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Concentration techniques tailored for the detection of SARS-CoV-2 genetic material in domestic wastewater and treatment plant sludge: A review

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ARTICLE INFO
Editor: Teik Thye Lim

Keywords:
SARS-CoV-2
Wastewater-based epidemiology (WBE)
Concentration methods
Detection and quantification
Ultrafiltration
PEG precipitation

ABSTRACT

Upon the outbreak of COVID-19 pandemic, detection and quantification of SARS-CoV-2 genetic material in domestic wastewater have led to an increase in the efforts to define and implement the wastewater-based epidemiology (WBE). This application provides valuable information to define local contamination monitoring, emergence of COVID-19 and its variants and many other aspects to cope with and control the pandemic. WBE surveillance, however, requires several consecutive steps such as sampling, pretreatment and concentration of samples, and detection and quantification of SARS-CoV-2 genetic material in wastewater. In this review paper, the literature regarding to all these applications reviewed considering their advantages, disadvantages as well as their applicability. A specific emphasis was placed on the last step, detection and quantification since it covers the most critical procedure for concentrating the virus before measurement. Evaluation of the existing data indicating ultrafiltration, polyethylene glycol (PEG) precipitation and electronegative membrane filtration (ENMF) were the most promising techniques for concentration. The ongoing studies are proposed to be continued within the context of standard methods. Future research needs are delineated and suggestions are made for details.

1. Introduction

Wastewater surveillance approach was first introduced to track pharmaceuticals and personal care products in the aquatic environment as well as illicit drug use in a community in 2001 [1]. During the last two decades, this approach has been used to predict initial concentration of pharmaceuticals by means of the analysis of their pharmaceutically active substances or metabolites excreted by humans in wastewater since those substances have persistence against degradation to some degree and cause problems in the environment. This approach has also been adapted to monitoring and assessment of the occurrence and re-emergence of pathogenic viruses such as poliovirus, hepatitis virus in wastewater as an indicator of pathogenic diseases and called a wastewater-based epidemiology (WBE) surveillance. WBE surveillance has been established as a tool to detect poliovirus during the global eradication programme since 2003 [2].

Owing to its well-known success in environmental surveillance for enterovirus infections, the global water sector has mobilized to search and utilize the WBE for SARS-CoV-2 surveillance [3]. At the early stage of ongoing COVID-19 pandemic, scientific data have proved that genetic material of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) should be present and can survive in wastewater as well as in wastewater treatment associated sludge. Therefore, WBE surveillance of SARS-CoV-2 genetic signal in sewersheds has been recommended as a promising and applicable tool to track COVID-19 pandemic by the working groups joined to International Water Research Summit held on April, 2020 [3]. According to remarks of the summit participants, WBE SARS-CoV-2 surveillance data could provide information on potential applications summarized in Table 1.

Similarly, Christian Daughton who was pioneer of the WBE concept [1] defined the potential applications of WBE surveillance for assessment and management of COVID-19 pandemic by (i) a qualitative, (ii) a semi-quantitative, and (iii) a quantitative approach [4–7]. Qualitative approach is used to assess whether SARS-CoV-2 outbreak is present or not. Semi-quantitative approach is utilized to monitor acceleration or deceleration of infection within an individual community. Quantitative approach reveals nationwide status and trends of outbreak due to intercomparison of infection levels across communities [5].

For all aforementioned potential applications, the heart of WBE surveillance is the detection and quantification of SARS-CoV-2 genetic

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https://doi.org/10.1016/j.jece.2021.106296
Received 17 May 2021; Received in revised form 26 August 2021; Accepted 28 August 2021
Available online 30 August 2021
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material in wastewater via standard protocols. In these protocols, sampling guidelines and detection and quantification methods must be clearly defined. Such methods consist of several steps (Fig. 1).

As outlined in Fig. 1, the main steps of WBE surveillance of COVID-19 genetic signals in sewersheds are: the design of the sampling program to collect the most representative samples, pretreatment of samples and finally concentration of SARS-CoV-2 genome from wastewater by an efficient method that is of utmost importance to gather informative and reliable data. Among them, the concentration or enrichment of SARS-CoV-2 genetic material from the samples is quite an important step without which SARS-CoV-2 genetic material determination cannot be made since virus concentrations in wastewater are below the detection limits of the determination methods. Presently, there are several methods being used for the concentration of SARS-CoV-2 genetic material in wastewater. In addition, the concentration or enrichment of SARS-CoV-2 genetic material from samples is a crucial step (Fig. 3), VIRADEL, skimmed milk precipitation (Fig. 3), VIRADEL, skimmed milk precipitation (Fig. 3), or aluminium driven flocculation (Fig. 3) without which SARS-CoV-2 genetic material determination cannot be made since virus concentrations in wastewater are below the detection limits of the determination methods. Presently, there are several methods being used for the concentration of SARS-CoV-2 genetic material in wastewater as well as to demonstrate the usability of WBE surveillance for the analysis trends/changes in occurrence. Considering the fact that composite sample is more homogenous and representative than grab samples, this type of sample was collected from influent of waste material in wastewater via standard protocols. In these protocols, sampling guidelines and detection and quantification methods must be clearly defined. Such methods consist of several steps (Fig. 1).

As outlined in Fig. 1, the main steps of WBE surveillance of COVID-19 genetic signals in sewersheds are: the design of the sampling program to collect the most representative samples, pretreatment of samples and finally concentration of SARS-CoV-2 genome from wastewater by an efficient method that is of utmost importance to gather informative and reliable data. Among them, the concentration or enrichment of SARS-CoV-2 genetic material from the samples is quite an important step without which SARS-CoV-2 genetic material determination cannot be made since virus concentrations in wastewater are below the detection limits of the determination methods. Presently, there are several methods being used for the concentration of SARS-CoV-2 genetic material in wastewater. Therefore, the present review aims to compile and evaluate the data published so far on sampling, concentration, and extraction for the detection and quantification of SARS-CoV-2 genetic material in wastewater. Therefore, the present review aims to compile and evaluate the data published so far on sampling, concentration, and extraction for the detection and quantification of SARS-CoV-2 genetic material in wastewater and wastewater treatment associated sludge. In this paper “wastewater” will refer to domestic wastewater unless otherwise is stated.

### 2. Sampling steps from collection to analysis

At the early stage of COVID-19 pandemic, primary purpose of collection of wastewater sample was to prove the presence of SARS-CoV-2 in wastewater as well as to demonstrate the usability of WBE surveillance for the analysis trends/changes in occurrence. Considering the fact that composite sample is more homogenous and representative than grab samples, this type of sample was collected from influent of wastewater treatment plants (WWTPs) for these purposes. Duration of the composite samples were ranged from 4 to 24 h. Some composite samples were prepared as a volume-weighted mixture of a few grab samples. Considering composite samples can inform only average concentration of the SARS-CoV-2 genetic material in wastewater as well as to demonstrate the usability of WBE surveillance for the analysis trends/changes in occurrence. Considering the fact that composite sample is more homogenous and representative than grab samples, this type of sample was collected from influent of wastewater treatment plants (WWTPs) for these purposes. Duration of the composite samples were ranged from 4 to 24 h. Some composite samples were prepared as a volume-weighted mixture of a few grab samples. Considering composite samples can inform only average concentration of the SARS-CoV-2 genetic material in wastewater during the sample collection period while grab samples allow monitoring the variation of SARS-CoV-2 signals in sewage throughout a day as well as for specific time-periods, snapshot samples were also taken from influent flow of WWTPs (Fig. 2). Some grab samples were also withdrawn from pumping stations and manholes located near hospitals, university or retirement home residents to find out a relation between the detected SARS-CoV-2 genetic material and the clinically confirmed cases. The fate of SARS-CoV-2 virus through WWTP was monitored using sewage sludge samples taken from different units as grab samples representing snapshots.

Both composite and grab samples used in the studies are defined in Tables 2 and 3 that will be given and discussed in the forthcoming sections. As seen in Fig. 2, grab samples were preferred over 24-hour composite samples. It should be noted that the representability and usability of composite and grab samples as a part of WBE SARS-CoV-2 surveillance was compared and discussed in a limited number of studies [8]. Therefore, further research is needed to define the most practical and reliable sampling protocols for the detection and quantification of SARS-CoV-2 genetic material in wastewater in terms of sample type, frequency, duration, and sampling location. Among these parameters, selection of suitable sampling location is of great importance, since samples collected from WWTP and its units mostly are considered to be representative, while semi-quantitative and quantitative approaches require the analysis of samples taken from not only WWTP but also at different spots all sewer network.

Another critical issue of a sampling protocol is transportation and storage conditions for virus survival. All collected samples must be

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**Table 1**
The WBE SARS-CoV-2 Surveillance Data [3].

| Use of the WBE surveillance data for | Provide information on |
|-------------------------------------|------------------------|
| Analysis of trends/changes in occurrence | Early signals on occurrence/re-emergence Tracking the impact of medical and social interventions |
| Assessment of community prevalence | Monitoring disease prevalence in the community Identification of affected and unaffected areas by SARS-CoV-2 |
| Viral evolution | Emergence of SARS-CoV-2 variants and their locations |
| Risk assessment | Risk to utility workers exposed to raw sewage/sludge |

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![Fig. 1. Main steps of WBE surveillance of COVID-19 genetic signals in sewersheds.](image-url)
shipped on cold chain using either refrigerator, cold packs, or ice [9]. Depending on storage period, the samples were either refrigerated at 4 °C for a few days or frozen at – 20, or particularly – 80 °C after RNA extraction for longer storage periods. To date a few studies addressed the influence of storage temperature on survival of SARS-CoV-2 RNA in wastewater [10,11]. Wu et al. [11] reported that SARS-CoV-2 RNA in the pasteurized samples (at 60 °C) could be refrigerated at 4 °C for more than two weeks (to 15 days) without significantly degrading. Presence of SARS-CoV-2 RNA in the refrigerated wastewater samples for a day was also confirmed in the study of Baldovin et al. [10].

Data of PCR (RT-qPCR) assays targeting E-Sarbeco, N2, and norovirus GII demonstrated the stability of SARS-CoV-2 RNA in wastewater samples at – 20 °C and – 75 °C for the time interval of 84 days [10].

3. Pretreatment of samples

Earlier data indicated that SARS-coronavirus (SARS-CoV) isolated from the sputum of a patient could be thermally inactivated and incubation at 60 °C for 30 min provided complete elimination of SARS-CoV [12]. Therefore, SARS-CoV-2 virus in wastewater or wastewater treatment sludge samples were thermally inactivated by pasteurization on water bath at 60 °C for 90 min to ensure the safety of laboratory personnel and the environment [11,13–16].

As detailed in Tables 2 and 3 given in the next section, some detection and quantification analyses were initiated by centrifugation and/or filtration to discard bacterial debris and coarse particles in wastewater. Centrifugation speed ranged from 1840 × g to 10000 × g for a timespan varying between 5 and 45 min [17]. In a few studies, filtration through either 0.22 µm polyester sulfone membrane [11,15,18] or 100 µm filter [19] was applied as a first step to remove coarse particles. Some concentration applications based on precipitation were also performed without centrifugation or filtration step [20–24]. It should be noted that the discarding bacterial debris and coarse particles in the sample by a separation technique such as centrifugation and/or filtration may cause negative interference since the partition of SARS-CoV-2 virus into the coarse particle is neglected. To the best of our knowledge, until now just a few studies have paid attention to this negative interference [8,25–27]. In a study, WWTP sludge samples were agitated at 100 rpm for 30 min before centrifugation step, so as to release SARS-CoV-2 virus into the aqueous phase [27]. The contribution of partition into solids separated was determined by analysis of this fraction in studies of Kitamura et al. [8], D’Aoust et al. [25] and Westhaus et al. [26]. The data indicated that the loss of SARS-CoV-2 genetic material by solid separation should not be disregarded as discussed in the following section.

4. Concentration, extraction, detection and quantification of SARS-CoV-2 genetic material in wastewater

Upon entering the sewer systems via human excretions i.e. stool and urine, SARS-CoV-2 genome becomes diluted under the detection limit [28]. Therefore, primary concentration of SARS-CoV-2 genetic material in wastewater is an essential step prior to RNA extraction to obtain more quantitatively accurate and consistent results. A number of techniques have been developed and tailored to concentrate the enteric, particularly non-enveloped viruses such as adenoviruses, noroviruses, and polioviruses from water as well as wastewater since 1960s. These techniques can be listed as polyethylene glycol (PEG) precipitation, ultrafiltration, virus adsorption-elution (VIRADEL) and skimmed milk or aluminium driven flocculation. Among them, PEG precipitation and ultrafiltration have already been recommended for the environmental surveillance of poliovirus circulation by World Health Organization [2] as well-documented and reliable concentration techniques. VIRADEL has been accepted by US EPA as a standard method for detection Cryptosporidium and Giardia in water [29]. Nevertheless, at the early stage of COVID-19 pandemic, no data existed on the potential use of these concentration techniques in the detection and quantification of SARS-CoV-2. Therefore, intensive and remarkable efforts have been made to tailor these techniques for the concentration of enveloped viruses particularly SARS-CoV-2 from wastewater within the context of WBE SARS-CoV-2 surveillance.

RNA extraction is another important step following viral concentration. In this step, RNA is isolated and purified without damaging from the concentrated sample. Organic extraction, silica-membrane based spin column technology, or paramagnetic particle technology has been commonly employed for this purpose [30]. Organic extraction may often create RNA contamination with proteins, other cellular materials, organic solvents such as phenol-chloroform, or ethanol [30]. Both silica-membrane spin column and paramagnetic particle technologies provide total intact RNA with low levels of contamination from proteins and other cellular materials while they cause significant levels of genomic DNA contamination [30]. Automated extraction kits based on these three techniques are also commercially available. Recently a list of commercial kits to be confidently used for SARS-CoV-2 RNA extraction has already been published by the Centers for Disease Control and Prevention of US [31]. Tables 2 and 3 outline the techniques used for SARS-CoV-2 RNA extraction.

Classical reverse transcription-PCR (RT-PCR) and RT-real time quantitative PCR (RT-qPCR) have been extensively and successfully used for detection and quantification SARS-CoV-2 RNA material. These molecular techniques are “gold standard methods”. CDC-US has published a list of primers/probes targeting different SARS-CoV-2 genes for these techniques [32]. The reverse transcription droplet digital PCR (RT-ddPCR) is another molecular technique with high specificity and sensitivity. The performance of RT-ddPCR in detection and quantification of SARS-CoV-2 in wastewater has been also compared with RT-qPCR in a few studies [25,33,34]. Tables 2 and 3 summarize molecular techniques used in the studies together with primers/probes targeting different specific SARS-CoV-2 genes.

In the forthcoming subsections, the data published so far from all over the world have been compiled, discussed and evaluated by classifying them into four main groups, namely ultrafiltration, PEG precipitation; electronegative membrane filtration; skimmed milk or aluminium driven flocculation.

4.1. Ultrafiltration

To the best of our knowledge, until now ultrafiltration (UF) based on size exclusion has been the most tested technique for concentration of SARS-CoV-2 genetic material from wastewater [8,10,15,18,19,26,27,33–46]. In these studies, UF applications were performed using low volume of pre-centrifuged and/or filtrated wastewaters varied between
Table 2
Ultrafiltration for concentration of SARS-CoV-2 in wastewater and sewage sludge.

| Ref  | Sample  | Pretreatment | Concentration | RNA Extraction | Detection and quantification |
|------|---------|--------------|---------------|----------------|------------------------------|
| [43] | G.S. - WWTP | Centrifugation at 4500 × g; 10 min (4 °C) | CeUF (30 kDa; 4750 × g; 10 min) | RNeasy PowerMicrobiome Kit® | RT-qPCR; (nucleocapsid (N) gene) |
| [43] | G.S. - WWTP | Centrifugation at 4500 × g; 10 min (4 °C) | CeUF (10 kDa; 4750 × g; 10 min) | RNeasy PowerMicrobiome Kit® | RT-qPCR; (nucleocapsid (N) gene) |
| [43] | C.S. – 2 WWTPs | Centrifugation at 4750 × g; 30 min (4 °C) | CeUF (10 kDa; 3500 × g; 15 min) | RNeasy PowerMicrobiome Kit® | RT-qPCR; (nucleocapsid (N) gene) |
| [38] | G.S. - WWTP | Centrifugation at 4600 × g; 30 min (4 °C) | CeUF (10 kDa; 1500 × g; 15 min) | TRizol-chloroform- miRNeasy® Mini Kit | RT-qPCR; (nucleocapsid (N) gene) |
| [35] | 24 h-C.S. 5 WWTPs | Centrifugation at 4654 × g; 10 min | CeUF (100 kDa; 1500 × g; 15 min) | RNeasy PowerMicrobiome Kit® | qRT-PCR; (N gene (N1 – N3); E gene) |
| [37] | 24 h-C.S. 2 WWTPs | Centrifugation at 3000 × g; 30 min (4 °C) | CeUF (10 kDa; 1500 × g; 15 min) | ZR® Viral RNA Kit | RT-qPCR (CDC N1 and N2) |
| [10] | G.S.WTP-Es | Filtration (0.22 µm polyether sulfone) | CeUF (10 kDa; 4000 × g; 15 min) | QIAamp® viral RNA mini kit | RT-PCR (ORF1b-nsp14 and N) |
| [8] | G.S. 2 WWTP-MH | Centrifugation at 1840 × g; 30 min | CeUF (30 kDa; 1840 × g; 30 min) | Sediment: The RNeasy PowerSoil kit® | RT-qPCR Mix™ for the NIID,N2 assay |
| [15] | 24 h-C.S. 11 WWTP-Es-MH | Pasteurization (60 °C; 90 min) | CeUF (30 kDa) | ABIpure Viral DNA/RNA Extraction kit | SARS-CoV-2: RT-qPCR Kit™ for the CDC,N1N2 |
| [34] | G.S. WWTP-HR | Not applied | CeUF (100 kDa; 1500 × g; 15 min) | Magnetic silica beads | RT-PCR |
| [40] | G.S. and C.S. 2 WWTPs | Centrifugation at 3500 × g; 5 min | CeUF (30 kDa; 3500 × g; 20 min) | NucliSENS® easyMAG™ platform | RT-digital droplet PCR (RT-ddPCR) |
| [47] | 24 h-C.S. 2 WWTPs | Centrifugation at 4500 × g; 30 min (4 °C) | CeUF (50 kDa) | Nucleospin™ RNA Virus kit | RT-qPCR (N1 and N3) |
| [41] | Sample I | Centrifugation at 4500 × g; 30 min (4 °C) | UF filter plate (10 kDa) | Nucleospin™ RNA Virus kit | RT-PCR (ORF1ab, N and S) |
| [48] | G.S. 11WWTPs 2 MH | Centrifugation at 3200 × g; 45 min | CeUF (10 kDa; 3200 × g; 25–40 min) | QiAmp cador Pathogen Mini Kit | RT-qPCR |
| [39] | 24 h-C.S. WWTP | Centrifugation at 3200 × g | CeUF (10 kDa) | QiAmp™ Viral RNA MiniKit | RT-qPCR |
| [49] | G.S. 3WWTPs | Filtration (0.45 µm filter) | CeUF (10 kDa; 3000 × g; 30 min) | QiAmp™ Viral RNA MiniKit | RT-qPCR |
| [44] | 24 h-C.S. 6WWTPs Samples II | Centrifugation at 4863 × g; 30 min | HFUF (150 kDa) | QiAmp™ Viral RNA MiniKit | RT-qPCR |
| [19] | [45] | Centrifugation at 4750 × g; 30 min | HFUF (30 kDa) | QiAmp™ Viral RNA MiniKit | RT- PCR |
| | | Centrifugation at 3200 × g; 120 min | HFUF (30 kDa; 3000 × g; 30 min) | QiAmp™ Viral RNA MiniKit | RT-qPCR |
| | | Centrifugation at 3200 × g; 120 min | HFUF (30 kDa; 100 kDa; 3500 × g; 15–30 min; 10 °C) | QiAmp™ Viral RNA MiniKit | RT-qPCR |
| | | Centrifugation at 10 kDa; 3000 × g; 25 min | HFUF (10 kDa) | QiAmp™ Viral RNA MiniKit | RT-qPCR |
| | | Centrifugation at 4654 × g; 30 min | HFUF (10 kDa) | QiAmp™ Viral RNA MiniKit | RT-qPCR |
| | | Centrifugation at 4654 × g; 30 min | HFUF (10 kDa) | QiAmp™ Viral RNA MiniKit | RT-qPCR |
| | | Centrifugation at 4654 × g; 30 min | HFUF (10 kDa) | QiAmp™ Viral RNA MiniKit | RT-qPCR |
| [26] | 24 h-C.S. 6 WWTP-Es | Liquid phase: Centrifugation at 4700 × g for 30 min | Universal Cartridge Kit™ | One-step multiplex RT-qPCR | |
| | | Solid phase: Centrifugation at 4700 × g for 30 min | Ultra Centrifugation | One-Step RT-qPCR Kit | LightCycler®Multiplex RNA Virus Master |
| | | Wash the pellets with deionized water; Centrifugation at 4700 × g for 5 min | | | |
| [23] | G.S. 3WWTPs | Centrifugation at 4600 × g; 30 min | QiAmp™ Viral RNA Mini Kit | RT-qPCR assays | |
| [43] | G.S. - WWTP | Centrifugation at 10 000 × g; 20 min (4 °C) | Ultra-centrifugation | RNeasy PowerWater™ | RT-qPCR assays |

WW: wastewater; G.S.: grab sample; WWTP: influent from wastewater treatment plant; CeUF: centrifugal UF; WWTP: wastewater treatment plant; C.S.: composite sample; WWTP-E: effluent from wastewater treatment plant; MH: manhole; HR: a mixture of wastewater samples collected from COVID-19 patients hospitalized in the local university hospital (60%) and from retirement home residents (40%); Sample I: mixture of 3 grab samples withdrawn from influent of WWTP, influent of UASB process after mechanical treatment, effluent of the UASB process, aeration tank and effluent of WWTP; Samples II: Water/wastewater grab and composite samples (3 WWTPs, an untreated surface water and finished drinking waters from 3 treatment facilities.
The protocols applied to wastewater and sewage sludge samples to detect and quantify SARS-CoV-2.

| Ref | Sample | Pretreatment | Concentration method | RNA Extraction | PCR assays |
|-----|--------|--------------|----------------------|----------------|------------|
| [11] | 24 h-C.S. WWTP | Pasteurization (60 °C; 90 min) Filtration (0.2 µm) | PEG precipitation | TRizol-chloroform method | RT-PCR (CDC N1, N2, and N3) |
| [16] | 24 h-C.S. 7 WWTPs | Pasteurization (60 °C; 90 min) Filtration (0.2 µm) | PEG precipitation | TRizol-chloroform method | N.A. |
| [21] | G.S. WWTP | Not applied | PEG precipitation | Direct-zol RNA Miniprep™ kit | One-step real time RT-PCR, SARS-CoV-2 nucleocapsid gene (N1 and N2) |
| [8] | G.S. 2 WWTPs-MH | Centrifugation (1840 × g; 30 min) | PEG precipitation | Wv: RNeasy Microbiome kit® Sediment: RNeasy PowerSoil kit® Magnetic silica beads NucliSENS® easyMAG™ platform | RT-qPCR Kit™ for the CDC,NIID assay RT-qPCR Kit™ for the CDC,N1N2 assay RT-PCR |
| [34] | G.S. WWTP-HP | Not applied | PEG precipitation | NucliSENS® easyMAG™ platform | RT-digital droplet PCR (RT-ddPCR) RT-qPCR assays |
| [43] | G.S.-WWTP | Centrifugation (10 000 × g; 20 min; 4 °C) | PEG Precipitation | RNeasy PowerWater™ | RT-qPCR assays |
| [40] | G.S. and C.S. 2 WWTPs | Centrifugation (3500 × g; 5 min) | PEG precipitation | Spin column-based nucleic acid purification Acid guanidinium thiocyanate phenol chloroform extraction | RT-qPCR |
| [15] | 24 h-C.S. 11 WWTP-E; MH | Pasteurization (60 °C; 90 min) Filtration (0.22 µm polyethyler sulfones) | PEG precipitation | the AllPure Viral DNA/RNA Extraction kit | RT-qPCR using GENESIG COVID-19 kits |
| [54] | G.S. | Seeding with gamma irradiated SARS-CoV-2, PEDV and MgV | PEG Precipitation* Aluminium-driven flocculation | Nucleopin RNA virus Kit™ magnetic beads | RT-qPCR using |
| [18] | G.S. WWTP | Centrifugation (4500 × g; 30 min) Filtration (0.22 µm membrane filters) | PEG precipitation | NucléoSpin® RNA Virus kit | RT-PCR (ORF1ab, N gene, S gene) |
| [41] | Sample I | Centrifugation (4500 × g; 30 min) Filtration (0.22 µm membrane filters) | PEG precipitation | NucléoSpin® RNA Virus kit | RT-PCR (ORF1ab, N gene, S gene) |
| [46] | Sample III | | PEG precipitation for the sludge samples | Universal Cartridge Kit™ | One-step multiplex RT-qPCR |
| [56] | G.S. 2 WWTPs | Centrifugation (5000 × g; 5 min) | PEG precipitation | QIAamp Viral RNA Mini Kit® | qRT-PCR assays RT-nested PCR assays |
| [48] | G.S. 7 WWTPs | Centrifugation (3000 ×g; 45 min) | PEG precipitation | QIAmp cador Pathogen Mini Kit | RT-qPCR |
| [24] | 24 h-C.S. 2 WWTPs | Not applied | PEG precipitation | NucléoSENS® miniMAG™ extraction system | One-step RT-qPCR assays |
| [27] | Sample IV | Shaking (100 rpm, 30 min, 4 °C for to transfer viruses into liquid phase Centrifugation (7471 × g; 30 min) | PEG precipitation* | Thermo NanoDrop 2000c™ | The RT-qPCR assays conditions: |
| [14] | 24-hour C.S. 5 WWTPs | Pasteurization (60 °C; 90 min) Centrifugation (1200 × g; 30 min) | PEG-dextran separation method | the NucléoSENS miniMAG™ Semi- automated extraction system with magnetic silica | Nested RT-PCR Real-time RT-(q)PCR (the E gene of the SARS Betacoronavirus and the RdRp gene of SARS-CoV-2) RT-qPCR RT-ddPCR |
| [25] | Sample V | Settling(4 °C, a hour) Decantation the supernatant Filtration (1.5 µm glass fiber filter and 0.45 µm GF6 mixed cellulose ester (MCE) filter) | PEG Precipitation | The RNeasy Power Microbiome Semi- kit One-step real time RT-qPCR, SARS-CoV-2 nucleocapsid gene (N1 and N2) | }
40 and 400 mL at centrifugation speeds between 3000 × g and 4000 × g. In these applications, centrifugal filters with different nominal molecular weight limit (NMWL) ranging from 10 to 150 kDa were utilized [17].

Ahmed and co-workers [36] performed a study to validate the RT-qPCR assay targeting N protein (assay name (AN): N Sarbeco and NIID_2019-nCOV_N) for the detection and quantification of SARS-CoV-2 genetic material. Two concentration methods namely, electronegative membrane filtration (ENMF) and UF using CenTricon® Plus-70 centrifugal filters with a NMWL of 10 kDa (3500 × g for 15 min) were compared using raw wastewater samples. In their study, RT-qPCR inhibition, confirmed by Sketa22 RT-qPCR assay, did not observed for the RNA samples in the concentrated sample. The amplification efficiencies 116% and 108% were obtained for N_Sarbeco and NIID_2019-nCOV_N, respectively. Both concentration methods tested for the detection and quantification of SARS-CoV-2 genetic material yielded inconsistent RT-qPCR results. Jafferali et al. [31] selected UF, double UF and RNA samples in the concentrated sample. The amplification efficiencies adsorption-extraction-ENMF with/without pre-centrifugation to assess respectively. Both concentration methods tested for the detection and quantification of SARS-CoV-2 genetic material yielded inconsistent RT-qPCR results. Jafferali et al. [31] selected UF, double UF and adsorption-extraction-ENMF with/without pre-centrifugation to assess.

In the study of Sherchan et al. [37], *Pseudomonas* bacteriophage Mu recovery efficiencies of ENMF and centrifugal UF (CeUF) with a NMWL of 100 kDa were determined as 54% (n = 15) and 56% (n = 15), respectively. UF provided the detection and quantification of SARS-CoV-2 RNA as 3.1 × 10^3 - 7.5 × 10^3 gc L^-1 from wastewater samples. Additionally, SARS-CoV-2 RNA were not detected in the effluent samples taken before and after chlorine disinfection.

Forés, et al. [44] tested two UF-based concentration approaches utilizing a CeUF device with a cut-off of 30 kDa and automated concentrating pipette using hollow fiber polysulfone PVH high-flow pipette ultrafilter tips (CP-HFP) with a cut-off of 150 kDa. Their wastewater samples were seeded with Bacteriophage MS2 (as non-enveloped RNA viruses) and Murine Hepatitis Virus-A59 (as enveloped betacoronaviruses; MHV) before pre-centrifugation application. While CeUF (26.34 ± 22.71%) and CP-HFP (27.72 ± 24.46%) applications yielded comparable recovery efficiencies for MS2, CP-HFP (7.51 ± 6.14%) failed to concentrate MHV from wastewater samples (CeUF: 24.07 ± 14.48%). The molecular analyses performed to detect and quantify SARS-CoV-2 (N1 gene) in liquid phase and the pellets obtained during pre-centrifugation, resulted in 23% detection of the whole in the pellets. Their data proved the partition of SARS-CoV-2 genome to solids in wastewater and suitability of centrifugation UF for wastewater surveillance of COVID-19 as a primary concentration technique. In the study of Gerrity [19], the hollow fiber UF (HFUF) was used as a primary concentration technique. In addition, HFUF concentrates were subjected to CeUF or PEG precipitation as a secondary concentration method. A comparison was made based on BCoV and PMMoV for each sequence. PMMoV recovery efficiencies were about tenfold lower than those of BCoV for all tested alternatives. Similar BCoV recovery efficiencies of 54% ± 11% and 55% ± 38% were obtained from HFUF and CeUF, respectively. On the other hand, application of either PEG (0.34%) or CeUF (2.1% ± 0.87%) as secondary concentration methods dramatically deteriorated the BCoV recovery efficiencies. Since only 0.18% ± 0.09% BCoV was recovered by CeUF from the pre-centrifuged solid extraction, the partition of BCoV to solids in wastewater was concluded to be insignificant. Westhaus et al. [26] investigated the partition of SARS-CoV-2 genetic material into the solids in the wastewater. Their molecular assay were comparatively performed by OneStep RT-qPCR using Luna Universal Probe One-Step RT-qPCR Kit (NewEngland Biolabs) or LightCycler®Multiplex RNA Virus Master (Roche) and the CFX96 Real-Time System, with a C1000 Touch Thermal Cycler (Bio-Rad). The quantification assays were conducted on both liquid and solid phases obtained from the pre-centrifugation steps of influent and effluent samples. Comparison of SARS-CoV-2 RNA in the solid phase (25 ge mL^-1) with the liquid phase of the influent sample (1.8 ge mL^-1) proved the partition of SARS-CoV-2 genetic material to solids in wastewater. Their data also confirmed that (i) re-partition of SARS-CoV-2 genetic material from the solid to the liquid phase during conventional activated sludge processes and (ii) poor SARS-CoV-2 genetic material removal performances of the tested three conventional activated sludge processes. Medema et al. [35] detected and qualified SARS-CoV-2 RNA concentrated by centrifugal ultrafilters with a cut-off of 100 kDa from the wastewater samples. They performed the real-time RT-qPCR assays targeting the nucleocapsid genes (N1 – N3) based on CDC-US [32] and the envelope gene (E) according to [50]. A correlation between the detected gene copies and the number of clinically reported Covid-19 cases was evident in their study. A similar correlation was also pronounced in the studies of Martin et al. [39], Gerrity et al. [19] and Trottier [47]. Also, Baldwin et al. [10] was reported the WBE detection power as at least equal to 1 clinically confirmed case out of 388-822 inhabitants based on their hospitalization data and their SARS-CoV-2 genetic material detections obtained from RT-qPCR assays targeting genes ORF1b-nsp14 and N.

Evaluation of above mentioned data indicated that UF, particularly double UF, yielded more sensitive results than other concentration techniques comparatively tested. It could be modified as a standard method owing to its consistence performance, satisfactory recovery rate, no need of eluting [51], and very short operation time (max 1 h) [52].
Together with this promising capability of recovery SARS-CoV-2 from wastewater, it should be kept in mind some disadvantages such as requirements of back-flushing, addition of blocking solution such as glycine or beef extract, and immobilization [51].

4.2. Polyethylene glycol precipitation

Polyethylene glycol (PEG) precipitation is an aqueous polymer two-phase separation based on liquid–liquid partition ([53]). This concentration method and its slightly modified applications such as overnight incubation [27,34,46,54] or dextran addition [13,14] have implemented to enrich SARS-CoV-2 genetic material in wastewater as well as treatment sludge at pH values of 6.5–7.5. Fig. 3 schematically illustrates the main steps of PEG precipitation adapted and applied for the recovery of SARS-CoV-2 genome from wastewater in the recent researches. As can be seen in Table 3, PEG precipitation is the second most applied technique to concentrate SARS-CoV-2 genetic material from wastewater [8, 11,13–16,18,21,24,25,27,34,40,41,46,48,54–57].

PEG precipitation has been refined by Wu et al. [11] to concentrate SARS-CoV-2 genetic material in wastewater. According to their protocol, pasteurization at 60 °C water bath for 90 min is the first step for the inactivation of the virus. The pasteurized samples are filtered through a 0.2 µm membrane to separate bacterial cells and debris. The filtrate is precipitated with PEG 8000 and NaCl for viral enrichment. After the concentration step, RNA is extracted by TRIzol-chloroform approach. Reverse transcription and real-time PCR with SARS-CoV-specific primers follow the RNA recovery step. 24-hour composite wastewater samples taken from an urban wastewater treatment plant in Massachusetts were used to establish and verify their protocol. Findings proved the feasibility of SARS-CoV-2 detection in wastewater matrix. In another research, the same protocol was used to detect SARS-CoV-2 genome in the 24-hour composite wastewater samples spanning seven southern California wastewater treatment plants (WWTPs) in May 2020 [16]. The viral ranged from 0.65% to 13.4% and the family Coronaviridae were detected in only a few reads of viruses. Barril et al. [21] tested eleven different concentration methods compiled from the literature to find out the most suitable and effective recovery method for SARS-CoV-2. The protocol of Wu et al. [11] was one of their preliminary screening methods used to evaluate the recovery efficiency. Their study was performed on grab samples taken from an effluent of WWTP in Argentina. Screening tests carried out using Feline Calicivirus (FCV) indicated that the protocol of Wu et al. [11] failed to detect FCV (not detected) in their tested conditions and high recovery efficiencies could be obtained by only two concentration methods namely PEG precipitation developed by Lewis and Metallo [60,61] and aluminium poly-chloride (PAC) flocculation as described in the study of Randazzo et al. [22]. Their results showed that both methods were efficient to recovery SARS-CoV-2 from wastewater matrix, but PEG precipitation exhibited higher limit detection (4.3 ×10⁵ gc L⁻¹) than PAC flocculation (4.3 ×10⁵ gc L⁻¹). Hasan et al. [15] concentrated SARS-CoV-2 RNA using the protocol of Wu et al. [11] for detection in the wastewater samples. The sampling program was planned to monitor SARS-CoV-2 for eleven WWTPs and different point sources as manholes and pumping stations in the United Arab Emirates. SARS-CoV-2 genome was detected in 85% of influent samples collected from WWTPs and the viral load varied between 7.5 × 10⁵ and 3.4 × 10⁶ gc L⁻¹. They concluded that (i) the ultrafiltration coupled with the commercial RNA kits provided more sensitive readings than those of the PEG/TRIzol method and (ii) there was a correlation between the SARS-CoV-2 viral load in wastewater and the number of clinically confirmed cases. The protocol of Wu et al. [11] was also tested and compared with electronegative membrane filtration (ENMF) and ultrafiltration (UF) by Kitamura et al. [8]. The study was realized on grab samples withdrawn from a manhole and the influent of two WWTPs in a metropolitan area in Japan. The recovery efficiency of three concentration methods were also compared with the RNA extraction implemented on the residue of solid and semisolid materials.

**Table 3**

| Concentration Method | Recovery Efficiency |
|----------------------|---------------------|
| PEG precipitation    | 85%                 |
| PAC flocculation      | 75%                 |
| ENMF and UF          | 50%                 |

**Fig. 3.** PEG precipitation tailored for the concentration of SARS-CoV-2 genetic material from wastewater.
remained after the primary centrifugation (1840 ×g; 30 min) step (referred to sediment). All tested concentration methods that was real-
ized using the supernatant, yielded null or low SARS-CoV-2 RNA con-
centrations. On the other hand, SARS-CoV-2 RNA genetic material
extracted in the sediment was efficiently quantified in the range 1.6 × 10^2 – 1.3 × 10^3 ge L⁻¹. Therefore, the sediment obtained from the
first centrifugation step using manhole samples recommended as a more
sensitive source for the WBE SARS-CoV-2 surveillance. Similarly,
the recovery performances of all three concentration methods were
compared for nonenveloped (F-specific coliphage MS2) and enveloped
(Pseudomonas phage φ6) virus surrogates and the acceptability of the
concentration method resulted in the highest Pseudomonas phage φ6
recovery to SARS-CoV-2 was searched [40]. In the study, PEG+TRizol
method using LS reagent and UF + QIAampTM Viral RNA Mini Kit yield-
ed the φ6 recovery ratio of 29.8–49.8% and 6.4–35.8%, respectively.
When ENMFF+ QIAamp™ Viral RNA Mini Kit performed, MS2 and φ6
recoveries found to be variable and their recovery rates decreased with
increasing TSS and UV250 in wastewater samples. Hence, its application
to enveloped viruses as a concentration method was recommended only
for the samples with low TSS and UV250. On the other hand, by applying
PEG + TRizol method using TRizol LS reagent and CDC N1 and N3
assay, SARS-CoV-2 RNA was successfully detected from the wastewater
samples. Therefore, they concluded that the combination of PEG pre-
cipitation with an appropriate RNA extraction such as acid guanidinium
thiocyanate-phenol-chloroform protocol developed by Chomczynski
and Sacchi [62,63] as well as the selection of suitable molecular process
was of great importance to achieve satisfying recovery performance for
the enveloped virus. UF and PEG precipitation were also compared using
a grab sample taken after decantation unit of a WWTP in French Grand
Est region and clinical samples in a study of Bertrand et al. [34]. Re-
cover rates of UF were determined as 55.8 ± 46.9% and 64.0 ± 41.6%
for SARS-CoV-2-RdRp-IP4 and SARS-CoV-2-E, respectively. These rates
were higher than those of PEG precipitation (SARS-CoV-2-RdRp-IP4
(23.5 ± 15.0%) and SARS-CoV-2-E (45.0 ± 44.6%)). Based on their
findings they deduced that overall performance of the ultrafiltration
using the Centricon® 70-Plus 100 kDa device coupled with RNA extrac-
tion performed phenol-chloroformisoamyl alcohol purification
approach was superior to that of the PEG precipitation.

A comparative study for PEG precipitation and alum flocculation
methods was made by Perez-Cataluna et al. [54] using an urban
wastewater sample seeded with gamma-irradiated SARS-CoV-2, porcine
epidemic diarrhea virus (PEDV), and mengovirus (MgV). Mean quanti-
tation level for SARS-CoV-2 at 95% and 50% confidence intervals
was determined as 1.7 genome equivalent (ge) mL⁻¹. D’Aoust et al. [25]
quantified SARS-CoV-2 viral RNA N1 and N2 gene regions in post-girt
chamber influent solids (PGS) and primary clarified sludge samples
(PCS). The samples were collected from the Robert O. Pickard Envi-
ronmental Centre, Quebec Water Resource Recovery Facilities (WRRFs)
serving communities with low COVID-19 incidence and prevalence in
Canada. In all samples, SARS-CoV-2 RNA copies was concentrated by
PEG precipitation. SARS-CoV-2 RNA signals in PGS and PCS were
detected and quantified by RT-ddPCR and RT-qPCR. A comparison of
RT-ddPCR and RT-qPCR results indicated that similar quantifiable
concentrations of RNA for PGS could be obtained by both assays but
signal inhibition was evident for the analysis of PCS using RT-ddPCR.
The frequencies of detection of N1 and N2 gene regions were deter-
minal as 92.7% and 90.6% (n = 6) in PCS and 79.2% and 82.3% (n = 5)
in PGS.

PEG precipitation with a modification of the protocol of Jones and
Johns [65] was implemented to concentrate the SARS-CoV-2 in the
wastewater samples taken from WWTPs located in Ishikawa and
Toyama Prefectures, in Japan, [56]. SARS-CoV-2 RNA was detected in
the wastewater samples (21of 45 samples) by the qRT-PCR assays
(CDCN2, CDCN3, and NIH assays). In the study, (i) application of
multiple qRT-PCR assays was recommended due to its complementary
property (ii) composite samples found to be more representative since a
correlation between SARS-CoV-2 RNA in the samples and the number of
cases confirmed was observed, and (iii) an increase in the detection
frequency was observed when the total number confirmed SARS-CoV-2
was higher than 10 per 100,000 capita in each prefecture. La Rosa et al. [14] modified the PEG-Dextran Method according to WHO [2] for
the detection of SARS-CoV-2 genetic material in wastewater samples taken
from WWTPs in Milan, Turin and Bologna in Italy. Spiking experiments
were run using Alphacoronavirus HCoV 229E (ATCC VR-740) to control
the recovery efficiency of the concentration and extraction procedure.
These control experiments yielded an average recovery of 2.04 ± 0.70%.
SARS-CoV-2 genome was quantified using the nested RT-PCR and real-time RT-PCR assays. Sample inhibition was determined as from null to 49.0% using RT-(q)PCR. An overall agreement of 65.0%
was reported for the nested RT-PCR and real-time RT-(q)PCR results.

Of the reviewed studies, a few reported insufficient performance of PEG precipitation for SARS-CoV-2 genetic material recovery from wastewater. Application of PEG precipitation for SARS-CoV-2 genetic material to wastewater generally resulted in positive but inconsistent data. Sometimes the other concentration techniques such as UF or alum driven flocculation exhibited superior performance over PEG precipitation. The sample volume, presence/absence of organic or inorganic inhibitors, and coagulation period, positively or negatively affected the recovery performance by PEG precipitation since there was no consensus on the application basis of this concentration technique. Reviewed data revealed that further studies are required for standardization of PEG precipitation for WBE SARS-CoV-2 surveillance to obtain consistent results and maximize recovery efficiency particularly in the extremely diluted conditions corresponding to low viral loads such as re-emerge or waning periods of outbreaks.

4.3. Virus adsorption-elution (VIRADEL)

Virus adsorption-elution (VIRADEL) utilizes either electronegative or electropositive membrane filters to concentrate viruses from wastewater. Separation of virus particles is realized by attachment onto a cellulose nitrate membrane filter with pore size 0.45 μm via salt-bridging which is sometimes supported by addition of MgCl₂ or NaCl during electrogene membrane filtration (ENMF) [53].

Ahmed et al. [43] applied ENMF as a concentration technique to a raw wastewater with/without different pre-conditioning applications namely acidified (2 N HCl; pH 4) and MgCl₂-supported (a final concentration of 25 mM). RNA recovery performances of these alternatives were also compared with those of CeUF, 8000 precipitation and ultracentrifugation using murine hepatitis virus (MHV). CeUFs were carried out using two different centrifugal filters: with a NMWL of 10 kDa (at 3500 × g at 4 °C for 30 min.) and with a NMWL of 30 kDa (at 4750 × g at 4 °C for 10 min). ENMF without preconditioning yielded the MHV recovery efficiency of 60.5 ± 22.2 (mean ± SD%). Addition of MgCl₂ improved the MHV recovery efficiency from 60.5 ± 22.2–65.7 ± 23.8% corresponding to the highest recovery efficiency obtained in the study. The lowest MHV recovery efficiency (26.7 ± 15.3) was obtained from the ENMF with pre-acidification. Based on their data, ENMF with/without added MgCl₂ recommended as an efficient concentration method for WBE SARS-CoV-2 surveillance. In another study [59], electronegative membrane-vortex followed by RNA extraction using the QIAamp™ Viral RNA Mini Kit (M1) and membrane adsorption-direct RNA (M2) were applied to the wastewater samples taken from influent and secondary-treated effluent (before chlorination) of a WWTP in Japan. Final concentration of MgCl₂ for the influent wastewater sample and secondary treated effluent samples was selected as 25 mM. An excess MgCl₂ dose of 500 mM was also added to the influent wastewater sample. All concentration applications were realized using mixed cellulose-ester membrane with pore size of 0.8 μm. Based on the geometric mean concentrations of PMMoV, M1 (2.6 × 10⁶ cfu L⁻¹) found to be more efficient method than M2 (1.3 × 10⁶ cfu L⁻¹). As a result, electronegative membrane-vortex followed by RNA extraction using the QIAamp™ Viral RNA Mini Kit was suggested as an applicable concentration method for WBE SARS-CoV-2 surveillance. Similarly, ENMF using nitrocellulose membrane with pore size of 0.45-μm was determined to be more efficient concentration method than UF using a centrifugal filters with a cut-off of 10 kDa at 2200 × g for 20 min [66]. In this study, grab samples were taken from after coarse and fine screening units (influent), activated sludge recirculation (secondary sludge) systems and after the disinfection units (effluent) of two WWTWs located in Mexico. Molecular assays were performed using RT-(q)PCR assays targeting the S protein and N protein. SARS-CoV-2 RNA level of secondary sludge was higher than those of influent samples and SARS-CoV-2 was negative in all effluent samples. These data confirmed the importance of solid-liquid interaction of SARS-CoV-2 in wastewater.
Centrifugation at 10000 ×g for 1 h at 4 °C

Elution of the pellet by adding 0.25 N glycine buffer (1:9 v v⁻¹) to pH 9.5

After transferring the supernatant to a new 50 mL falcon tube, pH adjustment to 3.5.

Addition of pre-flocculated skimmed-milk solution (1% w v⁻¹) to the supernatant (0.05% w v⁻¹ of skimmed-milk)

Slowly stirring for 3 h at room temperature

Centrifugation at 8000 ×g for 30 min at 4 °C to precipitate the flocs

Carefully removal of the supernatant

Dissolution of the pellet in phosphate buffer solution (pH 7.2)

Storage at −70 °C for further analysis

Addition of PAC* at the ratio of 1:100 v v⁻¹ to the sample

pH adjustment to 6.0

Gently agitation for 30 min at room temperature

Centrifugation at 1700 ×g for 20 min

Re-suspension of pellets into 10 mL of 3% beef extract (pH 7.4)

Shaking for 20 min at 80 rpm

Centrifugation at 1900 ×g for 30 min

Re-suspension of the pellet in phosphate buffer solution (pH 7.2)

Storage at −70 °C for further analysis

Fig. 4. Skimmed milk flocculation tailored for the concentration of SARS-CoV-2 genetic material from wastewater.

Fig. 5. Aluminium driven flocculation tailored for the concentration of SARS-CoV-2 genetic material from wastewater (PAC: aluminium poly chloride solution of 9%).
5. Conclusions and further research needs

To date many research groups have proven the presence of SARS-CoV-2 in wastewater and applicability of WBE surveillance for COVID-19 pandemic. WBE SARS-CoV-2 surveillance approach seems to be a promising and plausible tool providing several information on (i) early signals on occurrence/re-emergence, (ii) monitoring disease prevalence in the community, (iii) identification of affected and unaffected areas by SARS-CoV-2, (iv) prediction of the level of infection in a community, and (v) emergence of SARS-CoV-2 variants and their locations [3]. This approach necessitates accurate detection and quantification of SARS-CoV-2 in wastewater. Although current efforts may provide a potential to define a representative sampling program and reliable molecular assays for accurate detection and quantification methods for measurement of SARS-CoV-2 in a wastewater, a standard protocol involving a step by step procedure directing from the collection of wastewater sample to quantification of SARS-CoV-2 has not been established yet. It should be pointed out that significant knowledge gaps exist on the sampling program including sample type, time, frequency, duration and location. Scientific data have revealed that the partition of SARS-CoV-2 into coarse particles separated by pre-centrifugation or filtration is significant for accurate quantification. A reliable pretreatment application considering the contribution of this partition should be developed to discard coarse particles. Comparative studies should be performed in order to clarify these critical points for general use listed in Table 1. Findings have already proved applicability of present concentration techniques for recovery of SARS-CoV-2 genetic material. Among the present techniques, UF, PEG precipitation and ENMF exhibit higher potential to be improved as standard methods. Their variability, reproducibility, and reliability of these concentration methods should be evaluated by intra and inter laboratory calibration studies in order to be approved as standard methods. Some of the methods have been shown to have the potential of being used as a measure of SARS-CoV-2 removal performance of the domestic wastewater treatment units. The studies in the field should be continued within the context of “standard methods”, so the unified procedures could be derived. As indicated in the literature evaluation some preconditioning applications may significantly enhance the sensitivity or detection level of the concentration methods. Therefore, the further researches are also expected to account for preconditioning processes and their basis of the application. In parallel with the development of the methods, comparative studies would be quite useful to assess the strength and weakness of the individual method against the properties of raw and treated wastewaters as well as treatment sludge. The literature reviewed in this paper did not specify the wastewater strengths except for a few studies. Therefore, a relation between SARS-CoV-2 genetic material and wastewater characteristics could not be established. Similar calibration studies should be done for both RNA extraction techniques and modern molecular assays. Primer/probes targeting different SARS-CoV-2 variants should also be defined for efficient and successful WBE surveillance.

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.

Acknowledgements

The authors extend sincere gratitude to all scientists, researchers, laboratory teams and other employees for their efforts who involved in the studies in this field. They also wish to acknowledge Istanbul Technical University for the acquisition of the latest literature regarding the COVID-19 pandemic used in this review.

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