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Solid-liquid distribution of SARS-CoV-2 in primary effluent of a wastewater treatment plant

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ABSTRACT

Distributions of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and fecal viral biomarkers between solid and liquid phases of wastewater are largely unknown. Herein, distributions of SARS-CoV-2, Pepper Mild Mottle Virus (PMMoV), and F-RNA bacteriophage group II (FRNAPH-II) were determined by viral RNA RT-qPCR. Comparison of viral recovery using three conventional fractionation methods included membrane filtration, a combination of mid-speed centrifugation and membrane filtration, and high-speed centrifugation. SARS-CoV-2 partitioned to the solids fraction in greater abundance compared to liquid fractions in a combination of mid-speed centrifugation and membrane filtration and high-speed centrifugation, but not in membrane filtration method in a particular assay, while fecal biomarkers (PMMoV and FRNAPH-II) exhibited the reciprocal relationship. The wastewater fractionation method had minimal effects on the solids-liquids distribution for all viral and phage markers tested; however, viral RNA load was significantly greater in solid–liquid fractions viral RNA loads compared with the than whole-wastewater PEG precipitation. A RNeasy PowerWater Kit with PCR inhibitor removal resulted

Abbreviations: SARS-CoV-2, severe acute respiratory syndrome virus; PMMoV, pepper mild mottle virus; FRNAPH-II, F-RNA bacteriophage group II; WBS, wastewater-based surveillance; WWTP, wastewater treatment plants; WBE, wastewater-based epidemiology; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; AQHRP, armored RNA quant RNase P standard; IPC, internal RT-qPCR positive control; eRNA, environmental RNA; SD, standard deviation; K_{ow}, octanol-water partition coefficient; K_{oc}, adsorption coefficient; p*I*, isoelectric point.

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in greater viral RNA loads and lesser PCR inhibition compared to a QIAamp Viral RNA Mini Kit without PCR inhibitor removal. These results support the development of improved methods and interpretation of WBE of SARS-CoV-2.

- Distribution of SARS-CoV-2 to liquid and solid portions was addressed.
- Addressing PCR inhibition is important in wastewater-based epidemiology.
- Fraction methods have minimal effect.

Specifications table

Subject area:	Environmental Science
More specific subject area:	Wastewater based epidemiology (WBE)
Name of your method:	Method development for SARS-CoV-2 RNA partitioning in wastewater
Name and reference of original method:	Oloye, F. F., Xie, Y., Asadi, M., Cantin, J., Challis, J. K., Brinkmann, M., … & Giesy, J. P. (2022). Rapid transition between SARS-CoV-2 variants of concern Delta and Omicron detected by monitoring municipal wastewater from three Canadian cities. Science of the Total Environment, 841, 156,741.
Resource availability:	Not applicable

Method details

Introduction

Accurate and efficient surveillance of infections in communities is critical for wastewater surveillance to support public health decision-making in response to the COVID-19 pandemic. Traditional surveillance approaches are limited by the capacity of clinical lab-testing, public willingness to present for testing, and poor physical biomarkers for self-monitoring [1]. Additionally, pre-symptomatic [2] and asymptomatic transmissions [3] complicate the efficacy of traditional clinical testing as a means to limiting community transmission and understanding the overall burden of disease because of under-sampling of affected populations. Wastewater-based surveillance (WBS) or wastewater-based epidemiology (WBE) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a complementary monitoring tool and has globally gained acceptance from public health authorities [4]. The WBE of SARS-CoV-2 has the advantage of capturing the whole community or targeted populations such as high-risk groups in nursing homes, within a given sewershed, as it includes all infected cases, not only symptomatic persons.

Wastewater is used water with impurities, such as faeces, solid matters, organic and inorganic materials, and other contaminants, which can be purified by appropriate methods. Samples of wastewater typically consist of multiple matrices and the SARS-CoV-2 are most likely to consist of a mixture of strains [5,6], hence, virus particles are dispersed in wastewater and its solid constituents. Therefore, the applicable concentration and detection methods for wastewater samples with different matrixes are variable, and there is a need to develop and validate an appropriate method for specific wastewater target from time to time.

Current approaches for enrichment of viruses are dependent on centrifugation time/rate or ultrafiltration [7,8]. Thus, to be able to compare between and among locations and times of collections under various flow conditions, numbers of viral particles in wastewater are normalized by biomarkers of the amount for faeces contained in a sample, such as the Pepper Mild Mottle Virus (PMMoV) and host-specific bacteriophages. This is done to account for differences in the fecal content across samples and improve the interpretation of inherently variable wastewater surveillance data for the purposes of WBE.

Due to poor understanding of environmental behavior of SARS-CoV-2 in wastewater, the WBE methodology is continuing to be developed and refined. For example, recent techniques have focused on extraction of RNA in supernatants after removal of large particles, using filtration or slow speed centrifugation[4]. In contrast, attention has shifted to analysis of solids isolated from wastewater or primary clarifier sludges [9] given recent findings indicating that the suspended SARS-CoV-2 tends to undergo adhesion-aggregation to the wastewater insoluble fraction, such as organic particles [9,10]. Additionally, there are still gaps in understanding regarding the distributions of SARS-CoV-2 and fecal viral biomarkers between wastewater solid and liquid contents, specifically given that there are few studies comparing use of chemical tracers and biomarkers (e.g., PMMoV) for normalization of SARS-CoV-2 WBE.

This study was designed in order to gain insight into the distribution of SARS-CoV-2 and fecal viral biomarkers, including PMMoV and FRNAPH-II, in solids and liquids inside the primary clarifier effluent. Primary clarifier effluent was used in this study because it is easier to work with effluent than influents. Influent required more reagents, which is not always available as it was the case at the beginning of the COVID-19 pandemic, when there was a backorder problem. Samples were collected from the City of Saskatoon's municipal wastewater treatment plant (WWTP) and fractionated by three methods: membrane filtration (0.45 μm), a combination of mid-speed centrifugation (6500 g for 20 min) and membrane filtration (0.45 μm), and high-speed centrifugation (30,000 g for 10 min). Amounts of viral RNA were determined by use of reverse transcription-quantitative polymerase chain reaction (RT-qPCR), using two commercial nucleic acid extraction kits. These results are intended to support the development of improved methods and interpretation of SARS-CoV-2 WBE and, thus, provide public health authorities with more accurate data to protect communities globally. This study aimed to: (1). To investigate the effect of different RNA extraction and sample preparation methods on the viral load in wastewater. (2). To compare the solid-liquid distribution of SARS-CoV-2 and fecal indicators in wastewater. (3). To assess PCR inhibition and removal effects on the viral load in wastewater. (4). To compare the total viral load of SARS-CoV-2 RNA recovered

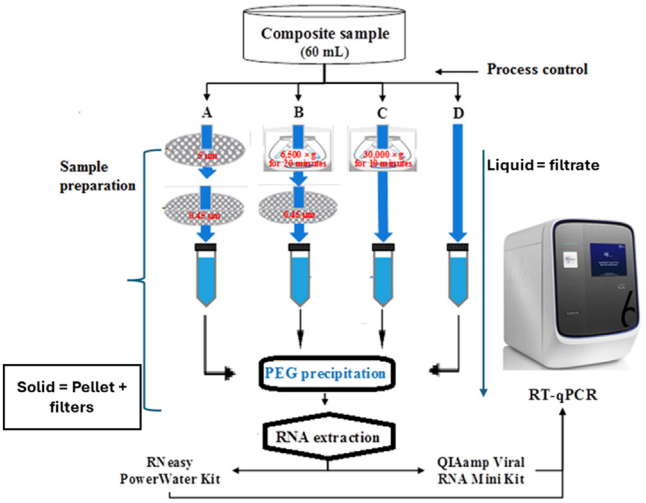

Fig. 1. Illustration of experiment design.

from non-processed whole wastewater and fractionated samples before RNA extraction. (5). To determine the effect of fractionation methods on the concentration of SARS-CoV-2 RNA in wastewater.

Materials and methods

Collection of samples

Primary clarifier effluent was collected after the grit chamber from the Saskatoon Wastewater Treatment Plant (Saskatoon, Saskatchewan, Canada; SWTP). The SWTP receives mostly domestic sewage from a population of approximately 300,000. The mean transit time for sewage from households to the SWTP is 4 to 6 h, and the hydraulic retention time of the plant is about 24 h. For every sampling day, one liter of a 24-h composite sample (about 60 mL grabbed every 15 min) was collected using an auto-sampler maintained at 4 ˚C. Samples were transported from the plant to the laboratory on ice, and divided into aliquots, after inactivation of the virus at 65 ˚C for 30 mins. A portion is immediately processed, and the remainder archived at −80 ˚C. For evaluation of methods of separating solid and liquid fractions and extraction of RNA, three additional 1 L composites were collected on November 11, 2020. Six more longitudinal samples between December 26, 2020, and January 5, 2021, were used for characterizing the distribution of viral RNA between liquids and solids. Typical characteristics of wastewater from the SWTP are listed in the Supporting Information (SI; Table S1).

Solid-liquid separation. Three methods of fractionation were tested (Fig. 1). *Method A* - Filtration: 60 mL of wastewater sample was sequentially filtered through a 5.0 μm followed by a 0.45 μm protein-low-binding Durapore® hydrophilic PVDF membrane (Millipore, USA) filters. The 5.0 μm and 0.45 μm PVDF membrane filters from one sample were pooled. *Method B* – Combined mid-speed centrifugation and filtration: 60 mL of wastewater sample was centrifuged in a fixed angle rotor at 6500 g for 20 min with the concentrate filtered through a 0.45 μm PVDF membrane. The pellet and filter were combined for RNA extraction. *Method C* – High-speed centrifugation: 60 mL of wastewater sample was centrifuged at 30,000 g for 10 min without brake. This centrifugation treatment was determined to pellet 85∼95% of the total suspended solids less than 0.3 μm in diameter [11]. *Method D* – no-fractionation control.

Frozen samples were thawed on ice. For a whole processing control, Armored RNA Quant RNase P standard (AQHRP, Asuragen, USA) was added at 3×10^3 gene copies per sample and incubated at 4 ˚C for 1 h before processing. Virus particles in the filtrate (*Method A and B*), concentrate (*Method C*), and whole (*Method D*) samples were enriched by PEG-8000 precipitation. Six grams PEG-8000 and 1.2 g NaCl were added to the liquid fraction.[21] Samples were agitated overnight (12–14 h) at 4 ˚C, on an orbital shaker, then centrifuged at 26,000 g at 4 ˚C for 1 h following incubation.

Extraction of environmental RNA (eRNA)

After preprocessing, RNA was immediately extracted from pellets and filters directly without elution of virus. QIAamp Viral RNA Mini Kit (Qiagen, USA) and RNeasy PowerWater Kit (Qiagen, USA) with PCR inhibitor removal procedure were tested side by side, using the sample collected on November 11, 2020. RNA in longitudinal samples was extracted, using only the Viral RNA Mini Kit. The Protocol of Viral RNA Mini Kit was modified slightly. The volume of lysis buffer AVL and ethanol was increased to four times the amount indicated in the manual. Buffer AVE was used for the elution of RNA for all samples.

RT-qPCR assays

Multiplex TaqMan-based one-step RT-qPCR assay was utilized for detection of SARS-CoV-2, Pepper mild mottle virus (PMMoV), F-Specific RNA Bacteriophages group II (FRNAPH-II), and AQHRP. Six-point, ten-fold serial dilution of synthetic SARS CoV-2 RNA standard (ATCC, USA; 2.9 to 2.9×10^5 copies/reaction), extracted RNA from AQHRP (2.5 to 2.5×10^5 copies/reaction), and synthetic PMMoV RNA standard (IDT, USA; 2.5 to 2.5×10^5 copies/reaction) were prepared for generating quantitative curves. Proportion of FRNAPH-II were calculated based on Ct values (Eq. (1)).

$$Proportion_i = \frac{(1+E)^{Ct_{MAX}-Ct_i}}{\sum_j^n (1+E)^{Ct_{MAX}-Ct_j}} \quad (1)$$

Where E, Ct_i and Ct_{MAX} are PCR efficiency of FRNAPH-II, Ct value of a given fraction, and maximum Ct value of fractions, respectively. Ctj stands for the sum of the Ct values.

RT-qPCR assays were performed using a QuantStudio™ 6 Flex thermal cycler (Thermo Fisher Scientific, CA), using automatic settings for threshold and baseline. Thermal cycling conditions consisted of Uracil-DNA glycosylases (UNG) incubation at 25 °C for 2 min, reverse transcription at 53 °C for 10 min, denaturation and Taq polymerase activation at 95 °C for 2 min, and 45 cycles of 95 °C for 3 s followed by 60 °C for 30 s (data collection). Sequences of primers and probes are listed in Table S2. RT-qPCR reactions were performed in duplicate for each sample and standard. Reaction mixtures are listed in Table S3.

Inhibition test

Novel RNA was designed and synthesized by Integrated DNA technologies (IDT, USA) as internal RT-qPCR positive control (IPC). Random RNA sequences were generated (GC: 45–55%) and checked by discontinuous Mega Blast against Nucleotide collection (nr/nt) and env_nt databases (Max E-value: 1E-10) [12]. Novel RNA sequence (5′-AACGCACAUAUACGGGUAGCAUAACUUUCGGAUGCAUCU AGUGAC AAUAAGGUGGUCUAUAGGCGGGAC-3′) was selected. Primers and probe (Assay 3 in Table S2) were designed using Primer3 [13]. RNA sample was spiked with known copy numbers (2.5×10^4 copies) IPC, as well as AVE buffer as a reference for assessing inhibition. Six-point, ten-fold serial dilutions of IPC RNA (25 to 2.5×10^6 copies/reaction) were prepared for generating standard curves for quantitation.

Two indicators (Eqs. (2) and (3)) were calculated to quantify the magnitude of inhibition during both reverse transcription and PCR:

$$\Delta Ct_i = \overline{Ct_i} - \overline{Ct_{Ref}} \quad (2)$$

$$IFQ_i(\%) = \frac{\left(\overline{C_{Ref,\,IPC}} - \overline{C_{i,\,IPC}}\right)}{\overline{C_{Ref,\,IPC}}} \times 100 \quad (3)$$

where, ΔCt_i and $\overline{Ct_i}$ are the delayed Ct value and averaged Ct value, respectively. $\overline{Ct_{Ref}}$ is the mean of Ct values of RNA-free references; IFQ_i and $\overline{C_{i,\,IPC}}$ are the inhibition factor of quantitation, and the averaged IPC concentrations of a given sample, respectively. $\overline{C_{Ref,\,IPC}}$ is the mean of IPC concentrations of RNA-free references.

To account for any contamination during the whole progress or key procedures, quality control, blank samples for sampling, extraction, and RT-qPCR were collected. Sample preparation, extraction, PCR setup, and thermal cycling were conducted in separated BSL-2 laboratories to minimize PCR cross-contamination. The UNG technique was applied to avoid potential contamination of DNA from previous RT-qPCR runs. Freshly prepared 10% bleach and 70% isopropanol were applied for disinfection and decontamination of the working area and waste disposal. All blank samples and negative controls were negative for all tested targets in this study. R^2 of standard curve and efficiency of RT-qPCR were 0.979 and 106.4% for N1 assay, 0.998 and 99.8% for N2, 0.999 and 97.0% for PMMoV, 0.989 and 101.5% for AQHRP, and 0.991 and 122.6% for IPC, respectively.

Statistics

Statistical analyses were performed using R Statistical Language v. 3.6.1 (R Core Team, 2019) and Origin 2021C. For all statistical tests, assumptions of normality and equal variance were tested by use of a Shapiro-Wilk test and a Fligner-Killeen test [14]. Mann-Whitney-Wilcoxon Test was used to compare differences of measurements between two groups. Kruskal-Wallis test was used to compare differences among measurements from three or more groups. On origin software two- and three-way ANOVA were used and the mean of different groups were compared using Bonferroni.

Method validation

The method applied for the RNA extraction has a significant effect on the overall viral load in wastewater (Fig. 2A). Also, observable differences exist in trends of the viral load when different assays were employed, for example, the patterns in Fig. 2A are different from that in Fig. 2B, which represent N1 and N2 assays, respectively. Similarly, the observation for the effect of sample preparation methods were different for each assay type. For instance, no significant difference in both RNA extraction method and the sample

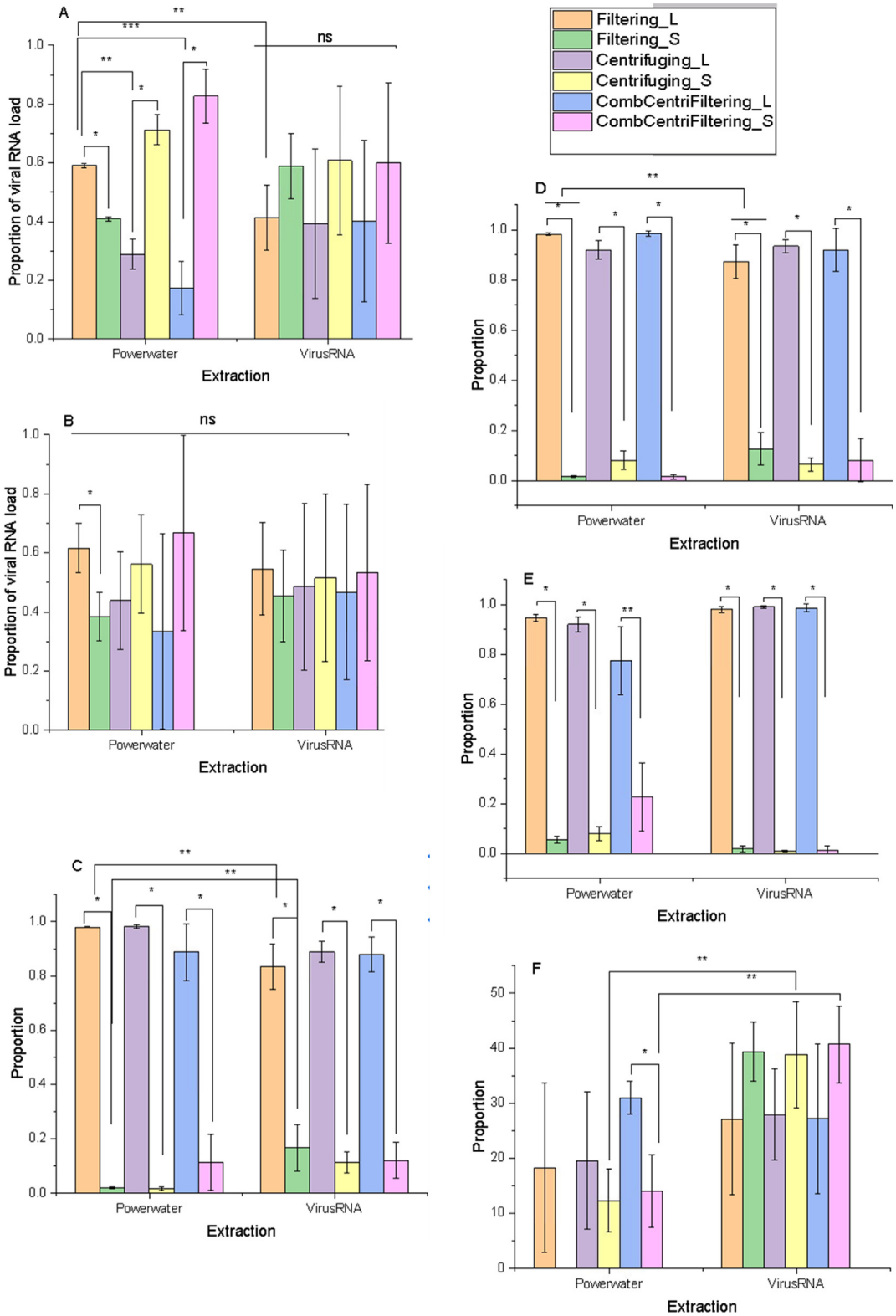

Fig. 2. Proportion of viral load and fecal biomarker in solid -liquid phases as a function of RNA extraction protocol and sample preparation methods, (A) SARS-COV-2 in N1 assay, (B) SARS-COV-2 in N2 assay, (C) Whole processing spike control, Armored RNA Quant RNase P standard (AQHRP), (D) Pepper mild mottle virus (PMMoV), (E) F-Specific RNA Bacteriophages (FRNAPH-II) and (F)) Inhibition factor of RT-qPCR of RNA extracted.

preparation method with N2 assays, while with N1 assay noticeable changes were evident between samples extracted with RNeasy PowerWater Kit, no such changes were seen by those extracted with QIAamp Viral RNA Mini Kit.

Methods of fractionation, such as filtration and centrifugation did not alter the viral load when the RNA was extracted using QIAamp Viral RNA Mini Kit with either N1 or N2 assays. Generally, overall load obtained preparing the samples with filtration, or centrifugation before RNA extraction was not significantly difference when N2 assays were used. However, with RNeasy PowerWater Kit using N2 assay was significantly different between solid fraction and liquid fraction with filtration method. Differences between preparation methods were obvious when the N1 assay was employed with RNeasy PowerWater Kit, where overall viral RNA prepared using filtration, and centrifugation were different from each other.

There was a statistically significant difference of solid-liquid distribution between SARS-CoV-2, and the set of fecal indicators tested (PMMoV, FRNAPH-II), and the whole process control (AQHRP) (Kruskal-Wallis test, p-value < 0.001). Proportions of SARS-CoV-2 were greater in solids (mean $\pm$ standard deviation (SD): N1, 62.3 $\pm$ 15.2%; N2, 54.7 $\pm$ 21.0%) than that in liquids (N1, 34.7 $\pm$ 15.2%; N2, 45.3 $\pm$ 21.0%; One-Tailed Mann-Whitney-Wilcoxon Test: N1 assay, p-value < 0.001; N2 assay, p-value $= 0.362$) when the samples were prepared using centrifugation and combined filtration with centrifugation method, however, for filtration fractionation process, the proportion of SARS-CoV-2 was greater. Alternatively, solids associated significantly smaller proportions of AQHRP (11.0 $\pm$ 7.3%, One-Tailed Mann-Whitney-Wilcoxon Test, p-value < 0.001) (Fig. 2C), PMMoV (7.7 $\pm$ 5.7%, p-value < 0.001) (Fig. 2D), and) FRNAPH-II (4.4 $\pm$ 7.2%, p-value < 0.001) (Fig. 2E) than liquid fractions. Within targeted non-enveloped virus, AQHRP had a more similar distribution to PMMoV than FRNAPH-II because FRNAPH-II did not show any significant differences in the pattern of the viral load when the RNA extraction method was change.

For the liquid fraction, there was no significant difference in PCR inhibition between the two extraction kits and among the fraction methods (Fig. 2F). However, PCR inhibitor removal significantly reduced the effects of PCR inhibitors from solids (Fig. 2F). The proportion of the inhibitor factor was lower with the RNeasy PowerWater kit compared to the other kits.

Comparison of the total viral load of SARS-CoV-2 RNA recovered from non-processed whole wastewater and fractionated samples before RNA extraction (Fig. 3A and B) showed that the two RNA extraction methods were significantly different from each other. The method with PCR inhibition removal steps recovered more SARS-CoV-2 RNA compared with the method without. Also, the non-process whole wastewater returned significantly greater SARS-CoV-2 RNA concentration compared to the combination of SARS-CoV-2 RNA from solid and liquid fractions combined. Although no significant difference was evident in the delayed count values between the two RNA extraction method (Fig. 3C), there were observable difference between delayed count values in centrifuge, and centrifuge followed by filtration method in the two RNA extraction method. The delayed counts values have no significant difference within the same extraction group regardless of the pre-processed methods.

Elevated proportions of SARS-CoV-2 RNA were found in solids as compared to the liquid fraction in N1 assay using method B and C, supporting previous studies that targeted solids for efficient detection. However, observation in method A was different, suggesting that the filter materials may not be affecting the overall concentration of SARS-CoV-2 RNA. For method A, the proportion of SARS-CoV-2 RNA in the liquid phase was more based on both the N1 and N2 assays, which is an indication that filtration has no effect on the SARS-CoV-2 RNA concentration. Nonetheless, other methods, such as centrifugation and combined centrifuging and filtration resulted in more SARS-CoV-2 RNA in solid portion, indicating that fractionation method affected determination of SARS-CoV-2 RNA. Therefore, the centrifugation force might be the factor that conditioned the virus to be precipitated to the solid portions, thus resulting in lower amount in liquid portion. Generally, enveloped viruses are believed to bind to particles in wastewater, because of the different binding site on their membrane [15,16]. Spike glycoproteins on the viral membrane contribute to adhesion-aggregation of virus onto surfaces of particles in wastewater [17,18]. There is evidence that viral glycoproteins interact with cholesterol as well as with HDL particles excreted in feces [19], therefore supporting the idea that virus could bind to the particles in wastewater. Furthermore, other universal lipids in wastewater [20] can enhance absorption. A significant proportion of SARS-CoV-2 RNA was encapsulated in municipal wastewater and non-negligible proportions of suspended SARS-CoV-2 were mostly due to mixing, diffusing, and resuspending in sewer pipelines. Therefore, the method employed for pre-processing samples before RNA extraction plays significant roles on the overall viral RNA concentration.

Observable trends in the proportion of solid and liquid with different preprocessing methods in N1 assay were absent in fecal indicators. All the tested fecal indicators had greater proportions in the liquid phase, which was different from what was observed when N2 assay was employed. The difference in the performance of SARS-CoV-2 RNA and the fecal indicators could be related to the structure and chemistry of each organism.

Isoelectric point (p*I*) can also alter partitioning behavior in wastewater. The p*I* values of non-enveloped PMMoV and FRNAPH-II Enterobacteria phage GA strains are 3.2–3.8 [21] and 2.1–2.8 [22], respectively. Predicted p*I* value of structural Spike, Envelope, Membrane and Nucleoprotein proteins, are 6.24, 8.57, 9.51, and 10.07, respectively [23], which are closer to the pH of primary effluent (7.7 - 8.2 Table S1) than those of PMMoV and FRNAPH-II. Therefore, the tendency of SARS-CoV-2 to aggregate and precipitate is greater than that of PMMoV and Enterobacteria phage GA. Other environmental factors, such as temperature, size distribution of particles, and lipid content [18], can play a significant role in partitioning of viruses in wastewaters, and thus should be considered when optimizing methods for extraction and enrichment.

For precise WBE, the ratio of solid-liquid fraction might be a fundamental parameter for estimating total SARS-CoV-2 from concentrations in solid or liquid fractions. Alternatively, efficient viral enrichment or extraction of RNA from whole wastewater without fractionation would be another solution for better detection of total viral RNA load in wastewater. The concentration recovered from whole wastewater without fractionation is significantly different from the combination of SARS-CoV-2 recovered from both solid and liquid combined together, and singly. Also, methods of extraction of RNA have more effect on the samples not pre-processed, compared with fractionated samples. Thus, the method of removing inhibitors in the Rneasy Powerwater kit could be one of the

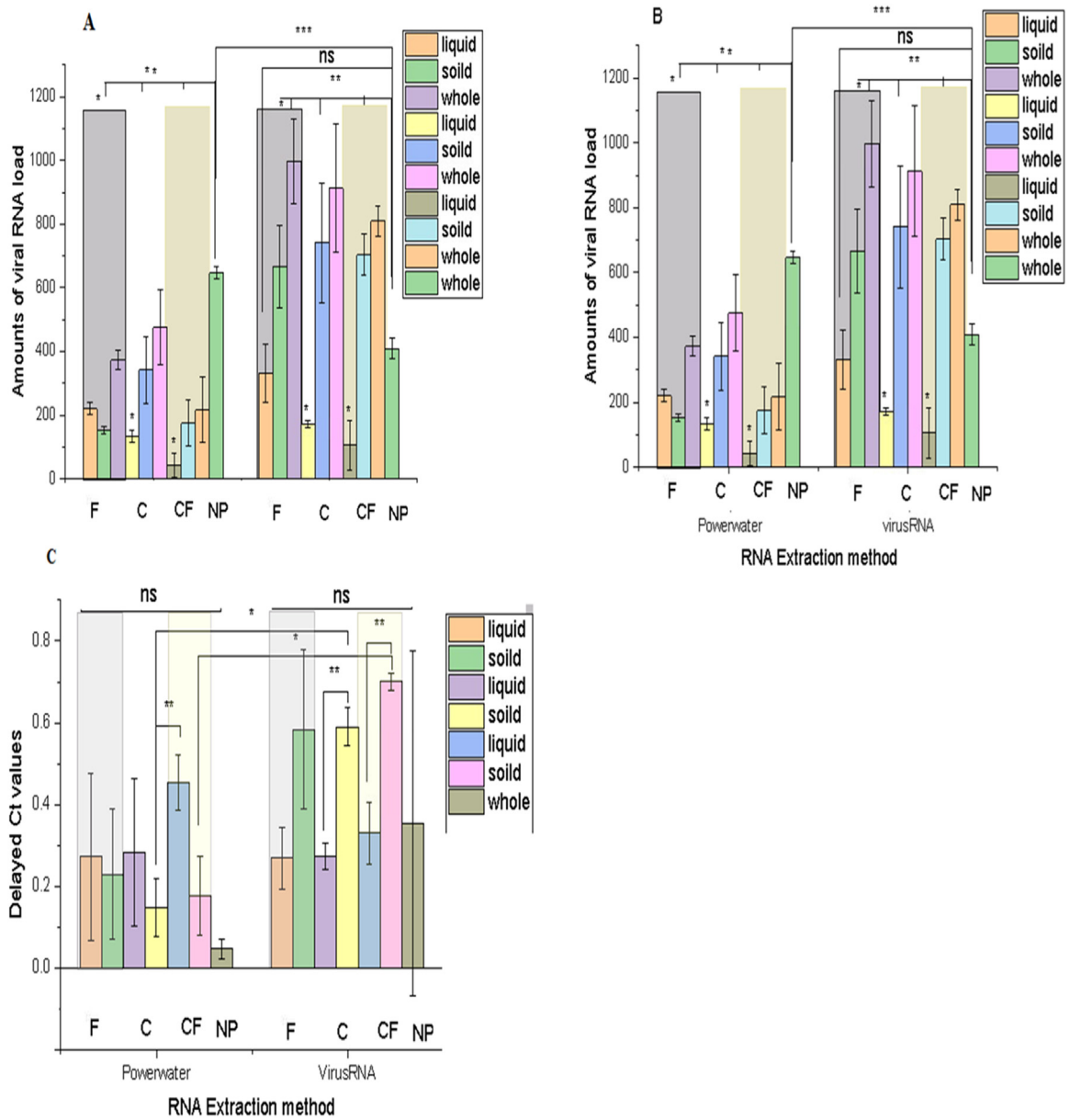

Fig. 3. Understanding the effect of fractionation on the amounts of total viral RNA load, by comparing non-fractionated samples with fractionated samples (A) SARS-CoV-2 in N1 assay, (B) SARS-CoV-2 in N2 assay, and (C) delayed counts between methods. F stands for filtation method, C- stands for centrifugation method, CF stands for combine centrifugation and filtration method, and NP stands for non-processing method.

reasons while the method returns more concentration. Thus, if the method is not properly optimized, under estimation of the total RNA will be recorded. This is because of matrix effect which could interfere with the extraction efficiency of the adopted method.

PMMoV from dietary [21] and human-feces-specific bacteriophages from host enteric bacteria [24] have been suggested for population normalization of WBE of SARS-CoV-2 [9]. PMMoV and FRNAPH-II have distinct distributions between solids and liquid phases different from that of SARS-CoV-2 in wastewater. PMMoV was more concentrated in the liquid fraction, which suggested that it might not be the best population indicator in all cases. For instance, a surrogate, Bovine coronavirus, of SARS-COV-2 was found to not be better than PMMoV when used as an internal standard in an earlier report [25,26]. Fecal indicator bacteria (FIB; for instance, *Escherichia coli, Enterococcus* and *Streptococcus* spp.) tend to sorb to solids similarly to SARS-CoV-2 [27], but they are poor indicators due to their instability in wastewater [28]. Nevertheless, F-Specific RNA Bacteriophages (FRNAPH-II) behaved closely like PMMoV and AQHRP, but not SARS-CoV-2.

The ideal fecal biomarker for population size normalization of SARS-CoV-2 WBE should have the same sources and similar environmental behaviors. Normalization with the viral load of PMMoV in filtrate [26] and solids [10] reduced noise and improved the

agreement of WBE virus trends with reported COVID-19 cases during short-term monitoring, during which water quality characteristics of municipal wastewater were relatively stable. Both PMMoV and FRNAPH-II are suitable to check sample-to-sample variability during processing [5]. Additionally, in addition to surrogate viruses, environmental behavior of chemical biomarkers has been studied in wastewater [29], and environmental behaviour of chemical tracers can be predicted based on their physicochemical properties such as aqueous solubility, octanol-water partition coefficient (K_{ow}), and adsorption coefficient (K_{oc}) [30]. Hence, a combination of viral and chemical biomarkers for population normalization can be beneficial for large-scale, long-term WBE of SARS-CoV-2. Variability in dietary biomarkers, such as artificial sweeteners and caffeine, as a result of differing or uncertain intake rates between communities should be considered for the selection of an appropriate population biomarker. Fecal biomarkers, for instance, FRNAPH-II, crAssphage, hormones, and sterols [29], might be more robust than dietary indicators alone.

Results of the study presented here suggest that accessing the solids fractions using facile and high-throughput methods such as centrifugation allow reasonable recovery of SARS-CoV-2. Fecal indicators are found not to partition to fraction in the same proportion as SARS-CoV-2. Thus, their appropriateness as normalizing factors requires further investigation. Tested methods of fractionation resulted in similar performance of viral recovery from solid and liquid, but less compared to non-processed whole wastewater. The evaluation of virus captures methods presented herein provides suggestions for method development based on available resources (i.e., filtration versus high-speed centrifugation versus raw unprocessed), which will be crucial to assist in pandemic response in the future. Enriching viruses in the field by use of filtration can work for remote sites without access to centrifuges and reduce the cost of shipment, preservation, and storage.

Non-fraction whole wastewater PEG precipitation is an efficient way to measure the total viral load but is limited by matrix effects and therefore requires proper optimization before use. Each sample has its uniqueness and sampling from the same plant day to day does not guarantee that the samples are the same. Hence, frequent optimization of the protocol for viral load quantification is necessary. Artificial, armed virus particles can also avoid contamination from a background in environmental samples. The inhibition factor has no significant effect on the liquid portion, but varied significantly in solid between RNA extraction methods, suggesting that matrix effects play more role in solid than in liquid. Removal of PCR inhibitors is a tradeoff between viral load and inhibition. The general RNA extraction kit without removal of PCR inhibitors is still workable for small volumes of wastewater.

Conclusion

Greater proportions of SARS-CoV-2 RNA were found in solid fractions versus liquid fractions of wastewater in N1 assay with viral enrichment done using centrifugation alone, or centrifugation followed by filtration method, which is contrary to the observation with filtration method alone. Also, RNA extracted with PCR inhibitor removal with non-processed samples showed significant difference from those without inhibitor removal. Therefore, matrix effect is strongly affecting concentration of SARS-CoV-2 RNA extracted. Differential solid-liquid distributions of SAR-CoV-2 from that of fecal biomarkers (PMMoV and FRNAPH-II) suggested the need for careful selection of indicators for population-size normalization. However, PMMoV and FRNAPH-II might be more suitable for normalization against the concentration of the liquid fraction. Fractionation methods resulted in a similar performance of viral recovery from solid and liquid in N2 assay. These results will support the development of improved methods and interpretation of WBE of SARS-CoV-2 and provide public health authorities with more accurate and efficient tools to better manage the pandemic and protect communities worldwide.

- The method applied for RNA extraction affects the viral load in wastewater, with observable differences in trends depending on the assay type and preparation method.
- Solid fractions had a higher proportion of SARS-CoV-2 RNA than liquid fractions, while the opposite was true for fecal indicators and process control.
- PCR inhibition removal reduced the effects of inhibitors from solids. Non-processed whole wastewater returned a significantly greater SARS-CoV-2 RNA concentration compared to the combination of solid and liquid fractions combined.
- The centrifugation force might be the factor that conditioned the virus to be precipitated to the solid portions.

Associated content

Supporting Information. Table S1, Characteristics of wastewater from Saskatoon Wastewater Treatment Plant, meteorological parameters, and epidemiology data. Table S2, Sequences of primers and probes. Table S3, Reaction mixtures of RT-qPCR assays.

Ethics statements

Not applicable.

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.

CRediT authorship contribution statement

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Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2024.102645.

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