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Case Report

Evaluation of SARS-CoV-2 concentrations in wastewater and river water samples

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1. Introduction

The epidemiological and sanitary crisis of the 20th century was primarily caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the causative agent of coronavirus disease 2019 (COVID-19). Detecting the genetic material of SARS-CoV-2 in stool and urine samples, as well as its environmental persistence, has made wastewater surveillance (WWS) a promising strategy for monitoring the dynamics of COVID-19 [1]. Wastewater-based epidemiology (WBE) is a well-established methodology for monitoring gastrointestinal viruses, such as hepatitis A and E viruses, poliovirus, astrovirus, and norovirus [2], and it has been successfully applied in the worldwide surveillance of SARS-CoV-2 [3–9].

Currently, SARS-CoV-2 surveillance through WBE is a complementary approach to clinical monitoring as asymptomatic cases are also prevalent [10], which is a key factor contributing to the rapid and global spread of the virus.
spread of SARS-CoV-2 [11,12]. Therefore, this feature is a potential tool for countries facing difficulties in implementing mass COVID-19 testing, such as in Brazil, where scenarios of underreporting have been observed [13,14]. Moreover, WWS can help detect fluctuations in SARS-CoV-2 viral load two weeks before its clinical occurrence [4], thereby helping policymakers in directing actions and population testing to specific areas.

However, there are some limitations to WBE for SARS-CoV-2 surveillance, including the methodologies used to concentrate and recover the virus [15], the wastewater sampling source and volume (particles or aqueous portions) [6,16,17], the RNA extraction methods that lack a gold standard [17], the primers or molecular diagnosis strategy used [3, 5,18,19], and the RT-qPCR kit used for testing [20]. In addition, the number of virus copies can vary depending on the recovery efficiencies of the methods [17,21,22]. It is also difficult to compare the data obtained from different methods because the variability in the viral copy number might reflect in differences in the quantitative data. Therefore, several studies have attempted to establish a simple, sensitive, and highly efficient method for concentrating SARS-CoV-2 from wastewater samples [3,5,18,21,23]. The most common strategies for this purpose include ultrafiltration, precipitation, and adsorption (virus adsorption–elution [VIRADEL]). Ultrafiltration is a particle size-exclusion technique with considerable advantages, including the absence of a pre-acidification step and long precipitation periods, thereby decreasing the possible instability of the viral particles [24,25]. However, other steps, such as the addition of a blocking solution and possible immobilization, may be required for this technique [26].

The concentration process can also be performed by separating molecules via precipitation with the help of substances that can interact with the viral capsid particles through their isoelectric point [27]. Generally, concentration by precipitation is used when a medium or large sample-sized aggregate is available [28]. However, the limitations of this technique include the use of toxic organic solvents, long precipitation time, and precipitation of non-viral proteins [29,30]. In contrast, VIRADEL methods are based on filtration through electrostatically charged membranes [31,32], in which the particles are separated using a cellulose nitrate membrane that adsorbs molecules by forming chemical bonds with a salt (usually MgCl₂ and NaCl) [15,33]. VIRADEL methods are known for their advantages such as the possible reduction of PCR inhibitors. However, these methods require a large volume of samples, in addition to the multiple steps involved, which can lead to viral loss [29,34].

In this context, researchers have worked extensively on the standardization of wastewater concentration methods. However, to the best of our knowledge, related studies on SARS-CoV-2 concentration in river water samples have not been performed yet. Thus, this study aimed to evaluate and validate the efficiencies of four concentration methods: vacuum filtration using an electronegative membrane with MgCl₂ addition; precipitation-based methods using aluminum hydroxide or polyethylene glycol (PEG) 8000; and ultrafiltration, using known concentrations of SARS-CoV-2 as recovery controls. To validate the recovery methods of viruses present in low filters, the samples were evaluated without the addition of SARS-CoV-2. The PEG 8000 precipitation method was evaluated under additional conditions to determine its efficiency in detecting SARS-CoV-2 in samples containing suspended solids.

2. Methodology

Fig. 1 presents an overview of the methodology used for the evaluation of four different concentration methods: vacuum filtration, aluminum hydroxide-based precipitation, ultrafiltration using centrifuge filters, and PEG 8000 precipitation.

2.1. Wastewater and river water sample collection

In this study, a single collection was performed for each sample matrix. Raw wastewater samples were collected on March 9, 2021, from a wastewater treatment plant (ETE Flamboyants; –12.937983873929847, –38.41128497439337) located in Salvador, Bahia State, Brazil. The wastewater sample collection area was located in an apartment complex. The Ipiranga River wastewater-enriched water sample was collected on February 26, 2021, from an urban area near Lauro de Freitas (–12.8517952071429, –38.29615719760099) in the municipality of Bahia State. This river receives clandestine ejection from untreated wastewater, which was the reason as to why the sample collection was performed near a wastewater ejection duct.

Wastewater samples were provided by collaborators from the Salvador Municipality Effluent Treatment Company, Empresa Baiana de Aguas e Saneamento (EMBASA). The sample collection methodology was based on grab collection from the influent collection tank of a treatment plant. The samples were collected in 2.5-L flasks and transported at 4 °C until storage in an ultrafreezer at –80 °C. The samples were maintained at low temperatures (–80 ± 2 °C) until further analyses.

2.2. Sample inactivation and suspended particle removal (pretreatment)

To increase the biosafety of the samples, thermal inactivation was performed, as described previously [35,36]. Prior to the concentration process, suspended particles from the samples were removed using filtration or centrifugation. The filtration protocol was performed using a vacuum filtration system and Whatman™ GF/C microfiber filters (1.2-µm pore size, 47-mm diameter; GE Healthcare Life Sciences™, Illinois, USA). The resulting flow-through was used for the concentration step. For centrifugation, the samples were centrifuged at 4500 × g for 30 min at 4 °C, and the supernatant was used in the following concentration procedures. The suspended particle residues were discarded.

2.3. SARS-CoV-2-spiked samples and biosafety

Spiked SARS-CoV-2 samples were obtained from nasopharyngeal swabs of SARS-CoV-2-positive patients from the Laboratory of Molecular Diagnosis, SENAI CIMATEC Health Technology Institute. The samples were mixed in a pool that was inactivated by heating at 60 °C for 60 min [35,36]. Two different pools of SARS-CoV-2-spiked samples were used for the experiment, because of the availability of SARS-CoV-2-positive samples. To each sample (40 mL), 90 µL of the SARS-CoV-2 pool was added. Quantification procedures for pools 1 and 2 are described in Section 2.5. In a volume of 90 µL, pool 1 quantification showed average copy numbers of 4.1 × 10^6 and 1.3 × 10^5 for N1 and N2 (primers/probes of SARS-CoV-2), respectively, whereas pool 2 quantification showed average copy numbers of 2.9 × 10^6 and 1.5 × 10^5 for N1 and N2, respectively. Pool 1 was used in all concentration methods tested for both sample matrices, i.e., wastewater and river samples, except for PEG 8000 precipitation, for which pool 2 was used.

2.4. Concentration methods

SARS-CoV-2 concentration was determined using three concentration approaches (a, b, and c) divided into four methods (1, 2, 3, and 4): (a) vacuum filtration using (1) an electronegative membrane with MgCl₂ addition; (b) precipitation approach using (2) aluminum hydroxide and (3) PEG 8000; and (c) ultrafiltration using (4) two devices with membranes of different porosities (10 and 50 kDa).

To assess each pretreatment step (filtration and centrifugation) and its associated concentration method, assays were performed using two aliquots of 40 mL for each sample matrix. Thus, all experiments were performed in duplicate and originated from the same sample matrix.
Fig. 1. Overview of the methodology applied for the evaluation of different concentration methods for detecting SARS-CoV-2 in river and wastewater samples. Created with BioRender.com.
2.4.1. Vacuum filtration method

The vacuum filtration method was performed as described by Ahmed et al. [21], with modifications to the MgCl\textsubscript{2} concentration used. To obtain a final concentration of 50 mM MgCl\textsubscript{2} after the removal of suspended particles, 800 μL of 2.5 M MgCl\textsubscript{2} solution was added to flasks containing 40 mL of the sample. The pH of each sample was adjusted between 5.5 and 6.0, and the samples were subjected to vacuum filtration through a negatively charged membrane made of mixed cellulose ester (0.45-μm pore size, 47-mm diameter; Merck Millipore, Massachusetts, USA). To extract the virus from the electronegative membrane after filtration, the membrane was washed with 25 mL of 0.14 M NaCl, placed in a 50-mL tube containing 1 mL of nuclease-free water and shredded with sterilized scissors. The shredded membrane was stored at −80°C until the final processing prior to RNA extraction. In the final processing, the filters were triturated using a TissueLyser II™ (Qiagen®, Hilden, Germany) with a 5-mm bead for 3 cycles of 1 h and 30 min, with 10-min intervals at a maximum frequency of 30 Hz. Subsequently, the samples were centrifuged at 12,000 × g for 10 min to separate the supernatant from filter residues. The samples were stored at −80 ± 2°C until RNA extraction.

2.4.2. Aluminum hydroxide-based precipitation

For the aluminum hydroxide-based precipitation method, the pH of the samples was adjusted to 6.0 after removal of suspended particles. To enable an Al(OH)\textsubscript{3} precipitate, a 1:100 (v/v) solution of 0.9 N AlCl\textsubscript{3} was added, and the sample pH was adjusted to 6.0 again. Subsequently, the samples were incubated at room temperature (25 ± 2°C) for 15 min on an orbital shaker (150 rpm). To pellet the precipitate, the samples were centrifuged at 17000 × g for 20 min, the supernatant was discarded, the pellet was resuspended in 2.5 mL of 3% beef extract solution (pH 7.4), followed by incubation for 10 min at 150 rpm. After discarding the supernatant, the pellet was resuspended in 1900 × g for 30 min. After discarding the supernatant, the pellet was resuspended in 1 mL of 0.01 M phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.8 mM KH\textsubscript{2}PO\textsubscript{4} at pH 7.2 [37]). The concentrated fractions were stored at −80 ± 2°C until RNA extraction.

2.4.3. Ultrafiltration using centrifuge filters

Ultrafiltration is used to concentrate small particles by separating them through a membrane to promote sample passage to the membrane apparatus. Ultrafiltration concentration assays were performed using Amicon Ultra-15 centrifuge filters (Merck Millipore, Massachusetts, USA) with two different pore sizes (10 and 50 kDa). The manufacturer recommends a maximum centrifugation force of 4000 × g for swing-bullet rotors, with centrifugation times varying from 15 to 60 min. Thus, in this experiment, centrifugation for all the tested filters was performed at 4000 × g for 30 min at 4°C. The centrifugation cycle was repeated at least thrice to filter all the volumes that were tested per sample (40 mL). Concentrated samples were recovered using the filters by pipetting, and the volumes ranged 250–400 μL and 150–250 μL for the 10 and 50 kDa filters, respectively. Volumes of the concentrated samples were adjusted to 400 μL by the addition of ultrapure nuclease-free water if required. The concentrated samples were stored at −80 ± 2°C until RNA extraction.

2.4.4. PEG 8000 precipitation

PEG 8000 precipitation assays were performed using filtered, centrifuged, and raw (untreated) wastewater and wastewater-enriched river water samples. For the assay, 40 mL of each sample was used, and 4 g of PEG 8000 (10% w/v) and 0.9 g of NaCl (2% w/v) were added as co-precipitants. Subsequently, the samples were homogenized until PEG 8000 and NaCl were completely dissolved. The samples were incubated overnight (16 h) under agitation at 4°C and then centrifuged at 4863 × g (maximum centrifuge force) for 60 min at 4°C to obtain a pellet containing viral samples. The supernatant was discarded, the pellet was resuspended in 0.01 M PBS solution (pH 7.2), and the final volume was adjusted to 1 mL. The samples were stored at −80 ± 2°C until RNA extraction. An additional step was performed to analyze the effect of potential PCR inhibitors co-concentrated in the wastewater and wastewater-enriched river water samples, which were concentrated with suspended particles (without pretreatment). The samples were cleaned using an OneStep PCR Inhibitor Removal Kit™ (Zymo Research, California, USA), according to the manufacturer’s instructions after RNA extraction (Fig. 2).

2.5. Viral RNA extraction and SARS-CoV-2 RT-qPCR

Viral RNA was extracted from concentrated samples using a MagMax™ Viral Pathogen II kit (Thermo Fisher Scientific™, Massachusetts, USA) and the KingFisher™ Duo Purification System (Thermo Fisher Scientific™, Massachusetts, USA), according to the manufacturer’s instructions. The extracted RNA was immediately subjected to one-step RT-qPCR or stored at −80 ± 2°C until further analysis. In the RT-qPCR assays, each marker was evaluated in duplicate per RNA sample, totaling four amplifications per sample matrix or method tested. The SARS-CoV-2-specific primers and probes were designed by the Centers for Disease Control and Prevention (CDC, USA), and N1 and N2 were designed by Integrated DNA Technologies (IDT, California, USA). RT-qPCR assays were performed using the TaqPath™ 1-Step RT-qPCR Master Mix (Thermo Fisher Scientific™, Massachusetts, USA), according to the manufacturer’s instructions. Briefly, the RT-qPCR mix consisted of 5 μL TaqPath™ 1-Step RT-qPCR Master Mix, 1.5 μL primer and probe mix, 8.5 μL nuclease-free water, and 5 μL template, resulting in a total volume of 20 μL. The reaction was performed on a QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific™, Massachusetts, USA). Only reactions with a cycle threshold (Ct) < 40 were considered positive. All protocols were executed as suggested by the CDC [38].

The limit of detection was 10 genome copies for N1 and N2 with Ct < 40.
values of 36.64 ± 0.30 and 37.60 ± 0.22, respectively. For the N1 and N2 targets, 2 × 10^5 copies/μL of 2019-nCoV positive control [38] was added as a positive control for the qPCR, and 1 μL of human specimen control (HSC; IDT, California, USA) was added as an RT-qPCR inhibition control. HSC (1 μL) was added to each sample before RNA extraction, and the human RNase P gene (RP) was amplified using RP primers and probes designed by the CDC (2020). To assess potential contamination during the concentration step and inhibition of qPCR assays, PBS was used as a blank control during the concentration and RNA extraction steps. To this blank control, 1 μL of HSC was added before RNA extraction, as performed for all tested samples. Inhibition was ruled out if the Ct value of RP amplification from the RNA samples was within the PBS blank control range of Ct ± standard deviation (SD) values plus a 2-Ct value higher than this range.

2.6. SARS-CoV-2 copy number and recovery efficiency

The number of SARS-CoV-2 copies was calculated using Equation (1) [39]. The variables in Equation (1) were obtained from a validated standard curve of n-CoV absolute plasmids [38], 10-fold serial dilutions (10^6 to 10^3 copies/μL), and a dilution curve of a 2-fold serially diluted sample pool. Both n-CoV and sample curves were obtained from three independent RT-qPCR experiments. These data were applied to Equation (1), which provided the estimated value of copy numbers.

\[
x_{0\text{ sample}} = 10^{(\text{Ts} - \text{Ta})} \times \text{EAMP} \times \log_{10} \text{EAMP}_{\text{sample}}/\text{EAMP}_{\text{abs} - \text{Cq}_{\text{sample}}}. \tag{1}
\]

Where \(x_{0\text{ sample}}\) is the number of target copies in the sample, \(\text{Ts}\) is the sample threshold, \(\text{Ta}\) is the absolute template (n-CoV standard curve) threshold, \(\text{EAMP}\) is the standard curve intercept, \(\text{Cq}_{\text{sample}}\) is the cycle threshold of the sample, \(\text{EAMP}_{\text{sample}}\) is the exponential amplification of the sample, and \(\text{EAMP}_{\text{abs}}\) is the exponential amplification of the absolute template. The exponential amplification was calculated as follows:

\[
\text{EAMP} = 10^{(-1/\text{slope})}.
\]

After generating the copy number data, recovery efficiencies were calculated as described by Ahmed et al. [21] and Barril et al. [23]. Owing to differences in detection between the tested concentration methods, the SARS-CoV-2 copy numbers determined in collected samples without spiked SARS-CoV-2 (raw samples) were not subtracted from the recovery efficiency analysis of artificially contaminated samples. Thus, recovery efficiencies higher than 100% refer to SARS-CoV-2 present in the samples at the time of collection.

2.7. Data and statistical analyses

The QuantStudio™ Design and Analysis Software v1.5.1 (Thermo Fisher Scientific™, Massachusetts, USA) was used to evaluate the standard curve and threshold data. The Ct, slope, intercept, threshold, and EAMP data were organized using Microsoft Office Excel™ (Microsoft Corporation, Washington, USA), and the SARS-CoV-2 copy number was calculated using Equation (1).

The average and SD values were obtained using column statistical analysis for descriptive statistics. Statistical analysis was performed using the efficiency data obtained from the N1 marker. Thus, the Shapiro–Wilk normality test was conducted to confirm if the samples and replicates deviated from a normal distribution. Subsequently, to compare the SARS-CoV-2 recovery efficiencies, three analysis groups were analyzed: (1) pretreatment (filtration or centrifugation) using the same concentration method, (2) concentration method in samples subjected to the same pretreatment, and (3) comparison of all pretreatment and concentration conditions tested for each sample matrix. Group 1 was analyzed using the non-parametric Wilcoxon test (p < 0.05). In contrast, Groups 2 and 3 were subjected to analysis of variance using the Friedman test (a non-parametric test) with post-hoc analysis using Dunn’s multiple comparison test (p < 0.05). All statistical analyses were performed using the GraphPad Prism 7.0 software (La Jolla, CA, USA).

3. Results

The preliminary experiments (data not shown) demonstrated a high inhibition of HSC extraction when RNA was extracted from samples containing suspended particles during the concentration step. Thus, suspended solid removal was incorporated into the main protocol, and most of the samples were concentrated only after the suspended particles were removed via filtration or centrifugation. The evaluation of qPCR inhibition by RP gene amplification did not show any significant inhibition of the qPCR assay for all the tested methods, except for wastewater concentrated through PEG 8000 without pretreatment, which presented 3-Ct values higher than those obtained using PBS as blank control (Table S1).

A high variation in recovery efficiency was observed in SARS-CoV-2-spiked samples using the tested methods. Considering both N1 and N2 markers, the efficiencies of spiked sample recovery for wastewater samples were higher when the ultrafiltration method was used with a 10- or 50-kDa cut-off filter after the removal of suspended particles via filtration or centrifugation (Table 1). However, the combination of centrifugation pretreatment and ultrafiltration using a 50-kDa cut-off filter showed the best results; the spiked sample recovery exhibited 113% efficiency for the N1 marker (Table 1). After concentration using ultrafiltration, higher recovery efficiencies were obtained when centrifugation pretreatment and concentration methods were conducted using an electropositive membrane with MgCl₂ addition. The other two concentration methods, i.e., PEG 8000 precipitation and aluminum-based adsorption–precipitation, resulted in lower recovery efficiencies, with the latter being the least efficient method for N1 marker analysis. Using the same concentration method, pretreatments of wastewater samples using filtration or centrifugation showed no significant differences (Fig. S1A). Statistical analysis of the concentrated wastewater samples after filtration showed significant differences between the

### Table 1

| Concentration methods efficiency evaluated by spike sample recovery. |
|---------------------------------------------------------------|
| **Concentration methods**                                      | **N1 (Efficiency Mean (%) ± SD)** | **N2 (Efficiency Mean (%) ± SD)** |
|                                                               | Filtration | Centrifugation | Filtration | Centrifugation |
| **Wastewater**                                                |            |                |            |                |
| Electronegative membrane + MgCl₂ – direct extraction         | 21.1 ± 3.0 | 40.4 ± 1.9     | 34.5 ± 6.1 | 73.8 ± 8.3     |
| Aluminum hydroxide-based precipitation                       | 4.5 ± 0.5  | 4.5 ± 1.1      | 5.7 ± 1.3  | 4.6 ± 0.9      |
| PEG 8000 precipitation                                       | 8.4 ± 1.3  | 9.8 ± 0.3      | 3.5 ± 0.2  | 3.9 ± 0.2      |
| Ultrafiltration – 10 kDa membrane                             | 57.9 ± 4.5 | 47.7 ± 2.0     | 46.9 ± 4.9 | 36.7 ± 16.2    |
| Ultrafiltration – 50 kDa membrane                             | 63.8 ± 27.9| 113.1 ± 5.0    | 46.2 ± 6.2 | 54.3 ± 3.3     |
| **River**                                                     |            |                |            |                |
| Electronegative membrane + MgCl₂ – direct extraction         | 4.7 ± 1.6  | 110.8 ± 30.4   | 3.9 ± 1.3  | 95.9 ± 26.8    |
| Aluminum hydroxide-based precipitation                       | 34.7 ± 1.0 | 33.4 ± 12.0^b  | 26.4 ± 0.9 | 28.3 ± 10.3^b  |
| PEG 8000 precipitation                                       | 57.8 ± 36.3| 47.8 ± 4.7     | 34.3 ± 11.0| 21.3 ± 2.8     |
| Ultrafiltration – 10 kDa membrane                             | 7.9 ± 3.3  | 45.2 ± 27.8    | 6.6 ± 2.8  | 39.3 ± 24.5    |
| Ultrafiltration – 50 kDa membrane                             | 2.9 ± 1.1  | 120.0 ± 0.8    | 2.4 ± 0.9  | 10.1 ± 0.7     |

^a Only one positive amplification in the quadruplicate.

^b Three positive amplification in the quadruplicate.
ultrafiltration (using 10- or 50-kDa cut-off membranes) and aluminum hydroxide-based precipitation methods (Fig. S2A). Similarly, for wastewater samples pretreated using centrifugation, significant differences were observed between the 50-kDa cut-off ultrafiltration and aluminum hydroxide-based precipitation methods (Fig. S2B). Finally, when all the combination methods (pretreatment and concentration) were compared (Fig. S3A), differences were observed only between the samples obtained via PEG 8000 precipitation (pretreatment via filtration) and ultrafiltration with a 50-kDa cut-off membrane (pretreatment via centrifugation).

Table 1 presents the SARS-CoV-2 recovery efficiencies in river water samples (which were artificially contaminated with the virus), association with centrifugation pretreatment, and concentrations when an electronegative membrane with MgCl₂ addition was used. Better efficiency performance was observed for SARS-CoV-2 recovery in spiked river water samples obtained by centrifugation pretreatment combined with PEG 8000 precipitation, followed by centrifugation in combination with ultrafiltration using a 10-kDa cut-off filter. The aluminum hydroxide-based precipitation method was capable of recovering SARS-CoV-2-spiked samples but did not exhibit amplification for both N1 and N2 markers for all replicates (Table 1). The same phenomenon was observed in the combination of filtration pretreatment and electronegative membrane with MgCl₂ that amplified only two replicates of the N1 marker. Given the partial amplification of the N1 marker using these methods, the results could not be statistically analyzed. Thus, filtration pretreatments in combination with an electronegative membrane using MgCl₂ and aluminum hydroxide-based precipitation methods were not statistically analyzed.

However, comparing different pretreatments for the same concentration method did not reveal any significant differences between pretreatments using filtration or centrifugation and recovery efficiencies for river water samples, as observed for wastewater samples (Fig. S1B). Comparison between concentration methods pretreated with the same strategy, i.e., filtration or centrifugation, showed significant differences only between the PEG 8000 and ultrafiltration methods using a 50-kDa cut-off membrane in the filtration pretreatment approach (Fig. S2C). For centrifugation pretreatment, differences were observed only between the electronegative membrane with MgCl₂ and ultrafiltration using a 50-kDa cut-off membrane (Fig. S2D). Finally, on comparing the recovery efficiencies of all methods and their associated pretreatment for river water samples, differences were observed only between centrifugation pretreatment associated with the electronegative membrane with MgCl₂ addition and ultrafiltration using a 50-kDa cut-off membrane after filtration pretreatment (Fig. S3B).

However, different results were obtained when wastewater samples without the artificial addition of SARS-CoV-2 (denoted as raw samples) were subjected to the same protocols as those described for wastewater-spiked samples. For raw wastewater samples, the combination of centrifugation pretreatment and ultrafiltration using a 10- or 50-kDa cut-off filter did not show any amplification for both N1 and N2 markers (Table 2). In contrast, the samples with suspended solids removed by filtration and ultrafiltration using 10- or 50-kDa cut-off filters showed positive amplification for both N1 and N2 markers, demonstrating the presence of SARS-CoV-2 in raw wastewater samples and the capability of this method to concentrate viral samples from wastewater (Table 2). Similar SARS-CoV-2 copy number results were obtained for raw wastewater samples using filtration pretreatment followed by filtration with an electronegative membrane and MgCl₂ addition or aluminum-based adsorption–precipitation concentration methods, but only for N2 markers. However, only two (2/4) and one (1/4) replicates were positive for N2. The use of PEG 8000 precipitation showed no amplification in the raw wastewater samples pretreated with filtration or centrifugation (Table 2). However, raw wastewater samples that were concentrated without solid removal were positive for both N1 and N2 markers after a clean-up step that specifically removed PCR inhibitors (Table 3).

| Table 2 | SARS-CoV-2 copy number in raw samples concentrated by different methods. |
|---------|---------------------------------------------------------------|
| **Concentration methods** | **N1 (mean of copy number (copies/L))** | **N2 (mean of copy number (copies/L))** |
| | Filtration | Centrifugation | Filtration | Centrifugation |
| **Wastewater** | | | | |
| Electronegative membrane + MgCl₂ – direct extraction | 9.5x10⁵ | N.d. | 1.1x10⁶ | N.d. |
| Aluminum hydroxide-based precipitation | 4.4x10⁵ | N.d. | 1.5x10⁶ | 1.8x10⁶ |
| PEG 8000 precipitation | 8.2x10⁵ | N.d. | 1.8x10⁶ | 2.1x10⁶ |
| Ultrafiltration – 50 kDa membrane | 6.9x10⁵ | 1.3x10⁶ | 2.6x10⁵ | 3.7x10⁵ |
| **River** | | | | |
| Electronegative membrane + MgCl₂ – direct extraction | 6.7x10⁵ | N.d. | 2.5x10⁶ | N.d. |

N.d.: Not detected.

a Only one positive amplification in the quadruplicate.
b Two positives amplifications in the quadruplicate.
c Three positive amplifications in the quadruplicate.

| Table 3 | Copy number obtained from samples inoculated or not with SARS-CoV-2 spiked sample concentrated by PEG 8000 precipitation without debris removal. The samples were or not submitted to the OneStep PCR Inhibitor Removal Kit (Zymo Research, CA, USA). |
|---------|---------------------------------------------------------------|
| **Concentration by PEG 8000** | **Spiked sample** | **Raw sample** | **Spiked sample** | **Raw sample** |
| **Wastewater** | | | | |
| Without PCR inhibitor removal | 2.0x10⁵ | N.d. | 1.3x10⁵ | N.d. |
| After PCR inhibitor removal | N.p. | 2.5x10⁵ | N.p. | 1.3x10⁵ |
| **River** | | | | |
| Without PCR inhibitor removal | 2.0x10⁵ | N.d. | 5.7x10⁵ | N.d. |
| After PCR inhibitor removal | N.p. | 4.0x10⁵ | N.p. | 1.1x10⁵ |

N.p.: Not performed.

N.d.: Not determined.

a Only one positive amplification in the quadruplicate.
b Three positives amplifications in the quadruplicate.

Analysis of raw river water samples showed that concentration by PEG 8000 precipitation and ultrafiltration using 10- or 50-kDa cut-off filters amplified the N1 and N2 targets in both pretreatments (Table 2). However, the methods associated with filtration pretreatment exhibited only one amplification in the quadruplicate tested, whereas the association with centrifugation pretreatment showed amplification of two and three replicates in the quadruplicate evaluated (Table 2). Centrifugation in combination with the electronegative membrane with MgCl₂ addition and aluminum hydroxide-based precipitation did not
show any amplification for either of the SARS-CoV-2 markers tested in the river water samples (Table 2).

Samples that were not pretreated (for suspended solid removal) were also subjected to PEG 8000-based precipitation. For both sample matrices, the results demonstrated the amplification of N1 and N2 markers only in samples spiked with SARS-CoV-2 that were untreated with the PCR inhibitor removal kit (Table 3). However, in wastewater samples, only one quadruplicate replicate was detected in the molecular assays for N1 and N2 markers. For wastewater-enriched river samples, all tested replicates were amplified for both SARS-CoV-2 markers. Inhibition was also observed in the amplification of RP in the wastewater samples that were not subjected to the inhibitor removal kit (Table S1). In contrast, raw wastewater and raw wastewater-enriched river water samples showed amplification only after removing the PCR inhibitor (Table 3). The wastewater-enriched river water samples amplified three replicates of the quadruplicate tested in the qPCR assays for N1 and N2 markers, whereas only one replicate was amplified for wastewater samples after the clean-up step.

4. Discussion

Most waterborne viruses are non-enveloped, and standard methodologies for viral concentration in wastewater are well-established for non-encapsulated viruses but not for enveloped viruses (such as coronaviruses) [21,40]. Therefore, standardization of an efficient concentration methodology for enveloped viruses remains a challenge in the scientific community [40], which underscores the importance of the present study.

Several methods have been used to concentrate SARS-CoV-2 in wastewater [3,18] and wastewater-enriched river water samples [7,41]. It is difficult to compare the data given the variable success rates of the methods for SARS-CoV-2 concentration. Our results showed a high variation in recovery efficiencies between the tested methods and that methods for SARS-CoV-2 concentration. Our results showed a high variation in recovery efficiencies between the tested methods and that methods for SARS-CoV-2 concentration. Our results showed a high variation in recovery efficiencies between the tested methods and that methods for SARS-CoV-2 concentration. Our results showed a high variation in recovery efficiencies between the tested methods and that methods for SARS-CoV-2 concentration. Our results showed a high variation in recovery efficiencies between the tested methods and that methods for SARS-CoV-2 concentration. 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Concentration using an electronegative membrane and ultrafiltration showed the best recovery efficiencies for MHV (>50%); their results were consistent with the results obtained in this study [21]. In addition, after ultrafiltration, the use of an electronegative membrane with MgCl2 addition resulted in the highest efficiency for the concentration of SARS-CoV-2-spiked wastewater samples. However, for the wastewater-enriched river water samples, no detailed investigation on methods to concentrate SARS-CoV-2 has been performed until now. Thus, this study is the first to investigate the concentration methods for SARS-CoV-2 in wastewater-enriched river water samples. Our results revealed that the electronegative membrane with MgCl2 addition was the most efficient method for these samples compared to the other methods used for SARS-CoV-2-spiked samples when centrifugation was used for pretreatment. For the separation to occur, the concentration obtained by ultrafiltration is independent of the availability of molecules present in the samples for possible chemical interactions, as it is based on a simple size-exclusion principle [29]. This feature may have facilitated the higher efficiency of this method for wastewater samples, compared to that when other methods are used, which require chemical interactions. However, when the electronegative membrane concentration method was employed instead of ultrafiltration, the wastewater-enriched river were more susceptible to osmotic pressure and electronegative (hydrophobic) interactions due to the addition of MgCl2 [32,42,43].

River water concentration is challenging because of low viral titers [44]. The most suitable volume and concentration method are discussed in the subsequent section. Haramoto et al. [6] attempted but failed to detect SARS-CoV-2 using a 5-L volume of river samples. They used an electronegative membrane with MgCl2 addition as the concentration method and six different qPCR/nested PCR assays and hypothesized that the absence of detection was due to the lower prevalence of COVID-19 in the region. Interestingly, the concentration of 2 L of water from two rivers in Quito, Ecuador showed a high number of copies for both N1 and N2 targets using an adapted skimmed milk flocculation method [41]. According to the authors, both rivers receive untreated wastewater, which explains the positive results and high copy numbers of SARS-CoV-2 RNA in their samples. Because the wastewater-enriched river water showed a different performance when compared to wastewater in terms of SARS-CoV-2-spiked sample recovery, the results of the present study suggest that the choice of the optimal concentration method should depend on the type of sample. When comparing the performance of the methods for river water samples based on different approaches, methods based on the principle of precipitation showed better results than those based on filtration.

Notably, the results of this study demonstrated that aluminum hydroxide-based precipitation was the least efficient method for recovering SARS-CoV-2 from wastewater-spiked samples, with an efficiency of less than 35% for river water-spiked samples. Aluminum ions are known inhibitors of PCR assays and are found in environmental samples such as wastewater [45]. Regarding the chemical properties of aluminum hydroxide used in precipitation methods, the ionic species Al(OH)4− is the predominant aluminum species in alkaline solutions. Moreover, other aluminum complexes as well as aluminum ions (Al3+) can be found in aqueous solutions, even at very low concentrations [46]. Hence, the use of a concentration method based on aluminum hydroxide precipitation, particularly in environmental samples, can result in a high content of Al3+ ions and lead to false-negative data due to the PCR inhibition process, as observed in the results obtained herein.

Another important finding of this study is related to the pretreatments utilized for removing suspended particles from both sample matrices. Other researchers have successfully used this strategy for SARS-CoV-2 investigations from wastewater samples [3–5,16,23,47]. Our results showed no significant differences in recovery efficiencies from the same concentration method with different pretreatments, indicating that the differences in recovery efficiencies were not attributed to the removal of suspended solids via filtration or centrifugation.

However, the removal of suspended solids from samples can affect SARS-CoV-2 detection and quantification. Westhaus et al. [47] compared the quantity of SARS-CoV-2 RNA copies obtained in separated aqueous and solid phases and observed that the solid phase contained 13.8-fold more viruses than the aqueous phase. Concentration tests performed withMHV showed that on average, 30% of the virus could be adsorbed onto these suspended particles [21]. Therefore, the concentration method performed using both the solid and liquid phases of samples can result in more viral titers, thereby reducing the occurrence of false-negative results.

Our results for the PEG 8000 precipitation the method for spiked samples with liquid and solid phases showed only one replicate in wastewater samples for N1 and N2 markers, whereas all tested replicates were amplified in river water samples for both markers. This indicates that the amount of solids in the samples affected qPCR detection because of the high quantity of inhibitors co-concentrated within the virus samples. D’Aoust et al. [46] reported that concentrated solid particles also increased the number of qPCR inhibitor molecules present in samples. Wastewater-enriched river water samples had significantly lower suspended particles than wastewater samples. No qPCR inhibition was observed in river water-spiked samples without PCR inhibitor removal. For raw wastewater-enriched river water samples, the clean-up step promoted the amplification of three replicates in a total of four, whereas for wastewater, only one replicate showed N1 and N2 amplifications. These results indicate that for wastewater-enriched river water samples, the clean-up step is sufficient to remove inhibitors that were co-concentrated during the concentration process; however, for
wastewater samples, the clean-up step alone is insufficient. Thus, using both solid and liquid phases of river water samples in the concentration step could be a good strategy if qPCR inhibitors are removed from the RNA samples. For wastewater, a higher amount of solid particles represents a risk in terms of qPCR inhibition, and removing the solids in the concentration step could improve detection, as inhibitors co-concentrated in higher amounts can be avoided.

In addition to the removal of large particles via filtration or centrifugation, some inhibitors are diluted into the aqueous part of the sample or into very small particles that cannot be removed by filtration or centrifugation techniques [43]. Thus, in some cases, the presence of inhibitors can be minimized but not completely eliminated, and they can co-concentrate with the virus [21,23]. Therefore, for better molecular detection of viruses in environmental samples, the removal of suspended solids should be combined with other methods to remove inhibitors that were co-concentrated in the aqueous phase, thus preventing false-negative results or underestimation of SARS-CoV-2 genome copy number [49].

Therefore, using internal and external controls, specific methods should be validated at the time of the experiment for efficient recovery of SARS-CoV-2 from different types of wastewater and other water bodies. Based on our results and those of previous studies, PEG 8000 precipitation and ultrafiltration are efficient approaches for concentrating SARS-CoV-2 from wastewater and river water samples [16,17,20].

5. Conclusions

The present study critically evaluated the recovery efficiency of four methods for SARS-CoV-2 concentration in high- and low-titer conditions in wastewater and wastewater-enriched river water samples. To the best of our knowledge, this is the first study that specifically evaluated the concentration methods for SARS-CoV-2 in river water samples. We found that the methods tested could efficiently recover SARS-CoV-2, with variations in the recovery percentages. Vacuum filtration using an electronegative membrane with MgCl2 addition showed good efficiency in high viral titer content but not in low-titer assays. In contrast, aluminum hydroxide-based precipitation showed a lower performance in both assays. Ultrafiltration is a good concentration method for both sample matrices in high- or low-titer conditions, in addition to co-concentrating many PCR inhibitors and removing the solid phase (suspended particles) from the analytes. Finally, PEG 8000 precipitation may be a good approach because it demonstrates high recovery efficiencies for river water samples and can concentrate SARS-CoV-2 from aqueous and suspended solids. Detecting SARS-CoV-2 in environmental samples is challenging owing to the variable nature of each sample; therefore, it is necessary to use specific methodologies that are best suited for each sample.

CRediT authorship contribution statement

Study conception and design: Maira Santos Fonseca, Bruna Aparecida Souza Machado, and Jailson Bittencourt de Andrade. Acquisition of data: Maira Santos Fonseca, Carolina de Araújo Rolo, and Edna dos Santos Almeida. Data analysis and interpretation: Maira Santos Fonseca, Bruna Aparecida Souza Machado, and Jailson Bittencourt de Andrade. Drafting of manuscript: Maira Santos Fonseca and Katharine Valéria Saraiva Hodel. Critical revision: Bruna Aparecida Souza Machado, Edna dos Santos Almeida, and Jailson Bittencourt de Andrade. All authors have read and approved the submitted manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cscecc.2022.100214.

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