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Review

Minimizing errors in RT-PCR detection and quantification of SARS-CoV-2 RNA for wastewater surveillance

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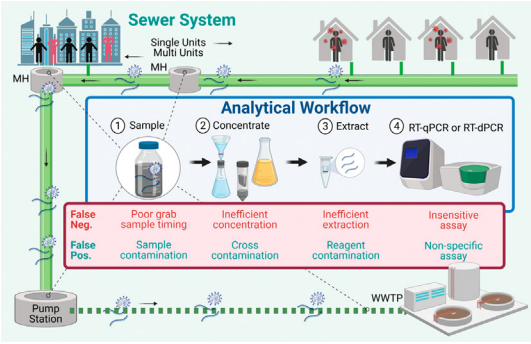
HIGHLIGHTS

- Harmonized QA/QC procedures for SARS-CoV-2 wastewater surveillance are lacking.
- Wastewater analysis protocols are not optimized for trace analysis of viruses.
- False-positive and -negative errors have consequences for public health responses.
- Inter-laboratory studies utilizing standardized reference materials and protocols are needed.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater surveillance for pathogens using reverse transcription-polymerase chain reaction (RT-PCR) is an effective and resource-efficient tool for gathering community-level public health information, including the incidence of coronavirus disease-19 (COVID-19). Surveillance of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) in wastewater can potentially provide an early warning signal of COVID-19 infections in a community. The capacity of the world's environmental microbiology and virology laboratories for SARS-CoV-2 RNA characterization in wastewater is increasing rapidly. However, there are no standardized protocols or harmonized quality assurance and quality control (QA/QC) procedures for SARS-CoV-2 wastewater surveillance. This paper is a technical review of factors that can cause false-positive and false-negative errors in the surveillance of SARS-CoV-2 RNA in wastewater, culminating in recommended strategies that can be implemented to identify and mitigate some of these errors. Recommendations include stringent QA/QC measures, representative sampling approaches, effective virus concentration and efficient RNA extraction, PCR inhibition assessment, inclusion of sample processing controls, and considerations for RT-PCR assay selection and data interpretation. Clear data interpretation guidelines (e.g., determination of positive and negative samples) are critical, particularly when the incidence of SARS-CoV-2 in wastewater is low. Corrective and confirmatory actions must be in place for inconclusive results or results diverging from current trends (e.g., initial onset or reemergence of COVID-19 in a community). It is also prudent to perform interlaboratory comparisons to ensure results' reliability and interpretability for prospective and retrospective analyses. The strategies that are recommended in this review aim to improve SARS-CoV-2 characterization and detection for wastewater surveillance applications. A silver lining of the COVID-19 pandemic is that the efficacy of wastewater surveillance continues to be demonstrated during this global crisis. In the future, wastewater should also play an important role in the surveillance of a range of other communicable diseases.

Contents

1. Introduction

Coronavirus disease-2019 (COVID-19), a global pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in 224,210,395 of diagnosed cases and 4,623,621 deaths globally by September 12, 2021 (Dong et al., 2020). SARS-CoV-2 is an enveloped respiratory virus with an RNA genome. Typical symptoms of COVID-19 may include any or a combination of the following: fever or chills, cough, shortness of breath or difficulty breathing, fatigue, muscle or body aches, headache, loss of smell/taste, chronic rhinorrhea, nausea or vomiting, and diarrhea in infected individuals (CDC, 2021a). Besides acute impacts, the virus can also cause debilitating late sequelae (CDC, 2020). COVID-19 is commonly detected in symptomatic individuals by collecting nasopharyngeal or throat swabs (Wölfel et al., 2020) and analyzing for SARS-CoV-2 RNA using reverse transcription-polymerase chain reaction (RT-PCR); however, antibody- and/or antigen-based tests are also used as a clinical surveillance tool (Ward et al., 2020). Notably, not all SARS-CoV-2-infected individuals develop the typical symptoms described above (Nishiura et al., 2020). Asymptomatic individuals may account for approximately 40–50% of infections (Oran and Topol, 2020). These individuals can transmit the virus to others in the community for >14 days (Gandhi et al., 2020; Oran and Topol, 2020).

Symptomatic and asymptomatic COVID-19 patients shed SARS-CoV-2 in their feces, saliva, nasal secretions, and urine (Cevik et al., 2021). A study found that analysis of fecal samples for SARS-CoV-2 can be more sensitive to infection than using samples from the respiratory tract, as feces carried a greater viral load, and positive results were consistently obtained over the course of the disease (Yuan et al., 2021). Intact or degraded SARS-CoV-2 viruses and RNA fragments reach sewer networks through toilets, showers, wash basins and sinks and can subsequently be detected in wastewater, both at the sub-catchment scale and in the influent to wastewater treatment plants (WWTPs). Recent studies have highlighted the potential of SARS-CoV-2 RNA surveillance in untreated wastewater to provide early detection or act as a warning system for COVID-19 circulation in a community (Ahmed et al., 2020a; Ahmed et al., 2020b; Kumar et al., 2020; La Rosa et al., 2020; Medema et al., 2020a; Miyani et al., 2020; Randazzo et al., 2020; Rimoldi et al., 2020; Wu et al., 2020; Chavarria-Miro et al., 2021; Gerrity et al., 2021). A caveat common to all these studies based solely on the detection of RNA (i.e., genetic material) is that wastewater surveillance measures the presence and quantity of SARS-CoV-2 RNA, not viable or infectious viruses from the individual building level to large metropolitan areas (i.e., WWTP influents).

This surveillance approach is currently used worldwide as a complementary tool for monitoring SARS-CoV-2 RNA in wastewater (Bivins et al., 2020). Wastewater surveillance programs are not novel or restricted solely to detection of COVID-19; wastewater surveillance for poliovirus and hepatitis A virus are past and current practices (Asghar et al., 2014; La Rosa et al., 2014; Alleman et al., 2021). SARS-CoV-2 wastewater surveillance is currently practiced in at least 55 countries (Supplementary Fig. 1). Over 260 organizations have announced that they are analyzing wastewater samples for SARS-CoV-2 RNA, with

samples being sourced from >2690 sites (including WWTPs, sewer catchments, and surface waters) (Naughton et al., 2021; https://arcg.is/1aummW).

Potential benefits of wastewater surveillance include detecting pre- and asymptomatic cases and the possibility of screening large populations with a single or few samples, independent of clinical testing availability and willingness (Thompson et al., 2020). With strategic facility-specific or sub-catchment sampling, wastewater surveillance can also identify COVID-19 transmission clusters within large geographic regions, and this information can facilitate focused and prompt action by local public health authorities (Stadler et al., 2020). Actions arising from positive results from wastewater surveillance might include public announcements to encourage individual testing, increasing the availability of testing, establishing fever clinics in specific locations, implementing stay-at-home directives, or mandating other mitigation measures such as face masks, social distancing, and capacity limits to reduce transmission (Randazzo et al., 2020). An important aspect of wastewater surveillance is the ability to gather data rapidly and cost-effectively from a large population of thousands of individuals, with enough potential sensitivity to detect a few infected individuals contributing SARS-CoV-2 RNA to the local wastewater system (Michael-Kordatou et al., 2020). This "pooling concept" can be particularly important for regions with low clinical COVID-19 testing rates, minimal resources, or unknown cases.

Wastewater surveillance of SARS-CoV-2 RNA involves a sequence of steps (Fig. 1), commencing with sampling from a location within a wastewater system (i.e., maintenance holes, pumping/lift stations, or facility influent) or, for near-source surveillance, (i.e., outlets of buildings and vessels), followed by virus concentration, RNA extraction, SARS-CoV-2 RNA detection (RT-PCR; positive and negative detection) and quantification (RT-qPCR; based on a standard curve or RT-dPCR; digital PCR without the need for a standard curve), and data analysis and interpretation. Many factors can affect the overall method performance and the reliability of the results (Ahmed et al., 2020d; Bustin et al., 2009; Medema et al., 2020b; Pecson et al., 2021). An optimized RT-PCR assay can theoretically detect a single SARS-CoV-2 RNA fragment (Bustin et al., 2009). Such a high sensitivity is particularly useful for SARS-CoV-2 surveillance in wastewater where the RNA target concentration can be extremely low (Ahmed et al., 2020a; Haramoto et al., 2020). However, increased analytical sensitivity coupled with poor RT-PCR assay specificity can increase the likelihood of false-positive errors. In this instance, a false-positive occurs when an RT-PCR result is positive for SARS-CoV-2 RNA, whereas it is absent in the wastewater sample.

While false-positive errors can be monitored, minimized, and eliminated through strict adherence to best QA/QC practices with appropriate controls, false-negative errors are more difficult to identify and mitigate. A false-negative error occurs when RT-PCR fails to detect SARS-CoV-2, but the target RNA is actually present in the wastewater sample. False-negative errors may arise from various factors inherent to a particular wastewater sample, including how it is collected, transported, and stored (i.e., freezing), the low abundance of the RNA target in wastewater, or the degradation of the RNA target in the sewer collection system. Other factors are related to sample processing and analysis, such as poor virus recovery and/or low effective sample volume (ESV) assayed, low RNA extraction efficiency, PCR inhibition in the RT-PCR assay, and poor assay sensitivity (Ahmed et al., 2020d; Medema et al., 2020b; Michael-Kordatou et al., 2020).

In this study, we discuss: (i) the implications of SARS-CoV-2 false-positive and false-negative errors for public health responses, (ii) factors that may cause false-positive and false-negative errors of SARS-CoV-2 RNA in untreated wastewater across the entire wastewater surveillance process, and (iii) strategies that can be used to minimize the likelihood and subsequent impacts of false-positive and false-negative errors on stakeholder confidence in SARS-CoV-2 wastewater surveillance. We provide a basic scientific framework that can aid in setting and developing appropriate QA/QC measures around sampling and laboratory workflows regarding wastewater surveillance for SARS-CoV-2 and other pathogens.

2. Definition of false-positive and false-negative errors in the wastewater surveillance context

Typically, for an analytical test such as RT-PCR, false-negative and false-positive errors are interpreted only regarding the presence or absence of the relevant analyte in the sample, as mentioned above. However, during wastewater surveillance, such as for SARS-CoV-2, the RT-PCR results are interpreted as relevant to two outcomes: (i) the presence or absence of SARS-CoV-2 RNA in a wastewater sample and,

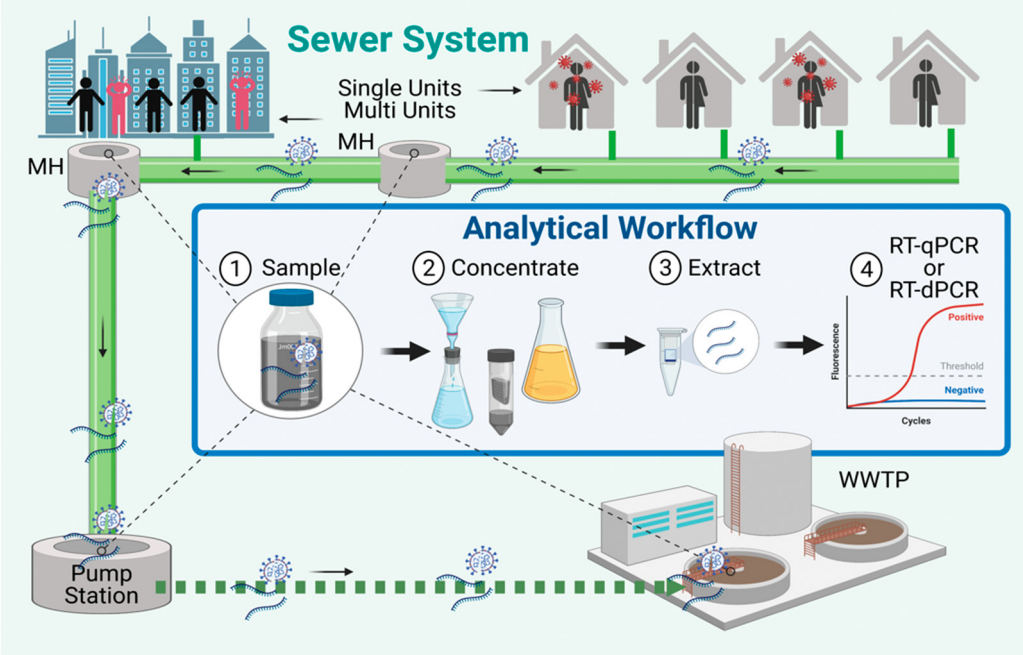

Fig. 1. A conceptual diagram of a typical sewer catchment with sampling points and analytical workflow of SARS-CoV-2 RNA surveillance in wastewater; MH: Maintenance hole.

subsequently, (ii) the presence or absence of SARS-CoV-2 infections in the community. Hence, two layers of error that must be considered. The first layer, which we termed RT-PCR false-positive or RT-PCR false-negative is pertinent to whether SARS-CoV-2 RNA is present or absent in a wastewater sample, and whether RT-PCR test results accurately reflect that status. The second layer, termed wastewater surveillance false-positive or false-negative, is pertinent to whether SARS-CoV-2 infection is present or absent in the community. In the ideal application of wastewater surveillance, an RT-PCR positive indicates that SARS-CoV-2 RNA is present in wastewater. Subsequently, an infection is present (via clinical testing) in the community, which is a true positive for both RT-PCR and wastewater surveillance. Or, conversely, an RT-PCR negative indicates that SARS-CoV-2 is absent in wastewater. Subsequently, SARS-CoV-2 infection is absent from the community, corresponding to a true negative for RT-PCR and wastewater surveillance.

Concordant and discordant false-negative and false-positive errors can occur for both layers of interpretation. For example, SARS-CoV-2 infection could be present in the community, but a wastewater sample might not contain detectable levels of SARS-CoV-2 RNA, therefore, an RT-PCR negative result would be an RT-PCR true negative but a wastewater surveillance false negative. Or, SARS-CoV-2 infection could be absent in the community, but the wastewater sample contains SARS-CoV-2 RNA owing to cross-contamination; therefore, an RT-PCR positive result could be an RT-PCR true positive but a wastewater surveillance false positive. The occurrences of false-positive and false-negative errors are not well documented in wastewater surveillance applications. Such errors can lead to erroneous decision making and inefficient expenditure of resources by public health officials.

3. Factors contributing to SARS-CoV-2 RT-PCR false-positive errors in wastewater surveillance

False-positive errors can arise from the introduction of contamination in field samples (Westhaus et al., 2020), the laboratory (Tahamtan and Ardebili, 2020), or even in the assay reagents (Mögling et al., 2020). They can also arise from poorly designed RT-PCR primers and probes that detect RNA not associated with SARS-CoV-2 (Westhaus et al., 2020). This section reviews potential sources of false-positive errors, which are typically identified by including negative controls at various stages of the sampling and laboratory workflows, and by sequence confirmation of amplified products.

3.1. Contamination in the field

The wastewater collection process presents multiple opportunities for sample contamination, which could result in false-positive errors. SARS-CoV-2 RNA can persist in untreated wastewater and tap water for approximately 10 days, at 37 °C or approximately 30–60 days, at 4 °C (Ahmed et al., 2020c). In regions with a high prevalence of COVID-19, SARS-CoV-2 RNA has been found on various surfaces in public places (Gholipour et al., 2020). Therefore, SARS-CoV-2 RNA can potentially accumulate on the surface of sampling equipment (e.g., tubes attached to autosamplers and, contaminated sampling bottles) and gear (e.g., telescopic sampler, pump, and multi-meters). Consequently, wastewater samples could become contaminated due to inadequate cleaning between equipment uses. A false-positive error can also occur when handling multiple samples at an individual collection location (near the influent or maintenance hole etc.) due to gloves and clothing being contaminated by accidental splashes or possibly aerosols. Improper storage during transport to the laboratory also could cause cross-contamination. Ice inadvertently contaminated by positive samples could potentially transfer SARS-CoV-2 RNA to another sample if the bottle lids were not properly sealed. Sampling guidance from government agencies such as USEPA, describe QA/QC protocols that are vital for assessing such contamination, including sample container cleanliness, equipment to be used, and sample negative controls (USEPA, 2017).

3.2. Contamination in the laboratory workflow

Sample-to-sample contamination may also occur in the laboratory during sample processing and storage (i.e., in a cold room or a refrigerator), sample concentration using the same equipment, RNA extraction from positive samples, or from positive control material (i.e., synthetic fragments, gamma-irradiated, or heat-inactivated SARS-CoV-2 reference materials), and/or RT-PCR amplification steps and amplicons generated from previous SARS-CoV-2 positive samples. Also, cross-contamination from an analyst's personal protective equipment to laboratory surfaces has been identified using RT-droplet digital PCR (RT-ddPCR) assays (Lv et al., 2020).

A shortage of skilled laboratory personnel due to the high demand for analysts has resulted in less experienced individuals working in laboratories, which could exacerbate cross-contamination issues and false-positive errors (Albano et al., 2020; Giri and Rana, 2020). Such issues may affect clinical diagnostics and wastewater surveillance. Some laboratories are analyzing numerous wastewater samples (up to 100 or more/week) for routine surveillance, and the testing rates may increase. As interest grows in routine wastewater surveillance, public health units that utilize the data for management actions demand faster results output. Both factors (sample numbers and rapid output) place increased pressure on laboratory personnel, which may lead to increases in errors potentially resulting in increases in contamination and false-positive errors.

3.3. Commercial reagent contamination

False-positives can also originate from the RT-PCR reagents themselves or due to poor assay specificity. The notion that commercial PCR reagents can contain unwanted contamination is not new. There are numerous reports of *E. coli* fragments in DNA polymerase preparations (Koponen et al., 2002) and human mitochondrial DNA fragments in deoxyribonucleotide preparations (Wilson et al., 2016). These contaminants are introduced during the manufacturing of reagents and, in some instances, can lead to false-positive errors. False-positive errors of SARS-CoV-2 assays in clinical specimens have been reported in Europe, where several laboratories have identified commercial primer and probe reagent batches contaminated with synthetic control materials (Mögling et al., 2020).

3.4. Assay specificity

Improper selection of SARS-CoV-2 target genes or design of primers and probes that are not specific to the target may also lead to false-positive errors. Previous testing of seven different RT-qPCR primer and probe sets on clinical samples found that all the assays, including the US CDC N1, N2, and N3 assays, were “highly specific” for SARS-CoV-2 with no observed cross-reactivity (Nalla et al., 2020). However, environmental sample matrices are much more complex and dynamic presenting unique challenges for RT-qPCR assays compared to clinical samples, and observations of specificity have not been consistent for wastewater testing. For example, Westhaus et al. (2020) noted that certain SARS-CoV-2 RT-PCR assays targeting the RdRP or M-gene, may also detect other endemic human coronaviruses, leading to false-positive errors for SARS-CoV-2. Gerrity et al. (2021) reported an instance of cross-reactivity between the E_Sarbeco assay and seeded bovine coronavirus. Pecson et al. (2021) reported significant cross-reactivity between a human coronavirus OC43 assay and seeded bovine coronavirus—two common SARS-CoV-2 surrogates used as sample process controls. Thus, it is essential that the specificity (i.e., amplification of only the target SARS-CoV-2 gene) of the selected SARS-CoV-2 target sequence and the RT-PCR oligonucleotides are adequately assessed to increase

confidence in SARS-CoV-2 RNA detection and minimize the potential for false-positives errors. Confirmation of assay specificity is particularly relevant for assays targeting emerging SARS-CoV-2 genome variants of concern, such as the B.1.1.7 (Alpha), P.1 (Gamma), B.1.351 (Beta), and B.1.617.2 (Delta) variants. These variants of concern are characterized by spike protein substitutions that can increase transmissibility and disease severity and may interfere with diagnostic assay performance (https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html#Concern). For example, early in the emergence of the Alpha variant, a S-gene "dropout" during RT-qPCR testing with certain kits indicated the presence of this mutation and the potential presence of the B.1.1.7 lineage (https://www.thermofisher.com/blog/behindthebench/solutions-for-surveillance-of-the-s-gene-mutation-in-the-b117-501yv1-sars-cov-2-strain-lineage/). Since then, RT-qPCR assays specific to new variants have been developed by targeting mutations in various genomic regions, predominantly the spike protein gene. For example, an allele-specific RT-qPCR assay for the Alpha variant was developed with "low cross-reactivity" and successfully used to test wastewater samples from communities in the United States (Lee et al., 2021). Another study reported the development of sensitive and specific RT-qPCR assays for the detection and quantification of both the Alpha and Beta variants of concern in wastewater (Yaniv et al., 2021). In the Netherlands, RT-ddPCR and an assay specific for mutation N501Y were used to detect the circulation of the Beta variant with proportions as low as 0.5% in a wastewater sample (Heijnen et al., 2021). Given the mutation rates inherent to viral replication, variants of concern are likely to continue to emerge. For example, more recently a RT-qPCR assay was developed targeting Gamma and Delta variants (Yaniv et al., 2021). Confirming the specificity of novel RT-qPCR assays for detecting and quantifying such variants in wastewater will be vital for effective future wastewater surveillance.

4. Factors contributing to SARS-CoV-2 RT-PCR false-negative errors in wastewater surveillance

SARS-CoV-2 false-negative errors in wastewater surveillance can result from inefficient RNA recovery in samples, PCR inhibition, or methods not optimized to detect trace quantities of target RNA. This section will review known and possible sources of false-negative errors for RT-PCR wastewater surveillance applications.

4.1. Variable shedding and low prevalence of SARS-CoV-2 infection in a community

Individuals infected with SARS-CoV-2 shed the virus and its genetic material via their sputum, nasopharyngeal secretions, saliva, urine, and feces (Cevik et al., 2021). SARS-CoV-2 RNA concentration in the feces of infected individuals is highly variable and ranges from 10^2 to 10^9 GC/g of feces (Wölfel et al., 2020; Zhang et al., 2020). The SARS-CoV-2 RNA detection rate in feces can range from 11 to 83% among COVID-19 positive adults (Wölfel et al., 2020) and up to 100% among children (Xu et al., 2020). Therefore, as the prevalence of COVID-19 decreases within a community, the number of individuals shedding the virus in their feces, urine, and nasal secretions and the subsequent amount of RNA shed into the sewer system will also decrease, thus reducing the amount of virus in the wastewater system.

The low and sporadic occurrence of SARS-CoV-2 RNA in wastewater due to low COVID-19 cases in the community makes RNA more challenging to detect. Therefore, it is critical to take steps to minimize the likelihood of false-negative errors. The effects of RNA target dilution, in large part due to mixing with other wastewater flows, stormwater infiltration, diurnal variation in shedding, and hydraulic residence time in the sewer collection system, can also affect the probability of detecting SARS-CoV-2 RNA, particularly in low prevalence conditions (Ahmed et al., 2020d). Thus, in communities where SARS-CoV-2 infection is low, RNA is less likely to be detected in wastewater. For example, in Australia, COVID-19 clinical test positivity was ~0.2% in late 2020 and early 2021 (Australian Government Department of Health; February 2021), and wastewater surveillance in some areas frequently resulted in non-detections for SARS-CoV-2. However, test positivity in many states within the United States still exceeded 5% in early 2021 (CDC, 2021a; https://www.cdc.gov/coronavirus/2019-ncov/covid-data/covidview/index.html), leading to more consistent detection of SARS-CoV-2 RNA in wastewater (Gonzalez et al., 2020; Gerrity et al., 2021; Wu et al., 2021).

4.2. Variability in wastewater composition

Despite mixing and dilution in sewer systems, it is incorrect to consider wastewater, as it arrives at a WWTP to be a well-mixed, evenly distributed liquor of everything discharged to the system during the day (Teerlink et al., 2012). Instead, sewers are dynamic flow systems, and the composition of wastewater arriving at a WWTP changes continually, reflecting variations in flow rates and substances discharged into the wastewater system (Ort et al., 2010b). Some wastewater systems can provide a degree of lateral flow mixing, such as those with storage capacity associated with pumping stations or flow equalization tanks. Turbulent flow regimes in sewer pipes can also induce lateral mixing, but this homogenization is often limited and may not be substantial relative to the overall volume of wastewater conveyed.

For a substance that is constantly or frequently discharged into a sewer network in relatively constant amounts, any random wastewater sample could reasonably be used to estimate a daily load. However, for a substance that is sporadically discharged into sewers in varying amounts temporally, either continuous sampling or multiple samples collected at different times of the day will be required to accurately estimate the total daily load (Ort et al., 2010a). Discontinuous surveillance using grab samples imparts a high probability of a false-negative error occurring or even disproportionately high concentrations that do not accurately reflect conditions in the broader community. Under low-prevalence conditions, if a sample is not collected at the precise moment that the wastewater "slug" containing a target substance (i.e., SARS-CoV-2 RNA) passes through the sampling point, "non-detection" may occur (Ort et al., 2010b). This concept is illustrated in Fig. 2, showing that some random wastewater volumes (orange ellipses) may contain detectable SARS-CoV-2 RNA, while others (brown matrix) may not. If a single grab sample is collected, the likelihood of it containing any of the daily SARS-CoV-2 RNA excretions of an individual person depends on the total daily volume of wastewater and the volume into which that individual's excretions may be dispersed. From an analytical perspective, the likelihood of detection also depends on the concentration of that individual's SARS-CoV-2 RNA excretions and the method limit of detection (MLOD; definition is given in Section 4.9). If no lateral mixing of wastewater has occurred, SARS-CoV-2 RNA will be present at high concentrations only in the wastewater volume into which it was discharged, such as a toilet flush. If a huge degree of lateral mixing has occurred (into many thousands or millions of L), the RNA concentration may be below the MLOD. Consequently, the degree of lateral mixing, substance dispersion volume, and MLOD are important factors determining the likelihood of positive detection in any single grab sample.

We use a simplified conceptual model to demonstrate the challenges of detecting SARS-CoV-2 RNA in a sewer system when the percentage of individuals excreting SARS-CoV-2 is relatively low. In the model scenario, a wastewater system serving 100,000 people with an average dry weather wastewater flow of 0.2 m^3/person/day will produce wastewater at a rate of 20,000 m^3/day. Thus, the probability that any single grab sample (relative to the detectable dispersion volumes) contains a detectable concentration of a specific individual's excreted SARS-CoV-2 can be estimated by the ratio of the average detectable dispersion volume (m^3/day) and the average dry weather flow (m^3/day). A detectable dispersion volume is the volume of wastewater into which a person's

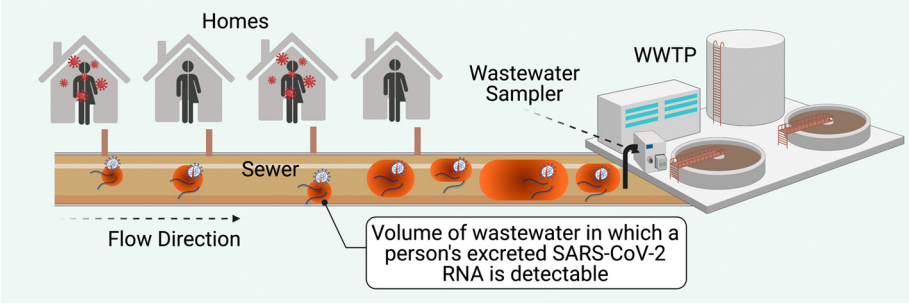

Fig. 2. Under low-prevalence conditions, some random sewage volumes (orange ellipses and circles) contain detectable SARS-CoV-2 RNA, while others (brown matrix) do not. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

total daily excretions are dispersed while remaining above the MLOD. Hypothetical values of 1, 10, or 100 m^3 were chosen to illustrate this point; detectable dispersion volumes will vary depending on the parameters of different sewer systems (e.g., SARS-CoV-2 RNA persistence given sewer travel times and temperatures, and mixing). The implication is that there is a greater likelihood of a positive detection for a larger detectable dispersion volume.

Since the dispersion volumes of multiple individuals may overlap, the probability $P(x)$ of any small-volume grab sample containing the detectable RNA excreted from any number of multiple individuals can be considered using the binomial distribution $P(x) \sim B(n, p)$, where x is the specified number of individuals for which excreted RNA can be detected in that sample, n = the number of individuals shedding SARS-CoV-2 RNA, and p = the probability that the sample contains a detectable concentration of a specific individual's excreted SARS-CoV-2 RNA (as given by the ratio above).

$$P(x) = \left(\frac{n!}{x!(n-x)!}\right) p^x (1-p)^{n-x}$$

Given the assumed parameters and model described above, the probability of a single wastewater sample being negative for SARS-CoV-2 RNA is given by the binomial distribution solution for $P(x = 0)$, as shown in Fig. 3a. If the proportion of people shedding SARS-CoV-2 is <0.01%, the likelihood of a grab sample not containing the RNA at a detectable concentration is above 90% for all three assumed mixing scenarios. In this case, $x = 0$, $n < 10$ people per 100,000, and p depends on the average detectable dispersion volume of each person's excretions.

In any of the circumstances considered in Fig. 3a, the likelihood of collecting a positive sample can be increased by collecting a larger number of grab samples. In this case, the probability (P) of never collecting a sample containing RNA $P(x = 0)$, can be modelled as an outcome of a second binomial distribution $B(n, p)$, where n = the number of samples collected and p = the probability of any sample being positive. Fig. 3b presents the probability (%) of all samples being negative for $n = 1$, 10, 100 or 1000 samples, where the average dispersion volume is assumed to be 10 m^3. For a population with 0.1% shedding RNA, the collection of 10 grab samples over a 24-h period gives <50% likelihood of returning one or more samples that contain the virus. For a population with only 0.01% shedding SARS-CoV-2, around 140 grab samples are required to give a 50% chance of a sample containing RNA. Thus, the capture of RNA and subsequent detection are unlikely when only a small proportion of the population is shedding SARS-CoV-2. This conceptualization is supported by observations during wastewater surveillance for SARS-CoV-2 RNA. For example, during a 24 h study of wastewater influent in Indiana, USA, only 50% of grab samples (collected every two hours) were positive for SARS-CoV-2 RNA when new COVID-19 cases averaged 16/day, but 100% of samples were positive when new cases averaged 65/day (Bivins et al., 2021a). During the same study, an increasing number of COVID-19 cases in each community was also associated with decreasing variation in SARS-CoV-2 RNA concentration over 24 h of surveillance (Bivins et al., 2021a).

To analyze many individual grab samples, an equivalent composite sample could be used to produce similar probability outcomes, if the degree of dilution provided by the "negative" composite sub-samples did not reduce the RNA concentration from "positive" composite sub-samples to <MLOD. These observations are meaningful only in circumstances where SARS-CoV-2 RNA is assumed to be a sporadically discharged substance—an assumption only applies where the proportion of concurrent excreters is very low. However, it may be relevant

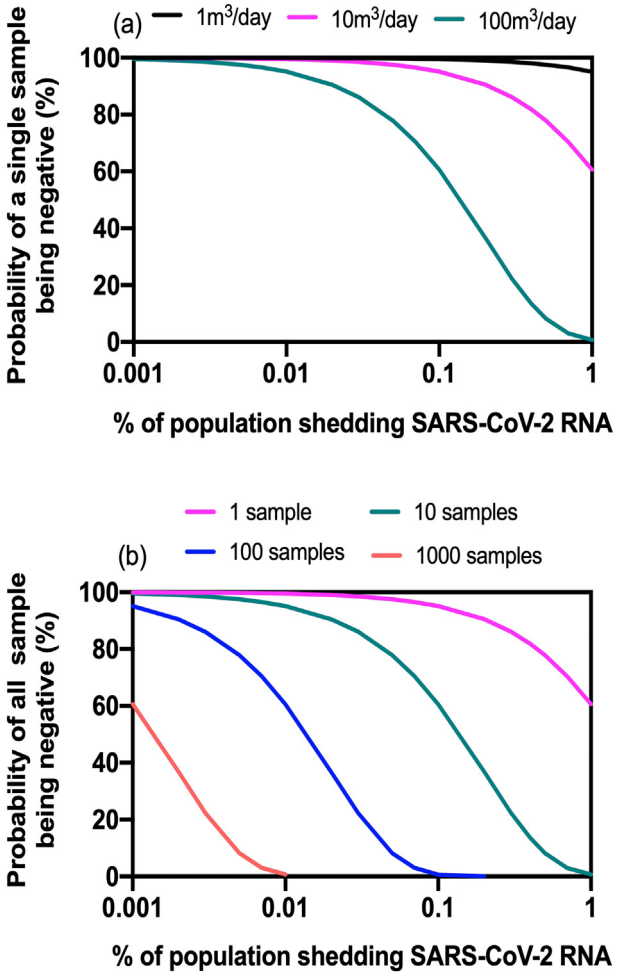

Fig. 3. a: Probability of a single small grab sample (relative to SARS-CoV-2 dispersion volume) being negative, assuming a sewer catchment population of 100,000 people, an average dry weather flow of 0.2 m^3/person/day, and dispersion volumes of 1 m^3/day, 10 m^3/day, or 100 m^3/day. 3b: Probability of all samples being negative for $n = 1$, 10, 100, or 1000 samples.

in any circumstance where wastewater surveillance is conducted for providing an "early warning" of the re-emergence of COVID-19, following near elimination of the virus. In such cases, it is important to recognise that failures to detect RNA in wastewater do not provide infallible evidence that RNA and COVID-19 infected people are not sporadically present in wastewater and the community, respectively.

Importantly, the model presented here is purely conceptual and was developed to illustrate the considerations for achieving representative wastewater sample for COVID-19 surveillance. Also, the model input parameters and outcomes may vary greatly considering different types of wastewater collection systems and shedding rates. An additional consideration is that the actual behavior of SARS-CoV-2 in wastewater collection systems remains poorly characterized. Enveloped viruses have been observed to partition favorably to solids, which likely changes their transport behavior from the dissolved species frequently used to characterize fate and transport in hydraulic systems (Ye et al., 2016). The adsorption of SARS-CoV-2 to surfaces such as suspended solids depends on both surface chemistry and solution chemistry, which further complicates the idealized conceptualization of fate and transport in wastewater (Liu et al., 2021). Additionally, SARS-CoV-2 and its RNA have been observed to exist in several forms in wastewater, including intact and ruptured viruses (Wurtzer et al., 2021).

4.3. Sampling approach matters

Since release of the first preprint paper on detecting SARS-CoV-2 in wastewater (Medema et al., 2020a), selecting the best sampling strategy has been a challenge for wastewater surveillance studies. As described above, poorly designed wastewater sampling strategies can easily contribute to false-negative errors (regardless of how good the analytical workflow is) (Ahmed et al., 2020d). Common sampling approaches include grab samples, which may be individual samples or composites of several grabs, or the use of an auto-sampler to collect a composite sample over a longer time, frequently 24 h (WHO, 2003; Miyani et al., 2020) or the use of passive samplers where an absorbent material such as swabs or medical gauze is placed in the wastewater and retrieved after hours to days, as a low-cost alternative to autosamplers (Schang et al., 2021).

In most scenarios, samples created from a composite of multiple sub-samples offer a more representative measure of viral concentration in wastewater (Matrajt et al., 2018). Ahmed et al. (2020e) revealed a greater variability in grab samples than in composite samples for both indicator and pathogenic viruses in untreated wastewater. Gerrity et al. (2021) noted that early-morning grab samples generally had lower concentrations than corresponding 24-h composite samples and demonstrated the potential trade-offs of sampling primary effluent, by which point wastewater flows have undergone greater mixing or dispersion but also significant dilution of peak loads.

Composite auto-samplers deployed at fixed sampling locations are becoming the most favored sampling approach in high-income countries (Hamouda et al., 2021). Depending on the wastewater system, either time-weighted or flow-weighted composite sampling may be more effective for capturing concentration spikes (Ort et al., 2010b). Only in systems where wastewater is homogenized and dilution is limited, grab sampling may result in representative samples (e.g., sampling from wastewater systems of airplanes and cruise ships) (Ahmed et al., 2020f; Albastaki et al., 2021). Sampling small wastewater collection networks (e.g., buildings or neighborhoods), where few toilet flushes occur for a day and large short-term fluctuations are expected, would require an increased sampling frequency or sampling to target the peak flow periods (e.g., during the morning hours) to capture these shedding events (Aymerich et al., 2017). Passive samplers may be useful alternatives in such cases, as they have demonstrated the ability to accumulate pathogens over time, often with greater sensitivity and efficiency than grab samples. Passive samplers are much less costly and can be much easier to deploy in drains or maintenance holes with low flow conditions than autosamplers and have been used for the detection SARS-CoV-2 RNA in wastewater (Hayes et al., 2021; Schang et al., 2021). However, quantification of SARS-CoV-2 RNA per volume of wastewater may be difficult to achieve using passive samplers. Furthermore, the mechanisms by which viruses attach to the materials in passive samplers are not well understood (Vincent-Hubert et al., 2021).

Sampling upstream of the WWTP at maintenance holes or pump station sites, or even building-level surveillance, such as on university campuses, can detect local disease transmission clusters that may be missed analyzing WWTP-influent samples due to viral RNA dispersion and dilution. The spatial resolution provided by wastewater catchment data could be valuable when maintenance holes draining areas with industrial activities prone to COVID-19 outbreaks, such as food processing plants, quarantine facilities, hospitals, or high-density living areas, such as college dormitories, are targeted for sampling. However, the intermittent nature of the signal becomes more pronounced as the catchment area gets smaller. Thus, high-frequency composite sampling or the use of passive samplers that sample for a prolonged period becomes especially important in these sampling schemes (Harris-Lovett et al., 2021). Regarding combined sewers, stormwater inflow and infiltration can further dilute viral signals in sewer catchments and loadings from industries and agriculture can potentially lead to false-negative errors due to interference in downstream analysis.

4.4. Pre-treatment and storage of wastewater samples

Current laboratory practices recommend that the concentration of wastewater samples be performed in a Biological Safety Level 2 (BSL-2) or Biological Containment 2 (BC2) laboratory with unidirectional airflow and BC3/BSL-3 precautions (CDC, 2021b). Pasteurization can be used to minimize biosafety risk and help ensure safe sample handling, especially in instances of sample manipulation that may generate aerosols (i.e., sample concentration) (Whitney et al., 2020; Wu et al., 2020). However, temperature, in general, can significantly influence microbial decay (Espinosa et al., 2020; Korajkic et al., 2019; Muirhead et al., 2020), with extended survival (minimal degradation) typically observed at lower temperatures (<15 °C).

Ahmed et al. (2020c) indicated that temperature is also a significant driver of enveloped viral RNA (MHV and gamma-irradiated SARS-CoV-2) decay rates in stored wastewater, with decay rates increasing at 25 °C, and being much greater at 37 °C when compared to 4 or 15 °C. Concerning cold temperatures, a recent study demonstrated that 28 days of storage at 4 °C caused linear decay of SARS-CoV-2 RNA, and thus a risk for false-negative errors; however, samples stored at −20 °C or −80 °C did not decay appreciably (Hokajärvi et al., 2021). The nature of the COVID-19 pandemic has created situations where there is a need to immediately collect and analyze a large number of samples, especially in areas where COVID-19 cases are rising. Owing to processing constraints, it is not always possible to immediately analyze samples; therefore, short- or long-term storage before analysis may be necessary. A strategy based on the other microbial targets could be stored for up to seven days at 4 °C and for longer terms at freezing temperatures of −20 °C or −80 °C. However, it is unclear whether storing bulk wastewater samples would lead to a higher rate of false-negative errors, which requires further investigation. Additionally, many of the studies performed have relied on seeding wastewater samples with exogenous control viruses. It is unclear whether such viruses, often the product of a pure culture, are representative of endogenous SARS-CoV-2, which has been expelled via the body fluids of infected individuals. Such virions may be present in various structural forms in wastewater and could be susceptible to decay and degradation due to temperature (Wurtzer et al., 2021). Findings from the limited studies available on pasteurization or cold storage of wastewater samples are discordant. For example, in Whitney et al. (2020), no significant reduction in SARS-CoV-2 RNA signal was observed when wastewater samples were pasteurized at 70 °C for 45 min, suggesting that at least some heat

pre-treatment conditions may not adversely affect the results. Similarly, Bivins et al. (2021a) observed no significant reduction in SARS-CoV-2 RNA numbers using RT-ddPCR following pasteurization at 60 °C for 90 min. In contrast, the effect of pasteurization was determined at 70 °C for 40 min on SARS-CoV-2 N gene concentrations in triplicate wastewater collected from three WWTPs in Southern California, USA (Fig. 4). Significant and consistent reductions in N1 and N2 concentrations were observed from the three WWTPs, with reductions from 1 to 3 $\log_{10}$ when the samples were concentrated by membrane adsorption coupled with the bioMerieux magnetic bead extraction kit (Fig. 4). Given the inconsistent observations on the effect of pasteurization on SARS-CoV-2 RNA copy numbers, definitive recommendations regarding the use of heat treatment cannot be made at this time. Considering the biosafety requirements of the analytical laboratory, such effects should be characterized locally, and an appropriate course of action should be taken.

Storage of wastewater samples at freezing temperatures results in a freeze-thaw cycle for the virus particles and can lead to SARS-CoV-2 RNA degradation, thus influencing the possibility of false-negative errors (Steele et al., 2021). For example, some researchers have reported ~90% loss of SARS-CoV-2 RNA signal following storage at −80 °C for one week (Weidhaas et al., 2020). However, longer storage at freezing temperatures does not seem to cause additional loss of the SARS-CoV-2 RNA signal (i.e., no appreciable decay within 58 days at low and ultra-low temperatures) (Hokajärvi et al., 2021). A recent study also reported no significant decay of the OC43 RNA signal (another betacoronavirus, and a recognized SARS-CoV-2 surrogate) at −80 °C over three weeks following multiple freeze-thaw cycles in an elution solution (containing 0.01% sodium hexametaphosphate, 0.01% Tween 80 and 0.001% Antifoam Y-20) (McMinn et al., 2021). Together, these observations suggest that while freeze-thaw is a potential contributor to SARS-CoV-2 RNA signal decay, the storage state and conditions are likely important and contribute to the magnitude of signal loss.

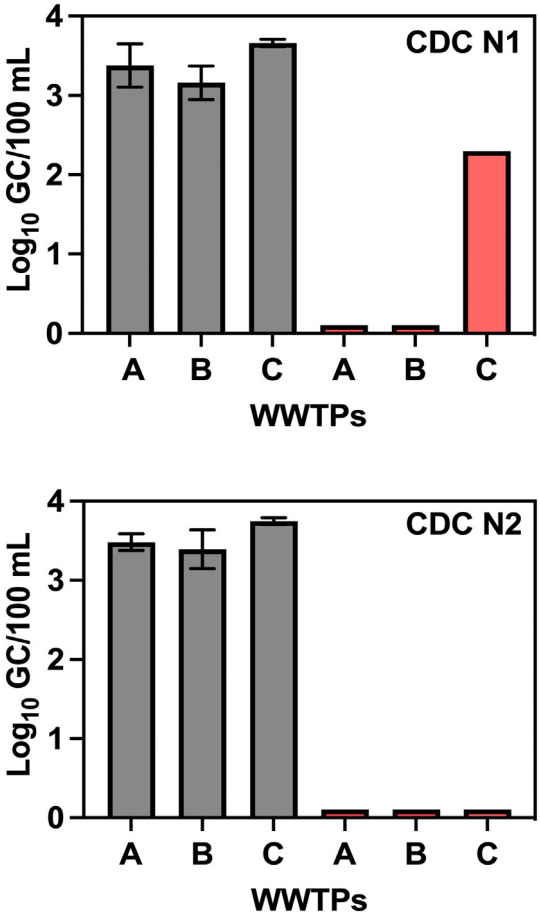

Fig. 4. SARS-CoV-2 concentrations measured by RT-PCR in wastewater samples that were untreated (gray bars) or pasteurized (salmon bars). The mean concentration in pasteurized samples was censored below the detection limit; values that were detected but not quantifiable (DNQ) were replaced with half the limit of quantification for statistical analysis. Samples in which the target was not detected (ND) were assigned a value of 1.0. Data were obtained partially from Steele et al. (2021).

4.5. Concentration strategies

Viral pathogens are typically diluted in wastewater, requiring the sampling of large volumes (e.g., >1 L) followed by a concentration step to obtain detectable amounts of viral nucleic acid (Ahmed et al., 2015). Therefore, efforts to concentrate SARS-CoV-2 RNA from wastewater samples can be a potential source of false-negative errors due to losses incurred during the concentration process. Several authors have reviewed and compared the concentration approaches used for enveloped viruses, and particularly for SARS-CoV-2 surveillance in wastewater (Ahmed et al., 2020e; Barril et al., 2021; Cervantes-Avilés et al., 2021; Philo et al., 2021; Rusiñol et al., 2020; Chik et al., 2021; LaTurner et al., 2021; Pecson et al., 2021). Many of these experiments relied on the use of exogenous virus controls seeded into wastewater to assess process efficiency and have been accompanied by various caveats and descriptions of limitations (Kantor et al., 2020). One important limitation, which was previously mentioned, is the uncertainty regarding the structural form and partitioning behavior of exogenous controls compared to endogenous SARS-CoV-2 shed to the wastewater. While seeded positive controls are useful, this uncertainty should be acknowledged in decisions regarding appropriate concentration methods.

Most of the available concentration methods have wastewater input volumes of 15 to 250 mL, using size exclusion (Gonzalez et al., 2020; Jafferali et al., 2020; Hasan et al., 2021), membrane adsorption (Jafferali et al., 2020), chemical precipitation (Wang et al., 2005; Randazzo et al., 2020; Torii et al., 2020), flocculation (Philo et al., 2021), ultracentrifugation (Prado et al., 2020), or combinations of these methods (Ahmed et al., 2020d; Gerrity et al., 2021; Pecson et al., 2021). The process detailed by Miyani et al. (2020) is the only large-volume concentration method documented in the literature where SARS-CoV-2 was concentrated from 45 L of wastewater using electropositive cartridges; however, this method is time-consuming and laborious.

Most of these techniques used for wastewater surveillance were originally developed for the concentration of non-enveloped enteric viruses, whose physiology and capsid structures significantly differ from enveloped respiratory viruses, such as SARS-CoV-2 (Philo et al., 2021). Recovery of enveloped viruses from wastewater using these methods can be highly variable, with recoveries ranging from 2 to 66% (McMinn et al., 2021). Furthermore, SARS-CoV-2 RNA recovery efficiency from wastewater samples can vary between and within WWTPs, even with the same concentration method, presumably due to variations in wastewater composition (Fig. 5). Seeding wastewater samples with gamma-irradiated SARS-CoV-2 resulted in estimated mean recovery efficiencies of 25.1 ± 11% (Fig. 5) (Ahmed et al., 2021). The percentage recovery variations within 10 WWTPs over the three sampling events ranged from 11.4 to 34.8% (WWTP J) and 7.0 to 14.7% (WWTP L). Such variations may induce false-negative errors when the concentration of SARS-CoV-2 is low in wastewater, may vary over time and between geographic locations.

The probability of observing false-negatives is further exacerbated by working with small wastewater sample volumes (≤100 mL) and likely represents a major limitation for many of the currently reported wastewater concentration methods. Consequently, virus concentration efficiency may be a significant impediment to wastewater surveillance applications where virus levels are low, and the recovery efficiency is highly variable (Chik et al., 2021; Pecson et al., 2021).

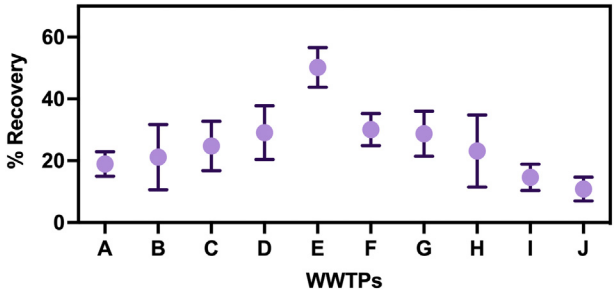

Fig. 5. Recovery (mean ± SD) of seeded SARS-CoV-2 RNA from 10 wastewater treatment plants (WWTP A-J) using a concentrating pipette Select™ (CP Select™). Each WWTP was sampled three times. Data were obtained partially from Ahmed et al. (2021).

Sample volume is another important factor that may induce false-negative errors by modulating the MLOD, especially when the concentration of the target viruses is relatively low in wastewater, as is the case for SARS-CoV-2, even in peak periods. Nevertheless, a few studies have reported the equivalent sample volume (ESV) analyzed using RT-PCR (i.e., considering both the volume of wastewater sampled and the volume of the concentrated sample used in the analysis). The ESV is the equivalent volume of the original wastewater sample matrix that is analyzed in a single RT-PCR reaction after accounting for the concentration achieved by the workflow. In a comparison study of concentration methods for SARS-CoV-2, a bag-mediated filtration system (positive filtration) enabled the highest ESV (15 mL, concentrated from a larger volume of wastewater), whereas up to 5.3 mL and 1.3 mL were assayed using ultrafiltration-based methods and PEG precipitation or skimmed milk flocculation (SMF) methods respectively (Philo et al., 2021).

In another study, Forés et al. (2021) reported similar ESVs for Centricon® and the Concentrating Pipette Select™ (CP Select™) ultrafiltration devices, resulting in concentration factors up to 333× and 250×, respectively. The MLOD is a function of the assay limit of detection (ALOD; Section 4.9), which is empirically determined for individual RT-PCR assays, and the ESV. Results in Fig. 6 show the MLOD estimations based on three different procedures, indicating that when low virus RNA concentrations are expected to occur in wastewater samples, the starting sample volume should be increased to minimize the possibility of false-negative errors. However, the impact of the concentration method on recovery efficiency and RT-PCR inhibition should be evaluated when analyzing large volumes of wastewater. Gerrity et al. (2021) processed 10 L of wastewater for SARS-CoV-2 detection using a combined sample concentration approach, with hollow fibre ultrafiltration (HFUF) followed by Centricon® centrifugal ultrafiltration. Despite achieving an ESV of ~50 mL with this combined approach, virus recovery based on seeded bovine coronavirus dropped to 2%. However, with HFUF or Centricon® alone, the ESV decreased to ~1 mL, and virus recovery increased to >50%. Therefore, when attempting to increase method sensitivity, one must simultaneously balance ESV and virus recovery, while also considering the practical aspects (e.g., time and cost) of sample processing.

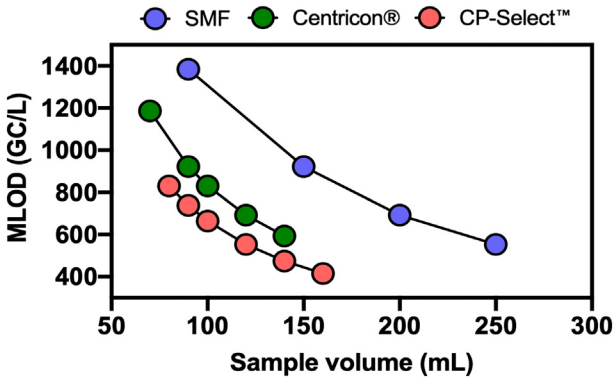

Fig. 6. Estimated method limit of detection (MLOD) according to the final volume of concentrated samples. The MLOD was calculated assuming 323 GC as the minimum number detected for a single RT-qPCR reaction using CDC N1 assay and a mean volume of viral concentrate of 300 μL for the CP Select™, 500 μL for SMF and 240 μL for Centricon®. A 100% recovery was assumed for the entire concentration, RNA extraction and detection process. SMF: skimmed milk flocculation. Data obtained partially from Forés et al. (2021).

A method comparison study revealed a 7-$\log_{10}$ range of recovery efficiency and ALOD values, demonstrating the broad range of outcomes possible with currently available protocols and highlighting the importance of characterizing performance before implementation (Pecson et al., 2021). Although, the study did not consider whether one approach surpassed another, it highlighted the importance of method characterization, including potential trade-offs between the sample concentration factor and virus recovery. Nonetheless, extremely low ESVs and virus recoveries have a greater potential to yield false-negatives or even artificially inflate-adjusted concentrations.

4.6. RNA extraction

Inefficient RNA extraction protocols can contribute to the lack of detection of SARS-CoV-2 false-negatives, and little is known about which protocols are best for wastewater surveillance applications. Due to the COVID-19 pandemic, an overwhelming demand for materials and reagents for SAR-CoV-2 testing has resulted in manufacturer shortages, potentially requiring laboratories to quickly modify method workflows to accommodate short-term supply issues (Fomsgaard and Rosenstierne, 2020). In some cases, researchers have been forced to process samples using a range of alternative/uncommon RNA extraction kits or to use methods lacking an RNA extraction step, all with varying levels of success (Fomsgaard and Rosenstierne, 2020; Merindol et al., 2020; Torii et al., 2020; Wee et al., 2020). Commercially available RNA extraction kits can vary in efficiency and consistency in isolating viral RNA and as well as efficacy in removing PCR inhibitors, even when sourced from the same supplier (Iker et al., 2013; Griffin et al., 2014; Zhang et al., 2018). Pivoting to alternative methodologies combined with potential inconsistencies in RNA recovery, even from commercial kits, the RNA extraction step remains a potential risk for false-negative detections due to low efficiency. Furthermore, with indications of the emergence of SARS-CoV-2 variants around the world, confounded by delays in vaccine distribution, manufacturer shortages of these critical reagents will likely remain an issue for ongoing wastewater surveillance efforts.

4.7. RT-PCR amplification inhibition

Municipal wastewater is a complex, often non-homogenous, mixture of substances beyond fecal waste, including pharmaceuticals, personal care products, stormwater, sediments, household detergents, industrial effluents, metals, and other substances. Some of these substances may completely or partially inhibit the RT-PCR amplification process, leading to a false-negative result. These substances are generally referred to as PCR inhibitors and include a heterogeneous and poorly defined group of chemical substances known to inhibit PCR-based methods, including multi-ringed polysaccharides (e.g., humic and fulvic acids), fats, proteins, metal ions (e.g., iron and aluminum), RNases, and others (Schrader et al., 2012). There are multiple mechanisms of PCR inhibition, including co-precipitation of inhibitors with nucleic acids, either degrading or sequestering target nucleic acids; binding of some inhibitors to nucleic acids or enzymes, inhibiting polymerase activity; or chelating metal ions (i.e., Mg^{2+}) necessary for optimal amplification performance. A full description of known mechanisms was reviewed by Schrader et al. (2012). For SARS-CoV-2 cDNA amplification, it has been demonstrated that the presence of such inhibitors can lead to false-negative errors in SARS-CoV-2 assays,

especially when viral concentrations are low (D'Aoust et al., 2021; Gonzalez et al., 2020; Graham et al., 2021).

4.8. RT-PCR assay selection

The SARS-CoV-2 RNA genome offers many possible targets for RT-PCR assays, including the nucleocapsid (N), envelope protein (E), RNA-dependent RNA polymerase (RdRP), open reading frame (ORF), membrane protein (M), and surface protein (S) genes. Multiple assays have been developed for each genetic region (D. Li et al., 2020; N. Li et al., 2020). Relatively poor assay performance could lead to false-negative errors. For example, Vogels et al. (2020) compared the performance of nine primer–probe combinations targeting several genes (i.e., E, N, ORF1, and RdRp) recommended by the World Health Organization (e.g., those developed by the Chinese Center for Disease Control and Prevention [China CDC], the US CDC, Charité Institute of Virology, Universitätsmedizin Berlin [Charité] (Corman et al., 2020), and Hong Kong University [HKU] (Chu et al., 2020)). This comparison was performed with standard reference materials and clinical samples (e.g., nasopharyngeal swabs, saliva, urine, and rectal swabs) seeded with the reference material. The authors demonstrated that, at low viral concentrations (1–10 viral RNA copies/μL), not all assays yielded positive results suggesting that some assays may be more prone to false-negative errors than others (Vogels et al., 2020). Most notably, the RdRp reverse primer had mismatches with the reference material attributed to the evolution of the virus, causing low analytical sensitivity.

These assays were developed for clinical testing, and many performed well with clinical samples; however, a few studies have evaluated their performance for wastewater analyses. This is crucial given that wastewater is enriched in other viruses and pathogens. A recent study reported that genes are transcribed by cells infected with SARS-CoV-2 at different rates, suggesting that assays targeting the N gene could help reduce the incidence of false-negatives (Kim et al., 2020). The US CDC N1 assay also outperformed the Charité assay and assays targeting the M gene and IP2/IP4 (RdRP) regions when applied to SARS-CoV-2 quantification from wastewater in Spain (Perez-Cataluna et al., 2021). In another study, the US CDC N1 assay resulted in a longer period of positive detection in wastewater than IP2 and IP4, the E gene regions, while the US CDC N2 assay provided inconsistent results (Chavarria-Miro et al., 2021).

Fig. 7 depicts results of an experiment using the Australian National Measurements Institute (NMI) SARS-CoV-2 reference materials (https://www.industry.gov.au/news/new-australian-standard-helps-covid-19-hotspot-detection), indicating the differences in assay performance based on paired testing of wastewater dilutions with the CDC N1 and N2 assays. Finally, a study conducted in Germany reported increased sensitivity and specificity of IP2/IP4 (RdRP) and M gene targets compared to N gene and E gene assays (Westhaus et al., 2020), but cautioned that these assays might detect other endemic human coronaviruses, potentially inflating reported performance metrics. Although no study to date has conducted a head-to-head comparison of all SARS-CoV-2 assays in wastewater samples, the limited information available clearly indicates that some assays may be more suitable than others.

4.9. RT-PCR assay performance characteristics

PCR performance characteristics, such as sensitivity or assay limit of detection or quantification (ALOD or ALOQ), calibration model parameters for RT-qPCR (amplification efficiency, slope, Y-intercept), and dynamic range play an important role in interpreting the assay robustness and reproducibility, thus minimizing the potential for ambiguous results and detection failure (Bustin et al., 2009; Johnson et al., 2014; Borchardt et al., 2021; Bivins et al., 2021b). ALOD is the concentration of a target that can be detected with a 95% probability (at least 95% of samples containing that quantity of target will be positive) (Bustin et al., 2009). ALOQ for RT-qPCR can be defined in several ways, but in general, it depends upon the standard deviation around the lower concentrations of the standard curve. A relatively common practice is to establish the ALOQ based on the coefficient of variation (CV) for measuring analytical standards near the ALOD. Acceptable CV levels are generally 10 to 35% (Forootan et al., 2017; Haugland et al., 2016). For example, an assay in which the 100 GC standard has a CV of 15% and the 10 GC standard has a CV of 75% would have a limit of quantification of 100 GC.

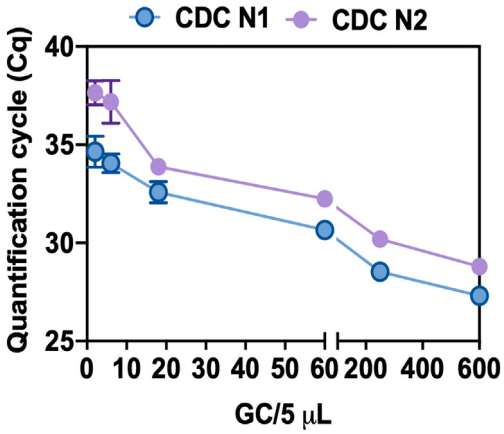

Fig. 7. Quantitation cycle (Cq) values of the US CDC N1 and N2 RT-PCR assays determined using the Australian National Measurement Institute (NMI) SARS-CoV-2 calibrant comprising six gravimetric dilutions at 600, 245, 60, 18.5, 6.5, and 2 GC/5 μL of RNA. The Bio-Rad CFX96 platform was used for the RT-PCR amplification of the N1 and N2 assays. Data source (unpublished): CSIRO Molecular Microbiology Laboratory, Queensland, Australia.

PCR-based assays with low ALOD and a wide dynamic range are ideal for wastewater surveillance applications. As previously described, MLOD is the method sensitivity or a concentration of a target that can be detected consistently after incorporating loss through the entire process from sample concentration to extraction, while the method limit of quantification (MLOQ) is the concentration of a target that can be quantified with an acceptable level of precision when present in a sample. Therefore, these values are calculated from the ALOD or ALOQ.

4.10. Mutations in the RT-PCR target regions

SARS-CoV-2 RNA can undergo strong selection pressures, high mutation rates or a combination of both, resulting in the formation of several prominent genetic variants since late 2020 (D. Li et al., 2020; N. Li et al., 2020; X. Li et al., 2020). Changes in key SARS-CoV-2 loci could result in reduced RT-PCR performance, leading to false-negative results depending on the location of the mutation relative to primers and probe-target regions. The rate of genetic change varies across the SARS-CoV-2 genome, with structural domains (i.e., spike, membrane, envelope, and nucleocapsid proteins) generally undergoing stronger selection pressure (due to host immunity) resulting in the accumulation of more mutations compared to nonstructural genes (i.e., ORF1a and 1b) (https://virological.org). One pertinent example is the emergence of the B.1.1.7 lineage (https://virological.org). However, accumulation of mutations within the probe and/or primer target regions is the utmost concern for wastewater surveillance, as these could, over time, lead to decrease in the sensitivity of RT-PCR detection, and an increased rate of false-negative errors. For example, a recent report indicated that single nucleotide polymorphism (SNP) in the nucleoprotein (N) gene interfered with some SARS-CoV-2 RT-PCR assays in clinical testing but not others (Ziegler et al., 2020). Another group reported the failure of the RT-PCR assay, targeting the E gene due to an SNP (Artesi et al., 2020). GISAID (Elbe and Buckland-Merrett, 2017) reports in the

Common Primer Check for High Quality Genomes show that, as of April 20, 2021, up to 1.8% of SARS-CoV-2 genomes had a least one mutation in the China-CDC-N primer region, whereas no genomes have been found to contain a mutation in the China-CDC-ORF1ab primer region. GISAID's Primer Checker tool (http://penelope.unito.it/sars-cov-2_detection/) allows the screening of SARS-CoV-2 genomes for mutations in the forward primer, reverse primer, and probe of various RT-PCR assays stratified by region and time scales.

4.11. PCR platforms

RT-PCR (both RT-qPCR, -dPCR and -ddPCR) has become a mainstream detection method for SARS-CoV-2 RNA in wastewater (Barcelo, 2020). However, like all technologies, RT-PCR is not without limitations; in particular, reliance on a standard curve for quantification and issues with PCR inhibition can be problematic. In recent years, digital PCR (dPCR) has emerged as an attractive alternative for environmental applications, as it offers absolute quantification without the need for a standard curve and may be more sensitive and less prone to inhibition (Cao et al., 2015). As applied to the detection of SARS-CoV-2, RT-dPCR outperformed RT-qPCR for clinical specimens, including nasopharyngeal samples (Falzone et al., 2020; Liu et al., 2020; Suo et al., 2020) and plasma from infected patients (Tedim et al., 2021), as it was found to be more sensitive and accurate.

While there are abundant examples in the literature comparing the performance of both technologies on clinical samples, analogous data for more complex matrices, such as wastewater, are limited. For example, a recent study documented the successful application of RT-dPCR technology to quantify SARS-CoV-2 from municipal wastewater but offered no direct comparison with RT-qPCR (Gonzalez et al., 2020). Notably, the potential for inhibition was assessed by adding a hepatitis G RNA control, resulting in approximately 50% recovery of the seeded material, suggesting that RT-dPCR may also be affected by interference from PCR inhibitors in wastewater. Another study reported the potential inhibition of RT-dPCR compared to RT-qPCR when applied to the quantification of SARS-CoV-2 from wastewater sludge samples (D'Aoust et al., 2021), but this inference was based on a higher frequency of target detection by the RT-qPCR, rather than the recovery of an exogenous control.

A direct comparison between RT-qPCR and RT-dPCR applied to aircraft and cruise ship wastewater samples indicated minimal differences in SARS-CoV-2 detection frequencies, with no inhibition observed with either approach. The authors concluded that potential differences in the detection frequency of SARS-CoV-2 may be considerably influenced by individual RT-PCR assay design rather than detection technology (Ahmed et al., 2020f). Another research group performed a comparative study using both CDC N2 RT-ddPCR and CDC N2 RT-qPCR for quantifying SARS-CoV-2 in influent wastewater from several WWTPs (Fig. 8). A positive signal was detected by RT-ddPCR at an earlier date when SARS-CoV-2 levels were presumably lower compared to RT-qPCR analyses. The capability for earlier detection was reinforced by comparing the analytical sensitivity of the two methodologies. Analysis indicated that the ALOD for N2 using RT-ddPCR was 0.25 copies/reaction whereas the ALOD for RT-qPCR was much greater at a concentration of 60 copies/reaction. While RT-ddPCR may be more sensitive than RT-qPCR for SARS-CoV-2 detection in wastewater, additional studies are needed to confirm these initial observations. Park et al. (2020) suggested relying on RT-ddPCR for quantification of reference materials and diagnosis in patients but utilizing RT-qPCR to analyze wastewater samples. A potential limitation of dPCR is the subjective thresholding that distinguishes positive partitions from negative partitions and the much narrower dynamic range of the measurement system.

4.12. Number of replicates (sub-sampling error)

Since the entire sample often cannot be analyzed, replicated subsamples may be used to detect or estimate the concentration of a target DNA/RNA present in a single sample. Technical replicates test the variability of the analysis method and involve taking multiple subsamples from the same sample and analyzing each subsample. In contrast, biological or matrix replicates test different independently obtained wastewater samples. In general, the analysis of the three biological replicates can provide robust results. However, even if three samples were obtained for RNA extraction in an environmental application, only a subsample (a portion) of the extracted material from each sample was used in any individual RT-PCR reaction. This sub-sampling practice can introduce errors in RNA detection (Taylor et al., 2019). If a 1-μL subsample of RNA is taken from a total volume of 100 μL of RNA containing a total of 500 GC of SARS-CoV-2 RNA, the expected number of GC in 1 μL of homogenous RNA subsample will be 5. However, if the quantity of SARS-CoV-2 RNA is 50 GC in a volume of 100 μL RNA, the expected number of GC in the 1 μL subsample will be 0.5. Assuming a theoretical ALOD of 1 GC, the latter scenario has a 50% probability of a false-negative error, assuming a single RT-PCR reaction. Therefore, a subsampling error is expected to be more pronounced in wastewater samples with low target RNA concentrations.

It has been reported that subsampling error contributes to more than 10% of the variance when the concentration of a target in a

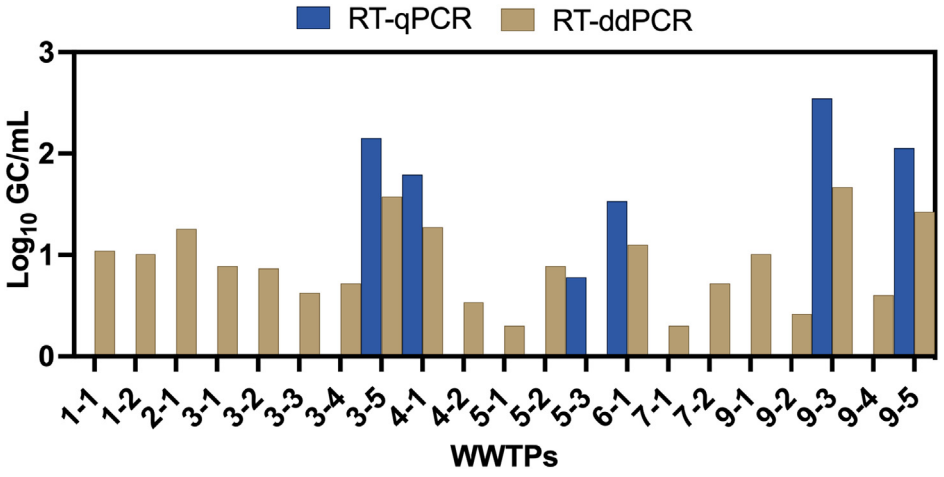

Fig. 8. SARS-CoV-2 (CDC N2) concentrations over time in gene copies (GC)/mL from seven distinct wastewater treatment plants (WWTPs) servicing in south–east Virginia. The WWTPs serve catchment populations ranging from 69,509 to 343,016 individuals. Weekly sampling events began May 14, 2020 and continued through July 14, 2020. WWTPs are identified first by the arbitrary facility number, followed by the corresponding sample collection event (e.g., WWTP-5-3 would refer to the third collection event for WWTP 5). Data was partially obtained from Ciesielski et al. (2021).

subsample is below 100 GC, and above 30% when the concentration is below 10 GC. Technical variability of RT-qPCR assay results is reported to increase when yielding Cq values >30 due to stochastic amplification, measurement uncertainty, and subsampling error (Taylor et al., 2019). For example, considering the detection rate of SARS-CoV-2 using the US CDC N1 assay in 523 wastewater samples in Queensland, Australia (Pers. Comm. Phil M. Choi; Queensland health), the Inclusion of six rather than three technical replicates resulted in a ~45% detection frequency increase, suggesting that sub-sampling error and low replicate numbers can contribute to false-negative errors.

4.13. Data reporting

Data reporting practices merit special consideration for highly infectious and transmissible viruses (and other biological agents), such as SARS-CoV-2, which present serious threats to global public health. For example, in instances where it has been documented that RT-PCR detection is not a result of extraneous contamination (i.e., false-positive), reporting detections below an established ALOQ such as detectable but not quantifiable (DNQ) (Layton et al., 2013) may be acceptable. In this case, the results below the ALOQ/MLOQ are classified as positive, thereby increasing the assay sensitivity, and reducing the incidence of false-negative errors. Alternatively, the SARS-CoV-2 wastewater surveillance dashboard in Finland denotes inconclusive results to avoid the implications of potential false-negative errors while emphasizing the importance of measures to avoid false-positive errors (Supplementary Fig. 2). Another important factor in data reporting is the lack of pertinent technical information in published articles regarding the sample processing workflow. Such information is crucial for interpreting the potential for false-negative and false-positive errors. Without information regarding the laboratory workflow, it is difficult to determine whether a sub-optimal result was due to sampling, poor virus concentration and/or RNA extraction efficiency, use of a less sensitive RT-PCR assay, or other factors.

5. Implications of SARS-CoV-2 false-positive and false-negative errors

When a wastewater surveillance program reports positive results to public health units, they may immediately contact and advise public health agencies and/or community leaders on the location. Local public health units may seek further wastewater testing to confirm the presence of SARS-CoV-2 before actions commence, or they may initiate targeted sampling/testing of localized human populations (i.e., upstream within the sewer systems). Confirmed positive detections lead to public announcements by government agencies, journalists, and others, revealing that SARS-CoV-2 was detected in wastewater. They may also advise a large segment of the population to seek testing for COVID-19 if they live within or have visited the area, and particularly if they have any COVID-19 symptoms. If community-wide clinical surveillance in response to positive wastewater surveillance results fails to identify active infections, the presumed wastewater surveillance false-positive errors may cause policymakers, public health officials, and the public to lose confidence in wastewater surveillance and question its reliability as an early warning system or useful source of information.

Conversely, it is possible for COVID-19 infections to be present in the community, and for SARS-CoV-2 RNA to be present in wastewater samples, yet in some cases wastewater sample testing yields negative results (RT-PCR and wastewater surveillance false-negative). Such false-negative errors can be just as problematic as false-positive ones. By failing to alert public health officials to a potential outbreak or by underestimating the extent to which SARS-CoV-2 is circulating in a community, wastewater surveillance false-negative errors may put communities and healthcare workers at unnecessary risk by providing a false sense of security. Also, such false-negative errors without clinical testing may increase in community transmission before any control measures can be put in place, with a concomitant rise in hospital admissions and deaths. This may in turn prompt more severe mitigation measures and subsequent economic impacts than if an outbreak had been detected earlier through wastewater surveillance results.

In many communities, the results of SARS-CoV-2 RT-PCR wastewater analyses are published on social media, internet dashboards, and/or in the media. False-negative errors may suggest to the public that SARS-CoV-2 infections are declining or absent in the community, which could result in greater apathy and disregard for public health mitigation measures. In addition, reporting false-negative errors, once discovered, may reduce public confidence in wastewater surveillance. In some jurisdictions, there may be legal consequences for reporting inaccurate results. This has not yet become apparent in practice; however, it should be considered where SARS-CoV-2 RT-PCR screening of wastewater is offered as a service by commercial laboratories.

6. Strategies to identify and mitigate SARS-CoV-2 false-positive and false-negative errors

To characterize and mitigate SARS-CoV-2 false-positive and false-negative errors, we recommend consulting the well-established Minimum Information for Publication of Quantitative Real-time PCR experiments (MIQE) guidelines which address data reporting for steps ranging from experimental design to data analysis (Bustin et al., 2009; Huggett et al., 2013). Nevertheless, such guidelines may need to be adapted to overcome specific challenges posed by environmental surveillance or the monitoring of pathogens, including SARS-CoV-2 wastewater surveillance. The recently published Environmental Microbiology Minimum Information (EMMI) Guidelines offer an excellent quality control and reporting framework for publication of environmental microbiology studies using quantitative PCR techniques, such as those for wastewater surveillance of SARS-CoV-2 (Borchardt et al., 2021).

6.1. Practices to identify and minimize false-positive errors

As previously discussed, contamination of an RT-PCR assay from extraneous nucleic acid sources is an important potential source of false-positive errors. Nucleic acid from equipment, other samples, and previously synthesized amplicons can contaminate samples or reagents. This section highlights key strategies for identifying and minimizing false-positive errors in wastewater surveillance applications.

6.1.1. Laboratory organization, management, and workflow

Laboratory organization, management, workflow, and QA/QC practices can help minimize the rate of false-positive errors in wastewater surveillance. Thorough cleaning of laboratory work areas with methods proven to destroy or disintegrate RNA on a routine basis (e.g., bleach and UV) is necessary (Huggett et al., 2020). It is also important to physically separate key steps in the RT-PCR workflow, creating autonomous work areas for sample processing (e.g., pre-treatment and concentration of samples), RNA extraction, RT-PCR master mix set-up (i.e., clean room), amplification, and post-RT-PCR (i.e., gel electrophoresis, sequencing, etc.). Each designated work area should be equipped with dedicated equipment, consumables, and personal protective equipment. Furthermore, cross-contamination between dedicated areas can be eliminated by implementing unidirectional laboratory workflow practices (Millar et al., 2002; Huggett et al., 2020).

6.1.2. Identification of contamination from field and laboratory environments

Controls should be used to ensure that contamination does not confound results. Contamination during sample collection can be identified by including a sample negative control (USEPA, 2014). A sample negative control is prepared by dispensing a sample of sterile water into a sample container in the field, which is subsequently transported to

the analytical laboratory together with the collected wastewater samples (Esbensen and Ramsey, 2015). An equipment negative control entails decanting a sample of laboratory-grade water over or through the sampling equipment before collecting a wastewater sample (Esbensen and Ramsey, 2015). In contrast, a transport negative control is a sample of sterile water that is transported from the laboratory to the sampling site, after which it is re-transported to the laboratory without having been exposed to the sampling environment. The transport negative control thus serves to verify that contamination was not introduced due to travel conditions.

A method negative control should always be included with each batch of wastewater samples tested to ascertain whether contamination was introduced in the laboratory during sample analysis (USEPA, 2014). The method negative control should be analyzed in parallel with the wastewater samples using the exact procedure. For the RT-PCR amplification step(s), multiple no-template controls (NTCs) (minimum of three) should be included with each thermal cycling instrument run. A reverse transcription NTC should also be included for two-step RT-PCR applications to identify whether complementary DNA (cDNA) or SARS-CoV-2 RNA is the source of contamination. It can also be helpful to watch for any spatial patterns in the occurrence of false-positives based on the RT-PCR experiment design, when interpreting results near or below a defined RT-PCR ALOD or ALOQ. Unexpected patterns in contamination detection based on experimental set-up or proximity to samples with a high target concentration or reference materials can suggest sample cross-contamination (Huggett et al., 2020). Finally, if false-positives are observed in control reactions, it may be useful to sequence these samples to confirm that they are SARS-CoV-2 genetic markers.

6.1.3. Sample handling minimization prior to concentration

From the time a wastewater sample is collected from the wastewater system to the time it is processed in the laboratory, it is vital to minimize contact with any materials or aerosols from the field and laboratory environments. The use of probes or any other materials that have come into contact with another sample can provide the opportunity to introduce contamination due to improper cleaning and/or exposure to aerosols. Wastewater samples should be immediately sealed and only opened during the composite preparation, aliquoting, and concentration steps. Practices that reduce the amount of time that samples are exposed to aerosols in the field and laboratory environment are recommended. In addition, it is important to avoid the insertion of any instrument probes into a sample to take measurements, such as temperature or turbidity. Instead, take measurements on a split sample after adequate homogenization and aliquoting.

6.1.4. Reagent screening and handling

The integrity of new batches of SARS-CoV-2 RT-PCR reagents (i.e., primers, probes, PCR master mix, and DNase/RNase free water) should be confirmed before use (Bustin et al., 2020; Huggett et al., 2020). This can be achieved by analyzing 10–12 NTC replicates alongside a positive control for each new lot of RT-PCR reagents. Moreover, the required amounts of RT-PCR master mix, primer-probe mix, and nuclease-free water should preferably be aliquoted for a single use to reduce the potential contamination associated with the frequent use of stock solutions over a prolonged time.

6.1.5. Plan a course of action

SARS-CoV-2 surveillance programs should consider developing a contingency plan to minimize interruptions in laboratory workflow due to contamination. While contamination response plans will likely vary by wastewater surveillance program, there are multiple practices that should be considered when false-positive errors occur (Table 1). When a false-positive is observed, it is important to confirm the amplicon by gel-electrophoresis and sequencing. It is also necessary to carefully consider which controls are yielding contamination. For example, if sample and method negative controls are negative but the RT-PCR NTC tests identify contamination, then troubleshooting should focus on the amplification work area and methodologies. In addition to verifying the presence of a SARS-CoV-2 false-positive and the responsible RT-PCR method step, it is necessary to expunge the contaminated reagent or equipment from the sample testing environment.

Table 1
Key elements of a successful false-positive mitigation plan.

Elements	Corrective actions
Verify the source of false-positive(s)	Confirm SARS-CoV-2 genetic marker(s) via gel-electrophoresis and/or sequencing. Use appropriate controls (e.g., field negative, method negative, and no-template) to identify sample collection, processing where contaminant (s) is/are introduced. RT-PCR method step(s) where contaminant(s) is/are introduced.
Expunge contamination from the test environment	Use aseptic technique and properly disinfect/sterilize all sample collection equipment, devices, bottles, and other laboratory consumables. Discard all unsealed consumables and reagents throughout entire method. Thoroughly clean all work area surfaces and equipment.
RT-PCR method updates (varies by source)	Strict enforcement of unidirectional laboratory workflow and dedicated equipment practices. Implement additional contamination monitoring for responsible step. Use different reagent lots. Use customized reagents that minimize carryover amplification contamination such as UNG. Additional analyst training.
Demonstrate absence of contamination	Use appropriate controls (e.g., field negative, method negative, and no template) to verify that contamination has been eliminated.

All unsealed samples, laboratory testing materials and reagents used throughout the entire analysis should be discarded. All work area surfaces (e.g., benchtops, floors, etc.) and laboratory equipment (e.g., microcentrifuges, microtube racks, etc.) should be treated with methods proven to degrade nucleic acids. Hydrogen peroxides or ethylene oxide (McEvoy and Rowan, 2019) gas sterilization may be necessary for pipettes and other equipment with working surfaces that are not amendable to surface cleaning. After all work areas and equipment are cleaned, it may be advisable to further confirm that the contamination source has been removed and to implement additional practices to prevent the reoccurrence of contamination.

Practices will vary by the source of false-positives; for example, using the NTC contamination example above, it may be useful to use an RT-PCR master mix formulation that includes a deoxynucleotide mix containing uracil instead of thymine along with a heat-labile uracil-N-glycosylase (UNG), which can be used to minimize potential carryover amplificon contamination during the RT-PCR amplification of SARS-CoV-2 (Blanchard et al., 2014; Mascuch et al., 2020). For other sources of contamination, it may be advantageous to seek additional analyst training or to implement additional control measures such as equipment or transport negative controls. Finally, it is crucial to demonstrate the absence of contamination leading to false-positive errors by systematically testing sample and method negative controls, and RT-qPCR NTCs before returning to wastewater sample testing.

6.2. Practices to minimize false-negative errors

Varying challenges associated with different factors can contribute to false-negative errors in wastewater surveillance for SARS-CoV-2, including sampling strategy, variability in community shedding and sewage composition, non-representative sampling, improper sample pretreatment and storage, lack of laboratory analyst proficiency, and

interferences originating from the wastewater sample itself. This section describes practices and potential strategies for identifying and minimizing the occurrence of false-negative errors in SARS-CoV-2 RT-PCR testing (Table 2).

6.2.1. Sample handling

Practices that limit exposure to suboptimal temperatures during transport and storage before concentration and RNA extraction are recommended. Temperature monitoring systems such as iButton (iButtonLink, Whitewater, WI, USA) can be included during transport to ensure samples are not subject to rapid or long-term fluctuations in temperature, which makes them more susceptible to RNA degradation. This is particularly crucial when sample transportation occurs over multiple days or under extreme temperature conditions. To minimize RNA degradation, samples should be cooled and stored at 4 °C, shipped as quickly as feasible, and processed within 48–72 h after collection (Tan et al., 2017).

Bulk wastewater samples should not be stored at −20 °C and −80 °C to avoid potential damage due to freezing and thawing (Calcott, 1978). However, concentrated samples that are caked on filters or that are otherwise ready for direct analysis (i.e., ultrafiltered samples) can be stored at −20 °C and −80 °C for longer periods (Salehi and Najafi, 2014), especially if stabilized in a storage buffer (i.e., guanidinium thiocyanate). RNA extracts can be stored at −20 °C or −80 °C, but multiple freeze-thaws should be avoided. In addition, it is ideal to avoid wastewater sample pasteurization, if possible, because there is currently conflicting information on its effect on SARS-CoV-2 RNA detection. While heat treatment may be necessary to ensure the safety of analysts in suboptimal laboratory conditions, this high-temperature treatment may reduce the ability to detect trace quantities of SARS-CoV-2 and should be evaluated before practice.

6.2.2. Sampling strategies

Numerous factors can influence the concentration of SARS-CoV-2 in a particular wastewater sample ranging from infection prevalence to individual fecal shedding trends, to the location in the wastewater collection/treatment system where samples are collected. It is therefore important to know and collect as much metadata as possible about the local wastewater system (e.g., relevant COVID-19 public health data from the local community, wastewater flow rate, pre-treatment steps such as chlorination or coagulant addition) to minimize or at least characterize the risk of false-negatives for wastewater surveillance applications. Information on peak flow periods, the impact of local precipitation (e.g., storm events, snow melts, etc.), and land use in service areas (i.e., residential vs. commercial vs. industrial) can be useful to customize wastewater sampling to maximize the likelihood of detecting SARS-CoV-2 in a community. For example, if a local wastewater facility service area includes industrial and residential sectors, it may be strategic to collect samples from a pump station associated with residential areas rather than collecting a WWTP-influent sample that contains mixed industrial and residential wastewater.

In many situations, composite sampling can help minimize false-negatives due to fluctuations in sewage composition over time. This is particularly important for detecting virus shedding from a very few individuals. A flow-weighted composite sample is highly recommended, as it accounts for the fluctuations' inflow at the influent of a WWTP. If this is not possible, a time-weighted composite sample should be the second choice. It may be necessary to conduct pilot experiments to optimize the time intervals and/or flow conditions for optimal composite sampling. If only grab samples are collected in the absence of autosamplers or a lack of logistics, sampling should occur when SARS-CoV-2 loads are expected to be high. It might also be useful to consider collecting multiple grab samples over a short duration (3–4 h) or deploying passive samplers to increase the probability of detecting SARS-CoV-2, especially in areas with low infection rates. During precipitation, larger volumes of wastewater may need to be sampled to compensate for the dilution factor. Analyzing a larger volume (i.e., 1–2 L) will also increase detection sensitivity when COVID-19 cases are low or absent, assuming there is not a corresponding reduction in virus recovery during sample concentration. Primary sludge may be an attractive alternative to untreated wastewater because of solid particles that contain a wide array of enteric viruses (Peccia et al., 2020; Graham et al., 2021). However, the occurrence of SARS-CoV-2 in primary sludge needs to be further characterized.

Table 2
Key elements of a false-negative mitigation plan.

Element	Corrective actions
Sampling	Use composite sampling where applicable; For grab sampling applications, multiple sampling events over a few hours during morning to midday period may be useful. Seeding wastewater samples with appropriate process control material. Use multiple sample replicates to confirm results. Collect multiple sample replicates to confirm results.
Sample storage	Proper storage conditions during transport and prior to processing. Avoid storing bulk samples and minimize freeze-thaw cycles where possible. Minimize storage time prior to concentration.
Virus concentration	Optimization of concentration method using appropriate surrogate and local wastewater. Include process control to assess recovery efficiency. Consider concentrating virus from both liquid and solid phases of wastewater.
RNA extraction	Assess RNA quality and yield before testing. Seed molecular process control to assess extraction efficiency. Validation of extraction method with appropriate surrogate.
PCR inhibition	Inhibition molecular process control or dilution to assess potential inhibition effects.
RT-qPCR or -dPCR amplification	Confirm assay sensitivity using local wastewater samples seeded with appropriate control material. Analyze multiple replicates for trace detections.

6.2.3. Virus concentration and RNA extraction

Any laboratory involved in or intending to participate in wastewater surveillance of SARS-CoV-2 should identify viable long-term options and evaluate virus concentration and RNA extraction methods based on performance and practical aspects (e.g., access to necessary equipment, labor, cost, and potential supply chain limitations) before implementation. SARS-CoV-2 should be concentrated from both liquid and solid phases of wastewater especially when small volume samples (i.e., <100 mL) of wastewater are processed in regions with low COVID-19 clinical cases.

Commercial RNA extraction kits often used in SARS-CoV-2 studies comprise of silica columns specifically designed for the efficient removal of polyphenolic compounds, humic/fulvic acids, tannins, and melanin (La Rosa et al., 2021; Graham et al., 2021); however, extractions based on nucleic acid solubility (precipitation) could be more effective in some scenarios. The performance of virus concentration and RNA extraction methods should be determined by seeding an appropriate sample process control in wastewater. If performance is not satisfactory in a series of samples, switching to alternative methods may reduce the likelihood of observing a false-negative due to poor virus concentration and RNA recovery. When possible, it is recommended that RNA recovery be monitored on a sample-specific basis, or at least routinely, due to potential shifts in wastewater composition over time.

6.2.4. Inclusion of sample processing controls

Systematic errors that introduce unwanted bias in SARS-CoV-2 measurements can lead to false-negative errors. For example, multiple studies have reported substances commonly found in environmental samples that readily bind to nucleic acids and, when bound, force a

shift in nucleic acid conformation (Cai et al., 2006). These interactions have been shown to interfere with nucleic acid recovery in surface waters polluted with wastewater and were undetectable with a molecular process control (Shanks et al., 2016). It is highly likely that this type of interference can occur in wastewater surveillance. To monitor this potential source of false-negative errors, it is recommended to include a sample process control (i.e., a surrogate virus) to obtain sample-specific information about the recovery efficiency of the entire process or even a specific step within the workflow (Ahmed et al., 2020e; McMinn et al., 2021). A sample process control should be included for at least 10–20% of the samples tested in a surveillance program. The biological and physical characteristics of the sample process control should be similar to SARS-CoV-2. In the absence of an appropriate surrogate, endogenous controls such as pepper mild mottle virus (Rosario et al., 2009) or crAssphage (Stachler et al., 2017) can also be used as an indicator of fecal strength or to monitor method performance.

6.2.5. Monitoring for PCR inhibition

Appropriate molecular process controls should be used to monitor the presence of suboptimal RT-PCR assay performance (Hata et al., 2011) and must be included in every wastewater sample tested (Murray et al., 2013). Pecson et al. (2021) demonstrated that the RT-qPCR methods generally passed regarding traditional inhibition controls, specifically seeding a molecular process control into a nucleic acid extract and assessing changes in Cq value. However, some samples still exhibited non-sigmoidal amplification curves which might indicate partial inhibition. Therefore, inhibition can be present in multiple ways by preventing the amplification of the intended target or biasing amplification. If inhibitors are suspected, efforts should be made to minimize inhibition to exclude false-negative errors. Multiple strategies exist for mitigating amplification inhibition in PCR-based experiments (Gibson et al., 2012).

In some cases, inhibition can be alleviated by utilizing a different RNA extraction approach capable of removing the specific inhibitor of concern. Others utilize inhibitor-tolerant designer polymerases such as Environmental Master Mix (Thermo Fisher Scientific, Grand Island, NY, USA), which has been shown to reduce the occurrence of amplification inhibition in environmental qPCR applications (Cao et al., 2012). Another widely applied approach is sample and nucleic acid dilution (Schrader et al., 2012). While dilution testing is guaranteed to help alleviate inhibition, it also directly reduces the concentration of the genetic target, potentially leading to a false-negative errors, and it is not useful when many samples are expected to provide a true-negative results. Therefore, sample or its nucleic acid dilution is not recommended or should be limited (e.g., 2-fold vs. 10-fold) for trace applications such as detecting low levels of SARS-CoV-2 in wastewater. Commercially available nucleic acid clean-up kits can be used to remove PCR inhibitors that are commonly found in complex matrices such as wastewater.

6.2.6. Assay optimization and selection considerations

RT-PCR assay protocols, such as those used for surveillance applications, should include optimized annealing temperatures and oligonucleotide concentrations, which may need to be determined empirically in each setting (Bustin et al., 2009; Huggett et al., 2013; Borchardt et al., 2021; Bivins et al., 2021b). In addition, RT-PCR performance characteristics such as ALOD and ALOQ should be established using appropriate reference materials and following practices outlined in the MIQE guidelines (Bustin et al., 2009; Huggett et al., 2013; The dMIQE Group and Huggett, 2020) before wastewater testing. Since the emergence of SARS-CoV-2, mutations and selection pressure on SARS-CoV-2 within the human population have led to new widespread variants in certain regions or worldwide (Peñarrubia et al., 2020). Although this phenomenon has not yet led to significant issues with the molecular assays commonly used for SARS-CoV-2 detection and quantification, it is important to monitor the genomic properties of local SARS-CoV-2 populations on a routine basis while keeping update on emerging lineages worldwide to allow for timely modification of primer and/or probe sequences to minimize detection biases and, in extreme cases, false-negatives.

6.2.7. Optimizing the experimental design for trace detection of a virus

To reduce the damage to fragile RNA templates, freezing and thawing prior before testing should be avoided. Where possible, it is ideal to test samples immediately after RNA extraction (no freezing before RT-PCR amplification). Material may also be lost due to the absorption of microtubes even when using low-retention plastics. If RNA extracts must be stored for prolonged periods before testing (>30 days), stability should be verified to ensure no degradation has occurred, potentially leading to a false-negative result (The dMIQE Group and Huggett, 2020). A molecular process control could be invaluable providing a reference with a known concentration to determine if the integrity of the RNA extract has significantly changed during storage. It can also be useful to optimize the number of replicates tested to increase the probability of detecting a trace quantity. This practice is important for wastewater samples and contamination controls (e.g., sample and method negative controls, and RT-qPCR NTCs). Increasing the sample volume may also be helpful if it does not result in reduced RNA recovery and/or a higher incidence of RT-PCR amplification inhibition. It is beneficial to explicitly state and empirically determine ALOD, MLOD, ALOQ and MLOQ definitions. This allows for the unambiguous interpretation of trace detections without any evidence of contamination in control experiments. Finally, it is useful to confirm trace detection in at least a subset of samples via sequencing.

Unlike many environmental microbiology research projects which aim to isolate, quantify, or characterize microbiota, the purpose of wastewater surveillance is to inform public health action. Such actions could include large expenditures of resources and the implementation of interventions that are associated with significant impacts on the public. Because of the magnitude of these actions and their potential ramification, wastewater surveillance program data should be of the highest quality. Likely sources of error or bias should be thoroughly explored, and protocols validated such that wastewater surveillance affords accurate information to public health officials. As described herein, this validation should include all aspects of the microbiology workflow from sampling to data analysis. In the absence of such verification, wastewater surveillance is unlikely to afford a reliable source of information.

7. Conclusions

- Wastewater surveillance is a rapidly developing and growing area of research and is already playing an important role in the COVID-19 pandemic. Wastewater surveillance strategies will also be vital for preventing new outbreaks and managing future pandemics and more traditional endemic pathogens such as seasonal flu, enteric pathogens and antimicrobial-resistant bacteria and their genes.
- Many wastewater analysis protocols are not optimized for trace analysis of viruses or other substances, making them susceptible to false-positive and false-negative errors, as discussed in this study. It is important to acknowledge these challenges when interpreting data and inferring the probability that individuals infected with SARS-CoV-2 are present in the community.
- Improvements are needed for reliable and rapid detection application. To detect SARS-CoV-2 or any other microbial target of public health interest with greater accuracy, it is essential to set coordinated guidelines on sampling, establish stringent QA/QC practices, and identify effective concentrations, efficient RNA extraction, and highly sensitive and specific RT-PCR assays for detection.
- While standardization of some aspects of the laboratory workflow may not be appropriate or possible due to context-specific variability in methods, instruments, and reagents, it is essential to seek broad inter-laboratory harmonization as much as possible.
- Additional research is also warranted, including inter-laboratory studies utilizing standardized reference materials and protocols to better

understand the variability associated with various aspects of the methods in use. In this way, underperforming laboratories could validate their performance against laboratories that demonstrate superior performance.

Disclaimer

The views expressed in this article are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. The U.S. Environmental Protection Agency through the Office of Research and Development provided technical direction but did not collect, generate, evaluate, or use the environmental data described herein.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149877.

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