WASTEWATER SURVEILLANCE OF SARS-COV-2 AT A CANADIAN UNIVERSITY CAMPUS AND THE IMPACT OF WASTEWATER CHARACTERISTICS ON VIRAL RNA DETECTION

by

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ABSTRACT

SARS-CoV-2 levels in the wastewater of a Canadian university campus and their residence buildings were monitored to identify changes, peaks, and hotspots of COVID-19 transmission and search for associations with campus events, social gatherings, long weekends, and holidays. Wastewater signals largely correlated with clinically confirmed cases, often increased following long weekends, and decreased after the implementation of lockdowns. Furthermore, the impact of wastewater parameters on SARS-CoV-2 detection was investigated, and the efficiency of ultrafiltration and centrifugation concentration methods were compared. Results indicated more sensitive results with the centrifugation method for wastewater with high solids content and with the ultrafiltration method for low solids content. Wastewater characteristics from the building sewers were more variable than overall campus wastewater. Statistical analysis was performed to manifest the observations. Overall, wastewater surveillance provided actionable information and was able to bring high-risk factors and events to the attention of the decision-makers, enabling timely corrective measures.

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LIST OF ABBREVIATIONS

ANFIS	Adaptive Neuro-Fuzzy Inference System
AS	Ammonium Sulphate Precipitation
BCoV	Bovine Coronavirus
cDNA	Complementary DNA
CF	Centrifugation Method
CoV	Coronavirus
COVID-19	Coronavirus disease 2019
Cq	Quantification Cycle
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphates
E-Protein	Envelope-Protein
HCoV	Human Coronavirus
HCoV-229E	Human Coronavirus 229-E
HCoV-OC43	Human Coronavirus OC43
IC	Internal Control
LoD	Limit of Detection
MERS-CoV	Middle Eastern Respiratory Syndrome-related Coronavirus

MHV	Murine Hepatitis Virus
M-Protein	Membrane Protein
mRNA	Messenger RNA
N-Protein	Nucleocapsid-Protein
NTC	Non-Template Control
ORF	Open Reading Frames
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol Precipitation
PMMoV	Pepper Mild Mottle Virus
qPCR	Quantitative Polymerase Chain Reaction
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic Acid
RTase	Reverse Transcriptase
RT-ddPCR	Reverse Transcriptase Digital Drop Polymerase Chain reaction
RT-dPCR	Reverse Transcriptase Digital Polymerase Chain reaction
RT-PCR	Reverse Transcriptase Polymerase Chain reaction
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus

S-Protein	Spike Protein
TS	Total Solids
UF	Ultrafiltration Method
VOC	Variant of Concern
VS	Volatile Solids
WBE	Wastewater-based Epidemiology
WHO	World Health Organization
WWTP	Wastewater Treatment Plant

1. INTRODUCTION

Wastewater-based epidemiology (WBE) is the surveillance of wastewater for epidemiological purposes and has been used for tracking viral and bacterial disease outbreaks as well as substance abuse for years (Asghar et al., 2014; Bishop et al., 2020; Du et al., 2017; Hellmér et al., 2014; McCall et al., 2020). WBE has been recently implemented globally to track the ongoing Coronavirus Disease 2019 (COVID-19) pandemic and monitor the overall abundance of viral signals in communities to guide public health decisions and interventions (Betancourt et al., 2021; Gibas et al., 2021; Prado et al., 2021; Westhaus et al., 2021). The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped respiratory virus with an RNA (ribonucleic acid) genome (Ahmed et al., 2022; Poon & Peiris, 2020). Symptoms develop after 2-14 days of exposure, followed by high viral shedding in bodily fluids and fecal matter (Betancourt et al., 2021; Harris-Lovett et al., 2021). Tracking SARS-CoV-2 in wastewater allows alerting the community of increased prevalence, allocating resources for testing and vaccination, and targeting public health messaging (Harris-Lovett et al., 2021). A key advantage of WBE over clinical testing is the representation of the entire population, including asymptomatic individuals, potentially accounting for 40 to 60 % of infections, infected individuals with mild symptoms that may not seek clinical testing, and marginalized groups with less access to clinical tests (Ahmed et al., 2022; Honda et al., 2021; Lavezzo et al., 2020; Murakami et al., 2020; Nishiura et al., 2020; Sharkey et al., 2021). Individual testing of the entire population is also more time-intensive, labor-intensive, and higher in cost compared to wastewater surveillance (Murakami et al., 2020). Additionally, the results of wastewater surveillance are not biased as the number of clinical COVID-19 cases might be (Honda et al., 2021; Murakami et al., 2020). WBE avoids the negative stigma and

discrimination that positive tested people might encounter as large populations are targeted (Honda et al., 2021; Murakami et al., 2020; Zhai & Du, 2020).

Knowledge about the presence and fate of SARS-CoV-2 and other enveloped viruses in municipal wastewater is still limited. Previous research reported that enveloped viruses adsorb onto solid particles in higher numbers than non-enveloped viruses (Ye et al., 2016). Furthermore, settled solids from wastewater treatment plants yielded higher SARS-CoV-2 concentrations than influent wastewater (Graham et al., 2021). Monitoring of the solids fraction in wastewater might therefore lead to greater detection of the enveloped SARS-CoV-2 virus. However, it is still not well understood how wastewater parameters and constituents may impact the concentration, extraction, and detection methods for SARS-CoV-2 RNA and whether some methods perform better under different wastewater qualities. Wastewater characteristics and strength change throughout the day but also in the sewers because of residence times, wastewater temperature, flow velocities and shear, and sewer operational conditions.

University campuses are considered hot spots for the transmission of COVID-19. Young people on campus tend to meet in larger groups and party against the advice of public health (Wilson et al., 2020). Vaccination rates among students are lower compared to the older population. Furthermore, residence buildings and classrooms increase population density and the risk of spreading the virus (Wilson et al., 2020). There has been an increased interest in using WBE as surveillance and potentially early warning tools at university and college campuses (Betancourt et al., 2021; Gibas et al., 2021; Harris-Lovett et al., 2021; Sweetapple et al., 2022). To set up wastewater surveillance, several key decisions must be made about sampling location, frequency, and sample type (i.e., composite or grab), as well as protocols that need to be established for sample concentration, RNA extraction, and detection methods (Gibas et al., 2021).

The overall objective of this research was to monitor the SARS-CoV-2 RNA levels in the wastewater of a Canadian university campus to provide actionable information and assist with decision-making to manage COVID-19 on campus effectively. The thesis begins with a literature review to introduce the reader to the topic of SARS-CoV-2 wastewater surveillance. Though wastewater-based epidemiology has been used for many years, its application for SARS-CoV-2 detection is still new and standard practices have not been developed. The literature review (second chapter) will give an overview of methods that have been applied since the beginning of the COVID-19 pandemic, including sample collection, concentration, extraction, and detection methods. Furthermore, common laboratory controls, as well as possible influences on the detection of SARS-CoV-2, are discussed. In the third chapter, the materials and methods that have been applied in this study are described in detail. Chapter 4 presents and discusses the results of the study, which has been published in the journal ES&T Water in a manuscript written by the author of this thesis (Bitter et al., 2022). The chapter describes SARS-CoV-2 trends, peaks, and hotspots that have been observed through the wastewater samples collected from the campus sewer and residence buildings, and discusses associations with campus events, social gatherings, long weekends, and holidays. Furthermore, the different SARS-CoV-2 concentration methods tested and the impact of wastewater parameters (temperature, pH, turbidity, UV absorbance, total solids, and volatile solids) on SARS-CoV-2 detection that were investigated are discussed. The chapter includes statistical analysis that was performed to manifest correlations between wastewater viral signals and reported cases, as well as wastewater characteristics. Chapters 5 and 6 summarize the conclusions gained from this research and recommendations for future research. The content of the study was presented at two different conferences, the 2021 Atlantic and Eastern Symposium on Water Quality Research (CAWQ) and the 2022 WEAO Technical Symposium.

2. LITERATURE REVIEW

2.1 Virus Taxonomy

2.1.1 Coronaviruses

Viruses exist in various shapes and sizes, ranging between 10-400 nm. Furthermore, differences exist in genome structure, chemical composition, reproduction, and range of host species (Twigg & Wenk, 2022). Viruses are composed of proteins, carbohydrates and lipids (Twigg & Wenk, 2022). They have a virus-coded protein capsid that surrounds the nucleic acid. The capsid is responsible for host cell recognition and binding mechanisms (Twigg & Wenk, 2022). SARS-CoV-2, the virus that caused the COVID-19 pandemic, is part of the coronavirus family (CoV). CoVs are a group of enveloped, single-stranded, positive-sense RNA viruses that are classed into four sub-groupings, known as alpha, beta, gamma, and delta (CDC, 2022b). Alpha- and beta-CoVs infect mammals, gamma-CoVs infect avian species and delta-CoVs infect both mammals and aves (Naqvi et al., 2020). The first human coronavirus (HCoV) was detected in the 1960s, but seven different HCoV types that are infectious to humans are known to date (CDC, 2022b; Zhang et al., 2022). The most common HCoVs cause mild diseases with mild symptoms, such as the common cold (229E, NL63, OC43 and HKU1) (CDC, 2022b; Naqvi et al., 2020). Others are more severe, such as the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) that caused an outbreak in 2002, and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) that was first reported in Saudi Arabia in 2012 (Zhang et al., 2022). The most recent outbreak (COVID-19) has been caused by SARS-CoV-2, which is closely related to SARS-CoV in terms of genomic and structural composition (Zhang et al., 2022). SARS-CoV, MERS-CoV and SARS-CoV-2 can cause severe respiratory illness and can result in death in comorbid individuals (Naqvi et al., 2020).

2.1.2 SARS-CoV-2

In December 2019, several pneumonia cases were detected in a province in China, all related to a wholesale food market (Wang H. et al., 2020). The cases were found to be caused by an unknown coronavirus and were first classified as “pneumonia of an unknown etiology” (Wang H. et al., 2020); A term that was first introduced in the 2003 SARS-CoV outbreak. More infected individuals were found quickly through an epidemiological investigation, which suggested person-to-person transmission (Wang H. et al., 2020). The newly discovered coronavirus was first named 2019-nCoV but was changed to SARS-CoV-2 on February 11, 2020, by the International Committee for the Classification of Viruses when similarities to SARS-CoV were detected (Wang H. et al., 2020). The World Health Organization named the disease caused by the SARS-CoV-2 virus Coronavirus Disease 2019 (COVID-19) (Wang H. et al., 2020).

SARS-CoV-2 is primarily transmitted via the airborne route through droplets generated by an infected person that is coughing or sneezing (Zhang et al., 2022). Infected individuals can transmit the virus for more than 14 days (Ahmed et al., 2022). The exponential spread of the virus has caused the implementation of several lockdowns from city to national levels worldwide in an attempt to contain the further spread of the virus. The virus primarily affects the respiratory system causing flu-like symptoms such as cough, fever or chills, and difficulty breathing (Naqvi et al., 2020). Additionally, symptoms can be fatigue, muscle or body aches, headaches, loss of smell and taste, nausea or vomiting, and diarrhea (Ahmed et al., 2022; Zhang et al., 2022). Not all infected individuals develop these symptoms, which makes it more challenging to contain the spread of the virus. The amount of asymptomatic individuals is unknown but estimated to be around 40 to 60 % of all infections (Ahmed et al., 2022; Nishiura et al., 2020). To confirm COVID-19 in an individual, nasopharyngeal or throat swabs are collected and analyzed using Polymerase Chain

Reaction (PCR) tests or serological tests that are based on antibody/antigen detection (Ahmed et al., 2022).

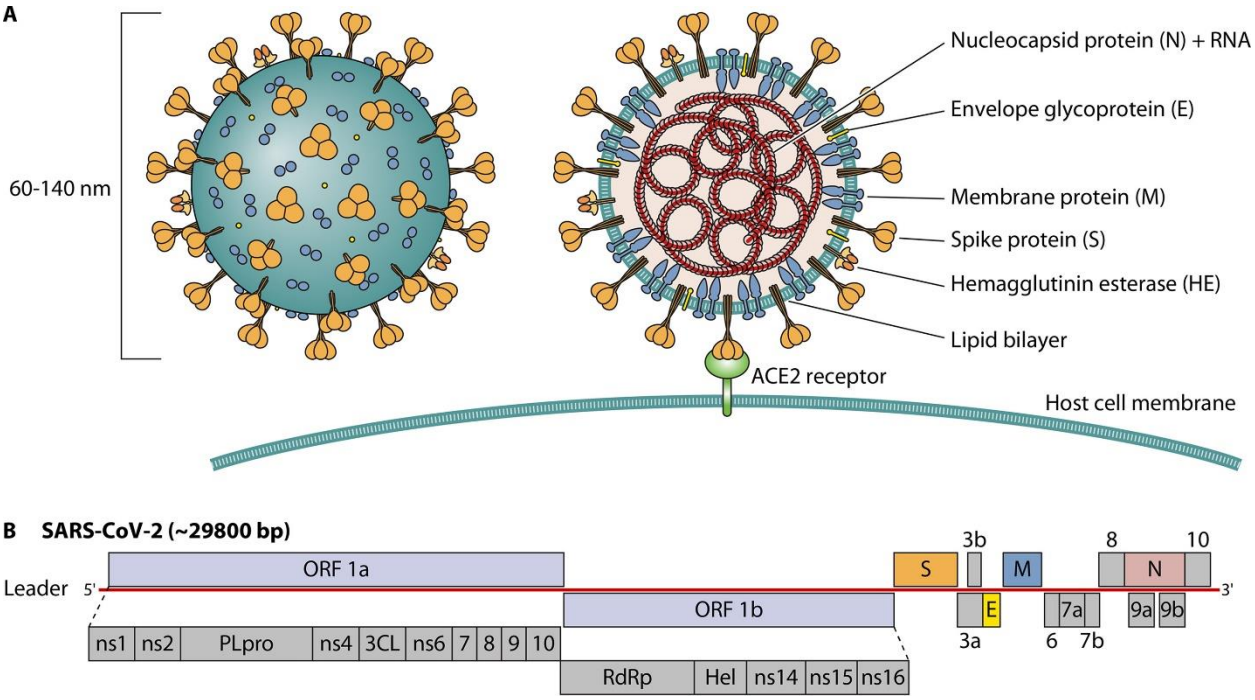


Figure 1: (A) Genomic Structure of the SARS-CoV-2 virus, (B) Genome organization and proteins (Safiabadi Tali et al., 2021)

Structurally, CoVs have the largest RNA viral genome, ranging from 26 to 32 kb in length (Naqvi et al., 2020; Wang H. et al., 2020). The genome of SARS-CoV-2 is comprised of a single-stranded positive-sense RNA, about 29.9 kb in length, encoding almost 30 proteins (Figure 1) (Safiabadi Tali et al., 2021). Some of their functions are known due to their similarity to other viruses, but others are still unclear (Safiabadi Tali et al., 2021). On the 5' end of the viral genome are two open reading frames (ORFs) that occupy approximately 71% of the entire genome (Safiabadi Tali et al.,

2021). They make up several non-structural proteins which are responsible for polyprotein processing, viral RNA replication, and mRNA (messenger RNA) synthesis (Safiabadi Tali et al., 2021). On the 3' end of the genome are the structural and accessory proteins (Safiabadi Tali et al., 2021). They are expressed from several nested subgenomic mRNA produced through a discontinuous transcription by the viral RNA-dependent RNA polymerase (RdRp) (Safiabadi Tali et al., 2021). The capsid-forming structural proteins that play an important role in pathogenesis include spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Naqvi et al., 2020; Safiabadi Tali et al., 2021; Wang H. et al., 2020). The S protein initiates the infection by binding to the host cell surface receptor. It determines the host tropism and transmission capacity (Naqvi et al., 2020; Safiabadi Tali et al., 2021; Wang H. et al., 2020). The spike protein is further classified into S1, responsible for receptor binding, and S2, responsible for cell membrane fusion (Wang H. et al., 2020). The E and M proteins help in the viral assembly and release of the virions (Naqvi et al., 2020; Safiabadi Tali et al., 2021). The N protein plays a vital role in the packaging of viral RNA into ribonucleocapsid (Naqvi et al., 2020; Safiabadi Tali et al., 2021). Knowing the structure and function of the SARS-CoV-2 virus is the basis for developing and improving detection methods in clinical and wastewater environments as well as in the development of immunization (Safiabadi Tali et al., 2021).

Throughout the COVID-19 pandemic, several waves of infections were recorded, driven by different variants of the SARS-CoV-2 virus. The mutations classified as variants of concern by the CDC, B.1.1.7 (Alpha), P.1 (Gamma), B.1.351 (Beta), B.1.617.2 (Delta), and B.1.1.529; BA.1; BA.1.1; BA.2; BA.3; BA.4; BA.5 (Omicron) are characterized by spike protein substitutions that change the transmissibility and severity of the disease. The mutations of the virus may also interfere with the detection and diagnosis of the virus (Ahmed et al., 2022).

2.2 Wastewater-Based Epidemiology

2.2.1 SARS-CoV-2 in Wastewater

Previous research on the removal and inactivation of viruses in wastewater treatment plants (WWTP) has focused on non-enveloped enteric viruses as they are abundant in wastewater and readily transmitted via the fecal-oral route (Twigg & Wenk, 2022). However, coronaviruses are enveloped viruses that are airborne and primarily transmitted via respiratory droplets. But besides detection of SARS-CoV-2 in an infected individual's saliva and nasal secretion, viral RNA has also been detected in feces and urine (Ahmed et al., 2022; Twigg & Wenk, 2022). The load of SARS-CoV-2 in feces of infected patients, symptomatic or asymptomatic, varies for each individual but can be in the range of $10^4 – 10^8$ copies per liter (Twigg & Wenk, 2022; Zhang et al., 2022). Once in the sewer system, the viral concentration is reduced to about $10^2 – 10^{6.5}$ copies per liter due to dilution with sewage (Twigg & Wenk, 2022). In some cases, viral shedding of SARS-CoV-2 can still be detected up to 10 weeks after the clearance of symptoms and negative throat swabs of an individual (Zhang et al., 2022). The SARS-CoV-2 RNA can survive in untreated wastewater for several days, depending on the environment's temperature and other factors. It can persist for approximately 10 days at 37 ℃ and 30-60 days at a temperature of 4 ℃ (Ahmed et al., 2022). While the infection risk of SARS-CoV-2 in wastewater is negligible, virus monitoring in wastewater presents an opportunity to use a wastewater-based epidemiology approach to determine the viral abundance in a community (Twigg & Wenk, 2022; Zhang et al., 2022).

2.2.2 History of Wastewater-Based Epidemiology

Wastewater-based epidemiology is an environmental surveillance approach that was first implemented to analyze chemical residues in wastewater to measure a population's consumption and exposure to certain chemicals, such as illicit and pharmaceutical drugs (Zhang et al., 2022).

For instance, a recent study in Spain and Portugal was performed to test wastewater samples for illicit drugs (amphetamine, methamphetamine, ecstasy, cocaine and cannabis) as well as licit substances of abuse (alcohol and tobacco) (Estévez-Danta et al., 2022). Another example is from the United States, where Bishop et al. (2020) tested wastewater for illicit drugs. WBE's use has been further extended to monitor the epidemiology of different viruses (Shah et al., 2022). For instance, Allemann et al. (2021) and Bero et al. (2022) have used WBE to test for poliovirus in Haiti and Mozambique, respectively. Chacon et al. (2021) used wastewater surveillance to test for several human enteric viruses, such as rotavirus, norovirus, enterovirus and hepatitis A in Costa Rica. Similarly, in China, wastewater surveillance was performed to test for norovirus and astrovirus, and in the United States to assess a hepatitis outbreak (Lin et al., 2021; McCall et al., 2021). Monitoring of sewage for the detection of pathogens in wastewater has been used for over 40 years all over the world and has helped to understand that small outbreaks and epidemics within a community can be predicted through wastewater surveillance (Shah et al., 2022). WBE can be a useful tool for monitoring public health by providing real-time or near real-time data (Zhang et al., 2022). Furthermore, it might predict the prevalence of certain diseases better than clinical data from hospitalizations can, as some infections do not require hospitalization or asymptomatic cases are present (Shah et al., 2022). For the ongoing pandemic, WBE is used to monitor the appearance and resurgence of COVID-19 (Shah et al., 2022). It allows for tracking the infections and trends within a community (CDC, 2022a). Furthermore, targeted sites can be screened to determine which sub-population to target for clinical testing and where to implement mitigation strategies (CDC, 2022a; Shah et al., 2022).

2.2.3 WBE and Clinical Surveillance as a Complementary Surveillance System

One of the challenges experienced during the COVID-19 pandemic has been the high demand for clinical testing, both PCR and serological testing (Shah et al., 2022). High coverage in a timely manner proved difficult during peak infection periods. WBE has been used as a complementary approach for clinical surveillance systems to maximize the probability of detecting COVID-19 cases in the community (Zhang et al., 2022). The advantage of wastewater surveillance is the inclusion of pre- and asymptomatic individuals, infected individuals not seeking medical help, or infected individuals in settings with low accessibility to clinical tests (Ahmed et al., 2022; Shah et al., 2022). It can therefore predict the overall abundance of SARS-CoV-2 in a particular catchment area more precisely. A study found that the virus persists longer and with a greater viral load in feces than it does in respiratory samples; therefore, the likelihood of viral RNA detection is higher in wastewater than in clinical surveillance (Shah et al., 2022; Wang X. et al., 2020). Screening a large population with only a few samples makes WBE a cost-effective and rapid approach (Ahmed et al., 2022; Shah et al., 2022). Using WBE at a building-specific or sub-catchment level can identify COVID-19 transmission clusters (Ahmed et al., 2022). Consequently, more focused mitigation and containment strategies can be applied. Overall, the results of wastewater surveillance can lead to the encouragement of individual testing, the establishment of clinics in certain areas, implementation of lockdowns or mandates for face masks, social distancing and capacity limits (Ahmed et al., 2022).

2.3 Sample Collection

2.3.1 Sampling Locations

WBE is a novel approach for detecting SARS-CoV-2 in a community. Sample collection, concentration, extraction and detection methods therefore vary. Various sampling locations that differ in catchment area size can be used for wastewater surveillance. Sampling from the influent of wastewater treatment plants is commonly done due to their large catchment area and easy access (Kumar et al., 2020; Prado et al., 2021; Westhaus et al., 2021; Wu et al., 2020; Wurtzer et al., 2022). The prevalence in an entire community or municipality can be predicted with only a few samples. On the other hand, the larger the catchment area is, the more likely the sample will be diluted from areas with no to low prevalence or due to stormwater inflows. Therefore, many research teams have decided to sample at a smaller scale, either at the neighborhood- or building-level, which can detect local disease transmission clusters that may be missed when sampling from WWTPs (Ahmed et al., 2022; Barrios et al., 2021; Sharkey et al., 2021). Sampling upstream of a treatment plant at maintenance holes or pumping stations may provide a better understanding of how SARS-CoV-2 infections are distributed within the sewershed (CDC, 2022a). Mitigation actions resulting from the data can therefore be more localized. The building-level sampling approach is popular for hospitals, university and college campuses (Betancourt et al., 2021; Gibas et al., 2021; Harris-Lovett et al., 2021; Sweetapple et al., 2022; Wilson et al., 2020). Active cases are detected through positive samples, which allow to strengthen public health measures. Stricter capacity limits, mask policies, physical distancing and isolation rules may be implemented as a result of the building-level wastewater surveillance. Raw sewage has been tested most commonly, but some studies have also focused on testing the virus at various stages of wastewater treatment plants (Balboa et al., 2021; D'Aoust et al., 2021; Foladori et al., 2022; Yanaç et al., 2022).

Furthermore, factors such as wastewater characteristics that can influence the SARS-CoV-2 detection should be considered when selecting a sampling location. Wastewater characteristics are more variable upstream from wastewater treatment plants as residence times are shorter, less mixing occurs and population varies (Amoah et al., 2022; CDC, 2022a; Kevill et al., 2022; Sweetapple et al., 2022). When selecting a sampling location, the type of sewers is also important, as combined sewers will have dilution from stormwater inflow and infiltration. Therefore, separate sewer systems are preferred. In addition to storms and other weather-related events, inflow from industrial or agricultural settings can cause dilution (Ahmed et al., 2022).

2.3.2 Sampling Methods

Besides sampling location, sampling methods vary across the research community, which may influence the measured viral concentration (Zhang et al., 2022). Grab, composite and passive samples are the most common approaches, each with its advantages and disadvantages. Grab samples are collected at one location at one point in time. Composite samples can either be multiple grab samples combined or can be collected using an autosampler set up over a longer period of time, commonly 24 hours (Ahmed et al., 2022). Autosamplers are either battery-operated or can be plugged in. A tube is placed into the wastewater flow to retrieve wastewater periodically, which is collected in a composite container/bottle. Passive samples are collected by placing an absorbent material into the wastewater flow and retrieving it after a few hours or days (Ahmed et al., 2022). A research team in Halifax has developed and tested a passive sampler specifically for detecting SARS-CoV-2 (Hayes et al., 2021).

As the viral RNA is shed sporadically into the sewer system in varying amounts, either continuous sampling or multiple samples throughout the day are necessary to estimate the daily viral load (Ahmed et al., 2022). Grab samples, as a discontinuous approach, may lead to inconsistent

results due to temporal changes in viral concentrations throughout the day (Zhang et al., 2022), either by producing false negatives by sampling at a time when infected individuals are not shedding or by having disproportionately high concentrations due to infected individuals using washroom facilities right at the time of sampling (Ahmed et al., 2022; Twigg & Wenk, 2022). The amount of lateral mixing of wastewater as well as the dispersion volume plays a significant role in that. If no to little mixing occurs, the SARS-CoV-2 RNA will remain at the concentration which it was discharged. On the other hand, if mixing occurs, the RNA will be diluted with the sewage and possibly be below the limit of detection, depending on the dispersion volume (Ahmed et al., 2022). The size of the catchment area also influences the type of sampling. The smaller the catchment area, the more intermittent the viral signal, and the more useful are continuous composite and passive samples (Ahmed et al., 2022). Grab samples should only be used at locations where wastewater is homogenized, and dilution is minimal (Ahmed et al., 2022). Either way, collecting a larger number of samples increases the probability of detecting positive samples (Ahmed et al., 2022). Rather than sampling many grab samples, which requires more time and resources, a composite sample may be preferred. Composite samples average the results of multiple aliquots in a given time period and therefore may provide a more accurate representation of average concentration across a longer time frame (Twigg & Wenk, 2022; Zhang et al., 2022). Passive samplers can be useful in small catchment areas where more fluctuations in signal strength are expected, as they accumulate the virus over time (Ahmed et al., 2022). Compared to autosamplers, they are much easier to install, lower in cost, and can be employed at low wastewater flow (Ahmed et al., 2022). Their disadvantage is the lack of quantification of the viral RNA per volume of wastewater and the limited knowledge about the viral absorbing mechanism (Ahmed et al., 2022).

2.4 Sample Concentration and RNA Extraction

Due to the dilution of pathogens in wastewater resulting in significantly lower concentrations, wastewater samples need to be concentrated for SARS-CoV-2 detection (Ahmed et al., 2022; Twigg & Wenk, 2022). However, most concentration methods used in WBE were developed for non-enveloped enteric viruses (Ahmed et al., 2022; Twigg & Wenk, 2022; Zhang et al., 2022). Their physiology and capsid structures significantly differ from enveloped respiratory viruses, which can affect the efficiency of these concentration methods (Ahmed et al., 2022). Common concentration methods for SARS-CoV-2 that have been evaluated to date are ultrafiltration, polyethylene glycol (PEG)-based separation, ultracentrifugation, skim milk flocculation and electronegative filtration (CDC, 2022a; Zhang et al., 2022). Within these methods, each laboratory also experiments with variable sample volume amounts used, filter sizes, centrifugation speeds, etc. Medima et al. (2020) were one of the first researchers to test for SARS-CoV-2 in wastewater samples in the Netherlands using an ultrafiltration method (Medema et al., 2020). First, larger particles such as debris and bacteria were removed by using centrifugation (Medema et al., 2020). Approximately 100-200 mL of the supernatant was then filtered through Centricon Plus-70 centrifugal ultrafilters (Medema et al., 2020). Since then, several studies have been performed to test various concentration methods for SARS-CoV-2 detection. Kevill et al. (2022) tested three different concentration methods, including polyethylene glycol precipitation (PEG), ammonium sulphate precipitation (AS) and a type of ultrafiltration method (CP select^{TM} Innova Prep® (IP)). They found no significant differences in viral copies with any of the three methods (Kevill et al., 2022). However, they did find that the viral recoveries were influenced by turbidity levels (suspended solids load), surfactant load and storage temperature (Kevill et al., 2022). Kitamura et al. (2021) also performed a study where they tested different concentration methods, including

electronegative membrane adsorption, polyethylene glycol precipitation, ultrafiltration and solid precipitation (Kitamura et al., 2021). Besides the solid precipitation method, all methods used the supernatant of the initial centrifugation, which resulted in lower viral copy numbers compared to the solids precipitation method suggesting the adsorption of the virus to the solid fraction (Kitamura et al., 2021). Various factors play into the decision of which concentration method to select. Depending on the sample type, some concentration methods may work better than others. For example, when using sludge samples rather than untreated wastewater samples, centrifugation is the most effective method to concentrate the sludge solids (CDC, 2022a). Membrane filtration methods are slow due to their filtration rates and centrifuge-based methods may have a volume constraint, which is why sample volume can be a deciding factor as well (CDC, 2022a). Outside of the sample characteristics, supply chain issues might dictate what concentration methods can be used (CDC, 2022a). Methods relying on commercial filtration products tend to have a problem with supply availability. Some concentration methods are more time-consuming, especially when wastewater samples have high turbidity. If constraints on the availability of laboratory personnel exist and processing times are preferred to be minimized, faster concentration methods may be selected (CDC, 2022a). Furthermore, financial and laboratory equipment constraints can influence the decision on which concentration method to use (CDC, 2022a). Selecting a concentration method needs to be done under the careful consideration of several factors.

Regardless of what concentration method is chosen, the concentrated viral SARS-CoV-2 RNA needs to be isolated from the sewage before molecular detection methods can be applied (CDC, 2022a). Wastewater is a complex mixture of constituents that interfere with molecular viral quantification methods (CDC, 2022a). Various commercial kits are available for the isolation of

the RNA that are specifically designed to purify nucleic acid from environmental samples (CDC, 2022a).

2.5 Methods of Detection

2.5.1 Measurement Methods for Clinical and Wastewater Surveillance

Following sample collection and preparation, various detection methods can be applied to qualify or quantify SARS-CoV-2 viral levels. The prevalence of COVID-19 in a community is determined both by clinical testing and wastewater surveillance. Clinical surveillance uses serological, molecular, or point-of-care detection methods. Wastewater surveillance, on the other hand, only uses molecular detection methods. Serological methods test for antibodies in a patient's serum or plasma and are therefore sometimes referred to as antibody testing (Zhang et al., 2022). The serological test identifies the antibody immune response of past or current infections rather than identifying the virus itself (Eftekhari et al., 2021; Zhang et al., 2022). Because of the nature of the test, it can not detect the infection at the beginning stages when antibodies have not formed yet (Zhang et al., 2022). For quick testing, several rapid antigen tests have been developed that can also be used by individuals to test themselves at home (Zhang et al., 2022). They indicate infection when viral proteins (antigens) are detected in respiratory tract samples (Zhang et al., 2022). Results can be achieved in 15 to 30 minutes (Zhang et al., 2022). The accuracy of these tests remains challenging, as many antigens develop only after several days of infection (Eftekhari et al., 2021). More reliable is molecular detection, which detects the RNA of the SARS-CoV-2 virus in the samples. Molecular detection is used both for clinical testing and wastewater surveillance. Results are reliable, but machines are costly and the process is time-consuming. Several different molecular methods based on the principles of polymerase chain reaction have been developed. For clinical purposes, isothermal nucleic acid amplification, nucleic-based metagenomic sequencing, and most commonly, reverse transcriptase polymerase chain reaction (RT-PCR) have been applied. For wastewater surveillance, reverse transcription digital PCR (RT-dPCR), reverse

transcription droplet-digital PCR (RT-ddPCR), or reverse transcription quantitative polymerase chain reaction (RT-qPCR) are used. The RT-qPCR method is the most widely applied method.

2.5.2 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a molecular technology that amplifies deoxyribonucleic acid (DNA) fragments. It was developed by Nobel laureate Kary Mullis in the 1980s (Applied Biological Materials Inc., 2022). Nucleic acid amplification is used in many research fields, such as DNA sequencing, DNA fingerprinting, medicine, forensics, diagnostics and detection of bacteria and viruses (Applied Biological Materials Inc., 2022; Bio-Rad, 2022). To begin the DNA amplification process, the viral nucleic acid of the collected sample first needs to be concentrated and extracted (Twigg & Wenk, 2022). Then amplification reactions are set up with PCR reagents, primers and probes that target specific nucleic acid sequences (Bio-Rad, 2022; Twigg & Wenk, 2022). The amplification process consists of 20 to 40 thermal cycles to replicate DNA (Applied Biological Materials Inc., 2022). The target sequence doubles with each thermal cycle resulting in exponential amplification (Applied Biological Materials Inc., 2022). The amplification begins with an initialization step and is then followed by three steps of thermal cycling: denaturation, annealing and elongation (Figure 2) (Applied Biological Materials Inc., 2022; Zhang et al., 2022). During initialization, the reaction is heated to 94-96℃ for approximately 2-10 minutes to activate the DNA polymerase and reagents and denature contaminants (Applied Biological Materials Inc., 2022). In the first step of thermal cycling (denaturation), hydrogen bonds between the DNA strands are broken due to high temperatures (94-98℃ for 20-30 seconds), creating single-stranded DNA (Applied Biological Materials Inc., 2022). During the annealing step, primers bind to the complementary sequence of the DNA template to guide the DNA polymerase. The optimal annealing temperature is dependent on the melting temperature of the primers used but is

approximately between 50-60℃ (20-40 seconds) (Applied Biological Materials Inc., 2022). After the primers establish a starting point, the DNA polymerase starts to incorporate deoxynucleotide triphosphates (dNTP's) in a 5' to 3' direction on the single DNA strands (Elongation) (Applied Biological Materials Inc., 2022). The result is a newly synthesized DNA strand complementary to the template strand. The temperature and extension time depends on the type of DNA polymerase enzyme and the target amplicon (Applied Biological Materials Inc., 2022). The commonly used Taq DNA polymerase works ideally at 72-78℃ (Applied Biological Materials Inc., 2022). After the last cycle, there is a final elongation step for 5-15 minutes at 72-78℃ to ensure that any remaining DNA strand is fully extended (Applied Biological Materials Inc., 2022). The instrument's software analyzes the final data. The amplification efficiency depends on several factors such as assay sensitivity, sample matrix and reagent concentrations (Twigg & Wenk, 2022).

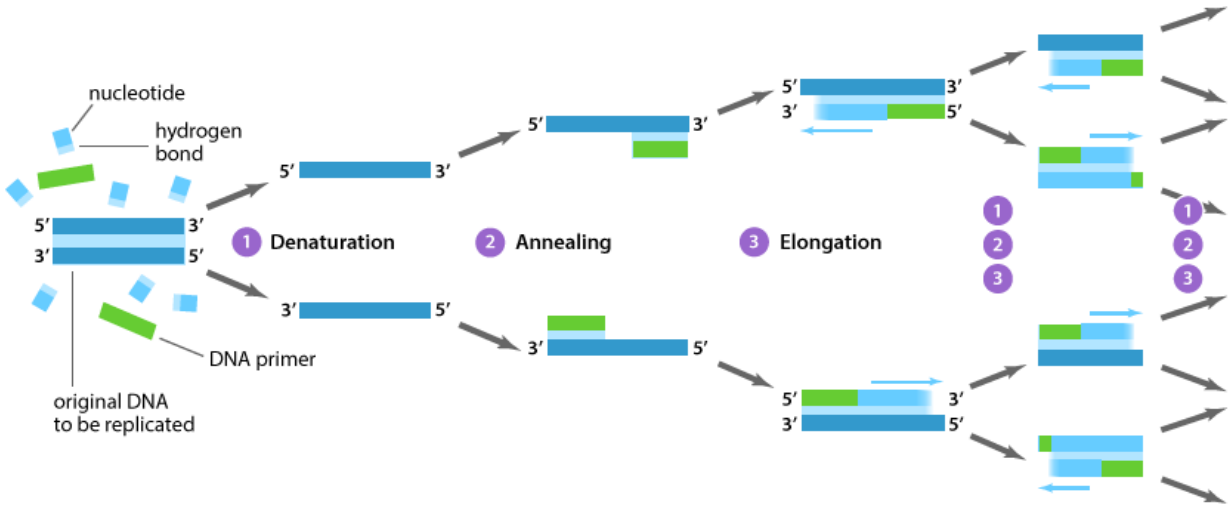


Figure 2: PCR Amplification Process including Denaturation, Annealing and Elongation (Applied Biological Materials Inc., 2022)

Some applications of PCR only require qualitative nucleic acid detection, while for others, it is essential to do quantitative analysis. Real-Time PCR can be used for both qualitative and

quantitative analysis (Bio-Rad, 2022). In real-time PCR, the amplification product is quantified after every thermal cycle in real-time. In comparison, conventional PCR only detects the amplified DNA product at the end of the process (Bio-Rad, 2022). Real-time PCR utilizes fluorescence signal that is emitted by specific probes or DNA binding dyes during the process, such as the TagMan probe and the SYBR Green methods (Applied Biological Materials Inc., 2022). As the data is collected after every cycle, the software generates an amplification plot in real-time (Applied Biological Materials Inc., 2022; Bio-Rad, 2022). The number of thermal cycles is displayed on the x-axis and the fluorescent signal on the y-axis (Figure 3). The fluorescent signal is proportional to the amount of amplified DNA (Bio-Rad, 2022). During the exponential phase, the PCR product doubles with each cycle, increasing the fluorescence signal. As the reaction proceeds, reaction components are eventually fully consumed. The reaction then slows down and reaches a plateau (Bio-Rad, 2022). During the first few cycles, fluorescence signals are below the detection limit and are shown as a baseline (Applied Biological Materials Inc., 2022; Bio-Rad, 2022). The cycle number at which the signal crosses the threshold value, which is given by the software or user, is called quantification cycle (Cq) (Bio-Rad, 2022). A low Cq value indicates a high amount of targeted nucleic acid is present, while a high Cq value indicates the opposite (Applied Biological Materials Inc., 2022; Bio-Rad, 2022). This correspondence allows relative quantification of DNA in a sample. Together with the use of standards, absolute quantification can be performed (Applied Biological Materials Inc., 2022). Software results are in units of copies per reaction or copies per reaction volume and should be converted to virus concentration per volume of unconcentrated wastewater samples for ease of comparison between samples (CDC, 2022a).

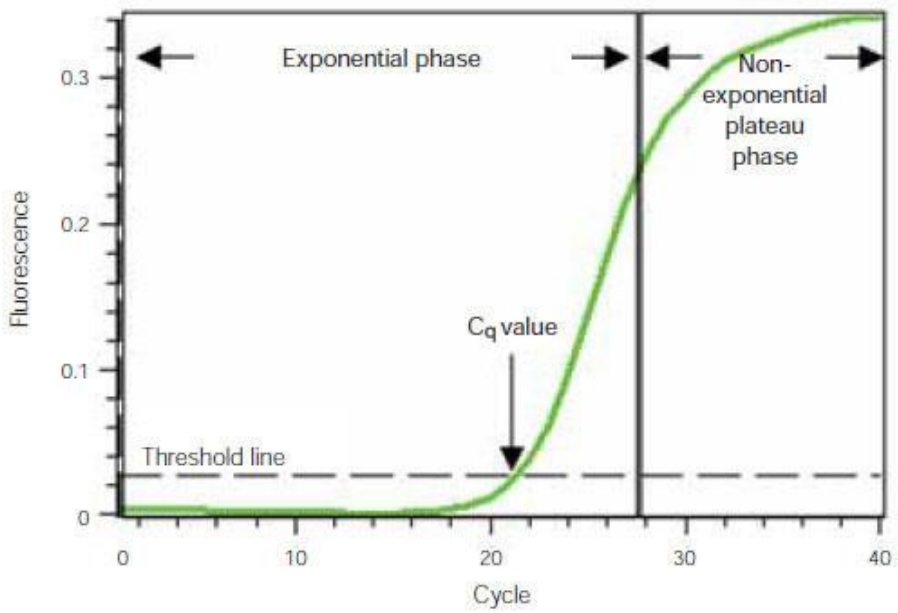


Figure 3: Amplification Plot (Bio-Rad, 2022)

For the detection of SARS-CoV-2, RT-qPCR has been most frequently used, both in clinical diagnostic settings and for wastewater surveillance (Twigg & Wenk, 2022; Zhang et al., 2022). RT-qPCR is used when the starting material is RNA; in this case, SARS-CoV-2 RNA. The RNA is first transcribed into complementary DNA (cDNA) by a reverse transcriptase (RTase) enzyme, which is then used as the template for the qPCR reaction (Applied Biological Materials Inc., 2022). The benefit of the RT-qPCR technique is the high sensitivity to low input RNA quantities (Applied Biological Materials Inc., 2022). There are two different types of RT-qPCR, which are the one-step and two-step RT-qPCR. For the one-step, the reverse transcription and PCR reaction occur in a single tube (Applied Biological Materials Inc., 2022; Bio-Rad, 2022). The advantage is that due to no transfer between tubes, contamination is limited, and it is less expensive and less time-consuming. It requires gene-specific primers and is therefore used when a known gene is targeted, such as in SARS-CoV-2 studies (Applied Biological Materials Inc., 2022). For the

two-step, the two reactions occur in two separate tubes (Applied Biological Materials Inc., 2022; Bio-Rad, 2022). One of the advantages here is that a greater range of RNA can be converted into cDNA. Additionally, primers from the first tube can be removed to avoid primer dimers present in the PCR reaction in the second tube (Applied Biological Materials Inc., 2022). On the other hand, the risk of contamination is increased due to the required transfer between tubes (Applied Biological Materials Inc., 2022). Both approaches have very similar protocols but only differ in the number of tubes and primers used (Applied Biological Materials Inc., 2022). RT-dPCR and RT-ddPCR are also based on the principles of RT-PCR and are available as 1-step or 2-step reactions. Digital PCR has been suggested as an alternative to RT-qPCR as it does not rely on standard curves and might be less prone to inhibition (Ahmed et al., 2022). There is yet no agreement on which detection method is better. Some studies have found digital PCR to be more sensitive in clinical samples, but additional studies are needed to determine sensitivity differences in wastewater samples (Ahmed et al., 2022). The detection of SARS-CoV-2 may be more influenced by the individual assay design rather than the detection technology (Ahmed et al., 2022).

2.5.3 Target Genes

Besides different types of samples and processing procedures, wastewater surveillance studies also vary in targeted genes selected for SARS-CoV-2 detection. As previously mentioned, the SARS-CoV-2 genome consists of several structural and non-structural proteins, which provide several possible targets for molecular detection methods. The CDC recommends using primers and probes that target the nucleocapsid genes (N1, N2 and N3) of the SARS-CoV-2 virus. The most commonly used targets are N1 and N2 (Barrios et al., 2021; Betancourt et al., 2021; D'Aoust et al., 2021; Nagarkar et al., 2022). Some studies also use the E protein for detection (Corman et

al., 2020; Domokos et al., 2022; Wurtzer et al., 2022). Other targets used in studies are the S gene, RdRp or ORF1ab region (Domokos et al., 2022; la Rosa et al., 2020; Wu et al., 2020). A study by Kumar et al. (2020) tested ORF1ab, N and S genes, which all detected similar levels of SARS-CoV-2 in the wastewater samples taken from wastewater treatment plant influent (Kumar et al., 2020). Arora et al. (2020) performed a study at the beginning of the pandemic using various gene targets. They had greater positive results using the E and N targets than the ORF1ab region, RdRp and the S gene (Arora et al., 2020). For comparison of sample results, the same target genes should be used as sensitivity differs (CDC, 2022a).

2.6 Laboratory Controls

Several levels of control can be introduced into wastewater surveillance research work that are essential to compare SARS-CoV-2 RNA wastewater concentrations over time and from different sources (CDC, 2022a). A matrix recovery control, also called process control, allows to determine viral recoveries or how much virus is lost during sample processing (CDC, 2022a). To determine viral recovery efficiencies, known amounts of exogenous virus controls are seeded into the wastewater samples and measured after all processes have been performed (Ahmed et al., 2022). Ideally, the control virus should have similar biological and physical characteristics as the SARS-CoV-2 virus (Ahmed et al., 2022). A research gap still exists due to the uncertainty of similarity between exogenous controls and endogenous SARS-CoV-2 in terms of structural form and partitioning behaviour (Ahmed et al., 2022). Furthermore, each surrogate virus might be influenced differently by various wastewater characteristics. SARS-CoV-2 surrogates are usually enveloped viruses, but non-enveloped viruses have been used as an alternative due to their common use as indicator quality control viruses in wastewater treatment (Twigg & Wenk, 2022). Controls that have been used in SARS-CoV-2 studies include murine hepatitis virus (MHV), bovine Coronavirus (BCoV), human coronavirus 229E (HCoV-229E), human coronavirus OC43 (HCoV OC43) or bacteriophage MS2 (Balboa et al., 2021; Graham et al., 2021; Kumar et al., 2020; Nagarkar et al., 2022; Wurtzer et al., 2022). MHV, BCoV, HCoV-229E and HCoV-OC43 are all enveloped positive-sense single-stranded RNA viruses such as the SARS-CoV-2 virus. MS2 is a bacteriophage with a non-enveloped positive-sense single-stranded RNA.

Another control tool is normalization, which is used to account for changes in wastewater characteristics, dilution (e.g., stormwater), or human fecal input over time (e.g., due to tourism or weekday commuters) (CDC, 2022a). Most commonly, SARS-CoV-2 concentrations are

normalized with the Pepper Mild Mottle Virus (PMMoV), which is highly abundant in wastewater as a fecal indicator. PMMoV can also be detected using RT-qPCR using a specific primer and probe set. Other normalization factors include, but are not limited to, flowrate, turbidity levels, and solids content. There is no consensus on the best normalization approach, which is why different research groups use different methods, making comparison between studies again difficult.

Due to wastewater being very complex and possibly containing compounds that could interfere with the amplification process, an inhibition assessment should be performed (CDC, 2022a). For that, the concentration of either the SARS-CoV-2 RNA or another spiked RNA should be compared to a dilution of that same virus (CDC, 2022a). If concentrations are as expected based on the dilution factor, no inhibition occurred. If inhibition occurs, the extraction step may need to be repeated, or extracts need to be diluted (CDC, 2022a). If PMMoV is measured for normalization purposes, it can also be used as an inhibition control by comparing its concentrations to its dilution.

In order to detect contamination of samples, negative controls are introduced to the process. A possible negative control is an extraction blank made by extracting RNA without adding any wastewater. They should be included in every extraction protocol to detect any potential contamination. A similar control, the non-template control, can be included in the PCR process. A non-template control is a well in the PCR plate that does not have the extracted wastewater sample but only the molecular reaction reagents. It should be included in every qPCR run in duplicates or triplicates to detect contamination. If no contamination is present, these reactions should not amplify at all.

2.7 Influences on Detection

Wastewater surveillance has its challenges due to low concentrations in wastewater samples caused by the dilution of feces in wastewater. Dilution can be caused by variations in daily water discharge, stormwater, or external inflow such as from industry or agriculture. But dilution might not be the only factor that challenges the detection of SARS-CoV-2 RNA. Several studies have touched on the importance of wastewater characteristics influencing SARS-CoV-2 detection, but knowledge about the presence and fate of SARS-CoV-2 in municipal wastewater is still limited.

The temperature of wastewater is a topic that has been discussed in several research papers. In general, the virus persists longer in wastewater with lower temperatures, approximately 10 days at 37 °C and 30-60 days at a temperature of 4 °C (Ahmed et al., 2022; Zhang et al., 2022). Many protocols developed for wastewater surveillance suggest short-term storage at 4 °C and long-term storage at up to -80°C. Other parameters and their influence on detection are lesser known. Amoah et al. (2022) published a study on the effect of selected wastewater characteristics on the estimation of SARS-CoV-2 viral load in wastewater using the Adaptive Neuro-Fuzzy Inference System (ANFIS). Considered wastewater characteristics were chemical oxygen demand, flow rate, ammonia, pH, permanganate value, and total solids (Amoah et al., 2022). They found a strong correlation between viral levels, ammonia, and pH (Amoah et al., 2022). Increased ammonia concentrations were associated with increased viral concentrations (Amoah et al., 2022). The highest SARS-CoV-2 concentrations were detected in wastewater samples with a pH between 7.1 and 7.4 (Amoah et al., 2022). They also found total solids content to influence RNA concentration but not consistently across all their sampled wastewater treatment plants (Amoah et al., 2022). Kevill et al. (2022) performed a controlled experiment to test the effect of turbidity on viral detection as a suspended solid load indicator. They used different concentration methods that

resulted in different levels of viral concentrations when turbidity levels differed (Kevill et al., 2022). Their precipitation method, using the solids fraction to concentrate viral RNA, yielded higher viral recoveries than their ultrafiltration-based method that concentrates viral RNA in the liquid phase (Kevill et al., 2022). They also tested different levels of surfactant load, which yielded similar results, with the precipitation method yielding higher recoveries (Kevill et al., 2022). They concluded that in environments with high turbidity and surfactant levels, precipitation methods should be used rather than ultrafiltration methods (Kevill et al., 2022). Graham et al. (2021) did not directly study the influence of wastewater characteristics but tested samples from a wastewater treatment plant influent and its settled solids. The settled solids yielded 350 to 3100 times higher viral RNA concentrations compared to the influent (Graham et al., 2021). A relationship between SARS-CoV-2 concentrations and solids content seems apparent, suggesting that concentration methods using the solids phase of wastewater samples such as centrifugation and precipitation yield higher viral RNA concentrations. Ye et al. (2016) studied the survivability, partitioning, and recovery of enveloped viruses and found that enveloped viruses adsorb to solids particles in higher amounts compared to nonenveloped viruses (Ye et al., 2016). This would explain why solid-based concentration methods yield higher viral concentrations. SARS-CoV-2, an enveloped virus, adsorbs to solids particles present in wastewater.

The literature review presented above points to many knowledge gaps in wastewater surveillance of SARS-CoV-2. Numerous factors can impact the accurate detection of the viral RNA and thus determine the success or failure of surveillance efforts. It is necessary to have a better understanding of these factors, their mechanisms and impacts, and the appropriateness of the chosen sampling, concentration, extraction, and detection methods for a specific SARS-CoV-2 surveillance application. Methods may need to be modified or optimized for different surveillance

applications, and the reliability and the usefulness of the obtained information may differ. Based on the research needs identified in the literature review, this study aims to monitor the SARS-CoV-2 RNA levels from the residences, buildings, and the sewer of a Canadian university campus to cover a range of upper sewershed sampling locations and investigate the associations between SARS-CoV-2 viral signal and case numbers, campus events, long weekends, and holidays. The campus presents a unique opportunity as it has its own small sewer system with no wastewater or stormwater coming in from the City, which allows better control of the SARS-CoV-2 surveillance and prevents the city wastewater or stormwater from impacting the campus viral signal. In addition, different SARS-CoV-2 concentration methods were tested in this study to determine whether some work better than others based on the differences in sampling locations and sample characteristics. The impact of wastewater parameters (i.e., temperature, pH, turbidity, UV absorbance, total solids, and volatile solids) on SARS-CoV-2 detection was also investigated to understand better the impact of wastewater characteristics and changes over time. The literature review also showed that many SARS-CoV-2 surveillance studies were published without any or very little statistical analysis during the pandemic, which may explain some of the contradictory research findings reported. A complete statistical analysis of the collected data over a year was carried out to establish the statistical significance of the observed data, trends, and associations.

3. MATERIALS AND METHODS

3.1 Wastewater Sampling

The university has its own campus and sewer system that is loop-shaped. There is no incoming wastewater from the city and collected wastewater from the entire campus can be sampled from a maintenance hole just before it exits the campus (hereafter referred to as location A). This provides a major advantage in monitoring SARS-CoV-2 levels on campus as it allows sampling only the people on campus with no external contamination or stormwater from the city. Wastewater surveillance started in September 2020 and is still being used. Initially, only campus wastewater was sampled with weekly grab samples, and wastewater surveillance was quickly increased to twice a week sampling with autosamplers and expanded to the residence buildings (hereafter referred to as location B) in addition to the campus wastewater (location A). Residence buildings were sampled from maintenance holes located adjacent to them, and it was possible to isolate the wastewater of the selected residence buildings. Sampling at location A started in September 2020 with grab samples, and from February on, both locations A and B were sampled with 24-hr composite samples. Only when problems with autosamplers occurred, such as low flow rates and clogged lines, grab samples were taken. The residence building sampling location (location B) was changed as needed to accommodate for summer closures and test the residences with the highest occupancy. For this thesis, 31 samples were collected and analyzed for location A; location B consisted of 11 samples for residence A, 3 samples for residence B, and 21 for residence C. Composite samples were chosen over grab samples as they represent more accurately the daily load of the virus being shed into the sewer sporadically (Ahmed et al., 2022). Teledyne Isco 3710 (United States) portable autosamplers were set up to collect 24 h composite samples. At the beginning of the sampling period, the autosamplers were set up once a week. From June on,

samples were collected twice a week. The battery-powered (charged after every use) autosamplers were mounted inside the manhole with the placement of the steel strainer at the end of the vinyl suction tubing within the wastewater flow, as seen in Figure 4. The controller was programmed to pump 100 mL of wastewater every 15 min, resulting in a 9.6 L representative sample, which was shaken well before 1 L was aliquoted for processing. Sample collection and autosampler installation can be seen in Figure 5. All samples were transported to the laboratory on ice for immediate processing.

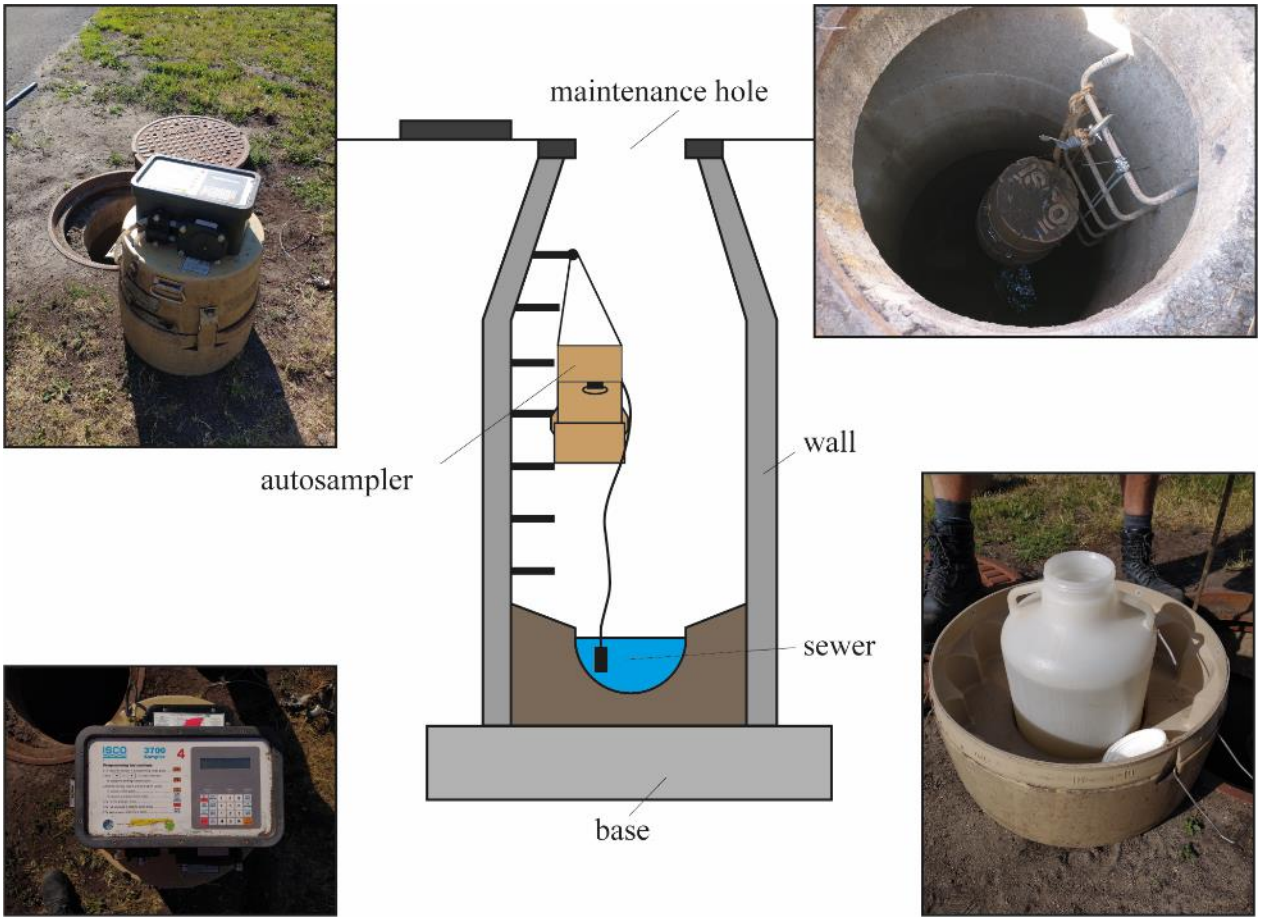


Figure 4: Autosampler Set-up and Installation (Teledyne Isco 3710 (United States))

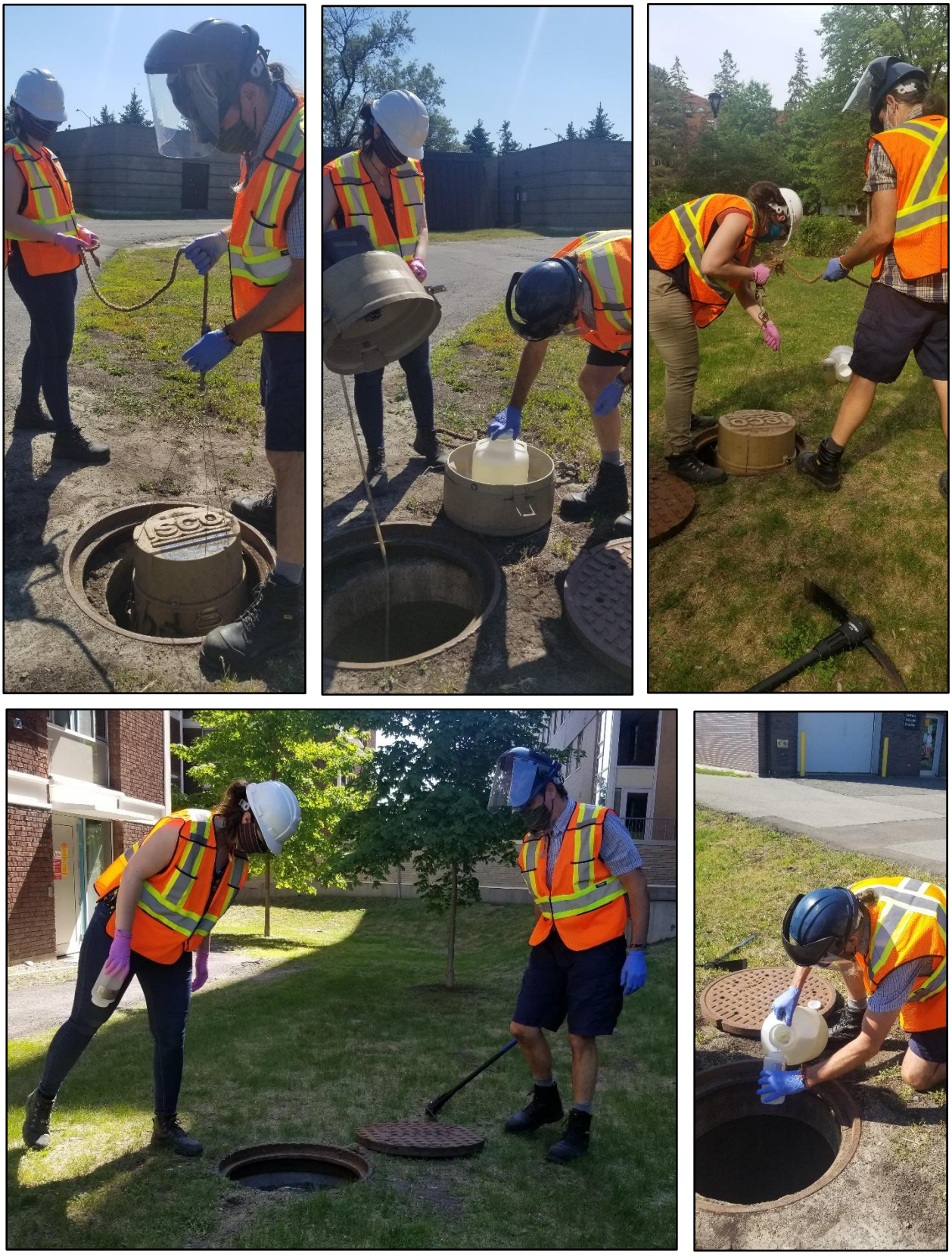


Figure 5: Sample Collection and Autosampler Installation on a Campus Maintenance Hole

3.2 Viral Concentration and RNA Extraction

Two different concentration methods were used in this study to analyze the liquid and solids fractions of the wastewater samples. The liquid fraction was concentrated using an ultrafiltration apparatus (Centricon Plus-70) with a pore size of 10 kDa (hereafter referred to as the ultrafiltration method). A 1 L of raw wastewater was well-mixed before aliquoting 200 mL into a 250 mL Nalgene centrifuge bottle. The samples were centrifuged at 4654g for 30 min (Thermo Scientific; Sorvall Legend RT+ Centrifuge). The supernatant was transferred to a sterile bottle without disturbing the pellet. A 70 mL portion of the centrate was then filtered with a Centricon Plus-70 at 3500g for 30 minutes. The process was repeated until all centrate was filtered through the unit. The filtrate was removed from the unit, and RNA was extracted. The protocol for solids analysis comprised concentrating 30 mL of well-mixed raw wastewater sample in a 50 mL conical centrifuge tube (FroggaBio TB50-25 Centrifuge tubes) (hereafter referred to as the centrifugation method) with a swing bucket rotor at 4280g for 20 min (Beckman Coulter; Avanti J-15R Centrifuge). The supernatant was carefully removed with a 25 mL disposable pipette (Celltreat Scientific TD-EX 20℃ Serological Pipet). The remaining solids were transferred to a 2 mL tube (Sarstadt AG&Co. KG Micro Tube) and centrifuged at 20,000g for 2 min (Thermo Scientific; Sorvall Legend Micro 21 Centrifuge). The supernatant was removed, and the resultant pellet (<250 mg) used for RNA extraction. All RNA extractions were done with the Qiagen RNeasy PowerMicrobiome RNA Extraction Kit according to the manufacturer's instruction with some modifications. Deviations included the addition of carrier RNA (Millipore Sigma Poly (A) Polyadenylic acid) at 10 µg per reaction and an increase from 1 to 20% Beta-mercaptoethanol in the lysis step. As well, 250 µL IRS solution was used instead of 100 µL with an increased incubation time of 10 min. RNA extractions were kept at 4 ºC if qPCRs were performed

immediately and then transferred to -20 ºC for long-term storage. All centrifugation steps were carried out at 4 ºC.

3.3 Molecular Detection of SARS-CoV-2

To quantify the SARS-CoV-2 RNA, one-step reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed using the BioRad CFX96 and CFX Opus thermocyclers and analyzed with the Maestro CFX software. The nucleocapsid genes N1 and N2 were used to identify the SARS-CoV-2 virus (CDC, 2020). Wastewater samples often contain substances that can inhibit the amplification process, ultimately resulting in delayed Cq and inaccurate quantification of the target (Gibas et al., 2021; Schneider et al., 2009). Therefore, RT-qPCR assessment of Pepper mild mottle virus (PMMoV) was performed as an inhibition control (Rosario et al., 2009). All test samples, standard curves, non-template controls (NTC), and blank extraction controls (negative control) were done in duplicate for each run. Each 20 µL reaction contained 5 µl Bio-Rad 4X Reliance One-Step Multiplex Supermix; 1.5 µl CDC RUO primers and probes (IDT Inc.); and either the sample (template), the standard, or the controls. Each reaction volume was adjusted with RNase-free water (Sigma). For gene copy quantification, the standard curves were generated initially with linearized plasmid (IDT) and were later shifted to using Strings DNA Fragments (Thermo Fisher Scientific). The standard curves had a limit of detection (LoD) at 95% confidence between 1 and 16.3 copies/reaction for the N1 and N2 gene. For PMMoV, the RNA extractions were diluted (1/10) and run alongside the main sample to observe the effect on Cq values. Each 20 µL reaction contained 5 µL 4X Reliance One-Step Multiplex Supermix, 500 nM each primer and 125 nM probe with the balance RNase free water. The standard curves were initially done with serially diluted gBlock (IDT) and later Strings DNA Fragments (Thermo Fisher Scientific). The standard curves for the linearized plasmid had an LoD at 95% confidence between 3.5 and 7.5 copies/reaction and the standard curves for the Strings DNA Fragments had an LoD between 3.8 and 7.8 copies/reaction. The thermal cycling protocol for all RT-qPCR runs consisted of 50 °C for

10 min for cDNA synthesis, Taq polymerase activation for 10 min at 95 ℃ followed by 45 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s. All primer and probe sequences, as well as the sequences for standard curves, are listed in Table 3 in Appendix A.

3.4 Viral Recovery

In this study, the virus recovery for the concentration methods was determined using the exogenous internal control (IC) MS2 bacteriophage (MS2). The IC should be easy to produce, easy to standardize, stable, and absent from the samples (Dreier et al., 2005). MS2 fits all but one of these criteria; it is present at trace amounts in wastewater but at levels ~1000 fold lower than our recovery spike. The MS2 was spiked into the wastewater samples before viral concentration to act as a recovery and internal process control. MS2 has been widely used as a surrogate for human enteric viruses in many studies on virus transport, disinfection, and fate (Dreier et al., 2005). The average percent recovery of MS2 spiked in the samples was 17.9% for the centrifugation method and 22.9% for the ultrafiltration method. Studies that also used MS2 as a surrogate in similar centrifugation processing methods reported recoveries of 36.0 ± 15.4% by Balboa et al. and a mean recovery of 57% by Kumar et al. (Balboa et al., 2021; Kumar et al., 2020). Various other surrogates have been applied as ICs with various recovery efficiencies, e.g. murine hepatitis virus (MHV) (6-33.5%), human Betacoronavirus-1 strain OC43 (0.2% to 4.3%), bovine coronavirus (BCoV) (0-75%), and HCoV-229E (Graham et al., 2021; Nagarkar et al., 2022; Wurtzer et al., 2022; Ye et al., 2016). Each surrogate virus behaves differently in wastewater and might be influenced differently by wastewater characteristics. Furthermore, each surrogate virus may have different partitioning characteristics (D'Aoust et al., 2021). SARS-CoV-2 and MHV are, for instance, abundant in the solids fraction of wastewater unlike nonenveloped enteric viruses (Alamin et al., 2022). For concentration methods including the solid phase, enveloped viruses might be better surrogates (Alamin et al., 2022). This study started processing with the ultrafiltration method, and therefore MS2 was spiked into the samples as it is known to partition more into the liquid phase. For consistency reasons, the use of MS2 for all samples in this study was continued, both for

ultrafiltration and centrifugation processed samples. There is no unified method or surrogate virus yet, and therefore a direct comparison of different studies is difficult.

3.5 Wastewater Characterization

The wastewater samples were characterized by measuring temperature, pH, turbidity, UV absorbance, and total and volatile solids. Once the samples were collected, the temperature was taken with an infrared thermometer; pH was measured with the Thermo Scientific Orion 5 Star pH Meter. For turbidity measurements, the samples were thoroughly mixed, transferred into a sample cell, and then inserted into the Hach 2100AN Turbidimeter (Figure 6). Readings were performed in three technical replicates for both biological replicates. The sample was then filtered through a 0.45 µm cellulose filter (Millipore S-Pak membrane Filters: 0.45 µm, 47 mm) (Figure 7). The filtrate was transferred into a 1 cm quartz sample cell and measured with the Jenway 6850 UV/Vis Spectrophotometer, which performed a full absorbance scan from 190 to 800 nm. Each sample was done in duplicate with two technical replicates each. To determine the total solids content, 50 mL of each sample was transferred with a disposable pipette (Celltreat Scientific TD-EX 20 ℃ Serological Pipet) to preweighed 75 mL dishes (Fisherbrand Aluminum Weighing Dishes) in duplicate. The dishes were then placed into a 105 ℃ oven (Barnsteadt Lan-Line L-C Oven) (Baird et al., 2017). After 24 h, they were weighed to determine the total solids content (TS). Subsequently, the dishes were placed into a furnace (Vulcan A-550 Ney) at 550 ℃ for 15 minutes and then weighed (Baird et al., 2017). The process was repeated until the change in weight was equal to or less than 0.5 mg (Baird et al., 2017). The solids lost to ignition were determined as volatile solids (VS).

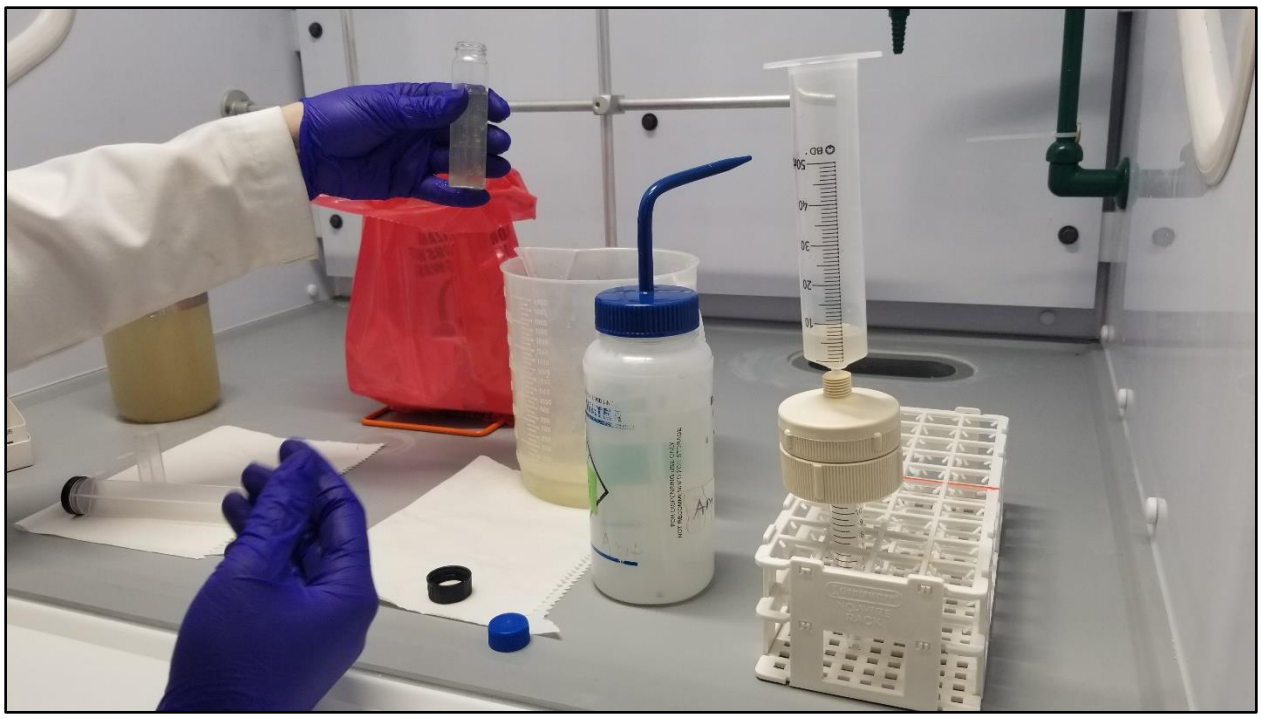


Figure 6: Turbidity Vial with Wastewater Sample

Figure 7: Preparation of Filter Unit for Absorbance Characterisation

3.6 Statistical Analysis

Statistical analysis using Pearson correlation was performed to examine the relationship between wastewater signals (both raw and PMMoV normalizd) and reported cases during noteworthy case spikes, as well as to investigate correlations between viral copies and wastewater parameters (TS, VS, turbidity, absorbance at 190 and 254 nm) for both processing methods (centrifugation and ultrafiltration). Furthermore, a statistical comparison of campus wastewater to the city wastewater, campus case numbers to the city case numbers, and campus wastewater to the city cases was included. Pearson correlation measures the statistical relationship, or association between two continuous variables, by determining the strength and direction of the correlation of the two variables. Data reflecting daily new cases at the city-wide, campus-wide, and residence-specific levels were used for statistical analysis between March 24 and August 4, 2021. In order to perform the analysis, daily case counts were aggregated in four ways: total cases 1 week (6 days) prior to, 3 days prior to, the week of (3 days before and after) and 1 week (6 days) after wastewater sample collection. Interpretation of the strength of Pearson correlation values include weak (< 0.29), moderate (0.30-.49), strong (0.50-0.80), and very strong (> 0.81). Correlations were deemed significant when $p < 0.05$ and were reflected in the tables as $*p < 0.05$, $** p < 0.01$. Nonsignificant correlations were interpreted as no correlation. Non-detects in viral data were treated as zero values for statistical purposes.

4. RESULTS AND DISCUSSION

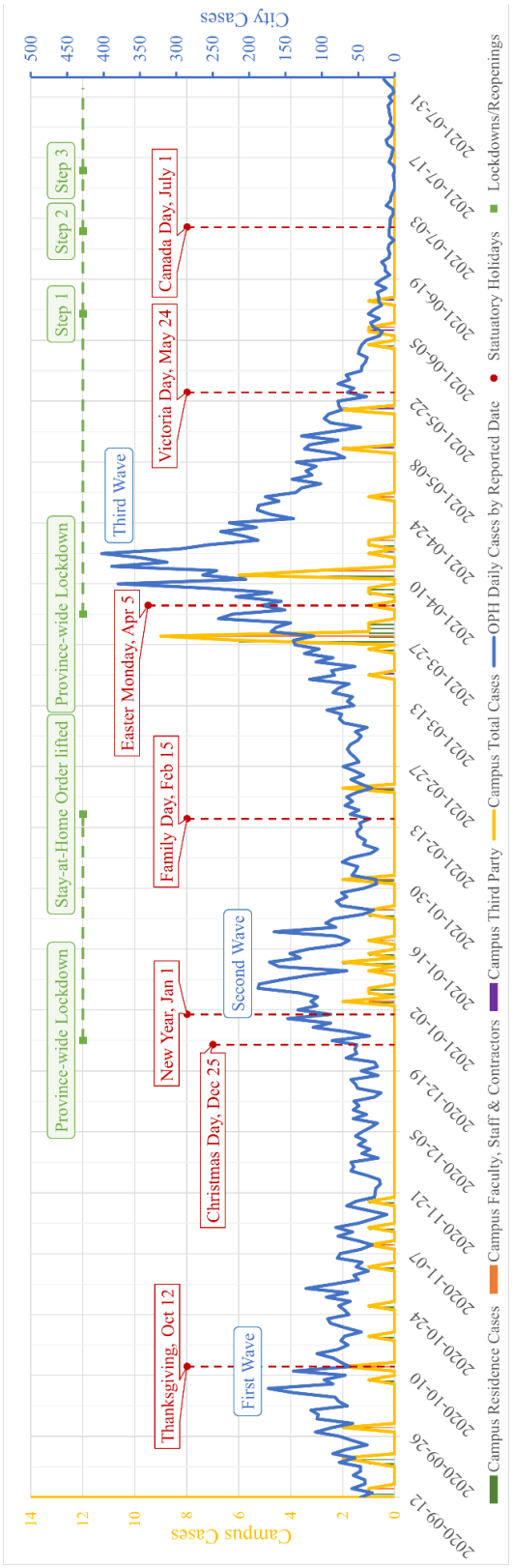


Figure 8: Reported University Campus Cases Compared to the Reported City Cases

4.1 Campus Cases in Comparison to City Cases

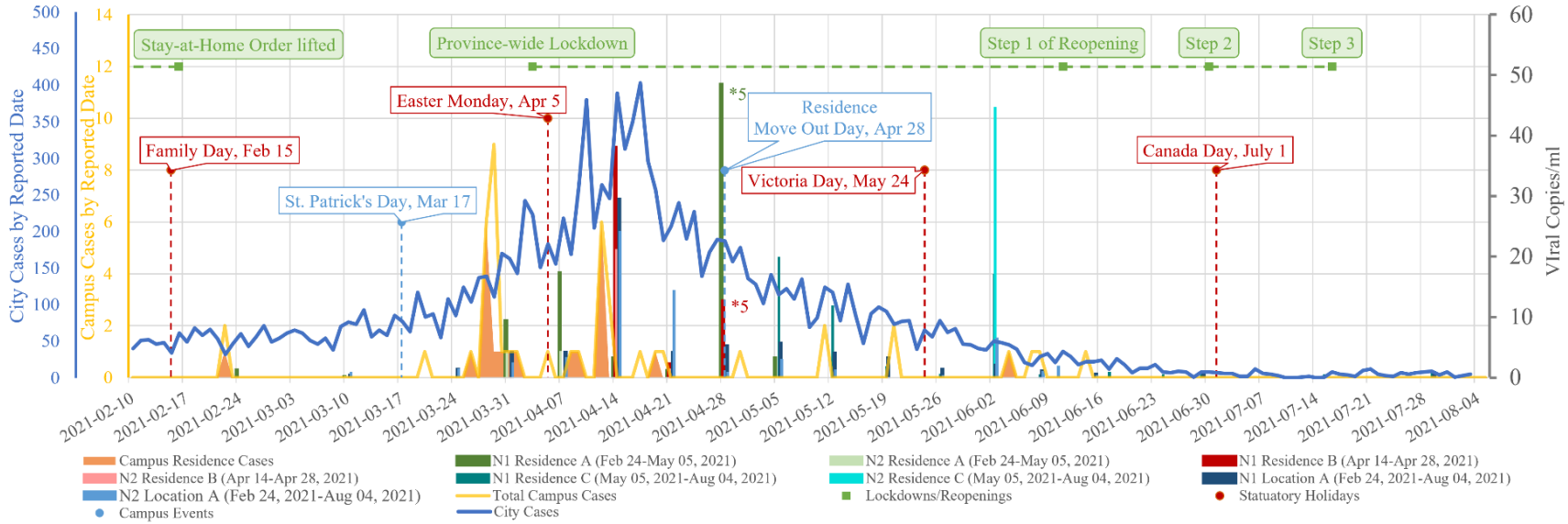
To put reported cases on campus in perspective, they were compared to the development of cases in the city. Case numbers were received from the local public health unit. Since September 2020, the city has seen a three-wave pattern of cases (Figure 8). The cases started rising in the middle of September and peaked on October 7, 2020 with 174 reported cases. Consequently, the city moved back to a stricter tier (modified Stage 2 tier) of the province-wide restriction plan on October second. The cases decreased during October and November but increased again by December. On December 26, 2020, a province-wide lockdown was put into place because of a province-wide increase in cases. Following Christmas and New Year's Eve, cases reached a new peak with over 180 reported cases per day due to family gatherings over the holidays that increased the risk of spread. On February 16, 2021, after the Family Day long weekend, the stay-at-home order was lifted, and the city moved into the less strict orange-restrict zone, based on the province's colour-coded system, because of declining cases, possibly initiated by the strict regulations during the lockdown. The colour-coded framework included five different zones: green-prevent, yellow-protect, orange-restrict, red-control, and grey-lockdown. The third and highest peak was reached at the beginning of April 2021. The city had already moved into the red-restrict zone on March 19, 2021, because of the rising case numbers. On April 3, another province-wide lockdown was put in place which included a stay-at home order. Following Easter weekend, the peak of the third wave was reached with just under 400 cases reported on a single day. The high case numbers were attributed to gatherings with friends and family in addition to the more aggressive variants. At the time of the study, WHO had classified four variants of concern (VOC), Alpha, Beta, Gamma and Delta (Roy et al., 2021). The Alpha variant originated in September 2020 and spread worldwide (Roy et al., 2021; WHO, 2021). The first cases in Canada were seen in December.

The Beta strain was first identified in May 2020, before being detected all over the world (Roy et al., 2021; WHO, 2021). The Gamma strain surfaced in November 2020 (Roy et al., 2021; WHO, 2021) The delta variant, the most contagious among the listed variants, originated in October 2021 (Roy et al., 2021; WHO, 2021). The Alpha strain was the most common variant in Canada and was a major driver of the third wave of COVID-19 in January 2021(Duong, 2021). The drastic decrease in case numbers after April correlated with the stricter lockdown and high vaccination rates. The lockdown was lifted on June 11, 2021, when the city entered Step 1 of the Reopening Plan. The cases continued to stay low and prompted the city to enter Step 2 of the Reopening Plan on June 30 and Step 3 on July 16.

The cases reported on the university campus followed a very similar trend with three flare-ups of cases closely following the city's timeline. Even though all classes were online at the time, the campus still represented a microcosm of the city. During the city's first wave in October 2020, several cases were reported on campus at the end of September and all of October. After the classes were over, students left the residences resulting in no reported cases on campus during late November and December. Once in the New Year, when the students returned, several cases were reported, mirroring the city's second wave. The concurrence between students returning from the holidays with family gatherings and the increase in campus cases showed that the risk of spreading the virus was increased during these family events. Cases were additionally reported on February 22, 2021, following the Family Day weekend (February 15). The next increase in cases on campus closely followed the third wave of the city. Two high spikes on March 28 and March 29 were reported, as well as a spike on April 12 a week after Easter weekend. A few cases were reported until mid-June, followed by a long period with no cases reported. The number of students living

on campus decreased drastically during the summer term that started in early May. During the three waves, a relationship between long weekends followed by an increase in cases was apparent, suggesting that gatherings with friends and family happened against the advice of the public health units, causing increased spreading of the virus. For the return of students to campus in the academic fall term, the university made vaccination and wearing a mask at all times a requirement to limit the spread of SARS-CoV-2.

Pearson correlation was performed between city-wide, campus-wide, and residence-specific case counts during the third wave to show the relationship of cases described in the previous paragraphs. Correlations between city-wide and campus-wide case counts were strong for all types of case aggregation, $r(26)=0.51\text{-}0.69$, $p<0.01$, showing that the campus embedded in the city behaves similarly as the city (Appendix A, Table 4). The strongest associations were observed when cases were aggregated a week after sample collection. Correlations with residence-specific samples were nonsignificant likely because of the small number of observations.



*Ultrafiltration Method (Feb 24, 2021-Mar 24, 2021), Centrifugation Method (Mar 31, 2021 - Ongoing)

Figure 9: Viral Copies/mL of the N1 and N2 Gene Targets Compared to Reported Cases on Campus and the City

4.2 Campus Cases in Comparison to Wastewater Signal

The university established wastewater surveillance to be able to detect the presence of SARS-CoV-2 on campus in support of clinical testing, as it also accounts for individuals who are asymptomatic and/or those with mild symptoms that may not seek clinical screening (Sharkey et al., 2021). Figure 9 displays the measured viral copies/mL of the N1 and N2 genes from location A and location B (residences A, B, and C). Location B changed throughout the sampling period because of changes in residence occupancy during the summer terms. The measured viral copies were compared to the reported cases on campus. Composite sampling on campus started on February 24th, 2021, for location A and location B (residence A). The samples were processed with the ultrafiltration method to analyse the liquid fraction, while the centrifugation method for solids analysis was first introduced on March 31. Until June 8, both methods were run in parallel for comparison purposes, and afterwards only the centrifugation method was used due to better sensitivity. Separate figures for the results of the centrifugation and the ultrafiltration methods can be found in Appendix B (Figures 15 & 16). Additionally, the PMMoV data were used to normalize the SARS-CoV-2 viral gene copies detected. The PMMoV normalized signals are displayed in Appendix C (Figures 17-20). Statistical analysis was performed for both the raw data and the normalized data to determine which had a better correlation with the case counts. On April 14, residence B was added but monitoring was stopped after 3 weeks because of issues with low flow. On May 5, location B was changed to residence C, as the remaining 200 students of the summer term were moved to this building. The entire sampling period occurred during the third wave of cases and beyond (Figure 9).

Several associations could be observed among reported cases, events, long weekends, and the wastewater surveillance results, which are described below. Some outliers and discrepancies were

also noticed, which are likely due to the contribution of asymptomatic cases, varying shedding rates, and nonresidents (Betancourt et al., 2021). Samples taken at location A allowed us to observe the overall COVID-19 trends (from students, faculty, and staff) on the entire university campus. The desired outcome was low concentrations and nondetects. For location B (residence samples), the desired outcome was nondetects. Even low concentrations of the viral genetic material would indicate the presence of a COVID-19 case in the building, symptomatic or asymptomatic, and could come from the residence students or from visitors, including visiting students from other buildings. Because the university was closed to in-person classes, staff, contractors, and graduate students were mainly present in the academic buildings. As these groups typically reside off-campus, positive cases were less likely to correlate with wastewater signal on campus (location A) after confirmation, as they stop shedding on campus when they isolate at home. Campus policy required to report any positive clinical test result if the person was on campus any of the 14 days prior to the test result.

On February 22, 2021, one week after the Family Day weekend, new cases were reported on campus, including students in residence. Two days later, on the sampling day, 1.25 viral copies/mL were detected with the N1 gene at residence A. Location A measurements were 0.33 and 0.47 copies/mL for the N1 and N2 genes, respectively. The higher flow rates at location A due to collecting the wastewater of the entire campus caused higher dilution and, therefore, lower viral copies. On March 10, low concentrations of viral copies at residence A (N1 0.08 & N2 0.19 copies/mL) but higher viral copies at location A (N1 0.35 & N2 0.82 copies/mL) were measured, indicating the presence of COVID-19 in the academic buildings. New cases on March 26 and March 28 until April 1 were reported in the residence commons. It was suspected that residence students celebrated St. Patrick's Day (March 17) together and caused the outbreak of cases in

residence. In addition to the residence cases, several campus cases were reported on March 29. Those also included student athletes that lived off-campus, and the dates of their last presence on campus were March 22 and 24. Therefore, they were no longer shedding into the campus wastewater thereafter. The March 24 samples indicated low viral copy numbers at location A, supporting the theory that the student athletes were shedding in the wastewater on the last days on campus before their positive test results. One week later, days after the reported cases in residence, viral copy numbers of 9.39 and 4.16 per mL were detected for N1 and N2 in residence A, respectively. On April 7, an even higher concentration was measured with 17.34 (N1) and 4.25 (N2) viral copies/mL in residence A and 4.18 (N1) and 3.27 (N2) at location A, either as a result of the cases reported a week before, preceded shedding of the residence cases reported on April 8, 9 and 12 or due to asymptomatic cases. It is likely that the cases at the beginning of April were a result of gatherings at Easter weekend. On April 13, several cases were reported among faculty members, staff, and contractors. Samples taken on April 14 included the additional residence building B that measured the highest viral concentration yet with 37.99 (N1) and 21.09 (N2) viral copies/mL. Location A measured 29.44 (N1) and 24.06 (N2) copies/mL. These higher numbers coincided with the peak of the third wave in the city and were just days after the reported residence and campus cases. The week after, numbers were declining again, concurrent with the lower case numbers.

An example of the usefulness of wastewater surveillance was the move-out event on April 28, 2021, at the end of the winter academic term. Several measures (i.e., social distancing, masks, rapid antigen tests) were taken on the move-out date to minimize the risk of transmission. However, students received help from family and friends during move-out who had access to the residence buildings and washroom facilities. This resulted in a high spike of viral copies in the

residence buildings indicating the high circulation of the virus among the off-campus crowd. On the move-out day, residence A detected 242.11 (N1) copies/mL and 63.65 (N1) copies/mL were detected in residence B. University administration would not be aware of such an increased risk of transmittance without wastewater surveillance.

At the beginning of the summer term, the remaining students on campus were moved to residence C, and therefore the sampling location was moved to residence C. The new location measured 19.33 viral copies/mL a week after the move-out event suggesting the lingering effects of the move-out. Only a few clinical cases were reported in this timeframe, but not within the residence, suggesting the presence of asymptomatic cases in the residence. Another peak in viral copies was measured on June 2 in residence C with 17.01 (N1) and 44.69 (N2) viral copies/mL, which was observed nine days after the Victoria Day long weekend. Afterwards, low to no viral copies were detected, following the same declining trend of reported cases in the city and campus. Because of the declining trend of cases in the city, the university allowed summer camps for children on outdoor campus property. Washrooms inside the buildings were used, but no increase in viral copies was measured.

The wastewater surveillance proved to be helpful to the university administration in managing COVID-19 on campus and several decisions were made based on the wastewater surveillance results. Voluntary rapid antigen tests within residences were encouraged, social distancing was enforced on the entire campus, masks were made mandatory, and isolation in locations that were deemed high risk was enforced. Leveraging the rapid antigen testing coupled with the wastewater sampling provided early indicators before encountering outbreaks. Furthermore, university administration acted on the increased risk experienced at the move-out event and implemented new rules to prevent new infections caused by the move-in and move-out dates. This is particularly

important for the move-in events as infected students would remain in the residences and infect other students, potentially leading to new outbreaks in the residence buildings. Under the new rules, parents and friends were not allowed in the residences during move-in and move-out and were asked to remain in their vehicles. After the implementation of the new rules, no spikes in viral levels were observed in the wastewater samples from the residence buildings. Overall, wastewater surveillance provided actionable information and will be continued in the future.

Pearson correlations between wastewater signals (centrifugation method) and the aggregated case counts were examined to manifest the described observations. Significant correlations are reflected in Table 1. City-wide case counts significantly correlated with all wastewater signal samples to a moderate to strong degree, regardless of case count aggregation method, which underlines the potential of sampling a smaller community within a city to still predict case development within the entire city. The highest correlation was found for comparisons with cases aggregated the week of and the week after. This highlights the use of wastewater surveillance as an early indicator. Residence-specific samples did not significantly correlate with residence case counts. This is likely due to the small sample sizes observed from residence A ($n = 6$). The results for campus-wide case counts were more nuanced. Campus-wide displayed a moderate to strong correlation with both normalized N1 and N2 samples at all aggregation counts. The raw scores only correlated with case count when counts were aggregated prior to wastewater sample collection. The strongest correlation was found with the normalized N1 and N2 data and cases aggregated a week after sample collection, which again underlines the use of wastewater surveillance as an early indicator.

Table 1: Correlations between Wastewater Signals (Centrifugation Method) and Case Counts

		N1		N2	
	n	Raw	Normalized	Raw	Normalized
Campus-wide Case Count					
Week prior	26	0.55**	0.48*	0.49*	0.46*
3 days prior	26	0.50*	0.42*	0.43*	0.41*
Week of	26	0.53**	0.49*	0.45*	0.47*
Week after	26	–	0.68**	–	0.66**
Residence A Case Count					
Week prior	6	–	–	–	–
3 days prior	6	–	–	–	–
Week of	6	–	–	–	–
Week after	6	–	–	–	–
City-wide Case Count					
Week prior	26	0.72**	0.62**	0.77**	0.62**
3 days prior	26	0.77**	0.68**	0.78**	0.68**
Week of	26	0.79**	0.74**	0.78**	0.74**
Week after	26	0.74**	0.79**	0.72**	0.79**

Note. Significant correlations reflected as * $p < 0.05$, ** $p < 0.01$, and – reflects nonsignificant correlation

The campus is embedded in the city's community which was also sampled by this laboratory. SARS-CoV-2 results from samples of the wastewater treatment plant in the city were compared to the campus data to analyse whether similar trends could be observed, both for raw and PMMoV normalized viral signals (Appendix D, Figures 21 & 22). Limited sampling data were available to compare the city wastewater samples to campus wastewater samples from March 31 to August 4, 2021. Correlations between raw N1, raw N2, and normalized N1 and N2 samples (centrifugation method) were all nonsignificant between the city and campus wastewater samples in this time period, likely because of the difference in residence times and different weather-caused dilution effects in the two sewer systems. The city is a metropolitan area and has a very large sewer with the highest sewage residence times reaching 36 h. The city wastewater samples also contained much higher quantities of PMMoV compared to the campus, and therefore the trends between campus and city varied much more (Figure 22).

4.3 Campus Wastewater Characteristics (Location A)

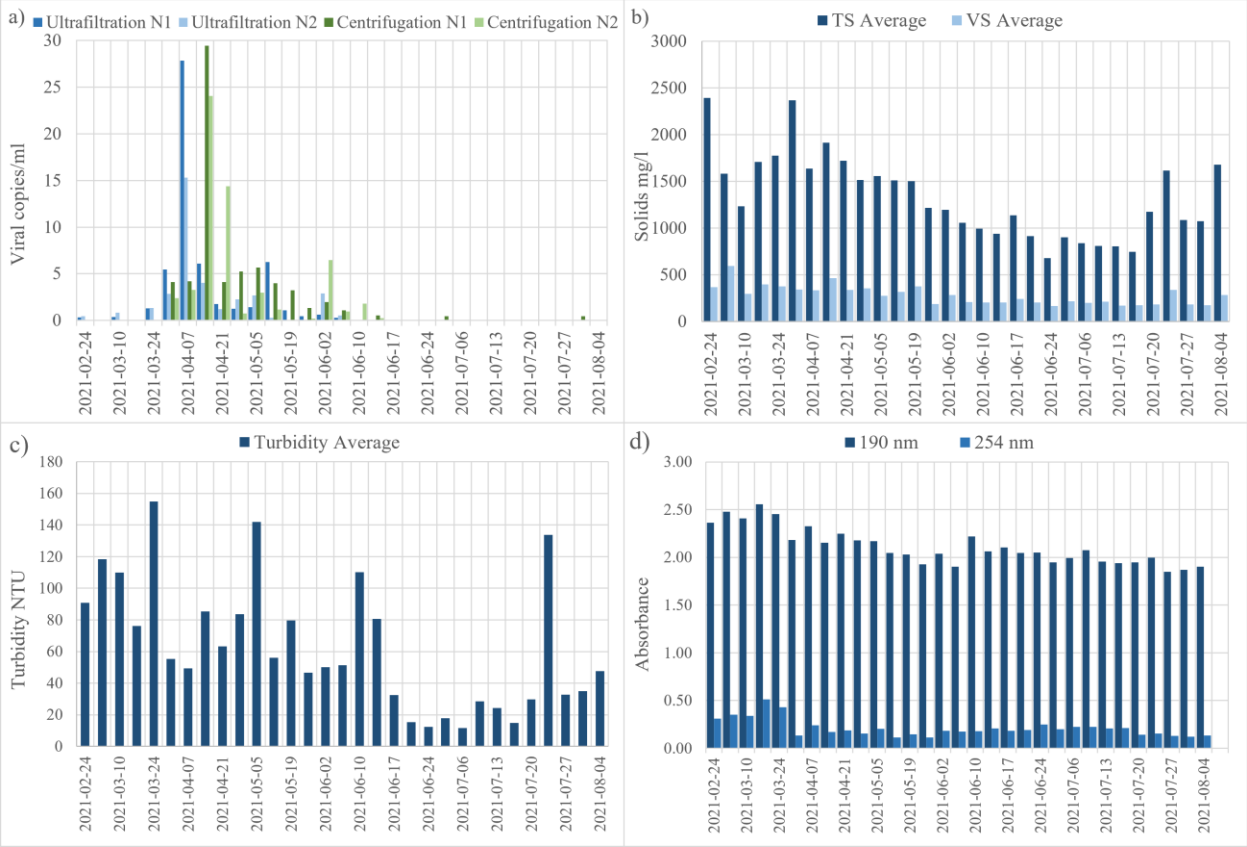


Figure 10: Wastewater Characteristics of Location A: a) Viral Copies N1 & N2 (Ultrafiltration & Centrifugation Method) b) Total and Volatile Solids c) Turbidity d) Absorbance (190 nm, 254 nm)

The persistence and the survival of SARS-CoV-2 in the sewer system are dependent on several factors, such as temperature and organic content (Anand et al., 2022). The virus and its RNA are exposed to a wide range of physical, chemical, and biological factors in the sewer system (Anand et al., 2022). Furthermore, wastewater characteristics can impact the efficiency of the concentration and extraction steps before qPCR and thus the detected viral signal. For these reasons, besides testing the wastewater samples for the viral genetic material, it is also important to analyse the physical and chemical wastewater parameters. Turbidity, total and volatile solids (TS & VS), as well as absorbance readings at wavelengths of 190 and 254 nm were measured to

investigate correlations with measured viral copies or trends over time (Figure 10). Temperature and pH data are provided in Appendix E (Figure 23 & 24). Absorbance scans from 190 nm to 350 nm for location A can also be found in Appendix E (Figure 25).

The highest measured viral copies at Location A with the ultrafiltration method were on April 7 with 27.86 copies/mL (N1) and 15.29 copies/mL (N2). With the centrifugation method, the highest viral copies were observed a week later, April 14, with 29.44 copies/mL (N1) and 24.06 copies/mL (N2) (Figure 10a), showing a difference in sensitivity of both methods. Sampling days before and after resulted in lower viral concentrations. The TS at the beginning of the sampling period up to the middle of May varied between 1232 and 2393 mg/L (Figure 10b). During the summer months, from May 25 to July 15, the TS content stagnated at a lower level between 680 and 1215 mg/L. Towards the end of the sampling period, the TS appeared to increase again. A similar trend could be observed with the VS, though the VS to TS ratio was consistently low with an average of 25.27% compared to typical raw wastewater (Figure 10b). This was suspected to be due to the construction work on campus that periodically dumped water into the sewer system, causing a high grit and sand content. The turbidity measurements did not show any particular trends (Figure 10c). Absorbance scans from 190 to 800 nm were performed to observe trends through the entire spectrum. Selected wavelengths indicating the presence of substances of interest were looked at in more detail to find a relationship to viral copies measured. Absorbance at 190 nm, the lower end of the spectrum, showed increased absorbance. At this wavelength, proteins are strongly absorbed (peptide bonds) and protein determination is often performed (Aitken & Learmonth, 2002). Absorbance at 254 nm is commonly used to characterize the organic constituents in a wastewater sample (Musikavong & Wattanachira, 2007; Szerzyna et al., 2017). Absorbance levels at the wavelength of 190 nm were slightly higher at the beginning of the sampling period. The same

could be observed with absorbance readings at 254 nm. From May on, the absorbance stayed at a consistently lower level (Figure 10d).

The association between wastewater parameters and viral signals was examined statistically for both sampling methods (centrifugation and ultrafiltration) at the campus-wide level (Table 2). Correlation with a significant difference could be observed for the raw and normalized viral signal with total and volatile solids for the centrifugation method. Correlation was also found for absorbance at 190 nm with all viral signals besides raw N1 signals. The ultrafiltration method only yielded a correlation between raw N1 and N2 data with absorbance levels at 190 nm. The raw N2 viral signal also correlated with absorbance from 254 nm. Turbidity values were too variable to have any statistical correlation with viral signals of either concentration method.

Table 2: Correlations between Viral Copies (Raw & PMMoV Normalized) and Wastewater Parameters by Processing Method for Location A

		N1		N2	
	n	Raw	Normalized	Raw	Normalized
Centrifugation Method					
Total solids	26	0.52**	0.45*	0.48*	0.43*
Volatile solids	26	0.71**	0.61**	0.64*	0.60**
Turbidity	26	–	–	–	–
Absorbance 190nm	26	–	0.45*	0.44*	0.45*
Absorbance 254nm	26	–	–	–	–
Ultrafiltration Method					
Total solids	11	–	–	–	–
Volatile solids	11	–	–	–	–
Turbidity	11	–	–	–	–
Absorbance 190nm	11	0.61*	–	0.68*	–
Absorbance 254nm	11	–	–	0.73*	–

Note. Significant correlations reflected as *$p < 0.05$, ** $p < 0.01$, and – reflects nonsignificant correlation

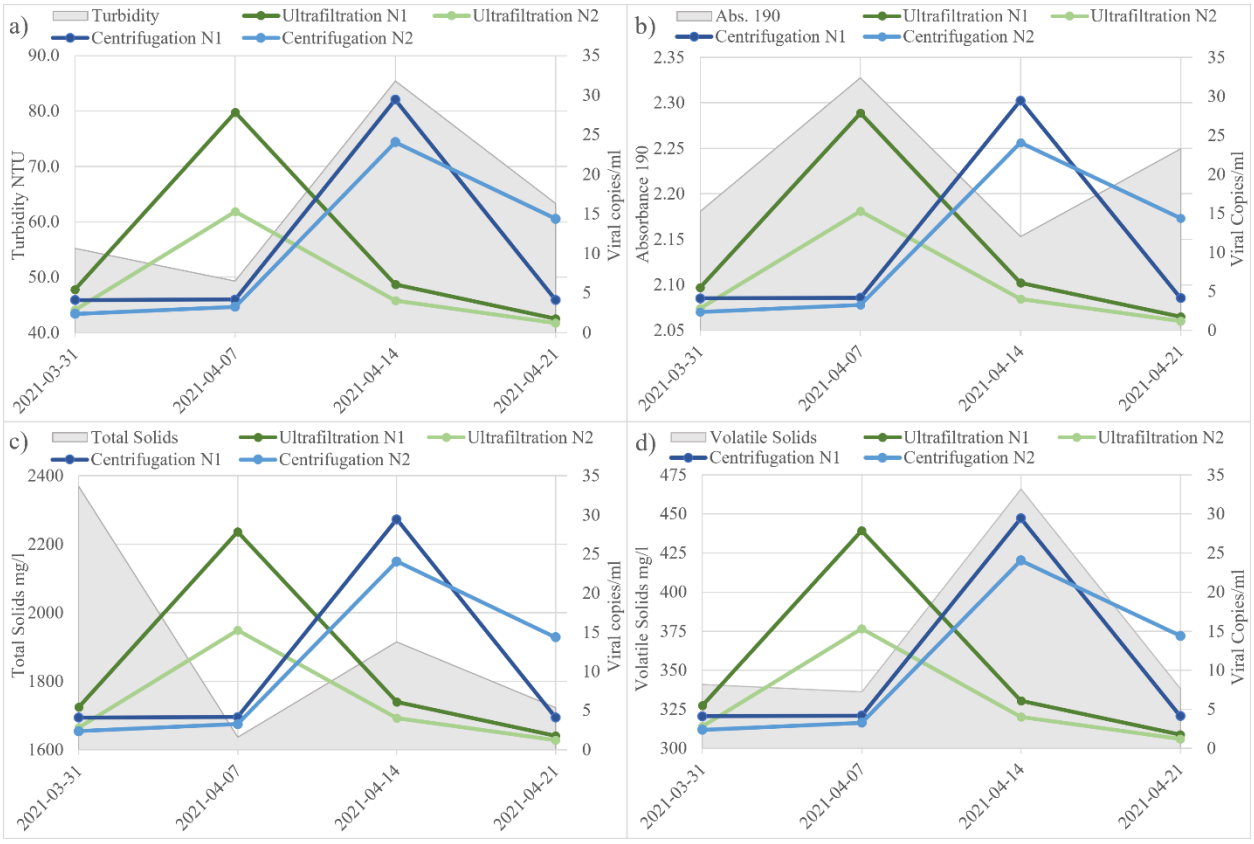


Figure 11: Comparison of Viral Copies to Wastewater Characteristics at Location A: a) Turbidity and N1&N2 b) Absorbance (190 nm) and N1&N2 c) Total Solids and N1&N2 d) Volatile Solids and N1&N2

To further investigate the relationship between the wastewater characteristics and the viral copies measured, it was necessary to closely analyze the timeframe with higher viral copies. The highest concentrations of the viral genetic material were measured between March 31 and April 21, 2021. Samples were taken weekly. On March 31, both methods quantified a similar number of viral copies at the lower end. On April 7, the ultrafiltration method yielded 27.8 viral copies/mL with the N1 gene and 15.2 viral copies/mL with the N2 gene. The centrifugation method, on the other hand, resulted in low values with 4.18 (N1) and 3.27 (N2) viral copies/mL. On April 14, the opposite was observed. The centrifugation method peaked with 19.4 (N1) and 24.0 (N2)

copies/mL, while the ultrafiltration method yielded low values with 6.09 (N1) and 4.04 (N2) copies/mL. On April 21, values for both methods decreased, with the centrifugation method still yielding higher numbers. To understand the difference in results between the ultrafiltration and centrifugation methods used for the liquid and the solids fractions, the viral copies were compared to the wastewater parameters. The turbidity was lower (49.3 NTU) on the day the ultrafiltration method yielded a higher value, and the values were much higher (85.5 and 63.3 NTU) when the centrifugation method yielded higher numbers (Figure 11a). The same trend could be observed with the total solids content with 1636 mg/L TS on April 7 (ultrafiltration method peak) and 1915 mg/L on April 14 (centrifugation method peak) (Figure 11c). These results suggest that the virus has a tendency to attach to solid particles, and when the TS content increases, more viruses attach to the solid particles, and therefore the centrifugation method is more sensitive and yields higher results. On the other hand, when the TS content is low, much of the viruses stay in the liquid fraction of the wastewater, and therefore, the ultrafiltration method captures higher numbers of viral copies. This is also manifested by the correlations found between viral data and total solids content presented in Table 2. Moderate to strong correlation was found between viral signals of the centrifugation methods and total solids, but no correlation was found with results from the ultrafiltration method. The viral RNA in the sample from March 31 was too low to be influenced by the TS content. The comparison with volatile solids captured the same trend and confirmed the findings (Figure 11d). The absorbance reading at the wavelength of 190 nm showed the opposite trend (Figure 11b). At high absorbance readings, the ultrafiltration method yielded high values and at low absorbance readings, the centrifugation method yielded higher values of the viral genetic material. One earlier study concluded that enveloped viruses such as mouse coronavirus, murine hepatitis virus (MHV), and the bacteriophage Phi6 partitioned to a larger extent to wastewater

solids in the influent of wastewater treatment plants than non-enveloped bacteriophages MS2 and T3 (Ye et al., 2016). The results of the study suggested that wastewater solids may contain coronaviruses at concentrations 1000 times those found in the influent on a per mass basis (Ye et al., 2016). Monitoring of the solids could therefore lead to more sensitive detection of the enveloped SARS-CoV-2. Another study compared SARS-CoV-2 results of influent samples to samples from settled solids of a wastewater treatment plant during an outbreak (Graham et al., 2021). Solids yielded 350 to 3100 times higher concentrations than the influent. Measuring the SARS-CoV-2 RNA concentrations in the settled solids appeared to be the more sensitive approach than measuring SARS-CoV-2 RNA in the influent (Graham et al., 2021).

4.4 Residence Wastewater Characteristics (Location B)

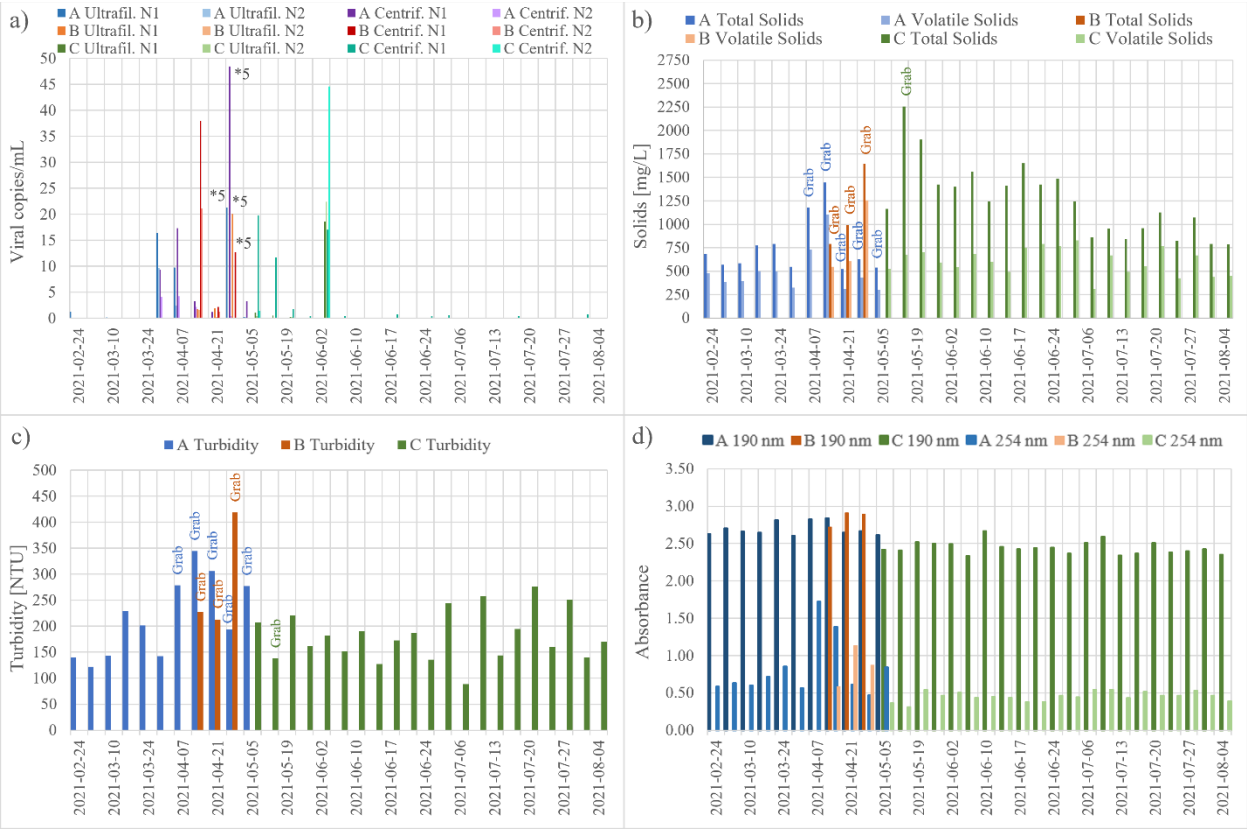


Figure 12: Wastewater Characteristics of the Campus Residences A, B & C: a) Viral Copies N1 & N2 (Ultrafiltration and Centrifugation Methods), b) Total and Volatile Solids, c) Turbidity, d) Absorbance (190 nm, 254 nm)

The same analysis was performed for the wastewater samples collected from campus residences (Figure 12). Considerable viral gene copies were detected from March 31 to June 2 during the third wave of cases in the city (Figure 12a). The highest values were measured on April 28, when the students moved out of the residence and many of their parents used the washroom facilities on campus. The centrifugation method yielded 242.11 copies/mL with the N1 gene, while the ultrafiltration method yielded 106.31 copies/mL (N1) for residence A. Residence B yielded 63.65 copies/mL (N1) and 100.07 copies/mL (N1) for the centrifugation and ultrafiltration method,

respectively. Compared to the campus wastewater, the residences generally yielded higher values, as sampling was performed right at the source. Once the wastewater reaches the sewer, it is more diluted with the wastewater coming from other residences and academic buildings and potentially stormwater depending on the weather.

Other research has shown that water quality differs significantly between sampling sites. Samples taken directly or close to the source vary more drastically than samples taken further away where the wastewater of several buildings is combined (Sharkey et al., 2021), which is supported by the findings of this study. The samples taken at the residences have higher variability than those taken from location A, as shown by the results of wastewater characterization. The total solids content at residence A was mainly within the range of 540 to 790 mg/L, with an average of 65.1% volatile solids. Outliers were on April 7 and April 14 with 1176 and 1447 mg/L, though these samples were grab samples because of issues with the autosampler. For the 3 weeks residence B was sampled, the TS increased from 790 to 1635 mg/L. The average VS content was 69.0%. Residence C yielded more inconsistent TS content. In May and June, the TS was on average 1447 mg/L ignoring the grab sample on May 12th with 2254 mg/L, while the TS in July and August was lower with an average of 902.3 mg/l. The VS was on average 50.3%, which was lower compared to the other two residences (Figure 12b). The turbidity values were variable throughout the whole sampling period in all residences (Figure 12c). Overall, higher turbidity values could be observed in April, simultaneous to higher viral gene copies. It should be noted that issues with the flow and autosampler occurred during the same time frame and grab samples were taken instead. Absorbance readings overall were consistent except for a few outliers (Figure 12d). Residence A yielded higher absorbance at 254 nm on April 7 and 14 with 1.72 and 1.38, respectively, compared to an average of 0.65. This coincides with the higher TS and VS values on the same dates.

Statistical analysis performed for residence A specific data did not show any correlation besides total solids with normalized N2 (centrifugation method) viral signals. This underlines the high variability in wastewater samples at the residence locations.

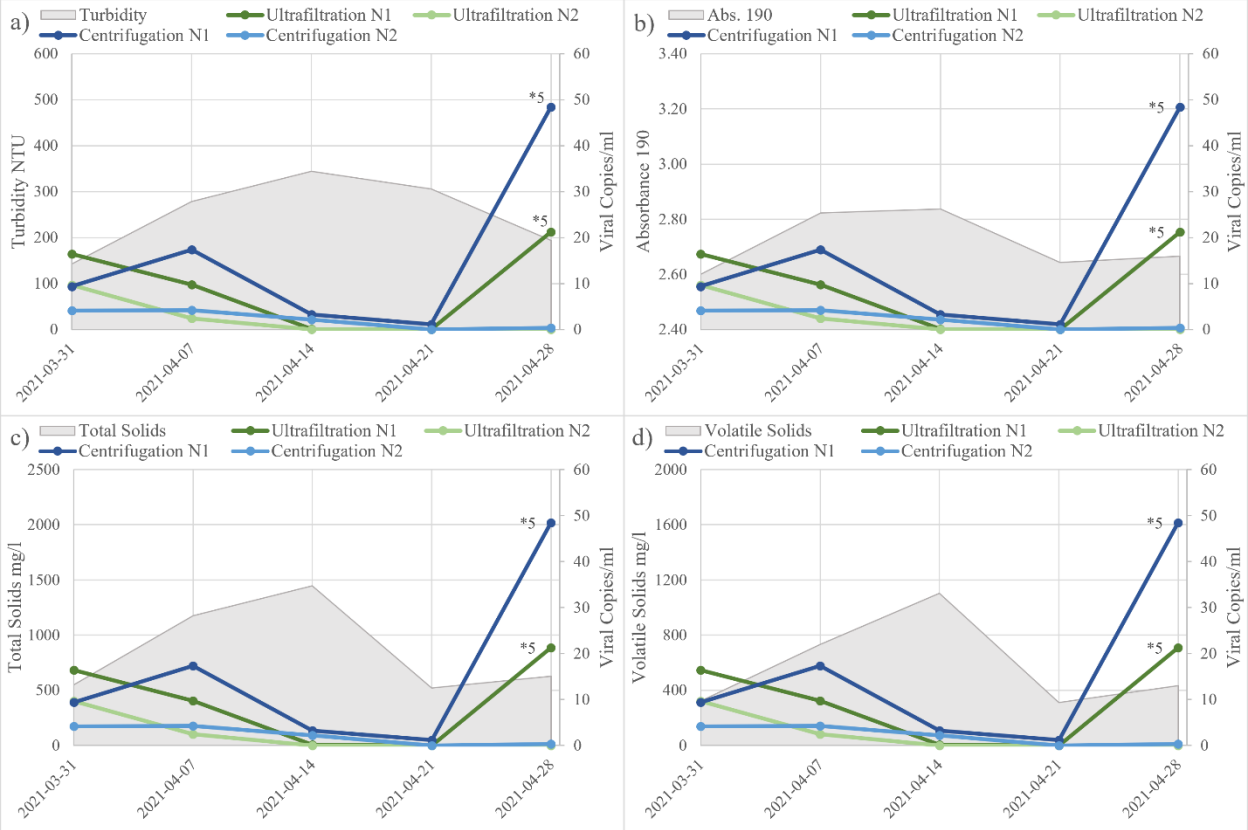


Figure 13: Comparison of Viral Copies (identical in all subplots) to Wastewater Characteristics at Residence A: a) Turbidity and N1&N2 b) Absorbance (190 nm) and N1&N2 c) Total Solids and N1&N2 d) Volatile Solids and N1&N2

Residence A was sampled during the third wave, and therefore, a closer look at the viral copies and the wastewater parameters was done for the dates from March 31, 2021, to April 28, 2021 (Figure 13). As previously mentioned, samples were not as well-mixed right at the source, and therefore, the data were more variable. The correlations found between the wastewater parameters and the viral copies at location A were not observed for the residence samples. Higher TS content

was not accompanied by higher viral copies using the centrifugation method for solids analysis. The viral copies obtained with both methods were overall lower compared to location A, with the exception of the move-out day on April 28, where the number of people using the facilities increased drastically. The variability within the N1 and N2 genes of both methods was higher; as a result, no notable difference between the ultrafiltration and centrifugation methods was observed. On April 7 and April 14, all wastewater parameters measured had increased values, but no valuable change in the viral copies was detected.

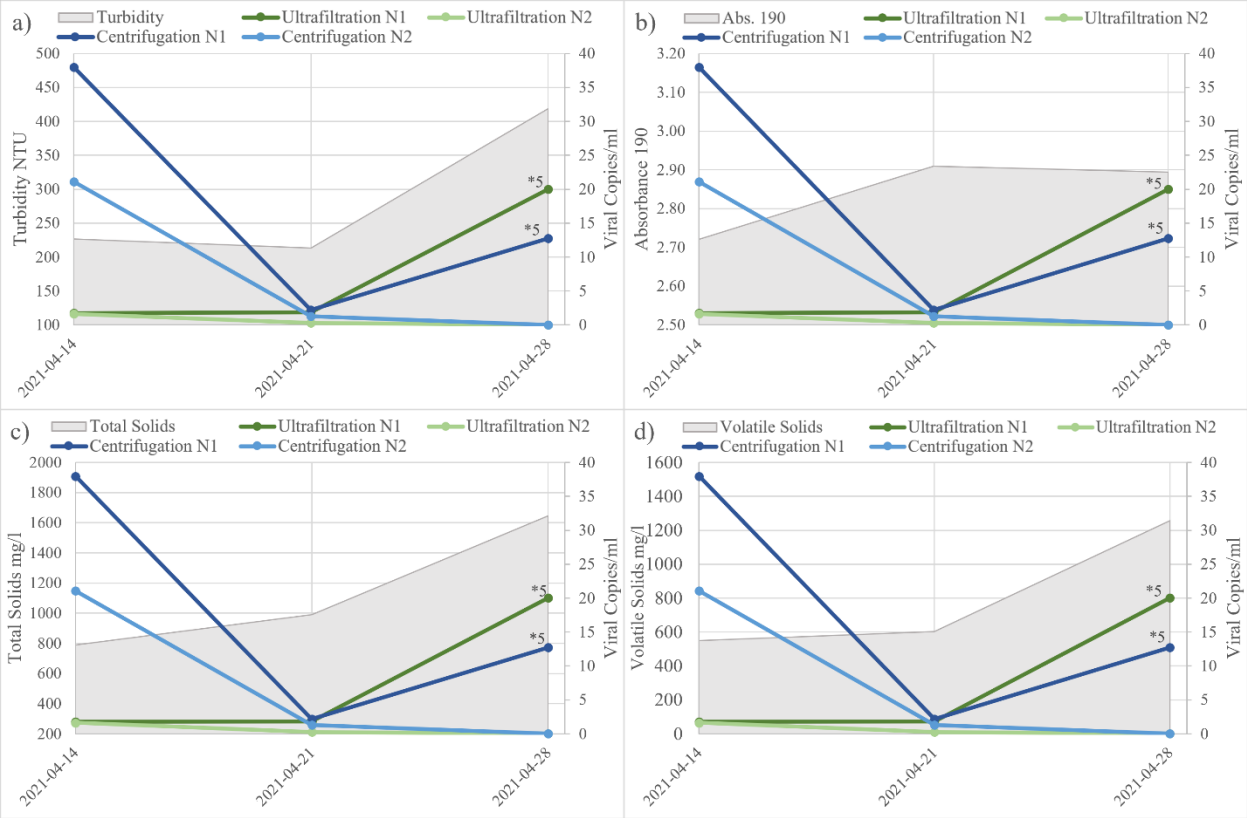


Figure 14: Comparison of Viral Copies to Wastewater Characteristics at Residence B: a) Turbidity and N1&N2 b) Absorbance (190 nm) and N1&N2 c) Total Solids and N1&N2 d) Volatile Solids and N1&N2

Residence B was sampled between April 14 and April 28 as well, but the correlations found at location A could not be observed in these samples (Figure 14). Several factors could be the reason for these results in both residences. First, the samples were taken close to the source so that residence time in the sewer was minimal and little to no mixing happened. Furthermore, most of the residence samples at that time were grab samples, as issues with the autosamplers occurred.

5. CONCLUSION

This study monitored SARS-CoV-2 levels in a university campus's wastewater for a year and investigated the correlations between viral signals, case numbers, campus events, and wastewater characteristics. Wastewater signals largely correlated positively with the clinically confirmed COVID-19 cases on campus. Long weekends and holidays were often followed by increased viral signals, confirming the increased risk through social gatherings on campus. The implementation of lockdowns in the city and online teaching on campus quickly decreased the case development. In spite of online teaching and restricted access to campus, the university represented a microcosm of the city and mirrored the three waves of cases that the city experienced, which was manifested with strong Pearson correlations. The variability of wastewater characteristics and their impact on the detection of SARS-CoV-2 RNA were also investigated. The ultrafiltration method analysing the liquid fraction of wastewater was more sensitive in detecting viral RNA when solids content of the wastewater was lower. On the other hand, the centrifugation method analysing the solids fraction of wastewater was more sensitive when solids content of the wastewater was higher. Wastewater characteristics collected from the residence buildings and sewer were different, and the variability was higher closer to the source. Overall, wastewater surveillance provided actionable information to the university administration, which was taken into consideration during the weekly COVID-19 management meetings. Wastewater surveillance was also able to bring high-risk factors and events to the attention of the decision-makers, which could have otherwise been missed, and provided an opportunity to take corrective measures for future events.

6. FUTURE RESEARCH DIRECTIONS

Wastewater-based epidemiology for SARS-CoV-2 detection is still a new approach and requires optimization in many different areas. More comparison studies of concentration, RNA extraction, and molecular detection methods may lead to developing standardized methodological approaches that make comparing results of various laboratories easier. Many different protocols are currently applied because of uncertainty about which assays are best to use. In addition, most studies show low viral recovery efficiencies with values below 50%. The research community needs a better understanding of where viruses are lost during the procedures and how they can be avoided. First, better control of viruses should be studied and second, ways to improve viral recoveries should be researched.

PMMoV is the most commonly applied normalization method, as it is a well-known fecal indicator for wastewater studies, but it is unclear whether it accurately displays trends in the viral spread. Other normalization methods should be further tested and compared to the raw data, and PMMoV normalized data. To do that, we also need to understand what affects the SARS-CoV-2 RNA in the complex wastewater matrix. Though some studies have tested various wastewater parameters and their effects on the concentration and detection methods, more research is needed.

Large networks have been established on regional and national levels to combat the COVID-19 pandemic. We should take advantage of these now established networks by extending their use to other research projects. Besides SARS-CoV-2 detection, laboratories can focus on other human viruses that can be simultaneously detected with PCR-based techniques, such as noroviruses, rotaviruses, influenza, etc. Their detection with improved WBE can lead to faster public health responses to mitigate virus outbreaks.

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Additional details, data and figures, including primer and probe sequences, statistical data for case counts, PMMoV normalized viral copies for campus and city data as well as additional wastewater characteristics can be found in the following appendices.

APPENDIX A: Primer and Probe Sequences & Additional Statistics

Table 3: Primer and Probe Sequences of N1&N2 Targets and PMMoV

Target	**Item**	**Sequence (5' → 3')**
N1	Primer-Forward	GAC CCC AAA ATC AGC GAA AT
	Primer-Reverse	TCT GGT TAC TGC CAG TTG AAT CTG
	Probe	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-MGBNFQ
	Linearized Plasmid	TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATG CAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGC CGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTG GCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAG ATTGTACTGAGAGTGCACCAAATGCGGTGTGAAATACCGCA CAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGC CATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGG GCCTCATCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGC TGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGT CACGACGTTGTAAAACGACGGCCAGTGCAACGCGATGACG ATGGATAGCGATTCATCGATGAGCTGACCCGATCGCCGCCG CCGGAGGGTTGCGTTTGAGACGGGCGACAGATATGTCTGAT AATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTT TGGTGGACCCTCAGATTCAACTGGCAGTAACCAGAATGGAG AACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGG TTTACCCAATAATACTGCGTCTTGGTTCACCGCTCTCACTCA ACATGGCAAGGAAGACCTTAAATTCCCTCGAGGACAAGGCG TTCCAATTAACACCAATAGCAGTCCAGATGACCAAATTGGC TACTACCGAAGAGCTACCAGACGAATTCGTGGTGGTGACGG TAAAATGAAAGATCTCAGTCCAAGATGGTATTTCTACTACC TAGGAACTGGGCCAGAAGCTGGACTTCCCTATGGTGCTAAC AAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAA TACACCAAAAGATCACATTGGCACCCGCAATCCTGCTAACA ATGCTGCAATCGTGCTACAACTTCCTCAAGGAACAACATTG CCAAAAGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTC AAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAA GAAATTCAACTCCAGGCAGCAGTAGGGGAACTTCTCCTGCT AGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCT GCTGCTTGACAGATTGAACCAGCTTGAGAGCAAAATGTCTG

		GTAAAGGCCAACAACAACAAGGCCAAACTGTCACTAAGAA ATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTA CTGCCACTAAAGCATACAATGTAACACAAGCTTTCGGCAGA CGTGGTCCAGAACAAACCCAAGGAAATTTTGGGGACCAGG AACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAA ATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATG TCGCGCATTGGCATGGAAGTCACACCTTCGGGAACGTGGTT GACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCAA ATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGAC GCATACAAAACATTCCCACCAACAGAGCCTAAAAAGGACA AAAAGAAGAAGGCTGATGAAACTCAAGCCTTACCGCAGAG ACAGAAGAAACAGCAAACTGTGACTCTTCTTCCTGCTGCAA TTTGGATGATTTCTCCAAACAATTGCAACAATCCATGAGCA GTGCTGACTCAACTCAGGCCTAAATCAGTTCTGGACCAGCG AGCTGTGCTGCGACTCGTGGCGTAATCATGGTCATAGCTGTT TCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACAT ACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAAT GAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCC GCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATG AATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGC GCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCG TTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTA ATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGA ACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTA AAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCC CCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGT CGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGC GTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTC GGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAA CCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTA TCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCAC TGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTA TGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACT ACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTG CTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTG ATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTG TTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCA AGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGT GGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATT ATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAA TGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTG GTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCT CAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCC CCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATC

		TGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCA CCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAA GGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCC ATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAG TTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTA CAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCA TTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATC CCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTC CGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACT CATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGC CATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACC AAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCT CTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAG CAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGG GGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAG TTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCAT CTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGA AGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGG AAATGTTGAATACTCATACTCTACCTTTTTCAATATTATTG AAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATA TTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCG CGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAA CCATTATTATCATGACATTAACCTATAAAAATAGGCGTAT CACGAGGCCCTTTCGTC
	Strings DNA Fragments	ATGTACTCATTCGTTTCGGAAGAGACAGGTACGTTAATAGT TAATAGCGTACTTCTTTTTCTTGCTTTCGTGGTATTCTTGCTA GTTACACTAGCCATCCTTACTGCGCTTCGATTGTGTGCGTA CTGCTGCAATATTGTTAACGTGAGTCTTGTAAAACCTTCTTT TTACGTTTACTCTCGTGTTAAAAATCTGAATTCCACCCATTC AGTACATCGATATCGGTAATTATACAGTTTCCTGTTTACCTT TTACAATTAATTGCCAGGAACCTAAATTGGGTAGTCTTGTA GTGCGTTGTTCGTTCTATGAAGACTTTTTAGAGTATCATGAC GTTCGTGTTGTTTTAGATTTCATCTAAACGAACAAACTAAA ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCG CATTACGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACC AGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCG GCCCCGTGGTCCAGAACAAACCCAAGGAAATTTTGGGGACC AGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCC GCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCG GAATGTCGCGCATTGGCATGGAAGTCACACCTTCGGGAACG TGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAG
N2	Primer-Forward	TTA CAA ACA TTG GCC GCA AA
	Primer-Reverse	GCG CGA CAT TCC GAA GAA
	Probe	FAM-ACA ATT TGC CCC CAG CGC TTC AG-MGBNFQ

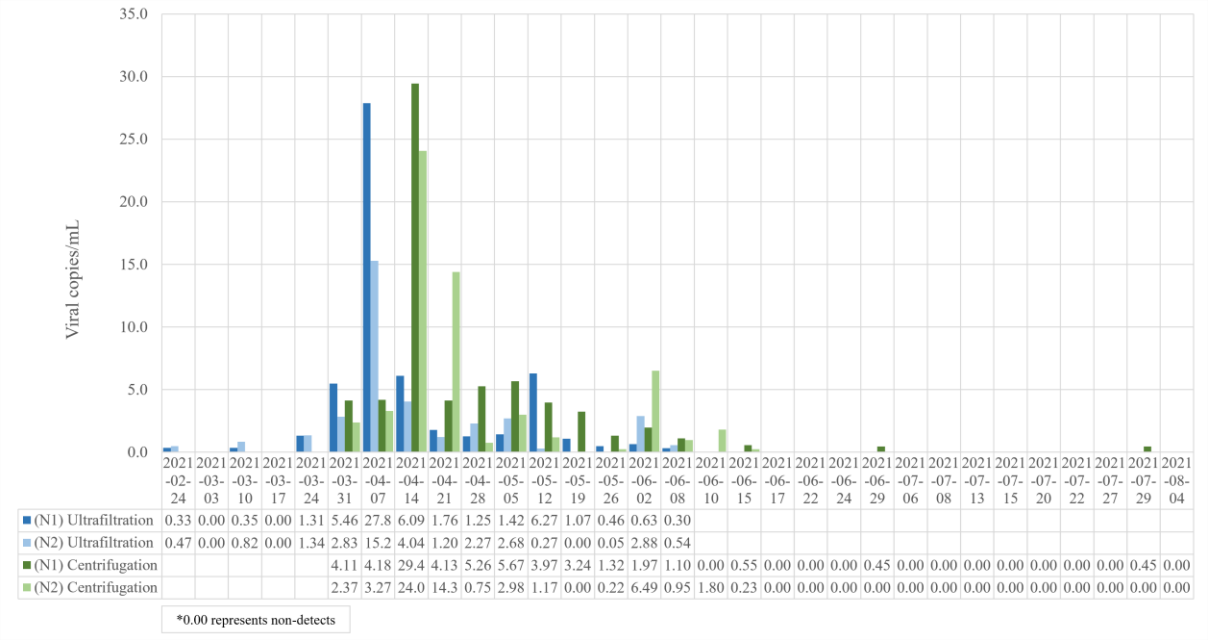
	Linearized Plasmid	Same as N1
	Strings DNA Fragments	Same as N1
PMMoV	Primer-Forward	GAG TGG TTT GAC CTT AAC GTT TGA
	Primer-Reverse	TTG TCG GTT GCA ATG CAA GT
	Probe	FAM-CCT ACC GAA GCA AAT G-MGBNFQ
	gBlock	5'-TGGCAGCAAAGGTAATGGTAGCTGTGGTTTCAAATGAG AGTGGTTTGACCTTAACGTTTGAGAGGCCTACCGAAGCAAA TGTCGCACTTGCATTGCAACCGACAATTACATCAAAGGAGG AAGGTTCGTTGAAGATTGTG-3'
	Strings DNA Fragments	5'-TGGCAGCAAAGGTAATGGTAGCTGTGGTTTCAAATGAG AGTGGTTTGACCTTAACGTTTGAGAGGCCTACCGAAGCAAA TGTCGCACTTGCATTGCAACCGACAATTACATCAAAGGAGG AAGGTTCGTTGAAGATTGTG-3'

Table 4: Correlations between Case Counts (City and Campus)

		City-wide Case Count			
	n	Week prior	3 days prior	Week of	Week after
Campus-wide Case Count					
Week prior	26	0.52**			
3 days prior	26		0.51**		
Week of	26			0.61**	
Week after	26				0.69**
Residence A Case Count					
Week prior	6	–			
3 days prior	6		–		
Week of	6			–	
Week after	6				–

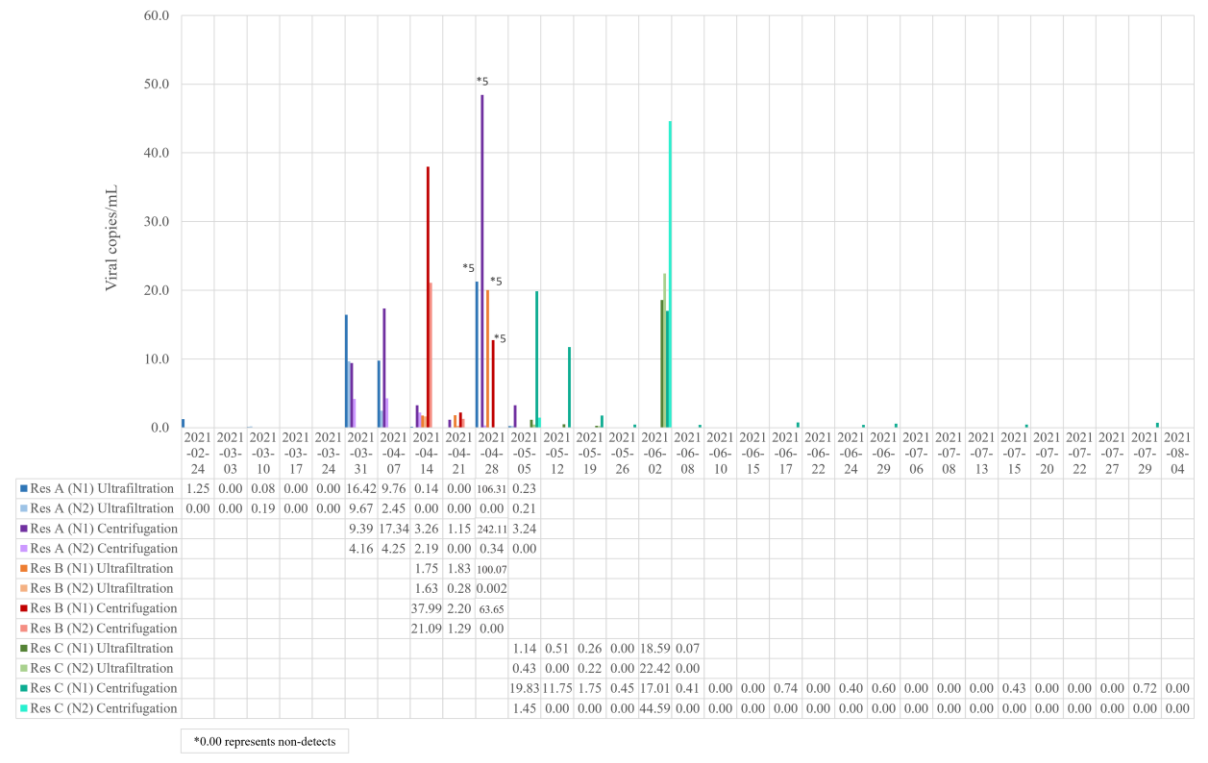
Note. Significant correlations reflected as * $p < 0.05$, ** $p < 0.01$, and – reflects nonsignificant correlation

APPENDIX B: SARS-CoV-2 Concentrations



	2021-02-24	2021-03-03	2021-03-10	2021-03-17	2021-03-24	2021-03-31	2021-04-07	2021-04-14	2021-04-21	2021-04-28	2021-05-05	2021-05-12	2021-05-19	2021-05-26	2021-06-02	2021-06-08	2021-06-10	2021-06-15	2021-06-17	2021-06-22	2021-06-24	2021-06-29	2021-07-06	2021-07-08	2021-07-13	2021-07-15	2021-07-20	2021-07-22	2021-07-27	2021-07-29	2021-08-04
(N1) Ultrafiltration	0.33	0.00	0.35	0.00	1.31	5.46	27.8	6.09	1.76	1.25	1.42	6.27	1.07	0.46	0.63	0.30															
(N2) Ultrafiltration	0.47	0.00	0.82	0.00	1.34	2.83	15.2	4.04	1.20	2.27	2.68	0.27	0.00	0.05	2.88	0.54															
(N1) Centrifugation						4.11	4.18	29.4	4.13	5.26	5.67	3.97	3.24	1.32	1.97	1.10	0.00	0.55	0.00	0.00	0.00	0.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.45	0.00
(N2) Centrifugation						2.37	3.27	24.0	14.3	0.75	2.98	1.17	0.00	0.22	6.49	0.95	1.80	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Figure 15: N1 & N2 Viral Gene copies/ml at Location A with the Ultrafiltration and Centrifugation Concentration Methods



	2021-02-24	2021-03-03	2021-03-10	2021-03-17	2021-03-24	2021-03-31	2021-04-07	2021-04-14	2021-04-21	2021-04-28	2021-05-05	2021-05-12	2021-05-19	2021-05-26	2021-06-02	2021-06-08	2021-06-10	2021-06-15	2021-06-17	2021-06-22	2021-06-24	2021-06-29	2021-07-06	2021-07-08	2021-07-13	2021-07-15	2021-07-20	2021-07-22	2021-07-27	2021-07-29	2021-08-04
Res A (N1) Ultrafiltration	1.25	0.00	0.08	0.00	0.00	16.42	9.76	0.14	0.00	106.31	0.23																			
Res A (N2) Ultrafiltration	0.00	0.00	0.19	0.00	0.00	9.67	2.45	0.00	0.00	0.00	0.21																			
Res A (N1) Centrifugation						9.39	17.34	3.26	1.15	242.11	3.24																			
Res A (N2) Centrifugation						4.16	4.25	2.19	0.00	0.34	0.00																			
Res B (N1) Ultrafiltration								1.75	1.83	100.07																				
Res B (N2) Ultrafiltration								1.63	0.28	0.002																				
Res B (N1) Centrifugation								37.99	2.20	63.65																				
Res B (N2) Centrifugation								21.09	1.29	0.00																				
Res C (N1) Ultrafiltration											1.14	0.51	0.26	0.00	18.59	0.07														
Res C (N2) Ultrafiltration											0.43	0.00	0.22	0.00	22.42	0.00														
Res C (N1) Centrifugation											19.83	11.75	1.75	0.45	17.01	0.41	0.00	0.00	0.74	0.00	0.40	0.60	0.00	0.00	0.00	0.43	0.00	0.00	0.00	0.72	0.00
Res C (N2) Centrifugation											1.45	0.00	0.00	0.00	44.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Figure 16: N1 & N2 Viral Gene Copies/ml at Location B with the Ultrafiltration and Centrifugation Concentration Methods

APPENDIX C: PMMoV Normalization

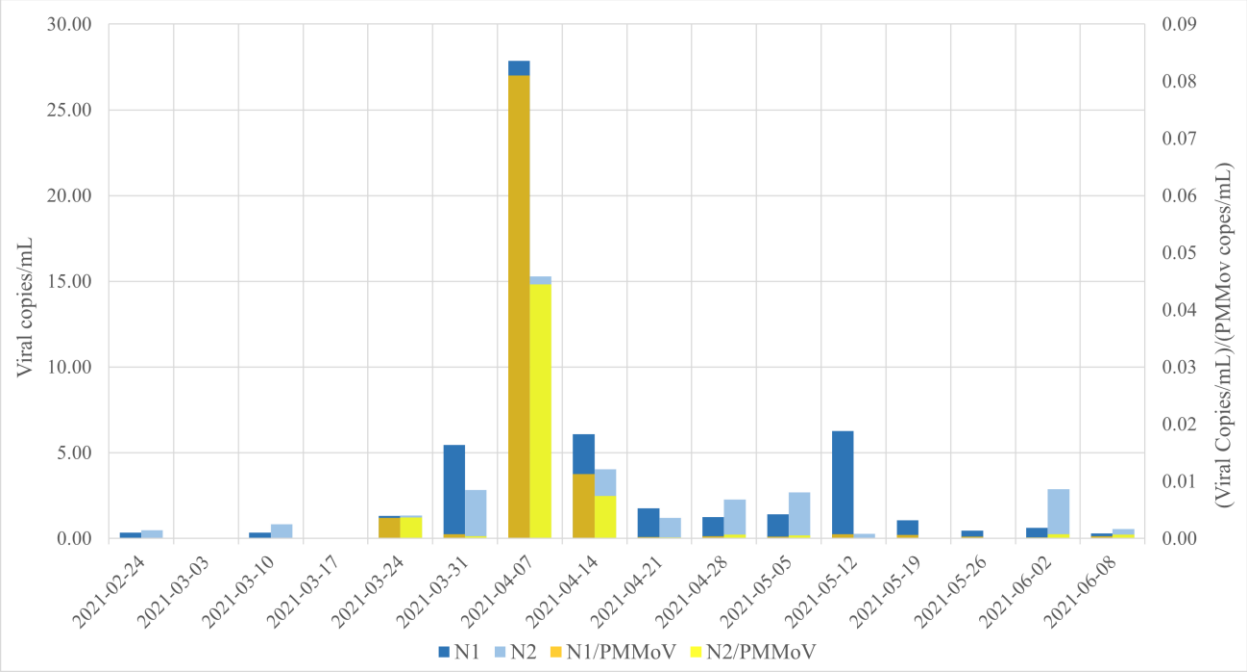


Figure 17: N1 & N2 Viral Gene Copies/ml Compared to Normalized N1&N2 Viral Gene Copies/ml with PMMoV at Location A with the Ultrafiltration Concentration Methods

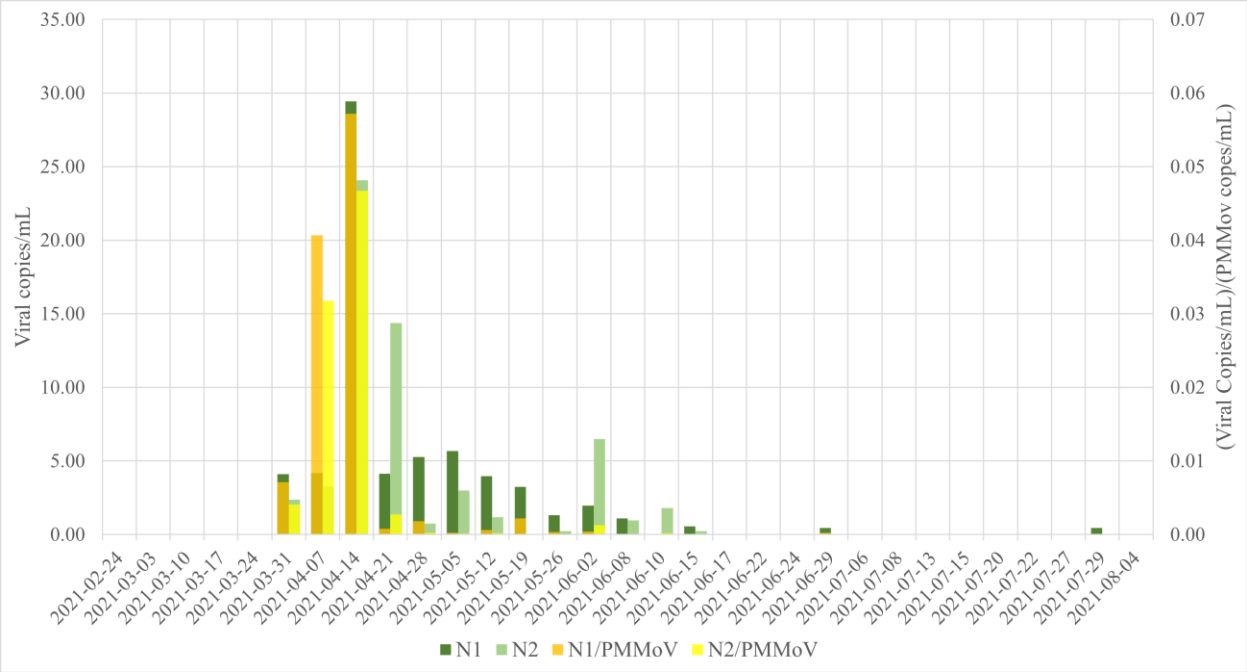


Figure 18: N1 & N2 Viral Gene Copies/ml Compared to Normalized N1&N2 Viral Gene Copies/ml with PMMoV at Location A with the Centrifugation Concentration Methods

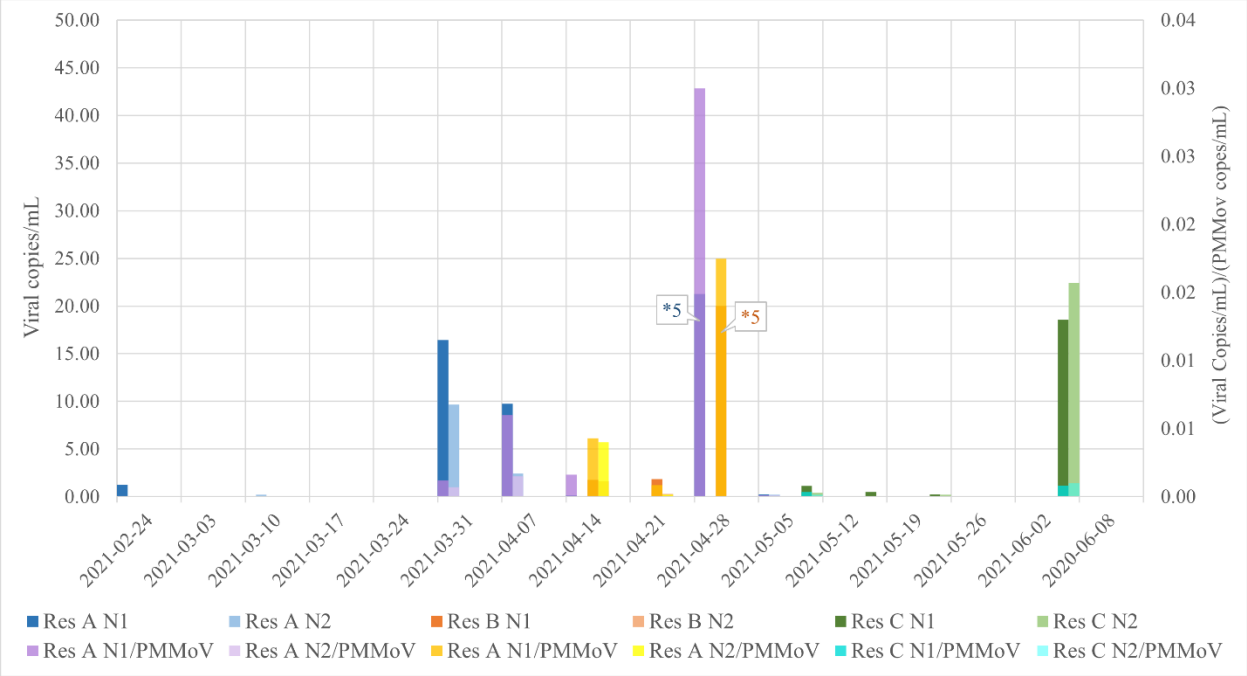


Figure 19: N1 & N2 Viral Gene Copies/ml Compared to Normalized N1&N2 Viral Gene Copies/ml with PMMoV at Location B with the Ultrafiltration Concentration Methods

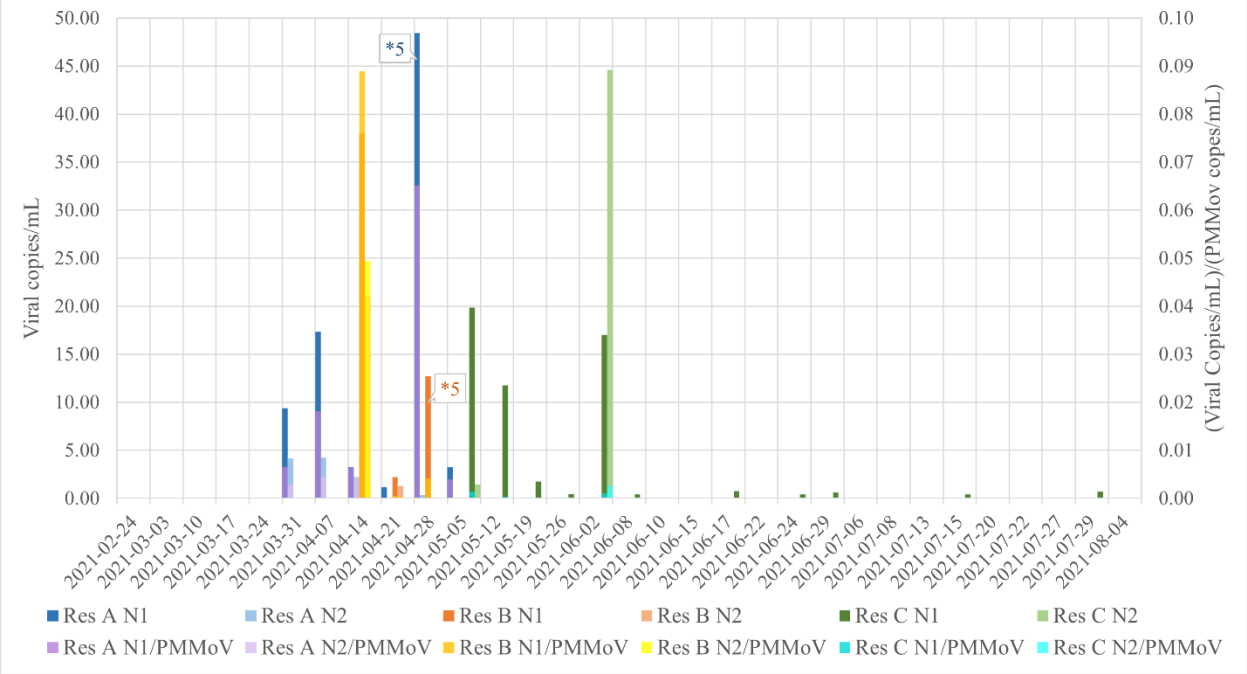


Figure 20: N1 & N2 Viral Gene Copies/ml Compared to Normalized N1&N2 Viral Gene Copies/ml with PMMoV at Location B with the Centrifugation Concentration Methods

APPENDIX D: Comparison of Campus Data (Location A) to City Data

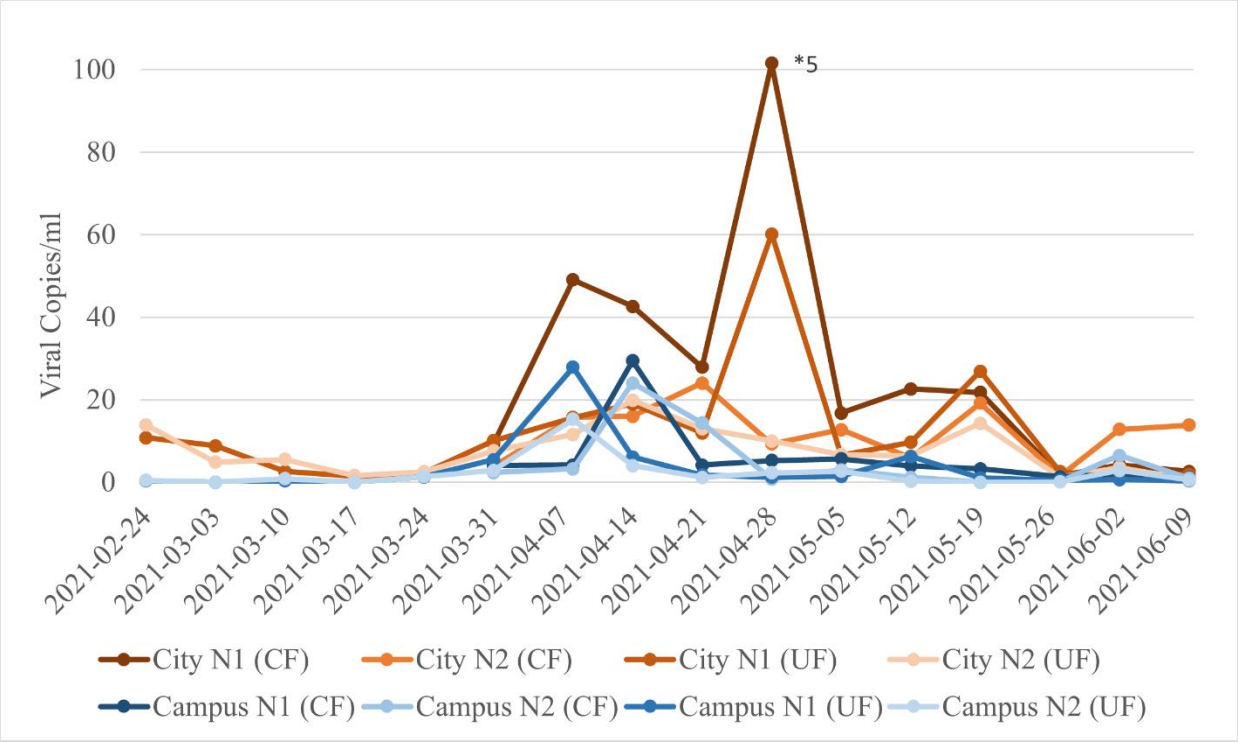


Figure 21: Comparison of Raw Viral Signals of the Campus and the City

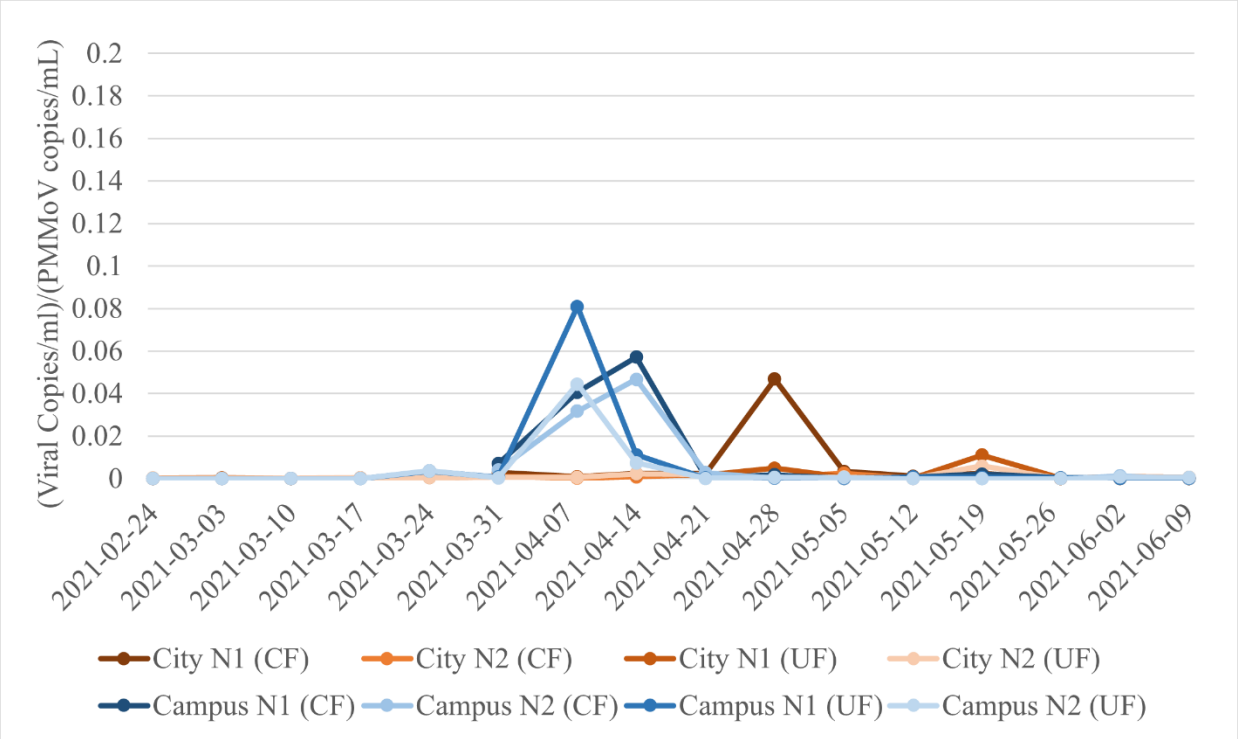


Figure 22: Comparison of PMMoV Normalized Viral Signals of the Campus and the City

APPENDIX E: Additional Wastewater Characteristics Data

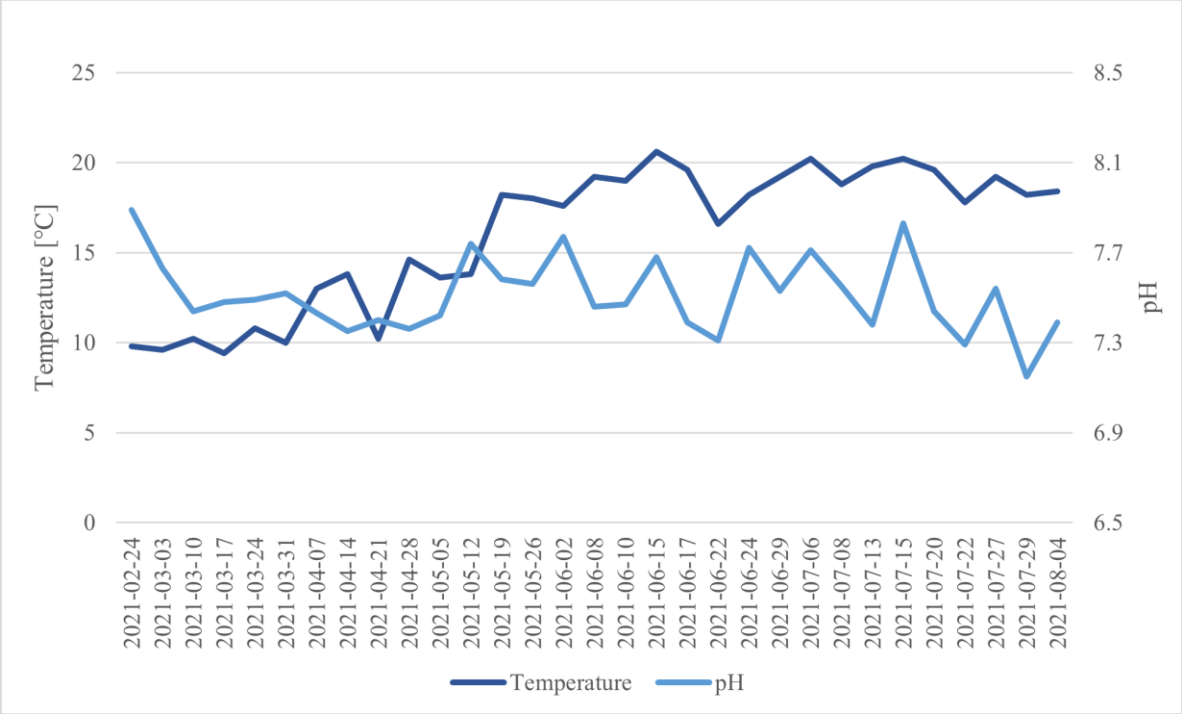


Figure 23: Temperature and pH Data for Location A

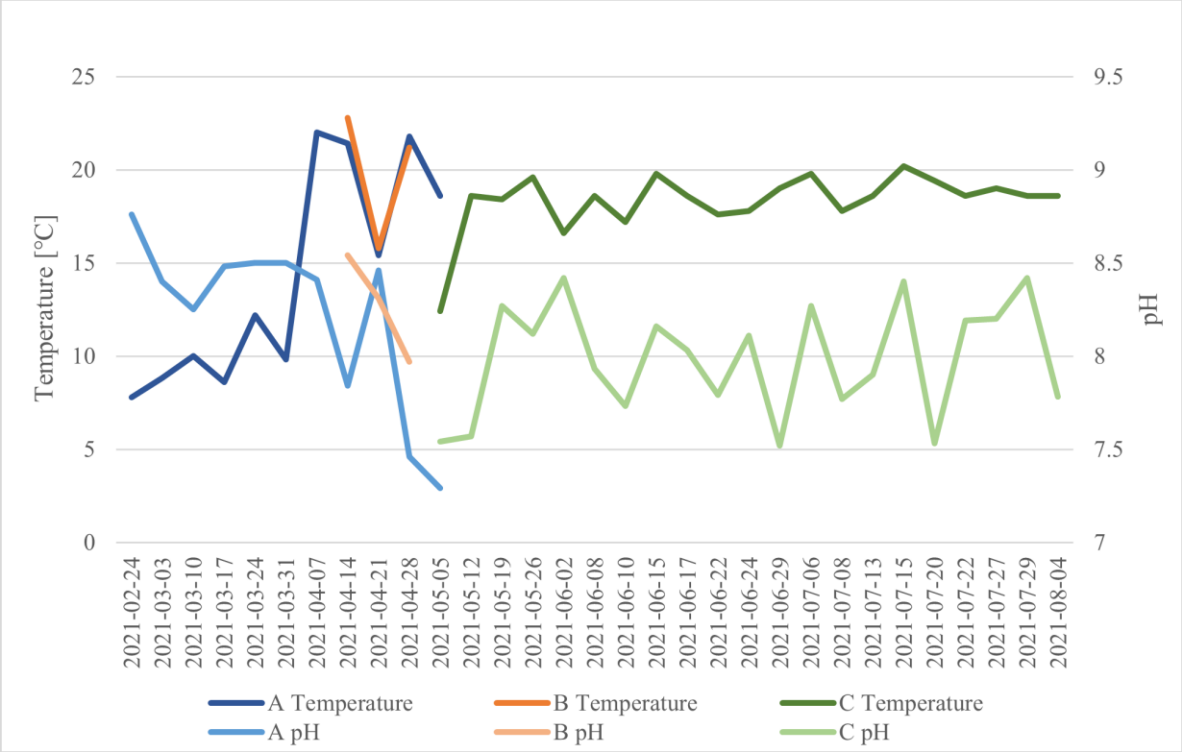


Figure 24: Temperature and pH Data for Location B (Residences A, B &C)

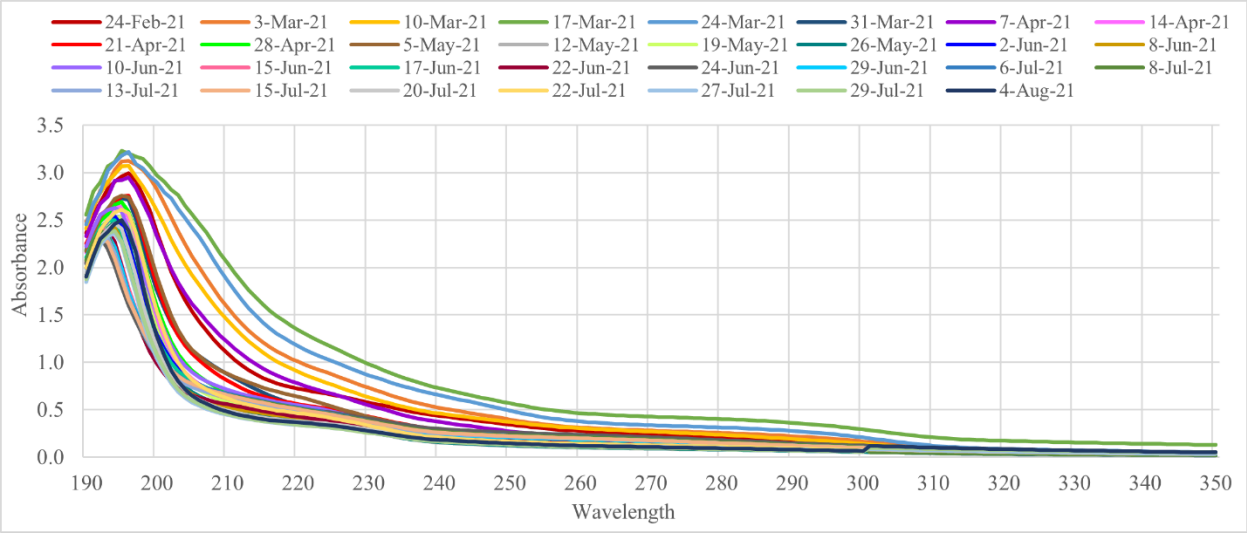


Figure 25: Absorbance Scans from 190 nm to 350 nm for Location A