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Recovery of SARS-CoV-2 from large volumes of raw wastewater is enhanced with the inuvai R180 system

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https://doi.org/10.1016/j.jenvman.2021.114296
Received 2 September 2021; Received in revised form 6 December 2021; Accepted 11 December 2021
Available online 15 December 2021
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Wastewater-based epidemiology (WBE) for severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) is a powerful tool to complement syndromic surveillance. Although detection of SARS-CoV-2 in raw wastewater may be prompted with good recoveries during periods of high community prevalence, in the early stages of population outbreaks concentration procedures are required to overcome low viral concentrations. Several methods have become available for the recovery of SARS-CoV-2 from raw wastewater, generally involving filtration. However, these methods are limited to small sample volumes, possibly missing the early stages of virus circulation, and restrained applicability across different water matrices. The aim of this study was thus to evaluate the performance of three methods enabling the concentration of SARS-CoV-2 from large volumes of wastewater: i) hollow fiber filtration using the inuvai R180, with an enhanced elution protocol and polyethylene glycol (PEG) precipitation; ii) PEG precipitation; and iii) skinned milk flocculation. The performance of the three approaches was evaluated in wastewater from multiple wastewater treatment plants (WWTP) with distinct singularities, according to: i) effective volume; ii) percentage of recovery; iii) extraction efficiency; iv) inhibitory effect; and v) the limits of detection and quantification. The inuvai R180 system had the best performance, with detection of spiked control across all samples, with average recovery percentages of 68% for porcine epidemic diarrhea virus (PEDV), with low variability. Mean recoveries for PEG precipitation and skinned milk flocculation were 9% and 14%, respectively. The inuvai R180 enables the scalability of volumes without negative impact on the costs, time for analysis, and recovery/inhibition. Moreover, hollow fiber ultrafilters favor the concentration of different microbial taxonomic groups. Such combined features make this technology attractive for usage in environmental waters monitoring.

1. Introduction

Surveillance of wastewater for epidemiological purposes has been previously used in public health, with the most important and successful example being the polio eradication program (GPEI, 2021). Given the ongoing Coronavirus disease 2019 (COVID-19) pandemic and accumulated reports of the presence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA in the stools of infected people and in raw wastewater (Gonzalez et al., 2020; Medema et al., 2020; Randazzo et al., 2020) the use of this matrix as a tool to monitor the emergence, prevalence, molecular epidemiology, and eventual phase out of SARS-CoV-2 in the community was prompted.
Wastewater-based epidemiology (WBE) of SARS-CoV-2 has thus been gaining track among scientists, stakeholders, and decision makers throughout the world to complement syndromic surveillance and clinical testing. Several factors impact the detection of SARS-CoV-2 in raw wastewater, particularly in the early stages of virus circulation, including, sample collection (composite vs grab samples; for grab samples, sampling time of the day is also critical) and optimized concentration methods. Although detection of SARS-CoV-2 may be performed directly on raw wastewaters with increased recovery percentages, ultimately optimization of concentration procedures is necessary in the early stages of virus circulation wherein low concentrations are expected (Gonzalez et al., 2020). Therefore, cost-effective, rapid, and efficient concentration methods are required for monitoring SARS-CoV-2 or any other pathogen in raw wastewater for the successful deployment of WBE.

Existing methods for the recovery of viruses were primarily developed for the detection of nonenveloped viruses. Knowledge gaps concerning the recovery efficiencies of enveloped viruses, such as SARS-CoV-2, remain. A study by Haramoto et al. (2020) showed recovery efficiencies to be largely different for both types of viruses, with methods performing better for the recovery of nonenveloped viruses. Blanco et al. (2019) determined similar recovery efficiencies using precipitation with 20% polyethylene glycol (PEG) following glass wool concentration for enveloped (Transmissible gastroenteritis virus (TGEV)) and nonenveloped viruses (Hepatitis A virus (HAV)). A recent study by Ahmed et al. (2020) showed recovery efficiencies varying between 26.7 and 65.7% for murine hepatitis virus (MHV) in raw wastewater with very low concentrations. PEG precipitation (Wurtzer et al., 2020), aluminum flocculation (Randazzo et al., 2020), skimmed milk flocculation. The performance of Fresenius Medical Care), with an improved elution protocol; ii) PEG precipitation (Chavarria-Miró et al., 2020) following glass wool concentration for enveloped (TGEV) and nonenveloped (HAV). A recent study by Ahmed et al. (2020) showed recovery efficiencies varying between 26.7 and 65.7% for murine hepatitis virus (MHV) in raw wastewater with very low concentrations. PEG precipitation (Wurtzer et al., 2020), ultracentrifugation (Wurtzer et al., 2020), PEG precipitation (Chavarria-Miró et al., 2020; La Rosa et al., 2020), aluminum flocculation (Randazzo et al., 2020), skimmed milk flocculation (Philó et al., 2021), and filtration through an electroporation membrane (Gonzalez et al., 2020; Haramoto et al., 2020). For an effective environmental surveillance to be put in place, not only for SARS-CoV-2 but also for potential future pandemics involving enveloped virus, it is paramount to have validated methods. Nonetheless, comparisons between published methods are difficult as they differ in many aspects including: i) seeding controls; ii) concentration methods; iii) extraction methods; iv) diagnostic and quantification molecular assays and genome targets; v) and mostly, the accepted performance levels. Some publications only mention the recovery efficiency (Ahmed et al., 2020; McMinn et al., 2021), others mention the recovery efficiency and the LoD but not LoQ (Gonzalez et al., 2020; Randazzo et al., 2020; Pérez-Catalahà et al., 2021), some mention LoQ but not LOD (LaTurner et al., 2021), while other studies show all data performance, including LoD, LoQ and recovery percentages (Philó et al., 2021). Additionally, different studies calculate the LoD and LoQ differently. The information collected from different studies should inform laboratories on method performance.

In the present study, we evaluated the efficiency of SARS-CoV-2 recovery from raw wastewater using three concentration methods: i) a newly developed hollow-fiber ultrafilter, inuvai R180 (inuvai, a division of Fresenius Medical Care), with an improved elution protocol; ii) PEG precipitation; and iii) skimmed milk flocculation. The performance of the three methods was compared in raw wastewater according to several characteristics, including: i) effective volume tested; ii) frequency and consistency of detection; iii) percentage of recovery; iv) extraction efficiency; v) inhibitory effect on reverse transcription-qPCR (RT-qPCR); and vi) concentration information (including, Limit of Detection (LoD) and Limit of Quantification (LoQ)). These three methodologies were chosen primarily because they allow for the concentration of large volumes of water which is of extreme relevance at the early stages of the epidemiological curve and because they allow for the concentration of different types of water, including treated wastewater and environmental waters, important when analyzing the entire SARS-CoV-2 urban water cycle.

This study benchmarks new and old methodologies for the detection of SARS-CoV-2 from raw wastewater for WBE applications.

2. Materials and methods

2.1. PEDV strain and cell lines

PEDV strain CV777 (kindly provided by Dr. Gloria Sanchez, IATA-CSIC) is an enveloped virus from the genus Alphacoronavirus and member of the Coronaviridae family, responsible for the porcine epidemic diarrhea. PEDV was propagated in Vero cell line (ATCC CCL-81, LGC Standards). Briefly, Vero cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco), supplemented with 100 units/mL of penicillin (Lonza), 100 units/mL of streptomycin (Lonza), and 10% heat-inactivated fetal bovine serum (Biological Industries). Cells were cultured in T175 flasks at 37 °C in 5% CO2. For infection with PEDV, cells were grown in T25 flasks and inoculated with 100 μL of viral stock. At 2 h post infection, DMEM supplemented with 0.3% tryptose phosphate broth, 100 units/mL of penicillin (Lonza), 100 units/mL of streptomycin (Lonza), and 10 μg/mL t trypsin, was added to the flasks. Flasks were then incubated at 37 °C in 5% CO2 for 4 days. PEDV were recovered following three cycles of freeze/thawing and centrifugation at 11000×g for 10 min. Quantification was performed by reverse transcription digital PCR (RT-dPCR) as described on section 2.5 using the primers and probes from Supplementary Table S1 (Zhou et al., 2017), following nucleic acid extraction as described on section 2.4. After absolute quantification by RT-dPCR (as described below), a stock solution was prepared in DNase/RNase free water to obtain a PEDV final concentration of 1.21×10^4 GC/L in wastewater. The same stock was used in all experiments described below.

2.2. Wastewater sample preparation

Twenty-four-hour composite samples were collected between July 6–10, 2020, from five wastewater treatment plants (WWTP) in Portugal (n = 8; n = 2 for Serzedelo, Gaia and Guia; n = 1 for Alcântara and Beirolas). The samples were transported to the laboratory, refrigerated, and processed within 8 h of collection. Samples were seeded with PEDV at a concentration of 1.21×10^4 GC/L (quantified as described below).

Seeded raw wastewater samples were aliquoted and concentrated using three methods: (i) hollow fiber with the newly developed inuvai R180 ultrafilters (inuvai, a division of Fresenius Medical Care, Germany) followed by PEG precipitation (method 1); (ii) direct PEG precipitation (method 2); and (iii) skimmed-milk flocculation (method 3). The inuvai R180 ultrafilter has a large membrane area (1.8 m²) and a fiber inner diameter of 220 μm, allowing for the concentration of large volumes of water, including wastewater, without problems such as clogging or compromising of the membrane structure. All methods were tested using the same initial volume of wastewater (1-L) for a more accurate comparison.

Method 1 employed the use of hollow fiber ultrafilters: 1-L of raw wastewater was filtered through inuvai R180 ultrafilters using a peristaltic pump with a flow rate of 250 mL/min. The elution was performed in three steps: (i) air forward push using 60 mL of air; (ii) backflush with 250 mL of elution buffer (1× PBS with 0.01% NaPP and 0.01% Tween 80/0.001% antifoam) at a flow rate of 140–280 mL/min; and (iii) forward flush using 50 mL of elution buffer. The final elution volume was 300 mL. Samples were further concentrated by precipitation with 20% (w/v) PEG 8000 (Sigma, Portugal) overnight (Blanco et al., 2019). Samples were centrifuged at 10,000×g for 30 min, the supernatant
skimmed milk flocculation, performed in accordance with Calgua et al. (2008). Briefly, a pre-flocculated solution of 1% (w/v) skimmed milk pH 3.5 was prepared in artificial seawater. The solution of skimmed milk was then added to a final concentration of 0.01% (w/v) to 1-L of previously acidified raw wastewater (pH 3.5). Samples were stirred for 8 h at room temperature and flocs were allowed to sediment for another 8 h. Supernatant was carefully removed without disturbing the sediment. The final volume (approximately 500 mL) was centrifuged at 7000 g for 30 min at 12 °C. The supernatant was carefully discarded, and the pellet resuspended in 0.2 M phosphate buffer at pH 7.5 to a final volume of 5 mL. All concentrates were stored at −80 (±10) °C until further analysis.

2.3. Nucleic acid extraction

Nucleic acid extraction was conducted using the QIAamp Fast DNA Stool mini kit (QIAGEN, Germany) from 220 μL of PEDV stock or concentrated raw wastewater samples according to the manufacturer’s instructions, recovering the nucleic acids in a final volume of 100 μL. Recovery efficiency for extraction was evaluated using Murine Norovirus 1 (MNV-1), added to the concentrates, as an extraction control. MNV was quantified using the assay described by Baert et al. (2008). Primers and probe information is provided on Supplementary Table S1.

2.4. Absolute quantification by RT-dPCR

RT-dPCR was used to determine the exact concentration of SARS-CoV-2 control (nCoV-ALL-Control plasmid, Eurofins Genomics, Germany) and PEDV. Controls were amplified using the AgPath-ID One-Step RT-PCR kit (Thermo Fischer Scientific) with the set of primers and probes described on Supplementary Table S1 (PEDV; E_Sarbeco and RdRp assays). The 15 μL reaction mixture consisted of 7.5 μL of 2 × RT-PCR buffer, 0.6 μL of 25 × RT-PCR enzyme mix, 800 nM of each primer, 200 nM of probe, 3.63 μL RNase/DNase-free water, and 3 μL of DNA (diluted 4-, 5-, 6- fold). The reaction mixture was then spread over the QuantStudio 3D Digital PCR thermal cycler. Amplification was performed as follows: i) SARS-CoV-2: 10 min at 45 °C, 10 min at 96 °C, 39 cycles of 2 min at 58 °C and 30 s at 98 °C, and final elongation step for 2 min at 98 °C; ii) PEDV: 10 min at 45 °C, 10 min at 96 °C, 39 cycles of 2 min at 60 °C and 30 s at 98 °C, and a final elongation step for 2 min at 60 °C. Reactions were performed in duplicate, and a non-template control (NTC) was included in each run.

2.5. Relative quantification of seeded material in wastewater

Relative quantification of SARS-CoV-2, PEDV and MNV-1 was carried out by RT-qPCR on all extracts using the AgPath-ID One-Step RT-PCR kit (Thermo Fischer Scientific). The final volume of 25 μL was composed of 12.5 μL of 2 × RT-PCR buffer, 1 μL of 25 × RT-PCR enzyme mixture, 800 nM of each primer, 200 nM of the probe, 6.05 μL RNase/ DNase-free water, and 5 μL of RNA. All RT-qPCR reactions were run on undiluted, 4- and 10-fold diluted extracts. RT-qPCR conditions were as follows: i) SARS-CoV-2 control: 10 min at 45 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 58 °C; ii) PEDV and MNV-1: 10 min at 45 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Standard curves, run with each PCR, for SARS-CoV-2 control (E_Sarbeco and RdRp assays), PEDV and MNV-1 were prepared in serial 10-fold dilutions in RNase/DNase-free water. Positive and NTC controls were also added to each PCR assay. Positive and NTC controls were also added to each PCR assay. Limits of detection (LoD) and quantification (LoQ) were determined in RNase/DNase-free water. The LoD was considered the lowest concentration of target that could be consistently detected (in more than 95% replicates tested) (Burd et al., 2010) and LoQ, the lowest concentration at which the performance of the method is acceptable, with a coefficient of variation below 35% (Klymus et al., 2020).

2.6. Recovery efficiency

The mean recovery efficiency of PEDV for each method was calculated using the copies quantified by RT-qPCR as follows (Eq. (2)):

\[
\text{Recovery efficiency (\%) = \frac{\text{Total nucleic acid copies recovered}}{\text{Total nucleic acid copies seeded}} \times 100}
\]  

(Eq. 2)

The mean and standard deviation for each method were also calculated.

2.7. Quality control

To minimize nucleic acid carry-over and cross-contamination, sample concentration, extraction procedures and RT-qPCR/RT-dPCR were performed in separate rooms of the laboratory. A process blank and extraction blank were included for each concentration method and each nucleic acid extraction, respectively. All spiked samples were tested in parallel with the corresponding unseeded samples to rule out or estimate the contribution of potentially native PEDV.

2.8. Data analyses

All data analyses were performed with SPSS Statistics 26 (IBM). Kruskal-Wallis test was conducted to compare the differences between the parameters estimated for the three methods and pairwise comparison was performed with Dunn’s test. In all cases, P-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Quantification of controls

Appropriate quantification of the controls used in spiking experiments and in standard curve for qPCR is extremely important, as it will influence downstream data interpretation. That is why we opted for RT-dPCR, with high precision and sensitivity, for the absolute quantification of controls. Digital PCR works by partitioning a unique sample into thousands of individual reactions running in parallel, being particularly useful for low-abundance targets or targets in complex matrices. Through Poisson statistics, the total number of target molecules is calculated, with no need for external reference standards (Monteiro and Santos, 2017). Several dilutions of SARS-CoV-2 control and PEDV, in duplicate, were quantified by RT-dPCR. The concentrations of the initial stocks for SARS-CoV-2 control were 1.94 × 10^8 GC/μL and 1.00 × 10^8 GC/μL for E_Sarbeco and RdRp assays, respectively. Concentration of PEDV as determined by RT-dPCR was 1.20 × 10^6 GC/μL.

3.2. Method comparison using PEDV as surrogates for SARS-CoV-2

All unseeded wastewater samples were negative for the presence of PEDV. Samples were chosen in a period with low number of daily COVID-19 cases (mean for entire country, and 374, between July 6 and 10, 2020) (DGVS, 2020). All process and extraction blanks were negative.
The effective volume tested within each method was the same (2.2 mL): all methods started with the same initial volume (1-L) of wastewater, followed by concentration steps prior to extraction and sediment resuspension in 5 mL of elution buffer; samples tested across the three methods were extracted using the same extraction protocol, and the same volumes and dilutions were analyzed by RT-qPCR. Nonetheless, the inuvai R180 ultrafilters (method 1) enabled the filtration of 2.5 – 5-L of raw wastewater. Increasing the initial volume of sample with the inuvai R180 ultrafilters would conduct to an increment of the effective volume assayed from 2.2 mL to 5.5 – 11 mL without further increases in the concentration time, the concentrate volume, costs for analysis, and RT-qPCR inhibition. On the other hand, increasing the volume of filtration in the skimmed milk flocculation method (and therefore, theoretically, increasing the effective volume assayed; method 3) would imply an increase of skimmed milk and artificial seawater, as well as of HCl to adjust the pH; the volume of concentrated matter and, therefore, of the concentrate would also increase, leading to a decrease in the efficiency of extraction and an increase of inhibitory effects on RT-qPCR. Additionally, increasing the processing volume would require the acquisition of larger volume sample containers, which would also take up more space in the laboratory. Concomitantly, increasing the processing volume when using solely PEG precipitation (method 2) implies increasing substantially the volume to be centrifuged, which increases the time spent in the concentration step and the costs due to the usage of larger amounts of PEG.

PEDV was used to compare concentration recoveries. The highest average percentage of recovery was obtained with the inuvai R180 system at 68% (±11%) for PEDV, with recoveries varying between 52 and 82% (Fig. 1A).

PEG precipitation had the lowest percentage of recovery for PEDV (9% (±5%)). Recovery with skimmed milk performed only slightly better (14% (±8%)) (Fig. 1A). There were statistically significant differences in the recovery percentage of PEG and skimmed milk compared to inuvai R180 (KW = 15.989, df = 2, p < 0.05), although no difference was observed between skimmed milk flocculation and PEG precipitation (p < 0.05).

The inuvai R180 was the single method that consistently led to nucleic acid detection in all samples. Concentration using PEG and skimmed milk led to the detection of PEDV in 50% of the samples.

The method using the inuvai R180 system led to detection by RT-qPCR of the highest mean genome copies, 4.25 GC/reaction for PEDV, respectively. Concentration with PEG (0.21 GC/reaction) and skimmed milk (0.28 GC/reaction) showed similar results (Fig. 1B).

Our recovery values using the inuvai R180 system were similar to those reported for MHV, while enabling an increase in the filtration volume (Ahmed et al., 2020). For PEG precipitation and skimmed milk flocculation the recoveries were slightly higher than those reported by Philo et al. (2021). The authors used a concentration of 14% (w/v) of PEG compared to 20% (w/v) PEG in our study. The use of higher concentrations of PEG, although implying increased costs, has been shown to increase the recovery of enveloped viruses from 31% to 51% (Blanco et al., 2019). In our study, recovery values for PEG precipitation were higher than those reported by Perez-Cataluna et al. (2021) when using similar nucleic acid extraction method (spin column). McMinn et al. (2021) developed a method for the recovery of coronavirus from raw wastewater also using hollow fibers as a primary concentration approach, followed by Concentrating Pipette Select™ (CP Select™), reporting overall recovery values for human coronavirus OC43 of 22%. Differences in recovery between our study and that of McMinn et al. (2021) may be attributed to the ultrafilter that used in our study (inuvai R180 vs Rexeed), coupled with an enhanced elution strategy with three steps that we adopted, and/or to the secondary concentration protocol. The inuvai R180 ultrafilter has a reduced nominal pore size (~5.5 nm with a correspondent cut-off ≤ 18.8 Kda) compared to the Rexeed 15 S, which has a more open pore structure. Additionally, the ultrafilter used in our study has a larger membrane area (1.8 m² for inuvai R180 vs 1.5 m² for Rexeed S15) and larger fiber inner diameters (220 μm for inuvai R180 vs 185 μm for Rexeed S15). In addition to the optimized elution and secondary concentration protocols, such features might help justify the differences registered in the recovery efficiencies of our study and McMinn et al. (2021). Table 1 summarizes the strong and weak points for the different methods used to concentrate SARS-CoV-2 from raw wastewater, including the ones tested in this manuscript.

The extraction efficiency using MNV as proxy averaged 70% (±19%) for inuvai R180 protocol. Extraction efficiencies for PEG precipitation and skimmed milk flocculation averaged 50% (±15%) and 36 (±13%), respectively. RNA extraction was conducted using QIAamp Fast DNA Stool mini kit (QIAGEN, Germany) that although being used for DNA extraction, can efficiently co-extract RNA. The reagents provided by the manufacturer as well as the columns provided are not specific for DNA. The handbook for the kit indicates that RNase should be used to remove RNA from the final eluate. Detection at a high rate demonstrated for MNV showed high efficiency, demonstrating therefore the application of this DNA extraction kit for the co-extraction of RNA.

Detection of PEDV using the inuvai R180 system was consistently achieved with the 1/4-fold dilution, while for undiluted spiked samples,
The table below compares the operational and theoretical advantages and disadvantages of different concentration methods tested in the study:

### Table 1

Operational and theoretical advantages and disadvantages of different concentration methods including those tested in this study.

| Concentration method     | Advantages                                                                 | Disadvantages                                                                 |
|--------------------------|---------------------------------------------------------------------------|------------------------------------------------------------------------------|
| inuvai R180 system       | • Concentrate viruses from both solid and liquid phase                     | • Time consuming                                                             |
|                          | • Concentration of large volumes of raw wastewater (up to 5-L, depending on the turbidity) without increase in time and cost for analysis | • Requires hollow-fiber ultrafilters                                         |
|                          | • Concentration of samples in the field                                     |                                                                              |
|                          | • Multiple samples processed at the same time if several filtration apparatuses are available |                                                                              |
|                          | • Easy to store and transport                                               |                                                                              |
|                          | • Low probability of clogging in volumes up to 3-L (particularly using volumes of just up to 1-L) |                                                                              |
|                          | • Possible application to other water matrices, with higher sample volumes, without further cost or time increase |                                                                              |
| PEG precipitation        | • Concentrate viruses from both solid and liquid phase                     | • Time consuming                                                             |
|                          | • Concentration of large volumes of raw wastewater (e.g. 1-L)              | • Cannot be applied in the field                                             |
|                          | • Only equipment necessary is centrifuge                                    | • Analysis of larger volumes implies increases in time for analysis, reagent costs (PEG particularly), and larger centrifuges |
|                          | • Relatively inexpensive                                                    |                                                                              |
|                          | • Concentrate viruses from both solid and liquid phase                     | • Can be performed onsite                                                  |
|                          | • Concentration of large volumes of raw wastewater, without time increase  | • Concentration volumes extremely variable                                  |
|                          | • Only equipment necessary is centrifuge                                    | • Clogging occurs for samples with high turbidity                          |
|                          | • Possible application to other water matrices, with higher sample volumes, without further cost or time increase | • Each unit is expensive, and several may be necessary for samples with high turbidity |
|                          | • Concentrate viruses from both solid and liquid phase                     | • Cannot be applied in the field                                             |
| Skimmed milk flocculation| • Rapid (depends on the turbidity of the water)                            | • Concentration of viruses only from the liquid phase                        |
|                          | • Only equipment necessary is a centrifuge                                 | • Concentration volumes extremely variable                                  |
|                          | • Rapid (depending on the turbidity of the sample)                         | • Clogging occurs for samples with high turbidity                          |
|                          | • Only equipment required is a filtration unit and a pump                  | • Upscaling to 90-mm membranes requires the use of expensive filtration units |
|                          | • Multiple samples processed at the same time if several filtration apparatuses are available | • Extra care is needed to minimize the risks of contamination, requiring washing and cleaning filtration units |

### Table 1 (continued)

Concentration method | Advantages                                                                 | Disadvantages                                                                 |
|---------------------|---------------------------------------------------------------------------|------------------------------------------------------------------------------|
| Aluminum chloride   | • The membrane is easily stored and transported to the laboratory         | • AlCl₃ highly hygroscopic, must be kept under the right conditions         |
|                     | • Rapid                                                                   | • Necessary to form Al(OH)₃ precipitate properly                            |
|                     | • Concentrates viruses from both solid and liquid phase                   | • pH adjustment is required                                                  |
|                     | • Concentration of large volumes of raw wastewater                        | • Cannot be applied in the field                                             |
|                     |                                                                            | • Requires the use of an orbital shaker, a relatively expensive equipment    |

Only 38% could be detected without inhibition. PEG precipitation was the single method that detected both targets from undiluted samples, although inhibition still occurred (as evidenced subsequently by testing the 4- and 10-fold dilution). As for the skimmed milk concentration method, detection in undiluted concentrates was found for 75% of the samples, although inhibition still occurred (as measured by the dilutions). These results indicate that inhibitory effects exerted upon RT-qPCR could be confirmed for the three methods under comparison. Difference between concentration using the combined R180 system (including elution step and PEG concentration) versus simply using PEG concentration may arise from the elution buffer used and high performance of the ultrafilter itself. Also, co-concentration of PCR inhibitors occurred for all three methods, although with a better extraction recovery using MNV obtained for the combined R180 system, which indicates that concentration using only PEG and skimmed milk potentiated higher co-concentration of inhibitors. Although the ultrafilters concentrated both liquid and solid phases and possible inhibitors were co-concentrated, many might have been directly eliminated during the filtration process and other may not have been eluted during the elution procedure. Therefore, one of the major driving forces for the different results, particularly when comparing both PEG-based methods, might have been the co-concentration of inhibitors.

Overall, our results showed that the inuvai R180 system coupled with an improved elution protocol is highly suitable for the detection of PEDV, used as a surrogate for SARS-CoV-2 in this study, exhibiting the highest percentage of detection and mean recovery value. Additionally, this method also showed greater extraction efficiency and larger volume processing without increased cost or time for downstream analyses. Furthermore, the performance of the inuvai system showed consistency across raw wastewater samples from different catchments/WWTP, including the Serzedelo WWTP, which is highly impacted by industrial effluents (tannery industry) and therefore an extremely complicated matrix to work with altogether, a result corroborated by the Pan-European Umbrella study (Gwik et al., 2021). In the Umbrella study, raw wastewater samples from different European countries were collected and sent for analysis in a centralized laboratory. In parallel, the same samples were also analyzed in each country for comparison of results. The centralized European laboratory was unable to recover SARS-CoV-2 RNA from Serzedelo raw wastewater presenting low recovery percentages (0.1%) and lower concentrations of crAssphage compared to the other samples analyzed. The same sample, analyzed by our group and using the inuvai R180 system, was positive for SARS-CoV-2 and the concentration of crAssphage was 3-log above that detected by the centralized laboratory. These results demonstrate the difficulty of working with this raw wastewater, highlighting the need to test method performance in raw wastewater from different origins.

Data from our study demonstrates the importance of validating concentration procedures using seeded controls. Although other studies have tested the efficiency of concentration and extraction methods, this
study showed the stability of the inuvai R180 system for the recovery of seeded controls in raw wastewater from WWTP with different composition particularities, including effluents from the tannery industry. A single concentration method may not necessarily be ideal to be used in waters from different backgrounds. The inuvai R180 system with improved three-step elution protocol was selected for monitoring SARS-CoV-2 in raw wastewaters and has been applied extensively for WBE for SARS-CoV-2 (Monteiro et al., 2022). This system is attractive as it enables the concentration of large volumes from different types of water, including raw and treated wastewater, drinking water, surface and bathing waters. Such feature enables the use of a single concentration method across different water types without loss in sensitivity, increasing costs or time for analysis, with a less challenging comparison of results. Moreover, a ‘one size fits all’ approach, that is having a single standardized method worldwide for the concentration of SARS-CoV-2, may not be the best approach due to several issues, including: (i) laboratories already have their own preferred methods with performances studied; (ii) the methods may not be useful for application in less economically developed countries; (iii) or simply because it is difficult to get a hold of laboratory materials/equipment (as it was the case of ultrafiltration ultrafilters or ultracentrifuges). Nonetheless, standards as to what should be asked in terms of method performance should be established so that laboratories could gather all the information about the methods to make a more informed choice. Wastewater surveillance has the potential to prevent the occurrence of new outbreaks (Peiser, 2020), and to help understand changes in the pandemic trends. Effective methods, with performance specifications detailed, are paramount for wastewater surveillance to be applied in accurately describing the transmission of SARS-CoV-2 in the community. This study expands the knowledge on analytical methods introducing a method with robust performance for SARS-CoV-2 detection in wastewater and establishing a step forward for the global application of WBE not only for this pandemic but also in future health crisis as the established protocol is modular with application to different taxonomic groups and water types.

3.3. RT-qPCR efficiency

After establishing the inuvai R180 system as gold-standard for primary concentration, the efficiency of the relative quantification method (RT-qPCR) was assessed by calculating the LoD and LoQ for the E_Sarbeco and RdRp assays.

The LoD was 3.99 GC and 5.52 GC per reaction for the E_Sarbeco and RdRp assays, respectively. This corresponded to a method LoD of $2.73 \times 10^3$ GC/L for E_Sarbeco and $3.79 \times 10^3$ GC/L for RdRp using the inuvai R180 system. As for the LoQ, the results were 66 GC and 178 GC per reaction for the E_Sarbeco and RdRp assays, respectively. This corresponded to a method LoQ of $4.56 \times 10^3$ GC/L for E_Sarbeco and $1.22 \times 10^3$ GC/L for RdRp assay.

The LoD obtained in our study were inferior to those obtained by Philo et al. (2021), Perez-Cataluna et al. (2021) reported similar LoD for E_Sarbeco assay, while also presenting method-dependence LoD. Gonzalez et al. (2020), testing the CDC assay (N1, N2, and N3), reported different theoretical limits of detection depending on the RT-qPCR assay used but the LoD were similar to those obtained in our study. A comparison between the performance of our method (evaluated through LoD and LoQ) and the method reported by McMinn et al. (2021) would have been useful, given that the authors have also used hollow-fiber ultrafilters for primary concentration, but such parameter information is missing on the former report. In fact, information on LoQ is missing from most publications with very few exceptions, such as LaTurner et al. (2021) who, while testing five different concentration methods, reported LoQ ranging from $2.76 \times 10^3$ to $8.39 \times 10^3$ GC/L. Philo et al. (2021) calculated their LoQ in nuclease-free water to be 100 gene copies per reaction for all CDC assays.

4. Conclusions

- The newly developed inuvai R180 ultrafilter, combined with a new elution method and PEG precipitation allowed for a higher percentage of recovery of PEDV, chosen as a surrogate for SARS-CoV-2.
- The inuvai R180 system performed at a high level for the detection of PEDV even in more difficult wastewater matrices, including those with a high input of industrial effluents
- The RT-qPCR targets have different sensitivity suggesting the choice of molecular targets for detection is crucial
- Uniformization of the adequate description of methods performance is necessary.

CredIt author statement

Silvia Monteiro: conceptualization, methodology, software, validation, formal analysis, investigation, writing – original draft, writing – review and editing, visualization. Daniela Rente: investigation. Mónica V. Cunha: writing - review and editing. Tiago A. Marques: formal analysis, writing - review and editing. Eugénia Cardoso: review and editing, sampling; Pedro Álvarez: review and editing, sampling; Joao Vilaca: review and editing, sampling; Jorge Ribeiro: review and editing, sampling; Marco Silva: review and editing, sampling; Norberita Coelho: review and editing, sampling; Nuno Broco: project administration, funding acquisition, review and editing; Marta Carvalho: project administration, funding acquisition, review and editing; Ricardo Santos: conceptualization, methodology, resources, formal analysis, writing – review and editing.

Funding

This work was supported by Programa Operacional de Competitividade e Internacionalização (POCI) (FEDER component), Programa Operacional Regional de Lisboa, and Programa Operacional Regional do Norte (Project COVIDETECT, ref. 048,467).

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.

Acknowledgements

We thank all the workers from Águas de Portugal Group who contributed with wastewater samples and those who contributed with critical discussion.

This work was funded by Programa Operacional de Competitividade e Internacionalização (POCI) (FEDER component) and Programa Operacional Regional de Lisboa (Project COVIDETECT, LISBOA-01-02B7-FEDER-048467).

Strategic funding of Fundação para a Ciência e a Tecnologia (FCT), Portugal, to eE3c,BioSL and CAEUl Research Units (UIDB/00329/2020, UIDB/04046/2020 and UIDB/00006/2020) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.114296.

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