Application of Passive Samplers for SARS-CoV-2 Wastewater Surveillance

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Abstract

SARS-CoV-2 wastewater surveillance is a promising tool for monitoring the spread of infection during pandemic outbreaks. 24-hour composite sampling of wastewater using autosamplers is the preferred means for wastewater surveillance sample collection. Autosamplers however require a significant capital cost and furthermore some sampling locations are not amenable to autosampler deployment because of a lack of space and lack of access to electricity. Grab sampling is an alternative to auto sampling for wastewater surveillance, however it may be less effective compared to 24-hour composite sampling due to the possibility to miss the collection of shed disease targets during critical shedding events. Torpedo-style passive samplers packed with medical gauze and tampon-style passive samplers are alternatives to grab sampling when deployment of autosamplers is not possible. Torpedo-style and tampon-style passive samplers are characterized as being easy to deploy and collect and have shown promise for disease surveillance using wastewater. Although passive samplers have shown the ability to detect SARS-CoV-2, they have not demonstrated the ability to quantify the viral load in the wastewater due to the fact that the collection of the liquid phase of the sampler is not consistent across the deployment period of a passive sampler. As SARS-CoV-2 disease targets have been shown to largely partition to the solids phase of wastewaters, it is hypothesized that mass fraction quantitation may enable passive samplers to quantify wastewater signals comparably to autosamplers. In this study, wastewater samples were collected from the same location over a period of three months from a sewer access point at the University of Ottawa using conventional 24-hour auto sampling. Two types of torpedo-style passive samplers and a tampon-style passive sampler were tested to assess whether passive sampler measurements of SARS-CoV-2 N1 and N2 gene targets can be used in the place of autosampler quantitated values.

When comparing the wastewater characteristics of centrifuged pellets collected by various passive samplers and a conventional autosampler, the results of this study showed that the torpedo-style passive sampler packed with two pieces of gauze (P2) collected significantly lower water content compared to the autosampler, and P2 collected significantly greater total solids and volatile solids compared to the autosampler. When measuring SARS-CoV-2 N1 and N2 signals, the results indicate that N1 and N2 gene region copy numbers from all of the samplers were not significantly distinct. However, the P2 sampler, a torpedo-style passive sampler packed with four pieces of gauze (P4), and the tampon-style passive sampler (T) captured a greater quantity of pepper mild

mottle virus (PMMoV) gene targets compared to the autosampler; where PMMoV is the most commonly measured fecal biomarker for wastewater surveillance of SARS-CoV-2. The greater quantity of PMMoV gene targets compared to the autosampler was likely due to proportionally higher total solids and volatile solids in the centrifuged pellet material captured. When N1 and N2 measurements were normalized against sample volume, pellet mass or PMMoV gene copy numbers, P2, P4, and T showed no significant differences compared to the autosampler. In contrast, differences were observed between passive samplers and the autosampler when PMMoV measurements were normalized against the matrix volumes or pellet mass. High statistical percentage differences were observed between all passive samplers and the autosampler. Overall, passive samplers are reliable, cost-effective devices for sampling disease targets in wastewater if results are expressed as copies/g or copies/copies PMMoV. These devices are feasible substitutes for autosamplers when detection and quantification of SARS-CoV-2 in wastewater are required. P2 passive samplers using units of measurement of copies/g are recommended for SARS-CoV-2 surveillance in the wastewater.

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List of Abbreviations

A	Autosampler
B2M	β-2 Microglobulin
BE	Beef Extract
CDC	Centers for Disease Control and Prevention
COSCa	COVID-19 Sewer Cage
COVID-19	Coronavirus Infectious Disease 2019
CrAssphage	Cross-Assembly Phage
Cts	Cycle thresholds
E	Envelope
EDDP	2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine
HCoV-NL63	Human Coronavirus NL63
HI-SCV-2	Heat-Inactivated SARS-CoV-2
HIV	Human Immunodeficiency Virus
HW Ratio	Hospitalization-to-Wastewater Ratio
LFB	Lateral Flow Biosensor
M	Membrane
MERS-CoV	Middle East respiratory syndrome coronavirus
MHV	Mouse Hepatitis Virus
N	Nuclocapsid
NGS	Next Generation Sequencing
P2	Passive Sampler with two pieces of medical gauze
P4	Passive Sampler with four pieces of medical gauze
PBS	Phosphate-Buffered Saline
PEG	Polyethylene Glycol
Phi6	Pseudomonas Phage
PMMoV	Pepper Mild Mottle Virus
PS	Primary Sludge
RdRp	RNA Polymerase
RT-ddPCR	Reverse Transcription Digital Droplet Polymerase Chain Reaction

RT-LAMP	Reverse Transcription Loop-Mediated Isothermal Amplification
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
S	Spike
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
T	Tampon
TS	Total Solid
TSS	Total Suspended Solid
TWAC	Time-Weighted Average Concentrations
WAS	Waste-Activated Sludge
WBE	Wastewater-based Epidemiology
WC ratio	Wastewater Viral Copy Numbers to Clinical Cases
WWS	Wastewater Surveillance
WWTP	Wastewater Treatment Plant
uOttawa	University of Ottawa
V2G	Volcano Second Generation
VOC	Variants of Concern
VS	Volatile Solid

Chapter 1 Introduction

1.1 Background

Surveillance of infectious diseases is essential to understand their occurrence in a population, understand trends and action community alerts and mandates by health authorities. Methods used for disease surveillance commonly rely on provider-initiated reports (passive surveillance) and health department-solicited reports (active surveillance)(Thacker et al., n.d.). However, event-based infectious surveillance methods may not record the occurrence of unknown cases, such as the Coronavirus Infectious Disease 2019 (COVID-19) pandemic (Mph et al., n.d.). Wastewater surveillance (WWS) as a type of environmental monitoring can be used to understand the characteristics of different infectious pathogens shed into wastewater by communities. WWS is a low-cost tool that has been used for tracking COVID-19 outbreaks in communities and subsequently compared to clinical testing. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the respiratory virus responsible for COVID-19 that is transmitted through respiratory droplets and aerosols. SARS-CoV-2 virus particles can be shed in sputum, saliva, urine, and feces (Hayes et al., 2021). By the time of writing this thesis (December 2022), SARS-CoV-2 had caused 600 million cases of COVID-19 and 6.6 million deaths (World Health Organization, 2022). Because body fluids are discharged into wastewater infrastructure through toilets, showers, washbasins and sinks, monitoring SARS-CoV-2 through wastewater surveillance has been implemented around the world to understand virus transmission and help governments communicate the incidence, prevalence and burden of the disease along with crafting public health policies. Studies show that 48% – 67% of people infected with COVID-19 had SARS-CoV-2 RNA in their stool, which can be shed into wastewater (Cheung et al., 2020). Therefore, it was reasonably assumed that SARS-CoV-2 titers measured in wastewater are on average proportional to the number of affected people within a catchment area (Rafiee et al., 2021). As such, with the broad global application of SARS-CoV-2 WWS, it was demonstrated that SARS-CoV-2 can be detected in wastewater samples before clinical cases are reported (Medema et al., 2020, D'Aoust et al., 2021, Wu et al., 2022), which identifies WWS as an early-warning system to reflect the real-time situation of the pandemic. In addition, wastewater data is understood to also provide a broad representation of community health, including asymptomatic or mildly symptomatic individuals who may have avoided and may now be non-eligible for clinical testing by using just a small number of samples. Therefore, data derived from WWS as a macro-scale surveillance system is

currently being combined with clinical data to understand the spread of the SARS-CoV-2 virus and COVID-19 infections in populations across the world and is being used to estimate the pandemic trends into the future.

Although SARS-CoV-2 WWS has been applied across the globe, several key challenges remain to be overcome to achieve highly sensitive, accurate, and reliable WWS measurements. SARS-CoV-2 surveillance in wastewater consists of several steps, including sample collection, the concentration of virus in the sample, qualitative and quantitative analyses, and data normalization. A key challenge in attaining high-quality wastewater data is that the quantitation of SARS-CoV-2 in wastewater is affected by differences in sampling time, sample volumes, and sampling frequency. Hence, establishing standardized sampling protocols and optimizing these protocols is essential for viral analysis and comparisons among monitoring locations. Methods used in recent studies to capture SARS-CoV-2 have focused on grab, composite, and automatic sampling. Grab sampling is the collection of a single volume of sample or a series of samples across a specified period of time and is used because of its convenience and low cost. Considering the inherent variability of wastewater flow and fecal shedding rates, 24 h composite samples are more representative of material flow in sewer networks compared to grab samples (Rafiee et al., 2021). 24 h composite wastewater samples are usually obtained using autosamplers, which are equipment operated for the collection of incremental sample volumes of wastewater at specified frequencies, usually operated across a period of 24 h when collecting for WWS initiatives. Although autosamplers operating for 24 h periods are the convention for WWS, their installation could be constrained by the high capital costs of the autosamplers themselves, lack of space to house the autosampler, lack of personnel to deploy or maintain the autosampler, lack of access to electricity to operate the autosampler or cold conditions that prevent the use of batteries. Hence, autosampler applications are limited to specific geo-locations and communities (Rafiee et al., 2021). Passive samplers are simple, alternative devices for sampling that can calculate the mean concentration of a virus in sewage and can be deployed to reveal changes in viral load over time, even for low-level disease outbreaks (Kevill et al., 2022). However, the effectiveness of passive samplers in comparison to other types of collection systems has not been fully explored. Furthermore, although passive samplers have shown the ability to detect SARS-CoV-2 and detect changes in the SARS-CoV-2 signal, they have not demonstrated the ability to directly quantitate the viral concentration in the wastewaters as these samplers collect the liquid phase of sampler inconsistently across the

deployment period of the sampler (J. Li et al., 2021, Wilson et al., 2022). Where passive samplers are believed to experience higher sorption of liquid during the early period of deployment and lower sorption of liquid during the latter period of deployment. As SARS-CoV-2 disease targets have been shown to largely partition to the solids phase of wastewaters, likely the fecal matter of wastewaters, it remains unknown if the quantitated viral target collected from passive samples quantitated using units of mass fractions or mass fraction units normalized with a fecal biomarker are comparable to quantitated 24 h autosampler collected samples (Ai et al., 2021, Kitamura et al., 2021). Therefore, a comparison of mass fraction viral signal measurements and mass fraction measurements normalized with a common fecal biomarker used for wastewater surveillance of SARS-CoV-2, Pepper Mild Mottle Virus (PMMoV), from various passive sampler types and autosamplers is required to understand the full extent of passive sampler utilization for SARS-CoV-2 and other solids partitioning viruses in wastewaters.

1.2 Aim of Study

The overall purpose of this study is to assess if SARS-CoV-2 viral measurements performed on wastewater samples collected by various types of passive samplers and a 24 h autosampler are comparable when applying mass fraction (copies of SARS-CoV-2/g of solids) and biomarker normalized mass fraction measurements (copies of SARS-CoV-2/copies of PMMoV). Although studies have reported the successful detection of SARS-CoV-2 when using passive samplers, systematic quantification, normalization, and comparisons between passive samplers and 24 h autosamplers remain lacking. PMMoV is the most commonly applied biomarker to date for SARS-CoV-2 measurements in wastewaters and was used in this thesis to normalize the SARS-CoV-2. The specific objectives of this study are as follows:

1. Characterize and compare wastewater characteristics between samples collected by various passive samplers and a conventional autosampler deployed to collect across 24 h.
2. Determine similarities and distinctions between SARS-CoV-2 and PMMoV measurements between samples collected by passive samplers and a conventional autosampler deployed to collect across 24 h.
3. Identify units of measurement and normalization methods to improve the comparability of passive sampler measurements with autosampler measurements.

4. Assess the effect of wastewater characteristics, specifically solids characteristics, on the SARS-CoV-2 signal and PMMoV signal from autosampler samplers and passive sampler samples.

1.3 Thesis Organization

This thesis is comprised of five chapters. Chapter 1 – Introduction presents background information on the significance of this research and the objectives of this study. Chapter 2 – Literature Review presents a literature review including a basic introduction to the SARS-CoV-2 virus, the history of wastewater surveillance and SARS-CoV-2 surveillance in wastewater, a review of factors that affect sampling efficiency, sampling methods employed for SARS-CoV-2 surveillance in wastewater, experimental protocols used for SARS-CoV-2 measurement in wastewaters, and the correlation between SARS-CoV-2 wastewater measurements and clinical data. Chapter 3 – Materials and Methods describes the sampling location, sampling time, experimental design, extraction method, and SARS-CoV-2 and PMMoV quantification methods used in this thesis. In Chapter 4 – Results and Discussion, the similarities and distinctions between various types of samplers and the characteristics of the wastewater samples they collected are presented and discussed. The effects of wastewater characteristics on the measurement of SARS-CoV-2 and PMMoV signals are examined. Particular attention is given to similarities and distinctions of SARS-CoV-2 and PMMoV data between passive samplers and autosamplers collecting samples across a period of 24 h when using different units of measurement. Finally, Chapter 5 – Conclusion states the conclusions and limitations of this study and offers suggestions for future work.

Chapter 2 Literature Review

2.1 SARS-CoV-2

Coronaviruses are a group of RNA viruses which could cause respiratory infections in mammals and animals. The coronavirus genome is comprised of a single-stranded RNA with genomes ranging in size from 26Kb to 32Kb (Yang et al., 2020). Six coronavirus strains have been detected since the 1960s, and three previously undefined coronaviruses emerged in the twenty-first century: severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003, Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 and SARS-CoV-2 in late December 2019. SARS-CoV, SARS-CoV-2 and MERS-CoV are all highly transmissible strains through the airways (Hamouda et al., 2021a). SARS-CoV-2 showed similarity to four endemic human coronaviruses that can be transmitted through the upper respiratory tracts, including human coronavirus NL63 (HCoV-NL63), HCoV-229E, HCoV-OC43 and HCoV-HKU1 (Lamers & Haagmans, 2022, Holmes et al., 2021). SARS-CoV-2 is a respiratory virus with an RNA genome belonging to the *Betacoronavirus* of the family *Coronaviridae* (Dong et al., 2020), which causes COVID-19 disease. SARS-CoV-2 is a highly transmissible and pathogenic coronavirus that causes symptoms including fever or chills, dry cough, fatigue, myalgia, headache, temporary loss of the sense of smell and taste, rhinorrhea, nausea or vomiting, and in severe cases, dyspnea (Ahmed et al., 2022, Hu et al., 2020, Hamouda et al., 2021b). Since the COVID-19 pandemic, there have been a total of 600 million cases of COVID-19 in at least 170 countries and territories, and the infected people were among all age groups (World Health Organization, 2022). However, the majority of confirmed cases were aged 30-79 years old, and most deaths occurred among patients over 60 years of age. High fatality rates were found for patients over 80 years (Tu et al., 2020). Up to the present, the main transmission route of SARS-CoV-2 is through respiratory aerosols, with the virus being transmitted through aerosol droplets. Studies also detected SARS-CoV-2 in the feces of patients who were confirmed to be infected, indicating that the virus can also exist and replicate in the digestive tract (Holshue et al., 2020).

2.2 History of WWS

2.2.1 WWS

Diseases caused by infectious pathogens are a threat to public health. WWS under the area of study of wastewater-based epidemiology (WBE) has been used to strengthen the surveillance of

pathogens and stop the transmission of infectious diseases. Many technologies have been used to monitor disease transmissions, such as clinical-based surveillance, questionnaire-based surveys, and clinical data from hospitals (Mao et al., 2020). However, these methods usually need large data and are usually limited by resources or high costs. WWS is an economical surveillance system that has been used to track disease targets in wastewater that overcomes the need to do numerous tests on individuals in large populations. Additionally, wastewater data have been shown to be characterized by low temporal representativeness and high spatial variability. In the last twenty years, WWS has been used to monitor various virus outbreaks globally, such as the Ebola virus in 2000, SARS-CoV in 2003, swine flu (Influenza A virus subtype H1N1) in 2009, MERS-CoV in 2012, Zika virus in 2015, Nipah virus in 2018, and SARS-CoV-2 in 2019 (Hamouda et al., 2021b, Mao et al., 2020). Apart from disease surveillance, WWS has also been used to monitor a number of pharmaceuticals (atenolol, citalopram, carbamazepine, oxazepam, metoprolol, 2-ethylidene-1,5-dimethyl-3, 3-diphenylpyrrolidine (EDDP) and morphine), illicit drugs (heroin, cocaine, ketamine, benzoylecgonine and methamphetamine), pesticides, and heavy metals (Cd, Co, Cu, Fe, Ni, Mn, Pb, Zn) (Vrana et al., 2005, Baz-Lomba et al., 2017).

WWS is a comprehensive technique that entails sample collection, storage, concentration, and viral genome extraction, followed by data analysis and quantification. To acquire accurate results, several challenges still need to be overcome. Endogenous and exogenous disease targets in wastewater could reflect the health conditions of people in an area in real time. However, once excreted in wastewater infrastructure, these targets become subject to external conditions and threats from the wastewater matrix that convolute their detection and quantification. Since wastewater is a complex matrix containing a wide range of chemical and biological substances, finding suitable and stabilized targets for specific diseases and suitable fecal or excreted biomarkers to normalize their quantity and ultimately successfully quantify the disease targets is challenging. The most common method used for pathogen quantification in wastewater is polymerase chain reaction (PCR). However, a series of factors in wastewater could cause PCR inhibition, such as humic acid, urea, heme, lactoferrin, collagen, melanin, indigo dye, tannic acid, and calcium ions (Geng & Mathies, 2015, Mao et al., 2020). Selecting suitable extraction methods can be used to minimize the PCR inhibition occurring in WWS.

2.2.2 SARS-CoV-2 WWS

SARS-CoV-2 is spread primarily by respiratory aerosols through close human interaction. However, studies found that SARS-CoV-2 can also infect gastrointestinal cells, which suggests that it could be excreted in feces into wastewater infrastructures. The first successful SARS-CoV-2 WWS detection of SARS-CoV-2 RNA in municipal wastewater was achieved in the Netherlands in February 2020 (Medema et al., 2020). To better understand the surveillance process of SARS-CoV-2 RNA, several factors have been studied that could affect the survival of the excreted disease target in wastewater, such as temperature, pH (Xagoraraki et al., 2014), suspended solids (Gundy et al., 2009), and the presence of other microorganisms. Temperature is one of the most significant factors and has been studied for its effect on viral RNA survival. In studies comparing SARS-CoV-2 to non-enveloped viruses based on sorption ability, SARS-CoV-2 exhibited affinity to wastewater particulates compared to non-enveloped viruses such as norovirus (Hokajärvi et al., 2021). As SARS-CoV-2 WWS has been applied as a long-term endeavour, the decay of stored viral RNA could affect measurements. The half-life of SARS-CoV-2 in repiratory sections in wet samples and air-dried sample at room temperature was 5 and 2.93 days, respectively. The half-life of SARS-CoV-2 in air-dried sample at 4℃ was 11.4 days (Guang & Hui, 2023). Therefore, maintaining proper storage conditions is essential to ensure the accuracy of SARS-CoV-2 wastewater measurements. Freezing and thawing of SARS-CoV-2 RNA samples causes degradation (Alygizakis et al., 2021).

2.2.3 Variants of Concern

SARS-CoV-2 RNA has mutated to more transmissible variants, termed variants of concern (VOC), and caused threats to public health, such as spreading more easily and decreasing the effectiveness of vaccines (Nasreen et al., n.d.). The main variants that have emerged now include B.1.1.7 (Alpha) in Britain, B.1.351 (Beta) in South Africa, B.1.617.2 (Delta), P.1 (Gamma) in Brazil, P.3 in the Philippines, AP.1 in Wales, B.1.616 in France, and B.1.1.529 (Omicron) (Boehm et al., 2021). WWS has also been used to detect SARS-CoV-2 variants B.1.1.7 (Canada) (Graber et al., 2021), P.1 (Brazil, Manaus), B.1.1.529 (Omicron), B.1.429 (USA, California), B.1.526 (USA, New York), A.23.1(Uganda), and B.1.525 (Unknown origin) (Bar-Or et al., 2021, Kirby et al., n.d.). SARS-CoV-2 variants genomes can be detected via next-generation sequencing (NGS) systems. Ai et al. (2021) quantified SARS-CoV-2 N1, N2 and E genes from influent samples and found that SARS-CoV-2 gene targets in wastewater were strongly correlated with COVID-19 cases (Ai et al., 2021).

2.3 Factors Affecting the Sampling Process

2.3.1 Sampling Location

Different sampling sites may be selected when monitoring SARS-CoV-2 in wastewater, such as buildings (e.g., long-term care facilities, hospitals, university campuses), selected sewersheds that isolate specific neighbourhoods (via sampling of specific sewer access points or pumping stations), and wastewater treatment plants. While monitoring SARS-CoV-2 RNA in the influent of wastewater treatment plants (WWTPs) enables a large population to be diagnosed with a single sample, surveillance at WWTPs lacks the geographic specificity of surveillance within sewer sheds and at specific buildings (Bivins et al., 2021). Therefore, identifying a suitable study site is essential to ensuring appropriate and accurate virus detection.

2.3.2 Partitioning of Disease Target in Wastewater Matrix

Although several studies proved that SARS-CoV-2 could be detected in liquid and solid fractions, it is believed that SARS-CoV-2 RNA can preferentially be detected in the solids portion of wastewater (Alamin et al., 2022). SARS-CoV-2 in settled solids occurs, at minimum, between 350 and 3100-fold higher concentration than in influent on a per mass basis and at between ~100 and ~1000 times concentration in primary solids than in influent (Graham et al., 2021). In addition, SARS-CoV-2 frequency of detection and copy numbers in pellets were higher than for influent (D'Aoust et al., 2021, Hokajärvi et al., 2021). Solids analysis also avoids the pre-concentration step that is needed for influent analysis (Graham et al., 2021). Therefore, isolating the solids from wastewater samples and analyzing the solids portion of the wastewater matrix may yield more sensitive results than testing wastewater influent without isolating the solids portion or analyzing the liquid portion of wastewater. Apart from influent samples, sludge samples are also used for SARS-CoV-2 WWS. In wastewater treatment plants, two types of sludge are generally formed: primary sludge and secondary sludge. Settled solids collected from primary sewage sludge (PS) has been widely chosen for analysis because it contains high concentrations of solids and a broad range of human viruses without the addition of aeration and retention for microbial degradation of the organic matter in the wastewater (Peccia et al., 2020). PS is mainly settled solids from the influent wastewater and is approximately 1 – 2% solids by weight compared to influent wastewater that is approximately 0.01 to 0.02% solids by weight. It has been shown that SARS-CoV-2 copy numbers for PS are higher than those for influent (Bilge Alpaslan Kocamemi et al., 2020).

2.3.3 Deployment Time of Samplers

The deployment time currently used for different types of wastewater samplers ranges from 4 – 96 h, with 24 h commonly used for WWS. Deployment time for different samplers can be determined according to the material used because the saturation time varies for different types of materials. Establishing an optimal deployment time for sampling is essential as deployment times affect the quantity of collected viral genomic copies. For example, as expected, when using Zetapor and Nylon membranes to detect SARS-CoV-2 in influent wastewaters, concentrations of SARS-CoV-2 virus collected for 24 h samples were shown to be higher than for samples collected for 4 h (Vincent-Hubert et al., 2022). Study shows that the SARS-CoV-2 concentration in gauze reaches saturation more quickly than for membranes and cotton buds (Habtewold et al., 2022). Besides, excessive exposure time could inhibit virus absorption in the wastewater. The effective sampling time for tampons and swabs is about 8 h. However, the PMMoV virus concentrations in gauze decrease sharply after 8 h, potentially due to the extended exposure time, which could increase the inhibition and reduce the ability to detect the virus (J. Li et al., 2022). However, similar cycle thresholds (Cts) between autosamplers and passive samplers when detecting the SARS-CoV-2 virus have been observed when the deployment times of the two types of samples are different. The sampling time for passive samplers varies from 48 –144 h, while it is only 24 h for autosamplers, indicating that increasing sampling time may not enhance virus detection (Wilson et al., 2022). Overall, although various packing materials of passive samplers showed different saturation time and different viral absorbance efficiency, and an optimal sampling time may exist for various sampler types, they may not affect the comparison between passive samplers and autosamplers. Hence, wastewater data are commonly related to clinical data, therefore 24 h deployment of samplers could strengthen the relation between these metrics.

2.4 Sampling Method

2.4.1 Automatic Sampling

Autosamplers are commonly used for WWS and are commonly deployed for 24 h periods to collect discriminant samples over incremental time periods, with the discriminant samples being mixed into a single composite sample for the set 24 h period. There are two kinds of autosampler collection methods: time-proportional and flow-proportional. Time-proportional autosampling collects fixed-volume samples at defined time intervals. In contrast, flow-proportional

autosampling collects variable volume samples in proportion to the wastewater flow rate at fixed time intervals or constant volume samples in proportion to the wastewater flow rate at varying time intervals. Many studies proved that autosamplers successfully detected and quantified SARS-CoV-2 in wastewater (Acosta et al., 2021, D'Aoust, Mercier,et al., 2021, D'Aoust, Towhid, et al., 2021, Gerrity et al., 2021, Wilson et al., 2022, Ma et al., 2022). Autosamplers are able to collect 24 h composite samples, either time-proportional or flow-proportional, which could catch daily shedding by a population. In addition, autosamplers collect samples across diurnal variations of wastewater flow and flux and therefore are more representative than grab samples for example. However, autosamplers can be constrained by (1) high capital costs, (2) the need for adequate space to be positioned and installed, (3) necessity for maintenance and resources, (4) the need for access to a power supply, especially in cold conditions, and (5) non-applicablility for very low wastewater flow conditions (Schang et al., 2021).

2.4.2 Grab Sampling

Grab sampling is a simple technique that involves filling a container with wastewater at one point in time. As such, grab sampling provides a "snapshot" measurement at a specific time (Schang et al., 2021). It is easy to deploy and use, requires low-technology equipment and is low-cost. However, it can report false negatives by missing the shedding events of individuals throughout time periods that are not samples. It also does not accurately nor proportionally attribute measurement to the entire population that contribute to the wastewater over the period of the day. Although grab sampling only presents the concentration in the water at the time of sampling, several studies observed good agreement between the SARS-CoV-2 RNA concentration of grab samples and 24-hour composite samples, which indicates grab sampling may be appropriate under certain conditions (Curtis et al., n.d., George et al., 2022, Augusto et al., 2022). To address the issues of grab samples only representing a single time, "snapshot" measurement, a series of grab samples can be collected multiple times a day and the grab samples can be combined into a composite sample. Composite sampling is generally more representative than grab sampling because it collects individual samples at defined time intervals (Liu et al., n.d.). However, its usefulness can also be limited due to long sampling periods, which is time-consuming, costly, and may increase safety risks.

2.4.3 Passive Sampling

Passive sampling is defined broadly as a sampling device that traps or retains analyte molecules by passive samplers from a medium (Vrana et al., 2005). Passive sampling has shown promise as a tool for measuring a wide range of pollutants since 1987. For example, passive samplers have been used to characterize organic contaminants, such as pharmaceuticals (Cristóvão et al., 2021), pesticides, etc., as particulate, dissolved and colloidal phases in the water column. Passive samplers can also be applied to detect metals such as Cd, Cu, Ni, Pb, and Zn species in the aquatic environment (Persson et al., 2001). The Moore swab method is an early form of passive sampler that uses a strip of gauze tied to a string and submerged in flowing water (Liu et al., n.d.). It was first used to trace Salmonella Paratyphi B from effluent sewage by Brendan Moore in 1948 and then used to collect microorganisms such as fecal-borne pathogens, poliovirus, and human norovirus in bodies of water. Recently, Moore swab as a method of passive sampling has been successfully validated for detecting SARS-CoV-2 in wastewater. Additionally, it was combined with RT-qPCR and it exhibited more sensitivity than grab sampling when monitoring SARS-CoV-2 RNA from a hospital with COVID-19 patients (Liu et al., n.d.). This method is more rapid and efficient as an early warning tool, and it can be deployed easily and performs well when SARS-CoV-2 is highly diluted in the sewershed. Also, the Moore swab method is cheaper than automatic and composite sampling methods, and its material can be purchased easily. However, the Moore swab method has disadvantages. It may not provide a positive result under the circumstances in which the SARS-CoV-2 was highly diluted in wastewater, and analysis based on normalization units of measurement is still limited.

Passive sampler devices have since improved upon the Moore swab sampler and have been designed to reduce ragging rates and increase the interaction between wastewater flow and adsorbent materials. Ragging is the solid materials accumulation in wastewater, such as food scraps, toilet tissues, and other santinary products. Also, devices are designed to prevent the material that is captured from being lost or destroyed by wastewater flow in the sewer line. Schang et al. (2021) used four designs of passive sampler units (colander, boat, matchbox, and torpedo) and found that the boat-style unit experienced the most significant ragging, followed by the matchbox-style unit. The colander-style unit was limited for sewage treatment plants because of its large size, and only the torpedo-style unit showed very little ragging. A study group at Monash University first designed a torpedo-style 3D-printed passive sampler holder, and many studies

(Wilson et al., 2022, Li et al., 2022) since then have used this device successfully to detect SARA-CoV-2 in wastewater. There are other types of passive sampling devices, such as the COVID-19 Sewer Cage (COSCa), which is a 10 cm diameter hollow sphere that minimizes over-saturation of the absorbent by solids and prevents the loss or damage of the adsorbent material as well (Hayes et al., 2021). At the same time, COSCa enhances virus detection sensitivity during low COVID-19 prevalence. Aside from COSCa, autoclaved stainless steel wire sieves with cotton gauze have also been used, proving that Moore swabs perform equally well compared to composite samples for monitoring SARS-CoV-2 (Rafiee et al., 2021).

2.4.3.1 Materials Used for Passive Sampling

Although passive samplers have been used for SARS-CoV-2 monitoring, much is yet to be understood with respect to different sampling materials. Various materials used in passive samplers have been employed to monitor SARS-CoV-2 in wastewater, such as electronegative membrane filters (Hayes et al., 2021, Schang et al., 2021, Habtewold et al., 2022, Liu et al., n.d., Wilson et al., 2022), cotton buds (Hayes et al., 2021, Habtewold et al., 2022), cheesecloth (Hayes et al., 2021), cellulose sponge (Hayes et al., 2021), tampons (Bivins et al., 2021, Kevill et al., 2022, Liu et al., n.d., Wilson et al., 2022), and medical gauze (Habtewold et al., 2022, Rafiee et al., 2021, Mangwana et al., 2022). The effectiveness of detecting SARS-CoV-2 in wastewater might vary according to the material that is used within the passive sampler. For example, cotton buds show lower positivity when monitoring for SARS-CoV-2 compared to other materials, such as gauze and membranes, and gauze had quicker saturation than membranes and cotton buds (Schang et al., 2021, Habtewold et al., 2022). Additionally, when comparing Ct values for grab sampling, grab-composite sampling, and passive sampling, gauze samples perform similarly to grab-composite samples and outperform grab samples, which indicates that cotton gauze might enhance the trapping of SARS-CoV-2 (Rafiee et al., 2021). Both gauze and tampon materials were sufficient to detect SARS-CoV-2 in the wastewater, when comparing Ct values for gauze and tampons, gauze showed a higher Ct value which may be due to the lower sorption capacity of gauze (Wilson et al., 2022).

2.4.3.2 Gaps of Knowledge with Respect to Passive Sampling

The main limitation of passive samplers is that the sampling volume collected by passive sampler during their deployment is unknown and sorption of liquids and collection of solids may not be steady across the deployment period of the passive samplers; with greater sorption occurring during the early deployment period and less sorption occurring during the latter period of deployment. Therefore, data from passive samplers cannot be expressed as concentrations (mass normalized per sample volume collected) and in turn cannot be compared to autosampler data that is most common for wastewater surveillance across and globe, as autosampler data is commonly expressed in concentration units. Although passive sampling has been validated for monitoring SARS-CoV-2 in many studies, its use in quantitating SARS-CoV-2 viral RNA in wastewater still needs to be understood and understood with respect to different units and normalization with fecal biomarkers. Therefore, further research on passive sampling and comparing the performance of various passive samplers to autosamplers is critical for furthering the science of SARS-CoV-2 surveillance.

2.5 RNA Extraction and Quantification

2.5.1 Concentration and Nucleic Acid Extraction

Viral concentrations and extraction methods affect the efficiency of viral recovery. Methods currently used for concentrating viruses include absorption-extraction, polyethylene glycol precipitation (PEG), direct flocculation, ultrafiltration, and centrifugation (Alygizakis et al., 2021). Although these methods successfully recover non-enveloped enteric viruses in wastewater, such as polioviruses, adenoviruses, noroviruses, and enteroviruses, it is unknown whether they can be used for SARS-CoV-2 surveillance. Ahmed et al. (2022) compared several concentration methods for SARS-CoV-2 surveillance in wastewater and found that the absorption-extraction method could provide rapid and straightforward recovery of SARS-CoV-2, while an electronegative membrane with $MgCl_2$ added prior to filtration showed the best recovery efficiency (Ahmed et al., 2022b). In addition, to select the most efficient recovery method, Kevill et al. (2022) compared four recovery methods for obtaining SARS-CoV-2 from passive samplers and found that elution of the virus via the polyethylene glycol (PEG) precipitation had the highest recovery efficiency, followed by direct nucleic acid extraction, beef extract (BE)-PEG precipitation, and finally the phosphate-buffered saline (PBS)-PEG precipitation. Centrifugation was commonly used for

SARS-CoV-2 analysis, which could precipitate and concentrate solids from liquid samples, and it was found to be more sensitive than filtration (Rafiee et al., 2021, Kevill et al., 2022).

2.5.2 Molecular Assays

PCR assays have been used widely for quantifying SARS-CoV-2 in wastewater because of their high sensitivity and specificity for SARS-CoV-2 detection. Therefore, understanding different assays and targets is essential. Commonly used gene targets for PCR analysis include replicase (ORF1a/ORF1b), nucleocapsid (N), envelope (E), spike (S), membrane (M), and RNA polymerase (RdRp) (Hu et al., 2020). The E-gene and N-gene regions were first used in a PCR assay, followed by RdRp and ORF1ab gene regions for SARS-CoV-2 detection in wastewater (Alygizakis et al., 2021). ORF1ab, N1, N2, and N3 gene regions were used by Rafiee et al. (2021) to detect SARS-CoV-2 in wastewater, and their results showed that the copy numbers for the assay targeting the N gene regions were higher than those for the ORF1ab gene regions, which supports the hypothesis that the N assay is more sensitive than the ORF1ab assay (Rafiee et al., 2021). Similar results were observed when comparing the N gene regions to the E gene regions. The N2 gene region showed lower Ct values compared to the N1 gene and the E gene regions, which shows that N gene regions are more sensitive than E gene regions for SARS-CoV-2 detection (Hamouda et al., 2021b).

Commonly used PCR quantification methods for monitoring SARS-CoV-2 include the reverse transcription quantitative PCR (RT-qPCR), reverse transcription digital PCR (RT-dPCR) and reverse transcription digital droplet PCR (RT-ddPCR). Each quantification method has its advantages and disadvantages. RT-qPCR employs antisense DNA primers and probes to amplify RNA because RNA cannot be amplified directly unless it is converted into a DNA form. Once in the form of cDNA, fluorescently-labelled probes and unlabeled primers can be used to amplify the DNA using a real-time PCR instrument (Hamouda et al., 2021b). However, RT-dPCR and RT-ddPCR may be undesirable because of their high cost. RT-dPCR is costly ($18 per sample), and sample turnaround time is longer (7 – 9 h to 3 – 4 h) per sample than RT-qPCR ($6 per sample) (Ma et al., 2022).

2.6 Normalization

Over the past few years, several viruses have been detected in wastewater, such as noroviruses, adenoviruses, polyomaviruses, and enteroviruses (Bivins et al., 2021). These viruses are normally

tracked indirectly via fecal indicators in wastewater. Significant variation in fecal viral load shedding could affect SARS-CoV-2 surveillance. Therefore, normalization needs to be applied to diminish sewershed variability. Several types of viruses were used to standardize the SARS-CoV-2 signal, such as cross-assembly phage (crAssphage), β-2 microglobulin (B2M), and PMMoV. CrAssphage is a gut-associated bacteriophage of Bacteroides with a double-stranded DNA genome. Studies showed that although the shedding rate of crAssphage per person is different, crAssphage-based normalization still can be applied because crAssphage load was consistent over time and space in large populations (Langeveld et al., 2023). B2M as a human protein-coding gene has also been used for normalizing SARS-CoV-2 in wastewater. B2M was first used as a biological marker for people infected with human immunodeficiency virus (HIV) or cancers. With the emergence of COVID-19, B2M was found to be shed from people into wastewater and then used to normalize SARS-CoV-2 in the wastewater (Zhan et al., 2022). PMMoV is a plant virus belonging to the Tobamovirus in the family Virgoviridae, and it is found in peppers and their processed products (Kitajima et al., 2018). In addition, it is a non-enveloped virus with a positive-sense, single-stranded RNA genome. As people digest vegetables that contain PMMoV, PMMoV can be detected in feces excreted by humans in the wastewater (Kitajima et al., 2018). PMMoV has been commonly used to normalize SARS-CoV-2 due to its high persistence in water compared to other fecal indicators (Greaves et al., 2020). Other studies also proved that PMMoV was widely used because it did not vary substantially across samples or plants and is found extensively in human feces but rarely in animal feces in wastewater (Kitajima et al., 2018, Graham et al., 2021). Studies also proved that SARS-CoV-2 normalizing by PMMoV could adjust the variation of viral RNA recovery across samples and adjust the fecal strength difference in wastewater (Wolfe et al., 2021). Additionally, the study also showed that normalizing by PMMoV could serve to correct for SARS-CoV-2 RNA degradation during sample storage (Simpson et al., 2021).

2.7 Correlation with Clinical Cases

Studies on the correlation between SARS-CoV-2 concentrations in wastewater and clinical cases are essential because they can be used to estimate COVID-19 case numbers, including asymptomatic patients, without the need for numerous clinical tests. Wastewater results can be used to confirm the trends and prevent the spread of COVID-19. Many studies have proved that SARS-CoV-2 can be detected before clinical cases are reported, and the lead days range from 0-

16 days (Medema et al., 2020, D'Aoust, Graber, et al., 2021, Xiao et al., 2022). Therefore, understanding the correlation between wastewater data and clinical case data is essential to estimate epidemic trends. Many parameters could affect the correlation between SARS-CoV-2 RNA concentration and clinical case numbers, such as the sampling method (X. Li et al., 2023). The wastewater viral copy numbers to clinical cases (WC ratio) as a quantitative control has been proposed to detect differences between wastewater data and clinical data (Xiao et al., 2022). A high WC ratio could indicate a situation whereby viral copy numbers are increasing but the clinical cases are not rising proportionally, or wastewater viral copy numbers are relatively stable but the clinical testing is decreasing. At the same time, a low WC ratio could indicate that clinical testing has captured the majority of infected people rather than wastewater surveillance. Therefore, changes in the WC ratio could reflect changes in clinical testing strategy or the onset of VOC when determining a public health response (D'Aoust et al., 2022). Longitudinal wastewater data of SARS-CoV-2 and daily confirmed case data have been evaluated to combine traditional clinical surveillance, predict trends, and track the effects of vaccination. Through meta-analysis, SARS-CoV-2 concentrations were found to correlate better with new cases (either daily new, weekly new, or future cases) than with active or cumulative cases (X. Li et al., 2023). However, other studies indicated that the variation in SARS-CoV-2 RNA concentrations is not proportional to the variation in clinical cases or incidence because wastewater is a complex matrix (Ai et al., 2021). To overcome variations in viral shedding and wastewater flow, a rolling average of confirmed cases can be employed instead of raw case numbers, which improves the correlation between wastewater data and clinical data (Ai et al., 2021, Zheng et al., 2022). A study comparing PMMoV and B2M for normalizing SARS-CoV-2 found that both indicators improve the correlation between COVID-19 cases and wastewater data when using volcano second generation (V2G)-qPCR chemistry (Zhan et al., 2022). Based on the WC ratio, a hospitalization-to-wastewater (HW) ratio has been proposed to evaluate the relationship between SARS-CoV-2 measurements and public health metrics during clinical testing is limited. The results showed that during periods with limited vaccination, wastewater is a strong predictor and indicator of disease incidence and burden, while during periods with peak vaccination, wastewater is a strong indicator for disease burden (Hegazy et al., 2022).

Chapter 3 Materials and Methods

This chapter describes the methodology that was used in this study, including the experimental design. It consists of the study site, sampling methods, laboratory analysis, and statistical analysis description.

3.1 Sampling Location

This sampling location was located within the City of Ottawa sewershed located at a Northern site of the University of Ottawa, Canada. This total student population residing on campus is around 40000 people. Sampling frequency at this location was conducted twice a week from July 1st to September 15th, 2021. Eighty-three wastewater samples were collected using an autosampler and three types of passive samplers: a torpedo-style passive sampler packed with two pieces of gauze (P2), a torpedo-style passive sampler packed with four gauzes (P4), and a tampon-style passive sampler (T). Samples were immediately transported to the laboratory for analysis after collection, where they underwent nucleic acid extraction and RT-PCR on the same day. During this study, samples were not collected on two days (A), three days (P4), and one day (T). The missing samples were due to blockage of the autosampler, a loss of the passive sampler into the sewer or insufficient/shallow wastewater flow.

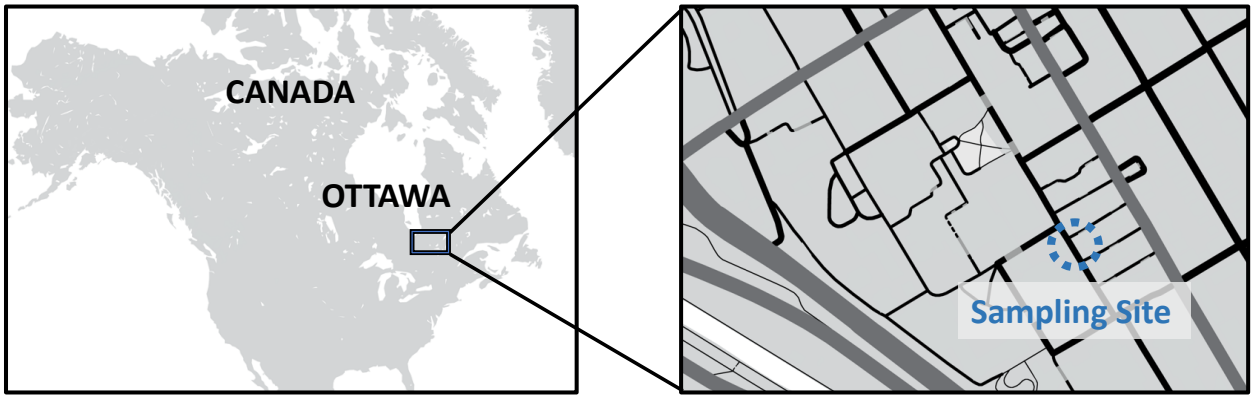


Figure 3.1: Sampling location at a Northern site (Colonel by building) of the University of Ottawa, Ottawa, Canada.

3.2 Sampling Plan

3.2.1 Autosampler

An ISCO 6700 series automatic sampler (Teledyne ISCO, Lincoln, NE, USA) was used in this study to collect 24 h time-weighted wastewater samples (4000 mL in total) twice a week. The

autosampler was located at a bus station on the northern site of the campus, where the sampling tube with a weighted sonde was fed through a pre-existing hole in a maintenance hole cover and placed into flowing wastewater. Four litres of wastewater were pumped into a sterile bucket using a defined procedure, and samples were stored at 4℃ in a refrigerator in the autosampler until they were collected. The rest of the wastewater was poured back into the maintenance hole.

Figure 3.2: Autosampler used in this study.

3.2.2 Passive Samplers

This study investigated three types of passive samplers: P2, P4, and T. All of the passive samplers were placed alongside the autosampler collection line in the sewer for 24 h. The torpedo-style sampler was a 3D-printed housing unit whose blueprints were provided by a research group at Monash University. The device has multiple points that allow wastewater to interact with medical gauze (Swisspers, China) inside to trap solid particles in the wastewater. Two or four pieces of medical gauze were chosen as the sampling material to see if the amount of gauze would affect

the solids capture efficiency. The tampon-style sampler comprised an organic cotton tampon (Thermofisher, Ottawa, Canada) tethered to a string that was put into the wastewater through the maintenance hole. Passive samplers were deployed every Monday and Wednesday and retrieved 24 h after each deployment, where upon the samples were put into plastic Falcon tubes for transportation to the laboratory.

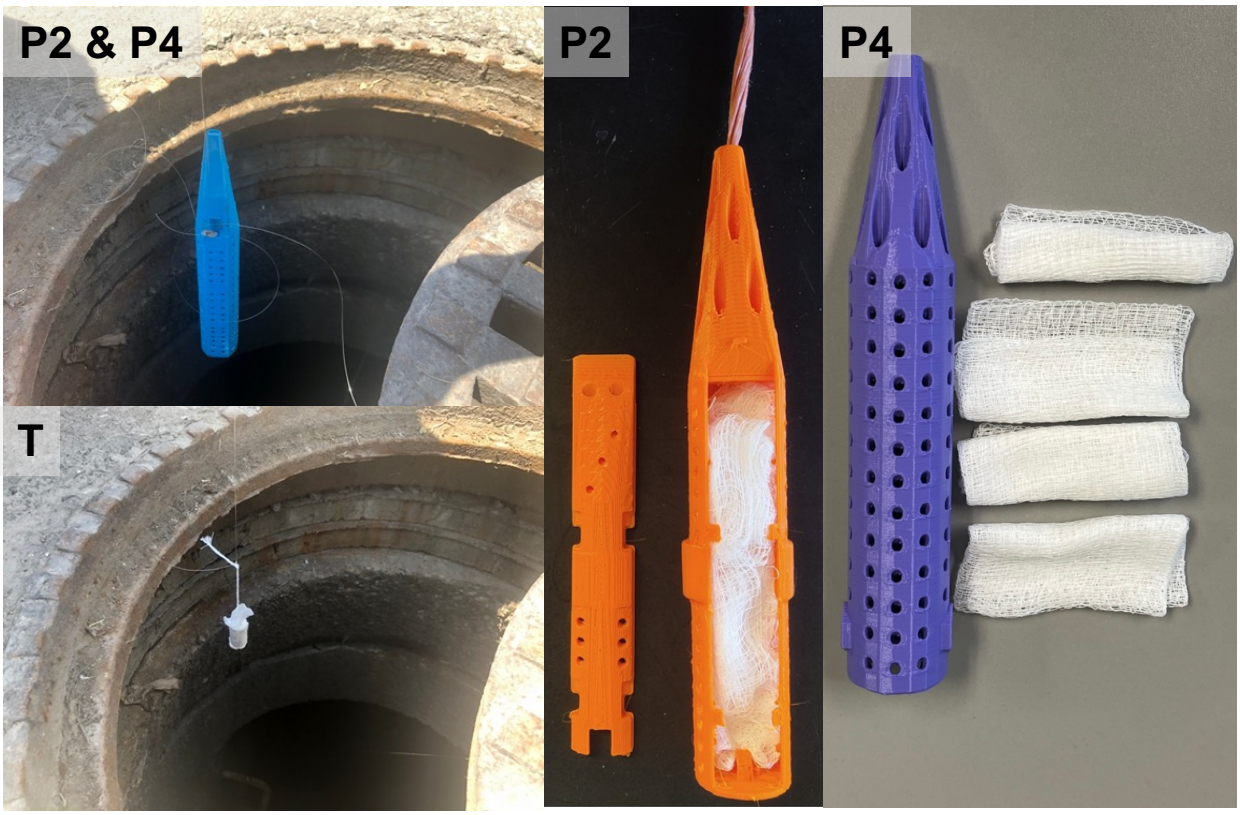


Figure 3.2S: Passive samplers used in this study. P2: a torpedo-style passive sampler packed with two pieces of gauze, P4: a torpedo-style passive sampler packed with four pieces of gauze, T: a tampon-style passive sampler.

3.3 Laboratory Analyses

3.3.1 Sample Transportation, Processing and RNA Extraction

The samples were transported to the laboratory immediately for analysis after collection. The autosampler samples were allowed to settle at room temperature for 30 min. The passive samples were placed in 300 mL of deionized water and stirred with a glass rod to release the absorbed solids. After squeezing the rest of the water out of the absorbent, the suspension was allowed to settle for 30 min, after which the supernatant from all of the samplers was decantated. The

dislodged solids were transferred to a 40 mL centrifuge tube and then concentrated at 10000 x g and 4℃ for 45 min. The supernatant was decanted to isolate the sedimented solids fraction. After this, samples were centrifuged once more at 4℃ for 5 min. After centrifugation, samples inside the 40 mL centrifuge tube were massed, and the total sample weights (pellet weights) of the samples were recorded. Then, 0.25 – 0.26 g of each sample was aliquoted and transferred to a Power Microbiome tube (Qiagen, Germany), mixed with 650 µL of PM1- β mercaptoethanol (1:100), vortexed, and frozen at -20℃ for two minutes. After that, samples were vortexed for two minutes, then dissolved in 600 µL of Trizol (Life Technologies, USA), vortexed, and incubated at 4℃ for 10 minutes. Then, 160 µL of chloroform (Life Technologies, USA) was added to each PowerBead tube, and the tubes were vortexed to mix and incubated at 4℃ for three minutes. They were then centrifuged at 15000 rpm for 15 minutes. After centrifugation, the top aqueous layer of each sample was transferred to a clean, labelled 2 mL tube to which was added with 200 µL IRS solution. After vortexing, the samples were incubated at 4℃ for 5 minutes and then centrifuged at 15000 rpm for 1 min. After transferring the supernatant to a 2 mL RB sample tube, the supernatant was processed using a Qiagen RNeasy PowerMicrobiome extraction kit (PN 26000-50, MD, USA) on a QIAcube Connect automated extraction platform with a modified methodology previously described for the RNeasy PowerMicrobiome Kit (Qiagen, Germantown, MD) (D'Aoust, Mercier, et al., 2021). Extraction blanks were run with each process. Finally, 100 µL of RNA-free water was used to elute the RNA. Samples that were not analyzed on the day they were retrieved from the sampler, run through extraction, but stored overnight at 4℃ before RT-PCR analysis.

3.3.2 RT-qPCR Analyses

N1 and N2 SARS-CoV-2 targets and the PMMoV viral genome were quantified using a singleplex one-step RT-qPCR through a CFX Connect qPCR thermocycler (Bio-Rad, Hercules, CA) according to established protocol (D'Aoust, Mercier, et al., 2021). A total of 7 µL of Master mix was prepared first, consisting of 2.5 µL 4×TaqMan Fast Virus 1-step mastermix (Thermo-Fisher, USA), 0.75 µL of 500 nM of each of the forward and reverse primers (IDT, Kanata, Canada), 0.75 µL 125 nM probe (IDT, Kanata, Canada), and 3 µL of nuclease-free water was added to 3 µL of RNA template. Samples were quantified using a five-point gradient of the EDX SARS-CoV-2 standard curve (Exact Diagnostics, USA). Detailed information for the sequence of each primer

and probe used in this study is shown in Table 3.1. To check for PMMoV inhibition, 1.5 µL samples diluted at 1:10 and 1:40 were compared with undiluted samples. A sample was considered positive if its amplified detected cycle threshold (Ct) was < 40 cycles for at least one of N1 and N2. The assay limit of detection (ALOD, ≥ 95% detection) was determined to be approximately 2 copies/reaction based on a previous assessment (D'Aoust, Mercier, et al., 2021). RNA extraction and RT-qPCR were performed in separate laboratories in Class 2 biosafety cabinets to avoid contamination. The concentrations per reaction of SARS-CoV-2 N1 and N2 viral signals and PMMoV viral signals were converted to copies per volume of wastewater and copies per g of solid weight for dimensional analysis. The PMMoV-normalized SARS-CoV-2 viral signals in this study were expressed as N1-N2 copies/copies of PMMoV. Samples below LOD were considered non-detect in this study. All samples were analyzed using technical triplicates, and samples with values greater than two standard deviations from the mean were discarded.

Table 3.1 Detailed descriptions of primers and probes used in this study.

Primer/Probe	Sequence	Reference
2019-nCoV_N1 forward primer (IDT)	GAC CCC AAA ATC AGC GAA AT	
2019-nCoV_N1 reverse primer (IDT)	TCT GGT TAC TGC CAG TTG AAT CTG	(CDC,2020)
2019-nCoV_N1 probe (IDT)	6-FAM-ACC CCG CAT /ZEN/ TAC GTT TGG TGG ACC-IBFQ	
2019-nCoV_N2 forward primer (IDT)	TTA CAA ACA TTG GCC GCA AA	
2019-nCoV_N2 reverse primer (IDT)	GCG CGA CAT TCC GAA GAA	(CDC,2020)
2019-nCoV_N2 probe (IDT)	6-FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-IBFQ	
PMMoV forward primer (ABI)	GAG TGG TTT GAC CTT AAC GTT GA	
PMMoV reverse primer (ABI)	TTG TCG GTT GCA ATG CAA GT	(Haramoto et al., 2013)
PMMoV probe (ABI)	6-FAM-CCT ACC GAA GCA AAT G-MGB	

3.3.3 Quantification of Total Solids and Volatile Solids

An experiment was conducted using an autosampler and two passive samplers (P2: a torpedo-passive sampler packed with two pieces of gauze, T: a tampon-style passive sampler) to evaluate the wastewater characteristics of the samples. A total of 18 wastewater samples collected on Aug 5th, Aug 10th, Aug 12th, Aug 19th, Aug 24th, and Aug 26th, 2021, were used to evaluate the water weight, total solids (TS) and volatile solids (VS) between samples collected by the autosampler and passive samplers. For all sample collection days, 0.1 g samples were aliquoted after ensuring 0.25 – 0.26 g solids were available for extraction. Prior to aliquoting the samples, aluminum weighing dishes (Fisher Scientific, PA, USA) were massed and labelled for each sample. The weighing dishes, including the pellet, were then massed and left in a furnace (VWR International, PA, USA) at 105℃ for three hours to remove the water fraction. To further explore the wastewater

characteristic of each material, the samples were left in an oven at 500℃ for 30 minutes. The resulting water content, TS and VS are shown in Table 4.1.

3.4 Statistical Analyses

Significant differences between wastewater characteristics of various passive samplers and the autosampler was determined using the student's t-test with a p-value of 0.10. Paired student's t-tests with a p-value of 0.10 or lower was also used to indicate statistical significance between daily samples collected by the autosampler and the various passive samplers when compared samples collected day-to-day.

Chapter 4 Results and Discussion

4.1 Characteristics of Wastewater Solids Collected by Passive Samplers and Autosamplers

Wastewater solids characteristics collected by various passive samplers and a conventional autosampler were compared. An autosampler and two types of passive samplers, a torpedo-style passive sampler packed with two sheets of gauze (P2) and a tampon-style passive sampler (T), were used to collect samples (n=18) from Aug 5th to Aug 26th, 2021 at the University of Ottawa. The torpedo-style passive sampler packed with four sheets of gauze (P4) was not measured because P4 sampler does not have enough solids for this study. Water weight, total solids and volatile solids content were compared between samples collected by the passive samplers and the autosampler (Table 4.1). Samples collected by P2 had a significantly lower water weight compared to A (84% ± 1% compared to 91% ± 1%, $p < 0.01$). The mean value of water weight for T compared to A was not statistically significant (89% ± 2% compared to 91% ± 1%, $p = 0.11$). Samples from P2 had the highest total solids content (16% ± 1%), followed by T (11% ± 1%) and A (9% ± 1%). There was significantly greater total solids content in the P2 sample and T sample compared to the A sample ($p < 0.01$, and $p = 0.09$ respectively) (Figure 4.1). For volatile solids content, P2 samples contained the highest volatile solids content (12% ± 1%), followed by T (10% ± 1%) and A (8% ± 1%). The volatile solids content showed the same trend as for total solids content, where P2 and T had significantly higher values than A ($p < 0.01$, $p = 0.05$, respectively). Hence, for all three wastewater solids characteristics measured in this study, P2 was statistically significant from A.

Table 4.1: Comparison of wastewater solids characteristics between passive samplers and the autosampler, samples were centrifuged and the centrifuged pellets were analyzed to characterize the wastewater solids of the collected samples. A *p*-value less than 0.10 indicates statistical significance. A: Autosampler, P2: Torpedo-passive sampler packed with two pieces of medical gauze, T: tampon-style passive sampler.

	Water weight (%)		**Total solids content (%)**		**Volatile solids content (%)**	
A	91% ± 1%		9% ± 1%		8% ± 1%	
P2	84% ± 1%		16% ± 1%		12% ± 1%	
T	89% ± 2%		11% ± 1%		10% ± 1%	
p- value	**P2 vs A**	**T vs A**	**P2 vs A**	**T vs A**	**P2 vs A**	**T vs A**
	<0.01	0.11	<0.01	0.09	<0.01	0.05

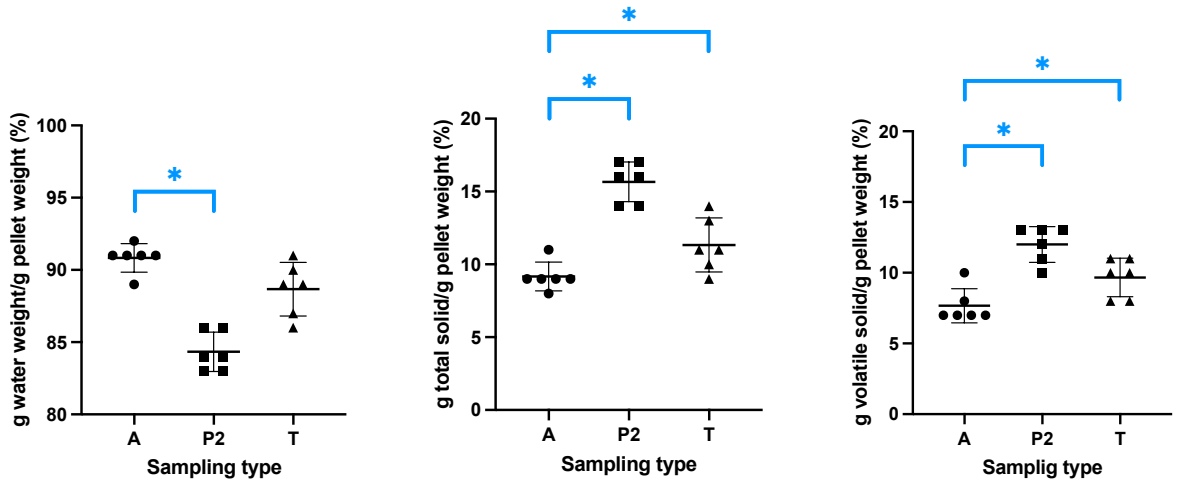


Figure 4.1: Comparison of wastewater solids characteristics between samples collected by passive samplers and the autosampler, samples were centrifuged and the centrifuged pellets were analyzed to characterize the wastewater solids of the collected samples. The mean with standard deviation is shown. Brackets with asterisks identify statistical distinction wastewaters collected by the autosampler and the passive samplers. A: Autosampler, P2: Torpedo-passive sampler packed with two pieces of medical gauze, T: tampon-style passive sampler.

The autosampler was programmed to collect 4 L wastewater samples. Two pieces of medical gauze were placed into the 3-D printed torpedo-style passive sampler (P2) to enable wastewater to interact with absorbent materials through multiple holes and trapped solids. The tampon was tied with a fishing line and placed directly in the wastewater. As wastewater only passed through the passive samplers, the total solids and volatile solids that they collected were proportionally higher than the autosampler. In addition, the water content of the samples collected was lower. The reason that T collected more water than P2 may be due to the unique structure of the tampon, which enables it to absorb and store water continuously until it becomes saturated. While after centrifugation, the water content of autosampler samples remains higher than passive sampler samples, therefore the inherent characteristic differences between autosampler samples and passive sampler samples is the reason for the observed statistical significance. Our results indicate that the affinity between solids in the wastewater and samplers differs according to the type of sampler, which could affect the virus adsorption efficiency and the virus concentrations. Hayes et al. (2021) found that the adsorption efficiency of heat-inactivated SARS-CoV-2 (HI-SCV-2)

increases with increasing viral concentrations in total suspended solid concentration (TSS) but would be inhibited in excessive TSS concentrations.

4.2 Comparison of SARS-CoV-2 and PMMoV Measurements Between Samples Collected by an Autosampler and Passive Samplers

Wastewater samples from the University of Ottawa were collected to identify similarities and differences between passive sampler and autosampler collected samples. A total of 83 wastewater samples were collected for 22 days. SARS-CoV-2 and PMMoV RNA for each sampler were quantified by RT-qPCR and normalized against the total sample mass collected or wastewater volume sampled. All of the samples were positive for SARS-CoV-2 and PMMoV.

Unit of copies/L is commonly used as a standardized unit for autosamplers for determining viral concentrations in wastewater. Although this is a common unit, it is difficult to extrapolate passive sampler measurements as copies/L due to the inconsistent sorption of liquid in the passive sampler across time. To overcome this limitation, in this study we assumed that the total solids concentration (i.e. the ratio of solids to liquid) of the wastewater sample collected by the passive samplers was identical to the TS of the autosampler that was deployed in the same location on that same day. This approach of employing the TS measurement from the collected autosampler that was deployed next to the passive sampler to enable this study to present the passive sampler measurement as copies/L (the common unit for expressing viral signal in wastewaters) was employed because the analytical methods for SARS-CoV-2 and PMMoV in this study are solids based methods. The results of VS/TS ratio of passive samplers and autosampler showed very small variation. Therefore, we used the autosampler TS measurement of the wastewater in the common sampling location to estimate the wastewater volume that passed through the passive samplers in a defined time (24 h) according to Eq 4.1.

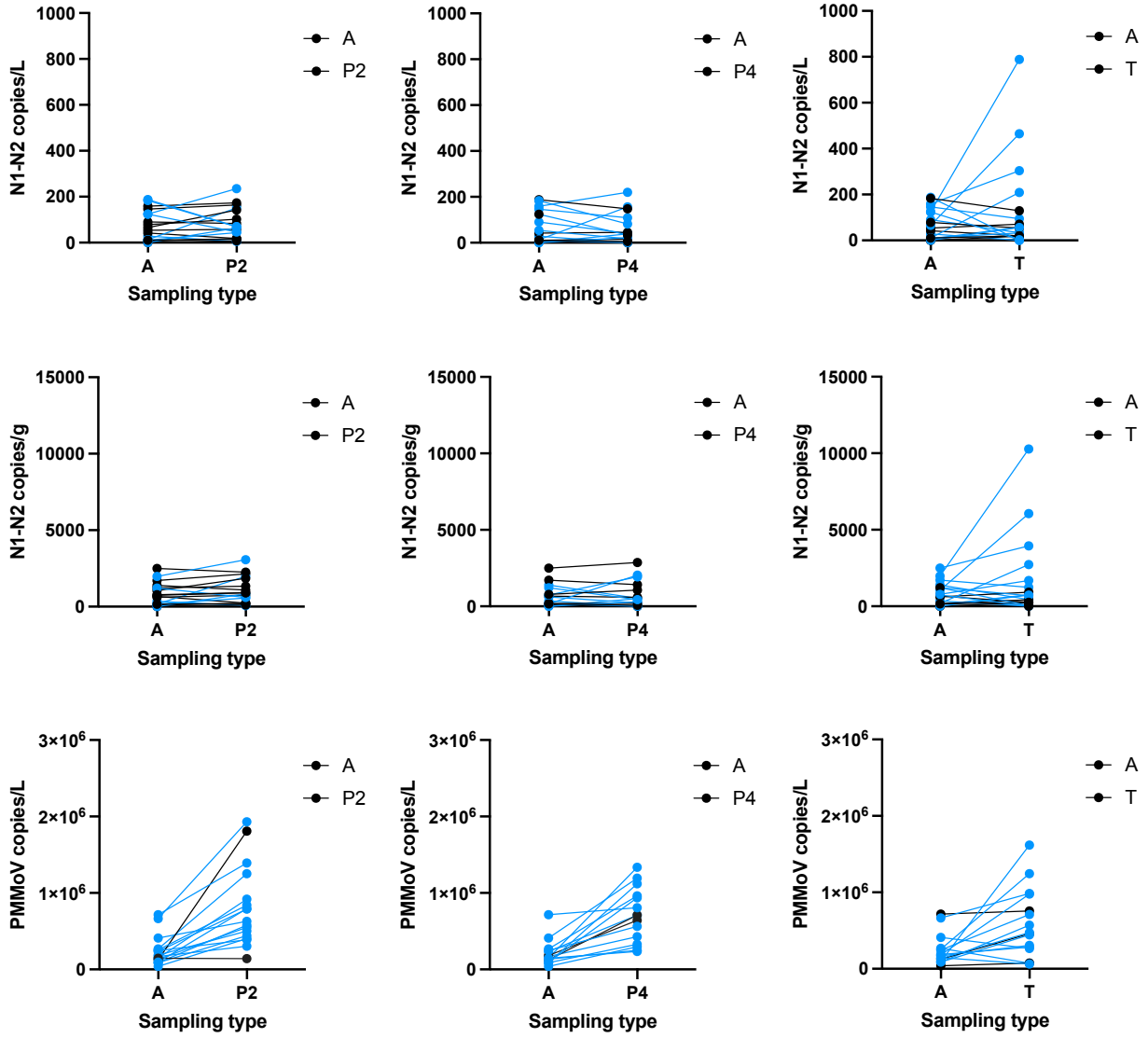
$$\frac{M_{Autosampler}}{V_{Autosampler}} = \frac{M_{Passive\ sampler}}{V_{Passive\ sampler}} \qquad \text{(Eq - 4.1)}$$

Here, $M_{Autosampler}$ is the extracted mass of solids collected by the autosampler, $V_{Autosampler}$ is the sample volume collected by the autosampler (programmed to collect 4 L), $M_{Passive\ sampler}$ is the extracted mass of solids collected by the passive sampler, and $V_{Passive\ sampler}$ is the calculated volume of liquid that corresponds to the collected mass of the passive sampler. Since average extracted mass was used on autosampler, the variance on mass will not affect the calculated volume of passive sampler. A day-to-day comparison of the passive sampler to the autosampler

measurements of the average N1 and N2 gene region targets and the PMMoV gene target was explored in this study (Figure 4.2, Table 4.2). In particular, technical triplicate data from samples collected on the same day by the passive samplers and the autosampler were compared to each other using the following common units for SARS-CoV-2 WWS: avg N1-N2 copies/L, avg N1-N2 copies/g, PMMoV copies/L, PMMoV copies/g and avg N1-N2 copies/copies of PMMoV. The percentage of daily samples collected by the P2 passive sampler with SARS-CoV-2 wastewater measurements which are not statistically distinct from the measurements of the autosampler samples collected on the same day were 61% when expressed as avg N1-N2 copies/L, 78% when expressed as avg N1-N2 copies/g and 58% when expressed as avg N1-N2 copies/copies PMMoV. Similar results were observed by Wilson et al. (2022), where N1 and N2 Ct values were compared between passive samplers and grab or composite samples, and no significant differences were observed. The difference between P2 measurements compared to A and P4 measurements compared to A could have been caused by the different quantity of medical gauze that were put into the torpedo-style housing unit. The increasing amount of medical gauze may have restricted the collection of trapped solids, thereby impeding solids capture. Because the passive sampler holder broke, data for P4 is absent for three sampling events, and thus, it is also possible that the missing events influenced the observed difference reported between A and P4, with no difference being reported between A and P2. The P2 passive sampler configuration demonstrates a higher percentage of daily samples being comparable to the autosampler measurements as compared to the P4 and T passives samplers used in this study when expressing the measurements of SARS-CoV-2 as avg N1-N2 copies/L (P2: 61%, P4: 44%, T: 42%), avg N1-N2 copies/g (P2: 78%, P4: 56%, T: 47%) or avg N1-N2 copies/copies PMMoV(P2: 58%, P4: 50%, T: 37%). It is noted that the highest percentage of daily samples being comparable to the autosampler measurements occurs for the P2 passive sampler when expressing the measurements as a function of solid mass, avg N1-N2 copies/g. Hence, P2 is identified in this study as a comparable means of sampling to autosamplers for SARS-CoV-2 WWS using mass fraction units.

PMMoV was used in this study as a fecal indicator to normalize SARS-CoV-2 to diminish the impacts of flow and population size because of its relative abundance. PMMoV was detected in all of the wastewater samples. The PMMoV measurements of the passive sampler samples collected on the same day showed a completely different trend when compared to the autosampler SARS-CoV-2 N1 and N2 measurements. The percentage of passive samplers which are not

statistically distinct from the autosampler was only 18% (P2), 27% (P4), and 44% (T), respectively when expressed as PMMoV copies/L, and 12% (P2), 20% (P4), 44% (T) when expressed as PMMoV copies/g (Table 4.2). Although PMMoV copies collected by the passive samplers were significantly higher than those for the autosampler, this did not affect the percentage of passive sampler samples are not statistically distinct from the autosampler samples when using measurements of N1-N2 copies/copies of PMMoV (P2: 58%, P4: 50%, T: 37%, respectively).



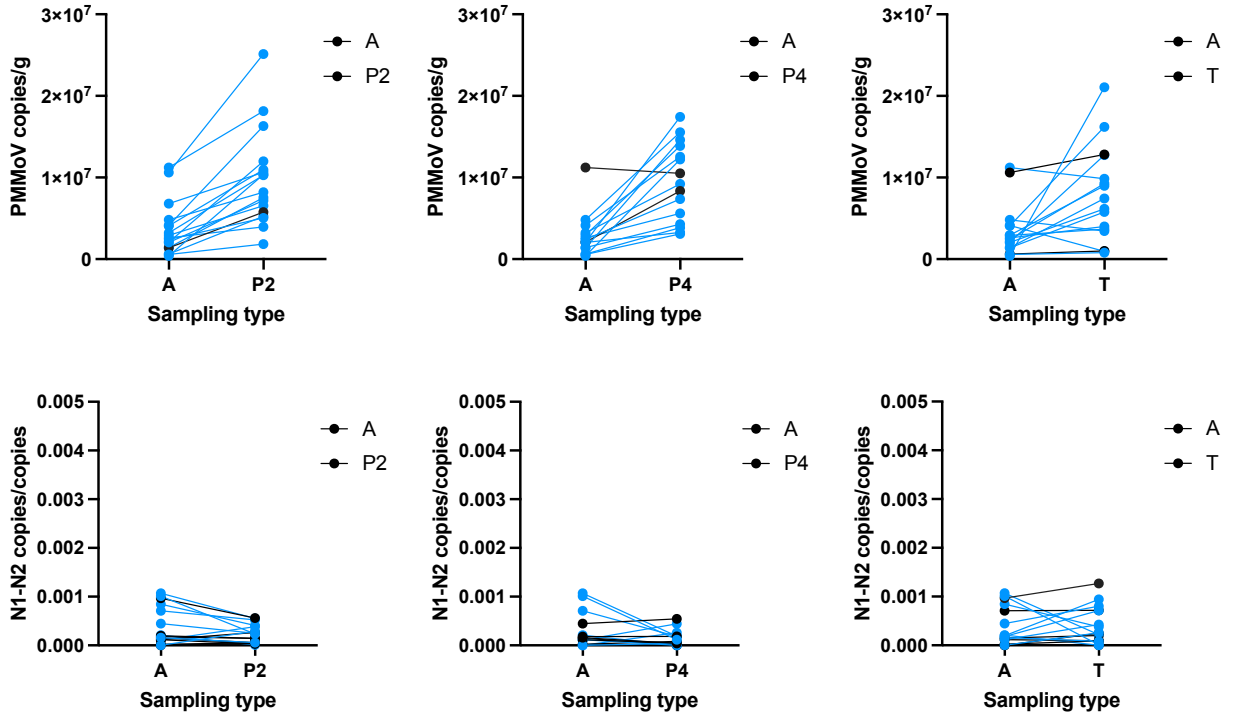


Figure 4.2: Day-to-day comparison between the passive samplers to the autosampler measurements of the average N1 and N2 gene region targets and the PMMoV gene target. Blue lines and points identify statistical significance between samples collected by the autosampler and the passive samplers, and black lines and points identify no statistical significance between passive samplers and the autosampler. A: Autosampler, P2: Torpedo-passive sampler packed with two pieces of medical gauze, P4: Torpedo-passive sampler packed with four pieces of medical gauze, T: tampon-style passive sampler.

Tale 4.2: Day-to-day comparison was used in this study, and paired t-test was used to determine statistical significance between samples collected by passive samplers and the autosampler, a *p*-value less than 0.10 indicates statistical significance. The percentage of passive sampler samples comparable to the autosampler samples identifies the proportion of samples that are not statistically distinct from the total samples. A: Autosampler, P2: Torpedo-passive sampler packed with two pieces of medical gauze, P4: Torpedo-passive sampler packed with four pieces of medical gauze, T: tampon-style passive sampler.

	Units of Measurement														
	N1-N2 (cp/L)			N1-N2 (cp/g)			PMMoV (cp/L)			PMMoV (cp/g)			N1-N2 (cp/cp of PMMoV)		
	P2 vs A	P4 vs A	T vs A	P2 vs A	P4 vs A	T vs A	P2 vs A	P4 vs A	T vs A	P2 vs A	P4 vs A	T vs A	P2 vs A	P4 vs A	T vs A
Total number of samples	18	16	19	18	16	19	17	15	16	17	15	16	19	16	19
Number of comparable samples	11	7	8	14	9	9	3	4	7	2	3	7	11	8	7
Percentage of comparable samples (%)	61%	44%	42%	78%	56%	47%	18%	27%	44%	12%	20%	44%	58%	50%	37%

The T passive sampler shows the highest average N1-N2 copies/L measurement (1.20 ± 2.83 copies/L), followed by P2 (0.95 ± 2.82 copies/L) and then P4 (0.41 ± 3.15 copies/L), which indicates that T collected higher N1-N2 copies than did the medical gauze or the autosampler (Table 4.3). Wilson et al. (2022) also observed that the N1 and N2 Ct values for the tampon are lower than for medical gauze, which indicates that the tampon is able to collect higher copy numbers of SARS-CoV-2 in wastewater. At the same time, in our study the tampon also showed higher variance compared to medical gauze and the autosampler (Figure 4.3). Similar to N1-N2 copies/L, N1-N2 copies/g of T passive sampler also shows the highest values (0.96 ± 2.28 copies/g), followed by P2 (0.87 ± 2.62 copies/g) and then P4 (0.51 ± 3.00 copies/g) (Table 4.2). The N1-N2 measurements for all of the passive samplers were higher than those for the autosampler (Figure 4.3), which shows that the passive samplers collected greater N1-N2 copies than did the autosampler for the same quantity of mass of solids collected, and hence for the extrapolated volume measurements applied to the passive samplers in this study. This difference is likely due to the differences observed in the wastewater characteristics of the centrifuged pellet between the autosampler and passive samplers (Figure 4.1). In particular, the higher volatile solids content of the passive samplers, and hence likely higher fecal matter content of the passive samplers, produce higher SARS-CoV-2 measurements.

Table 4.3: Comparison between passive samplers and the autosampler using N1-N2 copies/L, N1-N2 copies/g, PMMoV copies/L, PMMoV copies/g, and N1-N2 copies/copies of PMMoV. The mean with standard deviation is shown. A: Autosampler, P2: Torpedo-passive sampler packed with two pieces of medical gauze, P4: Torpedo-passive sampler packed with four pieces of medical gauze, T: tampon-style passive sampler.

	Units of Measurement				
	N1-N2 (cp/L)	N1-N2 (cp/g)	PMMoV (cp/L)	PMMoV (cp/g)	N1-N2 (cp/cp of PMMoV)
(P2-A)/A	0.95 ± 2.82	0.87 ± 2.62	3.42 ± 3.63	4.04 ± 5.76	- 0.19 ± 0.92
(P4-A)/A	0.41 ± 3.15	0.51 ± 3.00	3.73 ± 3.46	4.97 ± 7.25	- 0.10 ± 1.36
(T-A)/A	1.20 ± 2.83	0.96 ± 2.28	2.63 ± 4.09	1.55 ± 1.80	0.68 ± 1.63

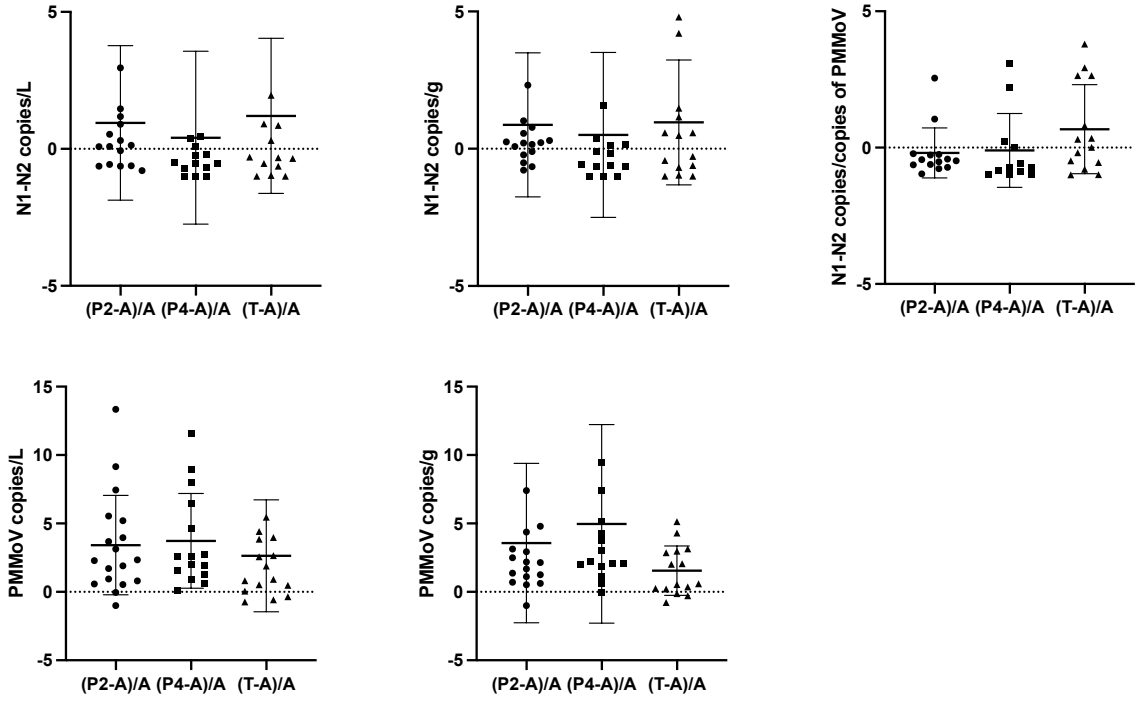


Figure 4.3: Relative differences between passive sampler and the autosampler measurements using units of N1-N2 copies/L, N1-N2 copies/g, PMMoV copies/L, PMMoV copies/g, and N1-N2 copies/copies of PMMoV. A: Autosampler, P2: Torpedo-passive sampler packed with two pieces of medical gauze, P4: Torpedo-passive sampler packed with four pieces of medical gauze, T: tampon-style passive sampler.

When PMMoV copy numbers were normalized using sample volumes, the trend was different compared to N1-N2 copies/L and N1-N2 copies/g. P4 showed the highest reading (3.73 ± 3.46 copies/L), followed by P2 (3.42 ± 3.63 copies/L), then by T (2.63 ± 4.09 copies/L) (Table 4.3). At the same time, the mean values of PMMoV copies/L for P2 and P4 were similar, and the measurements for T were lower than those for P2 and P4. Since the sample volumes taken by the autosamplers and passive samplers were similar, the statistical differences between autosamplers

and passive samplers were determined by PMMoV copies. The results indicate that PMMoV copies for all three types of passive samplers were higher than those for the autosampler (Figure 4.3). Previous studies have also observed this relationship, where PMMoV Ct values produced by cotton swabs and electronegative membranes passive samplers were shown to be significantly lower than measurements for autosamplers (Wilson et al., 2021). When using PMMoV copies/g as a standardized unit, P4 passive sampler still showed the highest measurement (4.97 ± 7.25 copies/g), followed by T (1.55 ± 1.80 copies/g) and then P2 (4.04 ± 5.76 copies/g) (Table 4.3). The difference in PMMoV copies/g between passive samplers and autosamplers was still caused by differences in PMMoV copies. The data indicates P2 captured the highest PMMoV copies (42,853 ± 32,560 copies/well), followed by T (37,029 ± 28,076 copies/well). P4 showed PMMoV copies similar with T (34,758 ± 19,911 copies/well), while PMMoV copies for A were significantly lower than for all other passive samplers (23,092 ± 42,217 copies/well). Total solids and volatile solids for the passive samplers may correlate better with PMMoV copies than the autosampler, and the total solids and volatile solids concentrations for the passive samplers were higher than those for the autosampler, therefore the passive samplers likely collected higher PMMoV copies than did the autosampler.

The trend for N1-N2 copies/copies PMMoV was different compared to the trend for N1-N2 copies/L and N1-N2 copies/g because the autosampler values were higher than those for P2 and P4 but they were not higher compared to T (Figure 4.3). The N1-N2 copies/copies of PMMoV measured for P2, P4, and T was -0.19 ± 0.92, -0.10 ± 1.36, and 0.68 ± 1.63, respectively. The negative values for P2 and P4 indicate that A collected higher N1-N2 copies/copies PMMoV than P2 and P4. This may have occurred because the PMMoV concentrations for P2 and P4 were 1.86 and 1.51 times higher than the autosampler, respectively, but N1-N2 copy numbers were similar (A: 8.05 ± 5.11, P2: 9.60 ± 6.46, P4: 9.24 ± 6.26, respectively). The N1-N2 copies/copies of PMMoV for the tampon were positive because the tampon collected about twice as many N1-N2 copies as did the autosampler but only 1.60 times higher PMMoV copies than did the autosampler. Altough N1-N2 copies/copies showed less distinction than N1-N2 copies/g in Figure 4.3, when use day-to-day comparison, among all the units of measurement, N1-N2 copies/g differed the least for the autosampler compared to N1-N2 copies/L and N1-N2 copies/copies. As solid-based method was used in this study, therefore P2 passive sampler with measurement units of N1-N2 copies/g

are recommended for SARS-CoV-2 wastewater surveillance when compared with autosampler measurements.

5 Conclusions

This study demonstrates that passive samplers can be used reliably to detect SARS-CoV-2 in wastewater. The deployment of passive samplers is simple and can be implemented for wastewater surveillance when automatic sampling is not feasible, particularly in remote, northern and rural communities, within sewersheds where autosamplers are not deployable, and within low-income countries. In addition, passive sampler configurations also need further consideration because the ragging rates of different types of passive samplers may affect their virus capture efficiency. The experimental results suggest that the water content in the centrifuged pellets of samples collected by the torpedo passive sampler packed with two pieces of gauze (P2) was significantly lower compared to the samples collected by the autosampler. Total solids and volatile solids of pellets collected by P2 and tampon passive sampler (T) were significantly higher than the autosampler. Wastewater solids characteristics of the centrifuged pellets from the various samplers, such as total solids and volatile solids, showed a greated impact on the measurement of PMMoV copies compared to SARS-CoV-2 N1-N2 copies. However, the significant differences in PMMoV copies for the passive samplers did not signifnacnlty affect the PMMoV-normalized N1-N2 copies.

Various units of measurement were used to compare the similarities and distinctions between the passive samplers and the autosampler. P2 showed SARS-CoV-2 measurements that were the most similar to the autosampler measurements among the three passive samplers. Copies/g units showed the best agreement between the passive samplers and the autosampler sampling compared to units of copies/L and copies/copies of PMMoV. These findings demonstrate that passive samplers may be used to detect SARS-CoV-2 in wastewater, and in particular P2 passive sampler designs with measurements expressed in unites of copies/g are recommended.

Wastewater flow rates were not measured in this study, and the volumes of water passing through the passive samplers while they were immersed were unknown. Although we used the mass-to-volume ratio based on autosampler measurements to calculate the volumes of wastewater samples sampled by the passive samplers, these results can still be optimized and further study is recommended. Also, the sewer residence time, surface areas of different materials and exposure time could affect the efficiency of solid absorption. Future studies are recommended to identfiy a suitable means to estimate the wastewater volumes sampled by passive samplers to diminish the variance in wastewater flow, solids content in the wastewater, and the number of people who are infected during outbreaks. If successful, autosamplers could be further used to measure wastewater

characteristics at given locations, with passive samplers becoming an adopted and accepted substitute for the autosampler.

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