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Caleb Baker Cantrell Date

Comparison of the Performance of Two Methods for Concentration of Wastewater for Outbreak Monitoring of COVID-19 in Metropolitan Atlanta, GA.

By

Caleb Baker Cantrell

Master of Public Health

Epidemiology

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Dr. Christine Moe
Committee Chair

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Dr. Marlene Wolfe
Committee Member

# Comparison of the Performance of Two Methods for Concentration of Wastewater for Outbreak Monitoring of COVID-19 in Metropolitan Atlanta, GA.

By

Caleb Baker Cantrell

B.S. Microbiology
Clemson University
2019

Thesis Committee Chair: Dr. Christine Moe
Committee Member: Dr. Marlene Wolfe

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
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# Abstract

Comparison of the Performance of Two Methods for Concentration of Wastewater for Outbreak Monitoring of COVID-19 in Metropolitan Atlanta, GA.
By Caleb Baker Cantrell

The use of wastewater surveillance for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which is the etiologic agent that causes the respiratory disease COVID-19 has rapidly expanded since the emergence of the virus in December of 2019. Studies have shown that rises in SARS-CoV-2 RNA within wastewater among sewersheds is indicative of increases in COVID-19 incidence among the population contributing to that sewershed. Advancements in concentration methods for SARS-CoV-2 RNA quantification have created the need for comparison studies to determine the association between these methods as well as reported COVID-19 cases. A prospective study was conducted among seven influent lines that collected wastewater for three publicly owned treatment works in the metropolitan Atlanta, GA area. This study sought to determine the association between wastewater sample SARS-CoV-2 RNA concentration found using membrane filtration (MF) and a magnetic hydrogel particle concentration method (Nanotrap® Magnetic Virus Particles), hereafter Nanotrap® particles, as well as the association between COVID-19 incidence and concentration of SARS-CoV-2 RNA in wastewater. The nonparametric correlation using Kendall's tau showed statistical correlation between the RNA concentration and COVID-19 incidence for Nanotrap® particles (tau = 0.26, $p < 0.001$) and for MF (tau = 0.56, $p < 0.001$). It was found that for every 1-log increase of RNA concentration using Nanotrap® particles resulted in a 0.55-log increase in COVID-19 incidence among all sewersheds; for every 1-log increase in RNA concentration using MF there was a 0.46-log increase in the incidence. Additionally, the relationship between the concentrations of SARS-CoV-2 RNA reported by Nanotrap® particles and by the MF method were significantly associated. Furthermore, the Nanotrap® particles yielded higher quantities of RNA compared to MF. These findings provide the foundation for comparison of the primary virus concentration methods used in wastewater surveillance of COVID-19 as the field evolves.

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## Introduction

The prevention of infectious disease spread relies on a multitude of key factors: infectious disease surveillance systems, laboratory analysis, epidemiological studies, widespread informatic systems to monitor and maintain the ever-growing amount of public health data, and implementation of effective prevention measures. Preventing the spread of infectious disease is a cornerstone of public health and epidemiology, and this relies on surveillance efforts to monitor diseases caused by pathogens and their transmission within a population. As defined by the Centers for Disease Control (CDC), public health surveillance is "the ongoing, systematic collection, analysis, and interpretation of health-related data essential to planning, implementation, and evaluation of public health practice, closely integrated with the timely dissemination of these data to those responsible for prevention and control." The importance of accurate and timely infectious disease surveillance is critical as the world currently deals with the COVID-19 pandemic. However, aggregating information on individual cases is challenging, expensive, and can be biased. Wastewater monitoring can complement existing surveillance efforts for COVID-19 as it can provide population-level data for public health officials to effectively monitor trends. This thesis will present a literature review of the current scope of surveillance for COVID-19, implementation of wastewater surveillance, and current methods for concentration of wastewater. Results from a comparison study of two primary concentration methods for wastewater are discussed as they were conducted for a COVID-19 wastewater surveillance program in metropolitan Atlanta, GA.

### Infectious Disease Surveillance: Active & Passive

The goals of public health, specifically infectious disease surveillance, are to describe the toll and up-to-date rates of a disease, monitor fluctuations in cases, and to help delineate spreading outbreaks or clusters from both novel and known pathogens[1]. Surveillance of infectious diseases is often broken down into active and passive systems. Active surveillance systems employ public health workers to actively contact and gather information regarding health conditions, and passive surveillance systems rely on reported case information from community healthcare providers to public health organizations[2]. Active

monitoring of disease requires immense resources, both human or financial. Therefore, the majority of infectious disease surveillance is passive: cases of diseases are diagnosed by healthcare professionals that then report data to public health agencies like county or state health departments or the CDC for diseases of interest. One of the major passive surveillance systems currently operational in the United States is the National Notifiable Disease Surveillance System (NNDSS) which is a collaborative effort between the CDC, state, and local health departments around the country. The CDC receives electronic reporting of disease cases from 3,000 health departments and processes them to determine trends throughout the regions of the United States[3]. However, a majority of infectious diseases that cause outbreaks, i.e., influenza, are not nationally notifiable. Additionally, the timeliness and quality of data reported to public health departments and the CDC are not uniform. Moreover, because it relies on existing healthcare infrastructure and access, passive surveillance is not able to report every case of disease and has a reduced ability to detect rare occurrences[2]. Certain infectious diseases can cause asymptomatic infections that prevent clinicians from properly diagnosing or result in infected individuals unknowingly spreading the disease. Moreover, increased case numbers can overwhelm surveillance efforts as diagnostic testing and case reporting can become backlogged. Additionally, financial input from governments is not always feasible or may be stopped for political or social reasons.

Active surveillance on the other hand devotes resources into tracking down every case. This requires an immense input from public health organizations within the surveillance system and collaborative efforts with clinicians and other community organizations to ensure any potential case is captured. This often provides the most complete epidemiological data as public health workers diagnosis cases, create reports for each case, track contacts of infected individuals, conduct laboratory tests, and report findings to governmental head organizations. However, the resources needed makes it unfeasible to have every single disease under active surveillance[1]. That is why a mixture of both passive and active surveillance systems have been implemented throughout the United States and abroad to monitor infectious disease. Challenges in these systems mean there is still need for a supportive surveillance system that can

detect various threats and be efficiently replicated in multiple environments as it is paramount for the future of public health here in the United States and worldwide.

The ongoing COVID-19 Pandemic

Currently, the emergence and on-going transmission of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, which causes the respiratory disease known as COVID-19, has put a strain on the public health sector along and has had significant economic and political impacts. In December of 2019, the first case of COVID-19 was reported in Wuhan, Hubei, China with further cases being reported in 114 countries by March 11th, 2020 when it was then declared a pandemic by the World Health Organization[4]. The SARS-CoV-2 virus that causes COVID-19 belongs to the *Coronaviridae* family, *Betacoronavirus* genus, and subgenus *Sarbecovirus*[5]. This virus is the ninth subtype of coronaviruses able to cause human infection and is closely linked to other SARS coronaviruses that have caused previous pandemics in 2001, 2003 (SARS-CoV) and the Middle East respiratory syndrome (MERS) coronavirus in 2012 that still has active transmission[6]. As of, April 11th, 2022, SARS-CoV-2 has caused over 481 million cases and resulted in 6,181,150 deaths worldwide[7,8]. This makes COVID-19 the seventh deadliest known pandemic behind both the HIV/AIDS pandemic which has killed an estimated 25-30 million and the Third Plague of 1855 that killed an estimated twelve million[9].

The clinical manifestations of COVID-19 are non-specific with a high prevalence of asymptomatic infections. The most commonly experienced symptoms that were observed at the start of the original outbreak in Wuhan, Hubei, China included fever, cough, and fatigue. Rarer symptoms included production of a thick sputum from damaged or diseased lungs, headaches, hemoptysis, and diarrhea[10]. A systematic review looking at reported clinical case data further cemented the top presented symptoms to be: fever, cough, fatigue, dyspnea, sputum presence, myalgia, shortness of breath, chest tightness, sore threat, headaches, diarrhea, and hemoptysis[11]. Since the original detection of the SARS-CoV-2 virus there have been mutations within the genome causing new lineages to evolve. Lineages that are classified as variants of concern (VOCs) can have altered symptom profiles from the wildtype in which milder symptoms are

present causing higher proportions of cases to be undiagnosed and higher transmissibility of the virus. VOCs may also increase the virulence of SARS-CoV-2, causing symptoms that are more severe and deadly. The changing symptoms, potential increase of virulence, transmissibility, and immune evasion seen in VOCs places critical emphasis on the need to effectively track outbreaks of COVID-19.

COVID-19 Surveillance.

Diagnosis and testing for COVID-19 infections is done on an individual basis at testing sites in hospitals, pop-up locations, clinics, and in correctional facilities for those incarcerated. These test results are aggregated for outbreak surveillance. Nasopharyngeal or oropharyngeal swabs are the most commonly collected samples that are processed within a laboratory to determine the status of infection. The current gold standard for confirmation of a COVID-19 infection is through the use of a Nucleic Acid Amplification Test (NAAT) which is routinely done using reverse transcription polymerase chain reaction (RT-PCR) for presumed positive COVID-19 individuals[12]. A secondary mode of testing for COVID-19 infections is an antigen-detection rapid diagnostic test (Ag-RDTs) which are rapid inexpensive detection kits that can be used in areas where NAAT RT-PCR testing is not readily available. The collection of individual swabs for testing relies on the ability and willingness of a person to subject themselves to a diagnostic test, and potential subsequent investigation by public health officials if their test is positive.

While the use of NAAT and Ag-RDTs diagnostic methods are reference standards for analyzing specimens; there are multiple barriers associated to expanding this mode of direct testing to adequately monitor COVID-19 on a broad population scale. First off, widespread access to testing is not uniform for all communities within the United States. Communities of color and minorities have been disproportionately affected by COVID-19 infections, and that burden is exacerbated by the lack of testing. These communities and the surrounding areas are also more likely to have higher percentages of other health disparities as a result of health inequities preventing them from accessing routine primary care and diagnostic testing[13]. Secondly, direct diagnostic testing for COVID-19 especially in the case of RT-PCR results can be untimely. Normal test results for RT-PCR analyzed samples are on average available 24-48

hours following sample collection[14]. However, surges in cases can push results back for days to weeks, and backlogs of sample results can happen which delay positive cases being reported on time. This backlog also affects reporting of cases to public health authorities for aggregation of the total population case numbers for specific areas. Lastly, direct testing for COVID-19 relies heavily on an individual experiencing a symptom that warrants a test if they are concerned, i.e., cough, fever, loss of taste or smell, however, asymptomatic cases of COVID-19 have been documented[15]. Initial reports showed that active surveillance of SARS-CoV-2 is complicated by the estimated 35% of infections being asymptomatic, and if the addition of pre-symptomatic individuals at the time of testing was included that estimate increased to 42.8%[16]. Reports of asymptomatic cases being as high as 48.9% further confirm the difficulty in utilizing direct diagnostic testing to adequately monitor COVID-19 infections on a large scale[17]. Furthermore, the relative cost for scaling mass testing of large populations requires immense financial input which is a strain, especially within developing countries[18]. All of these barriers have led to a need for a surveillance system to enhance the current approaches to effectively track COVID-19 cases at a population level, while remaining sensitive, reliable, cost effective, and time efficient[19].

Potential Novel Avenues for COVID-19 Surveillance

One approach to fill gaps and enhance current surveillance efforts for COVID-19 is by analyzing wastewater for SARS-CoV-2. There is evidence that infected individuals shed SARS-CoV-2 RNA in their feces alongside respiratory droplet shedding which means that the virus is likely to be reliably shed into wastewater[20]. Currently, within the United States there are over 14,000 publicly owned treatment works (POTWs) that collect and treat wastewater from more than 236 million people[21]. The large percentage of households in the United States connected to sewer and consistent shedding of the virus into wastewater by infected people makes wastewater a prime source of samples for large scale surveillance of COVID-19. This approach, commonly known as wastewater-based epidemiology (WBE), works by quantifying SARS-CoV-2 RNA found within sampled wastewater from POTWs to estimate the prevalence of COVID-19 within the geographical catchment basin for that particular POTW. The quantification of SARS-CoV-2

RNA within wastewater has proven to be a strong indication of the prevalence of COVID-19 infections within a POTW catchment basin, and can bolster public health efforts to effectively track COVID-19 cases[22–29]. This type of disease surveillance is not novel, but rapid work on developing and optimizing methods for wastewater analysis of a respiratory virus has never been done before.

Fecal Shedding & Detection of Enteric & Non-Enteric Viruses in Wastewater

Human feces can contain a wide variety of microorganisms, including enteric and non-enteric viruses, that can persist in wastewater and the environment for days to weeks. Enteric viruses have been the focus of wastewater monitoring in the past – they are one of the leading causes for gastroenteritis in the world and cause over 200,000 deaths every year as their primary transmission route is fecal-oral[30]. The concentration of these viruses shed by a single infected human can range from $10^5$ to $10^{11}$ virus particles per gram of stool[31]. The extremely high concentration and ability to persist in the environment make enteric viruses a practical target for wastewater surveillance.

A key implementation of WBE for pathogenic disease using wastewater was implemented in 1989 in Israel. For polio surveillance, the main indicator of a rise in cases is usually through detection of cases of acute flaccid paralysis, but the occurrence of this severe form of polio is rare[32]. This warranted a more robust surveillance system to detect potential increases and catch outbreaks early. Infected individuals shed infectious poliovirus in their feces, so Israeli authorities analyzed wastewater from their POTWs monthly for poliovirus. The surveillance efforts led to a detection of a silent polio epidemic in 2013 which in turn facilitated public health action from government officials to prevent a rise in infection[33]. The successful application also led further systems to be put into place for polio surveillance for focused eradication efforts.

Detection of other enteric viruses such as adenoviruses, astroviruses, norovirus, and hepatitis A have also been documented in wastewater. These viruses are commonly found at high titers within wastewater and can be detected throughout the year[34–37]. A positive association was found between the rise

of these viral concentrations in influent wastewater samples and clinical cases that hospitals received in the following weeks[38].

However, unlike enteric viruses, respiratory viruses such as influenza A, SARS, and MERS are not commonly associated with a fecal-oral or enteric route of replication or transmission. The first example of a respiratory virus that has been surveilled through wastewater is COVID-19. Yet, respiratory viruses along with other types of viruses such as West Nile virus, Dengue virus, Zika virus are shed in fecal matter and have been detected in wastewater[39–43]. The concentration and detection of these viruses within wastewater could prove to be a more sensitive temporal marker of infection in a population as they have been detected in wastewater prior to clinical detection. Recently, a statistically significant association, was found between the wastewater viral RNA concentrations and clinical cases of respiratory syncytial virus (RSV) reported by sentinel surveillance in 2021[44]. Another study detected influenza A RNA in wastewater and observed an association between the fluctuations of viral concentration and clinical cases seen at a major university[45]. The detection of respiratory viruses along with others that are not routinely known to transmit through the fecal-oral route suggest that wastewater may be broadly utilized for infectious disease surveillance.

Fecal Shedding & Detection of SARS-CoV-2 in Wastewater

When diarrhea was noted as a possible symptom of COVID-19[10,46], it raised questions about the levels of viral shedding in feces (as fecal shedding had been noted for MERS and SARS-CoV-1[20,47]). Furthermore, there was concern that presence of SARS-CoV-2 in feces could indicate a fecal associated route of transmission for COVID-19. However, due to the lack of infectious SARS-CoV-2 particles cultured from stool, this transmission pathways appears unlikely[48]. Although concerns about this transmission pathway have diminished, one of the first initial reports of SARS-CoV-2 RNA detected in stool found that anal swabs of infected hospitalized patients were positive in later days of an infection compared to an oral swab[49]. A secondary study also reported anal swabs that were positive for SARS-CoV-2 RNA, but did not observe positive anal swabs when oral swab samples came back as negative[50]. Results from a systematic review show that persistent shedding of SARS-CoV-2 RNA has been reported for up to 22 days in feces,

and the average time until no detection of SARS-CoV-2 RNA in stool was 4.8 days longer than respiratory samples[51]. Further studies devoted to quantifying the viral titers of SARS-CoV-2 RNA shed by infected individuals found that up to $10^7$ genomic copies per mL (gc/mL) could be shed in stool[46]. Additionally, there are reports that SARS-CoV-2 RNA can be present within stool on average four to five days prior to symptom onset or positive nasopharyngeal swabs[52]. Asymptomatic patients have also been documented actively shedding SARS-CoV-2 RNA within their stool[53]. The active shedding from symptomatic, presymptomatic, and asymptomatic COVID-19 cases means that SARS-CoV-2 shed into wastewater may provide a broad snapshot of the rates of disease in the community.

Utilization of WBE to Monitor COVID-19

Early WBE studies in Australia, the Netherlands, Japan, the United States and Italy confirmed the presence of SARS-CoV-2 RNA in wastewater collected from POTWs and found that changes in RNA concentration were positively associated with COVID-19 clinical cases in the following days[22–26]. This provided the foundational evidence to launch further investigation by additional municipalities, universities, and governments into the feasibility of WBE for COVID-19. As of April 12th, 2022, the adoption of wastewater monitoring for COVID-19 now spans 64 countries, 276 universities, and over 3,300 sampling location sites[54].

The research and academic environments around this topic in the pandemic are extremely dynamic and changing daily. This thesis will highlight current studies in different geographical regions of the United States and countries worldwide has been made to showcase the successful implementation of WBE for COVID-19 detection, population prevalence estimations, and tracking of new variant case surges.

On a local level, wastewater surveillance of university and college dormitory halls have provided an early warning system for campus health officials to mitigate COVID-19 spread and provide increased safety during semester sessions. Presymptomatic and asymptomatic testing has been provided to students at most universities throughout the United States[55]. However, universities have incurred a substantial cost

by providing testing to their students, staff, and faculty. Adoption of wastewater surveillance could provide a significant savings to universities while providing equally reliable and useful information about COVID-19 cases on their grounds. Implementation of wastewater surveillance at a Southeastern public university garnered widescale data for its population at 1.7% the cost of receiving the same level of data using clinical testing[56]. In addition to the low cost, the method is highly sensitive. Single asymptomatic individuals have been detected within resident hall populations greater than 150 through initial positive wastewater analysis and secondary rapid clinical testing[57]. The sensitivity to detect one to two cases within resident halls using wastewater monitoring led to swift testing; the same experience was reported in a study at Emory University[58] . These methods have also been used at scale - implementation of a highly automated WBE system using wastewater autosamplers, high throughput concentration and extraction methodologies, and campus wide alert system allowed for 85% of all COVID-19 infections to be diagnosed early on a campus with an onsite population of over 7,000 students[29]. Although the scale for university-based wastewater surveillance is smaller than programs at the city level, they have provided crucial information in protecting and mitigating spread of COVID-19 on their campuses. The unique knowledge of the campus population in each dorm and on campus grounds has also allowed for high-quality assessment of the relationship between COVID-19 cases and the concentration of SARS-CoV-2 RNA. This can help to formulate better estimation calculations for large scale WBE systems that analyze wastewater from POTWs in municipalities with tens or hundreds of thousands of citizens.

In the United States and abroad, large scale population surveillance for COVID-19 has been implemented using WBE methods at numerous municipalities. Municipalities in the United States and abroad often require multiple POTWs to effectively treat the millions of gallons per day (MGD) of wastewater generated by the community. The increased population sizes seen in comparison to universities does not change the core methodologies for wastewater surveillance, and wastewater from these larger populations can still be positive for the presence of SARS-CoV-2 RNA even with very few cases providing evidence for the sensitivity WBE for COVID-19 surveillance[27,59]. A surveillance program conducted in two

parishes of Louisiana detected SARS-CoV-2 RNA at their POTWs when the cumulative case count at the time was less than 6,500 for a population size of 240,000[60]. In Virigina, a large-scale wastewater surveillance system for COVID-19 was devised for a population of 1.7 million that contributed wastewater to nine major POTWs. A rise in the viral titers was seen during all stages of the phased reopening, showing that the increase in recorded COVID-19 infections for the region was not due solely to increased clinical testing. Furthermore, the WBE system was able to show spatial trends and a heterogenous spread of COVID-19 that clinical testing was unable to display[61].

Implementations of WBE for COVID-19 in universities[29,56–58] and cities[28,60–62] has been so successful that the CDC is supporting a national wastewater system for aggregating the data and is funding state programs to support data generation. Compiled data from partner states currently conducting WBE is shown on the CDC's National Wastewater Surveillance System (NWSS). These results are shown as percent changes along with proportion detections at each site over the last 15-days. The CDC recommends a set of methods that are well supported by research for the concentration, extraction, and quantification of SARS-CoV-2 RNA in wastewater samples[63]. The primary concentration methods of SARS-CoV-2 RNA from wastewater samples is crucial for the recovery of viral RNA for quantification that is used to accurately estimate the level of COVID-19 within the population. Current concentration methods recommended by the CDC for adequate SARS-CoV-2 RNA recovery from wastewater are: ultrafiltration, filtration through an electronegative membrane with pre-treatment of either $MgCl_2$ or acidification, polyethylene glycol (PEG) precipitation, skim milk flocculation, and ultracentrifugation. While these concentration methods are successful in capturing SARS-CoV-2 RNA there are new methodologies like Nanotrap® Magnetic Virus Particles (Ceres Nanoscience), hereafter referred to as Nanotrap® particles, and use of wastewater solids for efficient recovery of viral particles.

## Analytical Methods for Detection of SARS-CoV-2 RNA in Wastewater

### *Overview*

Because many pathogenic targets like SARS-CoV-2 are rare in wastewater, concentration methods are most often the first step following sample collection to begin the process of quantifying RNA concentration. Extensive research has been conducted to determine the viral recovery rates and feasibility of use for a multitude of methods[64–68]. Initial surveys of laboratories at the start of the pandemic found that PEG precipitation and membrane filtration were heavily favored for the primary concentration methods of SARS-CoV-2 RNA[69]. Polyethylene glycol, specifically PEG 6000 or 8000, and NaCl are precipitants that when added to an aqueous solution affect the solubility of SARS-CoV-2 viruses causing it to precipitate out of solution. Membrane filtration relies on utilization of specific micron sized pores that prevent the flowthrough of SARS-CoV-2 viruses when samples are vacuumed through. Less common methods used during the pandemic have been ultrafiltration and skim milk flocculation. Ultrafiltration of samples involves centrifugation at 4,000 - 10,000 xg to force a sample through a filter to concentrate viral particles. Skim milk flocculation works similarly to PEG by using skim milk powder to act as the flocculation agent for SARS-CoV-2 to allow for precipitants to form that contain the viral particles.

Recovery rates for different control viruses spiked into wastewater have often been used for comparing the effectiveness of different concentration methods. However, quantitative measurement of recovery is not always the most valid comparator for performance - reports of recovery rates for inactivated or irradiated SARS-CoV-utilizing identical methods found a recovery rate of 27.5% and 11.1% across wastewater matrices[70,71]. An investigation into ten of the most common concentration protocols in 2020 reported that ultrafiltration was not able to concentrate surrogate feline calicivirus spiked wastewater samples to allow for detectable RNA levels using RT-qPCR[72]. Furthermore, recovery rates of the fecal indicator marker Pepper mild mottle virus (PMMoV) and the surrogate virus Bovine Coronavirus (BCoV) were shown to be under 10% in spiked samples using ultrafiltration methods[66]. Improvements to primary concentration methods show that ultrafiltration has the highest recovery percentage for SARS-CoV-2 in

sample volumes of 30mL or less[73]. For larger sample volumes, 50mL or more, a study reported that electronegative membrane filtration was most sensitive for detection of SARS-CoV-2 RNA genes along with laboratory process controls[74]. Lastly, developments of concentration methods found that solids within wastewater influent and sludge contain increased levels of SARS-CoV-2 RNA and may prove to be a more sensitive marker for use to quantify the concentration of RNA in wastewater[27,75,76].

The effectiveness of a wide-range of primary concentration methods allows for more flexible adoption of WBE based on a laboratory's need and current equipment owned, but can provide challenges for comparability of results. Herein this study, two primary concentration methods for SARS-CoV-2 RNA were used. Electronegative membrane filtration (MF) and a novel hydrogel magnetic bead concentration protocol (Nanotrap® particles) were compared to determine the effectiveness of MF versus the new method that has the ability to increase throughput of samples due to its ease of use, and the relationship between results obtained using the two methods.

*Electronegative Membrane Filtration*

The gold standard procedure for analyzing and enumerating fecal coliforms in drinking water and in wastewater is done using a membrane filtration (MF) technique proposed by Goetz and Tsuneishi in 1951 and has been widely used in environmental microbiology work[77]. Membrane filtration methodologies have been successful in concentrating Norovirus, Rotavirus, and other enteric viruses in environmental water samples[65,73,78,79]. Samples are acidified to a pH below a virus' isoelectric point, subsequently vacuum filtered through an electronegatively charged membrane along with a multivalent salt, DNA or RNA is extracted and then analyzed using RT-qPCR to determine the viral concentration. The addition and adsorption of a multivalent salt on the electronegative membrane filter, i.e. $MgCl_2$, $AlCl_3$, $Al_2(SO_4)_3$, improves the attachment of a virus onto the membrane filter[80]. For a virus, their isoelectric point is the pH of the aqueous solution at which the virion's net charge is neutral (i.e. 0). In pH environments above a virus' isoelectric point, they display a net positive charge due to deprotonated carboxyl groups. In contrast, viruses in aqueous solutions that are at a lower pH than their isoelectric point results in a net positive charge from

the protonation of amine groups[81]. Adjustment of a sample's pH therefore can improve the binding of virions to a filter when using MF. However, MF is sensitive to organic material contained in environmental samples as this material collects on sample filters alongside SARS-CoV-2 RNA which can affect the quantification as a result of RT-qPCR inhibition[82]. The organic material, normally in the form of solids, is often removed prior to the vacuum filtration step to avoid clogging the filter by allowing the material to settle or through less than ten-minute centrifugation steps at lower xg than ultrafiltration.

One of the first studies looking at the recovery rate of SARS-CoV-2 RNA within wastewater using an MF approach utilized murine hepatitis virus (MHV) as a surrogate coronavirus due to the biosafety hazard of working with SARS-CoV-2. Seven total methods were analyzed with three MF methods: one with acidification of the wastewater sample to a pH of 4, no pre-treatment, and addition of a bivalent cation $MgCl_2$. Overall, the highest percent recovery of MHV at 65.7% for all methods was the inclusion of $MgCl_2$[65]. This primary study has been cited extensively for providing critical information on the use of MF with inclusion of $MgCl_2$ as a reliable and accurate WBE primary concentration method at the start of the COVID-19 pandemic. As noted, MF and the recovery rates of spiked samples is susceptible to RT-qPCR inhibition due to the high presence of organic material within wastewater samples. This makes comparison of recovery rates difficult, but studies looking at the rates of recovery of surrogate viruses provides evidence that MF is a viable method for concentration SARS-CoV-2 RNA[61,66,67,74,83]. Furthermore, although the surrogate viruses utilized are enveloped viruses among the same family of coronavirus or are animal respiratory viruses that does not confer the same isoelectric point and/or structure to allow for 100% adsorption to an electronegative membrane. However, MF is beneficial for wastewater monitoring due to its ability to analyze samples in a relatively quick timeframe along with the equipment needed being routinely used in POTWs laboratories for fecal coliform analysis.

*Nanotrap® particles as a Primary Concentration Tool for SARS-CoV-2 RNA*

Magnetic hydrogel particles, develop and marketed as Nanotrap® particles by Ceres Nanosciences, have been used for sample concentration and purification and have been applied for primary concentration

of wastewater. Several programs have shown effective use of Nanotrap® particles for workflows quantifying SARS-CoV-2 from wastewater. The particles contain high reactive affinity baits that are able to bind to targeted biomolecules such as proteins, viruses, and peptides[84]. Applications of Nanotrap® particles prior to SARS-CoV-2 RNA concentration were used to detect cancer biomolecules, hormones, and HIV-1 viral proteins that exist within blood in extremely low concentrations[84–86]. Infectious disease surveillance applications, prior to COVID-19, also centered around concentration of influenza, RSV, and respiratory pathogens within clinical diagnostic samples. The particles were able to bind to influenza viral particles contained in different diagnostic samples such as nasopharyngeal swabs, saliva swabs, aspirates, and nasopharyngeal washes. Furthermore, the Nanotrap® particles were able to detect virus at 1 plaque forming units (pfu) /mL in influenza spiked samples[87]. The high binding affinity and increased detection of pathogens at extremely low viral loads in samples makes Nanotrap® particles a viable tool for SARS-CoV-2 RNA detection in wastewater.

Enhanced recovery of SARS-CoV-2 RNA in diagnostic clinical samples has been reported following concentration of using Nanotrap® particles [88,89]. This enhanced recovery of viable RNA allows for greater accuracy in samples when following RT-qPCR or sequencing of samples. Furthermore, the sample volume used in current Nanotrap® particle methods are 5-10x lower than that of MF. Successful WBE applications of Nanotrap® particle technology to monitor COVID-19 infections have utilized sample volumes as low as 5 - 10mL[28,29,90]. Furthermore, these studies were able to detect single asymptomatic cases in buildings containing over 200 residents. A very high throughput version of the protocol can be run using automation of Nanotrap® particles, but manual methods are also available and efficient. The automation of wastewater concentration using Nanotrap® particles has greatly reduce the sample turnaround time from collection to quantified results allowing for early warning systems to be implemented[29]. The current literature, as discussed, is rapidly expanding in the field of wastewater surveillance for COVID-19 and pathogens in general. However, the Nanotrap® particles methodology is one that currently is still being researched as an alternative tool for the primary concentration of RNA in wastewater.

Wastewater Surveillance Importance and Key Notes

Wastewater monitoring has proven to be an effective tool for COVID-19 surveillance. Sampling of wastewater allows for testing of a particular pathogen of interest without needed to actively test every single individual for the disease. The ability to test whole populations can help to reduce the resources needed for mass testing along with providing a data on an outbreak that is not biased by access to healthcare. Samples from defined catchment areas that see a rise in SARS-CoV-2 RNA concentration or other infectious pathogen can be a crucial piece of information used by public health departments when designating diagnostic or preventative supplies or human power. The analytical methods of MF and the novel Nanotrap® particles have been proven to be viable and reliable primary concentration methods for SARS-CoV-2 RNA in wastewater. Utilization of these concentration methods in wastewater monitoring systems for COVID-19 can provide accurate results that can be used to enhance the public health sector's response to the pandemic currently and in future pathogenic surveillance.

Study Goals

We conducted a wastewater monitoring program throughout Atlanta, Georgia to both provide information on COVID-19 and optimize methods for analysis. This study looked to determine the performance of Nanotrap® particles technology as a primary concentration method in comparison to MF. Wastewater samples were collected weekly from a total of nine influent lines with distinct catchment basin areas feeding into three POTWs. Concentration of SARS-CoV-2 in the wastewater samples was done using both a concentration method of MF with addition of $MgCl_2$ and an automated Nanotrap® particles protocol that utilized a Kingfisher Apex machine to increase throughput. SARS-CoV-2 RNA was quantified using RT-qPCR and results were compared to clinical cases of COVID-19 reported to the Georgia Department of Public Health (GDPH) for the areas of interest. The goal of this study was to: (1) determine if there was a significant association between SARS-CoV-2 RNA concentration in wastewater, using Nanotrap® particles, and reported clinical cases of COVID-19; (2) assess the association between the SARS-CoV-2 RNA concentration, using MF, and the reported clinical cases of COVID-19 for the time period of

9/14/2021 to 11/01/2021; and (3) compare the relative performance of the two virus concentration methods and their relationships to reported COVID-19 cases.

## Materials & Methods

### *Sample Collection*

Wastewater samples were collected in partnership with the City of Atlanta's Department of Watershed Management (DWM) from the Utoy Creek Water Reclamation Center, RM Clayton Water Reclamation Center, and the South River Water Reclamation Center (WRC). Both the Utoy Creek WRC and South River WRC collect wastewater influent from primarily the lower southeast region of Fulton County while RM Clayton WRC receives wastewater from a central portion of Fulton County. Shown in Figure 1 are the catchment basin zones for each influent line along with the location of the WRCs. An average daily flow in million gallons per day (MGD) was recorded by each WRC and their influent lines, and is displayed in Table 1. A one-liter grab sample was taken on a weekly basis from three influent lines at each WRC before primary filtration by pumping wastewater from the influent line using a peristaltic pump for collection. The wastewater grab samples were then transported on ice to the laboratory for processing on the day of collection. Initial concentration and extraction of each sample was completed within eight hours of collection.

*Table 1. Descriptive Statistics for the sampling collection from influent lines of Utoy Creek, South River, & RM Clayton Water Reclamation Centers (WRCs), and their estimated service population provided by the Department of Watershed Management using 2020 census block data and average Million Gallons per Day (MGD) flowrate.*

| | Utoy Creek WRC | | | South River WRC | | | RM Clayton WRC | | | All Three WRCs |
|---|---|---|---|---|---|---|---|---|---|---|
| **Influent Line** | ***Old Winn Dixie*** | ***Phillip Lee*** | ***South Fulton*** | ***Flint*** | ***Intrenchment*** | ***Jonesboro*** | ***Nancy Creek*** | ***Proctor Creek*** | ***Peachtree Creek*** | ***All Lines Combined*** |
| Average MGD | 1 | 11 | 4 | 3.2 | 13 | 1 | 1 | 4 | 25 | 63.2 |
| Total Estimated Population Served | X | 37,552 | 39,377 | 29,519 | 79,502 | 5,427 | 41,698 | 51,433 | 178,077 | 462,585 |
| Sample Collection Dates | 9/14/2021 – 2/21/2021 | | | 9/14/2021 – 2/21/2021 | | | 11/15/2021 – 2/21/2021 | | | - |
| # of Samples Collected | 22 | 22 | 22 | 22 | 20 | 22 | 12 | 12 | 12 | 166 |
| *Analyzed by MF* | 7 | 7 | 7 | 7 | 7 | 7 | NA | NA | NA | 42 |
| *Analyzed by Nanotrap® particles* | 22 | 22 | 22 | 22 | 20 | 22 | 12 | 12 | 12 | 166 |
| MF Analysis Dates | 9/14/2021 – 11/01/2021 | | | 9/14/2021 – 11/01/2021 | | | NA | | | - |
| Nanotrap® particles Analysis Dates | 9/14/2021 – 2/21/2021 | | | 9/14/2021 – 2/21/2021 | | | 11/15/2021 – 2/21/2021 | | | - |

*X: Data not available, NA: Not applicable*

*Total Samples Collected from each WRC*

Grab samples were collected on a weekly basis starting on March 23rd, 2021 and collection has continued past February 21st, 2022 for all influent lines at both Utoy Creek and South River WRC. Collection of samples from the three influent lines at RM Clayton WRC was conducted weekly on the same days as the other WRCs starting November 15th, 2021 and has continued past the study time period. This study analyzes the sample collection time period of September 14th, 2021 until February 21st, 2022 for all available samples. The total number of samples included in this study and the timeframe for both the entirety of the study and for the comparison of the two concentration methods is shown in Table 1.

*Identifying Influent Line Catchment Basins*

An ArcGIS igraph file of the Atlanta sewer system was provided by the DWM that contained the location of all manholes along with the topography. Members of the study team analyzed the flow of wastewater from each manhole to the next using Rstudio. Each individual manhole point was then analyzed to determine which influent line was being fed by that location. Secondly, a shapefile polygon was formed for each influent line by creating a concave hull around the manholes that were determined to feed wastewater into that respective influent line. Manual edits of any overlap and unfilled areas for the polygons was done. A buffer was then created around the manually edited boundary to use for the area to determine how many clinical cases reported from the GDPH dataset fell into these zones.

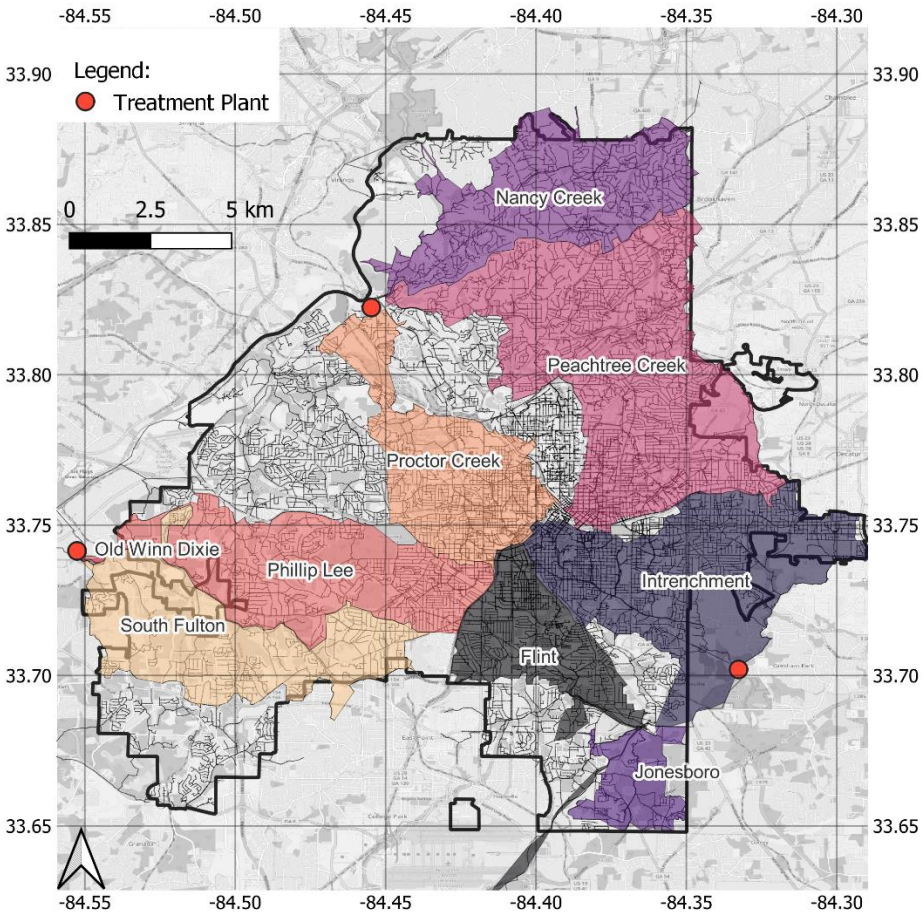


*Figure 1. (created by study team member Stephen Hilton). Sewar catchment basin areas for the nine influent lines: Jonesboro, Flint, Intrenchment, South Fulton, Old Winn Dixie, Phillip Lee, Nancy Creek, Proctor Creek, and Peachtree Creek. Orange dots represent the location of the WRCs.*

*Exclusion of Influent Lines: South Fulton & Old Winn Dixie*

The South Fulton influent line was excluded from this study's analyses due to physiochemical characteristics that prevented the concentration methods from working efficiently due to abnormal pH values in comparison to the other influent lines (data not shown). The Old Winn Dixie line was excluded due to the limited manhole location data contained within the ArcGIS igraph file that was used to delineating the proper geographic catchment basin area.

## Sample Processing

*Electronegative Membrane Filtration Concentration and RNA Extraction*

One liter grab samples dating from September 14$^{th}$ to November 1$^{st}$, 2021 were concentrated using an electronegative membrane filtration (MF) method. Prior to analysis, samples were stored at 4°C until

processing could be conducted on their respective collection day. A 300mL aliquot of each sample was measured and then centrifuged at 5000 rpm for five minutes to remove solids (Thermo Scientific SORVALL RC 6+). The remaining volume of sample was placed back into 4°C storage for further analysis using other concentration methods and to serve as emergency backup. Following initial centrifugation, samples were poured into their individually labeled Erlenmeyer flasks that contained 0.71g $\pm$ 0.02g of powdered $MgCl_2$ to create a final concentration of 25mM. Thorough mixing to ensure suspension of the powdered $MgCl_2$ was done before pH balancing the samples to 3.5 $\pm$ 0.1. This was done by adding 5% HCl solution or 5% NaOH solution and testing with a pH probe (Fisher Scientific. Fisherbrand accumet AB15 plus. pH meter CAT#13-620-631). A whole process control of Bovine Respiratory Syncytial Virus (BRSV) at a concentration of $10^4$ gc/mL was added after the addition of the $MgCl_2$. Finally, prior to vacuum filtration, each sample was placed back into 4°C storage for thirty minutes to allow for ample interaction time of the $MgCl_2$ with the sample solids and any potential viral components.

After incubation, 150mL of sample was passed through a sterile 0.45µm pore size electronegative membrane filter paper (Millipore. CAT#HAWP04700) that was on a membrane filtration cup (Fisher Scientific. CAT#01-812-55) using vacuum filtration. The remaining 150mL of sample was kept at 4°C as backup in case of contamination, spillage, or any other unforeseen errors during the concentrating step of sample processing. If a reduction in sample flowthrough was observed then the remaining sample volume was pipetted off and returned to the excess sample sitting at 4°C, and a secondary filter was placed onto the membrane filtration cup. The same volume of sample was then pipetted back, and filtration was continued. If the volume of sample was within 10% of the total volume added, 15mL or less, then a secondary filter was not added. Barring any additional blockages or issues with sample flowthrough, the filter(s) was then placed into a 2mL collection tube and extracted using the Qiagen RNeasy mini extraction kit (CAT# 74106).

Immediately following MF, 800µL of Buffer RLT from the Qiagen RNeasy Mini Kit was added to sample tubes containing their respective membrane filters and placed onto a benchtop shaker for ten minutes. The samples were then centrifuged at maximum speed before proceeding with the protocol as

specified by the manufacturer. An RNA extraction control was created using a blank 2mL collection tube and 800µL of Buffer RLT and was extracted under the same procedure as samples. A 100µL of RNA was obtained for each extracted sample and were stored at -20°C until RT-qPCR could be performed.

*Nanotrap® particles KingFisher Concentration & MagMAX Kingfisher Extraction*

The Ceres Nanosciences Nanotrap Wastewater Protocol was used to concentrate samples and extraction done using MagMAX kits for the WRC influent line grab samples from the first to final collection dates, September 14th, 2021 to February 21st, 2022. The study herein utilized Ceres Nanoscience Nanotrap® Magnetic Virus Particles and Nanotrap Enhancement Reagent 1 (ER1). A 10mL sample aliquot was analyzed using this methodology, and aliquots were obtained directly from the collection bottle(s) unless the collected sample was extremely turbid in which case they were centrifuged before concentration. A five-minute 5000rpm cycle was used for grab samples requiring centrifugation and aliquots of the supernatant were analyzed. A two-step sample process is completed using this protocol in which the first step is concentration of viral particles followed by an RNA extraction step. All steps were automated using a Kingfisher Apex system.

The 10mL sample aliquot was split among two 24-well samples plates, and a $10^4$ gc/mL BRSV whole process control was added to one of the two sample plates excluding the control. Microbiological grade water (Corning Catalog #46-000-CM) served as the negative control for the entirety of the analysis. Each sample and control well received 50µL of ER1 (Ceres Nano Catalog #10111-10) for improved SARS-CoV-2 RNA detection. After addition of ER1, 75 µL of Nanotrap® particles (Ceres Nano Catalog #44202) was added. Lastly, a lysis plate was created using the MagMAX Microbiome Lysis Solution (Thermo Fisher Catalog #A42361), and the concentration of each sample was handled through automation in a Kingfisher Apex system. The Kingfisher Apex system utilizes magnetic components to move the Nanotrap® particles through the steps of binding, washing, and elution[91]. After concentration, a 500µL lysate of each sample including controls was then extracted using the MagMAX Kingfisher procedure. The extraction procedure consists of four steps: wash 1, binding, wash 2, and elution. The two wash steps consist of 1mL of MagMAX

wash buffer for the first, and 80% ethanol for the secondary wash step. A binding plate was created using 400µL of the lysate from initial concentration, 10µL proteinase K, 530µL MagMAX binding solution, and the addition of 20µL MagMAX DNA/RNA binding beads. The elution plate contained only 60µL of MagMAX elution buffer. An automated extraction of each sample and control was handled by the Kingfisher Apex System and the system produced roughly 60µL of purified viral RNA for RT-qPCR analysis that was stored at -20°C if RT-qPCR could not be run on the day.

*Quantitative Real-time RT-PCR Analysis*

The presence and quantity of SARS-CoV-2 RNA and the control BRSV RNA were determined using real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR) using the $N_1$ nucleocapsid primer and probe developed by the Centers for Disease Control[92] and BRSV primer developed by Boxus et al[93]. A 20µL volume reaction was used for all amplification reactions in one step on a BioRad CFX machine with the following thermocycling conditions: reverse transcription at 50°C for 15 minutes with a two-minute initiation step at 25°C, then 95°C for two minutes for PCR heat activation, and 45 cycles at 95°C for three seconds to denature and 55°C for thirty seconds to anneal (Table 3). The $N_1$ SARS-CoV-2 assay consisted of 5µL TaqPath, 1.5µL of $N_1$ primer and probe mix shown in Table 4, 5µL of extracted RNA sample, and 8.5µL of molecular grade $H_2O$. The final concentration expressed within the $N_1$ wells was 300nM and 150nM for primers and probes respectively. A similar assay for BRSV was used with 5µL TaqPath, 2µL of B primer and probe mix shown in Table 5, 5µL of extracted RNA sample, and 8µL of molecular grade $H_2O$. The final BRSV primer and probe concentrations per well were 400nM and 200nM respectively. An inactivated SARS-CoV-2 RNA template (ATCC, Manassas, VA) was used to create ten-fold serial dilutions for a standard curve from $2\times10^5$ to 20 gc/µL for the $N_1$ assay, however, no standard curve was designed for the BRSV whole process control as this was a quality control indicator. All samples including no template controls, positive controls, and standards were run in duplicates. A duplex RT-qPCR protocol was created and utilized for all samples following December 1$^{st}$, 2021 in which both $N_1$ and the

BRSV assay were combined and run under the same thermocycler conditions as the single-plex protocol. Prior to analysis, RNA samples retrieved from -20°C were thawed, vortexed briefly, and subsequently centrifuged to ensure homogeneity. Sample cycle threshold ($C_T$) values were recorded and subjected to a quality control and quality assurance (QA/QC) check to determine if a secondary RT-qPCR run was necessary before final storage at -70°C.

*Table 1. Biorad CFX Single-Plex & Du-Plex RT-qPCR Thermocycler Conditions for $N_1$ and B primers*

| Step | Time | Temperature (°C) | Cycles |
|---|---|---|---|
| Reverse Transcription | 2 min | 25 | 1 |
| | 15 min | 50 | 1 |
| PCR initial heat activation | 2 min | 95 | 1 |
| Denaturation | 3 seconds | 95 | 45 |
| Annealing/Extension | 30 seconds | 55 | |

*Table 2. RT-qPCR $N_1$ Master Mix: Initial Concentration and Concentration per Well*

| $N_1$ Assay Mix | N (µL / well) | Initial Concentration | Final Concentration / Well |
|---|---|---|---|
| Molecular Biology Grade $H_2O$ | 8.5 µL | - | - |
| 4X TaqPath Master Mix | 5.0 µL | 4X | 1X |
| IDT $N_1$ Primer and Probe Mix | 1.5 µL | 13.3X | 1X |
| Primers | - | 4 µM | 300 nM |
| Probe | - | 2 µM | 150 nM |
| Template RNA | 5 µL | - | - |
| Total Volume | 20 µL | - | - |

*Table 3. RT-qPCRR B Master Mix: Initial Concentration and Concentration per Well*

| BRSV Assay Mix | B (µL / well) | Initial Concentration | Final Concentration / Well |
|---|---|---|---|
| Molecular Biology Grade $H_2O$ | 8.0 µL | - | - |
| 4X TaqPath Master Mix | 5.0 µL | 4X | 1X |
| BRSV Primer and Probe Mix | 2.0 µL | 10X | 1X |
| Primers | - | 4 µM | 400 nM |
| Probe | - | 2 µM | 200 nM |
| Template RNA | 5 µL | - | - |
| Total Volume | 20 µL | - | - |

*Limit of Detection for RT-qPCR*

A master standard curve was created using the combined data from all standard curves run for all RT-qPCR analyses to determine the SARS-CoV-2 RNA concentration at a given $C_T$ value and then dimensional analysis was used to calculate the $N_1$ gene copies per volume. The limit of detection (LOD) for each assay, single and duplex, was determined by combining the standard curves for all plates and finding the concentration at which 95% showed a detectable result. The LOD for single plex assay was found to be 20 gc/µL following aggregation of all data regarding the standard curve in which 95% of the standard curve 20gc/µL wells were detected. A switch was made to a duplex protocol that combined both the $N_1$ and BSRV assays, and the LOD was found to be 10 gc/µL using the same calculation methods as single plex.

*Data Quality Assurance / Quality Check*

An Rstudio script was created by the study team to perform quality assurance and quality checks (QA/QC) on all RT-qPCR $C_T$ sample values. A QA/QC decision making tree is shown in Figure 2, and if QA/QC for a sample was flagged then the RNA for that sample was rerun through RT-qPCR. In short, $C_T$ values had to be within a two $C_T$ difference of each other for a sample's RT-qPCR result to be considered valid. If not, then the sample was rerun for a secondary RT-qPCR result. For samples that had both $C_T$ values under 36 then they were quantifiable positives, and for samples with duplicates above 36 then they were considered non-quantifiable positives. Negative samples were those that showed one positive duplicate greater than 36 and the other showing no $C_T$ value due to the limit of detection, or when both duplicates did not display a $C_T$ value. Any rerun samples with $C_T$ values that had values with a difference greater than two had their average taken and reported as such. Lastly, all samples' BRSV duplicates had to report a $C_T$ value or the sample was rerun through RT-qPCR.

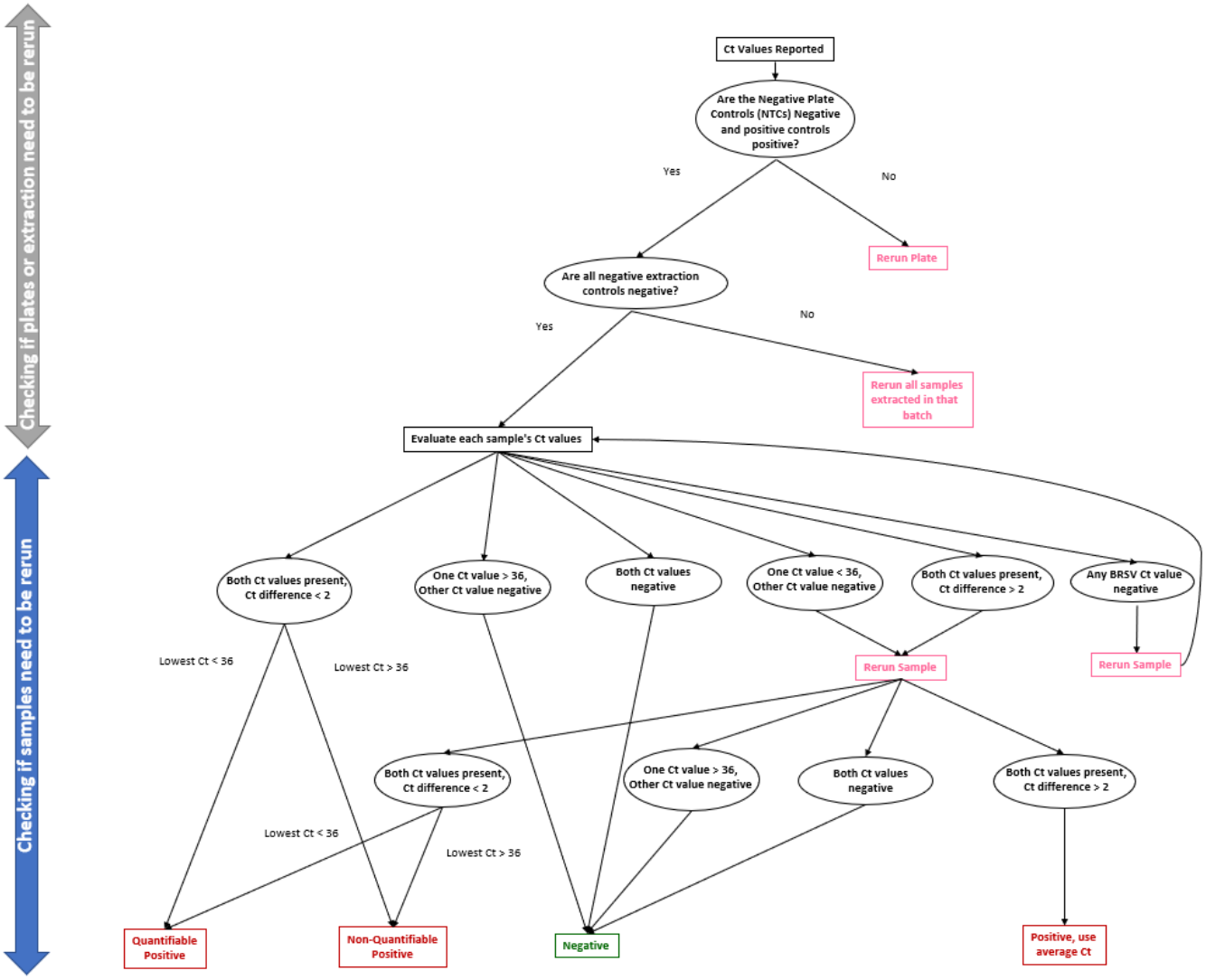


*Figure 2 (created by study member Stephen Hilton). Quality Assurance and Quality Check Decision Making Tree for RT-qPCR $C_T$ results for samples, no template controls, standards, and positive controls*

Clinical COVID-19 Case Data

Reported PCR positive clinical COVID-19 cases were compiled into a dataset from the Georgia Department of Public Health (GDPH) for each person under investigation (PUI). Each PUI that had a recorded address was geocoded to the catchment basin that best fit with their latitude and longitude. Furthermore, the date of symptom onset, date of symptom resolution, and positive PCR test date were used to filter PUIs for the specific time period of sampling from September 6$^{th}$, 2021 to the furthest available date of PUI information, January 31$^{st}$, 2022. In addition to this filtering, a seven-day rolling average of PUIs

was created using the positive PCR test date given by the GDPH for statistical analyses versus the SARS-CoV-2 RNA concentration in the weekly wastewater samples collected based on these dates.

Statistical Analysis

All statistics were computed using RStudio (version 1.3.1093). Prior to the statistical tests, the gene copies per 100mL (cp/100mL) and the $\log_{10}$ cp/100mL was tested for normality using the Shapiro-Wilks test. The nonparametric Kendall's tau correlation and linear regression models were then used to test the various null hypotheses: (1) that the distribution were the same between the SARS-CoV-2 RNA concentration in wastewater that was concentrated by Nanotrap® particles and COVID-19 incidence for each sewershed and when all data was aggregated; (2) that there was no significant correlation between wastewater SARS-CoV-2 RNA concentration using MF and the COVID-19 incidence for four of the influent lines; (3) there was not a statistical significant correlation between the reported SARS-CoV-2 RNA cp/100mL found by Nanotrap® particles versus MF. The $\log_{10}$ of the 7-day rolling average and the daily SARS-CoV-2 RNA cp/100mL were used to compute these statistical tests. A Wilcoxon signed rank test was also performed to determine whether the measurements of cp/100mL between Nanotrap® particles and MF were statistically significantly different.

## Results

*Sampling & Testing for the study period*

Over the course of the sampling timeframe from 9/14/2021 to 2/21/2022, samples were successfully collected at all three influent lines for both Utoy and South River WRC except on two dates, 11/22/2021 and 12/29/2021. Sample collection on those dates was excluded due to the Thanksgiving and Christmas holidays, respectively. For the influent lines at RM Clayton WRC, collection did not start until 11/15/2021, and no samples were collected on the same dates as the other WRCs. In total, 22 samples for each influent line were collected from Utoy and South River WRC and twelve samples from the influent lines of RM Clayton WRC.

On 10/08/2021, logistical and human power constraints within the laboratory prevented analysis of that collection date's samples using MF, and were only concentrated using the Nanotrap® particles. On 2/07/2022 and 2/14/2022, collection from the Intrenchment influent line was prevented by construction. All samples for the entirety of the time period were concentrated using Nanotrap® particles with no known missed samples. Quality assurance and quality control was conducted following RT-qPCR for all concentrated and extracted RNA samples to ensure no contamination was present among PCR negative controls. Concentration and extraction of each grab samples was completed within eight hours of collection.

*Testing for Normality of Wastewater Data*

A Shapiro Wilks test was conducted to assess the normality of the wastewater data prior to using the nonparametric Kendall's tau to assess the correlation of data. It was found that the wastewater data was not normally distributed for cp/100mL ($p < 1\times10^{-15}$), but was normally log distributed ($p\text{-value} = 0.052$, $p > 0.05$). The non-normal distribution provides the rationale for using nonparametric Kendall's tau as the statistical test is primarily used for data that is not normally distributed.

*COVID-19 Incidence & Concentration of SARS-CoV-2 RNA using Nanotrap® particles*

There was a total of 27,111 PUIs in total for all sewersheds. From here on, "COVID-19 incidence" refers to the seven-day moving average of PUI reported in the sewershed. A steep increase in the incidence of COVID-19 was seen at the tail end of November and extended past January for all influent line areas compared to the incidence in September and October. The smoothed trendline of cp/100mL showed similar increases as the incidence increased for all sewersheds as well (Figure 3).

Out of all 150 total samples analyzed by Nanotrap® particles or MF, 82% (123) wastewater concentrate samples were quantifiable positives, 11.3% (17) were non-quantifiable positives, and 6.67% (10) were below the LOD for SARS-CoV-2 RNA. For Nanotrap® particles, 122 samples were analyzed, 83.6% (102) were quantifiable positives, 9.8% (12) were non-quantifiable positives, and 6.5% (8) were below the LOD. The total number of samples analyzed by MF was 28 with 75% (21) as quantifiable

positives, 17.8% (5) as non-quantifiable, and 7.1% (2) below the LOD. The concentration of SARS-CoV-2 RNA in the samples ranged from below the LOD to $1.22\times10^5$ cp/100mL with a median of $3.33\times10^3$ cp/100mL. The highest reported cp/100mL was seen at Proctor Creek on 12/20/2021. Higher cp/100mL were seen in samples preceding and during the peaks of COVID-19 incidence at the end of November through the middle of January (Figure 3). The range of COVID-19 incidence was 0 – 259 with a median of 6.71, and peak rates were seen in the first week of January of 2022 (Table 6).

*Table 6. Ranges for each influent line's concentration of SARS-CoV-2 RNA in wastewater samples along with the COVID-19 incidence within the respective catchment basin for the entire study time period. ND: non-detect for RT-qPCR for quantification of gene copies per 100mL (cp/100mL). COVID-19 Incidence depicted as average number of reported cases per 7 days*

| Influent Line | Range of cp/100mL | Range of COVID-19 Incidence |
|---|---|---|
| All combined | ND – $1.22\times10^5$ | 0 – 259 |
| Flint | ND – $4.98\times10^4$ | 1.29 – 47.0 |
| Jonesboro | ND – $2.10\times10^4$ | 0 – 8.00 |
| Intrenchment | ND – $3.01\times10^4$ | 2.86 – 122 |
| Phillip Lee | ND – $8.06\times10^4$ | 1 – 67 |
| Nancy Creek | ND – $7.44\times10^4$ | 2.14 – 57.1 |
| Peachtree Creek | ND – $3.42\times10^4$ | 12.9 – 259 |
| Proctor Creek | ND – $1.22\times10^5$ | 2.57 – 82.6 |

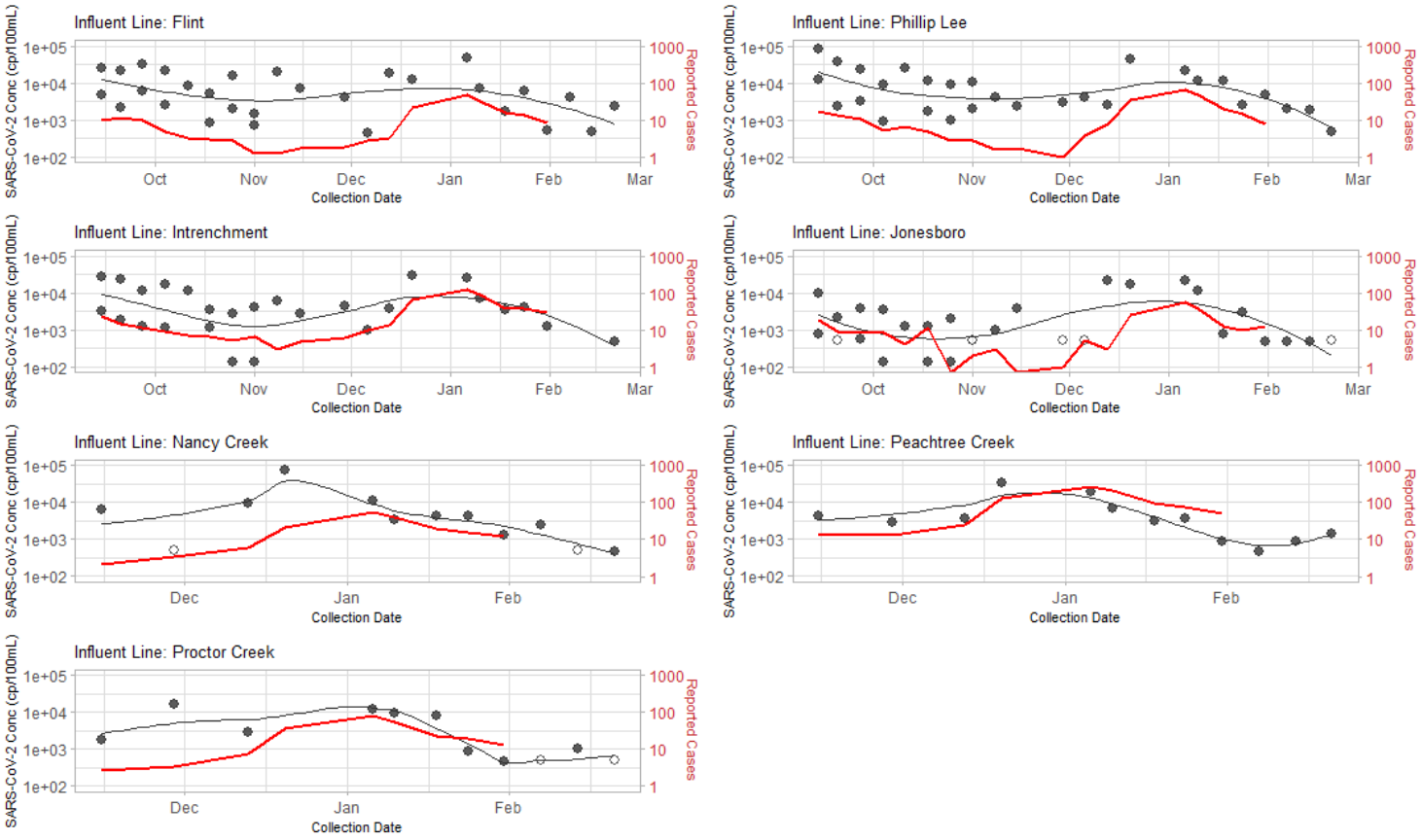


*Figure 3. SARS-CoV-2 RNA Concentration per 100mL found using Nanotrap® particles primary concentration on each collection date for each influent line. Starting in the top left is Figure 3A: Flint, 3B: Phillip Lee, 3C: Intrenchment, 3D: Jonesboro, 3E: Nancy Creek, 3F: Peachtree Creek, 3G: Proctor Creek. Shown in red is the incidence of COVID-19 for the specific catchment zone. Shown in black is the loess y~x smoothed cp/100mL trendline. ●: quantifiable positive RT-qPCR result ○: non-detect RT-qPCR result*

*Association of COVID-19 Incidence & SARS-CoV-2 RNA concentration in Wastewater Using Nanotrap® particles*

The three highest incidences of COVID-19 were reported in the Peachtree Creek catchment basin, and the highest cp/100mL wastewater samples were found to be in Nancy Creek, Proctor Creek, and Phillip Lee. Jonesboro had the lowest quantifiable cp/100mL and incidence of COVID-19 of all catchment basins (Figure 4).

There was a statistically significant positive association ($p < 0.001$) using Kendall's tau (tau = 0.26) among the cp/100mL for samples concentrated using Nanotrap® particles and the COVID-19 incidence when all influent line data was aggregated. However, when each influent line was analyzed separately, only Phillip Lee had statistically significant association ($p < 0.05$) with a (tau = 0.42) between COVID-19 incidence and SARS-CoV-2 RNA cp/100mL of wastewater samples (Table 7).

When all influent line data was aggregated, there was a significant linear relationship between the $\log_{10}$ wastewater values and $\log_{10}$ COVID-19 incidence ($p < 0.001$). The linear relationship between the two suggests that 1-log increase in SARS-CoV-2 RNA concentration is associated with a 0.56-log increase in the incidence of COVID-19. When each individual influent line was analyzed, the only significant linear relationship was seen in Phillip Lee ($p < 0.05$) with a 0.63-log increase in incidence for every 1-log increase in SARS-CoV-2 RNA concentration within the wastewater.

*Table 7. Value of Kendall's Tau & associated p-value for correlation analysis of aggregated data from all influent lines along with each individual influent line's respective values.*

| Influent Line | Kendall's Tau | p-value |
|---|---|---|
| All Combined | 0.26 | 0.00010 |
| Flint | 0.23 | 0.17 |
| Intrenchment | 0.30 | 0.07 |
| Jonesboro | 0.25 | 0.14 |
| Phillip Lee | 0.42 | 0.012 |
| Nancy Creek | 0.29 | 0.30 |
| Peachtree Creek | 0.39 | 0.14 |
| Proctor Creek | 0.33 | 0.21 |

*Table 7. Linear regression coefficients and associated p-value found using a linear model function of COVID-19 incidence versus cp/100mL reported by Nanotrap® particles concentration with no bootstrapping. Shown is the aggregated data of all influent lines along with each individual line.*

| Influent Line | Intercept | Slope | $R^2$ | p-value |
|---|---|---|---|---|
| All combined | -1.3 | 0.56 | 0.89 | 0.00062 |
| Flint | -0.012 | 0.20 | 0.062 | 0.30 |
| Intrenchment | -0.22 | 0.36 | 0.12 | 0.14 |
| Jonesboro | -1.7 | 0.39 | 0.027 | 0.50 |
| Phillip Lee | -1.6 | 0.63 | 0.32 | 0.011 |
| Nancy Creek | 0.067 | 0.28 | 0.12 | 0.35 |
| Peachtree Creek | -0.31 | 0.57 | 0.30 | 0.13 |
| Proctor Creek | 0.27 | 0.25 | 0.12 | 0.36 |

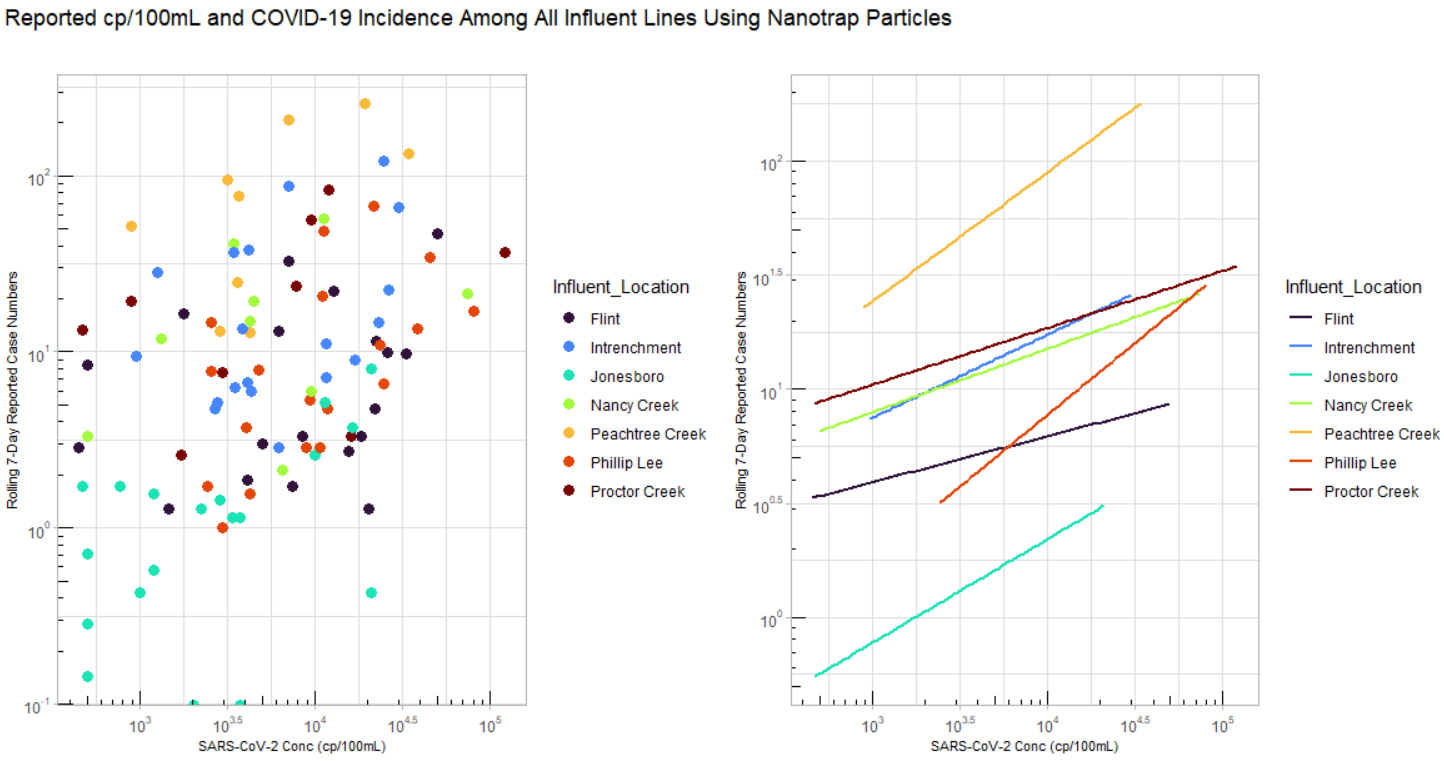


*Figure 4. Aggregated data from all available Nanotrap® particle concentration results for all influent lines and the associated COVID-19 incidence for the wastewater sample. From left to right: Only the datapoints for each influent line by color & linear regression lines without datapoints shown.*

*Association of COVID-19 Incidence & SARS-CoV-2 RNA in Wastewater using MF Concentration*

Four influent lines: Jonesboro, Intrenchment, Flint, and Phillip Lee were used to assess the association between COVID-19 incidence and the cp/100mL of SARS-CoV-2 RNA in wastewater following MF concentration. Intrenchment had the highest reported incidence for the time period of September 14th, 2021 to November 1st, 2021, but a wastewater sample from Phillip Lee had the highest quantified SARS-CoV-2 RNA in wastewater following MF concentration at $1.3 \times 10^4$ cp/100mL (Figure 5).

When all data was aggregated for the four influent lines, there was a significant association between the wastewater results and COVID-19 incidence ($p < 0.001$, tau = 0.52). However, when analyzed separately, the association was only significant at Intrenchment ($p < 0.01$, tau = 0.88) (Table 8).

Analysis of the linear relationship of MF concentrated wastewater sample SARS-CoV-2 RNA concentration and COVID-19 incidence showed that overall, a 1-log increase in SARS-COV-2 RNA concentration resulted in a 0.47-log increase in COVID-19 incidence ($p < 0.001$). Two of the four influent lines, Flint and Intrenchment, when analyzed separately were found to have positive linear associations that were significant ($p < 0.05$) (Table 9).

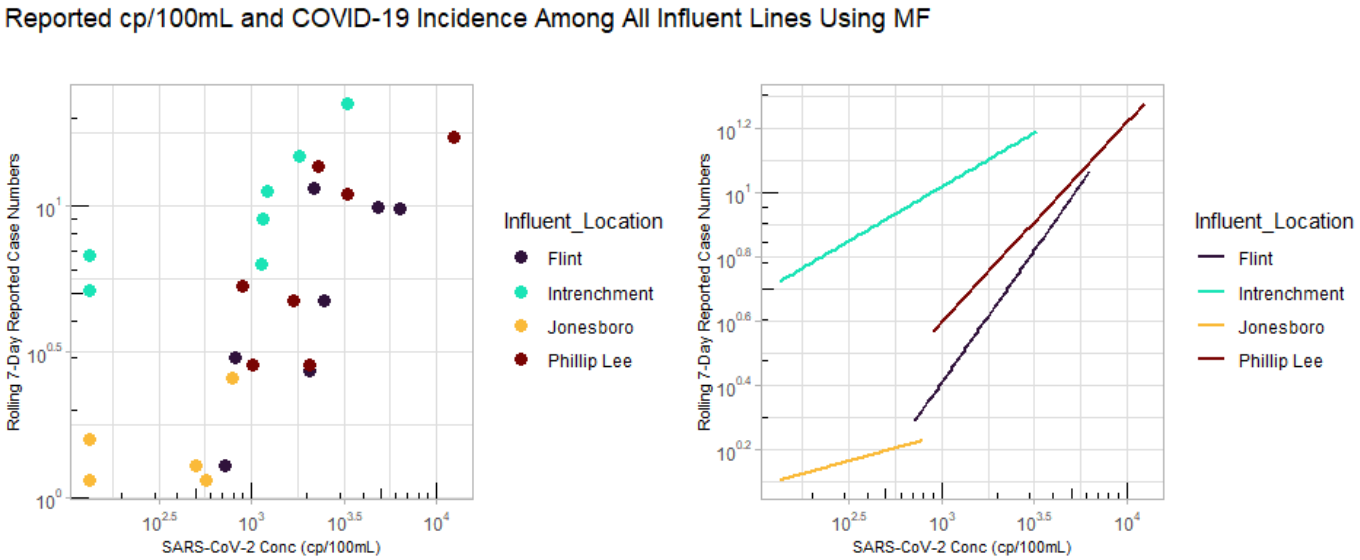


*Figure 5. Aggregated data from all available MF concentration results for four of the influent lines and the associated COVID-19 incidence for the wastewater sample. From left to right: Only the datapoints for each influent line by color & linear regression lines without datapoints shown.*

*Table 8. Value of Kendall's Tau & associated p-value for correlation analysis of aggregated data from the four influent lines along with each individual influent line's respective values.*

| Influent Line | Kendall's Tau | p-value |
|---|---|---|
| All Combined | 0.52 | 0.00026 |
| Flint | 0.52 | 0.1 |
| Intrenchment | 0.88 | 0.0060 |
| Jonesboro | 0.22 | 0.60 |
| Phillip Lee | 0.49 | 0.13 |

*Table 9. Linear regression coefficients and associated p-value found using a loess function of COVID-19 incidence versus cp/100mL reported by MF concentration with no bootstrapping. Shown is the aggregated data of all influent lines along with each individual line.*

| Influent Line | Intercept | Slope | $R^2$ | p-value |
|---|---|---|---|---|
| All combined | -0.74 | 0.47 | 0.31 | 0.00072 |
| Flint | -2.0 | 0.82 | 0.67 | 0.025 |
| Intrenchment | 0.0079 | 0.34 | 0.66 | 0.026 |
| Jonesboro | -0.24 | 016 | 0.16 | 0.16 |
| Phillip Lee | -1.3 | 0.62 | 0.55 | 0.057 |

*Comparison of Membrane Filtration versus Nanotrap® particles Concentration Methods*

Both MF and Nanotrap® particles were used to concentrate wastewater samples for RT-qPCR analysis and quantification of SARS-CoV-2 RNA from 9/14/2021 – 11/01/2021 for influent lines: Flint, Phillip Lee, Intrenchment, and Jonesboro at the Utoy and South River WRCs. A Wilcoxon signed rank test indicated that the distribution of SARS-CoV-2 RNA concentration reported using Nanotrap® particles was statistically different than that of MF ($p < 1\times10^{-6}$). The Nanotrap® particles method resulted in a higher average concentration, $1.0\times10^{4}$ cp/100mL, for all four influent lines compared to MF average concentration of $1.1\times10^{3}$ cp/100mL, and did not have any RT-qPCR results below the LOD (Figure 6).

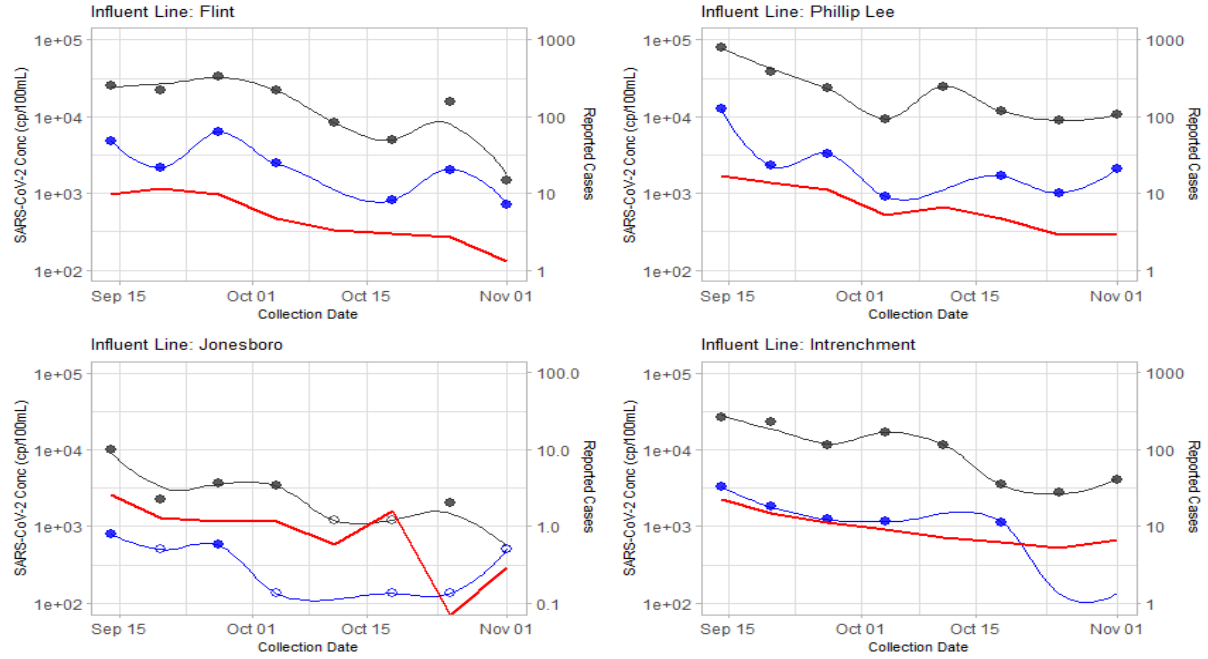


*Figure 6. SARS-CoV-2 RNA Concentration per 100mL reported by both methods for 9/14/2021 to 11/01/2021 for Flint (6A), Phillip Lee (6B), Jonesboro (6C), Intrenchment (6D). Shown in black are reported cp/100mL found using Nanotrap® particles. Shown in blue are reported cp/100mL using MF. Shown in red is the incidence of COVID-19 for the specific catchment zone during that timeframe. ●: quantifiable positive RT-qPCR result ○: non-quantifiable RT-qPCR result*

Association between the reported concentrations using both methods showed a significant correlation (tau = 0.75) between the cp/100mL of Nanotrap® particles and cp/100mL of MF ($p < 1\times10^{-7}$) when all available data was aggregated for the comparison time period. The Flint influent line showed a

significant correlation ($p < 0.01$, tau = 0.91), as well as the influent lines of Intrenchment (tau = 0.78) and Phillip Lee (tau = 0.71) both showing significant correlation ($p < 0.05$). Jonesboro was the only influent line that did not have a statistically significant association between the reported cp/100mL of both concentration methods (Table 10).

In similar fashion, there was a significant linear relationship between Nanotrap® particles cp/100mL and MF cp/100mL at all influent lines aside from Jonesboro. The overall aggregated linear relationship was shown to be that for every 1-log increase in cp/100mL reported by MF there was a 0.82-log increase in cp/100mL seen in Nanotrap® particle concentrated samples ($p < 1\times10^{-7}$). In addition, the cp/100mL of Nanotrap® particles was seen to be 1.5 logs higher for samples on average when all data is aggregated. Both Flint and Phillip Lee linear relationships were significantly correlated ($p < 0.01$) as well as Intrenchment ($p < 0.05$) (Table 10).

*Table 10. Linear regressions & Kendall's tau statistics for the influent lines: Flint, Jonesboro, Intrenchment, and Phillip Lee. Statistics were computed using the $log_{10}$ cp/100mL reported by MF and Nanotrap® particles for each sample.*

| Influent Location | Intercept | Slope | $R^2$ | p-value | Kendall's Tau | p-value |
|---|---|---|---|---|---|---|
| All combined | 1.5 | 0.82 | 0.67 | $1\times10^{-8}$ | 0.75 | $1\times10^{-8}$ |
| Flint | -0.027 | 1.2 | 0.81 | 0.0060 | 0.90 | 0.0043 |
| Jonesboro | 2.2 | 0.61 | 0.65 | 0.55 | 0.48 | 0.15 |
| Intrenchment | 2.6 | 0.33 | 0.077 | 0.028 | 0.78 | 0.015 |
| Phillip Lee | 1.4 | 0.86 | 0.81 | 0.0058 | 0.71 | 0.024 |

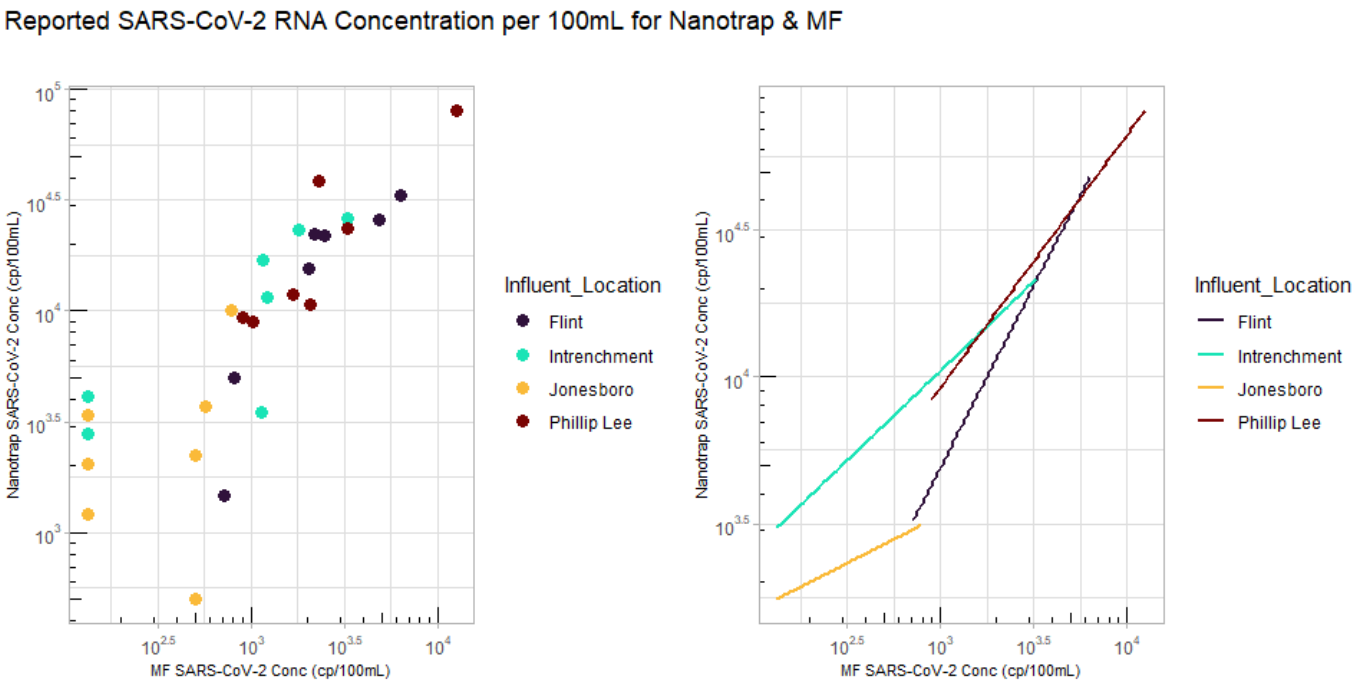


*Figure 7. Report cp/100mL from both concentration methods for the four influent lines: Flint, Intrenchment, Phillip Lee, and Jonesboro. From left to right: Only the datapoints for each influent line by color & Linear regression lines without datapoints shown.*

## Discussion

*The Importance of Wastewater Monitoring & Study Importance*

Wastewater monitoring for infectious disease surveillance has shown to be a reliable and accurate system to provide early warning detection of fluctuations in COVID-19 incidence[27–29,58]. The utilization of wastewater allows for a broader population to be monitored for COVID-19 without the need for individual testing, bypasses the barrier of unequal access to testing sites[13], and captures asymptomatic infections. Methodologies that are reliable, quick, and sensitive for analyzing the concentration of SARS-CoV-2 RNA in wastewater have rapidly expanded since the first systems were in place. In this study, we compared two methods commonly used for concentration of wastewater; samples from influent lines at three POTWs in the metropolitan Atlanta, GA area were concentrated using both a MF with pre-acidication and added $MgCl_2$ approach and a magnetic particle concentration method, the Nanotrap® particles from Ceres

Nanosciences. This study was useful in providing the framework for future work to compare primary concentration methods used in wastewater surveillance of COVID-19.

*Overall Observations for Each Method & Their Relationship to COVID-19 Incidence*

Overall, the Nanotrap® particles method was closely associated with trends in COVID-19 incidence. During the study time period, there were increases in incidence in late November to January among the catchment basins that were also seen in increases of measured SARS-CoV-2 RNA in wastewater across all sewersheds being surveilled. Although fewer results are available from MF, wastewater samples concentrated by both MF and Nanotrap® particles showed similar trendlines to each other as well as the incidence of COVID-19.

Although when data from all influent lines were analyzed together the relationship between Nanotrap® particles and COVID-19 incidence was statistically significant, when analyzed individually there was only a significant association between the concentration of SARS-CoV-2 RNA and the incidence at one influent line. Similar results were seen for the concentration of SARS-CoV-2 RNA when MF was used, in that influent lines together showed a relationship that was only seen in one influent line individually. When separated, only Intrenchment was significantly associated between the RNA concentration and incidence rate. The linear relationships for both methods when their respective data was aggregated showed significant positive associations between the incidence and SARS-CoV-2 RNA concentration in wastewater but again not all influent lines showed these same results when analyzed individually.

These results show that SARS-CoV-2 RNA concentrations in wastewater are significantly associated with COVID-19 incidence and there is a positive linear relationship between these two variables using multiple concentration methods. Results also suggest that sample size may have been insufficient to show this at each individual line. These findings are consistent with other studies that have conducted wastewater surveillance using Nanotrap® particles [28,29,90] and for MF[57,94]. However, significant correlation

was not seen for all influent lines when analyzed separately. The significance between SARS-CoV-2 RNA concentration in wastewater and COVID-19 incidence using both methods provide evidence of their ability to effectively track the relationship.

*Relationship between the MF & Nanotrap® particles Reported Concentrations*

Primary concentration of wastewater samples is one of the most critical steps in accurately quantifying SARS-CoV-2 RNA in wastewater surveillance systems. Variability of results between methods has been commonly seen[64–68]. This study looked to determine what association was present between samples concentrated by MF which is a commonly used primary concentration method in WBE systems, and the novel Nanotrap® particles method. A significant correlation exists between the SARS-CoV-2 RNA concentration found using Nanotrap® particles and for MF. The aggregated data of the four influent lines analyzed shows that as one method results in an increase of SARS-CoV-2 RNA so does the other. On a case-by-case basis, only Jonesboro did not show a significant association between the two concentration methods. The value of Kendall's tau for the other three influent lines were all above 0.7 which can be considered a strong correlation[95]. There was also a significant linear relationship between the two methods at all locations aside from Jonesboro. The results for Jonesboro, and the lack of statistical significance may be due to RT-qPCR inhibition of samples analyzed using MF as potential organic material collected on the filter preventing accurate quantification as two samples were below the LOD compared to one for Nanotrap® particles

The Wilcoxon signed rank test analysis of the cp/100mL shown by Nanotrap® particles compared to MF shows that the distributions were statistically different ($p < 1\times10^{-6}$). This nonparametric analysis was used as the Shapiro-Wilks test showed non-normal distributions for the $\log_{10}$ cp/100mL along with the fact that wastewater data is not normally distributed[27]. Additionally, this validates the linear relationship finding that the concentration of SARS-CoV-2 RNA when using Nanotrap® particles was inherently higher than that of MF, and reported statistically different cp/100mL measurements for wastewater samples. The

intercept, which was the Nanotrap® particles SARS-CoV-2 RNA concentration, of the linear lines of the combined and all influent lines except for Flint were positive.

The higher concentration reported by Nanotrap® particles could provide improved sensitivity of the wastewater surveillance efforts as none of the samples were below the LOD for RT-qPCR compared to two for MF. This is interesting to note as the sample volume used in MF was 15x higher than Nanotrap® particles, but did not yield concentrations as high as Nanotrap® particles, and could be attributed to RT-qPCR inhibition from the organic material collected on the filters altering RNA extraction. The association and linear relationship between both methods is crucial to understand as surveillance efforts are expanded and adaptations to current surveillance system methods are done to determine the optimal concentration method. Associations between the methods is also necessary to understand to support data interpretations for surveillance programs that decide to switch concentration methods. The higher recovery of SARS-CoV-2 RNA in wastewater using Nanotrap® particles may be vital as mitigation efforts are reduced, vaccination rates increase, and the incidence of COVID-19 decreases resulting in lower wastewater concentrations; Nanotrap® particles could prove to give better ascertainment of SARS-CoV-2 RNA in wastewater when other concentration methods cannot.

*Practical Considerations & Experience from Usage of Both Methods*

The usability of both methods for routine laboratory analyses for wastewater monitoring of COVID-19 is important as not all laboratories are the same functionally nor do all have the capacity to conduct multiple protocols. Both methods are comparable, however, as they can be conducted hands-on without steep learning curves. The incubation wait time for both the manual Nanotrap® particle protocol and the MF protocol used are similar at 20 minutes and 30 minutes respectively. However, there are drawbacks to MF as turbidity can play a tremendous role in the time it takes for a sample to be vacuum filtered through a membrane as well as the potential for PCR inhibition. Additionally, both MF Nanotrap® particles require the use of an RNA extraction kit. These kits are not uniform, and have their own wait times along with the necessary training required to properly perform them. For Nanotrap® particles, a major

drawback is the price of equipment such as the magnets needed for separation of the magnetic particles from suspension. Additionally, automation is recommended by Ceres Nanoscience to increase the throughput when using Nanotrap® particles, and the machine that was used for this study was a large financial investment. This cost could dissuade implementation of Nanotrap® particles for other wastewater surveillance systems. However, overall Nanotrap® particle methods are generally quicker, require a smaller sample size alleviating the need for large amounts of storage space, and when done manually can analyze five to ten more samples at a time than MF. In the end, the use of either depends on the circumstances, but as reported in the study, concentration using Nanotrap® particles resulted in higher SARS-CoV-2 RNA concentration than MF.

*Limitations of the Study*

It is known that more frequent sampling of wastewater can support strong associations between COVID-19 incidence and SARS-CoV-2 RNA concentration in wastewater[27]. The lack of statistically significant data for the majority of influent lines and analyses calculated may be due to the limited frequency of sampling or the limited total sample size (as results are significant when pooled across the city). Four of the influent lines were sampled and concentrated using Nanotrap® particles for the entirety of the time period resulting in 22 collected and analyzed samples. The other three influent lines only had twelve weeks of sampling, and resulted in a drastic reduction in sample size for analysis. Even when the Nanotrap® particles data was aggregated, only 132 data points were available, and does not compare to studies assessing correlation using higher frequency sampling[27–29]. On the other hand, the Nanotrap® particles data was much larger than the available MF data which had less than the optimal number of datapoints. The lack of higher frequency in samples was the largest limitation of this study, and future studies should have increased sampling occurrence. An increase to two times a week would be beneficial for future work if daily sampling is not feasible due to logistical constraints. The increased frequency allows for better estimations of COVID-19 incidence as well as reduce the variability of wastewater data that can arise from single weekly samples[96].

While the frequency of sampling was the most limiting factor for the study, the ascertainment of clinical COVID-19 cases was a secondary limitation due to multiple assumptions. The provided dataset from the GDPH was crucial in providing over 27,000 cases that were assumed to be presently shedding viral RNA within the community at their given address. However, the reported clinical cases were only geolocated into the area by their reported address, and does not guarantee that they were presently shedding SARS-CoV-2 RNA within the wastewater for their respective catchment basin. In addition to this assumption, delineation of the catchment basins was manually conducted and the boundaries around the manhole locations could have excluded clinical cases from catchment basins they were contributing to. Furthermore, another assumption was that transplants cases due to work or personal travel in the catchment basins were not actively shedding into the wastewater. Lastly, the barrier of access to clinical testing can be one that disproportionately affects lower socioeconomic areas[13], and this study was conducted in areas thought to be of lower socioeconomic status. Therefore, the clinical data for these areas cannot be considered a gold standard for the incidence of COVID-19, and the reported cases are likely to be a gross underestimate of the true COVID-19 burden in these geographic areas. This along with the assumptions described above could have reduced the number of clinical cases for accurate analysis of the correlation between the COVID-19 incidence and SARS-CoV-2 RNA concentration in this study.

*Future Directions*

Continued wastewater surveillance of all influent lines in this study is being adopted by the GDPH at the treatment plant level. Wastewater sampling frequency should be increased in the future to assess a larger sample size among each influent line for both concentration methods to determine the association between COVID-19 incidence and SARS-CoV-2 RNA concentration. This could be done in a single sewershed in conjunction with increased access to clinical testing by GDPH or in another area with a government public health office. Higher access to clinical testing in the form of more testing locations that is free as well as more complete information regarding their housing location can allow for better case ascertainment for comparison to wastewater data for each sewershed. If increased access to clinical testing

was provided, the frequency of individuals going to get test could increase and provide better reports of the total number of COVID-19 cases. The collaboration would allow for increased ascertainment of clinical cases, and provide an insightful comparison between heavy investment of active individual testing and the use of broad scale monitoring by wastewater surveillance. Additionally, investigations into the presence of VOCs in the sewersheds should be performed to determine whether increases of identified VOCs can be monitored in wastewater as their emergence warrants immediate public health action. The concentration of these VOCs does not require a separate method, and both MF and Nanotrap® particles could be used. Utilization of new forms of PCR such as digital droplet PCR are able to differentiate and quantify specific VOCs RNA if the segments of RNA for the VOCs are known and selected for. Wastewater surveillance could provide a feasible way to track the transmission of these VOCs, and enhance the public health sector's continued response to the ever-changing dynamics of the COVID-19 pandemic throughout the world.

*Key Points from This Study*

The limitations of this study regarding wastewater sampling frequency along with the complexities of COVID-19 case ascertainment could have contributed to reduced statistically significant associations on an individual catchment basin basis. However, there was significant association found for all three study goals when data was aggregated regarding the relationships between SARS-CoV-2 RNA concentration in wastewater samples and COVID-19 incidence for each method respectively along with the association between the two primary concentration methods. This provides evidence that wastewater surveillance using Nanotrap® particles concentration is a reliable and sensitive methodology and Nanotrap® particles should be considered a principal concentration method for SARS-CoV-2 RNA in wastewater. Additionally, the use of MF in this study was during a shortened time period, but the results show that this method was still correlated with increased COVID-19 incidence when the available data was combined. The future of wastewater surveillance is bright, and more work must be done to continue the comparison of concentration methods as well as refining each to improve their recovery of SARS-CoV-2 RNA.

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