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Wastewater-Based Epidemiology for Community Monitoring of SARS-CoV-2: Progress and Challenges

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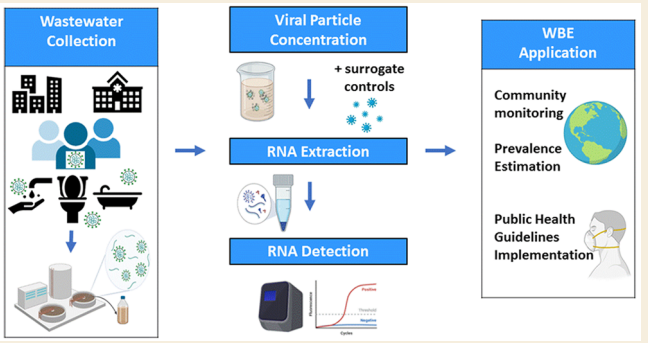
ABSTRACT: Wastewater-based epidemiology (WBE) is useful for the surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in communities, complementing clinical diagnostic testing of individuals. In this Review, we summarize recent progress and highlight remaining challenges in monitoring SARS-CoV-2 RNA in wastewater systems for community and environmental surveillance. Very low concentrations of viral particles and RNA present in the complicated wastewater and sewage sample matrix require efficient sample processing and sensitive detection. We discuss advantages and limitations of available methods for wastewater sample processing, including collection, separation, enrichment, RNA extraction, and purification. Efficient extraction of the viral RNA and removal of interfering sample matrices are critical to the subsequent reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for sensitive detection of SARS-CoV-2 in wastewater. We emphasize the importance of implementing appropriate controls and method validation, which include the use of surrogate viruses for assessing extraction efficiency and normalization against measurable chemical and biological components in wastewater. Critical analysis of the published studies reveals imperative research needs for the development, validation, and standardization of robust and sensitive methods for quantitative detection of viral RNA and proteins in wastewater for WBE.



KEYWORDS: *wastewater-based epidemiology, surveillance, SARS-CoV-2, Covid-19, RNA, RT-qPCR, controls*

1. INTRODUCTION TO WASTEWATER BASED EPIDEMIOLOGY OF SARS-COV-2

The current severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has severely damaged the state of global public health, economies, and overall societies.[1] This damage is unprecedented and worsening as the number of SARS-CoV-2 infections continues to rise in many countries. With new variants emerging and spreading globally, the control of SARS-CoV-2 remains challenging. Additionally, genetic variants, notably Alpha (B.1.1.7), Beta (B.1.351), and Gamma (P.1), are the main variants that are becoming the predominant strains in many countries. These variants have demonstrated increased transmissibility, disease severity, and mortality, and they may also reduce the effectiveness of current therapeutics and vaccines.[2−6] Recently, the Delta (B.1.617.2) variant is emerging and is responsible for the recent surge in local cases in many countries.[7] As of July 25, 2021, SARS-CoV-2 and new variants have infected over 192,000,000 cases and over 4.1 million deaths across the globe.[1]

SARS-CoV-2 is the seventh and newest strain of coronavirus from the *Coronaviridae* family to cause human illness.[8,9] It is an enveloped positive sense single-stranded RNA (+ssRNA) virus. The viral genome is enclosed within a viral capsid coated with a bilayer lipid envelope. The RNA genome contains ∼30 000 nucleotides, coding for four structural proteins (envelope (E), nucleocapsid (N), membrane (M), and spike (S) protein) and 25 nonstructural proteins.[10,11] The main route of transmission of SARS-CoV-2 in the population is either by direct contact with an infected individual or via respiratory droplets and aerosols generated during medical procedures.[12] Compared to the original SARS-CoV-2 strain identified in 2019, the Alpha, Beta, and Gamma variants spread more easily as they have changes to their S protein which is responsible for attaching and entering human host cells. Examples of S protein mutations include the N501Y and the E484K mutations.[2−5]

According to the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), the estimated incubation period of SARS-CoV-2 ranges from 1−14 days with a median period of 5−6 days between the time of

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infection and the onset of symptoms.[1,12,13] However, the incubation period may vary from person to person and the incubation period can be extended up to 27 days.[14] Thus, to contain the SARS-CoV-2 pandemic, accurate and timely diagnostic testing is critical to guide quarantine and self-isolation of patients and potential carriers. However, clinical testing is subject to local and regional policies which can restrict the availability and accessibility of clinical testing. Even within a given jurisdiction, clinical testing policies may change over time. When cases rose sharply, clinical testing was often limited to symptomatic patients and close contacts to manage the demand for testing and shortages in diagnostic resources. Furthermore, some individuals show unwillingness to be tested. One United States study estimated that only 32% of SARS-CoV-2-infected individuals sought medical care.[15] Presymptomatic, asymptomatic, and mild cases significantly contribute to the spread of SARS-CoV-2 and are mostly undetected during clinical surveillance. As a result, clinical testing for SARS-CoV-2 underestimates and inconsistently estimates the true scale of the pandemic. Public health officials must make critical decisions with limited surveillance data on guidelines for quarantine and lockdown restrictions for communities.[16] Wastewater-based epidemiology (WBE) can overcome some of these limitations by capturing data from most individuals (those using toilets, not including those using diapers) in the community.[17−21]

WBE is a concept where wastewater is monitored for genetic signals of SARS-CoV-2 to seek understanding of the presence and scale of infection in a community.[16−19] WBE is feasible because wastewater is a composite biological sample of the entire community. Clinical reports show that 16−73% of patients exhibit diarrhea in addition to respiratory symptoms.[22,23] When gastric, duodenal, or rectal epithelial cells are infected with SARS-CoV-2, infectious virions can be released into the rest of the gastrointestinal tract.[24] Thus, studies have estimated that SARS-CoV-2 sheds into the feces in 27−89% of infected patients.[22,23] Domestic wastewater also contains bath, shower, and laundry wastewater, meaning that respiratory secretions will also be present. To date, at least 21 countries have reported the presence of the SARS-CoV-2 RNA in wastewater systems including untreated wastewater, treated wastewater, and sludge (Table S1), but no infectious SARS-CoV-2 has been detected in wastewater and no case of SARS-CoV-2 transmission via contact with sewage or sewage-contaminated water has been reported.[25,26] In addition, it has been reported that conventional disinfection of water ensures inactivation of SARS-CoV-2.[27] Several WBE studies have reported the occurrence of local community transmission of SARS-CoV-2 before the first notified clinical SARS-CoV-2 case.[28−32] D'Aoust et al. observed increases of >400% of SARS-CoV-2 RNA in wastewater 48 h before the reported >300% increases in SARS-CoV-2 cases.[33] Two studies in the United States reported that WBE for SARS-CoV-2 foreshadowed new clinical case reports by 2−8 days,[21,34] and another study reported that viral titer trends in wastewater appeared 4−10 days earlier in wastewater than in clinical data.[35] Thus, testing SARS-CoV-2 RNA in wastewater can provide an early indication about the presence of SARS-CoV-2 in a community. These reports are mostly obtained from retrospective data analysis. Real time advanced warning can only be obtained by frequent wastewater sampling, rapid wastewater analysis, and result reporting to public health authorities. Furthermore, WBE is not limited to sampling wastewater at wastewater treat plants (WWTPs). Betancourt et al. and Gibas et al. have also studied university campus dormitories.[36,37] Localized WBE of SARS-CoV-2 may supplement other tools for managing opening of schools, workplaces, and high-risk communities.

Wu et al. estimated that 0.1−5% of the population of Massachusetts was SARS-CoV-2 positive based on the SARS-CoV-2 RNA level in sewage samples, whereas the clinical prevalence at the same time was only 0.026%.[38] Accurate estimation of population prevalence of infection depends upon knowledge about numerous factors that are not known, including rates of virus shedding, daily production of stool per capita, as well as percentage of SARS-CoV-2 patients who shed virus in their stool.[39] WBE can also potentially be used to monitor the effectiveness of public health interventions. A study in the U.K. reported that clinical testing underestimated the prevalence of SARS-CoV-2 and that large reductions in SARS-CoV-2 RNA in wastewater coincided with lockdown restrictions.[40] A study in the UAE also reported a drop in SARS-CoV-2 viral load in wastewater samples, which corresponded with the reduction of clinical cases reported in the population after precautionary measures implemented by the UAE government.[41] Furthermore, sequencing and phylogenetic analysis of SARS-CoV-2 RNA in wastewater allows for understanding sources of infection and transmission dynamics (the travel time and distribution of water and sludge through treatment tanks) as well as the detection of SARS-CoV-2 variants. A study in the U.K. used WBE to detect prevalent variants and identified the increasing dominance of the S protein G614 variant using whole genome sequencing (WGS).[40] Thus, WBE can provide important supplementary, objective information for public health officials to coordinate and implement actions to slow the spread of infection.

1.1. Overall Process for WBE of SARS-CoV-2

WBE monitoring of SARS-CoV-2 is challenging due to the lack of standardized procedures and methodologies.[21,42−44] The process generally includes wastewater sampling, virus concentration, RNA extraction and detection, and data interpretation. To make WBE studies more comparable, it is imperative to develop a standardized approach for WBE including a robust sampling design, methods with an improved viral recovery and RNA extraction efficiency from wastewater, and sensitive RNA detection.[39,45] To understand viral recovery and accurately determine the abundance of SARS-CoV-2 RNA in wastewater for WBE, a viral recovery control is required. This control is a non-SARS-CoV-2 virus (surrogate) spiked into a wastewater sample at a known concentration prior to sample processing. This control is used to determine the amount of virus lost during sample processing and the change of SARS-CoV-2 concentrations in wastewater over time. The recovery is measured as the amount of surrogate measured divided by the amount of surrogate spiked into the sample.

To evaluate the effect of viral recovery on the detection of SARS-CoV-2, an interlaboratory study was conducted using a variety of methods in different laboratories. The Water Research Foundation (WRF) funded a study involving 32 U.S. laboratories using a total of 36 individual standard operating procedures.[44] The laboratories were provided with two raw wastewater samples expected to contain native SARS-CoV-2 and spiked betacoronavirus OC43. The study reported that 80% of the recovery-corrected results of SARS-CoV-2 fell within a band of ±14.12 genome copies/L with higher reproducibility observed within a single operating procedure

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(standard deviation of 1.35 genome copies/L), although the spiked OC43 revealed a 7 log10 range of recovery efficiency. These results suggest normalizing the results using a recovery control is critical for presenting accurate results of WBE, and the same method or laboratory should be selected to track SARS-CoV-2 trends at a given facility. The Canadian Water Network (CWN) also organized an interlaboratory study among eight laboratories.[43] Wastewater samples spiked with low and high levels of gamma-irradiated inactivated SARS-CoV-2 and human coronavirus were provided to the eight laboratories. Overall, all eight laboratories accurately distinguished the high spike (1800 ± 200 gene copies/mL) from the low spike (18 ± 2 gene copies/mL) of inactivated SARS-CoV-2 with a 1.0 log10 range for both low and high spikes excluding nondetects. As expected, interlaboratory variability of the results was greater than intralaboratory variability. Thus, a consistent method within the same laboratory is required to explore temporal trends of SARS-CoV-2 in wastewater for a given system. Table S2 summarizes the SARS-CoV-2 studies that have included surrogates as recovery controls. However, these studies used different preparation procedures, and thus, the recovery results cannot be directly compared. Furthermore, many WBE studies of SARS-CoV-2 generally lack recovery controls and details on recovery experiments. Kantor et al. have further detailed the challenges in measuring recovery of SARS-CoV-2 from wastewater and highlighted recommendations for future studies.[42]

In the following sections, we will discuss reported methods for WBE of SARS-CoV-2. Figure 1 describes the multiple steps required, including sampling, virus concentration, RNA extraction and detection, and data interpretation for WBE of SARS-CoV-2. We also present different methods and requirements for controls. Understanding the advantages and difficulties in current methods may help to develop a robust protocol for the quantitative analysis of viral RNA in wastewater for WBE.

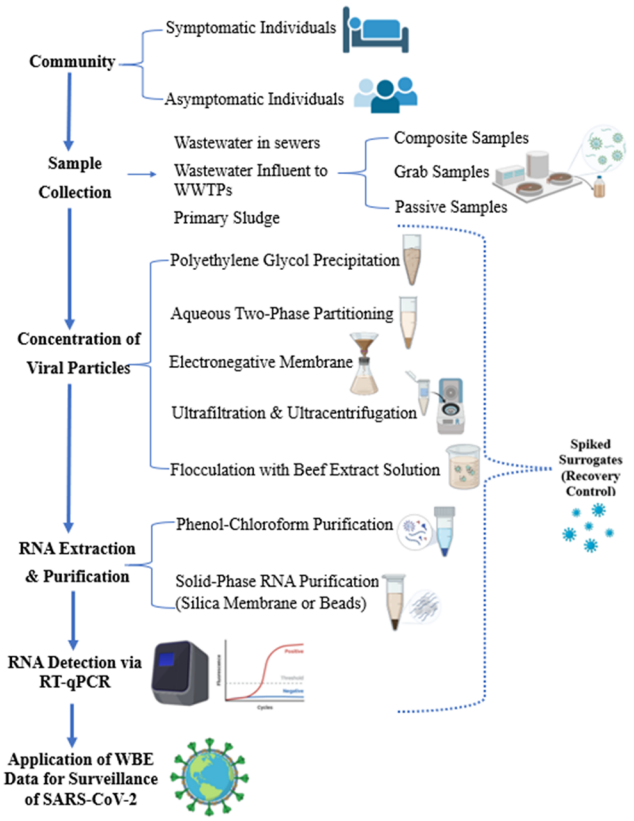

Figure 1. Various methods reported in the literature for sampling, virus concentration, RNA extraction and detection, and data interpretation for WBE of SARS-CoV-2

2. WASTEWATER SAMPLING

Two types of wastewater samples are suitable for surveillance of SARS-CoV-2: untreated wastewater and primary sludge. Studies have shown that changes in SARS-CoV-2 RNA concentrations in untreated wastewater and primary sludge samples correlate with clinically reported trends.[21,46,47] Untreated wastewater, sampled from WWTPs prior to any primary treatment, includes human waste, drainage from households containing saliva and shower/bath water, as well as nonhousehold sources. Wastewater has also been sampled directly from sewers to isolate a population from a specific building or area within a sewer network.[36,37] Primary sludge, sampled after the sedimentation process, contains more biological solids. It has been reported that the concentration of SARS-CoV-2 could be 2−3 orders of magnitude higher in primary sludge than in untreated wastewater.[21,48,49] Therefore, primary sludge samples may potentially reduce the sample volume required to concentrate and detect the virus. However, primary sludge samples also present challenges for detection of SARS-CoV-2 RNA. For example, the chemicals and treatment methods used at the WWTPs or the addition of recycled waste streams can significantly affect the performance of WBE laboratory procedures (viral concentration, RNA extraction, and detection). In addition, every WWTP will have its own dynamics about primary sludge sampling to allow it to be directly related to the daily temporal input of SARS-CoV-2 into the WWTPs. Furthermore, it is important to note that individuals in diapers will likely not be captured in the wastewater system. This has importance for studying wastewater samples from hospitals and nursing homes.

Grab and composite samples are commonly used for WBE. Grab samples represent the wastewater conditions at the exact time of collection and are highly influenced by daily fluctuations in wastewater flow and composition. Among all the publications pertaining to WBE summarized in this Review, less than one-quarter of studies used only grab samples (Table S1). Composite samples are collected by pooling multiple grab samples at a specified frequency over a set period, typically 24 h for wastewater surveillance. Composite samples represent the average wastewater characteristics during the period of collection and are used in most WBE studies (Table S1).[21,25,28,29,32,34,38−40,45,46,48] Sampling frequency depends on the purpose of WBE. Sampling once per week for monitoring the presence of SARS-CoV-2 in wastewater may be sufficient, while monitoring trends of infection and detection for early warning may require daily sampling. There is little data available describing how rapidly wastewater concentrations may change under various epidemic scenarios.[16,21]

The CDC recommends that samples be kept at 4 °C and processed within 24 h. Aliquots of samples should be kept at −70 °C and multiple freeze−thaw cycles should be avoided.[50] A few studies have demonstrated that SARS-CoV-2 is stable at 4 °C for 14 days.[51,52] However, one study found linear decay of SARS-CoV-2 RNA over 28 days at 4 °C in simulated wastewater samples containing SARS-CoV-2 from the

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nasopharyngeal swab of a SARS-CoV-2 patient.[53] It is recommended that samples be processed within 48−72 h to avoid the decay of SARS-CoV-2 RNA in wastewater.[39] Some studies have also stored samples immediately at −20 °C.[28,48,54−57] Hokajarvi et al. compared the stability of SARS-CoV-2 RNA in wastewater samples stored at −20 and −75 °C and found that wastewater samples stored at these two temperatures remained stable for 29−84 days.[53] However, further investigation is warranted to reduce RNA degradation and subsequent loss of signal as well as to improve the estimation of SARS-CoV-2 cases in the community.

Relevant details (time, date, location, flow rate, temperature, precipitation, water quality, pH, and community demographics) should be recorded during sample collection. Furthermore, the Water Research Foundation (WRF) recommends collecting 0.5−1 L for analysis and storage for future analysis.[58] As untreated wastewater contains various pathogens, researchers should follow CDC personal protective equipment (PPE) guidelines when collecting wastewater samples and pasteurize samples prior to analysis. For example, thermal treatment of wastewater samples (56 °C for 30 min or 60 °C for 90 min)[28,38] prior to analysis could be used because it reduces the infectivity of SARS-CoV-2 by over 5 log without affecting RNA integrity.[59,60]

3. CONCENTRATION OF VIRAL PARTICLES AND RNA FROM WASTEWATER

Wastewater contains complex chemical and biological compounds, which can cause low viral recovery with poor reproducibility, hindering the efficient concentration and detection of SARS-CoV-2. Low recoveries may result in false-negative results, preventing researchers and clinicians from deducing associations between waterborne viruses and specific outbreaks. Furthermore, extraneous particulate and dissolved constituents that get co-concentrated with the target may affect downstream RNA extraction and detection. Therefore, it is critical to selectively concentrate viruses and effectively reduce/eliminate nontarget materials. Concentration methods must be simple, fast, cheap, and capable of processing large volumes of wastewater. Commonly used concentration methods for WBE of SARS-CoV-2 include polyethylene glycol (PEG) precipitation, aqueous two-phase partitioning (PEG-dextran system), filtration with electronegative membranes (EM), ultrafiltration, ultracentrifugation, and flocculation with beef extract solution (Table S1).

3.1. Polyethylene Glycol (PEG) Precipitation

PEG precipitation, in combination with a high salt concentration, is a common method for concentrating viruses, DNA, RNA, and proteins from environmental samples. The high concentration of salts in solution neutralizes the charge of biomolecules. PEG mediates the aggregation and precipitation of biomolecules out of solution based on their molecular weights, with higher molecular weight precipitants being more efficient.[61] Currently, PEG 8000 and PEG 9000 are commonly used to concentrate SARS-CoV-2 from wastewater.[62,63]

PEG precipitation is relatively simple, inexpensive, and can handle large volumes of wastewater (∼1 L) to concentrate viruses from both liquid and solid matrices.[64] However, it is time-consuming (4−6 h) and may co-concentrate inhibitors, hampering subsequent RT-qPCR detection. Several studies have reported that PEG precipitation provided acceptable recoveries of surrogate viruses or SARS-CoV-2 (Table S2). However, the reported recovery ranges from 0.08 to 63.7%. The dramatic difference in recoveries is likely attributed to the different compositions of the samples and different surrogates used (Table S2). Some studies used the whole wastewater sample containing both the solid and liquid (supernatant) fraction, whereas others selectively used either the solid or supernatant fraction. Additionally, different viruses may vary in recovery as they differ in size, stability, surface characteristics, and solids-association. Four studies used the whole wastewater sample to concentrate viruses and reported a high recovery ranging from 44 to 63.7% (Table S2).[62,65−67] On the contrary, most studies using only the supernatant for concentration showed lower recovery ranging from 0.08 to 33.3%,[68,69] except for one study with a 57% recovery of the F-phage (Table S2).[70] This is a significant aspect to consider as studies have reported that the solid fraction of wastewater contains a higher abundance of SARS-CoV-2 than the supernatant.[25,27] Therefore, large particles and debris removed from the supernatant should not be discarded, but instead should be resuspended in beef extract solution or another solution to release the absorbed virus. The resulting supernatant could be combined with the supernatant from the initial centrifugation for subsequent concentration. In contrast to these studies, Wu et al. reported the opposite finding. They tested SARS-CoV-2 RNA signals in the unfiltered wastewater sample, filtrate collected by filtration through a 0.22 μm membrane, and solid materials on the filter after PEG precipitation. Unlike the unfiltered or solid fraction, the strongest and most consistent SARS-CoV-2 signal was detected from the PEG-precipitated filtrate fraction.[38] However, these results may be attributed to differences in the solid content of the wastewater samples.

PEG precipitation has been used to concentrate SARS-CoV-2 particles and RNA from wastewater samples in ten studies as summarized in Table S1. These ten studies have reported positive SARS-CoV-2 RNA signals in a portion of untreated wastewater samples. Positive detection of SARS-CoV-2 RNA in some treated wastewater samples was also reported,[32,69] indicating incomplete removal of SARS-CoV-2 RNA during wastewater treatment. Of these 10 studies, only two studies included both the supernatant and solid fractions of wastewater in their analysis.[32,71] The remaining studies pre-removed sediment and large particles, and only the supernatant or filtrate were used for subsequent concentration and detection of SARS-CoV-2. Because any viruses adsorbed onto the solid particles may have been discarded, the results may underestimate viral load. Therefore, it is imperative to include a control for measuring the recovery of a concentration method to account for the complexity and variability of wastewater. A control is necessary to compare the concentration of SARS-CoV-2 RNA in samples collected at different locations and different times. Even with a control being essential, uncertainty remains about the behavior of a spiked control versus the behavior of SARS-CoV-2 viral particles and RNA that is native to the wastewater sample but associated with solids.

3.2. Aqueous Two-Phase Partitioning (PEG-Dextran System)

The PEG-dextran aqueous two-phase system includes a PEG rich upper phase and a dextran rich lower phase. The phases can provide a protective environment for the biological activity of biomolecules and allow for selective partitioning. This technique has been widely used to concentrate proteins, membranes, viruses, enzymes, nucleic acids, and other

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biomolecules.[72] The partition coefficient of the virus depends on several variables such as hydrophobicity, molecular size, electrochemistry, molecular conformation of the virus, as well as solution conditions such as pH, buffer concentration, ionic strength, temperature, and virus concentration.[73] An optimized aqueous PEG-dextran two-phase separation method was developed for the concentration of Poliovirus from water samples and documented in the 2003 WHO Guidelines for Environmental Surveillance of Poliovirus.[74] Poliovirus is a nonenveloped virus, whereas SARS-CoV-2 is an enveloped virus. Regardless, this method has been used by research groups to concentrate SARS-CoV-2 from untreated wastewater (Table S1). La Rosa et al. enriched viral RNA using PEG-dextran from wastewater samples collected in Milan and Rome, where 50% (6/12) of samples positively detected SARS-CoV-2 RNA.[28] In the samples collected from Turin and Bologna, 37.5% (15/40) of samples tested positive.[75] To our knowledge, recovery controls using the PEG-dextran system for WBE of SARS-CoV-2 have not been mentioned, further highlighting the need for these studies to include recovery controls as part of the WBE process for consistent data acquisition and accurate interpretation.

3.3. Electronegative Membranes (EMs)

Electronegative membranes (EMs) have been widely used to concentrate viruses from wastewater samples. EM concentration is relatively simple and rapid (<40 min to process a sample) and cheaper (compared to ultrafiltration). Lu et al. reported less co-concentration of RT-qPCR inhibitors by EMs compared to PEG precipitation.[76] The common pore size of EM is 0.45 μM, which potentially allows for concentration of viruses in wastewater samples without the need for a precentrifugation step to eliminate large particulate matters and debris. Viruses are often negatively charged in wastewater samples. Acidification of wastewater samples and addition of cationic salts (Al^{3+} or Mg^{2+}) can facilitate the capture of viruses onto EMs.[77] At an acidic pH, the virus becomes positively charged and is electrostatically attracted to the EM. The addition of cationic salts allows for the formation of a salt bridge between the negatively charged virus and the negatively charged membrane, resulting in close contact between the virus and membrane. This close contact can increase hydrophobic interactions, facilitating adsorption. The viruses on EMs can then be eluted or directly used for extraction of RNA.[77]

Ahmed et al. have reported acceptable EM viral recoveries of 26.7−65.7% using the Murine Hepatitis Virus (MHV) surrogate, whereas the other two studies reported a lower recovery (0.96−6.6%) of the Beta Coronavirus (BCoV) and Bovine Respiratory Syncytial Virus (BRSV) (Table S2).[62,68,78] The large differences observed in these studies may be attributed to the different surrogates used as well as different wastewater matrices (Table S2). In addition, LaTurner et al. only used the sample supernatant to concentrate the virus and RNA, resulting in extremely low recovery of BCoV (0.96%).[68]

The recovery of EMs can also be affected by sample pretreatment. Ahmed et al. found that addition of $MgCl_2$ to 50 mL of untreated wastewater spiked with MHV resulted in an EM recovery of 65.7 ± 23% as compared to 26.7 ± 15% obtained by adjusting the sample pH to 4. It is intriguing that the study also found that the recovery of MHV with the EMs was 60.5 ± 22% in samples without the addition of either $MgCl_2$ or acid.[62] Ahmed et al. suggests that the original wastewater may have sufficient cations to facilitate the adsorption of virus onto the EMs. The addition of $MgCl_2$ further increased virus adsorption and recovery. A possible reason for acidification resulting in the lowest recovery is that acidification may have damaged the viral envelope of MHV. This may have caused the release of viral RNA and subsequent degradation by ubiquitous ribonuclease (RNase) present in wastewater. Studies have demonstrated that acidification of the sample may damage viral integrity and infectivity.[79,80] Considering that variability of recovery is large with all methods, EM filtration with $MgCl_2$ pretreatment is feasible for concentration of SARS-CoV-2 for WBE.

EMs have been utilized in concentrating SARS-CoV-2 from wastewater samples in at least five studies (Table S1). Ahmed et al. concentrated SARS-CoV-2 from wastewater samples pretreated with acid followed by direct RNA extraction. They successfully detected SARS-CoV-2 in 22% of their samples.[81] Another study by Kitamura et al. in Japan concentrated SARS-CoV-2 using an EM in both the supernatant and solid fraction of preacidified wastewater samples. In the supernatant, 19% (6/32) of samples were SARS-CoV-2 positive while 56% (18/32) of the samples were positive in the solid fraction.[47] Studies have also demonstrated the use of EM and samples pretreated with 2.5 M $MgCl_2$ to concentrate SARS-CoV-2 from wastewater samples. Sherchan et al. successfully detected SARS-CoV-2 in 13% of their samples, whereas Haramoto et al. reported positive detection in 20% of samples.[46,82] Gonzalez et al. pretreated samples by adding $MgCl_2$ in combination with acidification to a pH of 3.5 before concentration using an EM. SARS-CoV-2 was detected in 49% (98/198) of wastewater samples.[78]

3.4. Ultrafiltration

Ultrafiltration performed using widely available small centrifuges has gained popularity for concentrating viruses from water samples because of its fast processing and widely available equipment. Ultrafiltration is based on size exclusion with the pore size ranging from 5 nm to 0.1 μm.[83] The SARS-CoV-2 virion is approximately 100 nm in diameter. Several types of ultrafiltration units have been evaluated for recovery. Amicon Ultra-15 and Centricon Plus70 ultrafiltration units were used to recover the MHV surrogate from wastewater and provided recoveries of 56% and 28%, respectively.[62] The Centricon Plus70 unit has a greater surface area than the Amicon Ultra-15 unit, which was suspected to cause nonspecific adsorption of the MHV onto the membrane. However, centrifugal ultrafiltration units may become costly especially when needing to process many samples for WBE. The units can handle small volumes of samples (≤70 mL). Other drawbacks may include co-concentration of RT-qPCR inhibitors and clogging due to turbidity of wastewater samples.[62] Prefiltration of particulate matter can be performed to decrease turbidity, but careful handling is required to avoid the loss of viral particles, because particulate solids also contain virus.[25,47] Studies have compared ultrafiltration to PEG precipitation and found that ultrafiltration has a comparable recovery efficiency: 28−58% vs 44%[62] and 33.0%−42.6% vs 59.4−63.7%.[65]

Ultrafiltration has been commonly used to concentrate SARS-CoV-2 from wastewater samples (Table S1). To date, at least 15 studies have used ultrafiltration to concentrate SARS-CoV-2 from wastewater samples in 13 different countries. All these studies removed large particles by filtration or

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centrifugation prior to ultrafiltration concentration of the virus from wastewater samples and successfully detected SARS-CoV-2 in some samples. The positivity rate ranged from 13% to 100% (Table S1). Among these studies, only two studies to our knowledge have included a control to evaluate the recovery of virus by ultrafiltration. Medema et al. used ultrafiltration to screen for SARS-CoV-2 in wastewater pre- and post-pandemic declaration in The Netherlands. Using a F-specific phage as a recovery control, this study reported a recovery of 73 ± 50% (n = 16) and detected SARS-CoV-2 in 75% of their samples.[29] Gerrity et al. in the United States used BCoV as the surrogate and reported a recovery of 55 ± 38%. All the wastewater samples detected positive for SARS-CoV-2 (46/46).[84] These results suggest that ultrafiltration is a good choice for concentration of SARS-CoV-2 from wastewater when the viral titers and RNA concentration are high in the supernatant of wastewater samples.

3.5. Ultracentrifugation

Ultracentrifugation performed at 100 000g has been used for decades to concentrate viruses from environmental samples. Its application in WBE is limited due to requirements of an expensive ultracentrifuge, operator training, and low recovery. To our knowledge, six studies have used ultracentrifugation to concentrate SARS-CoV-2 from wastewater. In France, Wurtzer and co-workers carried out two studies using ultracentrifugation. The first study demonstrated 100% positive detection of SARS-CoV-2 in all untreated wastewater samples and 75% (6/8) positive detection in treated wastewater samples.[85] In the second study, they observed a reduction in SARS-CoV-2 detection in wastewater, which corresponded to the decrease in clinically reported cases following the implementation of lockdown measures.[86] Other studies (performed in Brazil, Chile, and Slovenia) also concentrated SARS-CoV-2 from wastewater using ultracentrifugation and reported a positive detection rate of 42−100% (Table S1). To determine the recovery efficiency, Green et al. used ultracentrifugation to concentrate inactivated SARS-CoV-2 from wastewater samples. Although the recovery was approximately 12%, this study reported positive detection of SARS-CoV-2 in 18/22 (82%) of their samples.[87] Further improvement of recovery using ultracentrifugation may be necessary for its application to accurately determine SARS-CoV-2 in wastewater for WBE.

3.6. Flocculation with Beef Extract Solution

Flocculation involves the addition of a chemical coagulant or acid to form large aggregates that can be easily separated. Thus far, two groups to our knowledge have used this method to concentrate SARS-CoV-2 from wastewater. Randazzo and co-workers used the aluminum flocculation-beef extract precipitation to concentrate SARS-CoV-2. In parallel, they used an enveloped surrogate virus (Porcine Epidemic Diarrhea Virus (PEDV)) and a nonenveloped surrogate virus (Mengovirus (MgV)) as recovery controls and obtained recoveries of 11 ± 2.1% and 11 ± 3.5%, respectively (Table S2). Randazzo et al. obtained an 80% (12/15) positive detection in the untreated wastewater samples collected in Valenica, Spain and a 50−64% positive detection for samples collected in Murcia, Spain (Table S1).[30,31] A study in the Czech Republic (Mlejnkova et al.) acidified and added beef extract solution to wastewater samples to directly precipitate SARS-CoV-2 and large particles. This study reported an 11.6% (13/112) positive detection of SARS-CoV-2 among all the collected wastewater samples.[88] Without a standardized recovery control or estimation, it is unclear whether differences in positive detection rates are due to matrix effect and/or viral load of the respective wastewater samples.

4. SARS-COV-2 RNA EXTRACTION FROM CONCENTRATED WASTEWATER SAMPLES

Extraction of the SARS-CoV-2 RNA requires multiple steps, including (a) viral lysis via breaking the viral envelope and capsid of the virions to release RNA, (b) RNA purification and removal of non-RNA material such as proteins, DNA, and other substances, and (c) RNA recovery by precipitation or elution.[89]

4.1. RNA Release and Maintenance of RNA Integrity

Viral lysis can occur through mechanical, chemical, or enzymatic approaches.[89−91] Chemical and enzymatic cell lysis is commonly used in commercially available kits. Chemical methods denature the lipid membrane or membrane proteins of target cells.[89] Examples of such chemicals include guanidine hydrochloride, guanidine thiocyanate, Triton X-100, and sodium dodecyl sulfate (SDS).[91] Enzymes are also used to break/hydrolyze the cell wall and membrane.[89,92] Examples of such enzymes include proteinase K, lysozymes, and lipases. It is crucial to maintain RNA integrity while releasing RNA because RNA is particularly susceptible to degradation in wastewater. RNA stability is affected by the pH, ionic conditions, and presence of ubiquitous RNases. Alkaline conditions hydrolyze RNA bases; therefore, a relatively low pH (~6.4) should be used to minimize RNA hydrolysis. RNA can also undergo strand scission when heated in the presence of divalent cations such as Mg^{2+} or Ca^{2+} at >80 °C for ≥5 min. To maintain the intact RNA whenever heating is used, a chelating agent, such as EDTA or sodium citrate, could be included in the extraction solution.[93,94] Importantly, RNase cleavage is the main cause of RNA degradation. RNases are abundant in wastewater and are difficult to completely remove or destroy. RNase is heat-stable and refolds following heat denaturation due to its small size and abundant disulfide bonds which help maintain the native structure.[95] 2-Mercaptoethanol and guanidinium hydrochloride or guanidinium thiocyanate are gold standard combinations to inactivate RNases. 2-Mercaptoethanol reduces the disulfide bounds of RNases, rendering it more susceptible to other denaturing agents such as guanidinium hydrochloride or guanidinium thiocyanate. The combination of guanidinium thiocyanate and 2-Mercaptoethanol are commonly used in commercial kits for extraction of SARS-CoV-2 RNA from wastewater samples (Table S1). Furthermore, to prevent RNase contamination, work areas must be kept clean and treated with decontamination solutions such as RNase AWAY.[90]

4.2. RNA Purification

After RNA release, the next step is to separate RNA from any non-nucleic acid materials such as proteins, salts, and other interfering substances in wastewater samples. Two purification methods using phenol-chloroform or solid phase extraction have been used to purify SARS-CoV-2 RNA from wastewater samples.

4.2.1. Phenol−Chloroform Purification. In phenol−chloroform purification (phenol/chloroform, 1:1), the proteins and DNA are removed by phenol and chloroform, while the RNA is kept in aqueous phase. The RNA in the aqueous phase is collected and precipitated from the supernatant by adding ethanol or isopropanol in combination with a high

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concentration of salt.[96] The resulting RNA pellet is washed, dried, and finally dissolved with RNase-free water. During purification, phenol can denature proteins rapidly while chloroform efficiently inhibits RNase activity. Thus, this phenol−chloroform mixture protects RNA during purification. TRIzol, a widely used commercial reagent for RNA extraction, applies the phenol-chloroform extraction method. This reagent separates RNA from DNA, proteins, and lipids after extraction with an acidic solution consisting of guanidinium thiocyanate, sodium acetate, phenol, and chloroform. This method is manually intensive, is time-consuming, and contains residual salts and organic solvents which can contaminate the extracted RNA, thus affecting downstream analysis. Two studies have used phenol−chloroform purification for the detection of SARS-CoV-2 RNA in wastewater. After PEG precipitation, Wu et al. added the TRIzol reagent containing phenol to the extract of SARS-CoV-2 RNA from wastewater samples, followed by the addition of chloroform to purify the extracted RNA. They positively detected SARS-CoV-2 in 10/14 samples collected at a major urban wastewater treatment facility in Massachusetts over the month of March 2020.[38] They estimated positive cases based on the presence of SARS-CoV-2 in wastewater, which was higher than clinically reported cases.[38] Hasan et al. also used the TRIzol reagent to extract RNA following PEG precipitation. However, using the same sample to compare, they reported detection results with a lower number of SARS-CoV-2 copies (2.6 gene copies/mL) for PEG/TRIZol than the ultrafiltration/ABIOpure Viral DNA/RNA Extraction kits (31.7 gene copies/mL).[41]

4.2.2. Solid-Phase RNA Purification. Solid-phase RNA purification methods using modified silica-based mini columns or beads are quick and efficient. In the presence of chaotropic salts such as guanidinium thiocyanate, sodium iodide, and guanidinium hydrochloride, silica can strongly bind to negatively charged RNA molecules through salt bridge formation by positive ions in the buffer and hydrophobic environment created by chaotropic salts.[97] Mini silica-based columns are commonly used for the extraction of SARS-CoV-2 RNA in wastewater samples (Table S1). Magnetic beads composed of a silica surface are also commonly utilized for RNA purification. After the magnetic beads adsorb the viral RNA, the RNA bound on the magnetic beads can be collected using a simple magnet and then washed to remove other matrices. The RNA is then eluted from the magnetic particles with an elution buffer. Compared to mini columns, this method eliminates the need for repeated centrifugation, vacuum filtration, or column separation. This procedure is simple and fast. As shown in Table S1, several commercial kits such as the NucliSENS miniMAG, NucliSENS easyMAG, and MagMax Viral/Pathogen kits use magnetic beads for extraction and purification of RNA for WBE of SARS-CoV-2.

Wastewater contains various compounds (calcium ions, bile salts, urea and related compounds, phenols, alcohols, polysaccharides, SDS, and other proteins including collagen, myoglobin, hemoglobin, and proteinases) that inhibit RT-qPCR reactions. The inhibitors present in wastewater are variable between wastewaters and can vary over time within a given wastewater. Thus, understanding and managing interferences is critical to establishing the credibility of quantitative WBE results. To enhance the removal of inhibitors during the purification step, other techniques are being developed. To check for the presence of matrix inhibition, a surrogate to control for RNA extraction is added into the viral concentrate and RNase free water in parallel prior to RNA extraction.[32,46,63,70,78] After RNA extraction and RT-qPCR, the presence of an inhibitor can be observed by comparing the cycle threshold (Ct) values obtained from the viral concentrate with the spiked surrogate and RNase free water with the spiked surrogate.[32,46,63,70,78] These controls have often been missed in the rush by some investigators to monitor SARS-CoV-2 in wastewater. Of the 38 studies regarding WBE of SARS-CoV-2, only 5 studies to our knowledge have investigated matrix inhibition. Sherchan et al., Kumar et al., and Gonzalez et al. used the *Pseudomonas* bacteriophage ø6, MS2 phage, and Hep G Armored RNA as matrix inhibitor indicators, respectively.[46,63,78] Fongaro et al. and Hata et al. used the Murine Norovirus as a matrix inhibitor indicator to determine the presence of RT-qPCR inhibitors.[32,70] Further studies are warranted to identify a proper surrogate and validate a standard control for RNA extraction and detection of SARS-CoV-2 RNA in wastewater to achieve practical and reliable WBE of SARS-CoV-2.

5. DETECTION OF SARS-COV-2 RNA USING REVERSE TRANSCRIPTION POLYMERASE QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

SARS-CoV-2 RNA can be specifically and sensitively detected using exponential amplification approaches. Feng et al. has critically reviewed the molecular methods for diagnostic testing of SARS-CoV-2.[98] RT-qPCR has been extensively validated and is currently the gold standard. RT-qPCR uses the reverse transcriptase enzyme to convert viral RNA to complementary DNA (cDNA) that is exponentially amplified and detected in real time after each cycle using the fluorescent reporters. The amount of PCR product is related to the amount of template in a sample. TaqMan probes are commonly used in RT-qPCR for clinical testing of SARS-CoV-2. The TaqMan probe contains a fluorescent reporter on the 5′ end and a quencher on the 3′ end. While the probe is intact, the quencher greatly reduces the fluorescence emitted by the adjacent reporter by fluorescence resonance energy transfer (FRET). During amplification, the probe anneals downstream from one of the primer sites on the target sequence and the probe becomes cleaved by the 5′ nuclease activity of the Taq DNA polymerase as the primer is extended. This cleavage results in the release of reporters emitting florescence. Specific hybridization between the probe and target is required to generate a fluorescent signal. Hence, the TaqMan probe provides good specificity. It also allows for the detection of several distinct sequences in one reaction tube by labeling different probes with different dyes. After the RT-qPCR reaction, a Ct value will be generated for each sample, which is inversely related to the amount of template in a sample. Based on a series of serial dilutions from standard with known copy numbers, a standard curve can be generated. For absolute quantification of the unknown SARS-CoV-2 copy number, the Ct value from the samples can be compared to the standard curve and extrapolated to determine the copy number in the wastewater sample. For quantification of SARS-CoV-2 RNA, SARS-CoV-2 RNA standards, not plasmid DNA standards, should be used to generate standard curve. SARS-CoV-2 RNA concentration estimates can be falsely skewed depending on whether plasmid DNA or RNA is used for standard curve generation. Chik et al. clearly demonstrated that using plasmid DNA as calibration standards can result in concentration values up to 2 orders of magnitude higher than when RNA is used.[43] This discrepancy may be because plasmid

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DNA is contained in a supercoiled formation during the early stages of RT-qPCR; therefore, there may be a falsely increased estimation of nucleic acids present in the reaction.[99]

The RT-qPCR targets of SARS-CoV-2 include the genes of structural proteins such as the envelope (E), nucleocapsid (N), and spike (S) protein as well as the RNA-dependent RNA polymerase (RdRP) gene and other regions in open reading frame 1ab (ORFab). The WHO provides a link to currently available methods for detection of SARS-CoV-2 on their web site (https://www.who.int/docs/default-source/coronaviruse/whoinhouseassays.pdf).[100] Table S3 summarizes these targets of SARS-CoV-2 used for RT-qPCR detection. The E and N genes are conserved in many coronaviruses, so they have been used as the targets for the detection of SARS-CoV-2 in wastewater. However, the S and RdRp genes are unique to SARS-CoV-2, and thus, they can be used to differentially detect SARS-CoV-2 from other coronaviruses.[9] The in-house assays are often designed to detect two or three regions of the SARS-CoV-2 genome. The detection of multiple genes can improve specificity and accuracy of the SARS-CoV-2 assays, eliminating the detection of other untested organisms or uncharacterized viruses. The WHO Foundation for the Innovation of Research Diagnostics (FIND) Web site (https://www.finddx.org/pipeline-2/) provides information on currently available RT-qPCR kits for detection of SARS-CoV-2.[101] These RT-qPCR assays are generally sensitive to detect as few as 1−10 copies/reaction;[98] however, the limit of detection (LOD) of RT-qPCR for wastewater is different depending on the sample matrix and the analytical processes employed. Currently, RT-qPCR is also the most commonly used method for detection of SARS-CoV-2 in WBE.

Table S1 summarizes the methods that were applied to investigate WBE of SARS-CoV-2. Sixteen WBE studies used a portion of or all the CDC N1, N2, and N3 segments of the N gene as the targets for the detection of SARS-CoV-2 in wastewater (Table S3). Fourteen studies used the RdRp and E gene as targets (Table S3), while other studies have used sequences developed by France, Japan, and China (Table S3). Some studies have used the following two kits which are not recommended by WHO, but are approved by the U.S. Food and Drug Administration for WBE of SARS-CoV-2. The Allplex system 2019-nCoV Assay kit has been used in one study by targeting the N, E, and RdRp genes,[69] while the TaqPath Covid-19 Combo kit has been used in two studies by targeting the ORF1ab, N, and S genes.[63,102]

Some studies have determined that when two or more targets are used for detection, the positive detection rate is consistent. For example, Wu et al. observed consistent detections using the N1, N2, and N3 genes, and Nemudryi et al. observed consistent detections using the N1 and N2 genes.[34,38] However, other studies have determined that the positive detection rate when multiple targets are used is inconsistent. Randazzo et al. found inconsistent positive detection rates when detecting N1(50%), N2(55%), and N3(64%) genes.[30] Medema et al. also reported inconsistent positive detection rates when detecting N1(58%), N2(0%), N3(33%), and E(21%) genes.[29] These differences may be due to the sensitivity of primer/probes used, interferences or inhibitors to PCR, and the instability of the virus and/or viral RNA in the wastewater samples from different sites. These factors will dramatically affect the positive detection rate when the abundance of SARS-CoV-2 is low in wastewater.

As new variants of SARS-CoV-2 including Alpha, Beta, Gamma, and Delta have become the predominant variants in many countries, it is imperative to develop RT-qPCR assays that can detect and differentiate the various SARS-CoV-2 variants for community surveillance. Graber et al. developed an assay using PCR probes to provide low cost, early detection of the Alpha variant in wastewater and demonstrated detection in a wastewater sample from Barrie, Ontario.[103] This is important because the number of Alpha variant related cases has exploded in Canada, with 45 000 new cases reported as of mid-April 2021. There is an urgent need to develop RT-qPCR assays that can specifically determine Beta, Gamma, and Delta variants in wastewater.

6. DATA INTERPRETATION

After SARS-CoV-2 RNA is quantified using RT-qPCR, the obtained Ct value can be converted to viral concentration per volume of unconcentrated wastewater or sludge sample based on a lab-generated standard curve. This conversion is related to the volume of template used in the RT-qPCR reaction and the overall recovery. For the comparison of samples collected at different times and locations, the SARS-CoV-2 concentration in the samples must be normalized by the recovery control of the concentration method. As shown in Table S1, after conversion, the amount of genomic copies/L in wastewater ranges from 0 to 4.6×10^7.

In addition, viral titers in sewage samples are determined by the concentration of fecal matter in the total flow of the WWTPs. However, the sewage flow rate is not stable and is impacted by several factors (e.g., dilution by stormwater for combined (sanitary plus stormwater) sewers, groundwater infiltration into leaky sanitary sewers, nondomestic contributions to the sewer network, diurnal variations affecting grab samples, etc.). When comparing viral levels across sampling locations over time, it is necessary to have a human fecal control, which is an organism or compound specific to human feces measured in wastewater to estimate human fecal content. The human fecal control concentration can be used to normalize viral concentrations. Several studies have shown that pepper mild mottle virus (PMMoV) is the most abundant RNA virus in human feces, and it is shed in large quantities into wastewater.[35,38] PMMoV is remarkably stable in the wastewater, and its concentration shows little seasonal variation.[104] Furthermore, like SARS-CoV-2, PMMoV is also a positive-sense single-stranded RNA virus, making it suitable as an internal standard to help control for wastewater sample-to-sample variability. These properties allow for the calibration of SARS-CoV-2 titers across samples. Wu and co-workers used PMMoV to calibrate the SARS-CoV-2 titer in wastewater samples.[35,38] To adjust for the SARS-CoV-2 viral titer for each sample, they calculated the deviation of PMMoV copies from the median PMMoV copies in all samples. The equation for calculating deviation factor is as follows: deviation factor = $1\hat{0}[k \times (\text{sample } C_T - \text{median } C_T)]$, where k is the slope of the standard curve and equals −0.2991 (amplification efficiency is 99.11% for the PMMoV primer set) based on the testing of PMMoV. Then the SARS-CoV-2 viral titers were divided by this deviation.

Based on the calibrated viral genomic copies/L in wastewater, the viral copies/person/day or the number of infected individuals in the tested community (prevalence) can be estimated. One study estimated the viral copies/person/day in the tested community in terms of viral genomic copies/L in

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wastewater.[78] In these studies, the SARS-CoV-2 load/person/day in a specific region was estimated using the RNA concentration in wastewater samples (copies 100 mL^{-1}), the population of the community, and the volume of wastewater entering the WWTP during a sampling event. This was calculated based on the following equation: LWWTP = CWWTP × $V \times f/P$, where LWWTP = population normalized SARS-CoV-2 loading to wastewater treat plant (WWTP) (copies per person in the catchment), CWWTP = RNA assay concentration in samples (copies 100 mL^{-1}), V = volume of wastewater entering wastewater treatment plant during sampling event (million gallons, MG), f = conversion factor between 100 mL and MG, and P = population within WWTP service area.

Three studies have estimated the prevalence of SARS-CoV-2 in a community based on WBE data.[41,81,84] The number of individuals infected by SARS-CoV-2 in a certain region was estimated using the measured viral loads in wastewater, the wastewater flow rate, the viral load in the stool of infected individuals, and the estimated daily production of stool per capita according to the following equation: number of individuals infected $N_{IF} = R_Q \times Q/F \times R_F\varepsilon$, where N_{IF} = estimated number of infected people in a specific location, R_Q = viral load in wastewater (viral gene copies/L) , Q = wastewater flow rate (L/day) , R_F = viral load in the stool (viral gene copies/g stool), F = daily production of stool per capita (g stool/capita·day), and ε = percent of COVID-19 patients who shed virus in their stool.

It is important to note that the SARS-CoV-2 shedding rates and related parameters used in these studies are not well documented, which can be expected to be variable from one community to another and to different stages of infection in each community.

7. DETECTION OF SARS-COV-2 PROTEINS

The detection of viral proteins may complement RT-qPCR as proteins may be present in higher numbers in samples and may have better stability.[10] Four structural proteins (nucleocapsid (N), spike (S), envelope (E), and membrane (M)) and 25 nonstructural proteins have been identified on the viral particle of SARS-CoV-2.[105] The copy numbers of M, N, E, and S proteins in each virus are reported to be ∼2000, ∼1000, ∼20, and ∼300, respectively.[10] These proteins serve as possible targets for SARS-CoV-2 detection. The N protein is conserved in the Coronavirus family and is expressed in abundance during infection; therefore, it is commonly used for detection. However, the detection of N proteins may be less specific and is limited for clinical testing because they are common in all members of the Coronavirus family. The S protein is an alternative target because it is divergent compared to other coronaviruses. The S protein is required for viral entry into host cells via the angiotensin-converting enzyme 2 (ACE2) receptors present on host cell surfaces. Therefore, it contributes to the virulence of SARS-CoV-2 making it a target of clinical significance.[106] Although the analysis of proteins can be more difficult, these techniques are not susceptible to nucleic acid variation in the primers or targeted regions. Hence, they can provide complementary information to RT-qPCR detection of viral RNA.

Proteins cannot be directly amplified; therefore, the detection of viral proteins requires indirect amplification or ultrasensitive techniques. Affinity ligands of high specificity are used to enable indirect amplification, for example, by using an ELISA or nucleic acid mediated assays. Affinity ligands for S and N proteins are available as monoclonal antibodies against the S protein and N protein of SARS-CoV-2.[98] Hence, antibody assays have been developed for the detection of viral proteins in clinical samples. A SARS-CoV-2 Antigen ELISA Kit was reported to detect recombinant SARS-CoV-2 N protein spiked in human serum (LOD of 1 ng/mL).[98] Neault et al. used oligonucleotide-linked antibodies unique to the viral protein target to develop a multiplex paired-antibody amplified detection (MPAD) method for the detection of the N protein of SARS-CoV-2. The linked oligonucleotides are used as templates for PCR amplification. Using a Western blot, they successfully used antibodies against the N protein to confirm the presence SARS-CoV-2 in wastewater samples. The amount of N protein was then measured using quantitative PCR. Compared to the RT-qPCR method for viral RNA, the MPAD method for the N-protein required fewer amplification cycles (Ct over 37 for RT-PCR vs 30 for MPAD on average).[107] Alternatively, Song et al. has described the use of aptamers as affinity ligands which recognized the receptor binding domain of the S protein. They developed two DNA aptamers that are potential affinity ligands for developing diagnostic assays.[108] Aptamers have not been applied to WBE of SARS-CoV-2. Further research will be required to develop affinity ligands with increased binding affinity and specificity, as well as to determine the potential application of these methods in SARS-CoV-2 WBE.

8. PERSPECTIVES AND OPPORTUNITIES

WBE has the potential to be a powerful and effective early warning tool for community-wide monitoring of viruses for public health surveillance; however, several challenges remain, providing opportunities for future research. With the implementation of proper quality assurance/quality controls, WBE can supplement but never replace clinical testing. While retrospective studies have demonstrated its potential for early warning, WBE for real time early warning cannot be realized without frequent (daily) sampling, rapid sample delivery, analytical turnaround, and reporting. The advancement of WBE highly depends on the performance of analytical processes. As the sample matrix of wastewater and sludge is extremely complicated and the concentrations of viruses and/or viral components are very low, especially at the start of a community outbreak, it is critical to extract the viruses and viral components from the sample matrix, concentrate, recover, and detect the molecular targets with high efficiency and sensitivity. Quantitative analyses for the purposes of characterizing trends require confirmation of consistent analytical performance in the face of many variables.

As previously discussed, many recent studies have focused on the RT-qPCR detection of the N, E, and S genes of the SARS-CoV-2 RNA that was extracted and concentrated from wastewater and sludge. However, complicated sample matrix materials from wastewater and sludge can inhibit RT-qPCR reactions, resulting in low sensitivity and/or false-negative detections. It is challenging to completely remove all potential RT-qPCR inhibitors and to efficiently extract the viruses and viral components from wastewater and sludge samples. It is important to examine RT-qPCR inhibition caused by the sample extract because of large variations in the sample matrix of wastewater and sludge collected at different locations. Only a few studies regarding WBE have included control experiments for evaluating the presence and effects of RT-qPCR

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inhibitors. Future studies should include sufficient controls so that possible false negative results due to RT-qPCR inhibition can be identified and corrected.

A variety of techniques and methods have been developed and tested for the collection and processing of wastewater and sludge samples. However, many published studies pertaining to WBE of SARS-CoV-2 did not include appropriate recovery experiments for estimating the efficiency of the collection, concentration, and extraction of SARS-CoV-2 and/or its RNA. The use of surrogate viruses as controls for determining recovery is necessary to verify consistent performance of a method across sample types and over time (e.g., changes in efficiencies of concentration of the targets and RNA extraction). Variable recoveries and extraction efficiencies have been reported from the use of different surrogate viruses added to the same wastewater samples. These variations could arise from differences in the size, stability, surface characteristics, and solids-association property of the different surrogate viruses. Recovery and extraction efficiency could also differ depending on the input concentration of the surrogate virus and the chemical characteristics of the wastewater sample. Despite the limitations and challenges, the use of a surrogate virus as a control and measurements of recovery should be included to help assess the validity of the WBE methodology. To compare the results between studies, researchers should use a consensus surrogate and a consistent input concentration of the surrogate virus.[43]

The number and characteristics of individuals contributing to a particular community sewer and wastewater flow may change over a surveillance period. This is particularly challenging for communities that have a high proportion of visitors. Tracking of all data sources that could explain anomalies by such variations is important. In all cases, appropriate normalization for the wastewater flow and the population surveyed is necessary to compare viral concentrations between wastewater samples over time. Studies have shown that easily measurable microbial and/or chemical components in human fecal materials could be used for the normalization purpose. However, many published studies have not included normalization against human fecal measurements, and thus, it is challenging to estimate the prevalence of infection in the community based on the apparent viral RNA concentrations in wastewater samples reported in these studies. Without an appropriate correction for the wastewater flow and the actual number of individuals contributing to the wastewater, the measured concentrations of SARS-CoV-2 RNA in wastewater alone cannot be meaningfully compared over time and between communities for assessing the levels of community infection. There is an urgent need to establish and standardize methods and procedures for the wastewater sample collection, concentration, extraction, and detection of SARS-CoV-2, including the emerging variants, to enable reliable and meaningful applications of WBE.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsenvironau.1c00015.

Comparison of the concentration, extraction, and detection methods used by various studies for WBE of SARS-CoV-2; common surrogates used in WBE of SARS-CoV-2 to estimate recovery efficiency; common primers and probes for the detection of SARS-CoV-2 (PDF)

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Notes

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