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Primary concentration – The critical step in implementing the wastewater based epidemiology for the COVID-19 pandemic: A mini-review

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HIGHLIGHTS

- Discuss the potential advantages and disadvantages of commonly used virus concentration methods
- Focus on the recently reported analytical studies of the SARS-CoV-2 RNA in wastewater samples
- Compare the performance of the reported studies based on efficiency, easy to access, and operate
- Recommend two methods of electronegative membrane filtration and PEG-based separation

GRAPHICAL ABSTRACT

Abstract

The recent outbreak of a novel coronavirus SARS-CoV-2 has posed a significant global public health threat and caused dramatic social and economic disruptions. A new research direction is attracting a significant amount of attention in the academic community of environmental sciences and engineering, in which rapid community-level monitoring could be achieved by applying the methodology of wastewater based epidemiology (WBE). Given the fact that the development of a mass balance on the total number of viral RNA copies in wastewater samples and the infected stool specimens is the heart of WBE, the result of the quantitative RNA detection in wastewater has to be highly sensitive, accurate, and reliable. Thus, applying effective concentration methods before the subsequent RNA extraction and RT-qPCR detection is a must-have procedure for the WBE. This review provides new insights into the primary concentration methods that have been adopted by the eighteen recently reported COVID-19 wastewater detection studies, along with a brief discussion of the mechanisms of the most commonly used virus concentration methods, including the PEG-based separation, electrostatically charged membrane filtration, and ultrafiltration. In the end, two easy and well-proven concentration strategies are recommended as below, aiming to maximize the practical significance and operational effectiveness of the SARS-CoV-2 virus concentration from wastewater samples.

Strategy 1: Prefiltration-Salt addition-Electronegative membrane filtration (for initial volume ≤ 50 mL).
Strategy 2: Preliminary-PEG-based separation-Overnight standing (for initial volume from 50 to 1000 mL).

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1. Introduction

The recent outbreak of a novel coronavirus SARS-CoV-2, also known as the COVID-19 disease, has posed a significant global public health threat and causes dramatic social and economic impacts. As of June 19, 2020, the World Health Organization (WHO) has reported more than 14,000,000 infected people globally (WHO, 2020). As a completely novel human coronavirus, the response of the global public health authorities and the academic communities to the COVID-19 demonstrated the challenges, the most immediate one is the relatively low viral particle concentration. As a result, the primary concentration method directly determines the next elution step to must adopt sodium citrate as the eluting reagent. Furthermore, due to the nature of soluble alginate filter materials, water samples with high turbidities, such as wastewater, have to conduct pretreatment by passage through a series of filters to reduce the clogging risk during the final virus concentration.

In summary, the characteristics of a primary concentration method can exert significant influence on the overall concentration process by determining if the subsequent extraction and detection method can be successfully applied to fulfill the goals of WBE. According to a recent review, some early developed methods that have been historically used to concentrate enteric viruses from wastewater samples may be subject to some technical issues, due to the SARS-CoV-2 virus is relatively unstable in the environment and is more susceptible to common oxidants (La Rosa et al., 2020a). Therefore, an effective concentration strategy has to be selected or developed in the first place. Given that many relevant techniques have been developed and refined over the past decades, this mini-review discusses the potential advantages and disadvantages of different concentration strategies with an emphasis on the primary concentration methods and the suitability for the COVID-19 WBE. Meanwhile, this mini-review puts a particular focus on several existing analytical studies of the SARS-CoV-2 virus in the wastewater matrix and evaluate their concentration strategies before the subsequent extraction and detection processes. At last, two concentration strategies are recommended to maximize the practical significance and operational effectiveness of the SARS-CoV-2 virus concentration from wastewater samples.

2. Commonly used primary concentration methods

A number of methods have been developed to effectively concentrate viral particles from water samples. Based on various concentrating mechanisms, we can classify all the different primary concentration methods into four broad types, namely (1) two-phase separation/partition precipitation, (2) particle exclusion, (3) Viruses ADSorption–Elution (VIRADEL), and (4) ultrafiltration. It is worth mentioning that the VIRADEL method using electrostatically charged microporous materials as filtration media is the most prevalent technique used today (Ikner et al., 2012), which can be further categorized as electronegative membrane filtration and electropositive membrane filtration based on surface charge differences (see Table 1). Along with the continuous evolution of the primary concentration methods, many of early methods have been scarcely used or phased out due to some inherent disadvantages, such as small treatment volumes (hydroextraction), poor recovery performance (cotton gauze pads), and excessive pretreatment processes (soluble membrane filtration). In this section, three of the most commonly used methods (i.e., PEG-based two-phase separation, VIRADEL method, and ultrafiltration) will be briefly discussed and compared on the basis of recovery performance, ease of operation, and consistency of concentrating outcome. Also worth noting is that the PEG-based two-phase separation and the VIRADEL method currently are the only two standard methods adopted by the World Health Organization (WHO/V-B03.03) and the U.S. Environmental Protection Agency (EPA Method 1615) respectively.

2.1. Polyethylene glycol (PEG) based two-phase separation

Using polyethylene glycol (PEG) based two-phase separation on concentrating viruses between two immiscible aqueous polymer phases was first proposed by Albertsson and Frick (1960). During the 1960s and 1970s, the method of aqueous polymer two-phase

| Nomenclature | Definition |
|----------------|-------------|
| COVID-19 | coronavirus disease 2019 |
| M genotype | mengovirus |
| MHV | murine hepatitis virus |
| PEDV | porcine epidemic diarrhea virus |
| PEG | polyethylene glycol |
| RSD | relative standard deviation |
| RT-qPCR | real-time quantitative polymerase chain reaction |
| SARS-CoV-2 | severe acute respiratory syndrome coronavirus 2 |
| U.S. EPA | U.S. Environmental Protection Agency |
| VIRADEL | Viruses ADSorption–Elution |
| WBE | wastewater based epidemiology |
| WHO | World Health Organization |
separation was continuously refined for the purpose of concentrating enteroviruses over a broad spectrum of water quality matrices, such as river water, tap water, groundwater, and wastewater (Shuval et al., 1969; Wallis et al., 1969). Based on the result reported by Shuval et al. (1969), after mixing with a combination of organic polymers (i.e., dextran and PEG), a sewage sample of several liters can be successfully concentrated for subsequent viruses detection with an average nominal concentration factor of 220-fold and an average recovery efficiency of 72%. The PEG-based separation method was eventually adopted by the WHO as a standard method for conducting environmental surveillance of poliovirus circulation (WHO, 2003). In summary, the PEG-based separation method has been extensively studied to concentrate and purify various viruses (e.g., bacteriophage T2, adenovirus, poliovirus, and ECHO virus) and found to be rapid, inexpensive, consistent, and non-destructive of viruses. In addition, commercial PEG Virus Preparation (VIRADEL) has been extensively studied to concentrate samples with a relatively small volume (as shown in Fig. 1a). As a result, a raw water sample must contain a moderate to large treatment volumes; inhibition of virus activity

2.2. Electrostatically charged membranes method

Unlike the PEG-based separation and electropositive membrane filtration method, the most common technique (i.e., VIRADEL) currently used for concentrating viruses from water (Farrah and Preston, 1985; Haramoto et al., 2004; Hata et al., 2015) is electrostatically charged membranes method. This phenomenon is mainly associated with the adsorption of metal ions to the electronegatively charged surface of cellulose nitrate HA membranes, thereby facilitating the attachment of polioviruses via salt-bridging (Ilner et al., 2012). Although the electronegatively charged membranes can effectively extract viruses from water samples, the addition of polyvalent salts makes it difficult for concentrating large volume samples. As a result, a number of early studies tried in situ charge modification of electronegative filters and successfully developed electropositive membrane filtration method, the most common technique (i.e., VIRADEL) currently used for concentrating viruses from water (Farrah and Preston, 1985; Haramoto et al., 2004; Sattar, 1980) or in situ charge modification of electronegative filters (e.g., NanoCeram and IMDS) are capable of continuously extracting enterovirus and norovirus from groundwater and surface water with the minimum specified volumes of 300 L and 1500 L, respectively (see Fig. 1b) (Fout et al., 2015). However, the presence of organic matter can lead to significantly reduced efficiency of virus concentration due to a preferential attachment of dissolved organic molecules over virus particles. In addition to the presence of organic matter, high water turbidity (≥50 NTU) can directly lead to the failure of sampling when applying the EPA Method 1615 with the recommended NanoCeram virus sampler (Fout et al., 2015). It is, therefore, inappropriate to apply the EPA Method 1615 directly to concentrate virus particles from raw wastewater samples that are usually turbid and contain a substantial amount of dissolved and suspended organic matter.

2.3. Ultrafiltration

Unlike the PEG-based separation and electropositive membrane filtration method, using ultrafiltration to extract viruses will not be subject to pre-conditioning of the water samples, which allows this method to be used over a broad range of water quality (Hill et al., 2007). The earliest study that used ultrafiltration to concentrate poliovirus from a water sample was designed using multiple layers of asymmetric cellulose acetate membranes to separate flow channels for raw water samples and driving solution, and achieved recovery efficiency of 95–100% from a large volume of water of 10 L (Sweet et al., 1971). Later studies using polysulfone hollow fiber ultrafilters demonstrated better resistance to pH and temperature variations, biological degradation, and showed less penetration of target virus particles due to the dense inner surface (Ilner et al., 2012). In terms of the ultrafiltration flow types, tangential crossflow ultrafiltration is a commonly used configuration, which can
enveloped viruses possess outer surface structures consisting of different functional groups, which may significantly impact their survival and partitioning behavior in an aqueous environment (Ye et al., 2016). An early study focusing on the evaluation of the survivability of an enveloped human coronavirus (HCoV) in the wastewater environment, reported that the time required for the HCoV titer to decrease by 99.9% (T99.9) is substantially shorter than the non-enveloped poliovirus 1 (PV-1) (Gundy et al., 2008). In terms of the primary concentration performance, as shown in Table 2, under the same conditions, most of the enveloped viruses showed relatively lower recovery rates compared to the non-enveloped viruses. For example, with the optimized ultracentrifugation method, Ye et al. (2016) reported mean recoveries of 25.1 ± 3.6% and 18.2 ± 9.5% for the enveloped murine hepatitis virus (MHV) and Pseudomonas phage φ6, respectively. Meanwhile, the non-enveloped Enterobacteria phage T3 and MS2 demonstrated significantly higher mean recoveries of 55.6 ± 16.7% and 85.5 ± 24.5%, respectively, when using the same ultrafiltration method. In Randazzo et al.’s (2020) study, the aluminum hydroxide precipitation method showed the closest primary concentration recovery when concentrating wastewater influent samples with spiked porcine epidemic diarrhea virus (PEDV), an enveloped virus member of the Coronaviridae family, and mengovirus (MgV), a non-enveloped member of the Picornaviridae family, of 11 ± 3.5 and 11 ± 2.1%, respectively. However, the overall low recovery rate (~3 to 11%) may hinder this method to be widely adopted for the detection of the SARS-CoV-2 virus.

Due to the characteristics of the enveloped viruses, including the SARS-CoV-2 virus, most of the concentration methods tend to result in a higher decay or inactivation for the enveloped viruses than the non-enveloped viruses. Another risk we face is the reduced viability and infectivity of the virus after a concentrating process. The increased sensitivity of the molecular detection method (i.e., RT-qPCR) has partially alleviated the problem of reducing the viability and infectivity of the viruses because the quantitative determination is no longer based on the traditional cell culture infectivity assays, which heavily relied on the viability, infectivity, and structural integrity of the concentrated viruses (Ikner et al., 2012). However, it is critical to recognize the technical capabilities/limitations of all the processes and techniques used so we can optimize each step involved to help minimize the loss of the original characteristics of the samples.

4. Primary concentration methods used for the COVID-19 WBE

Based on the review of all the currently published COVID-19 studies, there are 18 relevant wastewater SARS-CoV-2 virus detection studies that have been conducted in different epidemic areas over the world, including France, Japan, Israel, Italy, Australia, Netherlands, Spain, India, and Singapore. After carefully identifying the methodologies of these articles, we found the majority of them (17 out of 18) applied primary concentration methods to concentrate the SARS-CoV-2 virus particles or the genomic fragments from the wastewater samples (see Table 3).

4.1. The applied PEG-based separation method

The PEG-based separation is the most used technique (7 out of 18) among all concentration methods, and all four studies that adopted this concentration method showed positive results regarding the SARS-CoV-2 detection in wastewater samples (Bar Or et al., 2020; Hata et al., 2020; La Rosa et al., 2020b; Wu et al., 2020). As summarized in Table 3, most of the studies that implemented the PEG-based separation method (6 out of 7) also had additional follow-up processes, such as ultracentrifugation and overnight standing, to further condense the obtained PEG layer. Although ultracentrifugation can significantly reduce the final water content in the obtained PEG layer, the initial cost to obtain an ultracentrifuge may pose a cost-prohibitive obstacle for many analytical laboratories, especially for those in the wastewater treatment plants that typically can only conduct routine wastewater
analyses. In comparison, overnight standing at 4 °C in a separation funnel maybe not as effective as the immediate ultracentrifugation. However, this approach is significantly easier to achieve because it only requires a refrigerator, which is readily available in most of the laboratories. In terms of the preconditioning process, most of the studies using the PEG-based separation method (3 out of 7) had applied the centrifugation method to remove sediment and large particles from raw wastewater samples. Similarly, centrifugation is a fast and effective method to precondition a raw wastewater sample, but it requires sizeable centrifuges when handling a relatively large volume of wastewater samples. Thus, we believe the prefiltration method adopted by Wu et al. (2020) using 0.2 μm membranes is more feasible and accessible than centrifugation. It should be noted that the prefiltration method may capture viruses that were previously attached to large particles during the preconditioning process, and therefore, retaining the resulted cake for the subsequent elution together with the separated PEG-layer is highly recommended.

4.2. The applied electronegative membranes filtration method

Among all the SARS-CoV-2 detection studies, the electronegative membranes filtration technique was adopted for primary concentration in 3 of the 18 studies. In terms of the preconditioning method, these three studies are highly representative of the current development of the electronegative membrane filtration technique. Specifically, Ahmed et al. (2020a) used the simplified acidification method to impart a positive electrical double layer around the negatively charged virus particles. Haramoto et al. (2020) added a high concentration of magnesium chloride (MgCl2) to facilitate the attachment of virus particles via salt-bridging. Based on the results reported by Ahmed et al. (2020a), the SARS–COV-2 RNA was occasionally detected in some of the wastewater samples, and none of the wastewater samples showed any RT-qPCR inhibition. In addition, Haramoto et al. (2020) tested the efficiency of the electronegative membranes filtration method by using pepper mild mottle virus (PMMoV) as a detection biomarker and resulted in high RNA results of 2.6 × 106 copies/L. As previously mentioned, using electrostatically charged membranes filtration to concentrate viruses from turbid water, such as raw wastewater, can be subject to a significant reduction of virus recovery efficiency due to the presence of organic matter and high turbidity, which can lead to a preferential attachment to the charged filters and raise the risk of detrimental clogging.

However, in these studies, there is no compelling evidence to suggest that the organic materials from wastewater have a severe impact on the virus adsorption. This phenomenon is probably attributed to the relatively small volume that both studies involved, therefore alleviated the adverse impact from the high organic content in the wastewater samples.

4.3. The applied ultrafiltration method

We found four studies that implemented the ultrafiltration technique as the primary concentration strategy. Among them, two studies chose 100 kDa as the molecular weight cut-off (Medema et al., 2020; Nemudryi et al., 2020), and the other two studies chose to adopt a much smaller molecular weight cut-off, such as 10 kDa and 30 kDa (Ahmed et al., 2020b; Wu et al., 2020). Interestingly, all these studies employed, to some extent, precondition step, which is unnecessary when using the ultrafiltration technique to conduct the primary concentration (Fout et al., 2015; Rajal et al., 2007). For example, Nemudryi et al. (2020) pretreated all wastewater samples by sequentially filtering through 20 μm, 5 μm (Sartorius Biolab Products), and 0.45 μm ( Pall Corporation) membrane filters, and then concentrated down the obtained supernatant from 500 mL to 150 mL using ultrafiltration. The extensive amount of preconditioning process not only increased the workload but also increased the chance of experimental batch effect and systematic error. Thus, the necessity of including precondition needs to be carefully identified when choosing ultrafiltration as the primary concentration method. It is worth noting that according to the RT-qPCR result reported by Medema et al. (2020), the detected gene copies in all the wastewater treatment plants showed evident increase along with the increase of the cumulative number of the reported COVID-19 clinical cases. Indeed, the strong correlation between the detected virus concentration in wastewater and the reported clinical cases is determined simultaneously by a combination of various critical factors, such as the viral shedding pattern in stool and viral decay rate in sewage (Hart and Halden, 2020). However, it should not be ignored the ultrafiltration method used by Hart and Halden (2020) is one of the essential requisites which played vital roles in this successful effort.

Collectively, all concentration methods showed positive results regarding the SARS-CoV-2 virus detection in wastewater. In terms of treatment volume, all studies tend to work with a small amount (e.g., less than 1 L), which is likely due to the risk of potential enteric transmission of the COVID-19 in a wastewater sample. Also, handling a large volume of wastewater in an epidemic area may pose a significant infectious risk to the research team members. Another critical factor in the wastewater virus concentration lies in the degree of the primary concentration efficiency (i.e., the recovery rate of the primary concentration step). A superior concentration efficiency indicates a relatively small portion of virus loss during the concentrating procedure, ensuring

### Table 2

Recovery performance of concentrating enveloped and non-enveloped viruses.

| Virus (type) | Initial sampling matrix | Concentration method | Recovery rate (%) | Reference |
|-------------|-------------------------|----------------------|-------------------|-----------|
| SARS-CoVa (enveloped) | Hospital and domestic sewage | Silica gel with Al(OH)3 (Electropositive filtration) | 0–21.4 | (Wang et al., 2005) |
| Bacteriophage f2 (non-enveloped) | WWTP influent (prior to primary settling tank) | Centrifugal ultrafiltration | 25.1 ± 3.6 | (Ye et al., 2016) |
| MHV (enveloped) | WWTP untreated influent | Al(OH)3 precipitation | 11 ± 3.5 | (Randazzo et al., 2020) |
| Enterobacteria phage T3 (non-enveloped) | WWTP secondary & tertiary effluent | 3.3 ± 1.6 | (Randazzo et al., 2020) |
| Enterobacteria phage MS2 (non-enveloped) | WWTP secondary & tertiary effluent | 6.2 ± 1.0 | (Randazzo et al., 2020) |
| PEDV (enveloped) | WWTP influent (prior to primary settling tank) | 85.5 ± 24.5 | (Randazzo et al., 2020) |
| MgV (non-enveloped) | WWTP influent (prior to primary settling tank) | 56.0 ± 16.7 | (Randazzo et al., 2020) |

a SARS-CoV stands for severe acute respiratory syndrome coronavirus.
b MHV stands for murine hepatitis virus.
c PEDV stands for porcine epidemic diarrhea virus.
d MgV stands for mengovirus.
PEG-based separation (20 mg/L) followed by ultracentrifugation at 14,000 g for 45 min

PEG-based separation (100 g/L) followed by ultracentrifugation at 14,000 g for 30 min

PEG-based separation (29% (w/w)) followed by stand overnight at 4 °C in a separation funnel

PEG-based separation (8% (w/v)) followed by ultracentrifugation at 12,000 g for 2 h

PEG-based separation (20% (w/v) PEG-6000)

PEG-based separation (80 g/L of PEG9000) and NaCl (17.5 g/L) followed by ultracentrifugation at 13,000 g for 1.5 h

Electronegative membrane filtration

Electronegative membrane filtration (HAWP09000 membrane 0.45 μm)

Electronegative membrane filtration (cellulose-ester membrane 0.8 μm)

Electronegative membrane filtration (HAWP04700 membrane 0.45 μm)

Ultrafiltration

Ultrafiltration with 100 kDa molecular weight cut-off for 15 min

Centrifugal ultrafiltration with 100 kDa molecular weight cut-off at 1500 g for 15 min

Centrifugal ultrafiltration with 10 kDa molecular weight cut-off for 15 min

(d) Centrifugal ultrafiltration with 30 kDa molecular weight cut-off at 4750 g for 10 min; (e) Centrifugal ultrafiltration with 10 kDa molecular weight cut-off at 3500 g for 30 min

Al(OH)3 precipitation (0.009 N) followed by centrifugation at 1700 g for 20 min

Ultrafiltration at 200,000 g for 1 h at 4 °C

(g) Ultrafiltration at 100,000 g for 1 h at 4 °C followed by resuspension of pellets in glycine buffer and then ultrafiltration at 12,000 g for 15 min at 4 °C

Not implemented any primary concentration method

| Type                      | Primary concentration method                                                                 | Initial volume (L) | Preconditioning method                                                                 | References                  |
|---------------------------|-------------------------------------------------------------------------------------------------|--------------------|----------------------------------------------------------------------------------------|-----------------------------|
| PEG-based two-phase separation | PEG-based separation (20 mg/L) followed by ultracentrifugation at 14,000 g for 45 min          | 0.25–1             | Centrifugation to remove sediment and particles                                          | (Bar Or et al., 2020)       |
|                           | PEG-based separation (100 g/L) followed by ultracentrifugation at 14,000 g for 30 min          | 0.08               | Centrifugation to remove sediment and particles                                          | (Hata et al., 2020)        |
|                           | PEG-based separation (29% (w/w)) followed by stand overnight at 4 °C in a separation funnel   | 0.25               | Centrifugation and retaining the resulted pellet for further elution                    | (La Rosa et al., 2020b)    |
|                           | PEG-based separation (8% (w/v)) followed by ultracentrifugation at 12,000 g for 2 h            | 0.04               |Prefiltration through 0.2 μm membrane                                                    | (Wu et al., 2020)          |
|                           | PEG-based separation (20% (w/v) PEG-6000)                                                     | 0.1                | Non-pretreated                                                                          | (Chavarria-Miró et al., 2020) |
|                           | (f) PEG-based separation (PEG 10% (w/v)) followed by ultracentrifugation at 10,000 g for 30 min | 0.05               | Centrifugation at 10,000 g for 20 min, pellet resuspension in beef extract and centrifugation at 10,000 g for 10 min | (Ahmed et al., 2020b)       |
|                           | PEG-based separation (80 g/L of PEG9000) and NaCl (17.5 g/L) followed by ultracentrifugation at 13,000 g for 1.5 h | 0.05               | Centrifugation at 4500 g followed by filtration of supernatant through 0.22 μm membrane | (Kumar et al., 2020)       |
|                           | Electionegative membrane filtration (HAWP09000 membrane 0.45 μm)                              | 0.1–0.2            | Adjusting the sample pH to 3.5–4 using 2.0 N HCl and centrifugation at 10,000 g for 10 min | (Ahmed et al., 2020a)       |
|                           | Electronegative membrane filtration (cellulose-ester membrane 0.8 μm)                         | 0.2                | Addition of 2 mL of 2.5 M MgCl2                                                         | (Haramoto et al., 2020a)    |
|                           | Electronegative membrane filtration (HAWP04700 membrane 0.45 μm)                              | 0.05               | (a) Acidification to pH 4.0; (b) non-pretreated; (c) addition of MgCl2                   | (Ahmed et al., 2020b)       |
|                           | Ultrafiltration with 100 kDa molecular weight cut-off                                            | 0.5                | Prefiltration sequentially through mixed membranes (20, 5, 0.45 μm)                     | (Nemudinyi et al., 2020)    |
|                           | Centrifugal ultrafiltration with 100 kDa molecular weight cut-off at 1500 g for 15 min         | 0.1–0.2            | Centrifugation to remove sediment and particles                                          | (Medema et al., 2020)       |
|                           | Centrifugal ultrafiltration with 10 kDa molecular weight cut-off                               | 0.015              |Prefiltration to remove sediment and particles                                           | (Wu et al., 2020)          |
|                           | (d) Centrifugal ultrafiltration with 30 kDa molecular weight cut-off at 4750 g for 10 min; (e) Centrifugal ultrafiltration with 10 kDa molecular weight cut-off at 3500 g for 30 min | 0.05               | Centrifugation of the sample at 4500 g for 10 min at 4 °C to obtain a supernatant       | (Ahmed et al., 2020b)       |
|                           | Al(OH)3 precipitation (0.009 N) followed by centrifugation at 1700 g for 20 min                | 0.2                | Adjusting the sample pH to 6.0                                                          | (Randazzo et al., 2020)    |
|                           | Ultrafiltration at 200,000 g for 1 h at 4 °C                                                     | 0.011              | Homogenization                                                                         | (Wurtzer et al., 2020)      |
|                           | (g) Ultrafiltration at 100,000 g for 1 h at 4 °C followed by resuspension of pellet in glycine buffer and then ultrafiltration at 12,000 g for 15 min at 4 °C | 0.05               | Non-pretreated                                                                          | (Ahmed et al., 2020b)       |
|                           | Not implemented any primary concentration method                                               | 0.5b               |Prefiltration sequentially through glass fiber filters of 0.7 and 0.2 μm                 | (Rimoldi et al., 2020)      |

Underlines are present to emphasize the specific methods that have been adopted.

a Murine hepatitis virus (MHV), was used to test the efficiency of seven wastewater virus concentration methods: (a, b, c) electronegative membrane with three different pretreatment options, (d, e) centrifugal ultrafiltration with two molecular weight cut-off, (e) PEG-based two-phase separation, and (g) ultrafiltration (Ahmed et al., 2020b).
b Sampling volume based on the container size mentioned in the reference (Rimoldi et al., 2020).

a higher sensitivity of the overall detection process. Most importantly, the final concentration of the virus in a wastewater sample has to be calculated by dividing the subsequent RT-qPCR result by the primary concentration efficiency. Technically, spiking of a surrogate virus (e.g., F-specific RNA phage) with known concentrations as an internal reference is the most common practice to obtain this critical efficiency information. After screening all the found studies, five different research teams did employ internal reference(s) to facilitate the analysis of the primary concentration efficiency, and the result is presented in Table 4.

4.4. The efficiency of primary concentration

As of now, we have found only one comprehensive study that systematically compares the efficiency of different primary concentration methods for the COVID-19 wastewater analysis (Ahmed et al., 2020b). As shown in Table 4, four types of primary concentration methods were conducted to concentrate the spiked murine hepatitis virus (MHV) in the wastewater influent. According to the reported concentration efficiencies, the electronegative membrane filtration method with the addition of magnesium chloride resulted in the highest mean recovery rate. It appears that the high recovery rate of the electronegative membrane filtration method was mainly associated with skipping over any prefiltration and pre-centrifugation step, which maximized the internal reference (i.e., the spiked MHV) to be adsorbed from both the liquid and solid fractions of the wastewater sample simultaneously (Ahmed et al., 2020b). Such approach will significantly improve the adaptability of this method when it comes to handling a highly turbid influent sample. Again, the risk of membrane clogging has to be carefully addressed when dealing with a medium-sized turbid influent sample (e.g., 500 mL). This is because the electronegative membrane filtration method requires a sample to pass through a membrane filter, which typically has a small pore size of less than 1.0 μm.

In terms of the PEG-based separation method, both studies showed moderate-to-high recovery efficiencies of 44% and 57%, respectively (Ahmed et al., 2020b; Hata et al., 2020). The relatively higher recovery rate of 57% reported by Hata et al. (2020) was achieved with a single-step pretreatments of centrifugation at 5000 g for 5 min to remove large particles from raw influent samples. In comparison, Hata et al. (2020) adopted a more complex pretreatment process of (1) ultracentrifugation at 10,000 g for 20 min, (2) resuspension of the obtained pellet in 0.05 M glycine, (3) ultracentrifugation at 10,000 g for 10 min, (4) Combination of the supernatants from both ultracentrifugation processes, and (5) pH neutralization by adding 2 M HCl. As the PEG-based separation method have been developed and refined since 1960s, different
pretreatments aiming to improve its efficiency and adaptability have also been proposed and applied, which may result in overly complicated procedures. As a result, particular attention should be paid to the additional pretreatment steps used in the overall concentration process because of the possibility of the unforeseeable loss of viruses along with the processing.

As mentioned previously in Section 3, the aluminum hydroxide precipitation method showed low recovery rates (~3 to 11%) in all wastewater samples, and the difference in recovery rate between the spiked enveloped and non-enveloped viruses in influent samples is statistically insignificant, indicating the method has a similar efficiency of concentrating enveloped and non-enveloped virus from an influent sample (Randazzo et al., 2020). Comparing to the aluminum hydroxide precipitation method, all other techniques showed better recovery rates. For example, Medema et al. (2020) reported a significantly higher recovery rate of 73 ± 50% by concentrating the F-specific RNA phages with centrifugal ultrafiltration (100 kDa molecular weight cut-off). However, the high recovery rate of 73% was subject to a noticeably large relative standard deviation (RSD) of ±50%, which may reduce the application implication of the centrifugal ultrafiltration method. It should be noted that the issue of a low average recovery rate can be compensated, to some extent, by applying a large initial volume. However, the issue of data scattering (i.e., large RSD) might have a more detrimental effect on the accuracy of the centrifugal ultrafiltration method.

By combining the previously published studies of virus detection in wastewater with the most recent reports regarding the COVID-19 WBE, this review will provide some specific suggestions, in the section entitled “5. Recommendations,” for which technique(s) should be chosen and what preconditioning approach(es) should be adopted when conducting the SARS-CoV-2 wastewater detection.

5. Recommendations

In 18 studies that we found relevant to the wastewater SARS-CoV-2 detection, the initial sampling volume was all relatively small. For example, a small volume of 40 mL prefiltred wastewater sample can be successfully concentrated by using the PEG-based separation combined with the ultrafiltration and subsequently detected via RT-qPCR to result in the SARS-CoV-2 N1 genomic fragments of 50 to 250 copies/mL of raw wastewater (Wu et al., 2020). Furthermore, Ahmed et al. (2020b) reported using 50 mL- aliquot influent samples can result in a superior virus recovery rate up to 65.7 ± 23.8% via the electronegative membrane filtration method. Thus, a large initial concentrating volume seems to have less of an effect on the development of a sufficient concentration method. For the purpose of ease-to-operate, both the PEG-based separation and the electronegative membrane filtration methods have been implemented without large-scale instruments and resulted in desirable recovery efficiencies, such as 57% and 65.7%, respectively (Ahmed et al., 2020b; Hata et al., 2020). Based on what we have summarized above, an ideal method of conducting the primary concentration of the SARS-CoV-2 virus RNA from wastewater samples should have the following performance characteristics, including a small to medium-sized concentrating capacity, an easy to access and operate procedure, a reliable and efficient performance, and, most importantly, strong adaptability to a broad range of water quality.

As a result, we highly recommend using the electronegative membrane filtration method with the addition of magnesium chloride as the primary concentration method when dealing with a small initial volume (i.e., less than 50 mL). In case of the potential clogging risk, when handling a medium-sized influent sample (i.e., 50 to 1000 mL), we suggest using the PEG-based separation method followed by overnight standing at 4 °C in a separation funnel as the alternative primary concentration method. Due to the typically high turbidity of an untreated influent sample, the removal of large particles, such as sand, debris, and hair, could be achieved by passing through filters with a large pore size (e.g., 5 or 20 μm), if needed. The recommended two primary concentration processes can be achieved without any large laboratory equipment. At the same time, the main consumables are limited to the filter membranes and chemicals (i.e., magnesium chloride for the electronegative membrane filtration method and polyethylene glycol for the PEG-based separation method, respectively) (see Fig. 2). In summary, these two proposed methods are easy to use, free of large laboratory equipment, proven concentration efficiency, and highly accessible over a broad range of research facilities.

6. Conclusion

Due to the recent outbreak of the COVID-19 pandemic, wastewater based epidemiology starts to attract a significant amount of attention. The fundamental idea is to quantitatively detect the SARS-CoV-2 virus RNA from wastewater samples and then use this information to conduct infection prevalence estimation. To accurately reflect the epidemic situation, the detection technique must be highly sensitive and reliable. Therefore, developing a simple, effective primary concentration method before the SARS-CoV-2 RNA extraction and detection processes is of great significance. This mini-review first discussed several commonly used primary concentration methods, including the PEG-based separation, electrostatically charged membrane filtration, and ultrafiltration. After that, a particular emphasis was put on the eighteen existing SARS-CoV-2 wastewater analytical studies. Based on the primary concentration methods implemented in these studies, we present the following conclusions:

### Table 4

| Primary concentration type | Selected surrogate virus | Concentration performance (%) | Reference |
|----------------------------|--------------------------|--------------------------------|-----------|
| **Al(OH)₃ precipitation**  | PEDV and MgV³⁺           | 11 ± 2.1 and 11 ± 3.5⁸        | (Randazzo et al., 2020) |
| **Centrifugal ultrafiltration** | F-specific RNA phage       | 73 ± 50                         | (Medema et al., 2020) |
| **PEG-based separation**   | F-specific RNA phage       | 57⁷                            | (Hata et al., 2020) |
| **Electronegative membrane filtration** | PMMoV⁴⁺                   | 71.6 ± 25.2                    | (Haramoto et al., 2020) |
| **Electronegative membrane filtration (w/ different pretreatments)** | MHV⁵⁺                      | 26.7 ± 15.3 (acidification)    | (Ahmed et al., 2020b) |
| **Centrifugal ultrafiltration (w/ different molecular weight cut-off)** |                           | 60.5 ± 22.2 (non-treatment)    |           |
| **PEG-based separation**   |                           | 65.7 ± 23.8 (addition of MgCl₂) |           |
| **Ultracentrifugation**    |                           | 56.0 ± 32.3 (30 kDa)           |           |
|                            |                           | 28.0 ± 9.10 (10 kDa)           |           |
|                            |                           | 44.0 ± 27.7                    |           |
|                            |                           | 33.3 ± 12.1                    |           |

-⁴⁺ PEDV and MgV stand for porcine epidemic diarrhea virus and mengovirus (VMC0).
-⁸ Recovery data of influent samples.
-⁷ Relative standard deviation not provided.
-⁵⁺ PMMoV stands for pepper mild mottle virus.
-⁶ MHV stands for murine hepatitis virus.
1. This review work highlights the importance of having an easy to access and operate primary concentration method because implementing the real-time wastewater surveillance has to be based on the high availability of wastewater data.

2. Due to the positive results obtained with small initial volumes in all the reported studies, the main drawback (i.e., small concentrating capacity) of the PEG-based separation and the electrostatically charged membrane filtration can, to some extent, be ignored.

3. As of now, the PEG-based separation method is the most prevalent method used for the COVID-19 wastewater based epidemiology.

4. The electronegative membrane filtration method can be carried out without any prefiltration and pre-centrifugation and still produce the most desirable concentration efficiency.

5. Although the electronegative membrane filtration method has been proved experimentally, future studies should be careful about the preferential adsorption of organic matter on the charged membrane surface and the potential risk of clogging when handling turbid samples.

6. Ultrafiltration can provide high recoveries and consistent performance, but an ultrafiltration system is usually large, immobile, and not readily available in most laboratories.

7. We recommend two simple and straight-forward primary concentration strategies:
   (1) Prefiltration – Salt addition – Electronegative membrane filtration (volume ≤ 50 mL).
   (2) Prefiltration – PEG-based separation – Overnight standing (volume from 50 to 1000 mL).

CRediT authorship contribution statement

Dingnan Lu: Conceptualization, Investigation, Writing - original draft. Zhuangrong Huang: Conceptualization, Investigation, Writing - original draft. Jiayue Luo: Investigation, Writing - original draft. Xiaoqi Zhang: Writing - review & editing. Sha Sha: Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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