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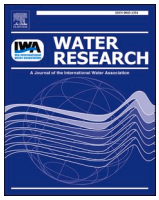

Highly efficient and sensitive membrane-based concentration process allows quantification, surveillance, and sequencing of viruses in large volumes of wastewater

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ABSTRACT

Wastewater-based epidemiology is experiencing exponential development. Despite undeniable advantages compared to patient-centered approaches (cost, anonymity, survey of large populations without bias, detection of asymptomatic infected peoples…), major technical limitations persist. Among them is the low sensitivity of the current methods used for quantifying and sequencing viral genomes from wastewater. In situations of low viral circulation, during initial stages of viral emergences, or in areas experiencing heavy rains, the extremely low concentrations of viruses in wastewater may fall below the limit of detection of the current methods. The availability during crisis and the cost of the commercial kits, as well as the requirement of expensive materials such as high-speed centrifuge, can also present major blocks to the development of wastewater-based epidemiological survey, specifically in low-income countries. Thereby, highly sensitive, low cost and standardized methods are still needed, to increase the predictability of the viral emergences, to survey low-circulating viruses and to make the results from different labs comparable. Here, we outline and characterize new protocols for concentrating and quantifying SARS-CoV-2 from large volumes (500 mL-1 L) of untreated wastewater. In addition, we report that the methods are applicable for monitoring and sequencing. Our nucleic acid extraction technique (the routine C: 5 mL method) does not require sophisticated equipment such as automatons and is not reliant on commercial kits, making it readily available to a broader range of laboratories for routine epidemiological survey. Furthermore, we demonstrate the efficiency, the repeatability, and the high sensitivity of a new membrane-based concentration method (MBC: 500 mL method) for enveloped (SARS-CoV-2) and non-enveloped (F-specific RNA phages of genogroup II / FRNAPH GGII) viruses. We show that the MBC method allows the quantification and the monitoring of viruses in wastewater with a significantly improved sensitivity compared to the routine C method. In contexts of low viral circulation, we report quantifications of SARS-CoV-2 in wastewater at concentrations as low as 40 genome copies per liter. In highly diluted samples collected in wastewater treatment plants of French Guiana, we confirmed the accuracy of the MBC method compared to the estimations done with the routine C method. Finally, we demonstrate that both the routine C method processing 5 mL and the MBC method processing 500 mL of untreated wastewater are both compatible with SARS-CoV-2 sequencing. We

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show that the quality of the sequence is correlated with the concentration of the extracted viral genome. Of note, the quality of the sequences obtained with some MBC processed wastewater was improved by dilutions or enzyme substitutions suggesting the presence of specific enzyme inhibitors in some wastewater. To the best of our knowledge, our MBC method is one of the first efficient, sensitive, and repeatable method characterized for SARS-CoV-2 quantification and sequencing from large volumes of wastewater.

1. Introduction

In December 2019, an outbreak of a novel coronavirus (SARS-CoV-2) was reported in China, and quickly spread worldwide. Since then, the ensuing disease (COVID-19) has become a pandemic that has led to several million deaths globally as of February 2023 (WHO). It has become evident that a quick and thorough assessment of the dynamics of SARS-CoV-2/COVID-19 infection within a population is crucial for implementing effective public health actions in response to the pandemic.

The time tested Wastewater-Based Epidemiology (WBE) which has been used to track the lifespan of previous pathogenic viruses such as poliovirus, enterovirus, adenoviruses and noroviruses in communities around the world (Lago et al., 2003; Katayama et al., 2008; McCall et al., 2020), proved to be equally effective to track SARS-CoV-2. Fecal shedding of SARS-CoV-2 with detectable viral RNA found in domestic waters has been reported and demonstrated by Kitajima et al. (2020), and Xiao et al. (2020). Furthermore, recent studies have identified a correlation between the number of viral genome copies in wastewater and the prevalence of infection within the population (Ahmed et al., 2020a; Medema et al., 2020a; Wurtzer et al., 2020). As such, WBE has been successfully adapted as a public health tool for tracking the spread of SARS-CoV-2 within communities (Maréchal et al., 2023). Worldwide Public Health departments have been diligently researching rapid and cost-effective tools for monitoring SARS-CoV-2 in wastewater samples. Regrettably, most protocols used for tracking SARS-CoV-2 in wastewater rely on viral extractions from small sample volumes. Consequently, when the virus is present at low levels (due to low community infection rates, or wastewater dilution from rainfall, for example), these protocols demonstrate reduced accuracy and therefore cannot achieve sufficient sensitivity (Zheng et al., 2023). Thus, there is a need for a more sensitive protocol for detecting and quantifying the virus. This protocol should have the capacity to detect SARS-CoV-2 RNA when the number of cases is low and should be applicable to monitor the emergence of specific variants. In addition, it should allow to quantify human markers that are used to calibrate the primary outcome concentrations from wastewater monitoring for use in epidemiological models such as cross-assembly phage, F-specific RNA bacteriophage genogroup II, or pepper mild mottle virus (Gyawali et al., 2021; Holm et al., 2022).

Finally, the method should be easily applicable to other respiratory viruses (Influenza or Respiratory Syncytial Virus) and to emerging viruses such as Dengue and West Nile arboviruses that are dangerously spreading in Europe together with their vectors (SPF, n.d.).

Prior to this work, various virus concentration methods were developed and used by scientists worldwide. These methods included ultrafiltration, ultracentrifugation, polyethylene glycol (PEG) precipitation, direct sludge extraction, and skimmed milk flocculation (Ahmed et al., 2020; Peccia et al., 2020; Medema et al., 2020; Ahmed et al., 2020a,b; Kabdaşlı and Tünay, 2021; Kaya et al., 2022). The development of highly sensitive methods for virus quantification requires processing large volumes of wastewater.

McMinn et al. described a concentration method applicable for 2L of wastewater (McMinn et al., 2021). However, the concentration process demonstrated a limited recovery rate of around 20%. More recently, Sylvia Monteiro and collaborators reported an impressive 68 ± 11% recovery rate after processing 1 liter of wastewater with the same Dead-End hollow fiber ultrafiltration-based method but did not evaluate the process for genome sequencing (Monteiro et al., 2022). Hence, there is a need for a deeply characterized, simple, sensitive, and affordable new virus concentration method for viral surveillance and genome sequencing.

In this article we describe our low-cost and brand limited C method dedicated to routine SARS-CoV-2 monitoring in wastewater and the complementary Membrane-Based Concentration (MBC) process that significantly improves the sensitivity of the quantification pipeline. In addition, we report that our methods are compatible with SARS-CoV-2 sequencing.

2. Material and methods

2.1. Sample collection

Samples of 24 h-composite untreated wastewater (influent) were collected weekly from Alsace region (France) and French Guiana wastewater treatment plants. Samples were shipped to the laboratory under cool conditions, stored at 4 °C, and used within one week of collection. Information on sampling points in Alsace is shown in Supplementary Table 1.

The conductivity was measured using Protavo® 907 MULTI (SE 615/1-MS, Knick), the pH was measured using a pH met er Accumet AE150 (Fisher Scientific) and the optical density (OD) at 600 nm was measured using an Eppendorf Biop hotometer.

2.2. Nucleic acid extraction (routine C method)

The following viral nucleic acid extraction protocol has been routinely used since November 2020 for monitoring 11 WWTPs in Alsace-France (réseau Obépine). 5 mL of untreated wastewater were mixed with 4.5 mL of lysis buffer (Guanidine Thiocyanate 10M / Tris-HCl 0.1M pH 6.4, 2% SDS, EDTA 0.038 M) and 450 µL of 2% DTT. The samples were incubated for 10 min at room temperature and then for an additional 10 min at 60 °C. 15 µL of polyadenylic acid (10 g/L), 5 mL of isopropanol, and 8 µL of silica particles (1.04 g/cm3) (MagPrep® Silica Particles, Sigma-Aldrich) were then added to capture viral RNA. The samples were incubated for 10 min with shaking at room temperature. The beads were subsequently collected and washed twice with washing buffer 1 (lysis buffer / ethanol 1:1 (v/v) and DTT 2%) and twice with washing buffer 2 (ethanol / Nuclease-Free Water 8:2 (v/v)). The beads were then incubated for 10 min at room temperature to allow ethanol evaporation. Elution was carried out for samples C by adding 50 µL of elution buffer (Tris-HCl-EDTA pH9 1M), then the samples were incubated for 10 min at 95 °C. A final purification step was performed using the OneStep-96 PCR Inhibitor Removal kit (#ZD6035, OZYME) following manufacturer's instructions. This final step was not essential for the viral extraction (results not shown in this paper) but was performed to improve the quantification and sequencing processes. 5 mL of ultrapure sterile water (Sigma) were extracted as a negative control at each batch of extraction. Purified samples were then stored at -80 °C until quantification by RT-qPCR assays.

2.3. Viral concentration method (MBC method)

For samples processed by the MBC method, 500 mL of untreated wastewater were concentrated using an Amicon® Stirred Cell 400ML (UFSC40001, Merck-Millipore), with a cut-off of 10 kDa membrane (Ultracel® regenerated cellulose membrane, PLGC07610, Merck-

Millipore), under a vacuum pressure set at 3 bar. The Ultracel® membrane was pre-rinsed before use, following manufacturer's instructions. To avoid the clogging of the membrane and the slowdown of the process, samples were decanted for 30 min. The liquid part was first poured into the Amicon, and the solid part was then added to the membrane at the end of process. The ultrafiltration was stopped when the final concentrate reached 5 mL.

After the collection of the membrane-based concentrate (MBC) sample, the membrane was rinsed by adding 4.5 mL of lysis buffer pH 4 (Guanidine Thiocyanate 10M / Tris-HCl 0.1M pH 6.4, 2% SDS, EDTA 0.038 M) into the cell. The lysis buffer was then collected and added to the 5 mL of MBC sample (total volume: 9.5 mL). For nucleic acid extraction, the protocol was adapted from the routine C method described in 2.2. After the first two steps of incubation (RT and 60 °C), a first step of purification with acid phenol pH 4.3 / chloroform 1:1 (v/v) was carried out. A volume of 9.5 mL of the phenol / chloroform mixture was added. Grease tubes (silicone grease / silica 90:10 (w/w)) were used to facilitate phase separation. The samples were centrifuged for 5 min at 3500 x g and a volume of 9.5 mL of supernatant from the aqueous phase was collected. The other extraction steps (RNA capture, washes, and elution) were then followed in the same way as for the C method. Elution volumes were 50µl or 100 µL (1st elution) and 50 µL (2nd elution) for MBC samples. The OneStep-96 PCR Inhibitor Removal kit (#ZD6035, OZYME) was used following manufacturer's instructions before the RT-qPCR assays. C samples used to calculate the efficiency of the MBC method were extracted following the same nucleic acid extraction protocol (with the phenol-chloroform step) for a better comparison.

2.4. Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR)

One-step RT-qPCR was performed with Master Mix Fast Virus 1-Step TaqMan™ kit (#4444434, Applied Biosystems – ThermoFischer Scientific) on a BioRad CFX96™ thermal cycler, software version 3.1 (Bio-Rad Laboratories) manually setting the threshold and baseline. Two RT-qPCR assays were chosen to quantify SARS-CoV-2: (I) E-Sarbeco assay (Corman et al., 2020) targeting the envelope protein E and (II) RdRp-IP4 assay targeting part of the ORF1ab. Primers and probes were synthesized and provided by Eurofins Genomics (sequences available in supplementary table 2). nCoV_E-Sarbeco and nCoV_IP4 set were used with 0.4 µM of each primer, 0.2 µM of probe in 10 µL final reaction volume with 5 µL of RNA sample, and 2.5 µL of Master Mix Fast Virus 1-Step TaqMan™ for each assay. The quantification of SARS-COV-2 genome copies per µL were then carried out using a standard curve. For the routine, a synthetic quantified SARS-CoV-2 RNA provided by Biorad (ref COV019) was diluted in a fourfold dilution series with concentrations from 1 to 1000 genome copies per reaction and run in duplicate wells on each plate.

The presence of PCR inhibitor in RNA extracts was assessed using a dengue RT-qPCR assay after spiking a known copy number of Dengue RNA (exogenous RNA, which is not present in the wastewater samples). In order to determine PCR inhibition, Dengue RNA was also added to Nuclease-Free Water sample and the resulting Ct value was used to set-up a reference point. Subsequently, the Dengue RT-qPCR assay was performed in 10 µL reaction mixtures using Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories), manually setting the threshold and baseline. Dengue set (Euliano et al., 2019) (sequences available in supplementary table 2) was used with 0.4 µM of each primer, 0.2 µM of probe in 10 µL final reaction volume with 5 µL of RNA sample and 2.5 µL of Master Mix Fast Virus 1-Step TaqMan™ and 6.8×10^3 genome copies / reaction of Dengue RNA. All samples were analyzed alongside two no template controls. All wastewater samples were within the 2-Ct values of the reference Ct.

Furthermore, F-specific RNA bacteriophages genogroup II (FRNAPH GGII) were quantified in all wastewater samples to determine the efficiency of viral genomes concentration. Briefly, for the FRNAPH GGII genome, the VTB4-Fph GII set published by Wolf et al. (2010) was used with 0.4 µM of each primer, 0.2 µM of probe in 10 µL final reaction volume with 2.5 µL of RNA and 2.5 µL of Master Mix Fast Virus 1-Step TaqMan™. On the BioRad thermal cycler, the RT was conducted at 50 °C for 5 min followed by an initial denaturation cycle at 95 °C for 20s and 49 cycles including denaturation step at 95 °C for 3s, and hybridization step at 58 °C for 30s.

A global overview of the analytical pipeline (step 2.1–2.4) is presented in Fig. 1.

2.5. LOD/LOQ determination

A synthetic quantified SARS-CoV-2 RNA provided by Biorad (ref COV019) was used. Standard curves were prepared from 1000 to 1 GC per reaction. Dilution points from 80 to 1 GC/reaction were run in 10 replicates to determine the Limit of Detection (LOD) of the RT-qPCR assay. A Ct value $\geq$ 40 was considered negative. The lowest concentration at which all replicates were positive was assumed to be the limit of detection (LOD). The limit of quantification (LOQ) was estimated at the lowest concentration where the Relative Standard Deviation (RSD) of the copy number measurements was below 25% (Hougs et al., 2019).

2.6. Viral recovery efficiency

Process recovery efficiency represents an important metric in viral signal quantification, as it allows comparison of results from study to study even if different concentration and quantification methods are used. In this study, the efficiency of virus recovery from the whole process (ultrafiltration / RNA extraction / RT-qPCR) was calculated using the following equation;

$$Recovery = \frac{(GC/L)\ in\ MBC\ sample}{(GC/L)\ in\ C\ sample} \times 100$$

2.7. SARS-CoV-2 RNA sequencing

NGS sequencing libraries were prepared using COVIDSeq Test kit (Illumina, Inc San Diego, CA, USA) with SARS-CoV-2 specific primers Artic v4.1 as described in Gerber et al. (2022). The SARS-CoV-2 enriched libraries were sequenced using NovaSeq 6000 SP Reagent Kit v1.5 (300 cycles) according to the manufacturer's instructions (Illumina, Inc San Diego, CA, USA). Sequencing data was analyzed using our in-house bioinformatic pipeline as described in Gerber et al. (2022) using Pangolin database v4.2 (O'Toole et al., 2021).

2.8. Statistical analysis

A normality test was performed using the Shapiro-Wilk test. Paired t-test and Wilcoxon tests were used to determine whether there were any statistically significant differences between the means of two dependent groups. One-way analysis of variance (ANOVA) was used to determine whether there were significant differences between the means of three or more independent groups. When p value was less than 0.05, the difference was considered significant. All statistical tests and figures were performed using GraphPad Prism version 9.

3. Results

3.1. Membrane-based concentration process is efficient to enrich enveloped and non-enveloped viruses from large volumes of wastewater

Concentrating viruses from wastewater samples is a crucial step, especially for detecting low-level virus RNA during the onset, the resurgence, and the deceleration of epidemics or in diluted wastewater due to rainfall. Twenty-three wastewater samples were collected from four different WWTPs in Alsace, France from February to October 2022 and were subsequently analyzed. A half-liter of each wastewater was

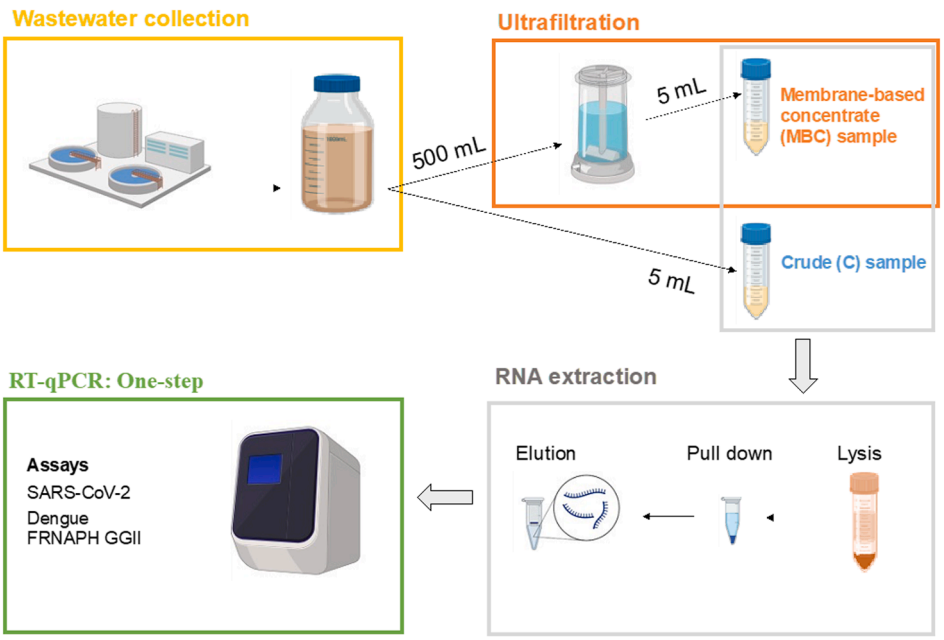

Fig. 1. Overview of the process used for quantifying SARS-CoV-2 in crude (C) and membrane-based concentrated (MBC) large volume of wastewater. 24h representative wastewater samples were collected from four different wastewater treatment plants (WWTPs) across Alsace, France. The MBC samples were concentrated from 500 mL to 5 mL through ultrafiltration using Amicon® Stirred Cell 400ML and Ultracel® regenerated cellulose membrane (10 kDa). Crude and MBC samples were subjected to RNA extraction and the RT-qPCR quantifications.

concentrated using ultrafiltration (Amicon® 10 kDa) to yield a final concentrate of 5 mL (MBC for membrane-based concentration). Viral RNA was then extracted using our in-house protocol and quantified by analyzing specific genes E-Sarbeco and RdRp-IP4 for SARS-CoV-2 and VTB4-Fph GII for FRNAPH GGII, using RT-qPCR. RNA amounts in membrane-based concentrated (MBC) samples were compared to their corresponding crude wastewater samples (C for crude).

As shown in Fig. 2A, the extracted amount of SARS-CoV-2 RNA, measured in genome copies (GC), ranged from 5.9×10^1 to 5.5×10^3 and from 3.8×10^3 to 2.5×10^5 for C and MBC samples, respectively.

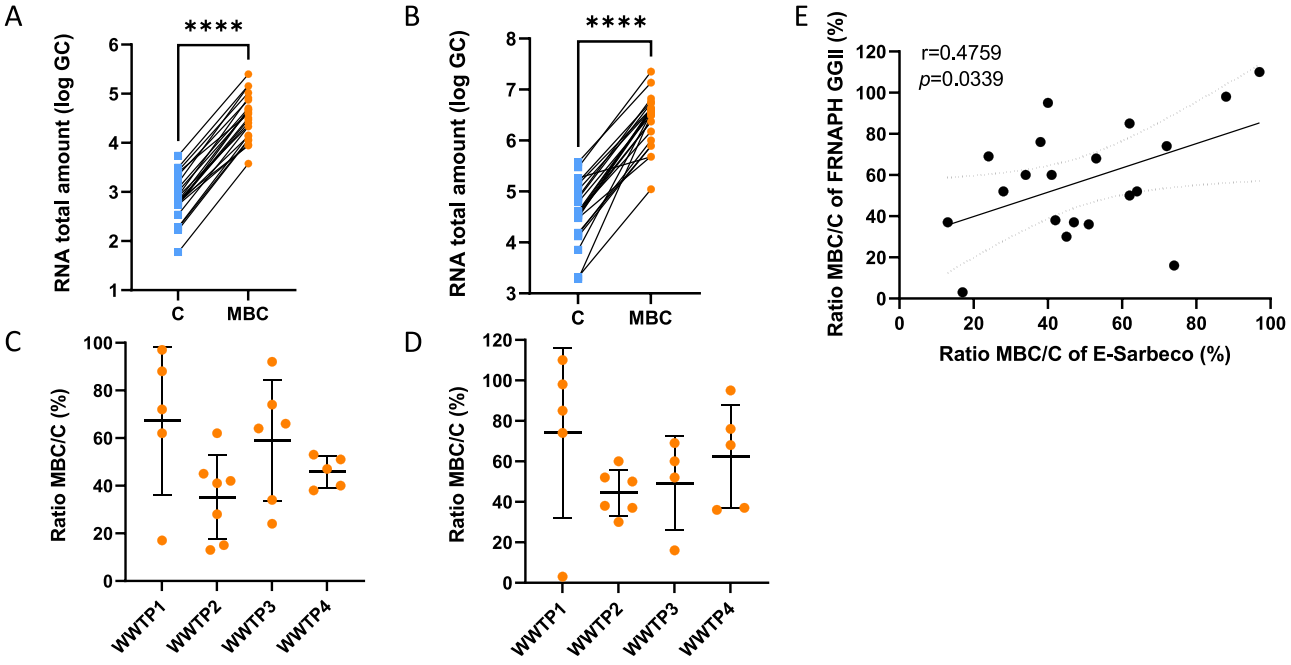

Fig. 2. Membrane-Based Concentration (MBC) is efficient for enveloped (SARS-CoV-2) and non enveloped (FRNAPH GGII) viruses. Total amount of SARS-CoV-2 (target gene E-Sarbeco) (A) and FRNAPH GGII (target gene VTB4-Fph GII) (B) genomes were quantified in 5 mL crude (blue) and 500 mL Membrane-based concentrated (orange) wastewater samples collected at different time points in four different treatment plants. Wilcoxon test was performed for statistical analysis. **** $p<0.0001$. Recovery (mean ± SD) of SARS-CoV-2 RNA (C) and FRNAPH GGII (D) from four wastewater treatment plants (WWTPs) using MBC. Means and standard deviations are shown on theses figures. Correlation between recoveries of SARS-CoV-2 and FRNAPH GGII in wastewater samples concentrated by ultrafiltration (E). Pearson's correlation coefficient (r) and the corresponding p-value are shown on this figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The process allowed an average increase of 51 ± 24-fold in SARS-CoV-2 quantities in MBC samples compared to C samples. Notably, some samples were enriched up to 97-fold, approaching the technique's 100% efficiency for the input of 500 mL. Based on our standard curves targeting the SARS-CoV-2 E gene and according to the JRC European guidelines (Hougs et al., 2019), the calculated limit of detection (LOD) of the routine C method is 10^4 genome copies per liter (GC/L), with detections possible up to 2×10^3 GC/L (Supplementary Figs. 1 and 2). In addition, comparisons with a commercial 5 mL-based method and the incidence rate confirmed that our open-access routine C method is suitable for SARS-CoV-2 monitoring (Supplementary Fig. 3A protocol 1 vs protocol 2 and Supplementary Fig 3B). Based on our standard curves targeting the SARS-CoV-2 E gene, the calculated limit of detection (LOD) of the MBC method is 50 GC/L (input 1L), with detections possible up to 10 GC per liter of wastewater processed. The high efficiency of the MBC process allowed for quantifications below 125 and up to 40 GC/L. As shown in supplementary Fig. 3A protocol 3, the MBC process proves valuable for quantifying SARS-CoV-2 when the concentrations in wastewater fall below the concentrations detectable with the routine C method. Notably, this high sensitivity of the MBC process was reachable for input volumes of 500 mL and 1L. However, MBC is suitable for larger volumes, potentially enabling quantification of even lower quantities of the virus.

To determine the efficiency of the MBC method for non-enveloped viruses, F-specific RNA phages of genogroup II / FRNAPH GGII RNA was concentrated and extracted using the same method. The extracted amount of FRNAPH GGII RNA ranged from 1.9×10^3 to 3.8×10^5 and 1.1×10^5 to 2.3×10^7 GC for C and MBC processed samples, respectively. The average increase of FRNAPH GGII in MBC samples compared to C samples was 57 ± 28-fold (Fig. 2B). Since the MBC efficiencies measured for FRNAPH GGII are comparable to those observed for SARS-CoV-2, the lowest detectable quantities are in the same range and below 50 GC/L.

The viral recovery rate was calculated as the ratio of the targeted virus (SARS-CoV-2 and FRNAPH GGII) concentration in MBC samples to its concentration in C samples. The mean recovery efficiency was 51 ± 24% and 57 ± 28%, for SARS-CoV-2 and FRNAPH GGII, respectively, using ultrafiltration method across samples from 4 WWTPs collected at different times between February and October 2022. However, we observed variations in the efficiency of viral RNA recovery between the different collection weeks for each WWTP and between the different WWTPs (Fig. 2C and D). The standard deviation S.D. of recovery efficiency within WWTPs ranged from 6.6 to 31.2% and from 11 to 42% for SARS-CoV-2 and FRNAPH GGII, respectively (Fig. 2C and D). These results suggest that the efficiencies of viral enrichment depend on the WWTP being sampled and on the week of collection. The heterogeneity of the recovery rates is therefore linked to the heterogeneity of the wastewater composition. However, the efficiency of our 500 mL MBC process was comparable to the one described for smaller volumes of wastewater (Bertrand et al., 2021) giving a better sensitivity to the MBC method.

Next, we compared the MBC process for SARS-CoV-2 and FRNAPH GGII. As shown in Fig. 2E, a good correlation could be found between the two viral concentrations after the MBC process. These results suggest that FRNAPH GGII can be used as a good internal control for normalization of the SARS-CoV-2 concentrations. In addition, it allows to take into consideration the efficiency of the MBC process when the SARS-CoV-2 concentrations are below the LOD of the routine C method.

3.2. MBC does not significantly increase PCR inhibition

The presence of PCR inhibitors in wastewater is detrimental to efficient and sensitive quantifications of viruses using molecular-based approaches. To ensure that PCR inhibitors are not concentrated along with the viruses during the MBC process, PCR inhibitions in both C and MBC wastewater samples were assessed. This assessment was carried out using a Dengue RT-qPCR quantification, after spiking RNA samples with a known copy number of Dengue RNA. Additionally, Dengue RNA was added to a nuclease-free water sample to establish a reference point for Ct values. An analysis of 23 C and MBC samples showed slight increases, but no significant PCR inhibitions were observed in the MBC samples (Fig. 3A). It is noteworthy that the quantified PCR inhibitions in C and MBC samples were far below the commonly acceptable thresholds for wastewater (ΔCt=2 or 78%). These findings suggest that our experimental conditions were suitable for viral genome quantifications in both crude (C) and membrane-based concentrated (MBC) wastewater samples.

3.3. MBC is highly repeatable

To determine the intra-assay precision of the entire SARS-CoV-2 quantification process, from the MBC to the final qPCR (ultrafiltration, RNA extraction and RT-qPCR) a wastewater sample collected in week 11 of 2022 from WWTP4 was separately analyzed six times. The Ct values of the SARS-CoV-2 E-Sarbeco gene from these six analyses were plotted against their corresponding mean (Fig. 3B). The relative standard deviation (RSD) to the mean was 1.8%. This RSD of 1.8% is far below the acceptable limit of 15%, as described by Biopharmaceutics Coordinating Committee of the US Food and Drug Administration (Bioanalytical Method Validation. Guidance for Industry, 2018). It is important to note that the RSD to the mean of the extraction part (C method) of the process alone was 1.5% (supplementary Fig. 2). These results demonstrate the high repeatability of our SARS-CoV-2 concentration, extraction, and quantification pipeline. Furthermore, they confirm that the variations in the recovery rate observed in Fig. 2C and D, were not linked to technical issues but to the heterogeneity of the wastewater.

3.4. MBC is suitable for epidemiological survey in wastewater

We next compared the dynamics of viral spread obtained with and without MBC in wastewater from three different WWTPs. SARS-CoV-2 (Fig. 4A–C) and FRNAPH GGII (Fig. 4D–F) genome copies per liter of crude wastewater quantified with both C and MBC methods followed the same trend over time. In addition, since the results obtained with our C method processing 5mL were comparable to the ones obtained with the commercial NucliSens® EasyMAG™ method for SARS-CoV-2 monitoring in wastewater, the MBC method processing 500 mL allowed for wastewater-based epidemiological survey when the quantities of virus fell below the limit of detection of the C method (supplementary Fig. 3A protocol 3).

To further document this point, we subsequently used wastewater samples collected between April and July 2021 in WWTP2 to perform MBC assays when SARS-CoV-2 concentrations dropped below the detection limit of the C method. As shown in Fig. 5A, when the virus was no longer detectable in crude samples, MBC allowed the quantification of SARS-CoV-2 for an additional 4 weeks. Thanks to MBC, we were able to quantify up to 40 GC/L of SARS-CoV-2 with a sample input of 1 L. This high sensitivity of the MBC process aligns perfectly with the detection range mentioned in Section 3.1. To further test the MBC process in highly diluted wastewater, we quantified SARS-CoV-2 in wastewater samples taken during the transition between the dry and the wet periods in Kourou and Saint Laurent du Maroni (French Guiana). As shown in Fig. 5B, the concentrations obtained with the C method were close to the LOD and therefore only estimated. The MBC process enables a more precise quantification of these low SARS-CoV-2 concentrations confirming that concentrations in proximity to the LOD, as measured with the C method, were at times either overestimated (SLM) or underestimated (Kourou). Of note, SARS-CoV-2 MBC concentrations are presented relative to the ratio GII MBC / GII C to take into consideration the efficiency of the MBC process.

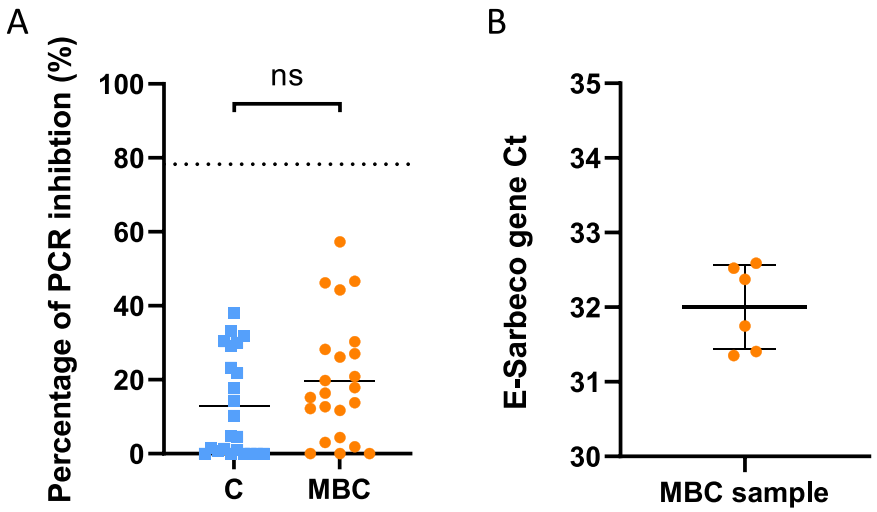

Fig. 3. Membrane-based concentration does not significantly increase PCR inhibitors and is highly repeatable. (A) PCR inhibitions have been compared for the crude wastewater (blue) and the membrane-based concentrate samples (orange). A Wilcoxon test has been performed to compare the two groups of samples. ns: not significant. (B) Six different Membrane-based concentrations were performed on the same wastewater and subjected to SARS-CoV-2 E-Sarbeco gene quantifications. Mean and standard deviation are shown on this figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

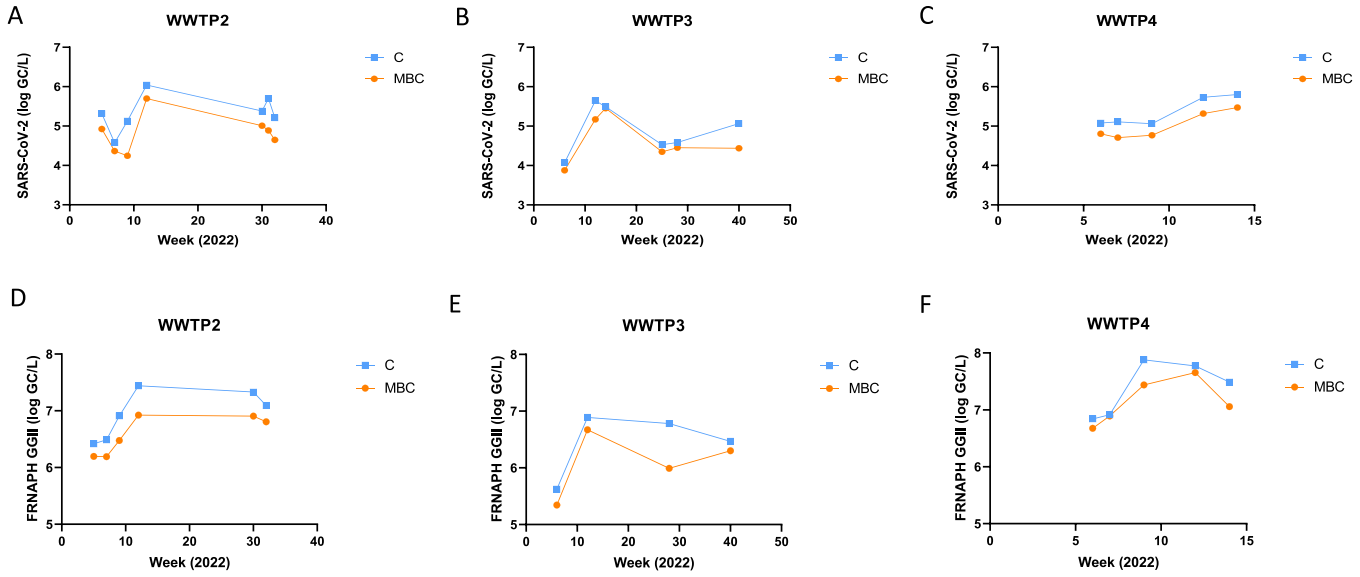

Fig. 4. Viral dynamic profiles are similar with the C and the MBC methods. Longitudinal comparison of SARS-CoV-2 (A,B,C) and FRNAPH GGII concentrations (D,E, F) (log genome copies per liter of wastewater) in C and MBC samples . Crude wastewater samples (routine C method) are represented in blue, and the membrane-based concentrated (MBC) samples represented in orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. MBC allows next generation sequencing of SARS-CoV-2

To investigate the impact of wastewater concentration on Next-Generation Sequencing (NGS), samples of increasing SARS-CoV-2 concentrations were sequenced with the Illumina short reads method COVIDSeq. As expected, the depth of SARS-CoV-2 RNA sequencing in crude wastewater was correlated with the concentration of the samples. The best breadths of coverage were observed for concentrations close to, or over 20 GC/µl in the final 50 µl samples obtained after extraction of 5 ml with the routine C method (Fig. 6A).

This optimal sequencing concentration of SARS-CoV-2 RNA in the extracted sample corresponds to 2.0×10^5 GC/L in the collected wastewater that has been observed only in high epidemic periods and only in 15% of the weekly based-quantified wastewater samples from 11 WWTPs in 2022. These results suggest that MBC could improve the sequencing when the viral concentrations in wastewater are low. We indeed confirm that MBC samples were compatible with NGS but we also observed that some MBC processed wastewater samples were difficult to sequence. These results further demonstrate that the heterogeneity of the origins and the compositions of the samples tested influence the quality of the sequence obtained. However, the dilution of those samples improved the depth of sequencing, suggesting that some NGS enzymes inhibitors present in MBC samples may impact the sequencing efficiency (Fig. 6B). Interestingly, the sequencing depth of the diluted MBC samples were better than the one observed with the crude extract of the same wastewater. These results suggest that the PCR inhibitors that did not significantly impact the RT-qPCR step of our process of quantification disfavored NGS sequencing with the COVIDSeq kit. To further

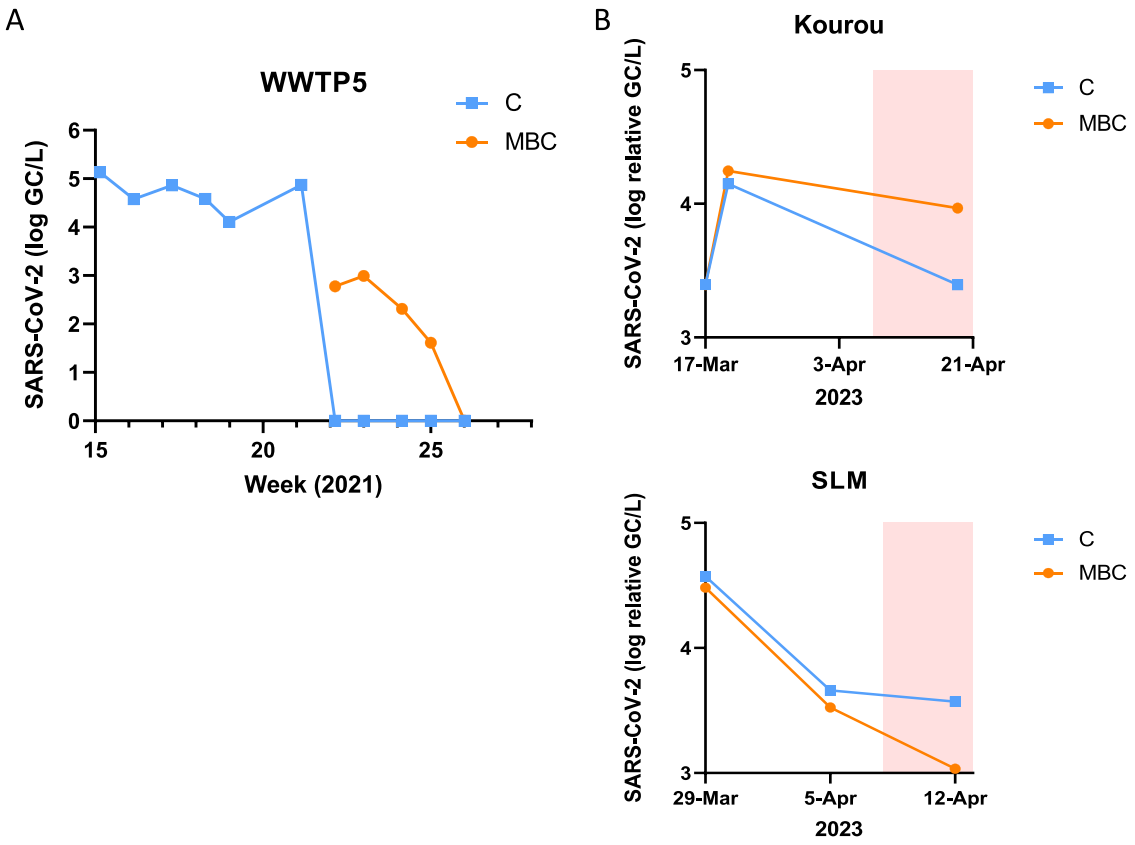

Fig. 5. Membrane Based-concentration allows quantifications of low viral concentrations. (A) Longitudinal quantifications of SARS-CoV-2 concentration in 5 mL crude (blue) and 500 mL MBC wastewater from WWTP5. Results are presented in log genome copies per liter. (B) Quantification of Sars-CoV-2 in two different wastewater samples from Guyana before and after the onset of the monsoon season (red highlighted aera). GII quantities in the C samples were set to 1 and MBC quantities of SARS-CoV-2 are presented in log GC/L relative to the ratio GII-MBC / GII-C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

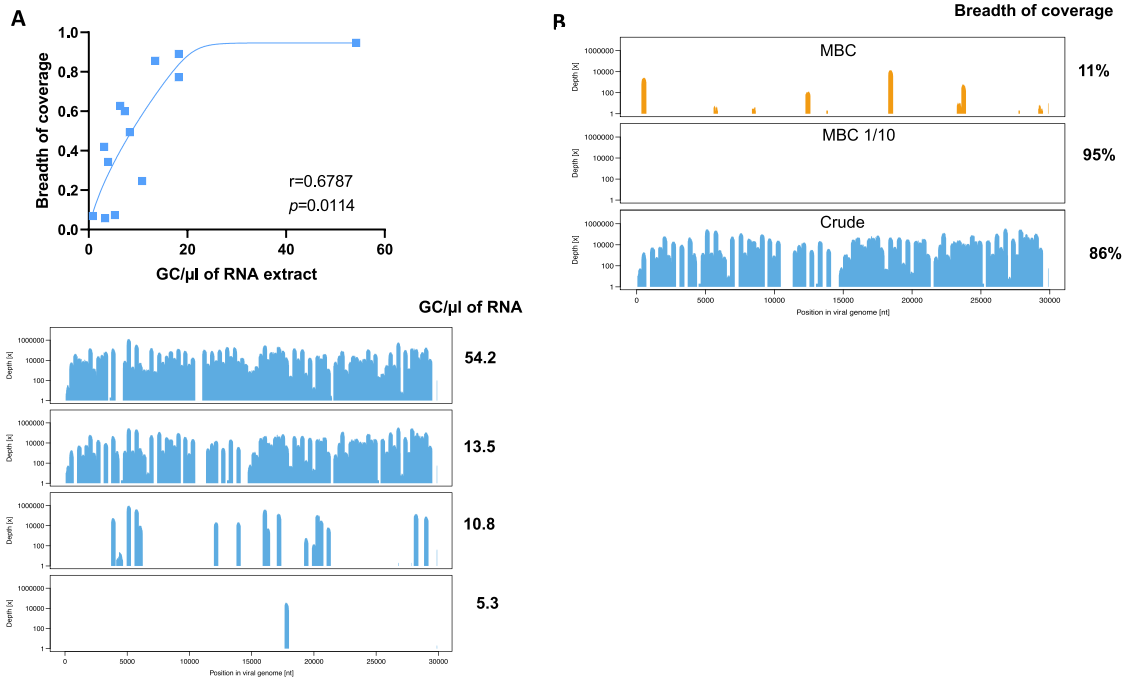

Fig. 6. Efficiency of wastewater extracted SARS-CoV-2 genome sequencing depends on RNA concentrations (A) and enzymatic inhibitions (B). (A) RNA extracted from different crude wastewater were quantified and subjected to COVID-seq Next-Generation Sequencing. Spearman's rank correlation results are presented (B) Crude (blue) and MBC (orange) RNA extracts were subjected to COVID-seq Next-Generation Sequencing. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

investigate this point, we performed NGS sequencing with the full COVIDSeq kit or after substitution of the COVIDSeq reverse transcriptase by the superscript IV reverse transcriptase. Bioinformatic analyses using Pangolin pipeline demonstrated that sequences obtained with MBC samples allow the identification of mutations in the SARS-CoV-2 circulating variant (Fig. 7).

Since SuperScript IV improved the depth of sequencing, variant calling highlighted more mutations present at low frequencies. Thereby, the gain of sequence coverage observed with the SuperScript IV was mitigated by those low-frequency mutations that may reflect a lower fidelity of the SuperScript IV compared to the COVIDSeq reverse transcriptase. Further investigations will be needed to improve this specific step of the method. Altogether, our results confirm that MBC is compatible with NGS sequencing but can still be optimized to limit the impact of the PCR inhibitors on the quality of the genome sequences.

4. Discussion

Wastewater-based epidemiology (WBE) has emerged as an efficient strategy to monitor in real-time the health status of a given population, as pathogenic viruses may be present in the excrement and fluids of infected individuals. The application of WBE was previously used to monitor pathogenic viruses around the world (Lago et al., 2003; Katayama et al., 2008; McCall et al., 2020), and recently implemented to monitor SARS-CoV-2/COVID-19. Most of the methods described in the literature have three fundamental stages for analyzing and quantifying SARS-CoV-2 virus: viral concentration, RNA extraction and quantification of target genes.

The concentration of viruses from wastewater samples is a crucial step driving the efficiency and the sensitivity of the techniques. Viral concentration methods were initially developed for analyzing and detecting non-enveloped viruses. Less is known about the recovery efficiency for enveloped viruses. There are significant structural differences between enveloped and non-enveloped viruses which could lead to differences in concentration recovery efficiencies (Wurtzer et al., 2021). Haramoto et al., 2009 demonstrated differences in virus recovery efficiencies between enveloped and non-enveloped viruses in lake water in Japan. To date, there is no standard methodology developed for the quantification and detection of SARS-CoV-2. Protocols differ according to sample volume, storage conditions, concentration method, RNA extraction method, and RNA quantification method.

To estimate SARS-CoV-2 recovery efficiencies, researchers have been using surrogate viruses such as process controls. For instance, bovine coronavirus (BCoV) was used by LaTurner et al. (2021) and Gonzalez et al. (2020), murine hepatitis virus (MHV) was used by Ahmed et al. (2020a), human coronavirus (HCoV-229E) was used by La Rosa et al. (2021), Phi6 was used by Sherchan et al. (2020), betacoronavirus OC43 was used by McMinn et al. (2021), porcine epidemic diarrhea virus (PEDV) was used by Monteiro et al. (2022) and recently inactivated SARS-CoV-2 was used by Zheng et al. (2023). However, there is a doubt about the ability to translate recovery efficiencies of spike-in process controls to SARS-CoV-2 (Ahmed et al., 2020b). In a recent publication, Zheng and colleagues suggested that endogenous SARS-CoV-2 in wastewater samples exhibits distinct affinities to flocculants and partitioning than exogenously spiked virus. Therefore, it is advised to use positive untreated wastewater samples to evaluate quantification methods and determine an optimal protocol for various applications (Zheng et al., 2023). Thereby, fecal contamination markers commonly found in wastewater such as PMMoV and FRNAPH GGII were used to evaluate concentration methods along with spiked SARS-CoV-2 (Alamin et al., 2022) In this study, we quantified SARS-CoV-2 and FRNAPH GGII in crude wastewater samples (C samples) and membrane-based concentrated samples (MBC). The mean recovery efficiency was 51 ± 24% and 57 ± 28% for SARS-CoV-2 and FRNAPH GGII, respectively, across 4 WWTPs demonstrating the efficiency of the MBC method to reduce the quantification limit of enveloped and non-enveloped viruses up to 100 folds for 500 mL of input. With 1 liter of input, MBC allowed to quantify SARS-CoV-2 RNA in wastewaters containing viral concentrations as low as 40 GC/L. However, large variations in viral RNA concentration efficiencies were observed within WWTPs. Considering the high repeatability of the method, these variations were not due to the technique but to the heterogeneity in the composition of the wastewater at the time of collection. Additional studies will be required to link this variability in MBC efficiency to specific water parameters. Physicochemical characteristics of the wastewater being sampled such as salinity, pH, temperature, or suspended solids will be investigated.

The volume of wastewater input in viral concentration process may impact reproducibility, sensitivity, variability and the instrument requirements (LaTurner et al., 2021). First, increasing the volume of wastewater input may influence processing time. While McMinn et al. reported very fast filtrations of large volumes, a nearly exponential relationship between input volume and processing time was reported for other filtration methods (McMinn et al., 2021; LaTurner et al., 2021). With our method, we have been able to concentrate viruses from 500 mL to 1 L of wastewater sample which was much larger volumes than ones reported to date (15 mL and 50 mL), (LaTurner et al., 2021; Ahmed et al. (2020a) within approximate times ranging from 1 to 4 h. Of note the use of multiple MBC systems allowed for the concentrations of multiple large volumes in parallel within the same time. Expanding the processed water volume will be a key area of future development for us

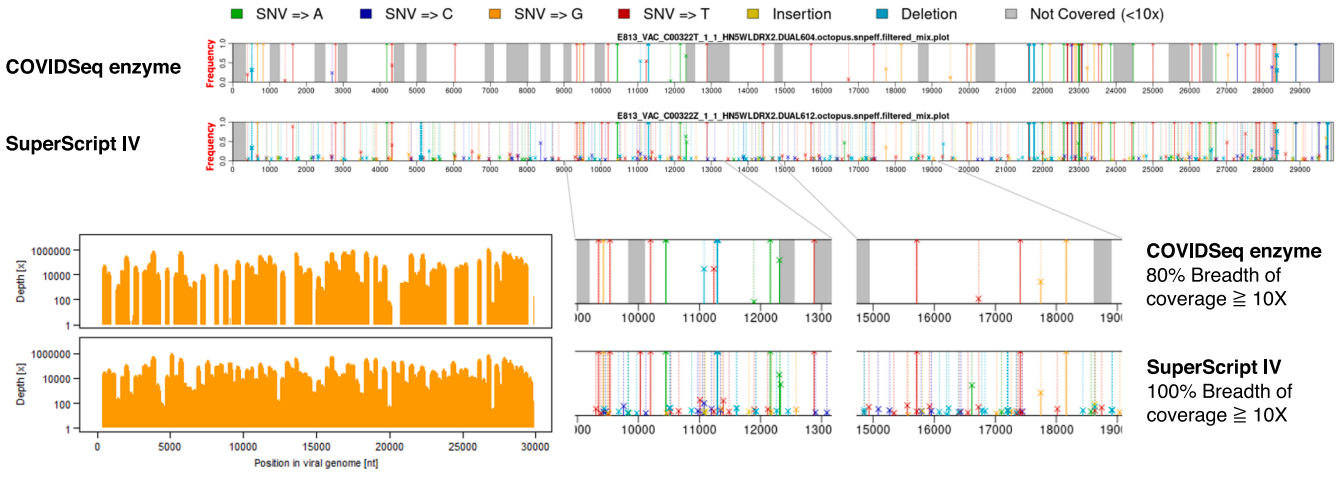

Fig. 7. Membrane-based concentrated samples are compatible for Next Generation sequencing. MBC RNA extracts were subjected to COVIDseq Next-Generation Sequencing with the complete COVIDSeq kit or with substitutions of the reverse transcription enzyme by SuperScript IV.

considering the existing limitations of the technique which are determined by the membrane filtration surface and the clogging that may occur with larger volumes of highly turbidity wastewater samples.

Our method for concentrating and quantifying SARS-CoV-2 RNA is reliable and cost-limited. Indeed, apart from the investment in the Amicon Stirred Cell (less than 2500€), the consumable cost of our ultrafiltration process of wastewater is about 3 times cheaper per mL of processed water than commercial ultrafiltration methods commonly used to quantify SARS-CoV-2. In addition, our in-house RNA extraction technique (routine C method) is about 30% cheaper than RNA extractions using commercial kits. Furthermore, our routine C method does not require sophisticated and expensive equipment such as automatons or high-speed centrifuges. Finally, limiting the use of commercial kits could be a crucial advantage during crisis period with limited access to kits. The routine C method presented here is brand-limited and easy to implement. It uses very common reagents and tools that can be found in most laboratories. However, further improvement would still be needed to limit the use of hazardous / toxic reagents.

Previous studies highlighted the need to concentrate both solid and liquid fractions of wastewater samples (Ahmed et al., 2020b). Indeed, pre-treatment steps such as pre-filtration to remove cells and larger debris led to losses of particles associated virus in the pellet. For instance, resultant pellets from using centrifugal filter devices (Centricon Plus-70 and Amicon Ultra 15) led to 30% loss of process control (MHV) during pre-filtration step (Ahmed et al., 2020b). With our method, both solid and liquid fractions of wastewater samples were processed. However, further investigations may be needed to guarantee an optimized extraction of the virus from the solid phase.

The Master Mix Fast Virus 1-Step TaqMan™ used in this study to quantify SARS-CoV-2 target genes is optimized to handle RT-PCR inhibitors from samples such as blood and faeces. Thereby, the co-concentration of PCR inhibitors from large volumes such as 500 mL of wastewater, had no significant influence on SARS-CoV-2 quantification with our method. However, the heterogeneity of the wastewater composition and the presence of PCR inhibitors impacted the quality of the sequences obtained by NGS. We indeed observed variability in the quality of the sequences generated with different MBC samples of wastewater. Our results suggest that inhibition of the NGS enzymes is the key point to be addressed to overcome this technical issue. Moreover, we describe sequences obtained with MBC samples that harbored very good breadth of coverage and allowed to identify SARS-CoV-2 mutations via the pangolin pipeline. The substitution of the COVIDSeq reverse transcriptase by the SuperScript IV further improved the coverage of the SARS-CoV-2 sequence to 100% but negatively impacted the sequence accuracy. These results suggest that COVIDSeq reverse transcriptase is more sensitive to inhibitors but has a higher fidelity. Altogether, MBC is compatible with NGS sequencing but more investigations are required to enhance the consistency of the sequencing quality, owing to the heterogeneous composition of wastewater. Wastewater is a very complex matrix that can affect the integrity of viral particles and viral genomes to be sequenced. Future work should identify the integrity of SARS-CoV-2 genome in wastewater and how it can be affected by concentration methods. Since the MBC protocol is not destructive, it can be applicable to quantify enveloped or non-enveloped viruses, bacteria, and parasites, and to evaluate the viability of the pathogens in wastewater. Finally, concentration by ultrafiltration may be further scaled-up. However, upscaling our MBC process require the development larger ultrafiltration systems with increased filtration surfaces.

5. Conclusions

A new MBC method has been developed to concentrate, quantify, and sequence SARS-CoV-2 in large volumes of wastewater.

- MBC average of SARS-CoV-2 concentration efficiencies exceeded 50 folds compared to crude wastewater, reaching up to 97 folds depending on the samples for 500 mL of input.
- MBC efficiency allows quantification of SARS-CoV-2 for concentrations below 100 and up to 40 GC/L.
- MBC does not significantly increase PCR inhibition compared to crude wastewater and is highly reproducible.
- MBC process is efficient for enveloped-viruses such as SARS-CoV-2 and non-enveloped viruses such as FRNAPH GGII.
- MBC process is suitable for wastewater-based survey of viral emergences.
- MBC is compatible with next generation sequencing but can still be improved to limit the variability of sequence qualities due to the heterogeneity of wastewater composition.

The routine C method is easy to implement, cheaper than the commercial kits, brand limited, open-access and suitable for viral survey in wastewater.

Authors Contribution

G.E.S., performed experiments, analyzed data, generated figures, and contributed to the writing of the article.

O.R., M.V.G., and C.W. conceived the experiments, supervised the work, analyzed the data, generated, and/or improved figures, provided fundings and wrote the article.

L.D.J. and J.V.A. performed experiments and analyzed data.

Z.G. performed NGS sequencing and analysis, contributed to scientific discussions and improved the writing.

D.D., R.O., and C.D., contributed to NGS sequencing and analysis.

N.C. processed data and generated figures.

V.M. and J-F.D., contributed to the scientific discussions, improved the writing, and contributed to provide fundings.

M. B., T.L., C.S., D.T., O.H. and C.V.L. contributed to scientific discussions and improved the writing.

S.R. and D.R. managed the scientific project and the pre-analytical stage including the sampling and logistic in French Guiana.

OBEPINE consortium supports French WWBE projects, organizes the logistic of WW sampling, contributes to global scientific orientations and contributes to provide fundings.

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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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120959.

References

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., Verhagen, R., Smith, W.J.M., Zaugg, J., Dierens, L., Hugenholtz, P., Thomas, K.V., Mueller, J.F., 2020a. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. Sci. Total Environ. 728, 138764 https://doi.org/10.1016/j.scitotenv.2020.138764.

Ahmed, W., Bertsch, P.M., Bivins, A., Bibby, K., Farkas, K., Gathercole, A., Haramoto, E., Gyawali, P., Korajkic, A., McMinn, B.R., Mueller, J.F., Simpson, S.L., Smith, W.J.M., Symonds, E.M., Thomas, K.V., Verhagen, R., Kitajima, M., 2020b. Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. Sci. Total Environ. 739, 139960 https://doi.org/10.1016/j.scitotenv.2020.139960.

Alamin, M., Tsuji, S., Hata, A., Hara-Yamamura, H., Honda, R., 2022. Selection of surrogate viruses for process control in detection of SARS-CoV-2 in wastewater. Sci. Total Environ. 823, 153737 https://doi.org/10.1016/j.scitotenv.2022.153737.

Bertrand, I., Challant, J., Jeulin, H., Hartard, C., Mathieu, L., Lopez, S., , Scientific Interest Group Obépine, Schvoerer, E., Courtois, S., Gantzer, C., 2021. Epidemiological surveillance of SARS-CoV-2 by genome quantification in wastewater applied to a city in the northeast of France: comparison of ultrafiltration- and protein precipitation-based methods. Int. J. Hyg. Environ. Health 233, 113692. https://doi.org/10.1016/j.ijheh.2021.113692.

Bioanalytical Method Validation. Guidance for Industry, 2018. Prepared by the biopharmaceutics coordinating committee of the US Dept. of health and human services food and drug administration/center for drug evaluation (CDER) in cooperation with the center for veterinary medicine (CVM).

Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K., Bleicker, T., Brünink, S., Schneider, J., Schmidt, M.L., Mulders, D.G., Haagmans, B.L., van der Veer, B., van den Brink, S., Wijsman, L., Goderski, G., Romette, J.L., Ellis, J., Zambon, M., Peiris, M., Goossens, H., Reusken, C., Koopmans, M.P., Drosten, C., 2020. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 25, 2000045 https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045.

Euliano, E.M., Hardcastle, A.N., Victoriano, C.M., Gabella, W.E., Haselton, F.R., Adams, N.M., 2019. Multiplexed adaptive RT-PCR based on L-DNA hybridization monitoring for the detection of Zika, Dengue, and Chikungunya RNA. Sci. Rep. 9, 11372. https://doi.org/10.1038/s41598-019-47862-6.

Gerber, Z., Daviaud, C., Delafoy, D., Sandron, F., Alidjinou, E.K., Mercier, J., Gerber, S., Meyer, V., Boland, A., Bocket, L., Olaso, R., Deleuze, J.F., 2022. A comparison of high-throughput SARS-CoV-2 sequencing methods from nasopharyngeal samples. Sci. Rep. 12, 12561. https://doi.org/10.1038/s41598-022-16549-w.

Gonzalez, R., Curtis, K., Bivins, A., Bibby, K., Weir, M.H., Yetka, K., Thompson, H., Keeling, D., Mitchell, J., Gonzalez, D., 2020. COVID-19 surveillance in Southeastern Virginia using wastewater-based epidemiology. Water Res. 186, 116296 https://doi.org/10.1016/j.watres.2020.116296.

Gyawali, P., Devane, M., Scholes, P., Hewitt, J., 2021. Application of crAssphage, F-RNA phage and pepper mild mottle virus as indicators of human faecal and norovirus contamination in shellfish. Sci. Total Environ. 783, 146848 https://doi.org/10.1016/j.scitotenv.2021.146848.

Haramoto, E., Kitajima, M., Katayama, H., Ito, T., Ohgaki, S., 2009. Development of virus concentration methods for detection of koi herpesvirus in water. J. Fish Dis. 32, 297–300. https://doi.org/10.1111/j.1365-2761.2008.00977.x.

Holm, R.H., Nagarkar, M., Yeager, R.A., Talley, D., Chaney, A.C., Rai, J.P., Mukherjee, A., Rai, S.N., Bhatnagar, A., Smith, T., 2022. Surveillance of RNase P, PMMoV, and CrAssphage in wastewater as indicators of human fecal concentration across urban sewer neighborhoods, Kentucky. FEMS Microbes 3, 1–12. https://doi.org/10.1093/femsmc/xtac003.

Hougs, L., Gatto, F., Goerlich, O., Grohmann, L., Lieske, K., Mazzara, M., Narendja, F., Ovesna, J., Papazova, N., Scholtens, I.M.J., Zel, J., 2019. Verification of analytical methods for gmo testing when implementing interlaboratory validated methods. Mahgoub, S.E.O., Nollet, L.M.L. Testing and Analysis of GMO-Containing Foods and Feed. CRC Press, Boca Raton, Florida, pp. 245–266. https://doi.org/10.1201/9781315178592-8. CRC Press, [2019].

Kabdaşlı, I., Tünay, O., 2021. Concentration techniques tailored for the detection of SARS-CoV-2 genetic material in domestic wastewater and treatment plant sludge: a review. J. Environ. Chem. Eng. 9, 106296 https://doi.org/10.1016/j.jece.2021.106296.

Katayama, H., Haramoto, E., Oguma, K., Yamashita, H., Tajima, A., Nakajima, H., Ohgaki, S., 2008. One-year monthly quantitative survey of noroviruses, enteroviruses, and adenoviruses in wastewater collected from six plants in Japan. Water Res. 42, 1441–1448. https://doi.org/10.1016/j.watres.2007.10.029.

Kaya, D., Niemeier, D., Ahmed, W., Kjellerup, B.V., 2022. Evaluation of multiple analytical methods for SARS-CoV-2 surveillance in wastewater samples. Sci. Total Environ. 808, 152033 https://doi.org/10.1016/j.scitotenv.2021.152033.

Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E., Rose, J.B., 2020. SARS-CoV-2 in wastewater: State of the knowledge and research needs. Sci. Total Environ. 739, 139076 https://doi.org/10.1016/j.scitotenv.2020.139076.

La Rosa, G., Mancini, P., Bonanno Ferraro, G., Veneri, C., Iaconelli, M., Bonadonna, L., Lucentini, L., Suffredini, E., 2021. SARS-CoV-2 has been circulating in northern Italy since December 2019: evidence from environmental monitoring. Sci. Total Environ. 750, 141711 https://doi.org/10.1016/j.scitotenv.2020.141711.

Lago, P.M., Gary, H.E., Pérez, L.S., Cáceres, V., Olivera, J.B., Puentes, R.P., Corredor, M. B., Jímenez, P., Pallansch, M.A., Cruz, R.G., 2003. Poliovirus detection in wastewater and stools following an immunization campaign in Havana, Cuba. Int. J. Epidemiol. 32, 772–777. https://doi.org/10.1093/ije/dyg185.

LaTurner, Z.W., Zong, D.M., Kalvapalle, P., Gamas, K.R., Terwilliger, A., Crosby, T., Ali, P., Avadhanula, V., Santos, H.H., Weesner, K., Hopkins, L., Piedra, P.A., Maresso, A.W., Stadler, L.B., 2021. Evaluating recovery, cost, and throughput of different concentration methods for SARS-CoV-2 wastewater-based epidemiology. Water Res. 197, 117043 https://doi.org/10.1016/j.watres.2021.117043.

Maréchal, V., Maday, Y., Wallet, C., Cluzel, N., Borde, C., 2023. Wastewater-based epidemiology: Retrospective, current status, and future prospects. Anaesth. Crit. Care Pain Med. 42, 101251 https://doi.org/10.1016/j.accpm.2023.101251.

McCall, C., Wu, H., Miyani, B., Xagoraraki, I., 2020. Identification of multiple potential viral diseases in a large urban center using wastewater surveillance. Water Res. 184, 116160 https://doi.org/10.1016/j.watres.2020.116160.

McMinn, B.R., Korajkic, A., Kelleher, J., Herrmann, M.P., Pemberton, A.C., Ahmed, W., Villegas, E.N., Oshima, K., 2021. Development of a large volume concentration method for recovery of coronavirus from wastewater. Sci. Total Environ. 774, 145727 https://doi.org/10.1016/j.scitotenv.2021.145727.

Medema, G., Been, F., Heijnen, L., Petterson, S., 2020a. Implementation of environmental surveillance for SARS-CoV-2 virus to support public health decisions: Opportunities and challenges. Curr. Opin. Environ. Sci. Health 17, 49–71. https://doi.org/10.1016/j.coesh.2020.09.006.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020b. Presence of SARS-coronavirus-2 in sewage. 10.1101/2020.03.29.20045880.

Monteiro, S., Rente, D., Cunha, M.V., Marques, T.A., Cardoso, E., Álvaro, P., Vilaça, J., Ribeiro, J., Silva, M., Coelho, N., Brôco, N., Carvalho, M., Santos, R., 2022. Recovery of SARS-CoV-2 from large volumes of raw wastewater is enhanced with the inuvai R180 system. J. Environ. Manag. 304, 114296 https://doi.org/10.1016/j.jenvman.2021.114296.

O'Toole, Á., Scher, E., Underwood, A., Jackson, B., Hill, V., McCrone, J.T., Colquhoun, R., Ruis, C., Abu-Dahab, K., Taylor, B., Yeats, C., du Plessis, L., Maloney, D., Medd, N., Attwood, S.W., Aanensen, D.M., Holmes, E.C., Pybus, O.G., Rambaut, A., 2021. Assignment of epidemiological lineages in an emerging pandemic using the pangolin tool. Virus Evol. 7, veab064. https://doi.org/10.1093/ve/veab064.

Peccia, J., Zulli, A., Brackney, D.E., Grubaugh, N.D., Kaplan, E.H., Casanovas-Massana, A., Ko, A.I., Malik, A.A., Wang, D., Wang, M., Warren, J.L., Weinberger, D.M., Omer, S.B., 2020. SARS-CoV-2 RNA concentrations in primary municipal sewage sludge as a leading indicator of COVID-19 outbreak dynamics. 10.1101/2020.05.19.20105999.

Sherchan, S.P., Shahin, S., Ward, L.M., Tandukar, S., Aw, T.G., Schmitz, B., Ahmed, W., Kitajima, M., 2020. First detection of SARS-CoV-2 RNA in wastewater in North America: a study in Louisiana, USA. Sci. Total Environ. 743, 140621 https://doi.org/10.1016/j.scitotenv.2020.140621.

SPF, 2023 Chikungunya, dengue, virus West Nile et zika en Nouvelle-Aquitaine. Point au 26 septembre 2023. [WWW Document]. URL https://www.santepubliquefrance.fr/regions/nouvelle-aquitaine/documents/bulletin-regional/2023/chikungunya-dengue-virus-west-nile-et-zika-en-nouvelle-aquitaine.point-au-26-septembre-2023 (accessed 10.11.23).

Wolf, S., Hewitt, J., Greening, G.E., 2010. Viral multiplex quantitative PCR assays for tracking sources of fecal contamination. Appl. Environ. Microbiol. 76, 1388–1394. https://doi.org/10.1128/AEM.02249-09.

Wurtzer, S., Marechal, V., Mouchel, J.M., Maday, Y., Teyssou, R., Richard, E., Almayrac, J.L., Moulin, L., 2020. Evaluation of lockdown effect on SARS-CoV-2 dynamics through viral genome quantification in waste water, Greater Paris, France, 5 March to 23 April 2020. Euro Surveill. 25, 2000776 https://doi.org/10.2807/1560-7917.ES.2020.25.50.2000776.

Wurtzer, S., Waldman, P., Ferrier-Rembert, A., Frenois-Veyrat, G., Mouchel, J.M., Boni, M., Maday, Y., , OBEPINE consortium, Marechal, V., Moulin, L., 2021. Several forms of SARS-CoV-2 RNA can be detected in wastewaters: Implication for

wastewater-based epidemiology and risk assessment. Water Res. 198, 117183 https://doi.org/10.1016/j.watres.2021.117183.
Xiao, F., Sun, J., Xu, Y., Li, F., Huang, X., Li, H., Zhao, J., Huang, J., Zhao, J., 2020. Infectious SARS-CoV-2 in feces of patient with severe COVID-19. Emerg. Infect. Dis. 26, 1920–1922. https://doi.org/10.3201/eid2608.200681.
Zheng, X., Wang, M., Deng, Y., Xu, X., Lin, D., Zhang, Y., Li, S., Ding, J., Shi, X., Yau, C.I., Poon, L.L.M., Zhang, T., 2023. A rapid, high-throughput, and sensitive PEG-precipitation method for SARS-CoV-2 wastewater surveillance. Water Res. 230, 119560 https://doi.org/10.1016/j.watres.2022.119560.