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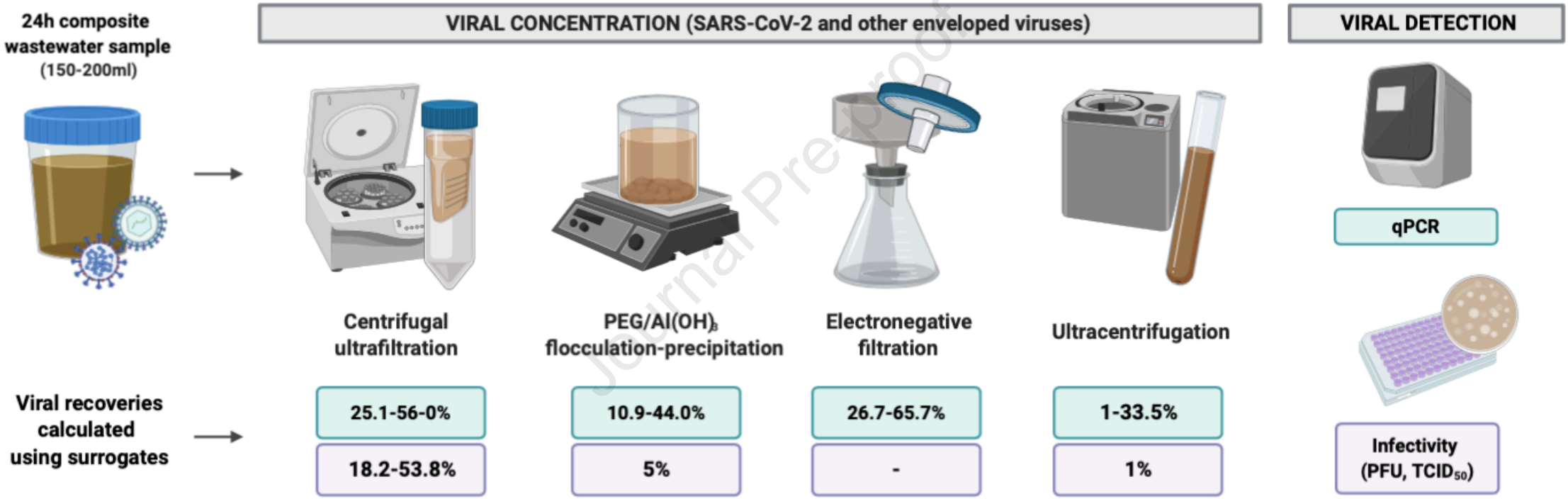

Concentration methods for the quantification of coronavirus and other potentially pandemic enveloped virus from wastewater

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Abstract

Since the novel SARS-CoV-2 was detected in faeces, environmental researchers have been using centrifugal ultrafiltration, polyethylene glycol precipitation and aluminium hydroxide flocculation to describe its presence in wastewater samples. High recoveries (up to 65%) are described with electronegative filtration when using surrogate viruses, but few literature reports recovery efficiencies using accurate quantification of enveloped viruses. Considering that every single virus will have a different behaviour during viral concentration, it is recommended to use an enveloped virus, and if possible, a betacoronaviruses as murine hepatitis virus (MHV), as a surrogate. In this review we show new data from a new available technology that provides a quick ultrafiltration protocol for SARS-CoV-2. Wastewater surveillance is an efficient system for the evaluation of the relative prevalence of SARS-CoV-2 infections in a community, and there is the need of using reliable concentration methods for an accurate and sensitive quantification of the virus in water.

Introduction

Many viruses that infect humans are excreted in large amounts through faeces and urine or skin desquamation, contributing to wastewater virome. Wastewater is a complex matrix that comprises a large variety of pathogenic and commensal viruses and provides important information about virus circulation, the introduction of emergent viruses and how they are transmitted among the population [1]. Waterborne viruses are generally non enveloped and excreted in high numbers by infected individuals with or without disease, and in some cases long after the resolution of symptoms [2]. The

study of excreted viruses is a very useful tool known as Wastewater-Based Epidemiology (WBE), which has the potential to act as a complementary approach for current infectious disease surveillance systems and an early warning system for disease outbreaks [3].

The incidence of emerging microbes is a serious health concern worldwide. The increase of human-livestock contacts [4], population mobility and trade networks [5,6], climate change [7] or the wild meat trade and loss of animal habitats [8] has raised the risk of a global pandemics. Since 1980, nearly 90 novel human pathogen species have been discovered, more than 70 of those corresponded to novel human viruses, that compared to other pathogens have the potential to evolve more rapidly, being 80 of these associated with nonhuman reservoirs [9,10]. Influenza viruses (H1N1, H7N1, H7N9), human immunodeficiency virus (HIV), ebola virus, coronaviruses as SARS-CoV, MERS, and the SARS-CoV-2 causing the COVID-19 pandemic have been the most significant.

SARS-CoV-2 was identified in China at the end of 2019 [11] and has become the first pandemic coronavirus (CoV). After the first case report of the presence of SARS-CoV-2 RNA in faeces [12], and because of the presence in the past of SARS-CoV-1 in feces and sewage [13–15], the scientific community started to investigate if this virus could spread into the environment. Specific stability of SARS-CoV-2 has only been tested in aerosols and surfaces [16], but it is known that enveloped virus are capable of retaining infectivity for days to months in aqueous environments [17–19]. On March 30th, SARS-CoV-2 was reported as detectable in wastewater three weeks before the first case was reported in the Netherlands [20]. On the following weeks, studies from Australia, China, Italy and Spain, reported the presence of SARS-CoV-2 and concentrations in raw wastewater to be between 10^4-10^6 GC/L [21–24].

One of the major challenges in SARS-CoV-2 research in wastewater samples is the lack of standardized protocols for its detection. From sample collection to virus concentration, there is still no consensus on the most efficient procedure. The way the sample is collected, or the virus is concentrated seems to be crucial in order to avoid false negative results or inaccurate reported concentrations. Although viral titers in composite samples are being reported to be lower than in non-composite ones, the persistent variability between non-composite replicates suggest using an autosampler that collects a volume proportional to flow as the best sampling strategy. Also, the fact that different studies use different nucleic acid extraction and detection methods made difficult to establish comparisons among different studies.

After conducting an extensive revision on the most commonly used methods for concentrating viruses from wastewater samples in the last two years, Bofill-Mas and Rusiñol (2020) described that viral concentration methods had been mostly focused on combinations of flocculation/precipitation strategies [25]. Traditionally, viral environmental surveillance has considered principally RNA enteric

viruses and also DNA viruses abundantly excreted in feces, urine o desquamation as adenoviruses, polyomaviruses and papillomaviruses, which are all non-enveloped virus [2]. In fact, in 2015, Wigginton and collaborators noticed that research should focus on the study of enveloped viruses in the urban water cycle as future pandemics could involve this type of viruses [26].

This review provides a brief on what it is known about the efficiency of viral concentration methods for CoV as well as for other enveloped viruses and new data of a comparative study analysing three concentration methods, skimmed milk flocculation, a new quick technology for ultrafiltration and a centrifugal ultrafiltration protocol.

SARS-CoV-2 in wastewater studies.

To date, the published SARS-CoV-2 surveillance studies use centrifugal ultrafiltration (CeUF) [20,21], methods including polyethylene glycol (PEG) or aluminium hydroxide ($Al(OH)_3$) flocculation-precipitation [22–24] to concentrate SARS-CoV-2 from untreated wastewater. Figure 1 summarizes the methods used in recently published studies to concentrate SARS-CoV-2 from wastewater samples.

As wastewater becomes a surveillance tool for potential incidence regrowth, the interest to understand the performance of the concentration methods used increases as well as the interest towards those methods developed and validated for non-enveloped viruses testing. Culturing SARS-CoV-2 requires BSL-3 laboratories and specially trained personnel, thus the use of surrogate CoV (e.g. non-human infectious CoV strains, or other enveloped viruses) should be considered for methods development or as positive control at this stage of research.

La Rosa *et al*. [27] recently published a review on CoV in water environments, including data on occurrence, persistence and survival. Also Carducci *et al*. [28] revised the current state of the art regarding CoV in water and highlighted the research gaps of the methods commonly used for sampling and concentration of enteric viruses which need to adapt to enveloped viruses. Both reviews are focused in the 4 available studies on human CoV that use two-step methodologies based on a pre-centrifugation and ultrafiltration [18], glass wool filtration and PEG elution [29,30] and electropositive filter media columns and PEG precipitation [31]. Kitajima *et al*. [32] reviewed the state of the knowledge regarding the potential role of wastewater in the transmission of SARS-CoV-2.

The mouse hepatitis (MHV), a surrogate for human CoV, has been used for persistence, survival and method comparison studies [18,33,34]. Ye *et al.* [18] compared, by means of MHV recoveries, three methodologies to concentrate enveloped viruses from wastewater samples, PEG precipitation and ultracentrifugation recovered approximately 5% of the spiked viruses whereas with ultrafiltration protocol the concentration was significantly higher (25%). The best performing method involved

removal of debris, prefiltering 250 mL of wastewater through a 0.22 μm PES membrane, followed by Centricon® Plus-70 10 kDa filtration. Recently, Ahmed *et al.* [34] have also evaluated six concentration strategies using MHV as a surrogate. The three filtration methods assayed provided highest mean recoveries: when $MgCl_2$ pre-treatment was included 65% of the MHV were recovered, when sample was directly filtered through 0.45-μm pore-size electronegative membranes, MHV recoveries were 60%, but when pre-acidifying the sample the mean recovery decreased to 27%. Between the two CeUF methods tested, the Amicon® Ultra-15 30KDa recovered 56% of the spiked surrogate and Centricon® Plus-70 10KDa recovered 28%. Finally, by means of PEG precipitation and ultracentrifugation, MHV recoveries were 44% and 33% respectively.

Although some enveloped viruses could be adequate surrogates for betacoronavirus concentration, only 5/15 published studies on SARS-CoV-2 occurrence in wastewater have used whole process controls, some non-enveloped virus including RNA phages [20] and Mengo virus [22], and an enveloped virus as porcine epidemic diarrhea virus (PEDV) [22]. The use of these controls prove that the protocol worked correctly and provide with an estimation of the recovery efficiency of the method for the control, although this could be different for the virus of interest. Highest recoveries were obtained with CeUF devices, like Centricon® Plus-70 30KDa, reaching 73% of the seeded F-specific RNA phages [20]. Randazzo *et al.* [22] used a surrogate CoV to calculate recovery. It is remarkable that with the $Al(OH)_3$ flocculation method a similar recovery (11%) was obtained for the enveloped virus, PEDV, and the non-enveloped virus, Mengo virus. Different viruses, even those sharing physical properties, use to show a different recovery when concentrated by the same method. To observe similar recovery values could have been a mere casualty or it could be that both viruses attached to flocs with similar efficiencies due to their negative charge when they are above the isoelectric point [35].

Preliminary data obtained by our research group in a study analysing different concentration methods for the detection of SARS-CoV-2 in wastewater from Catalonia (Spain), using MS2 as a process control, showed no statistically significant differences (*p*-value of the ANOVA test: 0,332) between the quantitative data (RT-qPCR) produced by the three viral concentration methods both for SARS-CoV-2 and for MS2. Four wastewater samples were concentrated using: the Skimmed Milk Flocculation (SMF) protocol [36] with an initial sample volume of 250 ml, the centrifugal ultrafiltration of 70 ml of the sample with Centricon® Plus-70 100 kDa (CeUF) [20] and a new and quick 80 ml ultrafiltration protocol using the automatic Concentrating Pipette (CP-Select™) from Innovaprep using 150 kDa ultrafiltration tips (www.innovaprep.com) (Figure 2). Debris were removed before the ultrafiltration by pelleting using centrifugation at 4750xg for 30 mins. A volume of the three concentration methodologies, the equivalent of 2ml of sewage was analysed at the qPCR.

Concentration of other enveloped viruses with pandemic potential in wastewater

On the lack of much data regarding CoV recovery efficiency when using commonly applied methods and until more data will be available, 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, the electropositive and electronegative filtration, CeUF, the organic flocculation and the PEG/$Al(OH)_3$ precipitation methods, have been used in different studies covering enveloped viruses' detection in environmental waters. Table 1, revises the concentration methods used until now for enveloped virus and summarises a selection of studies reporting recovery efficiencies.

It has been reported that higher percentage of enveloped viruses adsorb to the solid fraction of wastewater compared to non-enveloped viruses [18] and it is believed that these suspended solids protect viruses from inactivation [19,37,38]. None of the published studies included the first step separated solids into the analysis, but most of them involved an initial step to remove wastewater solids and then focused on recovering the viruses from the liquid phase.

Despite the proposed viral concentration methods for SARS-CoV-2 or generally for CoV, extensively reviewed by others, the organic flocculation, has been also used for the concentration of viruses in water including enveloped viruses. The enveloped virus, bovine viral diarrhea virus (BVDV), presented mean recoveries of 15% when tested with qPCR and 0,7% when tested for infectivity, but acid pH (for approximately 16h) that is used in the SMF protocol seems to reduce the infectivity, as the Log_{10} ratio RT-qPCR/infectivity for that virus was 2.03 [36,39,40]. The same observation has been described for PEG precipitation methods which disrupt the lipid bilayers and thus are not optimal for recovering infective enveloped viruses [18,41].

When testing viral recovery methods, it is relevant to consider how recovery rates are calculated and at this point the quantification of viral stocks used for spiking is of relevance since different values may be obtained when the quantification is done directly from viral stocks used for spiking or when quantifying after adding viral stock into a similar matrix from which recovered viruses will be quantified. Different enzymatic inhibition could be observed depending on the matrix in which viruses are embedded. On the other hand, if recovery is calculated according to infectivity by means of plaque forming unit's quantification assays, viral aggregation phenomena could lead to an under quantification of viral stocks. Disaggregation protocols before spiking should be considered to correct this effect [42]. Finally, direct quantification of viral stocks without pre-purification or enzymatic pre-treatment may overestimate the real amount of infectious viruses as the presence of free RNA may be quantified in viral suspensions from cell cultures [43].

Future research directions and conclusions

Agents causing novel infections are often zoonotic, crossing from the natural host into the human population. Hence, a one-health surveillance approach of virus-infected animals as well as humans is required. Structural and biochemical differences between enveloped viruses suggest that the same methods would not exhibit the same recoveries between them. As there is a potential for new outbreaks, the molecular detection of SARS-CoV-2 RNA in wastewater and the correlation between its concentration and reported prevalence of COVID19 may be a sensitive monitoring tool to evaluate the prevalence of the virus in a community, becoming a potential source of epidemiological data and public health risks information [20].

In order to face off novel outbreaks important public health organizations such as CDC, ECDC or WHO, highlight the role of scientific research to combat infectious disease, especially those emerging or re-emerging disease that may reappear in a more threating form. CDC establishes that detection and identification should be prioritized by expanding research on ecologic and environmental factors influencing disease emergence and transmission, meanwhile the ECDC highlights as a general surveillance objective, detect and monitor food- and waterborne and zoonotic outbreaks with respect to source, time, population and place in order to provide a rationale for public health actions. On the other hand, one of the WHO actions is to provide an integrated global alert and response system for epidemics and other public health emergencies for an effective international coordinated response. More scientific research is needed to identify viral transmission routes, characterizing protocols and early detection strategies for a better understanding of the factors involved in disease emergence, prevention, and elimination. In order to furnish health management models with wastewater surveillance data, more research should focus on optimizing and evaluating concentration methods able to recover enveloped potentially pandemic viruses or their surrogates from environmental samples.

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Table 1. Concentration methods and mean recoveries for enveloped viruses

Sample type	Family, genera and virus		Concentration method	Mean recovery ± SD (detection method)	ref
Waste water	*Coronaviridae, Betacoronavirus*	MHV	Ultrafiltration (Centricon® Plus-70 100kDa)	25,1 ± 3,6% (PFU)	[18]
			PEG/NaCl flocculation-precipitation	5% (PFU)	
			Ultracentrifugation	1% (PFU)	
			Ultrafiltration (Centricon® Plus-70 30KDa)	28,0 ± 9,10% (qPCR)	[34]
			Ultrafiltration (Amicon® Ultra-15 30KDa)	56,0 ± 32,3% (qPCR)	
			Electronegative filtration (pre-acidification)	26,7 ± 15,3% (qPCR)	
			Electronegative filtration (direct filtration)	60,5 ± 22,2% (qPCR)	
			Electronegative filtration (pre-treated $MgCl_2$)	65,7 ± 23,0% (qPCR)	
			PEG/NaCl flocculation-precipitation	44,0 ± 27,7% (qPCR)	
			Ultracentrifugation	33,5 ± 12,1% (qPCR)	
		SARS-CoV	Positive charged filter media + PEG elution	1,02% ($TCID_{50}$)	[31]
		PEVD	$Al(OH)_3$ flocculation-precipitation	Influent 10,90 ± 3,54% (qPCR) Effluent 3,29 ± 1,58% (qPCR)	[22]
	Cystoviridae, Cystovirus	Phi 6	Ultrafiltration (Centricon® Plus-70 100kDa)	18,2 ± 9,5% (PFU)	[18]
			PEG/NaCl flocculation-precipitation	5% (PFU)	
			Ultracentrifugation	1% (PFU)	
	Orthomyxoviridae, Alphainfluenzavirus	Influenza A (H5N1)	Ultrafiltration (Centricon® Plus-70 30KDa)	Influent 53,8% (qPCR) Effluent 42,7% (qPCR)	[44]
Surface water	*Coronaviridae, Alphacoronavirus*	TGEV	Glass wool (electropositive filtration) + 20% PEG elution	51,3 ± 10,5% (qPCR)	[30]
	Coronaviridae, Betacoronavirus	BCoV	Glass wool (electropositive filtration) + 10% PEG elution	Low turbidity 0,5 NTU: 25,8 ± 21,3% (qPCR) Medium turbidity 125 NTU: 9,2 ± 2,4% (qPCR) High turbidity 447 NTU: 19,5 ± 27,1% (qPCR)	[29]
	Flaviviridae, Pestivirus	BVDV type 1	Glass wool (electropositive filtration)+ 10% PEG elution	Low turbidity 0,5 NTU: 12,9 ± 5,4% (qPCR) Medium turbidity 125 NTU: 12,9 ± 13,3% (qPCR) High turbidity 447 NTU: 21,1 ± 5,3% (qPCR)	[29]
		BVDV	Skimmed Milk flocculation	15 ± 1,6% (qPCR) 0,7 ± 0,13% ($TCID_{50}$)	[36]
	Orthomyxoviridae, Alphainfluenzavirus	Influenza A (H5N1)	Glass wool (electropositive filtration)+ 10% PEG elution	River water 1% ($TCID_{50}$) Rain water 3,63-13,79 % (qPCR)	[45]

	Ultrafiltration (Hemoflow F80S)	Lake water 0,01-7,89% (qPCR) Surface water 5,4% (qPCR)	[44]
Influenza A (H5N3)	Pre-filtration + borosilicate glass membrane GF/F (electropositive filtration)	Riverwater: 4.7 ± 0.05% (qPCR) Seawater: 16.7 ± 0.04% (qPCR)	[46]
	Electronegative filtration (SMWP membranes)	Riverwater: 1.5 ± 0.01% (qPCR) Seawater: 5.00 (qPCR)	

MHV: murine hepatitis virus; PEVD: porcine epidemic diarrhea virus; TGEV: transmissible gastroenteritis virus; BVDV: bovine viral diarrhea virus; BCoV: Bovine coronavirus.

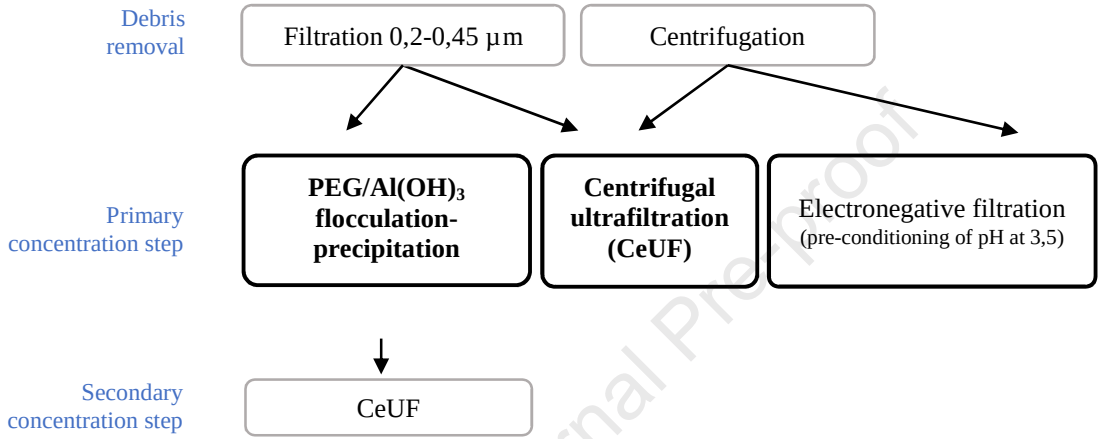
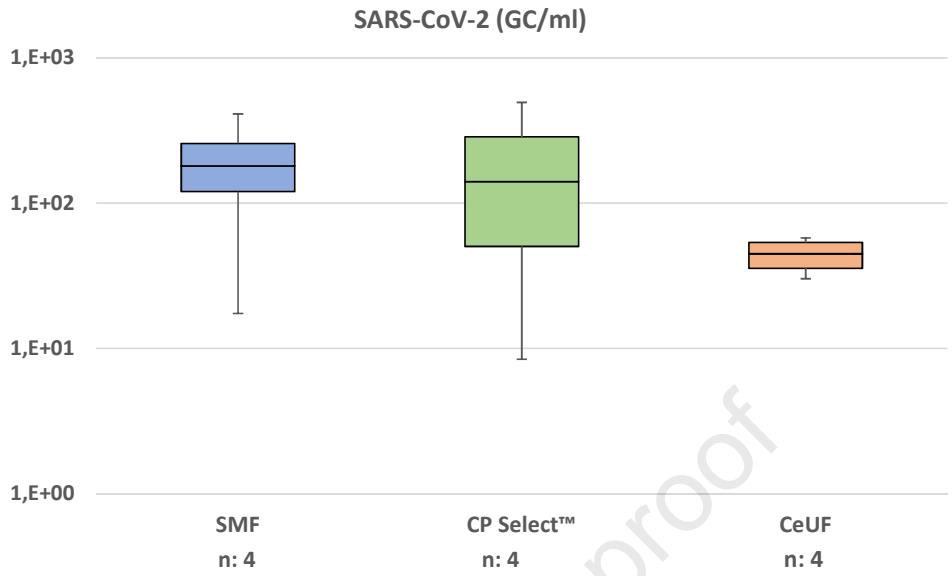


Figure 1: Summary of the diferent strategies used in the published literature to concentrate SARS-CoV-2 from wastewater samples. PEG/$Al(OH)_3$ flocculation-precipitation based methods [22–24] centrifugal ultrafiltration methods [20,21] and electronegative filtration [21] have been used to date, in the published studies for SARS-CoV-2.



Percentage of recovery using MS2 as process control

	SMF	CP Select™	CeUF
Sample 1	23%	50%	8%
Sample 2	37%	43%	23%
Sample 3	32%	66%	23%
Sample 4	24%	45%	12%

Figure 2. Barplots of the concentrations of naturally occuring SARS-CoV-2 in 4 sewage samples by using three different concentration methods: Skimmed Milk Flocculation (SMF), InnovaPrep concentrating pipette with single-use ultrafiltration tips 150KDa (CP Select^{TM}) and centrifugal ultrafiltration with Centricon Plus 70 100KDa (CeUF).

Highlights

There are efficient methods to concentrate enveloped virus such as coronaviruses
More data on the recovery of the specific pathogen of interest is needed
Viral surrogates, ideally betacoronavirus, may be used as SARS-CoV-2 process control
Recovery calculation needs an accurate quantification of the viral stock