INNOVATION IN PASSIVE SAMPLING FOR VIRAL DETECTION IN WATER RESOURCES: TOWARDS ACCESSIBLE AND SENSITIVE MONITORING TECHNIQUES

By

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Dalhousie University is located in Mi'kma'ki, the ancestral and unceded territory of the Mi'kmaq. We are all Treaty people.

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ABSTRACT

In response to the escalating challenges of viruses, this research focuses on advancing passive sampling methods to enhance public health security through improved viral surveillance in water resources. Effective surveillance hinges on the deployment of efficient sampling techniques that yield accurate and timely insights. Traditional sampling techniques, such as grab and composite methods, struggle to capture temporal variations and often underestimate viral levels with grab samples, while there are logistical challenges with processing large volumes or significant costs and technical limitations of autosamplers in remote regions. This research focuses on leveraging various adsorbent materials to determine the most effective yet accessible method for capturing viruses in water resources. This work aims to overcome the constraints of current sampling methods by enhancing pathogen detection with novel passive sampling and innovative molecular techniques, enabling rapid, sensitive, and accessible viral monitoring.

The use of accessible adsorbent materials like gauze, sponges, cheesecloth, and electronegative cellulose-nitrate membrane filters were evaluated for capturing SARS-CoV-2 from wastewater. These materials, especially electronegative membrane filters, demonstrated their potential for effective sampling of the virus in low-prevalence regions. However, bench-scale batch adsorption experiments revealed that these membrane filters align with pseudo-first-order kinetics reaching adsorption capacity within 24 to 48 hours. Also, the presence of total suspended solids in wastewater influenced the equilibrium adsorption dynamics of the filters, potentially due to the inhibitory effects of organic matter on subsequent analyses. Granular activated carbon (GAC) emerged as an enhanced adsorbent; in field-scale comparisons, GAC adsorbed SARS-CoV-2 in wastewater more effectively than electronegative filters. GAC's adsorption peaked at ~60 hours, suggesting its feasibility for longer deployments. The introduction of a multiplex RT-qPCR assay for simultaneous virus detection represents an advancement in surveillance, offering a rapid, economical alternative to other detection methods. The application of GAC-based passive sampling to a freshwater lake further confirmed the method's versatility and efficacy over grab sampling techniques for viral detection in diverse matrices. As such, GAC presents a scalable and convenient alternative for capturing viruses in water and wastewater, urging further investigation into adsorptive properties of GAC and further application for improved water safety.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ABS	Acrylonitrile Butadiene Styrene
ACN	Acetonitrile
AdV	Adenovirus
ALOD	Assay Limit of Detection
ATCC	American Type Culture Collection
BHQ	Blackhole Quencher
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
C_e	Equilibrium Concentration
CFU	Colony-Forming Units
COSCA	COVID-19 Sewer Cage
COVID-19	Coronavirus Disease 2019
CrAssphage	Cross-Assembly Phage
C_q	Cycle Quantification
C_t	Cycle threshold
DI Water	Deionized Water
ddPCR	Digital-Droplet Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
dPCR	Digital-Polymerase Chain Reaction
EMMI	Environmental Microbiology Minimum Information
EnV	Enterovirus
EtOH	Ethanol
FIO	Fecal Indicator Organisms
GC	Gene Copies
gBlock	GeneBlock®
GAC	Granular Activated Carbon
GISAID	Global Initiative on Sharing all Influenza Data
GU	Genomic Units
HCL	Hydrochloric Acid
HI-SCV-2	Heat-Inactivated SARS-CoV-2 Reference Material
INFA/INFB	Influenza A/B
IAC	Internal Amplification Control
LAMP	Loop-Mediated Amplification
LDPE	Low-Density Polyethylene
$LOD_{95\%}$	Limit of Detection at 95% Confidence Interval
LOQ	Limit of Quantification
MBG	Minor Groove Binder
MeV	Measles Virus
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments

MLOD	Method Limit of Detection
MS2	Bacteriophage Emesvirus Zinder
NCBI	National Center for Biotechnology Information
nM	Nanomolar
NFW	Nuclease-Free Water
NGS	Next-Generation Sequencing
NV	Norovirus
NS	Nova Scotia
NTC	Non-Template Control
PCR	Polymerase Chain Reaction
PFU	Plaque-Forming Units
PFO	Pseudo-First-Order
PLA	Polylactic Acid
PMMoV	Pepper Mild Mottle Virus
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PSO	Pseudo-Second-Order
PVDF	Polyvinylidene Fluoride
QA-QC	Quality Assurance and Quality Control
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RV	Rotavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SEM	Scanning Electron Microscope
SNV	Single Nucleotide Variant
$TCID_{50}$	50% Tissue Culture Infectious Dose
TOC	Total Organic Carbon
TSS	Total Suspended Solids
UV	Ultraviolet
VOC	Variants of Concern
WBE	Wastewater-based Epidemiology
WHO	World Health Organization
WWS	Wastewater Surveillance
WWTF	Wastewater Treatment Facility

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CHAPTER 1 INTRODUCTION

1.1 Research Rational

1.1.1 Significance of Viral Contamination in Water Resources

Water is an indispensable resource for life and is increasingly threatened by microbial contamination, notably from viruses, which pose significant public health concerns (1). The emergence and re-emergence of viruses in water resources is emphasized by the global population surge from 6.1 billion in 2000 to 8 billion in 2022 (2). These dynamics underscore escalating demands for water resources and heightened vulnerabilities of water security. Viral contamination, particularly through wastewater effluents, demands urgent and effective wastewater management strategies to mitigate viral spread, given that minimal viral concentrations (1–10 infectious virions per litre) can pose substantial risks to water safety. The critical role of water in the transmission, spillover, and spread of viruses (3–6), along with their ability to persist in the environment (3,7,8), emphasizes the need for vigilant monitoring and management. Addressing these challenges requires integrated strategies that combine effective water quality surveillance, wastewater treatment, and climate adaptation measures.

1.1.2 Challenges in Viral Surveillance

The presence of SARS-CoV-2, the virus causing COVID-19, in the gastrointestinal tracts of infected individuals, leads to viral shedding in stool and, to a lesser extent, urine, which then enters the wastewater system (9–11). Shedding can occur in both symptomatic and asymptomatic individuals, with viral concentrations in stool ranging from 10^2 to 10^9 gene copies per gram. As such, wastewater surveillance and wastewater-based epidemiology efforts have been increasingly reported in response to the COVID-19 pandemic. These techniques offer cost-effective and comprehensive methods to assess the spread of infections across large regions, providing spatially relevant, anonymous, and early detection of viral presence. Wastewater surveillance can inform public health responses by monitoring viral loads within communities, capturing entire populations at a single sampling point. This is especially valuable in resource-constrained regions where access to health services may be limited. However, the relationship between viral

concentrations in wastewater and actual viral prevalence in the population remains uncertain, necessitating further research to understand this relationship. The complexity of wastewater and dilution of low viral loads in large wastewater systems make conventional concentration-dependent methods ineffective and impractical for routine use.

Likewise, monitoring viral pathogens in water resources presents significant challenges that hinder effective water quality monitoring and management. The surveillance of viruses in recreational and source water environments is often challenged by low viral loads in large bodies of water (12,13). Robust virus monitoring in surface and groundwater systems often requires collecting and concentrating large volumes of water (14–16), a time-intensive and challenging process, especially in resource-limited settings. Moreover, the absence of a standardized surveillance framework that accommodates various virus types and water qualities likely leads to an underestimation of viral presence in water systems.

Furthermore, viral detection methods also face obstacles to accurate detection within water systems. Traditional culture-based methods for viral detection, are dependent on viral-host interactions, labour-intensive, and limited in breadth concerning the types of organisms detectable, providing limited information for decision-making (17). Advanced genomic techniques like quantitative polymerase chain reaction and next-generation sequencing, offer a promising alternative for viral detection (18,19). However, their inability to distinguish between viable and non-viable particles challenges their use in risk assessments. Moreover, the variability in viral deactivation processes, influenced by water quality and treatment processes, calls for improved detection strategies capable of identifying infectious viruses across diverse samples.

1.1.3 Passive Sampling: An Emerging Solution

Passive sampling has emerged as a promising solution for viral surveillance, particularly highlighted by the COVID-19 pandemic's drive for advancements in water-based microbial monitoring (20). This sampling approach, increasingly adopted for tracking community-level viral loads such as SARS-CoV-2 in wastewater, offers significant advantages. Passive sampling allows for continuous monitoring without the need for pumping systems or sophisticated infrastructure, making it an ideal tool for higher-risk, targeted areas like schools, prisons, and healthcare facilities. The cost-

effectiveness and simplicity of passive samplers require minimal training for deployment and recovery. Also, the adaptability of the samplers enables their application at various scales, from local communities to entire regions, providing valuable data for public health decisions. While alternative methods like autosamplers and grab sampling remain common, passive samplers stand out in their ease of use, affordability, and ability to circumvent typical issues of other methods. Their versatility across water systems positions them as a promising approach, potentially encouraging wider adoption of viral surveillance. This broader application aims to address the current underestimation of viral prevalence in water resources, with the hope that increased use of passive samplers will enhance our understanding and mitigation of viruses in aquatic environments.

1.2 Research Significance

The passive sampling methodologies established through this research have been recognized for their innovative approach to overcoming traditional limitations associated with viral detection in water resources. The adoption of these methods extends globally, from regions as diverse as Rwanda, Louisiana, New Zealand, and the United Kingdom, among others, highlighting the international impact and practical applicability of this work. A comprehensive list of locations where these methods have been implemented is provided in Appendix A, showcasing the global reach and significance of this research contribution.

1.3 Research Objectives

It was hypothesized that the adoption and optimization of passive sampling techniques for the surveillance of viruses in water and wastewater, particularly SARS-CoV-2 and other respiratory viruses, can significantly enhance the sensitivity, selectivity, and practicality of viral detection, thereby providing a more accurate assessment of viral prevalence and dynamics in water systems. This, in turn, will contribute to more informed public health decisions and strategies for managing water quality.

As such, the specific objectives of this research were as follows:

1. Assess and refine the effectiveness of various adsorbent materials in capturing viruses in water and wastewater, aiming to establish an enhanced passive sampling strategy for improved viral surveillance.

2. Design and validate a multiplex RT-qPCR approach to simultaneously detect and quantify four relevant respiratory viruses in wastewater, integrating it with passive sampling to enhance the scope of viral detection in wastewater.
3. Assess the real-world efficacy and practicality of molecular detection methods coupled with passive sampling techniques for viral surveillance in targeted sewersheds and a recreational freshwater lake, aiming to inform public health strategies and enhance water safety measures.

1.4 Organization of the Thesis

The chapters of this thesis are arranged in journal-style manuscripts, and the structure of each chapter is prepared with self-contained introductions, methods and materials, results, discussion, conclusions, and reference sections. Chapter 1 provides an introduction to the thesis rationale, research questions and objectives**.** Chapters 2 - 8 were prepared as manuscript submissions for publication in peer-reviewed journals. Chapter 9 presents the overall conclusions of this thesis and outlines areas for future research.

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CHAPTER 2 FROM CAPTURE TO DETECTION: A CRITICAL REVIEW OF PASSIVE SAMPLING TECHNIQUES FOR PATHOGEN SURVEILLANCE IN WATER AND WASTEWATER

This chapter is reprinted with permission from the following referred journal article:

Hayes, E. K., & Gagnon, G. A. (2024). From Capture to Detection: A Critical Review of Passive Sampling Techniques for Pathogen Surveillance in Water and Wastewater. Water Research, 122024.

E.K.H. carried out meta-data collection and analysis, wrote the paper, and prepared the figures.

2.1 Abstract

Water quality, critical for human survival and well-being, necessitates rigorous control to mitigate contamination risks, particularly from pathogens amid expanding urbanization. Consequently, the necessity to maintain the microbiological safety of water supplies demands effective surveillance strategies, reliant on the collection of representative samples and precise measurement of contaminants. This review critically examines the advancements of passive sampling techniques for monitoring pathogens in various water systems, including wastewater, freshwater, and seawater. We explore the evolution from conventional materials to innovative adsorbents for pathogen capture and the shift from culture-based to molecular detection methods, underscoring the adaptation of this field to global health challenges. The comparison highlights passive sampling's efficacy over conventional techniques like grab sampling and its potential to overcome existing sampling challenges through the use of innovative materials such as granular activated carbon, thermoplastics, and polymer membranes. By critically evaluating the literature, this work identifies standardization gaps and proposes future research directions to augment passive sampling's efficiency, specificity, and utility in environmental and public health surveillance.

2.2 Introduction to Passive Sampling

Water quality monitoring and surveillance is necessary to prevent possible threats to human health, including outbreaks related to pathogen contamination (e.g., bacteria, protozoa, and viruses) in recreational and drinking water resources. Effective microbial

monitoring relies on both the collection of representative samples and accurate measurement of the contaminant of concern. Traditional sampling methods, primarily involving discrete grab samples, have been instrumental in initial pathogen detection efforts. Yet, grab sampling exhibits significant limitations, particularly in its inability to capture temporal variations, often leading to the underestimation of contamination levels. Therefore, to capture a representative sample when using grab sampling an increased sampling frequency is needed or the use of automatic sampling systems to capture composite samples over certain time or flow-weighted intervals. While composite sampling offers a partial solution to the constraints of grab sampling, automatic samplers often entail prohibitive costs and logistical challenges, particularly in remote sampling regions. As such, advancements in sampling technology have led to the development of passive samplers, adept at continuously capturing contaminants over extended periods and detecting transient or low levels of contaminants.

Passive sampling can broadly be defined as the use of material to adsorb or absorb contaminants from mediums like water or wastewater until equilibrium is reached or the sampling duration concludes, without the necessity of active force (e.g. peristaltic pumps), to drive the collection process [1–3]. While passive sampling for chemicals often involves capturing dissolved contaminants through specific chemical interactions [4], the capture of pathogens is suspected to rely on adsorbents that trap particulate matter, to which pathogens are adhered to in solution [5,6]. Commonly, passive sampler's will rely on adsorption principles to capture contaminants to the adsorbent's surface, although the effectiveness of these types of materials can often be easily influence by an environments water chemistry [7]. Sampler's that operate based on absorption, trap contaminants within a materials internal structure, with effectiveness determined by the sorbents porosity and solubility [1]. Several sorbents and adsorbents, ranging from solvents, chemical reagents, activated carbon, silica gels, polymers, common household materials like gauze, cheesecloth, tampons, and even in some applications organisms like plants and fish have been used to passively capture chemical and biological contaminants [8–11]. Ultimately, the dynamics that drive the capture of chemical or biological contaminants by passive samplers are crucial to the effectiveness of these samplers [1]. Therefore, understanding

these dynamics is essential for data interpretation and estimating analyte concentrations over a sampling period.

The objective of this review is to critically examine current knowledge of passive sampling technologies for pathogen surveillance, highlight key research developments, analyzing the challenges and constraints encountered across various scenarios and identify gaps that need to be addressed By doing so, we seek to outline avenues for future research and foster advancements in this field.

2.3 Critical Review Scope and Methods

Herein we present the findings from a comprehensive review of literature published from November 1916 to March 2024. Articles for the review were identified through several databases, including PubMed, ProQuest, Scopus Science Direct, and JSTOR Collection. The inclusion criteria targeted research articles, scholarly journals, books, theses, conference proceedings, working papers, and technical and health-related reports. We excluded review papers, non-original work, and non-English publications. Initially, our research yielded a total of two hundred articles, with an additional 96 articles identified through the snowball approach. After removing duplicates, the remaining articles were screened based on their abstracts, key words, and conclusions to ensure the studies aligned with the review focus. The screening process used adhered to the preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines outlined by Moher et al., (2009) [12]. This screening resulted in 185 articles meeting our initial inclusion criteria. These were subjected to an in-depth, independent review for data extraction, which was systematically compiled in Microsoft Excel (version 2402). Of these, 180 articles were incorporated into our review and a standardized dataset was compiled from each article (Figure 2-1). The 5 articles excluded at this stage, despite initially meeting the inclusion criteria, were found upon closer inspection to fall short of the specific requirements for our review. All statistical analyses and figure preparation was conducted in RStudio (version 4.2.3), utilizing packages like tidyverse, scale, and ggtext to facilitate data processing and visualization [13–15].

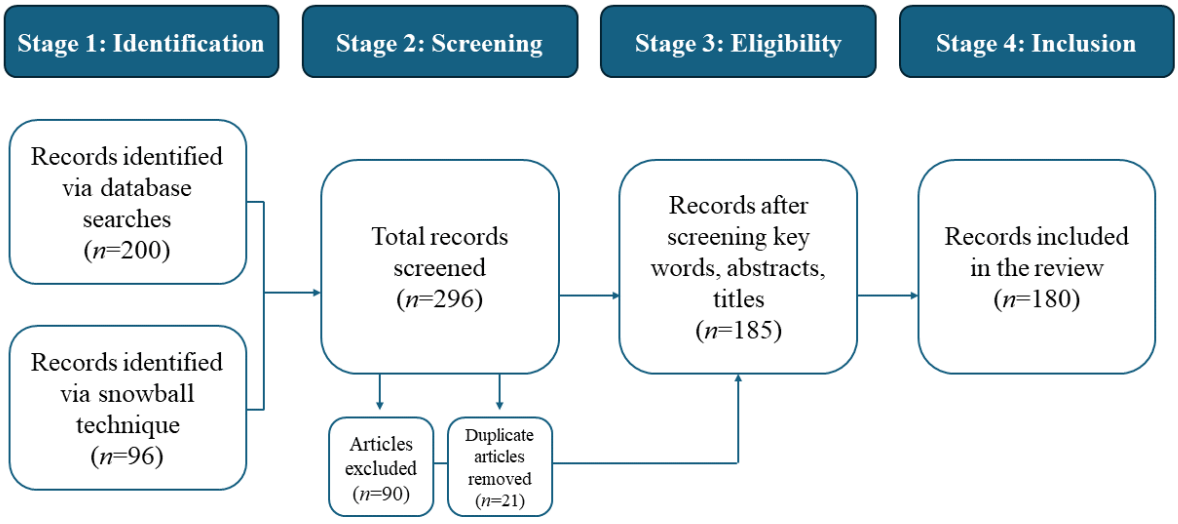

Figure 2-1. Diagram of the article screening process at each stage of the (PRISMA) guidelines.

2.4 Temporal & spatial distribution of publications

The evaluation of publication trends from 1916 to 2024 reveals that the volume of research articles published on passive sampling is connected with periods of significant public health events (Figure 2-2). The first reported use of passive sampling to monitor pathogens in wastewater was reported in 1916 [16], however, it wasn't until 1948 that the sampling method became more widely used [17]. A pronounced increase in the number of articles (n = 13) during the Poliovirus epidemics between 1948 and 1955 [18–21] and an even more substantial surge (n = 54) coinciding with the COVID-19 pandemic between 2019 and 2023 [22–25], indicating a reactive pattern in the scientific community's focus on passive sampling methodologies in response to emerging infectious disease threats. The historical parallel between these public health events and the associated scientific responses illustrates the central role of global health in shaping research priorities within this field.

Markedly, passive sampling methods have undergone a significant transformation over several decades. Initially, in the early to mid-twentieth century, applications were predominantly reported within the medicine and public health domains, peaking in the late 1960's. For example, between 1910 and 1999, approximately 100 articles were published using passive sampling, of these articles 71% were published in medicine and public health fields, 1% in food-related domains and 28% in science and engineering. However, in

subsequent decades there was a diversification of applications, particularly in science and engineering. Of the articles published between 2000 and 2024, 79% were in science and engineering, and 15% and 6% were in public health and food-related domains, respectively. The increase in research in science and engineering fields corresponded with the advancement of materials selection and investigations of capture mechanisms of pathogens on passive sampler devices.

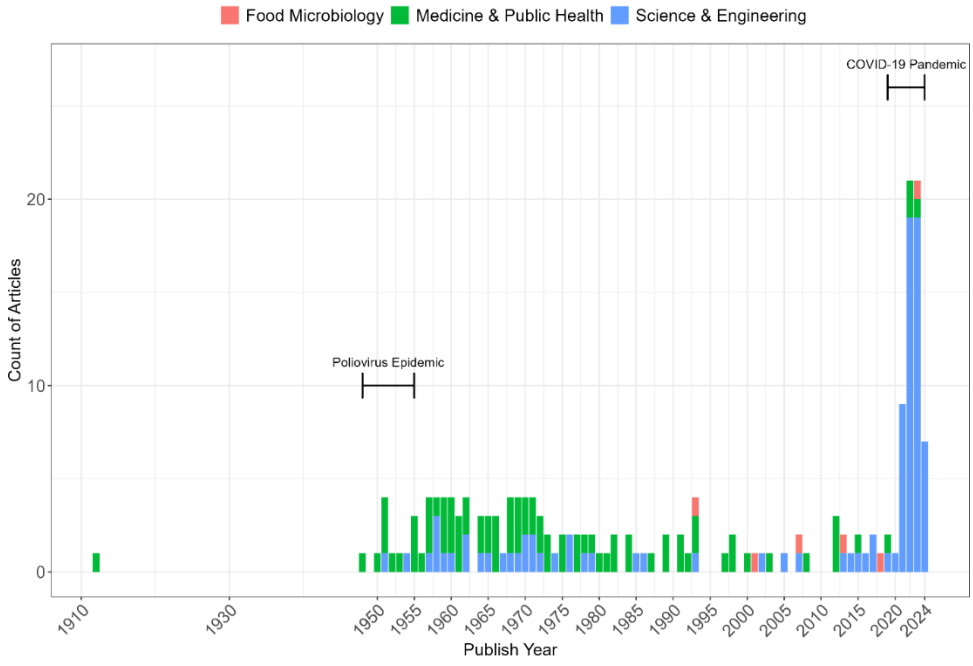

Figure 2-2. Stacked bar chart of all articles passive sampling related articles published from November 1916 to March 2024. Each bar represents the total number of articles published per year, with segments stacked to indicate the contribution from three research domains: food microbiology, medicine and public health, and science and engineering. Black bars on the plot show the approximate duration of two notable public health events; the poliovirus epidemic and the COVID-19 pandemic.

The initial adoption of passive sampling was predominantly driven by its applications in public health, a focus that emerged from the critical need to monitor diseases and inform public health interventions. The historical significance of waterborne diseases, exemplified by the cholera outbreak of 1854 in London, United Kingdom, alongside the poliovirus epidemics of the mid-20th century, highlighted the importance of vigilant monitoring within water systems [20,26]. Early use of passive sampling driven by the medical and public health fields saw little application in other research applications until recently. Passive sampling's utility in broader research domains, especially in food

safety and irrigation water monitoring, has expanded in recent times. This expansion is likely driven by heightened food safety concerns and regulatory demands in the context of global food security [27]. The shift towards engineered applications is evidenced by the significant increase in publications, reflecting the evolving global research priorities, possibly driven by technological advancements and societal needs.

2.5 Analyzing Geographical Evolution in Passive Sampling: Critical Lessons for Future Applications

The evolution of passive sampling research underscores the methods role in global water quality monitoring. By analyzing historical trends, we can better understand the driving forces behind research advancements and identify areas for future development. The data in Figure 2-3 reveals distinct publication trends geographically over, with a noticeable increase in articles from regions like Canada [24,28–38] and Oceania [23,39–46] in the twenty-first century. The data also suggests that while historically dominant regions continue to contribute significantly to the literature, there is a growing body of work emerging from underrepresented areas. This trend reflects a more inclusive approach to research and a collaborative effort to tackle global issues.

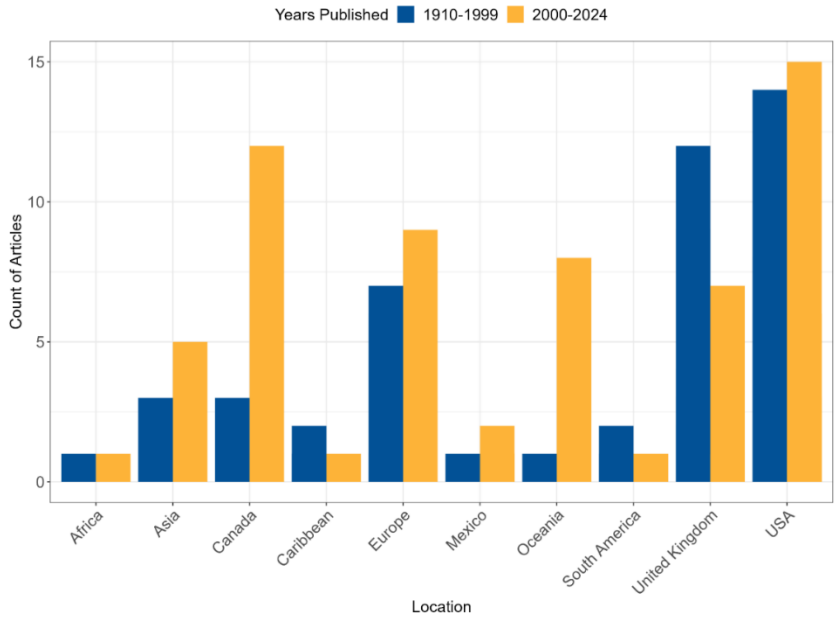

Figure 2-3. Geographical distribution of publications across different global regions, contrasting two periods: 1900-1999 and 2000-2024.

Historically, the research landscape for passive sampling was dominated by economically developed regions such as the United States, United Kingdom, and Europe. This concentration can be attributed to early public health initiatives and regulatory milestones, such as, the pioneering work of Dr. John Snow in 1854 [26] and the early establishment of water quality standards in the United States [47]. This focus was supported by key legislative developments including the United States Environmental Policy Act of 1969 [48], the Clean Water Act amendments of 1972 [49], and subsequent amendments to the Safe Drinking Water Act [50–52]. The geographical shift in publications likely reflects broader regulatory advances and heightened environmental awareness. Specifically, based on the timing of publications in regions like Canada [24,28–33,53–56], Asia [57–66] and Oceania [23,39–43,46], it appears that following the adoption of environmental acts [67–70], these regions saw an uptake in passive sampling efforts. Indicating that the evolution of environmental policy globally has been a driver of the advancement of passive sampling methods.

It is apparent from this critical review that there has been a range of passive sampling applications adopted globally to meet regional sampling needs. In 1946, Brendan Moore used gauze samplers to identify sources of infections responsible for clusters of paratyphoid fever in Devon, England [17]. In more recent applications, passive samplers were applied to monitor SARS-CoV-2 in wastewater during the Tokyo Olympics village, showcasing the methods application in regional responsiveness to public health needs [65]. Similarly, in Australia, passive sampling informed targeted public health interventions by monitoring SARS-CoV-2 in sewersheds with low COVID-19 cases [42]. While in Canada, passive samplers have been used to monitor viruses and bacteria in freshwater, aiming to contribute to water safety guidelines and decision making [31,55]. These global applications emphasize the scalability of passive samplers, allowing their application in diverse geographical regions, adapting to various environmental and socio-economic conditions. Importantly, the majority of passive sampling applications reported in the literature have been primarily conducted in high-income settings. However, the accessibility of passive sampling makes it a valuable resource for monitoring pathogens, especially in regions where clinical surveillance is limited or in other environmental contexts to monitor for the emergence or re-emergence of pathogens of concern. In

conclusion, while passive sampling has been used in high-income regions, its expansion into low- and middle-income areas could significantly improve global pathogen surveillance efforts.

2.6 Past Research Priorities by Water Matrices: Identifying Gaps and Future Opportunities for Passive Sampling in Pathogen Surveillance

Despite its relatively long history, passive sampling is still developing, and applications of the method are predominantly focused on wastewater (68% of all articles published) (Figure 2-4). This focus overlooks the method's potential in other water matrices such as freshwater (25%), seawater (3%), drinking water (1%) and irrigation water (2%) where pathogen surveillance is equally if not even more so critical. The underestimation of viral and bacterial pathogens in these environments poses significant public health risks, underscoring the need for a broader application of passive sampling techniques.

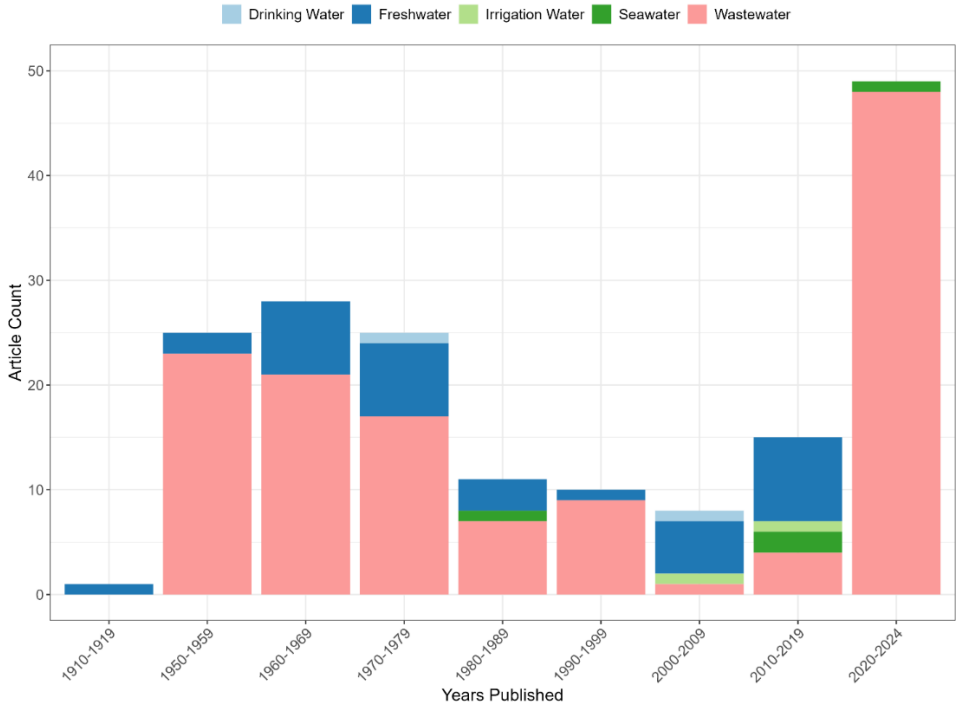

Figure 2-4. Count of passive sampling-related publications by decade from 1910 to 2024, with each bar segmented to indicate the types of water systems studied.

While the majority of passive sampling studies concentrate on wastewater, it is crucial to recognize the risk that viruses pose in other water matrices and how passive

sampling may serve to reduce the risk of underestimating the true viral presence in these environments. The extensive focus on wastewater highlights the prevailing trends and current scope of research, underscoring the effectiveness of passive sampling in tracking pathogens from this matrix for assessing public health interventions. However, this focus also reveals a significant underrepresentation of studies in other water matrices such as drinking water, seawater, and irrigation waters, where passive sampling could offer crucial insights for pathogen surveillance.

Historically, the intersection between water and wastewater systems, driven by gaps in wastewater management infrastructure, has shaped much of the research focus for passive sampling in freshwater systems [71]. Early studies using passive samplers in freshwater, reveal the challenges of managing pathogens in non-potable and potable water systems due to difficulties in accurately detecting pathogens like Aeromonas and Campylobacter [72,73]. Additionally, the work by Escartin et al. (2002) on Salmonella transmission from ornamental fountains highlighted the need for broader environmental assessments that consider both conventional and unconventional sources of pathogens [74]. While passive sampling in irrigation waters has demonstrated the presence of pathogens like Shiga toxin-producing *Escherichia coli* (*E.coli*), Salmonella, and Listeria monocytogenes [75,76]. However, the effectiveness of passive sampling in irrigation waters needs further refinement to reliably detect low-level pathogens [77]. Improving these methods will better safeguard irrigation waters and, consequently, food supplies.

The scarcity of research on pathogens in offshore marine waters is notable, given the ocean's vastness and ecological significance. Marine environments poses unique challenges for pathogen surveillance, such as the impact of high pH and salinity on viral infectivity and stability [78]. To date, passive sampling in marine environments has predominantly targeted viruses in nearshore areas, with sampling often being linked to recreational or aquaculture activities [79–81]. While recent work has begun to extended the scope of passive sampling into marine coastal areas, these initial studies emphasize a critical gap in our understanding of pathogen dynamics across broader marine environments [80,82]. The untapped potential of passive samplers in offshore and deep-sea areas remains vast. These environments, crucial to global ecological and climate

systems, could reveal significant insights into pathogen transmission and survival mechanisms, previously obscured by the logistical and financial hurdles of other sampling methods. Moreover, innovating and tailoring passive sampling tools for these complex contexts could revolutionize marine epidemiology.

Pathogens are ubiquitous in freshwater, with studies reporting notable findings of pathogen occurrence in surface water, groundwater, and drinking water [83–85]. For example, enteric pathogens were found in 15% of groundwater samples across the USA and Canada [86], and viral indicators were detected in a third of water supply samples in the USA [87]. These results challenge World Health Organization guidelines for pathogen-free drinking water [88,89], emphasizing the need for more effective pathogen surveillance methods. Many water and wastewater treatment systems are designed to reduce the concentration of organic matter, suspend solids, and pathogens, however, numerous pathogenic viruses tend to be more resistant to the removal and disinfection processes [90–93]. Despite technological advances, current strategies for monitoring pathogens typically rely on FIO [68,94]. However, FIO have been found to provide poor representations of temporal and spatial variations and fail to reliably indicate the presence and persistence of pathogens, hindering effective water safety management [95–97]. The ability of passive samplers to provide in-situ concentration of pathogens allows for the monitoring of low levels for specific pathogens of concern, eliminating the need to measure FIO. As well, the scalability of passive samplers to be deployed in a variety of locations in different configurations, positions them to be a promising alternative for monitoring treatment efficacy.

The evolution of passive sampling for pathogens across different matrices reveals considerable progress and persistent challenges. Historically, freshwater systems have offered valuable insights into pathogen dynamics due to their ties with wastewater contamination; however, few applications of passive sampling have been proposed to improve water safety in freshwater locations, despite significant limitations in existing surveillance methods. Marine water research, while expanding, is primarily focused on nearshore environments, leaving the vast offshore and deep-sea areas underexplored. In irrigation waters, passive sampling has demonstrated the presence of significant pathogens

but requires methodological advancements and rigorous testing for practical applications. The advancement of passive sampling reflects a complex interplay of scientific innovation, regulatory changes, and public health needs. Recognizing its full potential across varied environmental matrices can lead to improved pathogen sampling strategies tailored to specific surveillance needs.

2.7 Advancing Passive Sampling: Lessons from the Evolution of Capture Materials and Detection Methods

Developing effective passive sampling strategies requires careful selection of materials for pathogen capture and precise detection methodologies [5]. This dual focus is fundamental for assembling a successful sampling strategy but also for determining the adequacy and limitations of current practices. Our critical review of 180 pertinent studies reveals significant advancements in both passive sampling capture materials and pathogen detection methods paired with these materials. Our review demonstrates a significant shift in material usage and detection approaches over time, trending towards more innovative and target-specific methods. Earlier decades saw the dominance of gauze and cheesecloth, while recent years have favored polymer-based and thermoplastic materials, reflecting efforts to enhance pathogen capture efficiency and specificity (Figure 2-5A). Detection methods have also evolved from culturing and biochemical assays [77,98,99] to molecular and next-generation sequencing (NGS) techniques [96,100–102], indicating a need for heightened sensitivity, rapid analysis, and broad-spectrum surveillance (Figure 2-5B). This transition marks a response to emerging pathogen threats and the need for more precise environmental monitoring.

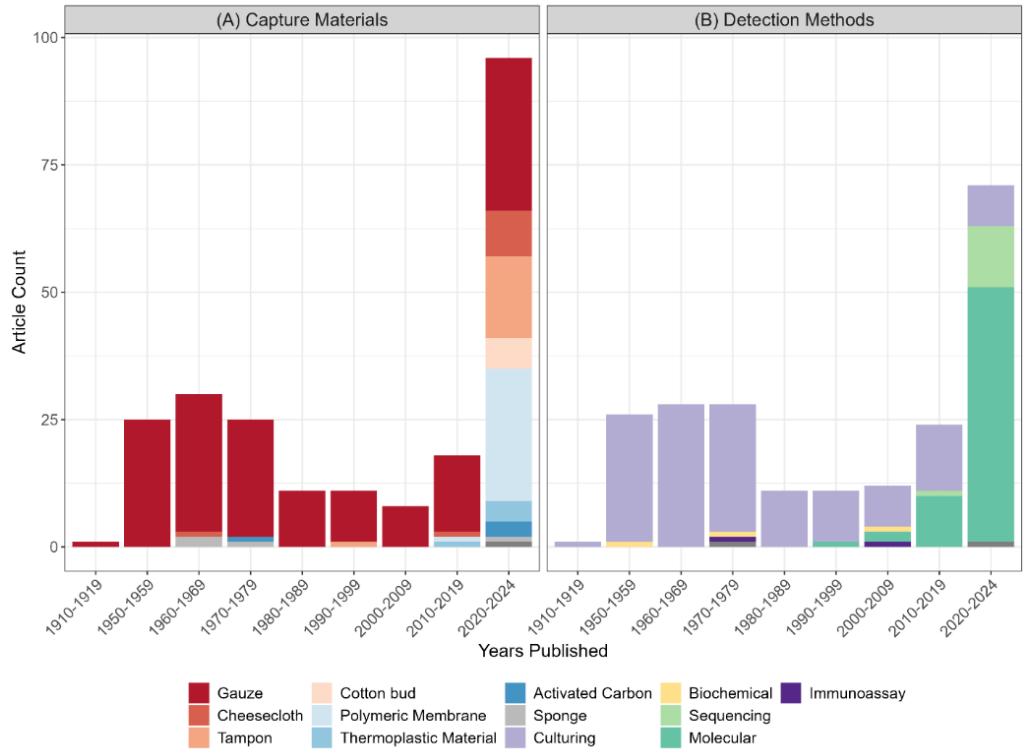

Figure 2-5. Distribution of published passive sampling methods from 1910 to March 2024, categorized by the capture and detection techniques reported. The left panel (A) displays the count of articles using various capture methods and the right panel (B) represents the count of articles by detection methods. The legend corresponds to the colours denoting each method.

2.7.1 Capture Materials

Cotton gauze and cheesecloth were the primary materials utilized for pathogen capture in water and wastewater contexts until 2020, underscored by the widespread adoption of the Moore Swab Method [17,103]. Introduced in 1948, the Moore Swab method marked a significant advancement in the detection of pathogens, particularly pathogenic bacteria like Salmonella [16,33,36,37,45,64,74,75,104–134]. While gauze and cheesecloth remain popular in passive sampling for their simplicity and widespread availability [135,136], researchers have begun to explore other common household materials such as tampons and cotton buds (q-tips), as well as more innovative materials like polymer membranes, activated carbon, and thermoplastics [23–25,28,43,57,137–140]. Tampons, often made of cotton, rayon, or a blend of these materials, are gaining recognition for their practicality in wastewater surveillance (WWS), due to their absorbency, cost-effectiveness, and easy access [5,28,32,43,135,138,139,141,142].

The advent of membranes in 2017 represents a critical shift in passive sampling research, offering enhanced pathogen capture efficiency while mitigating the adsorption of inhibitory substances, a noted shortfall of traditional gauze-based methods. Vincent-Hubert et al. (2017), pioneered this approach, developing a passive sampling method to capture norovirus and herpesvirus in seawater using electropositive membranes [79]. As a result of these findings, numerous polymeric membranes have since been employed for passive sampling of viruses, most notably electronegative cellulose-nitrate membranes, specifically, for capturing SARS-CoV-2 in wastewater [5,23,24,28,40,43,53,54,135,137,142–146]. Schang et al. (2021) highlighted the enhanced detection capabilities by electronegative cellulose-nitrate membranes compared to gauze and cotton buds [23]. Conversely, Hayes et al. (2021) observed gauze, cheesecloth and electronegative cellulose-nitrate membranes to have comparable efficiency for detecting SARS-CoV-2, although the membranes showed improved reproducibility over other materials evaluated [24]. Ifeoluwa, O. (2023) also described higher *E.coli* detection rates when using gauze in wastewater compared to PVDF membranes, cotton buds, and nylon membranes [146]. Some studies have also shown improved efficacy by combining electronegative membranes, cotton gauze, and cotton buds within a 3D-printed Torpedo-style sampler [23,43]. Kevil et al. (2022) found that tampons, outperformed SG81 Si-cellulose ion exchange membranes in capturing wastewater associated enveloped and non-enveloped viruses [142]. Despite some of the described advantages of polymeric membranes, these materials still encounter obstacles during deployment, such as fouling due to their hydrophobicity, limited chlorine tolerance, and a balance between permeability and selectivity for detecting a broader range of targets [147].

Of the 180 articles reviewed, only 5% of studies employed a material other than gauze, or cheesecloth, cotton buds, tampons, or membranes. Hayes et al. (2022) demonstrated improved adsorption of SARS-CoV-2 by granular activated carbon (GAC) in wastewater compared to electronegative cellulose-nitrate membranes [29]. Breulmann et al. (2023) assessed the suitability of polypropylene plastic ropes and polyethylene-based strips against cotton gauze strips for SARS-CoV-2 detection in wastewater [148]. Their findings indicated that while polyethylene-based samplers were the most practical, polypropylene ropes had the greatest overall detection frequency. Aguayo-Acosta et al.

(2023) proposed utilizing nanostructured polymeric membrane-based passive samplers as a potential advancement for WWS, suggesting that further refinement could enhance mechanical resilience, minimize fouling, and achieve greater selectivity [147].

The effectiveness of materials like thermoplastics, activated carbon and other engineered adsorbent materials described in literature underscores the benefits of exploring other novel capture materials for improve passive sampling. The transition from gauze samplers to more advanced capture materials with tailored properties illustrates the ongoing innovation and responsiveness to the complexities of environmental monitoring, thus highlighting the dynamic and evolving nature of this research.

2.7.2 Detection Methods

Our review revealed that detection technologies have remarkably evolved from traditional methods like microscopy, serology, and culture to advanced genomic techniques. This shift began with the inception of polymerase chain reaction (PCR) in 1983 [149] and the subsequent advent of fluorescent probes for gene count quantification in 1993 [150]. In our analysis of 180 articles, advanced methodologies like NGS and real-time and quantitative PCR methods (including qPCR, RT-qPCR, dPCR, and ddPCR) stood out for their precise quantification capabilities and comprehensive pathogen detection, respectively.

The integration of genomic-detection techniques with passive sampling has opened new avenues for environmental pathogen surveillance. Studies like those by Cha et al. (2024) and Mejías-Molina et al. (2023) exemplify the integration of NGS with passive sampling, both studies showcasing the ability of NGS to capture changes in genetic diversity within wastewater for tracking viral evolution and identifying emerging threats [145,151]. Cha et al. (2024) revealed that gauze can effectively concentrate pathogens from wastewater, thereby enriching shotgun sequencing analyses for more detailed genetic insights, especially in localized settings [151]. Similarly, Mejías-Molina et al. (2023) illustrated how electronegative membranes facilitated NGS-based detection and characterization of viral diversity in building-level sewersheds, proving valuable in targeted surveillance initiatives like nursing homes or university campuses [145].

The sensitivity, and specificity offered by PCR-based methods makes them particularly suitable for pathogen surveillance in the environment [152]. Particularly, the unique ability of dPCR and ddPCR techniques to provide absolute quantification without relying on external standards makes this tool suitable for detecting a diverse array of pathogens. The COVID-19 pandemic has particularly highlighted the relevance of these genomic-based detection methods in WWS for tracking of disease, signifying a methodological shift within the field (Figure 2-5B) [102,153]. For instance, Corchis-Scott et al. (2021) successfully employed RT-qPCR to identify the SARS-CoV-2 B.1.1.7 (Alpha) variant in building-level WWS using tampons [32]. Hayes et al. (2023) leveraged multiplex RT-qPCR analysis coupled with GAC samplers in targeted sewersheds for the simultaneous detection and quantification of SARS-CoV-2, INFA, RSV and Measles RNA [30]. While other studies, such as, Rafiee et al. (2021) utilized RT-qPCR for qualitative SARS-CoV-2 detection in wastewater [58], and Cha et al. (2022) used ddPCR for quantitative analysis of SARS-CoV-2 and pepper mild mottle virus (PMMoV) concentrations captured on gauze samplers deployed in building-level sewersheds [154].

Advancements in genomic methods offer improved accuracy in pathogen detection but come with challenges. Factors influencing these methods include the nature of the passive sampling material, the extraction process for virus recovery, and potential inhibitors that impede downstream analysis. Notably, the presence of environmental inhibitors like humic acids, heavy metals, and detergents, can affect PCR reactions and lead to false negatives or underestimation of viral loads [155–157]. Therefore, the choice of sampling material may impact viral recovery; materials that retain more solids or organics, such as tampons, gauze, and certain membranes, may experience more inhibition. Therefore, selecting appropriate materials to minimize inhibitory effects while maximizing virus recovery is important when designing a passive sampling strategy. Various approaches have been developed to address this issue, including pre-treatment methods like filtration and inhibitor-removal kits, which improve SARS-CoV-2 detection in passive wastewater samples [28]. Using internal controls in qPCR assays helps identify inhibitors and ensure accuracy, while optimizing nucleic acid extraction protocols enhances RNA purity and concentration [57]. Despite the benefits of advanced molecular detection methods, their higher costs can sometimes pose barriers, particularly in low-

resource settings. This underscores the need for diversified approaches, such as integrating passive sampling with simpler, cost-effective technologies like Immunoassays and Loop-Mediated Isothermal Amplification (LAMP) assays, which offer rapid detection and significant predictive value [138].

Traditional culturing techniques remain central to bacterial pathogen detection, as shown by their continued adoption in global water regulations and standards. Routine monitoring prioritizes culturable indicator organisms like Total coliforms and *E.coli* over direct virus monitoring [51,68,69,158], despite culturing limitations, such as being time-intensive and unable to capture a full spectrum of viral threats [158–160]. While genomic detection has revolutionized pathogen monitoring, current methods still fall short in differentiating between viable and non-viable particles, which is crucial for assessing risk. This gap between modern research potential and regulatory frameworks underscores the need for new monitoring strategies that embrace genomic-based detection. For this change to occur a comprehensive validation and benchmarking process is essential to ensure that genomic-based methods provide reliable, reproducible results and are adaptable across various settings.

2.8 Critical Insights into Deployment Durations for Effective Passive Sampling

A review of current literature underscored significant variability in the deployment durations of passive samplers, with historical trends and recent advancements reflecting diverse approaches tailored to specific pathogens and environmental contexts. Moore's seminal work in 1948 established a baseline with 48-hour deployments for gauze swabs [17], and subsequent studies have adhered to similar timeframes ranging from 24 to 78 hrs [104,105,127,134,161–163] to even up to 96 hrs [21,98,164], and 168 hrs [108,165]. Notably, modern applications in wastewater indicate a shift towards a broader range of deployment periods, driven by specific sampling goals and material capture efficiencies.

Here, we aim to report the most common deployment times that have been reported in literature for passive sampling of pathogens across matrices. Frequent deployment durations for gauze [5,53,65,135,143,144,146,154,166,167], cheesecloth [24,135,168], tampons [5,32,66,135,144,169,170], cotton buds (q-tips) [25,146,171], cellulose composite membranes [5,25,29,42,46,53,55,80,135,137,144,145,171], nylon netting

[79,80,146], glass wool filters [5,135] and various polymeric membranes [79,80,146] were reported to be ~24 to 48 hrs for capturing bacteriophages, enteric and respiratory pathogens in wastewater. However, a recent study revealed deployments of ~3 hrs for tampons deployed in sewersheds on a university campus to capture daily SARS-CoV-2 signals [138]. Conversely, other studies report extended deployment periods in wastewater, such as upwards of 96 hrs for GAC-based samplers [29,30] and up to 28 days for polypropylene plastic ropes and polyethylene-based plastic strips [148]. These longer durations are particularly notable in large-scale, city-level wastewater monitoring, emphasizing the scalability of passive sampling methods with certain capture materials.

In seawater, where salinity poses unique challenges for pathogen capture, Vincent-Hubert et al. (2021) demonstrated that nylon netting, LDPE, and PVDF effectively captured SARS-CoV-2 from seawater and wastewater within 4 hrs [81]. Nylon nets and electropositive membranes were also found to capture viruses in coastal seawaters within 4 to 8 hrs [79,81], while LDPE was favored for deployments of 48 to 360 hrs due to the materials durability [79]. Vincent-Hubert et al. (2017) also noted that for deployments of ~24 hrs, zetapor material was more adapted to capture norovirus, while for deployments of ~2 weeks, nylon and zetapor membranes displayed similar norovirus adsorption, and then for herpesvirus, PVDF was noted to be more adapted [79]. Freshwater studies reveal similar variability in deployment times reported, with common durations for gauze-based sampling ranging from 24 to 48 hrs for detecting pathogens such as norovirus [172], Salmonella [37,74,100,115,123,162,173,174], Pseudomonas [60,63], Listeria [75,76,100], *E.coli* [74,75,77,100,175], Campylobacter [73,176], Coliforms [74], Cholera [177], Enteroviruses and Mycobacteria [37,61,106,162,173,176,178,178]. Longer gauze-based sampling durations (72 to 120 hrs) were also used for pathogens like Salmonella [33,106,109,119,161], *E.coli* [77,119], and Plesiomonas [73]. Membrane-based passive samplers also exhibited a range of deployment durations reported, with electropositive membranes being deployed for 48 hrs in irrigation waters for the detection of norovirus [82]. While, electronegative membranes were reported for durations ranging from 4 to 96 hrs to detect *E.coli* [55], and for 24 hrs to detect SARS-CoV-2 in freshwater [171]. Whereas, Tampons were used to detect Campylobacter in freshwater, with deployment

durations ranging from 72 to 120 hrs [179] and GAC-based samplers reporting over 168-hrs deployment durations for viral detection in freshwater [31].

The rate at which a passive sampler captures pathogens, known as the sampling rate, is an important factor influencing the overall efficiency of pathogen detection [7]. This rate is typically defined as the volume of water from which the analyte is extracted per unit of time (e.g., mL/h or mL/min). Sampling rates are influenced by several factors, including the material of the sampler, the nature of the target pathogen, and environmental conditions [7]. Based on the concentration gradient of pathogens in the aqueous phase and on the collection phase (i.e., sampling material), pathogens can adsorb and/or absorb until an equilibrium is reached. As such, understanding when a passive sampler reaches equilibrium, is vital in obtaining the most accurate information. Deploying samplers past this point can lead to inaccurate data that doesn't reflect the entire sampling period. However, traditional adsorption models commonly used to characterize adsorption are primarily designed for organic and inorganic pollutants, and may inadequately address the complexities of pathogen capture [180–182]. Pathogens exhibit unique adsorption behaviours due to their size, structure, and interaction with the sampler material [183], necessitating more sophisticated models to accurately predict the complex dynamics of microbial adsorption. For example, while chemical pollutants' adsorption are well-defined by physicochemical interactions, pathogens such as viruses and bacteria can exhibit a range of adsorption dynamics influenced by factors like electrostatic interactions and surface hydrophobicity [6,184]. These complexities can further complicate adsorption dynamics, as pathogens may partition into or interact with compounds commonly found in environmental samples [185]. This discrepancy underscores the need for a deeper exploration of mechanisms to predict the capture of pathogens through passive sampling, particularly further research is needed on how adsorption characteristics differ between microbial organisms and other pollutants. Understanding the rate at which a target analyte adheres to the sampler surface and reaches equilibrium (adsorption kinetics) and the volume of water effectively sampled per unit time (sampling rates) is essential for accurate quantification and optimal deployment durations.

Studies by Hayes et al. (2022a and 2022b), Habtewold et al. (2022), Li et al. (2022), Rao, G. (2023), and Shakallis et al. (2024) have begun to fill this gap [25,29,43,46,54,186].

These researchers have investigated the adsorption dynamics specific to pathogen capture, revealing varied efficacies and dynamics of various materials under diverse conditions. Habtewold et al. (2022) demonstrated reliable viral particle capture with electronegative membranes, with longer deployments (up to 48 hrs) increasing viral RNA detection due to in situ concentration effects [25]. Likewise, Li et al., (2022) found that electronegative membranes deployed over 48 hrs in wastewater had a linear uptake, with sampling rates of 1 mL/hr for PMMoV, 0.3 mL/hr for enterovirus, 33.1 mL/hr for adenovirus 40/41 [43]. While, tampons and cotton-buds (q-tips) demonstrated non-linear uptake with reaching equilibrium after ~8 hrs or less, and gauze showed a rapid uptake followed by a decline in uptake up to 48 h suggesting desorption or decreased recovery efficiency with increasing exposure duration, highlighting the importance of regular calibration to avoid biofouling or decreased recovery efficiency with increasing exposure. Similarly, Hayes et al. (2022a) examined the capture of SARS-CoV-2 by electronegative membranes finding adsorption followed a pseudo-first-order model, with maximum concentrations achieved within 24 hrs in both deionized water (rate constant of ~0.001/hr) and wastewater (rate constant of ~0.05/hr), recommending 24 to 48-hr deployments for targeted sewershed sampling [54]. Hayes et al. (2022b) also evaluated the adsorption of SARS-CoV-2, PMMoV, and CrAssphage by GAC in wastewater, demonstrating maximum adsorption at ~60 hrs in wastewater for all three targets [29]. Finding that adsorption best followed a pseudo-second-order model, with second order rate constants calculated to be $\sim 3\times10^{-9}$/hr, $\sim 2\times10^{-6}$/hr and $\sim 1\times10^{-8}$/hr, for SARS-CoV-2, PMMoV, and CrAssphage, respectively. Conversely, Shakallis et al. (2024) investigated the capture of seeded bacteriophage MS2 by electronegative membranes followed a pseudo-first-order reaction model in deionized water and wastewater, finding adsorption equilibrium being reach within 3 hrs (rate constant of ~16/min) and 10 mins (rate constant of ~17/mins), respectively [46]. This work also described that adsorption was dependent on virus concentration but independent of time deployed, with higher initial concentrations leading to increased adsorption and with adsorption being lowest in alkaline matrices and greatest in more acidic conditions. However, calibration of sampling rates has been described to be time-consuming and costly, and their measurement cannot usually be optimized for each passive sampling system, target analyte, and all environmental conditions [187,188]. As a result, sampling

rates measured in the laboratory only consider conditions for a specific sampling setting, therefore significant inaccuracies may arise when using these measurements for field deployments.

Unlike these other studies, Rao, G. (2023) characterized the capture dynamics of gauze for several viral targets from wastewater over 48 hrs using the Redlich-Peterson isotherm model, observing that the highest viral RNA concentrations were observed on the gauze between 9-12 hours in the presence of stable densities of target microbes under controlled laboratory conditions [186]. Rao, G. (2023) also found that after 24 hrs, maximum adsorption capacities (q_{max}) were calculated to be ~1×10^{13}, ~5×10^{11}, ~4×10^{12}, and ~1×10^{11} gene copies per gram of gauze for Bovine coronavirus, murine hepatitis virus, Φ6, and Zika virus, respectively. However, it was observed that equilibrium capacity of the gauze increased with higher viral target concentrations, regardless of TSS levels in wastewater. Based on these findings, the authors suggested the rate of adsorption equals the rate of desorption for gauze samplers. Likewise, Hayes et al. (2022a) demonstrated SARS-CoV-2 adsorption from wastewater with low (~118 mg/L) to medium (~265 mg/L) TSS levels to electronegative membranes in were best characterized by Freundlich isotherm models with a max adsorption of ~7000 GC/cm^2 observed under medium TSS conditions. This work also noted an excess of total suspended solids (TSS) in wastewater may impede viral recovery, while moderate TSS levels may enhance recovery. Therefore, measuring TSS levels in sampler eluates may be a feasible option to assess inhibitory impacts to viral detection when wastewater TSS levels are not measurable. Whereas, Hayes et al. (2022b) described the capture of SARS-CoV-2 under common wastewater conditions to the study region onto GAC's surface, using a Hybrid Freundlich-Langmuir equilibrium isotherm model to calculated a q_{max} value of ~2.5 10^9 GU/g, indicating physiochemical and multilayer adsorption at lower viral concentrations and more monolayer adsorption processes as adsorption capacity is reached [29].

In conclusion, optimizing passive sampling for pathogen surveillance requires understanding the process of adsorption and sampling rates, tailored to the specific conditions of each deployment to ensure accurate and reliable data. However, the kinetics and sampling rates needed to move toward more standardized deployment strategies, are not yet well defined making this a challenging undertaking. Nonetheless, by leveraging

insights from current literature, we can begin to establish preliminary guidelines for deployment durations. For example, a consistent theme in the literature is that shorter deployments (≤24 hrs) are ideal for capturing transient signals and minimizing biofouling, though they may underestimate pathogen concentrations in dynamic environments. In contrast, longer deployments (>24 hrs) may provide a better representation of pathogen presence over time, but the adsorption capacity and sampling rates of the sampler must be considered to avoid inaccurate data.

2.9 A Critical Assessment of Reported Detection Capabilities in Passive Sampling Studies

This review analyzed 180 articles, with sixty-two providing quantitative data on genomic units detected. The data illustrates the variability in pathogen detection across materials, target pathogens, and environmental matrices (Figure 2-6). Gauze and cheesecloth demonstrated a wide range of viral concentrations in wastewater, for example, detections of SARS-CoV-2 spanned from ~10^4 to ~10^7 GC/sampler, with enteric viruses showing a similar pronounced range of detection and even in some instances detection upwards of ~10^{10} GC/sampler for Bacteriophages and Adenovirus. Tampons also demonstrated detection capabilities in wastewater for respiratory viruses (ranging from ~10^1 to ~10^9 GC/sampler) and enteric viruses (from ~10^3 to ~10^6 GC/sampler). Polymeric membranes exhibited a broad detection range, with high maximum concentrations being detected for respiratory viruses in wastewater (~10^8 GC/sampler), seawater (10^7 GC/sampler) and freshwater (~10^4 GC/sampler). GAC, although used less frequently, detected high concentrations of enteric and respiratory viruses up to ~10^9 GC/sampler and minimum concentrations of ~10^2 in freshwater and ~10^1 to ~10^8 GC/sampler in wastewater. Thermoplastics, also less studied, showed promise in detecting enteric virus in wastewater and seawater (between ~10^2 to ~10^4 GC/sampler) and respiratory viruses in wastewater upwards of ~10^3 GC/sampler.

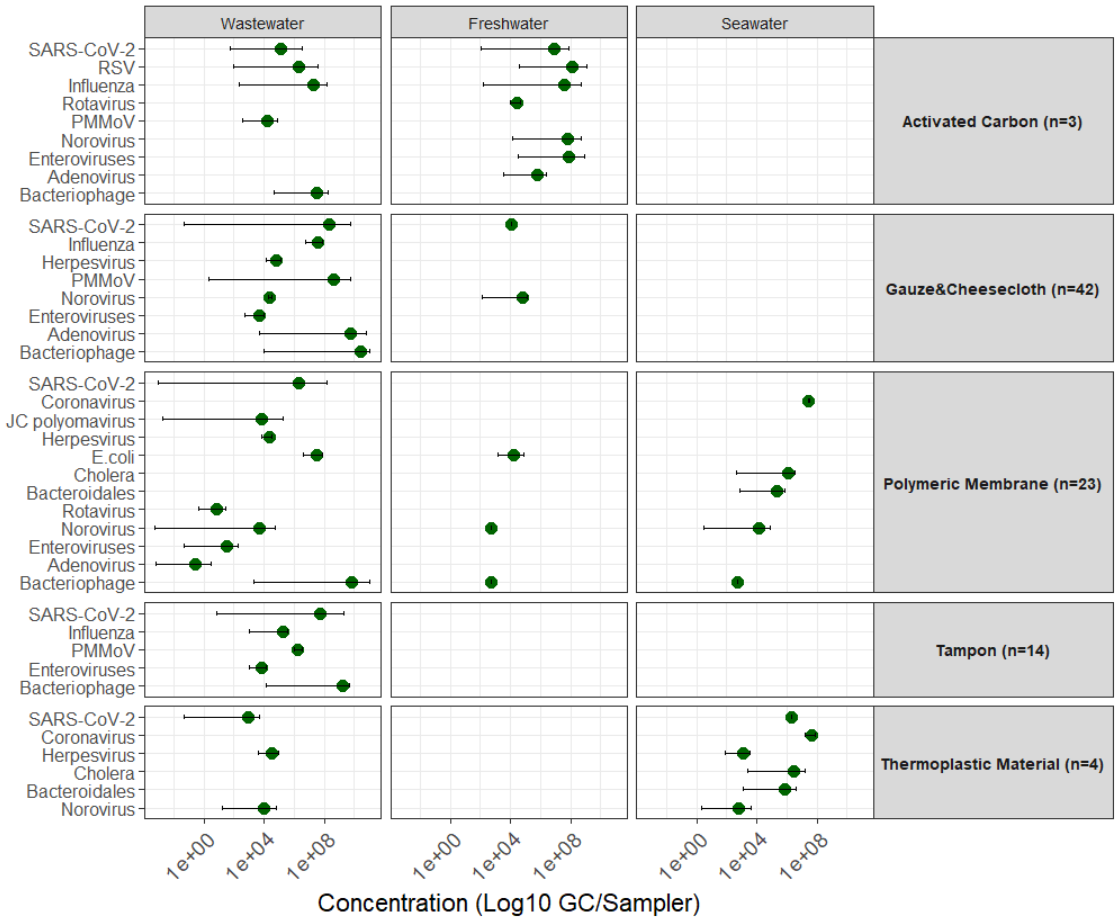

Figure 2-6. The minimum and maximum gene concentrations (GC/sampler) captured at the time of collection by each material category reported in the literature by molecular detection methods (e.g., qPCR/RT-qPCR and ddPCR/RT-ddPCR) are shown by black bars for each target pathogen and water matrix of the given capture materials. The average concentration reported for each target is shown by the green-coloured circles. The number of reported applications for each material (n=) is in the titles.

It is important to acknowledge that the direct comparison of viral concentrations across sampling events is challenging due to variations in material properties, deployment durations, and adsorption kinetics. Environmental factors such as pathogen shedding rates, survival, and degradation influenced by temperature, salinity, and organic matter, along with methodological differences like unknown sample volumes, sample storage conditions, nucleic acid extraction and purification procedures, further complicate the assessment of material performance [155,186,189–191]. The adsorption and detection efficiency of a passive sampler is also dependent on the specific pathogen and environmental conditions, which were not consistently reported for direct comparisons. Despite these challenges, comparing the pathogen concentrations found in literature

provides valuable insights into the detection capabilities of various passive sampling materials and highlights gaps in our understanding and applications of pathogen surveillance with passive samplers. Future studies can use this data as a baseline to reinforce findings within similar ranges or to provide insights into potential issues method issues when observing concentrations outside the reported ranges.

Table 2-1 provides an overview of pathogen detection across passive sampling studies and allows for a broad comparison with traditional sampling methods reported in literature. In wastewater, passive sampling methods have demonstrated sensitivity comparable to, or even exceeding, traditional grab sampling techniques for detecting SARS-CoV-2 RNA [24,28,58,192]. As well, materials such as gauze and electronegative membranes have shown promising results, often outperforming 24-hr composite samples in terms of detection sensitivity [23,28,58,154]. Markedly, West et al. (2023) reported that tampon samplers detected SARS-CoV-2 RNA more frequently than grab sampling when viral RNA concentrations were below the study's limit of detection [192]. This indicates a potential advantage of passive sampling in scenarios with low viral load in wastewater. Additionally, passive sampling offers several other advantages to grab sampling and autosampling, including ease of deployment, lower operational costs, and in-situ concentration, which may enhance virus detection probability [136]. These attributes suggest that passive sampling is a compelling alternative to traditional methods for pathogen detection in wastewater. Conversely, Bivins et al. (2022) suggests while passive samplers, particularly electronegative membranes, have potential to produce time-weighted averages or semi-quantitative data for SARS-CoV-2 RNA in wastewater [136], they found an overall weak linear relationship between viral concentrations from passive and autosampling across five different wastewater settings. Further highlighting the limited understanding of passive samplers to produce quantitative data comparable to autosamplers and the need for future research to continue to explore paired studies that evaluate passive sampling to autosampling methods.

Table 2-1. Summary of published data on pathogen detection in wastewater, freshwater, and seawater using passive and conventional sampling techniques. Maximum pathogen concentrations are presented as cycle quantification (Cq) values, total gene copies detected per passive sampler (GC/sampler), or total gene copies per sample volume (GC/sample) for grab or composite samples.

Matrix	Pathogen	Sample	Volume	Max Conc.	Reference
Wastewater	SARS-CoV-2	Grab	1 L	32.1 Cq/sample	[58]
		24 hr Composite	8 L	31.4 Cq/sample	
		Gauze	n/a	30.4 Cq/sampler	
		Electronegative cellulose-nitrate membrane	10 mL	1.1×10^{3} GC/sampler	[23]
		24 hr Composite	50 mL	2.3×10^{1} GC/sample	
		Tampons	n/a	5.0×10^{5} GC/sampler	[192]
		Grab	200 mL	2.6×10^{5} GC/sample	
Freshwater	Norovirus	Gauze	n/a	3.5×10^{3} GC/sampler	[172]
		Electropositive cellulose membranes			[82]
	Norovirus	GAC		4.8×10^{8} GC/sampler	[31]
	Enterovirus			8.8×10^{8} GC/sampler	
	Rotavirus			4.7×10^{4} GC/sampler	
	Adenovirus			2.6×10^{6} GC/sampler	
	Norovirus	Grab	2 L	6.6×10^{4} GC/sample	[193]
	Rotavirus		10 L	1.2×10^{4} GC/sample	[194]
	Adenovirus		2 L	1.3×10^{5} GC/sample	[195]
	Enterovirus		1 L	9.8×10^{3} GC/sample	[196]
	PMMoV		2 L	4.0×10^{6} GC/sample	[197]

Matrix	Pathogen	Sample	Volume	Max Conc.	Reference
Seawater	Noroviruses	Grab	10 L	2.8×10^{9} GC/sample	[198]
	Adenovirus		18 L	5.4×10^{7} GC/sample	[199]
	Enterovirus		0.5 L	3.3×10^{7} GC/sample	[101]
	Coronavirus	Nylon netting	n/a	8.0×10^{7} GC/sampler	[80]
		Electropositive cellulose membranes		3.2×10^{7} GC/sampler	
	Norovirus	LDPE		3.1×10^{0} GC/sampler	[81]
		Electropositive cellulose membranes		9.4×10^{0} GC/sampler	
		Nylon netting		3.9×10^{0} GC/sampler	

Another significant consideration is the detection of pathogens present at low concentrations, because even at low levels, many pathogens can still pose significant public health risks [195,200,201]. Traditional autosamplers often miss these low concentrations. While high concentrations of target viruses can be detected in relatively small volumes of wastewater samples (<100 mL) [68,69,159], detecting viruses in freshwater, seawater, and drinking water often requires much larger volumes (10–1000 L or more) due to lower viral concentrations [159,160,202]. However, the collection and concentration of such large water volumes is resource-intensive and logistically challenging, often leading to an underestimation of viral presence as a result of these practical constraints. Given these challenges, additional tools are required to enhance the detection of pathogens, particularly in recreational freshwater and drinking water systems. Passive sampling is positioned to advance the detection of pathogens in water resources, however, the current limitations of producing quantitative data and the lack of understanding on viability of pathogens adsorbed to samplers will need to be further investigated to move towards a greater acceptance of passive sampling in water safety frameworks.

2.10 Towards Better Reporting: A Critical Review of Practices to Guide Future Passive Sampling

The units reported in literature for passive sampling are highly variable, with articles using culturing methods most often reporting results in CFU/mL and CFU/swab, with occasional use of MPN/mL, PFU/mL, PFU/swab, and TCID50/mL, with qualitative reporting being another common strategy. In contrast, molecular methods predominantly use units to report absolute quantification, in units of GC/sampler (sometimes referred to as GC/swab) and GC/volume, followed by GC/surface area of material, GC/gram of material, and GC/days sampled. Although qualitative reporting is less common in molecular methods, it does occur when standard curves cannot be generated.

The choice of reporting units in passive sampling studies significantly affects data interpretation and comparability. Qualitative data, although useful for specific targets where standard reference materials are unavailable to generate quantification curves, limits the ability to monitor changes in pathogen prevalence or load over time, restricting trend analysis and correlation with environmental or health outcomes. Volumetric reporting units (e.g., CFU/mL) align with many regulatory standards, facilitating comparisons to established water quality criteria and supporting disinfection technologies [68,69]. However, in passive sampling contexts where the volume of water interacting with the sampler is most often unknown, volumetric reporting can be misleading, potentially distorting perceived pathogen concentrations. Units based on total gene copies per sampler (e.g., GC/sampler) provide absolute concentrations of pathogen load on the sampler at the time of collection, rather than estimating the concentration of a pathogen in a given volume in the water systems. As a result, passive sampler concentrations do not enable comparisons to traditional volumetric measurements and therefore at present are unable to be integrated with existing water quality frameworks. However, the ability to generate quantitative data is crucial for supporting public health decision-making and improving water quality.

To enable passive sampling results to offer actionable insights for public health and water quality decision-making, future research must establish clear correlations between pathogen concentrations detected through passive sampling (e.g., GC/sampler) and traditional volumetric metrics (e.g., CFU/mL). Researchers can better understand these

correlations by conducting parallel sampling studies with active and passive sampling in various environments through bench-scale and field-scale experiments. Integrating passive sampling results with epidemiological data and comparing baseline values between culturing and genomic units will also improve data reliability. Additionally, implementing long-term monitoring programs using passive samplers will provide valuable data on temporal trends in pathogen levels and their changes with seasonality, climate events, and public health responses. Through this work, researchers can better understand risk thresholds from passive sampling results and make informed comparisons across locations.

A significant challenge that appears from the current body of literature is the lack of uniformity in reporting units and methodologies. This heterogeneity makes accurate cross-study comparisons challenging and limits the ability to draw robust conclusions about the efficacy of different passive sampling strategies. To achieve clear and consistent reporting, we suggest detailed metadata must be provided across several categories. For instance, future studies should aim to include the type of study (bench-scale or field-scale), sampling locations (wastewater, freshwater, marine water, irrigation water, drinking water), sampling dates and times (time of year and geographical location), and target virus (seeded/surrogate or endogenous). As well, environmental context requires background information on the site, including historical data on pollution and land use, where possible. Sample collection details should cover sample environment flow rate (whether known or unknown), specific deployment duration (particularly if variable among detections and/ or capture materials), and specific matrix conditions such as temperature, pH, TSS, dissolved organic carbon, total organic carbon, turbidity, conductivity, etc., during sampling. Passive sampler details should specify the sampler media, its preparation, deployment method, and any additional details to address constraints over sampling. Sample processing information should describe the elution methods, volume used, and sample storage conditions. While data analysis should detail quantification methods (or rationale for lack of quantification), and any data normalization approaches, used for reporting concentrations measured. Finally, reporting should include results presentation formats and units, detection limits, and the reporting of uncertainty and error. By including the above-mentioned details,

future researchers will ensure improved reliability in data reporting and enhanced cross-study comparisons for passive sampling.

2.11 Conclusions

This review critically evaluates the evolution of capture materials and detection methods for passive sampling pathogens in wastewater, freshwater and seawater. This review demonstrates the effectiveness of a variety capture materials, based on context-specific applications in various matrices for pathogenic bacteria and viruses. The results of this review demonstrate that passive sampling in wastewater is most commonly cited throughout literature, with many articles showing improved or comparable sensitivity of passive sampling methods to conventional grab and autosampling methods, most notably for the detection of SARS-CoV-2. However, freshwater and seawater applications of passive sampling are presently limited, and only a few studies reporting the utility of capture materials such as GAC, thermoplastics, and polymer membranes. Although, these initial studies emphasize the potential of passive sampling to overcome technical constraints associated with large volume sampling for pathogens, particularly in recreational freshwater and drinking water. Likewise, advancements over the last few decades in pathogen detection methods have significantly improved surveillance, with modern molecular techniques offering greater sensitivity and specificity than traditional. However, future research is needed to better understand how molecular detections from passive samplers can be correlated to viability.

To continue to advance this field of work, future research should focus on refining existing passive sampling protocols for improved cross-study direct comparisons of capture methods in more diverse environmental matrices. By providing more detailed methods and potentially standardizing the way information is shared on capture materials, deployment durations, detection methods and data reporting strategies, we can gain a better understanding of how to best use passive samplers. As we continue to explore novel methods and applications for passive sampling, we can begin to align passive sampling to regulatory benchmarks, ultimately leading to this method become a more widely accepted tool for managing water-related pathogens.

2.12 References

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CHAPTER 3 A NOVEL PASSIVE SAMPLING APPROACH FOR SARS-COV-2 IN WASTEWATER IN A CANADIAN PROVINCE WITH LOW PREVALENCE OF COVID-19

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E.K.H. developed the method, carried out data collection, and data analysis, wrote the paper, and prepared the figures.

3.1 Abstract

The overall objective of this work was to develop a simple and effective passive sampling protocol for the detection of SARS-CoV-2 in sewer catchments at targeted institutional-level sampling sites in a region of low COVID-19 prevalence. We developed a new 3D-printed sampling cage and assessed four commercially-available materials (cotton gauze, cotton cheesecloth, cellulose sponges, and electronegative filters) for RNA adsorption in the cage. We determined that cheesecloth and electronegative filters provided an effective approach for collecting and measuring SARS-CoV-2 in wastewater. We also compared the performance of three elution mixtures (a commercially-available lysis buffer, a Tween®20-based buffer, and a 1:1 acetonitrile: water mixture) for the detection of heat-inactivated SARS-CoV-2 reference material (HI-SCV-2) spiked into municipal wastewater at $1.0x10^3$ genomic units per millilitre (GU mL^{-1}). The highest mean RNA concentrations were achieved using the cheesecloth ($7.0\times10^4 \pm 3.7x10^4$ GU per eluate) and electronegative filters ($2.3\times10^4 \pm 2.5\times10^4$ GU per eluate) in combination with the Tween®20-based buffer with positive detections in all three biological replicates for both material types. We deployed passive samplers at two sewer catchments (Locations A and B) to compare the performance of each passive sampler material type in the field. Over 15 sampling events at each site, we demonstrated that both cheesecloth (Location A) and electronegative filters (Location B) coupled with a Tween®20-based elution technique could be utilized for the reliable detection of SARS CoV-2. These results have demonstrated a quick and effective passive sampling approach for SARS-CoV-2 detection

in targeted locations in wastewater collection systems, which may have long-term applicability as global vaccination programs evolve.

3.2 Introduction

Since the onset of the coronavirus 2019 (COVID-19) pandemic, the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been identified in both respiratory and gastrointestinal tracts,[1] with viral shedding reported in feces and urine of both symptomatic and asymptomatic individuals through all stages of infection.[2] The global spread of COVID-19 has led to the progression of wastewater-based epidemiology (WBE) as a macro-scale surveillance tool that is sought to aid in public health decision-making.[3–9] The WBE approach is relatively new and was developed based on the analysis of biomarkers and pollutants in wastewater to obtain both quantitative and qualitative data on the activity of individuals in a wastewater catchment area.[10–14] For example, recent studies have shown that SARS-CoV-2 RNA was detected in sewage samples before any reported cases, suggesting that virus monitoring could be feasible before cases are documented through the health surveillance system.[8,15–17] This approach also offers a broader viral surveillance method within the populous at a relatively low cost compared to individual clinical laboratory tests.

Although the WBE approach has been applied for SARS-CoV-2 monitoring, much is yet to be understood surrounding sampling methods. Conventionally, samples for WBE have been taken through grab or 24-hour composite sampling techniques.[18] Grab sampling, although simple and convenient, provides only a snapshot of representation of a population's wastewater system. Similarly, composite sampling offers a final volume that is more representative of a given population over time but can be time-consuming and costly. Further, composite sampling may not be a reliable monitoring approach in areas of low COVID-19 prevalence as composite samples can dilute SARS-CoV-2 signals.[19,20] Alternatively, sampling upstream of WWTPs at sewershed pump stations, manholes, or institution-level sites (e.g., commercial properties, airports, university campuses, etc.) can target specific areas of interest. Consideration of the challenges of each sampling approach is critical for developing effective wastewater monitoring programs, which could become increasingly more relevant as vaccination programs begin to take shape globally.[21,22]

A passive sampling approach provides a cost-effective and relatively easy option to grab and composite sampling. This approach involves the use of a passive sampling device which is deployed at a location in the sewershed for a predetermined period, and contaminants in the wastewater are allowed to interact with an adsorbent material housed inside the device,[23,24] which results in the concentration of the virus. A passive sampling technique commonly referred to as the "Moore swab" has been previously used to extract enteric pathogens from cotton gauze in water.[25,26] Recently, the Moore swab approach was used for passive sampling of SARS-CoV-2 in wastewater and it was determined that passive samplers were at least as sensitive as conventional sampling for detecting SARS-CoV-2 in wastewater.[24] Another benefit of the passive sampling approach for WBE is that these devices can be deployed at smaller scales at sewer locations or pump stations for targeted monitoring at a specific location or building where grab or composite sampling may not be feasible.

The Moore swab method involves the use of cotton gauze as a swab and has been used in the collection of microorganisms in water and wastewater, including poliovirus,[27] and human norovirus.[28] Sikorski and Levine (2020) noted that the types of materials that have been used range from cheesecloth to cotton gauze.[25] Specific to SARS-CoV-2 monitoring, Schang et al. (2020) tested medical gauze, laboratory-grade electronegative filter paper and cotton buds for passive sampling of viruses in wastewater.[24] Additionally, Liu et al (2020) have successfully used cotton gauze in the passive sampling of wastewater to measure SARS-CoV-2.[29] A more recent study successfully implemented the use of tampon swabs in the detection of SARS-CoV-2 RNA in wastewater.[30] Although these materials have proven to be effective, there is an opportunity to further optimize the selection of adsorbent material for passive sampling for SARS-CoV-2 in wastewater. Schang et al. (2020) also stated that further research is needed for laboratory methods, particularly concerning elution and extraction of the virus from the various adsorbent materials.[24] For example, a mixed elution buffer consisting of phosphate buffer solution, Tween 80 and antifoam emulsion was used for the recovery of SARS-CoV-2 RNA from adsorbent material, but it is recognized that several chemical agents could be used to increase elution from specific swabbing materials. Thus, there is a need to comparatively

evaluate the elution efficacy of the various buffers or mixtures that have been considered in the literature.

The overall objective of this work was to develop a simple and effective passive sampling protocol for the detection of SARS-CoV-2 in sewer catchments at targeted institutional-level sampling sites in a region of low COVID-19 prevalence. Our specific aims were to 1) design and construct a 3-D-printed passive sampling device that protects the enclosed adsorbent material from being lost, torn, or obstructed by larger wastewater debris; 2) assess four materials for maximum recovery of heat-inactivated SARS-CoV-2 reference material from deionized water and wastewater; 3) compare the performance of three elution mixtures for maximum recovery of SARS-CoV-2 surrogate RNA (heat-inactivated SARS-CoV-2) from spiked wastewater; and 4) conduct field-scale testing at an institutional level using this passive sampling protocol to monitor SARS-CoV-2 in a region of low COVID-19 prevalence.

3.3 Materials and Methods

3.3.1 Reagents and materials

Heat-inactivated SARS-CoV-2 (HI-SCV-2) (ATCC® VR1986HK™) was sourced from American Type Culture Collection (Virginia, USA). DI water was obtained from a Milli-Q system (Reference A+, Millipore) and contained a total organic carbon (TOC) concentration of < 5 µg L^{-1} and a resistivity of 18.2 MΩ cm. Ethanol (EtOH) and acetonitrile (ACN) were purchased from Fisher Scientific (Ottawa, ON, CA). Cellulose sponges, cotton gauze, and cheesecloth were acquired from a local pharmacy, and electronegative filter membranes (4.7 cm, 0.1 µm or 9.0 cm, 0.22 µm cellulose nitrate membrane filters) were purchased from Fisher Scientific (Ottawa, ON, CA) and Sigma-Aldrich (St. Louis, MO, USA). For preliminary work, a premade elution buffer comprised of 0.075% Tween®20 + 25 mM Tris HCl was obtained from Innovaprep Technologies (Drexel, MO, USA). For subsequent experiments, this mixture was made using Tween®20 and Tris-HCl sourced from Sigma Aldrich (Ottawa, ON, CA); 75 µL of Tween®20 and 250 µL of a 0.1 M Tris-HCl intermediate was added to DI water for a total volume of 100 mL. Magnetic binding beads (20 g L^{-1}), RNA extraction kits, and SARS-CoV-2 assay kits were obtained from LuminUltra Technologies Ltd (Fredericton, NB, CA). Samples were

stirred on a Fisherbrand™ Isotemp™ magnetic plate stirrer (Fisher Scientific, Ottawa, ON, CA). Bovine serum albumin (BSA), used to reduce inhibition in RT-qPCR reactions, was purchased from Alfa Aesar by Thermo Fisher Scientific (Tewksbury, MA, US) to make a 1 mg mL^{-1} BSA solution (10 mg lyophilized BSA in 10 mL DI water).

3.3.2 The passive sampling device: COVID-19 Sewer Cage (COSCa)

To build upon the Moore swab concept as a passive sampling approach for SARS-CoV-2 in low prevalence areas, the COVID-19 Sewer Cage (COSCa) (Supplemental Material Figure S1) was developed to minimize over-saturation of solids on the adsorbent material (e.g., swab) and to prevent loss or damage of the adsorbent material itself. The COSCa is a 10-cm diameter hollowed sphere with 26 holes, with each hole having a 1.5-cm diameter to foster non-restrictive flow. The COSCa was designed and exported from Fusion 360 software (2018) and sent to a material jetting 3D printer (Any Cubic Mega Zero 2.0) and was printed with acrylonitrile Butadiene Styrene (ABS) plastic, an engineered thermoplastic with a high melting point that can withstand high autoclave temperatures. The COSCa was printed with solid walls to provide sufficient mass for complete submersion in stagnant waters or in moderate-flow catchments.

3.3.3 Wastewater collection for method development

Four 1-L wastewater samples (24-hr influent composite) were collected from a wastewater treatment facility (WWTF) in Moncton, New Brunswick, Canada on different calendar days in November and December 2020. Six 1-L wastewater samples (24-h influent composite) were collected from two WWTFs in Halifax and Dartmouth, Nova Scotia, Canada between March, and April 2021. Samples were transported to Dalhousie University on ice and kept at 4 °C for up to 24 hours before initial RNA extraction to determine background levels of SARS-CoV-2. The remaining sample volumes were stored at -20 ºC until used in passive sampling experiments.

3.3.4 Bench-scale experimental set-up for HI-SCV-2 RNA recovery in DI water and wastewater

For each bench-scale experiment, 500 mL samples were prepared. DI water and wastewater samples were spiked with HI-SCV-2 (1×10^3 GU mL^{-1}) in triplicate and left stirring continuously at 100 rpm at room temperature for 30 min to equilibrate before

adsorbent material was added. To simulate deployment in sewer catchments, adsorbent material was placed inside each COSCa, which was then suspended in the prepared water samples from a biohazard waste bag holder and continuously stirred on a stir plate at 100 rpm for 24 h at room temperature. After 24 h, the material was extracted from the COSCa and placed into 50-mL falcon tubes for subsequent RNA extraction. For each sample batch, a single matrix sample was left unspiked to serve as a blank.

3.3.5 Bench-scale evaluation of swab materials for SARS-CoV-2 detection in different water matrices

Four swab materials were assessed for SARS-CoV-2 absorbance: 100% cotton gauze, 100% cotton cheesecloth, cellulose sponges, and electronegative filter membranes. The gauze, cheesecloth, and sponges were chosen as passive sampling materials as these inexpensive materials were readily available from a local pharmacy. The materials were evaluated for HI-SCV-2 RNA concentration in laboratory experiments in two different water matrices: DI water, and municipal wastewater that previously tested negative for SARS-CoV-2 using the LuminUltra magnetic bead-based protocol (described below). All samples in this section were eluted using a lysis buffering agent (Lysis Buffer Concentrate, LuminUltra Technologies Ltd) and extracted for SARS-CoV-2 RNA using the magnetic bead-based protocol. The lysis buffer was selected for preliminary experiments as it was a readily-available component in the RNA extraction kit used in this study.

Bulk samples of gauze and cheesecloth were cut to approximately 7.6 × 183 cm and folded four times, based on the approach described by Sikorski and Levine (2020).[25] The sponge was cut into pieces of approximately 1 × 2.5 × 4 cm. Due to the fragility of electronegative filter papers, filter holders were designed and 3D-printed for each device. Filters were inserted between two attachments and placed inside the COSCas to allow contact with the wastewater while maintaining the integrity of the filter. For each laboratory-controlled sample, three filters were placed adjacent to each other within a 3D-printed electronegative filter holder inside a COSCa device. The placement of different adsorbent materials inside the COSCa passive sampler and experimental set-up are shown in Figure 3-1.

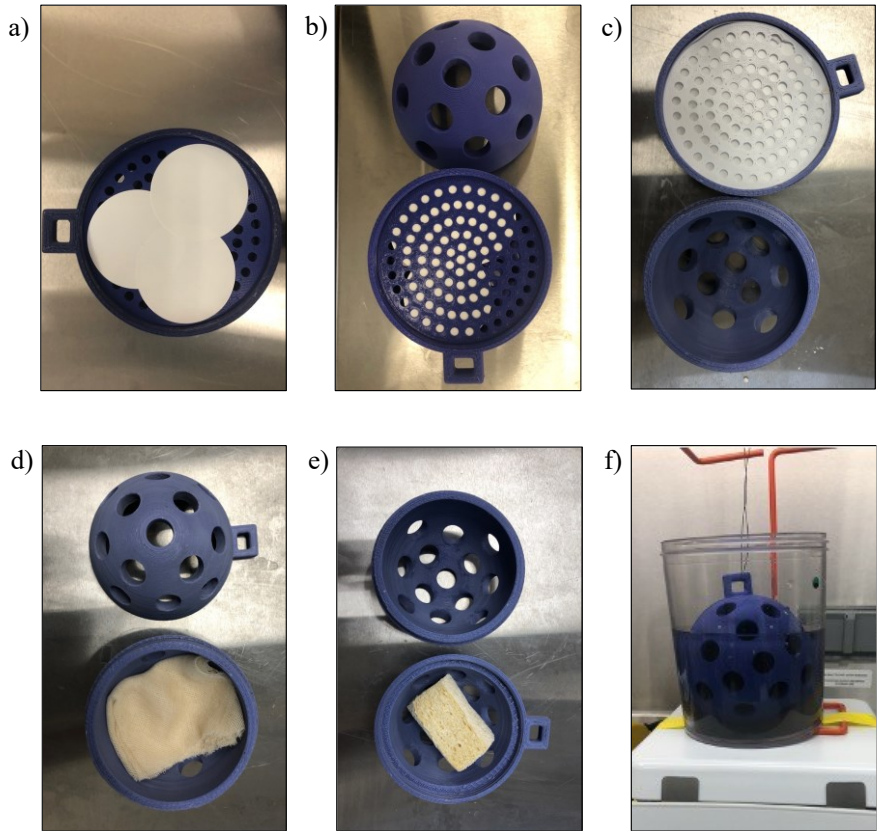

Figure 3-1. Placement of different adsorbent materials inside the COVID-19 Sewer Cage (COSCa) passive sampler. a) arrangement of three 4.7-cm electronegative filters; b) filters secured inside the COSCa insert; c) 9.0-cm filter inside a COSCa insert for field sample collection; d) gauze/cheesecloth and e) cellulose sponge placement inside the COSCa; and f) laboratory bench-scale COSCa passive sampling experimental set-up.

3.3.6 Bench-scale evaluation of elution mixtures for maximum concentration of SARS-CoV-2 from swabs

For assessing different materials in the detection of SARS-CoV-2 in different water matrices, swabs were eluted with a lysis buffer, as described. Two materials from the previous experiment (cheesecloth and electronegative filters) were used to compare the elution efficacy of the lysis buffer with that of two additional mixtures: a Tween®20-based buffer and a 1:1 acetonitrile: water mixture. Following a 24-hour stirring period, each sample (cheesecloth or filter) was immediately eluted by adding 2 mL of elution mixture, shaking vigorously by hand for 1 min, and incubating for 1 min at room temperature. Residual liquid was pressed out of the adsorbent material by kneading, and the eluate was transferred to a separate falcon tube. This process was repeated twice more for a total elution volume of 6 mL, 1 mL of which was used for RNA extraction using the magnetic

bead-based method described in Section 3.3.8 “RNA extraction”. This 6-mL elution volume was determined using a scaled-down approximation of elution buffer volume used in a Moore swab processing method described by Liu et al (2020).[29]

3.3.7 Comparing the performance of cheesecloth and electronegative filters in COSCa passive samplers at two sewer catchments

Two sewer catchments at targeted institutional-level sampling sites were chosen for this study: Location A, a university residence and Location B, a business center comprised of five adjacent buildings (Figure S2). These sites were selected after news releases indicated that there was at least one known case of COVID-19 at a university residence on the same campus as Location A, as well as known cases amongst the five buildings that service Location B. Multiple COSCas were not deployed at the same sewer location as we did not want to lose sampling equipment in the sewer system and harm sewer infrastructure. To compare the performance of cheesecloth (7.6 x 183 cm) and electronegative filters (9.0-cm diameter) in the detection of SARS-CoV-2 in wastewater using a passive sampling device, Location A was regularly sampled using a COSCa containing cheesecloth swabs and Location B was sampled using a COSCa containing filters. Fifteen sampling events were conducted at each location between January and May 2021.

For each sampling event, a COSCa was deployed for 24, 48 or 72 h, depending on site accessibility. A paired grab sample (125 mL) was collected with most COSCa samples at Location A; however, grab samples were not feasible at Location B due to manhole depth restrictions. Following each deployment period, the COSCa was retrieved, and the swab or filter was immediately placed inside a sterile 50-mL Falcon tube and transported to the lab on ice for analysis. All COSCa samples obtained through field experiments were eluted with a Tween®20-based elution buffer. To conserve reagents, single aliquots for each field sample eluate were extracted for SARS-CoV-2 RNA and analyzed via RT-qPCR. In cases when inhibition was expected (e.g., sample eluate with high solids content), extracted RNA was diluted 1:1 with BSA. For cheesecloth samples containing a large amount of solids, the eluate was diluted (up to 5-fold) to facilitate RNA extraction. Sample eluate and RNA dilutions for each sampling event are summarized in Supplementary Material Tables S1 and S2. For redeployment, the COSCa was disinfected with EtOH, a

new swab or filter was placed inside, and the COSCa was then lowered and placed directly into the wastewater flow.

3.3.8 RNA extraction

RNA extraction for raw wastewater and passive sampler swabs was carried out using a magnetic bead-based RNA extraction procedure.[31] A volume of 1 mL of sample (wastewater or COSCa eluate) was used to perform this extraction protocol, resulting in a total of 50 µL of eluted RNA for RT-qPCR analysis. This RNA extraction method was selected for several reasons: A. the LuminUltra GeneCount SARS-CoV-2 Wastewater RT-qPCR Assay Kit utilizes a commercially-available patent-pending method based on a simple and rapid extraction that produces results within a couple of hours; B. this magnetic bead-based extraction protocol was used in our regular wastewater surveillance program and offered direct comparison between sampling approaches; C. the passive sampling swabs required a direct RNA extraction method that could process low-volume particulate-laden samples, as other commonly used RNA extraction method involve large sample volumes and extensive sample pre-processing (i.e., preconcentration, filtration, etc.). The RNA extraction protocol was followed according to the manufacturer's instructions and is summarized in the Supplementary Material.

3.3.9 RT- qPCR analysis

All RNA samples were processed by RT-qPCR on a GeneCount® Q-16 instrument (LuminUltra Technologies Ltd, Fredericton, CA). The primers and probes sequences used were published by the US CDC as shown in Table 3-1.[32] For the analysis of SARS-CoV-2, 20-µL reactions were prepared using the GeneCount SARS- CoV-2 Screening kit (LuminUltra Technologies Ltd, Fredericton, CA), containing 15 µL of Master Mix and 5 µL of template RNA. When inhibition was expected, 2.5 µL template RNA was diluted with 2.5 µL BSA solution. Thermal cycling reactions were carried out as follows: a pre-denaturation step at 55 °C for 10 min followed by a second pre-denaturation step at 95 °C for 1 min. The two pre-denaturation steps were followed by 45 cycles of 95 °C for 10 sec and 55 °C for 45 sec, along with a final hold step at 50 °C for 1 min. Positive detections were indicated by cycle threshold (Ct) values under 40. The RT-qPCR upper Ct value detection threshold is 40 cycles, which corresponds to 1.4 copies per reaction.

Table 3-1. Sequences for primers and probes of viral surrogates were used in this study.

Organism	Sequence Type	Sequence (5' – 3')
SARS-CoV-2 N2 Gene	N2 Forward primer	TTACAAACATTGGCCGCAAA
	N2 Reverse primer	GCGCGACATTCCGAAGAA
	N2 Probe	ACAATTTGCCCCCAGCGCTTCAG

3.3.10 Quantitative analysis of SARS-CoV-2 from passive sampling material

In this work, quantitative analysis is carried out to assess the relative performance of each swab material type and elution mixture. RNA concentrations that reflect the amount of viral RNA eluted from the adsorbent material (total genomic units per 6 mL eluate) were calculated using Equation 1. Recovery of HI-SCV-2 was calculated using Equation 2. A flowchart showing both calculations is shown in Figure S3.

Eq. 1

$$\text{RNA concentration (GU per eluate)} \approx \text{sample concentration (GU mL}^{-1}\text{)} \times 6 \text{ mL eluate}$$

Eq. 2

$$\text{RNA Recovery (\%)} \approx \frac{100 \times \text{sample concentration (GU mL}^{-1}\text{)} \times 6 \text{ mL eluate}}{\text{Spiked concentration (1,000 GU mL}^{-1}\text{)} \times \text{Sample volume (500 mL)}}$$

3.3.11 Quality control

All passive sampling experiments and RNA extractions were performed in a Thermo Scientific 1300 Series A2 biosafety cabinet, with RT-qPCR assays being prepared in a separate laboratory to minimize contamination. All materials were sterilized in an autoclave to eliminate any pre-contamination, and blank samples were run with all RNA assays to ensure no contamination occurred during sample extractions and preparation. Extracted RNA was analyzed with the RT-qPCR immediately after extraction, with all experiments being performed in triplicate. Standards outlined in MIQE guidelines were consulted for evaluating qPCR-based experiments. Internal positive and negative controls were implemented in each RNA extraction and qPCR assay. The LuminUltra qPCR system utilizes a master standard curve incorporated into the software. Mean Ct values are provided for each assay. To assess sampling efficacy in bench-scale experiments, biological replicates were performed in triplicate while technical replicates were omitted to conserve reagents and materials.

The method limit of detection (MLOD) determined by Parra et al (2021) for the direct extraction of SARS-CoV-2 RNA from raw wastewater was experimentally determined as 40 GU mL^{-1}, and the method recovery efficiency for Accuplex, a SARS-CoV-2 surrogate, from wastewater, was reported as ~12%.[31] However, these values for MLOD and recovery efficiency are not directly applicable to this passive sampling approach for two reasons: A) a representative MLOD requires calculations of recovery efficiencies based on adsorption kinetics data, which are beyond the scope of this work; and B) a different surrogate was used to determine recovery efficiency and MLOD for the direct magnetic beads extraction method. To compare the performance of each method parameter, relative recovery was calculated for each adsorbent material type and elution mixture using the HI-SCV-2 surrogate, as shown in Figure S3.

3.3.12 Statistical Analysis

A Welch two-sample *t*-test (two-tailed, $\alpha = 0.05$) was performed to evaluate the statistical significance between mean HI-SCV-2 RNA concentrations obtained from experiments carried out using different adsorbent materials and elution buffers in both DI water and wastewater. All experiments were performed using three biological replicates and standard deviation was used to determine error bars. All statistical analyses were performed using R (version 4.0.4) software.[33]

3.4 Results and Discussion

3.4.1 Comparison of adsorbent materials for the detection and recovery of HI-SCV-2 from different water matrices

A controlled bench-scale experiment was conducted using four adsorbent materials and two different water matrices (DI water and municipal wastewater, both spiked with HI-SCV-2) to evaluate the detection and recovery of HI-SCV-2. RNA concentrations (GU per 6 mL eluate), recoveries (%), and percent positive detections are shown in Figure 3-2. In the controlled experiment using DI water spiked to 1.0×10^3 GU mL^{-1}, HI-SCV-2 RNA was recovered from all four materials. The electronegative filters resulted in the highest mean RNA concentration ($8.0\times10^2 \pm 3.7\times10^2$ GU per eluate) followed by cheesecloth ($4.6\times10^2 \pm 6.3\times10^1$ GU per eluate). The sponge resulted in the lowest mean RNA concentrations ($3.2\times10^1 \pm 5.6\times10^1$ GU per eluate). Ct values for RNA extracted from

cheesecloth, gauze, and electronegative filters in DI water ranged from 36.8 to 37.2, 36.8 to 37.5, and 35.6 to 37.3 respectively. The Ct value for the single positive detection in the sponge replicates was 39.5, which is approaching the qPCR limit of detection.

To further explore the performance of each material in recovering HI-SCV-2, the experiment was carried out in municipal wastewater that previously tested negative for SARS-CoV-2 and was spiked to 1.0×10^3 GU mL^{-1} with HI-SCV-2. In this matrix, the highest mean HI-SCV-2 RNA concentration was recovered from the cheesecloth ($1.7\times10^3 \pm 3.1\times10^2$ GU per eluate). The electronegative filters resulted in the second highest mean RNA concentrations ($1.4\times10^3 \pm 3.6\times10^2$ GU per eluate), but there was no statistically significant difference ($p = 0.331$) in the recovered concentrations from the cheesecloth and filters. Although the use of a cellulose sponge was promising due to its absorbance capacity, the surrogate was not detected in any of the sponge replicates. Ct values for RNA extracted from cheesecloth, gauze, and electronegative filters in wastewater ranged from 34.6 to 35.2, 35.3 to 35.6, and 34.8 to 35.5 respectively. In these initial experiments, recoveries were below 1% for all passive sampling material types eluted with the lysis buffer in both matrices.

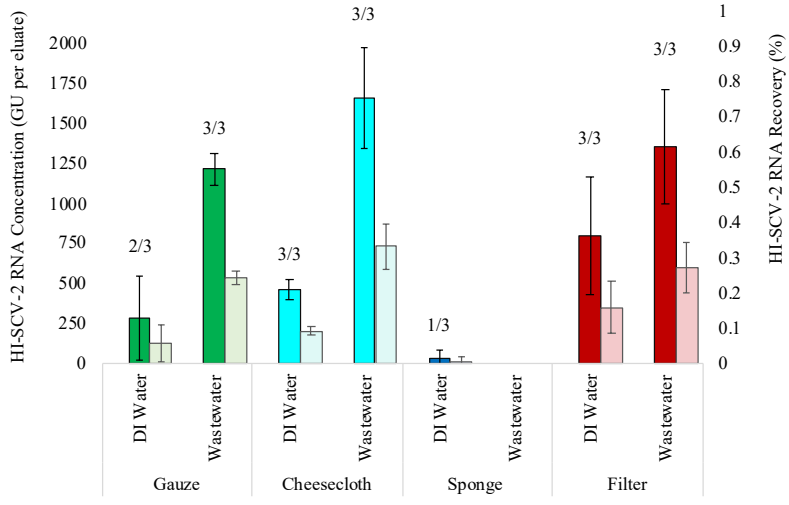

Figure 3-2. Mean concentrations (bold-coloured bars) and recovery of HI-SCV-2 RNA (lightly-coloured bars) from bench-scale passive sampler experiments using four different adsorbent materials: cotton gauze, cheesecloth, sponge, and electronegative filters in spiked DI water (1.0×10^3 GU mL^{-1}) and spiked wastewater (1.0×10^3 GU mL^{-1}) that previously tested negative for SARS-CoV-2. Each material was incubated for 24 hrs and eluted with 6 mL of lysis buffer. The number of detections for each material type is shown at the top of each bar (n=3).

The recovery of SARS-CoV-2 may be impacted by the presence of solids in wastewater. Retention of solids can improve RNA recovery, as SARS-CoV-2 partitions to solids,[34–36] which can act as a vehicle for viral transport. In contrast, excessive retention of solids can *impede* recovery by inhibiting the RNA extraction process. The optimal passive sampling material should balance the benefit of retaining virus-laden particles and the negative impact of solids on efficient RNA extraction. The cheesecloth and gauze samples retained more solids from wastewater than did the filters. The concentration of HI-SCV-2 RNA recovered using cheese cloth and gauze was not significantly different in DI water ($p = 0.373$) nor the wastewater matrix ($p = 0.091$). The comparable performance of these two readily-available materials suggests that either may be used as an alternative when laboratory-grade materials (e.g., electronegative filters) are not available, which may be relevant for wastewater surveillance programs in remote areas. In some sampling locations where solids content is expected to be high, a filter may be a preferred choice for passive sampling to allow efficient RNA extraction. As such, the solids content of wastewater should be considered when selecting passive sampling materials.

The affinity of the solids in the wastewater to the passive sampling material also plays a role in the adsorption of the virus. While COVID-19 virion sizes range from 0.07 to 0.09 μm,[37] researchers have found that cellulose nitrate membranes were capable of recovering viruses despite pore sizes exceeding that of the viruses.[38] This phenomenon is understood to be the result of multiple reactive sites covering the filters causing viral adhesion or the adsorption of the virus-laden particles that may adhere to the membrane's surface. Consequently, due to the nature of wastewater matrices, high solid content is often observed, which may result in preferential adsorption of organics to the filter membrane thus reducing viral adsorption efficiencies. However, the quantity of solids retained over 24 hours did not impact RNA extraction processes in bench-scale experiments.

The results of these experiments indicate that electronegative filters and cheesecloth resulted in the highest mean HI-SCV-2 RNA concentrations in spiked DI water and wastewater, respectively. Although cheesecloth may be used as a quick and effective passive sampling material for SARS-CoV-2 detection in municipal wastewater, particularly when electronegative filters may not be readily available, the use of laboratory-grade materials provides reproducibility and consistency in results that

household materials may not. The cheesecloth and the electronegative filters were selected for further investigation in subsequent bench-scale experiments and field studies because of the comparable performance of these materials in both matrices.

3.4.2 Comparison of three elution techniques in the analysis of SARS-CoV-2

To optimize laboratory methods for this passive sampling approach, two other elution mixtures in addition to the lysis buffer were tested, including a Tween®20-based elution buffer and a 1:1 acetonitrile: water mixture. All samples were run in biological triplicates for cheesecloth swabs and electronegative filters in municipal wastewater spiked to $1.0x10^3$ GU mL^{-1} with HI-SCV-2 (Figure 3-3). All elution mixtures resulted in positive detections for both material types. The Tween®20-based elution buffer resulted in the highest mean RNA concentrations for both materials with positive detections in all three replicates. The cheesecloth resulted in a mean RNA concentration of $7.0\times10^4 \pm 3.7 \times10^4$ GU per eluate, while a mean concentration of $2.3\times10^4 \pm 2.5\times10^4$ GU per eluate was obtained with the filters. There was no statistical difference in mean RNA concentrations between the cheesecloth and filters eluted with the Tween®20-based buffer ($p = 0.149$). In wastewater, mean recoveries of HI-SCV-2 increased from 0.3 to 13.9 % and from 0.3 to 4.5 % using the Tween20®-based elution buffer to elute cheesecloth and filters, respectively. All other recoveries in this series of experiments were below 2%. For wastewater, Ct values for RNA extracted from cheesecloth and electronegative filters using the Tween20®-based elution buffer ranged from 28.1 to 29.6 and 29.4 to 34.1 respectively. By comparison, Ct values for all other elution mixtures were less reliable and ranged between 31.3 (one filter eluted with 1:1 acetonitrile: water mixture) and 37.1. In addition to the material type and characteristics, the relative recovery of viral RNA is highly dependent on the performance of the elution buffer.

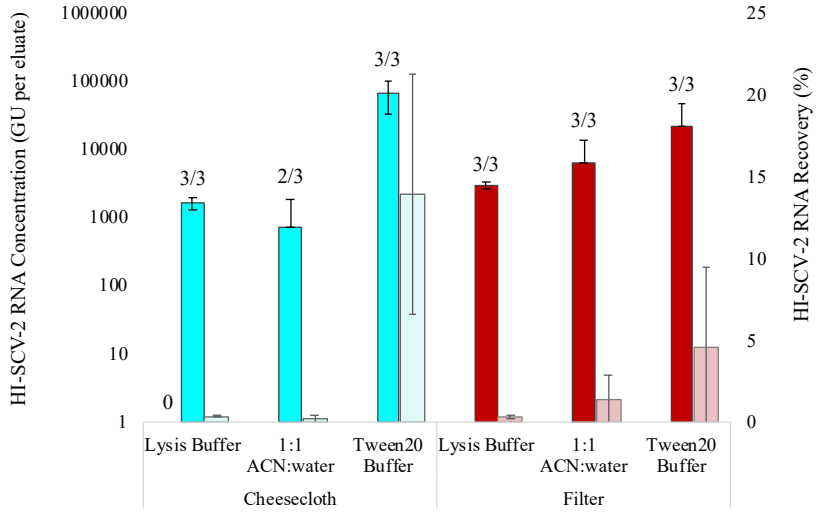

Figure 3-3. Mean concentrations (bold-coloured bars) and recovery (lightly-coloured bars) of HI-SCV-2 RNA eluted from cheesecloth and electronegative filters using three different elution mixtures: lysis buffer; 1:1 acetonitrile (ACN): water mixture; and a Tween®20-based elution buffer. Triplicate swabs for each elution mixture were tested in wastewater spiked to 1.0×10^{3} GU mL^{-1} with HI-SCV-2, incubated for 24 h, and eluted with 6 mL. Error bars indicate standard deviation. Number of detections for each material type and elution mixture is shown at the top of each bar.

Many factors of an elution mixture can impact the elution efficiency of viruses from materials, including differences in pH, salinity, or the use of a surfactant.[39] Tween®20 is a non-ionic polysorbate surfactant widely used in biochemical applications and is known for being a gentle surfactant at lower concentrations to prevent premature cell lysis.[40] In other work, Tween®20 has been successfully implemented for ultrafiltration techniques, significantly increasing microbial recovery efficiencies.[41] Tween® 80, has been utilized for its capability to elute viruses from filtration media,[42] and most recently, SARS-CoV-2 from passive sampling material.[24,29] The use of either Tween®20 or Tween®80 are often interchangeable, with the main difference of the two being the composition of fatty acids.[43] Liu et al., (2020) and Schang et al., (2020) used 0.05% Tween®80 mixed with sterile phosphate buffer solution and 0.001% Y-30 antifoam emulsion for elution of SARS-CoV-2 from cotton gauze collected from passive samplers.[24,29]

In this study, quantitative analysis of viral RNA was carried out to assess the performance (i.e., relative recovery efficiency) of each adsorbent material type and elution mixture. Although recovery assessment for other quantitative viral RNA extraction methods is often carried out using the spike-and-recovery approach with a surrogate, this

may not accurately represent recovery efficiency, as many surrogates used to assess SARS-CoV-2 extraction efficiency do not appreciably partition to the solids fraction of wastewater when seeded as SARS-CoV-2 does naturally.[44] This difference in behaviour between SARS-CoV-2 and its surrogates may introduce variability in, and impact the interpretability of, results for not only controlled recovery studies, but for our assessment of relative recovery as well. As such, field studies to assess the optimized passive sampling approach (i.e., a combination of materials and elution buffers) were a critical next step from bench-scale experiments to optimize the detection and quantitation of SARS-CoV-2. Based on the performance of both the electronegative filters and cheesecloth from our laboratory-controlled experiments, these two material types in combination with the Tween®20-based elution buffer were used in subsequent field experiments.

3.4.3 Detection of SARS-CoV-2 in two sewer catchments using cheesecloth and electronegative filters in COSCa passive samplers

Over 15 sampling events at each location, SARS-CoV-2 was detected on seven separate occasions at Location A (COSCa with cheesecloth swabs) and five sampling events at Location B (COSCa with electronegative filters) (Figure 3-4). Based on results from bench-scale experiments, the Tween®20-based buffer was used for viral elution from cheesecloth swabs and filters in all field experiments. Detection levels varied at Location A, ranging from 2.6×10^2 to 1.6×10^4 GU per eluate and from 1.8×10^3 to 6.1×10^3 GU per eluate at Location B. SARS-CoV-2 was not detected in any of the grab samples paired with the passive samples collected from Location A.

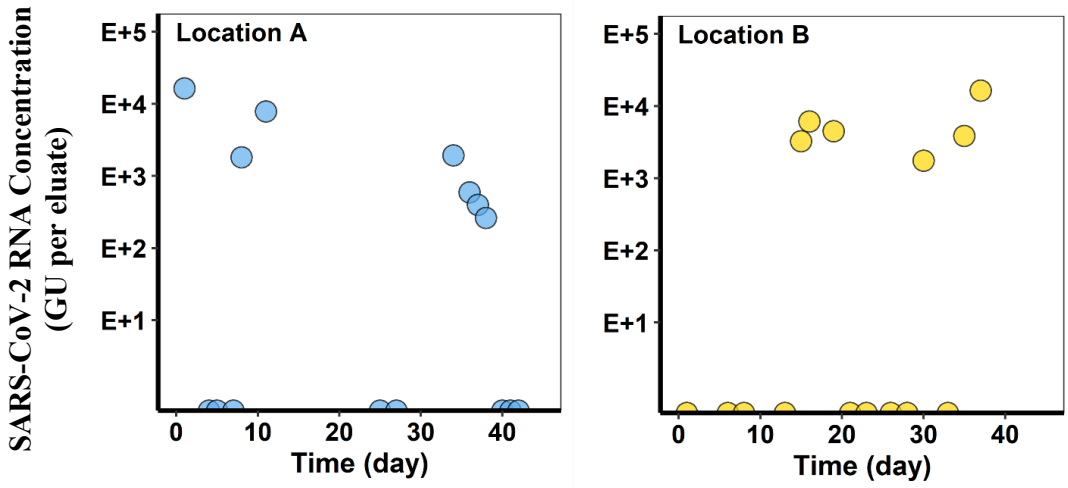

Figure 3-4. Mean SARS-CoV-2 RNA concentrations (GU per 6-mL eluate) from two sewer catchments using the COSCa devices with cheesecloth (Location A) and electronegative filters (Location B) over 15 sampling events. All samples were eluted with a Tween®20-based buffer. Data points on the x-axis indicate non-detects from RT-qPCR analysis.

At Location A, there were three positive SARS-CoV-2 RNA detections in the first six sampling events. The viral signal was not detected in the next two sampling events but reappeared in the following four consecutive sampling events. For these positive detections, there was a decline in the eluate viral RNA concentration from 1.9×10^{3} to 2.6×10^{2} GU mL^{-1}, and the signal was eventually no longer detected in the last three sampling events. At Location B, three consecutive positive detections were observed following four non-detects. The signal appeared again in two of the four remaining sampling events. SARS-CoV-2 RNA concentrations ranged from 1.8×10^{3} to 6.1×10^{3} GU mL^{-1} at Location B. Although the sampling sites in this study were targeted based on known cases in the areas at the time, the actual number of cases in each location, and the contributing population of each catchment, were unknown.

The results of passive sampling at these two sewer catchments demonstrate that both cheesecloth (Location A) and electronegative filters (Location B) are effective materials for detecting SARS-CoV-2 in wastewater when eluted with a Tween®20-based buffer. Furthermore, this COSCa sampling approach successfully detected changes in viral presence in two small contributing populations, with distinct resolution in viral RNA concentrations observed across two orders of magnitude. However, the adsorption capacity

of the passive sampling materials used in this study may have been exceeded, as maximum mean RNA concentrations do not exceed 7×10^4 GU per eluate in bench-scale and field experiments. To determine the maximum absorbance capacity of these passive sampling materials, further research investigating the adsorption kinetics of the COSCa passive sampling materials is required.

Our results also demonstrated the lack of sensitivity of grab sampling when paired with passive sampling. However, the suitability of this passive sampling approach may ultimately depend on specific site characteristics and water quality parameters. The deployment of these devices is ideal for low-flow locations, such as manholes and thus, are best suited to target specific buildings, designated catchment areas within the sewershed, or remote communities.

3.5 Conclusions

In assessing the performance of four adsorption materials and three elution mixtures for the analysis of SARS-CoV-2 in municipal wastewater using our 3D-printed passive sampling devices, the results of this study show that cheesecloth and electronegative filters in combination with the Tween®20-based elution buffer resulted in the highest mean concentrations of a SARS-CoV-2 surrogate in bench-scale studies. When deployed at two targeted locations within the sewer catchment, both cheesecloth (Location A) and electronegative filters (Location B) allowed the reliable detection of SARS-CoV-2 in wastewater. Furthermore, this passive sampling approach revealed fluctuations in viral presence in the two small contributing populations at these locations.

This work demonstrates the effectiveness of passive sampling to detect SARS-CoV-2 in wastewater, and the lack of sensitivity of grab sampling in low prevalence areas when grab samples were collected along with COSCa samples. During prolonged periods of low COVID-19 prevalence, detection in wastewater using grab and composite sampling strategies can be inconsistent and ineffective. To overcome these challenges, the COSCa provides a solution that can foster more direct and targeted analysis when the number of COVID-19 cases is low, which may have increased relevance as vaccination programs expand. The potential use of the described passive method provides added sensitivity and a straightforward approach to concentrating samples during collection. The passive

sampling approach outlined offers a quick and effective wastewater monitoring tool for SARS-CoV-2 detection in targeted locations that may provide an early warning signal during low COVID-19 prevalence.

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CHAPTER 4 OPERATIONAL CONSTRAINTS OF DETECTING SARS-COV-2 ON PASSIVE SAMPLERS USING ELECTRONEGATIVE FILTERS: A KINETIC AND EQUILIBRIUM ANALYSIS

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E.K.H. designed and carried out experiments, analyzed data, prepared figures and wrote the paper.

4.1 Abstract

In developing an effective monitoring program for the wastewater surveillance of SARS-CoV-2 ribonucleic acid (RNA), the importance of sampling methodology is paramount. Passive sampling is an effective tool to detect SARS-CoV-2 RNA in wastewater. However, the adsorption characteristics of SARS-CoV-2 RNA on passive sampling material are not well understood, which further obscures the relationship between wastewater surveillance and community infection. In this work, adsorption kinetics and equilibrium characteristics were evaluated using batch-adsorption experiments for heat-inactivated SARS-CoV-2 (HI-SCV-2) adsorption to electronegative filters. Equilibrium isotherms were assessed or a range of total suspended solids (TSS) concentrations (118, 265, 497mg L^{-1}) in wastewater, and a modelled q_{max} of 7×10^3 GU cm^{-2} was found. Surrogate adsorption kinetics followed a pseudo-first-order model in wastewater with maximum concentrations achieved within 24 h. In both field and isotherm experiments, equilibrium behaviour and viral recovery were found to be associated with wastewater and eluate TSS. Based on the results of this study, we recommend a standard deployment duration of 24 to 48 hours and the inclusion of eluate TSS measurement to assess the likelihood of solids inhibition during analysis.

4.2 Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (COVID-19) [1], has been detected in the feces of

symptomatic, and asymptomatic patients [2]. As such, SARS-CoV-2 ribonucleic acid (RNA) may be recovered from municipal wastewater samples to monitor the prevalence of the virus in sewersheds [2]. Through the implementation of standardized methods for analyzing and interpreting wastewater data, and collaboration between public health, wastewater surveillance (WWS) has the potential to be a powerful tool for informing public health actions [3,4]. Currently, an approach that incorporates standardized sampling techniques, analytical protocols, and data interpretation methods for WWS of SARS-CoV-2 is needed. Therefore, ongoing investigation and development of these WWS components are crucial for applying this tool to better understand COVID-19 prevalence in our communities.

In developing an effective monitoring program for the WWS of SARS-CoV-2, the importance of sampling methodology is paramount. Although grab and 24-hour composite sampling are the conventional sampling techniques used for wastewater collection, they have disadvantages [5]. Grab samples capture wastewater in a sewershed at a single time point and, thus, are less representative of the sewershed population. While 24-hour composite sampling is more representative of the contributing population over time, dilution of the target in the sample results in low viral concentrations, requiring sensitive detection methods with sufficiently low detection limits. Furthermore, expensive auto-sampling equipment contributes to the high costs associated with this sampling method and the use of this technology is not always feasible at targeted manhole locations. By contrast, a passive sampling approach may provide a sample that is more representative of the contributing population, as the viral target is concentrated through particulate accumulation during sample collection. For this sampling technique, the adsorbent material is placed in wastewater flow for a predetermined amount of time to preferentially collect solid particles that adhere to the absorbent material [6–8]. This approach offers a cost-effective, flexible, and simple alternative to conventional sampling methods for detecting SARS-CoV-2 in wastewater, especially when viral loads in wastewater are low.

Since the onset of the COVID-19 pandemic, three novel passive sampling approaches for WWS of SARS-CoV-2 RNA have been developed [6,9,10], all deriving from the Moore Swab method which applied medical gauze in raw water to extract enteric pathogens [11]. Schang et al., (2021) successfully utilized a passive sampling device with

cotton buds, gauze, and electronegative filters, to detect SARS-CoV-2 RNA in municipal wastewater [6]. Bivins et al., (2021) employed tampons for passive sampling of SARS-CoV-2 RNA in wastewater collection systems. Hayes et al., (2021) designed a 3D-printed passive sampling device that housed cotton cheesecloth or an electronegative filter, both of which were capable of passively adsorbing SARS-CoV-2 RNA in laboratory-controlled experiments and municipal sewersheds [10]. Of the two adsorbent materials evaluated in Hayes et al., electronegative filters were the preferred adsorbent material for passively capturing SARS-CoV-2 RNA in wastewater, as the laboratory-grade filters provide reproducibility and consistency. As electropositive filters have yet to be evaluated for SARS-CoV-2 adsorption in wastewater, we used electronegative membrane filters as they are successful in the monitoring of SARS-CoV-2 in both bench-scale work and field studies [12,13], were readily available, and are cost-effective. This is supported in various other work where electronegative membranes have been used for concentrating enteric viruses [14,15] and SARS-CoV-2 RNA [16,17] from wastewater. While it is known that enveloped viruses, such as SARS-CoV-2, have high adsorption efficiency to the electronegative filter [16] and the solid fraction of wastewater [18], the viral adsorption kinetics and equilibrium conditions of these filters are not well understood.

It is hypothesized that the use of electronegative filters to passively sample for SARS-CoV-2 in wastewater is constrained in quantifying viral concentrations due to limitations in adsorption capacity and inhibitory processes during downstream analysis. While wastewater solids capture is crucial to recover associated viral units, excess solids present during sample processing can impede RNA extraction and amplification. This work aims to conduct batch-adsorption isotherm experiments to better understand the kinetic and equilibrium adsorption behaviour of a viral surrogate in the presence of suspended solids in wastewater when exposed to an electronegative filter. The adsorption isotherms assessed in these experiments were used to inform optimal passive sampler deployment periods and estimate the concentration range for which this material can detect SARS-CoV-2 RNA when deployed in municipal sewersheds. The specific objectives of this work were to (1) determine the adsorption equilibrium capacity over a 24-h period for a 90-mm electronegative filter collecting viral surrogate spiked into municipal wastewater; (2) investigate the impact of total suspended solids (TSS) concentration in wastewater on

viral adsorption and recovery equilibrium behaviour; (3) determine the adsorption kinetics of a viral surrogate spiked at a known concentration into municipal wastewater collected onto a 90-mm electronegative filter; and (4) compare sample deployment conditions, including duration, SARS-CoV-2 RNA recovery range, and TSS characteristics of both laboratory and field samples to identify optimal deployment conditions for detection of SARS-CoV-2 RNA in wastewater using passive sampling techniques.

4.3 Materials and Methods

4.3.1 Reagents and materials

Heat-inactivated SARS-CoV-2 (HI-SCV-2) (ATCC® VR1986HK™) was sourced from American Type Culture Collection (Virginia, USA). Deionized (DI) water was acquired from a Milli-Q system (Reference A+, Millipore) and contained a resistivity of 18.2 MΩ cm and a total organic carbon (TOC) concentration < 5 µg L^{-1}. Whatman® electronegative nitrocellulose membrane filters, 0.2-µm pore size, 90-mm diameter were purchased from Sigma-Aldrich, (St. Louis, MO). The elution buffer used in this study was comprised of 0.075% Tween®20 + 25 mM Tris HCl sourced from Sigma Aldrich (Ottawa, ON, CA). This buffer was utilized based on previous work that examined three elution mixtures, of which the 0.075% Tween®20 + 25 mM Tris HCl buffer resulted in the greatest SARS-CoV-2 surrogate RNA recovery from electronegative membranes [10]. Samples were stirred on an orbital shaker table from Sigma-Aldrich (St. Louis, MO). Magnetic binding beads (20 g L^{-1}), RNA extraction kits, and SARS-CoV-2 RT-qPCR assay kits were obtained from LuminUltra Technologies Ltd (Fredericton, NB, CA). Ethanol (EtOH) was purchased from Fisher Scientific (Ottawa, ON, CA). To reduce inhibition during RT-qPCR reactions, Bovine serum albumin (BSA) (1 mg mL^{-1}) was utilized. The BSA solution was made from 10 mg lyophilized BSA from Alfa Aesar, Thermo Fisher Scientific (Tewksbury, MA, US) in 10 mL of DI water. All TSS measurements were taken using a Sartorius Entris^{TM} Analytical Balance (Fisher Scientific).

4.3.2 Wastewater collection for method development

For adsorption experiments, 10 1-L wastewater samples (24-h influent composite) were collected from a wastewater treatment facility (WWTF) in Nova Scotia, Canada on 10 different calendar days between February and July 2021. Samples were transported to

Dalhousie University on ice and kept at 4 °C for up to 24 hours before initial RNA extraction to determine background levels of SARS-CoV-2 RNA. All wastewater samples used in the batch-adsorption experiments in this study tested negative for SARS-CoV-2 RNA before experimental use.

4.3.3 Field Application of a 3D-Printed Passive Sampler for Field-Scale Comparison

Field samples were collected using a 3D-printed passive sampler, containing 90-mm (0.2-µm pore size) electronegative filters for SARS-CoV-2 RNA detection in wastewater [10]. All field samples were eluted using the same 0.075% Tween®20 + 25 mM Tris HCl-based buffer used in the bench-scale experiments. The 3D-printed passive sampling device was suspended in the wastewater flow by a 3/16" nylon rope attached to a steel crossbar under the manhole cover. After 24 – 72 h, the sampling device was retrieved from the wastewater, and the electronegative filter was removed for analysis while a new filter was inserted into the device and suspended in the wastewater flow until the following sampling period. Samples were transported to Dalhousie University on ice and analyzed immediately using a magnetic beads-based RNA extraction protocol and RT-qPCR techniques [20]. For this study, 23 sampling events took place over 15 weeks at three different sewershed manholes (Locations A, B, and C) during Nova Scotia's "third wave" of COVID-19 cases. Location A receives wastewater from an urban area largely comprised of residential homes. Location B collects from a large commercial property containing apartments, retailers, and restaurants. Location C is located directly outside a large university residence building. Additional information about the sampling conditions is presented in Supporting Information Table S1. These locations were selected based on access to sites from April 2021 to August 2021, when there was an increase in clinical cases of COVID-19 in Nova Scotia, as described by Parra-Guardado et al. (2021) [19]. Further details on specific caseloads during the study period are located in Figure S1; however, the authors do not have specific clinical information for the sewershed areas throughout the study period.

4.3.4 Total Suspended Solids Measurements - Wastewater

Wastewater

TSS concentrations in wastewater were measured by filtering 250 mL of each wastewater sample through a standard glass fibre filter and drying the residue retained on the filter at 103 to 105 ºC [20]. The increase in the filter mass corresponded to the quantity of suspended solids in the solution. Each sample was measured in triplicate, and the average TSS concentration was calculated. TSS concentrations were measured in a range of wastewater samples. The samples with the lowest, highest, and average TSS concentrations (118, 497, 265 mg L^{-1}, respectively) across all samples were identified and used as the matrices for the batch-adsorption experiments.

Filter Eluate

All electronegative filters used in the passive sampling experiments, both laboratory and field-based, were eluted with 6 mL of a 0.075% Tween®20 + 25 mM Tris HCl-based buffer. TSS concentrations in the filter eluate were measured by filtering 2 mL of the 6-mL eluate volume through a standard glass fibre filter after a brief 10-second vortex to homogenize the eluate. Each 2-mL aliquot was measured via a 10-mL graduated cylinder to avoid pipette tip clogging in eluates with high solid concentrations. Similar to the method performed to measure TSS in wastewater, after filtration, each glass fibre filter was dried at 103 to 105 ºC and the corresponding mass increase was measured. This TSS concentration was determined for both experimental and field study eluates to compare the TSS in the laboratory and field samples. Each sample was measured in triplicate and the average concentration was reported as mg per eluate (6 mL).

4.3.5 Equilibrium adsorption isotherm models

For each batch-adsorption experiment, 90-mm (0.2-µm pore size) electronegative cellulose nitrate filters were placed in 100-mL samples prepared with wastewater or DI water in sealed 250-mL Erlenmeyer flasks. Samples were spiked with HI-SCV-2 and stirred continuously on an orbital shaker table at 150 rpm at room temperature. Spiked wastewater samples were stirred for 1 h before adsorption experiments were conducted. The electronegative filters were exposed for 24 h in either matrix to ensure sufficient viral interaction with the adsorbent. All tests were run in biological triplicate and results were reported as average SARS-CoV-2 RNA concentration in GU cm^{-2}. Each filter was eluted

with a 0.075% Tween®20 + 25 mM Tris HCl-based buffer; the eluate was used to evaluate TSS concentrations adsorbed and for RNA extraction using a magnetic beads-based method [19]. All RNA samples with initially negative results were diluted up to 1:10 with a 1 mg mL^{-1} BSA solution to assess for false negatives due to inhibition.

The distribution of HI-SCV-2 between the liquid phase and the adsorbent is a measure of the equilibrium condition in the adsorption process. This work used the Langmuir and Freundlich isotherm models to mathematically describe the equilibrium adsorption behaviour of the system [21–24]. Langmuir and Freundlich isotherms are the most widely used models to represent equilibrium in surface adsorption and both have parameters relating to the number of binding sites and adsorbate concentration [25]. Both models were applied to investigate the adsorption equilibrium of HI-SCV-2 onto electronegative filters in three wastewater matrices of varying TSS concentrations (118, 265, and 497 mg L^{-1}) and DI water. HI-SCV-2 was spiked in all matrices at concentrations ranging from 1×10^1 to 5×10^4 GU mL^{-1} (1×10^1 1×10^2, 1×10^3, 1×10^4, and 5×10^4) in DI water and (1×10^1, 5×10^1, 1×10^2, 5×10^2, 1×10^3, 5×10^3, 1×10^4, and 5×10^4) in wastewater.

The Langmuir isotherm model is based on the assumption of homogeneous mono-layer adsorption, implying molecules adsorb only at specific localized sites on a surface, and when these sites are saturated, no interactions between adsorbed molecules may occur. The Langmuir model equation is defined as Eq (1):

$$q_e = \frac{q_{max} K_L C_e}{1 + K_L C_e}, \tag{1}$$

where q_e is the adsorption capacity at equilibrium (GU cm^{-2}), q_{max} is the maximum adsorption capacity (GU cm^{-2}), K_L is the Langmuir equilibrium constant, related to the affinity of binding sites and energy of adsorption, and C_e is the equilibrium concentration (GU mL^{-1}).

The Freundlich isotherm model defines the distribution of adsorption energy onto a heterogeneous surface and describes a reversible and non-ideal adsorption process [25]. This empirical model considers that multilayer adsorption is feasible, such that saturation of the adsorbent will not occur [26]. The empirical nature of the Freundlich model is restrictive in offering reliable insight into adsorption mechanisms at the surface level and

is commonly favoured in biological adsorption [27,28]. The Freundlich model is represented by Eq (2):

$$q_e = K_f C_e^{\frac{1}{n}}, \quad (2)$$

where K_f is the Freundlich equilibrium constant (GU cm^{-2}) and $1/n$ is the adsorption intensity which can vary based on the material's heterogeneity.

The average relative error (ARE) was calculated for both models. This was selected to minimize the fractional error distribution across large ranges of concentration, based on the work of Ayawei et al., (2017). The equation to calculate ARE is given by the following, Eq (3):

$$ARE = \frac{100}{n} \sum_{i=1}^{n} \left[\frac{q_{e,i,calc} - q_{e,i,meas}}{q_{e,i,meas}} \right], \quad (3)$$

where, $q_{e,\ i,calc}$ is the theoretical concentration of adsorbate on the adsorbent (calculated from the isotherm models) and $q_{e,\ i,meas}$ is the experimentally determined concentration of the adsorbate on the adsorbent.

4.3.6 Adsorption Kinetic Isotherm

To understand the interactions occurring in HI-SCV-2 adsorption to electronegative filters and provide insight into the mechanisms of HI-SCV-2 adsorption onto electronegative filters, Lagergren's pseudo-first-order (PFO) model and Ho's pseudo-second-order (PSO) model were evaluated. Batch-adsorption experiments were run in the same experimental setup as described in Section 4.3.4.; however, the filters were exposed to only a single wastewater matrix (TSS = 118 mg L^{-1}) and a DI matrix, both spiked with HI-SCV-2 (1×10^3 GU mL^{-1}). The spiked HI-SCV-2 concentration and wastewater matrix were selected for this experiment based on the results of the adsorption equilibrium isotherms, indicating optimal viral spike and TSS concentrations. Filter exposure periods of 1, 2, 4, 6, 8, 12, 24, 36, and 48 h were used for wastewater, and 2, 8, 12, 24, 36, 48, and 72 h for DI water. Due to the unpredictable nature of the viral surrogate in wastewater (22),

each matrix was modelled up to the time it was perceived that equilibrium had been reached, 24 h and 72 h for wastewater and DI water, respectively.

The PFO kinetic model assumes that the adsorption rate is proportional to the difference between the adsorbed concentration and the number of available sites [25] and can be written as Eq. (4)

(4)

$$Log\ (q_e - q_t) = \log q_e - K_1 t,$$

where q_e and q_t are the amounts of HI-SCV-2 (GU cm^{-2}) at equilibrium and at time t, respectively, and K_1 is the PFO equilibrium rate constant (1 $hour^{-1}$).

The PSO kinetic model assumes that the rate-limiting step in adsorption depends on the collision between solute molecules with unoccupied sites at the adsorbent surface [25]. The PSO model can be written as Eq. (5).

(5)

$$\frac{t}{q_t} = \left[\frac{1}{K_2 q_e^2}\right] + \frac{1}{q_t} t,$$

where K_2 is the rate constant of the equation (1 $hour^{-1}$).

4.3.7 Material Characterization

The surface morphology of the electronegative filters was characterized by a scanning electron microscope (SEM) with a Zeiss (Jena, Germany) SIGMA 300 VP scanning electron microscope with an acceleration voltage of 5 kV, a current probe of 220 pA, and a working distance of 12 and 15 mm. The samples were allowed to dry completely for 24 h at room temperature, mounted on aluminum specimen stubs with double-sided adhesive tape, and sputter-coated with gold/palladium (80/20) in argon using a Leica ACE600 sputter coater with a current of 30 mA until a thickness of 15 nm was reached. Three filters were evaluated: (a) a filter collected from a wastewater sample after a 24-h sampling period; (b) a filter collected from a wastewater sample after a 24-h sampling period and eluted with a 0.075% Tween®20 + 25 mM Tris HCl-based buffer; and (c) an unexposed filter. All filters were exposed to their respective conditions as whole filters (90 mm), then cut into sections, and analyzed using SEM technology in triplicate.

4.3.8 Data Analysis

All tests were evaluated in biological triplicate. RNA concentrations that reflect the amount of viral RNA per square cm of filter (GU cm^{-2}) were calculated using Eq. (6). Mean viral concentrations were calculated and standard deviation among replicates was used to represent error bars. Recovery of HI-SCV-2 RNA was calculated using Eq. (7) [10]. Microsoft® Excel for Microsoft version 2109 (2021) was used for all data analysis. Graphs were produced using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA.

(6)

$$\text{RNA (GU cm}^{-2}\text{)} \approx \frac{\text{sample concentration (GU cm}^{-2}\text{)} \times \text{eluate volume (6 mL)}}{\text{Filter surface area (63.6 cm}^{-2}\text{)}}$$

(7)

$$\text{RNA Recovery (\%)} \approx \frac{100 \times \text{sample concentration (GU mL}^{-1}\text{)} \times \text{eluate volume (6 mL)}}{\text{Spiked concentration (GU mL}^{-1}\text{)} \times \text{sample volume (100 mL)}}$$

4.3.9 RNA Extraction

A magnetic beads-based RNA extraction method was used for all sample analyses [19]. A 1-mL volume from each filter eluate was extracted for RNA according to the manufacturer's instructions. The final volume of eluted RNA from the extraction process was 50 µL, which was subsequently used for RT-qPCR analysis.

4.3.10 RT-qPCR

RNA samples were processed by RT-qPCR on a GeneCount® Q-16 instrument (LuminUltra Technologies Ltd, Fredericton, CA). Each RT-qPCR reaction contained 15 µL of Master Mix (667 nM of forward primer, 667 nM of reverse primer, and 167 nM of probe) and 5 µL of template RNA. The sequences of the primers and probes used were published by the US CDC, and are presented in Table 4-1 [30]. Thermal cycling reactions were carried out as follows: a pre-denaturation step at 55 °C for 10 min followed by a second pre-denaturation step at 95 °C for 1 min. The two pre-denaturation steps were followed by 45 cycles of 95 °C for 10 sec and 55 °C for 45 sec, along with a final hold step at 50 °C for 1 min. Positive detections were indicated by cycle threshold (Ct) values

under 40, and all viral concentrations were reported as genomic units per millilitre (GU mL^{-1}) and converted to genomic units per centimetre square (GU cm^{-2}). The RT-qPCR upper Ct value detection threshold is 40 cycles, which corresponds to 1.4 copies per reaction.

Table 4-1. Sequences for primers and probes of viral surrogates were used in this study.

Organism	**Sequence Type**	**Sequence (5' – 3')**
SARS-CoV-2 N2 Gene	N2 Forward primer	TTACAAACATTGGCCGCAAA
	N2 Reverse primer	GCGCGACATTCCGAAGAA
	N2 Probe	ACAATTTGCCCCCAGCGCTTCAG

4.3.11 Quality Control

All batch-adsorption experiments and RNA extractions were performed in a Thermo Scientific 1300 Series A2 biosafety cabinet. To assess contamination, each sample batch was run with an unspiked sample to serve as a blank. Standards outlined in Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [31] and Environmental Microbiology Minimum Information (EMMI) guidelines [32] were referred to for evaluating qPCR-based tests. Negative controls were implemented in each RT-qPCR assay; however, RT-qPCR technical replicates were omitted to conserve reagents and materials. Biological replicates were performed in triplicate. The master mix utilized contains MS-2 bacteriophage as an internal amplification control (IAC) to confirm amplification capability; samples were only considered if the IAC passed. All RNA samples were analyzed using RT-qPCR the same day as RNA extraction and then stored at -76°C for any subsequent analysis. The LuminUltra GeneCount® Q-16 RT-qPCR system relies on a master standard curve, ranging from 1×10^1 to 1×10^4 copies per reaction. Each point on the curve was run in duplicate and was constructed using a serial dilution of SARS-CoV-2 RNA reference material (ZeptoMetrix, Buffalo, USA). The GeneCount® Q-16 has an R^2 value of 0.948 and an efficiency of 85%. The instrument's efficiency has been thought to be impacted by factors such as lyophilization; however, it has been shown to be reliable and generate accurate and reproducible results in previous work [10,19].

All RT-qPCR assay and quality control measures used in this work have been previously reported in Parra-Guardado et al., (2021) [19]. The experimentally determined

method limit of detection (MLOD) of the RNA extraction protocol for HI-SCV-2 spiked in wastewater was 5×10^{1} GU mL^{-1}. However, Parra-Guardado et al. (2021) reported SARS-CoV-2 RNA concentrations as low as 1.7 GU mL^{-1} in field samples. Maximum recovery efficiency was reported as 86.9% but was found to be dependent on matrix characteristics (e.g., TSS) and RNA dilution to mitigate inhibition. A process control, which accounts for varying RNA extraction efficiencies in a wastewater matrix, was not utilized in this work because there are currently no known process controls identified as exhibiting similar adsorption characteristics as the viral target for passive sampling experiments using electronegative filters.

4.4 Results and Discussion

4.4.1 Equilibrium Adsorption Isotherms

It is well understood that SARS-CoV-2 RNA is shed in fecal material and has a high partitioning affinity to solid particles in wastewater [18,33,34]. As a result, TSS concentrations are expected to impact viral adsorption and recovery from passive samplers. To examine the influence of TSS concentrations on the adsorption behaviour of HI-SCV-2 to 90-mm electronegative filters, the viral surrogate was spiked to DI water and wastewater containing increasing concentrations of TSS (low: 118 mg L^{-1}, medium: 265 mg L^{-1}, high: 497 mg L^{-1}), surrogate concentrations ranged from 1×10^{1} to 5×10^{4} GU mL^{-1}. Figure 4-1 shows the experimental equilibrium adsorption capacity, q_e, as a function of initial viral surrogate concentration and TSS variations. Due to poor viral recoveries (< 1%) in the DI water matrix, the adsorption capacity was excluded from Figure 4-1.

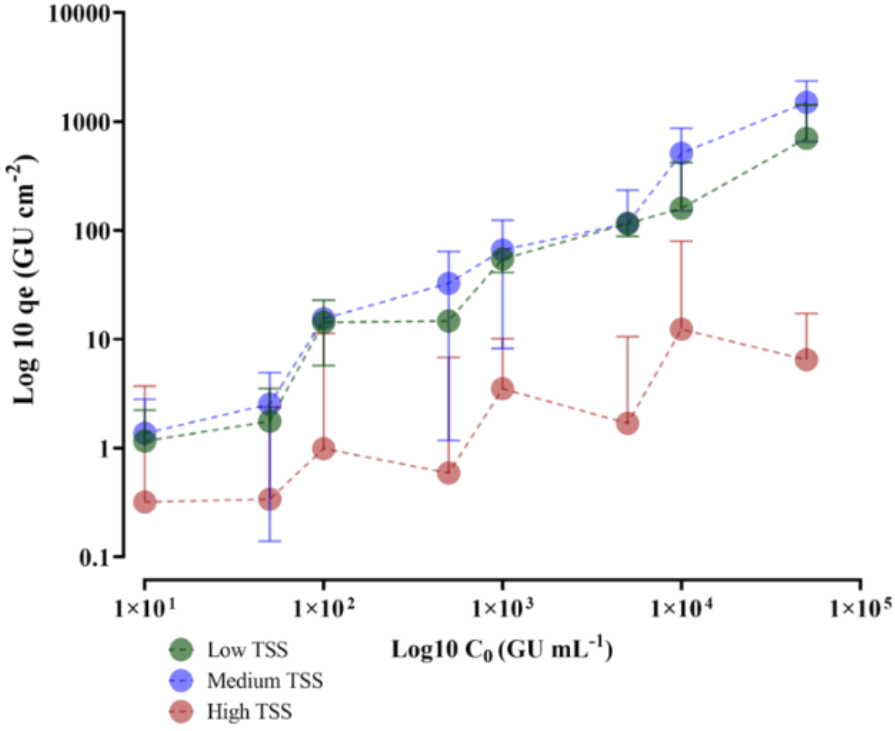

Figure 4-1. The effect of initial spiked HI-SCV-2 concentration, Log10 C_0 (GU mL^{-1}) on equilibrium adsorption capacity, Log_{10} q_e (GU cm^{-2}) to the filter. The points represent the mean concentrations for each test group and a dotted line is used to interpolate between spiked concentrations represented on the horizontal axis. Vertical error bars on each data point indicate the standard deviation of replicates (n=3); some error bars are not shown because they are smaller than the symbol.

At low initial surrogate spike concentrations, minimal differences in observed adsorption capacity were found as a function of TSS concentrations. However, when initial viral concentrations exceeded 1×10^3 GU mL^{-1}, the low and medium TSS samples showed an increase in viral adsorption and recovery, which suggests that surrogate viruses were associated with solid particles captured by the filter. Based on this observation, it could be expected that further increases in TSS would provide additional viral adsorption and improved viral recovery; however, all samples with high concentrations of TSS showed minimal recovery of viral RNA. Although the surrogate association with wastewater solids is inherently different than shed viruses in environmental matrices [35], HI-SCV-2 has been reliably detected in the solids-rich fraction of 50-mL wastewater samples when extracted after a 1-h incubation period [19]. Comparably, the results of this work indicate that a synthetic surrogate-TSS system approximates real-world conditions.

The results of this experiment indicate that a direct study of viral adsorption is limited by challenges in the viral recovery process, which is subject to inhibition during RNA extraction and amplification [36,37]. Both medium and low TSS matrices had a maximum HI-SCV-2 recovery of 10% when spiked to 1×10^{2} GU mL^{-1}. In the high TSS matrix, maximum HI-SCV-2 recovery was 2% when spiked at 1×10^{1} GU mL^{-1}, but the average recovery regardless of initial HI-SCV-2 concentration was only 0.4%. Likewise, equilibrium isotherm experiments with DI water also resulted in low viral capacities, which were likely caused by poor adsorption of the virus onto the filter surface rather than inhibitory processes during extraction. These results are supported by previous work, where it has been reported that virus recovery in wastewater is affected by excessive solid concentrations (> 400 mg L^{-1}) that may cause inhibitory actions in downstream analysis [38]. The reduced RNA recovery observed at higher TSS concentrations may be a consequence of solid particles inhibiting RNA extraction and amplification. There appears to be an intrinsic trade-off between maximum adsorption capacity and viral recoveries with TSS concentrations. It has been previously observed that the adsorption of enteroviruses to electronegative adsorbent filters was enhanced in raw water containing moderate levels of solids compared to solids-free water samples [39]. Sobsey and Glass (1984) suggested that additional virus adsorption sites were created by the solids that accumulate on filter micropores, which was supported in this work through SEM analysis, which displays a coating of adsorbed particles on and between the electronegative filter micropores (Figure S2). Moreover, the results of the equilibrium batch-adsorption isotherms identify the apparent interference TSS has on viral detection; when a matrix has high TSS concentrations, viral quantification may appear low or even absent, regardless of viral concentration.

Langmuir and Freundlich isotherm models (Figure S3) and their adsorption fitting parameters were determined (Table 4-2). Minimization of the ARE was used to optimize the model fit. Adsorption isotherms for the three TSS concentrations were fit equally well by both models, however, the Freundlich model provided a marginally better fit to the medium and high TSS adsorption isotherms, while the Langmuir model provided a slightly better fit to the low TSS concentration adsorption isotherm. The DI water adsorption isotherm was fit equally well by both models. The Langmuir isotherm model is widely

applied in a variety of adsorptive systems [22,26,40–42] because it offers an estimation of a theoretical maximum adsorptive capacity (q_{max}). The filter q_{max} was determined by the Langmuir model to be 7.0×10^3 GU cm^{-2} in the medium TSS solution, while the q_{max} for the high and low TSS matrices were 6.3×10^0 and 2.8×10^2 GU cm^{-2}, respectively. The q_{max} in the low TSS system could be reflective of limited associations between the surrogate virus and the minimal suspended solids, while the q_{max} in the high TSS matrices is likely due to inhibition during downstream processes. The Freundlich model constants, *n,* and K_F are characteristic of the particular adsorption system, where *n* is an indicator for the degree of surface heterogeneity and if adsorption is favourable $n>1$; if adsorption is unfavourable, $n<1$. When *n* deviates from 1, this demonstrates that there is heterogeneity at the adsorption surface [28]. Heterogeneity likely occurs at the filter surface from solids in the matrix, as the deposition of solids onto the filter can create a complex organic-rich layer for additional TSS capture.

Table 4-2. The Langmuir and Freundlich equilibrium isotherm constants; ARE, *n* and K_{LF} in wastewater matrices of low (118 mg L^{-1}), medium (265 mg L^{-1}), and high (497 mg L^{-1}) TSS concentrations.

Langmuir isotherm constants				Freundlich isotherm constants		
TSS (mg mL^{-1})	K_L	q_{max} (GU cm^{-2})	ARE	K_F	*n*	ARE
Low	0.000128	286	35.1	0.018	0.975	54.0
Medium	0.00000751	7020	41.1	0.125	1.15	35.9
High	0.00021	6.3	62.6	0.008	2.89	40.2

The adsorption of solids, and associated viruses, to the passive sampler filter, is likely to vary based on the physical and chemical nature of the TSS and may be highly variable across wastewater systems. Although sewersheds are dynamic systems with fluctuations in viral shedding, flow regimes, and water quality parameters throughout the day, the results of this work offer insight into the surface association between SARS-CoV-2 RNA, suspended solids, and the filter. Further, this work found that viral recovery is dependent on the presence of fecal matter and TSS; however, excessive TSS prevented the recovery and amplification of SARS-CoV-2 RNA. This finding has important implications for interpreting the analysis of field samples that have a high TSS concentration.

4.4.2 Kinetic Adsorption Isotherms

Understanding the effect of sampler deployment duration on recovered viral concentrations is important in developing a sampling plan that effectively captures the total viral signal over a pre-determined period. The medium TSS wastewater matrix yielded the highest q_{max} in the equilibrium adsorption experiments and was therefore used as the representative test matrix in the kinetic adsorption isotherm study. Figure 4-2 demonstrates the capacity of 90-mm electronegative filters to adsorb HI-SCV-2 spiked at 1×10^3 GU mL^{-1} over a 48-h exposure period in two separate matrices (wastewater with a TSS concentration of 265 mg L^{-1} and DI water). Virus adsorption improved with increasing contact time in both matrices up to 24 h of exposure where no greater increase in adsorption was observed. In wastewater, the maximum HI-SCV-2 RNA concentration (5.82×10^2 GU cm^{-2}) was recovered after 24 h. At most time points, HI-SCV-2 RNA recovered concentrations were found to be an order of magnitude lower in the DI water matrix than in the wastewater matrix. This is consistent with results in the batch-adsorption equilibrium isotherms, where viral recoveries in DI water were lower than from the wastewater matrix with medium TSS concentrations.

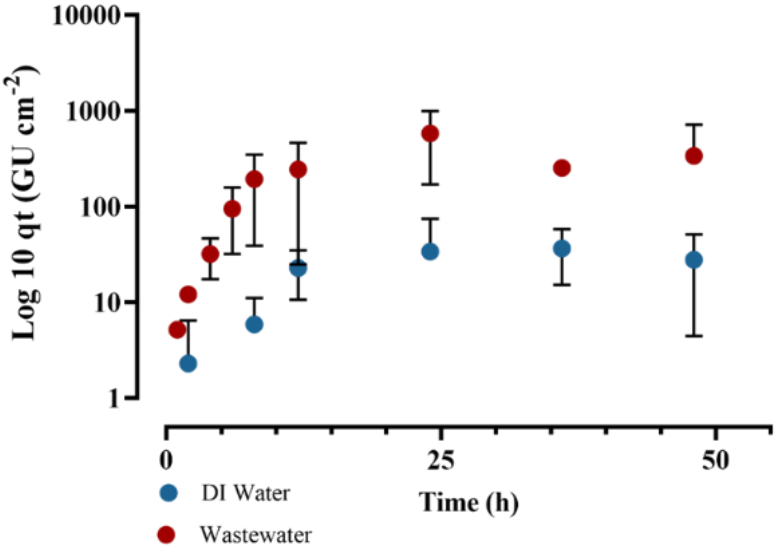

Figure 4-2. The adsorption capacity of HI-SCV-2 to the filter at time (h) is reported on the y-axis as Log10 q_t (GU cm^{-2}) for wastewater (TSS concentration: 265 mg L^{-1}) and DI water over a 48-h exposure period. Initial spiked concentrations were 1×10^3 GU mL^{-1}. Error bars on each data point indicate standard deviation (n=3): some error bars are not shown because they are shorter than the size of the symbol.

To investigate the kinetic mechanisms of HI-SCV-2 adsorption to the filter in a medium TSS wastewater solution, PFO and PSO order kinetic models (Figure S4) were fit to experimental data. The resulting kinetic parameters are displayed in Table 4-3. The calculated q_e results indicate the adsorption at equilibrium, fit by the model, and the $K_{1,2}$ calculations indicate the rate constant for either model. Based on the R^2 values, the adsorption mechanism in the wastewater matrix is best represented by the PFO kinetic model; while recoveries were low in the DI matrix, the data moderately followed a PSO model.

Table 4-3. The kinetic parameters for PFO and PSO models in wastewater (TSS concentration: 265 mg L^{-1}) and DI water.

Matrix	**Reaction**	R^2	q_e (GU cm^{-2})	$K_{1,2}$
Wastewater	PFO	0.94	638	0.05
DI Water	PSO	0.66	45	0.001

The use of a PFO model to fit experimental data has become increasingly common due to limitations based on the PSO model assumptions [43]. A PSO reaction rate suggests that adsorption is governed by chemical interactions between adsorbent and adsorbate. Under PSO conditions, the adsorption rate is dependent on adsorption capacity, and not on the concentration of adsorbate [44]. In contrast, PFO kinetics are understood to be driven by the assumption that the rate of change of solute uptake with time is directly proportional to the difference in initial spiked concentration and amount of solute adsorbed over time.

It has been demonstrated that the adsorption mechanism between passive samplers and a water phase follows first-order models, and in general, this sampling strategy is effective at detecting episodic events when analyte concentrations in water matrices are variable [45,46]. Likewise, the results of this work align with Habtewold et al., (2021) who assessed SARS-CoV-2 RNA adsorption to electronegative filters in a pilot-scale municipal wastewater facility in Guelph, Ontario, Canada in February 2021. Habtewold et al., (2021) described a linear up-take of SARS-CoV-2 RNA to the filters for up to 48 h with little variability observed in viral accumulation between 48 and 96 h, although the wastewater temperature during these experiments was not noted by the authors. Here, we found little variation in viral concentration between 24 and 48 h (under room temperature ~23 ± 3°C).

Adsorption capacities after 24 hours may have been limited by viral decay due to the temperature sensitivity of the viral surrogate [47,48]. This work indicates that electronegative filters provide a suitable approach to reach maximum concentrations of HI-SCV-2. Based on these experimental results, it can be inferred that sampling times equal to or greater than 24 h will likely achieve a maximum adsorption capacity in real-world scenarios, such that deployments over 24 h may not yield greater viral recoveries. This is advantageous for WWS programs as 24-hour deployments are convenient for wastewater operating staff and will capture daily population dynamics.

4.4.3 Application of Experimental Results to Field Study Context: Implications for Passive Sampling Deployment at Targeted Sewershed Locations

Throughout 15 weeks, 86 passive sampling events were conducted, 23 at Locations A and B, and 40 sampling events at Location C (Figure 4-3). Detected SARS-CoV-2 RNA concentrations ranged from 2.53×10^{0} to 4.88×10^{3} GU cm^{-2} across all three sites, and collectively, the mean and median concentrations for all positive samples were 8.91×10^{2} and 2.30×10^{2} GU cm^{-2}, respectively. At Locations A and B, clusters of positive detections within a small range of concentrations can be observed. However, SARS-CoV-2 RNA concentrations never exceeded 1×10^{4} GU cm^{-2} throughout the entire sampling period. There were only three positive detections at Location C, with minimum and maximum SARS-CoV-2 RNA concentrations of 2.5×10^{0} and 4.0×10^{1} GU cm^{-2}, respectively. Non-detects during this period may be attributed to population dynamics (e.g., active cases moving out of the catchment area), differences in viral shedding rates, or downstream analysis inhibition. All samples were deployed in the sewersheds for either 48 or 72 h, except for a single sampling occurrence at Location B that had a deployment of 24 h. These deployment durations were chosen based on the availability of operating staff. Regardless of the deployment period, SARS-CoV-2 RNA concentrations in the field did not exceed the Langmuir modelled q_{max} of 7.0×10^{3} GU cm^{-2} and were consistently above the batch-adsorption lowest detectable RNA concentration (1.4×10^{0} GU cm^{-2}).

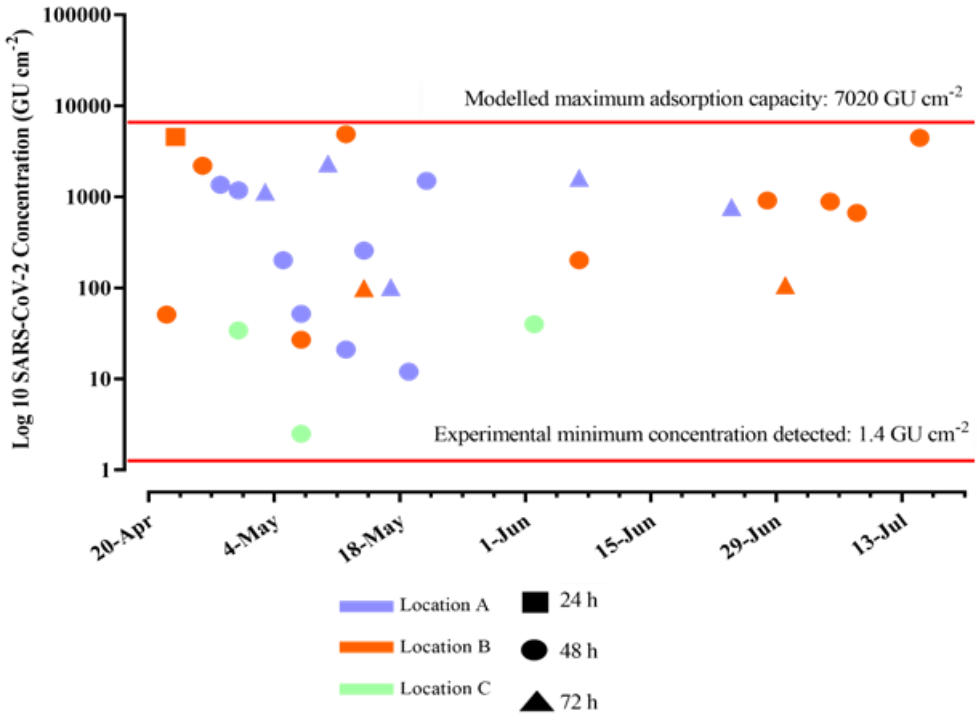

Figure 4-3. Log_{10} SARS-CoV-2 RNA concentrations (GU cm^{-2}) from three sewer catchments in Halifax, NS are shown on the y-axis. Sampler deployment duration is shown in geometric shapes (24-, 48-, and 72-h in-sewer periods). Non-detects are not shown on the x-axis. The top red line indicates the modelled maximum adsorption capacity (q_{max}, 7.0×10^{3} GU cm^{-2}) in the medium TSS equilibrium isotherm. The lower red line displays the experimental minimum concentration (1.3×10^{0} GU cm^{-2}) observed in the medium TSS equilibrium adsorption isotherm. During the sampling period, the number of active cases in each sewershed was unknown [49].

Adsorption and recovery of SARS-CoV-2 RNA in the field do not exceed the modelled adsorption maximum identified in the surrogate-based laboratory experiments. Maximum adsorption capacity plateaus have also been observed in the passive sampling of norovirus in freshwater using electropositive filters [50] and SARS-CoV-2 RNA in wastewater using electronegative filters [51]. The assessment of the laboratory and field data suggests that deployment durations greater than 48 h are unlikely to result in additional viral recovery due both to the adsorptive capacity of the filter and the inhibitory effect of high TSS interference. Due to these challenges, considerations should be made for inhibition during sample collection and analysis to reduce the likelihood of false-negative results.

4.4.4 Deployment Techniques: The Role of TSS in Sample Analysis

To better understand the role of TSS in the inhibition of SARS-CoV-2 RNA detection, TSS concentrations from the filter eluates were measured from both laboratory and field-based samples. Eluate TSS was evaluated, rather than bulk wastewater TSS, as it provides a more accurate indication of potential inhibition of RNA extraction and amplification from solids. In Figure 4-4, average eluate TSS concentrations and SARS-CoV-2 RNA concentrations (if detected) are shown for 70 of 85 samples collected in the field. This figure also displays the minimum and maximum eluate TSS concentrations from the equilibrium batch-adsorption experimental data.

There appears to be a clear relationship between TSS concentration in the eluate and the ability to detect SARS-CoV-2 RNA. All samples that had SARS-CoV-2 RNA detections were within an eluate TSS concentration range of 1×10^2 to 1×10^3 (mg per eluate) and fell within the eluate TSS ranges observed for the low and medium wastewater matrices in the equilibrium isotherms. However, SARS-CoV-2 RNA was not detected in any sample eluates having TSS concentrations in the range of 1×10^3 to 1.5×10^3 (mg per eluate), which was within the eluate TSS range of the batch-adsorption high TSS matrix where the greatest impact on viral recovery was observed.

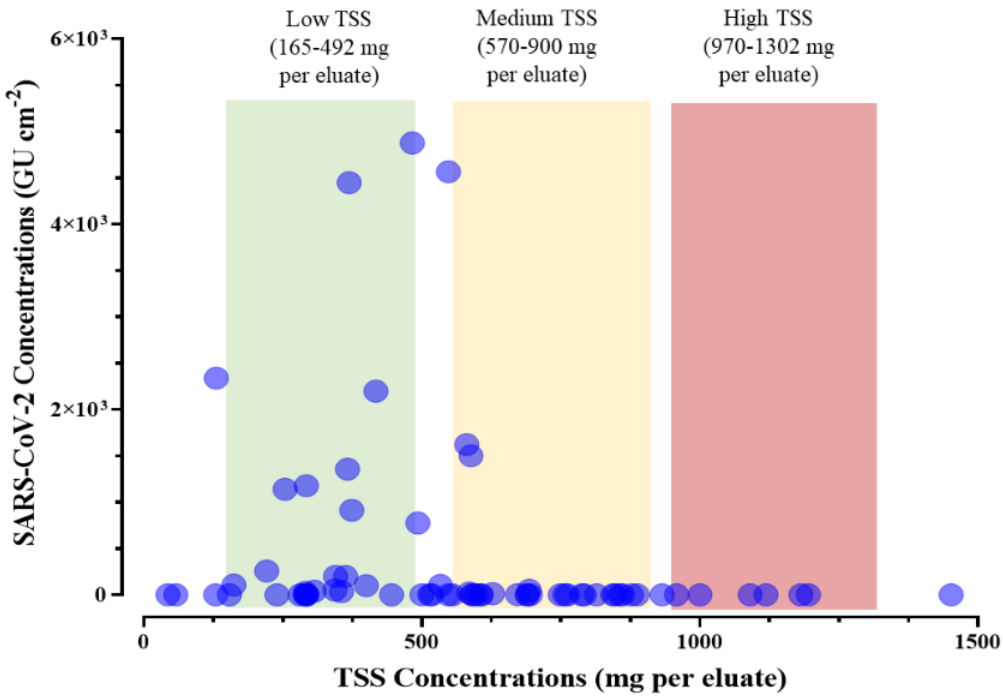

Figure 4-4. SARS-CoV-2 RNA concentrations from Locations A, B, and C are reported on the y-axis (GU cm^{-2}). TSS concentrations were measured per 6 mL filter eluate and are reported on the x-axis as mg per eluate. To highlight the effect of TSS on SARS-CoV-2 RNA recovery in a field setting, sites are not differentiated. The minimum and maximum filter eluate TSS concentration range for low, medium, and high TSS matrices from the batch-adsorption equilibrium isotherms are superimposed in green, yellow, and red bars, respectively.

In the batch-adsorption isotherms, HI-SCV-2 recovery was thought to be adversely affected by inhibition due to the high TSS concentrations in the sample eluate, which is related to high TSS concentrations in the wastewater matrix. A similar inhibitory occurrence could likely have adversely affected SARS-CoV-2 RNA detections in field samples with similarly high TSS concentrations. The comparison of the experimental and field eluate TSS concentrations suggests that inhibitory mechanisms could result in the under-detection of SARS-CoV-2 RNA in high TSS wastewater samples. This has important implications for the interpretation of passive sampling-derived field data.

It is recommended that suspended solid concentrations in the filter eluate are determined when developing a passive sampling WWS program. Based on this work, measuring the TSS concentration in the viral eluate is an effective means of predicting potential downstream inhibition; when eluate TSS concentrations are above 1×10^3 mg per eluate, the likelihood of false negatives may be increased. Evaluating eluate TSS

concentrations before RNA extraction or analysis can inform researchers when to take corrective action in mitigating inhibition during downstream analysis through RNA extract dilution.

A limitation of this work is that physiochemical characteristics of wastewater that are likely to influence viral interactions, such as pH, temperature, and flow rates [52–55], were not evaluated. Consequently, further research on the impact of these characteristics on SARS-CoV-2 RNA adsorption is required to improve data interpretation of passive sampling results. However, this paper establishes useful parameters for passive sampling deployment and highlights the importance of eluate TSS in identifying samples that may result in false negatives.

4.5 Conclusions

In this work, we investigated the adsorption of HI-SCV-2 and SARS-CoV-2 RNA to electronegative membranes in laboratory batch-adsorption isotherm experiments and in-field applications, respectively. The adsorption of HI-SCV-2 was best described by a PFO rate model and Freundlich isotherm model for wastewater with TSS concentrations ~ 265 mg L^{-1}. Comparatively, the Langmuir isotherm model had a similarly good fit, indicating a modelled q_{max} HI-SCV-2 concentration of 7×10^3 GU cm^{-2}. TSS concentrations, in water samples with low (118 mg L^{-1}) and medium (265 mg L^{-1}) TSS, were found to facilitate HI-SCV-2 adsorption to the filter and at higher (497 mg L^{-1}) TSS concentrations, RNA extraction, and amplification efficiencies may be impacted. In the kinetic isotherm experiments, a maximum HI-SCV-2 concentration was achieved after 24 h of deployment.

In field experiments, detected SARS-CoV-2 RNA concentrations were all within the modelled maximum adsorptive capacity of the filter established via equilibrium batch-adsorption experiments. Regardless of sample deployment duration, SARS-CoV-2 RNA concentrations did not exceed this modelled maximum adsorptive plateau. Evaluating TSS concentrations in the filter eluate of both the bench- and field-study samples demonstrated the capability of eluate TSS to aid in predicting potential sample inhibition in downstream analysis.

Based on this work, it is recommended that when utilizing a passive sampling approach for the detection of SARS-CoV-2 RNA in wastewater, samplers should be

deployed for 24 to 48 hours. Additionally, the maximum adsorption capacity of the filter should be considered when interpreting results, as viral loads that exceed capacity will not be accurately quantified in field samples. Most importantly, TSS concentrations should be measured in the filter eluate to determine if the absence of viral signal could be a function of solids inhibition during analysis. This study provides valuable insight into the effective field-scale deployment of passive samplers for capturing SARS-CoV-2 RNA in wastewater and will inform decision-making for real-world sewershed deployments. This study is the first of its kind to evaluate SARS-CoV-2 RNA adsorption onto electronegative filters from wastewater in passive sampling bench-scale studies. Future work may involve similar kinetic and equilibrium analyses for different adsorbent materials to investigate other suitable materials that may have a higher adsorption capacity for SARS-CoV-2 RNA in wastewater using passive sampling.

4.6 Acknowledgements

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CHAPTER 5 DETECTION OF THE SARS-COV-2 OMICRON VARIANT: A RETROSPECTIVE ANALYSIS THROUGH WASTEWATER IN HALIFAX, CANADA

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E.K.H. carried out sampling and sample analysis, evaluated data, prepared figures and wrote the paper.

5.1 Abstract

This study evaluates the efficacy of wastewater surveillance (WWS) for the early detection of the Omicron variant of SARS-CoV-2 in a university setting in Halifax, Canada. Utilizing an allele-specific RT-qPCR assay, that targets a distinctive Omicron-Lambda mutation (N: P13L; C28311T), we retrospectively analyzed wastewater samples collected from four university residences between 01 September and 31 December 2021. We analyzed 276 passive wastewater samples from four university residences and 51 composite wastewater samples from the wastewater treatment facility (WWTF) which is located downstream of the university. Our findings reveal the presence of the C28311T mutation in wastewater collected before the clinical identification of the Omicron variant in the province. Retrospective analysis of SARS-CoV-2-positive samples using the C28311T RT-qPCR assay showed detections in wastewater collected at the university on 05 November 2021 and 06 November 2021 and in the WWTF samples on 26 November 2021. SARS-CoV-2 N2 RNA was detected in 51 campus samples and 20 treatment facility samples (18 and 39% detection rate, respectively). The study emphasizes the utility of passive sampling for its cost-effectiveness and minimal maintenance, enabling rapid testing and prompt health interventions within an institutional setting. The comparison between the localized approach at the university and the broader community surveillance at the WWTF illustrates the nuanced understanding provided by targeted WWS. While the WWTF samples reflect a community-wide perspective with less variability, the university's targeted surveillance captures localized outbreaks, offering actionable insights for campus management. These findings underscore the strategic value of integrating

passive wastewater sampling into public health strategies for variant detection and outbreak prevention, particularly in institutional settings with high-density populations.

5.2 Introduction

Response to COVID-19 has become increasingly complex due to the emergence of multiple variants of SARS-COV-2, the virus responsible for COVID-19. These variants emerge through mutations in the virus's genetic material and while many mutations have negligible effects, others significantly increase public health risks.[1] In the first two years of the pandemic, the World Health Organization (WHO) identified five key variants of concern (VOCs). Among these, Omicron (B.1.1.529) was first identified in South Africa on 24 November 2021 and was quickly declared a VOC by the WHO on the 26th of November in 2021.[2,3] Subsequent investigations revealed Omicron's presence in Europe 10 days before the official identification in South Africa,[4] raising questions about its origin and evolution.[5] Retrospective analysis of wastewater in France,[6] and the Netherlands[7] revealed the presence of the Omicron variant in wastewater samples dated back to mid-November of 2021. These findings predate the initial identification of the variant in South Africa, suggesting an earlier-than-anticipated spread of the variant globally. This early circulation is further evidenced by wastewater sequencing in Utah, USA which identified the Omicron variant in samples from 19 November 2021, nearly 10 days before its detection through clinical sequencing. Following the variant's emergence, prevalence escalated across different sewersheds from December 2021 into January 2022, mirroring the uptick in clinically diagnosed cases. [8]

Omicron is distinguished by its high number of mutations, contributing to its enhanced transmissibility and infectivity.[9] At the time, these mutations facilitated a rapid spread of the Omicron variant, leading to significant increases in case numbers globally, despite the ongoing vaccination efforts. The variant's emergence and subsequent dominance highlighted the adaptability of the virus and the persistent challenges in pandemic management,[10] including the impact of reduced clinical testing capacities, the prevalence of asymptomatic or mild infections, and general pandemic fatigue, which all complicated efforts to accurately monitor and control the spread of the virus.[11]

Wastewater surveillance (WWS) emerged as a valuable tool for monitoring SARS-CoV-2 at the population level during the COVID-19 pandemic, [12,13] including its use in the detection of VOCs like Omicron in community wastewater. [11,14,15] Among the advancements in WWS, passive sampling has emerged as a particularly promising technique.[16] Numerous studies have utilized passive samplers containing sorptive materials such as tampons,[17,18] cottons buds (q-tips), cotton gauze or cheesecloth,[19,20] electrostatically charged membranes,[19–23] and, more recently, materials such as polyvinylidene fluoride and granular activated carbon have been reported to detect viruses in wastewater.[24,25] Passive sampling offers a simplified, cost-effective approach for monitoring viral RNA in wastewater systems, enabling the capture of representative samples over extended periods without the need for complex infrastructure or frequent manual sampling.[16] The adaptability and efficiency of passive sampling make it particularly effective for the timely and sensitive detection of SARS-CoV-2 within localized community settings, serving as an early warning tool that compares favourably with traditional sampling methods.[26] Corchis-Scott et al. (2021) highlights the use of tampon-based samplers to monitor COVID-19 cases in a university residence outperformed grab samples.[27] Similarly, gauze-based passive samplers targeting wastewater from a hospital admitting COVID-19 patients demonstrated more consistent SARS-CoV-2 detections then grab samples.[22] Additional studies have shown passive samplers can provide comparable data to autosamplers[26,28,29] and outperform grab sampling methods in detecting viral RNA in wastewater.[30,31]

While the majority of WWS efforts have focused on collecting samples from wastewater treatment facilities (WWTFs),[32,33] targeted surveillance in specific settings such as universities, healthcare facilities, and residential communities can offer valuable insights into the prevalence of current and emerging VOCs. [34–38] These settings enable more efficient tracking of incident cases and allow interventions to be implemented promptly upon the detection of the virus in wastewater systems. University campuses present a unique opportunity for monitoring COVID-19 infections due to their geographically diverse student populations, which include individuals who may travel internationally multiple times during their academic programs.

A noteworthy implementation of a campus wastewater monitoring program at Emory University (Atlanta, Georgia) led to the detection of SARS-CoV-2 from campus facilities, which in turn facilitated the identification of previously undetected COVID-19 cases within those premises.[39] Likewise, Gibas et al. (2021) illustrated the efficacy of WWS in emergency response at the University of North Carolina, where positive viral signals in campus wastewater prompted a swift action plan.[40] Consequently, an overnight lockdown was put into effect within merely 36 hours of sample collection, with students undergoing COVID-19 testing the next morning. The study underscored the high sensitivity of wastewater testing in detecting asymptomatic carriers, effectively identifying the presence of an asymptomatic individual in residential buildings accommodating 150 to 200 students, thus demonstrating the method's effectiveness in uncovering asymptomatic COVID-19 cases. Similarly, the University of Windsor's WWS program effectively prevented an outbreak by identifying the Alpha variant in wastewater samples, building on the demonstrated success of similar initiatives.[34] Wright et al. (2022) demonstrated that combining WWS with clinical testing effectively enhances campus health monitoring, evidenced by a significant correlation between SARS-CoV-2 RNA in wastewater and clinical COVID-19 tests on a university campus in the Fall of 2020.[41]

The objective of this research was to determine the efficacy of passive sampling techniques in WWS for the early detection of SARS-CoV-2 VOCs within a localized population. By leveraging such methodologies, this research seeks to highlight the potential of wastewater surveillance as a non-invasive, comprehensive tool for pre-emptively identifying circulating VOCs, thereby informing public health responses and mitigating community transmission.

5.3 Materials and Methods

5.3.1 Description of University Residence Sampling Sites

The university campus where sampling was carried out is largely surrounded by residential neighbourhoods and is adjacent to two large teaching hospitals and other health profession departments. Four University residence buildings were selected for wastewater monitoring in this study: Residence A, Residence B, Residence C, and Residence D.

During the Fall 2021 semester, the capacity of each residence was estimated to be 376, 573, 352, and 261 occupants, for residence buildings A, B, C and D, respectively.[42] However, due to COVID-19 precautions, the university reduced its residence capacity to 80% for the 2021/2022 academic year.[43] These residences were chosen as part of the University's Health and Safety plan to monitor and detect potential infections within the residence community during the return to campus activities for the Fall 2021 period.[44] Passive wastewater samples were collected at the confluence of two sewer lines outside of each residence building. Each sampling point received flow from a sanitary sewer line directly exiting the target building and flow from a stormwater sewer line. Because the sanitary sewer line intersected a stormwater sewer line at the collection point, the viral target in the collected wastewater samples was susceptible to dilution during rain or snow melt events.[45] Descriptions of each residence sampling location are provided in the Supplemental Material (Figure S1).

5.3.2 Passive Sampling at University Residence Buildings

Wastewater samples were collected using a 3D-printed passive sampler containing 90-mm (0.2-µm pore size) electronegative filters.[30] Two 47-mm diameter filters (0.1-µm pore size) were used when 90-mm filters were unavailable. Samplers were deployed in each sewershed for durations ranging from 24 and 72 hrs. Hayes et al. (2022) demonstrated that electronegative filters would achieve close to maximum adsorption capacity for SARS CoV-2 following 24-h of exposure to wastewater.[21] The variability in deployment durations was primarily due to the availability of management staff and operators, as well as logistical considerations. However, wastewater systems are dynamic and despite the difference in deployment durations, the data obtained may be considered semi-quantitative, reflecting overall changes in viral detections and concentrations accumulated on the sampler.

Samples were collected at 9:30 AM on each sampling date and processed the same afternoon. Results were available the same evening by approximately 5:00 PM. Generally, wastewater testing results were communicated to the university within eight hours of sample collection. The first positive detection of SARS-CoV-2 RNA in any of the residence building passive wastewater samples resulted in triggering a daily sampling

strategy, and following the persistence of SARS-CoV-2 RNA detection (≥ 3 consecutive days of positive RNA signal) daily wastewater sampling was triggered at all four residence buildings. Following 3 consecutive days of positive wastewater signal the university issued recommendations for more frequent COVID-19 rapid testing for asymptomatic residents.

A passive sampler was deployed at Residence A before the study period, in January of 2021. At Residence C and Residence D, passive samplers were deployed on 05 September 2021 and Residence B on 15 September 2021. Throughout the sampling period, the residence WWS strategy involved sample collection three times a week on Mondays, Wednesdays, and Fridays, except when the university was closed. Samples were routinely collected and immediately redeployed at 9:30 AM on the day of collection. For safety, the university's facilities management staff were on-site during sample collection to lift the manhole covers and to ensure safe levels within the sewer catchments using confined space gas monitors. Sampling continued until 23 December 2021, when the University closed for winter break.

5.3.3 Composite Sampling at the Wastewater Treatment Facility

To benchmark campus WWS data, 24-h composite samples were collected by utility personnel from a WWTF in Halifax, Nova Scotia, Canada between 01 September and 31 December 2021. All composite wastewater samples were collected from the influent wastewater stream, post-screening, and pre-grit removal. Samples did not undergo any additional treatment before collection. The 1-L wastewater samples were collected three times per week on Mondays, Wednesdays, and Fridays. Following collection, the 1-L composite wastewater samples were transported to the University laboratory on ice and processed within the same day as sample collection. The processing method for these samples followed the protocol described by Parra-Guardado et al. (2021).[46] Specifically, a 50-mL aliquot of raw wastewater influent was centrifuged for 5 minutes to obtain a 500-µL pellet. This pellet was eluted with 2 mL of a Tween®20-based elution buffer, and 1 mL of the eluate was then extracted for RNA using a direct magnetic bead-based extraction method. The WWTF receives flow from a combined sewer network that collects the wastewater from residential and commercial locations, including the University campus

buildings. The facility services an area with an estimated 117,000 inhabitants, with a mean influent flow rate of approximately 108,000 m^3 per day. A service map of the wastewater treatment catchment area is shown in Figure S2.

5.3.4 Reagents

Deionized (DI) water was produced by a Milli-Q system (Reference A+, Millipore) with a total organic carbon (TOC) concentration <5 µg L^{-1} and resistivity of 18.2 MΩ cm^{-1}. Electronegative filter membranes (4.7-cm, 0.1-µm or 9.0-cm, 0.2-µm cellulose nitrate membrane filters) and ethanol (EtOH) were purchased from Fisher Scientific (Ottawa, ON, CA). The viral elution buffer was made using Tween®20 and Tris-HCl sourced from Sigma Aldrich (Ottawa, ON, CA). The mixture consisted of 75 µL of Tween®20 and 250 µL of a 0.1 M Tris-HCl intermediate solution added to DI water for a total volume of 100 mL. Magnetic binding beads (50 g L^{-1}), RNA isolation kits, and SARS-CoV-2 assay kits were obtained from LuminUltra Technologies Ltd (Fredericton, NB, CA). Bovine serum albumin (BSA) used to make a 1 mg mL^{-1} solution (10 mg lyophilized BSA in 10 mL DI water) was obtained from Alfa Aesar by ThermoFisher Scientific (Tewksbury, MA, US). All primers to detect the C28311T mutation associated with the Omicron variant were purchased through Integrated DNA Technologies (IDT, Coralville, IA, USA), and the TaqMan MGB probe was purchased from ThermoFisher Scientific (Burlington, ON, CA).

5.3.5 RNA Extraction

All passive samples were eluted using 6 mL of 0.075% Tween®20 + 25 mM Tris HCl-based buffer; 1 mL of this eluate was used for subsequent RNA extraction. RNA extraction of influent wastewater and passive sampler filter eluate was carried out using a magnetic bead-based RNA extraction methodology described by Parra-Guardado et al. (2021) and Hayes et al. (2021), respectively.[30,46] Briefly, a 1-mL aliquot from either sample type (composite sample solids pellet or filter tween-based eluate) was used to perform the extraction protocol according to the manufacturer's instructions (LuminUltra Technologies Ltd). For particularly soiled passive samples, the sampler eluates would be diluted 1:2 with nuclease-free water (NFW) to mitigate potential inhibition of RNA extraction or RT-qPCR analysis. Soiled eluates were identified based on visual inspection for excessive particulate matter. The extracted RNA (50 µL) was processed using RT-

qPCR. Samples from the WWTF were extracted in duplicates. As the volume of passive sampler eluates was only 6 mL, samples were extracted in single aliquots to conserve raw eluates for subsequent analyses.

5.3.6 SARS-CoV-2 N2-Gene RT-qPCR Assay

All RNA samples for the monitoring program, including those from passive samplers and the WWTF, were processed by RT-qPCR on a GeneCount® Q-96 instrument (LuminUltra Technologies Ltd, Fredericton, CA). The probe and primer sequences for targeting the SARS-CoV-2 N2 gene used in this study are shown in Table 1. The 20-µL reactions were prepared using the GeneCount SARS-CoV-2 Screening kit (LuminUltra Technologies Ltd, Fredericton, CA), containing 15 µL of Master Mix and 5 µL of template RNA. The RT-qPCR Master Mix utilized for the N2 assay contains MS2 bacteriophage as an internal amplification control (IAC). Thermocycling conditions were performed as follows: 15 min at 50 ºC, 2 min at 95 ºC, and 45 cycles of 10 s at 95 ºC and 45 s at 55 ºC; and a final hold step for 45 sec at 55 ºC. All RT-qPCR analyses performed in the GeneCount® Q-96 instrument included at least two no-template control (NTC) containing NFW. Each sample was analyzed at a single RNA dilution; if the first aliquot resulted in a non-detect, a second dilution was analyzed to minimize the likelihood of false negatives due to inhibition during analysis.

A positive control, four-point standard curve ($10^2 – 10^5$ copies μL^{-1}) was carried out using a plasmid containing the target N genes for SARS- CoV-2 (IDT, Coralville, IA, USA) and a serial 10-fold with NFW (~2×10^5 copies μL^{-1}). Plasmid control was prepared and stored based on manufacturer recommendations. All points on the curve were run in duplicate and averaged to create one standard curve used to calculate copies mL^{-1} through cycle quantification (Cq) values. The efficiency of the N2 assay standard curve was ~96%, with an R^2 value of 0.99 and y-intercept of ~38.4.

5.3.7 SARS-CoV-2 C28311T Mutation RT-qPCR Assay

Retrospective analysis of RNA samples was carried out for the detection of the C28311T allele frequency, a single nucleotide variant specific for both Lambda and Omicron VOCs using a GeneCount® Q-96 instrument (LuminUltra Technologies Ltd, Fredericton, CA).[14] The C28311T RT-qPCR assay was performed using an allele-specific

forward primer and probe combined with the CDC N1 reverse primer.[47] The RT-qPCR assay employed in this study is founded on a primer extension approach, specifically designed for the detection of the SARS-CoV-2 Omicron variant C28311T mutation in wastewater samples. The C28311T RT-qPCR assay targets a distinctive mutation (N:P13L; C28311T) of the Omicron variant in the N1 amplicon region, observed in the B.1.1.529 genomes deposited in GISAID in December 2021.[48] The single nucleotide variant, C28311T, during the time of this study, was present in >97% of B.1.1.529 (Omicron; BA.1 and BA.2) and C.37 (Lambda) GISAID-deposited sequences and found in <0.5% of other sequences. Importantly, Lambda VOC prevalence in Canada throughout the pandemic was <0.01% (32/239,025 GISAID-deposited sequences as of 12 January 2022).

The sequences of the primers and probes, as well as their working concentrations, for the C28311T RT-qPCR assay are shown in Table 1. All C28311T RT-qPCR reactions were prepared using 3 µL of RNA template in a final volume of 20 µL. The thermocycling parameters were carried out as follows: 5 min at 50 ºC, followed by 20 s at 95 ºC, and 45 cycles of 3s at 95 ºC and 45 s at 55 ºC. All samples were assessed in technical duplicates, each run at two separate RNA dilutions for a total of four reactions per sample. A 6-point standard curve ($10^6 – 10^1$ copies μL^{-1}) was generated from Twist synthetic SARS-CoV-2 RNA control 48 (B.1.1.529/BA.1) for C28311T analysis. The standards were prepared in single-use aliquots through a 10-fold dilution series of the stock solution (~1×10^6 copies μL^{-1}). The standard curve had an R^2 value of 0.99, an efficiency of 96% and a y-intercept of 39.1. A total of 70 N2-positive and 142 randomly selected N2-negative samples across all sampling sites between 01 September and 31 December 2021 were analyzed using the C28311T assay.

5.3.8 SARS-CoV-2 Alpha (B.1.1.7) and Delta (B.1.617) RT-qPCR Assays

Here, we describe the use of allele-specific RT-qPCR strategies to target mutations in the N gene of SARS-CoV-2 to allow for quantification of mutations associated with the Alpha (B.1.1.7) and Delta (B.1.617) variants of concern. These assays are capable of discriminating single nucleotide variants in wastewater samples and, therefore, were employed to determine the presence or absence of the these variants and distinguish between the presence of these other strains in samples that were positive using the Omicron

(C28311T) assay. The Delta variant was chosen as it was the predominant VOC in Canada before the rapid spread of Omicron in November 2021.[49] The Alpha and Omicron variants share a common mutation: the deletion of the S gene del (69-70).[50] However, the Alpha variant has had a <1% presence in Canada as of 14 November 2021.[49] Therefore, assessing the Alpha variant through specific targeting of a mutation independent of the Omicron variant (i.e., the D3L mutation, as used in this work) allowed us to further rule out the potential presence of the variant in our samples.

The Alpha RT-qPCR assay was carried out based on previous work by Graber et al., (2021) who implemented an allele-specific B.1.1.7 allele (D3L) RT-qPCR assay using a recently designed forward primer combined with the N1 probe and reverse primer (Table 5-1).[51] The D3L forward primer used for this work incorporates a deletion mutant (A28271Del) which has been previously described as the dominant single nucleotide variant in the B.1.1.7 lineage. The paired non-B.1.1.7 alleles (D3) assay was not performed in this work, as the work was implemented to confirm the absence of the B.1.1.7 lineage. RT-qPCR reactions were prepared with TaqMan® Fast Virus 1-Step Master Mix (ThermoFisher, Ottawa, Canada) in duplicate reactions, each with 1.5 µL of RNA template in a final reaction volume of 10 µL. Thermal cycling conditions were as follows on the LuminUltra GeneCount® Q96, RT at 50°C, 5 min, followed by polymerase activation and template denaturation at 95°C for 20 s, and 45 cycles of denaturation (95°C for 3s), then annealing/extension (55°C for 45s). Each sample set analyzed on the RT-qPCR was run with at least two positive and negative controls. Only if the negative and positive controls passed (i.e., Cq values >40 and 38, respectively) were the sample results considered acceptable. The positive control (10^3 copies μL^{-1}) and standard curve utilized a synthetic B.1.1.7 RNA template (GISAID accession ID EPI_ISL_710528, Twist Biosciences); an average positive control Ct value of 30.4 was observed. The standard curve assay was performed with a six-point (10^1 – 10^6 copies μL^{-1}) serial dilution of the B.1.1.7 RNA template using NFW. Efficiencies, linearity, and y-intercepts were ~91%, 0.99 and 38.6 respectively for the D3L standard curve.

RT-qPCR for the Delta variant (B.1.617) mutation was carried out on all RNA positive for N2 from 01 September to 31 December 2021 at the University residence and

the region WWTF sites. The RT-qPCR assay was performed based on the workflow described by Yaniv et al. (2021),[52] each reaction contained 5 µL of RNA sample with 5 µL TaqMan® Fast Virus 1-Step Master Mix (ThermoFisher, Ottawa, Canada), 7.6 µL of nuclease-free water, and 0.5 µM of each forward and reverse primer, and 0.2 µM of probe for a total of 20 µL solution mix (Table 1). Reactions were performed on the LuminUltra GeneCount® Q96 instrument under the following thermal cycling parameter, 5 min at 50 °C followed by 20 s at 95 °C for reverse transcription and 40 cycles were performed at 95 °C for 3 s followed by 30 s at 60 °C. 60 °C for annealing and amplification (30 s). All samples, standards and controls were performed in technical duplicates and the high concentration between replicates was reported as not all replicates were detected for each sample. Nuclease-free water served as a negative control in each RT-qPCR reaction. A known-positive DNA gene block template (10^3 copies μL^{-1}) functioned as a positive control (Table S1). Sample results were only considered valid if negative and positive controls passed (i.e., Cq values >38 and <37, respectively). Using the DNA gene block template positive control, a six-point ($10^1 - 10^6$ copies μL^{-1}) standard curve was generated. Through linear regression of RT-qPCR resulting cycle threshold values plotted against log copy numbers the amplification efficiency was determined to be ~92%, with an R^2 value of 0.99 and a y-intercept of 40.88.

Table 5-1. RT-qPCR oligonucleotides for SARS-CoV-2 detection, including Omicron-Lambda allele-specific primer extension RT-qPCR assay targeting the N:P13L: C28311T mutation, the SARS-CoV-2 N2 gene, Alpha (B.1.1.7) and Delta (B.1.617) variant mutations. Each oligonucleotide is provided with its specific sequence and designated working concentration in nanomolar (nM). All primers and probes were purchased through Integrated Technologies (IDT, Coralville, IA, USA) and stored based on manufacturer's recommendations.

Item	Sequence Type	Conc. (nM)	Sequence (5' – 3')
C28311T	Probe	500	CCAAAATCAGCGAAATGAACT
	RP	500	TCTGGTTACTGCCAGTTGAATCTG
	FP	125	CCGCATTACGTTTGGTGGACCC
N2	Probe	667	TTACAAACATTGGCCGCAAA
	RP	667	GCGCGACATTCCGAAGAA
	FP	167	ACAATTTGCCCCCAGCGCTTCAG
Delta (B.1.617)	Probe	200	TGGATGGAAAGTGGAGTTTATTCTAGT
	RP	500	GGCTGAGAGACATATTCAAAAGTG
	FP	500	GTTTATTACCACAAAAACAACAAAAG
Alpha (B.1.1.7)	Probe	125	ACCCCGCATTACGTTTGGTGGACC
	RP	500	TCTGGTTACTGCCAGTTGAATCTG
	FP	500	CATCTAAACGAACAAACTAAATGTCTCT

5.3.9 Quality Control

Samples were analyzed for N2 gene detections via RT-qPCR directly after sample RNA was extracted. Following RT-qPCR analysis, RNA samples were stored at -80ºC for up to two months before being retrospectively analyzed for C28311T. Extraction blanks were included during RNA extraction to assess contamination during sample processing. All extraction blanks presented no detectable levels of SARS-CoV-2 RNA. Furthermore, to minimize contamination during sample processing, RNA extractions and RT-qPCR analyses were performed in separate laboratories, each equipped with a certified biosafety cabinet. NTCs were implemented into each RT-qPCR assay, and a failed NTC (Cq value $\leq$ 38) resulted in a re-analysis of all samples in the respective run. To alleviate inhibition, each sample was diluted up to three times (1:1, 1:5 and 1:10) using a BSA solution (1 mg mL^{-1}). An additional criterion for the acceptance of SARS-CoV-2 results was the passing of the internal amplification control (MS2 bacteriophage, acquired from LuminUltra Technologies Ltd). Amplification controls were used to validate successful amplification in the RT-qPCR reaction, thereby preventing false negative results that may be caused by

inhibitory compounds. However, under conditions where the IAC failed, samples that were non-detect for SARS-CoV-2 were re-analyzed by RT-qPCR with RNA dilutions. No contamination was observed during the study period at any of the sampling locations.

Standards outlined in Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines[53] and Environmental Microbiology Minimum Information (EMMI) guidelines[54] were referenced for evaluating RT-qPCR-based tests (Table S2). Parra-Guardado et al. (2021) reported the experimentally determined method limit of detection (MLOD) of the N2 RNA extraction protocol for heat-inactivated SARS-CoV-2 spiked in wastewater as 5×10^{1} gene copies (GC) mL^{-1} and observed N2 RNA concentrations from field samples as low as 1.7 Gc mL^{-1}.[46] The SARS-CoV-2 RNA concentration of 1.7 GC mL^{-1} observed by Parra-Guardado et al. (2021) was selected as the MLOD for all WWTF composite wastewater samples in this study.[46]The recovery efficiency of the wastewater processing and RNA extraction protocols were determined to be ~87% by Parra-Guardado et al. (2021). For wastewater collected via passive samplers, a SARS-CoV-2 RNA concentration of 89 GC mL^{-1}, as experimentally determined by Hayes et al. (2022), [21] was designated as the MLOD for this study. The RNA concentrations below these MLODs were considered non-detects. A process control to account for varying RNA extraction efficiencies was not utilized in this study, as there are no established process controls known which exhibit similar adsorption characteristics to SARS-CoV-2 for passive sampling experiments using electronegative filters. As well, considering the small catchment areas of the university residence sampling sites in this study, the use of a fecal indicator was not implemented. Small catchment areas experience significant daily fluctuations, making normalization of SARS-CoV-2 signals using fecal indicators (e.g., PMMoV) ineffective in mitigating viral signal variability.[55,56] These indicators exhibit location-specific variability and exhibit fluctuations due to the influence of individual dietary patterns. However, fecal indicators can still be useful for checking sample integrity and extraction performance, helping to identify potential outliers.

5.3.10 Statistical Analysis and Data Reporting

All composite wastewater samples collected from the WWTF were analyzed for SARS-CoV-2 N2 and C28311T RNA in two biological replicates, while samples collected via passive sampling were processed as single aliquots. Two technical replicates were

analyzed for each extracted RNA sample. The mean target RNA concentration from the technical duplicates was reported for all sample types analyzed. RNA concentrations obtained from the WWTF samples are reported as GC mL^{-1} of the 1-L composite wastewater sample collected and were calculated using Eq. (1) based on the methods outlined in Parra-Guardado et al. (2021).[46] For samples collected from passive samplers, RNA concentrations are reported as GC mL^{-1} of the 6-mL sampler eluate and were calculated using Eq. (2)[30]. Plots were generated using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California US) and RStudio (version 4.2.3), utilizing packages such as tidyverse, scale, and ggtext.[57–59] A two-sample Welch's t-test assuming unequal variances was performed to compare the slopes of the linear trends in RNA concentrations between the N2 and C28311T targets.

Eq. (1)

$$RNA\ Conc.\ (GC\ mL^{-1}) \approx \frac{Reaction\ concentration\ \left(\frac{GC}{\mu L}\right) \times 50\ \mu L (Extraction\ vol.)}{50\ mL\ (original\ sample\ vol.)}$$

Eq. (2)

$$RNA\ Conc.\ (GC\ mL^{-1}) \approx \frac{Reaction\ concentration\ \left(\frac{GC}{\mu L}\right) \times 50\ \mu L (Extraction\ vol.)}{6\ mL\ (original\ sample\ vol.)}$$

5.4 Results and Discussion

5.4.1 University Action Plan for Campus Wastewater Monitoring Program

During the Fall 2021 academic semester, the University implemented a multifaceted public health strategy to ensure campus safety, which included on-campus vaccination, a comprehensive WWS program, and an extensive asymptomatic rapid antigen testing initiative.[60,61] Tailored to university settings, this strategy aimed to mitigate the spread of COVID-19 on campus. As part of this strategy, between 01 September and 31 December 2021, 272 passive wastewater samples from four campus residences, alongside 53 composite samples from the downstream WWTF, were analyzed. Notably,

SARS-CoV-2 RNA (N2 gene) was detected in 51 samples from the university residences and 20 WWTF samples (Table 5-2).

Table 5-2. Summary of passive and composite wastewater samples collected from 01 September 2021 to 31 December 2021, at the university residences and the WTTF, along with the count of SARS-CoV-2 N2 RNA detections observed at each sampling site.

Month	Residence A	Residence B	Residence C	Residence D	Total	WWTF
Sept	0/12	0/6	0/10	0/10	0/38	0/13
Oct	1/16	6/15	0/16	1/16	8/63	7/13
Nov	11/24	1/25	0/25	0/25	12/99	5/13
Dec	11/20	15/19	5/18	0/15	31/72	8/14
Total	23/72	22/65	5/69	1/66	51/272	20/53

Figure 5-1 demonstrates the consecutive detections in passive wastewater samples collected from sewershed locations outside of the university Residence A and Residence B, marked by red and blue dotted lines, respectively. The sudden increase of detections at Residence A and B during October and November 2021 coincides with the return of students post-summer break, while a following increase in December points to an extended phase of transmission within the university dormitory community, likely exacerbated by increased indoor social interactions due to colder weather. The detection clusters of the N2 gene in November and December of 2021 played a crucial role in activating the university's rapid response protocols. The initial detections at Residence A and Residence B prompted immediate, campus-wide testing efforts, leading to the identification of six COVID-19 cases.[62] This underscores the effectiveness of WWS in supporting public health actions by providing early warnings of potential outbreaks.

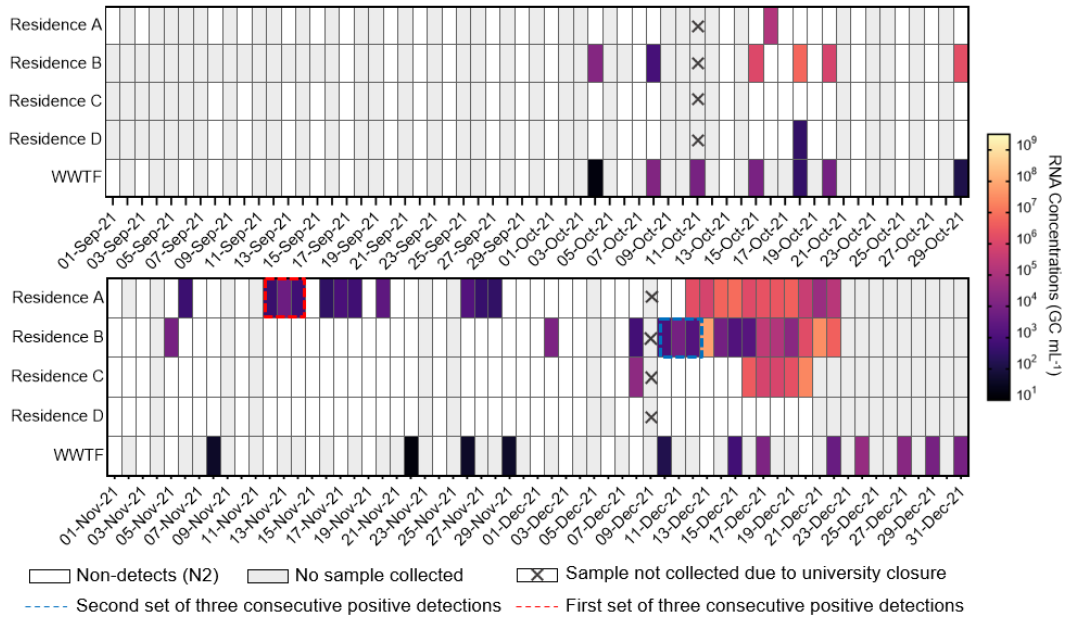

Figure 5-1. Time series of SARS-CoV-2 (N2 Gene) RNA concentrations in wastewater from Dalhousie University residences and the local WWTF (01 September – 22 December 2021). Red and blue dotted lines highlight consecutive detections at Residence A and Residence B, respectively. Concentrations are shown in $\log_{10}$-scale; MLODs are 89 GU mL^{-1} for passive samples and 1.7 GU mL^{-1} for composite samples.

In response to the increased viral load detected in the residence wastewater samples, where three or more consecutive viral detections were observed in November and December 2021, the university launched a targeted communication and testing strategy. The University reported to the communities of each residence, targeting all student residents and support staff, including those in residence life, operations, custodial, security, and food services. Rapid antigen test kits were made widely available in residence lobbies and other strategic high-traffic areas on campus, with support personnel providing guidance and clear instructions for follow-up actions. These efforts were instrumental in identifying an additional 23 COVID-19 cases by 17 December 2021,[63] highlighting the importance of integrated surveillance and response strategies in managing potential outbreaks.

Throughout the semester, the campus completed 2,025 in-person COVID-19 tests and distributed approximately 108,929 self-tests,[64] alongside the collection of 273 passive wastewater samples from sewersheds directly outside the four university residence buildings. This integrated approach, featuring WWS as a complementary strategy to other COVID-19 mitigation strategies, mirrors the strategies employed by numerous institutions

worldwide.[34,40,65–67] Other studies, such as those by Betancourt et al. (2021) and Gibas et al. (2021), emphasize the critical role of sewer sampling within university residences in identifying clusters for targeted testing and facilitating early isolation to prevent outbreaks.[40,65] However, it is important to acknowledge that the efficacy of WWS cannot be merely evaluated by the number of detected cases or the speed of outbreak detection. Various factors, including campus density, the proportion of remote versus in-person participation, and adherence to public health guidelines, may significantly influence the overall effectiveness of any strategy. At the University of Notre Dame, the introduction of tampon-based passive samplers for building-level WWS underscored the complexity of such interventions. The study highlighted limitations including methodological performance variability across different wastewater sources and the potential for non-resident RNA shedding, complicating data interpretation.[17] Likewise, researchers at the University of Calgary, reported that the younger demographic's higher asymptomatic rates and voluntary case reporting further complicated the efficacy of WWS, emphasizing the critical role of campus dynamics and public health compliance in shaping the outcomes of these surveillance efforts.[68]

5.4.2 Retrospective Omicron Detection in Wastewater by Allele-Specific RT-qPCR

Following the initial detection of Omicron cases in Nova Scotia on December 13, 2021,[69] we conducted a focused retrospective analysis on 71 wastewater samples that had previously tested positive for the SARS-CoV-2 N2 gene in attempts to detect the Omicron C28311T mutation using an allele-specific RT-qPCR assay. The singleplex single nucleotide variant RT-qPCR assay is sensitive to a single nucleotide variation in the N gene, indicative of the N: P13L non-synonymous amino acid change, a mutation characteristic of the SARS-CoV-2 Omicron variant.[70] This retrospective analysis confirmed the presence of the C28311T mutation in 46/71 wastewater samples collected from university Residences A and B and WWTF locations during the study sampling period (Figure 5-2). Mean RNA concentrations measured were approximately 1.1×10^7 GC mL^{-1} for Residence A, ~3.0×10^6 GC mL^{-1} for Residence B, and 1.3×10^6 GC mL^{-1} for the WWTF. The frequent detection of the C28311T mutation in these wastewater samples indicates that there was possibly a substantial presence of the Omicron variant across the sampled locations. This is supported by research that has demonstrated wastewater to

effectively capture the emergence and dominance of the Omicron variant, aligning with epidemiological projections.[71]

Importantly, all 142 N2-gene negative samples, collected from 01 September and 31 December 2021, lacked the C28311T mutation, suggesting that the absence of the mutation is consistent with the negative N2 gene results and the absence of this mutation circulating during this period.. Additional RT-qPCR analysis for other VOCs using identified no Alpha (D3L mutation) or Delta (Δ157-158 mutation) variants in Omicron-positive samples from the university residences. In contrast, 5/20 of the N2-positive samples from the WWTF showed the Δ157-158 mutation, highlighting variant diversity in the broader community.

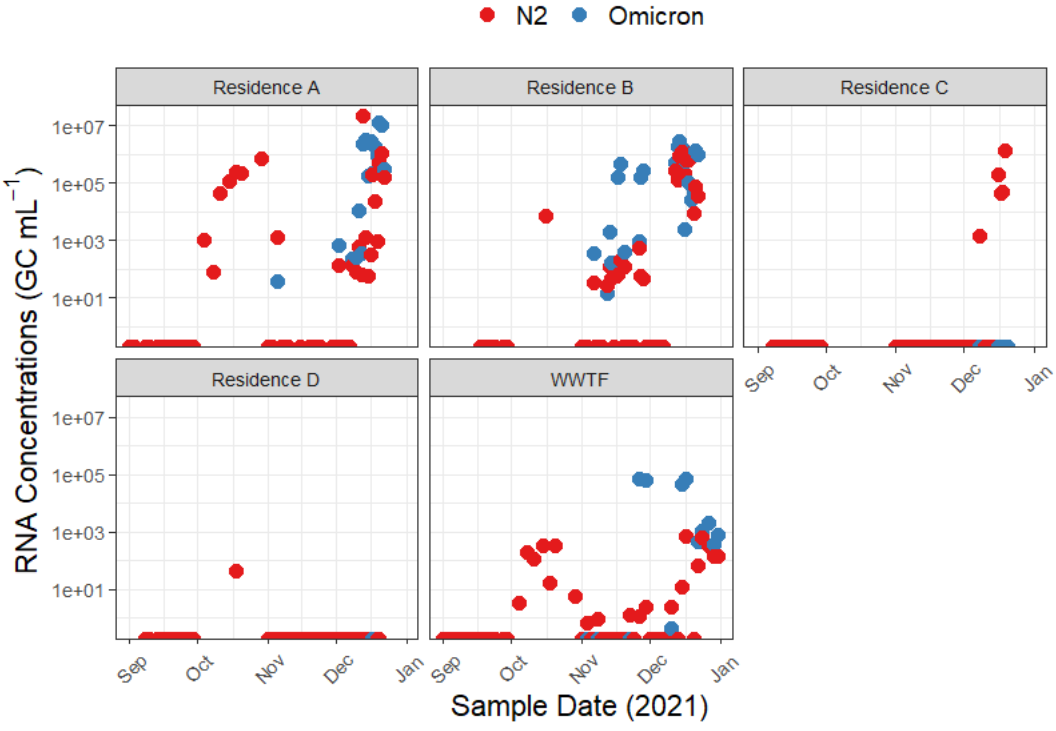

Figure 5-2. Time series of SARS-CoV-2 N2 (red) and C28311T (blue) gene copies in wastewater from September 2021 to January 2022. Passive samples from four university residences and composite samples from a WWTF are displayed. Sampling dates are shown on the x-axis, while the y-axis presents the average concentration in GC mL^{-1}.

Figure 5-2 illustrates the temporal distribution of SARS-CoV-2 N2 RNA and C28311T RNA detections in passive wastewater samples from four university residences and the influent stream of a downstream WWTF, spanning from 01 September 2021 to 31 December 2021. The initial detections of the C28311T mutation in wastewater predated

the first clinically confirmed Omicron cases in the region on 13 December 2021,[72] with early C28311T detections emerging from Residence A on 5 November 2021 (average RNA concentration ~3.6×10^{1} GC mL^{-1}), and then at Residence B the following day, 6 November 2021 (average RNA concentration ~4.9×10^{2} GC mL^{-1}). The first C28311T detection at the WWTF was not observed until 26 November 2021 (RNA concentration ~7.0×10^{4} GC mL^{-1}). Throughout the study, periods of non-detects followed by clusters of SARS-CoV-2 RNA detections were observed at the WWTF for both C28311T and N2 target genes. As well, the variance between SARS-CoV-2 N2 and C28311T RNA concentrations at the university residences compared to the WWTF highlights the influence of different sampling scales and methods. In this study, composite wastewater samples from the WWTF generally showed more gradual fluctuations in viral concentrations over time compared to the university residences. However, the university residences exhibited significantly higher RNA concentrations for more N2 and C28311T. For instance, the WWTF samples displayed a maximum C28311T RNA concentration of ~9.1×10^{4} GC mL^{-1} on 15 December 2021, with more consistent and gradual changes observed throughout the sampling period. In contrast, Residence A showed a surge in C28311T RNA concentrations with peaks reaching up to ~2.1×10^{7} GC mL^{-1} on 20 December 2021, while Residence B displayed a peak RNA concentration of ~3.0×10^{7} GC mL^{-1} on 14 December 2021. This pattern can be attributed to greater dilution effects and population heterogeneity often noted at WWTF compared to smaller catchment sampling.[73] Whereas, the passive samplers deployed at the university residences adeptly capture transient surges in viral load, which may be indicative of acute localized outbreaks that may be missed when sampling at WWTFs.[74,75] However, the variations in RNA concentrations across sampling sites may also reflect differences in population density, infection rates, or sampling methodologies. Nonetheless, the contrast in detections between sampling locations underscores the importance of sampling resolution in WWS, suggesting that passive sampling at a granular level may be critical for early outbreak detection and targeted public health responses.

The peak C28311T RNA concentration at Residence A, on 21 December 2021, suggests the variants escalating presence on campus since its initial detection in early November 2021. This trend suggests not only the rapid spread of the variant but also the

possibility of its sustained presence on campus since early November 2021. However, the marked increase in RNA levels in December 2021 may reflect a more cumulative effect of widespread exposure among residents, compounded by the prolonged shedding of viral RNA in wastewater after infection, resulting in persistently high viral loads in the residence's wastewater.[76] The Omicron variant has been characterized to have a lower minimal infective dose compared to the wild-type SARS-CoV-2 leading to potentially more rapid transmission and widespread infection.[77] Champredon et al. (2024) observed that the Omicron variant's spread in Canada took less than a month to become dominant, compared to the three to four months required for the Delta variant to become the prevalent strain.[78]

The temporal distribution of SARS-CoV-2 detections offers valuable insights into the potential epidemiological trends on campus and the surrounding community during the sampling period. The episodic fluctuations in viral RNA concentrations at the university residences align with potential individual cases or more widespread outbreaks on campus. For example, Residences A and B observed an increase in N2 gene detections in October 2021, possibly coinciding with the influx of the student population returning to campus in September 2021. However, during this period, detections of the C28311T allele were absent in all wastewater samples, suggesting the predominance of other SARS-CoV-2 strains at this time. Conversely, Residence C and D presented the fewest number of N2 detections and no C28311T detections, possibly due to effective containment measures or low residence occupancy. protocols. The absence of detections at Residences C and D may be attributed to differences in student population infection rates among the residents, and varying levels of adherence to health protocols. While the number of students was unknown in each residence during the time of study, the university's COVID-19 protocols at the time limited guests in the residences.

While the C28311T RNA concentrations at the WWTF began to rise sharply in late November 2021 into December 2021, the N2 RNA concentrations showed a more gradual increase during this period. Statistical analysis comparing the slopes of the linear trends for RNA concentrations ($p = 0.00021$) confirmed that the increase in C28311T RNA concentrations was significantly faster than that of N2 RNA concentrations during this

sampling period. Discrepancies between the C28311T detections and N2 detections may be a reflection of the rapid emergence and spread of the C28311T mutation, or the technical nuances between these RT-qPCR assays impacting their sensitivity. For instance, the N2 assays sensitivity may have been impacted by the multiplexing of the assay with an additional target for internal amplification control.[79] We also noted that inhibitors inherent to wastewater impacted the assays differently, where the N2 assay often required template RNA to be diluted to 1:10 during RT-qPCR reactions to alleviate inhibition, whereas the C28311T assay required no dilution or only a 1:1 dilution. Additionally, there is potential for the N2 assay to reflect an N gene dropout effect, where additional mutations may lead to decreased sensitivity or failure of the assay.[80] While there are no specific reports of the C28311T mutation causing an N2 dropout, the high mutation rate in the N gene and the documented impact of other mutations suggest that it may be a plausible concern. These factors highlight the importance of using a multi-target RT-qPCR approach to ensure comprehensive detection of evolving variants and to mitigate the risk of false negatives. A limitation of this study was that additional SARS-CoV-2 targets (e.g., N1 gene) were not evaluated, which could provide additional insights into the detections observed in this work. Furthermore, the absence of public clinical data for the catchment area limited a more granular analysis of the fluctuating viral signals observed in this study. However, the utility of integrating WWS data with broader epidemiological information is evident. Such integration would be vital for crafting targeted interventions in the future, specifically during the academic year's beginning and end, when population densities and social behaviours may change.

5.4.3 Significance of C28311T Detection and other VOC-associated Mutations in Wastewater

The findings from this WWS program indicate the presumptive presence of the Omicron variant, based on the detection of the C28311T mutation in Nova Scotia as early as 5 November 2021. This detection notably precedes the initial identification of the Omicron variant in South Africa on 24 November 2021.[2,3] However, it is important to recognize that the C28311T mutation targeted in this study is also associated with the SARS-CoV-2 Lambda variant. Although, the presence of the Lambda variant was low in Canada (<0.01%, 32/239,025 GISAID-deposited sequences as of 12 January 2022) during

the study period,[81] the possibility of residual circulation of Lambda or other non-named variants that share this mutation cannot be entirely excluded. Therefore, without confirmatory sequencing data the C28311T mutations observed in this work should be considered presumptive for the presence of the Omicron variant.

The absence of the Alpha and Delta variants in the university residence wastewater aligns with observations by Lee et al. (2021), who identified the predominance of a singular SARS-CoV-2 strain within an institutional context during their study period.[35] Similarly, research conducted at the University of Cambridge, UK, demonstrated that the vast majority of SARS-CoV-2 genomes sequenced from student samples were part of a single genetic lineage. [82] This was attributed to cases arising from a singular event, such as a social gathering off-campus, indicating a constrained entry of the virus into the campus community.

Retrospective wastewater sample analyses have been instrumental in identifying the presence of VOCs before confirmation by clinical epidemiological testing. For example, Joshi et al. (2021) identified Delta variant mutations in untreated wastewater samples more than a month before variant's clinical emergence in the region.[83] Similarly, early indications of the Omicron variant were detected in community wastewater samples across multiple U.S states, with the earliest detection on November 21, 2021, predating the first clinically reported Omicron case on December 1, 2021, by the CDC.[84] As well, a study by Novoa et al., (2022) revealed a significant correlation between the predominant variants identified in wastewater samples and the individuals testing positive clinically for those variants within the same geographic region.[85] These examples, along with our observations, substantiate the utility of WWS for retrospective identification of variants, offering a critical window for timely public health interventions.

5.4.4 Limitations and Interpretation

While RT-qPCR is recognized for its precision in detecting SARS-CoV-2, serving as a cornerstone for clinical diagnostics,[86,87] this study extends its application to the surveillance of SARS-CoV-2 in wastewater, specifically for targeting the Omicron mutation N: P13L; C28311T. The development of RT-qPCR assays for identifying mutations indicative of VOCs in wastewater represents a significant advancement in viral surveillance.[15,51,88–92] Despite its demonstrated effectiveness, it is important to

acknowledge the inherent limitations of RT-qPCR methods. For example, RT-qPCR assays are limited to predefined genetic targets, which may miss novel mutations not incorporated in the assay. As well, RT-qPCR's sensitivity to primer and probe designs can also result in missed detections due to minor genetic variations.[93] Although RT-qPCR is valuable for estimating viral loads, it does not offer the complete genomic context that sequencing does, limiting the depth of insight into the viral genome's evolution and interaction of mutations.

Importantly, the effectiveness of allele-specific RT-qPCR for detecting SARS-CoV-2 VOCs in wastewater relies on the validation before implementation to establish the assay's sensitivity and specificity.[94] Equally critical is the adoption of robust quality assurance and quality control (QA-QC) measures to uphold the RT-qPCR results reliability.[53] Our study ensured the reliability and accuracy of the allele-specific RT-qPCR assay for SARS-CoV-2 detection in wastewater by strictly adhering to QA-QC practices, including the use of extraction blanks, conducting sample analyses in dedicated laboratory spaces, internal amplification controls, positive-template control validation, and routine no-template controls in RT-qPCR runs.

The sensitivity and cross-reactivity of the C28311T allele specific RT-qPCR primers used in this study have validated in previous research, showing negligible cross-reactivity and providing high confidence in estimating the frequency of the C28311T mutation, which was shown to be the dominant circulating Omicron lineage in 2021 and 2022.[70] The C28311T mutations was present in over 97% of the B.1.1.529 (Omicron; BA.1 and BA.2) and C.37 (Lambda) GISAID-deposited sequences (as of 12 January 2022; outbreak.info), while it is found in less than 0.5% of other sequences. The cross-reactivity of the allele specific RT-qPCR primers was further tested against RNA templates containing non-Omicron/Lambda sequences (i.e., ancestral/wild type, Delta, and Alpha variant sequences). The C28311T assay performed well across a linear range using Omicron/Lambda RNA templates, but was unable to amplify until over 1000 gene copies when using non-Omicron/Lambda template RNA.

While genomic sequencing remains vital for confirming the presence and identity of VOCs, its application in routine WWS is hindered by several practical challenges. These include high operational costs, the necessity for specialized technical expertise, and extended processing times.[88,89] Moreover, the complex nature of wastewater samples,

characterized by their propensity for RNA degradation and dilution, poses additional complexities for adequate genomic sequencing methods.[95] Given these constraints, allele-specific RT-qPCR analysis emerges as a viable alternative for ongoing monitoring of VOC's in wastewater. Although RT-qPCR may not provide the comprehensive genomic insights provided by sequencing, when properly validated, its sensitivity, speed, and cost-effectiveness compared to sequencing make it a valuable tool for timely viral detection.

5.5 Conclusions

The utility of WWS in university residences, as demonstrated in this study, highlights its practicality as an approach to offer actionable insights for campus health management. The success of the WWS strategy employed was notably enhanced through passive sampling techniques, recognized for their low maintenance, cost-effectiveness, and extended monitoring capabilities, which played a pivotal role in the success of this surveillance initiative. This approach enables the rapid acquisition of test results, facilitating swift interventions to mitigate the spread of COVID-19 infections on campus. Additionally, this study revealed the emergence of the C28311T mutation, associated with the Omicron variant, through retrospective examination of samples. This analysis revealed the temporal and spatial variability of the C28311T mutation across both the university and broader community's wastewater throughout the study period. The findings of this study emphasize the value of allele-specific RT-qPCR assays in deepening our understanding of the evolutionary dynamics of variants as detected through WWS. This illustrates the program's capability to provide a better understanding of COVID-19 dynamics and assist in the implementation of targeted remedial actions.

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CHAPTER 6 ADSORPTION OF SARS-COV-2 ONTO GRANULAR ACTIVATED CARBON (GAC) IN WASTEWATER: IMPLICATIONS FOR IMPROVEMENTS IN PASSIVE SAMPLING

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E.K.H. designed and carried out experiments, analyzed data, prepared figures, and also wrote and reviewed the paper.

6.1 Abstract

Based on recent studies, passive sampling is a promising method for detecting SARS-CoV-2 in wastewater surveillance (WWS) applications. Passive sampling has many advantages over conventional sampling approaches. However, the potential benefits of passive sampling are also coupled with apparent limitations. We established a passive sampling technique for detecting SARS-CoV-2 in wastewater using electronegative filters. However, it was evident that the adsorption capacity of the filters constrained their use. This work intends to demonstrate an optimized passive sampling technique for SARS-CoV-2 in wastewater using granular activated carbon (GAC). Through bench-scale batch-adsorption studies and sewershed deployments, we established the adsorption characteristics of SARS-CoV-2 and two human feacal viruses (PMMoV and CrAssphage) onto GAC. A pseudo-second-order model best-described adsorption kinetics for SARS-CoV-2 in DI water and SARS-CoV-2, CrAssphage, and PMMoV in wastewater. In both laboratory batch-adsorption experiments and in-situ sewershed deployments, the maximum amount of SARS-CoV-2 adsorbed by GAC occurred at ~60 hrs in wastewater. In wastewater, the maximum adsorption of PMMoV and CrAssphage by GAC occurred at ~60 hrs. In contrast, the adsorption capacity was reached in DI water seeded with SARS-CoV-2 after ~35 hrs. The equilibrium assay modelled the maximum adsorption quantity (q_{max}) in wastewater with spiked SARS-CoV-2 concentrations using a Hybrid Langmuir-Freundlich equation, a q_{max} of 2.5×10^{9} GU/g was calculated. In paired sewershed deployments, it was found that GAC adsorbs SARS-CoV-2 in wastewater more effectively

than electronegative filters. Based on the anticipated viral loading in wastewater, bi-weekly sampling intervals with deployments up to ~96 hrs are highly feasible without reaching adsorption capacity with GAC. GAC offers improved sensitivity and reproducibility to capture SARS-CoV-2 RNA in wastewater, promoting a scalable and convenient alternative for capturing viral pathogens in wastewater.

6.2 Introduction

Effective monitoring strategies and early detection of SARS-CoV-2, the virus that causes COVID-19, play critical roles in reducing transmission and mitigating outbreaks. Wastewater surveillance (WWS) has emerged as a complementary approach to clinical surveillance for identifying viral infections within communities. SARS-CoV-2 RNA has been found in sewage samples before increases in reported clinical cases, suggesting that WWS might give an early warning of viral prevalence when combined with clinical cases [1–5]. Selecting the optimal sampling approach is critical for reliable viral detection in WWS, as highlighted by Bivins et al. (2022) [6]. Conventional wastewater sampling methods include periodic grab samples and composite sampling, which present significant limitations and challenges for targeted wastewater sampling at community- or building-level sewersheds. For example, composite sampling will provide a representative sample over time but is often expensive, and frequently results in dispersion and dilution of the viral target at low concentrations [7]. While grab sampling offers simplicity and practicality, it offers only a snapshot of viral presence in the wastewater. Accordingly, passive sampling for WWS has become increasingly prevalent due to its ease of use, cost-effectiveness, and ability to concentrate viral targets over time [8–13]. Since the onset of the pandemic, hundreds of peer-reviewed articles have been published on SARS-CoV-2 detection in wastewater [14]. However, few articles have applied and investigated passive sampling techniques [6]. Most current publications for passive sampling of SARS-CoV-2 RNA in wastewater utilize single-material adsorbents, such as cotton gauze, cheesecloth, tampons, cellulose sponges, and electronegative filters or other synthetic polymer membranes [9,11,15,16]. Electronegative filters have demonstrated ample uptake and detection of SARS-CoV-2 RNA in both bench-scale and field experiments. However, continued development of optimized sampling techniques is still required; in published work to date, viral uptake by electronegative filters did not exceed ~48 h [12,13,17] and

adsorbed SARS-CoV-2 RNA concentrations did not surpass 7×10^3 genomic units (GU) per cm^2 [17]. Despite the increased popularity of passive sampling as an alternative to conventional sampling techniques, recent publications have shown the limitations of the application of passive samplers for SARS-CoV-2 detections in wastewater [13,16,17]. One of the identified challenges for passive sampling is to increase the quantitative interpretation of this sampling approach to improve decision-making.

This work investigates granular activated carbon (GAC) as an alternative media for passive sampling to capture SARS-CoV-2 RNA in wastewater. The highly porous nature and considerable surface area of GAC has made it a promising adsorbent to selectively remove pollutants in several water and wastewater applications [18,19] and could improve viral capture adsorption capacity. Hijnen et al. (2010) observed up to 1.1-Log, and 1.3-Log to 2.7-Log removal of *Escherichia coli (E. coli), Cryptosporidium parvum, and Giardia lamblia* (oo)cysts, respectively in water treated with GAC for 12 min[20]. Similarly, Kenney et al. (2008) and Li et al. (2014) observed considerable *E. coli* removal in deionized (DI) water (7-Log after 500 min) and stormwater (2-Log after 45 min), respectively [21,22]. Camper et al. (1985) observed that GAC could readily adsorb enteric pathogens such as *Yersinia enterocolitica*, *Salmonella typhimurium*, and *enterotoxigenic E. coli* from river water [23]. Recent findings have also demonstrated that activated carbon can remove inactivated SARS-CoV-2 from RNase-free water [24]. Although previous studies have illustrated the capability of GAC to remove pathogens from numerous water streams, its use has yet to be applied to capture SARS-CoV-2 in wastewater for sampling purposes.

We hypothesize that the capability of GAC to adsorb SARS-CoV-2 and other relevant target pathogens will be more significant than the electronegative filters currently used in the passive sampling of wastewater systems. Therefore, the overall objective of this study was to understand the kinetic and equilibrium behaviour of GAC in the adsorption of viral targets such as SARS-CoV-2 to optimize our passive sampling approach and maximize its utility by using GAC. To inform passive sampler deployments, we utilized adsorption isotherms to assess the mechanisms of SARS-CoV-2 RNA adsorption onto GAC. Lastly, this work used both bench-scale and field-scale experiments

to compare the performance of GAC and electronegative filters to capture SARS-CoV-2 RNA in wastewater using passive sampling techniques.

6.3 Materials and Methods

6.3.1 Reagents

The DI water utilized in batch-adsorption experiments was produced by a Milli-Q system (Reference A+, Millipore) (resistivity of 18.2 MΩ cm and total organic carbon (TOC) concentration < 5 μg L^{-1}). Whatman® electronegative nitrocellulose membrane filters, 0.22 μm, 90-mm diameter, were sourced from Sigma-Aldrich (St. Louis, MO). A Tween®20-based elution buffer was made from 0.075% Tween®20 + 25 mM Tris HCl obtained from Sigma Aldrich (Ottawa, ON, CA). For bench-scale batch-adsorption work, samples were stirred on an orbital shaker table from Sigma-Aldrich (St. Louis, MO). Total nucleic acid extraction kits and SARS-CoV-2 RT-qPCR assay kits were acquired from LuminUltra Technologies Ltd (Fredericton, NB, CA). The pepper mild mottle virus (PMMoV) RT-qPCR assay and CrAssphage qPCR assay reagents were purchased from Integrated Technologies (IDT®, Iowa, USA). Ethanol (EtOH) was purchased from Fisher Scientific (Ottawa, ON, CA). Bovine serum albumin (BSA) was purchased from Alfa Aesar by ThermoFisher (Tewksbury, MA, US) to make a 5 mg/mL BSA solution.

6.3.2 Adsorbate and adsorbent

Bench-scale experiments were performed using heat-inactivated SARS-CoV-2; this surrogate was received from the American Type Culture Collection (Virginia, USA) at ~3.75×10^5 copies per μL [25]. For this work, we used FILTRASORB 300 (Calgon Carbon Corporation) GAC media; the physical properties are in Table S1 of the supplemental information. Prior to use, the activated carbon was washed with distilled water to remove any impurities and minimize interferences by soluble organic residues, then let to dry overnight. For sewershed deployments, a 4.5 cm by 4.5 cm, 25-μm nylon heat sealable mesh sleeve accommodated the GAC within a 3D-printed passive sampler developed by Hayes et al. (2021) [11]; Table S2 details this preparation. Scanning electron microscopy (SEM) was used to investigate the physical characterization of the GAC and nylon (Table S3).

6.3.3 Passive Sampler Adsorbent Processing

The electronegative filters used in this work's comparative field study portion follow previously described protocols by Hayes et al. (2022) [11]. The elution procedure for GAC followed that of the filters; however, when processing the GAC samples, the media was first processed by cutting the nylon mesh, releasing GAC into a 50-mL falcon tube, and then adding 6-mL of 0.075% Tween 20 + 0.25 mM Tris-HCl. The GAC and elution buffer were mixed by hand vertically up and down for ~30 seconds, and the elution buffer was added to a new tube for subsequent nucleic acid extraction, taking care not to transfer GAC.

6.3.4 Molecular Methods

Total nucleic acid extraction for all targets analyzed utilized a commercial magnetic bead-based extraction method obtained from LuminUltra Technologies Ltd (Fredericton, NB, CA). Samples extractions followed the manufacturer's protocol; additional method details for the nucleic acid extraction protocol can be found in the work of Parra et al. (2021) [26]. Single-plex, probe-based, one-step RT-qPCR and qPCR were performed using the GeneCount Q-96 thermocycler instrument (LuminUltra Technologies Ltd, Fredericton, NB, CA). Nuclease-free water served as non-template-controls (NTCs) in each reaction, and the median point of each standard curve was employed for each positive assay control. All samples, standards, and controls were assessed in technical duplicates, reporting the average of each duplicate. All RT-qPCR and qPCR assay characteristics, reaction cycling parameters, target primers, probe sequences, and reaction concentrations are in Table S4.

6.3.5 Batch-Adsorption Experimental Setup

In the batch adsorption experiments, 0.5 g of GAC within a 4.5 cm × 4.5 cm nylon bag was incubated in 100-mL adsorbate solutions (wastewater or DI water). The wastewater for bench-scale experiments was composed of 24-h composite influent samples collected from two wastewater treatment facilities (WWTFs) in Nova Scotia (NS), Canada. Initial concentrations for target analytes were measured before use in batch-adsorption experiments; additional wastewater characteristics are in Table S5. All batch-adsorption experiments were kept under vigorous stirring using an orbital shaker table

(Sigma-Aldrich, MO) at a continuous speed of 150 rpm at ~20 °C. Nucleic acid extractions and qPCR analysis were performed within 24-h of incubation.

6.3.6 Batch-Adsorption Isotherm Experiments

Adsorption isotherm data was used to evaluate the adsorption capacity of GAC; kinetic isotherms were performed in 100 mL of wastewater and DI water seeded with an initial SARS-CoV-2 concentration of 5.0 x 10^4 GU/mL. Surrogates were not available for fecal biomarkers, PMMoV, and CrAssphage; these markers' kinetic adsorption was assessed by apparent analyte background concentrations found in municipal wastewater collected from the WWTFs. Kinetic adsorption was evaluated up to 96 h, with samples analyzed at time points of 2, 4, 8, 10, 12, 24, 30, 35, 48, 56, 60, 72, and 96 h. Equilibrium adsorption isotherms were performed using 100 mL of wastewater spiked with SARS-CoV-2 to the following concentrations (GU/mL), 1×10^1, 5×10^1, 1×10^2, 2.5×10^2, 5.0×10^2, 1×10^3, 1×10^4, 5.0×10^4, 1×10^5, 2.5×10^5, 5×10^5, 1×10^6, 2.5×10^1, 5.0×10^6, with samples shaken continuously for 72 h to reach near-equilibrium conditions.

6.3.7 Batch-Adsorption Experiments, Theoretical Models

The adsorption capacity of GAC was evaluated by considering the amount of analyte bound to the GAC at a presumed equilibrium, calculated according to Eq. (1):

Eq. 1

$$q_e = \frac{(C_0 - C_e)V}{m}$$

Where, where q_e (GU/g) is the concentration adsorbed by GAC; C_0 and C_e (GU/mL) are the initial and equilibrium SARS-CoV-2 concentrations at time (t), respectively; V (mL) is the volume of wastewater in each flask and m (g) is the mass of GAC in each reaction [27].

6.3.8 Adsorption Kinetics Isotherm

Kinetic models can determine the adsorption mechanism and the adsorption efficiency of an adsorbent. In this study, the adsorption data of SARS-CoV-2 by GAC in wastewater and DI water were fitted through two kinetic models, including Lagergren's pseudo-first-order (PFO) and the Ho and McKay pseudo-second-order (PSO) models [28]. The PFO and PSO kinetic models are shown in Eq.2 and 3, respectively.

Eq. 2

$$ln(q_e - q_t) = ln\, q_e - k_1 t$$

The PFO equation describes q_e as the concentration of adsorbate at equilibrium and q_t as the adsorbate amount time t, and the PFO equilibrium rate constant is shown by K_1. The PFO model assumes that the adsorption rate is proportional to the difference between adsorbate and available sites on a surface plane [27,28].

Eq. 3

$$t/q_t = \frac{q_e^2\, k_2 t}{1 + q_e t}$$

The PSO equation is described by the same q_e and q_t definitions as those used in the PFO model; however, K_2 is the rate-limiting constant in the PSO model. The constants q_e and K_2 can be revealed from the y-intercept and slope of t/q_t against t.

6.3.9 Adsorption Equilibrium Isotherms

The equilibrium condition and sorption mechanisms, surface properties, and adsorbent affinities in the adsorption process can be mathematically determined. Historically, four main adsorption isotherm models have been applied: Langmuir, Freundlich, Temkin, and Dubinin Radushkevich [27,29]. However, these two-parameter models have not accurately described adsorption interactions in more complex systems. Three or more parameter models generally fit experimental data better than two-parameter models [30]. As such, a four-parameter Hybrid Freundlich-Langmuir model was utilized in this work to describe adsorption mechanisms by GAC (Eq. 4):

Eq. 4

$$q_e = \frac{q_{max} \cdot b \cdot C}{1 + b \cdot C} + k \cdot\ C^{1/n}$$

Where q_e is the adsorbed amount of SARS-CoV-2, and C is the equilibrium concentration of SARS-CoV-2 in the liquid phase of the reaction (GU/mL), q_{max}, b, k, and n are the hybrid Langmuir–Freundlich constants.

Environmental systems generally do not follow a linear relationship regarding adsorption mechanisms [31]. Thus, the linearization of isotherm models frequently creates inherent biases in the distribution of errors in experimental data [29]. To limit this bias,

non-linear regression and error minimization techniques were employed between experimental data and the convergence criteria of predicted data. In this work, the hybrid fractional error function (HYBRID) equation was utilized to determine the minimum fraction of error at both high and low adsorbate concentrations [18] (Eq. 5).

Eq. 5

$$HYBRID = \frac{100}{n-P}\sum_{i=1}^{n}\left[\frac{(q_{e,i,meas}-q_{e,i,calc})^2}{q_{e,i,meas}}\right]$$

Where q_{meas} and q_{calc} are the quantities of adsorbate measured and calculated, respectively, constraint n is the number of data points, and p, is the number of isotherm parameters.

6.3.10 Field Deployment Procedures

Both 90-mm electronegative filters and 1-g GAC media were deployed in field-based experiments using 3D-printed passive samplers [11]. For the field studies, samplers were deployed parallel at three university residence building sewersheds (Locations A, B, and C); one sampler housed an electronegative filter, and the other sampler contained GAC media. Samplers were deployed during two periods when COVID-19 cases were prevalent in the community [32] (Figure S3). Samples were collected from 15 December 2021 to 30 March 2022, with January 2022 being omitted from the study, as no students were housed in the residences, so no samples were collected. Samplers were collected from each sewershed at least three times each week and deployed for durations between 24 and 96 hours.

6.3.11 Quality Assurance

Nucleic acid extraction and RT-qPCR preparation were carried out in different laboratories to reduce potential contamination. A negative sample control was incorporated during incubation and nucleic acid extraction, and all controls were negative. PCR inhibition in extracted samples was assessed using template serial dilutions. If samples analyzed with dilutions had cycle quantification (Cq) values greater than two cycles from the reference control, the sample result was considered to have been affected by inhibition [33]. All samples presumed to be affected by inhibition were re-run with a minimum of two dilutions at 1:1 and 1:5 ratios of the target template and DI water. For

assessing qPCR-based assays, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [34] and the Environmental Microbiology Minimum Information (EMMI) guidelines were used [35].

6.3.12 Data Analysis

For SARS-CoV-2, the assay limit of detection (ALOD) was approximately five gene copies, with 95% confidence in detection. The method limit of detection (MLOD) for this work with 95% confidence was ~25 GU/mL (Figure S4). Samples were considered positive for target analytes if there was amplification in at least one replicate within 45 cycles. Quantification was considered if amplification was observed in all replicates, with replicate concentrations above the ALOD. A Welch two-sample t-test (two-tailed, = 0.05; 95% confidence level) was used to assess the statistical significance of the mean values in both laboratory and field experiments [34,35]. Statistical analysis was performed using Microsoft Excel for Microsoft version 2109 (2021), and graphs were generated using GraphPad Prism v.4 for Windows, San Diego, CA.

6.4 Results and Discussion

6.4.1 SARS-CoV-2 Adsorption Kinetics

The relationship between time and SARS-CoV-2 adsorption by GAC in wastewater and DI water is shown in Figure 6-1. Experimental datasets were fit to non-linear regression analysis and plotted against the calculated 95% confidence and prediction limits. All data points fell within the prediction limits, and most points were within or near calculated confidence intervals. A maximum SARS-CoV-2 RNA concentration of 9.2×10^6 GU/g was recovered from the wastewater matrices after 60 h, ~92% recovery from the initially spiked concentration. Three main events can describe the adsorption kinetics of SARS-CoV-2 by GAC in wastewater over a 96-h incubation period.

- A relatively fast adsorption rate was observed within eight hours of exposure; this may be due to physical solid adsorption interactions between adsorbent and adsorbate [36]. Often, the adsorbent has more surface area available at the start of a reaction; thus, a more significant concentration gradient between the adsorbate in the aqueous and solid phase often occurs [36–38].

- A slower but steady rise in adsorption is observed with increased time, with only 55% of the initial SARS-CoV-2 concentration adsorbed after 24 h. Enhanced adsorption over time has been described as a result of the increased kinetic energy of the adsorbate with increased agitation time [39,40].
- As time progressed, adsorption kinetics gradually plateaued, and, finally, the adsorption capacity began to approach equilibrium at ~60 h. As systems reach equilibrium, mass transfer of the adsorbate to the solid phase in the solution becomes increasingly limited and leads to less absorbance [41].

Conversely to the adsorptive behaviour observed in wastewater, equilibrium was reached faster in DI water. It took about 35 h to reach a maximum SARS-CoV-2 RNA concentration of 9.8×10^6 GU/g GAC. Across all time points, the mean SARS-CoV-2 RNA concentrations adsorbed by GAC in DI water were significantly less than concentrations observed in wastewater ($p = 0.0015$). The difference in adsorption kinetics between wastewater and DI water is consistent with previous work; it was found by Hayes et al. (2022) that SARS-CoV-2 RNA concentrations adsorbed from electronegative filters in DI water were an order of magnitude lower compared to concentrations adsorbed in wastewater [17]. The relationship between SARS-CoV-2 adsorption and the solid fraction of wastewater is well established, and considerable portions of enveloped viruses such as coronaviruses may readily adsorb to solids and organic matter within water samples [4,42–44]. Wastewater contains many different organic compounds, suspended solids, and colloids that may compete for adsorption sites, interfering with the uptake kinetics of the virus. Thus, characterizing adsorption kinetics using viral surrogates can help inform the selection of deployment durations and translate counts from passive samplers into quantitative or semi-quantitative data. While GAC can be deployed, and effectively concentrate SARS-CoV-2 RNA for prolonged periods, shorter sampling periods would also be feasible and useful as early warning detection methods.

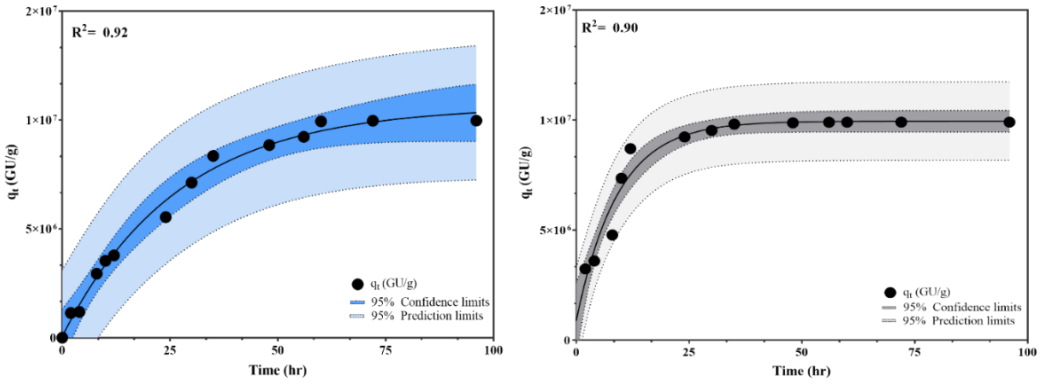

Figure 6-1 The adsorption of spiked SARS-CoV-2 by GAC over a 96-h duration in wastewater (Left) and DI water (Right). Data is shown on a linear scale for either plot, the 95% confidence and prediction limits are shown by dark and light-shaded bars, solid black circles show experimental data, and the R^2 values are shown in the top left.

6.4.2 Kinetic Adsorption for Two SARS-CoV-2 Biomarkers

Bench-scale kinetic batch-adsorption experiments investigated the adsorption of two human feacal indicators (PMMoV and CrAssphage) commonly used for normalizing SARS-CoV-2 RNA concentration in municipal wastewater. The adsorption of PMMoV and CrAssphage by GAC followed similar adsorption trends over time to that observed for SARS-CoV-2 in wastewater; both targets approach adsorption capacity at ~60 h (Figure 6-2). When compared, the experimental data for SARS-CoV-2 in wastewater (Figure 6-1) shows a difference in its relative rate of change over 96 h, however, all targets reach an adsorptive plateau between 50 h and 60 h. Adsorbed PMMoV RNA concentrations did not exceed 1×10^5 GU/g, whereas the maximum measured CrAssphage DNA concentration was 2×10^7 GU/g. High concentrations of CrAssphage DNA have been frequently observed in wastewater due to the increased fecal shedding associated with the bacteriophage [45]. In contrast, considerable variability in PMMoV RNA concentrations in wastewater has been noted because of the dietary and seasonal fluctuations in the virus's infectivity [46].

Due to the fluctuating dynamic of a sewershed, resulting from precipitation, shifting waste streams, and other unpredictable human activities, the quantity of feacal matter contributed to the sewer may change over time and may influence viral loads when sampling [14]. As a result, biomarkers, like, PMMoV and CrAssphage, have been widely used to estimate faecal contribution by a given population [47,48]. However, the lack of consensus on reliable population estimating methods remains an ongoing challenge to

expanding the use of WWS. Only two other studies have investigated PMMoV for normalization of SARS-CoV-2 when utilizing passive sampling [12,13], both of which reported in-situ accumulation of PMMoV versus SARS-CoV-2 over time. The study presented here is the first to observe laboratory-controlled adsorption of PMMoV and CrAssphage over time for passive sampling in wastewater. Based on these results, using either PMMoV or CrAssphage to normalize SARS-CoV-2 concentrations in wastewater may be acceptable when deploying GAC passive samplers. However, a comparison between recoveries of the fecal indicators and SARS-CoV-2 under varying sample compositions is required to understand whether one biomarker improves normalization more than the other.

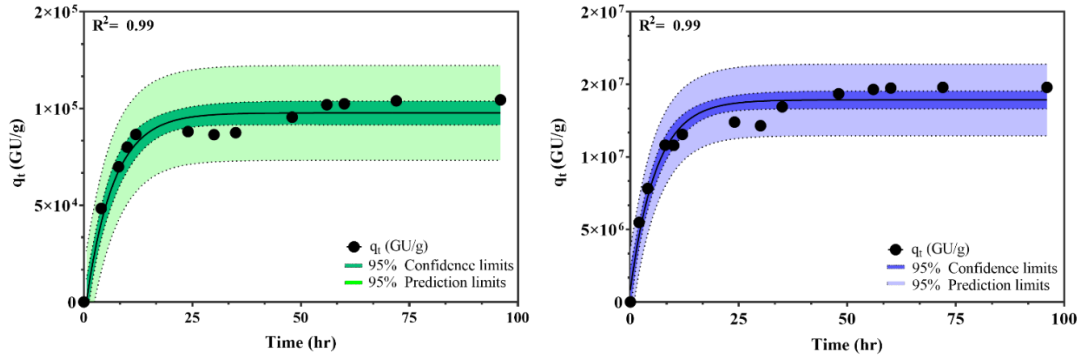

Figure 6-2 The adsorption of PMMoV (Left) and CrAssphage (Right) by GAC in wastewater over 96-h. Initial RNA and DNA concentrations measured for PMMoV and CrAssphage were 5.3×10^2 and 8.0×10^4 GU/mL, respectively. 95% confidence and prediction limits are presented by dark and light-shaded bars, respectively; solid black circles show experimental data and R^2 values for each dataset are shown on the plots.

6.4.3 Modelling of Adsorption Kinetic Processes

To investigate the mechanisms that drive adsorption processes, PFO and PSO kinetic models were used to evaluate kinetic data obtained in both DI water and wastewater (Figure S5). The PFO and PSO rate constants, $K_{1,2}$ equilibrium adsorption capacity, q_e (GU/g), and correlation coefficients (R^2) were calculated from the linear plots of the PFO and PSO kinetic models (Figure S5) and are listed in Table 6-1. The correlation coefficient for the PFO kinetic model was low in all cases, and a significant difference in equilibrium adsorption capacity (q_e) was observed between the experimental and

calculated datasets, indicating a poor PFO fit. However, the PSO model yielded an R^2 closer to 1, and the theoretical q_e values agree well with the experimental data. The kinetic rate constants ($K_{1,2}$) were higher in the case of the PFO model equations compared to the PSO-modelled data. The reaction rate is often determined by adsorbate concentrations and qualified by the difference in orders; however, the rate constant may still estimate the relative rate of a reaction [49].

Table 6-1 The PFO and the PSO adsorption rate constants ($K_{1,2}$), q_e (GU/g), and coefficient of determination (R^2).

Model	**Parameter**	**GAC Experimental Conditions and Targets**			
		SARS-CoV-2 in wastewater	**SARS-CoV-2 in DI water**	**PMMoV in wastewater**	**CrAssphage in wastewater**
PFO	K_1	0.09	0.13	0.05	0.06
	q_e (GU/g)	2.2×10^{7}	1.1×10^{7}	7.0×10^{4}	9.2×10^{6}
	R^2	0.79	0.98	0.90	0.92
PSO	K_2	3.3×10^{-9}	2.3×10^{-8}	1.6×10^{-6}	1.2×10^{-8}
	q_e (GU/g)	1.3×10^{7}	1.1×10^{7}	1.1×10^{7}	1.7×10^{7}
	R^2	0.91	0.99	0.99	0.99

For this study, the PSO model was used to explain and predict particle adsorption mechanisms by GAC. The PSO model assumes that chemical adsorption is the rate-limiting phase by which adsorption occurs through chemical bonds that tend to maximize their arrangement on the surface plane of the adsorbent [36,50]. The PSO kinetic model has been broadly applied in literature [51], with numerous studies demonstrating the applicability of the kinetic equation to fit environmental data [50]. The PSO equation is favoured as it can describe kinetic processes other than surface reaction [52], like intraparticle diffusion-driven kinetic sorption [50,50]. Thus, the applicability of the PSO equation to describe multi-level interactions makes it an ideal model for understanding viral adsorption mechanisms in wastewater.

6.4.4 SARS-CoV-2 Adsorption Equilibrium Isotherms

To assess the amount of SARS-CoV-2 taken up by GAC, the effect of initial SARS-CoV-2 RNA concentration was evaluated in batch-adsorption isotherm experiments using seeded municipal wastewater. This study fitted equilibrium data to a four-parameter Hybrid Langmuir-Freundlich model employing mathematical transformation. The

capacity of GAC to adsorb SARS-CoV-2 increased with increasing initial concentrations until, eventually, an equilibrium was reached, as shown in Figure 6-3. The average viral recovery by GAC, regardless of initial surrogate concentration, was ~95% and the maximum adsorption capacity (q_{max}) of SARS-CoV-2 by GAC was calculated to be 2.5×10^9 GU/g, based on the Langmuir portion of the Hybrid model equation.

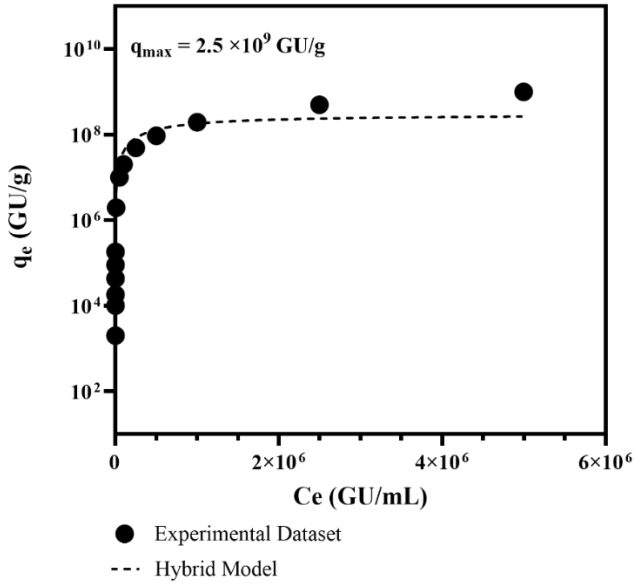

Figure 6-3 Hybrid-Langmuir-Freundlich isotherm calculated modelled data (dotted line) and corresponding environmental data collected from batch-adsorption experiments (black dots). A calculated maximum adsorption quantity (q_{max}) was 2.5 $\times10^9$ GU/g.

This hybrid isotherm is an empirical modification to reduce the error between experimental and predicted equilibrium datasets, satisfying high and low adsorbate concentration boundaries common in heterogeneous systems like wastewater [18,30]. The model efficiently reduces to a Freundlich isotherm at low sorbate concentrations and a Langmuir isotherm at high adsorbate concentrations [31]. Table 6-2 shows the best-fit parameter for the Hybrid model constants *k*, *n*, and *b*, found to be 7.4×10^{-1}, 1.2×10^{0}, and 1.6×10^{-6}, respectively. The Hybrid model constants evolve in the same manner as the conventional Freundlich and Langmuir model constants. The *k* constant denotes the adsorption capacity of the adsorbent irrespective of the model employed, and the value of *n* signifies the magnitude of adsorption intensity for a given system. The constants q_{max}

and *b* describe the maximum adsorption of the adsorbate and the related affinity between the adsorbent surface and the target adsorbate, respectively.

Table 6-2. Parameter values and correlation coefficient for the Langmuir-Freundlich Hybrid model calculated for SARS-CoV-2 adsorption by GAC in wastewater at ~25ºC.

Model	**Parameter**	**GAC in Wastewater**
L-F Hybrid model	q_{max} (GU/g)	2.5×10^{9}
	n	1.2×10^{0}
	k	7.4×10^{-1}
	b	1.6×10^{-6}
	HYBRID Error Function	6.5×10^{1}

6.4.5 Detection of SARS-CoV-2, PMMoV, and CrAssphage using GAC in Building-level Sewersheds

Due to partitioning differences, synthetic surrogates may not accurately represent the in-situ recovery of viruses from wastewater [43,53]. Therefore, we deployed passive samplers at sewersheds outside three university dormitories in Halifax, NS, over four months (December 2021 to March 2022) to evaluate the in-situ uptake over time of SARS-CoV-2, PMMoV, and CrAssphage by GAC. All samples were analyzed for SARS-CoV-2, PMMoV, and CrAssphage, and mean concentrations for each target were calculated for samples deployed for 24, 48, 72, and 96 h periods (Figure 6-4). All targets appeared to follow similar adsorption patterns, observing a slow and continuous adsorptive behaviour until approaching an equilibrium plateau between 48 to 72 h. The maximum SARS-CoV-2 RNA concentration was 1×10^{5} GU/mL after 96 h, and the maximum CrAssphage DNA and PMMoV RNA concentrations observed were 1.5×10^{4} and 4.1×10^{7} GU/mL, respectively, after 96 h. Similar to the bench-scale results, recovered CrAssphage DNA concentrations were significantly higher than SARS-CoV-2 and PMMoV ($p < 0.05$), and PMMoV RNA concentrations were consistently lower than those observed for SARS-CoV-2.

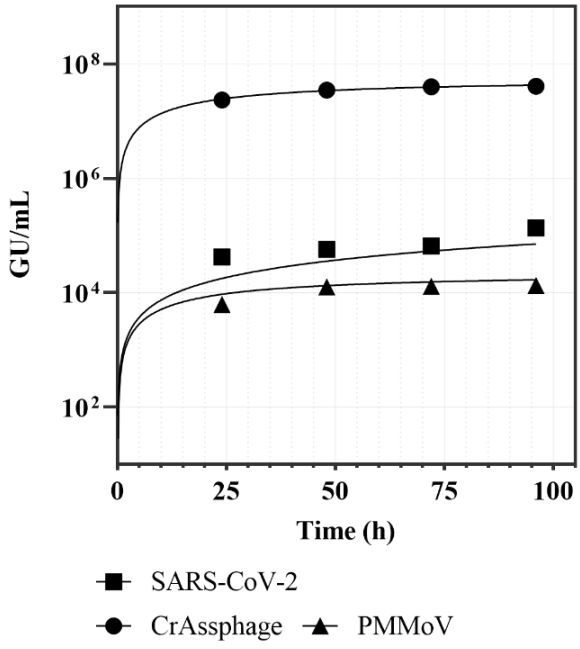

Figure 6-4 In-situ kinetic adsorption of GAC passive samples across three sewersheds, with the average GU/mL observed for SARS-CoV-2, PMMoV, and CrAssphage targets at 24, 48, 72, and 96 h deployment durations: each target analyte data was fit to a non-linear curve.

Multiple studies have used PMMoV and CrAssphage to normalize fecal sewage contribution at WWTFs [54,55]. However, few studies have measured biomarkers to normalize SARS-CoV-2 RNA concentrations collected from building-level sewersheds, likely due to variable upstream population size and dynamics not realized at WWTFs with larger contributing populations. These results indicate that when employing GAC, PMMoV and CrAssphage may be suitable fecal indicators to normalize SARS-CoV-2 concentrations in wastewater. Furthermore, these field deployments describe the capability of GAC's highly dynamic surface area to adsorb pathogens beyond SARS-CoV-2. Thus, providing a scalable method for future applications involving other relevant contaminants of concern.

6.4.6 The use of GAC Versus Filters for the Detection of SARS-CoV-2 in Building-level Sewersheds

Across 16 separate sampling events, pairs of passive samplers were deployed in parallel for 24 h, 48 h, and 96 h periods across three sewershed locations (A, B, and C). Two passive samplers were deployed at each location, one sampler containing a 90-mm

electronegative filter and the other having 1 g of GAC. Forty-eight samples were collected and analyzed for each adsorbent media. Of the filter samples, 45% had positive signals of SARS-CoV-2 RNA, while the paired GAC samples had a positive detection frequency of 85% (Figure 6-5). There were 18 instances where SARS-CoV-2 RNA was detected in the GAC samples but not in the paired filter samples, whereas no instances occurred where SARS-CoV-2 RNA was detected from the filter sample and not the paired GAC sample. The mean SARS-CoV-2 RNA concentrations for the extracted eluate of the positive paired filter and GAC samples were $2\times10^2 \pm 4\times10^2$ GU/mL and $2.5\times10^4 \pm 7.4\times10^4$ GU/mL, respectively. The values compared here reflect the GU per mL of extracted eluate since the filter and GAC surface areas are unknown and cannot be compared. Positive SARS-CoV-2 RNA concentrations observed in GAC samples were significantly greater than those detected in paired filter samples ($p < 0.0001$).

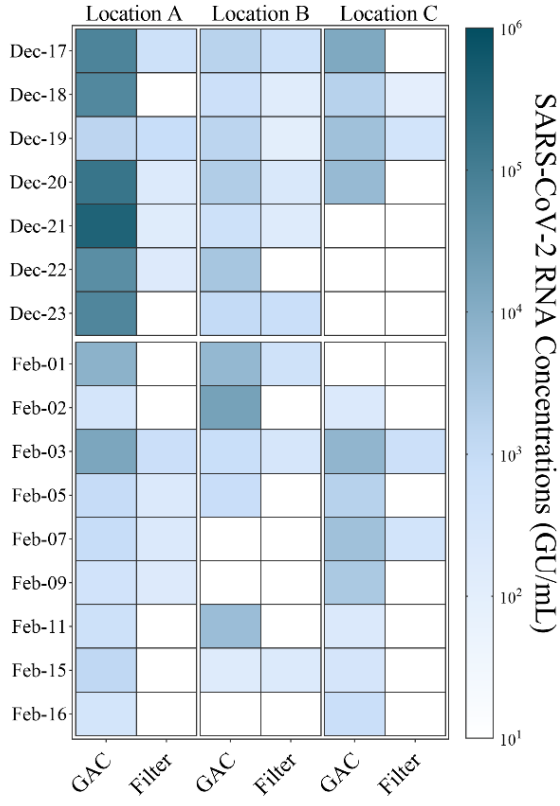

Figure 6-5 SARS-CoV-2 RNA concentrations (GU/mL) for paired passive samples collected using GAC and filters across three sewershed locations (A, B, and C) during two separate sampling periods (December 2021 and February 2022) in Halifax, NS.

The increased detection frequency and viral concentrations when using GAC demonstrate this method's sensitivity in capturing SARS-CoV-2 RNA in wastewater. The improved adsorption capacity of GAC was observed in both the experimental and field portions of this work. The use of GAC permits viral capture during extended deployment periods (~60 h) and the detection of higher viral concentrations, with a modelled q_{max} of 2.5×10^9 GU/g. In contrast, electronegative filters reach adsorption capacity around 48 h and are unlikely to adsorb concentrations greater than 7.0×10^4 GU/mL based on model calculations [17]. Discordance between adsorbents used for passive sampling of SARS-CoV-2 in wastewater is common [11,12,16]. Li et al. (2022) described that in field deployments of 24 h at upstream sewer utility holes, electronegative filters had 82% positive detections of SARS-CoV-2 RNA, whereas paired tampon samples had only 47% positive detections [13]. The authors described this misalignment as a result of a discrete SARS-CoV-2 signal in sewers and the tampon material reaching adsorption capacity faster than the filters. Schang et al. (2022) describe a higher proportion of electronegative membranes (41% and 80%) having SARS-CoV-2 RNA detections than gauzes (31% and 78%) and cotton buds (25%) [16].

In comparison, Habtewold et al. (2022) saw similar SARS-CoV-2 RNA detections between membranes (80%) and filters (78%) but at much lower detection frequencies with cotton buds (50%) [12]. Differences between adsorbent's viral recoveries may result from various factors shown in wastewater sampling, adsorbent processing, and viral detection methods. For instance, potential viral loss may occur due to saturated adsorption capacities or from untargeted adsorption of organics and suspended solids in wastewater that may cause inhibition in the downstream analysis [12,13,17]. The mechanism of adsorbate removal (i.e., mechanical, chemical, or direct extraction) and how the eluate is processed for molecular analysis may also influence overall viral recovery. In the present study, far less fouling and accumulation of suspended solids were noted with GAC samples, and as such, less inhibition was observed in downstream molecular analysis. Thus, improving the overall detection incidence and sensitivity compared to the filters that were considerably impacted by their ability to accumulate high amounts of solids.

6.4.7 Future Implications for Passive Sampling in Wastewater using GAC

Passive sampling provides a unique opportunity for WWS upstream of the WWTFs, capturing more viral signals and identifying infected populations even when community prevalence is low. However, substantial limitations currently accompany the potential advantages of passive sampling. Presently, material-based adsorbents such as synthetic membranes [11–13,16], cotton gauze [3,56,57], and tampons [9,13,46] are the main adsorbents utilized to detect SARS-CoV-2 in wastewater by passive samplers [6]. Therefore, this study is the first to apply GAC to capture and recover SARS-CoV-2 in wastewater. GAC offers a more accessible approach to WWS, as it is widely available and often more cost-effective than other sampling methods (e.g., auto sampling) and even other adsorbents (e.g., electronegative filters). Activated carbon exists in various forms, including biochar, and can originate from many biomasses, such as agricultural waste, pulp, paper products, and animal waste [58]. Accordingly, its ubiquity allows for an abundance to minimize supply chain disruptions and foster usage in underdeveloped regions looking to employ WWS initiatives. Scaling WWS methods to include low-resource settings is vital to secure a more equitable future in this field of work [59]. Most passive sampler applications have yielded semi-quantitative wastewater results for COVID-19 surveillance. The findings of this work suggest that GAC could improve the spatial resolution of WWS and scale toward quantitative data for future public health interventions.

6.5 Conclusions

We have presented an enhanced passive sampling procedure using GAC; the adsorption behaviour of GAC was shown through several laboratory-controlled batch-adsorption experiments and sewershed deployments. Adsorption kinetics revealed that GAC does not approach equilibrium until after ~60 h of deployment in wastewater. Based on batch-adsorption experiments performed using DI water showing GAC adsorption capacity was reached at ~30 h, the composition of wastewater is likely a driving force of adsorption. Further, the concentration of SARS-CoV-2 was not noted to impact viral adsorption capacity until exceptionally high viral concentrations and a modelled maximum adsorption capacity was determined to be 2.5×10^9 GU/g based on a Hybrid Langmuir-

Freundlich isotherm equation. GAC demonstrated a significant capacity to detect SARS CoV-2 relative to electronegative filters in field comparison studies.

The adsorption of SARS-CoV-2 and related biomarkers, PMMoV and CrAssphage, were abundant in both bench-scale and field-scale applications, with either target following similar adsorptive trends of SARS-CoV-2 over time. These results demonstrated that PMMoV and CrAssphage might be suitable fecal indicators to normalize SARS-CoV-2 concentrations in wastewater. Also, the highly adsorbent surface of GAC likely permits its application to a host of different contaminants. Further, paired targeted sewershed deployments of GAC and electronegative filters showed increased SARS-CoV-2 detection frequency and higher observed RNA concentrations in the samplers containing GAC.

Activated carbon is an abundant adsorbent easily obtained at a relatively low cost. When coupled with widely scalable building-level passive sampling techniques can result in a low-barrier, next-generation technology that can be used to monitor viral infection in communities. The benefits of GAC make it a widely scalable resource that has the potential to promote a more equitable solution for WWS.

6.6 References

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CHAPTER 7 SIMULTANEOUS DETECTION OF SARS-COV-2, INFLUENZA A, RESPIRATORY SYNCYTIAL VIRUS, AND MEASLES IN WASTEWATER BY MULTIPLEX RT-QPCR

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E.K.H. designed and carried out experiments, analyzed data, prepared figures and wrote paper.

7.1 Abstract

A multiplex quantitative reverse transcription polymerase chain reaction (RT-qPCR)-based method was designed for the simultaneous detection of Influenza A, SARS-CoV-2, RSV, and Measles virus. The performance of the multiplex assay was compared to four monoplex assays for relative quantification using standard quantification curves. Results showed that the multiplex assay had comparable linearity and analytical sensitivity to the monoplex assays, and the quantification parameters of both assays demonstrated minimal differences. Viral reporting recommendations for the multiplex method were estimated based on the corresponding limit of quantification (LOQ) and the limit of detection at 95% confidence interval (LOD) values for each viral target. The LOQ was determined by the lowest nominal RNA concentrations where %CV values were $\leq$ 35%. Corresponding LOD values for each viral target were ~15 and ~25 gene copies per reaction (GC/rxn), and LOQ values were within 10 to 15 GC/rxn. The detection performance of a new multiplex assay was validated in the field by collecting composite wastewater samples from a local treatment facility and passive samples from three sewer shed locations. Results indicated that the assay could accurately estimate viral loads from various sample types, with samples collected from passive samplers showing a greater range of detectable viral concentrations than composite wastewater samples. This suggests that the sensitivity of the multiplex method may be improved when paired with more sensitive sampling methods. Laboratory and field results demonstrate the robustness and sensitivity of the multiplex assay and its applicability to detect the relative abundance of four viral targets

among wastewater samples. Conventional monoplex RT-qPCR assays are suitable for diagnosing viral infections. However, multiplex analysis using wastewater provides a fast and cost-effective way to monitor viral diseases in a population or environment.

7.2 Introduction

In the collective fight against the COVID-19 pandemic, effective monitoring strategies and early virus detection are critical in reducing transmission and mitigating outbreaks [1]. Wastewater surveillance (WWS) has emerged as an alternative, complementary monitoring strategy to understand community viral loads. WWS offers a monitoring technique that provides a spatially relevant, anonymous signal for viral prevalence in communities or subpopulations independent of an individual's medical-seeking behaviour and detects viral presence in advance of clinical indicators [2]. This relatively non-invasive early detection monitoring strategy has provided advanced and localized knowledge to inform public health responses [3]. Due to its ability to assess populations virus loads in large populations from a single sample, WWS is particularly valuable in resource-constrained regions where individual-level testing is not widely available [4].

Apart from severe accurate respiratory virus type 2 (SARS-CoV-2), many respiratory viruses co-circulate each season, including Influenza types A/B (INFA and INFB) and respiratory syncytial viruses subtypes A and B (RSV-A and RSV-B) [5,6]. These viruses can cause acute upper and lower respiratory in children and adults, leading to hospitalization and death [7]; thus, timely diagnosis of viral infection is critical for appropriate patient management and public health interventions [8]. Measles virus (MeV) is another etiological agent of public health concern that often presents as a rash, with complications in unvaccinated individuals that can include encephalitis or death [9]. Although there is no sustained circulation of MeV in the United States or Canada [10], and with measles/mumps/rubella (MMR) vaccines being part of childhood immunization, infections may still occur from travellers arriving from areas where MeV is endemic, exposing vulnerable populations (e.g., under-immunized communities or individuals with waning immunity). Multiplexed viral detection in wastewater could provide simultaneous baseline monitoring for viruses of concern and may provide early detection of threats to community health.

Several quantitative polymerase chain reaction (qPCR) techniques are available to detect and identify pathogenic viruses [11–13]. However, many of these methods require time-consuming sample processing due to single-plex RT-qPCR reactions or are limited in sensitivity and specificity for complex matrices such as wastewater. For instance, the Centre for Disease Control (CDC) recently developed a multiplexed clinical RT-qPCR assay to simultaneously detect SARS-CoV-2, INFA, and INFB [14]. However, there are considerable challenges in applying clinical multiplex strategies to complex matrices such as wastewater, where common qPCR interferences are highly abundant and viral concentrations are much lower than those in clinical specimens. To address these challenges, this study aimed to (a) develop a simple and accurate multiplex RT-qPCR assay to quantify SARS-CoV-2, INFA, RSV-A, and MeV, and (b) verify the analytical and detection performance of the multiplex RT-qPCR assay based on laboratory-controlled and field testing.

7.3 Methods and Materials

7.3.1 Wastewater and Passive Sample Collection

Composite 24-hour wastewater samples were collected from the untreated influent stream within a local wastewater treatment facility (WWTF) in Halifax, Nova Scotia. The WWTF receives mainly residential and some commercial wastewater, serving a population of ~55,000. Wastewater samples (~250 mL) were sub-sampled in sterilized polypropylene bottles from a 24-h composite autosampler sampler once a week. Passive samplers were collected three times a week (Monday, Wednesday, and Friday). Passive samples were deployed and collected from maintenance hole sites within the same catchment area that serves the WWTF. The adsorbent media used for the passive samplers consisted of 3 g of granular activated carbon (GAC) housed inside a 3D-printed sampling device [15]. All samples were collected and immediately transported on the ice at 4^{o}C to be processed within the same day.

7.3.2 Passive Sampling and Wastewater Processing Methods

Passive samplers were processed identically to the work of Hayes et al. (2022). Viral elution of the samplers was conducted by submerging used GAC media in 6 mL of a 0.075% Tween-20 + 25 nM Tris HCl-based buffer sourced from Sigma-Aldrich (Ottawa,

ON, CA). Once mixed, the GAC and Tween-20 buffer were shaken by hand in 50-mL falcon tubes for approximately 1 minute. Then, the supernatant was pipetted into a separate 15-mL falcon tube, and 1 mL of this supernatant was used for subsequent RNA extraction.

Composite wastewater samples were processed as per the work of Parra et al. (2021) [16]. The 250-mL samples collected from the WWTF were mixed by inversion, then poured into 50-mL aliquots and centrifuged in falcon tubes for 5 minutes at 5000 rpm. After centrifugation, the supernatant was discarded from each falcon tube, and 2 mL of 0.075% Tween-20 + 25 nM Tris-HCl buffer was mixed in with the solids-rich pellet remaining in each tube. After the Tween-20 buffer addition, the sample tube was mixed by inversion for 5 seconds and then left to sit at room temperature for 5 minutes, allowing the resuspended particular to settle. Lastly, 1 mL of the uppermost Tween-20 buffer supernatant layer was used for subsequent RNA extractions.

7.3.3 RNA Extraction

Viral RNA was isolated from composite and passive sample concentrates as described in Section 7.3.2, using LuminUltra Technologies Ltd. (Fredericton, NB, CA) commercially available SARS-CoV-2 Advanced Wastewater Testing kit that employs a direct magnetic-beads-based approach for RNA isolation. All RNA extractions were performed with reagents provided by LuminUltra Technologies Ltd. For both wastewater and passive samples, 1 mL of the eluate was extracted according to the manufacturer's instruction, resulting in a final volume of 50 µL of RNA extract. Extracts were stored at 4ºC for up to 24-h until subsequent RT-qPCR analysis and then at -80ºC following analysis. Further details on the RNA extraction protocol performed can be found in Table S3.

7.3.4 RT-qPCR Reaction and Thermocycling Parameters

The multiplex RT-qPCR assay was constructed to detect SARS-CoV-2, INFA, RSV-A, and MeV simultaneously. All primers and probes were manufactured by Integrated DNA Technologies (IDT; Coralville, IA, US). Dual-labelled oligonucleotide probe sequences were labelled at the 5′-end with fluorescent reporter dyes and quenched with Blackhole Quencher 1 or 2 (BHQ 1 or 2) at the 3′-end (Table 7-1). RT-qPCR reactions comprised 20-µL mixtures, consisting of 3 µL of isolated RNA, five µL of TaqMan Fast Virus 1-Step Multiplex Master Mix (ThermoFisher, Tewksbury, MA, US), and primer and probe

concentrations listed in Table 1. Samples were analyzed using a Gene Count Q-96 thermocycler instrument (LuminUltra Technologies, Ltd.). Thermal cycling conditions were carried out as follows: 2 min at 25°C, 15 min at 50°C, 2 mins at 95°C, 45 cycles of 15 s at 95°C, and 30 s at 60°C.

Table 7-1. Oligonucleotide sequences and respective primer/probe working concentrations.

Target	Genes	Sequence (5'–3')	Concentration (nM)	Amplicon Length (bp)	Ref.
INFA	Matrix protein (M1)	**F1:**CAAGACCAATCYTGTCACCTCTGAC	400	106	[14]
		R1:GCATTYTGGACAAAVCGTCTACG	600		
		F2:CAAGACCAATYCTGTCACCTYTGAC	400		
		R2:GCATTTTGGATAAAGCGTCTACG	200		
		P:TGCAGTCCTCGCTCACTGGGCACG	200		
SARS-CoV-2	Nucleocapsid (N1)	**F:**CTGCAGATTTGGATGATTTCTCC	100	92	
		R:CCTTGTGTGGTCTGCATGAGTTTAG	200		
		P:ATTGCAACAATCCATGAGCAGTGCTGACTC	200		
RSV-A	Nucleoprotein (N)	**F:**GCTCTTAGCAAAGTCAAGTTGAATGA	500	82	[6]
		R:TGCTCCGTTGGATGGTGTATT	500		
		P:ACACTCAACAAAGATCAACTTCTGTCATCCAGC	200		
MeV	Nucleoprotein (N)	**F:**ATATATCGTAGAGGCAGGATTAG	500	119	[31]
		R:AGGACTCAAGTGTGGATAAC	500		
		P:AAACTATGTATCCTGCTCTTGG	200		

7.3.5 Experimental Comparison of the Linearity, Efficiency, and Repeatability of the Monoplex and Multiplex RT-qPCR Approaches

The performance of the multiplex RT-qPCR assay was validated analytically using synthetic viral controls (Table S1). Four standard quantification curves were constructed by preparing viral control solutions per the manufacturer's recommendations (Twist Bioscience, San Francisco, CA) and then serially diluted each synthetic target reference RNA material to known concentrations between ~10^6 – 10^0 log copies per µL of RNA template. The repeatability of the assay was accounted for by evaluating each template RNA concentration in the standard curves in technical triplicates. Quantification precision and linearity were estimated from the coefficient of correlation (R^2) value obtained from the linear regression of each standard curve, with R^2 criteria for adequate validation being $\geq$ 0.9. The Pearson's correlation coefficient (r) values were evaluated to measure the statistical association and strength between the monoplex and multiplex assays. Amplification efficiency (Ɛ) was determined from the slope of the standard quantification curve where $Ɛ = 100 \times (10^{-1/slope}-1)$ [17]. The quantification parameters for the monoplex and multiplex RT-qPCR assays were compared based on the slope and y-intercept values of the regression lines.

7.3.6 Multiplex RT-qPCR Limit of Detection and Limit of Quantification

The limit of detection (LOD) and quantification (LOQ) for the multiplex RT-qPCR assay were evaluated using synthetic RNA template controls. In total, 12 replicates of each serial dilution spanning 1 GC/rxn to 1000 GC/rxn were evaluated, using six technical replicates per run and two independent experiments. The LOQ and LOD were estimated mathematically using a logistic regression model (Eq. 1), where the fraction of detected replicates was assigned as the model response and the known RNA template concentrations (GC/rxn) as the model predictor [18]. The model was fit using an iteratively reweighted least squares approach, where $\hat{y}$ serves as the prediction parameter and β_i as the corresponding model parameters. The quantification capacity of the multiplex assay was evaluated by determining the smallest amount of the reference standard that produced amplification at a 95% confidence level, defined as the LOD for each target virus. The LOQ was the lowest number of copies deduced from a standard curve with a percent coefficient of variation (%CV) value no greater than 35% [18].

$$\hat{y} = \frac{1}{1 + e^{\beta_0 - \beta_1 x}} \quad \textbf{Eq. (1)}$$

7.3.7 In silico Specificity

The primers and probes targeted N1, M1, N, and N genes of SARS-CoV-2, INFA, RSV-A and MeV, respectively. *In silico* specificity was analyzed by comparison of the reference sequences of the synthetic controls and entries from the GenBank database of the NCBI (http://www.ncbi.nlm.nih.gov). Reference sequences from nucleotide BLAST software (version 1.2.4) were aligned to target gene primer sequences (Table S2) using the multiple sequence alignment tool, Clustal Omega (version 1.2.2) [19].

7.3.8 Evaluation of Multiplex Assay Efficacy to detect SARS-CoV-2, RSV, and MeV from Seeded Wastewater Samples

Bench-scale experiments to evaluate viral recovery from seeded wastewater samples utilized three 250-mL, 24-hr composite samples collected from a local WWTF in April 2022. To ensure little to no virus background levels were present in the wastewater, each sample was left at room temperature for approximately one month and then autoclaved at 121ºC with a pressure of 15 psi for 60 mins. Before spiking the wastewater with a viral surrogate, each sample was evaluated for viral background signal by extraction and analysis via the methods described in Section 7.3.3 and Section 7.3.4, respectively. This initial wastewater screening showed no indication of target viruses in the wastewater.

To determine the effect of viral concentration and extraction procedures, recovery efficiencies were assessed by seeding synthetic viral surrogates (Table S1) for all four viruses into 100-mL aliquots of wastewater at three concentrations: 1×10^2 GC/mL, 1×10^3 GC/mL, and 1×10^5 GC/mL. Before RNA extraction, the seeded wastewater samples were mixed thoroughly by inverting sample vials repeatedly and were left to incubate for one hour at 4ºC. For each of the seeded wastewater samples, three biological replicates in 1 mL aliquots were taken for RNA isolation and purification (further details in Section 7.3.3). Technical duplicates were analyzed for each biological replicate during RT-qPCR analysis. The average of each technical duplicate was used to calculate the overall mean ± standard deviation of the biological replicates (mean ± SD, *n=3*). A paired *t-test* analysis (two-tailed, $\alpha = 0.05$, 95% confidence) was conducted to compare the statistical significance between mean viral concentrations used to calculate percent recovery values

calculated for each viral target. The percentage recovery for the spiked viral controls was calculated using Eq. (2) for each target virus.

$$\%\, Recovery = \frac{C_{sample}V_{sample}}{C_{seeded}V_{seeded}} * 100 \quad \textbf{Eq. (2)}$$

Where C_{sample} is the mean detection concentration from the standard curves, C_{seeded} is the estimated spiked wastewater concentration, V_{sample} is the volume of sample adjusted for the amount of RNA used in the RT-qPCR reaction, V_{seeded} is the fraction of volume assayed relative to the volume processed.

7.3.9 Simultaneous detection of SARS-CoV-2, INFA, RSV-A and MeV in Wastewater from Composite and Passive samples

The detection performance of the Multiplex RT-qPCR method was assessed by evaluating field samples that met the criteria of detecting at least one of four viral targets. In total, 44 samples were collected across composite and passive sample types; eleven 24-h composite wastewater samples were collected from the WWTF, and 33 passive samples were collected across three targeted sewershed locations (11 passive samples collected per site). All samples were collected between 01 May 2022 and 01 July 2022, with composite wastewater samples collected once a week and paired passive samples collected three times a week for either 48-h or 72-h deployment periods. Wastewater and passive samples were processed as per Section 7.3.2, and RNA extracts were concentrated and analyzed using the methods described in Section 7.3.3 and Section 7.3.5, respectively. Biological replicates were omitted for field samples to conserve reagents and materials; however, technical duplicates were performed to evaluate variability within the multiplex RT-qPCR assay for each sample.

7.3.10 Quality Assurance-Quality Control (QA-QC)

To assess qPCR-based testing and design, criteria from the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [17] and the Environmental Microbiology Minimum Information (EMMI) guidelines were followed [20]. The MIQE checklist for essential and desirable reported information is presented in Table S3. All materials used were either purchased pre-sterilized or sterilized

in an autoclave to eliminate any pre-contamination of samples. To reduce potential cross-over contamination during seeded wastewater experiments and RNA extraction procedures, a Thermo Scientific 1300 Series A2 biosafety cabinet was used. A Mystaire MY Model PCR Prep Station Class 100 laminar flow enclosure was used to minimize contamination while preparing RT-qPCR reactions. Unilateral flow was ensured between steps and autonomous working areas were maintained throughout this work with dedicated areas defined for sample processing, RNA extractions, RT-qPCR master mix preparation, and amplification steps. Each working area contained laboratory equipment, as well as disposable consumables, reagents, and personal protective equipment. All laboratory working surfaces were decontaminated with 1% bleach for 30 minutes of contact time, followed by rinsing with DNase/RNase-free water and then exposed to ultraviolet light for 90 minutes [21].

During RNA extraction in both bench-scale and field-scale analysis, at least one process blank consisting of DNase/RNase-free water was introduced to assess potential contamination. At least one no template control (NTC) and positive template controls for each target were always run in parallel with each RT-qPCR plate during RT-qPCR analysis. NTCs comprised DNase/RNase-free water, whereas positive controls consisted of synthetic RNA template controls (Table S1).

RT-qPCR reagents were all prepared in single-use aliquots to prevent cross-contamination or freeze-thaw degradation of stock solutions. During the field-scale implementation of the multiplex RT-qPCR method, the integrity of a newly purchased batch of RT-qPCR reagents (i.e., primers, probes, and mastermix) was confirmed before use by analyzing ten no-template control replicates in conjunction with at least one positive template control [22]. Results were not used if process blanks or no template controls were amplified or if the positive controls did not amplify. Inhibition effects were monitored in the RNA extracts from wastewater by comparing undiluted and DNase/RNase-free water-diluted RNA templates during RT-qPCR analysis (1:0, 1:1, and 1:5 dilution factors were applied per sample) [23].

7.4 Results and Discussion

7.4.1 Experimental Comparison of the Linearity, Efficiency, and Repeatability of the Monoplex and Multiplex RT-qPCR Approaches

When assessing the analytical sensitivity of monoplex and multiplex RT-qPCRs, inverse correlations were observed between the log quantity of template RNA and the detected Cq values for all four gene targets ($R^2 > 0.95$) (Figure 7-1). As indicated by the slopes, the amplification of samples exhibited an equivalent rate of change in fluorescent emission intensity per amount of template. Precision in the log-linear relationship between template RNA concentrations and amplification thresholds indicates efficient reverse transcription and amplification [24]. The standard quantification parameters for monoplex and multiplex assays demonstrated minimal differences, indicating comparable amplification efficiencies. A strong correlation (Pearson's r, $0.90 < r < 0.99$) was also observed between the mean Cq values in the monoplex and multiplex assays.

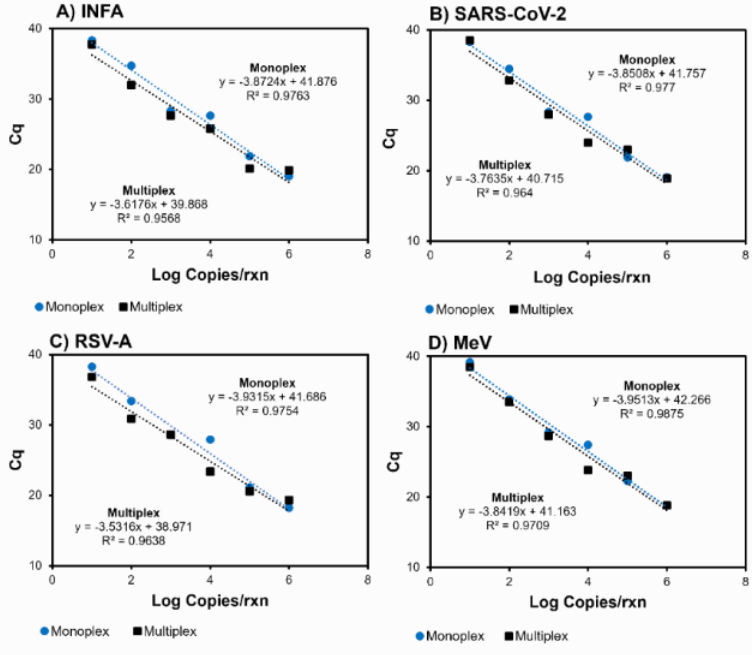

Figure 7-1. Standard quantification curves of multiplex (black squares) and monoplex (blue circles) were generated using simple linear regression for the cycle threshold values versus the amounts of template RNA in each reaction (log copies/rxn) for both monoplex and multiplex RT-qPCR assays. Plots are listed by virus, INFA (A), SARS-CoV-2 (B), RSV-A (C), and MeV (D). Symbols represent the mean value of all replicates ($n = 3$), and dotted lines linear regression analysis.

7.4.2 In silico Specificity

With RNA viruses, sequence diversity in the RT-PCR target regions occurs between different genotypes or strains and can affect detection sensitivity [25]. Therefore, the accurate design of primer/probe sets is advantageous, especially for precise detection from complex environment samples. The specificity of each multiplex RT-qPCR primer/probe sequence was tested *in silico* against various viral genotypes and strains available from NCBI GenBank (Table S2). *In silico* analysis showed some heterogeneity in gene targets; specifically, the RSV-A gene target was conserved in several RSV-A strains. However, mutations in the reverse primer prevented adequate sequence alignment for RSV-B and human orthopneumovirus genotypes. The INFA gene aligned well with most A-type genotypes but not INFB genes. The MeV gene target was conserved in the A, D8, D4 and C1 genotypes; however, primer and probe mutations cause misalignment in all other genotypes. For the SARS-CoV-2 N1 gene target, mutations in the reverse primer were only observed in early lineages. Ideally, methods for detecting viral gene targets should be periodically updated and re-evaluated to account for genetic diversities observed in recently circulating viruses. While this *in silico* approach does not account for all the *in vitro* parameters that can affect qPCR reactions, the findings of this analysis are valuable in identifying potential limitations in target amplifications and guiding further experimental evaluations on the impacts of these mismatches on RT-qPCR performance.

7.4.3 Multiplex RT-qPCR Limit of Detection and Limit of Quantification

The analytical sensitivity of the multiplex assay was further evaluated by determining the LOQ and LOD_{95} values for each viral target. Figure 7-2 demonstrates the fraction of positive detections at temple RNA concentrations ranging from 1 GC/rxn to 1000 GC/rxn. For each viral target, the LOQ was determined to be between 10 GC/rxn and 15 GC/rxn, and the $LOD_{95\%}$ for each target was between 15 GC/rxn and 25 GC/rxn. The LOQ was determined from the inter-experiment data in which the lowest nominal RNA concentration where the %CV was still $\leq$ 35 %. Although there is no general agreement on %CV standards for acceptable qPCR validation, percentages between 10% and 35% have been frequently applied for evaluating variance when Cq values are calculated to gene copy numbers [18,26]. The variance between sample replicates became

increasingly significant when RNA template concentrations decreased below 10 or 15 GC/rxn in the current study. However, this is commonly observed in RT-qPCR analysis and has been attributed to more pronounced stochastic effects in quantification as target concentrations lessen. The LOD findings in this study align with the Poisson distribution assumption that LOD should not be less than three GC/rxn for PCR applications. [17,27]. Numerous WWS studies implementing RT-qPCR analysis have evaluated LOD using serially-diluted RNA reference standards [28–30], reporting LOD values in the range of 1 GC/rxn, 50 GC/rxn, and 100 GC/rxn. Although the results of the current study align with previous findings, statistical models and analytical methods may be highly variable between studies as detection thresholds are both assay and target-dependent; therefore, no one study can be directly comparable.

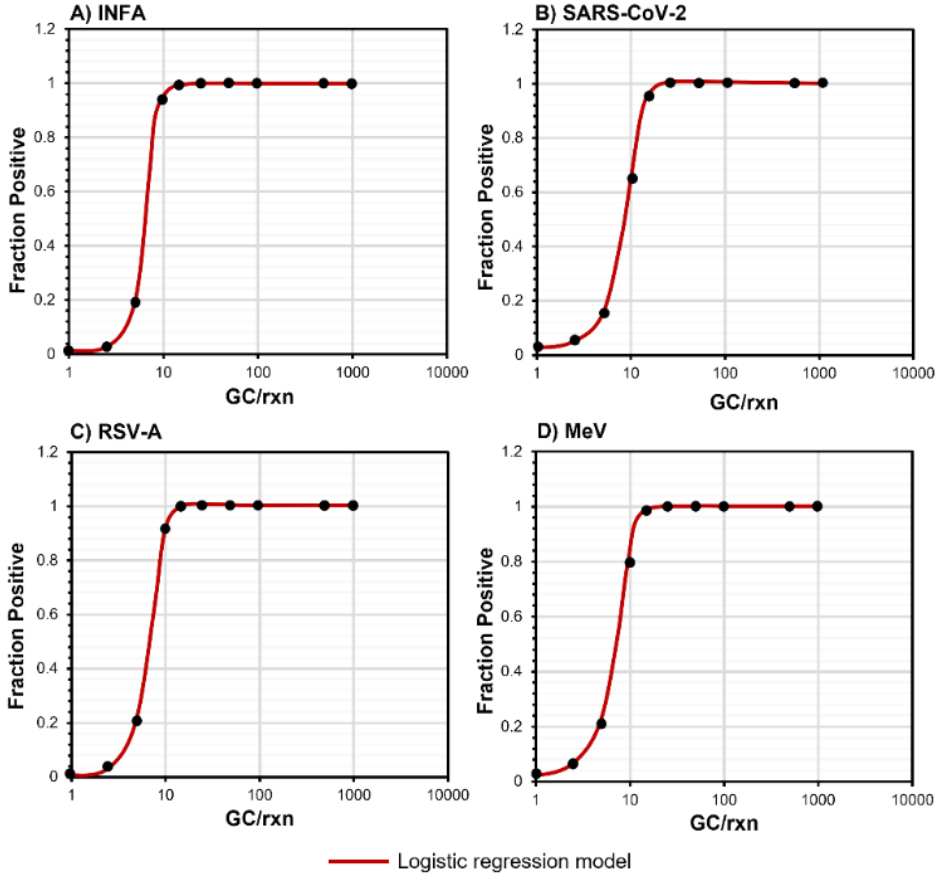

Figure 7-2. Experiment estimation of the LOQ and $LOD_{95\%}$ determinations for INFA (A), SARS-CoV-2 (B), RSV-A (C), and MeV (D) using logistic regression models with a detection limit of 95%. The y-axis indicates the fraction of detected replicates (*n=12*) and the x-axis shows the RNA concentration per 20 µL multiplex RT-qPCR reaction volume.

When using this multiplex assay and making sample reporting decisions, we take into account the LOD and LOQ results as identified in this study. To ensure accurate reporting, virus concentrations below the LOQ should be considered inconclusive and reported as non-detect. For concentrations between the LOQ and LOD, a qualitative report (i.e., "<LOD") is recommended, while for detections exceeding the LOD, a quantitative result should be provided. Previous studies have recommended when using RT-qPCR analysis, Cq thresholds between 40 [16,31,32] and 45 cycles [33] be used for defining positive amplification of viral targets. However, variations during analysis may result in differences in reporting Cq thresholds among laboratories. Setting quantification thresholds based on Cq values may result in biased reporting due to the dependence of Cq amplification on instrument efficiency [34].

Analytical detection limits may not sufficiently capture the impact of RT-qPCR efficiency, nucleic acid preparation errors, and sample variation. However, obtaining representative process controls for diagnostic analysis can be challenging due to economic, technical, or biosafety constraints. As a result, binomial regression models, as demonstrated in this study, have been used in conjunction with analytical data to establish a minimum nucleic acid concentration and relative intervals of confidence associated with a detection probability (e.g., 95-100%) [35].

7.4.4 Evaluation of Multiplex Assay Efficacy to Detect SARS-CoV-2, RSV, and MeV from Seeded Wastewater Samples

Recovery efficiencies of SARS-CoV-2, INFA, RSV-A and MeV were determined through bench-scale experiments seeding wastewater samples with virus surrogates for each viral target at three concentrations (10^5, 10^3, and 10^2 GC/mL). At a spike concentration of 10^5 GC/mL, viral recoveries for SARS-CoV-2, INFA, RSV-A, and MeV were 78% ± 4% (mean ± SD, *n=3*), 85% ± 6%, 85% ± 4%, and 72% ± 8%, respectively (Figure 7-3). Viral recovery of 63% ± 5%, 72% ± 7%, 80% ± 5%, and 53% ± 11%, were observed for SARS-CoV-2, INFA, RSV-A, and MeV at a spike concentration of 10^3 GC/mL. At the lowest viral concentration (10^2 GC/mL), viral recoveries were 47% ± 4%, 67% ± 2%, 69% ± 6%, and 49% ± 8% for SARS-CoV-2, INFA, RSV-A, and MeV, respectively. The reduced recoveries observed with decreasing spiked virus concentrations suggest increasing variability and lower reproducibility at lower virus concentrations.

Similar viral recoveries from wastewater have been reported using comparable methods, including mean recoveries of a heat-inactivated SARS-CoV-2 (~10^3 GC/mL) between 11% to 40% [16,36]. Farkas et al. (2022) observed a similar phenomenon when evaluating virus recovery from wastewater and described that the differences between virus structure, shape, size, genetic material, and inactivation mechanisms might influence detection variability [37]. A degree of variation between RT-qPCR replicates, experimental runs, instruments, and laboratories is intrinsically expected [38]. Therefore, future investigation is required to understand the effects of viral properties on virus recovery from wastewater.

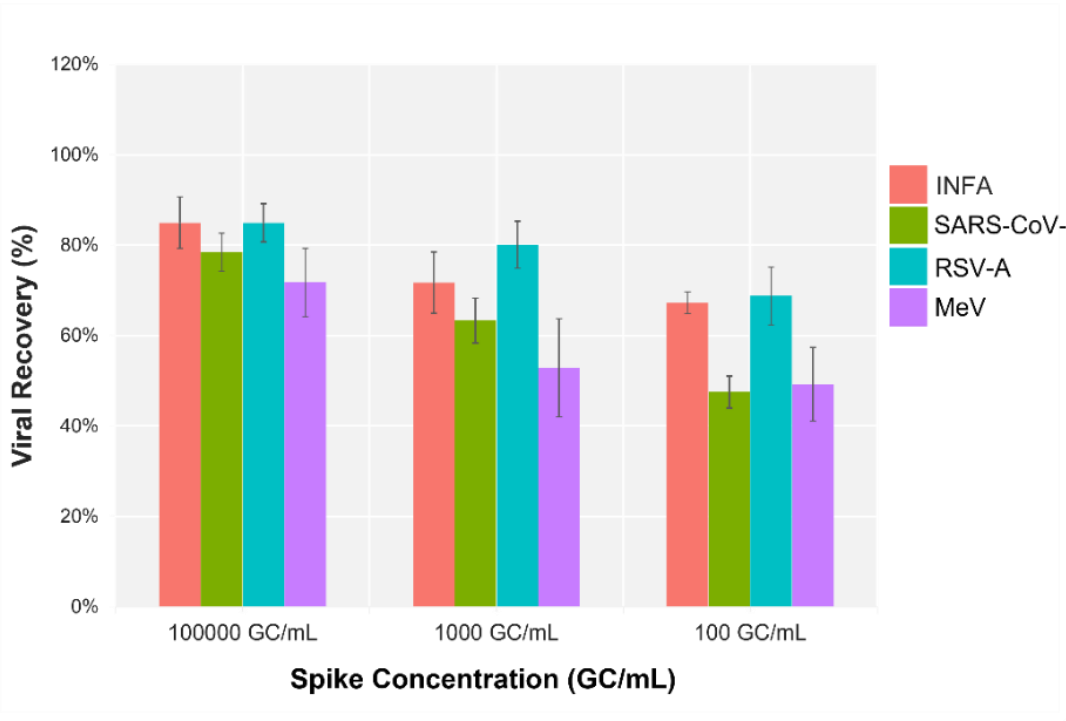

Figure 7-3. Viral RNA recovery (%) for three virus spike concentrations (10^5, 10^3, and 10^2 GC/mL). Detections are shown for INFA, SARS-CoV-2, RSV-A and MeV with red, green, blue, and purple bars, respectively. The bars express the mean viral recovery (*n=3*), and the error bars indicate the standard deviation between replicates.

In this study, viral recovery did not differ significantly (p-value $\geq$ 0.05) between all four viral targets at each of the spiked concentrations. However, further optimization could be considered, particularly for SARS-CoV-2 and MeV, where recovery was lower.

The potential for inhibition in downstream RT-qPCR analysis has been commonly referred to in WWS efforts [22], as these methods are prone to interferences in sensitivity due to inhibitory compounds inherent to wastewater (e.g., humic/fulvic acids, salts,

chemicals, or biological material) [38,39]. To assess potential inhibition in our samples, we analyzed both diluted and non-diluted RNA extracts [23]. Our findings, illustrated in Figure S1, indicate a linear decrease in viral recovery for diluted extracts, suggesting minimal interference from inhibitory compounds present in the wastewater. However, monitoring for full or partial inhibition during RT-qPCR analysis can be often uncertain, labour-intensive and costly [39,40], and is a limitation of this work. For example, the dilution of RNA extracts has been shown to help assess inhibition, but when targets are present at low levels, dilution may result in false-negative results. Much uncertainty also exists in selecting, validating, quantifying, and interpreting various endogenous and exogenous controls to normalize RNA concentrations most effectively from wastewater [22]. Optimizing inhibition monitoring strategies and methods may be necessary to reduce inhibitory effects in real-world RT-qPCR applications.

In summary, our multiplex RT-qPCR assay effectively detected SARS-CoV-2, INFA, RSV-A, and MeV at both high and low concentrations in wastewater with minimal interference from inhibitory compounds. However, it's important to note that the recovery efficiencies of spike tests may not accurately reflect viral interactions in field samples [41], nor the full range of matrix effects observed in real-world environments [42].

7.4.5 Simultaneous Detection of SARS-CoV-2, INFA, RSV-A and MeV in Wastewater from Composite and Passive Samples

To assess the multiplex RT-qPCR assay's detection performance, we analyzed passive from three sewershed locations and composite wastewater samples from the receiving WWTF. Thirty-three passive and eleven composite wastewater samples were collected between 01 May 2022 and 01 July 2022. From these samples, 81.8% (27/33) passive and 81.8% (9/11) composite wastewater samples had at least one viral detection. Across all targets, there were 69 quantifiable results ($\geq$ LOD) and seven qualitative detections between the analytical LOD and LOQ values. Figure 7-4 demonstrates the range of quantifiable viral concentrations for SARS-CoV-2, INFA, and RSV-A. Importantly, no MeV genes were detected in any of the wastewater or passive samples analyzed. However, this is not unexpected as Canada has no sustained circulation of this virus [10]. For composite wastewater samples, the minimum and maximum quantifiable virus concentrations were as follows: SARS-CoV-2 (2.0×10^1 to 2.1×10^3 GC/mL), INFA

(8.5×10^1 to 3.5×10^4 GC/mL), and RSV-A (1.5×10^1 to 4.2×10^3 GC/mL). Across all three passive sampling locations, the minimum and maximum virus concentrations were as follows: SARS-CoV-2 (1.0×10^1 to 1.1×10^5 GC/mL), INFA (3.5×10^1 to 2.7×10^7 GC/mL), and RSV-A (2.6×10^1 to 6.4×10^6 GC/mL).

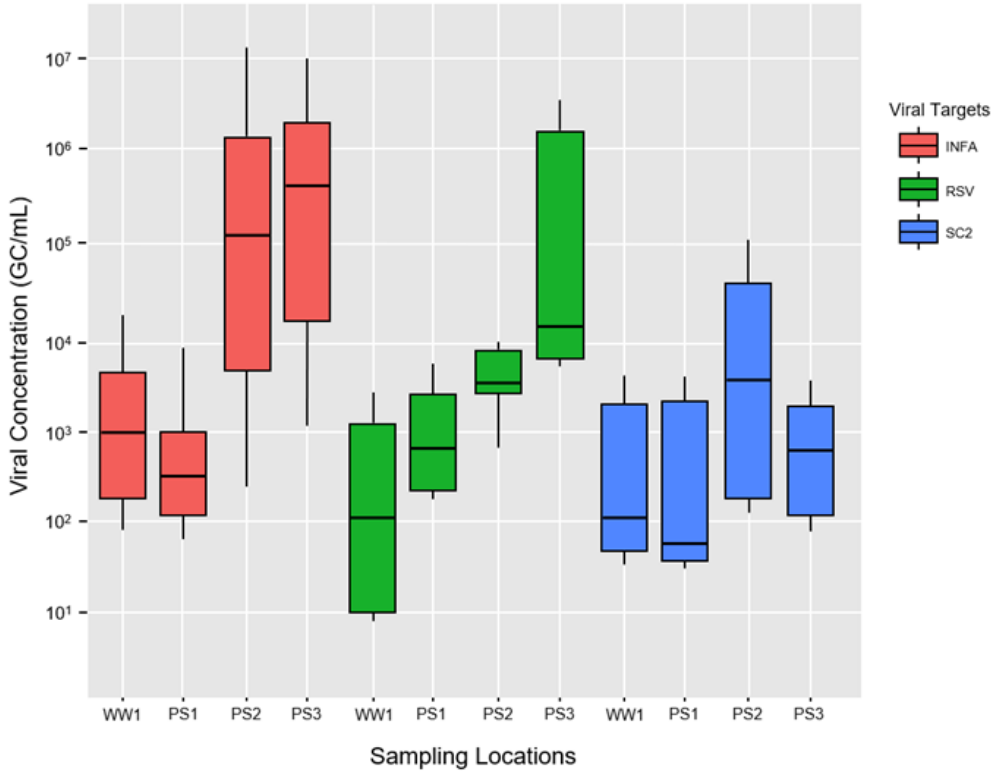

Figure 7-4. Boxplot of log viral concentrations (GC/mL) for SARS-CoV-2, INFA, RSV, and MeV at four sampling locations across two months, including wastewater samples collected at the WWTF (WW1), and passive samples (PS) from three sewersheds (PS1, PS2, and PS3). For each viruses distribution values, the lower and upper box boundaries indicate the 25th and 75th percentiles, respectively, the horizontal line inside each box denotes the median, and the lower and upper error lines show the 10th and 90th percentiles, respectively.

These findings highlight that the multiplex RT-qPCR methods produced similar results in field samples, inherently more prone to significant interferences in downstream analysis than those analyzed in controlled bench-scale experiments. The relationships between adsorption, inactivation, and interference mechanisms are often difficult to replicate to those experienced in the field. The concentrations of SARS-CoV-2, INFA, and RSV-A obtained from 24-h composite samples collected at the WWTF were significantly lower ($p < 0.05$) than the passive samples deployed within the WWTF catchment. Previous

studies have reported similar results for passive and aqueous samples, attributing the passive sampler's ability to concentrate viruses from large volumes over several days [15,43,44]. In contrast, composite wastewater samples typically collect a pre-determined portion of flow over a limited period, usually not exceeding 24 hours. Kevill et al. (2022) found comparable recovery rates for INFA, INFB, SARS-CoV-2, human adenovirus, norovirus GII, and MeV in cotton and tampon-based passive samplers, compared to liquid composite wastewater samples, using monoplex RT-qPCR analysis [45]. While several molecular detection methods have been used to identify viruses in wastewater through passive or liquid sampling methods, Hayes et al. (2022) is the only study to report viral RNA detection using GAC-based passive samplers [15]. Therefore, more research is needed to determine the most effective sampling method.

As previously noted, different factors can pose challenges that contribute to the uncertainty of interpreting results in WWS. Important considerations when interpreting real-world WWS results include sample collection and processing methods, community shedding dynamics, sewage composition, analyst expertise, and matrix interferences. However, since the goal of WWS is to monitor viral trends over time at each sampling location, many of these variables will remain consistent across time points concerning population infection dynamics [46,47]. This work aims to serve as an initial step to establishing multiplex analysis of the four viruses, but further real-world validation is necessary to compare virus concentrations with infection and shedding prevalence.

To our knowledge, no one-step RT-qPCR assay has been published to detect SARS-CoV-2, INFA, RSV-A and MeV from wastewater. We believe the methods described herein may greatly assist our understanding of wastewater's role in disease surveillance. In contrast to other published monoplex detection strategies, this multiplex assay provides comparable sensitivity and enhanced throughput for environmental virus surveillance. It can detect viruses that are prevalent in high numbers, such as SARS-CoV-2, INFA, and RSV-A, and identify regions affected by low-prevalence diseases like MeV that might otherwise go undetected. The multiplex assay’s ability to detect various viral concentrations with minimal interferences from common wastewater inhibitors highlights the robustness of this method when combined with the described sampling techniques,

RNA isolation, and purification methods. The detection of low viral signals in highly dilute wastewater systems will be of utmost importance, particularly when targeting viruses not presently circulating (e.g., MeV). Future WWS efforts should validate the current methods against presently circulating viruses and should investigate other relevant pathogens.

7.5 Conclusions

This study aimed to evaluate a novel multiplex RT-qPCR assay's performance for detecting and quantifying SARS-CoV-2, INFA, MeV and RSV-A in wastewater. The assay was evaluated through bench-scale experiments and field sampling, and the results indicate that this multiplex RT-qPCR analysis is feasible for routine surveillance of viral pathogens in both high and low concentrations in wastewater. The multiplex assay is robust and sensitive, providing a cost-effective approach to monitoring multiple diseases that may not be detected through individual-level testing. While further optimization may be necessary, this work will be a valuable step toward understanding disease epidemiology and establishing appropriate WWS strategies.

7.6 Acknowledgments

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CHAPTER 8 ENHANCED DETECTION OF VIRUSES FOR IMPROVED WATER SAFETY

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8.1 Abstract

Human viruses pose a significant health risk in freshwater environments, but current monitoring methods are inadequate for detecting viral presence efficiently. We evaluated a novel passive *in-situ* concentration method using granular activated carbon (GAC). This study detected and quantified eight enteric and non-enteric pathogenic viruses in a freshwater recreational lake in paired grab and GAC passive samples. Results found that GAC passive sampling had a higher detection rate for all viruses compared to grab samples, with adenovirus found to be the most prevalent virus, followed by RSV, Norovirus, Enterovirus, Influenza A, SARS-CoV-2, and Rotavirus. GAC in-situ concentration allowed for the capture and recovery of viral gene copy targets that ranged from one to three orders of magnitude higher than conventional ex-situ concentration methods used in viral monitoring. This simple and affordable sampling method may have far-reaching implications for reducing barriers associated with viral monitoring across various environmental contexts.

8.2 Introduction

Human pathogenic viruses are responsible for a substantial portion of human morbidity and mortality [1,2]**.** Woolhouse & Gaunt (2007) found that two-thirds of the 87 novel pathogens first detected between 1980 and 2005 were due to viruses [3,4]. Viral prevalence in freshwater environments is a known path for human pathogen transport and infection due to public exposure through recreational activities and drinking water sources [5–7]. Recreational waters and drinking water sources are susceptible to contamination by pathogenic viruses from multiple pathways [8], most commonly through undertreated or untreated wastewater discharge and surface runoff into receiving bodies containing human and animal fecal matter [9–11]. Viral presence and persistence in wastewater also suggest

potential hazards linked to its agricultural reuse. Using contaminated wastewater or freshwater to irrigate crops could indirectly transmit viruses through product handling or consumption [12]. Most research to date has focused on the detection of enteric viruses in freshwater sources, as these viruses are known to replicate in the gastrointestinal tract of infected hosts, shed in high volumes in fecal matter for prolonged durations, and be transmitted through fecal-oral exposure pathways in contaminated water [5,6]. However, recent work has revealed that several respiratory viruses are also shed through the gastrointestinal tract. Viruses can persist and remain viable in water sources for several days to weeks, depending on water quality characteristics and environmental conditions [13–15]. This is a significant development, as the majority of emerging human viruses, upwards of 85%, are known to be non-enteric RNA-stranded viruses, i.e. severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and Influenza A and B (INFA/INFA) [3,4]. The COVID-19 pandemic illustrated the importance of non-enteric viral tracking in aqueous environments, particularly wastewater effluents, to understand respiratory viruses' fate, transport, and infectivity in environmental reservoirs [12,16]. There have been significant advancements in detecting and identifying viruses in the environment through molecular-based methods, such as quantitative polymerase chain reaction (qPCR), which can be used to evaluate the presence of genes of interest (i.e., viral or indicator genes). However, despite improvements in viral detection methods, the occurrence, abundance, and persistence of enteric viruses in freshwaters remain understudied. In the case of non-enteric viruses, they are nearly entirely uninvestigated due to the lack of routine monitoring. Virus monitoring in freshwaters remains challenging due to the inefficient, time-intensive, and costly methods currently available to recover and concentrate viruses from aqueous environments [17].

There is a need for rapid, simple, and cost-effective monitoring of established and emerging viruses, both enteric and non-enteric, in freshwaters to improve public health protection and anticipate possible future pandemic threats [4,8,18,19]. Due to the health risks of pathogenic viruses in freshwater, many jurisdictions have established drinking and recreational water microbial guidelines to protect the public from infection. Water quality guidance recommends using fecal indicator organisms (FIOs) to characterize fecal contamination and, by extension, viral presence in freshwaters [17,20–22]. For context,

Canadian drinking water guidelines recommend treatment to a 4-Log reduction standard for enteric viruses in both surface and groundwater sources [20,21]. Further, Canadian guidelines recommend that routine microbiological monitoring in treated drinking water be limited to Total coliforms and *Escherichia coli* (*E. coli*) without standard monitoring for individual viruses [18]. Recreational water quality guidance in Canada, and more broadly, is also limited to monitoring *E.coli* and enterococci [21]. However, these FIOs are known to be poor surrogates for viral abundance, with weak correlations reported between enteric viruses and FIOs, such as enterococci and E.coli [23–25]. FIOs also offer no details on viral specificity, which depends on the disease burden of the population producing the wastewater contribution, differing rates of inactivation during treatment, and environmental degradation processes specific to different viruses [21,26–29]. While FIOs can indicate potential risk of exposure to pathogens in freshwater environments, they are not reliable for monitoring viral presence or exposure risk to viruses in freshwater environments. This is problematic for source water management and drinking water treatment approaches because different viruses have varying survival and inactivation susceptibilities and require different levels of disinfection [28,30].

Viral monitoring in freshwater is challenging because of the low concentrations of viruses distributed heterogeneously in large bodies of water [8,19,31]. While these low concentrations are difficult to monitor, they are significant enough to cause human disease [32,33]. To ensure accurate results, robust sampling methods typically involve collecting and concentrating large volumes of water, ranging from 10 L to over 1500 L. Current guidance and methodologies recommend filtering at least “a few hundred litres” of surface water sources intended for drinking water, at least 1500 L of groundwater, and up to 1000 L of recreational water for virus concentration [17,22,34]. Various *ex-situ* concentration methods have been developed to concentrate trace amounts of viruses from large volumes of water, but are often time-intensive and cumbersome [17,19,35]. Although downstream processing and analysis are critical components in the overall detection process, the initial water sampling technique for virus monitoring in freshwater environments frequently serves as a limiting factor, particularly in settings with constrained resources. Bofill-Mas & Rusinol [19] reviewed 59 research articles for viral concentration procedures and found precipitation/flocculation, centrifugation, and filtration (ultra-, electronegative, and

electropositive) to be the dominant processes used in recent research. However, these concentrations require a substantial volume of water for analysis and a significant amount of time and energy for concentration. For example, Schijven et al. 2019 developed a Quantitative Microbiological Risk Assessment process for the evaluation of the health risk of adenovirus in drinking water, which required the collection of thirty-five water samples, each approximately 600 L, that was passed through an ultrafiltration unit before elution of the filters for preparation for qPCR [36]. Because of the laborious and impractical methods available for virus concentration, viral detection in drinking and recreational waters is costly and rarely done.

The need for rapid, affordable, and simple viral monitoring to understand the spread of SARS-CoV-2 has led to the refinement of passive sampling techniques initially developed in 1948 to monitor poliovirus in drinking water sources [37]. Passive sampling is an *in-situ* concentration method which deploys adsorbent materials to concentrate target analyte based on diffusion-driven adsorption/sorption processes [38,39]. The recent pandemic response led to the evaluation of several different adsorbent media for capturing a range of viral targets from water and wastewater matrices. Hayes et al. (2022 and 2023) utilized granular activated carbon (GAC) to effectively capture and recover SARS-CoV-2, INFA, Respiratory Syncytial Virus (RSV), Measles (MeV), Pepper Mild Mottle Virus (PMMoV), and CrAssphage from wastewater [38,40]. Compared to other adsorbent media, enhanced sensitivity and reproducibility were demonstrated when using GAC. This passive approach is advantageous as it allows for prolonged deployment in an aqueous environment, resulting in time-integrated measurements. This work aims to evaluate the effectiveness of passive sampling in capturing and concentrating other viruses of concern in freshwater environments, building on the advancements made in SARS-CoV-2 sampling methods.

This research utilizes established qPCR techniques coupled with a novel GAC passive sampling program to evaluate the presence of both enteric and non-enteric viruses in freshwater. Viruses can adsorb to particles rather than remain detached and free-floating in water [41,42]. The application of passive sampling in this context provides an *in-situ* particulate concentration in the water column by capturing small fractions of suspended or settling particles and adsorbing free-floating viruses if present [39,43]. Others [42, 43]

have shown that activated carbon can readily adsorb enteric viruses and coliphages, including Adenovirus (AdV), Rotavirus (RV), Norovirus (NV), and Bacteriophage MS2, from a range of source waters. While much of this research has focused on activated carbon for point-of-use water treatment for virus sequestration, this current study seeks to exploit the adsorptive nature of activated carbon as an *in-situ* concentration technique for viral monitoring. This work aims to investigate a novel GAC-based passive sampling technique for viral detection in a freshwater recreational lake. Passive samplers were deployed to detect the presence of 8 common enteric and non-enteric pathogenic viruses, including AdV, Enterovirus (EnV), INFA, NV, RSV, SARS-CoV-2, MeV, and RV through *in-situ* concentration to address the challenges of virus capture and concentration in freshwater.

8.3 Materials and Methods

8.3.1 Sampling Location and Sample Collection Methods

To study the occurrence of the selected viruses in a recreational freshwater lake, samples were collected from two locations in June, July and August of 2022. The study lake is located in a populated urban area of Nova Scotia. The lake is ~1.3 km long, 500 m wide, and 11 m deep and is surrounded by mixed residential/commercial properties, recreational facilities (canoe/kayak clubs) and several roadways. There are no known wastewater inputs to the lake other than potential recreational swimming or potential fecal material from wild and domestic animals. Two sampling locations were monitored to evaluate the viral abundance throughout the lake. At the first site (Site 1), passive samplers were deployed from a floating dock adjacent to a popular recreational beach, with the passive sampler suspended using nylon rope approximately 1.5 m below the surface. For the second site (Site 2), the passive sampler was secured to the shoreline again using nylon rope and deployed approximately 3 m from the water's edge; this sampler rested on the bottom of the lake (immediately adjacent to the sediment layer) at a depth of approximately 1 to 2 m below the surface. The passive sampler was engineered with a density that naturally facilitated its submersion, eliminating the need for supplementary weights for stable suspension in the lake. Generally, Site 2 had less recreational activity than Site 1.

Passive sampling was conducted using an adapted version of the 3D-printed passive sampler developed by Hayes et al. 2021 [61]. For each deployment, 3 grams (g) of GAC

was placed in a heat-sealable nylon mesh sleeve with ~25-µm pores and was put in the passive sampler to capture viral targets [38]. Passive samplers were deployed for one week, a duration found to optimally balance effective analyte adsorption with the GAC adsorption capacity [38]. Following week-long deployments, samplers were collected and placed in sealable plastic bags for transport to the lab and a new sampler was deployed for the subsequent week's sampling. Simultaneously with the deployment and retrieval of the passive samplers, grab samples were collected from the exact locations at approximately the same depth as the passive samples. Grab samples used for nucleic acid extraction were collected in sterilized 500 mL Nalgene bottles, and those used for water quality characterization were collected in acid (5% HCL) washed 1 L Nalgene bottles. These volumes were chosen based on Health Canada's sampling recommendations of between 200 and 500 mL for FIO analysis in recreational waters [21]. This recreational water guidance does not specify recommendations for viral monitoring protocols. Passive and grab samples were placed in coolers packed with ice until they were delivered to the lab, where they were stored at 4 °C. In total, 20 passive samples and 33 grab samples were collected across both Site 1 and Site 2 during the three-month sampling period.

8.3.2 Sample Processing

GAC Passive Sampling

Viral RNA was desorbed from GAC using a modified elution protocol adapted from Hayes et al. 2022 [38]. Briefly, GAC was removed from the passive sampler and eluted with 6 mL of a Tween20®-based buffer solution; a 1 mL aliquot of the eluate was then placed in a bead beating tube containing 500 µL of lysis buffer (BioGX, Birmingham, AL, USA). The resulting lysate was transferred to a sterile Eppendorf tube and stored at -20 °C while awaiting RT-qPCR analysis.

Grab Sampling

Grab samples were processed by concentrating a ~100 mL aliquot of the 500 mL sample on a 0.8 µm acrylic copolymer filter membrane (Cole-Parmer, Vernon Hills, IL, USA) using a sterile syringe filter. Using sterile tweezers, the filter membrane was placed in a bead-beating tube containing 500 µL of lysis buffer (BioGX, Birmingham, Alabama,

USA). The resulting lysate was transferred to a sterile Eppendorf tube and stored at -20 °C while awaiting RT-qPCR analysis.

8.3.3 Nucleic Acid Extraction

Nucleic acids were extracted from passive and grab samples within 24-h of sample collection and then stored at -80ºC until subsequent RT-qPCR analysis. To minimize contamination during nucleic acid extraction and RT-qPCR preparation, a Thermo Scientific 1300 Series A2 biosafety cabinet (Thermo Fisher Scientific, Oakwood, OH, USA) and a Mystaire MY Model PCR Prep Station Class 100 laminar flow enclosure were utilized, respectively.

8.3.4 RT-qPCR Reactions and Thermocycling Parameters

The isolated RNA/DNA was utilized for viral detection of SARS-CoV-2, INFA, RSV-A, MeV, EnV, RV, and NV through RT-qPCR techniques. Primer, probe sequences, working concentrations, and the thermocycling conditions used for each viral target are listed in Table S2. Primers and probes for each assay were produced by Integrated DNA Technologies (IDT; Coralville, IA, USA). RT-qPCR reactions comprised 20 µL mixtures, consisting of 3 µL of isolated nucleic acid and five µL of TaqMan Fast Virus 1-Step Multiplex Master Mix (ThermoFisher, Tewksbury, MA, US). Samples were analyzed using the Gene Count Q-96 thermocycler instrument (LuminUltra Technologies, Ltd., Fredericton, NB, CA).

8.3.5 Water Quality Analysis

In-situ pH, dissolved oxygen, conductivity, total dissolved solids, and temperature were measured using a YSI Professional Plus sonde. All laboratory water quality characterization of grab samples was completed within 48-h of sample collection. Concentrations of dissolved and total organic carbon (DOC, TOC) were quantified using a Total Organic Carbon Analyser (Shimadzu, TOC-VCPH). Turbidity was measured using a HACH 2100AN 32 turbidimeter. Ultraviolet absorbance at 254 nm (UV_{254}) and actual colour were measured on a HACH Spectrophotometer. Total aluminum, iron, and phosphorus were analyzed via inductively coupled plasma—mass spectrometry (ICP-MS) using an X-Series II ICP-MS (Thermo Fisher Scientific, Oakwood, OH, USA).

8.3.6 Statistical Analysis

Concentrations of viral target detections between the two sampling sites were compared using the paired samples Wilcoxon test with a significance level of $\alpha = 0.05$. All statistical analyses and generation of figures were completed using R Studio (version 4.2.3) and packages including tidyverse, scales, janitor and ggtext [62–65]. A corresponding Cq value characterized all samples, and gene concentrations were calculated based on the respective calibration curves generated for each viral target (Table S3). To determine the performance of each assay, the slopes (S) of the regression lines were used to calculate the amplification efficiency (Ɛ) of each calibration curve, according to the formula $Ɛ = 10^{|-1/s|} - 1$. Total gene copies (GC) recovered in passive and grab samples were computed by Eq. (1).

Eq. (1)

$$Total\ GC = \frac{\frac{GC}{rxn} * \frac{rxn}{3\ \mu L} * (500\ \mu L + 1000\ \mu L)}{1000 \mu L} * (6000\ \mu L)$$

8.3.7 Quality Assurance-Quality Control (QA-QC)

Standards outlined in the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Table S4) and environmental microbiology minimum information (EMMI) guidelines were consulted to ensure the reliability of the RT-qPCR results [66,67]. All consumables were either purchased pre-sterilized or were autoclave sterilized. All RT-qPCR reagents were prepared as single-use aliquots to ensure the reliability of RT-qPCR results and prevent potential issues such as cross-contamination or degradation of stock solutions. The quality and functionality of a freshly acquired batch of RT-qPCR reagents, including primers, probes, and mastermix, were verified before utilization. This verification process involved analyzing ten replicates of no-template controls alongside at least one positive template control [68]. Each workstation included its laboratory equipment, along with all laboratory supplies, reagents, and personal protective equipment. Ultraviolet light (90-minute exposure) and DNase/RNase-free water were used on all lab work surfaces after decontamination by 1% bleach for ~30 minutes. To ensure methodological integrity, unidirectional workflow was implemented, accompanied by the establishment of distinct autonomous working areas. For nucleic acid

extractions, a Thermo Scientific 1300 Series A2 biosafety cabinet was used, and for qPCR reaction prep a Mystaire MY Model PCR Prep Station Class 100 laminar flow enclosure was used. Several controls were used throughout each sample processing and analysis procedure, including a concentration control to monitor the process efficiency of each analysis (bacteriophage MS2), a negative nucleic acid extraction control, and positive and negative RT-qPCR template controls. DNase/RNase-free water served as the process and template negative controls, and synthetic reference material for each virus was used for positive controls during RT-qPCR analysis (Table S5). Quantitative results were reported based on a Cq value threshold of ≤ 37 cycles. Results below this threshold were considered non-detect. Any results obtained from samples where process blanks or no template controls were amplified, were excluded from the analysis and rerun.

8.4 Results and Discussion

8.4.1 Prevalence of Human Viruses in Freshwater Environments

Across three months, 20 passive sampling events and 33 grab sample events occurred at two locations in a freshwater lake in Nova Scotia, Canada. Grab and passive samples were analyzed by RT-qPCR methods to determine the presence of SARS-CoV-2, Influenza A, RSV, Measles, Adenovirus, Enterovirus, Rotavirus, and Norovirus. The general water quality of the lake is shown in Table S1; briefly, the water temperature in the lake ranged from 14.1 to 25.5 °C, DOC ranged from 2.2 to 2.5 mg L^{-1}, and pH ranged from 5.7 to 7.9. Importantly, these water quality parameters would indicate a healthy lake within the region and did not exhibit any signs of significant water quality inconsistencies or contamination.

Grab samples were found to have a 0.38% detection rate for viruses included in this study. There was a single grab sample detection for RSV in June of the study period. GAC passive samples were found to have a 38.8% total positive detection rate, with seven of the eight viruses included in this study being detected. No MeV was present in the passive samplers, which was expected given that Canada has no sustained circulation of this virus [44]. As shown in Figure 8-1, AdV was the most prevalent, with an overall positive detection of 80%, followed by RSV, NV, EnV, INFA and SARS-CoV-2, each with positive detections of 60%, 55%, 50%, 40% and 20%, respectively. RV was detected at the lowest prevalence with a positive detection of 5%. These detection frequencies are particularly

notable in the study lake because there is no centralized municipal wastewater effluent inflow to the lake. Human viral inputs are limited to direct human vectors during recreation, overland runoff during rain events, subsurface discharge from nearby septic systems, or possible unregulated direct discharge from shoreline residences.

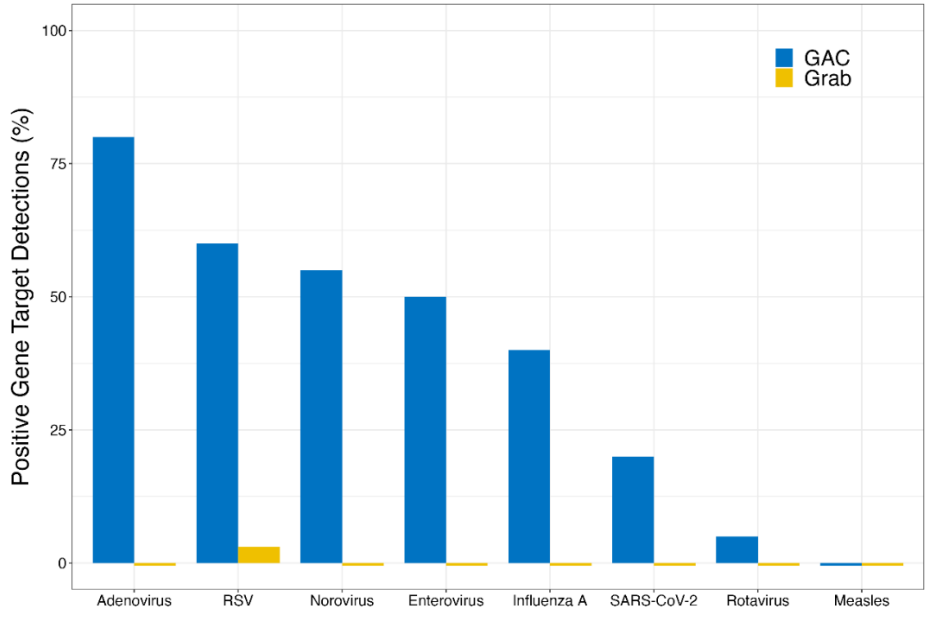

Figure 8-1. Percent positive gene target detections for the eight viruses using the two sampling methods. The detection frequencies were computed using the total number of samples from both locations.

The findings presented in Figure 8-1 indicate the positive detection rates for the two sampling locations. Statistical analysis found no significant differences in concentrations of the target genes between the two locations ($p < 0.05$, Wilcoxon signed rank test), except for the RSV target. At Site 1, the RSV gene concentrations were detected at approximately 1.3×10^7 GC (CI = 6.4×10^5 to 5.7×10^8 GC) more than at Site 2 ($p = 0.03$). The cause for this spatial variation for RSV is unknown, as RSV has not been previously studied in freshwater.

The absence of viral detection in relatively small volumes of freshwater highlights why most recreational water guidance relies on *E. coli* and enterococci monitoring to estimate fecal contamination. Monitoring of FIOs in this study found *E. coli* and enterococci concentrations to have a geometric mean of 14.9 CFU per 100 mL and 46.1 CFU per 100 mL, respectively. The low *E. coli* concentrations and undetectable viral concentrations in grab water samples indicate mild fecal contamination and limited viral presence. However,

passive sampling revealed high viral prevalence in the same water body, highlighting the uncertainty of using grab samples alone for monitoring viral occurrence in freshwaters. Grab samples may not always reflect the actual microbial load due to spatial and temporal variations in microbial distribution. Results indicate that passive samplers provide an effective in-situ concentration of otherwise undetectable levels of viral presence. However, meaningful interpretation of this data will require methods for correlating viral loads accumulated on the sampler with corresponding human health impact.

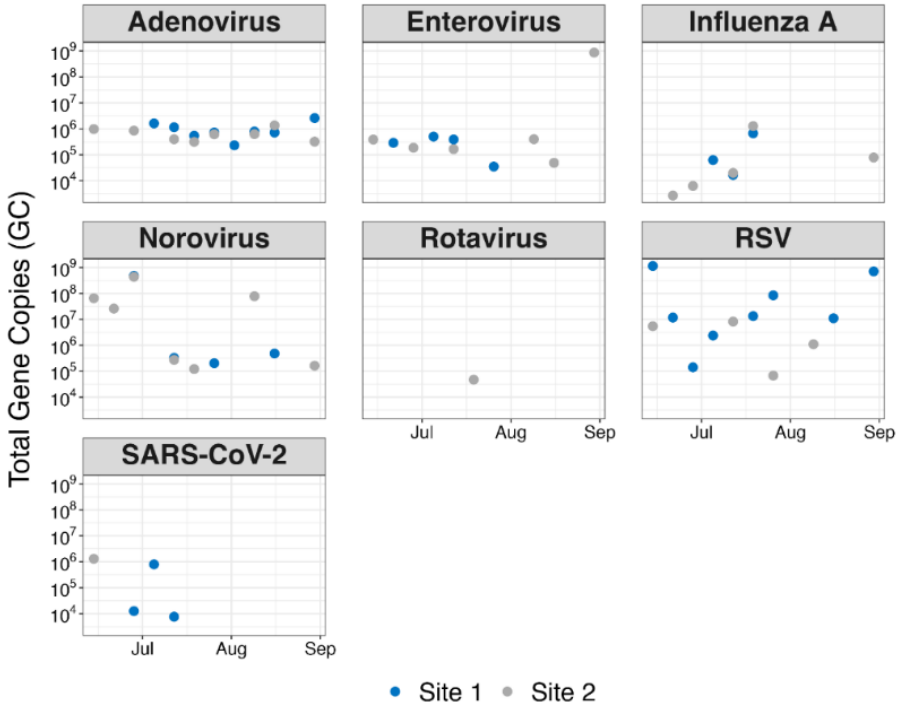

Figure 8-2. Concentrations of total viral gene copies detected from the GAC passive sampler. The concentrations obtained from each of the two sample locations are shown and denoted by the points' colours. Samplers at both sampling sites were deployed and collected weekly. MeV is not shown, as it was not detected in any samples.

The total number of gene copies detected for each virus per sampling event is shown in Figure 8-2. The maximum total gene copies recovered during the sampling period for each virus were as follows: INFA (4.2×10^5 GC), RSV (3.9×10^8 GC), SARS-CoV-2 (4.5×10^5 GC), AdV (8.7×10^5 GC), RV (1.5×10^4 GC), NV (1.6×10^8 GC) and EnV (2.8×10^8 GC). Because the volume of lake water in contact with the GAC and the adsorption kinetics over time for the viruses are unknown, we cannot translate these findings to an aqueous concentration in the lake. However, understanding the magnitude

of viral abundances and therefore fluctuations is noteworthy to understand viral fate and transport in freshwaters. Further, these results provide a basis for comparative analysis against conventional concentration methods, shedding light on their limitations in efficiency in capturing viruses. Table 8-1 summarizes eight recent studies of viral monitoring in surface and groundwaters globally and includes location, water body type, the volume of water sampled, positive detection rate, maximum concentration (in GC L^{-1}), total gene copies (generated for comparison to this study), and method of concentration used.

When compared to the studies shown in Table 1, the passive sampling method found similar positive detection frequencies of AdV and NV as Pang et al. (2019) and Vergara et al. (2016), with these viruses having the highest detection rates of all viruses studied, in both surface waters and groundwaters [45,46]. Most research on enteric viral presence in freshwater bodies has detected AdV up to 4-log higher concentrations than other enteric viruses [47,48]. The passive sampling results align with past findings of pervasive AdV and a lower abundance of RV. RV abundance has also fluctuated seasonally, with lower abundance observed during warmer months [49,50]. Li et al. (2023) found that RV gene concentrations reduced by over 10-fold during summer months compared to winter months [31]. This may account for the low detections of RV in this work, or the virus may not have been widespread during this sampling period. In general, the GAC passive sampler collected orders of magnitude more enteric viral gene targets than other studies in Table 1. A recent groundwater study by Stokdyk et al. (2020) reported very low detection frequencies for all enteric viruses analyzed. However, when adjusted for sampling volume, total gene copies detected approached the order of magnitude observed in the current study [51]. Stokdyk et al. (2020) collected and concentrated upwards of 1800 L of water to quantify viruses of interest. GAC passive sampling may provide a cost-effective and robust method of in-situ viral target concentration for tracking enteric virus presence and abundance in freshwaters.

While comparative research is available for enteric viruses, data on the prevalence of non-enteric viruses in freshwaters, such as SARS-CoV-2, INFA, RSV and MeV, are limited or non-existent. While RSV has been detected in wastewater [40,52], to our

knowledge, this is the first study to evaluate RSV prevalence in a freshwater environment. Likewise, reports on the occurrence of INFA viruses in freshwater environments are still limited. Current research has primarily focused on avian virus subtypes in surface waters, often with low recoverable viral loads [53,54]. Only two previous studies have documented the detection of SARS-CoV-2 in freshwater environments [55,56]. Mahlknechtt et al. (2021) found that SARS-CoV-2 concentrations in surface waters varied based on seasonality and wastewater discharge events, with temporal fluctuations reflecting virus epidemiological trends [56]. Hemalatha et al. (2022) reported no detection of SARS-CoV-2 in peri-urban or rural lakes, while urban lakes exhibited a prevalence of SARS-CoV-2 consistent with clinically reported infections [55]. The GAC passive sampling results show that both RSV and INFA are highly abundant in the study lake, with total gene copies detected in the same range as AdV and NV. Comparative data are scarce for non-enteric viruses in freshwaters; therefore, the results of this study emphasize the need for further research to enhance our understanding of respiratory virus prevalence and behaviour in these environments.

Table 8-1. Aggregated published data for the detection of pathogenic viruses in freshwater environments.

Region	Water Source	Virus	Volume Collected and Processed (L)	Sample Type	% Pos.	Maximum Gene Copies L^{-1}	Total GC Detect.	Virus Conc. Method
India [55]	Fresh-water Lake	SARS-CoV-2	1	Grab	75%	9.9×10^{4}	9.9×10^{4}	Ultrafiltration
Germany [53]	Fresh-water Lake	Avian INF	10	Grab	69%	N/A	N/A	Ultrafiltration
Asia [46]	Freshwater Lake	NV	10	Composite	75%	4.5×10^{3}	4.5×10^{4}	Ultrafiltration
		AdV			60%	5.4×10^{3}	5.4×10^{4}	
Asia [57]	Fresh-water River	NV GI	1	Grab	13%	6.6×10^{4}	6.6×10^{4}	Adsorption-elution
		NV GII			2%	6.8×10^{2}	6.8×10^{2}	
		AdV			39%	3.4×10^{4}	3.4×10^{4}	
		Sapovirus			5%	1.6×10^{3}	1.6×10^{3}	
		Polyomavirus			2%	5.0×10^{2}	5.0×10^{2}	
		Torque teno virus			3%	1.8×10^{3}	1.8×10^{3}	
Alberta, Canada [45]*	Fresh-water River	NV	20	Grab	75%	4.2×10^{0}*	8.4×10^{1}	Adsorption-elution
		RV			100 %	4.5×10^{0}*	9.0×10^{1}	
		Sapovirus			75%	4.3×10^{0}*	8.6×10^{1}	
		Astrovirus			92%	3.8×10^{0}*	7.6×10^{1}	
		EnV			58%	2.6×10^{0}*	5.2×10^{1}	
		AdV			92%	4.4×10^{0}*	8.8×10^{1}	
		Polyomavirus			83%	2.9×10^{0}*	5.8×10^{1}	
Mexico [56]	Ground-water	SARS-CoV-2	0.125	Grab	44%	3.8×10^{4}	4.8×10^{3}	None

Region	Water Source	Virus	Volume Collected and Processed (L)	Sample Type	% Pos.	Maximum Gene Copies L^{-1}	Total GC Detect.	Virus Conc. Method
Minnesota, USA [51]	Ground-water	AdV	140-1783	Grab	2.50 %	6.4×10^{2}	1.1×10^{6}	Ultrafiltration
		EnV			0.90 %	2.3×10^{0}	4.1×10^{3}	
		NV			0.50 %	$2.2\text{x}10^{2}$	3.9×10^{5}	
		RV			1.50 %	2.3×10^{2}	4.1×10^{5}	
Alberta, Canada [58]	Ground-water	AdV	500	Grab	3.00 %	8.6×10^{1}	4.3×10^{4}	Adsorption-elution
		NV			<1%	N/A	≤ 60	
		RV			3.00 %	2.2×10^{1}	1.1×10^{4}	
		Polyomavirus			<1%	6.8×10^{1}	3.4×10^{4}	
		Reovirus			1.00 %	3.4×10^{1}	1.7×10^{4}	

**Data is presented in terms of median concentration, and only one of six rivers from the study is presented.*

The results from this study show that GAC serves as an effective adsorbent media for *in situ* concentration of viruses in freshwater lakes, either through direct viral adsorption or, more likely, through the adsorption of particle- and sediment-bound viruses. Although the exact mechanisms driving adsorption between GAC and each viral target in these environments are largely still unknown, previous work has observed the ability of GAC to serve as a non-selective media for viral capture in aqueous environments. Cormier et al. (2014) reported that activated carbon could remove upwards of 6 Log PFU^{-1} of MS2 from seeded seawater and freshwater [35]. The capture and recovery of SARS-CoV-2, PMMoV, and CrAssphage have been shown in both deionized water and wastewater samples [38], and RSV and INF viruses have been detected using GAC in wastewater [40]. GAC has also been used as an adsorptive media for viral capture in drinking water point-of-use filtration devices, with a known capacity for removing enteric viruses upwards of 99.9% [59]. The results of our work align with these previous reports, showing measurable concentrations of both double-stranded (RV) and single-stranded (EnV, INFA, NV, and SARS-CoV-2) RNA viruses, in enveloped and non-enveloped form, as well as a non-

enveloped double-stranded DNA virus (AdV). This study demonstrates the application of passive sampling in freshwater systems to understand viral occurrence.

The present study highlights the range of recoveries for different viral concentration methods and the influence of various sampling conditions. Therefore, factors such as cost, ease of use, need for recovery controls, and a method's ability to achieve the study's specific objectives should be considered when selecting a method [60]. This approach can lead to more practical and adequate decision-making in monitoring viral contamination in various environmental samples. To ensure the safety of recreational and drinking water supplies, it is crucial to consider the limitations of grab samples and explore alternative methods, such as passive samplers, for monitoring viral occurrence in freshwater environments.

8.4.2 Summary of Future Research Needs

Information on the concentration of viruses in natural waters is critical to understanding the risk of infection and the effectiveness of controls to limit exposure. However, current knowledge on the occurrence of viruses in freshwater environments is largely limited to enteroviruses, and much of this work is constrained to academic studies and occasional commercial research, with an overreliance on FIOs for policy development, water treatment standards and public health guidelines. There is a limited understanding of the spatial and hydrological influence of viral abundances in freshwater, regardless of the concentration method used. Conventional concentration methods often rely on water collected at a single point in time and from a single location. Passive sampling methods offer valuable insights into time-integrated viral concentrations, offering a more accurate spatial and temporal representation of viral abundance in water sources. The emerging information obtained through passive sampling is currently of great interest and has generated ongoing discussions in the research field [43]. Future research should work to enhance our understanding of viral dynamics throughout the deployment phase and also establish a baseline for evaluating the efficiency of passive samplers in direct relation to volume-based metrics, providing valuable insights for future monitoring and management efforts.

Applying GAC-based passive sampling could advance viral monitoring in recreational and drinking water sources to inform water quality management better. To fully leverage the utility of passive sampling for viral monitoring, future studies need to investigate temporal and spatial dimensions of passive sampling in freshwater bodies. To establish effective environmental surveillance and guide policy on viral monitoring, future work must establish passive sampling procedures for fecal contamination and viral abundance in freshwater environments. This can inform public health decisions and refine drinking water treatment technologies.

8.5 Conclusions

Despite advances in drinking water and wastewater treatment, water-related pathogenic viruses remain a public health concern globally. We have presented GAC passive sampling as a potentially viable, simple, and cost-effective way of simultaneous *in situ* concentration of a range of enteric and non-enteric viruses in freshwaters. Future work is needed to characterize better adsorptive mechanisms, the role of equilibrium and kinetics in viral or soil-bound viral uptake, survival and transport, and the relationship between passive sampling and viral loads and exposure risks to the public. Our findings may have far-reaching implications for reducing barriers associated with viral monitoring across various environmental contexts.

8.6 Data Availability Statement

All data and code needed to reproduce the analyses are available online (https://github.com/djredden/lake_viruses/tree/main).

8.7 Author Contributions

E.H. and G.G. conceived the project, and G.G. supervised the project. D.R. collected field samples, and E.H. and M.G. analyzed all grab and passive samples via RT-qPCR. D.R. refined sample elution from GAC, and E.H. and M.G. developed the multiplex methodology. M.F., E.H., and M.G. wrote the manuscript, and M.F. provided significant editing during the writing process. D.R. provided data visualization assistance and statistical analysis. All authors have read and agreed to the submitted version of the manuscript.

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CHAPTER 9 CONCLUDING STATEMENTS

This thesis explored the development and application of novel passive sampling techniques for the detection of viruses from wastewater and freshwater systems. This work aimed to enhance the accessibility and sensitivity of viral monitoring techniques through innovative passive sampling techniques. A summary of key findings follows.

Cotton cheesecloth and electronegative cellulose-nitrate membrane filters have proven effective in detecting SARS-CoV-2 in wastewater, notably through the use of a novel 3D-printed cage designed for sewershed deployments. These findings underscored the viability of these materials in capturing SARS-CoV-2, especially in communities with a low prevalence of COVID-19. Moreover, the use of electronegative membrane filters, combined with allele-specific RT-qPCR, has enabled the retrospective detection of the SARS-CoV-2 omicron variant within a university surveillance program. These results highlight the adaptability and potential of passive samplers for monitoring viral evolution and emerging threats through wastewater.

Investigation into the adsorption behaviour of SARS-CoV-2 by electronegative cellulose-nitrate membrane filters has revealed insights into the mechanisms driving effective viral adsorption in wastewater. The adsorption process aligns with the Pseudo-First Order rate model and Freundlich isotherm, suggesting a heterogeneous adsorption surface suitable for environments with moderate levels of total suspended solids (TSS). This work also indicated that TSS concentrations can impact the effectiveness of the membrane filters, with higher TSS potentially hindering downstream RNA extraction and amplification processes. Furthermore, field and bench-scale analysis demonstrated that optimal SARS-CoV-2 RNA capture is attainable within 24 to 48 hours of filter deployment. This duration is likely to ensure efficiency without exceeding the filters' maximum adsorption capacity.

Given the identified constraints in the adsorptive capacity of the electronegative cellulose-nitrate membrane filters, subsequent exploration into alternative materials led to the discovery of granular activated carbon (GAC) as an improved media for passive sampling and capture of SARS-CoV-2 from wastewater. In comparison to cellulose-nitrate membrane filters, GAC showed improved sensitivity and consistency in detecting SARS-CoV-2 from wastewater and demonstrated the capability to adsorb other microbial targets

like PMMoV and CrAssphage. Given its affordability and availability, GAC presents a scalable and accessible option for viral surveillance in wastewater and freshwater environments.

This work also evaluated a novel multiplex RT-qPCR assay designed to simultaneously detect four respiratory viruses, (SARS-CoV-2, RSV, Influenza A, and Measles) to enhance our viral surveillance capabilities. The sensitivity of this multiplex assay was comparable to traditional monoplex assays, as demonstrated by bench-scale experiments. The findings suggest that while monoplex assays are still widely used, the introduction of multiplex detection methods such as the one described in this work offers a rapid and efficient alternative for routine viral monitoring in water systems. Multiplex detection used in conjunction with sensitive sample collection techniques, such as GAC-based passive sampling, enhance the overall effectiveness of wastewater surveillance of viral pathogens.

Finally, this work extended the innovative GAC-based sampling approach to the surveillance of viruses in a freshwater lake, demonstrating its capabilities across diverse water systems. The use of GAC for *in situ* concentration of various enteric and non-enteric viruses, not only highlights the method's effectiveness in freshwater but also the method's simplicity and improved detection capabilities compared to traditional grab sampling techniques. The improved virus detection rates with GAC emphasized the importance of further investigations into the adsorptive processes at play and the potential correlation between passive sampling data and actual viral load exposure risks. The broad applicability of GAC passive sampling in advancing virus monitoring and potentially reducing public health risks associated with viral contamination was evident in both freshwater and wastewater systems.

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APPENDIX A: KNOWLEDGE TRANSLATION THROUGH THE GLOBAL ADOPTION OF PASSIVE SAMPLING METHODS DEVELOPED IN THIS THESIS

Table A.1: List of international and domestic locations where the passive sampling methods developed in this research were adopted.

Context	Institution	Location
Academic	DVGW Water Tech Center	Germany, Europe
Academic	Louisiana State University	Louisiana, United States
Academic	Sorbonne University	France, Europe
Academic	Dalhousie University – Faculty of Medicine	Rwanda, Africa
Academic	Dalhousie University - CWRS	Nova Scotia, Canada
Academic	Laval University	Quebec, Canada
Academic	Queens University	Ontario, Canada
Academic	Memorial University	Newfoundland, Canada
Academic	St. Francis University	Nova Scotia, Canada
Academic	Acadia University	Nova Scotia, Canada
Academic	Cape Breton University	Nova Scotia, Canada
Academic	Bangor University	Whale, United Kingdom
Academic	University of West Virginia	Virginia, United States
Academic	University of Saskatchewan	Saskatchewan, Canada
Utility	Municipality of Colchester	Nova Scotia, Canada
Utility	The Richmond Public Works Department	Nova Scotia, Canada
Utility	Greater Moncton Wastewater Commission	New Brunswick, Canada
Utility	Department of Municipal & Community Affairs, Government of the Northwest Territories	Northwest Territories, Canada
Utility	Taiga Environmental Laboratory, Department of Environmental & Natural Resources	Northwest Territories, Canada
Utility	Atlantic First Nations Water Authority	Nova Scotia, Canada
Government	Dept. of Community & Govern. Services	Nunavut, Canada
Government	Public Health Department of the James Bay Cree Territory	Quebec, Canada
Policy	Centre for Disease Control and Prevention	Atlanta, United States
Public Health	Nova Scotia Health Authority	Nova Scotia, Canada

Context	Institution	Location
Public Health	British Columbia Centre for Disease Control	British Columbia, Canada
Public Health	Wastewater Surveillance Unit, National Microbiology Laboratory, Public Health Agency of Canada	Manitoba, Canada
Industry	Institute of Environmental Science & Research Limited	Wellington, New Zealand
Industry	LuminUltra Technologies Ltd.	New Brunswick, Canada
Industry	CBCL, Ltd.	Nova Scotia, Canada

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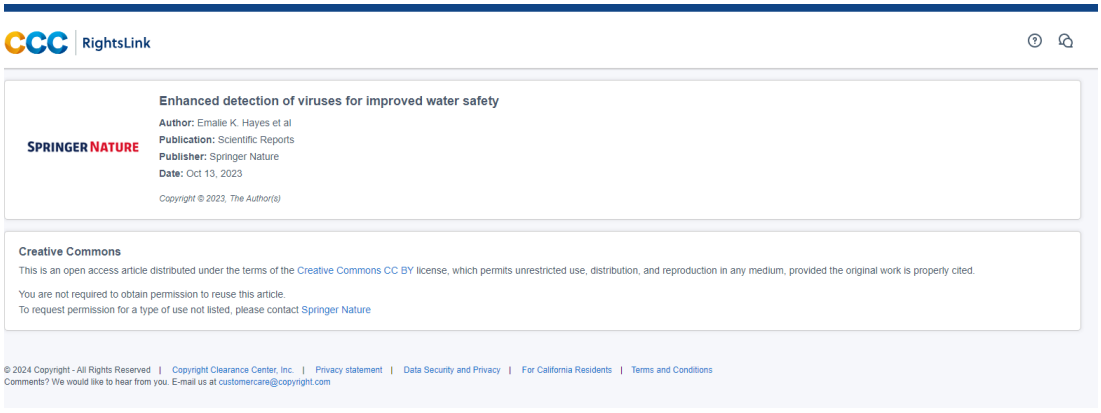

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Operational Constraints of Detecting SARS-CoV-2 on Passive Samplers using Electronegative Filters: A Kinetic and Equilibrium Analysis

Emalie K. Hayes, Crystal L. Sweeney, Megan Fuller, Genevieve B. Erjavec, Amina K. Stoddart, and Graham A. Gagnon*

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SUBJECTS: Adsorption, Computer simulations, Genetics, SARS-CoV-2, Wastewater

the wastewater surveillance of SARS-CoV-2 ribonucleic acid (RNA), the t. Passive sampling has been shown to be an effective tool to detect SARS-CoV aracteristics of SARS-CoV-2 RNA on passive sampling material are not well- p between wastewater surveillance and community infection. In this work, adsorption kinetics and equilibrium characteristics were evaluated using batch-adsorption experiments for heat-inactivated SARS-CoV-2 (HI-SCV-2) adsorption to electronegative filters. Equilibrium isotherms were assessed or a range of total suspended solids (TSS) concentrations (118, 265, and 497 mg L^{-1}) in wastewater, and a modeled q_{max} of 7×10^3 GU cm^{-2} was found. Surrogate adsorption kinetics followed a pseudo-first-order model in wastewater with maximum concentrations achieved within 24 h. In both field and isotherm experiments, equilibrium behavior and viral recovery were found to be associated with

Adsorption Isotherms

Log qt (GU cm^{-2})

Wastewater | DI water

Time

Sampling in the Field

•Deployment of ~ 24 h

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Adsorption of SARS-CoV-2 onto granular activated carbon (GAC) in wastewater: Implications for improvements in passive sampling

Author: Emalie K. Hayes,Amina K. Stoddart,Graham A. Gagnon
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Simultaneous detection of SARS-CoV-2, influenza A, respiratory syncytial virus, and measles in wastewater by multiplex RT-qPCR

Author: Emalie K. Hayes,Madison T. Gouthro,Jason J. LeBlanc,Graham A. Gagnon
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APPENDIX C: CHAPTER 2 SUPPORTING MATERIAL

Table S1 Supplemental references used in the meta-analysis, however, not included in the main text of the manuscript.

No.	Reference – Passive Sampling Review
1	Lambert-Slosarska, K. 2023. Use of passive samplers for the capture of SARS-CoV-2 and other viruses from wastewater—Bangor University. MScRes.
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APPENDIX D: CHAPTER 3 SUPPORTING MATERIAL

RNA Extraction

A volume of 1 mL of sample (wastewater or COSCa eluate) was combined with 6.5 mL of a lysis buffer solution, vortexed at 3000 rpm for 30 sec, and incubated at 30 °C for 10 min. Following incubation, 3.5 mL EtOH was added and thoroughly mixed, then 100 µL of the binding beads mixture was added to the lysed sample, vortexed at 3000 rpm for 30 sec and incubated at 30 °C for 10 min. To precipitate the magnetic beads, a magnet was applied, and the supernatant was discarded. The magnetic beads were washed three times with 1 mL of a wash solution and again three times with 1 mL of another wash solution. Between each wash, the magnetic beads were vortexed for 30 sec, precipitated and the supernatant was discarded. Once washed, the magnetic beads were then left to dry at room temperature for 1 h to evaporate residual EtOH. To elute the RNA from the magnetic beads, 50 µL of preheated (60 °C) elution buffer was added to the magnetic beads, then vortexed at 1500 rpm for 30 sec and incubated at 60 °C for 5 min. Using a magnet, the magnetic beads were separated from the elution, and the eluted RNA was collected and transferred to a separate tube for RT-qPCR analysis.

The LuminUltra RT-qPCR software requires input of the 1 mL RNA extraction sample volume as the RNA concentration calculation is based on the initial amount of sample processed. The concentration is calculated from the standard curve in the software which takes into account the 5-µL volume used in the reaction, 50 µL extracted, and the original 1 mL processed: $y = -3.74x + 40.3$ where y = Ct and x = concentration.

Figure S1. The COVID-19 Sewer Cage (COSCa), is a passive sampling device for monitoring SARS-CoV-2 in municipal wastewater.

Figure S2. Sewer catchment sampling locations for monitoring SARS-CoV-2 in municipal wastewater using a 3D-printed passive sampling device and two types of adsorbent material. The map in this figure was created using © OpenStreetMap contributors (openstreetmap.org).

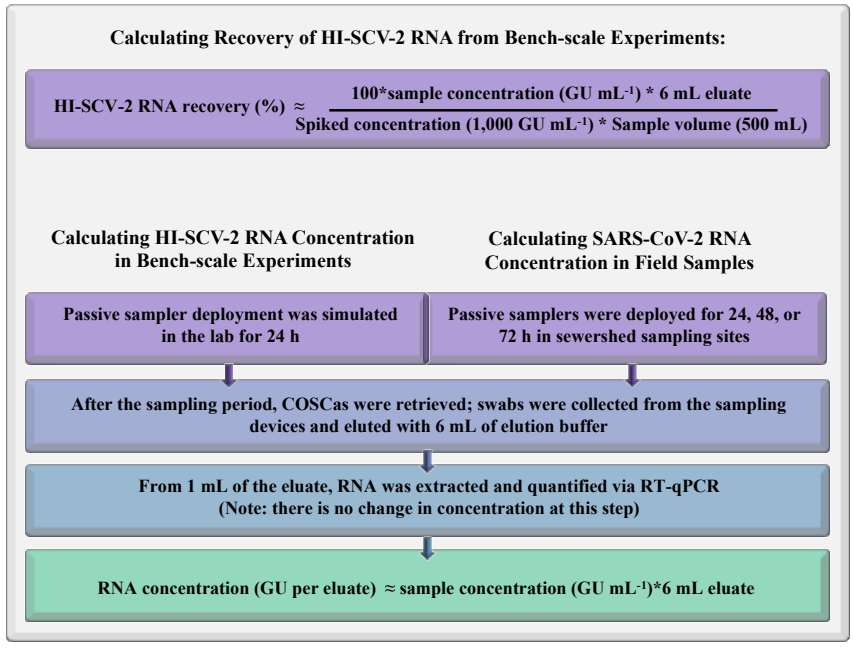

Figure S3. Calculation of RNA recovery (bench-scale experiments) and concentration (bench-scale experiments and field samples) from passive sampling material.

Table S1. Sample eluate and RNA dilutions for each sampling event at Location A.

Sampling Event	SARS-CoV-2 (GU per eluate)	Days	Deployment (hrs)	Eluate Dilution	RNA Dilution
1	16380	1	24	None	None
2	0	4	72	None	None
3	0	5	72	None	None
4	0	7	24	None	None
5	1812	8	72	None	None
6	7860	11	72	None	None
7	0	25	48	None	None
8	0	27	168	None	None
9	1932	34	48	1:5	None
10	591	36	24	1:5	None
11	396.6	37	28.5	None	None
12	262.2	38	48	None	None
13	0	40	24	None	None
14	0	41	24	None	None
15	0	42	24	1:1	None

Table S2. Sample eluate and RNA dilutions for each sampling event at Location B.

Sampling Event	SARS-CoV-2 (GU per eluate)	Days	Deployment (hrs)	Eluate Dilution	RNA Dilution
1	0	1	48	None	None
2	0	6	144	None	None
3	0	8	48	None	None
4	0	13	144	None	None
5	3276	15	48	None	1:1
6	6120	16	48	None	1:1
7	4512	19	72	None	1:1
8	0	21	48	None	1:1
9	0	23	48	None	1:1
10	0	26	72	None	1:1
11	0	28	48	None	1:1
12	1752	30	48	None	1:1
13	0	33	72	None	None; 1:1
14	3852	35	48	None	1:1
15	0	37	48	None	1:1

APPENDIX E: CHAPTER 4 SUPPORTING MATERIAL

Descriptions of COVID-19 case data in Nova Scotia, characterization of electronegative filters using SEM analysis, experimental adsorption equilibrium and kinetic isotherm model fitting, and additional field sampling data.

Nova Scotia Active COVID-19 Case Data

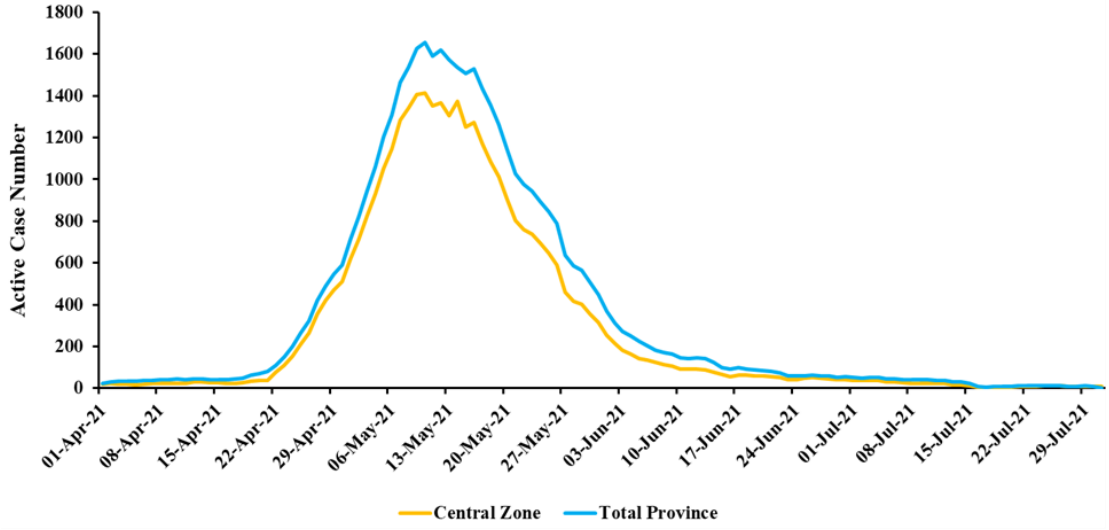

Figure S1. The average active COVID-19 cases throughout April, May, June, and July are presented for the NS central zone and the entire NS province. The Nova Scotia census data from 2016 show that the population in the central zone was 424, 037 and the total province's population was 923,727 [26].

Characterization of the Electronegative Filter

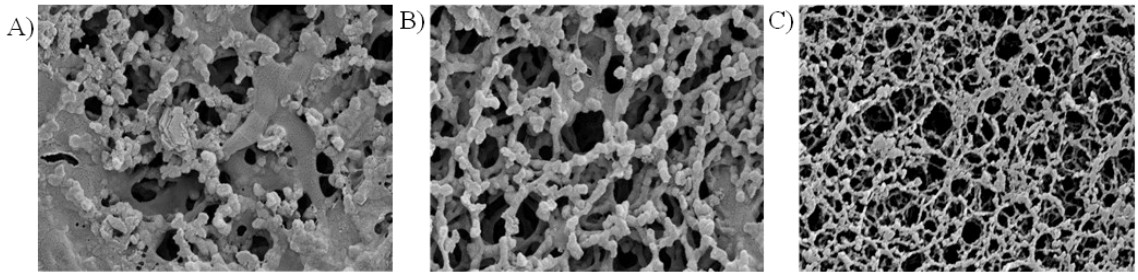

Figure S2. SEM images of gold sputter-coated electronegative filters: A) a filter collected from a wastewater sample after a 24-h sampling period (Mag: 10KX, 1µm); (B) a filter collected from a wastewater sample after a 24-h sampling period and eluted with a 0.075% Tween®20 + 25 mM Tris HCl based buffer (Mag: 10KX, 1 µm); and (C) an unexposed filter (Mag: 4KX, 2 µm). In the image (A), discrete particles are identifiable on the filter, and organic material can be seen between the fibres as a coating. Once eluted, there is significantly less organic coverage on and between the fibres of the material, as presented in the image (B). Image (C) shows the pristine matrix of a filter that has not been exposed to wastewater.

Equilibrium Isotherm Models

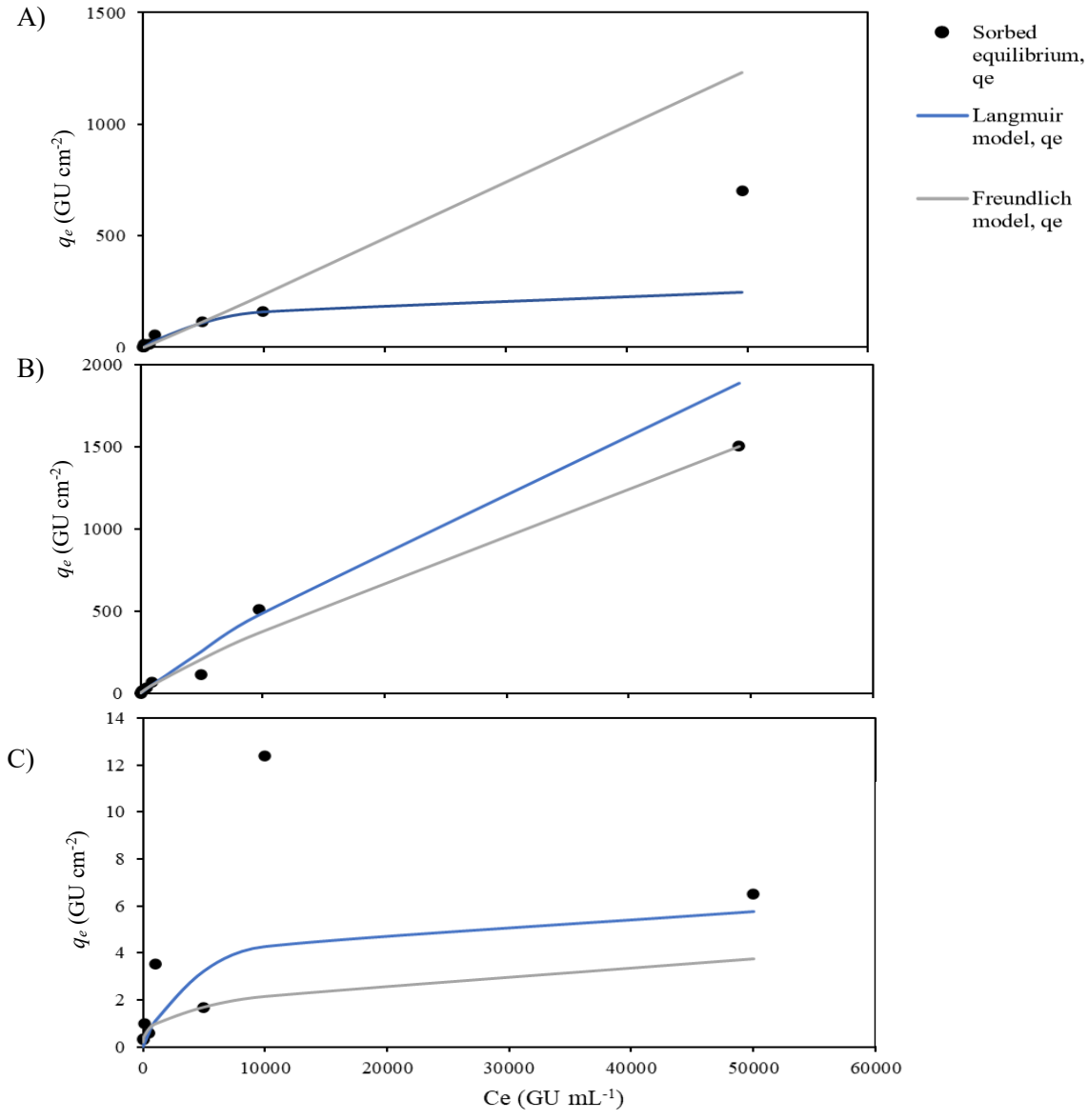

Figure S3. The sorbed equilibrium data (black circles), Langmuir (blue lines), and Freundlich (grey lines) equilibrium models (qe, GU cm^{-2}) are plotted across all graphs. HI-SCV-2 adsorption to an electronegative filter was assessed over 24 hours and a spiked concentration range of 1x10^{1} to 5x10^{4} GU mL^{-1}. Graph (A) represents Low TSS (118 mg L^{-1}) wastewater; Graph (B) displays the medium containing TSS (265 mg L-1) wastewater, and Graph (C) exhibits the high TSS (497 mg L^{-1}) comprising wastewater.

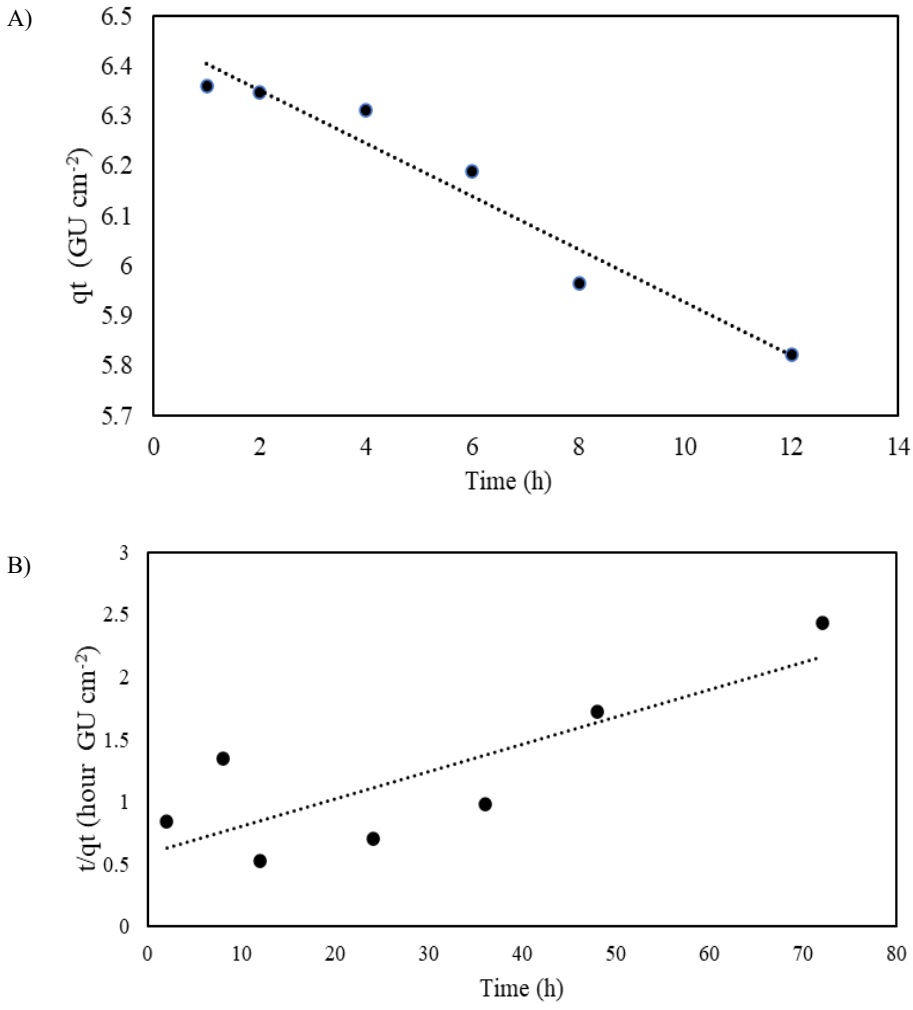

Figure S4. Laboratory-controlled batch-adsorption experiments were performed, and graph (A) displays the Pseudo-First-Order rate model for HI-SCV-2 adsorption to electronegative filters over a 24-h period in wastewater (TSS: 265 mg L-1). Graph (B) shows the Pseudo-Second-Order rate model for HI-SCV-2 adsorption to electronegative filters over 72-h in DI water spiked to 1x103 GU mL-1. Each matrix was spiked to 1x103 GU mL-1 with HI-SCV-2.

Field Sampling at Targeted Sewershed Locations: Additional Data

Table S1. For each sampling sewershed (Locations A, B, and C), the date of deployment and retrieval are indicated. Total deployment duration (h), corresponding cycle threshold (Ct), and final RNA concentrations (GU mL-1 & GU cm-2) are presented. The pass or failure of the RT-qPCR internal control is shown as either a Y or N (yes or no). Only samples are given that had positive detections, samples below the limit of detection were excluded.

Sampling Location	Date Deployed	Date Retrieved	Sampling Time (h)	Ct Value	Conc. (GU mL^{-1})	Conc. (GU cm^{-2})
Sewershed A	26-Apr-21	28-Apr-21	48	31.09	1.44E+04	1.36E+03
Sewershed A	28-Apr-21	30-Apr-21	48	33.54	1.25E+04	1.18E+03
Sewershed A	30-Apr-21	3-May-21	72	34.02	1.21E+04	1.14E+03
Sewershed A	3-May-21	5-May-21	48	32.72	2.14E+03	2.02E+02
Sewershed A	5-may-21	7-May-21	48	34.89	5.58E+02	5.26E+01
Sewershed A	7-May-21	10-May-21	72	34.67	2.48E+04	2.34E+03
Sewershed A	10-May-21	12-May-21	48	36.35	2.28E+02	2.15E+01
Sewershed A	12-May-21	14-May-21	48	34.1	2.73E+03	2.58E+02
Sewershed A	14-May-21	17-May-21	72	35.6	1.09E+03	1.03E+02
Sewershed A	17-May-21	19-May-21	48	36.13	1.31E+02	1.24E+01
Sewershed A	19-May-21	21-May-21	48	31.24	1.59E+04	1.50E+03
Sewershed A	4-June-21	7-Jun-21	72	34.6	1.72E+04	1.62E+03
Sewershed A	21-Jun-21	24-Jun-21	72	34.36	7.76E+02	7.32E+01
Sewershed B	20-Apr-21	22-Apr-21	48	34.93	5.46E+02	5.15E+01
Sewershed B	22-Apr-21	23-Apr-21	24	33.91	4.84E+04	4.57E+03
Sewershed B	23-Apr-21	26-Apr-21	72	34.28	2.33E+04	2.20E+03
Sewershed B	5-May-21	7-May-21	48	35.94	2.92E+02	2.75E+01
Sewershed B	10-May-21	12-May-21	48	34.66	5.17E+04	4.88E+03
Sewershed B	12-May-21	14-May-21	48	35.63	1.06E+03	1.00E+02
Sewershed B	4-Jun-21	7-Jun-21	72	32.71	2.14E+03	2.02E+02

Sampling Location	Date Deployed	Date Retrieved	Sampling Time (h)	Ct Value	Conc. (GU mL^{-1})	Conc. (GU cm^{-2})
Sewershed B	25-Jun-21	28-Jun-21	72	30.26	9.68E+03	9.13E+02
Sewershed B	28-Jun-21	30-Jun-21	48	33.73	1.14E+03	1.08E+02
Sewershed B	30-Jun-21	5-Jul-21	72	30.3	9.40E+03	8.87E+02
Sewershed B	5-Jul-21	8-Jul-21	72	32.56	7.08E+03	6.68E+02
Sewershed B	12-Jul-21	15-Jul-21	72	33.3	4.45E+03	4.20E+02
Sewershed C	28-Apr-21	30-Apr-21	48	35.59	3.64E+02	3.43E+01
Sewershed C	5-May-21	7-May-21	48	39.82	2.68E+01	2.53E+00
Sewershed C	31-May-21	2-Jun-21	48	35.33	4.26E+02	4.02E+01

APPENDIX F: CHAPTER 5 SUPPORTING MATERIAL

Sampling Sites

In this study, four university residences were evaluated (Figure S1), each one of the residences contained a meal hall facility, communal washrooms on each floor, and multiple laundry rooms[1–3]. However, Residence D is unique as it encompasses multiple student services (International Centre, Welcome Centre and Recruitment Office, Admissions Office, and the Student Health and Wellness Centre)[4]. All of the student services contribute to the same sewershed locations as the student housing in Residence D. At each of the residences, grey water and black water exit the buildings via the sanitary sewer lines which are accessed outside of the building nearby. Residence B is sectioned into six houses and contains a complex network of multiple sanitary sewer lines. Residences A, C and D are individual buildings with one accessible sanitary sewer line. All sampling sites are combined sewer systems; however, the proportion of stormwater is unknown. Halifax WWTF was constructed in 2007 and services the Halifax peninsula and surrounding area (Figure S2). The facility has a capacity of 340,000 m^3 per day and utilizes an advanced primary treatment process[5].

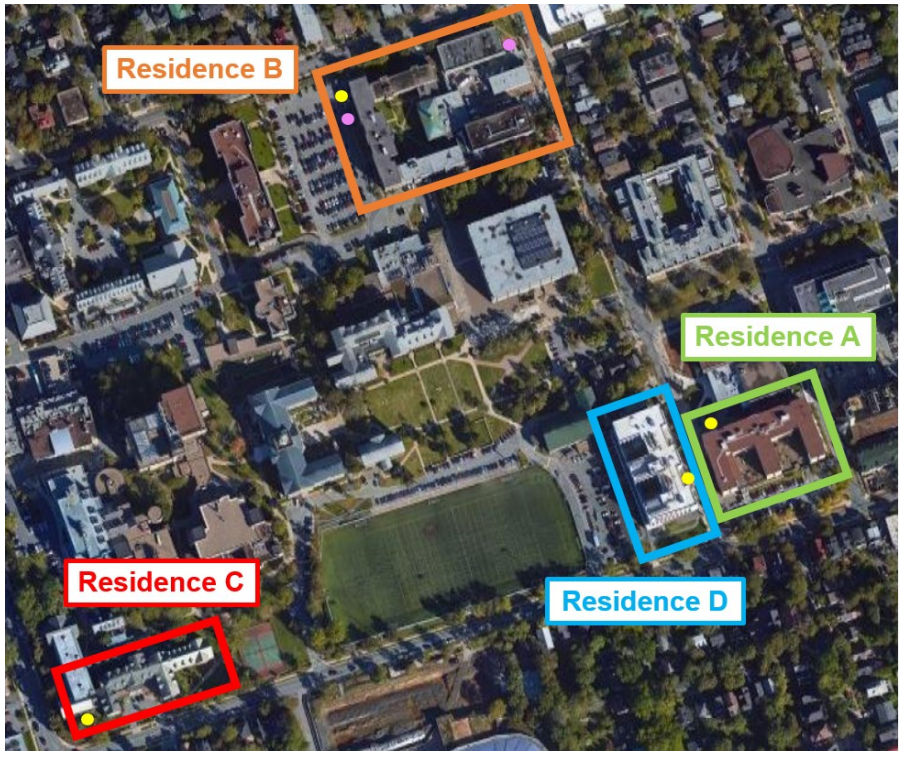

Figure S1. Satellite map of the four university residences. Yellow circles denote the approximate locations of passive sampling sites deployed at the start of the university Fall semester (05 to 15 September 2021). Pink circles denote sampling sites added on 12 December 2021 at Residence B.

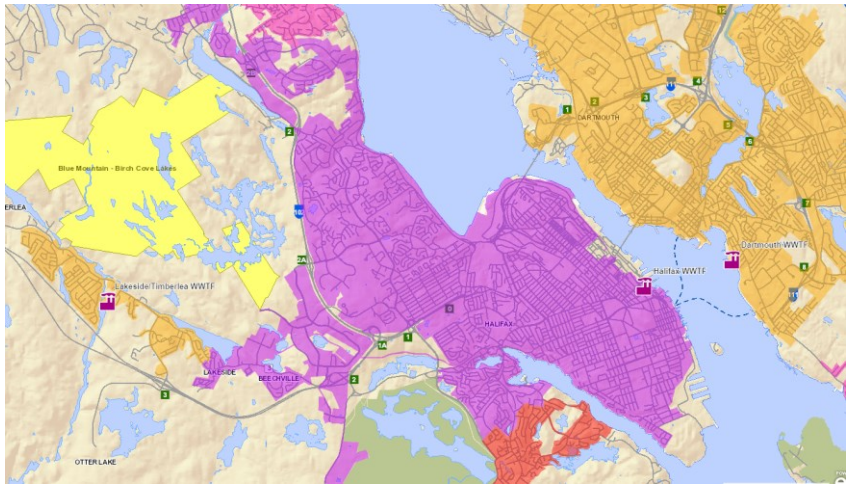

Figure S2. Catchment Area of Halifax, NS WWTF. The map portrays the contributing region (highlighted in purple) for wastewater inflow to the WWTF.

Table S1. Sequences of SARS-CoV-2 Alpha (B.1.1.7) and Delta (B.1.617) VOCs. All primers and probes were purchased through Integrated Technologies (IDT, Coralville, IA, USA) and stored based on manufacturer's recommendations.

Assay Information			**Ref**
Alpha (B.1.1.7)	Twist Control 14 Alpha (103907)	GISAID accession EPI_ISL_710528 and assigned to the B.1.1.7 (α) Pango lineage	[6]
Delta (B.1.617)	Pos DNA gblock Δ157-158	TTGTTATTAAAGTCTGTGAATTTC AATTTTGTAATGATCCATTTTTGG ATGTTTATTACCACA AAAACAACAAAAGTTGGATGGA AAGTGGAGTTTATTCTAGTGCGA ATAATTGCACTTTTGA ATATGTCTCTCAGCCTTTTCTTAT GGACCTTGAAGGAAAACAGGGT AATTTCAAAAATCTTAGGG	[7]

Table S2. The Minimal Information for Publications on Quantitative Real-Time PCR Experiments (MIQE) checklist of essential and desirable information that should be reported to enable the reviewer to judge the validity of the paper and the reader to repeat the experiment and reproduce the results.

Category	**Item**	**Paper Location**	**Author Comments**	**Check**

Sample	Type	Methods	Passive and 24 Composite samples	✓
	Method of dissection/procurement	Methods and Supplemental	No dissection, synthetic template manufacturers, and sample collection are described	✓
	Processing procedure	Methods	Pre-RNA extraction processing (Sampler elution & Composite centrifugation)	✓
	If frozen, how and how quickly?	Methods	RNA frozen, -80C prior to C28311T analysis	✓
	If fixed, with what and how quickly?	N/A	No fixing performed (i.e., no formalin-fixed procedures)	N/A
	Storage conditions and duration	Methods and Supplemental	Template control storage conditions are described in SI based on manufacturers recommendations.	✓
Extraction	Method or instrument	Methods	Commercial kit (LuminUltra Technologies Ltd).	✓
	Reagents/kits/modifications	Methods		✓
	DNAse or RNAse treatment	N/A	No specific treatment was completed; purification steps were included in the commercial kit used (LuminUltra	N/A

			Technologies Ltd).	
	Evidence for lack of contamination (DNA or RNA)	Supplemental and Methods	Non-template controls, and Extraction Blanks performed. No amplification observed in any.	✓
	Nucleic acid quantification	Supplemental	RT-qPCR analysis, and multiplex RT-qPCR analysis	✓
	RNA integrity	Supplemental		✓
Reverse transcription	Complete reaction conditions, including all components and their concentrations	Supplemental and Methods		✓
	RNA amount and reaction volume	Methods		✓
	Priming oligo sequence(s)	Supplemental		✓
	Cq values with and without reverse transcriptase	N/A	One-step RT-qPCR was used in this study	N/A
qPCR target	Sequence accession number	Supplemental and Methods	Primer sequences and template sequences used	✓
	Amplicon length	Supplemental and Methods		✓
	In silico specificity (BLAST)	Validated in previous work not performed in this study		✓
	Location by exon/intron	Supplemental		✓
	Identify the splice variants amplified	N/A		×
	All primer/probe sequences	Supplemental		✓

	Location and identity of any oligonucleotide modifications	Supplemental		✓
	Complete reaction conditions, including all components and their concentrations	Methods & Supplemental		✓
qPCR protocol	cDNA/DNA amount and reaction volume	Methods		✓
	Instrument identification and complete thermocycling parameters	Methods & Supplemental		✓
	Evidence for PCR specificity (gels, sequencing, or melting curves)	Validated by other researchers not in this study		×
qPCR validation	Template inhibition data (template titrations)	Supplemental	Extract dilutions were evaluated for inhibition, no inhibition was expected.	✓
	For SYBR Green I reactions, the Cq of the no template control	Methods	Didn't include data where no template control failed	✓
	Calibration curves with slope and intercept	Methods		✓
	PCR efficiency from the slope	Methods		✓
	r^2 of the calibration curve	Methods		✓
	Evidence for the linear dynamic range	Methods		✓
	Evidence for the limit of detection	Validated in previous publications – cited in methods		✓

	For multiplexed assays, the efficiency and limit of detection of each assay		Multiplex assay not used	N/A
	qPCR analysis method/software	Methods		✓
Data analysis	Method of Cq determination	Methods		✓
	Results of no template controls	Methods		✓
	Justification of number and choice of reference genes	Supplemental and methods		✓
	Normalization method	No normalization periods		×
	Number and stage (reverse transcription or qPCR) of technical replicates	Supplemental and Methods		✓
	Intra-assay variation in terms of concentration, not Cq	Not performed		×
	Statistical methods/software	Methods		✓

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1. Dalhousie University. Howe Hall. *Residence* https://www.dal.ca/campus_life/residence_housing/residence/halifax-campus/res-buildings-halifax/howe-hall.html.

2. Dalhousie University. Risley Hall. *Residence* https://www.dal.ca/campus_life/residence_housing/residence/halifax-campus/res-buildings-halifax/risley-hall.html.

3. Dalhousie University. Shirreff Hall. *Residence* https://www.dal.ca/campus_life/residence_housing/residence/halifax-campus/res-buildings-halifax/shirreff-hall.html.

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5. Halifax Water. Service Information. *Where Does My Wastewater Go?* https://hwc.maps.arcgis.com/apps/InformationLookup/index.html?appid=fe494fffcd144087a142dce3703afa8b.

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7. Yaniv, K., Ozer, E. & Kushmaro, A. *SARS-CoV-2 Variants of Concern, Gamma (P.1) and Delta (B.1.617), Sensitive Detection and Quantification in Wastewater Employing Direct RT-qPCR*. http://medrxiv.org/lookup/doi/10.1101/2021.07.14.21260495 (2021) doi:10.1101/2021.07.14.21260495.

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APPENDIX G: CHAPTER 6 SUPPORTING MATERIAL

GAC Adsorbent Characterization

Table S1 Physical properties of Calgon Carbon Corporation FILTRASORB 300 GAC [1].

Specifications	**FILTRASORB 300**
Iodine Number, mg/g	900 (min)
Moisture by Weight	2% (max)
Effective Size	0.8-1.0 mm
Uniformity Coefficient	2.1 (max)
Abrasion Number	78 (min)
Apparent Density (tamped)	0.56 g/cc
On 8 mesh	15% (max)
Through 30 mesh	4% (max)
Total pore volume, mL/g	0.709
Micropore, mL/g	0.378-0.408
Mesopore, mL/g	0.063-0.378
Micropore average diameter, nm*	0.841
Porosity	0.608
Particle diameter, nm	0.85-1.7

Design and Preparation of GAC for Passive Sampling

To functionalize activated carbon in its granular form, we utilized nylon mesh bags, which permitted the use of GAC in the field and improved user replacement of media on site. As shown in Table S3, the GAC housed inside the nylon bag can be easily prepared and placed inside a 90-mm 3D-printed sampler without difficulty for field deployments. Table S3 highlights the simple workflow of preparing and collecting passive samplers that utilize GAC. Batch-adsorption experiments evaluated GAC's adsorption capacities and characteristics in wastewater. Figure S1 shows a schematic diagram of the orbital shaker setup utilized and the steps to prepare GAC before incubation in water matrices.

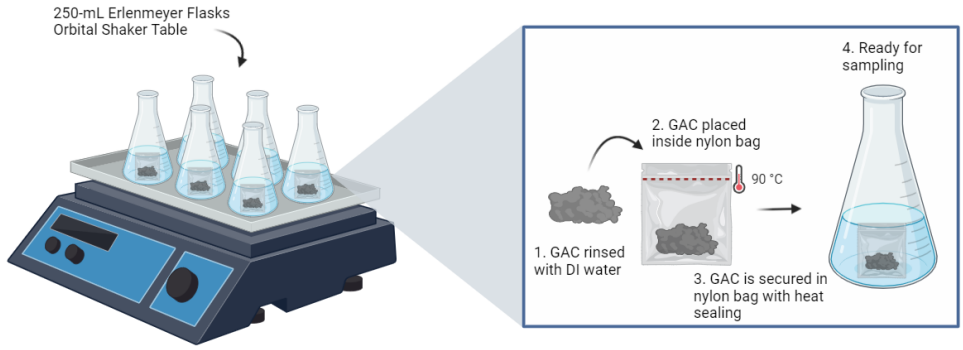


Figure S1 Schematic diagram for the bench-scale batch adsorption experiments using an orbital shaker table and GAC heat sealed inside a 4.5 cm x 4.5 cm nylon mesh within Erlenmeyer flasks.

Scanning Electron Microscope

Before being examined under the scanning electron microscope (SEM), untreated granular activated carbon (GAC) fragments and small cut nylon pieces were mounted to carbon adhesive tape using a silver conductive liquid. A Zeiss (Jena, Germany) SIGMA 300 VP SEM (acceleration voltage of 5 kV, 220 pA probes, and working distances of 12 and 15 mm) was utilized for all SEM imagery. As SEM images were taken before wastewater adsorption, the images can be compared qualitatively (Table S2). The GAC particles consisted mainly of fragments >25 µm in size; when compared with the nylon mesh at 105X magnification, it is evident that the GAC particles evaluated could not pass through the nylon weave structure. At 300X and 10,000X magnification, the surface of GAC appears irregular and porous. The dynamic structure of GAC is likely composed of micropores, mesopores, and macropores.

Molecular Methods

For SARS-CoV-2 detection, each reaction contained 15 µL of Master Mix (LuminUltra) and 5 µL of template RNA, for a total reaction volume of 20 µL. Serial dilutions of Twist synthetic SARS-CoV-2 RNA standard control were run to produce standard curves (10^6 – 10^1 copies μL^{-1}) used to quantify SARS-CoV-2 gene copies. The SARS-CoV-2 assay standard curve efficiency was ~94%, with a y-intercept of 38.3 and an R^2 value of 0.99. For pepper mild mottle virus (PMMoV) detections, reactions were prepared to contain 5 µL of RNA sample with 10 µL qScript 1-Step Sough Master Mix, 200 nM of each forward and reverse primer, and 80 nM of probe for a total of 20 µL solution mix. A known-positive DNA GeneBlock (IDT®, Iowa, USA) template was serial diluted (10^7 – 10^1 copies μL^{-1}) and utilized to construct standard curves for quantifying PMMoV gene copies. The efficiency of the PMMoV assay standard curve was ~90%, with an R^2 value of 0.99 and a y-intercept of ~37.1. For CrAssphage reactions, 25 µL was used, consisting of 2X Taqman Environmental Master mix (ThermoFisher Scientific), 3 µL template DNA, 5 µg of BSA, 100 nM for both forward, and reverse primers, and 80 nm of probe. A standard curve was produced by diluting a reference DNA gBlock® (IDT®, Iowa, USA) with known concentrations (10^5 – 10^1 copies μL^{-1}). CrAssphage qPCR performance parameters were 0.99 for an R^2 value, 40.8 for the y-intercept, and ~92% for the PCR

efficiency. The RT-qPCR and qPCR thermocycling parameters and reagent concentrations and sequences can be noted in Table S4.

Table S4 RT-qPCR and qPCR cycling parameters and target primers, probe sequences, and reaction concentrations.

Organisms	Sequence (5'–3')	Cycling parameters	(nM)	Ref.
CrAssphage	F:CAG AAG TAC AAA CTC CTA AAA AAC GTA GAG R: GAT GAC CAA TAA ACA AGC CAT TAG C P: AAT AAC GAT TTA CGT GAT GTA AC	10 min at 95°C, 40 cycles of 15 s at 95°C, 60 s at 60°C	1000 1000 100	[2]
PMMoV	F: GAG TGG TTT GAC CTT AAC GTT TGA R: TTG TCG GTT GCA ATG CAA GT P:FAM-CCT ACC GAA GCA AAT G	10 min at 50°C, 10 min at 95°C, 45 cycles of 30 s at 95°C, 60 s at 53°C, 60 s at 72°C, 10 min at 72°C	200 200 80	[3]
SARS-CoV-2 N2	F:TTACAAACATT GGCCGCAAA R:GCGCGACATT CCGAAGAA P:ACAATTTGCCC CCAGCGCTTCAG	10 min at 55 °C, 1 min at 95 °C, and two-step cycling 10 s at 95 °C and 45 s at 55 °C for 45 cycles, with a final hold step at 50 °C for 1 min	667 667 167	[4]

Wastewater Characteristics

Wastewater collected from two wastewater treatment facilities was utilized for the bench-scale experiments of this work. Table S5 describes the average wastewater characteristics collected by treatment facility operators at two WWTFs in NS, Canada.

Table S5 Average concentrations of the wastewater characteristic for the municipal wastewater at either WWTF in Halifax, NS.

Facility Parameter	**Treatment Facility 1**	**Treatment Facility 2**
Average daily flow (m^3/day)	10,794	32,941
Average TSS (mg/L)	147.9	214
Average BOD_5 (mg/L)	79.3	133
Average NH_3-N (mg/L)	14.5	21

Temperature (°C)	11.6	10.2
pH	7.0	7.2
Point of autosampler placement	Post-screening	Post-screening

Passive Sampler Deployments and Collections at Sewershed Locations

This work's comparative field study portion occurred at three university residence sewersheds (Figure S2, Locations A, B, and C). Paired passive samplers containing either electronegative filters or GAC media were collected three times a week from each sewershed location and deployed 24 to 96 hours. Sample collection occurred at 9:30 AM, and all samples were processed within 24-hrs or otherwise stored at 4° C until sample analysis. For safety reasons, at least two persons were present throughout the sample collection. Before sampling, each sewage catchment was tested with a confined space gas sensor to ensure safe working conditions. Although all sewershed locations receive sanitary and storm sewage, the storm sewer lines at the sample locations monitored in this study did not receive wastewater from upstream sewer systems. Samplers were placed at each building's sanitary sewage line and the stormwater drainage line.

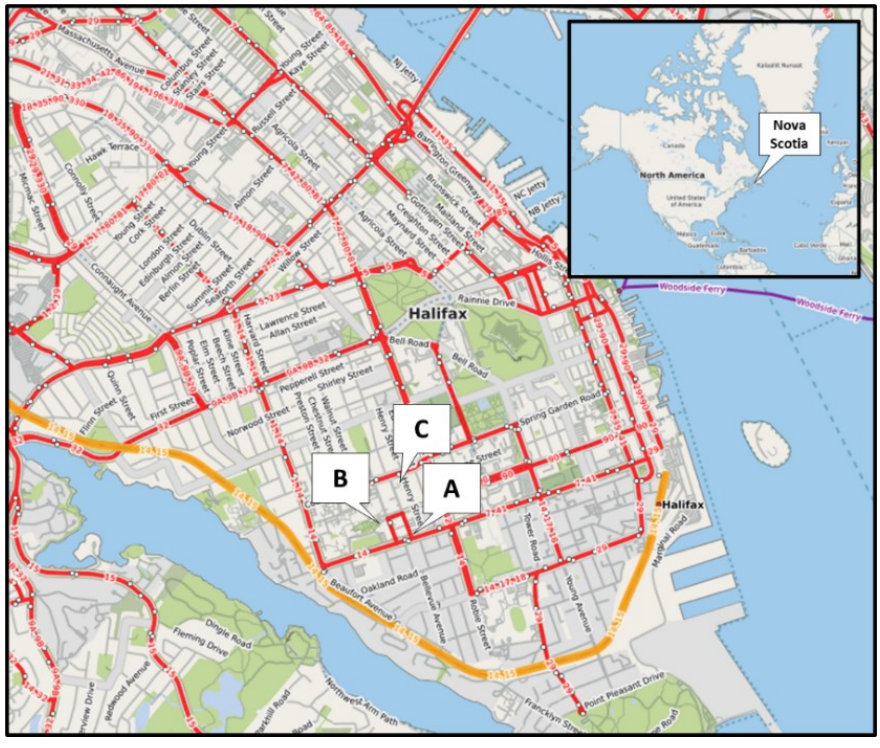


Figure S2 Three targeted sewer catchment locations (A, B, and C) in Halifax, NS, Canada, for monitoring SARS-CoV-2 in sewage using a 3D-printed passive sampling device and two types of adsorbent material. The map in this figure was created using © OpenStreetMap contributors (openstreetmap.org).

Nova Scotia COVID-19 Clinical Case Data

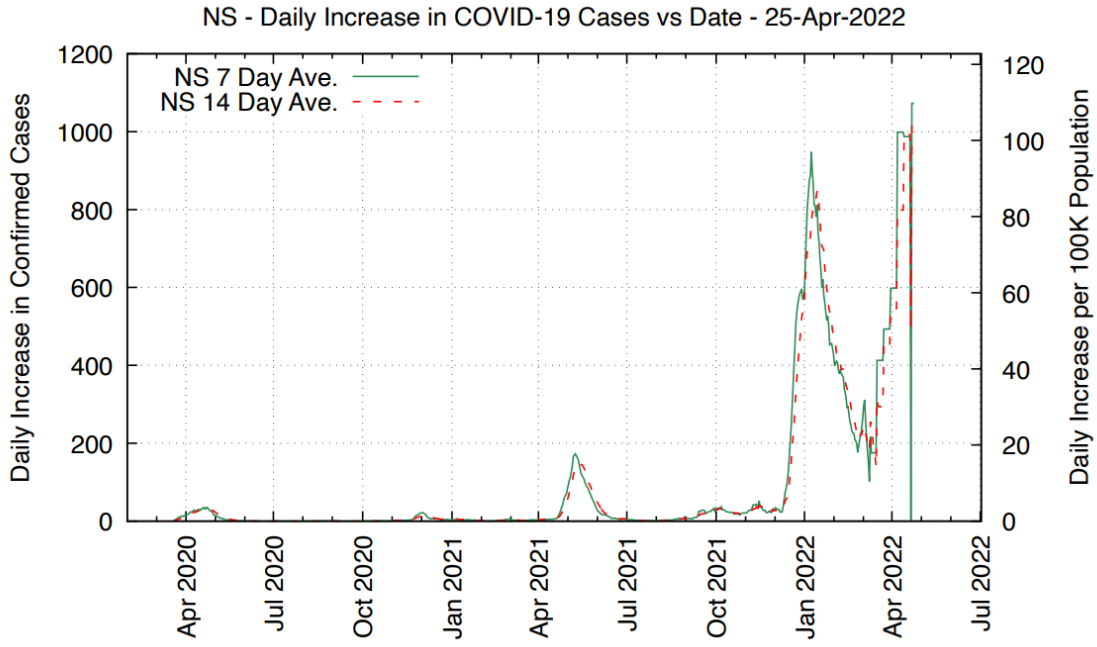


Figure S3 Nova Scotia's daily increase in COVID-19 clinically reported COVID-19 cases versus date. Data are up to 25 April 2022 and are based on reported values from Health Canada [5].

Experimental Method Limit of Detection

The MLOD was calculated mathematically by a logistic regression model (Figure S4). The response was measured as the percentage of replicates that yielded positive SARS-CoV-2 detections and the predictor being known as spiked SARS-CoV-2 concentrations in a series of test wastewater samples. The model (Eq. S1) was fit using iteratively reweighted least squares fit analysis and can be presented in full by Forootan et al. (2017), where $\hat{y}$ are β_i are the corresponding prediction and model parameters [6].

Eq. S1

$$\hat{y} = \frac{1}{1 + e^{-\beta_0 - \beta_{1x}}}$$

Adsorption Kinetic Models

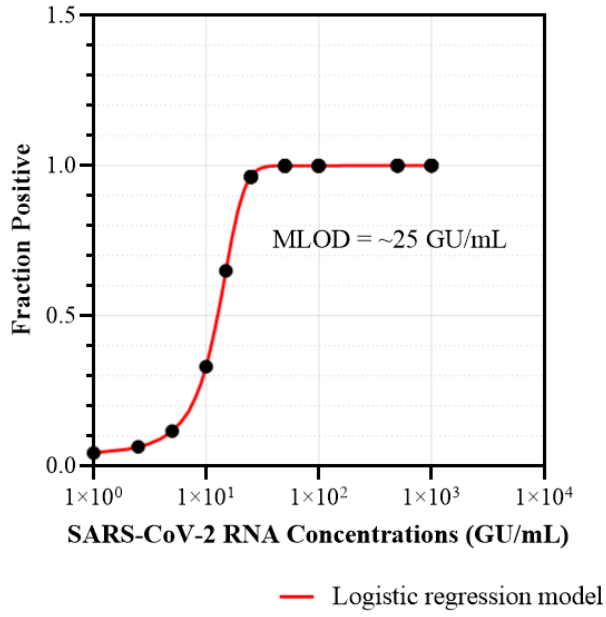


Figure S4 Experimental MLOD for GAC was determined by the fraction of positive detections fitted to a logistic regression model with a 95% detection limit (β_0 = -3.35 and β_1= 0.265).

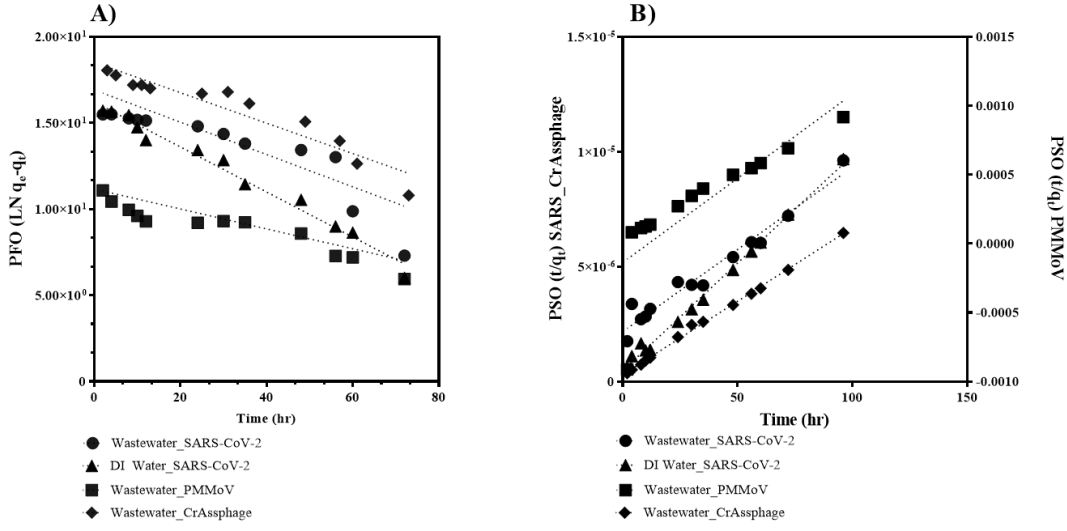
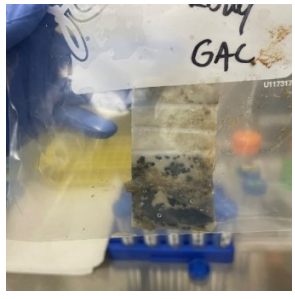


Figure S5 Batch-adsorption pseudo-first-order (A) and pseudo-second-order (B), kinetic model results for SARS-CoV-2 surrogate adsorption by GAC in wastewater and DI water, and biomarkers, PMMoV and CrAssphage in wastewater.

Table S2 Three stages of sample preparation for GAC deployment in passive sampler devices; A) pre-deployment installation of 1 g of GAC housed inside a 4.5cm x 4.5 cm nylon mesh, B) post-deployment collection of GAC, and C) post-deployment GAC sample processing.

No.	Descriptions	Images
A) Pre-Deployment Installation and Preparation		
A.1)	GAC is weighed via an analytical scale and then placed directly inside the nylon encasement. Nylon is sealed with heat, ensuring to leave enough space for the GAC to move freely.	
A.2)	GAC heal sealed in a nylon bag is placed directly inside the 3D-printed passive sampler. No particular direction or orientation is required.	
B) Post-Deployment Collection		
B.1)	GAC is collected on-site in a plastic zip-lock bag	

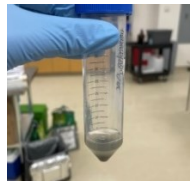
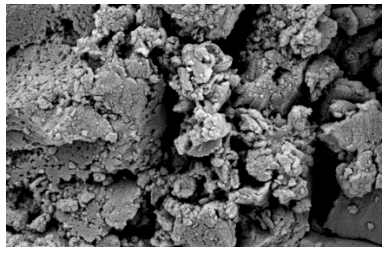
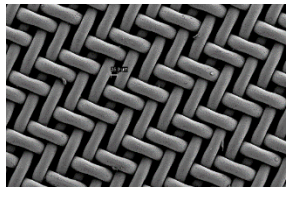
B.2)	Then carefully placed in a 50-mL falcon tube with forceps in the lab	
C) Post-Deployment Processing		
C.1)	GAC processed eluate, composed of Tween20®-based buffer (~6 mL)	

Table S3 Scanning electron microscopy images of gold sputter-coated granular activated carbon at A) 300X and B) 10 KX magnification and C) the 25 µm nylon heat-sealable mesh at 105X magnification.

ID	Descriptions	Images
A	GAC at 300X magnification	
B	GAC at 10 KX magnification	
C	25 µm nylon heat-sealable mesh at 105X magnification	

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[6]Forootan A, Sjöback R, Björkman J, Sjögreen B, Linz L, Kubista M. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol Detect Quantif 2017;12:1–6. https://doi.org/10.1016/j.bdq.2017.04.001.

APPENDIX H: CHAPTER 7 SUPPORTING MATERIAL

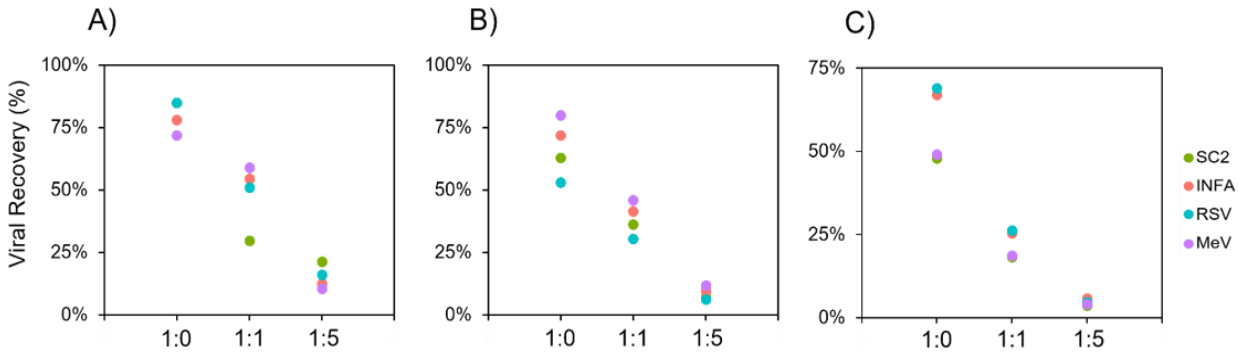

Figure S1. Percent viral recovery of SARS-CoV-2, INFA, RSV and MeV targets under three separate template dilution factors of 1:0, 1:1, and 1:5. Data is grouped by spiked gene concentrations (GC/mL), including, A) 100000, B) 1000, and C) 100.

Table S1. Synthetic viral RNA reference material used in verification and validation of RT-qPCR assays.

Company	**Name**	**Accession # / Designated Strain**	**Specification Range provided by Manufacturer**	**Storage Conditions**
Twist Bioscience	Influenza A H1N1 (2009)	NC_20643 NC_026431 NC_026432 NC_026433 NC_026434 NC_026435 NC_026436 NC_026437 NC_026438	~1×10^6 copies/µL	-90 °C to -70°C
	Measles virus	NC_001498.1		
	SARS-CoV-2 Control 48	Omicron B.1.1.529, BA.1 lineage **GISAID ID:** EEPI_ISL_6841980 **GISAID NAME:** Hong Kong/HKU-211129-001/2021		
ATCC®	Human respiratory syncytial virus (RSV) A2	(ATCC® VR-1540DQ™) / MW582527.1	~1 x 10^6 copies/µL	-80°C

Table S2. All target templates were found in selected based on the NCBI nucleotide BLAST program. Primer specificity stringency was evaluated based on at least 2 total mismatches to unintended targets, including at least 2 mismatches within the last 5 base pairs at the 3' end. For each viral gene target, nucleotide collections were evaluated across several similar organisms. Dots indicate alignment with a reference sequence, and underlined bases represent specific genotype mutations.

RSV Genotype	**Forward Primer (5'-3')** GCTCTTAGCAAAGTCAAGTTGAATGA	**Reverse Primer (5'-3')** TGCTCCGTTGGATGGTGTATT	**Probe (5'-3')** ACACTCAACAAAGATCAACTTCTGTCATCCAGC
Human RSV A/Maryland.USA/Long/1956 **Accession**: OK649668.1	.....	.....	.....T...
Human RSV A Canada/1995 **Accession**: OK649659.1	.....	.....	.....
Human RSV A /USA/2022 **Accession**: OP890335.1	.....	.....A....	.....A.....
Human orthopneumovirus strain RSVB/Vietnam/2015 **Accession**: MH828511.1	.....	.A..A.....A..A.....	...T.A..T..G.....G..G.....
Human RSV B/USA/2022 **Accession**: OP890341.1	.....	.A..A.....A..A.....	...T.A..T..G.....G..G.....
Human RSV B /Australia/2019 **Accession**: OP975389.1	.....	.A..A.....A..A.....	CATTAA.TA.GGATCAGCTGCTGTCATC.AGC.
Human orthopneumovirus **Accession**: MW587044.1	.....	.A..A.....A..A.....	.....A..T..G.....G..G.....
Human RSV MinL **Accession**: KJ817800.1	.....	.....	.....
Human RSV MinB **Accession**: KJ817799.1	.....	.....	.....

SARS-CoV-2 Genotype	**Forward Primer (5'-3')** CTGCAGATTTGGATGATTTCTCC	**Reverse Primer (5'-3')** CCTTGTGTGGTCTGCATGAGTT TAG	**Probe (5'-3')** ATTGCAACAATCCATGAGCAGTGCTGACTC
SARS-CoV-2 strain Omicron (B.1.1.529) **Accession**: OM570259.1	.....	.GAAA..CAC..C...T. CG..T.	.....
SARS-CoV-2 strain Delta **Accession**: OM470973.1	.....	.A.G....A.....T.AC..... ..C	.....
SARS-CoV-2 strain B.1.1.7 **Accession**: OW996043.1	.....	.A.G....A.....T.AC..... ..C	.....
SARS-COV-2 strain Gamma **Accession**: OM442897.1	.....	.G..TG.....G....T..C TC	.....
Measles Genotype	**Forward Primer(5'-3')** TGGCATCTGAACTCGGTATCAC	**Reverse Primer(5'-3')** TGTCCTCAGTAGTATGCATTGC AA	**Probe(5'-3')** AGGACTCAAGTGTGGATAAC
B3 strain/Marseille.France/2019 **Accession**: OP951103.1	.....	....	.CAG.....A.A..A...TC
D9/Yunnan.CHN/18.14 **Accession**: OM584357.1	.....	....	.A.G....GACAC.....C.
H1/Yunnan.China/16.19 **Accession**: MZ067549.1	.....	....	....TG....A...T.C...
A/Yokohama.Japan/47.19 **Accession**: LC537226.1	.....	....	....TG.....A.A..G.G
D9/Yunnan.China/18.14 **Accession**: OM584357.1	.....	....	.A.G....GACAC.....C.
B2/Engela.NAM/30.09 **Accession**: JQ627689.1	.....	.....T..... ..	C...G..C..CC.A...CCG

Influenza Genotype	Forward Primers(5'-3')	Reverse Primer(5'-3')	Probe(5'-3')
	F1:CAAGACCAATCYTGTCACCTCTGAC F2:CAAGACCAATYCTGTCACCTYTGAC	R1:GCATTYTGGACAAAVCGTCTACG R2:GCATTTTGGATAAAGCGTCTACG	TGCAGTCCTCGCTCACTGGGCACG
Influenza A H1N1(Anser brachyrhynchus/South Korea/2019) **Accession**: OQ296907.1	F1:..... F2:.....	R1:.....G..... R2:.....C....C.....	.TGT..T.A.....C.T..C.A
Influenza A H3N1(Anser fabalis/China/2020) **Accession**: OP341283.1	F1:..... F2:.....	R1:.....T.....G..... R2:.....C.....	.TGT..T.A.....C.T..C.A
Influenza A H5N6 (goose/Hebei /2019) **Accession**: OP601604.1	F1:..... F2:.....	R1:.....C..... R2:.....C....C...C.....	.TGTA.T.A.....C.T..C.A
Influenza A H5N1 (duck/Bangladesh/2021) **Accession**: OP030705.1	F1:..... F2:.....C.....	R1:.....T.....G..... R2:.....C.....	.TGT..T.A.....C.T..C.A
Influenza A H1N1(Mallard(Anas platyrhynchos)/South Korea/2021) **Accession**: ON495908.1	F1:..... F2:.....C.....	R1:.....G..... R2:.....C.....	.TGT..T.A.....C.T..C.A

Table S3. LuminUltra Technologies Ltd., SARS-CoV-2 Advanced Wastewater Testing Kit detailed instructions.

Order	Description of Step
1	In a 15-mL centrifuge tube, 6 mL of a lysis buffer concentrate and 250 µL of Lysis Supplement 1A were added to 1 mL of wastewater sample. The mixture was gently inverted five times and immediately incubated at 30 °C for 10 min.
2	After incubation, 3.5 mL EtOH was added to the lysed sample; the tube was gently inverted five times to mix thoroughly and then spiked with 40 µL of magnetic beads. The mixture was gently inverted five times and incubated again at 30 °C for 10 min.
3	Following the second incubation step, the magnetic beads were precipitated to the side of the sample tube with a magnet, and the supernatant was discarded.
4	The magnetic beads were then washed with two separate wash buffer solutions, with each wash, the beads were rinsed in 1 mL of solution by gently pipetting the solution and beads together.
5	Once the beads and wash solution were homogenized, a magnet was used to precipitate the beads and discard the wash supernatant.
7	This process was repeated three times with 1 mL of the wash I solution, twice with 1 mL of the wash 2 solution, and once with 1 mL of ethanol (EtOH); with washing steps occurring in that order.
8	Subsequently, any excess EtOH was pipetted from the tubes, and the samples were left at room temperature to allow any excess EtOH to evaporate.

9	To elute the viral RNA from the magnetic beads, 50 µL of elution buffer was mixed in with the beads, and incubated at 60 °C for 5 min.
10	Lastly, the magnetic beads were precipitated with a magnet and separated from the elution buffer in the tube.

Table S4. The Minimal Information for Publications on Quantitative Real-Time PCR Experiments (MIQE) checklist of essential and desirable information that should be reported to enable the reviewer to judge the validity of the paper and the reader to repeat the experiment and reproduce the results.

Category	**Item**	**Paper Location**	**Author Comments**	**Checklist**
Sample	Type	Section 4.3.1		✓
	Method of dissection/procurement	Table S1 & Section 4.3.1	No dissection, synthetic template manufacturers, and wastewater sample collection are described	✓
	Processing procedure	Section 4.3.2	Pre-RNA extraction processing	✓
	If frozen, how and how quickly?	Section 4.3.4 & Section 4.3.4	No freezing of wastewater or passive samples before RNA extractions or analysis	✓
	If fixed, with what and how quickly?	N/A	No fixing performed (i.e., no formalin-fixed procedures)	N/A
	Storage conditions and duration	Table S1, Section 4.3.4 & Section 4.3.1	Template control storage conditions are described in SI based on the manufacturer's recommendations.	✓
Extraction	Method or instrument	Section 4.3.4 & Table S3	Commercial kit (LuminUltra Technologies Ltd)	✓

	Reagents/kits/modifications	Section 4.3.4 & Table S3	Additional step-by-step methods described in the SI	✓
	DNAse or RNAse treatment	N/A	No specific treatment was completed; purification steps were included in the commercial kit used (LuminUltra Technologies Ltd).	N/A
	Evidence for lack of contamination (DNA or RNA)	Section 4.3.10	RNA extract dilution during RT-qPCR analysis	✓
	Nucleic acid quantification	Methods & results	RT-qPCR analysis, monoplex & multiplex analysis	✓
	RNA integrity	Table S1	Reported for reference RNA material – quantified by manufacturer	✓
Reverse transcription	Complete reaction conditions, including all components and their concentrations	Section 4.3.4		✓
	RNA amount and reaction volume	Table 4-1 & Section 4.3.4		✓
	Priming oligo sequence(s)	Table 4-1		✓
	Cq values with and without reverse transcriptase	N/A	One-step RT-qPCR was used in this study	✓
qPCR target	Sequence accession number	Table S1	Included in SI/methods for primer sequences and template sequences used	✓
	Amplicon length	Table 4-1		✓
	In silico specificity (BLAST)	Table S2		✓

	Location by exon/intron	Table 4-1		✓
	Identify the splice variants amplified	N/A		×
	All primer/probe sequences	Table 4-1		✓
	Location and identity of any oligonucleotide modifications	Table 4-1	Probe fluorophore modifications	✓
	Complete reaction conditions, including all components and their concentrations	Table 4-1 and Section 4.3.5		✓
qPCR protocol	cDNA/DNA amount and reaction volume	Section 4.3.4 & Table S1		✓
	Instrument identification and complete thermocycling parameters	Section 4.3.4		✓
	Evidence for PCR specificity (gels, sequencing, or melting curves)	Table S2		✓
qPCR validation	Template inhibition data (template titrations)	Section 4.3.10	Extract dilutions were evaluated for inhibition, no inhibition was expected.	✓
	For SYBR Green I reactions, the Cq of the no template control	Table S1		✓
	Calibration curves with slope and intercept	Section 4.4.1 & Section 4.3.5		✓
	PCR efficiency from the slope	Section 4.4.1 & Section 4.3.5		✓

	r^2 of the calibration curve	Section 4.4.1 & Section 4.3.5		✓
	Evidence for the linear dynamic range	Section 4.4.1 & Section 4.3.5		✓
	Evidence for the limit of detection	Section 4.4.2 & Section 4.3.6		✓
	For multiplexed assays, the efficiency and limit of detection of each assay	Section 4.4.2 & Section 4.3.6		✓
	qPCR analysis method/software	Throughout methods sections	GeneCount thermocycler and software for RT-qPCR	✓
Data analysis	Method of Cq determination	Section 4.3.5	Construction of standard curves with known concentrations of RNA reference material	✓
	Results of no template controls	Section 4.3.10		✓
	Justification of number and choice of reference genes	Introduction		✓
	Normalization method	Section 4.4.3		✓
	Number and stage (reverse transcription or qPCR) of technical replicates	Throughout methods sections		✓
	Intra-assay variation in terms of concentration, not Cq	Throughout methods sections		✓

APPENDIX I: CHAPTER 8 SUPPORTING MATERIAL

Table S1. Median values and standard deviations for select water chemistry parameters were measured between May and November 2022 at the two sample locations at the freshwater lake.

	Units	Median	Standard Deviation
Total Aluminum	$\mu g\ L^{-1}$	7.04	3.05
Colour	Pt-Co	10	11.15
Conductivity	$\mu s\ cm^{-1}$	844	91.97
Dissolved oxygen	$mg\ L^{-1}$	7.65	1.16
Dissolved organic carbon	$mg\ L^{-1}$	2.21	0.1
Total Iron	$\mu g\ L^{-1}$	37.65	16.17
Total dissolved solids	$mg\ L^{-1}$	597.5	38.98
Total organic carbon	$mg\ L^{-1}$	2.21	0.08
Temperature	°C	19.75	3.49
Total Phosphorus	$\mu g\ L^{-1}$	8.64	8.31
Turbidity	NTU	0.76	0.74
UV_{254}	cm^{-1}	0.05	0.01
pH	-	7.5	0.38

Table S2. Oligonucleotide sequences for the respective primers, and probes used for each virus. The working concentrations for each qPCR assay. Oligonucleotide probe sequences were labelled at the 5′-end with fluorescent reporter dyes and quenched with a Blackhole Quencher 1 or 2 at the 3′-end.

Targets	Genes	Sequences (5'–3')	Concentration (nM)	Amplicon size (bp)	Cycling Conditions	Ref.
INFA[1]	Matrix protein (M1)	**F1:**CAAGACCAATCYTGTCACCTCTGAC	400	106	2 min at 25°C, 15 min at 50°C, 2 mins at 95°C, 45 cycles of 15 s at 95°C, and 30 s at 60°C	[1]
		R1:GCATTYTGGACAAAVCGTCTACG	600			
		F2:CAAGACCAATYCTGTCACCTYTGAC	400			
		R2:GCATTTTGGATAAAGCGTCTACG	200			
		P:TGCAGTCCTCGCTCACTGGGCACG	200			
	Nucleocapsid (N1)	**F:**CTGCAGATTTGGATGATTTCTCC	100 200	92		

SARS-CoV-2[1]			200			
		R:CCTTGTGTGGTCTGCATGAGTTTAG				
		P:ATTGCAACAATCCATGAGCAGTGCTGACTC				
RSV-A[1]	Nucleoprotein (N)	**F:**GCTCTTAGCAAAGTCAAGTTGAATGA	500 500 200	82		
		R:TGCTCCGTTGGATGGTGTATT				
		P:ACACTCAACAAAGATCAACTTCTGTCATCCAGC				
MeV[1]	Nucleoprotein (N)	**F:**ATATATCGTAGAGGCAGGATTAG	500 500 200	119		
		R:AGGACTCAAGTGTGGATAAC				
		P:AAACTATGTATCCTGCTCTTGG				
EnV	Polyprotein (PP)	**F:**GATTGTCACCATAAGCAGC	400 400 100	148	5 min at 50°C, 20s at 95°C, 40 cycles of 3 s at 95°C, and 30 s at 60°C	[2]
		R:GCCCTGAATGCGGCTAATC				
		P:CGGAACCGACTACTTTGGGTGTCCGT				
AdV[2]	Hexon structure gene of species F (type 40/41)	**F:**TCCGACCCACGATGTAACCA	250 250 100	112		[3]
		R:CACGGCCAGCGTAAAGCG				
		P:ACAGGTCACAGCGACT				
RV[2]	non-structural protein 3 (NSP3) gene) species A	**F1:** ACCATCTACACATGACCCTCTATG **F2:** ACCATCTTCACGTAACCCTCTATG	250 250 100	83		
		R: ACATAACGCCCCTATAGCCATTT				
		P:AATAGTTAAAAGCTAACACTGTC				
NV[2]	ORF2	**F:**CCAATGTTCAGATGGATGAGATTCTC	250 250 100	92		
		R:TCGACGCCATCTTCATTCACA				
		P:ATCGCCCTCCCACGT				
MS2	Maturation & structural	**F:**GTCCATACCTTAGATGCGTTAGC	400 400	160	30 min at 55°C, 3 min	[4]

	protein (*mat* and *cp* genes)	**R:**CCGTTAGCGAAGTTGCTTGG **P:**ACGTCGCCAGTTCCGCCATTGTCG	250		at 94°C, followed by 45 cycles 15 s at 94°C and 1 min at 60°C	

[1] Multiplex RT-qPCR assay previously validated in the work of Hayes et al., (2023) for the simultaneous detection of SARS-CoV-2, INFA, RSV, and MeV [1]. [2] Multiplex qPCR assay for the detection of human rotavirus, enteric adenovirus, and human norovirus, formerly validated by [3].

Table S3. Viral calibration curve information for each viral target.

INFA		**SARS-CoV-2**		**MeV**		**RSV**	
R2	0.95	R2	0.96	R2	0.97	R2	0.96
y-int	38.78	y-int	40.71	y-int	41.16	y-int	38.97
Slope	-3.17	Slope	-3.76	Slope	-3.84	Slope	-3.53
Efficiency (%)	106.71	Efficiency (%)	84.48	Efficiency (%)	82.14	Efficiency (%)	91.90
EnV		**Norovirus**		**Adenovirus**		**Rotavirus**	
R2	0.99	R2	0.99	R2	0.99	R2	0.99
y-int	37.48	y-int	40.34	y-int	39.22	y-int	40.32
Slope	-3.13	Slope	-3.38	Slope	-3.28	Slope	-3.50
Efficiency (%)	108.37	Efficiency (%)	97.66	Efficiency (%)	101.5	Efficiency (%)	92.8

Table S4. The Minimal Information for Publications on Quantitative Real-Time PCR Experiments (MIQE) checklist of essential and desirable information that should be reported to enable the reviewer to judge the validity of the paper and the reader to repeat the experiment and reproduce the results.

Category	**Item**	**Paper Location**	**Author Comments**	**Checklist**
Sample	Type	Methods	Passive and grab samples	✓
	Method of dissection/procurement	Methods and Supplemental	No dissection, synthetic template manufacturers, and sample collection are described	✓

	Processing procedure	Methods	Pre-RNA extraction processing	✓
	If frozen, how and how quickly?	Methods		✓
	If fixed, with what and how quickly?	N/A	No fixing performed (i.e., no formalin-fixed procedures)	N/A
	Storage conditions and duration	Methods and Supplemental	Template control storage conditions are described in SI based on manufacturers recommendations.	✓
Extraction	Method or instrument	Methods	Commercial kit	✓
	Reagents/kits/modifications	Methods		✓
	DNAse or RNAse treatment	N/A	No specific treatment was completed; purification steps were included in the commercial kit used (LuminUltra Technologies Ltd).	N/A
	Evidence for lack of contamination (DNA or RNA)	N/A	RNA extract dilution during RT-qPCR analysis	X
	Nucleic acid quantification	Supplemental	RT-qPCR analysis, and multiplex RT-qPCR analysis	✓

	RNA integrity	Supplemental		✓
Reverse transcription	Complete reaction conditions, including all components and their concentrations	Supplemental		✓
	RNA amount and reaction volume	Methods		✓
	Priming oligo sequence(s)	Supplemental		✓
	Cq values with and without reverse transcriptase	N/A	One-step RT-qPCR was used in this study	✓
qPCR target	Sequence accession number	Supplemental	Included in SI / methods for primer sequences and template sequences used	✓
	Amplicon length	Supplemental		✓
	In silico specificity (BLAST)	Validated in previous publications		✓
	Location by exon/intron	Supplemental		✓
	Identify the splice variants amplified	N/A		×
	All primer/probe sequences	Supplemental		✓
	Location and identity of any oligonucleotide modifications	Supplemental		✓
	Complete reaction conditions, including all components and their concentrations	Methods & Supplemental		✓
qPCR protocol	cDNA/DNA amount and reaction volume	Methods		✓

	Instrument identification and complete thermocycling parameters	Methods & Supplemental		✓
	Evidence for PCR specificity (gels, sequencing, or melting curves)	Validated in previous publications		✓
qPCR validation	Template inhibition data (template titrations)	Supplemental	Extract dilutions were evaluated for inhibition, no inhibition was expected.	✓
	For SYBR Green I reactions, the Cq of the no template control	Methods	Didn't include data where no template control failed	✓
	Calibration curves with slope and intercept	Supplemental/previously validated in other published work		✓
	PCR efficiency from the slope	Supplemental		✓
	r^2 of the calibration curve	Supplemental		✓
	Evidence for the linear dynamic range	Supplemental		✓
	Evidence for the limit of detection	Validated in previous publications		✓
	For multiplexed assays, the efficiency and limit of detection of each assay	Supplemental		✓
	qPCR analysis method/software	Supplemental & Methods		✓
Data analysis	Method of Cq determination	Methods		✓

	Results of no template controls	Methods		✓
	Justification of number and choice of reference genes	Supplemental		✓
	Normalization method			X
	Number and stage (reverse transcription or qPCR) of technical replicates	Supplemental		✓
	Intra-assay variation in terms of concentration, not Cq	Validated in previous publications		✓
	Statistical methods/software	Methods		✓

Table S5. Synthetic RNA reference material used in RT-qPCR verification and validation.

Company	**Name**	**Accession # / Designated Strain**	**Manufacturer Specifications**	**Storage Conditions**
Twist Bioscience	Influenza A H1N1 (2009)	NC_20643 NC_026431 NC_026432 NC_026433 NC_026434 NC_026435 NC_026436 NC_026437 NC_026438	~1×10^6 copies/µL	-90 °C to -70°C
	Measles virus	NC_001498.1		
	SARS-CoV-2 Control 48	Omicron B.1.1.529, BA.1 lineage **GISAID ID:** **E**EPI_ISL_6841980 **GISAID NAME:** Hong Kong/HKU-211129-001/2021		

ATCC®	Human respiratory syncytial virus (RSV) A2	(ATCC® VR-1540DQ™) / MW582527.1	~1 x 10^6 copies/µL	-80ºC
Integrated DNA Technologies gBlocks	Bacteriophage MS2	NC_001417	~1×10^{13} copies/µL	-20ºC
	Norovirus	X86557	10 ng/uL	
	Adenovirus	D13781		
	Rotavirus	X81436		
	Enterovirus	MW473684.1		

Supplementary References

[1] Hayes EK, Gouthro MT, LeBlanc JJ, Gagnon GA. Simultaneous detection of SARS-CoV-2, influenza A, respiratory syncytial virus, and measles in wastewater by multiplex RT-qPCR. Science of The Total Environment 2023:164261. https://doi.org/10.1016/j.scitotenv.2023.164261.

[2] Coudray-Meunier C, Fraisse A, Martin-Latil S, Delannoy S, Fach P, Perelle S. A Novel High-Throughput Method for Molecular Detection of Human Pathogenic Viruses Using a Nanofluidic Real-Time PCR System. PLOS ONE 2016;11:e0147832. https://doi.org/10.1371/journal.pone.0147832.

[3] Lee D-Y, Leung K, Lee H, Habash M. Simultaneous Detection of Selected Enteric Viruses in Water Samples by Multiplex Quantitative PCR | SpringerLink. Water Air Soil Pollut 2016;227. https://doi.org/10.1007/s11270-016-2811-5.

[4] Gendron L, Verreault D, Veillette M, Moineau S, Duchaine C. Evaluation of Filters for the Sampling and Quantification of RNA Phage Aerosols. Aerosol Science and Technology 2010;44:893–901. https://doi.org/10.1080/02786826.2010.501351.