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Case Report

Evaluation of viral concentration and extraction methods for SARS-CoV-2 recovery from wastewater using droplet digital and quantitative RT-PCR

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ABSTRACT

The ongoing pandemic caused by the emergence of SARS-CoV-2 has resulted in millions of deaths worldwide despite the various measures announced by the authorities. Wastewater-based epidemiology has the ability to provide a day-to-day estimation of the number of infected people in a fast and cost-effective manner. However, owing to the complex nature of wastewater, wastewater monitoring for viral genome copies is affected by the extensive viral fragmentation that takes place all the way to the sewage and the analytical lab. The aim of this study was to evaluate different methodologies for the concentration and extraction of viruses in wastewaters and to select and improve an option that maximizes the recovery of SARS-CoV-2. We compare 5 different concentration methods and 4 commercially available kits for the RNA extraction. To evaluate the performance and the recovery of these, SARS-CoV-2 isolated from patients was used as a spike control. Additionally, the presence of SARS-CoV-2 in all wastewater samples was determined using reverse transcription quantitative PCR (RT-qPCR) and reverse transcription droplet digital PCR (RT-ddPCR), targeting three genetic markers (N1, N2 and N3). Using spiked samples, recoveries were estimated 2.1–37.6% using different extraction kits and 0.1–2.1% using different concentration kits. It was found that a direct capture-based method, evaluated against a variety of concentration methods, is the best in terms of recovery, time and cost. Interestingly, we noticed a good agreement between the results provided by RT-qPCR and RT-ddPCR in terms of recovery. This evaluation can serve as a guide for laboratories establishing a protocol to perform wastewater monitoring of SARS-CoV-2. Overall, data presented here reinforces the validity of WBE for SARS-CoV-2 surveillance, uncovers potential caveats in the selection of concentration and extraction protocols and points towards optimal solutions to maximize its potential.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus emerged in Wuhan, People's Republic of China in the end of 2019 as a novel member of the coronaviruses family with the capacity to cause a pneumonia-like illness [1]. The virus rapidly spread around the globe before COVID-19 was declared a pandemic on March 11th, 2020 (https://www.who.int/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19—11-march-2020). The extent of the virus prevalence in virtually all countries and the high hospitalization and death rates observed [2] quickly created the necessity for the containment of the virus. Nasopharyngeal swabs destined to detect viral genome mainly via PCR and lateral flow technology testing have been in widespread use [3]. However, it is practically impossible to test every individual in a certain area in order to find out the exact number of infected individuals at any given time point. Furthermore, people are already contagious and may spread the virus before symptoms arise and tests turn out positive [4]. When SARS-CoV-2 virus was found to be secreted in the feces of infected individuals and travel all the way to the sewage, waste-water based epidemiology (WBE) immediately offered these two key components of global & timely virus surveillance [5,6].

Indeed, it has been demonstrated through predictive modelling, that a correlation between SARS-CoV-2 viral load and clinical cases reported

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at the community level exists [7,8] and that this load can be used to warn about imminent outbreaks [9,10] and predict hospitalizations and intensive care unit (ICU) admissions [11].

The main analytical steps from sample receipt until SARS-CoV-2 RNA quantification are the wastewater concentration from large to small volumes and the subsequent nucleic acids extraction and significant viral genome losses may take place during these steps. In order to control for such losses several viral surrogates have been proposed and used in respective viral quantification studies [12]. To name a few, enterovirus (EV) [13], murine hepatitis virus [14,15], bovine coronavirus (bovi-neCoV) [15,16], pepper mild mottle virus (PMoV) [12,15,16], porcine epidemic diarrhea virus (PEDV) [17], mengovirus (MgV) [17], bacteriophages such as phage φX174, F-specific coliphage [12], and SARS-CoV-2 itself [18,19]. However, each surrogate virus is characterized by different structural and biochemical properties and as a result a very careful selection of the most appropriate surrogate virus for each method adopted is very important in order to obtain the most accurate results possible. Ideally, the virus must be as close as possible to the structure of the virus that needs to be quantified.

Many wastewater concentration methods previously published for other viruses have been used for SARS-CoV-2 as well. The most widespread methods used for SARS-CoV-2 are based on polyethylene glycol (PEG) based precipitation and ultrafiltration. Actually, there are several studies that have compared these two methods head-to-head [20–24]. One study has compared five different variations of the PEG precipitation [15], while another has compared only two different ultrafiltration methods [25]. Another commonly used method for the initial concentration step was based on viral adsorption on electronegative membranes and subsequent extraction from them [16,26]. Studies that compared all the 3 aforementioned methods and direct viral extraction [13,27] or centrifugation [14] and more comprehensive studies that compared 8 [18], 11 different methods [28] or even 14 different method variations [29] have contributed to the data galore on wastewater pre-concentration. Most studies have utilized RT-qPCR for the detection of the viral genetic material, while only a few have tested the performance of droplet digital PCR (RT-ddPCR) in wastewater. Summaries of concentration methods comparisons have also been published [30–32].

The RNA extraction step is usually carried out by utilization of commercial kits that bind nucleic acids and finally elute them in a small volume. This step may account for significant viral gene copies losses as well and the chemistry behind each kit is crucial for the extent of these losses. There are a few studies that have compared at least four different extraction kits and their yields were reported to be highly variable [12,29,33,34].

Lately, studies that have compared many steps of the process from initial wastewater pre-treatment, sample volume, concentration protocols, RNA extraction protocols and PCR steps included in the final viral genetic material detection have been reported [35–37].

The aim of the present study was to describe the performance comparison of five wastewater concentration methods based on 4 different physicochemical properties (PEG precipitation, ultrafiltration, adsorption-extraction and direct capture) and the subsequent comparison of four different RNA extraction commercial kits. Both RT-qPCR and RT-ddPCR technologies were utilized for the measurement of viral levels and their concordance was tested and confirmed.

2. Methods

2.1. Wastewater sampling

Six daily composite flow proportional raw wastewater samples (21–23/11/21 for concentration step and 14, 19 & 20/12/21 for extraction step) were collected from the wastewater treatment plant of Attica, the region of Greece that includes Athens metropolitan area and suburbs. Further physicochemical information regarding these samples can be found in Supplementary file 1. The raw wastewater samples which were collected for the comparison of the different concentration and extraction methods, were placed in pre-cleaned high-density polyethylene (HDPE) 2 L bottles and transported at 4 °C to the laboratory. Biosafety guidelines were followed during sampling, transportation and the analytical procedures. The various concentration and extraction protocols are presented in detail below.

2.2. SARS-CoV-2 viral spike control

Nasopharyngeal swabs collected from 10 SARS-CoV-2 positive individuals with high viral loads were collected and buffers were pooled to a total of 20 mL. Virus was then inactivated at 65 °C for 1 h and centrifuged at 5,000g for 5 min. The supernatant was collected and used as a spike control in the recovery experiments. The inactivated SARS-CoV-2 sample was used to spike one concentration and one extraction replicate in order to evaluate the recovery and process efficacy during the aforementioned procedures. The spike sample was processed according to each concentration and extraction protocol as well as the three replicates of the real samples.

RNA was extracted from the inactivated SARS-CoV-2 pooled clinical specimen using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany). The inactivated virus was diluted ten-fold and was processed according to the manufacturer’s instructions. The concentration of the inactivated virus was measured with droplet digital PCR (RT-ddPCR) as described earlier and was determined to be 10⁶ copies/μL. Eluted RNA was used in order to quantify the spike control gene copies and for the construction of a reference curve used for the RT-qPCR experiments.

2.3. Wastewater sample concentration

The different concentration methods are depicted in Fig. 1A. Concentration occurred three different days following sample collection. Process replicates were performed in triplicate plus one spike sample for each concentration method. In order to compare these five concentration protocols, 5 μL of inactivated SARS-CoV-2 (10⁶ copies/μL) were added in 50 mL of a wastewater sample before each process. These different concentration approaches were compared with respect to the percentage yield for inactivated SARS-CoV-2. All the replicates of the various concentration methods were processed with the same extraction and clean-up protocol (Manual Enviro Wastewater TNA kit - Promega, United States).

2.3.1. PEG precipitation with salt addition

50 mL of a wastewater sample were centrifuged (Remilab, India) at 4,700 rcf for 30 minutes at 4 °C. 3.5 g Polyethylene Glycol 8000 (molecular biology grade, Promega, United States) and 0.8 g NaCl (Lach-Ner, Czech Republic) were added in a 50 mL empty and sterile centrifuge tube (Sarstedt, Germany) and mixed with the supernatant of the wastewater sample. After complete dissolution, the mix was again centrifuged at 10,050 rcf for 2 hours at 4 °C. The supernatant was discarded. The viral pellet in the centrifuge tube was reconstituted to 500 μL nuclease-free water (molecular biology grade, Promega, United States). Also, 500μl of Promega Protease Solution (Promega, United States) were added to the concentrated sample, mixed and proceeded with the extraction protocol.

2.3.2. PEG modified glycine

1 g glycine (Sigma Aldrich, United States) was added to 50 mL of wastewater sample and was mixed for 30 min in a magnetic stirrer device (VELP Scientifica, Italy). Subsequently, the samples were processed according to PEG protocol.

2.3.3. Direct capture - promega

Manual Enviro Wastewater TNA kit (Promega, United States) was used according to manufacturer’s instructions. The protocol started with the addition of 0.5 mL Protease Solution in 50 mL of a wastewater
sample that were placed into 50 mL sterile centrifuge tubes (Sarstedt, Germany) and were incubated for 30 min at room temperature. The samples were centrifuged at 3,000 rcf for 10 min and the supernatant was decanted into two clean sterile centrifuge tubes (25 mL each). 6 mL of Binding Buffer 1, 0.5 mL of Binding Buffer 2 and 24 mL of Isopropanol (Fisher Chemical, United Kingdom) were added in each tube and were mixed well. The mixture from each tube was poured in one PureYield Binding Column which were placed to the Vac-Man Laboratory Vacuum Manifold (Promega, United States) and the total nucleic acids remained on the membrane of the PureYield Binding Columns. 5 mL Inhibitor Removal Wash and 20 mL of RNA Wash Solution (RWA) were passed through the PureYield Binding Column by vacuum. Finally, 1 mL of Binding Buffer 1, 0.5 mL of Binding Buffer 2 and 24 mL of Isopropanol were added to the concentrated sample, mixed and proceeded with the extraction protocol.

2.3.4. Adsorption-extraction with electronegative membranes

This method began with centrifugation of 50 mL of wastewater sample (4100 g, 10 min, 4 °C) and the supernatant was transferred to new sterile centrifuge tube. 2.5 M of MgCl₂ were added to the sample for a final concentration of 25 mM. The adjustment of the sample pH to 2.3.5. Ultrafiltration

50 mL wastewater sample was subjected to centrifugation at 4,700 rcf (Remilab, India) for 30 min at 4 °C to remove debris, bacteria and large particles. The supernatant of each sample was concentrated, using Amicon Ultra-15 centrifugal filter devices with a cut-off of 10 kDa (Merck Millipore, The Netherlands). The ultrafilters were centrifuged (4,750 rcf) for 15 min at 4 °C. The resultant concentrated samples (≈500 μL) were collected from the concentrate collection cup with a pipette. RNA was extracted from these samples immediately after concentration. Also, 500 μL of Promega Protease Solution (Promega, United States) were added to the concentrated sample, mixed and proceeded with the extraction protocol.

2.4. RNA extraction

Using the optimal concentration method (Direct capture), four different extraction methods are depicted in Fig. 1B. RNA extraction occurred on three different days following sample collection. For each extraction method, the concentrated samples were processed in triplicates plus one spike sample. Two hundred μL of the concentrated sample was used for each of the following kits (following manufacturer’s instructions), namely: (i) Water DNA/RNA Magnetic Bead kit (IDEXX, United States of America), (ii) RNeasy PowerWater kit (Qiagen, Germany), (iii) AllPrepPowerViralDNA/RNA kit (Qiagen, Germany) and one mL of concentrated sample was used for Manual Enviro Wastewater TNA kit (Promega, United States). In order to compare these four RNA extraction protocols, 5 μL of inactivated SARS-CoV-2 (10⁶ copies/μL) were added in replicate samples.

2.5. Quantification

2.5.1. Reverse transcription - droplet digital polymerase chain reaction (RT-RT-ddPCR)

A one-step five-plex RT-RT-ddPCR assay was performed for the simultaneous detection of three different regions of the nucleoprotein (N) gene (N1, N2, N3) of SARS-CoV-2, a synthetic RNA of known concentration as an RNA exogenous control (RNA-EC) and Beta-2-Microglobulin (B2M) transcripts as an endogenous RNA internal control (RNA-IC), as previously described [38]. One-step five-plex RT-RT-ddPCR assay was performed using one-step RT-RT-ddPCR Advanced Kit for Probes (Bio-Rad, California, USA) and probe-mixing
multiplexing strategy that resulted in five unique fluorescence footprints. RT-RT-ddPCR reaction mix was partitioned into nanoliter-sized droplets using the Bio-Rad QX200™ Droplet Generator and Reverse transcription was performed in the T100™ Thermal Cycler. Following PCR, the 96-well plate was read in the QX200 Droplet Reader (Bio-Rad, California, USA) and the absolute copy number of the five different target transcripts was calculated using the QuantaSoft analysis software (Bio-Rad), according to the Poisson distribution [38].

2.5.2. Reverse transcription - quantitative polymerase chain reaction (RT-qPCR)

SARS-CoV-2 RNA was analyzed using N1 and N2 RT-qPCR primer and probe sets (Water SARS-CoV-2 RT-PCR Test, IDEXX, United States of America) as described elsewhere [11]. All PCR reactions were performed in Touch Real-Time PCR Detection System (Bio-Rad, United States) using automatic settings for threshold and baseline and each RNA extract was analyzed in duplicates.

The standards used contained 10^6 copies/μL. Standard curve was conducted using an 5 series dilution, starting with 10^6 copies/μL to 10 copies/μL. In order to accurately assess whether inhibitors are acting upon collected samples, 1:10 dilutions were made from the RNA extract directly prior to plate analysis. Non-template controls confirmed PCR integrity.

Finally, each PCR batch (n = 6) included a five-point calibration curve, i.e., 5 × 10^5, 5 × 10^4, 5 × 10^3, 5 × 10^2, 5 × 10^1 copies of inactivated SARS-CoV-2. The average slope was −3.12 (±0.07, SE) and the average intercept was 39.40 (±0.47, SE).

\[
\text{RNA copies per L} = \text{genome copy number} \times \left( \frac{\text{RNA}^{\text{total}}}{\text{RNA}^{\text{PCR}}} \right) \times \left( \frac{\text{concentrate}^{\text{total}}}{\text{concentrate}^{\text{extracted}}} \right) \times \left( 1000 \text{ mL/L} \cdot \text{wastewater} \right)
\]

Equation 1. Formula providing the RNA copies per liter of initial raw wastewater where: RNA^{total} is the total volume of RNA eluted from each extraction method; RNA^{PCR} is the volume of purified RNA tested in PCR; concentrate^{total} is the total volume of wastewater concentrate; concentrate^{extracted} is the volume of wastewater concentrate from which RNA was extracted; wastewater is the volume of original wastewater sample processed with the different concentration methods.

Detailed Cq values and back-calculation for all process and technical replicates are listed in Supplementary file 2.

2.5.3. Quantification of virus recovery

Using the standard curves for N1 and N2 genes established after testing the SARS-CoV-2 RNA standard, mean C(q)-values measured in all samples were converted into genome copies. Recovery was calculated based on the copies measured in the spiked control samples, the endogenous copies of each sample and the theoretical copies that were spiked in each replicate.

Fig. 2. N1 & N2 SARS-CoV-2 log copies per liter of initial raw wastewater measured by a) RT-qPCR and b) RT-ddPCR on three different days using the five different concentration methods.
Equation 2: Formula providing the % recovery of the spiked virus from each sample.

2.6. Statistical analyses

All statistical analyses reported in this study were performed using GraphPad Prism software (version 6.01).

3. Results & discussion

3.1. Quantification of SARS-CoV-2 RNA in wastewater with different concentration methods

SARS-CoV-2 viral RNA as determined by RT-qPCR and RT-ddPCR for each of the five different concentration methods is illustrated in Fig. 2. RNA copies per liter of initial wastewater sample were calculated for each method in order to compare the concentration efficiency of the endogenous virus in the wastewater samples.

According to the results above, the direct capture method consistently detects more viral copies compared to the other concentration methods employed, while the PEG precipitation methods provide comparable results and work better than the filtration and adsorption methods when it comes to the endogenous virus detection. Head-to-head comparisons of PEG-based precipitation and ultrafiltration methods have shown that PEG methods yield more genome copies than ultrafiltration based methods when a surrogate enveloped virus was used (BRSV) [29]. Furthermore, in line with our results the ultrafiltration method yields higher genome copy numbers than the adsorption-based methods as was also the case in a previous study comparing ultracentrifugation and adsorption-based methods [16]. Of note, in that study, different RNA extraction kits were used for each one of the two concentration methods.

Overall, the direct capture by using Enviro Wastewater TNA kit (Promega, USA) consistently yielded the highest viral genome copies. This direct capture method is based on a silica column affinity capture of compromised virions or free RNA associated with other compounds) is one of the two technologies deemed to be comparable (p values: 0.106; 0.035; 0.068; two-tailed).

3.2. Quantification of SARS-CoV-2 RNA in wastewater with different extraction methods

SARS-CoV-2 viral RNA as determined by RT-qPCR and RT-ddPCR for each of the four different extraction methods is presented in Table 1. RNA copies per liter of initial wastewater sample were calculated for each method in order to compare the extraction efficiency of the endogenous virus.

The QIAGEN viral kit consistently recovers the greatest number of viral copies followed by the Promega and IDEXX kits, while the QIAGEN Water kit recovers the least number of copies compared to the other kit as far as the endogenous virus (which could be any form of intact or compromised virions or free RNA associated with other compounds) is concerned. Notably, our best performing kit was the worst performing in a study comparing this kit with 3 other RNA extraction kits/protocols (but with a constant concentration protocol) for the detection of endogenous viral species [33].

A paired t-test was performed in order to compare the performance of RT-qPCR and RT-ddPCR for each one of the three days wastewater samples were processed with the extraction kits. In all three days the results of the two technologies were deemed to be comparable (p values: 0.158; 0.081; 0.086; two-tailed t-test).

3.3. Recovery of SARS-CoV-2

3.3.1. Recoveries based on RT-qPCR results

In order to compare the recovery of each method, spiking experiments were performed using SARS-CoV-2 as an external control on 3 different days. Per cent recoveries of the SARS-CoV-2 viral standard for each concentration method and each extraction kit were calculated for all 3 days based on RT-qPCR results (N1 & N2 genes) and are presented

Table 2

| Concentration method | DAY 1 | DAY 2 | DAY 3 | AVERAGE |
|----------------------|-------|-------|-------|---------|
| Recovery (SD)        |       |       |       |         |
| PEG precipitation    | 0.1289 (0.0165) | 0.1067 (0.0123) | 0.0864 (0.0142) | 0.1073 |
| PEG & Glycine        | 0.1373 (0.0082) | 0.2703 (0.0217) | 0.3031 (0.0042) | 0.2369 |
| Ultrafiltration      | 0.0104 (–) | 0.0118 (0.0000) | 0.0115 (0.0065) | 0.0079 |
| Adsorption-extraction| 0.0202 (0.0044) | 0.0042 (0.0028) | N/A | 0.0122 |
| Direct capture       | 1.82 (0.06) | 1.63 (0.01) | 2.73 (0.02) | 2.06 |
| Extraction method    | DAY 1 | DAY 2 | DAY 3 | AVERAGE |
| Recovery (SD)        |       |       |       |         |
| IDEXX                | 6.31 (0.0012) | 7.07 (0.0039) | 12.7 (0.0054) | 8.7 |
| QIAGEN Viral         | 22.0 (0.0035) | 14.8 (0.013) | 25.6 (0.0122) | 20.8 |
| QIAGEN Water         | 1.02 (0.0016) | 0.90 (0.0013) | 4.41 (0.0028) | 2.11 |
| Promega              | 39.3 (0.0071) | 36.0 (0.0239) | 37.6 (0.0258) | 37.6 |

Table 1

| N1 & N2 SARS-CoV-2 copies per liter (standard deviation values in parentheses) of initial raw wastewater measured by RT-qPCR and RT-ddPCR on three different days and with all four different extraction kits by using the optimal concentration method (direct capture). |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| Extraction method | DAY 1  | DAY 2  | DAY 3  | AVERAGE |
| | Recovery (SD) | Recovery (SD) | Recovery (SD) | Recovery (SD) | |
| IDEXX             | 33290 (5682) | 8000 | 132449 (18541) | 8000 | 175949 (25934) | 8667 |
| QIAGEN Viral      | 193358 (16897) | 9733 | 419011 (62391) | 42133 | 491813 (58861) | 30667 |
| QIAGEN Water      | 32686 (7975) | <1LOD | 48660 (6025) | 8000 | 88530 (13037) | 8000 |
| Promega           | 46323 (6841) | 91200 (7625) | 230464 (22965) | 33173 (24859) | 171471 (25934) | 25387 |

**Recovery (%) = \frac{\text{copies of spiked control samples } - \text{endogenous copies of each sample}}{\text{theoretical copies that were spiked in each replicate}} \times 100**
in Table 2.

Fig. 3a shows the % recoveries for each concentration method as determined by RT-qPCR. One-way ANOVA was performed to compare the 5 methods used and it was decided that the direct capture has significant larger recoveries (p ≤ 0.001) than any other of the four methods, while those methods didn’t differ significantly (p > 0.05) when a Tukey’s multiple comparison post hoc test was performed.

As a result, the direct capture method was deemed to be the optimal for the wastewater initial concentration and was also used for the concentration of all samples when the comparison of the extraction kits was undertaken. While the direct capture method has only been compared to the PEG-based concentration method before [39], these methods have been shown to yield recoveries throughout a large range such as from 0.001% up to 78% in the case of the PEG precipitation [15]. In one study comparing concentration methods while using the same extraction kit for all of them it was demonstrated that adsorption and ultrafiltration outperformed PEG precipitation [14]. This may contradict our results, but an enveloped surrogate virus was used instead of SARS-CoV-2 in that study (MHV) and therefore, different viruses used for the calculation of recoveries may provide an altered picture as to which method works better. Indeed, another study using SARS-CoV-2 as a spike control showed that PEG precipitation yielded higher recoveries than ultrafiltration [23].

Fig. 3b shows the % recoveries for each extraction kit as determined by RT-qPCR. One-way ANOVA was performed to compare the 4 methods used and it was decided that the Promega kit was found to have significant larger recoveries (p ≤ 0.0001) than the IDEXX and the QIAGEN RNA extraction kits. In all three days the results of the two technologies were deemed to be comparable (p values: 0.288; 0.623; two-tailed).

Furthermore, the recoveries of the two QIAGEN kits differed significantly (p ≤ 0.001) as well. Finally, lower degrees of significance yielded the comparisons of the QIAGEN Viral kit with the Promega (p ≤ 0.01) and the IDEXX kit (p ≤ 0.05).

The Promega kit yields at least two times the recoveries of the other kits and as a result matches with the direct capture concentration method. As opposed to the concentration method comparisons, there are not any head-to-head comparisons of RNA extraction kits using a recovery spike control virus available in the literature. Our results show that the Promega kit is the most compatible for the intact enveloped form of the virus, whereas the other kits exhibit significant losses of the intact virus.

### 3.3.2. Recoveries based on RT-ddPCR results

Per cent recoveries of the SARS-CoV-2 viral standard for each concentration method and for each extraction kit were calculated for all 3 days based on RT-ddPCR results and are presented in Table 3.

Fig. 4a shows the % recoveries for each concentration method as determined by RT-ddPCR. One-way ANOVA was performed to compare the 5 methods used and it was decided that there are not any significant differences between the methods compared in terms of recovered virus. Even though the comparisons among the concentration methods and the RNA extraction kits did not reach the same levels of significance as was the case for the RT-qPCR results, the relative % recoveries remained the same, thus providing a confirmation for the RT-qPCR results and for the actual performance of each method and kit.

### 3.3.3. Comparison of RT-qPCR and RT-ddPCR recoveries

A paired t-test was performed in order to compare the recoveries based on RT-qPCR and RT-RT-ddPCR for each one of the 3 days the concentration methods were evaluated. In all three days the results of the two technologies were deemed to be comparable (p values: 0.288; 0.277; 0.623; two-tailed).

Furthermore, a paired t-test was performed in order to compare the recoveries of RT-qPCR and RT-RT-ddPCR for each one of the 3 days wastewater samples were processed for the evaluation of the four extraction kits. In all three days the results of the two technologies were
deemed to be comparable (p values: 0.143; 0.159; 0.069; two-tailed).

There are a few studies that have compared the performance of SARS-CoV-2 detection by RT-qPCR and RT-ddPCR in wastewater. Most of them have reported a moderate to good agreement between the results provided by the two technologies [23, 26, 40]. However, at low genome copy numbers there are studies that find RT-ddPCR is more sensitive than RT-qPCR [21, 26, 41], while others have reported RT-qPCR to be more sensitive than RT-ddPCR [42] or inconclusive results [40].

It should be noted that some of the percent recoveries reported here seem to be particularly low. However, in each study different wastewater samples with matrices varying in great extent in composition are expected to produce very different results. Additionally slight variations of each protocol may account for different recoveries reported as well.

4. Conclusion

In the present study the performance of five different wastewater concentration methods and four commercially available RNA extraction kits has been evaluated utilizing as detection tools RT-qPCR and RT-ddPCR. One of the most crucial factors that need to be taken into consideration when it comes to choosing a viral concentration method and the subsequent RNA extraction commercial kit is the nature of the virus (mainly if it is enveloped or not) and the form it is present in wastewater (fully intact virions, partially compromised virions, viral RNA free or associated with proteins or other compounds). Viral genome copy yields largely depend on these factors and the intrinsic physico-chemical characteristics each method and kit are based on may favor and preferentially enrich any of the aforementioned forms or a combination thereof. Accordingly, the present study was divided in two parts, one aiming for the absolute viral quantification in copies/liter of initial wastewater and a second aiming for the calculation of the recovery of a SARS-CoV-2 spike control. While the latter represents a model of intact virions spiked in wastewater, the former is the actual world scenario where the genetic material of the virus shed from carriers ends up in wastewater in a continuum of viral/RNA species. As a result, the direct capture method and the QIAGEN Viral kit (followed by the Promega one) are the optimal performing combinations for the isolation of the endogenous SARS-CoV-2, whereas the direct capture method and the Promega kit (followed by the QIAGEN Viral) are the best solutions for the isolation of the intact enveloped SARS-CoV-2 virus. From the observed reversal between the performance of the Promega and the QIAGEN Viral kit from the endogenous to the spiked enveloped virus detection it can be presumed that there is a significant proportion of compromised virions in the influent wastewater analyzed as is expected to be the case in such a complex matrix.

Detection using either RT-qPCR or RT-ddPCR seems to not largely affect the conclusions presented here. However, the detection of low viral loads very close to the limits of detection and quantification were beyond the scope of this study. Future studies-and especially as the pandemic wanes-could focus on identifying the best combination of isolation and detection strategies that could detect and quantify trace amounts of SARS-CoV-2.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cscee.2022.100224.

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