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An alternative approach for bioanalytical assay optimization for wastewater-based epidemiology of SARS-CoV-2

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HIGHLIGHTS
• Development of an analytical procedure for detection of SARS-CoV-2 RNA in wastewater
• Extraction recovery was evaluated in influent wastewater.
• Precision measured with dPCR used as a proxy for qPCR.
• qPCR of the N2 gene fragment showed high in-sample stability of SARS-CoV-2 on average.

GRAPHICAL ABSTRACT

ABSTRACT

Wastewater-based epidemiology of SARS-CoV-2 could play a role in monitoring the spread of the virus in the population and controlling possible outbreaks. However, sensitive sample preparation and detection methods are necessary to detect trace levels of SARS-CoV-2 RNA in influent wastewater (IWW).

Unlike predecessors, method optimization of a SARS-CoV-2 RNA concentration and detection procedure was performed with IWW samples with high viral SARS-CoV-2 RNA loads. This is of importance since the SARS-CoV-2 genome in IWW might have already been subject to in-sewer degradation into smaller genome fragments or might be present in a different form (e.g. cell debris,...). Centricon Plus-70 (100 kDa) centrifugal filter devices resulted in the lowest and most reproducible Ct-values for SARS-CoV-2 RNA. Lowering the molecular weight cut-off did not improve our limit of detection and quantification (approximately 100 copies/μL for all genes).

Quantitative polymerase chain reaction (qPCR) was employed for the amplification of the N1, N2, N3 and E-gene fragments. This is one of the first studies to apply digital polymerase chain reaction (dPCR) for the detection of SARS-CoV-2 RNA in IWW. dPCR showed high variability at low concentration levels (10^3 copies/μL), indicating that variability in bioanalytical methods for wastewater-based epidemiology of SARS-CoV-2 might be substantial. dPCR results in IWW were in line with the results found with qPCR. On average, the N2-gene fragment showed high in-sample stability in IWW for 10 days of storage at 4 °C. Between-sample variability was substantial due to the low native concentrations in IWW. Additionally, the E-gene fragment proved to be less stable compared to the N2-gene fragment and showed higher variability. Freezing the IWW samples resulted in a 10-fold decay of loads of the N2- and E-gene fragment in IWW.

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1. Introduction

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is an enveloped non-segmented positive-sense RNA virus, which is associated with the pathogenesis of the coronavirus disease 2019 (COVID-19) in humans (Huang et al., 2020). Due to the partly asymptomatic transmission and the high infectivity of this virus (Chen et al., 2020; Gao et al., 2020), it is crucial to have timely and accurate figures on the spread of SARS-CoV-2 in defined population groups for controlling possible viral outbreaks. Currently, the extent of SARS-CoV-2 circulation has been monitored by diagnostic testing of primarily symptomatic patients and contact tracing to also isolate asymptomatic patients (Peccia et al., 2020; Vandamme and Nguyen, 2020). However, a major limitation with these methods is that they depend on participation of individuals, even when they have no symptoms or only mild aspecific symptoms of COVID-19. Lack of recognition of symptoms or refusal to participate in detection or quarantine measures allows further spread of the virus in the general population. In Belgium, contact-tracing is primarily done manually through regional call centers and through the implementation of a smartphone application. Participation is heavily influenced by personal, social and public trust and requires additional efforts to connect with lower educated and vulnerable population groups (Vandamme and Nguyen, 2020). Contact-tracing efforts could potentially be biased by reporting and concealment bias and requires from each individual to keep track of their contact list. Additionally, if contact-tracers fail to track down an individual’s contacts swiftly (for example due to the prolonged incubation period or time to perform diagnostic testing), it could have limited effect on the spread of this highly infectious virus (He et al., 2020).

Wastewater-based epidemiology (WBE) employs the analysis of influent wastewater (IWW) on human (metabolic) excretion products and has been used as an alternative approach to investigate the circulation and spread of infectious diseases at the population level (Fig. 1) (Mao et al., 2020; Sims and Kasprzyk-Hordern, 2020). Infectious disease biomarkers (e.g. viral genomes) are released, pooled and transported in the wastewater system. The abundance of pathogens in IWW reflects the spatio-temporal changes and spread of infection at the population level and does not depend on participation at the individual level (Corpuz et al., 2020). For this reason, WBE is an efficient alternative approach for the prevention of infectious disease outbreaks, to track down possible hotspots and to evaluate the effectiveness of large scale anticontagion interventions within different communities (Ahmed et al., 2020a; Hamouda et al., 2021; Medema et al., 2020b). In order for WBE to monitor infectious diseases, the corresponding pathogen should preferentially be causing an enteric infection. Alternatively the pathogen or its genome should be excreted at sufficient levels in the faeces, urine or other excretions that end up in the wastewater (Hamouda et al., 2021; Pan et al., 2020; Zhang et al., 2020). In this light, several research papers indicated the potential of WBE in surveilling the transmission of the recently emerged SARS-CoV-2 at the population level (Ahmed et al., 2020a; La Rosa et al., 2020b; Wu et al., 2020).

Thanks to some advantages of this epidemiologic approach, WBE could aid in filling some knowledge gaps. Asymptomatic individuals infected with SARS-CoV-2 also shed the virus via their stool in the sewers (Lescure et al., 2020; Zhang et al., 2020) enabling WBE to track down the extent of transmission of the virus in different catchments and to predict future disease outbreaks in communities. Additionally, WBE could potentially be used as an early-warning tool for emergence of SARS-CoV-2 in the general population (Ahmed et al., 2021; Medema et al., 2020a; Orive et al., 2020). WBE is especially useful to invigilate the emergence of sporadic cases as it is highly inefficient to test thousands of individuals when detection rates are low. In this light, it is crucial to have sensitive and robust bioanalytical methods to quantitively measure SARS-CoV-2 in IWW (Alygizakis et al., 2021).

The surveillance of wastewater on viral RNA loads of SARS-CoV-2 proposes some analytical challenges. These primarily include the low detection levels of the virus in IWW and the wastewater matrix potentially harboring a wide array of organic matter and heavy metals that could interfere with the molecular methods of assaying viruses (Ahmed et al., 2020d; Alygizakis et al., 2021; Gibson et al., 2012). Therefore, it is crucial to have reproducible concentration methods and sensitive instrumental techniques to accurately measure viruses in IWW (Corpuz et al., 2020). At this moment, there has been a broad range of concentration methods for the extraction of SARS-CoV-2 RNA in IWW. Pre-existing concentration methods for SARS-CoV-2 RNA primarily include ultracentrifugation, ultrafiltration, charged filter membranes or PEG precipitation (Ahmed et al., 2020a; Ahmed et al., 2020c; Hasan et al., 2021; Jafferali et al., 2021; Kitajima et al., 2020; Medema et al., 2020b). Nevertheless, in most of these studies, efficiency of recovery (RE) was optimized by spiking non-enveloped human enteric viruses to IWW and only a limited number of studies investigate RE through spiking of enveloped surrogate viruses (e.g. coronavirus, the murine hepatitis virus and the bacteriophage pseudomonas virus phi6) (Ahmed et al., 2020c; Alygizakis et al., 2021; Corpuz et al., 2020; La Rosa et al., 2020a; McMinn et al., 2021; Ye et al., 2016). However, even with enteric surrogate viruses, RE of concentrations methods are mostly determined by the virus and the matrix composition. In this light, different coronaviruses (CoV) may have quite distinctive structural and physical properties compared to some of the proposed surrogate viruses and RE observed in these studies may not be representative for SARS-CoV-2 and the structures in IWW that contain SARS-CoV-2 RNA (Haramoto et al., 2018). Furthermore, it is not exactly known in what complex the SARS-CoV-2 RNA is present in IWW (packaged in virus particles, cellular fragments...). In this light, a hypothesis that the SARS-CoV-2 RNA is not present as naked RNA due to the poor stability of RNA in IWW. The abovementioned spiking experiments with viruses, although useful, are probably not fully representative for evaluation of stability and extraction of real IWW. In this light, a...
handful of studies confirmed their presented results in IWW with the native concentrations of SARS-CoV-2 RNA (i.e. concentrations present without seeding the virus to IWW) (Ahmed et al., 2020a; Hasan et al., 2021; Jafferli et al., 2021; Torli et al., 2021).

The aim of this study was to compare a broad range of bioanalytical procedures for the concentration of SARS-CoV-2 RNA in IWW. This bioanalytical assay (combination of sample concentration, RNA extraction and PCR detection) was optimized with IWW originating from eight Belgian wastewater treatment plants (WWTPs) with confirmed native levels of SARS-CoV-2 RNA, in combination with spiking with an animal Coronavirus. Additionally, in-sample stability at different storage conditions was further investigated. Finally, this study applied, as one of the first, digital polymerase chain reaction (dPCR) for assaying SARS-CoV-2 RNA in IWW, in a direct comparison with traditional quantitative polymerase chain reaction (qPCR).

2. Materials and methods

2.1. Sampling

Method optimization was done with IWW samples acquired from eight different Belgian WWTPs with population equivalents ranging between 25,000 to 200,000 inhabitants and sanitary wastewater (SAW) from a company that had a high number of positive COVID-19 cases (approximately 17% of the employees) (Outters, 2020). Daily IWW samples were collected in the preamble (2nd of August 2020), peak (20th of November 2020) and tail (20th of January 2021) of the second wave of the COVID-19 pandemic. Locations are not further specified due to anonymity constraints, however, matrix compositions differ substantially between the locations of interest to demonstrate the robustness of the methodology. Additionally, catchment areas with distinctive geographical characteristics (e.g. cities versus towns) were considered to test the suitability of the bioanalytical method. Although it is virtually impossible to determine the broad range of matrix interferences present in IWW, matrix compositions will vary significantly between days and locations due to spatio-temporal differences in disposal and excretion of compounds in the sewer system (Corpuz et al., 2020). It should be noted that in-sewer degradation in the SAW could potentially be less substantial compared to the IWW samples because of minor average hydraulic residence times at the subcatchment level (Ahmed et al., 2020b). Previously, Choi et al. indicated that biotransformation of WBE biomarkers could be substantial due to the influence of hydrochemical parameters and presence of biofilms (Choi et al., 2020). SARS-CoV-2 RNA decay could potentially be more substantial during in-sewer transport due to the presence of biofilms and hydrochemical processes, as indicated by Ahmed et al. (2020b). Additionally, the SARS-CoV-2 genome is more diluted in IWW compared to the SAW, which could result in different decay rates (Ahmed et al., 2020b).

For method optimization, the applicability of the sample concentration and extraction methods was tested in IWW from the second wave of the COVID-19 pandemic in Belgium where the number of positive test cases was substantial. This period was deliberately chosen for the assessment of suitability of the method because higher number of test cases will result in higher excretion of SARS-CoV-2 RNA. Additionally, the samples acquired during the preamble and tail of the COVID-19 pandemic also demonstrate the applicability of the method in areas with low prevalence levels of COVID-19.

Daily 24-h composite IWW samples were collected time- or flow proportionally in order to obtain samples that were representative for an entire day. For time-proportional sampling, 10-minute intervals were applied to compile daily samples to accurately capture viral RNA loads over the 24-h period (Ort et al., 2010). Average residence time was less than 12 h in all locations. After sample collection, samples were transported immediately at 4 °C to the laboratory and analyzed within 24 h.

2.2. Sample concentration

Centrifuge Plus-70 Centrifugal filters (100 kDa and 30 kDa), Amicon® Ultra Centrifugal filters (50 kDa and 10 kDa), Macrosep Advance Centrifugal devices with Omega Membrane (100 kDa and 30 kDa) and Vivaspin 20 ultrafiltration units (100 kDa and 50 kDa) were purchased from Millipore (Burlington, US), Pall (New York, US) and Sartorius (Göttingen, DE), respectively. PEG 8000 and sodium chloride were acquired from Promega (Madison, US).

Virus concentration is necessary because of the low levels of SARS-CoV-2 RNA in wastewater. The analytical procedure needs to be sensitive enough to detect viral loads in the beginning or at the tail of the COVID-19 peak when only a limited number of SARS-CoV-2 infections are present in the catchment area. Several ultrafiltration methods (for protocols, see Figs. S1–S2) with different centrifugal devices with varying molecular weight cut-offs (MWCO) and loading volumes were tested for the concentration of viral RNA loads in IWW in order to obtain high extraction efficiencies. Additionally, PEG precipitation was also tested as an alternative for sample concentration. Conditions of the PEG precipitation (e.g. initial volume, final concentration of PEG and NaCl, ...) were given in Fig. S3. It should be noted that co-concentration of PCR inhibitors could also occur when using these concentration methods which could affect the assay’s sensitivity (Ahmed et al., 2020d). The composition of the IWW matrix is highly variable and contains a range of heavy metals, RNases and polysaccharides that could interfere with qPCR amplification (Corpuz et al., 2020; Gibson et al., 2012). It is logistically impossible to determine the exact composition of the collected IWW samples, however, due to the complexity of the IWW matrix it is highly expected that substances present in IWW will result in some degree of inhibition.

Several studies are already available addressing different methods for concentrating viruses in wastewater through spiking the IWW samples pre- and post-extraction with a surrogate control virus (Ahmed et al., 2020b; Alygizakis et al., 2021; Corpuz et al., 2020; La Rosa et al., 2020a). However, the complex of the SARS-CoV-2 RNA present in IWW (virus particles, cellular fragments, ...) remains uncertain and the RE of the SARS-CoV-2 genome in IWW might potentially deviate from the controls used in these studies (Kitajima et al., 2020). For this reason, RE was determined in this study with an alternative approach (i.e. with native SARS-CoV-2 concentrations) to further verify the usefulness of different ultrafiltration filters for concentrating SARS-CoV-2 RNA. The protocol with the lowest and most reproducible cycle threshold (Ct) levels for the amplification of the different SARS-CoV-2 gene fragments was chosen for sample concentration. This approach was chosen since spiking IWW with enteric enveloped viruses may not be representative for RE of SARS-CoV-2 RNA in IWW due to different structural properties of these surrogate viruses or the in-sewer degradation of viral SARS-CoV-2 genome. However, during method optimization, IWW samples were spiked in parallel with porcine coronavirus (PRCV) to investigate whether the RE of this seeded surrogate control virus was in line with the SARS-CoV-2 results when optimizing the different concentration protocols. Table 1 summarizes the design of experiment with the varying extraction protocols in order to obtain the most suitable sample concentration method of viral RNA loads in the wastewater matrix.

In the final protocol, samples were firstly centrifuged at 4625g for 30 min at 4 °C to remove solids and debris. The supernatans was

| Condition | Amicon | Centricon | Macrosep | Vivaspin | PEG 8000 |
|-----------|--------|----------|----------|----------|----------|
| Loading volume (mL) | 15, 30 | 50, 100 | 20, 40 | 20, 40 | 90 |
| Concentrate volume (mL) | 1 | 1.5 | 1 | 1 | 1.5 |
| Molecular weight cut-off (MWCO) (kDa) | 10, 50 | 30, 100 | 30, 100 | 50, 100 | – |
transferred to a Centrifuge Plus-70 centrifugal filter for sample concentration. The sample was centrifuged for 15 min at 2500g at 4 °C in these ultrafiltration devices. Subsequently, the filter cup was centrifuged for an additional 2 min at 1000g at 4 °C to collect the sample concentrate. Finally, the sample concentrate was extracted and standardized at a volume of 1.5 mL.

2.3. RNA extraction

Similar to the sample concentration protocols, different commercially available manual RNA extraction kits were compared in order to obtain the lowest and most reproducible Ct-values for both SARS-CoV-2 and PRCV. The QIAamp Viral RNA minikit, the RNeasy plus minikit and the RNeasy Powermicrobiome kit were obtained from QIAGEN (Hilden, DE). Automated RNA extraction was done with the Maxwell® RSC Instrument with the PureFood GMO and Authentication kit, both from Promega (Madison, US). Initially, a selection of IWW samples was processed with these three manual RNA extraction kits. These RNA extraction kits need to be capable of isolating trace levels of SARS-CoV-2 RNA from IWW. The RE depends heavily on the composition of the RNA extraction kit. The number of washing steps to remove PCR inhibitors may vary between the kits and different elution solvents are used for the extraction of SARS-CoV-2 RNA from the concentrate. These commercialized kits have been proposed in other WBE applications for the extraction of SARS-CoV-2 in IWW (Alygizakis et al., 2021).

For each RNA extraction kit, each sample concentration method was considered. The Ct-values obtained with the automated Maxwell PureFood GMO and Authentication RNA extraction kit were compared with the results from the manual RNA extraction kits to investigate whether it was possible to increase the throughput of the bioanalytical assay (i.e. capability of analyzing larger amounts of samples rapidly).

In the final method, extraction was performed with the automated Maxwell PureFood GMO and Authentication RNA extraction kit. 200 μL of the concentrate was added to 200 μL cetyltrimethylammonium bromide buffer and 40 μL proteinase K and the total volume was incubated for 10 min at 56 °C. This mixture was transferred to the sample well together with 300 μL lysis buffer. The final elution volume with this RNA extraction kit was 50 μL.

2.4. Molecular methods for assaying SARS-CoV-2

2.4.1. qPCR

SARS-CoV-2 and PRCV primers were obtained from Integrated DNA Technologies (IDT, Coralville, US) (Centers for Disease Control and Prevention, 2020; Corman et al., 2020). Table 2 summarizes the sequences for the primers and probes used for qPCR. PRCV used as a whole process control was obtained from Ghent University (kind gift of Prof. H. Nauwynck, Merelbeke, Belgium). SARS-CoV-2 RNA used as a positive qPCR control was obtained from the Institute of Tropical Medicine Antwerp (kind gift of Prof. K. Ariën, ITG, BE). The EURM-019 reference standard for the construction of the calibration curve was obtained from the Joint Research Centre (JRC, European Commission). The Laboratory of Microbiology, Parasitology and Hygiene possesses the necessary permits for this research.

qPCR was performed with the LightCycler® 96 instrument from Roche (Bazel, CH). All qPCR amplifications were performed in 20 μL reaction mixtures using a 2× Sensifast™ Probe No-ROX One-Step kit from Bioline (Cincinnati, US). Each reaction mixture contained 20% v/v of the extracted RNA (4 μL template). The final concentration of the primer and probes in the different qPCR mixtures was given in Table 2. A six-point calibration curve with a concentration between 105 and 103 copies/μL was constructed in ultrapure DEPC-treated water for quantification of the different gene fragments of interest in IWW. The lower limit of quantification (LLOQ) was defined as the concentration in the lowest point of the calibration curve and was 103 copies/μL for all gene fragments. All qPCR reactions were performed in duplicate. For each qPCR run, two negative controls and a positive control were included. qPCR settings were as follows: 10 min for reverse transcription at 45 °C, 2 min at 95 °C for polymerase activation followed by 45 cycles of 5 s at 95 °C for denaturation and 30 s at 60 °C for annealing and extension. Concentrations (in copies/μL) were only considered in this study (i) when comparing the results measured in this study with the results found by others, (ii) when comparing the performance of qPCR and dPCR and (iii) when assessing in-sample stability. The same sample concentration and RNA extraction method was applied to compare qPCR with dPCR and to assess in-sample stability allowing the use of this metric.

2.4.2. dPCR

In IWW samples with low RNA concentrations, qPCR could potentially be affected by inhibitors present in the sample, resulting in poor amplification efficiency, less precision and the need for relative quantification, which might lead to the occurrence of false-negative results (Klein, 2002; Kuypers and Jerome, 2017; Suo et al., 2020). It is hypothesized that dPCR could potentially be more tolerant to PCR inhibitors compared to qPCR (Baker, 2012; Dingle et al., 2013; Sedlak and Jerome, 2013; Suo et al., 2020). These PCR inhibitors are potentially highly present in IWW and could be co-concentrated during sample preparation (Ahmed et al., 2020d; Gibson et al., 2012). Therefore, dPCR was tested for the detection of SARS-CoV-2 RNA in IWW to further compare the sensitivity of both PCR methods. An advantage of dPCR is that absolute quantification is performed through Poisson calculations.

| Target gene fragment | Primer/probe | Final concentration (nM) | 5′ | Sequence | 3′ |
|----------------------|--------------|--------------------------|---|----------|---|
| SARS-CoV-2           |              |                          |   |          |   |
| Nucleocapsid (N1)    | 2019-nCoV_N1-F | 200                      | None | GACCCAAAAATACCCGGAAT | None |
|                      | 2019-nCoV_N1-R | 200                      | None | TTCTGATACGGCGACTTCG | None |
|                      | 2019-nCoV_N1-P | 200                      | FAM  | ACCCGCTATATCTGTGTGGAC | /ZEN/3IaBkFQ/ |
| Nucleocapsid (N2)    | 2019-nCoV_N2-F | 200                      | None | TTACAAACATCCGCCAAA | None |
|                      | 2019-nCoV_N2-R | 200                      | None | GCCGGATACCTCCGAAAG | None |
|                      | 2019-nCoV_N2-P | 200                      | FAM  | ACAATTCGCCACCGCCCTCAC | /ZEN/3IaBkFQ/ |
| Nucleocapsid (N3)    | 2019-nCoV_N3-F | 200                      | None | GGGCCGGTGTACCAAGAAA | None |
|                      | 2019-nCoV_N3-R | 200                      | None | GTGACGAGGGGCTGGATG | None |
|                      | 2019-nCoV_N3-P | 200                      | FAM  | AYACATAGGCGCCCGAATGGCTG | /ZEN/3IaBkFQ/ |
| Envelope (E)         | E_Sarbeco_F   | 400                      | None | ATATGTCGAGCTGACACACA | None |
|                      | E_Sarbeco_R   | 400                      | None | ATATGTCGAGCTGACACACA | None |
|                      | E_Sarbeco_P1  | 200                      | FAM  | ATATGTCGAGCTGACACACA | /ZEN/3IaBkFQ/ |
| PRCV                 | PRCV_1_F      | 200                      | None | AGCTTCTGACGTTCAAGGAATTG | None |
|                      | PRCV_1_R      | 200                      | None | CATACGACGTCATGACACAAA | None |
|                      | PRCV_1_P      | 100                      | HEX   | TCGACGACACCAATATACCGGC | /ZEN/3IaBkFQ/ |
A calibration curve prepared with the same RNA extracts was used with both qPCR and dPCR for all gene fragments of interest to evaluate the precision with dPCR at different concentration levels as a proxy for validation of the quantitative results. For each dPCR run a non-template control was included to determine the signal-to-noise (S/N) threshold. dPCR was done with the QIAacuity One 5-Plex from QIAGEN (Hilden, DE). Additionally, IWW originating from the seven locations previously described in 2.1. were analyzed simultaneously with dPCR and qPCR to directly compare sensitivity for both assays. Samples were assayed with the QiAcuity One-Step Viral RT-PCR kit and concentrations were 400 nM for primers and 200 nM for probes. Eight μL template RNA was diluted to 40 μL with dPCR master mix. dPCR settings were as follows: 40 min for reverse transcription at 50 °C, 2 min at 95 °C for polymerase activation followed by 40 cycles of 5 s at 95 °C for denaturation and 30 s at 60 °C for annealing and extension.

Positive IWW samples from three locations were diluted with ultra-pure water (i.e. no dilution, ½ dilution and ¼ dilution) to further investigate the tolerance of the different PCR assays to potential matrix interferences. These diluted samples were analyzed in parallel with both qPCR and dPCR. Detection was performed with PCR assays for the N1, N2 and E gene. Additionally, different volumes of template RNA (1, 5 and 10 μL) from the same locations were loaded to investigate if further diluting the RNA template in the dPCR reaction wells influences the sensitivity with dPCR. These samples were not diluted before sample concentration.

2.5. Stability experiments

During transport in the sewer system, it is possible that the SARS-CoV-2 genome is broken down in smaller RNA fragments containing the SARS-CoV-2 gene fragments. Therefore, in-sample stability was not investigated through standard addition with SARS-CoV-2 since stability of the corresponding gene fragments might not be comparable with the fragments found in IWW. This study only considered in-sample stability and in-sewer stability should be further investigated as medium to low stability could substantially influence the concentrations of SARS-CoV-2 RNA in the sewer system. The aim of the in-sample stability experiments was to determine suitable storage conditions for the collected IWW samples.

For each IWW sample, viral loads for each gene fragment were quantified at each time point and expressed as a relative percentage of the native concentration present in the corresponding IWW samples at the starting point of this stability study. The mean and relative standard deviation (%RSD) of all IWW samples were considered for each gene fragment of interest.

3. Results and discussion

3.1. Sample concentration

Fig. S4 summarizes the results of the different sample concentration methods for each RNA extraction kit. As indicated by Medema et al., most WBE applications on SARS-CoV-2 only perform the experiments in single (Medema et al., 2020a). This study performed the experiments in duplo because of the high variability expected at the LLOQ. The choice of the final protocol also took the reproducibility of the duplicates into consideration. For this initial comparison, a fresh IWW sample (stored at 4 °C) from location 2 from the 2nd of August 2020 was divided in different aliquots which were analyzed with different sample concentration methods for SARS-CoV-2 and seeded PRCV. At this time, the number of confirmed cases of COVID-19 was on the rise in this specific location, but was still considerably lower compared to the second wave of the pandemic. The Centricon Plus-70 centrifugal filters resulted in the lowest Ct-values of PRCV and SARS-CoV-2 for all gene fragments of interest. While PEG precipitation resulted in detection levels of SARS-CoV-2 above the LLOQ for the E-gene fragment, detection levels for the N1 and N2 gene fragment were generally low or these gene fragments were not detectable by PEG precipitation.
fragments remained undetected. This was in line with previous studies that reported poor recoveries of SARS-CoV-2 RNA with PEG precipitation, possibly due to the higher co-concentration of PCR-inhibitors (Alygizakis et al., 2021; Hasan et al., 2021). Ct-values measured with the other ultrafiltration methods were also low, as presented in Fig. S4. However, the Ct-values observed with PRV were considerably lower with the ultrafiltration methods compared to the PEG precipitation. For this reason, PEG precipitation was excluded from further method optimization. The N3-gene fragment resulted in poor detection levels with both RNA extraction kits and across all sample concentration methods. For this reason, further amplification of SARS-CoV-2 gene fragments in IWW mainly focused on the N1-, N2- and E-gene fragment.

The effect of different MWCO and different loading volumes was tested with the different ultrafiltration methods in order to obtain the lowest and most reproducible Ct-values for the different qPCR assays. SAW from a company with high prevalence of SARS-CoV-2 infections was used for the further optimization of the method and to verify the results shown in Fig. S4, as illustrated in Fig. 3A. Overall, increasing the sample volume resulted in the lowest Ct-values for the gene fragments of interest with the different sample concentration methods. However, higher loading volumes (i.e. two times the maximum capacity of the filter) often resulted in blockage of the centrifugal filter membrane and, therefore, the IWW sample was only loaded once to prevent this. Blockage of the filter could also potentially lead to higher concentrations of PCR-inhibitors which could negatively influence the sensitivity with qPCR. The use of lower sample volumes also increases the throughput of the bioanalytical assay, since higher loading volumes require multiple centrifugation steps. Lower MWCO did also not result in more sensitive detection of SARS-CoV-2; only the Amicon centrifugal filters with a MWCO of 10 kDa showed minor improvements. Although only two different MWCO were tested for each of the individual filtration methods, results were consistent for all centrifugal devices (with the exception of the Amicon filters). Larger MWCO could also potentially result in minimizing co-concentration of PCR inhibitors. Concentration with the Centricon Plus-70 centrifugal filters resulted in the lowest Ct-values; the other centrifugal filters were comparable. It is hypothesized that the Centricon Plus-70 centrifugal filters are potentially more suitable due to a higher RE, a higher concentration factor and less co-concentration of PCR inhibitors, but more research is needed to confirm this hypothesis. Other studies also found acceptable RE of surrogate viruses or seeded SARS-CoV-2 in IWW with some of these ultrafiltration methods. Loading volumes ranged between 50 and 500 mL in these studies. However, the variation of the RE with these sample concentration methods was quite substantial (Ahmed et al., 2020d; Alygizakis et al., 2021; Medema et al., 2020b).

Structural properties of the SARS-CoV-2 RNA found in the SAW might differ from the viral loads measured in IWW due to in-sewer degradation of viral RNA during sewage transport. It should be noted that only one SAW sample was acquired from the corresponding company, however, performance of the different centrifugal filters was in line with the results in Fig. S4. Although the effect of different MWCO was consistent throughout the different filters, it should be noted that the small sample size is a limitation for the interpretation of these results.

A final selection of sample concentration methods was tested in IWW samples (50 mL) collected from eight different Belgian WWTPs. These samples were processed with the Centricon (100 kDa) and the Macrosep (100 kDa) centrifugal filters to confirm the results. The effect of direct extraction was also considered because of the high incidence of SARS-CoV-2 infections at the time of sample collection. The Maxwell PureFood GMO and Authentication kit was used for RNA extraction. Fig. 4 illustrates the Ct-values for the different SARS-CoV-2 gene fragments under investigation for the different sample concentration methods. The use of no concentration step resulted in poor detection levels of SARS-CoV-2 RNA with Ct-values above the LLOQ only detected in a few locations, mainly for the N2-gene fragment. This verifies the need for a concentration step to detect the low concentration levels of SARS-CoV-2 RNA in IWW. The use of the Centricon centrifugal filters resulted in the lowest Ct-values for the N1- and E-gene fragment. This was also confirmed by the variations in positive rates between the different ultrafiltration methods. Results for the N2-gene fragment were comparable between the Centricon and Macrosep centrifugal devices. Fig. 4 also indicates that the proposed methodology proved to be applicable in monitoring SARS-CoV-2 RNA levels in catchment areas with quite distinctive geographical and socio-economic characteristics. In the final protocol, ultrafiltration with Centricon filters was chosen for sample concentration. It should also be noted that variability between duplicates was greater in locations with viral concentrations at LLOQ levels. The variability at the LLOQ levels is further explored in Section 3.3.2.

![Fig. 3. Optimization of MWCO and loading volumes with the different sample concentration methods in SAW using (A) the Viral RNA extraction kit and (B) the Maxwell PureFood GMO and Authentication kit. The colour of each cell represents the Ct-value, the y-axis the different sample concentration protocols and the x-axis the different PCR assays. Cells indicated with a red asterisk have higher Ct-values than the lowest point of the calibration curve and could therefore not be quantified. However, in these cells a positive signal was still detected. No signal was detected in cells with a black cross. Side-by-side cells for each location represent duplicate Ct-values.](image-url)
3.2. RNA extraction

Fig. S4 compares the different RNA extraction protocols for both SARS-CoV-2 and PRCV. The use of the Powermicrobiome kit resulted in low detection levels of the different SARS-CoV-2 gene fragments in IWW. Therefore, this RNA extraction method was excluded at an early stage. The Viral RNA and RNeasy extraction kit showed comparable results, with slightly higher detection levels observed with the Viral RNA extraction kit for both SARS-CoV-2 and PRCV. Concentration factors were similar between both kits (Table S1), but slightly higher compared to the Maxwell PureFood GMO and Authentication kit and the Powermicrobiome kit. The Viral RNA extraction kit also recovered higher viral RNA loads in frozen IWW compared to the RNeasy extraction kit. However, in frozen IWW viral loads were almost always lower than the LLOQ, as further discussed in Section 3.4.

3.3. Molecular methods for assaying SARS-CoV-2

3.3.1. qPCR

A surrogate coronavirus (i.e. PRCV) was used as a whole process control. There was an expected repetitive drop in the Ct-value for the SARS-
CoV-2 gene fragments and PRCV_1-gene fragment when amplifying a 10-fold dilution series proving the applicability of qPCR for the detection of the gene fragments of interest. SARS-CoV-2 gene fragments were not detected in the negative controls and the positive control tested always positive. The concentrations (copies/μL) measured in this study with qPCR were in the same order of magnitude as the results found by others (Hasan et al., 2021; Hata et al., 2021; Medema et al., 2020b; Wu et al., 2020).

3.3.2. dPCR

Up till now, investigation of SARS-CoV-2 gene fragments with dPCR remains underexplored (D’Aoust et al., 2020; Peeson et al., 2021). IWW samples from the tail of the second wave of the COVID-19 pandemic from the different locations described previously were analyzed with both qPCR and dPCR. The latter method could potentially be more sensitive and precise in measuring viral RNA loads in IWW because of the theoretical higher tolerance to PCR inhibitors, potentially because dPCR employs end-point PCR. As described earlier, it is expected that the IWW samples from the different locations have quite distinctive matrix compositions due to different excretion profiles and disposal of chemicals. The positive partition rate was generally low in the IWW samples (<0.05%). At the low concentration levels observed in these samples, the 95% confidence interval (CI) with dPCR was also quite broad, as can be expected (Table 3). Concentrations measured with qPCR were in the same order of magnitude as those with dPCR for all locations, with the exception of location 3 and 4 for the E-gene fragment (i.e. 10-fold higher with qPCR). Overall this indicates that sensitivity of both molecular assays is comparable. D’Aoust et al. also reported that dPCR did not result in superior detection of SARS-CoV-2 RNA compared to qPCR (D’Aoust et al., 2020). In their study sensitivity was even lower compared to qPCR.

As illustrated in Table S3, loading 1 μL of SARS-CoV-2 RNA to the dPCR reaction well resulted in poor detection, while sensitivity was considerably higher when loading 5 μL and 10 μL SARS-CoV-2 RNA. The detection levels with 5 μL template were slightly higher compared to the loading volume of 10 μL, but more research is required. It is hypothesized that further increasing the RNA template volumes could also be accompanied with more prevalent matrix interferences and PCR inhibition. However, these results should be interpreted with caution due to the limited sample size (N = 3) and volume parameters (i.e. 1 μL, 5 μL and 10 μL).

Additionally, the effect of diluting IWW samples with ultrapure water was also taken into consideration to evaluate the tolerance of dPCR to matrix interferences. Positive IWW samples from three locations were either: (i) not further diluted, (ii) diluted with a factor of 2 or (iii) diluted with a factor of 4 before sample concentration. The same diluted IWW samples were analyzed with qPCR to provide a comparison between both assays. The results presented in Table S4 illustrate that diluting the samples with qPCR resulted in poor detection levels for the different gene fragments. Diluting the IWW samples with a factor of 2 resulted in improved sensitivity with dPCR. This could potentially indicate that dPCR is more tolerant to matrix interferences compared to qPCR. It should be noted that the dilution factor was taken into account when calculating the concentrations presented in Tables S3 and S4. This experiment and the results shown in Table 3 potentially highlight the usefulness of dPCR for the wastewater-based epidemiology of SARS-CoV-2, however, the presented results should be further verified with larger sample sizes and IWW originating from other locations with distinct matrix compositions.

A calibration curve prepared from the same RNA extracts was processed with both qPCR and dPCR. A major advantage of dPCR is that it allows absolute quantification and, thus, no calibration curve is needed. The different calibration points were amplified with dPCR as a proxy to validate variability at different concentration levels and Ct-levels with qPCR. This is of importance because native concentrations of SARS-CoV-2 RNA in IWW are in the low copies/μL range. Important to note is that precision is generally higher with dPCR compared to qPCR because of the higher tolerance to PCR inhibitors and the use of absolute quantification (Kuypers and Jerome, 2017; Suo et al., 2020). Therefore, precision observed with dPCR is used as a proxy for qPCR, but most likely the actual variability with qPCR will be higher due to methodological differences.

Fig. 5 combines the result for the calibration curve observed with qPCR with the results of dPCR. At low concentration levels (10^{-3} to 10^{0} copies/μL), the width of the CI tends to be rather broad. For the E-gene fragment, no positive partitions were measured in the reaction well containing the 10^{0} copies/μL calibration point. The width of the CI for the N1- and N2-gene fragment at this concentration level was 79.0% and 95.7% respectively (see also Table S2). This further evidences the high variability observed at Ct-values around the LLOQ with qPCR and could potentially explain why only one single well out of the side-by-side duplicates tested positive for SARS-CoV-2. Of course, the high variability observed in WBE applications for SARS-CoV-2 has further implications for the analysis of temporal trends in SARS-CoV-2 infections, especially in catchment areas with low prevalence of COVID-19. This uncertainty is further explored in Section 4.

3.4. Stability

In WBE applications that measure chemicals (e.g. pharmaceuticals), stability is evaluated according to McCall et al. (2016). Generally, WBE biomarkers are classified as either high (<20% transformation), medium (20–60%), low (60–100%) or variable (i.e. different results found in WBE studies) stability over a pre-defined time period. However, the variability observed with native concentrations measured at the LLOQ levels (see Section 3.3.2.) complicates this assessment.

The N2-gene fragment and E-gene fragment were detected in concentrations above the LLOQ in 100% and 87.5% of the IWW samples, respectively. The relative standard deviation (%RSD) was relatively high for both SARS-CoV-2 gene fragments, as illustrated in Fig. 6. Additionally, it appears that the variability for the E-gene fragment tends to increase towards the end of the time period, with the highest %RSD values observed at day 10. On average, the N2-gene fragment shows high sample stability for the entire sampling period, which is in line with the findings of others (Ahmed et al., 2020b; Hokajärvi et al., 2021; Medema et al., 2020a). The E-gene fragment showed medium to low

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Table 3

Comparison of qPCR and dPCR results for all gene fragments of interest for seven IWW samples collected in the tail of the second wave. CI = 95% confidence interval. Matrix composition (i.e. number of PCR inhibitors) was different between the different samples.

| Sample | Real-time qPCR | Digital PCR |
|--------|----------------|-------------|
|        | N1 (copies/μL) | N1 (copies/μL) | CI (%) | N2 (copies/μL) | N2 (copies/μL) | CI (%) | E (copies/μL) | E (copies/μL) | CI (%) |
| 1      | 0.17           | 0.17         | n.d.   | 0.70           | 95.7           | 0.54   | 109.1          | 0.27           | 168.6   |
| 2      | n.d.           | n.d.         | n.d.   | 0.13           | 274.4          | n.d.   | –              | 0.26           | 168.6   |
| 3      | 0.46           | 0.13         | 0.50   | 1.33           | 64.9           | 1.00   | 79.0           | 0.13           | 274.4   |
| 4      | n.d.           | n.d.         | 13.57  | n.d.           | –              | n.d.   | –              | 0.13           | 274.4   |
| 5      | 0.08           | n.d.         | n.d.   | 0.14           | 274.4          | n.d.   | n.d.           | –              | –       |
| 6      | 0.20           | n.d.         | n.d.   | 1.77           | 56.3           | 0.53   | 109.1          | 0.40           | 274.4   |
| 7      | 0.17           | 0.17         | 0.74   | 1.27           | 56.3           | 0.53   | 109.1          | 0.40           | 274.4   |
in-sample stability during the time period, while others reported relatively high in-sample stability for this gene fragment (Hokajärvi et al., 2021).

Most of the stability studies use surrogate viruses to investigate the stability of SARS-CoV-2 in wastewater or often do not include all gene fragments of interest (Casanova et al., 2009; Gundy et al., 2009; Medema et al., 2020a). Additionally, most of the available stability studies assess in-sample stability by seeding SARS-CoV-2 or another virus in IWW (Ahmed et al., 2020b; Hokajärvi et al., 2021). However, the genome of SARS-CoV-2 could potentially be degraded to smaller fragments during in-sewer transport. To our knowledge, only Hokajärvi et al. and Medema et al. investigated decay of native SARS-CoV-2 RNA loads in IWW (Hokajärvi et al., 2021; Medema et al., 2020a) and variability reported by Hokajärvi was also substantial.

Freezing the samples drastically influences the in-stability of viral RNA loads of all gene fragments of interest, with a 10-fold decrease in SARS-CoV-2 gene fragment copies. This was also observed during method optimization (Fig. S4) where detection levels were considerably higher in fresh IWW samples (kept at 4 °C) compared to frozen IWW samples. During sample transport and storage, IWW samples should be kept at 4 °C to minimize in-sample degradation of viral RNA loads and IWW samples should be analyzed within three days after sample collection.

4. Uncertainties

At this moment, an ideal external control standard with the same properties as SARS-CoV-2 for quantification is missing (Alygizakis et al., 2021). In WBE that measures chemicals, fixed amounts of deuterated analogues of the analytes of interest are added as internal standard to each IWW sample for relative quantification (van Nuijs et al., 2018). This internal standard compensates for matrix interferences and potential losses during sample preparation. In this study PRCV was used as a whole process control, but this surrogate might not entirely reflect the structural properties of the SARS-CoV-2 genome in IWW. In traditional WBE, isotope-labelled analogues are used for the quantification of chemical biomarkers. However, for biological applications, such controls are unavailable which could potentially lead to high variability with the current bioanalytical assays. Therefore, further methodological and molecular assay validation for SARS-CoV-2 RNA in IWW is required to enhance the accuracy and precision of WBE for SARS-CoV-2. This also further emphasizes the need for surrogate CoV (e.g. PRCV) as whole process control to ensure overall quality of these bioanalytical assays. The presence of a whole process control is especially of importance because of the high variability in the composition of the matrix. The fraction of PCR inhibitors could vary within a single WWTP over time and is potentially very different between WWTPs. In this study, the %RSD at the low detection levels was still considerable, as indicated with dPCR. The high variability observed in the LLOQ range also addresses the need for replicates.

To our knowledge, limited information is available on the in-sewer degradation of the SARS-CoV-2 genome with only Ahmed et al. investigating in-sewer stability through the use of microcosm experiments (Ahmed et al., 2020b). This study showed acceptable persistence of SARS-CoV-2 RNA in untreated wastewater at 4 °C and 15 °C for several days, which is generally much higher than the hydraulic retention times in wastewater collection systems. However, this was tested in the absence of biofilms and may therefore not be representative of different sewer structures. Low to medium stability can severely impact the
concentration of the SARS-CoV-2 RNA in IWW. Additionally, fragmentation of the genome during in-sewer transport could potentially affect RE with the different concentration methods found in literature and lead to high variation between WWTPs due to different sewer structures and presence of biofilms (Ahmed et al., 2020b; Kitajima et al., 2020). In the future, sequencing of the SARS-CoV-2 genome in IWW is required to identify the different fragments of the SARS-CoV-2 genome in IWW. This would add valuable information on the state of SARS-CoV-2 RNA in IWW.

In the final protocol, solids were removed during the precentrifugation of the IWW samples. However, adsorption of the SARS-CoV-2 genome to the pellet could affect RE. In this study, SARS-CoV-2 was detected to some extent in solids (data not shown), but the overall importance needs to be further explored.

5. Conclusions

The present study proposes an alternative approach to assess RE of SARS-CoV-2 gene fragments in IWW with different ultrafiltration protocols. Native concentration levels of the different SARS-CoV-2 gene fragments measured in IWW from different Belgian WWTPs with the different sample concentration methods used to optimize RE of SARS-CoV-2 RNA in IWW. The bioanalytical assay proved to be capable of measuring low concentrations of SARS-CoV-2 RNA present in the samples from different IWW sources. The present study is among the first to apply dPCR for the quantification of SARS-CoV-2 RNA in IWW and dPCR results were comparable with the qPCR results.

The variability observed with the sample concentration methods for SARS-CoV-2 remains substantial due to the lack of an ‘ideal’ external control standard with similar properties to SARS-CoV-2. This control standard would also be able to robustly correct for differences in matrix composition between days and locations. At this moment, there is also a lot of uncertainty regarding the state of SARS-CoV-2 genome (fragments) in IWW due to potential in-sewer degradation. More research on variability of SARS-CoV-2 in IWW and potential transformation of SARS-CoV-2 RNA in the IWW is necessary to further investigate the applicability of WBE.

Although WBE can already aid in filling some critical knowledge gaps in the epidemiological surveillance of SARS-CoV-2, future research should aim to further validate and standardize bioanalytical assays, especially with regards to methodological uncertainties. This is especially of importance when the number of WBE applications on data triangulation with other epidemiological information sources outpace the number of WBE studies that investigate intrinsic methodological uncertainties.

CRediT authorship contribution statement

Tim Boogaerts: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. Lotte Jacobs: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. Naomi De Roeck: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. Siel Van den Bogaert: Data curation, Writing – review & editing. Bert Aertgeerts: Writing – review & editing. Lies Vanhaute: Writing – review & editing. Alexander L.N. Van Nuijs: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing, Supervision, Funding acquisition. Peter Delpuec: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing, Supervision, Funding acquisition.

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.scitotenv.2021.148043.

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