DEVELOPMENT AND OPTIMIZATION OF VIRUS CONCENTRATION AND DETECTION METHODS FOR TRACKING SARS-COV-2 AND ITS VARIANTS IN WASTEWATER

by

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ABSTRACT

MD ARIFUL ISLAM JUEL. Development and Optimization of Virus Concentration and Detection Methods for Tracking SARS-COV-2 And its Variants in Wastewater. (Under the direction of DR. MARIYA MUNIR)

Wastewater-based epidemiology (WBE) has garnered significant attention as an early warning tool for detecting and predicting the course of COVID-19 cases within a community, working alongside public health data. This monitoring approach has been employed in various settings, including municipal wastewater treatment centers, universities, and community living spaces, to track COVID-19 trends. To effectively conduct WBE surveillance, it's imperative to quantify viral copies precisely and reliably from wastewater. The accuracy of SARS-CoV-2 quantification hinges on the selection of an efficient and dependable virus concentration method. The concentration of samples plays a pivotal role, particularly when the viral load in untreated wastewater falls below the threshold detectable by Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) analysis.

The first objective of my dissertation, discussed in Article 1, is the performance evaluation of a rapid ultrafiltration-based virus concentration method using InnovaPrep Concentrating Pipette (CP) Select and how it compares with electronegative membrane filtration (HA) method. The criteria of the evaluation were based on the SARS-CoV-2 detection sensitivity, surrogate virus recovery rate, and sample processing time. Results suggested that the CP Select concentrator was more efficient at concentrating SARS-CoV-2 from wastewater compared to the HA method. About 25% of samples that tested SARS-CoV-2 negative when concentrated with the HA method produced a positive signal with the CP Select protocol. The optimization of the CP Select protocol by adding AVL lysis buffer and sonication increased Bovine Coronavirus (BCoV) recovery by 19%, which compensated for viral loss during

centrifugation. Filtration time decreased by approximately 30% when using the CP Select protocol, making this an optimal choice for building surveillance applications where quick turnaround time is necessary.

The second objective of my dissertation, discussed in Article 2, aims to develop and optimize a large-volume concentration method for increased sensitivity in detecting SARS-CoV-2, particularly during periods of low COVID-19 infection. Most current virus concentration methods have inherent limitations, as they can only process small volumes of wastewater, typically ranging from 20 to 250 mL. While small-volume methods are effective for detecting and quantifying SARS-CoV-2 during high community infection, they may lack informativeness during the early stages of community infections. In this study, we filtered 3 liters of wastewater through a hollow ultrafilter (UF) and then further concentrated the first eluate using the electronegative membrane filter, also known as the HA filter. The optimized combination method, UF-HA_Soni, resulted in a 100% positive detection rate for SARS-CoV-2 during low COVID-19 infection period. In contrast, the UF and HA methods used individually achieved detection rates of 63% and 9%, respectively. During high COVID-19 infection periods, no significant difference in SARS-CoV-2 detection was observed. However, the hollow UF method yielded a higher mean SARS-CoV-2 concentration. Additionally, the UF method successfully recovered 33.2% of Bovine Coronavirus (BCoV), which was significantly greater than any of the alternative methods. Our analysis of virus partitioning revealed that 26% of SARS-CoV-2 viruses were attached to solid particles, with the majority found in smaller suspended particles separated by centrifugation, as opposed to the larger gravity-settled solids.

The third objective of my dissertation as discussed in Article 3 is to evaluate the application of the digital droplet PCR (ddPCR) to detect and quantify SARS-CoV-2 variants

in wastewater. We used two mutation assays targeting the S gene (N764K and N856K) to detect and quantify SARS-CoV-2 Omicron and Delta variants. With these two assays, we first detected the Omicron variants on December 6, 2021, in the wastewater sample from Mecklenburg County. This detection preceded the first clinical detection on December 10, 2021. The relative abundance of Omicron VOCs determined by RT-ddPCR in wastewater showed a strong and positive correlation with clinically reported VOCs ($r = 0.98$, $p < 0.0001$). This surveillance method for variant analysis provided near real-time insights into the transmission dynamics of Omicron variants, facilitating swift administrative interventions, including awareness, preparedness, and control measures.

Key Words: Virus concentration, Large volume filtration, SARS-CoV-2, ddPCR, wastewater based epidemiology, COVID-19, Omicron

DEDICATION

This dissertation is dedicated to my parents, wife, daughter, and sons. Thank you very much for your support and keeping faith on me.

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LIST OF ABBREVIATIONS

BCoV	Bovine Coronavirus
COVID -19	Coronavirus Disease 2019
CP Select	Concentrating Pipettes Select
CDC	Centers for Disease Control and Prevention
Cq	Cycle of Quantification
ddPCR	Digital Droplet PCR
D-HFUF	Dead End Hollow-fiber ultrafiltration
EMF	Electronegative Membrane Filtration
IR	Incidence Rate
LOD	Limit of Detection
MHV	Murine Hepatitis Virus
NGS	Next Generation Sequencing
NCDHHS	North Carolina Department of Human Health and Services
PEG	Polyethylene Glycol
PMMoV	Pepper Mild Mottle Virus

RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SMF	Skimmed Milked Flocculation
UF	UltrafilterUF-HA Hollow Ultrafilter – Electronegative Membrane Filtration
UF-HA_Soni	Hollow Ultrafilter – Electronegative Membrane Filtration_Sonication
VOC	Variants of Concern
WWTP	Wastewater Treatment Plant
WBE	Wastewater Based Epidemiology
WHO	World Health Organization

1 INTRODUCTION

SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), which emerged in mid-December 2019, initiated the ongoing global pandemic that began in March 2020. The world has witnessed millions of deaths and hundreds of millions of COVID-19 infections since then (Kitajima et al., 2020). This virus primarily spreads through inhalation of aerosol droplets, contact with infected persons, and high-touch surfaces. Another potential mode of transmission is associated with wastewater that is fecal-oral transmission (Buonerba et al., 2021). The presence of SARS-CoV-2 in anal swabs and in feces collected from some infected patients, suggested the potential transmission of the virus through aerosols produced during toilet flushing (Kang et al., 2020). Some articles have reported successful culture of live infectious SARS-CoV-2 from stool samples of COVID-19 patients, supporting this hypothesis (Wang et al., 2020; Xiao et al., 2020). However, when infectious fecal matter enters wastewater and reaches treatment plants, the level of infectivity diminishes due to natural degradation of the intact viral capsid (as depicted in Figure 1), although it remains quantifiable using RT-qPCR or RT-ddPCR (Bivins et al., 2020).

Numerous studies have detected and quantified SARS-CoV-2 in untreated wastewater collected from various sources such as sub-sewersheds, wastewater treatment plants (WWTPs), university resident halls, medical wastewater, sludge from treatment plants, as well as wastewater from aircraft and cruise ships (Medema et al., 2020; Tiwari et al., 2021; Tran et al., 2021; Gibas et al., 2021). While these references indicate the persistence of SARS-CoV-2 in

untreated wastewater (Bivins et al., 2020), they do not definitively confirm virus transmission (Bivins et al., 2020)

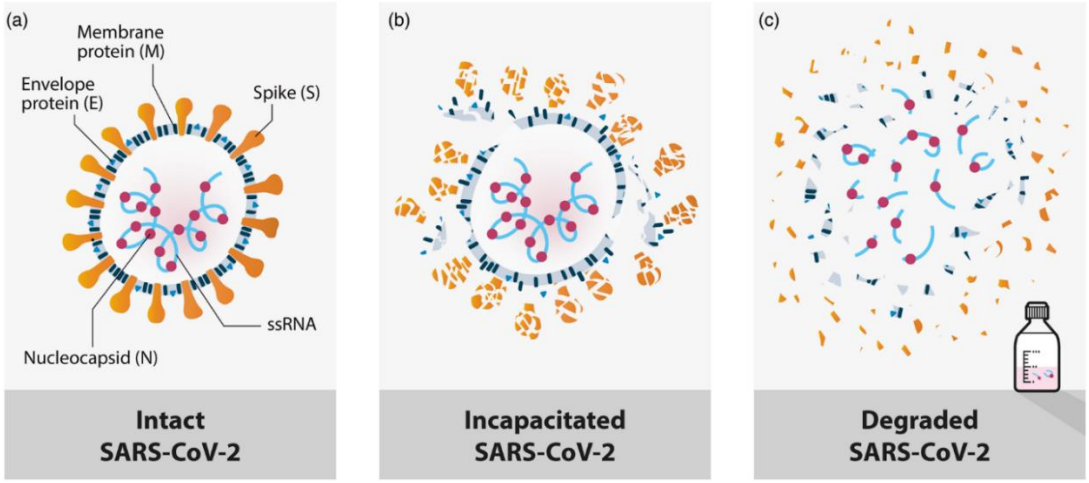

Fig. 1. 1 Three stages of SARS-CoV-2 that can be detectable through RT-qPCR/ddPCR, Source: Water Quality Research Journal. Published online May 26, 2020.

Wastewater Based Epidemiology (WBE) is a public health tool that uses wastewater to monitor human pathogenic viruses that are shed through sneezes and feces of infected patients and mixed with domestic wastewater (Kitajima et al., 2020). The first WBE concept was proposed to estimate the drug abuse situation in a community by analyzing the pharmaceutical concentration in wastewater in 2001 (Daughton, 2001). Previously, this concept was successfully used for the monitoring of poliovirus, hepatitis virus, and antimicrobial resistance bacteria (Hendriksen et al., 2019). The principle is that any substance excreted by humans in wastewater could be traced back to its initial source concentration if the substance or its metabolites are stable in wastewater to some degree. This raised a question of whether SARS-CoV-2 RNA are stable enough in wastewater to implement this concept for tracking COVID-19 infection in the community. Bivins et al., (2020) reported that both infectious and non-infectious SARS-CoV-2 RNA virus are persistent in $4^{O}C$ for 7 days after the sample is collected

from the wastewater treatment plant (WWTP). Intact viruses degrade faster at room temperature; however, both incapacitated and degraded viruses are still detectable in wastewater through RT-qPCR or ddPCR quantification (Hill et al., 2020).

This WBE tool has recently been used to monitor Corona Virus Diseases (COVID-19) outbreak in the community by quantifying SARS-CoV-2 viruses from wastewater (Ahmed et al., 2020; Kitajima et al., 2020). Since COVID-19-infected individuals shed viruses through feces irrespective of symptoms onset, surveillance of this disease through wastewater testing covers both symptomatic and non-symptomatic COVID-19 patients. Thus, wastewater surveillance reflects a true representation of the infection scenario in the community. Multiple studies reported that this WBE tool can track 1 to 2 weeks of an early signal before the COVID-19 outbreak hits the community which can allow administrators to take preventive measures before the possible outbreaks (Peccia et al., 2020; Barua et al., 2022).

The successful application of the Wastewater-Based Epidemiology (WBE) tool hinges on several critical factors, with precise quantification of viral copies from wastewater being one of the key elements (Lu et al., 2020). This process entails navigating various variables that can significantly impact the accuracy of virus quantification. Virus concentration method is highly important, particularly in regions with low COVID-19 prevalence where viral titers in wastewater are not high enough to be detectable. Researchers have employed several common methods for virus concentration from wastewater, including Polyethylene Glycol (PEG) precipitation (La Rosa et al., 2020; Polo et al., 2020), centrifugal ultrafiltration (Nemudryi et al., 2020; Wu et al., 2020), and electronegative filtration (Ahmed et al. 2020; Haramoto et al., 2020). These methods were mostly developed for non-enveloped enteric viruses such as adenovirus, norovirus, hepatitis A, norovirus, enterovirus, adenovirus, and hepatitis A (Kitajima et al., 2020). Mostly, they are fall into three main categories: centrifugation-

ultrafiltration (CeUF), membrane (electronegative/electropositive) filtration, PEG/NaCl based precipitation (Polo et al., 2020).

Ultrafiltration works based on size exclusion using centrifugal filters with different molecular cut-off ranging from 10 to 100 kDa (Kitajima et al., 2020). This method is widely used for concentrating SARS-CoV-2 virus from wastewater that has been reported in about 43% of recently published WBE articles (Buonerba et al., 2021). The advantage of this method is comparable virus recovery, fast processing and widely available equipment though it is not true for ultracentrifuge-based filtration equipment. However, sample processing cost is comparatively higher than other methods. Electronegative membrane filtration, with or without modification, is usually used for concentrating enteric viruses in treated or untreated wastewater (Haramoto et al., 2018). Due to the net negative charge on the surface of most enteric viruses at neutral pH they easily get adsorbed on the filter surface either by electrostatic forces or making salt bridging with the help of cations such as $MgCl_2$ (Ikner et al., 2012). Though most enveloped virus is sensitive to pH variation, nevertheless, the SARS-CoV-2 showed stability in a wide range of pH. This information allows the HA method to be useful for concentrating SARS-CoV-2 virus as well with a modification of pH. About 13% of SARS-CoV-2 WBE published articles so far used this method successfully for virus detection and quantification. Simple setup, low installation, and processing cost as well as comparable virus recovery make this one of the most popular methods. However, the problem of processing turbid samples and low-sample volume processing are the disadvantages of this method (Juel et al., 2021).

The precipitation method is one of the most inexpensive, simple, and capable of processing a large volume of sample (up to 1L) that has been used for concentrating virus from environmental samples for a long time (Wu et al., 2020; Kumar et al., 2020; La Rosa et al.,

2020). Polyethylene glycol (PEG 8000 and PEG 9000) based precipitation are commonly used compared to the $AlCl_3$ based precipitation method. $AlCl_3$/PEG polymers trap solvent and separate virions (proteins) from the solvent phase by precipitating proteins once their saturation solubility exceeds (Lewis and Metcalf, 1988). In around 27% of SARS-CoV-2 WBE articles, this method has been reported as a virus concentration method (Buonerba et al., 2021). A diverse virus recovery efficiency reported so far ranging from 0.08 to 69% (Kumblathan et al., 2021) which is mainly because of the variation of the protocols. Incubation time and centrifugation are the main steps of variation that different labs follow. The drawback of this method is long sample processing (4 to 24 h) and co-precipitation of PCR inhibiting materials (Kumblathan et al., 2021).

In addition, Ultracentrifugation and Skimmed Milked Flocculation (SMF) were also used to concentrate environmental samples (Randazzo et al., 2020). Multiple virus methods can produce comparable results and can be used for SARS-CoV-2 surveillance (Pecson et al., 2020). However, based on the literature review, Ultrafiltration, HA (HA filtration), or PEG precipitation-based methods were mostly used successfully for concentrating SARS-CoV-2 from wastewater. That option can be narrowed down after analysing multiple factors including rapid data reporting, cost, throughput, and sensitivity. When rapid data reporting is necessary, such as for monitoring college dorms, the PEG precipitation method may not be suitable as it require a long time to process. In that case, a concentration method with rapid sample processing is desired. One of the objectives of this dissertation is to evaluate virus concentration method focussing on rapid data turnaround.

Furthermore, optimized protocol should always be used as any changes in the protocol may result different performance, for example, 5 different PEG precipitation protocols that was being reported in different articles were tested, only one of them showed 62% recovery while

the recovery rate for other modifications was very low ranging 0 to 10% (Baril et al. 2021). When there is a need to quantify viruses from river or effluent from wastewater treatment plant, a large volume filtration-based concentration method can be adopted. For example, Dead-End Hollow Ultrafiltration (DEHU) which can filter 10 L followed by a secondary concentration using CP Select method (McMinn et al., 2021). This would allow for concentrating very low virus titered sample. We also aim to evaluate the effectiveness of the large volume-based concentration method for increased SARS-CoV-2 detection sensitivity.

The effectiveness of wastewater-based epidemiology in predicting COVID-19 outbreaks has been well-established, primarily relying on the analysis of liquid wastewater samples. However, studies have highlighted the partitioning of SARS-CoV-2 viruses in both liquid and solid phases (Graham et al., 2021; Kim et al., 2022; Wolfe et al., 2021). Some reports indicate that approximately 50% of SARS-CoV-2 viruses are distributed between these phases (Breadner et al., 2023; Juel et al., 2021). Other studies reported no significant difference in SARS-CoV-2 quantification when separating solids from the liquid (Pecson et al., 2021; Ai et al., 2021; Fores et al., 2021). This variability in recovery rates across studies may stem from differences in wastewater matrix composition at various locations or collection sites, or due to methodological disparities. The degree of association of SARS-CoV-2 virus with solid particles can vary depending on the size of the solid particles. Greaves et al., (2022) found that higher percentage of six different fecal pollution biomarker were associated with smaller suspended particles than large solid particles. Usually, the size of the large settleable solid particle is ≥180 μm whereas it is ≤0.45 μm for the smaller suspended particles (Greaves et al., 2022). In addition to liquid solid partitioning analysis, Investigations have delved into whether SARS-CoV-2 viruses are predominantly absorbed in larger settleable solid particles or in smaller suspended particles, necessitating centrifugation for separation. This information will

be useful for considering pre-concentration steps, such as solid removal, of different filtration-based virus concentration methods.

Numerous variants of SARS-CoV-2 have emerged, recognized as Variants of Concern (VOC) due to their heightened transmissibility, pathogenicity, and ability to evade vaccine-induced or natural immunity. The Alpha, Beta, and Gamma variants prevailed as dominant VOCs in early 2021, contributing to increased COVID-19 cases worldwide until mid-2021 (Wurtzer et al., 2022). However, these variants gradually gave way to the Delta variant, maintaining dominance until December 2021, when a new VOC emerged: Omicron. For screening new mutations in pathogenic bacteria and viruses, Next-Generation Sequencing (NGS) with targeted sequencing stands as the gold standard technology (Lou et al., 2022; Deng et al., 2020). Conversely, PCR-based methods, such as RT-qPCR and RT-ddPCR, serve as alternative techniques for quantifying specific mutations in wastewater (Bloemen et al., 2022; Heijnen et al., 2021). These methods, while less costly and time-consuming than NGS, offer high sensitivity in VOC detection (Lou et al., 2022). An additional objective of this dissertation is to elucidate the transmission dynamics of Omicron variants by assessing the relative proportions of circulating strains in wastewater Objectives of the Study

1.1 Objectives

The main objectives of this dissertation were to develop and optimize different virus concentration methods for increased molecular detection of SARS-CoV-2 viruses and its variants. The objectives of this research work are defined broadly as follows:

(i) To optimize and compare different virus concentration methods for SARS-CoV-2 for improving detection sensitivity and quick data turnaround. This study has already been published in the Science of the Total Environment journal (Article 1).

(ii) To develop and optimize large volume filtration-based virus concentration method for increased detection sensitivity of SARS-CoV-2 from wastewater. The manuscript based on this study has already been prepared to submit to a peer-reviewed journal (Article 2)

(iii) To determine the transmission dynamics of SARS-COV-2 Omicron and Delta variants circulating in wastewater. The manuscript based on this study has already been prepared to be submitted to a peer-reviewed journal (Article 3).

2 ARTICLE 1: PERFORMANCE EVALUATION OF VIRUS CONCENTRATION METHODS FOR IMPLEMENTING SARS-COV-2 WASTEWATER BASED EPIDEMIOLOGY EMPHASIZING QUICK DATA TURNAROUND

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2.1 Introduction

Wastewater-based epidemiology (WBE) is a widely used approach that has been rapidly adopted by the environmental science and engineering academic community as part of the response to the COVID-19 pandemic. WBE has been demonstrated to be an effective early warning tool for rising case numbers, when combining COVID-19 wastewater surveillance data and public health data. As it can provide evidence of both symptomatic and asymptomatic COVID-19 cases, WBE has been applied to detect COVID-19 cases in college residence halls (Betancourt et al., 2021; Gibas et al., 2021; Harris-Lovett et al., 2021; Scott et al., 2021), schools (Gutierrez et al., 2021; Crowe et al., 2021), nursing homes (Spurbeck et al., 2021), and other group living settings. Precise and accurate quantification of viral copies in wastewater is a prerequisite for a successful WBE surveillance project. Detection sensitivity is dependent on the choice of an effective and reliable virus concentration method prior to RNA extraction and quantification.

Virus concentration is crucial in the wastewater especially when viral titers are very low, as is seen in building-based surveillance (Corchis-Scott et al., 2021; Gibas et al., 2021). Polyethylene glycol (PEG)-based precipitation was initially widely used to concentrate the virus with successful signal detection (La Rosa et al., 2020; Wu et al., 2020a; Kumar et al., 2020). This method, however, requires a long processing time. Other methods such as Electronegative Membrane Filtration which is also known as HA method, and Ultrafiltration have been used successfully to concentrate viruses from wastewater prior to RNA extraction

in a variety of application contexts worldwide (Ahmed et al., 2020a; Medema et al., 2020; Nemudryi et al., 2020; Wu et al., 2020b; Wurtzer et al., 2020). Skimmed milk flocculation is suggested as a promising method for resource limited environments based on its detection consistency and simplicity (Philo et al., 2021). Another study focused on a two-step concentration procedure to process large wastewater volumes (McMinn et al., 2021). Among the available methods, the HA method has previously been reported to be one of the most efficient methods of virus concentration based on surrogate virus recovery rate (Ahmed et al., 2020a)). However, Jafferali et al. (2021) recently reported that ultracentrifuge-based methods showed better efficiency in spike recovery and quantification of SARS-CoV-2, citing qPCR inhibition as a potential pitfall of the HA method.

We previously reported outcomes of building level surveillance WBE for a large urban college campus during Fall 2020 using HA as the method of concentration (Gibas et al., 2021). However, to shorten the timeline from sample collection to reporting, we have tested and adopted an alternative concentration method using the InnovaPrep CP Select rapid concentrator. The CP Select is an automatic system that allows the user to concentrate bacteria or virus particles by passing a liquid sample through either hollow or ultrafiltration based concentrating pipette tips. It can process large volumes (up to 5 L) depending on the turbidity of the sample and can concentrate to volumes as small as 150 uL (https://www.innovaprep.com). Rusiñol et al. (2020) investigated three rapid concentration methods: skimmed milk flocculation (SMF), InnovaPrep CP Select automated ultrafiltration using (150 kDa) filter tips, and centrifugal-ultrafiltration (CeUF) based Centricon plus-70 (100 kDa) using MS2 as the surrogate virus spiked into wastewater samples. The higher MS2 recovery (51%) in that study was achieved using the InnovaPrep quick concentrating pipette (CP) to SMF (29%) and CeUF (16.5%). Limited replication in that study did not allow for a

firm conclusion, and the use of MS2, a non-enveloped virus, as a surrogate was not optimal as a benchmark for recovery of an enveloped virus like SARS-CoV-2. Gonzalez et al. (2020) reported the use of the CP Select concentrator for COVID-19 surveillance in the southeastern Virginia area and performed a comparison of viral surrogates from treatment plant influent wastewater, in which the CP Select reported an average BCoV recovery of 5.5% compared to 4.8% with HA.

The characteristics of wastewater collected from congregate living facilities such as university residence halls, schools, and nursing homes are somewhat different from the highly pooled wastewater treatment plant (WWTP) influent. Building level wastewater has a higher variability in viral load, fecal matter content and suspended solids concentration compared to WWTP influent samples. Corchis-Scott et al. (2021) reported that the pepper mild mottle virus (PMMoV), a fecal indicator, in residence hall wastewater varied in concentration across 4 orders of magnitude with a coefficient of variation (CV) of 2.83. In comparison, the signal varied more modestly for influent samples from five different WWTPs, with concentrations falling within only one order of magnitude (CV of 0.38). Because our surveillance system relies on raw building-level wastewater, we have evaluated the CP Select specifically in the building surveillance context with a direct comparison to the established HA method. We aimed to determine how the optimized CP Select method performs compared to the HA method in terms of filtration time, BCoV recovery, and sensitivity of SARS-CoV-2 detection and quantification. We also investigated whether, due to the complex nature of the wastewater, RNA extracted from wastewater might contain inhibitors to RT-qPCR amplification, by evaluating inhibition under each concentration protocol. These optimizations resulted in a CP Select protocol providing increased viral recovery and suitable for rapid reporting of results from building-level SARS-CoV-2 WBE.

2.2 Experimental method

2.2.1 Sample collection

In conjunction with the COVID-19 Wastewater Surveillance being conducted on the UNC Charlotte campus (Gibas et al., 2021), we collected samples from thirty-seven sites that were used to monitor a combination of dormitories, Greek village housing and neighbourhood sites consisting of on-campus non-residential buildings. Wastewater samples were collected thrice weekly via HACH AS960 and ISCO GLS Compact autosampler devices located at a building plumbing cleanout or at a manhole accessed externally. At each of these sites, an autosampler was placed on flat ground at higher elevation than the sample stream. A total of 53 wastewater samples were collected during five separate sampling events between October 2020 and March 2021 for this study.

2.2.2 Sample volume processing/filtration threshold

Ten samples were used to test the impact of turbidity on sample processing time (Table 2.1). VWR/BDH Chemicals pH test strips and the HACH 2100Q Portable Turbidimeter were used to determine pH and turbidity, respectively. The maximum value that can be accurately determined using the HACH 2100Q Portable Turbidimeter is 1000 NTU. Any value that exceeds this limit was listed as >1000 NTU. HA filtration was routinely used as the virus concentration method for SARS-CoV-2 surveillance as previously reported (Gibas et al., 2021). When 40-50 mL wastewater samples were processed, turbid samples require a long processing time, due to clogging of filter pores. In preliminary tests, the InnovaPrep CP Select concentrator was capable of processing 125-150 mL wastewater samples, regardless of

turbidity. We compared the filtration capability of both HA and the CP Select protocols systematically, by processing 40 - 100 mL volumes of 10 different samples using each method. We chose 5 samples which were turbid and 5 which were visually clear, excluding samples that exceeded the measurement threshold for turbidity. Processing time was recorded for each input volume, and downstream outcomes in viral surrogate recovery as well as in the qPCR detection step were compared.

2.2.3 Virus concentration and RNA Extraction

Bovine Coronavirus or BCoV (BOVILIS® Coronavirus, Merck Animal Health, NE, USA), a surrogate of human coronavirus, was spiked into the wastewater as a process control prior to sample concentration. The concentration of BCoV was previously determined (2.2x10^5 copies/mL) using ddPCR and spiked in at a concentration of 1µL per mL of wastewater. Samples were then processed via HA filtration as previously described (Gibas et al., 2021). Briefly, wastewater samples were acidified to adjust the pH in the range of 3.5 - 4.0 followed by the addition of 100X MgCl2, 6H20 (2.5M) in a ratio of 1:100 (Ahmed et al., 2020a; Ciesielski et al., 2021). 40 - 100 mL aliquots of adjusted wastewater were filtered through a 0.45 µm pore size, 47 mm diameter electronegative membrane filter (HA, Millipore) coupled with a disposable filter funnel (Pall corporation, NY, USA) until all liquid appeared to have passed through the filter. After filtration, the membrane filter was folded and resuspended in a 2 mL sterile tube containing 1000 µL of AVL lysis buffer with carrier RNA (Qiagen). The membrane filter suspended in the lysis buffer was incubated at room temperature for 10 minutes followed by vortexing for 15 sec to facilitate the recovery of adsorbed virus particles from the filter. For sample processing with the CP Select concentrator, wastewater samples were centrifuged for 10 mins at 10000×g to remove solid debris. 10% Tween-20 was added to

the supernatant in a ratio of 1:100 before concentration, as recommended by the manufacturer to increase virus recovery. 40 to 150 mL samples were then filtered through a single use 0.05 µm PS Hollow Fiber Filter Tips (InnovaPrep) using the automatic CP Select™ (InnovaPrep). Viral particles attached to the filter tips were recovered by eluting with 0.075% Tween-20/Tris elution fluid using Wet Foam Elution™ technology (InnovaPrep) into a final volume ranging from 250 µL to 500 µL. Following the HA or CP Select concentration step, we then used the QIAamp viral mini kit (Qiagen, Valencia, CA, USA) for RNA extraction from 200 µL of concentrated sample. RNA was extracted following the manufacturer-recommended protocol. Extracted RNA was eluted with AVE buffer into a final volume of 60 µL. All extracted RNA was stored at -80℃ until quantification.

2.2.4 Detection and Quantification method using RT-qPCR

Quantitative reverse transcription PCR (RT-qPCR) was used to detect and quantify SARS-CoV-2 and Bovine Coronavirus from extracted RNA. The CDC recommended N1 (Nucleocapsid) primer and probe set (Corman et al., 2020) was used for SARS-CoV-2 quantification while a primer/probe set published by Decaro et al., (2008) was used for Bovine Coronavirus quantification. All amplification reactions were carried out in one step, with a reaction volume of 20 µL. The SARS-CoV-2 assay consisted of 10 µL iTaq universal one step reaction mix (Bio-Rad, Hercules, CA), 0.5 µL iScript reverse transcriptase (Bio-Rad), 500 nM primers along with 125 nM probe (IDT), and 5.0 µL extracted RNA template. The reaction mix then was amplified using a CFX96 qPCR thermocycler (Bio-Rad, Hercules, CA) with the following thermocycling conditions: reverse transcription at 50°C for 15 min with initiation at 25°C for 2 minutes, followed by polymerase activation at 95°C for 2 min and 44 cycles of denaturation at 95°C for 3 s, followed by annealing at 55°C for 30 s (CDC, 2020). Single stranded RNA based SARS-CoV-2 positive control from Twist Bioscience was used to

generate a standard curve using a series of ten-fold serial dilutions with concentrations ranging 10^5 to 10 copies per reaction. All samples were run in triplicate along with a series of three positive and negative controls. The limit of detection (LoD) of assay was determined following the same protocol as described in Gibas et al., (2021). An extended dilution series of SARS-CoV-2 positive control in a range from 10^5 to 1 copy/reaction in 6 replicates was amplified following the protocol for generating the standard curve as described above. The LoD of the RT-qPCR assay is determined as the lowest concentration at which all the replicates were positive with a less than 1 quantification cycle (Cq) variation among the replicates (Francy et al., 2012). The LoD of the assay was determined as 5 copies/reaction. The LoD of the method was then calculated by multiplying this concentration with the concentration factor which had been previously calculated considering the sample volume processed for the respective methods. Any samples to be considered as SARS-CoV-2 positive must have the concentration above the limit of detection with a minimum of two replicates agreement.

The BCoV assay was similar to the N1 assay, with the primer and probe concentrations at 600 nM and 200 nM, respectively. Thermal cycling parameters were the same used in the Decora et al. (2008) protocol, except the annealing temperature was set at 55°C instead of 60°C. This change improved the primer efficiency from 85% to 102.5%. For BCoV recovery quantification, a standard curve was generated using a serially diluted BCoV vaccine, in the concentration range of 10^5 to 1 copies/reaction. All the primer and probe sequences and the standard curves are included in supplementary file (Table S5 and Figure S1, respectively). All samples were run in triplicate along with a series of three positive and negative controls. The BCoV recovery efficiency was calculated based on the following equation:

$$Recovery\ efficiency(\%) = \left(\frac{Total\ BCoV\ copies\ recovered}{BCoV\ copies\ spiked}\right) \times (100)$$

2.2.5 CP Select protocol optimization

The addition of AVL lysis buffer with carrier RNA (Qiagen) following concentration on SARS-CoV-2 detection was investigated. Eluted concentrated samples from the CP Select concentrator, as discussed in section 2.3, were divided into two parts. AVL lysis buffer with carrier RNA was added into one part at a ratio of 1:1, while the other part was processed without adding the buffer. RNA was extracted from both aliquots using the QIAmp Viral RNA extraction kit, and results were compared with RT-qPCR analysis targeting the N1 gene. The modified CP Select protocol was used in the comparison with the HA method.

2.2.6 Virus attachment to solid debris

We investigated the possible impact of the centrifugation step on viral recovery. As we separate out solids from the wastewater by centrifugation prior to the filtration with the CP Select concentrator, it is likely that some fraction of viral components may end up settling with the pellet at the centrifugation step (Forés et al., 2021). To quantify the amount of virus settled with the pellet during centrifugation, BCoV spiked wastewater were incubated about an hour at 4°C to attach viruses with the debris properly, then the pellets generated from 80 mL wastewater samples after centrifugation at 10000×g for 10 minutes were extracted using an AllPrep Power Viral DNA/RNA Kit (Qiagen, Cat. No. / ID: 28000-50). Both BCoV recovery and SARS-CoV-2 were quantified from both pellet and supernatant extracts, following the same qPCR protocol used for liquid samples.

2.2.7 Effect of sonication on virus recovery

To assay for increased virus recovery, we tested the effect of sonication, which is known to improve recovery in municipal wastewater treatment plant samples with the PEG and

AlCl3 precipitation method (Strubbia et al., 2019; Q. Wu & Liu, 2009). A separate set of samples (n=10) were subjected to sonication treatment for 1 minute prior to the centrifugation step, and then processed and quantified as previously described. Equal aliquots of the same set of samples without sonication treatment were processed for comparison. Both BCoV recovery and SARS-CoV-2 (N1 gene) quantification results were considered for this comparison. A summary of sampling sets and sampling volumes used in different experimental setup for the comparison of HA and CP select method was provided in the supplementary Table S1.

2.2.8 RT- qPCR inhibition

Wastewater is considered as a complex matrix containing a variety of high molecular weight compounds such as humic acids, polysaccharides and proteins that cause interference during RT-qPCR amplification (Schlindwein et al., 2009). This effect may be greater with high concentrations of suspended solids. Though most of the inhibitory substances seem to be removed during the RNA extraction process, residual substances may interfere with the amplification reaction. 10 samples with a 60 mL sample volume were selected to test for the presence of inhibition. RT-qPCR inhibition was assayed by running a VetMAX™ Xeno™ Internal Positive Control - VIC™ Assay (Catalog no. A29767, Applied Biosystems) which has previously been used to test wastewater samples (Greenwald et al., 2021). A known concentration (250 copies/reaction) of VetMAX™ Xeno™ Internal Positive Control (Catalog no-29761, Applied Biosystems) was spiked into RNA extracted from the wastewater and into DNase/RNase free water. VetMAX™ Xeno™ Internal Positive Control - VIC™ Assay was prepared in the same manner as SARS-CoV-2 assay described in section 2.4, only, we added 0.8 µL of premix VetMAX™ Xeno™ - VIC™ Assay instead of N1 primers/probe mix. RT-qPCR was run following the same thermocycling condition as SARS-CoV-2 protocol. All samples were processed together in the same plate to avoid introduction of nuisance variables.

The Cq value found in the DNase/RNase water acts as a reference standard for the wastewater sample. If a higher Cq value is measured in wastewater samples compared to the reference Cq value, it is assumed that there is some degree of inhibition due to the composition of the wastewater sample. Typically, a delayed Cq of 2 or greater in wastewater samples relative to the reference Cq value is considered to have RT-qPCR inhibition (Staley et al., 2012; Ahmed et al., 2020b).

2.2.9 Data analysis

All the figures were plotted using Excel 2016 (Microsoft). One-way anova test, t-test and regression analysis were performed using Minitab® 19. P values less than 0.05 were considered statistically significant while greater than 0.05 were considered insignificant or alternative hypotheses are valid. All the RT-qPCR data were analysed using CFX Maestro™ Software (Biorad).

2.3 Results and discussion

2.3.1 Optimization of CP Select protocol

The automated CP Select concentrator is a relatively new method that has only recently begun to be widely adopted for filtration of wastewater samples. Though there are manufacturer-recommended protocols for concentration of virus from wastewater, which were initially followed, we tested several modifications aimed at improving the performance of the concentration workflow to increase recovery of SARS-CoV-2. Using the manufacturer-recommended protocol we were able to detect SARS-CoV-2 successfully by filtering 100 to 150 mL of wastewater; however, quantification was not as robust as with our established HA protocol which uses a 40 mL input volume (Supplementary Table S2). We had previously

observed improved results with HA filtration upon addition of AVL lysis buffer to the filtered sample. Therefore, we tested the impact of adding an AVL lysis buffer with carrier RNA to the concentrated samples eluted from the CP Select as described in Section 2.3. This addition to the manufacturer-recommended protocol significantly improved detection. SARS-CoV-2 was detected in all three replicates from the eluent with added lysis buffer, and not detected in the replicates without the lysis buffer (Supplementary Table S3). This optimization step was included in the protocol and when compared with the HA method, as described in Section 3.4, the modified CP Select protocol performed better.

2.3.2 Time comparison of HA and CP Select concentration methods

A side-by-side comparison of the HA and the CP Select methods was designed, as shown in Table 2.1, to understand how the viral concentration method would impact wastewater sample processing time. There are two components to the processing time - preprocessing and filtration (concentration). Preprocessing consists of sample pH adjustment and MgCl2 addition for the HA method, while centrifugation is used as a preprocessing step for the CP Select protocol. The second step in the protocol is filtration (concentration) itself. We found that for filtration of 40 mL samples, which is the typical input for the HA protocol in our previous work (Gibas et al., 2021), the CP Select method gave no clear advantage over HA in filtration time. However, for larger samples of 60 mL and above, the CP Select outperformed the HA method significantly. For 60 mL samples, the average time to concentration with the CP Select was 9.25 min, compared to over 30 min for the HA method. For 100 mL samples, the HA method could not be used to process most samples, while the CP Select continued to successfully filter samples in under 30 min. In addition, when the lab team compared the time required to complete both the preprocessing and the filtration for 20 samples, 3 h were required for processing 40 mL using the vacuum manifold HA approach,

while only 2 h were required for processing 80 mL when using the CP Select concentrator; this is considering that 6 vacuum manifold stations were available to be used in parallel, and only 4 InnovaPrep stations could be used in parallel. Given this, the CP Select is the practical choice for larger total input volume in routine processing.

Table 2.1 Filtering volume time comparison between HA and CP Select method.

Sample ID	pH	Turbidity (NTU)	40 mL filtering (min)		60 mL filtering (min)		100 mL filtering (min)	
			HA	CP Select	HA	CP Select	HA	CP Select
S1	7	26.7	1	5	2	10	Over 30	20
S2	7	27.3	1	1	2	1	Over 30	5
S3	7.5	13.2	1	1	1	10	6	10
S4	9	53	1	2	Over 30	10	Over 30	20
S5	7.5	15.6	1	1	1	10	2	30
S6	7	10.1	1	1	2	1	2	5
S7	7.5	11	-	1	-	1	-	14
S8	7	1000	Over 30	2	Over 30	7	Over 30	30
S9	8.5	347	Over 30	1	Over 30	10	Over 30	20
S10	7.5	97.8	7	2	Over 30	10	Over 30	14

- Data is not available

2.3.3 Surrogate virus recovery for HA and CP Select concentration methods

Surrogate virus recovery data is essential to test virus concentration methods as well as process control for the surveillance system especially when RNA of the target organisms cannot be quantified exactly or is difficult to determine. A known concentration of a surrogate virus is spiked into the wastewater before processing and quantified using RT-qPCR following RNA extraction to determine what percentage of the spiked input is recovered from the system, and how much is lost during the sample processing steps. Based on the type of virus concentration method and the RNA extraction process, RNA recovery percentages vary widely. This is often a determining factor for selecting potential virus concentration methods from among different alternatives (LaTurner et al., 2021).

Several different viruses have been used as process controls in WBE studies, including Murine Hepatitis Virus (MHV) (Ahmed et al., 2020a), Beta Coronavirus OC43 (Pecson et al., 2021; Sherchan et al., 2020) Feline calicivirus (Barril et al., 2021), Human coronavirus (HCoV 229E) (Betancourt et al., 2021; La Rosa et al., 2020), Bovine respiratory syncytial virus (Gonzalez et al., 2020), BCoV (Gonzalez et al., 2020; Jafferali et al., 2021), and Phi 6 (Pecson et al., 2021; Sherchan et al., 2020), MS2 (Rusiñol et al., 2020). Non-enveloped viruses like MS2, when used as a process control, showed higher recovery than enveloped viruses. Enveloped viruses have a lipid layer in the outer membrane making it more susceptible to pH, temperature, and organic solvent (Ye et al., 2016; Polo et al., 2020). We selected BCoV as a process control because it is as an enveloped virus similar to SARS-CoV-2 and belonging to the same Coronaviridae family (LaTurner et al., 2021) as recommended by Pecson et al. (2021) and Sherchan et al. (2020).

For surrogate virus recovery analysis, wastewater samples (n = 10) were processed using the same input volume of wastewater (40 mL, 60 mL, and 100 mL) for both the concentration methods side by side. Fig. 2.1 shows the mean BCoV recovery from wastewater concentrated using HA and the CP Select for different sampling volumes. Both methods showed a wide range of BCoV recovery, due to high variability of sample characteristics such as turbidity. The sample volume also has a role in the variation of BCoV recovery for both concentration methods. The HA method showed an average BCoV recovery of 17.3% when 40 mL wastewater was filtered which was higher than that for 60 mL and 100 mL sampling volume (Fig. 2.1(b)). This is similar to the BCoV recovery rate found by Jafferali et al. (2021) and is higher than the reported value of 4.8% by Gonzalez et al. (2020); however, this is lower compared with MHV recovery reported in (Ahmed et al., 2020a). It might be because of the different structure or isoelectric point of MHV compared to BCoV. For the CP Select method, 60 mL sampling size seemed to be optimum based on the BCoV recovery result shown in Fig. 2.1(a). The CP Select method yielded an average of 36.81% BCoV recovery from the 60 mL sampling volume, the highest among all other sampling volumes. Similar results reported in other studies using MS2 and OC43 recovery (Forés et al., 2021; McMinn et al., 2021). In this study, when comparing the BCoV recovery between the two methods under consideration, the CP Select method showed higher recovery than HA in terms of both median value and average recovery value, as shown in Fig. 1, however, it was not statistically significant (P value of 0.12). Forés et al. (2021) also compared two rapid concentration method – CeUF based Centricon plus® 70 and CP Select and found similar performances in terms of MS2 recovery and SARS-CoV-2 quantification, although higher MHV recovery was reported with the Centricon plus® 70.

The effective volume assayed in the RT-qPCR reaction is another way to evaluate the efficiency of concentration methods. The CP Select method allowed the use of up to 5 mL equivalent wastewater per reaction, with a minimum of 1.33 mL, while the range of effective volume for the HA method was 0.66 - 1.67 mL. Similarly, use of the CP Select also resulted in higher concentration factors ranging 160-600× in comparison to 40-100× with the HA method.

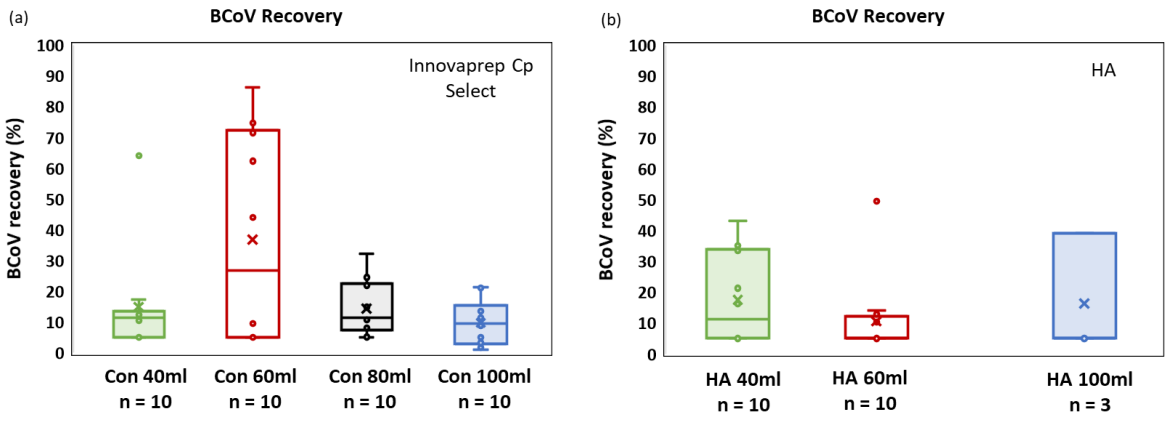

Fig. 2.1. The effect of sampling volume on the BCoV recovery from wastewater samples processed with the CP Select and HA method. (a) Percentage BCoV recovery for the CP Select method; (b) percentage of BCoV recovery for the HA method. The 'box' symbol (□) of the boxplots represents lower (Q1) and upper quartile (Q3) data with median value; 'cross' symbol (×) indicates the average BCoV recovery data. 'Whiskers' symbol (⊥) indicates the data variability outside of the lower and upper quartile with minimum and maximum value.

2.3.4 Performance comparison based on SARS-CoV-2 detection and quantification

For the 40 mL sampling volume, three samples were detected as SARS-CoV-2 positive using the HA protocol while four samples were positive when the CP Select protocol was used. When a 60 mL sample volume was used as input, no additional positives were detected using the HA protocol, but two additional samples were detected as positive with the CP Select

protocol. Detection was also more robust following CP Select processing with the larger sample; all three qPCR replicates were positive in more samples in contrast to HA-processed samples not showing detectable amplification in all replicates (Supplementary Table S4). Also, the variation among Cq values for each sample using the CP Select method was lower.

Table 2.2 SARS-CoV-2 detection from wastewater sample concentrated by HA and CP Select methods.

Sample ID	40 mL sample		60 mL sample	
	SARS-CoV-2 detection		SARS-CoV-2 detection	
	HA	Cp Select	HA	Cp Select
S1	-	-	-	-
S2	-	+	-	+
S3	-	-	-	-
S4	+++	+++	-	+++
S5	-	++	+++	+++
S6	-	-	-	+
S7	++	+++	++	+++
S8	-	+	-	++
S9	++	+++	+++	+++
S10	+	+	-	+
SARS-CoV-2 positive	3 out of 10	4 out of 10	3 out of 10	5 out of 10

- Not detected
+ SARS-CoV-2 detected in one replicate out of three
++ SARS-CoV-2 detected in two replicates out of three
+++ SARS-CoV-2 detected in three replicates out of three

The LoD for the CP Select assay workflow was in the range of 1.5×10^3 to 3.75×10^3 copies/L for 100 mL to 40 mL wastewater samples processed, respectively, while it was 3.0 ×

10^3 to 7.5×10^3 copies/L for the HA method; twice the LoD of the CP Select method. Fig. 2.2 (a) and (b) which show the variability in viral copy number detected from the same set of samples using the HA and CP Select workflows. When a 40 mL sample was processed using the HA, SARS-CoV-2 quantification ranged from 104 to 4.2×10^5 genome copies/L while it was 1.5×10^3 - 9.3×10^4 genome copies/L using the CP Select method. The lower end of the quantification range of the latter method is due to the samples that did not amplify and could be considered non-detected with the HA method. However, no significant differences were observed between these two methods in SARS-CoV-2 quantification for high titer wastewater samples ($P = 0.51$). This described trend was also observed for the 60 mL data set. Other studies also reported similar range of SARS-CoV-2 concentration both in the university resident hall's wastewater and WWTP's influent using the CP Select method. For example, A range of 2.4×10^4 - 4×10^4 copies/L of SARS-CoV-2 gene was reported in the residence hall wastewater using the CP Select method during the surveillance at the University of Windsor (Corchis-Scott et al., 2021) while it was in the range from 10^3 to 2×10^5 copies/L for WWTP's influent samples (Forés et al., 2021; Lynch et al., 2021). Although these data are mostly similar to the concentration found in this study, however, there is highly likely to have a different concentration of SARS-CoV-2 in wastewater as it mostly depends on the density of the COVID-19 infected people staying in those area during the sampling time, demographic location, pattern of the sewerage system, and wastewater characteristics (Barua et al., 2021).

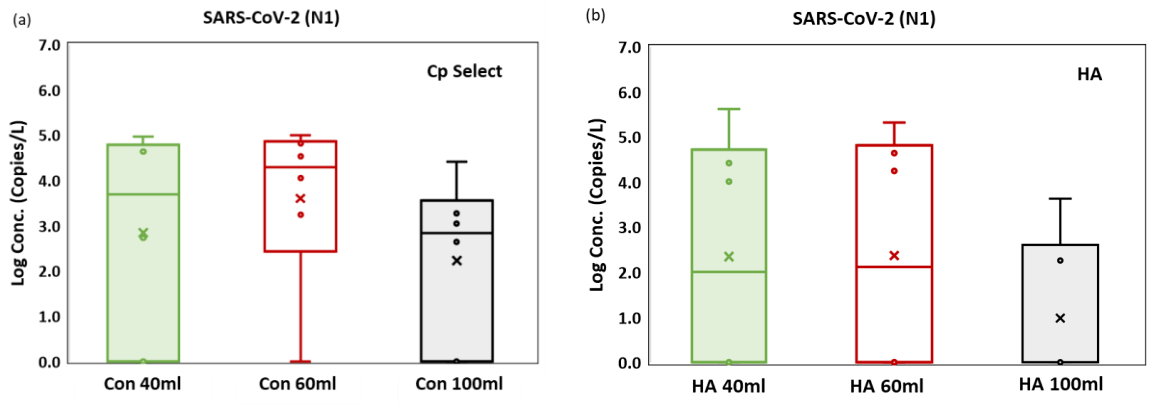

Fig. 2.2 Effect of sample volume size on the performance of CP Select concentrator and HA in terms of SARS-CoV-2 quantification. (a) SARS-CoV-2 quantification from concentrated samples using Innovaprep CP Select concentrator; (b) SARS-CoV-2 quantification from concentrated samples using HA. The 'box' symbol (□) represents lower (Q1) and upper quartile (Q3) data with median value; 'cross' symbol (×) indicates the average SARS-CoV-2 quantification data. 'Whiskers' symbol (⊥) indicates the data variability outside of the lower and upper quartile with minimum and maximum Log transformed SARS-CoV-2 concentration.

SARS-CoV-2 detection and quantification performance was further evaluated for the CP select method using a larger input volume of wastewater, on the assumption that concentrating more copies of the virus, would allow for better quantification of low viral titer samples. A separate set of samples (n = 20) were then processed using the two-concentration methods side by side, followed by RNA extraction, and quantification. 100-150 mL wastewater was filtered through the CP Select concentrator, while 40 mL (the volume routinely used in our surveillance protocol) was filtered through the HA filter. Out of 20 wastewater samples, SARS-CoV-2 was detected in 8 samples processed with the HA filtration, while 12 samples were positive when processed using the CP Select (Fig. 2.3). By concentrating viruses from a larger volume of wastewater, the CP Select method resulted in more sensitive detection. Five samples reported negative using the routinely followed HA method were detected as SARS-CoV-2

positive when processed with the CP Select method, while in only one case did HA filtration detect a positive where the CP Select did not. The SARS-CoV-2 was detected in the additional CP Select derived samples had higher Cq values (i.e. at lower viral copy numbers) which indicated that the workflow using the CP Select concentration step is capable of capturing viruses from low-titer wastewater samples that may be missed using the HA method.

Overall, the CP Select method is more sensitive than HA method as the higher number of positive samples obtained by this method than obtained by the HA method. The CP Select method is beneficial in situations where detection sensitivity and quick data reporting is important. The tradeoff for this method is the cost effectiveness where the CP Select protocol doubles the material and the reagent cost per sample as compared with the HA method. However, the ease of operation of the CP Select also reduces the number of lab workers and time needed to process the samples.

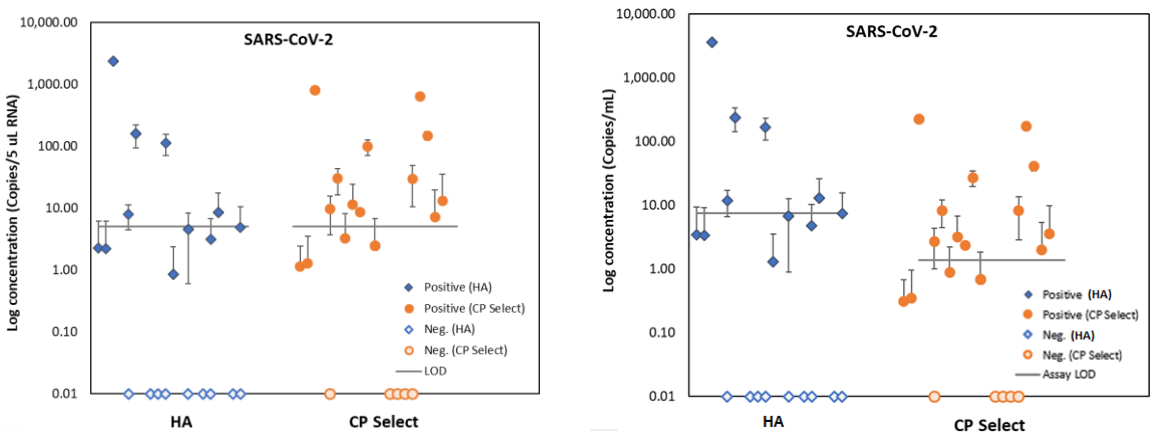

Fig. 2.3 Quantification of SARS-CoV-2 from wastewater concentrated by HA and Innovaprep CP Select protocol. Error bars indicate the standard deviation among replicates.

2.3.5 Virus attachment to solid debris

To determine whether a significant amount of virus remained in the pellets following centrifugation step, we quantified recovery of BCoV and natural SARS-CoV-2 from both the pellet and the supernatant of centrifuged samples (Fig. 2.4). A significantly smaller fraction of BCoV was recovered from the pellet than from the supernatant, with a P-value of 0.015 ($P < 0.05$). However, SARS-CoV-2 behaved differently from BCoV in centrifugation, with similar recovery fractions in the supernatant and the pellet (P value of 0.857). This difference may be due to the viral structure itself; the structure of the spike protein may result in SARS-CoV-2 attaching more strongly to a solid surface compared to BCoV. Ai et al. (2021) reported a similar trend with 0.2% BCoV recovery from the pellets while the SARS-CoV-2 recovery was found to be 10%. Similar to the SARS-CoV-2 partitioning results found in our analysis Forés et al. (2021) reported about 23% SARS-CoV-2 recovery from the pellet, and Ye et al. (2016) observed about 24% MHV partitioning to the solid. However, another study reported no significant difference in SARS-CoV-2 quantification results due to separating solids from the liquid (Pecson et al., 2021), although the pellet material was not directly assayed. The variation in recoveries observed in different studies may be due to the variability in the wastewater matrix at different locations or collection sites, or due to the differences in the methodological approaches.

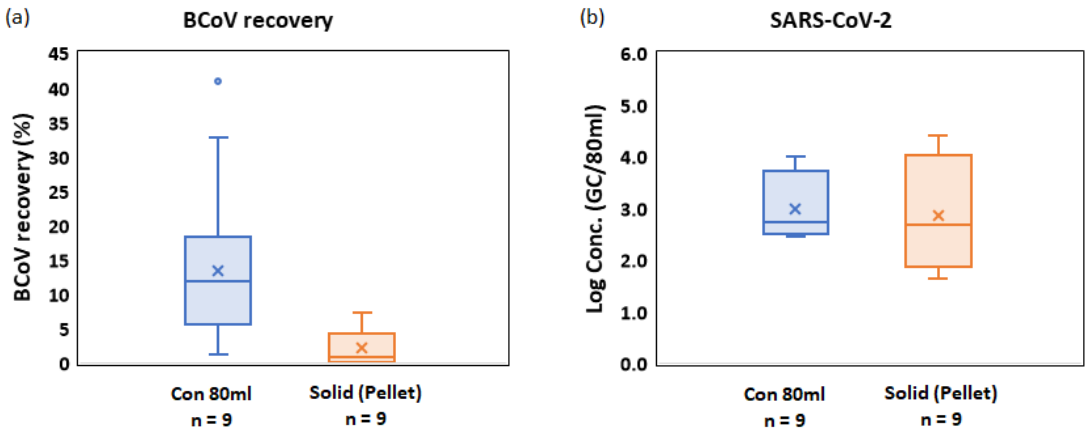

Fig. 2.4 Fraction of viral material partitioned to the supernatant and solid debris fraction for CP Select processed samples, which are centrifuged prior to concentration to remove debris. (a) percentage of BCoV recovery and (b) SARS-CoV-2 quantification.

2.3.6 Effect of sonication on virus recovery

In the previous section, we observed that a fraction of viral material is adsorbed by suspended solids and settled with the pellet during centrifugation step. To counter this effect, we tested the impact of a very short sonication step (1 min) prior to centrifugation of wastewater samples. The sonication step acts to disrupt the attachment of viral material to solids but was kept short to minimize damage to the viral RNA itself. Sonication treatment has been previously shown to increase viral recovery by causing desorption of viral particles from organic substances and release of viral particles from host cells (Corpuz et al., 2020; Strubbia et al., 2019).

Table 2.3 The effect of sonication treatment on BCoV recovery and SARS-CoV-2 detection.

Sample ID	pH	Turbidity (NTU)	BCoV recovery				SARS-CoV-2	
			Without Sonication		With Sonication		Without Sonication	With Sonication
			Avg Cq	Recovery (%)	Avg Cq	Recovery (%)	Avg Cq	Avg Cq
S31	7.5	46.5	33.7	3.2	29.8	50.4	36.2*	35.0
S32	7.5	>1000	35.1	1.2	ND	-	35.2*	ND
S33	8	390	33.5	3.8	33.5	3.8	ND	34.9
S34	7.5	338	35.1	1.3	34.5	1.8	ND	40.0
S35	7.5	>1000	35.2	1.1	32.6	7.1	35.5	37.5
S36	8.5	38.2	34.2	2.3	30.0	59.8	34.7	32.3
S37	8	>1000	33.0	5.2	31.6	14.5	36.5	35.7
S38	8	58.2	31.7	12.9	30.6	28.8	35.5	35.9
S39	8	978	ND	-	ND	-	ND	ND

Results of the sonication experiment are shown in Table 2.3. BCoV recovery improved for most samples after addition of the sonication treatment. Average recovery increased from 3.85% to 23.74%. Due to the variability of material collected during our ongoing sampling operation and available for testing the group of samples for this analysis were very turbid (Table 2.3) compared to some of the samples used previously (Table 2.1), and initial BCoV recovery from these samples was somewhat lower than typical. Along with improved BCoV recovery from a majority of samples, SARS-CoV-2 detection also improved with sonication treatment, with Cq values being lower in many instances, and detection of the virus in samples which had previously appeared to be negative (Table 2.3). The sonication step may partly solve a problem common to all ultrafiltration-based concentration methods, in which some part of

the virus is lost with the pellet during centrifugation. We subsequently adopted the sonication step as part of our standard CP Select virus concentration protocol used for the routine SARS-CoV-2 wastewater-based monitoring at UNC Charlotte.

2.3.7 qPCR inhibition

RT-qPCR detection of the VetMAX™ Xeno™ Internal Positive Control spiked into the extracted RNA is shown in Fig. 2.5. An average Cq of 8 NTC replicates was used as the reference point (Cq = 32.62). Most samples did not appear to be affected by inhibitors in the RT-qPCR step using either protocol, as nearly all Cq values fall within 2 Cq of the reference line. One sample processed with the CP Select did show a delayed Cq, which was not replicated when the sample was processed using HA, but overall, the difference between the two methods did not meet a threshold for statistical significance when all values were compared. Cq values for all other samples processed with both methods were within the 1 Cq variation from the reference value. This suggests there is no consistent and significant inhibition to RT-qPCR amplification for extracted RNA from samples processed with either of the two filtration methods.

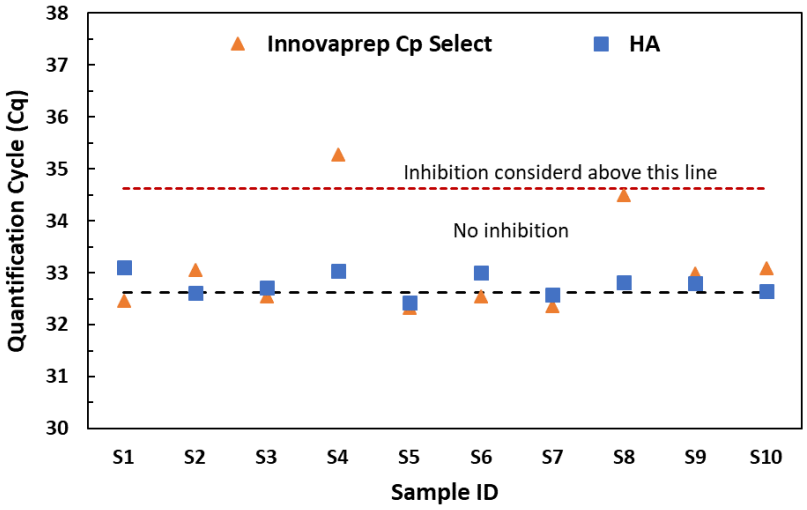

Fig. 2.5 RT-qPCR inhibition test comparing results for samples concentrated with CP Select and with the HA method. Across all samples, differences in Cq did not rise to the level of statistical significance.

2.4 Conclusion

We have developed an optimized protocol for use of the InnovaPrep CP Select concentrator, in a routine building wastewater surveillance program on a university campus. The CP Select method resulted in a BCoV recovery rate of approximately 37%, which is higher than BCoV recovery from samples processed using an HA protocol. The CP Select is capable of processing up to 150 mL of wastewater within 30 min, while the HA method fails at larger volumes and operates optimally with 40 mL input. This allows for a higher effective volume of wastewater to be assayed with the CP Select relative to HA, which in turn results in increased sensitivity for detection and quantification of SARS-CoV-2 from wastewater. Overall, the processing time for handling a typical day's collected samples in a surveillance scenario was decreased by 33% (from 3 h to 2 h). We found that use of a lysis buffer (AVL) significantly improved the performance of the InnovaPrep manufacturer recommended protocol for

wastewater and have introduced that modification to our routine work. One observation in use of an ultrafiltration-based protocol was that viral material may be lost with the pellet in the required centrifugation step, however, in combination with a brief sonication treatment, we were able to achieve higher recovery fractions. We did not observe significant differences in qPCR inhibition when the CP Select protocol was used, relative to the HA protocol. In general, the CP Select concentrator is advantageous for concentrating low viral titer wastewater samples, especially when rapid data reporting is necessary, and use of this protocol can also improve recovery and detection sensitivity.

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2.6 Appendices

Supplementary Table S1: Summary of sampling set and sampling volume used in different experimental setup for the comparison of HA and CP select methods.

Sample ID	No. of samples	Volume (ml)	Objectives and sections covered
S1 to S10	10	40 – 100	Comparison based on filtration time (section 3.2)
		40 - 100	Volume based comparison in terms of BCoV recovery and SARS-CoV-2 detection and quantification (section 3.3)
		80	Virus attachment to solid debris (section 3.5)
		60	qPCR inhibition determination (section 3.7)
1 to 20	20	40*/150*	Impact of higher sample volume on CP Select in terms of SARS-CoV-2 detection and quantification (section 3.3, Figure 4)
31 to 39	9	60	The effect of sonication treatment on the performance of CP Select method (section 3.6)

*40 ml used in HA while 125-150 ml used in the CP Select method.

Supplementary Table S2: Comparison between two concentration methods. In this analysis, 40 mL wastewater was filtered using HA and 125 to 150 mL processed with the CP Select Concentrator. The AVL lysis buffer was not added to the concentrated sample from the CP Select protocol. Water quality includes in the Table.

Sample name	pH	Turbidity (NTU)	HA			CP Select		
			Cq	Mean Cq	Copies/L	Cq	Mean Cq	Copies/L
Greek-3_10.16.20	7.0	488	ND 35.74 35.9	35.82	1.46E+04	ND 35.25 34.48	34.87	2.31E+03
Holshouser_10.16.20	8.0	45.2	35.65 35.79 35.95	35.80	1.48E+04	ND 36.23 ND		
Greek-3_10.14.20	-	-	ND 34.89	35.16	2.35E+04	35.15 34.81	34.85	2.31E+03

			35.42			34.59		
Holshouser_10.14.20	8.5	-	Negative			Negative		
Greek-3_10.12.20	-	-	34.42 36.77 36.51	35.9	3.31E+04	38.40 ND 38.48	38.44 *	1.14E+03
Holshouser_10.12.20	-	-	Negative			Negative		
Lynch_10.16.20	7.5	183	Negative			Negative		
Scott_10.16.20	8.5	200	Negative			Negative		
Sanford_10.16.20	8.5	87.7	ND ND 36.22			Negative		
Hunt_10.16.20	-	-	Negative			Negative		

* 50 ml wastewater was concentrated to 250 µL in the concentrator. Red Color indicates out of the LoD

Supplementary Table S3: The effect of addition of lysis buffer to the concentrated eluted sample from the CP Select method.

Sample ID	With of AVL Lysis buffer				Without AVL lysis buffer			
	Rep.# 1	Rep.# 2	Rep.# 3	Mean Cq	Rep.# 1	Rep.# 2	Rep.# 3	Mean Cq
Hawthorn	ND	ND	ND	-	ND	40.24	36.65	38.45
Holshouser	35.47	37.39	36.31	36.39	ND	ND	36.36	-
Belk_West	32.87	32.58	33.39	32.95	ND	39.19	ND	-

Red Color indicates out of LoD.

Supplementary Table S4**:** Effect of sample volume size on the performance of HA and CP Select methods in terms of SARS-CoV-2 quantification.

	HA filtration (40 mL)					CP Select (40 mL)				
	Cq Value					Cq Value				
I.D.	Rep. #1	Rep. #2	Repl. #3	Mean Cq	SD	Rep. #1	Rep. #2	Repl. #3	Mean Cq	SD
S1	-	-	-	-		-	-	-	-	
S2	-	-	-	-		36.14	-	-	-	
S3	-	-	-	-		-	-	-	-	

S4	31.13	31.16	31.12	31.14	0.02	31.67	32.41	32.78	32.28	0.56
S5	-	-	-			39.10	37.04	-	38.07	1.45
S6	-	-	-	-		-	-	-	-	
S7	34.60	-	35.40	35	0.57	32.90	33.15	33.54	33.2	0.32
S8	-	-	-	-		37.09	-	-	-	
S9	36.62	-	36.02	36.32	0.42	33.22	33.14	34.05	33.47	0.50
S10	37	-	-	-		37.46	-	-	-	

	HA filtration (60 mL)					CP Select (60 mL)				
	Cq Value					Cq Value				
I.D.	Rep. #1	Rep. #2	Repl. #3	Mean Cq	SD	Rep. #1	Rep. #2	Repl. #3	Mean Cq	SD
S1	-	-	-	-		-	-	-	-	
S2	-	-	-	-		37.12	-	-	-	
S3	-	-	-	-		-	-	-	-	
S4	-	-	-	-		32.42	32.61	32.63	32.55	0.11
S5	36.31	41.24	41.24	39.60	2.85	34.70	35.02	35.54	35.08	0.42
S6	-	-	-	-		37.23	-	-	-	
S7	34.60	-	35.40	35	0.57	31.75	32.35	31.90	32.00	0.31
S8	-	-	-	-		37.33	-	37.16	37.24	0.12
S9	31.75	31.17	31.66	31.53	0.31	32.97	32.80	33.60	33.12	0.42
S10	-	-	-	-		37.04	-	-	-	

	HA filtration (100 mL)					CP Select (100 mL)				
	Cq Value					Cq Value				
I.D.	Rep. #1	Rep. #2	Repl. #3	Mean Cq	SD	Rep. #1	Rep. #2	Repl. #3	Mean Cq	SD
S1						-	-	-	-	
S2						-	-	-	-	
S3	-	-	-			-	-	-	-	
S4						32.53	33.06	32.71	32.77	0.27
S5	38.48	34.00	ND	36.24	3.17	41.79	35.41	-	38.60	4.51
S6	38.89	42.44	ND	40.66	2.51	-	-	-	-	
S7						37.26	37.87	36.40	37.18	0.73
S8						-	-	-	-	

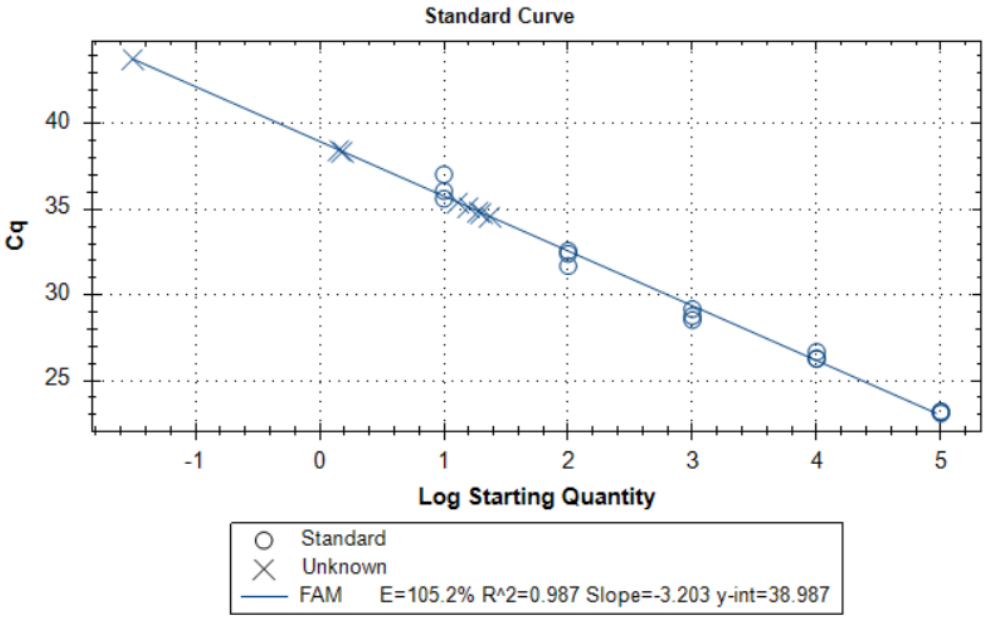
S9		37.23	37.17	35.66	36.69	0.89
S10		-	-	36.83	-	

Grey color column indicates those samples did not pass through the HA filter during processing.

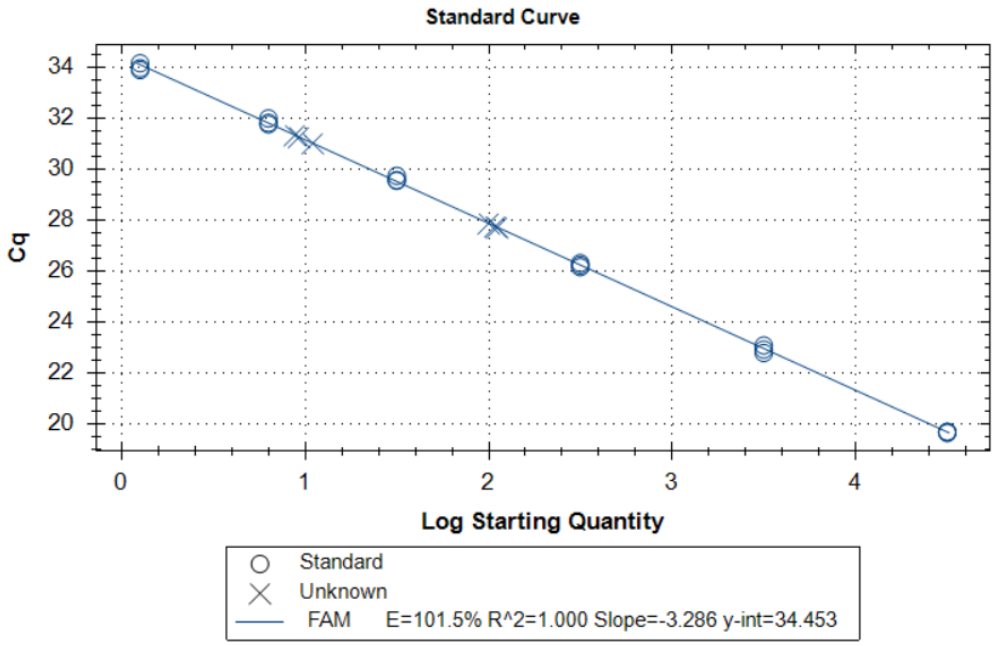
Supplementary Table S5: Primers and probe sequences used in this study.

Assay	Primer/Probe	Sequences	References
CDC N1	Forward	GACCCCAAAATCAGCGAAAT	Lu et al., 2020
	Reverse	TCTGGTTACTGCCAGTTGAATCTG	
	Probe	FAM-ACCCCGCAT/ZEN/TACGTTTGGTGGACC-3IABkFQ	
BCoV	Forward	CTGGAAGTTGGTGGAGTT	Decaro et al., 2008
	Reverse	ATTATCGGCCTAACATACATC	
	Probe	FAM-CCTTCATATCTATACACATCAAGTTGTT-BHQ1	

(a)



(b)



Supplementary Figure S1: Standard curve used in this study: (a) SARS-CoV-2 (N1) (b) BCoV

Supplementary Table S6: Water quality of the wastewater samples used in the Fig.4

Sample ID	pH	Turbidity (NTU)
1	8	65.1
2	7.5	77.5
3	7.5	46.4
4	7.5	59.6
5	8.0	60.9
6	8.5	342
7	-	-
8	7.5	854
9	7.5	>1000
10	7.5	61.5
11	7.0	43.5
12	8.0	25.4
13	8.5	131
14	7.0	14.7
15	8.0	739
16	9.0	265

17	7.5	86
18	7.0	>1000
19	7.0	>1000
20	7.5	64.7

3 ARTICLE 2: DEVELOPMENT OF LARGE VOLUME FILTRATION-BASED VIRUS CONCENTRATION METHOD FOR INCREASED DETECTION SENSITIVITY OF SARS-COV-2 FROM WASTEWATER

3.1 Introduction

Wastewater Based Epidemiology (WBE) is a public health tool that uses wastewater to monitor human pathogenic viruses that are shed through sneezes and feces of infected patients and mixed with domestic wastewater. This tool has recently been used to monitor Corona Virus Disease (COVID-19) outbreak in the community by quantifying SARS-CoV-2 viruses from wastewater (Ahmed et al., 2020; Hohl et al., 2020; Sherchan et al., 2020). Since COVID-19-infected individuals shed viruses through feces irrespective of symptoms onset, surveillance of this disease through wastewater testing covers both symptomatic and non-symptomatic COVID-19 patients. Thus, wastewater surveillance can reflect a true representation of the infection scenario in the community. Multiple studies reported that this WBE tool can track a COVID-19 outbreak 1 to 2 weeks in advance, thus allowing administrators to take preventive measures before possible outbreaks (Barua et al., 2022; Peccia et al., 2020).

The successful implementation of this WBE tool depends on many factors; one of them is precise viral copies quantification from wastewater (LaTurner et al., 2021; Miura et al., 2021). Concentrating viruses from wastewater prior to quantification is imperative as viruses shed through feces become low in concentration after mixing with a high volume of wastewater (Michael-Kordatou et al., 2020; Randazzo et al., 2020). There are several common virus concentration methods such as PEG precipitation (La Rosa et al., 2020; Polo et al., 2020), centrifugal ultrafiltration (Nemudryi et al., 2020; F. Wu et al., 2022), and electronegative membrane filtration a.k.a HA method (Ahmed et al., 2020; Barua et al., 2022) which have been used for SARS-CoV-2 surveillance. These methods were successful in SARS-CoV-2 detection

and quantification in wastewater during the high COVID-19 infection rate in the community. The inherent limitation of most of the current methods is the small volumes used for processing the wastewater, which ranged from 20 – 250 mL (McMinn et al., 2023). While small volume-based virus concentration method can be successful for detecting and quantifying SARS-CoV-2 viruses during high community infection, these methods may not be informative especially during the early stages of community infections because of low COVID-19 prevalence where viral titers in wastewater are not high enough to be detectable (McMinn et al., 2023). Estimating viral levels during the early stage of infection is crucial for public health decision-making to better support and protect the community and allocate resources for monitoring more effectively.

Most of the large volume-based virus concentration methods have been used for surface water filtration which requires processing up to 100 L of water (Cuevas-Ferrando et al., 2021; Korajkic et al., 2022; Liu et al., 2012). Because of the low concentration of different bacterial and viral targets in water and wastewater, multi-step concentration methods may require detecting targets of interest. However, multi-step large volume concentration method needs to be optimized and improved for evaluating the sensitivity of virus and bacterial targets. The successful application of hollow UF based multi-step concentration method was studied for detecting enteric pathogens such as noroviruses, rotaviruses, adenovirus. (Wu et al., 2023). So far, very little attention has been paid to the use of large-volume concepts for the increased detection sensitivity of SARS-CoV-2 from wastewater samples. One study found an increased detection sensitivity of SARS-CoV-2 when 2 L of wastewater samples were filtered using a dead-end hollow fiber ultrafilter (UF) followed by the Innovaprep CP Select™ (McMinn et al., 2023). A limitation of this study is the low number of wastewater samples (n = 2) used. However, this concept has recently been challenged in another study demonstrating no

significant improvement in detection sensitivity when a large volume of wastewater (1 L) was used compared to a small volume (30 mL) (Zheng et al., 2022). So, more rigorous studies are needed to investigate the applicability of the large volume-based virus concentration for increased detection sensitivity. The objective of this paper was to evaluate the effectiveness of the hollow UF based large volume concentration method in detecting viruses from low titered wastewater samples in compared to electronegative membrane filter (HA) based small volume concentration method.

The success of wastewater-based epidemiology for predicting COVID-19 disease outbreaks has already been established, and in most cases, liquid wastewater samples have been used for the analysis. However, SARS-CoV-2 viruses have been reported to be partitioned in both liquid and solid (Graham et al., 2021; Kim et al., 2022; Wolfe et al., 2021). Some articles reported that around 50% of SARS-CoV-2 viruses were partitioned between the solid and liquid phases (Breadner et al., 2023; Juel et al., 2021). This study also analyzed the partitioning of SARS-CoV-2 viruses between liquid and solid phases. In addition, whether SARS-CoV-2 viruses are absorbed most in (1) large settleable solid particles or in (2) smaller suspended particles where centrifugation is needed for separation, was also investigated. Usually, the size of the large settleable solid particle ranges from 20 - 180 µm where around 0.45 µm for the smaller suspended particles (Greaves et al., 2022). This information will be useful for considering pre-concentration steps, such as solid removal, of different filtration-based virus concentration methods.

3.2 Experimental Methods

3.2.1 Sample Collection

Wastewater samples were collected from Sugar Creek (SC) and Mallard Creek (MLC) Wastewater Treatment Plant (WWTP) on a weekly basis from January 2021 to February 2022. The sewershed boundary of Sugar Creek WWTP covers uptown Charlotte including the CLT international airport, major hospitals, industries, and serves a population of 180,000 while the UNC Charlotte campus, and hospitals are covered by the Mallard Creek WWTP sewershed boundary and serves a population size of 120,000.

3.2.2 Liquid sample processing

We aimed to develop a two-step concentration technique that enabled filtering large volumes of wastewater which consists of primary and secondary concentrations. A dead-end hollow fiber ultrafilter (UF) (Rexeed 15S, Chester, PA), which has a higher surface area, was used to filter 2-3 L of wastewater as a primary concentration. The filtration procedure using the hollow UF was followed based on the protocol described in Huiyun et al., (2023). Before filtration, a stock solution (55000 GC/µL) of Bovine Coronavirus (BCoV) was mixed well with wastewater at a concentration of 25 µL/L wastewater. Wastewater samples (2-3 L) were filtered through hollow UF using a peristaltic pump at a speed of 300 rpm (Fig. 3.1). Viruses from the filter surface were recovered in 200 mL of a previously prepared buffer solution (0.01% Tween 80, 0.01% sodium hexametaphosphate, 0.001% Antifoam Y-20). 1 mL out of the 200 mL of 1st eluate was directly extracted which was denoted as UF (Fig. 3.2). The rest of the 1st eluate (concentrated samples) were aliquoted for the secondary concentration. As a part of the secondary concentration, 20 mL of the aliquoted samples was filtered through the

electronegative membrane filter (HA) following the same protocol as described in Gibas et al., (2021). The combination method was denoted as UF-HA (Fig. 3.2).

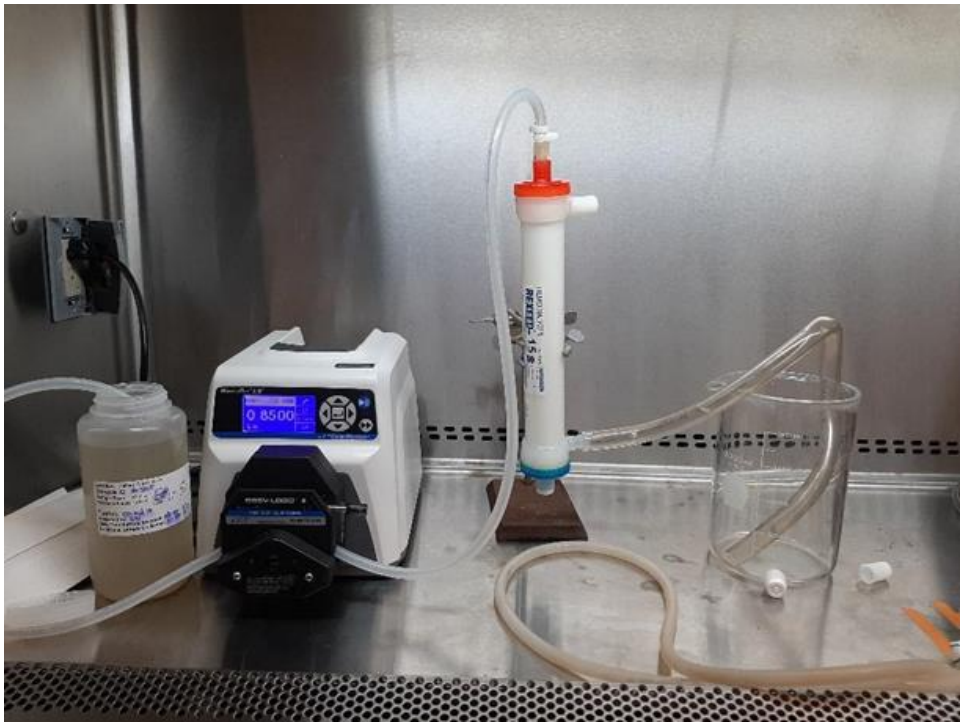


Fig. 3.1 Hollow UF filtration setup for concentrating 2-3 L of wastewater samples.

When the first eluate from the hollow UF was subjected to do secondary concentration by filtering through the HA filter, solids substances clogged the pores of filters resulting in a longer filtration process. In addition, we hypothesized that the inhibitory substances could be carried over to the extracted RNA which could cause PCR inhibition. To reduce the filtration time and inhibition, we centrifuged the aliquoted 20 mL 1st eluate at 3000 x g to remove solids before secondary concentration through the HA method. Because SARS-CoV-2 viruses have been reported to be partitioned almost equally into solid and liquid, solid separation can lead to losing part of the viral load. Our previous study found that a brief sonication can recover SARS-CoV-2 viruses that are attached to solid surfaces (Juel et al., 2021). As part of the modification, we sonicated samples for 1 min before centrifugation followed by filtration

through the HA membrane. This modification of the combination method was denoted as UF-HA_Soni (Fig. 3.2).

As part of the small-volume method, 50 ml of raw wastewater was filtered through HA filter for direct comparison with the corresponding combined large-volume filtration method. Both the direct extraction of 1 mL of 1st eluate and filters were extracted using the RNeasy PowerWater kit (Qiagen) following the manufacturer's protocol. Viral RNA/DNA were eluted in 100 µL nuclease-free water. The flow chart of the experimental design of the hollow UF-based large-volume concentration method and solid sample analysis is shown in Fig. 3.2.

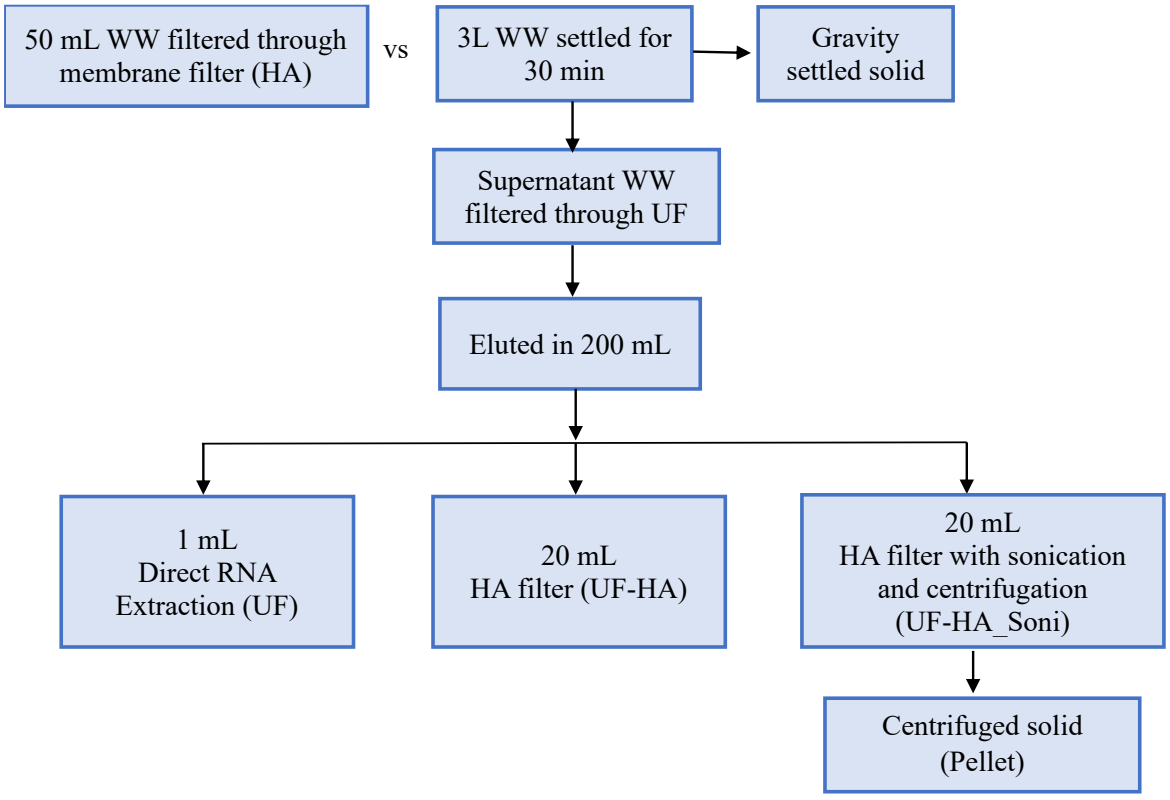


Fig. 3.2 Experimental workflow for large volume filtration method with Hollow ultra-fiber filter and electronegative membrane filter (HA).

The experimental design for the optimization of the hollow UF-based large-volume concentration method is summarized in Table 3.1.

Table 3. 1 Experimental design for the optimization of the secondary concentration method for increased SARS-CoV-2 detection sensitivity. Initially, 3 L of wastewater were filtered through D-HFUF and eluted in 200 mL buffer solution (1st eluate).

SL	Volume of 1st elute	Purpose	Modification	Level of concentration	Symbol
1	1 mL	Direct extraction	None	Primary	UF
2	20 mL	Filter through HA	According to method described in previous section	Secondary	UF - HA
3	20 mL	Filter through HA	1 min sonication followed by centrifugation at 3000 x g before filtration	Secondary	UF – HA_Soni

3.2.3 Processing of solid samples

Both the gravity-settled solids and centrifuged solids samples were analyzed and compared with the concentrated liquid samples. Wastewater samples (3L) were allowed to sit for 30 mins for gravity settling. After separating the supernatant liquid, the gravity-settled solids were centrifuged at 20000 x g for 30 mins at 4ºC. The resultant pellet was kept in a -80ºC freezer until RNA extraction. In the second step, 20 mL of the concentrated liquid was centrifuged at 3000 x g for 10 mins at 4ºC to settle the suspended solid into pellets (Fig. 2). RNA was extracted from both types of solids using the Allprep DNA/RNA kit (Qiagen) following the manufacturer's protocol.

3.2.4 Detection and quantification using RT-qPCR

Quantitative reverse transcription PCR (RT-qPCR) was employed to detect and quantify SARS-CoV-2 and Bovine Coronavirus from the extracted RNA. The amplification of SARS-CoV-2 viruses was conducted using the N1 (Nucleocapsid) primer and probe set recommended by the CDC. The amplification reaction had a total volume of 20 µL, comprising 10 µL of iTaq universal one-step reaction mix from Bio-rad (Hercules, CA), 0.5 µL of iScript reverse transcriptase from Bio-rad, 500 nM of primers, 125 nM of probes, 0.4 µL of bovine serum albumin (20 mg/mL), 5 µL of template RNA, and nuclease-free water. The amplification was carried out in a CFX96 instrument (Bio-rad, Hercules, CA) with the following thermal conditions: 50 ºC for 15 minutes, 25 ºC for 2 minutes, 95 ºC for 2 minutes, followed by 44 cycles at 95 ºC for 3 seconds and 55 ºC for 30 seconds. A standard curve was created by using a 10-fold dilution series ranging from 10^5 copies to 10 copies per reaction. Each sample was tested in triplicates, with both positive and negative controls on every plate. The limit of detection was determined as 5 copies per reaction, following the procedure outlined in Gibas et al. (2021). To evaluate the potential RT-qPCR inhibition, VetMAX™ Xeno™ internal positive control (from Applied Biosystems) was spiked into RNA extracted from wastewater. The VIC™ Assay (Catalog no. A29767, Applied Biosystems) was multiplexed with N1 assay to amplify the spiked control based on the procedure discussed in Juel et al (2021). We found inhibition (measured by more than 2 delayed Cq from the control) for solid samples which was resolved by running fivefold diluted RNA.

The BCoV target was amplified based on the protocol described in Decora et al., (2008). The primer and probe concentration were 600 nM and 200 nM, respectively. The thermal cycling parameters were similar to those employed in the protocol by Decora et al. (2008), except the annealing temperature that was adjusted to 55 °C instead of 60 °C. For quantifying

BCoV recovery, a standard curve was established through serial dilution of a BCoV vaccine, ranging from 10^5 to 1 copy per reaction. All the primer and probe sequences, as well as the standard curves, are provided in the supplementary file (Table S1 and Figure S1 & S2, respectively).

3.3 Data analysis

All the figures were plotted using Excel 2016 (Microsoft). One-way anova test, t-test and regression analysis were performed using Minitab® 19. P values less than 0.05 were considered statistically significant while those greater than 0.05 were considered insignificant or alternative hypotheses are valid. All the RT-qPCR data were analyzed using CFX Maestro™ Software (Bio-Rad).

3.4 Result and Discussion

Wastewater samples collected from mid-November 2021 to February 2022 were divided into two parts – samples collected during low COVID-19 infection and high infection period. The low and high COVID-19 infection period was classified based on the COVID-19 incidence rate (IR) which is defined as the number of COVID-19 cases per 10000 residents. A low infection rate was considered for the IR lower than 2.4 while a high infection rate was considered for the IR higher than 2.4. Incidence rate data was collected from the North Carolina Department of public health and Human Services website. Detailed information on the IR for all the studied samples is shown in supplementary file (Table S3 and S4). To prevent any future outbreak, it is crucial to quantify SARS-CoV-2 viruses during the early stage of the infection where virus titer in wastewater is low.

3.4.1 SARS-CoV-2 detection sensitivity during low and high COVID-19 infection period

The HA method was not successful in detecting SARS-CoV-2 in samples that were collected during low COVID-19 infection period - only 9% of wastewater samples resulted in SARS-CoV-2 positive detection. The hollow UF-based primary method resulted in 63.63% (n = 11) SARS-CoV-2 positive detection (Fig. 3.3(a)) which showed comparatively better virus recovery than the HA method. However, neither of the primary methods was as successful as the combination of the two methods. SARS-CoV-2 detection sensitivity was increased significantly (ANOVA, $p<0.0001$) with the UF-HA_Soni (modification with sonication and centrifugation) method which resulted in 100% SARS-CoV-2 positive detection (Fig. 3.3(a)). This increased detection sensitivity is attributed to the higher concentration factor that was produced from filtering 3 L of wastewater samples through hollow UF followed by secondary concentration with the HA method. The combination of UF and HA methods yields a 300X concentration factor while the HA method alone yields 50X. However, the non-modified version of the combination method (UF-HA) didn't improve the detection sensitivity. The higher bacterial genome and inhibitory substances can be the factors for lower performance with the UF-HA method. This problem was resolved with the modified version (UF-HA_Soni) where sonication and centrifugation steps were added. The sonication step was hypothesized to recover viruses that were attached to the solid surface and the centrifugation step reduced inhibitory substances by separating the solid before filtration (Juel et al., 2021). These two steps significantly increased the SARS-CoV-2 detection rate from 36% to 100%. However, a considerable percentage of SARS-CoV-2 was also found in the separated solid by centrifugation that is further discussed in the later section.

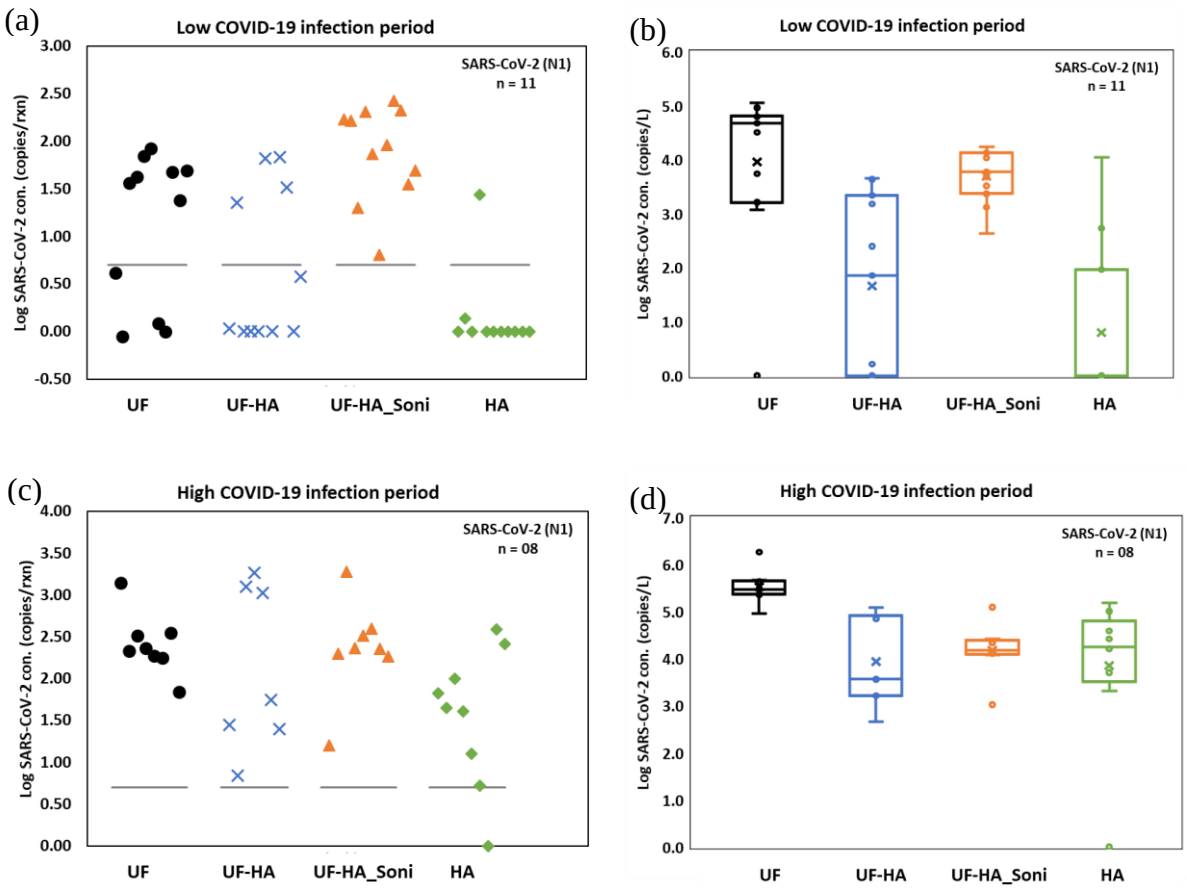


Fig. 3.3 SARS-CoV-2 detection sensitivity of hollow UF and HA filter-based virus concentration methods from wastewater collected during low COVID-19 infection and high infection period. The line symbol (-) used in figure (a) and (c) used to indicate the LOD (5 copies/rxn).

On the other hand, during the high COVID-19 infection period, both the primary methods (UF, HA) and their combination method (UF-HA_Soni) were able to detect SARS-CoV-2 viruses in about 100% of wastewater samples (Fig. 3.3(c)). This higher detection rate is expected as there was an abundance of viruses present in wastewater samples during the high COVID-19 infection period. In terms of concentration, though there was no significant difference observed among them (ANOVA, $p = 0.06$), the UF method resulted in higher mean SARS-CoV-2 recovery compared to all other alternatives. In this case, the addition of the

secondary concentration method along with the primary method didn't bring any additional benefits in terms of SARS-CoV-2 detection and quantification.

3.4.2 BCoV recovery

Bovine coronavirus, a surrogate of human SARS-CoV-2, was spiked in wastewater samples before processing through the tested methods. The average BCoV recovery was found to be 33.8% for UF which is significantly higher than all the other alternatives (Annova, P = 0.002). The BCoV recovery for the hollow UF found in our study seems to have better agreement with the recently published article (McMinn et al., 2021). The lowest mean recovery of 2.5% was observed with the HA method. The observed BCoV recovery results with these two methods support the SARS-CoV-2 quantification data for wastewater samples processed with the hollow UF and HA methods. A higher mean SARS-CoV-2 concentration (>1 log copies/L) was observed with the UF method compared to the HA method, especially during the high COVID-19 infection period. The mean BCoV recovery of the combination method was 6.5 – 7.5% (Fig. 3.4). The modified UF-HA_Soni showed higher BCoV recovery than the non-modified version, but the difference was not statistically significant. Since each step of the sample processing loses some percentage of viruses from the total spiked viruses, the combination of primary and secondary concentration methods may not report a higher percentage recovery compared to the UF method (the primary method) which shows maximum recovery. However, the combined method may accumulate more viruses from natural wastewater samples which can eventually increase the detection sensitivity as evidenced by the SARS-CoV-2 detection result, especially during the low COVID-19 infection period.

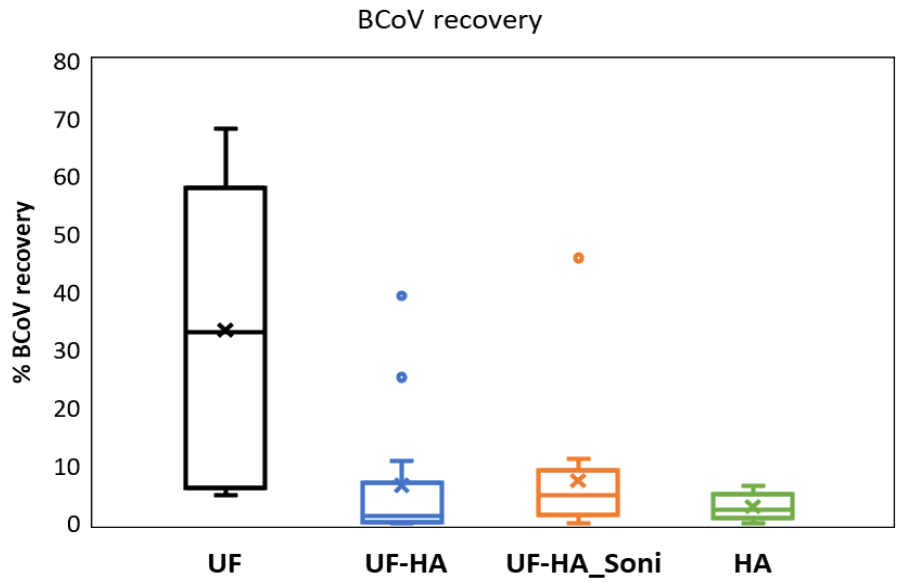


Fig. 3.4 BCoV recovery from the hollow UF and HA filter-based virus concentration methods.

3.4.3 Partitioning of SARS-CoV-2 viruses into liquid and solid

As part of the analysis of SARS-CoV-2 partitioning into liquid and solid phases, total solid generated from the 3L of wastewater was considered. The total number of SARS-CoV-2 gene copies quantified from the resulting solid were compared to the total number of gene copies quantified from the 3L of liquid. Fig. 3.5 shows the relative percent of SARS-CoV-2 viruses partitioned into total settled solid and total centrifuged solid. The liquid phase carried a significantly higher percentage of SARS-CoV-2 viruses than the solid phase (gravity settled solid plus centrifuged solid) ($p = 0.04$). On average, 73.5% of SARS-CoV-2 viruses were partitioned into liquid phase compared to 26.5% into solid phase. So, solid removal through gravity settlement and centrifugation step can cause around 26.5% viral loss. Out of this 26.5% viral loss through solids removal, 21.6% was contributed from the centrifuged solids which is significantly higher than the gravity settled solid ($p = 0.049$). This indicates the SARS-CoV-2 viruses are more inclined to attach with smaller suspended particles compared to large solid

particles. This data also suggests that solid separation from wastewater sample through gravity settlement can be performed with a negligible viral loss. This step can reduce filter clog in filtration-based virus concentration method. In accordance with the findings of this study, Greaves et al. (2022) similarly reported that the highest percentage (40% to 80%) of six distinct molecular fecal pollution targets were linked to smaller suspended particles (0.45 µm).

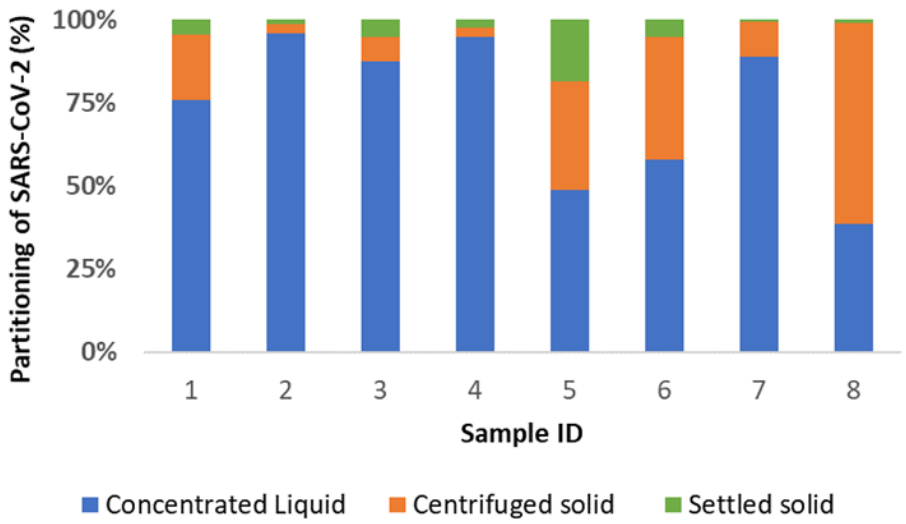


Fig. 3.5 SARS-CoV-2 viruses partitioned into gravity-settled solid, centrifuged solid, and liquid part. Both the gravity-settled solid and centrifuged solid were separated from the 3 L of wastewater samples.

The SARS-CoV-2 partitioning result found in this study is congruent with the results reported in previously published articles (Breadner et al., 2023; Forés et al., 2021). However, this finding is opposite from Kim et al., (2022) where a three order of magnitude higher SARS-CoV-2 concentration in solid (copies/g dry solid) was reported when compared to the liquid(copies/mL). However, this comparison based on the mass equivalent basis may not be appropriate because of the different units of expression as practically one g dry solid may not be generated from one mL of liquid sample though it is dependent on the degree of suspended

solid in wastewater. In our analysis, only around 0.5 g dry gravity settled solid were recorded from the 3 L of wastewater samples (Table S4) which is very minimal. In that case, the expression of SARS-CoV-2 copies per g dry solid can be overestimated if it is compared with the copies present in one mL of liquid.

As SARS-CoV-2 viruses were detected in solid samples, in addition to liquid samples, the solid can also be used as samples for tracking COVID-19 disease monitoring. For that purpose, centrifuged solid generated from the direct wastewater samples may be considered as better option of sampling compared to the solid collected from the primary clarifier as higher SARS-CoV-2 gene copies were found in the centrifuged solid than in the gravity-settled solid.

3.5 Conclusion

The combination of hollow UF and HA method generated a higher concentration factor that resulted in increased virus detection sensitivity. This method is effective in tracking the early COVID-19 infection point as it increases the SARS-CoV-2 detection sensitivity from the low virus-titrated wastewater samples that happens especially during the low COVID-19 infection period. However, for monitoring SARS-CoV-2 abundance in wastewater during the high COVID-19 infection period, the hollow UF method can be more effective for quantifying SARS-CoV-2 viruses from the wastewater samples. The higher BCoV recovery with the hollow UF method compared to the combination method and HA method supports these results. The addition of brief sonication and centrifugation steps in the combination method significantly increased the PCR amplification signal. From the SARS-CoV-2 partitioning analysis, it was observed that a significantly higher percentage of SARS-CoV-2 viruses were partitioned into liquid phase compared to the solid phase. It was also observed that a higher

percentage of SARS-CoV-2 were attached to smaller suspended particles that were separated through centrifugation compared to the gravity settleable solid.

Overall, the modified hollow UF and HA filtration-based combination method might be effective in detecting SARS-CoV-2 virus, especially during the low COVID-19 infection period and the hollow UF method can be used to monitor the abundance of SARS-CoV-2 viruses during the high COVID-19 infection period. This information can aid public health departments in understanding the extent of virus transmission within the community and in implementing essential measures to curb the outbreak.

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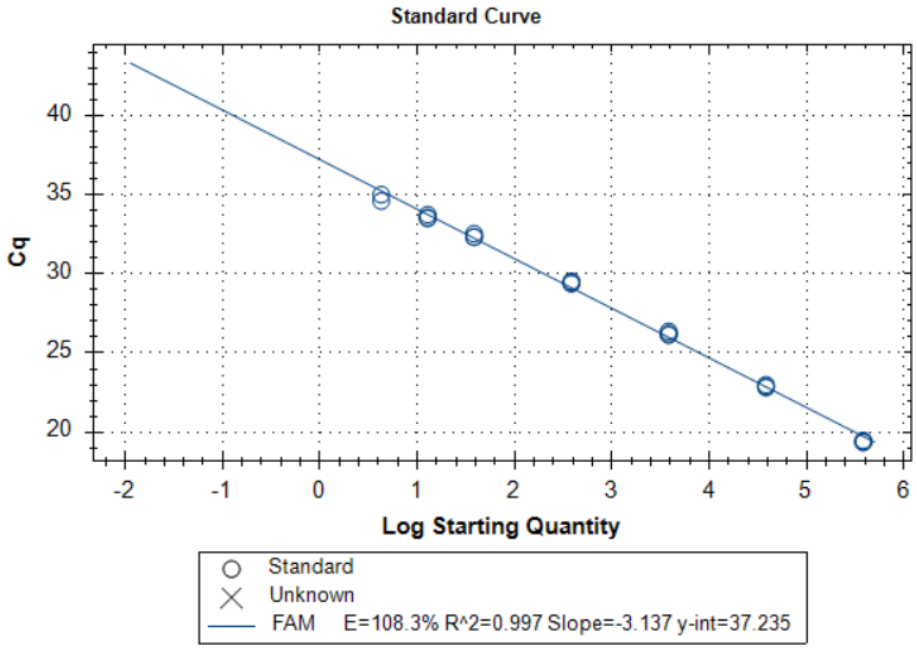
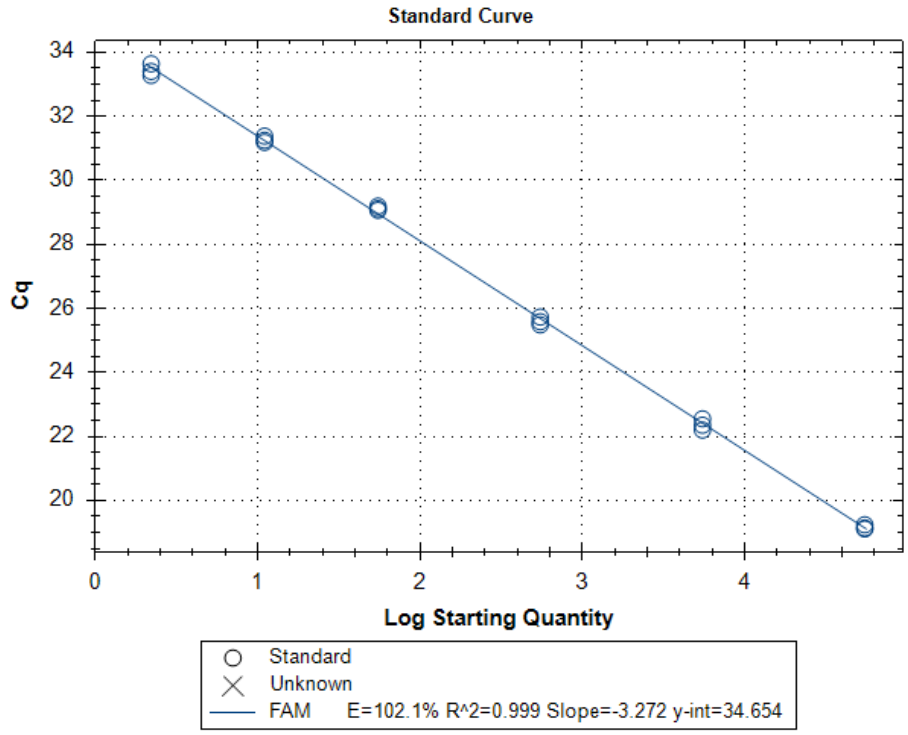
3.7 Appendices

Supplementary Table S1: Primers and probe sequences used in this study.

Assay	Primer/Probe	Sequences	References
CDC N1	Forward	GACCCCAAAATCAGCGAAAT	Lu et al., 2020
	Reverse	TCTGGTTACTGCCAGTTGAATCTG	
	Probe	FAM-ACCCCGCAT/ZEN/TACGTTTGGTGGACC-3IABkFQ	
BCoV	Forward	CTGGAAGTTGGTGGAGTT	Decaro et al., 2008
	Reverse	ATTATCGGCCTAACATACATC	
	Probe	FAM-CCTTCATATCTATACACATCAAGTTGTT-BHQ1	



Supplementary Figure S1: Standard curve used for the quantification of SARS-CoV-2 viruses.



Supplementary Figure 2: Standard curve used for the quantification of Bovine Coronavirus (BCoV).

Supplementary Table S2: Classification of low COVID-19 infection period based on the incidence rate and SARS-CoV-2 quantification result (copies/rxn).

Date	WWTP	New COVID-19 cases number	Incidence rate	UF	UF-HA	UF-HA with sonication	HA
11/22/2021	SC	12.96	0.71	<LOD	1.07	168.84	ND
11/29/2021	SC	23	1.26	<LOD	22.74	163.85	<LOD
12/6/2021	SC	27	1.48	36.23	<LOD	19.87	<LOD
12/13/2021	SC	29.02	1.59	42.35	ND	205.35	27.58
2/14/2022	SC	20.08	1.10	69.30	ND	73.45	<LOD
2/21/2022	SC	14.97	0.82	84.55	65.54	6.41	ND
11/22/2021	MLC	21	1.08	<LOD	ND	91.20	ND
11/29/2021	MLC	15.96	1.33	<LOD	67.75	265.16	ND
12/6/2021	MLC	15.96	1.33	47.77	32.88	211.45	ND
12/13/2021	MLC	27.96	2.33	24.12	ND	35.43	<LOD
2/21/2022	MLC	15	1.25	49.23	<LOD	49.40	ND

Supplementary Table S3: Classification of high COVID-19 infection period based on the incidence rate and SARS-CoV-2 quantification result (copies/rxn).

Date	WWTP	New COVID-19 cases number	Incidence rate	UF	UF-HA	UF-HA with sonication	HA
12/20/2021	SC	87.05	4.77	N/A	28.08	15.85	66.13
1/24/2022	SC	91.98	5.04	325.79	1839.19	1935.2	12.73
1/31/2022	SC	75.92	4.16	188.31	55.19	325.50	ND
2/7/2022	SC	43.98	2.41	175.75	24.95	397.45	388.85
12/20/2021	MLC	62.04	5.17	N/A	6.88	196.68	44.32
1/10/2022	MLC	198	16.50	1388.72	ND	1875.55	99.46
1/24/2022	MLC	204	10.08	212.76	1244.13	34.56	40.16
1/31/2022	MLC	60	5.00	232.49	1057.49	232.31	5.24
2/7/2022	MLC	45	3.75	351.49	0.00	228.58	257.74

Supplementary Table S4: Total dry weight of solid samples generated from the 3L of wastewater.

SL	Sampling date	WWTP	Total dry solid weight (g)
1	11/22/2021	SC	0.48
2	11/22/2021	MLC	0.42
3	11/29/2021	SC	0.51
4	11/29/2021	MLC	0.41
5	12/6/2021	SC	0.5
6	12/6/2021	MLC	0.53
7	12/13/2021	SC	0.33
8	12/13/2021	MLC	0.36

4 ARTICLE 3: DYNAMICS OF SARS-COV-2 OMICRON AND DELTA VARIANTS CIRCULATING IN WASTEWATER IN THE CHARLOTTE AREA

4.1 Introduction

The SARS-CoV-2 virus remains a public health threat due to rapidly evolving transmission and infection capabilities since its emergence in November 2019, despite the development of effective vaccines and implementation of mass vaccination programs (WHO, 2022). Several successive variants of concern (VOCs) have emerged with higher transmissibility, pathogenicity, and immune evasion capabilities. The Alpha, Beta, and Gamma variants were dominant in early 2021 and were responsible for increased COVID-19 infections and hospitalizations in different parts of the world until mid-2021 (Tegally et al., 2023; Wurtzer, Waldman, et al., 2022). These variants were gradually replaced by the Delta variants, which caused the third worldwide wave of infections and was the dominant variant until late 2021 when the Delta variants were displaced by Omicron (Tegally et al., 2023). The World Health Organization (WHO) declared Omicron (B.1.1.529) as SARS-CoV-2 Variant of Concern (VOC) on 26th November 2021 after its first detection in South Africa on 24th November 2021 (CDC COVID-19 Response Team, 2021; Parums, 2022). The first Omicron case was identified in the USA on December 1, 2021, rapidly spreading across the country (CDC COVID-19 Response Team, 2021). The Omicron variants were initially considered less virulent in vaccinated individuals but have spread widely among the younger population (Brüssow, 2022; Ferré et al., 2022; Wurtzer, Waldman, et al., 2022). The Omicron variants have a shorter incubation time and reproduce faster than Delta due to structural differences in the spike protein, which lead to immune evasion (Zhang et al., 2021).

Following the introduction of the original omicron strains, new variants have continued to emerge on a regular basis, and COVID-19 is becoming part of the endemic infectious disease

burden, with hospitalization rates often 2-3 times those of influenza. For public health preparedness, it is critical to monitor the emergence and spread of Variants of Concern (VoC) within the community. This helps in understanding how these VoCs are transmitted and allows us to implement necessary public health protection measures. Currently, the surveillance of VoCs primarily relies on the complete genome sequencing of patient samples. However, this method is relatively costly, labor-intensive, time-consuming, and often inaccessible in many regions worldwide. In addition, sequencing of SARS-CoV-2 from wastewater samples is challenging due to limited coverage and fragmented genomes (Davis et al., 2021; Lou et al., 2022). PCR-based methods, such as RT-qPCR and RT-ddPCR, can be an alternative and potentially more efficient approach for tracking the emergence and dissemination of VoCs within a community from wastewater samples (Bloemen et al., 2022; Heijnen et al., 2021). Rare mutation detection is always a challenge because of difficulties in differentiating between two highly similar sequences, one of which is significantly more abundant than the other. In digital droplet RT-PCR (RT-ddPCR), 40 µL reaction mix is partitioned into 10000 – 20000 tiny droplets. PCR reactions take place individually within these isolated droplets, enabling the detection of rare mutations and distinguishing closely similar sequences based on the kinetics of probe binding. Because of this feature, ddPCR offers higher sensitivity and resistance to inhibitors in wastewater samples compared to RT-qPCR (Barua et al., 2022; Ciesielski et al., 2021).

However, few surveillance programs have used RT-ddPCR for SARS-CoV-2 VOC detection. This study aims to detect and quantify Omicron and Delta variants in wastewater using RT-ddPCR targeting spike protein mutations at positions 764 and 856 (N764K and N856K). This study also aims to determine the transmission dynamics of the Omicron variants by assessing the relative proportion of the strains circulating in Charlotte, North Carolina. The

detection of new variants in wastewater poses challenges due to the presence of highly similar sequences, with one being more abundant than the other. During the early period, the relative proportion of a new variant is usually low compared to the wild-type mutation. This study aims to address this challenge by proposing a large-volume filtration method for virus concentration, which is hypothesized to enhance the sensitivity of detecting new VOCs. Proper virus concentration techniques are crucial to ensure the detectability of new variants in wastewater samples.

4.2 Experimental methods

4.2.1 Sample collection

Wastewater samples were collected from Sugar Creek and Mallard Creek Wastewater Treatment Plant (WWTP) on a weekly basis from January 2021 to February 2022. The sewershed boundary of Sugar Creek WWTP covers uptown Charlotte including the CLT international airport, major hospitals, industries, and with a population size of 180,000 while the UNC Charlotte campus, and hospitals are covered by the Mallard Creek WWTP sewershed boundary with a population size of 120,000. Wastewater samples were also collected from various sewer access sites within the UNC Charlotte campus.

4.2.2 Virus concentration and Nucleic Acid extraction

Two different virus concentration methods were used for the virus concentration. Electronegative membrane filtration, i.e., HA method was used from the beginning of the sample collection according to our previous publication (Barua et al., 2022; Gibas et al., 2021; Juel et al., 2021). In short, 5 microliters of Bovine coronavirus (55000 copies/µL) were spiked to 50 ml of wastewater followed by the addition of HCl to control pH between 3.5 and 4.0.

Magnesium Chloride (MgCl2.6H2O) was then added with a final concentration of 25 nM for increasing breeze between cations and negatively charged SARS-CoV-2 particles. Then, wastewater samples were filtered using a vacuum manifold through a 0.45µM pore-sized cellulose-based membrane filter (HA) coupled with a single-use filter funnel (Pall corporation).

In parallel to the HA method, the dead-end hollow fiber ultrafilter (UF) method, which has a higher wastewater filtration capacity, was also applied for virus concentration. This method followed the protocol outlined in Wu et al. (2023). Three liters of untreated wastewater were filtered through hollow UF (Rexeed 15S, Chester, PA) using a peristaltic pump operating at 300 rpm. Viruses from the filter surface were recovered in a 200 mL previously prepared buffer solution (0.01% Tween 80, 0.01% sodium hexametaphosphate, 0.001% Antifoam Y-20) by running a peristaltic pump (Cole Parmer, Vernon Hills, IL) in an anticlockwise-clockwise-anticlockwise direction for 1 min in each interval, repeated three times followed by elution into a beaker. In owing to further concentration of viral materials, 20 mL of eluted samples were sonicated for 1 min followed by separating solid by centrifuging at 3,000 x g for 10 min. The resulting supernatant was then filtered through the HA method described above. The filters from both methods were stored at -80 ºC and later extracted using the RNeasy PowerWater Kit (Qiagen) following the manufacturer's protocol. Viral RNA/DNA were eluted in 100 mL nuclease-free water.

4.2.3 Detection and quantification using RT-qPCR

The amplification reaction consisted of a 20 µL volume, including 10 µL of iTaq universal one-step reaction mix (Bio-Rad, Hercules, CA), 0.5 µL of iScript reverse transcriptase (Bio-Rad, Hercules, CA), 500 nM primers, 125 nM probe, 0.4 µL of bovine serum

albumin (20 mg/mL), 5 µL of template RNA, and nuclease-free water. Amplification was carried out using CFX96 (Bio-Rad, Hercules, CA) under the following thermal conditions: 50 ºC for 15 minutes, 25 ºC for 2 minutes, 95 ºC for 2 minutes, followed by 44 cycles at 95 ºC for 3 seconds and 55 ºC for 30 seconds (CDC, 2020). A standard curve was generated using a 10-fold dilution series ranging from 10^5 copies to 10 copies per reaction. All samples were run in triplicates, with positive and negative controls included on each plate. The limit of detection was determined as 5 copies per reaction, following the protocol described in Gibas et al. (2021). To assess any potential interference from extracted RNA in the PCR reaction, VetMAX™ Xeno™ internal positive control (Applied Biosystems) was spiked into the wastewater-derived RNA. Multiplexing VIC™ Assay (Catalog no. A29767, Applied Biosystems) was used alongside the N1 assay (Juel et al., 2021) to amplify the spiked control.

4.2.4 Detection of SARS-CoV-2 variant by RT-ddPCR

Both Delta and Omicron variants were quantified from wastewater samples utilizing the QX200 AutoDG Droplet Digital PCR System (Bio-Rad, USA). S:N764K and S:N856K are the two characteristic mutations associated with the Omicron lineage. While the N856K spike mutation is only found in 21K (Omicron) or BA.1, the spike mutation of N764K is common in both BA.1 and BA.2 (21L Omicron) (covarints.org). Both the N764K and N856K duplex assays were designed by Bio-Rad. The probes targeting the Omicron variants were labeled with FAM fluorophore whereas the probes targeting the Delta variants were labeled with the HEX fluorophore as a reporter. Details of the assay information and thermal profile is given in Tables 4.1 and 4.2.

A one-step RT-ddPCR approach was employed to quantify the mutations. A 22 µL reaction mixture was prepared, consisting of 5.5 µL of 4× One-step RT-ddPCR Super Mix

(Bio-Rad), 2.2 µL of One-step RT-ddPCR Reverse transcriptase (Bio-Rad), 15 mM DTT, 0.9 µM forward primer, 0.9 µM reverse primer, 0.25 µM probe, and 6 µL of wastewater extract or standard. Droplet generation was carried out following the manufacturer's instructions, and the droplets were subsequently amplified in a C1000 thermal cycler using the following temperature profile: reverse transcription at 50°C for 60 minutes, inactivation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 s and annealing-extension at 55°C for 1 min, followed by deactivation at 98°C for 10 minutes, and hold at 4°C. After completion of the RT-ddPCR cycling, the plate was transferred to a QX200 instrument (Bio-Rad), and the droplets were analyzed as per the manufacturer's instructions. Data acquisition and analysis were performed using QuantaSoft V1.74.0917 (Bio-Rad).

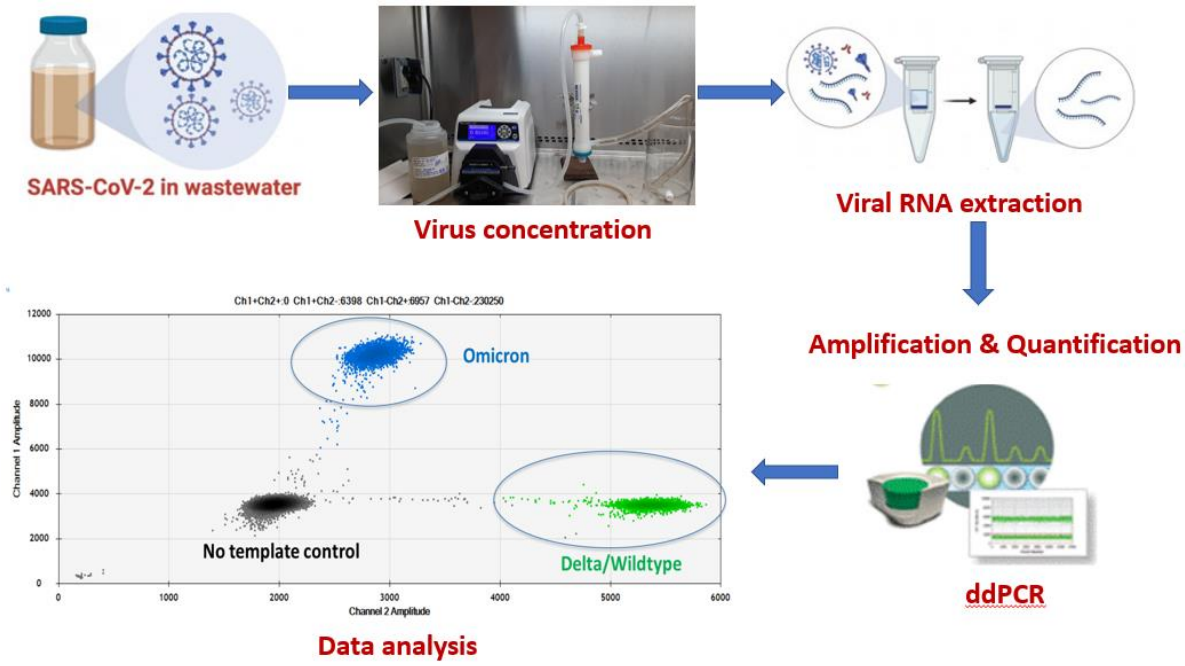


Fig. 4.1 Experimental workflow for detecting variants from wastewater.

4.2.5 Control study for the mutation assay validation

The assay specificity for quantifying Delta and Omicron variants from samples was determined in a controlled study. The Delta and Omicron-specific synthetic positive controls from Twist Bioscience and two other controls from clinical samples identified from the genomic analysis were amplified using these two assays. The Omicron positive controls were amplified successfully by the FAM fluorophore of both assays that target N764K and N856K mutation while the HEX fluorophore targeting wild type (Delta) showed negative. The limit of the detection of these two assays was determined as ≥ 2 positive droplets with a minimum of 10,000 acceptable droplets.

Table 4.1 Target sequences and mutation assay characteristics

Assay name	Reference Genome	Mutant/Wildtype Allele	Amplicon Length
N764K[1]	GTGATTCAACTGAATGCAGCAATCTTTTGTTGCAATATGGCAGTTTTTGTACACAATTAAA[**C/A**]CGTGCTTTAACTGGAATAGCTGTTGAACAAGACAAAAACACCCAAGAAGTTTTTGCACAA	A/C	70
N856K[2] (Catalog no. 10049047)	AATATGGTGATTGCCTTGGTGATATTGCTGCTAGAGACCTCATTTGTGCACAAAAGTTTAA**[C/A]**GGCCTTACTGTTTTGCCACCTTTGCTCACAGATGAAATGATTGCTCAATACACTTCTGCAC	A/C	60

[1] https://www.bio-rad.com/digital-assays/assay-detail/dMDS900687606

[2] https://www.bio-rad.com/digital-assays/assay-detail/dMDS761081950

Table 4. 2 Determining the assay specificity in discriminating Omicron and Delta variants.

Controls Name	Omicron (Copies/rxn)		Delta (Copies/rxn)	
	N764K (FAM)	N856K (FAM)	N764K (HEX)	N856K (HEX)
Twist control (Omicron)	8844	7634	Negative	Negative
Clinical Omicron Positive	1223	1075	Negative	Negative
Twist control (Delta)	Negative	Negative	9614	8932
Clinical Delta Positive	Negative	Negative	1245	1155

4.2.6 Statistical analysis

The Pearson and Spearman rank correlation statistics were applied to compare the relative abundances of the Omicron lineage estimated from the clinical specimen using the sequencing techniques and from the wastewater samples using the ddPCR duplex assay technique. A p-value less than 0.05 was considered statistically significant. All the figures were plotted using Excel 2016 (Microsoft). One-way anova test, t-test and regression analysis were performed using Minitab® 19. P values less than 0.05 were considered statistically significant while those greater than 0.05 were considered insignificant or alternative hypotheses are valid. All the RT-qPCR and RT-ddPCR data were analyzed using CFX Maestro™ Software (Bio-Rad) and QuantaSoft, version 1.7 (Bio-Rad), respectively.

4.3 Result and Discussion

4.3.1 Dynamics of Omicron Variants at Wastewater

The predominant SARS-CoV-2 variant circulating in the USA in late 2021 was the Delta variant. The first clinical case of Omicron variants was detected in Mecklenburg County on December 10, 2021. Following this detection, there was a significant increase in clinical cases in the Mecklenburg area within a week (Fig. 4.2). Building upon the success of wastewater-based epidemiology in controlling SARS-CoV-2 outbreaks in university dorms (Gibas et al., 2021) and municipality areas (Barua et al., 2022), we applied the same concept to detect Omicron variants in wastewater samples collected from WWTPs and university dorms. The first Omicron variants were detected in Sugar Creek WWTP samples collected on December 6, 2021, which was about a week in advance of the first clinical case detection. The UNC Charlotte dorms' wastewater samples showed the presence of this variant on December 08, 2021, however, it was not detected in the Mallard Creek WWTP samples on the same week, first detected on December 13, 2021, though the plant receives wastewater samples from the University area. The reason may be the dilution of the Omicron gene copies with the other wastewater flow while traveling to the WWTP. The sewershed boundary of the Sugar Creek WWTP covered uptown Charlotte and CLT International Airport which suggests a link with national or international travelers for carrying this variant from other countries or states of the USA (Fig 4.2). Mallard Creek being farther apart from the CLT International Airport showed the presence of this new variant one week later from Sugar Creek which agrees with the conclusion that the communities located at a greater distance away from the international airport showed the delayed emergence of omicron variant (Hubert et al., 2022).

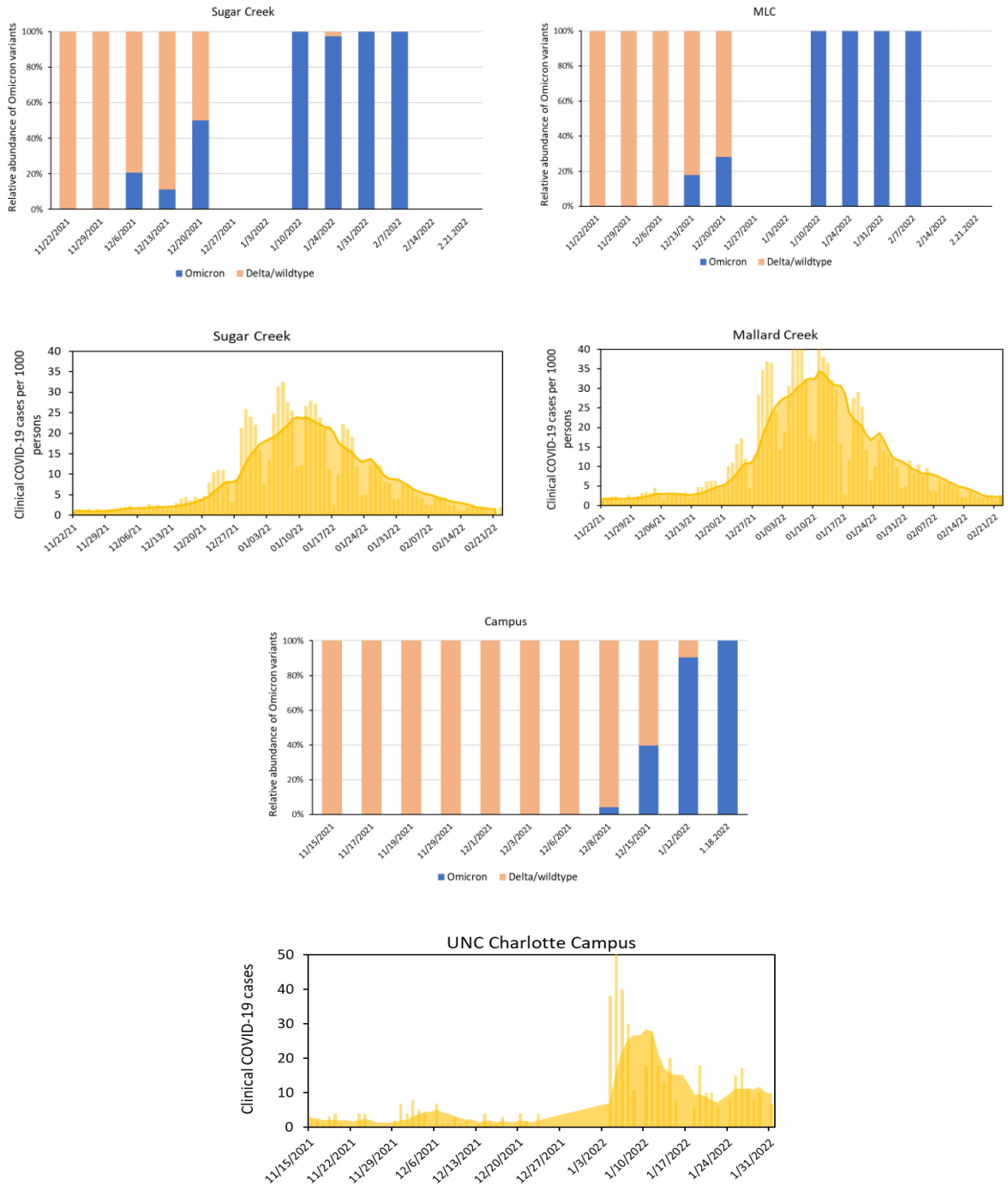


Fig. 4.2 Relative abundance of Omicron and Delta VOCs circulating in wastewater that represent Mecklenburg County and UNC Charlotte campus. COVID-19 case counts were adjusted based on the boundary of the sewersheds that belong to a WWTP.

However, a similar pattern of the clinical case spike was observed in these regions irrespective of the different first detection dates of these variants. The COVID-19 cases started rising within two weeks of the first Omicron variants detection in wastewater corresponding to each area.

Furthermore, the relative abundances of the Omicron lineage started displacing the dominant Delta variants. The pattern of transition from Delta to Omicron was similar for all three regions. In early January 2022, the Omicron variants in wastewater became dominant over Delta variants in all three regions. The COVID-19 incidence rate (Number of cases per 10,000 residents) also reached its peak during the same week when Omicron became the dominant variant over Delta. This relationship established the linkage between the Omicron variants and the 4th wave of COVID-19 clinical cases.

Other studies found a link to the rise of the COVID-19 clinical case counts with the emergence of the omicron variant (BA.1) during December and January in different parts of the world (Amnemarie Adusei, 2022; Hubert et al., 2022). The wastewater data revealed that the prevalence of the Omicron variant took only 4±1 week to reach almost 100% of lineages in Mecklenburg County which is the most rapid displacement of any new variant over wild strains. For example, the Alpha variant (B.1.1.7) took an average of 8 ± 2 weeks to become dominant over the wildtype SARS-CoV-2 Wuhan strains in Spain (Carcereny et al., 2021; Radu et al., 2022). In the greater Paris area, the displacement rate for Omicron (BA.1) was 6.0% /day which is higher than the Delta (5.2% /day) and Alpha VOC (2.8% /day) (Wurtzer, Levert, et al., 2022).

4.3.2 Relationship between wastewater VOCs, N1, and clinically reported VOCs

We determined the relationship between the SARS-CoV-2 positive detections by RT-qPCR targeting N1 gene and mutation detections (either Delta or Omicron) by RT-ddPCR. All

samples showing positive detections for mutations using RT-ddPCR were also positive with N1 using RT-qPCR. However, 13% of samples showing positive detections with N1 were negative for both Delta and Omicron variants. The reason may be due to the emergence of the new SARS-CoV-2 mutations whose genome sequence doesn't match with the currently used assay's target sequence. Those 13% samples were collected in February 2022 when the abundance of the Omicron (BA.1) lineage was reduced (Fig. 4.3) and BA.2 lineage, a new mutation, emerged that supports our hypothesis. This circumstance can be applied in the future for investigating any potential new mutations when there will be a discrepancy between N1 positivity and mutation detections using the corresponding assay.

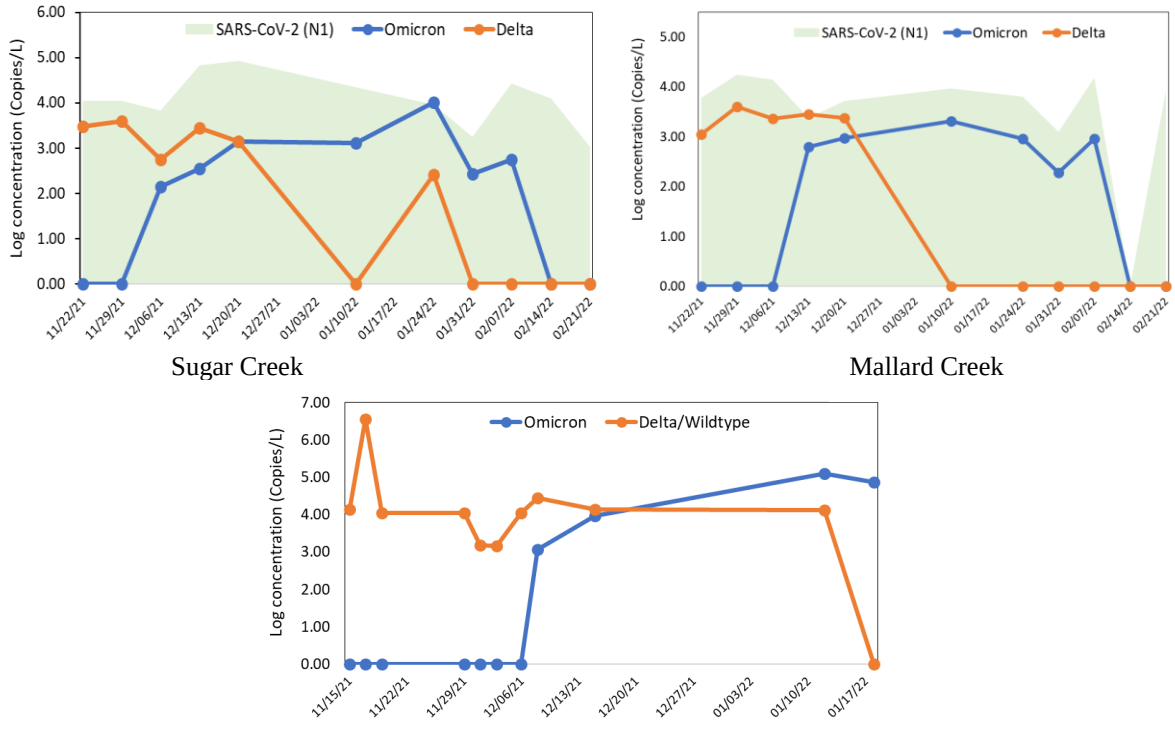


Fig. 4.3 Temporal variation of SARS-CoV-2 (N1), Delta and Omicron (BA.1) VOCs from November 2021 to February 2022 at Sugar Creek (Left side), Mallard Creek (right side) WWTP and UNC Charlotte campus (Bottom).

We also determined the relationship between SARS-CoV-2 VOCs quantified from wastewater samples and from clinical samples. The relative abundance of the Omicron variant determined by the RT-ddPCR technique from wastewater samples reached over 90% during early January of 2022 (Fig. 4.3). The clinical sequencing data for the North Carolina state also showed a similar dominance (Over 80%) of Omicron lineage (B.1.1.529 and BA.1) in the 1st week of January 2022 which supports the wastewater data. As the clinical VOCs data were reported for the whole Mecklenburg County (NCDHHS website) the variants data estimated for the Sugar Creek and Mallard Creek WWTP sewerage boundary were averaged first and then the correlation statistics were applied. The Pearson correlation showed a strong, positive, and statistically significant correlation between the percentage of Omicron lineage over the time found in the clinical specimens and in wastewater samples ($r = 0.98$, $p = < 0.0001$). A similar strong correlation was also observed with the Spearman correlation statistics ($rho = 0.94$, $p = <0.0001$). These analyses support the wastewater-based variant analysis (Omicron) using the RT-ddPCR technique that reflects the variants circulating in the community determined by the sequencing process.

When comparing the two variants detection methods, the sequencing technique is considered the gold standard method for variant analysis, however, the ddPCR method is cost-effective and less time-consuming compared to the sequencing techniques (Bloom et al., 2021; Lou et al., 2022). Also, the ddPCR method is more sensitive for determining SARS-CoV-2 variants, especially from wastewater samples compared to the sequencing process (Lou et al., 2022; Wurtzer, Levert, et al., 2022). This method can create a robust platform for variants surveillance which can reduce pressure on the sequencing process. This ddPCR technique can also be used for determining any future mutation detections by designing proper primers/probes from the revealed genome sequences of new strains.

4.3.3 Impact of virus concentration method on SARS-CoV-2 VOCs detection

The ddPCR duplex assay showed sensitivity to the virus concentration method for detecting both Omicron and Delta variants from the wastewater samples. As part of the optimization of variants detection from wastewater, two different methods were used for concentrating viruses from wastewater – Electronegative membrane filter (HA) and hollow UF. The concentrated samples eluted from the hollow UF were further concentrated by passing 20 mL through the HA filter. The following Fig 4.4 shows the Omicron variants detection result concentrated by HA and the combination of hollow UF and HA filter was denoted by UF-HA. Out of the 15 sampling events starting from November 15, 2021, to February 14, 2022, viruses concentrated from the UF-HA method resulted in 86% Omicron variants detection compared to 40% detection with the HA method. Similarly, for the Delta variants detection, in contrast to 91% positivity with the UF-HA, only 33% of samples showed positive with the HA. This higher detection sensitivity with the UF-HA is attributed to the higher concentration factor. The combination of hollow UF and HA method yields a 300X concentration factor while the HA method alone yields 50X. The larger concentration factor is beneficial for detecting target genes especially when less viral materials are present in the wastewater samples (Juel et al., 2021). This becomes clearer when wastewater-generated VOCs data was plotted against the incidence rate (number of cases/10000 residents) shown in Fig. 4.4 Both the UF-HA and HA methods resulted in positive Omicron detection consistently when the incidence rate was higher than six. However, for the low incidence rate (less than five) the HA method was not consistent for detecting Omicron variants - only 3 out of 13 samples showed omicron-positive detection. On the other hand, the UF-HA concentration method consistently resulted in positive Omicron detection even when the incidence rate was lower than five. Delta variants were also detected consistently during the lower incidence rate when the UF-HA method was applied for the virus

concentration, but it was not consistent with the HA method. This indicates that the increased variants detection sensitivity is associated with the virus concentration methods that have comparatively larger concentration factors.

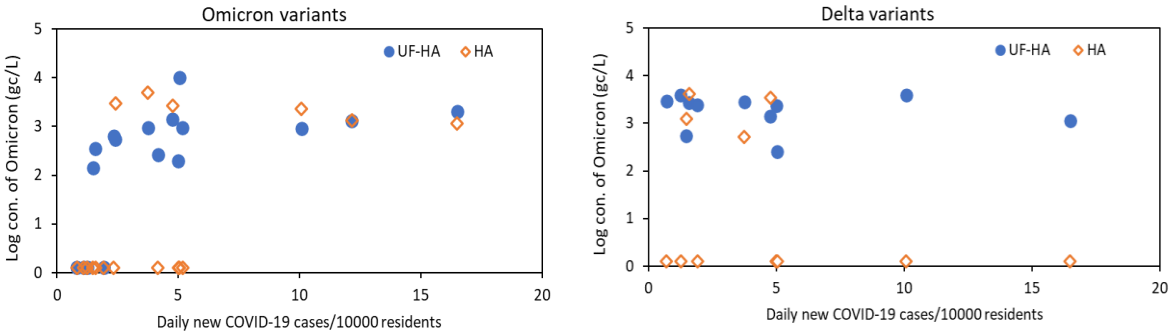


Fig. 4.4 Impact of virus concentration method on SARS-CoV-2 VOCs detection.

4.4 Conclusion

Omicron variants started circulating in the Mecklenburg County sewersheds in the 2nd week of December 2021. Omicron variants were dominant over all other SARS-CoV-2 strains from early January 2022 and were responsible for the 4th wave of COVID-19 cases in Mecklenburg County. The percentage lineage of Omicron variants quantified by RT-ddPCR from wastewater was strongly and positively correlated with the clinical Omicron variants data. The virus concentration method largely impacts the variants detection result for the wastewater samples. The combination of hollow UF and HA method-based large volume concentration method showed higher variants detection sensitivity than the HA method, especially during the low COVID-19 incidence rate. The ddPCR based variant detection technique enables a near real-time transmission dynamic of Omicron and Delta variants which can help the administration to take quick necessary public interventions such as awareness, preparedness, and control measures. It can also be applied for tracking new mutations by designing a new

assay provided that genome sequences of the new strains are available. Because of a reduced turnaround time, this method enables real-time SARS-CoV-2 VOCs surveillance which can be beneficial for keeping track of variants propagation over time and can help the public health department to make proper decisions for outbreak control.

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5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Wastewater Based Epidemiology (WBE) is a public health tool that uses wastewater to monitor human pathogenic viruses in a defined population. Given the nationwide efforts to implement COVID-19 wastewater-based epidemiology for accurate data on community infection levels, the virus concentration method is of great importance, especially in low-prevalence COVID cases. The purpose of this work was to identify a suitable virus concentration method for qPCR or ddPCR quantification of virus particles. Since the application of WBE for COVID-19 outbreaks in congregate settings, such as residence halls, schools, and nursing homes, differs from city-level surveillance, this study analyzed various virus concentration methods suitable for building-level and WWTP-based surveillance covering large populations. This study optimized and compared the rapid InnovaPrep CP select concentration method with the electronegative membrane filter-based HA method, emphasizing quick data turnaround and enhanced SARS-CoV-2 detection sensitivity for building-level wastewater. Additionally, the two-step hollow UF and HA filtration-based concentration method was developed and optimized for detecting SARS-CoV-2 particularly in low-titered wastewater samples. In this dissertation, the transmission dynamics and prevalence of the Omicron and Delta variants in Mecklenburg County was also studied using ddPCR duplex mutation assays targeting mutation in the S gene (N764K and N856K). The key conclusions from the studies are as follows:

- The CP Select concentrator protocol was notably more efficient at concentrating SARS-CoV-2 from wastewater in comparison to the HA method. Approximately 25% of the samples that initially tested negative for SARS-CoV-2 with the HA method produced

a positive signal when processed with the CP Select method. Moreover, the CP Select method reduced processing time by 30%, rendering it an optimal choice for building surveillance applications that require rapid results.

- The results of SARS-CoV-2 partitioning indicated that roughly 50% of SARS-CoV-2 viruses were distributed between the liquid and solid phases. Among the solid portion, a significantly higher percentage of SARS-CoV-2 viruses were found attached to small particles compared to the larger settleable solids. However, the inclusion of a sonication step reduced viral loss during the centrifugation process.
- The optimized two-step large volume concentration method (UF-HA_Soni) successfully concentrated viruses from low-titered wastewater samples, enabling 100% SARS-CoV-2 detection. In contrast, the HA method achieved only 9% detection, and the UF method reached 63% detection. This method is suitable for tracking viruses during the early stages of a COVID-19 outbreak in a community, which is crucial for controlling the virus's spread and preventing further outbreaks.
- Utilizing the ddPCR duplex mutation assay, both Omicron and Delta variants were effectively detected in wastewater. Similar to the SARS-CoV-2 results, a significantly higher percentage of Omicron and Delta variants were also detected using the optimized combination method (UF-HA_Soni) compared to the HA method.
- The relative abundance of the Omicron variants exhibited a strong positive correlation with clinically reported VOCs ($r = 0.98$, $p < 0.0001$). This data suggests that both new and existing mutations circulating in the community can be efficiently monitored by the ddPCR mutation assay at a reduced cost.

5.2 Broader Impact

WBE is a public health tool that uses wastewater to monitor human pathogenic microorganisms such as SARS-CoV-2 viruses to infer the risk of illness in the population. WBE can be used to detect the presence of the virus in a community before symptoms appear in individuals. This can help public health officials identify and isolate outbreaks before they spread further, which can help to slow the spread of the disease. One of the main advantages of WBE for COVID-19 surveillance is that it can be used to monitor large populations, including those that may be difficult to reach or test using traditional methods. Another advantage of WBE for COVID-19 surveillance is that it can detect viral shedding in individuals who are asymptomatic or pre-symptomatic, which is important for understanding the spread of the disease. Additionally, the results can be used to monitor the effectiveness of these interventions over time.

Successful implementation of WBE for disease monitoring hinges on factors such as method optimization and quality control, pivotal for generating reliable public health data. This dissertation mainly focused on the development and optimization of the virus concentration methods and quality control approach. The primary objective, detailed in Article 1, aimed to optimize, and compare virus concentration methods with an emphasis on swift data processing and heightened virus detection sensitivity. This study targeted improving the existing SARS-CoV-2 surveillance protocol used at UNC Charlotte, reducing sample processing time to enable same-day data reporting. The existing protocol took two days to finish the cycle beginning from sample collection to data reporting. The objective was to reduce the sample processing time without compromising molecular detection sensitivity so that the data reporting can be done on the same day. This goal was achieved after adopting the newly developed and optimized virus concentration protocol in UNC Charlotte Campus COVID-19 monitoring. This

study has a direct application in disease monitoring in college dorms, schools and other congregate living facilities where rapid data reporting can reduce the risk of rapid infectious virus spreading and control outbreak.

The second article's impact on disease monitoring among larger populations is significant work. Most commonly used methods can only process small volumes of wastewater, making it difficult to detect pathogens such as viruses during the early stage of community infections or in low illness prevalence areas. However, estimating viral level during the early stage of infection is crucial for public health decision-making to control the spread of disease in mass level. The newly developed two-step large volume-based concentration method addresses this limitation by detecting and quantifying low-titered pathogens from wastewater. Moreover, it enhances the detection sensitivity for identifying rare mutations, a difficulty posed by highly similar sequences. This optimized concentration method proves beneficial for early-stage infection estimation and mutation tracking, critical for effective public health decision-making on disease spread control at a mass level.

The dissertation also explores the application of droplet digital polymerase chain reaction (ddPCR) in detecting and quantifying newly emerged SARS-CoV-2 mutations in wastewater samples. The surveillance of VoCs primarily relies on the complete genome sequencing of patient samples. However, this method is relatively costly, labor-intensive, time-consuming, and often inaccessible in many regions worldwide. In addition, sequencing of SARS-CoV-2 from wastewater samples is challenging due to limited coverage and fragmented genomes (Davis et al., 2021; Lou et al., 2022). The ddPCR based mutation assay technique enables near real-time transmission dynamic variants like Omicron and Delta variants, facilitating prompt public interventions. It also has the potential to track new mutations if

genome sequences of the new strains are available, offering a valuable tool for monitoring emerging variants.

In addition, as part of the NC Wastewater Pathogen Research Network project, I was fortunate to participate in the setup of the laboratory, analytical, and epidemiological research capacity for the State of North Carolina and beyond and responded rapidly to the COVID-19 pandemic through coverage of over twenty municipal wastewater treatment plants since Fall 2020. Now, the wastewater surveillance data for COVID-19 surveillance in the State of North Carolina has been incorporated into the dashboard on the website of the CDC National Wastewater Surveillance System and NC Department of Health and Human Services (NC DHHS) dashboard. Policymakers were able to use this information to make evidence-based decisions and implement measures to safeguard public health. This setup will also help manage endemic disease burden or any newly emerging pandemic within the community.

Overall, this work holds direct implications for public health through passive disease surveillance and advances the field of WBE. It empowers health authorities with efficient surveillance tools, provides insights into transmission dynamics, and equips communities with early warnings, ultimately strengthening our collective ability to combat infectious diseases.

5.3 Recommendations

1. **Sequencing Attempt:**

   We attempted to sequence SARS-CoV-2 from wastewater samples that were concentrated using hollow UF, HA filtration, and their combination methods. We utilized VarSkip (Variant Skip) Short primers in conjunction with Oxford Nanopore technology to determine the lineage percentages of the Omicron and Delta variants in wastewater samples. Unfortunately, due to technical issues and multiple freeze-thaw

cycles of the RNA, the sequencing results were less promising. Future studies should explore the impact of different combinations of large-volume filtration methods on the sequencing process.

2. **Monitoring Disease Outbreaks:**

   We hypothesize that this optimized combination method could be adapted to monitor other disease outbreaks in specific regions or communities. The feasibility of this expanded scope can be assessed through appropriate methodological planning.

3. **Sonication's Impact:**

   During this study, we found that the sonication step improved viral recovery from the solid phase. However, the impact of different sonication time intervals on viral recovery was not investigated. Future research can explore this aspect.

4. **Solid Particle Sizes:**

   Our study revealed that small solid particles, separated through the centrifugation step, contained a higher percentage of SARS-CoV-2 gene copies compared to larger solid particles settled due to gravity. In future studies, the association of SARS-CoV-2 viruses with solid particles of varying sizes can be examined by segregating them based on their size.

6 LIST OF PUBLICATIONS

6.1 List of Articles

Paper 1: Juel, MAI., et al., (2021), Performance evaluation of virus concentration methods for implementing SARS-CoV-2 wastewater-based epidemiology emphasizing quick data turnaround", Science of the Total Environment, 801, 149656. https://doi.org/10.1016/j.scitotenv.2021.149656

Paper 2: Juel, MAI., et al., Development of large volume filtration-based virus concentration method for increased SARS-CoV-2 detection sensitivity (Submission phase in Science of the Total Environment)

Paper 3: Juel, MAI., et al., Dynamics of SARS-COV-2 Omicron and Delta variants circulating in wastewater in the Charlotte area. (Submission phase in Frontier in Environmental Science/ES&T Water)

Paper 4: Barua, BV*., **Juel, MAI***., et al., (2022), Tracking the Temporal Variation of COVID-19 Surges Through Wastewater Based Epidemiology During the Peak of the Pandemic: A Six-Month Long Study in Charlotte, North Carolina. Science of the Total Environment, 814, 152503. (*Equally contributed 1st author) https://doi.org/10.1016/j.scitotenv.2021.152503

Paper 5: Wu, H., **Juel MAI.,** et al., (2023), Temporal and spatial relationships of CrAssphage and enteric viral and bacterial pathogens in wastewater in North Carolina. Water Research (2023), 239, 120008. https://doi.org/10.1016/j.watres.2023.120008

Paper 6: Gibas, C., Lambirth, K., Mittal, N., **Juel, MAI**., et al., (2021), Science of the Total Environment Implementing building-level SARS-CoV-2 wastewater surveillance on a university campus. Science of the Total Environment (2021), 782, 146749 https://doi.org/10.1016/j.scitotenv.2021.146749

Paper 7: Khanal, N., **Juel, MAI**., et al., Optimization of SARS-CoV-2 RNA Extraction Protocols for Enhanced Viral RNA Yield in Wastewater Samples. (Manuscript in preparation).

Paper 8: Brazell, L. R., Stetz, S., Hipp, A.,….**Juel, MAI**., ... & Gibas, C. (2021). Environmental screening for surface SARS-CoV-2 contamination in urban high-touch areas. medRxiv, 2021-05. https://doi.org/10.1101/2021.05.04.21256107

Paper 9: Agan, M. L., Taylor, W. R., Willis, W. A.,... Juel, MAI., ….. & Gibas, C. J. (2022). Wastewater as a backdoor to serology? medRxiv, 2022-11. https://doi.org/10.1101/2022.11.11.22282224

6.2 List of Conference presentation (Selected)

1. **Juel, MAI.,** et al., (2023). Large-volume filtration-based virus concentration method for increased SARS-CoV-2 detection sensitivity from wastewater. 25th NC WRRI Annual Conference, Raleigh, NC. 2023

2. **Juel, M.A.I**., Schlueter, J., Gibas, C., Munir, M. (2022). Wastewater-based surveillance of SARS-CoV-2 Omicron variants circulating in the community. Third Annual Graduate Research Symposium, CEE, UNC-Charlotte, NC. (First place)

3. **Juel, MAI.,** et al., (2022). Wastewater-based surveillance of SARS-CoV-2 Omicron variants circulating in the community. NC One Water, December 2022. (3rd place)

4. **Juel, MAI.,** et al., (2022). Comparison of two Methods for Implementing SARS-CoV-2 Wastewater-based Epidemiology. WEF Public Health and Water Conference in Cincinnati, OH, March 21-24, 2022

5. **Juel, MAI.,** et al., (2021). Surveillance of COVID-19 Outbreak in Local Community through Quantitative Analysis of SARS-CoV-2 in Wastewater. 2nd Graduate Research Symposium of the Civil and Environmental Engineering Department, 2021. (2nd place)

6. **Juel, MAI.,** et al., (2021). A QA/QC approach for implementing SARS-C0V-2 wastewater-based epidemiology. GPSG Graduate Research Symposium, UNC Charlotte, 2021. (2nd Place)

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