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Evaluation of two rapid ultrafiltration-based methods for SARS-CoV-2 concentration from wastewater

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Abstract:	Quantitative measurements of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in raw wastewater have been implemented worldwide since the beginning of the pandemic. Recent efforts are being made to evaluate different viral concentration methodologies to overcome supplier shortages during lockdowns. A set of 22-wastewater samples seeded with murine hepatitis virus (MHV), a member of the Coronaviridae family, and the bacteriophage MS2, were used to characterize and compare two ultrafiltration-based methods: a centrifugal ultrafiltration device (Centricon®Plus-70) and the automated concentrating pipette CP-Select™. Based on the recovery efficiencies, significant differences were observed for MHV, with Centricon® Plus-70 (24%) being the most efficient method. Nevertheless, concentrations of naturally occurring SARS-CoV-2, Human adenoviruses and JC polyomaviruses in these samples did not result in significant differences between methods suggesting that testing naturally occurring viruses may complement the evaluation of viral concentration methodologies. Based on the virus adsorption to solids and the necessity of a pre-centrifugation step to remove larger particles and avoid clogging when using ultrafiltration methods, we assessed the percentage of viruses not quantified after ultrafiltration. Around 23% of the detected SARS-CoV-2 would be discarded during the debris removal step. The CP-Select™ provided the highest concentration factor (up to 333x) and the lowest LoD (6.19 x103 GC/L) for MHV and proved to be fast, automatic, highly reproducible and suitable to work under BSL-2 measures.
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detection.

To date, the published SARS-CoV-2 surveillance studies have been conducted by applying mainly centrifugal ultrafiltration devices (Centricon and Amicon) [Medema et al., 2020, Ahmed et al., 2020], and other methods including polyethylene glycol (PEG) or aluminium hydroxide (Al(OH)3) flocculation-precipitation are also being used [Randazzo et al., 2020, Zhang et al 2020, LaRosa et al., 2020]. On the first revision of this manuscript, we already included a paragraph stating that: “Ultrafiltration is an interesting method since samples do not need preacidification….Centricon® devices 10, 30 and 100kDa as well as Amicon Ultra-15 have been successfully applied for virus concentration”. Thus, we selected the 30kDa filters because they were the most frequently used even in pre-published studies.
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Abstract

Quantitative measurements of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in raw wastewater have been implemented worldwide since the beginning of the pandemic. Recent efforts are being made to evaluate different viral concentration methodologies to overcome supplier shortages during lockdowns. A set of 22-wastewater samples seeded with murine hepatitis virus (MHV), a member of the *Coronaviridae* family, and the bacteriophage MS2, were used to characterize and compare two ultrafiltration-based methods: a centrifugal ultrafiltration device (Centricon® Plus-70) and the automated concentrating pipette CP-Select™. Based on the recovery efficiencies, significant differences were observed for MHV, with Centricon® Plus-70 (24%) being the most efficient method. Nevertheless, concentrations of naturally occurring SARS-CoV-2, Human adenoviruses and JC polyomaviruses in these samples did not result in significant differences between methods suggesting that testing naturally occurring viruses may complement the evaluation of viral concentration methodologies. Based on the virus adsorption to solids and the necessity of a pre-centrifugation step to remove larger particles and avoid clogging when using ultrafiltration methods, we assessed the percentage of viruses not quantified after ultrafiltration. Around 23% of the detected SARS-CoV-2 would be discarded during the debris removal step. The CP-Select™ provided the highest concentration factor (up to 333x) and the lowest LoD (6.19 $x10^3$ GC/L) for MHV and proved to be fast, automatic, highly reproducible and suitable to work under BSL-2 measures.

Reviewer #1: Dear Editor,

I believe that the content of the manuscript does not comply with the aim of this highly impact journal "....for publication of novel, hypothesis-driven and high-impact research on..."In addition, the authors did not reply to all my comments, while the given answers are still not well addressed, i.e. is seems that the rationale behind this work is the shortage of a specific product in the market (1st paragraph of response), which may be true, however there are many other companies that provide filters with the same specs. In addition, the authors did not comment on the following: "There are some studies revealing the role of not only the concentration of suspended solids, but also the content of organic matter, nutrients, etc.; factors that should also be considered when dealing with wastewater samples. In addition, a significant constrain when implementing such analyses that may influence the recovery rates is the presence of inhibitors and this is not discussed in the manuscript, although the authors tested wastewater samples from different plants and in some cases recovery rates presented high variability. The presented results are in line with already published data and new contributions are limited to the CP-Select commercial concentration method. "Conclusions" section is absent. All in all, the approach is rather poor, the methodology not well justified, the results inadequately presented, and the discussion is very weak...." I regret to say that to my opinion the scientific quality of the manuscript does not fit with the scientific value of the papers published in this journal.

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Reviewer #3: On the whole I'm satisfied by how Authors managed the first round of review and upgraded their paper.

Nevertheless, I still share the same concern of Rev.1 about the (unsupported) choice of different molecular weight cut offs of tested devices. However, I'd say that the manuscript could be accepted in its current form, after a short paragraph is added in the Discussion, plainly stating and debating this methodological important limitation and maybe expliciting the possibility/need of further comparison among devices with the same MWCO (independently from their stock availability during the ongoing pandemic times).

As answered above, the manuscript main aim is not to compare methods but to provide an alternative ultrafiltration-based method to ultrafiltration devices more commonly used for SARS-CoV-2 concentration. The CP-Select ™ uses a new elution concept based on the use of pressurized eluent. Thus, our aim hasn't been to compare two different ultrafiltration systems because of their different pore sizes but two different ultrafiltration systems reported to be useful for SARS-CoV-2 detection that differ in the pore size, the concentration factor and the elution system among other features. Moreover, we choose this alternative since it is a rapid method that can be perform into a BL2 cabinet and could be performed in the point of use if needed. We have proved that the method provides equivalent results to the ones obtained by using Centricon devices. Moreover, we believe the method could be easily used in routine testing.

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Keywords: SARS-CoV-2, wastewater, viral concentration method, ultrafiltration, viral recovery

1. Introduction

There is increasing evidence that untreated wastewater is a promising unbiased indicator of the presence of SARS-CoV-2 virus in the population as it has been reported by different research groups as a possible way to monitor trends and the approximate overall prevalence of COVID-19 in the population (Kitajima et al., 2020; Medema et al., 2020a)

Given the coronavirus pandemic impacts, the method to detect SARS-CoV-2 RNA in wastewater had, by necessity, to be developed and implemented at warp-speed. One of the major challenges in SARS-CoV-2 research in wastewater has been the lack of standardized protocols for its detection. The way the virus is concentrated seems to be crucial in order to avoid false negative results or inaccurate reported concentrations.

On the lack of much data regarding coronavirus recovery efficiency when using common methods for viral concentration, we should rely on what it is known for other enveloped viruses considering that every single virus will have a different behaviour during viral concentration. Alone or combined, electropositive and electronegative filtration, centrifugal ultrafiltration, organic flocculation and PEG/$Al(OH)_3$ precipitation methods have been used in different studies targeting enveloped viruses' in environmental waters as recently reviewed (Rusiñol et al., 2020).

Preliminary data obtained by our research group in a study evaluating different concentration methods for the detection of SARS-CoV-2 in wastewater showed no significant differences between skimmed milk organic flocculation and Centricon® Plus-70– and CP-Select™ ultrafiltration devices (Rusiñol et al., 2020). Centricon® Plus-70 ultrafilters have been described as a useful method for SARS-CoV-2 concentration from wastewater. Ultrafiltration is an interesting method since: i) samples do not need preacidification, ii) nor a long time of precipitation, which could not favour the stability

of enveloped viruses, and iii) their concentration relies mainly on their size. However, and due to COVID-19 pandemic, there has been a shortage of these ultrafiltration devices. For this reason, this study was focused on the evaluation of two ultrafiltration methods described as useful for SARS-CoV-2 concentration from wastewater. Centricon® Plus-70 30kDa devices and the Concentrator Pipette CP-Select™ from Innovaprep were tested to concentrate raw wastewater samples ~~in~~ artificially spiked with MS2 bacteriophage and Murine Hepatitis Coronavirus (MHV) ~~raw wastewater samples~~ and presenting also naturally occurring SARS-CoV-2, Human adenoviruses (HAdV) and JC polyomaviruses (JCPyV). Centricon® of different cut-off size (10, 30 and 100kDa) have been applied to concentrate SARS-CoV-2 (Medema et al., 2020a; Rusiñol et al., 2020). In this issue 30kDa were the filters of election, trying to favour viral retention while avoiding the retention of smaller molecules that could act as enzymatic inhibitors. Regarding filter tips to be coupled to CP-Select™, the smaller pore size tips available (150kDa) were used. The novelty of this method resides in the use of a pressurized eluent in the form of wet foam.

2. Material and methods

2.1.Viruses and cell lines

Bacteriophage MS2 (ATCC 23631), a model for non-enveloped RNA viruses and Murine Hepatitis Virus-A59 (MHV-A59), a model for enveloped betacoronaviruses (like SARS-CoV-2), were propagated using the following protocols. Bacteriophage MS2 was cultured in *Salmonella typhimurium* strain WG49 (NCTC 12484) following ISO 10705-1 indications. MHV-A59 and DBT murine cell line were kindly provided by Wigginton Group Research, Michigan University, Michigan. MHV were propagated by infecting confluent monolayers of DBT cells following previously described instructions

(Leibowitz et al., 2011). Viruses were clarified from the supernatant by centrifugation at 3,000xg for 15 min and the supernatants were kept at - 80ºC.

2.2.Sample collection

A total of 22 24-hours-composite raw wastewater samples (500 ml) were collected between March and September 2020 from 6 WWTPs, located in Catalonia (Spain) (Table 1). The selected WWTPs treat urban and industrial wastewater from approximately 20% of the Catalan population. Samples were either shipped to the laboratory under cool conditions or alternatively stored after collection at - 20ºC.

Additionally, to determine the relation between the viral recovery and wastewater physicochemical characteristics, the turbidity was measured using a turbidimeter HI98703 (Hanna Instruments Inc.), the pH was measured using a pHímeter 902/4 (Nahita Inc.) and the ~~D~~BOD$_5$ values were provided by WWTP managers.

2.3.Viral concentration methods

An aliquot of 200ml of each wastewater sample was seeded with 10^7 GC/ml of MS2 and MHV (1:100, v/v). Samples were centrifuged at 4,750x*g* for 30 minutes in order to remove suspended solids that may interfere with the ultrafiltration. The resulting supernatant was divided into two aliquots of 100 ml and subjected to two different viral concentration methods:

1) Concentration Pipette CP-Select™ using Hollow Fiber Polysulfone PVP high-flow pipette ultrafilter tips (CPT) with a cut-off of 150 KDa (InnovaPrep) and 2) Centricon® Plus-70 centrifugal ultrafiltration (CeUF) devices, with a cut-off of 30 KDa (Millipore). CP-Select™ method began with filtration of 80 ml of supernatant through single-use CPT. Viral particles were eluted with 0.075% Tween 20/Tris using *Wet Foam Elution*™ canisters (Innovaprep) into a final volume of between 240 µl and 600 µl.

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The CeUF devices were pre-rinsed before use, following manufacturer instructions, and then 70 ml of supernatant w~~ere~~as centrifuged at 3,000x*g* for 30 minutes. Viruses were eluted inverting the CeUF device and centrifuged at 1,000x*g* for 3 minutes to obtain the final concentrate of approximately 280-900 µl.

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2.4.Nucleic acid extraction and q(RT)PCR quantification

Viral nucleic acids (NA) were extracted using the QIAmp Viral RNA Mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol in an automated QIAcube platform (Qiagen, Inc., Valencia, CA). The volume of the concentrates used for the extraction were 140 µL and the elution volumes were 60 - 80 µL. A negative control of the viral nucleic acid extraction was added per batch of samples.

Specific real-time qPCR and RT-qPCR assays previously described were used to quantify SARS-CoV-2 N1 and N2 (probes, primers and cycling conditions described in the CDC-006-00019 CDC/DDID/NCIRD/ Division of Viral Diseases protocol), MS2 bacteriophage (Pecson et al., 2009), MHV (Ahmed et al., 2020), HAdV (Hernroth et al., 2002) and JCPyV (Pal et al., 2006) by using TaqMan™ Environmental Master Mix 2.0 and RNA UltraSense™ One-Step RT-qPCR System (Invitrogen) for DNA and RNA viruses respectively. Quantification was performed in a StepOne plus Real-Time PCR System (Applied Biosystems, USA). Undiluted and 10-fold dilutions of the nucleic acid extracts were analysed in duplicate. All the qPCR and RT-qPCR assays included non-template controls to demonstrate that the mix did not produce fluorescence and bovine serum albumina (BSA) (1mg/ml), was added to RT-qPCR assays to reduce PCR inhibitors. The standards for viruses were prepared using synthetic gBlocks® Gene Fragments (IDT) and quantified with a Qubit® fluorometer (Thermo Fisher Scientific) except for SARS-CoV-2 standard which was constructed using the EURM-019 single stranded RNA fragments of SARS-CoV-2, provided by the European Commission Joint

Research Centre. For all the standards, ten-fold dilutions were prepared from 10^0 to 10^7 copies per reaction.

As for enzymatic inhibition we performed previous tests, when setting up qPCR for N1 and N2 assays for SARS-CoV-2 detection, by adding known amounts of target RNA into wastewater. Inhibition was reduced when including BSA to the qPCR master mix. Every tested sample was previously spiked with MS2 bacteriophages that were used as a process control as well as for controlling inhibition by analysing tenfold dilutions of every nucleic acid extraction.

2.5.LOD/LOQ determination

The limit of detection (LoD) of the whole method (including ultrafiltration, extraction and RT-qPCR detection) was calculated by running six replicate tenfold dilutions of target DNA/RNA suspensions around the detection end point (2.5, 5, 25 and 50 GC/reaction), for each analysed virus. The concentration that produced at least 95% positive replicates was assumed to be the LoD of the qPCR assay, which was transformed to LoD of the entire method using the sample volume tested in each of the methodologies. The limit of quantification (LoQ) was estimated using the procedure described by Foorotan and colleagues (Forootan et al., 2017).

2.6.Evaluation of viral recovery

Viral recovery percentage was calculated according to experimental values obtained by spiking samples with MS2 and MHV viral stocks, shaking for 10 min and using as input viral concentration the direct quantification of the viral stock added:

$$Virus\ recovery\ (\%) = \frac{Concentrate\ Titer\ (GC/ml)\ X\ Sample\ Volume\ (ml)}{Inoculum\ Titer\ (GC/ml)\ \ X\ \ (Sample\ Volume\ (ml)/100)} X100$$

To shed some light into the role that the matrix into which viral stock is embedded may play when calculating viral recoveries, four different quantification strategies were conducted: 1) direct quantification of the viral stocks; 2) quantification of raw wastewater spiked with known concentrations of the viral stocks; 3) same as 2 but after debris removal, and 4) quantification of the viral stocks in a concentrated wastewater sample. All these quantifications were assayed in triplicate.

2.7.Virus attachment to suspended solids

To investigate the percentage of coronaviruses which could remain attached to suspended material and not be properly quantified using ultrafiltration methods, viruses present in pellets obtained after centrifugation of 9 raw wastewater samples were further eluted in 3.5 ml of glycine buffer pH9.5 for 30 minutes and after the addition of 3.5 ml of 2xPBS centrifuged at 3000x*g* for 20 minutes. The resulting supernatant (6.5 - 7.5 ml) was filtered using Amicon® Ultra-15 Centrifuge Filters Ultracell® 50KDa (Merck Millipore) and eluted for further viral quantification. Simultaneously supernatants obtained after first centrifugation were further concentrated as described in section 2.3 using Centricon® Plus-70 devices.

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2.8.Tween-20 addition in the pre-concentration step before ultrafiltration with CP-Select™

CP-Select™ manufacturer recommends the addition of Tween-20 before ultrafiltration in order to increase viral recovery. The appropriateness of including this step to the CP-Select™ concentration protocol step was evaluated in 3 selected wastewater samples (100 ml). Prior to ultrafiltration, 5% Tween 20 (1:100, v/v) was added to raw wastewater and processed as described above.

2.9.Data visualization and statistical analysis

Data visualization, plotting and statistical test was done using R version 4.0.2 (R Core Team, 2020). For each virus, Wilcoxon signed rank tests for paired data were used to evaluate whether there were statistically significant differences between both ultrafiltration methods. To evaluate potential associations between viral recovery and raw wastewater turbidity we run Pearson's correlation coefficient tests.

3. Results

3.1.Comparison between CP-Select™ and Centricon® Plus-70 devices

The MS2 phage, a non-enveloped RNA virus frequently used as a process control in environmental studies (Coulliette et al., 2014; Ikner et al., 2011; Ye et al., 2016) and the MHV, an enveloped RNA surrogate for human coronavirus (Ahmed et al., 2020; Casanova et al., 2009; Ye et al., 2016), were seeded to calculate viral concentration methods recovery efficiencies.

Mean recovery values for MS2 and MHV in wastewater are represented in Figure 1. No statistically significant differences were observed between concentration methods regarding MS2 recovery (p-value = 0.75) but CeUF provided significant highest mean recoveries for MHV (p-value = 0.004). However, no statistical differences were observed between the two methods when naturally occurring viruses were quantified (Figure 2): SARS-CoV-2 (p-value of 0.27 and 0.73 for N1 and N2, respectively), HAdV, JCPyV (p-values > 0.05). CeUF provided higher mean recovery percentages for MHV whereas CP-Select™ provided higher recovery rates for MS2.

Table 2 summarizes equivalent sample volumes analyzed and the resulting concentration factors by using CP-Select™ or CeUF methods as well as the limits of detection and

quantification ($LoD_{95\%}$ and LoQ), calculated mean recoveries, standard deviations and coefficients of variation of the compared concentration methods based on MS2 and MHV quantifications. By using the concentrating pipette, a higher concentration factor was obtained, and a larger sample volume was analyzed in each RT-qPCR reaction.

After addition of Tween-20 into wastewater previously to concentration with CP-Select™, no statistical differences were observed when adding Tween-20 (p-value = 0.105), obtaining mean values of 50.7 and 20.9 GC/ml SARS-CoV-2 with and without Tween-20 addition respectively. However, the ultrafiltration time when adding Tween-20 was reduced.

3.2.Viral stock quantification

When evaluating if calculation of viral recoveries could be biased by the effect of the matrix in which viral stocks were embedded, no significant differences were observed when quantifying MS2 stocks directly or within different wastewater matrices (p-values >0.05) (Figure 3). On the other hand, MHV stock quantification showed a matrix effect suggesting that the way the viral stock, used for spiking recovery assays, is quantified may influence recovery values obtained. In this study, the recoveries represented in Figure 1 were calculated according to the direct quantification of the MHV used for spiking whereas MHV stock quantification in wastewater matrices would have showed higher viral recoveries (data not shown).

3.3.Virus attachment to suspended solids

Seeded MS2 and naturally occurring SARS-CoV-2 (N1 gene) were quantified from sample concentrates and in the generated pellets at the debris removal step (Figure 4). For MS2, similar fractions were measured from the pellet (49%) and the supernatant (51%). For the naturally occurring SARS-CoV-2 (N1 assay), those samples that could be

quantified showed more variability. In samples 1-9, most of the detectable SARS-CoV-2 fraction (mean values of 77%) was measured in the supernatant whereas the remaining 23% was detected in the pellets.

The turbidity of the wastewater samples was highly variable, ranging from 106 to 830 NTU (Nephelometric Turbidity Units). Weak correlations were observed between sample turbidity and viral quantifications obtained by using the CP-Select™ method (Pearson's correlation coefficients of 0.2 and 0.4 for MS2 and MHV, respectively) and inverse relation with sample turbidity was observed when using CeUF (Pearson's correlation coefficients of 0.2 and 0.1 for MS2 and MHV, respectively). No correlations between viral concentrations and pH and BOD_5 were observed (<0.3).

4. Discussion

In the actual pandemic scenario, viral concentration methods showing acceptable performance for both enveloped and non-enveloped viruses have received increased attention. As recently reviewed, a wide variety of strategies are being used to study viral presence in wastewater (Corpuz et al., 2020) but few of those concentration methodologies has been implemented for SARS-CoV-2 surveillance (Rusiñol et al., 2020). When comparing methodologies, ultrafiltration achieves higher MHV recoveries (25%) than PEG precipitation (5%), but the ultrafiltration devices are less used than flocculation/precipitation methods (Ye et al., 2016). This has been mainly caused by the shortage of supplies and the lack of readily material in many countries during lockdowns. Nevertheless, the one-step centrifugal ultrafiltration techniques enable the detection of viruses from relatively small sample volumes (70—80 mL).

Three ultrafiltration devices: the Centricon® Plus-70 (Medema et al., 2020b), the Amicon® Ultra-15 (Ahmed et al., 2020) and the new automatic Concentrating Pipette

(CP-Select™) from Innovaprep (Gonzalez et al., 2020; Rusiñol et al., 2020) have been successfully used to detect SARS-CoV-2 from wastewater. The first two devices have also been used to concentrate other human enteric viruses from water (Qiu et al., 2016; Sidhu et al., 2018). Viruses are retained based on size exclusion and backwashed from the ultrafilters. Both CeUF devices (Centricon® and Amicon®) contain an Ultracell® regenerated cellulose membrane that results in 19 cm^2 and 7.6 cm^2 respectively, whereas the CP-Select™ with Hollow Fiber Polysulfone ultrafiltration tips has a surface are of 98 cm^2, which is 5 to 13 times larger than those of the other CeUF devices. To our knowledge this is the first study that compares the performance of the CP-Select™ with Centricon® Plus-70 to concentrate SARS-CoV-2 and other viruses from wastewater samples. It should be noticed that this system includes a wet foam elution step which according to the manufacturer's improves viral elution from filter cells.

When applying ultrafiltration to wastewater, samples need to be pre-centrifuged to remove larger particles and avoid clogging. The resulting supernatant (70 - 80ml) is then passed in a single-step through the ultrafilter. Viruses have been reported to adsorb to the solid fraction of wastewater (Ye et al., 2016). According to our results, 23% of total detected SARS-CoV-2 would be discarded during the debris removal step while higher percentage of the detectable MS2 (49%) would be retained in the pellet. Ye et al. (2016) reported MHV to adsorb to the solid fraction of wastewater samples in higher percentages (26%) than MS2 (6%) while Ahmed et *al.*, reported similar loss for seeded MHV (30%) at the pre-filtration step (Ahmed et al., 2020). According to our results and considering the need of easy and fast method for SARS-CoV-2 detection in wastewater as an early warning tool, a straightforward and routinely adopted method shouldn't consider including viruses attached to the debris. This would ~~suppose~~imply e an extra elution step, from the debris, and addition to the wastewater sample~~, a procedure which has not a 100%~~

~~efficiency~~, which would suppose an addition of only a percentage of viruses attached to solid material. Thus, in our opinion, this step is not worth doing for routine testing and only when very high sensitivities and accurate quantifications are needed. Regarding the two ultrafiltration methods evaluated in this study, significant differences were only observed for MHV for which CeUF devices performed better than CP-Select™. In contrast, for naturally occurring SARS-CoV-2 both methods provided similar results showing that, as expected, each single virus behave differently under the same concentration procedure. Despite MHV is also a member of the Genus *Betacoronavirus* (as SARS-CoV-2), it did not show equivalent recovery rates to CeUF. Interestingly, however, the concentration of naturally occurring SARS-CoV-2 from wastewater using both concentration methods resulted in equivalent outcomes. This suggest that the best way to compare concentration methods for SARS-CoV-2 could be testing real environmental samples since, as observed for other viruses and other concentration methods, each virus has a particular behaviour for each of the methodologies applied. The way the MHV stock was quantified seemed to affect the recovery value obtained thus pointing at a clear effect of the matrix into which the viral stock is suspended. This could be probably due to different RNA protection/degradation phenomena or to the presence/absence of enzymatic inhibition in the different matrices assayed. This is another reason to consider when evaluating viral concentration methods and another argument in favour of using naturally occurring virus to complement concentration methods comparison studies, although this strategy does not allow the estimation of recovery rates.

Overall, CeUF devices were confirmed as an efficient ultrafiltration procedure for SARS-CoV-2 as it has been previously reported by others (Ahmed et al., 2020; Medema et al., 2020b). Moreover, CP-Select™ with Hollow Fiber Polysulfone tips showed to be useful

for SARS-CoV-2 concentration from wastewater as well as for the concentration of other wastewater occurring viruses independently of the turbidity of the samples. It is worth mentioning that equipment fits into a BSL-2 cabinet which makes this procedure strongly recommended for viruses requiring biosafety containment. In turn, CeUF devices should be used in a superspeed centrifuge that is difficult to fit into BSL-2 facility especially in routine laboratories that require extreme security measures to avoid spill overs.

Also, CP-Select™ provides with good concentration factor and equivalent LoD, LoQ and variance than CeUF devices. The use of Tween-20, as it has been recommended by manufacturers, has not proven to increase SARS-CoV-2 recovery although it has been observed it may help to filtrate samples reducing the time required for ultrafiltration.

CP-Select™ is a handy equipment that can be applied without previous debris elimination or by only using syringe filters or vacuum filtration devices. This device allows concentration at the point-of-use by simply connecting the CP-Select™ equipment to a power supply. The number of methods available for SARS-CoV-2 concentration from wastewater is increasing, as well as data on their performance, which will be relevant for researchers and routine laboratories in order to make a good election on their SARS-CoV-2 testing strategies. Detection of other potential pandemic enveloped viruses, that could emerge soon, would require optimized and well characterized viral concentration methods.

Conclusions:

- Ultrafiltration devices (Centricon® and CP-Select™) performed equally for different naturally occurring viruses, including SARS-CoV-2, whereas for the spiked MHV, used as a model of enveloped viruses of the genus betacoronavirus, the CeUF achieved higher recoveries.
- The way the viral stock is quantified may influence recovery values calculations.

- Up to 23% of detected SARS-CoV-2 adsorb to the solid fraction and is not considered in the further detection by quantitative PCR.
- The CP-Select™ fits into a BSL-2 cabinet enabling to work under biosafety containment

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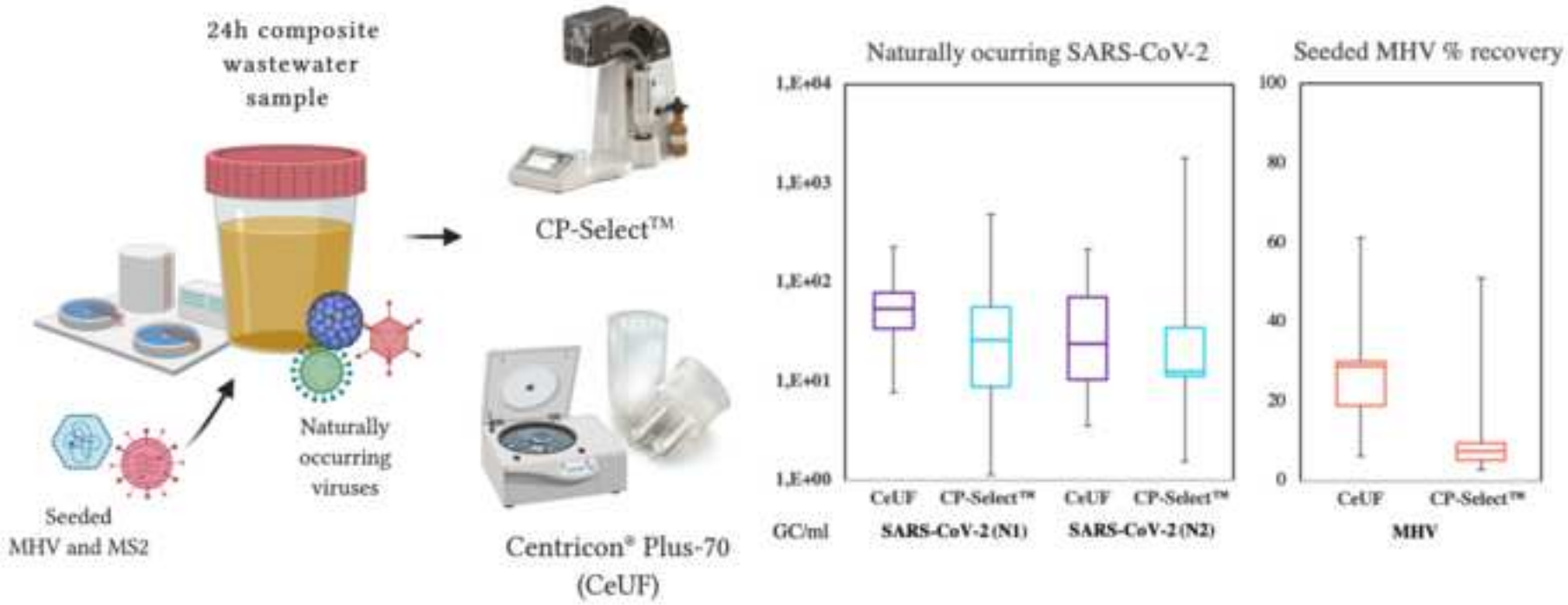
424

Graphical Abstract



Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

- Centricon® and CP-Select™ performed equally for naturally occurring SARS-CoV-2
- Higher MHV recoveries were calculated using centrifugal ultrafiltration devices
- Naturally occurring viruses complement concentration methods comparison
- A 23% of detected SARS-CoV-2 adsorb to the solid fraction of wastewater
- CP-Select™ fits into a BSL-2 cabinet enabling to work under biosafety containment

Evaluation of two rapid ultrafiltration-based methods for SARS-CoV-2 concentration from wastewater

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Abstract

Quantitative measurements of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in raw wastewater have been implemented worldwide since the beginning of the pandemic. Recent efforts are being made to evaluate different viral concentration methodologies to overcome supplier shortages during lockdowns. A set of 22-wastewater samples seeded with murine hepatitis virus (MHV), a member of the *Coronaviridae* family, and the bacteriophage MS2, were used to characterize and compare two ultrafiltration-based methods: a centrifugal ultrafiltration device (Centricon® Plus-70) and the automated concentrating pipette CP-Select™. Based on the recovery efficiencies, significant differences were observed for MHV, with Centricon® Plus-70 (24%) being the most efficient method. Nevertheless, concentrations of naturally occurring SARS-CoV-2, Human adenoviruses and JC polyomaviruses in these samples did not result in significant differences between methods suggesting that testing naturally occurring viruses may complement the evaluation of viral concentration methodologies. Based on the virus adsorption to solids and the necessity of a pre-centrifugation step to remove larger particles and avoid clogging when using ultrafiltration methods, we assessed the percentage of viruses not quantified after ultrafiltration. Around 23% of the detected SARS-CoV-2 would be discarded during the debris removal step. The CP-Select™ provided the highest concentration factor (up to 333x) and the lowest LoD (6.19 $x10^3$ GC/L) for MHV and proved to be fast, automatic, highly reproducible and suitable to work under BSL-2 measures.

Keywords: SARS-CoV-2, wastewater, viral concentration method, ultrafiltration, viral recovery

1. Introduction

There is increasing evidence that untreated wastewater is a promising unbiased indicator of the presence of SARS-CoV-2 virus in the population as it has been reported by different research groups as a possible way to monitor trends and the approximate overall prevalence of COVID-19 in the population (Kitajima et al., 2020; Medema et al., 2020a).

Given the coronavirus pandemic impacts, the method to detect SARS-CoV-2 RNA in wastewater had, by necessity, to be developed and implemented at warp-speed. One of the major challenges in SARS-CoV-2 research in wastewater has been the lack of standardized protocols for its detection. The way the virus is concentrated seems to be crucial in order to avoid false negative results or inaccurate reported concentrations.

On the lack of much data regarding coronavirus recovery efficiency when using common methods for viral concentration, we should rely on what it is known for other enveloped viruses considering that every single virus will have a different behaviour during viral concentration. Alone or combined, electropositive and electronegative filtration, centrifugal ultrafiltration, organic flocculation and PEG/$Al(OH)_3$ precipitation methods have been used in different studies targeting enveloped viruses' in environmental waters as recently reviewed (Rusiñol et al., 2020).

Preliminary data obtained by our research group in a study evaluating different concentration methods for the detection of SARS-CoV-2 in wastewater showed no significant differences between skimmed milk organic flocculation and Centricon® Plus-70 and CP-Select™ ultrafiltration devices (Rusiñol et al., 2020). Centricon® Plus-70 ultrafilters have been described as a useful method for SARS-CoV-2 concentration from wastewater. Ultrafiltration is an interesting method since: i) samples do not need preacidification, ii) nor a long time of precipitation, which could not favour the stability

of enveloped viruses, and iii) their concentration relies mainly on their size. However, and due to COVID-19 pandemic, there has been a shortage of these ultrafiltration devices. For this reason, this study was focused on the evaluation of two ultrafiltration methods described as useful for SARS-CoV-2 concentration from wastewater. Centricon® Plus-70 30kDa devices and the Concentrator Pipette CP-Select™ from Innovaprep were tested to concentrate raw wastewater samples artificially spiked with MS2 bacteriophage and Murine Hepatitis Coronavirus (MHV) and presenting also naturally occurring SARS-CoV-2, Human adenoviruses (HAdV) and JC polyomaviruses (JCPyV). Centricon® of different cut-off size (10, 30 and 100kDa) have been applied to concentrate SARS-CoV-2 (Medema et al., 2020a; Rusiñol et al., 2020). In this issue 30kDa were the filters of election, trying to favour viral retention while avoiding the retention of smaller molecules that could act as enzymatic inhibitors. Regarding filter tips to be coupled to CP-Select™, the smallest available pore size tips (150kDa) were used. The novelty of this method resides in the use of a pressurized eluent in the form of wet foam.

2. Material and methods

2.1.Viruses and cell lines

Bacteriophage MS2 (ATCC 23631), a model for non-enveloped RNA viruses and Murine Hepatitis Virus-A59 (MHV-A59), a model for enveloped betacoronaviruses (like SARS-CoV-2), were propagated using the following protocols. Bacteriophage MS2 was cultured in *Salmonella typhimurium* strain WG49 (NCTC 12484) following ISO 10705-1 indications. MHV-A59 and DBT murine cell line were kindly provided by Wigginton Group Research, Michigan University, Michigan. MHV were propagated by infecting confluent monolayers of DBT cells following previously described instructions

(Leibowitz et al., 2011). Viruses were clarified from the supernatant by centrifugation at 3,000x*g* for 15 min and the supernatants were kept at - 80ºC.

2.2.Sample collection

A total of 22 24-hours-composite raw wastewater samples (500 ml) were collected between March and September 2020 from 6 WWTPs, located in Catalonia (Spain) (Table 1). The selected WWTPs treat urban and industrial wastewater from approximately 20% of the Catalan population. Samples were either shipped to the laboratory under cool conditions or alternatively stored after collection at - 20ºC.

Additionally, to determine the relation between the viral recovery and wastewater physicochemical characteristics, the turbidity was measured using a turbidimeter HI98703 (Hanna Instruments Inc.), the pH was measured using a pHmeter 902/4 (Nahita Inc.) and the BOD_5 values were provided by WWTP managers.

2.3.Viral concentration methods

An aliquot of 200 ml of each wastewater sample was seeded with 10^7 GC/ml of MS2 and MHV (1:100, v/v). Samples were centrifuged at 4,750x*g* for 30 minutes in order to remove suspended solids that may interfere with the ultrafiltration. The resulting supernatant was divided into two aliquots of 100 ml and subjected to two different viral concentration methods:

1) Concentration Pipette CP-Select™ using Hollow Fiber Polysulfone PVP high-flow pipette ultrafilter tips (CPT) with a cut-off of 150 KDa (InnovaPrep) and 2) Centricon® Plus-70 centrifugal ultrafiltration (CeUF) devices, with a cut-off of 30 KDa (Millipore). CP-Select™ method began with filtration of 80 ml of supernatant through single-use CPT. Viral particles were eluted with 0.075% Tween-20/Tris using *Wet Foam Elution*™ cans (Innovaprep) into a final volume of between 240 µl and 600 µl.

116 The CeUF devices were pre-rinsed before use, following manufacturer instructions, and
117 then 70 ml of supernatant was centrifuged at 3,000x*g* for 30 minutes. Viruses were eluted
118 inverting the CeUF device and centrifuged at 1,000x*g* for 3 minutes to obtain the final
119 concentrate of approximately 280-900 µl.

120 *2.4.Nucleic acid extraction and q(RT)PCR quantification*

121 Viral nucleic acids (NA) were extracted using the QIAmp Viral RNA Mini kit (Qiagen,
122 Inc., Valencia, CA) according to the manufacturer's protocol in an automated QIAcube
123 platform (Qiagen, Inc., Valencia, CA). The volume of the concentrates used for the
124 extraction were 140 µL and the elution volumes were 60 - 80 µL. A negative control of
125 the viral nucleic acid extraction was added per batch of samples.

126 Specific real-time qPCR and RT-qPCR assays previously described were used to quantify
127 SARS-CoV-2 N1 and N2 (probes, primers and cycling conditions described in the CDC-
128 006-00019 CDC/DDID/NCIRD/ Division of Viral Diseases protocol), MS2
129 bacteriophage (Pecson et al., 2009), MHV (Ahmed et al., 2020), HAdV (Hernroth et al.,
130 2002) and JCPyV (Pal et al., 2006) by using TaqMan™ Environmental Master Mix 2.0
131 and RNA UltraSense™ One-Step RT-qPCR System (Invitrogen) for DNA and RNA
132 viruses respectively. Quantification was performed in a StepOne plus Real-Time PCR
133 System (Applied Biosystems, USA). Undiluted and 10-fold dilutions of the nucleic acid
134 extracts were analysed in duplicate. All the qPCR and RT-qPCR assays included non-
135 template controls to demonstrate that the mix did not produce fluorescence and bovine
136 serum albumina (BSA) (1mg/ml), was added to RT-qPCR assays to reduce PCR
137 inhibitors. The standards for viruses were prepared using synthetic gBlocks® Gene
138 Fragments (IDT) and quantified with a Qubit® fluorometer (Thermo Fisher Scientific)
139 except for SARS-CoV-2 standard which was constructed using the EURM-019 single
140 stranded RNA fragments of SARS-CoV-2, provided by the European Commission Joint

Research Centre. For all the standards, ten-fold dilutions were prepared from 10^0 to 10^7 copies per reaction.

As for enzymatic inhibition we performed previous tests, when setting up qPCR for N1 and N2 assays for SARS-CoV-2 detection, by adding known amounts of target RNA into wastewater. Inhibition was reduced when including BSA to the qPCR master mix. Every tested sample was previously spiked with MS2 bacteriophages that were used as a process control as well as for controlling inhibition by analysing tenfold dilutions of every nucleic acid extraction.

2.5.LOD/LOQ determination

The limit of detection (LoD) of the whole method (including ultrafiltration, extraction and RT-qPCR detection) was calculated by running six replicate tenfold dilutions of target DNA/RNA suspensions around the detection end point (2.5, 5, 25 and 50 GC/reaction), for each analysed virus. The concentration that produced at least 95% positive replicates was assumed to be the LoD of the qPCR assay, which was transformed to LoD of the entire method using the sample volume tested in each of the methodologies. The limit of quantification (LoQ) was estimated using the procedure described by Foorotan and colleagues (Forootan et al., 2017).

2.6.Evaluation of viral recovery

Viral recovery percentage was calculated according to experimental values obtained by spiking samples with MS2 and MHV viral stocks, shaking for 10 min and using as input viral concentration the direct quantification of the viral stock added:

$$Virus\ recovery\ (\%) = \frac{Concentrate\ Titer\ (GC/ml)\ X\ Sample\ Volume\ (ml)}{Inoculum\ Titer\ (GC/ml)\ \ X\ \ (Sample\ Volume\ (ml)/100)} X100$$

To shed some light into the role that the matrix into which viral stock is embedded may play when calculating viral recoveries, four different quantification strategies were conducted: 1) direct quantification of the viral stocks; 2) quantification of raw wastewater spiked with known concentrations of the viral stocks; 3) same as 2 but after debris removal, and 4) quantification of the viral stocks in a concentrated wastewater sample. All these quantifications were assayed in triplicate.

2.7.Virus attachment to suspended solids

To investigate the percentage of coronaviruses which could remain attached to suspended material and not be properly quantified using ultrafiltration methods, viruses present in pellets obtained after centrifugation of 9 raw wastewater samples were further eluted in 3.5 ml of glycine buffer pH9.5 for 30 minutes and after the addition of 3.5 ml of 2xPBS centrifuged at 3000x*g* for 20 minutes. The resulting supernatant (6.5 - 7.5 ml) was filtered using Amicon® Ultra-15 Centrifuge Filters Ultracell® 50KDa (Merck Millipore) and eluted for further viral quantification. Simultaneously supernatants obtained after first centrifugation were further concentrated as described in section 2.3 using Centricon® Plus-70 devices.

2.8.Tween-20 addition in the pre-concentration step before ultrafiltration with CP-Select™

CP-Select™ manufacturer recommends the addition of Tween-20 before ultrafiltration in order to increase viral recovery. The appropriateness of including this step to the CP-Select™ concentration protocol step was evaluated in 3 selected wastewater samples (100 ml). Prior to ultrafiltration, 5% Tween 20 (1:100, v/v) was added to raw wastewater and processed as described above.

2.9.Data visualization and statistical analysis

Data visualization, plotting and statistical test was done using R version 4.0.2 (R Core Team, 2020). For each virus, Wilcoxon signed rank tests for paired data were used to evaluate whether there were statistically significant differences between both ultrafiltration methods. To evaluate potential associations between viral recovery and raw wastewater turbidity we run Pearson's correlation coefficient tests.

3. Results

3.1.Comparison between CP-Select™ and Centricon® Plus-70 devices

The MS2 phage, a non-enveloped RNA virus frequently used as a process control in environmental studies (Coulliette et al., 2014; Ikner et al., 2011; Ye et al., 2016) and the MHV, an enveloped RNA surrogate for human coronavirus (Ahmed et al., 2020; Casanova et al., 2009; Ye et al., 2016), were seeded to calculate viral concentration methods recovery efficiencies.

Mean recovery values for MS2 and MHV in wastewater are represented in Figure 1. No statistically significant differences were observed between concentration methods regarding MS2 recovery (p-value = 0.75) but CeUF provided significant highest mean recoveries for MHV (p-value = 0.004). However, no statistical differences were observed between the two methods when naturally occurring viruses were quantified (Figure 2): SARS-CoV-2 (p-value of 0.27 and 0.73 for N1 and N2, respectively), HAdV, JCPyV (p-values > 0.05). CeUF provided higher mean recovery percentages for MHV whereas CP-Select™ provided higher recovery rates for MS2.

Table 2 summarizes equivalent sample volumes analyzed and the resulting concentration factors by using CP-Select™ or CeUF methods as well as the limits of detection and

quantification ($LoD_{95\%}$ and LoQ), calculated mean recoveries, standard deviations and coefficients of variation of the compared concentration methods based on MS2 and MHV quantifications. By using the concentrating pipette, a higher concentration factor was obtained, and a larger sample volume was analyzed in each RT-qPCR reaction.

After addition of Tween-20 into wastewater previously to concentration with CP-Select™, no statistical differences were observed when adding Tween-20 (*p*-value = 0.105), obtaining mean values of 50.7 and 20.9 GC/ml SARS-CoV-2 with and without Tween-20 addition respectively. However, the ultrafiltration time when adding Tween-20 was reduced.

3.2.Viral stock quantification

When evaluating if calculation of viral recoveries could be biased by the effect of the matrix in which viral stocks were embedded, no significant differences were observed when quantifying MS2 stocks directly or within different wastewater matrices (*p*-values >0.05) (Figure 3). On the other hand, MHV stock quantification showed a matrix effect suggesting that the way the viral stock, used for spiking recovery assays, is quantified may influence recovery values obtained. In this study, the recoveries represented in Figure 1 were calculated according to the direct quantification of the MHV used for spiking whereas MHV stock quantification in wastewater matrices would have showed higher viral recoveries (data not shown).

3.3.Virus attachment to suspended solids

Seeded MS2 and naturally occurring SARS-CoV-2 (N1 gene) were quantified from sample concentrates and in the generated pellets at the debris removal step (Figure 4). For MS2, similar fractions were measured from the pellet (49%) and the supernatant (51%). For the naturally occurring SARS-CoV-2 (N1 assay), those samples that could be

quantified showed more variability. In samples 1-9, most of the detectable SARS-CoV-2 fraction (mean values of 77%) was measured in the supernatant whereas the remaining 23% was detected in the pellets.

The turbidity of the wastewater samples was highly variable, ranging from 106 to 830 NTU (Nephelometric Turbidity Units). Weak correlations were observed between sample turbidity and viral quantifications obtained by using the CP-Select™ method (Pearson's correlation coefficients of 0.2 and 0.4 for MS2 and MHV, respectively) and inverse relation with sample turbidity was observed when using CeUF (Pearson's correlation coefficients of 0.2 and 0.1 for MS2 and MHV, respectively). No correlations between viral concentrations and pH and BOD_5 were observed (<0.3).

4. **Discussion**

In the actual pandemic scenario, viral concentration methods showing acceptable performance for both enveloped and non-enveloped viruses have received increased attention. As recently reviewed, a wide variety of strategies are being used to study viral presence in wastewater (Corpuz et al., 2020) but few of those concentration methodologies has been implemented for SARS-CoV-2 surveillance (Rusiñol et al., 2020). When comparing methodologies, ultrafiltration achieves higher MHV recoveries (25%) than PEG precipitation (5%), but the ultrafiltration devices are less used than flocculation/precipitation methods (Ye et al., 2016). This has been mainly caused by the shortage of supplies and the lack of readily material in many countries during lockdowns. Nevertheless, the one-step centrifugal ultrafiltration techniques enable the detection of viruses from relatively small sample volumes (70 – 80 mL).

Three ultrafiltration devices: the Centricon® Plus-70 (Medema et al., 2020b), the Amicon® Ultra-15 (Ahmed et al., 2020) and the new automatic Concentrating Pipette

(CP-Select™) from Innovaprep (Gonzalez et al., 2020; Rusiñol et al., 2020) have been successfully used to detect SARS-CoV-2 from wastewater. The first two devices have also been used to concentrate other human enteric viruses from water (Qiu et al., 2016; Sidhu et al., 2018). Viruses are retained based on size exclusion and backwashed from the ultrafilters. Both CeUF devices (Centricon® and Amicon®) contain an Ultracell® regenerated cellulose membrane that results in 19 cm^2 and 7.6 cm^2 respectively, whereas the CP-Select™ with Hollow Fiber Polysulfone ultrafiltration tips has a surface are of 98 cm^2, which is 5 to 13 times larger than those of the other CeUF devices. To our knowledge this is the first study that compares the performance of the CP-Select™ with Centricon® Plus-70 to concentrate SARS-CoV-2 and other viruses from wastewater samples. It should be noticed that this system includes a wet foam elution step which according to the manufacturer's improves viral elution from filter cells.

When applying ultrafiltration to wastewater, samples need to be pre-centrifuged to remove larger particles and avoid clogging. The resulting supernatant (70 - 80ml) is then passed in a single-step through the ultrafilter. Viruses have been reported to adsorb to the solid fraction of wastewater (Ye et al., 2016). According to our results, 23% of total detected SARS-CoV-2 would be discarded during the debris removal step while higher percentage of the detectable MS2 (49%) would be retained in the pellet. Ye et al. (2016) reported MHV to adsorb to the solid fraction of wastewater samples in higher percentages (26%) than MS2 (6%) while Ahmed et *al.*, reported similar loss for seeded MHV (30%) at the pre-filtration step (Ahmed et al., 2020). According to our results and considering the need of easy and fast method for SARS-CoV-2 detection in wastewater as an early warning tool, a straightforward and routinely adopted method shouldn't consider including viruses attached to the debris. This would imply an extra elution step, from the debris, and addition to the wastewater sample, which would suppose an addition of only

a percentage of viruses attached to solid material. Thus, in our opinion, this step is not worth doing for routine testing and only when very high sensitivities and accurate quantifications are needed. Regarding the two ultrafiltration methods evaluated in this study, significant differences were only observed for MHV for which CeUF devices performed better than CP-Select™. In contrast, for naturally occurring SARS-CoV-2 both methods provided similar results showing that, as expected, each single virus behave differently under the same concentration procedure. Despite MHV is also a member of the Genus *Betacoronavirus* (as SARS-CoV-2), it did not show equivalent recovery rates to CeUF. Interestingly, however, the concentration of naturally occurring SARS-CoV-2 from wastewater using both concentration methods resulted in equivalent outcomes. This suggest that the best way to compare concentration methods for SARS-CoV-2 could be testing real environmental samples since, as observed for other viruses and other concentration methods, each virus has a particular behaviour for each of the methodologies applied. The way the MHV stock was quantified seemed to affect the recovery value obtained thus pointing at a clear effect of the matrix into which the viral stock is suspended. This could be probably due to different RNA protection/degradation phenomena or to the presence/absence of enzymatic inhibition in the different matrices assayed. This is another reason to consider when evaluating viral concentration methods and another argument in favour of using naturally occurring virus to complement concentration methods comparison studies, although this strategy does not allow the estimation of recovery rates.

Overall, CeUF devices were confirmed as an efficient ultrafiltration procedure for SARS-CoV-2 as it has been previously reported by others (Ahmed et al., 2020; Medema et al., 2020b). Moreover, CP-Select™ with Hollow Fiber Polysulfone tips showed to be useful for SARS-CoV-2 concentration from wastewater as well as for the concentration of other

wastewater occurring viruses independently of the turbidity of the samples. It is worth mentioning that equipment fits into a BSL-2 cabinet which makes this procedure strongly recommended for viruses requiring biosafety containment. In turn, CeUF devices should be used in a superspeed centrifuge that is difficult to fit into BSL-2 facility especially in routine laboratories that require extreme security measures to avoid spill overs.

Also, CP-Select™ provides with good concentration factor and equivalent LoD, LoQ and variance than CeUF devices. The use of Tween-20, as it has been recommended by manufacturers, has not proven to increase SARS-CoV-2 recovery although it has been observed it may help to filtrate samples reducing the time required for ultrafiltration.

CP-Select™ is a handy equipment that can be applied without previous debris elimination or by only using syringe filters or vacuum filtration devices. This device allows concentration at the point-of-use by simply connecting the CP-Select™ equipment to a power supply. The number of methods available for SARS-CoV-2 concentration from wastewater is increasing, as well as data on their performance, which will be relevant for researchers and routine laboratories in order to make a good election on their SARS-CoV-2 testing strategies. Detection of other potential pandemic enveloped viruses, that could emerge soon, would require optimized and well characterized viral concentration methods.

Conclusions:

- Ultrafiltration devices (Centricon® and CP-Select™) performed equally for different naturally occurring viruses, including SARS-CoV-2, whereas for the spiked MHV, used as a model of enveloped viruses of the genus betacoronavirus, the CeUF achieved higher recoveries.
- The way the viral stock is quantified may influence recovery values calculations.
- Up to 23% of detected SARS-CoV-2 adsorb to the solid fraction and is not considered in the further detection by quantitative PCR.

- The CP-Select™ fits into a BSL-2 cabinet enabling to work under biosafety containment

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Table 1. Characteristics of the selected wastewater treatment plants (WWTP). Mean values and standard deviations. BOD_5: biological organic demand.

WWTP	Number of samples	Design capacity (Hab. Eq.)	Turbidity (NTU)	pH	BOD_5 (mgO_2/L)
1	10	2843750	816±17	7.39±0.13	364±72
2	2	451250	218±2.31	7.54±0.15	390±72
3	2	285666	113±8.14	8.17±0.21	69±30
4	3	196167	165±4.36	7.62±0.10	217±63
5	2	165450	106±1.15	7.55±0.20	316±126
6	3	99166	222±5.86	7.80±0.15	191±47

Table 2. Characterization of the concentration methods: volume of wastewater sample analyzed in each reaction, mean concentration factor, estimated 95% limit of detection ($LoD_{95\%}$) and limit of quantification (LoQ) and mean recovery values for each of the seeded viruses.

	CP-Select™	**CeUF**
Sample volume analyzed per reaction	1,56-2,92 ml	0,91-2,19 ml
Concentration factor	133-333x	77-250x
$LoD_{95\%}$ (CI)*	MS2: 5,14 $x10^3$ *(3,02 $x10^3$ -9,40 $x10^3$)* MHV: 6,19 $x10^3$ *(2,43 $x10^3$-1,58 $x10^4$)*	MS2: 5,67 $x10^3$ *(3,22 $x10^3$-1,03 $x10^4$)* MHV: 6,61 $x10^3$ *(2,59 $x10^3$-1,68 $x10^4$)*
LoQ*	MS2: 2,32 $x10^3$ MHV: 2,35 $x10^4$	MS2: 3,56 $x10^3$ MHV: 2,51 $x10^4$
Mean recovery ± SD (CV)	MS2: 27,72 ± 24,46% (0,65) MHV: 7,51 ± 6,14% (0,68)	MS2: 26,34 ± 22,71% (0,66) MHV: 24,07 ± 14,48% (0,58)

*$LoD_{95\%}$ and LoQ values are given in genome copies detected per liter of the original wastewater sample. CI: confidence interval; SD: Standard deviation; CV: coefficient of variation.

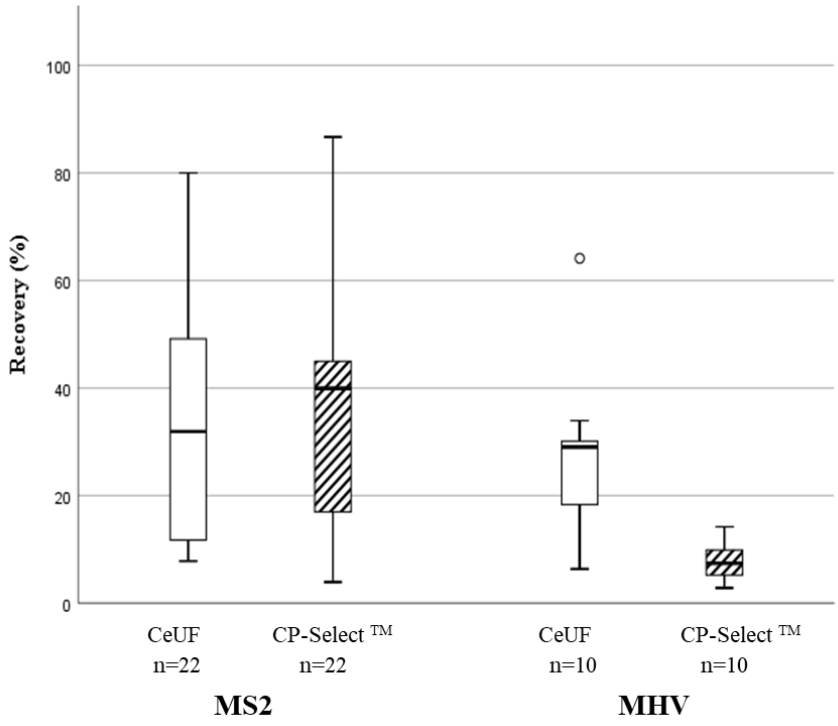


Figure 1. Barplots of the mean recovery values (%) of MS2 and MHV by using two different ultrafiltration methods: InnovaPrep concentrating pipette with single-use ultrafiltration tips 150KDa (CP Select™) and centrifugal ultrafiltration with Centricon® Plus-70 30KDa (CeUF).

Figure 2 Click here to access/download;Figure;Figure_2_v2.docx

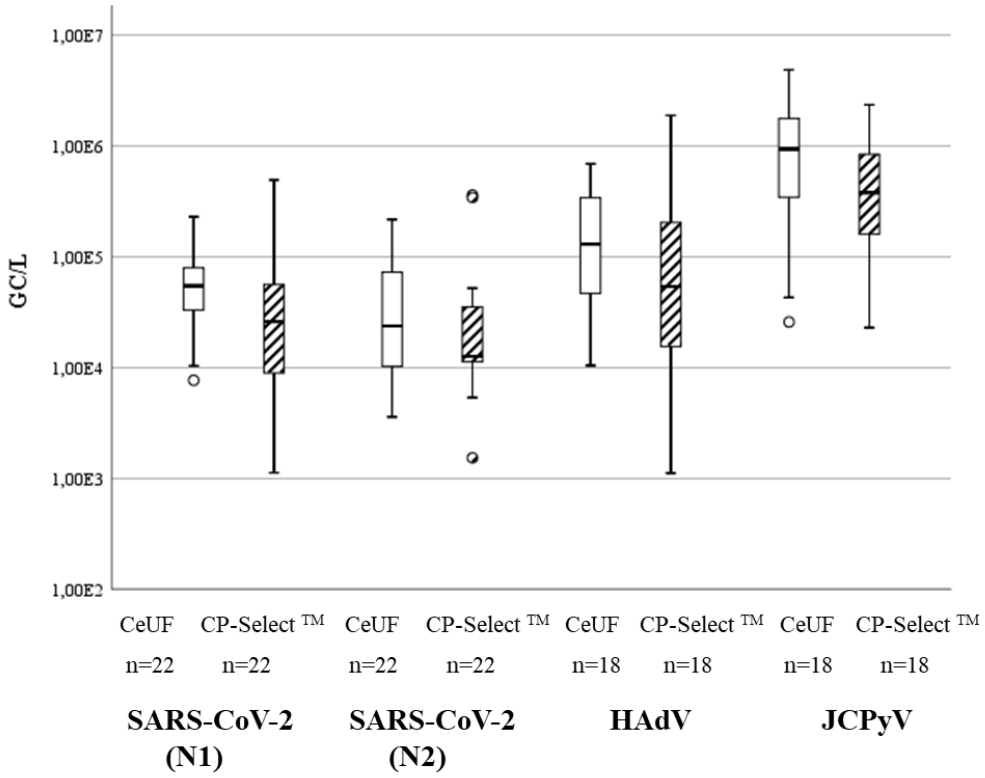
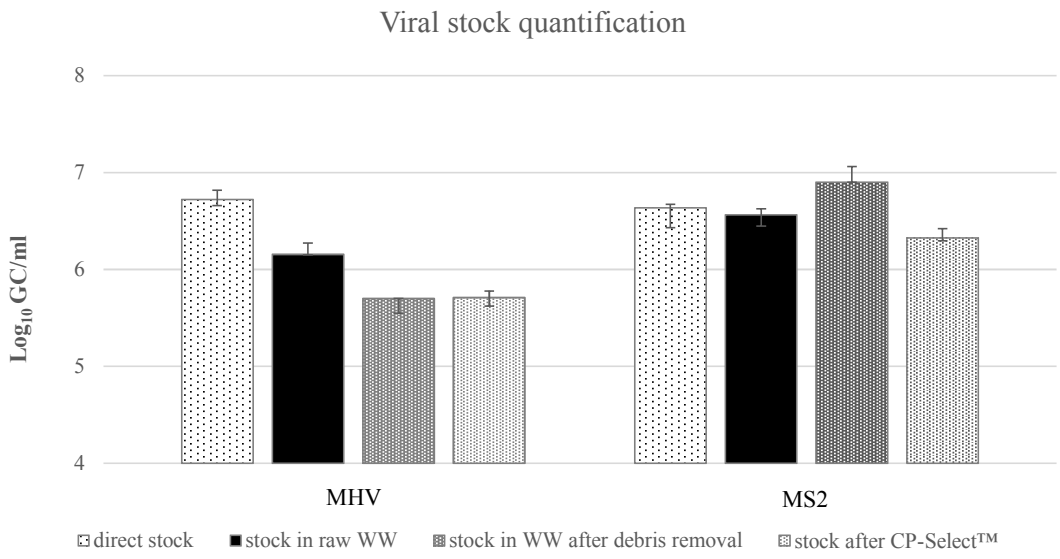


Figure 2. Barplots of the concentrations of naturally occurring SARS-CoV-2 (N1 and N2 assays), HAdV and JCPyV (expressed in genome copies per liter) by using two different ultrafiltration methods: InnovaPrep concentrating pipette with single- use ultrafiltration tips 150KDa (CP Select™) and centrifugal ultrafiltration with Centricon® Plus-70 30KDa (CeUF).

Figure 3. Mean concentration values of the viral stocks, using 4 different quantification strategies.



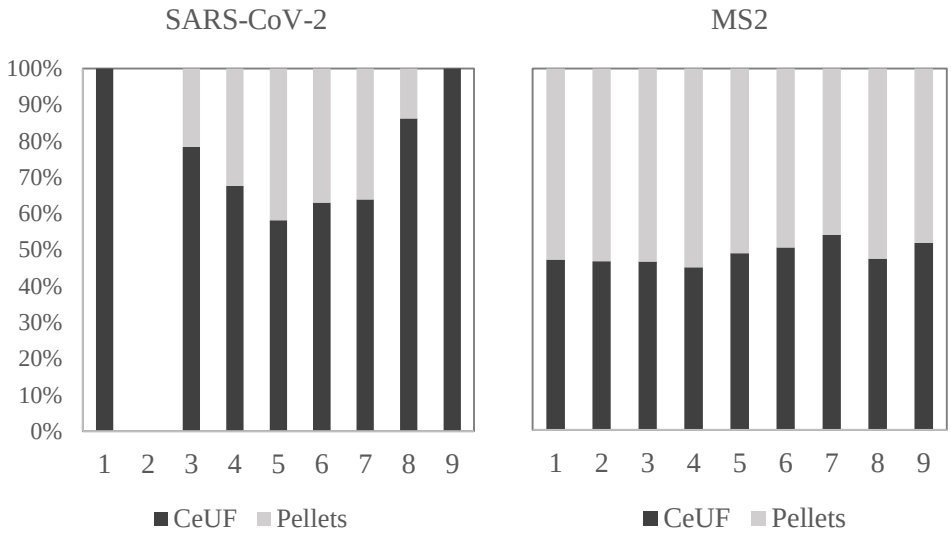


Figure 4. Detection of naturally occurring SARS-CoV-2 (N1 assay) and seeded MS2 in the pellet or supernatant fractions of nine wastewater samples after 4,700x*g* 30 minutes centrifugation expressed as the percentage of total viruses detected.

CRediT authorship contribution statement

Eva Forés: Sampling, Methodology, Formal analysis, Writing-Original draft preparation
Bofill-Mas S: Methodology, Formal analysis, Writing- Original draft preparation, Conceptualization, Writing- Reviewing and Editing
Itarte M: Methodology, Formal analysis.
Martínez-Puchol S: Methodology.
Hundesa A: Methodology.
Calvo M: Data statistical analysis
Borrego C.M.: Sampling, Reviewing and Editing
Corominas LL: Sampling, Reviewing and Editing
Girones R: Reviewing and Editing
Rusiñol M: Methodology, Formal analysis, Conceptualization, Writing-Original draft preparation, Supervision

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: