graham et al., 2021) or solids (Kitamura et al., 2021), while others have suggested that the liquid fraction had higher yields (Forés et al., 2021). As our surveillance approach sampled wastewater from across the sewershed, the presence of solids in wastewater samples from different sites were highly variable, which would limit our ability to test for sludge or solids. This study therefore focused on concentrating the liquid fraction of wastewater. Additionally, as solid fractions are likely to have more PCR inhibitors (Li et al., 2021), testing of the liquid fraction may provide the added advantage of reduced inhibition.

In summary, this study evaluated key parameters for PEG-precipitation and ultrafiltration methods, which can inform the development of an optimal wastewater processing protocol for laboratories conducting wastewater testing. In our setting, we found that both PEG precipitation and ultrafiltration would be useful for wastewater testing and can support high-throughput wastewater processing in a country-wide wastewater surveillance programme.

CRediT authorship contribution statement

Diyar Mailepessov: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Sathish Arivalan:** Investigation, Methodology. **Marcella Kong:** Investigation, Methodology. **Jane Griffiths:** Investigation, Methodology. **Swee Ling Low:** Investigation, Methodology. **Hongjie Chen:** Investigation, Methodology. **Hapuarachchige Chanditha Hapuarachchi:** Investigation, Methodology, Writing – review & editing. **Xiaoqiong Gu:** Investigation, Methodology. **Wei Lin Lee:** Methodology, Writing – review & editing. **Eric J. Alm:** Methodology. **Janelle Thompson:** Methodology, Writing – review & editing. **Stefan Wuertz:** Methodology, Writing – review & editing. **Karina Gin:** Methodology. **Lee Ching Ng:** Conceptualization, Methodology, Supervision, Project administration, Writing – review & editing. **Judith Chui Ching Wong:** Conceptualization, Methodology, Supervision, Project administration, Writing – original draft, 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.

Appendix A. Supplementary data

Supplemental materials to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154024.

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