# Assessing the utility of passive sampling for building-scale SARS-CoV-2 wastewater-based surveillance to inform public health action

by

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# Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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# Statement of Contributions

Blake R. Haskell is the sole author for Chapters 1 and 3, which were written under the supervision of Prof. Mark Servos. This thesis contains one manuscript which has been accepted for publication as follows:

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# Abstract

Wastewater-based surveillance (WBS) is an effective public health tool that has been used to detect human viruses for decades. Most recently it has been applied to monitor SARS-CoV-2 RNA in municipal wastewater to track the prevalence and spread of COVID-19 in communities during the pandemic. Much of this work has been performed at the wastewater treatment plant (WWTP) prior to treatment, where it has been shown that WBS is closely related to clinical infections and hospitalizations. The application of WBS has gradually expanded to include upstream sites within the sewershed where neighbourhood and building-scale surveillance can be performed. However, sampling in these upstream environments introduces additional challenges for sampling and interpretation. The intermittent flow and composition of wastewater in the sewers close to the source greatly influences the variability and ability to accurately detect and quantitate the target viral fragments. One approach to circumvent some of these challenges is to employ a passive sampling approach where a chosen material is immersed in the sampled medium and left to passively accumulate a target analyte of interest over time. The sampling material is consistently exposed to the sampling environment which may reduce the likelihood of false negative detections. In this thesis, a two-tiered, trigger-based wastewater surveillance program was developed on the University of Waterloo (Waterloo, Ontario) campus residences during an active public health emergency (COVID-19 pandemic). The objective was to determine if WBS using passive sampling can be used to inform institution-level public health action.

Preliminary pilot studies validated a passive sampling method capable of detecting SARS-CoV-2 RNA in a municipal sewage system. Three candidate materials held in plastic frames (e.g., torpedo shaped perforated tubes) were tested for their efficacy in this application, including electronegative membrane filters and standard tampons. Cotton gauze was selected as the sampling medium for routine surveillance as it was able to detect the virus the most consistently and retained more suspended solids than the other materials tested. Twenty-four-hour passive samples were then collected three days per week over an eight-month surveillance period at selected utility holes associated with residences on the University of Waterloo campus. Two nucleocapsid gene targets (N1 and N2) of SARS-CoV-2 as well as the endogenous fecal indicator pepper mild mottle virus (PMMoV) were washed from the samplers, concentrated, extracted, and then quantified using real-time quantitative polymerase chain reaction (RT-qPCR). PMMoV is an endogenous indicator associated with human feces which has been used to normalize SARS-CoV-2 concentrations and account for dilution effects. The SARS-CoV-2 results were reported to the University health team in near real time (<12 h from sample collection). The developed workflow prioritized surveillance population coverage and minimal resource usage to address a variety of complex stakeholder needs and best support public health decision-making.

The period of the study included two contrasting exposure scenarios prior to and after the rapid emergence of the SARS-CoV-2 Omicron (B.1.1.529) variant. In the fall of 2021, community viral burden was low and a tiered sampling network was able to isolate individual clinical cases at the building-scale. In the winter of 2022 wastewater signals were quickly elevated by the emergence of the highly transmissible Omicron variant. The high prevalence of SARS-CoV-2 shifted surveillance objectives from isolating cases to monitoring trends. Throughout the surveillance period, comparisons between detection results and reported clinical cases revealed that passive samplers positively identified all but one of 203 infected individuals over eight months. In one instance surveillance led to the pre-symptomatic detection of a single individual at a site monitoring over 1300 students. WBS detected the infected individual two days in advance of clinical testing confirmation, demonstrating the efficacy of the tiered passive sampling approach in supporting public health action. Remarkably, SARS-CoV-2 concentrations on passive samplers were significantly correlated with confirmed clinical cases within the upstream sewershed. The strongest correlations were observed when clinical cases were assumed to have been shedding for 10 days from reporting illness. Additionally, comparisons between SARS-CoV-2 concentrations detected in campus sewers and in municipal wastewater influent suggest that the spread of COVID-19 on the campus was similar to that of the broader community. Periods of increasing and decreasing viral burden were captured at both sampling scales and closely mirrored each other in winter 2022. These results add to the mounting evidence that passive samplers are capable of producing semi-quantitative data that reflects the prevalence of disease within the sewer catchment. Alongside routine surveillance, methodological refinement occurred in parallel with routine surveillance efforts with the goal of maximizing data insight and actionability. This included routinely evaluating samples for evidence of RT-qPCR inhibition which saw a marked increase when students returned to campus and wastewater production increased. Modifications to the workflow were made to reduce inhibition and increase confidence in surveillance results. PMMoV concentrations on passive samplers were not reflective of upstream population differences and normalization of SARS-CoV-2 with PMMoV did not improve correlations with a clinical dataset. The results suggest that saturation of the material occurred during the exposure period thus limiting the utility of PMMoV as a normalizer to account for dilution effects.

This investigation demonstrates that passive sampling can be used as an effective tool to guide highly localized public health action. Spatially refined wastewater surveillance can support pandemic management decision making by acting as an early warning system, providing a basis for targeted health intervention and possible clinical testing, and is able to track spatiotemporal variations in viral RNA concentrations. The utility of a tiered surveillance approach described in this thesis demonstrates the importance of developing versatile methodologies that can be applied at varying spatial scales to address emerging public health challenges.

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# Dedication

This thesis is dedicated to the frontline workers, first responders, medical professionals, researchers and yes, even fish biologists, who supported our communities throughout the pandemic. Let this period of endurance and collaboration be a reminder of the great things we can achieve when we work together.

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# Chapter 1
# Wastewater-based surveillance and SARS-CoV-2

The novel coronavirus-19, or severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the virus responsible for the COVID-19 disease. The virus originated in Wuhan, China in 2019 and was later declared a global pandemic by the World Health Organization (WHO) in March 2020 (World Health Organization, 2020). Individuals who become infected with SARS-CoV-2 can experience a wide array of symptoms including headaches, fever, malaise, shortness of breath, and diarrhea (Gao et al., 2020; Zhang et al., 2020). As of February 2023, there have been over 756 million confirmed cases of COVID-19 worldwide, resulting in more than 6.8 million deaths globally (World Health Organization, 2023). In response to the COVID-19 pandemic, researchers around the globe began to explore the use of wastewater-based surveillance (WBS) to track disease prevalence in communities. WBS exploits the phenomenon of fecal shedding – a process where an individual infected with SARS-CoV-2 releases both intact and fragmented virus through their stool which can later be detected downstream in wastewater (Ahmed et al., 2020; Hrudey et al., 2022; Kitajima et al., 2020; Xiao et al., 2020). WBS demonstrated its use as a public health tool during the re-emergence of Poliovirus in the early 2000s (Aguiar-Oliveira et al., 2020; Hovi et al., 2001; Manor et al., 1999) and its continued use to monitor illicit drug consumption (Choi et al., 2018; Huizer et al., 2021; O'Keeffe, 2021). Over the last three years, in what has been an unprecedented period of collaboration, the COVID-19 Wastewater Coalition was created under the Canadian Water Network to rapidly disseminate knowledge between researchers and accelerate progress in developing a useful, scientifically rigorous framework for WBS (Canadian Water Network, 2023b; Manuel et al., 2021). Now, three years later, the outcomes of these efforts have been fully realized with integrated WBS programs established in all thirteen Canadian provinces and territories (Canadian Water Network, 2023). Support for WBS has expanded because of its ability to closely track trends in clinical cases, forecast key public health metrics, and fill a critical knowledge gap especially when clinical testing capacity is reduced (Cheng et al., 2023; Hrudey et al., 2022; Manuel et al., 2022).

Along with municipal wastewater programs under development at the beginning of the pandemic, researchers began exploring the application of WBS in upstream settings where targeted public health intervention would be desirable. A key focus for such efforts included an array of congregate living facilities such as long-term care homes, homeless shelters, prisons, daycares, universities and even the Olympic Village at the Tokyo 2020 Olympic games, each presenting their own unique challenges related to pandemic management (Akingbola et al., 2022; Harris-Lovett et al., 2021; Hrudey et al., 2022; Kitajima et al., 2020, 2022; Ouslander & Grabowski, 2020). Maintaining operations in these facilities where vulnerable and largely

sedentary populations reside was logistically challenging (Harris-Lovett et al., 2021; Ouslander & Grabowski, 2020; Ryan et al., 2021). It was thought that similar methodologies used downstream at the wastewater treatment plant (WWTP) could be applied in these upstream settings to provide building-scale public health data in near real-time. Institutions could reap the benefits of spatially refined surveillance by following up positive wastewater detections with targeted clinical testing or mitigating public health measures. Once a clinical case or cases were identified through testing, the individual(s) could be moved to isolation so that the facility can resume normal operations. While this solution to address institutional health challenges was promising, sampling in these environments introduced unique, site-specific challenges for practitioners of WBS globally (Bivins et al., 2022a; Harris-Lovett et al., 2021; Kitajima et al., 2020; Schang et al., 2021). Of primary concern was the need for a suitable method to address the pulse-input flows characteristic of upstream sampling sites. Given the intermittent nature of human toileting patterns, discrete sampling methods risk missing an infected individual's input into the system during non-sampling intervals. This could result in a type II error by failing to detect the target despite its presence in the system during the sampling window. Addressing this limitation should be a top priority for upstream WBS programs where positive detections are used to guide targeted public health action.

In tandem with the development of upstream WBS programs, in spring 2021 the Department of Housing at the University of Waterloo (the University) was preparing to reopen on-campus residence buildings to the general population. At the time, a massive COVID-19 vaccination program was underway in Canada whereby the end of July 2021, 67.2% of college-aged students in Ontario had received one dose of a Health Canada approved COVID-19 vaccine (Public Health Agency of Canada, 2023). On campus, several public health measures were developed to protect human health while maintaining facility operations heading into the fall term. These measures included implementing occupancy limits, restrictions on shared bathroom and dining facilities and a framework for case management and contact tracing. However, under shifting provincial public health guidance there was still concern for the efficacy of existing tools to detect asymptomatic cases in residence. SARS-CoV-2 variants of concern (VOC) also introduced uncertainty. These distinct viral lineages arise from naturally occurring mutations in the virus which can alter its physical and immunological properties (Volz et al., 2021; World Health Organization, 2023b). VOCs are lineages that have been classified on their potential to adversely impact human health, such as increased transmissibility or associated disease severity (World Health Organization, 2023b). Reports of waning vaccine longevity and immunity under emerging VOCs furthered concerns for case load management in the fall semester (Mallapaty, 2021; Pilishvili et al., 2021; Shrotri et al., 2021; Spurbeck et al., 2021; Thomas et al., 2021) which prompted the University to require students to have a second dose of a COVID-19 vaccine before November 1, 2021. The University also did not employ any invasive testing requirements at any time, meaning no student was ever required to take a clinical PCR test. Asymptomatic individuals or infected individuals

intentionally evading public health protocols also birthed potential for widespread transmission in these close-quarters communities. Given these challenges, WBS served as an attractive tool for campus decision makers to monitor disease prevalence on campus. It was hypothesized that building-scale WBS could provide an additional layer of evidence to support the implementation or potential easing of public health restrictions in communities. It also provided the potential for early detections to be identified and isolated to minimize transmission potential within afflicted communities. However, the University was no exception to the previously described challenges with building-scale surveillance. A suitable method needed to be developed and scaled using existing wastewater infrastructure and be designed to maximize actionability of the data. This institutional need coupled with knowledge gaps in building-scale WBS literature serve as the basis for this thesis.

## 1.1 Overview of SARS-CoV-2

SARS-CoV-2 is a coronavirus belonging to the *Coronaviridae* family (Hasöksüz et al., 2020). Coronaviruses are enveloped, positive-sense, single stranded, RNA viruses that have been shown to infect a variety of animals and humans (Hasöksüz et al., 2020). SARS-CoV-2 is the seventh coronavirus known to infect human beings (Andersen et al., 2020; Nalbandian et al., 2021). The viral genome hosts a highly conserved nucleocapsid (N) gene which is responsible for encoding proteins that associate with the viral RNA gene and form the ribonucleoprotein core (Chang et al., 2006). Two regions of interest on the N-gene form the basis for diagnostic tests developed by the U.S. Centre for Disease Control (CDC) and are known as N1 and N2 (Centers for Disease Control and Prevention, 2021).

Human-to-human transmission of the virus occurs primarily through the transfer of aerosolized liquid particles caused by coughing, sneezing, and speaking at a conversational distance (World Health Organization, 2021). An individual can become infected through the inhalation of an infectious particle. To a lesser extent, people may also become infected by touching their eyes, nose, or mouth after touching a surface contaminated with infectious viral particles (World Health Organization, 2021). Studies suggest that individuals are most infectious immediately prior to the development of symptoms and in the early stages of infection, though this timeline can vary depending on the severity of the disease (Cevik et al., 2021; Jones et al., 2021; World Health Organization, 2021). In addition to the stage of infection, environmental conditions have also been shown to influence the transmissibility of the virus. Due to the mechanism of transmission, areas which are poorly ventilated or crowded can enable the aerosols to remain suspended in the air for a longer period of time (World Health Organization, 2021). Poor ventilation allows for infectious particles to travel farther, beyond a conversational distance, thus increasing the zone of transmission. Exposure time also plays a role in transmissibility, with longer periods of time in these settings increasing the risk of infection

(World Health Organization, 2021; Yu et al., 2020). These transmission dynamics have provided the basis for many of the public health measures enacted in countless jurisdictions (including the University) over the past three years, including social distancing, personal masking, aerosol barriers and building capacity limits.

Individuals who become infected with SARS-CoV-2 can experience a wide array of symptoms with varying degrees of severity (Gao et al., 2020; W. Zhang et al., 2020). Recent research has shown that symptoms can persistent well beyond the acute phase of infection and can affect multiple organ systems (Nalbandian et al., 2021). Chronic kidney disease, chest palpitations and thromboembolism have all been documented as chronic symptoms of COVID-19 (Nalbandian et al., 2021). These reasons in part make minimizing transmission a top priority for health care systems with finite resources. While symptomatic cases make up the majority of all infections globally, a meta-analysis on SARS-CoV-2 including nearly 30 million individuals undergoing clinical COVID-19 testing found that 40.5 ± 7.5% of all cases are asymptomatic (Ma et al., 2021). These individuals do not experience symptoms associated with COVID-19 despite still being able to transmit the virus to others (Jones et al., 2021; Lee et al., 2020).

## 1.2 Viral shedding in humans

Throughout the course of infection, a person infected with SARS-CoV-2 will release the virus through various bodily functions in a process known as viral shedding (Zapor, 2020). The virus leaves the body as both intact SARS-CoV-2 viruses and inert viral fragments through various shedding pathways (Zapor, 2020). For SARS-CoV-2, shedding primarily occurs via the pharynx and gastrointestinal (GI) tract, though urine, saliva and other bodily fluids can produce detectable amounts of virus as well (Crank et al., 2022; Karia & Nagraj, 2020). Infection of the pharynx enables the detection of the virus via a nasopharyngeal swab to be used in clinical molecular testing. Alternatively, infection of the GI tract can cause the excretion of the virus embedded in feces (Cevik et al., 2021; Gao et al., 2020; Holshue et al., 2020; Zhang et al., 2020). This process is known as gastrointestinal shedding or fecal shedding. Fecal shedding occurs in at least 50-66% of all infected people, whether they are symptomatic or asymptomatic (Chan et al., 2021; Lo et al., 2020a; Xiao et al., 2020). Fecal viral shedding provides the basis for non-invasive population-level testing methods to be discussed in the next section.

The rate at which viral shedding occurs is highly variable. One of the most influential factors is the stage of infection, where peak respiratory shedding occurs anywhere between symptom onset and day five of infection, coinciding with period of peak transmissibility (Cevik et al., 2021; Jones et al., 2021). While the majority of cases produce peak shedding within this timeframe, respiratory viral shedding up to six days in advance of symptom onset has been documented (He et al., 2020). Fecal shedding is less predictable. It has

been reported that fecal shedding variability is much more erratic than respiratory shedding, highlighting the complexity of factors which govern this process (Walsh et al., 2020; Zheng et al., 2020). Fecal viral shedding has been shown to last significantly longer than respiratory viral shedding in approximately 70% of patients, sometimes persisting weeks and even months following infection and particularly for individuals with pre-existing health conditions (Cevik et al., 2021; He et al., 2020; Morone et al., 2020; Natarajan et al., 2022). Recent research has shown that shedding rates may also be variant-specific and change as new mutations in the virus occur (Prasek et al., 2023). Still, fecal shedding patterns generally follow the same trends observed in clinical settings wherein the peak viral load is reached in the first week following infection (Cheung et al., 2020; Natarajan et al., 2022; Zhang et al., 2021).

The presence of symptoms and their severity plays a major role in an individual's viral load and consequently, the viral load in which they excrete into wastewater. One study reported that patients experiencing gastrointestinal symptoms, such as diarrhea, had a 2.4-fold increased likelihood of excreting detectable levels of SARS-CoV-2 RNA in their stool, compared to individuals with no GI symptoms (Zhang et al., 2021). Cheung et al. (2020) came to similar conclusions, reporting that patients with diarrhea had a higher stool RNA positivity than those without. Symptom prevalence and severity may also be sex-linked. Sierpinski et al. (2020) found that non-hospitalized females infected with COVID-19 had a significantly higher incidence of GI symptoms compared to non-hospitalized males. This suggests that a greater proportion of females shed detectable levels of SARS-CoV-2 in stool than males (Sierpiński et al., 2020). Physical health at the time of infection may also mediate shedding dynamics. As one example, obesity and diabetes have been associated with an increased severity of COVID-19 symptoms and consequently a prolonged period of viral shedding (Zhong et al., 2022). Age plays an indirect role in shedding dynamics as well. Since younger adults tend to be in better overall health than senior cohorts, they may tend to exhibit less severe symptoms associated with COVID-19. One study administering over 700 clinical tests to college-aged students found that 79.2% of all COVID-19 cases had no symptoms at all (Schmitz et al., 2021) – a significantly higher proportion than the previously cited global estimate of 40.5 ± 7.5% (Ma et al., 2021). This suggests that younger populations, such as undergraduate level students, may have a lower incidence of symptoms and consequently produce lower levels of SARS-CoV-2 RNA in their stool. This highlights the utility of a WBS approach to monitoring disease prevalence in communities where asymptomatic transmission is expected to be high.

In clinical settings, COVID-19 vaccinations have been shown to significantly reduce the risk of developing severe symptoms, especially in young adults (Zhong et al., 2022). This in turn translates to a reduced viral load in stool where detectable. Most recently, the outcomes of vaccination campaigns have been realized in WBS applications where the immunity gained from vaccines has translated to a significantly

reduced viral load and positivity measured in wastewater (Bivins & Bibby, 2021). However, much of the work to characterize the effects of vaccines on viral loads in wastewater, and their associated implications for surveillance is on-going. Certainly, viral shedding is a dynamic process with considerable variability between individuals and within populations.

## 1.3 Wastewater-based surveillance

Wastewater is a complex matrix of greywater and blackwater that is produced by a host of human activities. Greywater is generated from sinks, washing machines, bathtubs, showers, and industrial processes which introduce chemicals such as disinfectants and detergents into the wastewater system (Global Water, 2022). Consequently, greywater contains few human pathogens. Alternatively, blackwater (also known as sewage, or brown water) comes from toilets and contains fecal matter and urine which is human pathogen-rich (Global Water, 2022). Both types of introduce inhibiting substances into the system which can interefere with the detection and quantification of a target analyte downstream in the RT-qPCR process. In wastewater, these substances can include humic and fluvic acids, heavy metals, proteins, salts and other chemical constituents making wastewater composition spatially and temporally diverse (Bitter et al., 2022; Bivins et al., 2022a). Wastewater captures various social, industrial, and biological processes in the form of biotic and abiotic constituents which can be measured (Choi et al., 2018; Hrudey et al., 2022; O'Keeffe, 2021). The study of wastewater-based epidemiology (WBE) aims to analyze these constituents to make inferences about human health (O'Keeffe, 2021). Most recently, however, the term wastewater-based surveillance (WBS) has been used to make the distinction between epidemiological research-related applications and on-going public health tracking and monitoring. The phenomenon of viral shedding is one such process that can be exploited for use in WBS. Fecal shedding inputs the SARS-CoV-2 into wastewater, thus enabling the detection and quantification of the virus downstream. With 86% of Canadians relying on municipal wastewater systems daily, wastewater is abundantly available as a sampling medium (Environment and Climate Change Canada, 2020) which allows for efficient monitoring disease prevalence in a significant portion of the population.

The fecal shedding of SARS-CoV-2 RNA is one example of a WBS application, though the concept is not new. One of the first applications of WBS occurred in 1954 where Bayer was able to trace a familial schistosome outbreak to a population of snails contaminated from sewage sources near a municipal dam (Bayer, 1954). Earlier studies sampling municipal water sources were able to identify the sources of several infectious disease outbreaks (Bivins et al., 2022a; Moore, 1948). In the late 1990s and early 2000s, WBS arose as a useful tool to monitor the re-emergence of Poliovirus in several countries and was shown to be highly sensitive (Aguiar-Oliveira et al., 2020; Hovi et al., 2001; Manor et al., 1999). For example, Hovi et al. (2001) reported being able to detect 1 out of 10,000 people shedding poliovirus RNA by analyzing a single

400 milliliter wastewater sample (Hovi et al., 2001). In Hawaii, Diemert and Yan embarked on a year-long monitoring campaign of *Salmonella* to fill data gaps in clinical infection rates (Diemert & Yan, 2020). These applications provided the foundational knowledge upon which WBS for SARS-CoV-2 has been built. Outside of infectious diseases, WBS has been applied to monitor illicit drug consumption, dietary patterns, human stress and a host of other endpoints to make inferences about human health and lifestyle (Huizer et al., 2021; O'Keeffe, 2021). The application of WBS has continued to be an effective as a public health tool during the COVID-19 pandemic.

## 1.4 Discrete methods for wastewater sampling

Wastewater samples are conventionally collected one of two ways. The first method is to use a composite sampler (also known as an autosampler) which is a device that can draw aliquots of wastewater for a specified time interval (Agriculture and Agri-Food Canada, 2020). The aliquots are combined to form a composite sample representative of the wastewater produced in the sampling window. This sampling approach can be time-weighted or flow-weighted, with the latter integrating pre-determined flow thresholds to trigger a sampling event (Agriculture and Agri-Food Canada, 2020; Augusto et al., 2022). These devices have been heavily used in wastewater surveillance for SARS-CoV-2, especially at the municipal level where sampling occurs at a wastewater treatment plant (WWTP) (Cheng et al., 2023; Dhiyebi et al., 2023; Hrudey et al., 2022). Composite samplers work well in these downstream applications where wastewater flow and composition are subject to less temporal variability. The major benefit of this sampling approach is the resulting quantitative data which has demonstrated its use to track and even lead COVID-19 case trends (Bibby et al., 2021; Cheng et al., 2023; D'Aoust et al., 2021). Similar benefits are sowed through the collection of grab samples – another common, discrete sampling method. Grab (or static) samples involve the collection of a single known volume of wastewater at a particular time and place (Agriculture and Agri-Food Canada, 2020). It represents only the wastewater passing through the system at that moment, similar to a "snapshot" in time. Grab samples also produce quantitative data but lack the time-weighted integration that composite samples can produce (Agriculture and Agri-Food Canada, 2020). Both composite samples and grab samples can be collected at any point of access throughout the wastewater system, including upstream of the WWTP. The method selection largely depends on the research question, logistical constraints and requirements for data analysis.

In this thesis, the term 'upstream sampling' refers to any wastewater sampling performed upstream of the WWTP. As the sampling location moves further upstream, away from the WWTP, the sampler moves closer to the source of the wastewater. This concept is employed in the sampling site selection process which allows practitioners of WBS to refine their surveillance efforts to highly specified spatial scales, isolating

wastewater from communities, a given street, or even a specific building. At upstream sampling sites where wastewater composition is more dynamic, the discrete nature of both composite and grab sampling risks missing the target analyte of interest (Rafiee et al., 2021; Schang et al., 2021; Wilson et al., 2022). This can result in the observance of false negatives, where the sampler did not detect the target analyte despite its presence in the system during the sampling window. This limitation has been cited in several studies investigating the detection and quantification of SARS-CoV-2 upstream of the WWTP (George et al., 2022; Habtewold et al., 2022; Rafiee et al., 2021; Schang et al., 2021). For this reason, many researchers and institutions have looked to passive sampling for its simplicity, affordability, and efficacy in these sampling environments.

## 1.5 Passive sampling

Passive sampling for infectious diseases has been practiced for several decades. Moore was one of the first to use a passive sampling approach to select for *Salmonella typhi* and was able to trace multiple human outbreaks back to the upstream fecal contamination of water (Moore, 1948). In his 1948 report, Moore cited that the most successful sampling method was a "strip of gauze, 4 feet by 6 inches in size, folded into eight thicknesses, secured with a string" – a sampling apparatus known today as the Moore swab, which is still widely used for environmental monitoring (Moore, 1948; Rafiee et al., 2021). Passive samplers are constantly exposed to the sampling medium – in this case, wastewater. This exposure increases the likelihood of the passive sampler to collect an analyte of interest, compared to the discrete sampling nature of conventional composite or grab samplers (Bivins et al., 2022a; Habtewold et al., 2022; Li, Verhagen, et al., 2022; Schang et al., 2021). This quality makes passive simply particularly advantageous in upstream sampling settings where fluctuations in wastewater flow and composition can be extreme (Corchis-Scott et al., 2021; Welling et al., 2022). Passive samplers also do not require electricity, can be made using readily available and affordable materials, and critically for surveillance applications, they often require less processing time compared to whole wastewater samples (Górecki & Namieśnik, 2002; Jain et al., 2022; Schang et al., 2021; Wilson et al., 2022).

### 1.5.1 Theory

In contrast to discrete sampling, passive sampling is based on the spontaneous free flow of analytes from the sampling medium (i.e., wastewater) to a collection medium (i.e., the passive sampler) (Górecki & Namieśnik, 2002). Unlike composite and grab samples which involve some action, passive samplers remain sampling without intervention or force – they are deployed and left to passively accumulate an analyte of

interest. This technique can be used on virtually any sampling medium in which the sampler can be immersed, including wastewater. The net flow of the target analyte from the sampling environment to the sampling medium occurs spontaneously until equilibrium (saturation) is reached or until the sampling session is terminated – whichever occurs first (Górecki & Namieśnik, 2002). To understand when this occurs, the kinetics of a given targets uptake and retention must be conceptualized as either an equilibirium or non-equilibrium operation.

In a non-equilibrium operation, the analyte of interest accumulates on the sampling material at a rate which is typically modeled using first-ordered kinetics (Salim & Górecki, 2019). Since the saturation point of the material is not reached, practitioners can estimate a time-weighted average of the analyte concentration on the sampling medium using a calibrated kinetic model provided two assumptions are met. The first is that the transfer of the analyte to the sampling medium is irreversible – that is, that no loss of the analyte occurs once it is captured (Górecki & Namieśnik, 2002). This is referred to as the "zero-sink" assumption (Górecki & Namieśnik, 2002). The second assumption is that the uptake rate of the target analyte is constant (linear) throughout the exposure period. If these assumptions are met, a calibration can be performed to empirically determine a suitable exposure duration based on the uptake rate and the sorption capacity of the sampling material (Salim & Górecki, 2019).

In an equilibrium operation, the concentration on the sampler will remain constant assuming the target analyte in the sampling medium remains constant (Górecki & Namieśnik, 2002). Once equilibrium is reached, the concentration of the target analyte on the sampling medium can be used to estimate the concentration of the target analyte in the sampling environment using an empirically determined partitioning coefficient (Salim & Górecki, 2019). Under equilibrium operations, however, changes in the medium being sampled (such as the constantly changing composition of flowing wastewater) can bias the estimated concentrations depending on the temporal scale over which those changes occur. Consequently, wastewater sampling for SARS-CoV-2 violates assumptions under both equilibrium operations which has largely limited the use of passive sampling for this purpose to generate qualitative data.

### 1.5.2 Limitations of passive samplers

Passive samplers are not without limitations. Primarily, it is difficult to produce truly quantifiable data from passive samplers. In the context of wastewater surveillance for SARS-CoV-2, the total volume of wastewater flowing through the sampler during the sampling period is unknown. Due to this restraint, passive sampling for SARS-CoV-2 RNA in wastewater violates the assumptions under both the equilibrium and non-equilibrium operations described above. The composition of wastewater also varies both spatially and

temporally. The biological and chemical constituents in wastewater that result from diverse upstream activities produce a wide range of physicochemical variation that can influence the uptake of the target analyte on the passive sampler (Bivins, et al., 2022a; Bivins, et al., 2022b; Habtewold et al., 2022; J. Li, Verhagen, et al., 2022). Excessive suspended solids and biocolloids in the wastewater can lead to adsorption and capture competition between analytes (Bitter et al., 2022; Hayes et al., 2021, 2022; Schang et al., 2021). Environmental factors such as temperature and pH may also influence the uptake of the target analyte on the sampling material and influence the quality and degradation of RNA in the sewer system (Bitter et al., 2022; Bivins et al., 2022a; Li et al., 2022; Li et al., 2023). Additionally, the concentration of SARS-CoV-2 in wastewater is dependent on both the viral load and fecal shedding rate of infected persons within the catchment, which as previously discussed is highly variable (He et al., 2020; Karia & Nagraj, 2020). The input into the system in conjunction with same-day fluctuations in water use patterns make the concentration of SARS-CoV-2 in the sampled wastewater all but constant.

Research on the calibration of passive samplers for SARS-CoV-2 uptake and retention is limited. This is primarily because there is also no standard approach to capture viral RNA using passive sampling. The pressure under which many SARS-CoV-2 surveillance programs were established over the last three years has left a patchwork of different collection, processing, and analytical methods to solve similar institutional-level issues (Harris-Lovett et al., 2021; O'Keeffe, 2021; Twigg & Wenk, 2022). The selection of the sampling material is another factor that has varied between groups. Researchers have tested various materials from sponges and cheesecloth to medical gauze and feminine hygiene products for their efficacy in capturing SARS-CoV-2 RNA in wastewater (Habtewold et al., 2022; Jain et al., 2022; Li et al., 2022; Rafiee et al., 2021; Schang et al., 2021). The basis for material selection has been variable depending on individual assessments of efficacy, affordability, and the intended application for surveillance. Additionally, there are logistical challenges to performing calibrations in-situ related to replication and sample collection. For example, the fouling or ragging of sampling units (a term to describe the entanglement and blockage of the sampler by in-situ debris) may become a significant confounding factor if multiple samplers are deployed at the same site. Multiple samplers would be a desirable experimental design if a suitable level of replication and statistical power is to be achieved. However even if a suitable in-situ calibration were performed, the presence of SARS-CoV-2 and its concentration in wastewater is subject to temporal variability which may limit findings. Similarly, bench-scale data may have limited applications to in-situ sampling environments given the absence of complex wastewater matrices that would be encountered in a real sewage system (Bivins et al., 2022a). Site-specific wastewater matrices may limit the applicability of findings between geographical regions. For these reasons, it has been extremely difficult to characterize the empirical kinetic qualities of passive samplers.

### 1.5.3 Passive sampling to produce semi-quantitative data

The physicochemical variations of flowing wastewater make it impossible to utilize passive sampling to measure SARS-CoV-2 concentrations accurately and precisely in these systems. Although, evidence to support that passive samplers can produce semi-quantitative (involving less than quantitative accuracy) data is mounting. Bivins et al., (2022a), performed an analysis of five studies that made comparisons between SARS-CoV-2 concentrations on passive samplers and in paired whole wastewater samples. They found that aggregating the datasets produced a weak linear relationship ($y = 0.40x + 3.0$, $r^2 = 0.41$) (Bivins et al., 2022). To account for the methodological differences between each study, they assessed each relationship individually and found that the linear fit of the studies ranged from 0.27 to 0.56. As a consequence of the diverse methods and environments across which these studies were performed, it is difficult to draw any universal conclusions from these findings. Nonetheless, it is promising that multiple studies have reported a significant relationship between the SARS-CoV-2 concentrations on passive samplers and the concentrations in wastewater.

Most studies utilizing a passive sampling approach have focused on reporting qualitative data (presence/absence). However, several studies have made attempts to produce semi-quantitative data using other endpoints. One approach is to estimate the volume of water passive through the sampler directly, based on flow data measured directly at the sampling site. Other studies have indirectly estimated wastewater volumes based on flush counts, water usage data and electricity usage (Acer et al., 2022; Sweetapple et al., 2022). As observed with varying success at the municipal level, endogenous indicators have also been used to normalize the amount of SARS-CoV-2 to the amount of fecal matter present in the system (Cheng et al., 2023; D'Aoust et al., 2021; Dhiyebi et al., 2023; Sakarovitch et al., 2022). This is primarily to account for the dilution effects that could influence the detection and quantification of SARS-CoV-2 in the sample. One of the most used endogenous indicators for SARS-CoV-2 has been pepper mild mottle virus (PMMoV). PMMoV is a plant virus belonging to the genus *Tobamovirus* (Kitajima et al., 2018). The virus infects species of bell and ornamental peppers and are consequently found in their processed food products, such as hot sauce (Kitajima et al., 2018). Like SARS-CoV-2, PMMoV is also shed through the gastrointestinal tract making it a good indicator of human feces (Corchis-Scott et al., 2021; J. Li, Verhagen, et al., 2022; Wilson et al., 2022). The global distribution of PMMoV, its correlation with fecal contamination, and its stability in water under various conditions make it a suitable endogenous reference candidate (Kitajima et al., 2018; Symonds et al., 2018). Theoretically, this is an effective approach to account for the variations in discharge that are most common in upstream applications. However, recent research demonstrates that the use of PMMoV to normalize the SARS-CoV-2 signal does not improve correlations with clinical cases or hospitalizations (Cheng et al., 2023; Dhiyebi et al., 2023). In the case of passive sampling, the quantification

of PMMoV harbours the same limitations as SARS-CoV-2. The equilibrium dynamics of the virus onto various sampling media are uncharacterized which limits meaningful interpretation of PMMoV-normalized passive sampling data.

### 1.5.4 Passive sampling applications for SARS-CoV-2 surveillance

At the onset of this investigation, several studies had utilized discrete sampling techniques to implement building-level WBS for SARS-CoV-2 on university campuses (Betancourt et al., 2021; Bivins & Bibby, 2021; Colosi et al., 2021; Gibas et al., 2021; Karthikeyan et al., 2021; Scott et al., 2021a; Wong et al., 2021). While the utility of grab and composite sampling in these environments often translated to meaningful public health action, practitioners of building-scale WBS highlighted several limitations to their use. Of primary concern is the ability of discrete methods to collect a representative sample. The success of close-to-source sampling, as achieved through building-scale WBS, is largely governed by periods of intermittent high and low flows related to human activity in the sewershed (Anderson-Coughlin et al., 2022; Fahrenfeld et al., 2022; Lee et al., 2023). These pulse-input events translate to significant temporal variability in wastewater composition which can have significant implications on detecting the virus. Grab samples are most subject to this variability given sampling occurs at a single point in time (Agriculture and Agri-Food Canada, 2020). Composite samplers aim to address this gap by sampling at regular time or flow-weighted intervals (Agriculture and Agri-Food Canada, 2020). However, even composite samplers may fail to capture the virus despite its presence in wastewater during the sampling window, particularly when disease prevalence in the surveillance catchment is low (Li et al., 2022; Rafiee et al., 2021; Schang et al., 2021). Early practitioners of upstream WBS also highlighted challenges with using composite samplers for routine surveillance. These devices typically require a power source to operate the pump, which is often not readily available at selected sampling points. Additionally, sample integrity to minimize RNA degradation prior to the sample being collected requires cool storage conditions which may not be offered by all composite samplers. One review paper reported that the average cost per automatic sampling unit was $3,000 – $5,000 USD (Harris-Lovett et al., 2021), with refrigerated sampling units costing even more. The same review reported that multiple WBS programs on university campuses experienced vandalism and theft of sampling units, thus impacting the reliability of reporting efforts (Harris-Lovett et al., 2021). These factors in combination often call for alternate approach for sampling of buildings where reliable and rapid reporting are called for.

In August 2021 the scientific literature on passive sampling for SARS-CoV-2 in wastewater was in its infancy. At the time, just two studies had piloted the use of passive sampling for SARS-CoV-2 and demonstrated potential for scalability in routine surveillance. Schang et al., (2021), were one of the first

groups to apply passive sampling for the detection of SARS-CoV-2. Their study investigated the efficacy of cotton buds, electronegative membranes and medical gauze for the capture and retention of SARS-CoV-2 RNA at various sewershed scales. Additionally, the group also tested four different material housing vessels – porous enclosures that prevent the ragging (entanglement and blockage by debris) of the sampler while still allowing wastewater and suspended solids to flow through it. They found that a novel 3D-printed torpedo shaped housing vessel had the lowest ragging incidence of the vessels tested. The authors reported that electronegative membranes produced the highest detection rate amongst passive sampling materials (41%), followed by gauze (31%) and cotton buds (25%) (Schang et al., 2021). Passive sampling materials also detected SARS-CoV-2 RNA in 13 of 33 sampling days where autosamplers did not, underscoring the potential for discordance between the two approaches in upstream applications (Schang et al., 2021). The authors highlight that passive samplers detected SARS-CoV-2 RNA more often than grab or composite samplers when wastewater concentrations fell below 1.8 SARS-CoV-2 gene copies per mL, indicating the superior performance by passive samplers at lower concentrations in wastewater (Schang et al., 2021). This is an important consideration for designing WBS programs, especially in environments where viral burden is expected to be low and when periods of high wastewater flow are expected.

Hayes et al. (2021) further expanded on passive sampling method development by comparing different materials using a novel 3D-printed sampling cage. They tested similar materials at both bench-scale and in-situ. The results demonstrate that both cheesecloth and electronegative membrane filters performed similarly in terms of total RNA retention and detection frequency. Gauze was also assessed in this investigation where the authors conclude that despite its superior performance for solid retention, SARS-CoV-2 concentrations in electronegative membranes were higher (Hayes et al., 2021). The authors suggest that while efficient solid retention is critical for detecting the virus, excessive retention can impede recovery through the accumulation of inhibitors. Hayes et al., (2021) also reported complete discordance between passive samplers and grab samples, echoing evasion concerns reported by Schang et al., (2021). Based on these early findings, this investigation aimed to determine if a passive sampling approach can be used to address institution-scale public health challenges to reliably detect SARS-CoV-2 and report in near real time.

## 1.6 Research Goals

The goal of this research is to determine if SARS-CoV-2 wastewater surveillance can be used on the University of Waterloo campus to inform public health action and response. This research aimed to address an urgent institutional problem related to public health management during an operationally challenging return to campus life. In parallel, the success of wastewater surveillance programs at the municipal level prompted questions about how methodologies could be applied to monitor highly targeted upstream populations. This thesis explores the application of this concept in the context of a post-secondary Canadian institution as pandemic dynamics shifted in real time. Additionally, this thesis describes the challenges and lessons learned from both a practical and analytical perspective that advance our understanding of the applications for WBS. Such efforts will be a critical part of pandemic preparedness in the future.

# Chapter 2

# Implementing an adaptive, two-tiered SARS-CoV-2 wastewater-based surveillance program on a university campus using passive sampling

Research presented in Chapter 2 has been published as:

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## 2.1 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus responsible for coronavirus disease 2019 (COVID-19) and the resulting pandemic. Once infected, up to two-thirds of individuals release the virus via the gastrointestinal tract in a process known as viral shedding (Chan et al., 2021; Lo et al., 2020b; Y. Zhang et al., 2021). SARS-CoV-2 embedded in feces can later be quantified downstream in wastewater using analytical methods which typically target specific regions (e.g., N1, N2) of the SARS-CoV-2 nucleocapsid gene in the extracted, purified RNA sample (US CDC, 2021; Zhang et al., 2022). This also applies to other targets of interest, such as pepper mild mottle virus (PMMoV) found in foods containing peppers, that has been used as an endogenous wastewater indicator of fecal material to normalize SARS-CoV-2 (Corchis-Scott et al., 2021; D'Aoust et al., 2021; Dhiyebi et al., 2023; Kitajima et al., 2018; Scott et al., 2021b; Symonds et al., 2018). Shedding of fragments of SARS-CoV-2 in feces has enabled wastewater-based surveillance (WBS) to be applied to monitor populations of interest around the globe (Akingbola et al., 2022; Corchis-Scott et al., 2021; Gibas et al., 2021; Godinez et al., 2022; Jain et al., 2022; Scott et al., 2021a). SARS-CoV-2 WBS programs are now well-established and have demonstrated the ability to accurately forecast key COVID-19 public health metrics which has increased acceptance and adoption by public health administrators and confidence in the approach by the general population (Cheng et al., 2023; Hrudey et al., 2022). As the utility of wastewater surveillance became realized, practitioners began to apply these surveillance principles to address institutional challenges in facilities with high population density. Prisons, long-term care homes, dormitories and other congregate living settings became a focus for applying wastewater surveillance programs with the potential to act as an early warning system for localized outbreaks (Akingbola et al., 2022; Betancourt et al., 2021; Hrudey et al., 2022). Although widespread clinical testing served as a primary approach for many institutions, this option proved to be challenging and costly in

the face of supply chain issues (Ouslander & Grabowski, 2020; Peccia et al., 2020). WBS offered an adaptive, scalable, and sensitive solution to monitor spatial and temporal trends in community viral burden (Bivins et al., 2022; Colosi et al., 2021; Corchis-Scott et al., 2021). The potential for WBS to act as a leading indicator of disease prevalence also drew interest from a variety of public health stakeholders, particularly when clinical testing efforts were limited or unavailable entirely (Bibby et al., 2021; Fahrenfeld et al., 2022).

Sampling upstream from the wastewater treatment plant (WWTP), especially at building-scale, introduces a series of unique challenges including high temporal variation due to pulse-inputs into the sewage system. Human behaviour within the sewer catchment drives wastewater flow, volume and timing. Toilet flushes, water appliance usage and showering directly influence the temporal variability of wastewater. Several studies have shown that SARS-CoV-2 concentrations in wastewater can fluctuate by orders of magnitude over a short period of time, which demonstrates the importance of sampling design to optimize the potential capture of the viral signal (Anderson-Coughlin et al., 2022; Betancourt et al., 2021; Welling et al., 2022). Use of discrete sampling methods, such as grab and composite samplers, may miss significant sewer inputs if they occur in the intervals between sample collection (George et al., 2022; Habtewold et al., 2022; Schang et al., 2021; West et al., 2023). An additional consideration is the lack of security and electricity required to operate automatic sampling units at some sites. Autosamplers are also often too costly for localized surveillance programs, especially if multiple units are required (Karthikeyan et al., 2021; Schang et al., 2021; Wright et al., 2022). As an alternative, passive sampling has been increasingly employed in upstream wastewater sampling to address these limitations (Acer et al., 2022; Bivins, Kaya, et al., 2022; Hayes et al., 2021; J. Li, Ahmed, et al., 2022). Passive sampling is a monitoring technique that uses sampling media to passively accumulate analytes of interest over time (Górecki & Namieśnik, 2002). The sampling medium remains immersed in the environment for the entire sampling period, which makes it less likely to miss significant fecal shedding events (Schang et al., 2021). Several studies have documented superior performance by passive samplers in the detection of SARS-CoV-2 when compared with composite and grab samples, especially at lower concentrations of SARS-CoV-2 in wastewater (Liu et al., 2022; Rafiee et al., 2021; Schang et al., 2021). Additionally, passive samplers have advantages for a diverse array of applications because they are affordable, simple to produce and use, do not require electricity and can be easily concealed (Habtewold et al., 2022; Jiang et al., 2022; Schang et al., 2021).

The University of Waterloo is home to Canada's fourth largest post-secondary housing operation with over 5,600 beds catering primarily to undergraduate students. Like many other institutions, concerns over the effectiveness of existing clinical tools to monitor pandemic status in the residences led to considerable uncertainty around managing the reopening of the campus while minimizing the risk to the community. Therefore, in the summer of 2021 a pilot upstream wastewater surveillance program for SARS-CoV-2 was deployed on campus residence buildings. The goal of the project was to determine if passive

sampling could be used as an effective tool to inform localized public health action and decision-making. The design of the surveillance program prioritized maximal surveillance coverage of the campus residences, efficient use of resources and optimization of actionability of the data. Twenty-four-hour passive samples were collected three times per week from late August 2021 to April 2022 using cotton medical gauze as a sampling medium. Results were rapidly reported within 12 hours of sample collection to a campus committee responsible for implementing public health interventions. The program was adapted as needed as knowledge of the wastewater system progressed, the pandemic trajectory changed, and public health requirements shifted. This trigger-based approach surveillance program is among the first to report on the use of passive sampling in a university setting. Efforts to maximize surveillance coverage and minimize resource usage are critical to informing future surveillance programs as funding and resource availability are both spatially and temporally variable.

## 2.2 Materials and Methods

### 2.2.1 University background

The University of Waterloo (Waterloo, ON, Canada) is a public research university in Canada with an annual enrolment of ~42,000 students, 90% of which are undergraduate students (University of Waterloo, 2022). The University operates 12 residences buildings across a ~400 ha campus, collectively housing over 5,600 students when at maximum capacity. Residence halls are categorized by two designations: (1) traditional-style residences which are characterized by washroom facilities shared by up to 25 individuals, and (2) apartment-style residences wherein three to four students share a private bathroom and kitchen. Residence operations closed as a result of the pandemic in March 2020 with the exception of some international students who remained on campus due to travel restrictions. The reopening of campus housing to the broader student community was scheduled for September 2021 at the onset of this study. Students were required to have at least one dose of a Health Canada approved COVID-19 vaccine by move-in day and to receive their second dose no later than November 1, 2021. Public health protocols were established for residents including active screening during move-in, mandatory mask wearing in public spaces, take-out only at cafeterias and foot-traffic decals. Gauze was used as the collection substrate in the passive samplers for the routine surveillance. Experiments conducted with passive torpedo samplers in the campus sewer demonstrated that although both gauze and electronegative membranes could capture a SARS-CoV-2 signal, significantly more solids and total RNA were collected on the gauze (Appendix A – Section 1). The gauze more consistently detected the SARS-CoV-2 and at higher total copy numbers.

### 2.2.2 Passive sampler design and use

The passive samplers were housed in torpedo-shaped vessels (15 x 3 x 3 cm) developed by Schang et al. (2021) that were 3D printed by WaterPuris Inc. (Kitchener, ON, Canada) using clear, durable V2 resin (Formlabs, Somerville, MA, USA). Modifications were made to the original print file to increase the thickness of the sampler walls to minimize in-situ warping. A fishing leader line was fed through the sampler and secured on the interior with a standard safety pin. The vessels were loaded with a loosely folded 2.76 x 2.76 cm sterile cotton gauze pad (Fisher Scientific, Mississauga, ON) then secured with a lid to create the sampling apparatus (Appendix A – Figure A-6). The exterior end of the fishing leader line was tied to a 45.3 kg test saltwater fishing line (Goture Co., San Francisco, CA, USA) which was affixed to a heavy-duty neodymium magnet hook with swiveling carabiner. The magnetic hook was then affixed to the interior of the utility hole rim to maintain the sampler in position. Samplers were typically deployed between 8-10 a.m. and remained in place for 24 hours. Upon collection, samples were transferred on ice to the laboratory for processing. Samplers were cleaned with a 10% bleach solution before redeployment to avoid cross-day contamination.

### 2.2.3 RNA concentration and extraction

In a sterile biological safety cabinet, each sample was transferred to an individual 50 mL sterile polypropylene centrifuge tube (Sigma Aldrich, MO, USA) containing 15 mL of Dulbecco's phosphate buffered saline (DPBS) (Fisher Scientific, Mississauga, ON) with added 0.01% v/v polysorbate-20 (Fisher Scientific, Mississauga, ON). The tubes were placed on a 16-tube vertical tube rotator (Fisher Scientific, Mississauga, ON) and spun at 40 rpm for 15 min at 4°C, then placed on a digital vortex mixer (Fisher Scientific, Mississauga, ON) at 2000 rpm for 30 s to release trapped solids. Gauze was then removed from the tubes using a pair of sterile forceps and squeezed on the interior wall of the centrifuge tube to ensure the original 15 mL DPBS/polysorbate-20 volume was retained. The sampling material was then discarded, and the remaining solution was centrifuged at 12,000 RCF for 30 min at 4°C with no brake. Following centrifugation, the supernatant was carefully discarded using a pipette.

A 140 – 150 mg sample of the generated pellet was sterilely placed into a 2 mL glass-style bead beating tube provided in the QIAGEN® RNeasy Power Microbiome® RNA extraction kit (QIAGEN®, Germany, No. 26000-50). Each tube then received 100 µL of TRIzol reagent (Fisher Scientific, Mississauga, ON) and 650 µL of PM1 (a kit-based reagent, QIAGEN®) mixed with beta 2-mercaptoethanol (Fischer Scientific, MA, USA) in a 99:1 ratio. The remainder of the extraction was performed in adherence to the manufacture's protocol using the QIAGEN® Qiacube® (QIAGEN®, Germany, Serial No.: 02278). A Bead Ruptor 12 Bead Mill Homogenizer (Fischer Scientific, MA, USA) was used for 5 minutes at 3.55 m/s to

homogenize the samples. Following extraction, the resulting purified RNA was eluted into a 100 µL suspension of RNAse-free water. An extraction blank (whole-process control) was included with each batch of samples to screen for contamination. Additionally, samples and extraction blanks were analyzed for total RNA content by measuring a one µL subsample of the RNA elution using the NanoDrop One spectrophotometer (ThermoFisher Scientific, Mississauga, Ontario).

### 2.2.4 Analytical methods

Extracted RNA underwent 1-step reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) for SARS-CoV-2 (N1, N2 gene targets) and PMMoV. Detailed assay information can be found in the Appendix A (Sections 2 and 3). In Fall 2021 N1 and PMMoV were quantified in a duplexed assay. All assays were then run on either the CFX96 Touch or the CDX Opus Real-Time PCR systems using CFX Maestro Software for CFX Real-Time PCR Instruments (Bio-Rad Laboratories, Hercules, CA, USA).

Based on experiments conducted in our lab and regional reports of an N1 dropout in late 2021 (Miller et al., 2021; Wang et al., 2022; Wollschläger et al., 2021), N2 was measured in all samples during Winter 2022 in a simplex assay to draw comparisons between the two targets. To evaluate the presence of inhibition, when possible, samples were plated in 1x and 10x dilution and compared using the corrected concentration of PMMoV on the sampler. The actual PMMoV concentration is measured in the undiluted sample and the theoretical or expected PMMoV concentration is derived from the 10x dilution. Samples were considered inhibited if the ratio of theoretical to actual PMMoV concentrations exceeded two. Inhibition ratios were used to assign confidence to negative samples and assist in interpreting results. An undiluted sample negative for SARS-Cov-2 which had no evidence of inhibition would be less likely to produce a false negative result compared to an inhibited sample. Diluted an inhibited sample may reduce inhibitory effects enough to allow a SARS-CoV-2 detection to be made, mitigating the false negative interpretation.

To define a positive sample, a one GC/reaction threshold was used as the absolute minimum concentration for a RT-qPCR detection to occur. Given that both the RNA elution and pellet were subsampled, the sampler detection limit was determined in order to identify the minimum number of GCs required to make a positive detection. This is assuming that there are no viral losses during the extraction process and that the resulting pellet and RNA elution were homogenous. The sampler detection limit was calculated using the following formula:

**Equation 1.** $$Sampler\ detection\ limit\ = \left(\frac{1\ GC}{PCR\ reaction}\right)\left(\frac{mass_{pellet}}{mass_{subsample}}\right)\left(\frac{100\ \mu L\ elution}{5\ \mu L\ plated}\right)$$

Individual PCR wells were categorized based on the amplified SARS-CoV-2 concentration. A sample was considered positive if at least two of three PCR wells amplified above the sampler detection limit, or if all three wells produced a Cq value regardless of concentration. For samples where two or less wells amplified SARS-Cov-2 below the sampler detection limit, these were classified as 'trace' detections.

### 2.2.5 Two-tiered sampling network

A thorough survey of wastewater infrastructure identified surveillance sites that were organized into a two-tiered network (Figure 2-1) which maintained 85% surveillance coverage of the on-campus residence population. Site information including upstream occupancy data is presented in Appendix A (Table A5). A decision-making framework (Figure 2-2) was developed to create thresholds for determining when building-scale (upstream) sampling would be triggered. If a sample was positive for SARS-CoV-2, both the upstream and building-cluster (downstream) sites were sampled the subsequent sampling day to isolate the probable source of the signal. If a sample produced a positive result in at least two of three technical replicates below the assay limit of detection (1 N-GC/reaction) it was classified as a trace detection. In these cases, upstream sampling was also triggered. Both tiers of the surveillance network were sampled until they each produced non-detections for three consecutive sampling days.

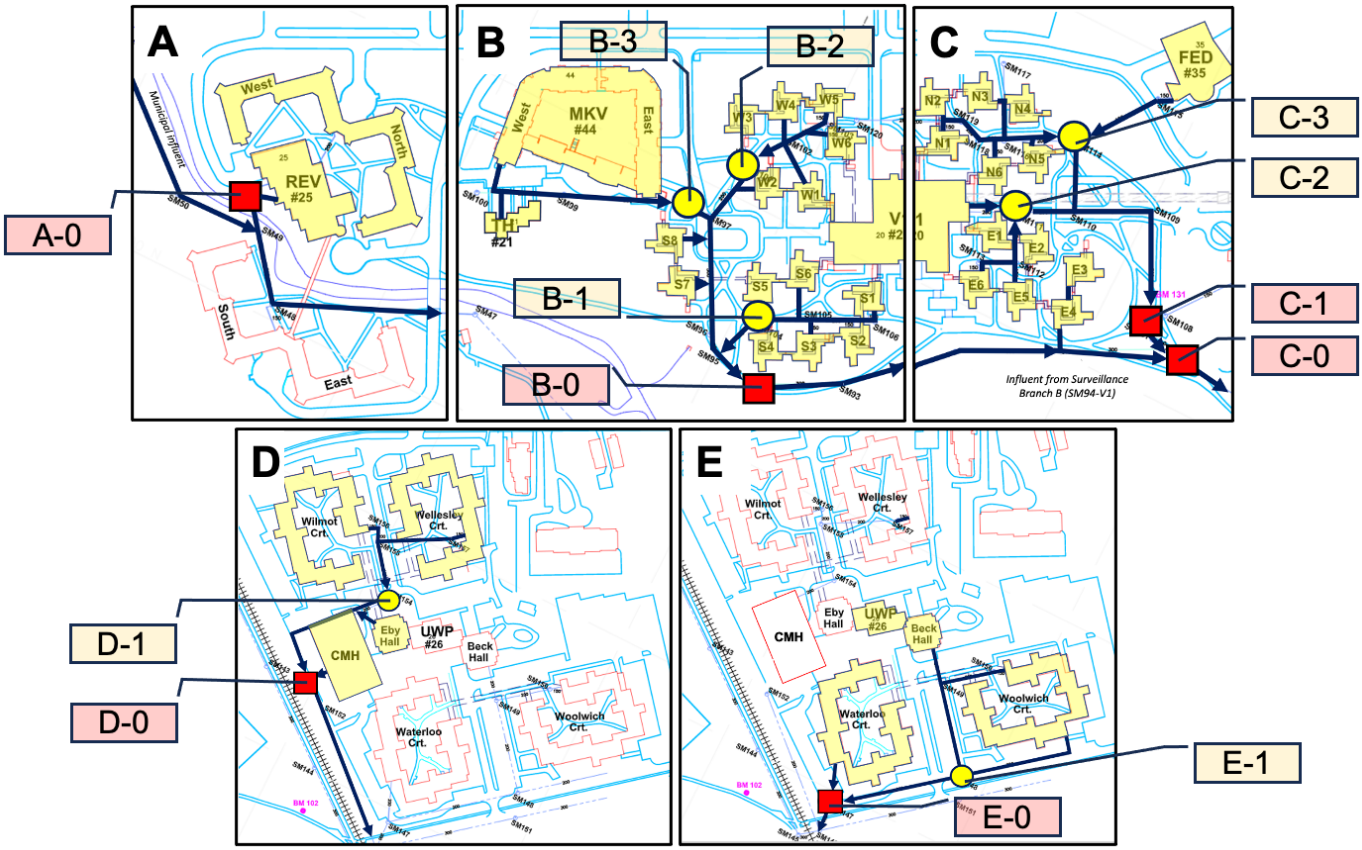


**Figure 2-1.** Two-tiered wastewater surveillance networks (Branches A-E) highlighting downstream sites (red squares) and associated upstream sampling sites (yellow circles). Campus residence buildings within each sewershed are highlighted in yellow. Sewers and flow direction are denoted by the blue arrows.

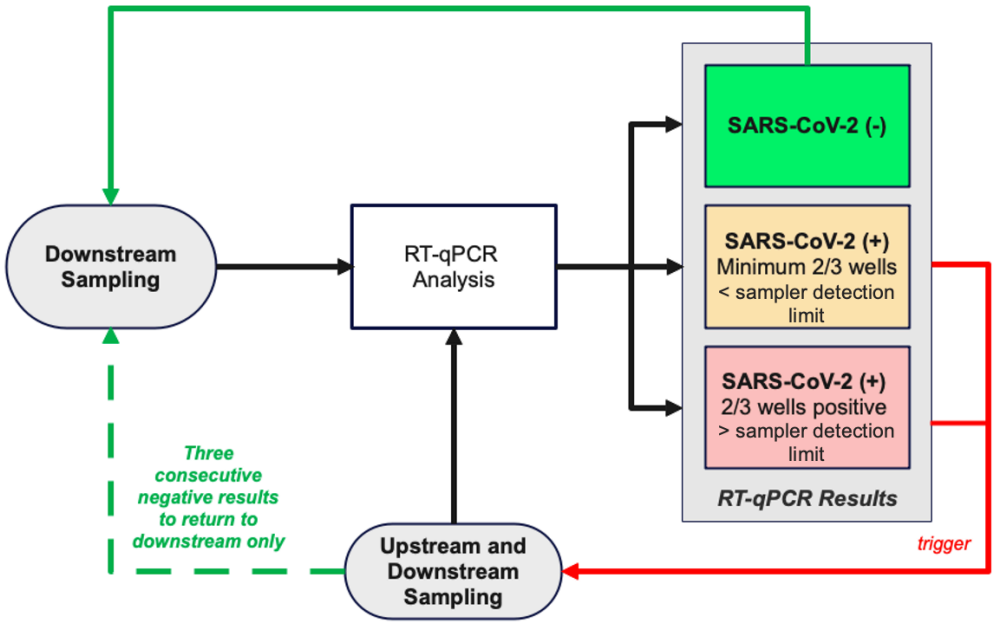


**Figure 2-2.** Decision-making framework for determining event-to-event sampling sites.

### 2.2.6 Sampling frequency

Twenty-four-hour passive samplers were deployed three times per week between August 2021 and April 2022. Surveillance period 1 (fall 2021) began on August 18, 2021, and ceased on December 17, 2021. Following the holiday break, surveillance period 2 (winter 2022) began on January 7, 2022, and ceased on April 29, 2022, following the final exam period.

### 2.2.7 Municipal wastewater sampling

Influent from the City of Waterloo WWTP located approximately 4 km northeast of the University campus was sampled as part of a larger WBS program. Samples collected using a 24-h composite sampler were processed in accordance with the methods described in Cheng et al., 2023. Briefly, polyethylene glycol (4 g) and NaCl (0.9 g) were added to a 40 mL influent sample that was centrifuged at 12,000g for 1.5 h after settling overnight. The pellet was analyzed using the same kit-based extraction method and quantified with similar RT-qPCR methods as described above (Cheng et al., 2023).

### 2.2.8 Clinical case data

Anonymized case data for each residence building was provided by the University of Waterloo Department of Housing after the project had concluded and was completely independent of wastewater surveillance collection. Clinical case data was collected solely based on students self-reporting illness as there was no mandatory clinical testing on the campus. Clinical cases therefore include only individuals who volunteered that they had tested positive (PCR test or rapid antigen test) and reported results to the University. It is possible that some students may not have reported illness to the University Housing unit.

To identify relationships between known clinical cases and passive sampler data, three assumed shedding periods were applied to the clinical dataset to account for fecal shedding variability. Cases were counted for each shedding period from the date of symptom onset (if known), or the day the student reported the illness to the University. In Fall 2021, in accordance with the Public Health guidelines at the time, students who were symptomatic or had tested positive were moved to an isolation facility outside of the surveillance catchment for a 10-day period. These cases were included in the tracker on the day the student was moved to isolation, as well as the day of return, and any additional days counted in the assumed shedding period. In Winter 2022, Public Health guidelines shifted to a five-day isolation period. During this time most students were ordered to “Stay-In-Place” to self-isolate in their assigned residence accommodations, contrary to moving to a dedicated isolation space as was done in Fall 2021. In these cases, students were included in the

tracker for the entire assumed shedding period as they were still contributing to the wastewater signal. Individual cases were counted for an assumed 5-day, 10-day and 21-day shedding period and compared with SARS-CoV-2 concentrations on passive samplers. In a separate analysis to account for the decay of detectable virus in stool over the course of infection, cases were assigned a contribution factor (CF) for each day in the shedding period based on an exponential decay formula (Equation 1). The formula aims to capture the theoretical decrease in fecal shedding load where *a* represents the initial contribution strength (100%), *r* is the assumed decay factor of either 25%, 50% or 75%, and *x* is the elapsed time in days. This analysis was performed under an assumed 10 and 21-day shedding period.

**Equation 2.** $CF = a(1 - r)^x$

### 2.2.9 Data analysis

Standard curve slope and y-intercepts from RT-qPCR were used to quantify viral targets using the instrument's recorded Cq value. Logarithmic transformation of wastewater data collected at both the WWTP and on campus fit a normal distribution, allowing for use of parametric tests. Correlation analysis was performed using the Pearson's product method or Spearman's rank method depending on data normality. A Wilcoxan rank-sum test was used to identify differences in median N1 and N2 concentrations within samples. A Pearson Chi-square test was used to compare detection frequencies between residence style types. To draw comparisons between target analyte concentrations in different residence style types, a one-way ANOVA was used. Concordance metrics were calculated using conventional formulas. All statistical analyses were performed using IBM SPSS Statistics 29 (SPSS Inc., Chicago, IL, USA) and visualizations were generated using Microsoft Excel Version 16 (Microsoft Corp., Redmond, WA, USA).

### 2.2.10 Reporting plan and reporting

The research team provided results to the Department of Housing and University Safety Office within 12 hours of sample collection in Fall 2021 with reporting three days per week. In Winter 2022 reporting was performed once per week given the project scope changes subsequently discussed. The research team and campus partners met weekly throughout the surveillance project to discuss results and response plans. In the event of a positive detection campus partners utilized several adaptive response strategies for targeted communities including direct email notification and public messaging. The research team and campus stakeholders hosted a public information session on the WBS program and worked jointly to develop and update an online dashboard relaying the status of wastewater surveillance in each residence complex. Clinical reporting and case management was managed and tracked internally by Department of Housing staff

throughout the project and performed independently of the WBS program. A project timeline including university COVID-19 policies is presented in the Appendix A (Figure A-7).

## 2.3 Results and discussion

To address an institutional public health need, a wastewater network was strategically sampled to gain building-scale information on SARS-CoV-2 prevalence. Motivated by the ability for WBS to detect asymptomatic cases, University decision-makers sought to follow up positive wastewater detections with targeted public health action to minimize risk to students and staff. The surveillance program was initially designed to end on November 1, 2021, coinciding with the double vaccination deadline for students living on-campus. However, results over the Fall 2021 semester would extend the surveillance program an additional six months. During this time, passive samplers were evaluated for their performance characteristics, concordance with known clinical cases and relation to the prevalence of COVID-19 in the broader community.

### 2.3.1 Passive sampler performance and inhibition

Solids captured by passive samplers generated a mean wet pellet weight of 0.670 ± 0.265 g with a minimum and maximum capture of 0.115 g and 2.178 g respectively, indicating that cotton gauze was able to collect a range of solid masses. Similarly, the elution extracts yielded a mean total RNA concentration of 973.6 ± 421.6 ng/µL with concentrations ranging between 76.3 and 3063.4 ng/µL. The mean pellet mass from samples collected in the two weeks prior to student move in were much lower compared to the remainder of the semester, likely attributed to the low wastewater flow observed during that period. Only 3% of the passive samplers in this study exhibited ragging, a term used to describe partial or full blockage of the sampling unit through the collection of in-situ debris. This is similar to Schang et al., (2021), who reported the ragging rate on their torpedo-style housing units to be less than 10% (Schang et al., 2021). Despite this finding, all samples tested positive for PMMoV indicating the presence of fecal matter in every sample. Gauze captured a mean PMMoV concentration of 6.79 ± 0.376 $\log_{10}$ gene copies (GC)/sampler across sampling sites. Mean PMMoV concentrations on passive samplers between downstream sites were not significantly different (One-way ANOVA, $F = 1.979$, d.f. = 4, 418, $P = 0.097$; Appendix A – Figure A-8) despite known upstream population differences. Additionally, while there was a significant relationship between log-transformed wet pellet mass and PMMoV GC/sampler, the relationship was weaker than expected (Pearson-product moment correlation, $r = 0.422$, $N = 575$, $P < 0.001$). Using the PMMoV concentrations derived from the diluted samples did not improve strength of the relationship, which indicated

that inhibition of PMMoV was not a significant factor. Together these results suggest that the amount of PMMoV captured and retained by the passive sampler is not reflective of upstream population differences. While the normalization of SARS-CoV-2 targets by PMMoV has been used successfully in on-campus surveillance programs using discrete sampling methods (J. Lee et al., 2023; Scott et al., 2021b) results of the study reported here suggest PMMoV may not be a suitable normalization target for SARS-CoV-2 when using gauze as a sampling medium. This conclusion is supported by Habtewold et al., (2022) who did not observe the linear accumulation of PMMoV in gauze samplers, and Li et al., (2022) who reported saturation of the material in eight hours, followed by viral losses. It is possible that normalization by PMMoV could be used in gauze-based applications for exposure durations under 24 hours, but further work is needed to characterize the equilibrium kinetics of the material in-situ. Although other normalizing metrics were considered for use in this study (including flush counts, electricity and water usage), this information was either unavailable or not feasible to collect during the rapid deployment of the current surveillance program. For these reasons subsequent data analyses were only performed on un-normalized SARS-CoV-2 data only.

In Fall 2021 there were reports of a potential dropout of the N1 target caused by mutations in the SARS-CoV-2 N-gene (Miller et al., 2021; Wang et al., 2022; Wollschläger et al., 2021). To safeguard against diagnostic evasion, N2 was quantified in simplex along with the N1/PMMoV duplex reaction in Winter 2022. Retrospective analysis using a Wilcoxon rank-sum test indicated a statistically significant difference between the median N1 and N2 signals in Winter 2022 (Wilcoxan signed rank test, $Z = 10.848$, $P < 0.001$) with a mean N1:N2 ratio of 0.714. However, the correlation coefficient between the log-transformed N1 and N2 measurements (Appendix A – Figure A-10) was 0.969 (Pearson's method, $r = 0.969$, $N = 277$, $P < 0.01$). While the N1 dropout observed at the onset of the Omicron wave (Miller et al., 2021; Wang et al., 2022; Wollschläger et al., 2021) might have caused part of this divergence, slight differences or bias in the assays cannot be ruled out. However, both N1 and N2 behaved similarly and were capable of tracking temporal trends in SARS-CoV-2 signal. The N1 duplexed assay was also able to detect all clinical cases known to the University in Fall 2021, assigning additional confidence to surveillance efforts during this period.

Samples collected in the two weeks preceding student move in did not indicate any presence of inhibition. However, persistent inhibition was observed after student move in at all sites, possibly reflective of the detergents, cleaners and other chemicals input to the wastewater system (Appendix A - Figure A-11). In Fall 2021, 90 of 203 (44.3%) tested samples were deemed inhibited which was of concern for the purposes of detecting low SARS-CoV-2 concentrations in wastewater. Reducing sample inhibition was a top priority for public health officials who sought to follow up positive detections with building-specific action and who relied on negative results to confirm the existing public health measures in place. Therefore, a methodological change was made to target a smaller pellet subsample mass to prevent overloading of the extraction column.

This change was made on September 27, 2021, which generally reduced the severity of inhibition in passive samples. In Fall 2021 there were two diluted samples that made a positive detection despite the same undiluted sample testing negative. These findings demonstrate the importance of assessing and mitigating inhibition even when the surveillance program is driven by qualitative passive sampling results. While this diagnostic evasion of SARS-CoV-2 is a concern, these two detections did not lead to the identification of any new cases and no subsequent positive detections were made in the days that followed. In Winter 2022, 87 of 288 (30.2%) tested samples demonstrated some degree of inhibition with no apparent temporal or spatial trends. Given the magnitude of SARS-CoV-2 concentrations measured in Winter 2022 and inhibition estimates derived by PMMoV concentrations on passive samplers, there was significantly less concern regarding the diagnostic evasion of N-gene targets during this surveillance period.

### 2.3.2 Surveillance in fall 2021 – low COVID-19 prevalence

A total of 291 samples were collected over 52 sampling days in Fall 2021. Of these samples, 12 (4.1%) tested positive for SARS-CoV-2, an additional 8 (2.8%) detected trace levels of SARS-CoV-2 but most samples (93.1%) tested negative for SARS-CoV-2 (Figure 2-3). The total number of samples positive for SARS-CoV-2 was variable across sites and ranged from zero to four. The first two positive SARS-CoV-2 detection of Fall 2021 (Figure 2-3 – Branches B, E) were made within the two weeks prior to student move in and were followed by negative results on subsequent sampling dates, indicating the presence of at least one transient non-student case in each surveillance catchment. Following student move in over the week of September 6, other positive and trace-level detections translated to eight separate triggers of upstream sampling. Four of the eight upstream sampling triggers were succeeded by non-detections at both the upstream and downstream sites. A positive downstream detection followed by non-detections at all surveillance sites indicated to public health decision-makers that the infected individual was no longer in the community and transmission risk was reduced. In two other instances, upstream sampling sites were able to refine the probable origin of the positive signal to a small building subunit within the sewershed. In both examples, upstream sampling was able to refine the origin of the signal from a population of 1550 students to just 292 (Figure 2-3 – Branch C). This enabled highly targeted public health intervention by means of notification, increasing student risk awareness and communicating best practices such as frequent hand washing and encouraging the use of face masks. Had invasive clinical testing been a component of the University's response to a positive wastewater detection, spatial refinement would minimize the number of clinical tests needed to identify the infected person(s) and consequently improving the ability to isolate individual(s) and optimize efficiency.

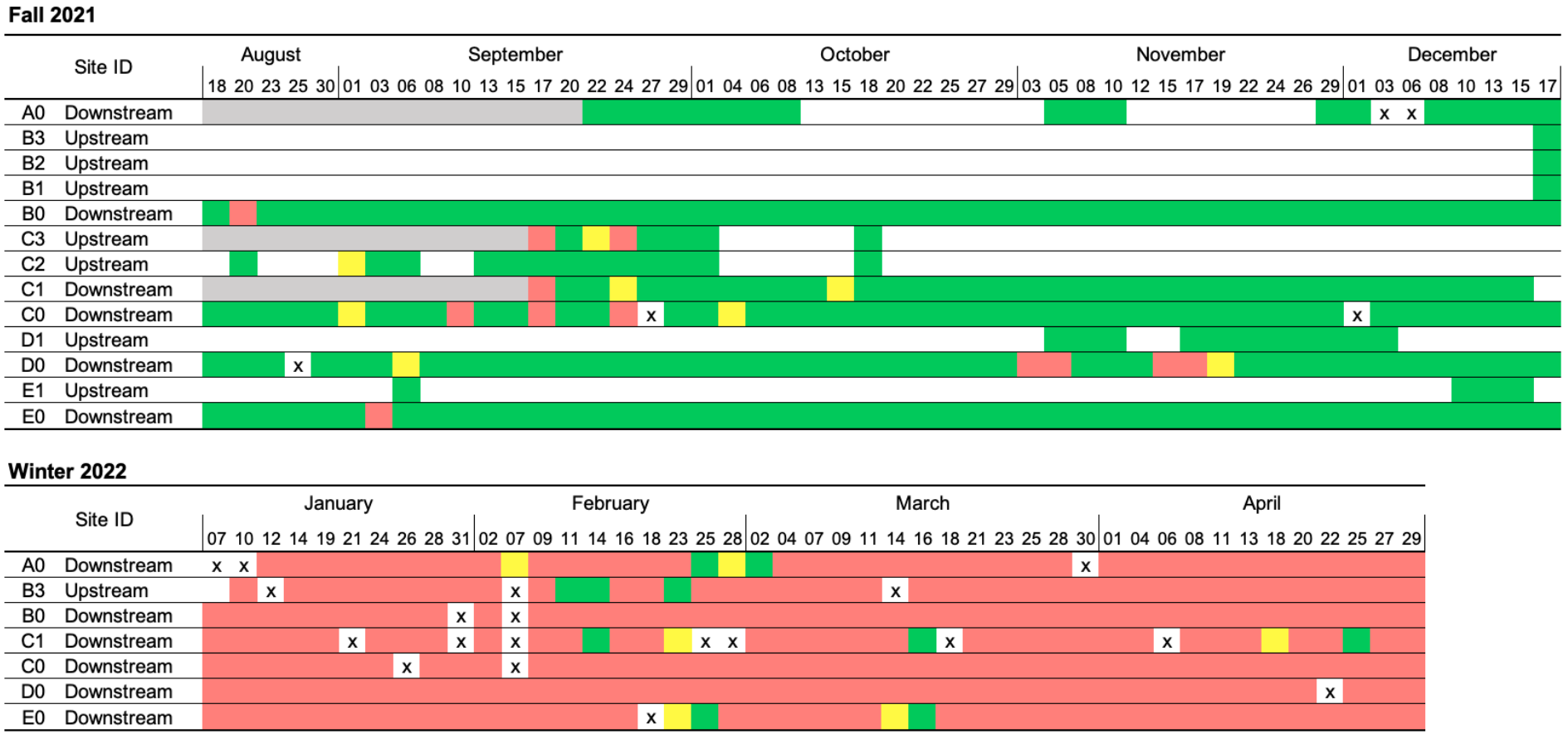


**Figure 2-3.** Qualitative results for all sites by surveillance period. Negative detections are highlighted in green, positive detections are highlighted in red, and yellow squares indicate a trace detection of SARS-CoV-2 RNA. Sites were not sampled on days that are coloured white. Grey blocks indicate the site was not included in the surveillance program for that respective period of time. Samples that were lost during collection or that could not be retrieved due to weather constraints contain an 'x'.

The remaining two upstream sampling events were linked to a clinical COVID-19 case known to the University. On November 3, 2021, the downstream site D0 produced $1.79x10^4$ N1 genomic copies (GC)/sampler – the maximum detection observed in Fall 2021 (Figure 2-4). The detection triggered upstream sampling the following sampling day where the upstream site tested negative for SARS-CoV-2, and the downstream site continued to test positive, though substantially reduced in signal (~79% reduction from November 3 – November 5). This combination of results prompted response action in two candidate residence buildings. Retrospective analysis of the clinical dataset revealed that a single student began experiencing symptoms on November 4 and reported a positive PCR test to the University on November 5. The following day the student was moved to an isolation space outside the surveillance catchment. This timeline indicates that SARS-CoV-2 was detected in wastewater 24 h in advance of the individual becoming symptomatic, two days before clinical test confirmation, and three days before the student was moved into a quarantine facility (Figure 2-4). This single case was detected at a sampling site collecting wastewater from nearly 1300 individuals, indicating a high degree of methodological sensitivity.

This example demonstrates the efficacy of the tiered approach in achieving an actionable degree of spatial surveillance while maximizing resource and personnel use. It also adds to mounting evidence that WBS has the potential to provide early detection and outbreak notification at the building-scale, especially in this setting where a comprehensive clinical testing program was not in place (Bibby et al., 2021; Corchis-Scott et al., 2021). However, it also highlights the challenges that convalescent cases within the surveillance catchment can pose to data interpretation. Upon the students return to their original accommodation following the 10-day isolation period, positive detections continued to be made until November 19. Fecal shedding duration of SARS-CoV-2 can be variable between individuals (Cevik et al., 2021; Xiao et al., 2020), with prolonged shedding periods confounding interpretation and reporting efforts (Cavany et al., 2022; Colosi et al., 2021; Corchis-Scott et al., 2021; Sellers et al., 2022). In the absence of mandatory clinical testing, there is no way to know whether the same individual contributed to the later positive detections or not. One interesting point of note, however, is that the quantification of SARS-CoV-2 on the passive sampler during the initial detection period closely mirrored what is known about viral shedding in clinical settings. While extended periods of fecal shedding have been documented, viral shedding tends to peak within five to six days of infection followed by a rapid decline (Cavany et al., 2022; Cevik et al., 2021; Natarajan et al., 2022; Y. Zhang et al., 2021). This suggests that passive samplers might be sensitive enough to capture the fecal shedding variability of a single individual while simultaneously monitoring a much larger community. Under the assumption that all detections made at D-0 in November were from the same person, this places the individuals fecal shedding duration at approximately 16 days which is close to the previously estimated mean fecal shedding duration of 17.2 days (Cevik et al., 2021). Due to the episodic nature of positive detections across sites throughout Fall 2021 and the low COVID-19 prevalence in the community, isolating the origin

of these signals worked as intended. While positive detections were informative for public health decision-makers, the widespread non-detections observed across campus in Fall 2021 validated the efficacy of existing public health measures in place.

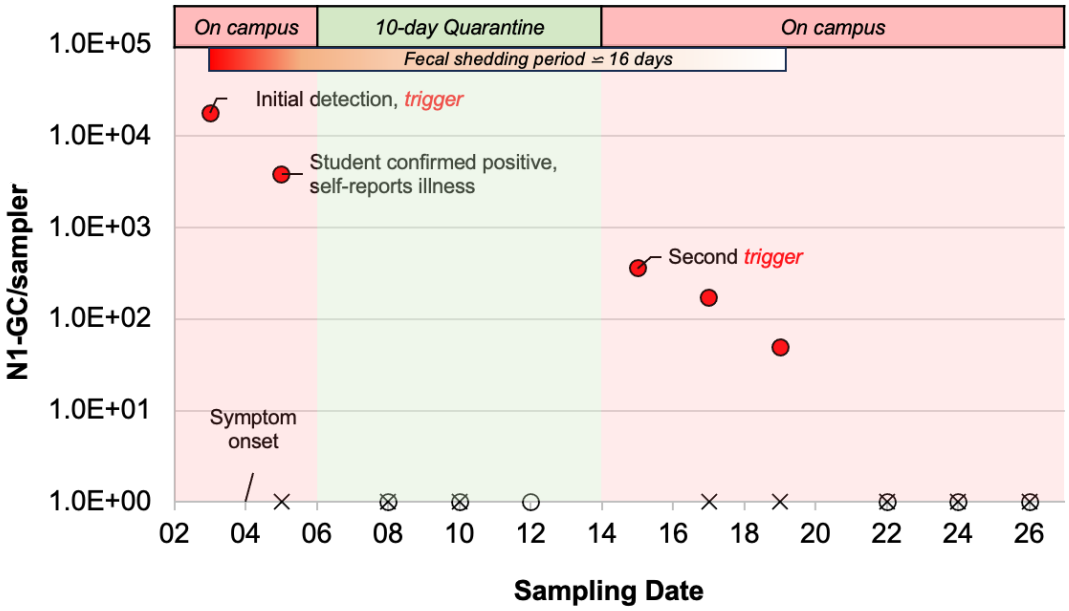


**Figure 2-4.** WBS results for Surveillance Branch D over November 2021. N1-GC per sampler on log scale from November 3 – 26, 2021. The downstream site is denoted by circles where positive detections are filled in red and non-detections are empty. The associated upstream site is produced only non-detections in the sampling period and are denoted by X marks. All non-detections were assigned a value of one (1) for graphical purposes. The green and red shaded areas signify when the student was living in their original accommodation and when they were moved a quarantine facility respectively.

### 2.3.3 Surveillance in winter 2022 – high COVID-19 prevalence

The arrival and spread of the SARS-CoV-2 Omicron (B.1.1.529) variant in Ontario demanded major scope changes to the surveillance project. While Omicron has a comparable period of communicability compared to other SARS-CoV-2 Variants of Concern (VOC), it has been shown to be significantly more transmissible (Public Health Ontario, 2023). This was reflected in the rapid increase in the numbers of clinical case and hospitalizations in Ontario that occurred in January 2021 (Cheng et al., 2023). Because few students were on campus during the Holiday Break, sampling was not conducted during this period. Although only a portion of the students returned during the first two weeks in January 2022 all sites tested positive for SARS-

CoV-2, some up to 10-fold greater than the strongest detection made in the Fall 2021. Given that upstream sampling no longer provided useful information on the probable origin of a positive signal, the two-tiered approach was no longer needed. Instead, only downstream sites were included in surveillance in an attempt to identify changes in the SARS-CoV-2 signal to inform the easing (or further implementation) of public health restrictions. In Winter 2022, there were a total of 295 samples were collected (Figure 2-5). In contrast to the previous surveillance period, 42 sampling days produced just 10 (3.4%) samples that were negative for SARS-CoV-2, 6 (2.0%) detected trace-levels of SARS-CoV-2, and 278 (94.2%) samples tested positive. Similar to other WBS programs on university campuses (Jain et al., 2022; J. Lee et al., 2023), all downstream sites at the University closely followed trends measured downstream at the municipal WWTP (Figure 2-5).

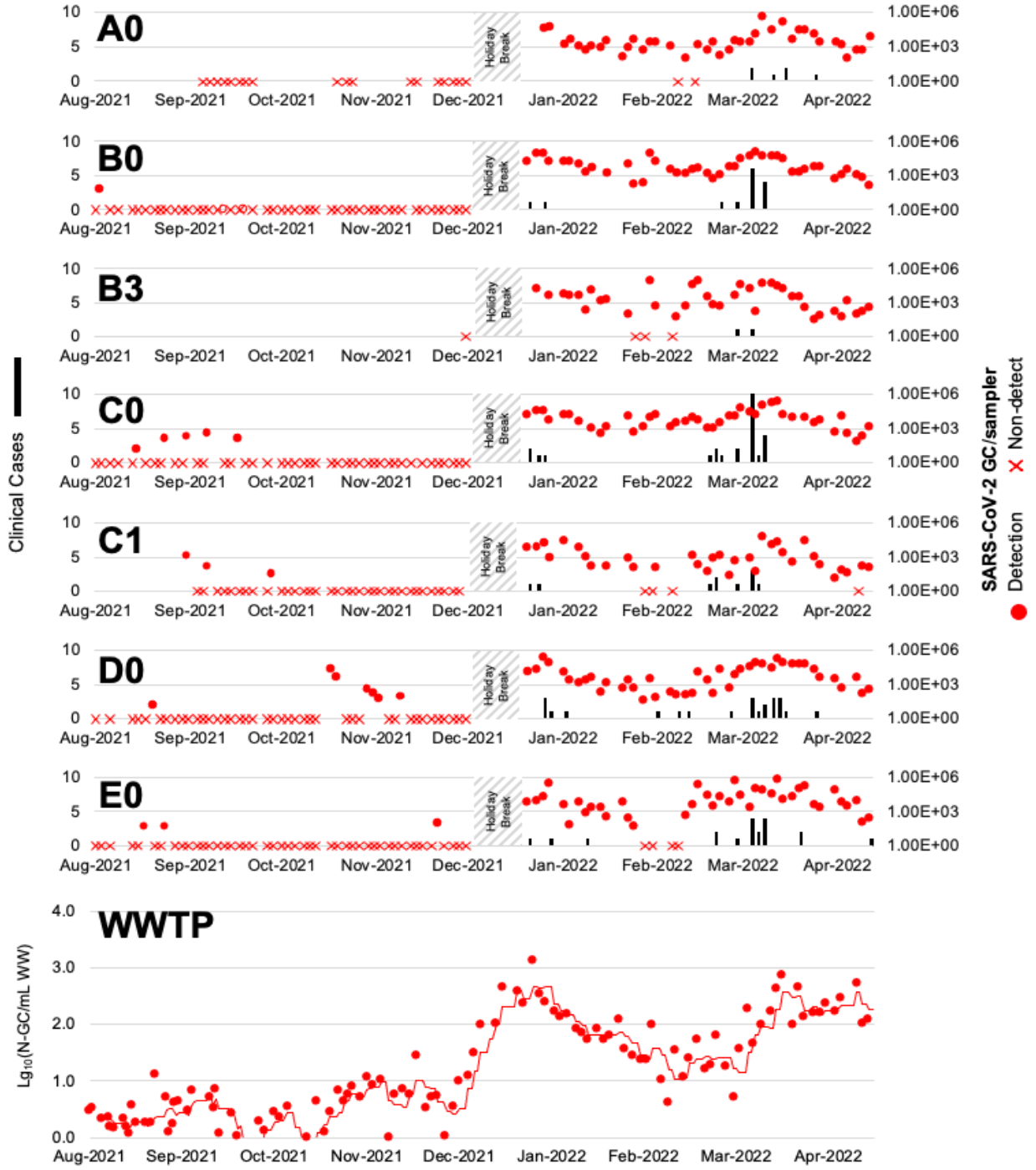


**Figure 2-5.** Wastewater surveillance data for downstream campus sampling sites (Panels A0, B3, B0, C1, C0, D0, E0) and the City of Waterloo WWTP (bottom panel). For campus data, the total number of new cases reported to the University (vertical black bars) is on the primary y-axis, and the log-transformed SARS-CoV-2 concentrations on passive samplers (red circles) and non-detections ('x' marks) are on the secondary y-axis. Municipal wastewater data (Panel WWTP) is presented as the log-transformed N-GC/mL of wastewater with the red line representing the one-week moving average. The holiday break is denoted in each panel where there were no on-campus sampling activities from December 18, 2021 – January 6, 2022.

### 2.3.4 Detections at traditional and apartment-style residence buildings

Several on-campus WBS programs have prioritized traditional-style residence buildings that shared bathroom and dining facilities on the assumption that they would pose a greater transmission risk to residents and consequently result in a greater detection frequency compared to apartment-style residence buildings (Anderson-Coughlin et al., 2022; Kotay et al., 2022; Scott et al., 2021a). In this study there was no statistical difference between the detection frequencies of traditional and apartment-style residence buildings, $X^2$, (1, N = 312, P = 0.721). To compare the overall SARS-CoV-2 burden between residence styles a one-way ANOVA was performed of the data from the sites measuring the two residence styles. Traditional-style residences had a significantly higher mean SARS-CoV-2 load compared to apartment-style residences for both log-transformed N1 (One-way ANOVA, F = 6.630, d.f. = 1, 158, P = 0.011) and N2 (One-way ANOVA, F = 4.285, d.f. = 1, 152, P = 0.040) data. Boxplots are presented in Appendix A (Figure A-12). While it is possible that traditional-style residences carry a greater COVID-19 burden, this result might also be attributed to other intrinsic factors unique to apartment-style residence buildings. For example, apartment-style residences on the campus had a lower population density compared to traditional-style buildings and access to ensuite appliances such as dishwashers and washing machines which introduce additional greywater into the sewage system (Anderson-Coughlin et al., 2022). The additional greywater could accumulate more inhibitors and cause an increased dilution effect, thereby reducing SARS-CoV-2 concentration on passive samplers at these sites. Nonetheless, these results suggest that residence style may not need to be a key consideration for WBS site selection.

### 2.3.5 Passive sampling concordance with epidemiological data

Unlike Fall 2021, interpretation of the wastewater signal in Winter 2022 was challenging given that most students were required to stay in place for self-isolation. As observed at site D0 in the Fall of 2021 and as other studies have reported, the presence of convalescent cases within the sewershed can confound the interpretation of wastewater data (Cavany et al., 2022; Colosi et al., 2021; Corchis-Scott et al., 2021; Sellers et al., 2022). There was strong concordance between same-day clinical case reports and detections in wastewater (Table 2-1; sensitivity = 98.2%) meaning the method was able to detect all but one clinical case known to the University. This single case was reported on the last day of surveillance in Fall 2021. It is possible that this student moved out or did not use the restroom facilities within their assigned accommodation during the sampling window, or that they did not shed a detectable amount of the virus. The method also demonstrated strong predictive power in accurately identifying the absence of clinical cases upstream (Table 2-1; negative predictive value (NPV) = 99.6%). A large number of positive

wastewater detections were made in the absence of a clinical case being reported, producing a positive predictive value (PPV) of 16.7% which is less than other upstream sampling programs have reported (Bivins et al., 2022; Welling et al., 2022). However, this result is not surprising given the limitations of the clinical dataset. The absence of mandatory clinical testing and voluntary reporting resulted in a high degree of uncertainty in estimating the methodological positive predictive ability. Other studies which paired wastewater surveillance with comprehensive clinical testing programs observed significantly higher PPVs (Bivins et al., 2022; Welling et al., 2022). Given these limitations and that college-aged students have a significantly higher proportion of asymptomatic cases compared to older cohorts (Schmitz et al., 2021), widespread diagnostic evasion was anticipated. One study which administered over 700 clinical tests on a university campus found that nearly 80% of all cases were asymptomatic (Schmitz et al., 2021). This is likely reflected to some degree in the discordance between wastewater testing and the clinical dataset. As previously mentioned, the indiscriminate nature of WBS does not allow for distinction between new COVID-19 cases and convalescent cases within the sewershed, particularly when sick individuals reside in the same building as the rest of the population under surveillance. Despite asymptomatic and unreported cases reducing the same-day PPV, the true value of the surveillance program was realized in these detections where discordance was followed up by targeted public health intervention. This is the case for more than 83% of all positive samples which had no known clinical cases associated.

**Table 2-1.** Comparison of same-day results between wastewater surveillance detection and the associated presence or absence of clinical cases upstream. Counts represent all sites across both surveillance periods.

| | | **Clinical Testing** | |
|---|---|---|---|
| | | + | - |
| **Wastewater** | + | 53 | 264 |
| | - | 1 | 267 |

Sensitivity (98.2%), Specificity (50.3%), PPV (16.7%), NPV (99.6%)

Recognizing the variability of fecal viral shedding, a 5-day, 10-day and 21-day shedding period were applied to clinical cases to identify any significant relationships with passive sampling data. The five and ten-day shedding periods were selected since they represent the required isolation periods for Fall 2021 and Winter 2022 respectively, as recommended by the provincial government at the time. A 21-day shedding period was also evaluated to account for the possibility of a longer shedding period and is similar to the 17-day mean shedding period reported by Cevik et al., 2021. Initially, cases were counted for the

entire shedding period. Clinical case counts could not be normalized by transformation, therefore, the Spearman's rank method was used to identify significant correlations between case counts and log-transformed SARS-CoV-2 concentrations on passive samplers (Table 2-2). The 10-day assumed shedding period produced the strongest correlation with both N1 and N2 followed by the 5-day and 21-day shedding periods (Table 2-2). However, this approach fails to capture the exponential reduction in SARS-CoV-2 concentrations in the stool of infected patients following peak shedding which occurs approximately five days after infection (Cavany et al., 2022; Cevik et al., 2021; Natarajan et al., 2022; Y. Zhang et al., 2021). Since nearly all individuals in our dataset were symptomatic, it was assumed that peak shedding occurred on the day illness was reported. Of the different conditions tested, an assumed 21-day shedding and a decay rate constant of 25% produced the strongest correlation for both log-transformed N1 and N2 concentrations (Table 2-2). Within the two tested shedding periods, correlation coefficients decreased as the decay rate constant increased. However, applying contribution factors (CFs) to clinical cases to account for signal decay over the shedding period did not improve correlation strength overall compared to whole case counts. This is likely a consequence of unreported cases in the sewershed contributing to the wastewater signal during periods when the decay analysis tracks the decline in known cases. Therefore, this analysis might be best suited to monitoring applications where more is known about the clinical dynamics of the population under surveillance. A better understanding of decay rates in SARS-CoV-2 fecal shedding might assist practitioners with the interpretation of wastewater data, particularly when convalescent cases are present in the sewershed and confound results.

**Table 2-2.** Spearman's rank correlation coefficients between log-transformed on-campus passive sampling data and known clinical case counts under various assumptions. All correlations are significant at the $P < 0.01$ level.

| Shedding Period | Decay Factor | N1 | N2 |
|---|---|---|---|
| 5-day | - | 0.480 | 0.471 |
| 10-day | - | 0.561 | 0.561 |
| 21-day | - | 0.475 | 0.455 |
| 10-day | 25% | 0.523 | 0.522 |
| 10-day | 50% | 0.502 | 0.495 |
| 10-day | 75% | 0.490 | 0.479 |
| 21-day | 25% | 0.528 | 0.521 |
| 21-day | 50% | 0.498 | 0.485 |
| 21-day | 75% | 0.483 | 0.466 |

### 2.3.6 Relationship between campus passive sampling and regional (quantitative) wastewater surveillance

To compare the SARS-CoV-2 burden on campus to the broader community, comparisons were made between log-transformed passive sampling data and SARS-CoV-2 concentrations in wastewater collected at the municipal WWTP. Logarithmic transformation produced an approximately normal distribution for WWTP data and for campus sampling data (Appendix A – Figures A-13 and A-10 respectively). There was no significant relationship between passive sampler and WWTP N1 concentrations in Fall 2021 due to the large number of non-detections for SARS-CoV-2 measured on campus and the comparatively low viral prevalence in the community (Pearson product-moment correlation, $r = 0.204$, $N = 8$, $P = 0.629$). Alternatively, the persistent positive detections on campus in Winter 2022 made for a more suitable comparison. All downstream sampling sites on campus measured two distinct peaks in SARS-CoV-2 concentrations in Winter 2022 – the first peak immediately following the return from the holiday break, and the second occurring towards the end of March where clinical cases reported to the University were at their maxima. These peaks closely coincide with the peaks observed in municipal wastewater samples collected at the City of Waterloo WWTP and were also observed in WBS programs at other universities during the same time period (Jain et al., 2022; J. Lee et al., 2023). Analysis of SARS-CoV-2 concentrations on passive samplers and in wastewater produced a weak, positive correlation for both N-gene targets (Table 2-3). While these measurements were not paired, similar linear relationships between passive and discrete sampling methods have been previously reported using a variety of sampling materials (Bivins, Kaya, et al., 2022; Habtewold et al., 2022; Schang et al., 2021).

**Table 2-3.** Pearson correlation coefficients between log-transformed on campus passive sampling data and log-transformed SARS-CoV-2 concentrations in wastewater measured at the WWTP during winter 2022. All correlations are significant at the $P < 0.001$ level.

| | | **Campus Passive Samplers** | |
|---|---|---|---|
| | | N1 | N2 |
| **Municipal WWTP** | N1 | 0.397 | 0.372 |
| | N2 | 0.403 | 0.386 |

These results suggest that drawing comparisons between building-scale and community-scale WBS might be dependent on disease-prevalence within the sewershed. This was largely due to the episodic nature of positive detections on campus in Fall 2021 which produced significant discordance with the WWTP dataset. Additionally, near-source sampling is known to produce significant day-to-day variation in SARS-CoV-2 levels due to variable flow regimes, fecal shedding dynamics and other factors which mediate viral input into the sewage system (Bivins & Bibby, 2021; Corchis-Scott et al., 2021; Rondeau et al., 2023). Downstream at the WWTP where wastewater is drawn from a larger catchment, wastewater composition may be less prone to temporal density variability. Alternatively, when disease prevalence in the community is comparatively high and detectable at both sampling scales, the results demonstrate that building-scale sampling may be redundant. SARS-CoV-2 concentrations in municipal wastewater closely resembled that of the campus through time, indicating that the municipal surveillance program might have been a suitable surrogate for the overall incidence of disease within on campus residence buildings. It is also important to recall that many students at the University who do not live in residence are living in the surrounding area serviced by municipal WWTP. The interaction between the University population and the wider community, and therefore transmission of COVID-19, may have contributed to this similarity in results. Nonetheless, there were several campus samples in Winter 2022 that tested negative for SARS-CoV-2 which validated known clinical information and provided useful spatial data for public health officials. These periods of lower disease prevalence would have otherwise not been detected had surveillance only been performed at the WWTP.

The correlation coefficients presented in Table 2-3 also support mounting evidence that passive sampling has the potential to generate semi-quantitative data (Bivins et al., 2022; Habtewold et al., 2022; Hayes et al., 2021). Despite several studies demonstrating a limited capacity for gauze to act as a passive sampling medium for SARS-CoV-2 (Habtewold et al., 2022; Li et al., 2022), the results demonstrate the material's efficacy over a 24-hour sampling period both in terms of epidemiological concordance and quantitative potential. However, it is still unclear what portion of this sampling window is most reflective of the SARS-CoV-2 concentrations observed on the passive samplers. This is largely due to the poorly characterized uptake and retention kinetics of the material and the site-specific characteristics known to govern these dynamics (Bivins et al., 2022). As previously discussed, standard cotton gauze likely becomes saturated with PMMoV within 8 hours of sampler deployment (Habtewold et al., 2022; J. Li, Verhagen, et al., 2022), however the saturation capacity (equilibrium) and breakthrough point for SARS-CoV-2 have not yet been determined. This is largely due to confounded results by the accumulation of inhibitors and the inability to characterize potential viral exchanges and losses when samplers are deployed in-situ (Habtewold et al., 2022; J. Li, Verhagen, et al., 2022). Other investigations have highlighted the utility of electronegative membrane filters in passive sampling for SARS-CoV-2, citing linear uptake of multiple

genetic targets of interest in excess of 24 hours (Habtewold et al., 2022; J. Li, Verhagen, et al., 2022). Such a material would provide the benefit of time-integrated sampling which can be used to make inferences about the population under surveillance and improve confidence in the sampling window. As the demand for WBS programs for SARS-CoV-2 begin to wind down, research should continue to characterize the equilibria kinetics of these materials to better interpret passive sampling data in future applications.

## 2.4 Conclusions

This investigation demonstrated the ability for passive sampling to act as a critical public health surveillance tool to support institution-level decision-making. The two-tiered sampling network proved extremely useful in minimizing time and resource usage without compromising the number of individuals under surveillance. It also enabled same-day analysis and reporting which was critical for stakeholders involved in the public health response. Most importantly, the tiered model was effective in isolating the origin of positive SARS-CoV-2 signals when COVID-19 prevalence in the campus community was low. This enabled decision makers to implement targeted communication-based public health measures that would have significantly reduced clinical testing needs had a mandatory program been in place. While the approach to WBS must remain site and context-specific, the results demonstrates that a tiered design may be most useful in resource-limited settings and especially when clinical testing is not available. The early detection potential of passive sampling was also realized in Fall 2021 when a single pre-symptomatic case was detected, leading to the prompt isolation of the student to prevent transmission. In Winter 2022 when COVID-19 burden was high, the tiered approach needed to be modified to maximize data actionability and temporally monitor trends in SARS-CoV-2. This provided decision-makers with an additional layer of evidence to support the public health restrictions. Beyond building-scale applications, passive sampling trends on campus in Winter 2022 closely mirrored regional pandemic dynamics and was significantly correlated with SARS-CoV-2 concentrations in the local municipal wastewater treatment plant. Wastewater surveillance filled a critical gap in the return to campus as an independent, holistic means to track the emergence of SARS-CoV-2 on the University campus. The WBS program was also insulated from many of the biases involved in clinical testing including test-seeking behaviours, student participation, and changes in test eligibility. Applying this research advances our knowledge in the detection and quantification of SARS-CoV-2 in wastewater and validates the ability of passive sampling data to produce actionable data related to human health. Further advancing these methodologies can be applied to detect a variety of genetic targets and will be an essential component to pandemic and healthcare preparedness in the future.

# Chapter 3
# Conclusions and implications for the next pandemic

As demonstrated in Chapter 2, passive sampling can be used as an effective tool to monitor disease prevalence at the building-scale and inform localized public health action. Passive sampling demonstrated its reliability and sensitivity by positively identifying nearly all known clinical cases over the surveillance period. In fall 2021, wastewater led clinical test results in at least one infected student providing a two-day advanced warning. In winter 2022, cotton gauze as a sampling medium demonstrated its propensity to capture quantifiable differences in SARS-CoV-2 RNA over time, expanding the materials previously understood utility for long-term surveillance applications. The workflow for processing passive samplers also produced results faster than conventional sampling methods and prioritized stakeholder end use of the data. Implementing passive sampling under the two-tiered model also offered several benefits to the surveillance program which should be considered in future monitoring endeavors. When implemented over the fall 2021 term, the tiered sampling approach demonstrated its capacity for scalability and efficient use of resources while surveilling a relatively large population. The decision-making framework for determining when and where to sample guided surveillance efforts to refine the origin of SARS-CoV-2 RNA to the building and sub-building level while maintaining a short turnaround time for results. This approach may be particularly advantageous in low-resource settings where sampling capacity is a key limiting factor. As demonstrated here, the utility of passive sampling WBS data was best realized in the absence of a clinical testing program. WBS supplemented existing public health data to implement rapid, targeted follow-up to a relatively small community of impacted students. Despite refined spatial resolution having limited utility in winter 2022, passive samplers demonstrated the ability to track trends in SARS-CoV-2 concentrations at all sampled sites. This was achieved in the absence of flow data or an endogenous indicator to account for dilution effects, further demonstrating the utility of cotton gauze to act as an effective passive sampling medium for SARS-CoV-2. This applies both in terms of epidemiological concordance and quantitative potential. Passive sampling data was significantly correlated with known clinical cases and with SARS-CoV-2 concentrations in municipal wastewater demonstrating the ability for passive samplers to generate semi-quantitative data. It also demonstrated that viral transmission on campus was similar to the broader community. In summary, passive sampling can support institution-scale public health action by:

1. **Reliably detecting the presence of SARS-CoV-2 RNA in wastewater to act as an early alert system**. Passive sampling circumvents many of the challenges associated with grab and composite

samplers for routine public health surveillance. Its sensitivity and concordance with epidemiological data make WBS data attractive to its end users and assign confidence to reporting.

2. **Providing an additional layer of evidence to support existing public health measures when negative results are obtained**. In terms of qualitative reporting, passive sampling may afford more confidence in negative results compared to those obtained by discrete sampling methods.
3. **Tracking trends in the viral load of afflicted communities, particularly when disease prevalence in the community is high.** Beyond qualitative applications, passive samplers can track measurable differences in the viral signal through time that are positively related to the SARS-CoV-2 burden within the upstream catchment.

By taking a passive sampling approach under a tiered, trigger-based sampling design, public health action and decision-making can be further supported by:

1. **Providing spatial information on where infected persons may reside.** Building-scale resolution of WBS can allow for highly targeted intervention, thus providing the most immediate utility for public health action.
2. **Identifying target populations for clinical testing, thereby minimizing associated resource usage.** When positive wastewater detections are followed up with clinical testing, building-scale surveillance resolution can help to minimize the number of associated tests required to identify the source or sources.
3. **Minimizing the number of sites required to surveil a given population.** By clustering upstream sites together, more people can be monitored with less effort. This has direct financial implications for WBS programs, most of which rely on specialized equipment and costly reagents.
4. **Maintaining a reasonable turnaround time to identify a positive detection to the building-level.** When a downstream site tests positive for SARS-CoV-2, the workflow and simplistic two-tiered design enables follow up testing at upstream sites to occur within a few days. This allows for timely follow up and the afforded confidence that the persistence of the signal is originating from a resident (as opposed to a transient source). This time could be shortened further depending on laboratory capacity and financial resources.
5. **Scaling to expand or reduce the scope of the surveillance program, as needed.** The tiered aspect of the surveillance program was only useful when disease prevalence in the community was comparatively low. The nested design of the sampling regime allows for sites to be easily added or removed as institutional and epidemiological conditions change.

## 3.1 Limitations

While the use of passive sampling to support spatially refined public health surveillance has been demonstrated, there are some limitations which should be considered. First, human behaviour plays a significant role in this study. Although routine surveillance efforts may assume that students use the restroom facilities within their assigned accommodation, this may not always be the case. Students move between residence buildings and the main campus frequently, especially when in-person classes resumed in February 2022. Another assumption is that all positive detections originate from residents within the sewershed. On a university campus, residence halls are often frequented by visitors, contractors and employees who may contribute to the wastewater profile. Both of these transient factors have implications for reporting results. Under the tiered model, either may have incidentally triggered an alert in one building while the infected person remains undetected in their original accommodation or elsewhere on campus.

Limitations exist on the analysis of fecal shedding periods and in the linear relationships reported between clinical cases and passive sampling data. Of primary restraint is the lack of a comprehensive clinical dataset. Self-reported illness, lack of clinical testing and asymptomatic infections introduce uncertainty in the case counts and are almost certainly reflected to some degree in the correlation outputs and concordance metrics presented in Chapter 2. While this is a limitation of the analysis, WBS is effective at informing public health action in the absence of clinical testing. Had a comprehensive clinical testing program been in place, WBS still would have provided relevant, independent information that would support interpretation of the spread of COVID-19. The data available to establish the relationships was often lacking during the pandemic. As so many practitioners of WBS have come to realize, what is demanded by an on-going public health crisis does not always align with the ideals of controlled experimental design. Tradeoffs had to be made in real time, under shifting public health guidance, to prioritize data actionability.

It is important to recall that passive sampling data involves less than quantitative accuracy and precision. While this study has demonstrated that gauze as a sampling medium is capable of tracking temporal SARS-CoV-2 trends in wastewater, it may not be a suitable sampling media for direct quantitative applications. For example, enumerating clinical cases in the sewershed using passive sampling data requires calibration of the material which is challenging for previously described reasons. The equilibria kinetics of cotton gauze in a complex matrix such as wastewater are largely uncharacterized and vary spatially. This imposes a limitation in understanding what portion of the sampling window the SARS-CoV-2 concentration on the passive sampler is most representative of. As previously discussed, the few existing calibration studies suggest cotton gauze saturates for PMMoV within eight hours, but results are universally confounded for SARS-CoV-2 (Habtewold et al., 2022; Li et al., 2022). If future calibration studies are able

to conclusively determine that gauze becomes saturated with SARS-CoV-2 within 24 hours, surveillance efforts could be modified to reflect the effective sampling window and target specific peak hours of the day where the most wastewater production is expected. Efforts to characterize these qualities would be of benefit to similar upstream WBS studies, both future and in retrospect.

## 3.2 Considerations for future WBS programs

WBS has grown considerably in the near century since its inception. Remarkably, Moore's original cotton swab design demonstrated its utility through time by its modern application in environmental monitoring, WBS and this very investigation. The rich history of WBS demonstrates the importance of documenting lessons learned for consideration in future public health crises to improve the power of wastewater surveillance as a monitoring tool.

### 3.2.1 Passive sampler design and processing

Since concluding routine surveillance in this study, WBS for SARS-CoV-2 using passive sampling has been applied in several bench-scale and in-situ applications to assess passive sampler performance and surveil populations of interest (Acer et al., 2022; Jain et al., 2022; Mangwana et al., 2022; West et al., 2023; Wilson et al., 2022). As observed with discrete sampling methods, the approaches to passive sampling have been variable between research groups. Differences in resource availability, workflow and data use have translated to variations in chosen sampling material, wash eluents and concentration methods (Appendix B; Table B-1). This study involved the use of cotton gauze as a sampling medium which has been previously described as having a potentially limited capacity for exposure durations over eight hours (Habtewold et al., 2022; J. Li, Verhagen, et al., 2022). However, none of those studies directly analyzed the solid fraction of swab eluates, instead opting for the filtration of the resulting liquid and performing RNA extraction directly from a filter paper. This may explain some of the discordance between results. However, it is true that other materials may be better suited depending on the application in question. For example, materials such as electronegative membrane filters which have been shown to linearly accumulate SARS-CoV-2 over extended sampling durations may be better suited to quantitative applications. This of course, is provided that the practitioner can meet the time and resource demands associated with processing the material. Particularly under a public health emergency, tradeoffs may need to be made to find the right balance of priorities, including priorities related to sampling material and subsequent processing. Practitioners of WBS should make their best attempt to understand these factors and address the research or surveillance objective at hand.

### 3.2.2 Analytical considerations

There are many analytical tools that can be used to detect SARS-CoV-2 and other viruses in wastewater, each with their unique advantages, limitations, and costs. This investigation used RT-qPCR to detect and quantify SARS-CoV-2 and PMMoV RNA concentrations on passive samplers. RT-qPCR is relatively affordable despite requiring commercially produced primers and probes, MasterMix and specialized equipment. RT-qPCR can take longer to perform compared to some other less sensitive techniques since the process involves repetitive thermal cycling to amplify the target sequence in a sample. Digital droplet PCR (ddPCR) as used by West et al., (2023) has a similar cycling time but higher costs associated with specialized reagents and equipment. The advantage of ddPCR is the superior precision it provides by splitting the sample into thousands of individual reactions to generate an absolute quantitative value and that it does not rely on standard curves for quantitation. However, this precision may often not be needed in passive sampling applications where qualitative data is collected. To minimize resource costs, one study employed the use of reverse transcription loop-mediated isothermal amplification (RT-LAMP) to detect the virus in a building-scale surveillance project (Bivins et al., 2022b). They report that despite RT-LAMP being approximately 20 times less sensitive than ddPCR, it may still be useful in resource-limited environments where WBS is desirable (Bivins et al., 2022b). The authors also cite that they were able to process and analyze samples within three hours, which is five to nine hours faster than the workflow described in this thesis. However, qualitative data would have limited use in building-scale surveillance when disease burden in the community is high, as observed in this study in winter 2022. RT-LAMP also reduces the probability of detection when viral signals are extremely low, because of reduced detection limits. There were several trace-level detections in fall 2021 that may have gone undetected with the use of a less sensitive methods. In keeping with the adaptive nature of WBS, practitioners may find it appropriate to use a combination of methods depending on pandemic conditions and resource constraints.

RT-qPCR has been widely used in passive sampling applications because of its relative sensitivity and affordability. In this investigation, two different RT-qPCR assays were used to quantify two N-gene targets and PMMoV. However, the results indicate that there may have been opportunity for further time and cost savings related to routine surveillance. As described in Chapter 2, the primary assay used throughout the eight-month surveillance period duplexed the N1 SARS-CoV-2 gene with PMMoV. As previously discussed, PMMoV has been widely used to normalize SARS-CoV-2 concentrations in wastewater, but its application in passive sampling at the time was untested. Conclusions from Chapter 2 and other studies published since indicate that gauze is not a suitable sampling media for PMMoV beyond eight hours of exposure. Therefore, surveillance programs using cotton gauze, and possibly other cotton-based materials would not benefit from quantifying PMMoV. Alternative normalization tools such as

wastewater production volume or flush counts may be a more appropriate alternative. If exposure conditions and sampling material are suitable, and PMMoV is a desired endpoint of the surveillance program, it should be quantified in simplex. As demonstrated in Chapter 2, discordance in the quantification between the two N-gene targets may have resulted from a combination of the natural dropout of the N1 gene in the virus circulating in the community, and biases in the duplexed assay. High concentrations of PMMoV in wastewater samples relative to SARS-CoV-2 may lead to binding competition for nucleotides within the RT-qPCR reaction. Assessing N-gene targets separately and mitigating inhibition improves the odds of detection and provides the best conditions for measuring temporal variations in SARS-CoV-2 concentrations. While optimizing assay performance should be a top priority for all WBS programs, this study was still able to demonstrate that N1 and N2 were reliably detected in samples and tracked similar temporal trends on SARS-CoV-2 concentrations on passive samplers. This suggests that either N-gene target could be suitable for long-term surveillance and that measuring both routinely may be redundant.

### 3.2.3 Infrastructure management recommendations

The design phase of the WBS program revealed several site-specific challenges which could limit the scope of similar surveillance programs in the future. These considerations should be taken into account when defining the population under surveillance, sampling frequency and feasibility, and logistical considerations with respect to sample collection. The first recommendation is to consistently maintain and update wastewater infrastructure blueprints. The on-campus residence buildings house a combination of old and newly constructed wastewater infrastructure reflective of an expanding residence program over the last several years. The sewer network survey undertaken in August 2021 revealed several discrepancies between existing blueprints and new infrastructure that had yet to be reflected on these documents. As reported by other institutions (Harris-Lovett et al., 2021), this included new and unmapped sewage and storm water infrastructure, unmarked maintenance access points, unmarked connections between buildings, and in some cases, blueprints for entire buildings were not initially available. A patchwork of sewer schematics were eventually obtained from several different campus stakeholders, but consolidating this information would be of great benefit to future WBS efforts undertaken on campus.

One persistent challenge in site selection for this study was the inability to isolate wastewater influent from individual buildings or building subunits. On the University campus there were several candidate sites that had confounding sources of wastewater. Strong-armed by utility hole locations and inaccessible cleanouts, the surveillance program often had to rely on building cluster-level surveillance, thereby broadening the list of potentially affected communities. While little can be done to modify existing wastewater infrastructure, the future construction or repair of building sewers for residence

accommodations, as well as the main campus, should incorporate accessible indoor cleanouts for future sampling endeavors. This recommendation comes at a time when the University aims to address a severe student housing shortage in the province (Lundy, 2023) through the recent announcement to expand the residence program by the addition of a new 500-bed facility set to open in 2026 (UW Media Relations, 2023). This will not only improve ease of access to sampling sites but also help to mitigate costs related to the number of samples required. Such infrastructure would also allow for a more feasible comparison of passive samples and composite samplers in-situ and could serve a wide variety of other research and surveillance interests as well.

## 3.3 The future of WBS

WBS has demonstrated its versatility as a public health tool over decades of being successfully implemented in diverse applications. At its core, WBS aims to fill the knowledge gaps associated with conventional public health tracking. It does so independent of human test-seeking behaviours and other factors that influence the quality and scope of clinical datasets. These characteristics have popularized the approach during the COVID-19 pandemic and will continue to attract public health practitioners into the future. As its rich history demonstrates, WBS is adaptive in scope and scale, and can be a responsive tool to solve novel problems. Research on WBS will continue to expand as new pathogens emerge and as new epidemiological research questions arise. In the context of SARS-CoV-2, adaptations were made at all levels of public health to address emerging VOCs. This included exploring new SARS-CoV-2 endpoints, optimizing existing assays and developing new ones. Congruently, other endpoints have been widely incorporated into existing WBS programs to maximize public health data gleaned from a single sample. This includes the use of WBS to track influenza, respiratory syncytial virus and the monkeypox virus.

A major part of the success of WBS during COVID-19 was the effective communication of its strengths, limitations and potential to support public health. Skepticisms of WBS during the initial stages of the pandemic were grounded in uncertainties around effectiveness, added value and financial costs. However, public understanding and acceptance of the approach has significantly broadened. WBS data became routinely reported alongside other public health metrics, and critically, were made accessible to all people. The public often relied on WBS to gain a better understanding of pandemic dynamics in their communities, especially when clinical testing was no longer reliable. Without public support, WBS would not be possible. It is critical that surveillance efforts remain transparent, accessible, and understandable to all stakeholders in order to maximize the benefits WBS can offer. This applies at all spatial surveillance scales whether at or upstream of the WWTP.

The suite of indicators that can be detected and monitored using WBS make it a powerful tool for a variety of end users in the absence of other non-invasive testing means. On campus, WBS results were relied on by both University decision-makers and student residents to understand public health risks within building-scale communities. WBS supported existing public health measures by providing an additional layer of evidence to detect and isolate individual cases. Passive sampling was able to deliver meaningful results in a fast and affordable fashion, demonstrating its utility at various sampling scales to address changing surveillance needs. These factors will only become more important considerations in the delivery of WBS programs as novel pathogens emerge and new institutional challenges arise. In the public health practitioner's toolbox, WBS stands superior in its scope of potential applications and versatility in sampling scale. Given its most recent successes, WBS will be a critical component of our response to the next pandemic.

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# Appendix A
# Supplemental Material for Chapter 2

## 1. Passive Sampler Validation Experiment

## 1.1 Introduction

Several pilot studies were performed in advance of the fall 2021 academic semester to assess the possibility of feasibility SARS-CoV-2 RNA in on-campus wastewater. These experiments were designed to compare the efficacy of three candidate passive sampling materials: cotton gauze, electronegative membrane filters and standard tampons, the latter of which was quickly abandoned due to logistical constraints in sampler processing. These materials were selected based on their availability, affordability, and early promise from previous research (Hayes et al., 2021; Rafiee et al., 2021; Schang et al., 2021). Unfortunately, the low SARS-CoV-2 viral load in wastewater during this time precluded meaningful comparisons between materials. Therefore, following the conclusion of the routine surveillance program, a method validation experiment was performed to (1) assess the efficacy of cotton gauze and electronegative membranes, and (2) to better understand the variability associated with passive samplers. In this experiment, multiple torpedoes were deployed along the same sewer channel to improve statistical power and make direct comparisons between the materials in-situ.

## 1.2 Materials and methods

### 1.2.1 Passive sampling material selection

Two materials were compared in this experiment: (1) cotton gauze pad, 7.62 $cm^2$ (Fischer Scientific, Mississauga, Ontario), and (2) electronegative cellulose nitrate membrane filter, 37 mm, 0.45 mM pore size (Sartorius AG, Göttingen, Germany).

### 1.2.2 Sampling locations

Three utility holes were identified along a sewer main that had no known infiltration points between sites. Therefore, passive sampling materials within and between sites were expected to capture the same number of SARS-CoV-2 N-gene fragments. This sewer line drains a large municipal subdivision before ultimately connecting to the campus Health Services building complex. These sites were selected primary

on the basis that they sample the same wastewater with the added benefit that a municipal water source would increase the odds of a positive detection, regardless of material type.

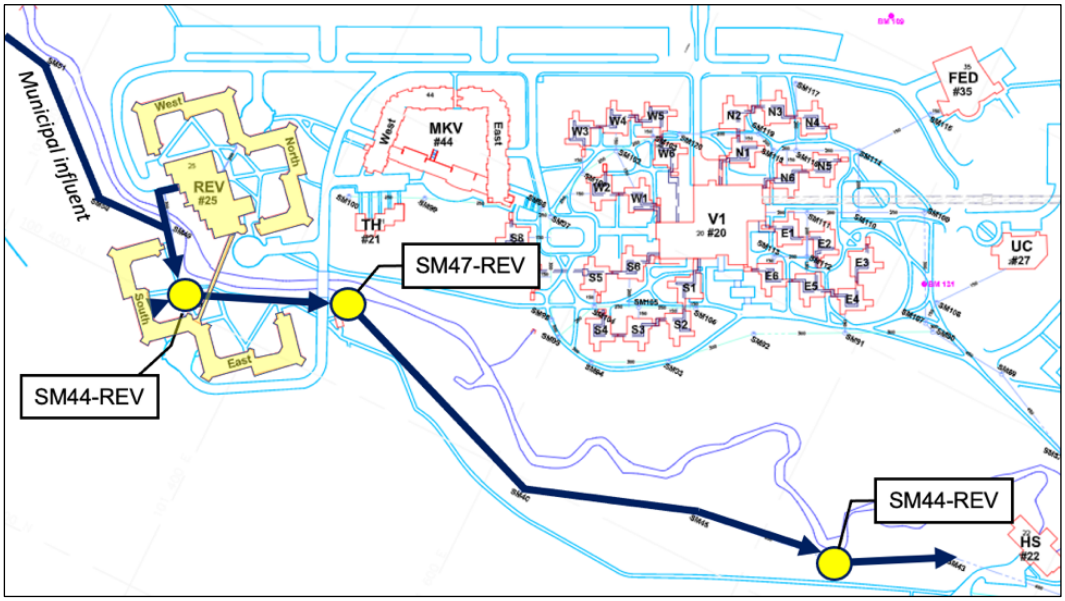


**Figure A-1.** Sampling sites for torpedo replication experiment. The blue arrows delineate the sewer channel and flow direction. Yellow circles indicate the locations of three sampling sites: SM44-REV (upstream), SM47-REV (midstream) and SM44-REV (downstream). The former two sites are separated by approximately 100 m of sewer pipe whereas the latter two are separated by approximately 500 m of sewer pipe.

### 1.2.3 Modified sampling apparatus

Passive sampler design was completed in accordance with the methods described in the main methods of this paper with one modification. To improve statistical power, three torpedo units were affixed in a line using fishing line to create a singular sampling unit (Figure A-2). Each torpedo housed one electronegative membrane and one gauze pad in a randomized position (front and back).


**Figure A-2.** Triple torpedo sampling apparatus (A) loaded with electronegative membrane filter and medical gauze, and (B) sampling apparatus immediately following collection.

### 1.2.4 Sampler processing and RT-qPCR

Sampler processing, RNA extraction and analytical methods were carried out in accordance with the methods described in the materials and methods section of this research paper. However, electronegative membranes were processed slightly differently compared to gauze. Membranes did not undergo the DPBS/Polysorbate-20 washing step, but rather, were directly adding into the 2 mL bead beating tube for RNA extraction.

### 1.2.5 Statistical analyses

A two-way ANOVA was used to compare quantified N-gene targets on a raw and normalized to PMMoV basis with site ID and material type as independent factors. The same analysis was repeated using total RNA per gram of pellet as the dependent variable. All assumptions of the parametric two-way ANOVA were met. All statistical analyses and visualizations were performed and created using IMB SPSS Statistics, Version 29.0 (Armonk, NY: IBM Corp.).

## 1.3 Results

Sampler unit collection revealed minimal ragging of the torpedoes increasing confidence in consistent wastewater exposure between materials and sampling sites. Both gauze and membrane filters were able to detect N1 and N2 at all three sites in this experiment. Gauze had a 100% detection rate for both N-gene targets across sites, whereas approximately 77% of membrane filters detected either N-gene target (Table A-1).

**Table A-1.** Binary detection results for passive sampling validation experiment. Positive detections indicate at least two of three technical replicates detected the respective SARS-CoV-2 N-gene target.

| | **N1** | | **N2** | |
|---|---|---|---|---|
| | Detected | Not Detected | Detected | Not Detected |
| Gauze | 9 | 0 | 9 | 0 |
| Membrane | 6 | 3 | 8 | 1 |

Raw N-gene quantification (Figure A-3) and total RNA (Figure A-4) on passive samplers were compared on a per gram of wet pellet basis to account for differences in material surface area. N-gene normalization by PMMoV was also explored (Figure A-5). Based on a two-way ANOVA, there is there is insufficient evidence to suggest an interactive effect between material type and site ID (Two-way ANOVA, $F = 2.670$, $P = 0.110$, d.f. = 2, 12). However, material types did differ with gauze producing significantly higher concentrations of SARS-CoV-2 GC per gram of pellet (Two-way ANOVA, $F = 26.423$, $P < 0.001$, d.f. = 1,12). Gauze still produced significantly higher ratios than membranes did when SARS-CoV-2 concentrations were normalized by PMMoV (Two-way ANOVA, $F = 11.095$, $P = 0.006$, d.f. = 1, 12). This suggests that given the methodology used, gauze outperforms membrane filters in collecting SARS-CoV-2 from wastewater over a 24-hour period. In addition to collecting the most SARS-CoV-2 RNA, gauze pads also collected more total RNA per gram than membranes did (Two-way ANOVA, $F = 42.70$, $P < 0.001$, d.f. = 1, 12). These were desirable characteristics for the material to have given that the collection and retention of suspended solids from wastewater was a top priority.

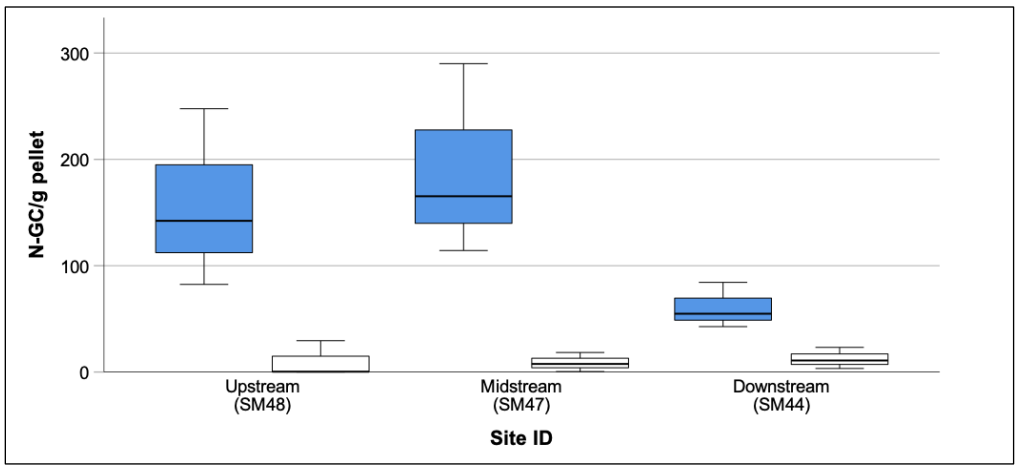


**Figure A-3.** Boxplot of raw N-gene copies per gram of pellet by site ID and material type. Gauze boxplots are shaded blue and electronegative membrane filter boxplots are white, where n = 3 for each box.

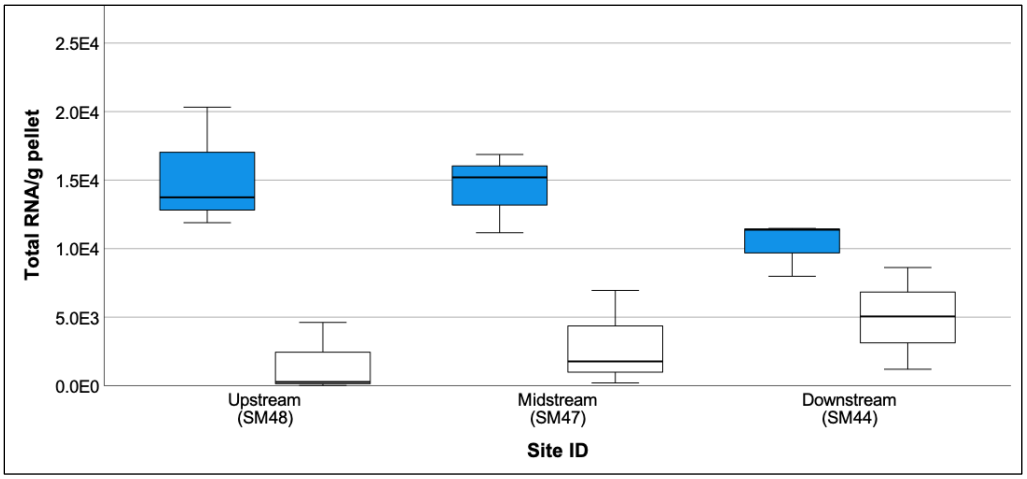


**Figure A-4.** Boxplot of total RNA (ng) per gram of pellet by site ID and material type. Gauze boxplots are shaded, and electronegative membrane filter boxplots are white, where n = 3 for each box.

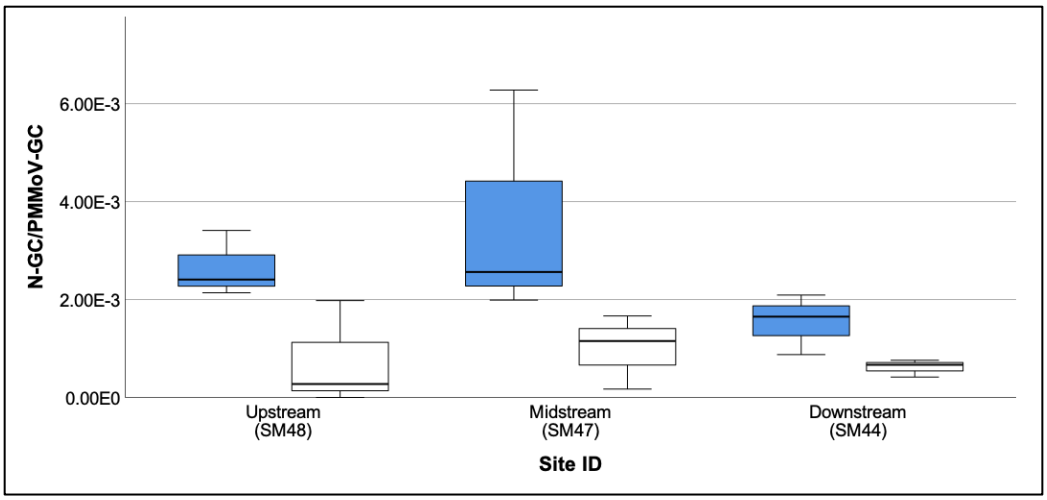


**Figure A-5.** Boxplot of N-gene copies normalized to PMMoV gene copies by site ID and material type. Gauze boxplots are shaded blue and electronegative membrane filter boxplots are white, where n = 3 for each box.

## 1.4 Conclusions

Cotton gauze was able to collect significantly more total RNA and SARS-CoV-2 gene fragments than electronegative membranes did. While the variability observed in cotton gauze was higher, the material was able to detect both N-gene targets at all sites where the electronegative membranes did not. The normalization of SARS-CoV-2 by PMMoV produced similar results. Given these findings, higher quantification of SARS-CoV-2, and a higher detection rate served as the primary basis for the selection of gauze as a passive sampling medium in the current study. However, the selection of substrate for passive sampling should be validated for each study as subtle differences in the sewers, materials, and methods may lead to differences in effectiveness of each in different WBS application scenarios.

## 2. Analytical Methods for RT-qPCR

Elution extracts were analyzed in triplicate using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) for two regions of the nucleocapsid (N) gene for SARS-CoV-2 (N1 and N2) using TaqPath™ 1-Step RT-qPCR Master Mix, GC (Life Technologies, Thermo Scientific, Mississauga, Ontario). N-gene assays were performed using the primers developed by the CDC (Centers for Disease Control and Prevention, 2021). The region of the coat protein of the endogenous fecal indicator Pepper Mild Mottle Virus (PMMoV) was also quantified through RT-qPCR using the same Master Mix (Table A-2). Reactions occurred in a 96-well plate (Bio-Rad Laboratories, Hercules, CA, USA) which was sealed with clear strip caps and mixed on a PCR plate vortex at 400 rpm for 30 seconds. All assays were then run on either the CFX96 Touch or the CDX Opus Real-Time PCR systems using CFX Maestro Software for CFX Real-Time PCR Instruments (Bio-Rad Laboratories, Hercules, CA, USA) with the cycling conditions described in Table A-3. Primer and probe sequences for all other genetic targets are presented in Table A-4.

The N1 and PMMoV targets were duplexed into a single assay where each reaction contained 5 mL of 4X Master Mix, 10 mM of primers, 5 mM of probes, 6 mL of nuclease-free water and 5 mL of RNA extract for a total reaction volume of 20 mL. The N2 assay was run in simplex with each reaction containing 5 mL of 4X Master Mix, 10 mM of primers, 5mM of probe, 7.5 mL of nuclease-free water and 5 mL of RNA extract for a total reaction volume of 20 mL. Appendix Tables 1-3 present additional information on primer and probe sequences and assay cycling conditions. Each plate had a minimum of six no-template controls (NTC) reactions, three no reverse-transcriptase control (NRT) reactions, a standard curve and positive controls. Standard curves were serially diluted where the starting material for N-gene targets was EDX SARS-CoV-2 Standard (Bio-Rad, No.: COV019). All other targets were quantified using dsDNA gBlock standards (Integrated DNA Technologies, Coralville, IA, USA) with all standards verified by dPCR (QIAcuity, Qiagen, Hilden, Germany). The N1 and PMMoV duplex assay was performed on all samples in both surveillance periods whereas the N2 simplex assay was performed only in winter 2022. All primer and probe dilutions were made, and concentrations verified using the NanoDrop One device (Thermo Scientific, Mississauga, Ontario). Standard starting material concentrations were routinely verified using digital PCR (QIAcuity, Qiagen, Hilden, Germany).

**Table A-2.** Synthetic oligonucleotides (gBlock) sequences for pepper mild mottle virus that was supplied by Integrated DNA Technologies (Coralville, IA, USA).

| Genetic Target | Sequence (5' to 3') |
|---|---|
| PMMoV | AGGTAATGGTAGCTGTGGTTTCAAATGAGAGTGGTTTGACCTTAACGTTTGA GAGGCCTACCGAAGCAAATGTCGCACTTGCATTGCAACCGACAATTACATCA AAGGAGGAAGGTTCGTTGAAG |

**Table A-3.** RT-qPCR cycling conditions for assays performed in this study. The denature and anneal/elongation steps were repeated for 45 cycles.

| Assay | Stage | | | | |
|---|---|---|---|---|---|
| | **Preheat** (°C; min) | **Reverse Transcription** (°C; min) | **Activation** (°C; min) | **Denature** (°C; min) | **Anneal/ Elongation** (°C; min) |
| N1/PMMoV duplex | 25; 2 | 50; 15 | 95; 2 | 95; 3 | 55; 30 |
| N2 simplex | 25; 2 | 50; 15 | 95; 2 | 95; 3 | 60; 30 |

**Table A-4.** Primers and probes used in this study were provided by Millipore Sigma.

| Primer/ Probe[1] | Final Concentration (nM) | Sequence (5' to 3') | Probe Reporter/ Quencher | Reference |
|---|---|---|---|---|
| N1-FP | 500 | GACCCCAAAATCAGCGAAAT | - | (Centers for Disease Control and Prevention, 2021) |
| N1-RP | 500 | TCTGGTTACTGCCAGTTGAATC TG | - | |
| N1-P | 125 | ACCCCGCATTACGTTTGGTGGA CC | 6-FAM/BHQ-1 | |
| N2-FP | 500 | TTACAAACATTGGCCGCAAA | - | |
| N2-RP | 500 | GCGCGACATTCCGAAGAA | - | |
| N2-P | 125 | ACAATTTGCCCCCAGCGCTTCA G | 6-FAM/BHQ-1 | |
| PMMoV-FP | 400 | GAGTGGTTTGACCTTAACGTTG A | - | (Zhang et al., 2006) |
| PMMoV-RP | 400 | TTGTCGGTTGCAATGCAAGT | - | |
| PMMoV-P | 125 | CCTACCGAAGCAAATG | Cy5/BHQ-1 | |

[1] FP = forward primer; RP = reverse primer; P = probe

## 3. RT-qPCR Quality Assurance and Control (QAQC)

Standard curves were run on each plate and had at least four points with triplicate analysis for each point. On select surveillance days where this condition was not met, an average standard curve was applied provided other QAQC measures were met. Average standard curves were created with ten replicates per standard point. Standard curve slopes typically ranged from -3.1 to -3.6 with a requirement for reaction efficiencies to fall between 90% and 110%. Standard curves also had an $R^2$ value of 0.98 or greater. All standard curve technical replicates as well as positive controls had a standard deviation of 0.5 Cq or less. Where QAQC standards were not met the plate was rerun. Run information for assays performed each sampling day is presented in Appendix C.

# 4. Supplemental Figures and Tables

**Figure A-6.** Passive sampling apparatus prior to deployment: A torpedo-shaped housing vessel (Schang et al., 2021) loaded with cotton gauze and secured by an elastic band.

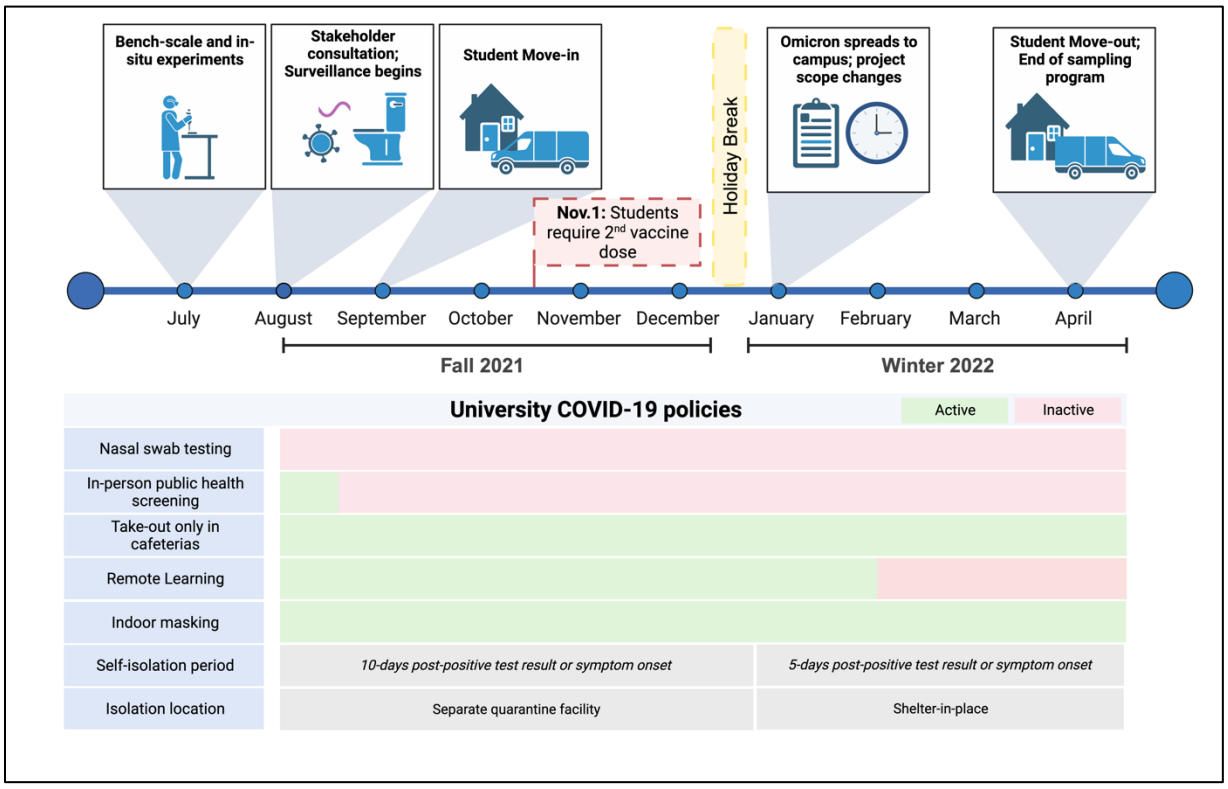


**Figure A-7.** Key events during implementation of the WBS program including COVID-19 policies administered by the University.

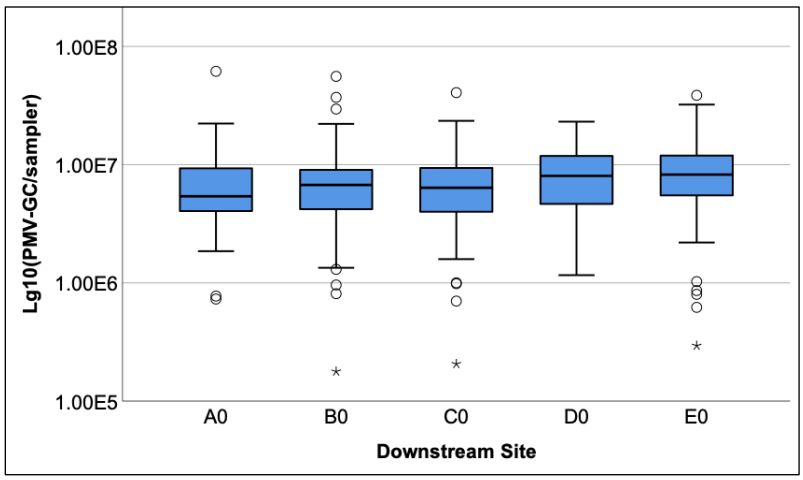


**Figure A-8.** Boxplot of transformed PMMoV GC/sampler on log scale between five downstream sites. Potential outliers are denoted by circles and asterisks.

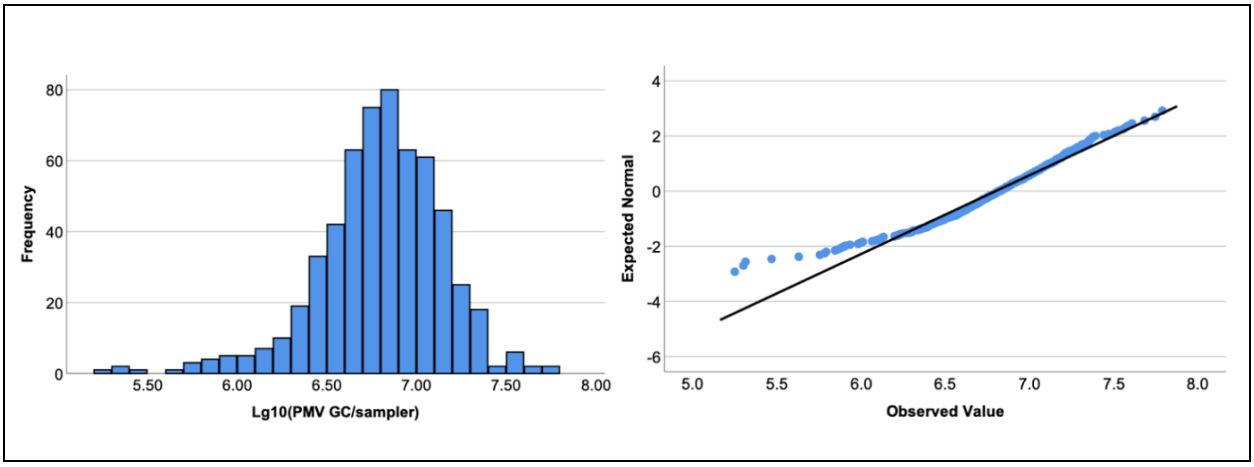


**Figure A-9.** Histogram (left) and Q-Q plot (right) of PMMoV GC/sampler for all samples.

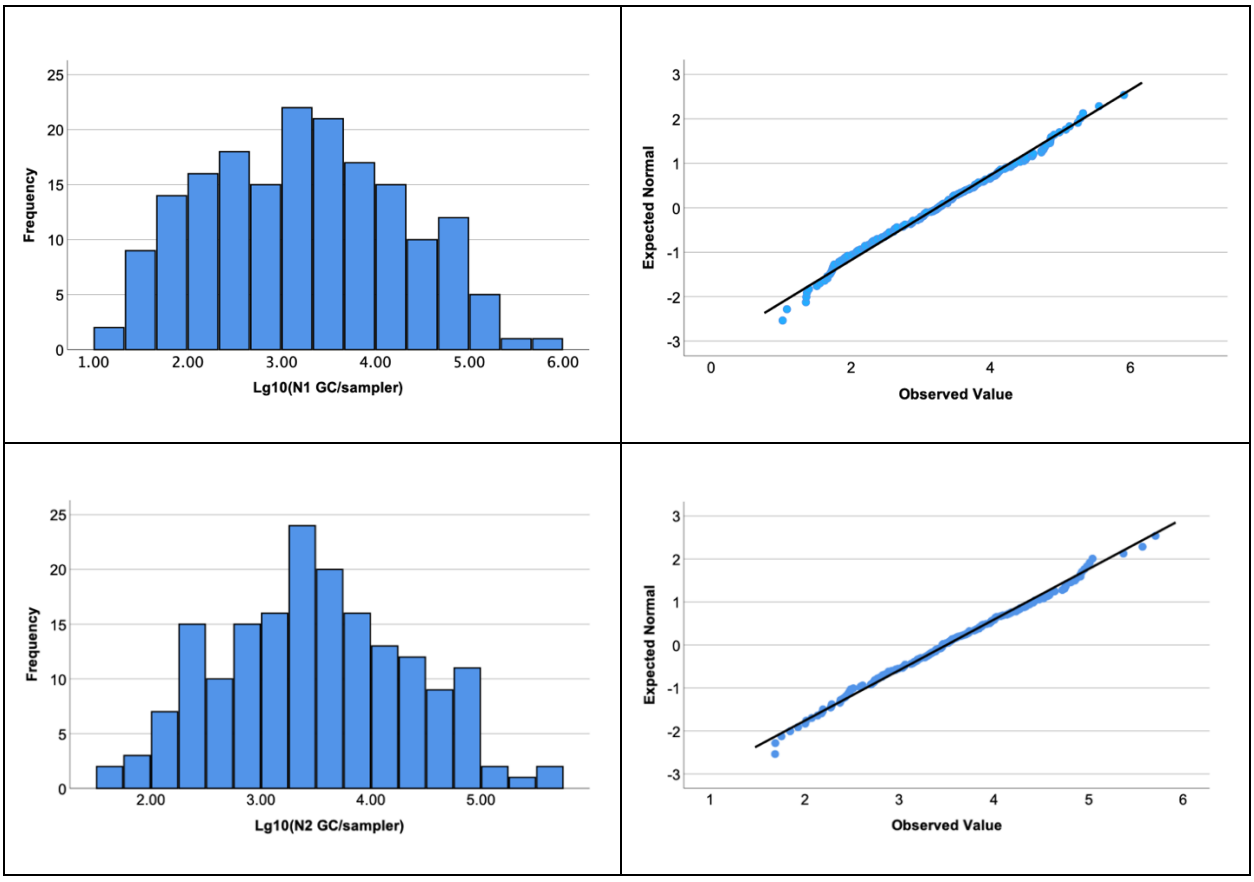


**Figure A-10.** Histogram (left) and Q-Q plot (right) of N1 GC/sampler (top panels) and N2 GC/sampler (bottom panels) for passive samplers.

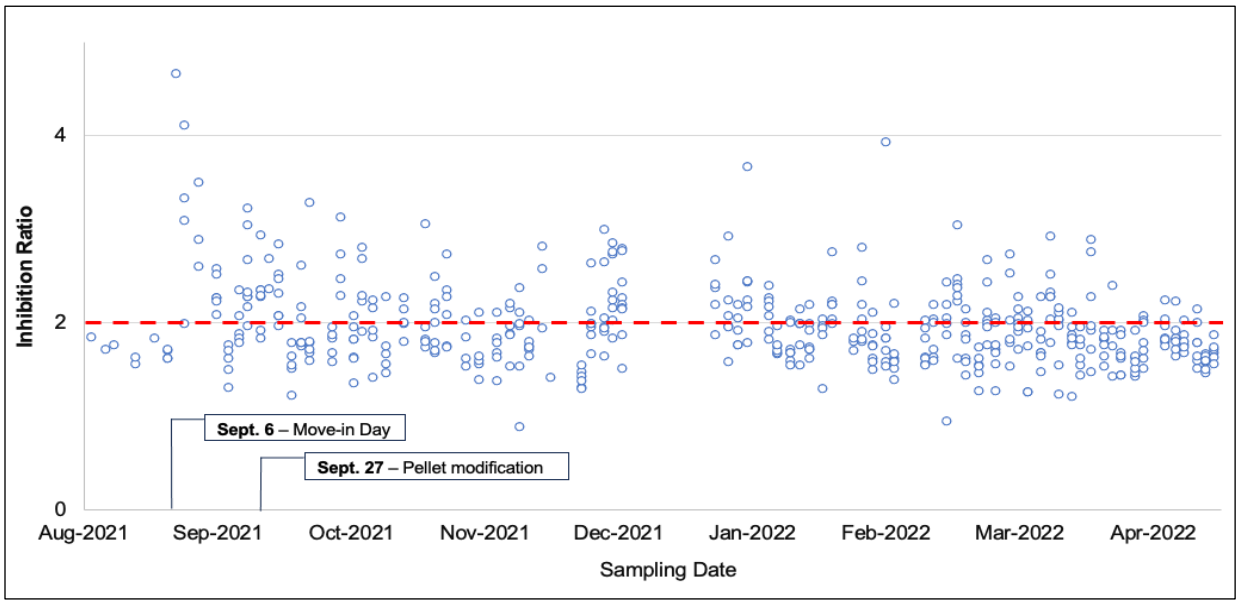


**Figure A-11.** Inhibition ratio of samples collected over fall 2021 and winter 2022. A sample was deemed inhibited if the inhibition ratio exceeded 2.

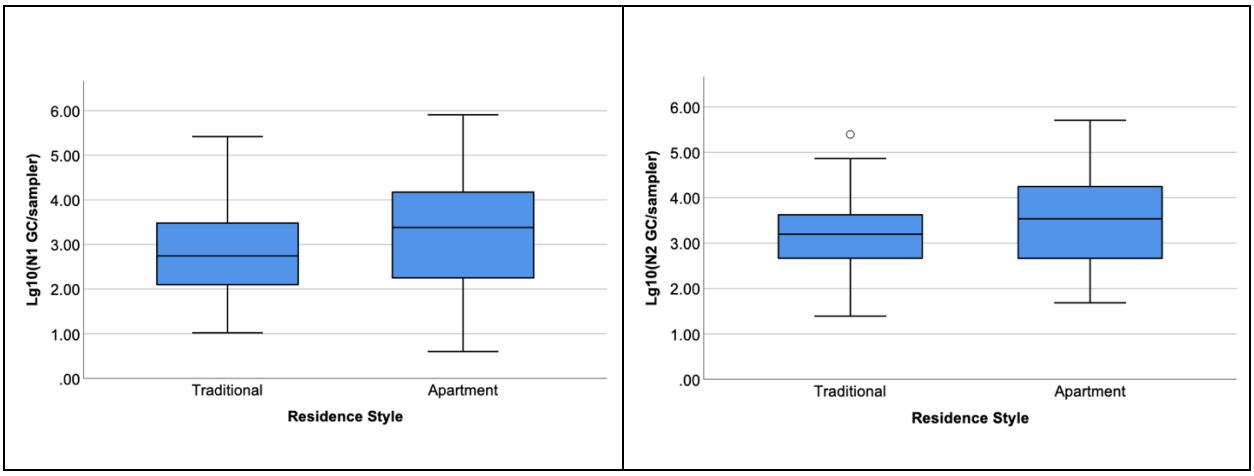


**Figure A-12.** Boxplots for N1 (left) and N2 (right) GC per sampler in log scale comparing traditional and apartment-style residence buildings. Potential outliers are noted by circles.

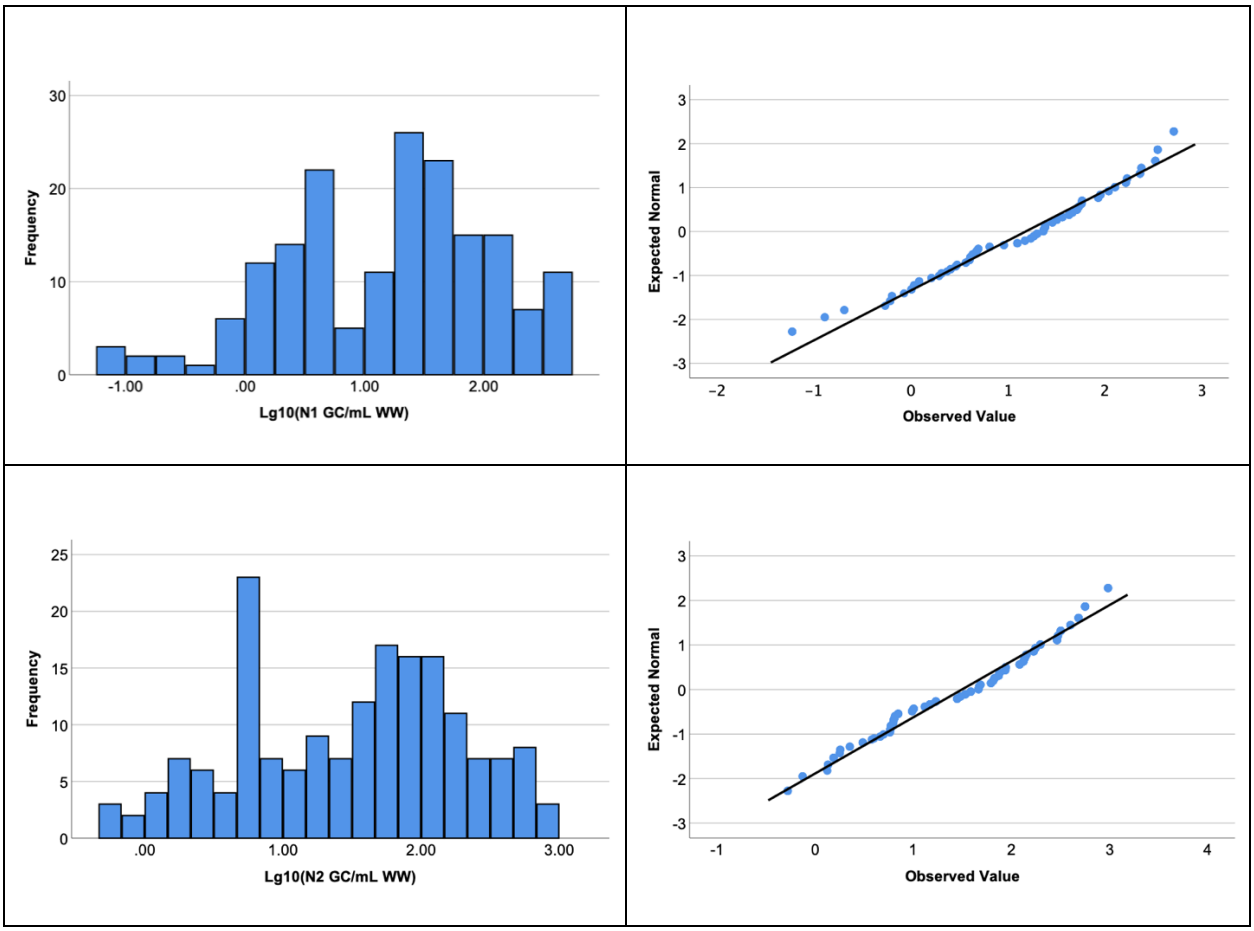


**Figure A-13.** Histograms (left) and Q-Q plots (right) of log-transformed N1 (top panels) and N2 (bottom panels) GC/mL of wastewater collected at the City of Waterloo WWTP

**Table A-5**. All wastewater surveillance sampling sites, their associated occupancies and infrastructure characteristics. All sampling sites were located within a gravity sewer system.

| Surveillance Branch | Designation | Upstream Occupancy | | Site Characteristics |
|---|---|---|---|---|
| | | Fall 2021 | Winter 2022 | |
| **A0** | Downstream | 331 | 443 | • 300 mm concrete<br>• Shallow channel; ~2 m from surface<br>• Adjacent to laundry facility |
| **B3** | Upstream | 288 | 315 | • 250 mm concrete<br>• Deep, curved channel; ~8 m from surface<br>• Distance to downstream site (B0): ~ |
| B2 | Upstream | 292 | 306 | • 150 mm concrete<br>• Shallow channel; <1 m from surface<br>• Y-junction (mixing point) |
| B1 | Upstream | 243 | 306 | • 250 mm concrete<br>• Shallow channel; ~2 m from surface<br>• Y-junction (mixing point) |
| B0 | Downstream | 920 | 1029 | • 300 mm concrete<br>• Shallow channel; ~2 m from surface |
| C3 | Upstream | 292 | 306 | • 200 mm concrete<br>Shallow channel; ~3 m from surface |
| C2 | Upstream | 194 | 204 | • 200 mm concrete<br>• Deep channel; ~5 m from surface<br>• Adjacent to cafeteria; site prone to pooling |
| C1 | Downstream | 486 | 510 | • 300 mm concrete<br>• Persistent pooling; major infiltration by tree roots<br>• Shallow channel; ~3 m from surface |
| **C0** | Downstream | 1552 | 1651 | • 450 mm concrete<br>• Deep channel; >15 m from surface<br>• Collects influence from C1 and B0<br>• Vertical y-junction (mixing point) |
| D1 | Upstream | 480 | 572 | • 200 mm concrete<br>• Shallow channel; < 1 m from surface |
| **D0** | Downstream | 1298 | 1394 | • 200 mm concrete<br>• Shallow channel; ~2 m from surface<br>• Y-junction (mixing point) |
| E1 | Upstream | 429 | 450 | • 200 mm concrete<br>• Shallow channel; <1 m from surface<br>• Y-junction (mixing point) |
| **E0** | Downstream | 643 | 675 | • 200 mm concrete<br>• Shallow channel; ~2 m from surface<br>• Y-junction (mixing point) |

# Appendix B

## Summary of relevant literature

**Table B-1.** Summary of publications reporting the use of passive sampling in building-scale wastewater surveillance of SARS-CoV-2 RNA. This table was taken from (Bivins et al., 2022b) with some modifications and adapted to include Haskell et al., 2023 (Chapter 2) and other most recently published data[1]. For 'Fraction used for analysis', swab sorbate refers to the solids and liquids retained by the sampler from wastewater and eluate refers to the resulting solution if the sampler is washed with an eluent.

| Citation | Setting | Location | Population | Sampling frequency | Material | Housing vessel | Exposure duration | Fraction used for analysis | Viral concentration method | Analytical methods |
|---|---|---|---|---|---|---|---|---|---|---|
| Haskell et al., 2023[1] | University | Waterloo, Ontario, Canada | 194 to 1552 | 3 days per week | Cotton gauze | Torpedo | 24 h | Swab sorbate + eluate; solids fraction | Centrifugation | RT-qPCR |
| Schang et al., 2021 | Municipal | Melbourne, Victoria, Australia | 260 to 2.6 M | NR | Cotton gauze; electronegative filter; cotton buds | Colander; boat; box; torpedo | 3 to 24 h | Direct extraction (cotton bud, electronegative filter); eluate (gauze) | Filtration via electronegative membrane | RT-qPCR |
| Hayes et al., 2021 | Office complex, University | Halifax, Nova Scotia, Canada | Unknown | 15 events over 5 months | Cotton gauze; cheesecloth; cellulose sponge; electronegative filter | COSCa | 24 h<br>48 h<br>72 h | Eluate | NA | RT-qPCR |
| Habtewold et al., 2022 | Municipal | Guelph, Ontario, Canada | NR | 3 events over 1 month | Cotton gauze; cotton bud; electronegative filter | Torpedo | 4 h<br>8 h<br>24 h<br>48 h<br>72 h<br>96 h | Direct extraction (cotton bud, electronegative filter); eluate (gauze) | Filtration via electronegative membrane | RT-qPCR |
| Corchis-Scott et al., 2021 | University | Windsor, Ontario, Canada | ~200 | 3 days per week to daily | Tampon | None | 20 h | Eluate | Ultrafiltration via CP Select | RT-qPCR |
| Bivins et al., 2021 | University | South Bend, Indiana, USA | 1627 | 1 day per week | Tampon | None | 3 h | Eluate solid fraction | NA | RT-LAMP[2] |

| Citation | Setting | Location | Population | Sampling frequency | Material | Housing vessel | Exposure duration | Fraction used for analysis | Viral concentration method | Analytical methods |
|---|---|---|---|---|---|---|---|---|---|---|
| Rafiee et al., 2021 | Municipal | Tehran, Iran | NR | 2 events over 5 months | Cotton gauze | Stainless steel wire cage | 16 h | Swab sorbate + eluate liquid fraction | PEG precipitation | RT-qPCR |
| Liu et al., 2022 | University | Atlanta, Georgia, USA | NR | 1 event per week | Cotton gauze | None | 24 to 72 h | Swab sorbate + eluate | Skimmed milk flocculation; PEG precipitation | RT-qPCR |
| Wang et al., 2022 | University | Atlanta, Georgia, USA | 91 to 600 | 1 event per week | Cotton gauze | None | 24 to 72 h | Swab sorbate + eluate | Skimmed milk flocculation; PEG precipitation | RT-qPCR |
| Kitajima et al., 2022 | Olympic village | Tokyo, Japan | NR | NR | NR | NR | NR | NR | NR | RT-qPCR |
| Hayes et al., 2022 | Bench scale; Residential; Office complex; University | Halifax, Nova Scotia, Canada | Unknown | 23 events over 15 weeks | Electronegative filter | COSCa | 24 to 72 h | Eluate | NA | RT-qPCR |
| Li et al., 2022 | Municipal | Queensland, Australia | 13,000 to 231,000 | 2 events | Electronegative filter; cotton buds; cotton gauze; tampon | Hair roller (tampon); torpedo (others) | Up to 48 h | Swab sorbate (tampon); eluate (cotton gauze); direct extraction (membrane, cotton buds) | Ultrafiltration (tampon); Filtration via electronegative membrane (gauze eluate) | RT-qPCR |
| Jain et al., 2022[1] | University; Municipal | Saskatoon, Saskatchewan, Canada | 125 to 399 | 5 days per week | Electronegative filter; cotton gauze | Torpedo | 24 to 72 hours | Swab sorbate + eluate | Magnetic bead-based separation | RT-qPCR |
| Wilson et al., 2022[1] | Bench-scale; Hospital | Edmonton, Alberta, Canada | Unknown | 2 days per week | Cotton gauze; tampons | Torpedo | 48 to 144 h | Swab sorbate + eluate | Centrifugation | RT-qPCR |
| Kevill et al., 2022[1] | Bench-scale | Bangor, North Wales, United Kingdom | 40,000 | 1 event | Tampons; electronegative filter | None | 1 h | Swab sorbate; Swab sorbate + eluate; Direct extraction | PEG Precipitaiton; | RT-qPCR |
| Acer et al., 2022[1] | University | Amherst, Massachusetts, USA | NR | Three to five times per week | Tampons | None | 24 h | Swab sorbate + eluate | Filtration via electronegative membrane | RT-qPCR |
| Mangwana et al., 2022[1] | University | Cape Town, South Africa | 6,500 | Twice per week over 28 weeks | Cotton gauze | Torpedo | 21 h | Swab sorbate + eluate; solids fraction | Centrifugation | RT-qPCR |

| Citation | Setting | Location | Population | Sampling frequency | Material | Housing vessel | Exposure duration | Fraction used for analysis | Viral concentration method | Analytical methods |
|---|---|---|---|---|---|---|---|---|---|---|
| West et al., 2023[1] | University; Long-term care home; Municipal | Detroit, Michigan, USA | < 500 (University); 500 – 2,000 (Long-term care home); 12,000 (Municipal) | 126 events over 5 months | Tampons | Stainless steel wire cage | 3 to 4 h | Swab sorbate + eluate | NA | ddPCR[3] |

[1] Publication not included in Bivins et al., 2022 table.

[2] Reverse transcription loop-mediated isothermal amplification

[3] Digital droplet polymerase chain reaction

# Appendix C

## Quality control and assurance metrics for routine RT-qPCR

**Table C-1**. RT-qPCR quality control and assurance metrics. Flags for analysis are highlighted in yellow.

| PCR Date | Target | Efficiency (%) | $R^2$ | Slope | Y-intercept | No. standard curve points | % NTCs amplified | % NRTs amplified | $Cq_{+control}$ (Mean) | $Cq_{+control}$ (SDev.) | Plate passes QAQC | Plate qualifiers |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2021-08-18** | N1 | 110 | 0.985 | -3.111 | 37.99 | 4 | 33 | 0 | 30.97 | 0.21 | NO | LC, R |
| **2021-08-20** | N1 | 90 | 0.985 | -3.578 | 40.68 | 4 | 33 | 33 | 32.60 | 0.05 | NO | LC, R |
| **2021-08-23** | N1 | 118 | 0.980 | -2.963 | 37.91 | 10 | 0 | 0 | 32.60 | 0.41 | NO | ASC, R |
| **2021-08-25** | N1 | 96 | 0.970 | -3.416 | 39.45 | 5 | 50 | 0 | 31.10 | 0.02 | NO | LC, R |
| **2021-08-25** | N2 | 100 | 0.987 | -3.334 | 39.30 | 7 | 0 | 0 | 32.08 | 0.13 | YES | R |
| **2021-08-30** | N1 | 110 | 0.995 | -3.103 | 37.91 | 4 | 17 | 0 | 31.44 | 0.21 | NO | LC, R |
| **2021-09-01** | N1 | 79 | 0.985 | -3.971 | 40.16 | 5 | 67 | 0 | 32.99 | 0.11 | NO | LC, R |
| **2021-09-01** | N1 | 115 | 0.987 | -3.006 | 37.98 | 4 | 17 | 0 | 31.73 | 0.20 | NO | LC, R |
| **2021-09-03** | N1 | 99 | 0.995 | -3.342 | 39.00 | 4 | 17 | 0 | 32.36 | 0.18 | YES | R |
| **2021-09-06** | N1 | 106 | 0.986 | -3.193 | 38.18 | 4 | 33 | 0 | 31.93 | 0.04 | YES | LC, R |
| **2021-09-08** | N1 | 92 | 0.985 | -3.539 | 38.94 | 4 | 67 | 0 | 31.86 | 0.18 | NO | HC, NR, RR |
| **2021-09-08** | N1 | 103 | 0.993 | -3.259 | 39.71 | 4 | 33 | 0 | 33.31 | 0.24 | YES | R |
| **2021-09-10** | N1 | 99 | 0.988 | -3.358 | 40.07 | 4 | 17 | 0 | 32.39 | 0.19 | YES | R |
| **2021-09-13** | N1 | 105 | 0.985 | -3.212 | 38.07 | 4 | 17 | 0 | 33.33 | 0.21 | YES | LC, R |
| **2021-09-15** | N1 | | | | | | | 0 | | | YES | R |
| **2021-09-17** | N1 | 108 | 0.987 | -3.153 | 38.52 | 4 | 0 | 0 | 32.31 | 0.24 | YES | R |
| **2021-09-20** | N1 | 101 | 0.989 | -3.295 | 38.83 | 4 | 8 | 0 | 31.45 | 0.05 | YES | R |
| **2021-09-22** | N1 | 100 | 0.992 | -3.331 | 37.91 | 5 | 17 | 0 | 30.99 | 0.21 | YES | R |
| **2021-09-24** | N1 | 95 | 0.995 | -3.458 | 38.86 | 5 | 0 | 0 | 31.80 | 0.24 | YES | R |
| **2021-09-29** | N1 | 107 | 0.995 | -3.161 | 39.30 | 4 | 0 | 0 | 32.87 | 0.31 | YES | R |
| **2021-10-01** | N1 | 92 | 0.987 | -3.529 | 39.48 | 4 | 33 | 0 | 32.36 | 0.15 | NO | LC, R |
| **2021-10-04** | N1 | 89 | 0.951 | -3.619 | 38.65 | 10 | 0 | 0 | 31.87 | 0.29 | NO | ASC, R |
| **2021-10-06** | N1 | 108 | 0.993 | -3.154 | 39.07 | 5 | 11 | 0 | 32.83 | 0.17 | YES | R |
| **2021-10-08** | N1 | 92 | 0.990 | -3.517 | 39.00 | 5 | 11 | 0 | 32.23 | 0.08 | YES | R |
| **2021-10-13** | N1 | 99 | 0.992 | -3.335 | 39.31 | 5 | 0 | 0 | 32.96 | 0.08 | YES | R |
| **2021-10-18** | N1 | 96 | 0.987 | -3.422 | 39.29 | 4 | 33 | 0 | 31.75 | 0.49 | NO | LC, R |
| **2021-10-20** | N1 | 104 | 0.992 | -3.226 | 38.06 | 4 | 0 | 0 | 32.01 | 0.04 | YES | R |
| **2021-10-20** | N2 | 101 | 0.992 | -3.309 | 40.24 | 7 | 0 | 0 | 33.50 | 0.21 | YES | R |

| PCR Date | Target | Efficiency (%) | $R^2$ | Slope | Y-intercept | No. standard curve points | % NTCs amplified | % NRTs amplified | $Cq_{+control}$ (Mean) | $Cq_{+control}$ (SDev.) | Plate passes QAQC | Plate qualifiers |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2021-10-22** | N1 | 94 | 0.988 | -3.471 | 39.84 | 4 | 0 | 0 | 33.16 | 0.19 | YES | R |
| **2021-10-25** | N1 | 100 | 0.985 | -3.315 | 39.69 | 5 | 9 | 0 | 32.98 | 0.22 | YES | R |
| **2021-10-27** | N1 | 102 | 0.993 | -3.272 | 38.70 | 4 | 0 | 0 | 32.91 | 0.10 | YES | R |
| **2021-10-29** | N1 | 92 | 0.991 | -3.544 | 39.67 | 4 | 0 | 0 | 32.58 | 0.08 | YES | R |
| **2021-11-01** | N1 | | | | | | | 0 | | | NO | NR |
| **2021-11-03** | N1 | 102 | 0.996 | -3.266 | 39.74 | 3 | 17 | 0 | 32.53 | 0.24 | YES | LC, R |
| **2021-11-05** | N1 | 105 | 0.994 | -3.208 | 39.77 | 3 | 0 | 33 | 31.97 | 0.23 | YES | R |
| **2021-11-05** | N2 | 100 | 0.989 | -3.333 | 40.22 | 7 | 0 | 0 | 33.83 | 0.25 | YES | R |
| **2021-11-08** | N1 | 101 | 0.996 | -3.303 | 39.64 | 4 | 0 | 0 | 32.51 | 0.11 | YES | R |
| **2021-11-10** | N1 | | | | | 10 | 0 | | 31.87 | 0.19 | NO | ASC, R |
| **2021-11-12** | N1 | 98 | 0.986 | -3.378 | 40.17 | 4 | 0 | 0 | 32.63 | 0.17 | YES | R |
| **2021-11-15** | N1 | 93 | 0.993 | -3.491 | 39.50 | 4 | 0 | 0 | 32.20 | 0.06 | YES | R |
| **2021-11-17** | N1 | 97 | 0.980 | -3.399 | 39.16 | 4 | 7 | 0 | 31.08 | 0.17 | YES | R |
| **2021-11-19** | N1 | 92 | 0.984 | -3.530 | 39.17 | 4 | 0 | 0 | 31.64 | 0.24 | YES | R |
| **2021-11-22** | N1 | 105 | 0.986 | -3.204 | 39.29 | 4 | 0 | 0 | 32.29 | 0.07 | YES | R |
| **2021-11-24** | N1 | 92 | 0.991 | -3.526 | 39.37 | 5 | 0 | 0 | 32.12 | 0.13 | YES | R |
| **2021-11-26** | N1 | 94 | 0.996 | -3.487 | 38.51 | 5 | 8 | 0 | 31.36 | 0.18 | YES | R |
| **2021-11-29** | N1 | 113 | 0.991 | -3.055 | 39.24 | 10 | 11 | 0 | 32.11 | 0.21 | NO | ASC, R |
| **2021-12-03** | N1 | 108 | 0.982 | -3.148 | 39.52 | 5 | 0 | 0 | 32.01 | 0.10 | YES | R |
| **2021-12-06** | N1 | 92 | 0.982 | -3.541 | 39.69 | 5 | 0 | 33 | 30.78 | 0.08 | YES | R |
| **2021-12-08** | N1 | 100 | 0.982 | -3.322 | 38.54 | 5 | 0 | 0 | 30.27 | 0.21 | YES | R |
| **2021-12-10** | N1 | 121 | 0.978 | -2.898 | 39.70 | 10 | 0 | 0 | 32.40 | 0.11 | NO | ASC, R |
| **2021-12-13** | N1 | 78 | 0.875 | -3.982 | 40.55 | 10 | 0 | 33 | 31.29 | 0.18 | NO | ASC, R |
| **2021-12-15** | N1 | 97 | 0.986 | -3.399 | 38.44 | 5 | 0 | 0 | 31.27 | 0.13 | YES | R |
| **2021-12-17** | N1 | 130 | 0.852 | -2.760 | 39.54 | 10 | 0 | 33 | 31.81 | 0.14 | NO | ASC, R |
| **2022-01-07** | N1 | 77 | 0.979 | -4.016 | 41.07 | 10 | 0 | 0 | 32.06 | 0.13 | NO | ASC, R |
| **2022-01-07** | N2 | 104 | 0.994 | -3.223 | 39.79 | 5 | 0 | 0 | 33.21 | 0.07 | YES | R |
| **2022-01-10** | N1 | 107 | 0.981 | -3.164 | 39.41 | 4 | 0 | 0 | 31.47 | 0.17 | YES | R |
| **2022-01-10** | N2 | 92 | 0.988 | -3.545 | 39.62 | 4 | 0 | 0 | 30.52 | 0.05 | YES | R |
| **2022-01-12** | N2 | 102 | 0.995 | -3.274 | 39.20 | 5 | 0 | 0 | 32.45 | 0.35 | YES | R |
| **2022-01-12** | N1 | 97 | 0.983 | -3.387 | 40.01 | 5 | 0 | 0 | 31.69 | 0.05 | YES | R |
| **2022-01-14** | N2 | 93 | 0.992 | -3.500 | 39.78 | 5 | 8 | 0 | 31.97 | 0.19 | YES | R |
| **2022-01-18** | N1 | 83 | 0.916 | -3.801 | 40.62 | 10 | 0 | 0 | 31.36 | 0.08 | NO | ASC, R |
| **2022-01-18** | N2 | 96 | 0.996 | -3.424 | 40.56 | 5 | 0 | 0 | 32.87 | 0.17 | YES | R |

| PCR Date | Target | Efficiency (%) | $R^2$ | Slope | Y-intercept | No. standard curve points | % NTCs amplified | % NRTs amplified | $Cq_{+control}$ (Mean) | $Cq_{+control}$ (SDev.) | Plate passes QAQC | Plate qualifiers |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2022-01-19** | N2 | 99 | 0.988 | -3.357 | 40.53 | 5 | 0 | 0 | 32.96 | 0.11 | YES | R |
| **2022-01-19** | N1 | 100 | 0.980 | -3.324 | 40.74 | 4 | 0 | 0 | 32.24 | 0.15 | YES | R |
| **2022-01-21** | N1 | 106 | 0.969 | -3.197 | 39.27 | 10 | 0 | 0 | 31.30 | 0.16 | NO | ASC, R |
| **2022-01-21** | N2 | 108 | 0.997 | -3.137 | 39.72 | 5 | 0 | 0 | 32.65 | 0.05 | YES | R |
| **2022-01-24** | N2 | 95 | 0.992 | -3.455 | 39.86 | 5 | 0 | 0 | 32.84 | 0.19 | YES | R |
| **2022-01-24** | N1 | 110 | 0.986 | -3.104 | 38.33 | 4 | 67 | 67 | 31.12 | 0.13 | YES | HC, R |
| **2022-01-28** | N1 | 105 | 0.960 | -3.209 | 36.85 | 10 | 0 | 0 | 29.99 | 0.15 | NO | ASC, R |
| **2022-01-28** | N2 | 104 | 0.987 | -3.236 | 39.54 | 4 | 0 | 0 | 32.72 | 0.18 | YES | R |
| **2022-01-31** | N1 | 108 | 0.990 | -3.153 | 39.54 | 4 | 0 | 0 | 31.68 | 0.12 | YES | R |
| **2022-01-31** | N2 | 98 | 0.992 | -3.363 | 39.39 | 5 | 0 | 0 | 31.84 | 0.01 | YES | R |
| **2022-02-02** | N2 | 99 | 0.995 | -3.337 | 39.72 | 5 | 0 | 0 | 32.36 | 0.14 | YES | R |
| **2022-02-02** | N1 | 95 | 0.990 | -3.451 | 39.94 | 4 | 0 | 0 | 31.35 | 0.14 | YES | R |
| **2022-02-07** | N1 | 99 | 0.986 | -3.345 | 38.49 | 5 | 0 | 0 | 30.63 | 0.18 | YES | R |
| **2022-02-07** | N2 | 106 | 0.989 | -3.180 | 39.51 | 5 | 0 | 0 | 32.21 | 0.12 | YES | R |
| **2022-02-09** | N2 | 99 | 0.987 | -3.353 | 39.87 | 4 | 0 | 0 | 32.06 | 0.09 | YES | R |
| **2022-02-09** | N1 | 96 | 0.993 | -3.429 | 40.68 | 4 | 0 | 0 | 32.04 | 0.17 | YES | R |
| **2022-02-11** | N2 | 108 | 0.993 | -3.136 | 39.55 | 5 | 0 | 0 | 32.29 | 0.22 | YES | R |
| **2022-02-11** | N1 | 104 | 0.998 | -3.230 | 39.21 | 4 | 0 | 0 | 31.07 | 0.21 | YES | R |
| **2022-02-11** | N2 | 106 | 0.996 | -3.189 | 37.48 |  | 0 | 0 | 30.04 | 0.16 | YES | R |
| **2022-02-14** | N2 | 96 | 0.995 | -3.435 | 39.43 |  | 0 | 0 | 31.54 | 0.11 | YES | R |
| **2022-02-14** | N1 | 103 | 0.981 | -3.245 | 36.91 |  | 0 | 0 | 29.61 | 0.26 | YES | R |
| **2022-02-14** | N1 | 88 | 0.905 | -3.634 | 39.94 | 10 | 0 | 0 | 31.25 | 0.24 | NO | ASC, R |
| **2022-02-16** | N1 | 98 | 0.987 | -3.366 | 40.46 | 5 | 0 | 0 | 33.03 | 0.12 | YES | R |
| **2022-02-16** | N2 | 93 | 0.993 | -3.490 | 40.07 | 5 | 17 | 0 | 33.02 | 0.28 | YES | R |
| **2022-02-18** | N2 | 90 | 0.990 | -3.580 | 39.77 | 5 | 8 | 0 | 32.53 | 0.20 | YES | R |
| **2022-02-18** | N1 | 110 | 0.992 | -3.113 | 39.87 | 5 | 0 | 0 | 32.95 | 0.36 | YES | R |
| **2022-02-23** | N2 | 109 | 0.995 | -3.121 | 38.29 | 4 | 0 | 0 | 32.34 | 0.14 | YES | R |
| **2022-02-23** | N1 | 107 | 0.993 | -3.169 | 40.06 | 5 | 8 | 0 | 32.41 | 0.06 | YES | R |
| **2022-02-25** | N2 | 96 | 0.990 | -3.421 | 40.05 | 4 | 11 | 0 | 33.28 | 0.27 | YES | R |
| **2022-02-25** | N1 | - | - | - | - | - | 0 | 0 | 32.11 | 0.08 | NO | NR |
| **2022-02-28** | N2 | 101 | 0.996 | -3.301 | 39.13 | 5 | 0 | 0 | 32.23 | 0.06 | YES | R |
| **2022-02-28** | N1 | 109 | 0.985 | -3.122 | 39.50 | 5 | 17 | 0 | 32.10 | 0.10 | YES | R |
| **2022-03-02** | N2 | 96 | 0.995 | -3.428 | 39.92 | 5 | 0 | 0 | 33.04 | 0.20 | YES | R |
| **2022-03-02** | N1 | 84 | 0.945 | -3.772 | 38.68 | 5 | 0 | 0 | 32.93 | 0.19 | NO | NR |

| PCR Date | Target | Efficiency (%) | $R^2$ | Slope | Y-intercept | No. standard curve points | % NTCs amplified | % NRTs amplified | $Cq_{+control}$ (Mean) | $Cq_{+control}$ (SDev.) | Plate passes QAQC | Plate qualifiers |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2022-03-04** | N2 | 105 | 0.995 | -3.216 | 40.08 | 4 | 0 | 0 | 32.54 | 0.12 | YES | R |
| **2022-03-04** | N1 | 109 | 0.981 | -3.134 | 39.15 | 5 | 8 | 0 | 32.13 | 0.15 | YES | R |
| **2022-03-07** | N2 | 103 | 0.991 | -3.259 | 38.48 | 5 | 0 | 0 | 31.63 | 0.03 | YES | R |
| **2022-03-07** | N1 | 91 | 0.948 | -3.547 | 39.90 | 10 | 11 | 0 | 32.13 | 0.11 | NO | ASC, R |
| **2022-03-09** | N2 | 107 | 0.988 | 3.156 | 38.93 | 5 | 0 | 0 | 32.80 | 0.22 | YES | R |
| **2022-03-09** | N1 | 106 | 0.991 | -3.187 | 39.27 | 5 | 17 | 0 | 32.36 | 0.24 | YES | R |
| **2022-03-11** | N2 | 108 | 0.988 | -3.140 | 39.46 | 5 | 0 | 0 | 33.45 | 0.08 | YES | R |
| **2022-03-11** | N1 | 110 | 0.991 | -3.112 | 39.91 | 5 | 11 | 0 | 32.73 | 0.19 | YES | R |
| **2022-03-14** | N2 | 97 | 0.981 | -3.390 | 40.00 | 4 | 0 | 0 | 32.38 | 0.12 | YES | R |
| **2022-03-14** | N1 | - | - | - | - | - | 0 | 0 | 32.46 | 0.26 | NO | ASC, R |
| **2022-03-16** | N2 | 104 | 0.998 | -3.224 | 38.40 | 5 | 0 | 0 | 31.62 | 0.04 | YES | R |
| **2022-03-16** | N1 | 98 | 0.985 | -3.384 | 39.18 | 5 | 0 | 0 | 31.79 | 0.04 | YES | R |
| **2022-03-18** | N2 | 108 | 0.991 | -3.155 | 38.91 | 5 | 0 | 0 | 32.59 | 0.19 | YES | R |
| **2022-03-18** | N1 | 90 | 0.937 | -3.583 | 40.58 | 10 | 0 | 0 | 32.12 | 0.21 | NO | ASC, R |
| **2022-03-21** | N2 | 99 | 0.995 | -3.341 | 40.26 | 5 | 0 | 0 | 33.63 | 0.02 | YES | R |
| **2022-03-21** | N1 | 72 | 0.921 | -4.252 | 43.08 | 10 | 17 | 0 | - | - | NO | ASC, R |
| **2022-03-23** | N2 | 98 | 0.996 | -3.377 | 39.62 | 5 | 0 | 0 | 32.78 | 0.14 | YES | R |
| **2022-03-23** | N1 | 109 | 0.983 | -3.129 | 39.91 | 6 | 0 | 0 | 32.83 | 0.13 | YES | R |
| **2022-03-25** | N1 | 102 | 0.983 | -3.271 | 39.45 | 5 | 0 | 0 | 32.72 | 0.15 | YES | R |
| **2022-03-25** | N2 | 93 | 0.993 | -3.490 | 39.65 | 5 | 8 | 0 | 32.15 | 0.19 | YES | R |
| **2022-03-28** | N2 | 103 | 0.994 | -3.252 | 39.84 | 5 | 0 | 0 | 33.18 | 0.15 | YES | R |
| **2022-03-28** | N1 | 110 | 0.984 | -3.113 | 39.99 | 5 | 0 | 0 | 32.83 | 0.34 | YES | R |
| **2022-03-30** | N2 | 98 | 0.991 | -3.374 | 40.58 | 5 | 0 | 0 | 33.60 | 0.25 | YES | R |
| **2022-03-30** | N1 | 99 | 0.982 | -3.344 | 40.28 | 6 | 0 | 0 | 32.15 | 0.23 | YES | R |
| **2022-04-01** | N2 | 110 | 0.993 | -3.110 | 39.89 | 5 | 0 | 0 | 33.43 | 0.10 | YES | R |
| **2022-04-01** | N1 | 110 | 0.981 | -3.107 | 40.06 | 7 | 0 | 0 | 32.59 | 0.27 | YES | R |
| **2022-04-04** | N2 | 97 | 0.997 | -3.409 | 39.55 | 5 | 0 | 0 | 32.42 | 0.08 | YES | R |
| **2022-04-04** | N1 | 91 | 0.980 | -3.562 | 40.14 | 6 | 0 | 0 | 32.43 | 0.25 | YES | R |
| **2022-04-08** | N2 | 95 | 0.996 | -3.440 | 39.57 | 5 | 0 | 0 | 32.52 | 0.20 | YES | R |
| **2022-04-08** | N1 | 96 | 0.980 | -3.425 | 39.82 | 7 | 0 | 0 | 32.40 | 0.42 | YES | R |
| **2022-04-11** | N2 | 104 | 0.989 | -3.241 | 39.74 | 4 | 0 | 0 | 32.88 | 0.24 | YES | R |
| **2022-04-11** | N1 | | | | | | 0 | 0 | | | NO | ASC, R |
| **2022-04-13** | N2 | 98 | 0.996 | -3.362 | 39.98 | 4 | 0 | 0 | 33.65 | 0.24 | YES | R |
| **2022-04-13** | N1 | 91 | 0.981 | -3.554 | 40.25 | 7 | 0 | 0 | 32.48 | 0.06 | YES | R |
| **2022-04-18** | N2 | 104 | 0.992 | -3.232 | 39.37 | 5 | 0 | 0 | 32.75 | 0.20 | YES | R |

| PCR Date | Target | Efficiency (%) | $R^2$ | Slope | Y-intercept | No. standard curve points | % NTCs amplified | % NRTs amplified | $Cq_{+control}$ (Mean) | $Cq_{+control}$ (SDev.) | Plate passes QAQC | Plate qualifiers |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2022-04-18** | N1 | 102 | 0.981 | -3.268 | 39.83 | 7 | 0 | 0 | 32.83 | 0.37 | YES | R |
| **2022-04-20** | N2 | 99 | 0.995 | -3.335 | 38.91 | 5 | 0 | 0 | 31.89 | 0.12 | YES | R |
| **2022-04-20** | N1 | 94 | 0.997 | -3.487 | 39.06 | 5 | 0 | 0 | 31.84 | 0.23 | YES | R |
| **2022-04-22** | N2 | 97 | 0.996 | -3.402 | 39.38 | 4 | 0 | 0 | 32.37 | 0.29 | YES | R |
| **2022-04-22** | N1 | 93 | 0.984 | -3.506 | 39.55 | 6 | 0 | 0 | 32.07 | 0.08 | YES | R |
| **2022-04-25** | N2 | 104 | 0.995 | -3.239 | 38.54 | 5 | 0 | 0 | 31.71 | 0.12 | YES | R |
| **2022-04-25** | N1 | 93 | 0.990 | -3.502 | 39.19 | 7 | 0 | 0 | 31.77 | 0.28 | YES | R |
| **2022-04-27** | N2 | 93 | 0.985 | -3.492 | 39.19 | 5 | 0 | 0 | 32.13 | 0.28 | YES | R |
| **2022-04-27** | N1 | 94 | 0.988 | -3.479 | 38.48 | 6 | 0 | 0 | 31.50 | 0.10 | YES | R |
| **2022-04-29** | N2 | 94 | 0.996 | -3.477 | 39.45 | 5 | 0 | 0 | 32.52 | 0.26 | YES | R |
| **2022-04-29** | N1 | 102 | 0.980 | -3.285 | 38.89 | 6 | 0 | 0 | 32.17 | 0.03 | YES | R |

ASC – An average standard curve from the last ten plates were used to quantify the target
LC – Low contamination; amplification of negative controls did not interfere with interpretation of results
HC – High contamination; amplification of negative controls confounded results
R – Qualitative results were reported
NR – No results were reported
RR – Assay was performed again (rerun)