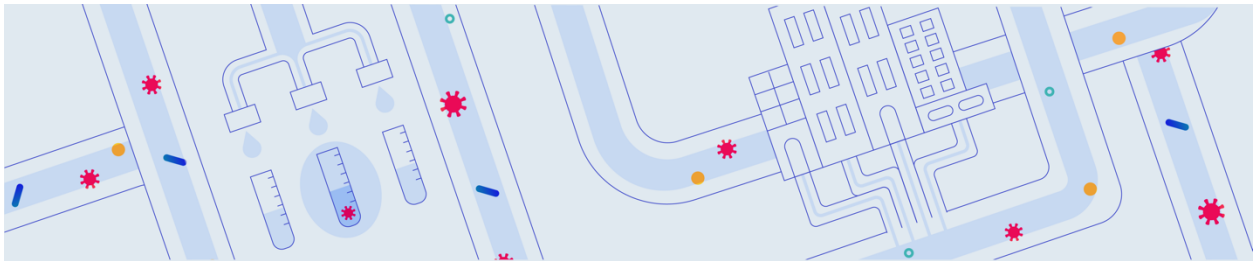

DHS/NIST Workshop: Standards to Support an Enduring Capability in Wastewater Surveillance for Public Health

Poster Abstracts

June 14-18th 2021

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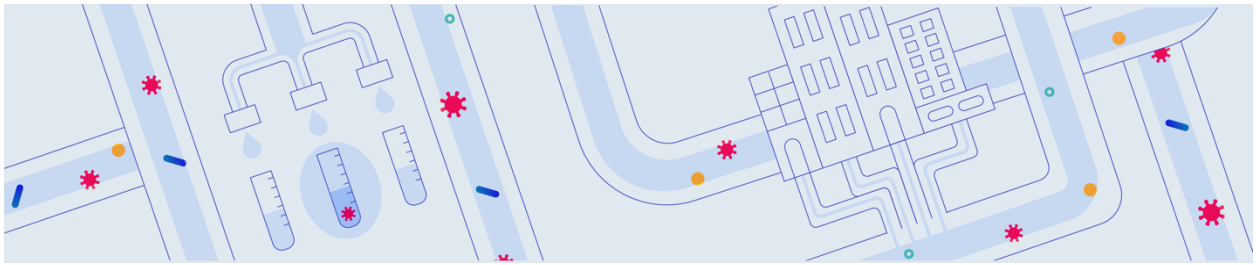

CEDAR-MC: Clinical and Environmental Dynamics of Antibiotic Resistance within Microbial Communities

George Hanna[1,2], Bashir Hamidi[1], Scott Curry[1], Cheryl Carmack[2], Alexander V. Alekseyenko[1]

[1]Medical University of South Carolina; [2]Charleston Waterkeeper

Introduction: Escape into the environment and the persistence of antibiotic resistance is an imminent threat to the healthcare advances attained in the 20th century. Microbial communities that co-exist with resistant bacteria may help uncover novel strategies for global antimicrobial control and curb emergence and maintenance of resistance. However, availability of clinically relevant specimens with complementary samples from the built and natural environment is a major obstacle to effective studies of the dynamics of resistance in the affected human populations and in their surroundings.

Methods: We bring together environmental and clinical measurements of the microbial communities with evidence for emerging resistance by linking existing local clinical and environmental surveillance programs. The clinical specimens are sourced from the Medical University of South Carolina (MUSC) infection surveillance culture program that routinely samples the MUSC patient population for clinically relevant pathogens. The environmental specimens are the result of partnership with a local non-profit, Charleston Waterkeeper, that performs water quality monitoring at popular recreational spots in the area.

Results: The surplus infection surveillance specimens are made available for research via a Living µBiome Bank system that enables nuanced electronic phenotyping of patient populations for just-in-time capture of clinical microbiology specimens. We show the feasibility of sequencing these clinical specimens for microbiota composition, which in combination with resistance status is invaluable in characterizing the broader structure of resistant microbial communities. The environmental specimens collected from May 2018 through October 2020 have been banked to be assessed for evidence of antibiotic resistance of their constituent microbiota.

Discussion: We continue to expand the resources and the biobanks associated with the clinical and environmental surveillance programs with an overarching goal to control infections by understanding the dynamics of emergence and maintenance of resistance. This project demonstrates the value of collaboration between academic healthcare and community-funded environmental monitoring programs.

Key words: antibiotic resistance, living biobank, water quality

Standards and control materials used: *Yes*

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A Step-By-Step Approach to Evaluating SARS-CoV-2 Methods: Adaptability for Future Use with Other Microorganisms

Brienna L. Anderson-Coughlin[1] and Kalmia E. Kniel[1]

[1]Center for Environmental and Wastewater-based Epidemiological Research/Department of Animal and Food Sciences, University of Delaware, Newark, Delaware

There is a breadth of research regarding enteric viruses in agricultural waters and using viruses such as pepper mild mottle virus (PMMoV) as fecal contamination indicators. Traditionally used in food safety research, PMMoV is now being employed for wastewater surveillance efforts during the COVID-19 pandemic. The purpose of this study was to determine how existing wastewater surveillance methods for SARS-CoV-2 could be adapted for other microorganisms, using PMMoV as a model. Wastewater was collected across New Castle County, Delaware, incubated, filtered, and concentrated. Viral concentrates were extracted, and detection performed via RT-qPCR for PMMoV and the N1 and N2 SARS-CoV-2 targets. Variability in recovery along with impacts of incubation (none or 60°C for 60 min) and refrigerated storage (0-hour or 24-hour) were evaluated. Delta cycle threshold (dCT), the number of cycles before completion (CT=40) at which the target amplified across the threshold (e.g., CT38=dCT2).

PMMoV was detected in all (n=48) replicates for incubated and non-incubated samples with 0- and 24-hour holds. SARS-CoV-2 N1 and N2 were detected in 95.8% (n=46) of incubated samples in each treatment of 0 and 24 hour holds, in 79.2% (n=19) of non-incubated with 0-hour hold, and 50% (n=12) of non-incubated with 24-hour hold. PMMoV detection in samples (n=6) was significantly ($p<0.05$) greater than N1 and N2. PMMoV increased by 3.16 ± 1.18 dCT in processed (9.25 ± 1.15 dCT) samples compared to unprocessed (6.09 ± 0.98 dCT). N1 and N2 increased by 0.77 ± 1.38 and 0.63 ± 1.32 dCT in processed (0.95 ± 1.37 and 1.04 ± 1.29 dCT) compared to unprocessed (0.18 ± 0.61 and 0.41 ± 0.90 dCT), respectively.

The detection and recovery efficacy of the method explored here confirmed the potential for its adaptation for use with additional organisms. These data provide evidence that with appropriate modifications, the methods and infrastructure created during the COVID-19 pandemic and currently used for SARS-CoV-2 surveillance, can be successfully utilized for viral, bacterial, and parasitic organisms of interest.

Key words: Wastewater surveillance, Method Adaptation, Viruses

Standards and control materials used: Positive and negative controls for this study consisted of plasmid gene sequences (IDTDNA: 10006625) and nuclease-free water (Qiagen: 129114), respectively.

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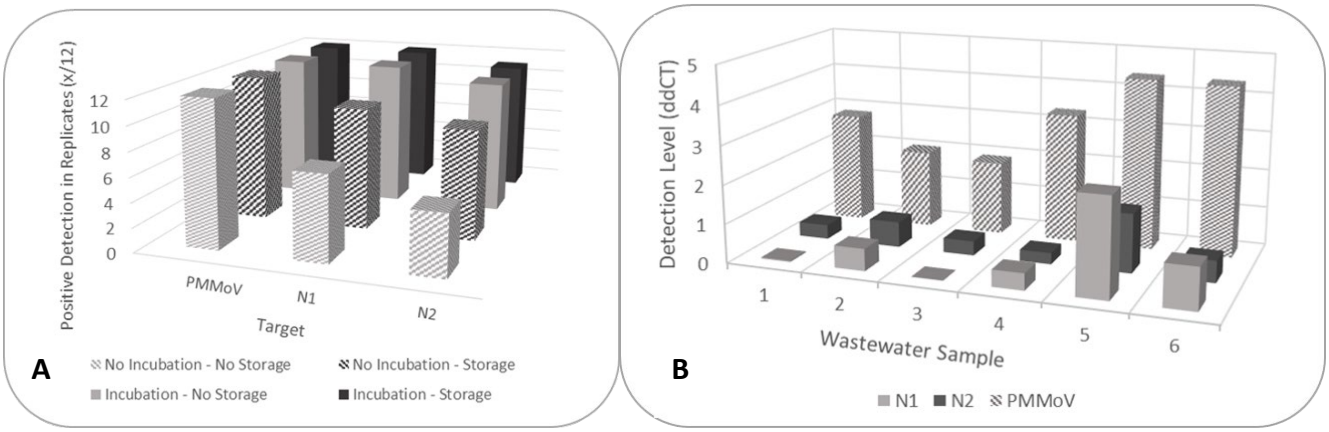

Figure 1. The detection of PMMoV and SARS-CoV-2 (N1 and N2 genes) from wastewater samples. A. The number of replicates (n=12) for each target in which positive detection occurred, treatments evaluated included the incubation (60C for 60 minutes) or no incubation and storage (4C for 24 hours) or no storage prior to completing wastewater filtration, concentration, nucleic acid extraction and detection. B. The detection level, represented by ddCT values (the difference in CT between unprocessed and processed samples), of targets for each of the six samples processed in triplicate. Large variation in recovery was observed across wastewaters and between PMMoV and SARS-CoV-2.

The impact of data on source control

Anne-li Steutel-Maron
Kando

This abstract will elaborate on how we should rethink wastewater for water reuse, and data's role in securing consistently high-quality wastewater. Raw wastewater is a valuable raw material. Having a consistent, stable, and high quality raw material is essential for maximizing its value. For wastewater, controlling the quality of inputs to the collection network is vital to realizing its value as a resource. Optimized 'source control' cannot rely on random sampling, as water-reuse requires high quality water that needs to rely on precise, reliable, and continuous flow of data, giving reuse plants and service providers a total understanding of their water quality.

Key words: waterreuse Key word 1; source control Key word 2; data

Standards and control materials used: **ISO 5667-10:2020**

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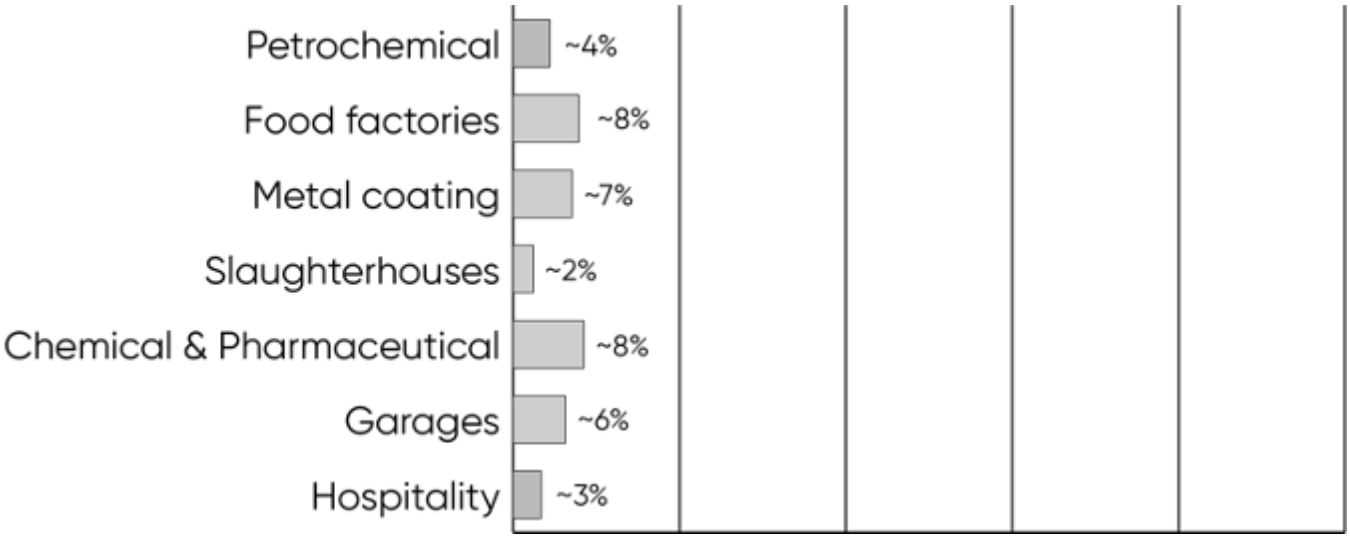

Figure 1. Pollution found with grab sampling

Figure 2. Pollution found with smart monitoring & sampling

Optimizing Sampling Strategies for Large Rural Regions

Anuj Tiwari, Aaron Packman, Charles Williams, Wilnise Jasmin, Rachel Poretsky, Wayne Duffus, Sarah Patrick, Leslie Wise, Charlie Catlett[1]

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Abstract

Wastewater Based Epidemiology (WBE) typically focuses on wastewater treatment plants (WWTPs) and the corresponding community. However, many public health questions require insight across many communities such as at the county or state level. What is the optimal sampling strategy in regions such as rural counties with dozens of treatment plants and private septic systems, or in entire states with many such counties?

The State of Illinois is a primarily rural region comprising 58k square miles with a population of roughly 16M, over half of whom live within 50 miles of Chicago. Outside of the Chicago area, Illinois has roughly 140 cities with populations greater than 10k and nearly 1,000 with populations under 10k. To understand and track infectious disease such as COVID-19 across these communities, a comprehensive WBE sampling plan would involve over 1,000 WWTPs and countless private septic systems.

In Sep 2020, we introduced a machine learning based COVID-19 Vulnerability Index (C19VI) using CDC's six themes: (a) socioeconomic status, (b) household composition & disability, (c) minority status & language, (d) housing type & transportation, (e) epidemiological factors, and (e) healthcare system factors[2]. This model uses an ensemble learning approach with recursive partitioning to optimally compute non-linear relationships between input themes. We refined the model in early 2021 with additional demographic and sequencing information to evaluate WBE sampling strategies across Illinois counties to support an expansion of WBE from currently several dozen to over 150 WWTPs. The vulnerability index supported the evaluation of various sampling strategies, such as selecting the largest population centers within each of the eleven COVID-19 "Restore Illinois" regions[3], and eventually the current strategy of sampling from the largest population center in each of Illinois' 102 counties. In this poster we outline the models and methods used, including various sampling strategies explored, the current strategy, and experiments planned to evaluate the sampling strategy as we expand to 150 WWTPs in 2021.

Keywords: COVID-19; Wastewater Based Epidemiology (WBE); Wastewater Sampling Strategy; COVID-19 Vulnerability Modeling; Machine Learning (ML)

---

[1] Tiwari and Catlett are from the University of Illinois Discovery Partners Institute; Packman is from Northwestern University; Poretsky is from the University of Illinois-Chicago; Jasmin is from the Chicago Department of Public Health; and Williams, Duffus, Patrick, and Wise are from the Illinois Department of Public Health.

[2] Tiwari, Anuj, Arya V. Dadhania, Vijay Avin Balaji Ragunathrao, and Edson RA Oliveira. "Using machine learning to develop a novel COVID-19 Vulnerability Index (C19VI)." *Science of The Total Environment* 773 (2021): 145650.

[3] Restore Illinois. Available at https://www.dph.illinois.gov/restore

Parameters influencing the methods of detection for SARS-CoV-2 RNA wastewater surveillance using RT-PCR quantification

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Abstract

The COVID-19 pandemic presented many challenges to public health units attempting to track infected individuals by performing individual clinical testing of large populations. Due to the lack of trained personnel and shortage of testing materials, many undiagnosed but infected individuals caused substantial viral spread within communities[1]. Thus, it is crucial that alternative surveillance methods are explored, which can provide public health organizations with information on the number of infected individuals. Such methods allow for real-time allocation of resources, staff, and restrictive measures to reduce further spread. Wastewater surveillance of viral RNA has emerged as a surprising approach to track and monitor the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in communities. Considering the novelty of the methods used, the aim of this study was to determine which parameters are suitable in order to store, pasteurize, and concentrate the viral particles found in raw wastewater influent. Six wastewater treatment plants (WWTPs) provided 500mL samples three times a week from different municipalities in the Durham Region, Ontario, Canada. Storage conditions were investigated by storing 30mL raw influent at 4◦C, -20◦C, -80◦C for 7, and 14 days and analyzed using a Three-Way ANOVA ($p<0.005$). Pre-treatment of the samples included pasteurization for 60 mins at 60◦C analyzed using t-tests ($p<0.005$), and concentrated

using polyethylene glycol (PEG) for either 0 or 12 hours and analyzed using a t-test ($p<0.005$). Combined, this study presents recommendations for developing reliable, accurate, sensitive and reproducible estimation of the evolution of the SARS-CoV-2 virus in wastewater.

Keywords: Covid-19, SARS-CoV-2, Wastewater, RT-qPCR, Pasteurization, Viral Concentration

Standards and control materials used: Sars-CoV-2 Standard (EDX), pMMoV gBlock (IDT)

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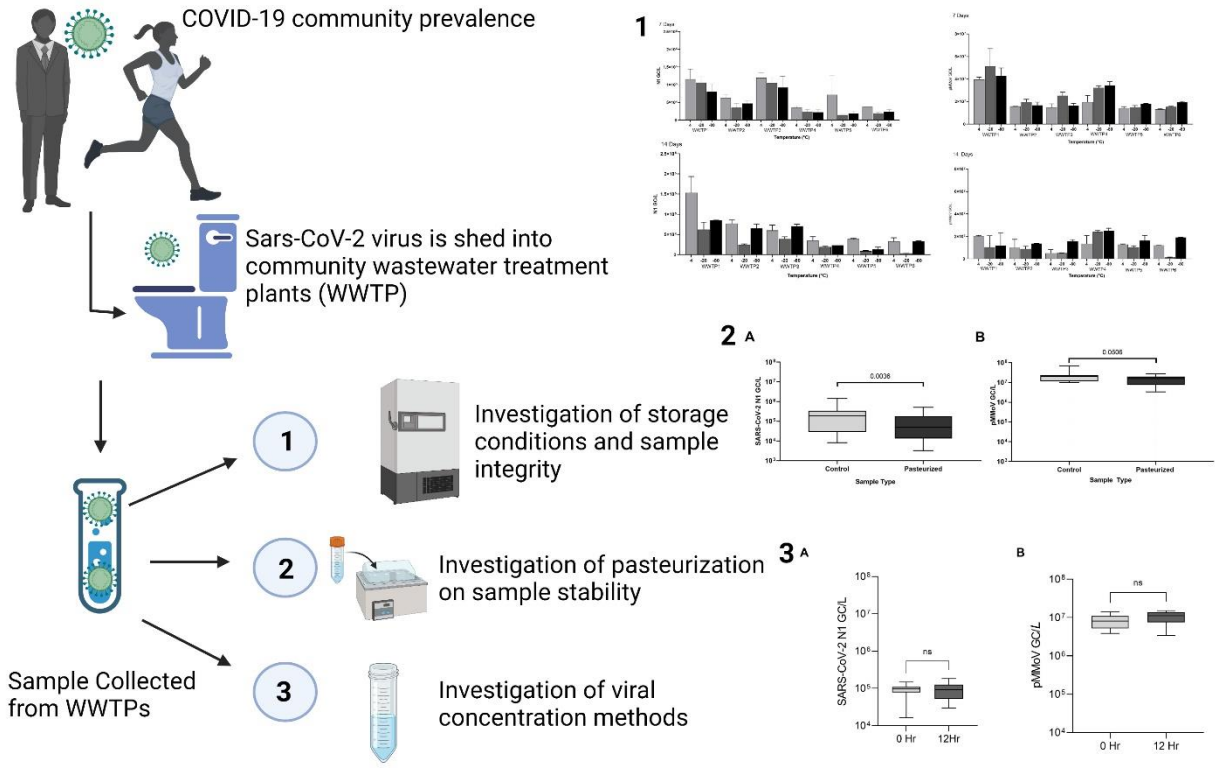

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Metagenomics for Monitoring Antibiotic Resistance in Water and Wastewater: Key Considerations and a Path Towards Standard Protocols

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*Corresponding Author

Environmental dimensions of antibiotic resistance are increasingly being recognized and are essential to a One-Health framework for combatting antibiotic resistance [1]. Strategies are emerging for comprehensive surveillance of resistomes (i.e., all antibiotic resistance determinants carried across a microbial community) in surface water, wastewater, and recycled water matrices to both establish a baseline and to monitor for changes in resistance patterns that occur over time [2]. Metagenomics, i.e., the study of an entire microbial community's genomic information via shotgun next-generation sequencing (NGS), is emerging as a powerful tool for characterizing these aquatic resistomes. Metagenomics is an attractive approach to monitoring because it theoretically allows for the simultaneous detection of all antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), as well as the microbial composition in an environmental matrix without *a priori* knowledge of targets, circumventing the narrow target scopes of both quantitative polymerase chain reaction and culturing. With short- and long-read sequencing technologies, the contextualization of ARGs (e.g., their positions on MGEs and taxonomic affiliation) are also further being realized [3]. However, NGS approaches to water quality monitoring have only been applied in the last decade and guidance is still needed with respect to sampling, DNA extraction, library preparation, sequencing platforms, sequencing depths required for unique matrices and targets, and metrics that are ultimately mined and derived from the raw sequencing data.

Here we conducted a comprehensive, systematic literature review of articles that use NGS to investigate the resistomes of surface water, wastewater, and recycled water. We identified 98 peer-reviewed papers meeting our search criteria and systematically compiled and compared sample processing workflows, sequencing approaches, and bioinformatic analyses to find commonalities in data generation and data reporting. We also performed a robust meta-analysis of raw sequencing data retrieved from each study's accompanied Sequence Read Archive (SRA) Bioproject to extract summary statistics as well as determine the intrinsic sequence diversities using Nonpareil [4]. The findings help inform a framework for standardized metagenomic monitoring of antibiotic resistance in aquatic environments.

Key words: metagenomics, water quality monitoring, antibiotic resistance, standardization

Standards and control materials used: Mock Community [5]

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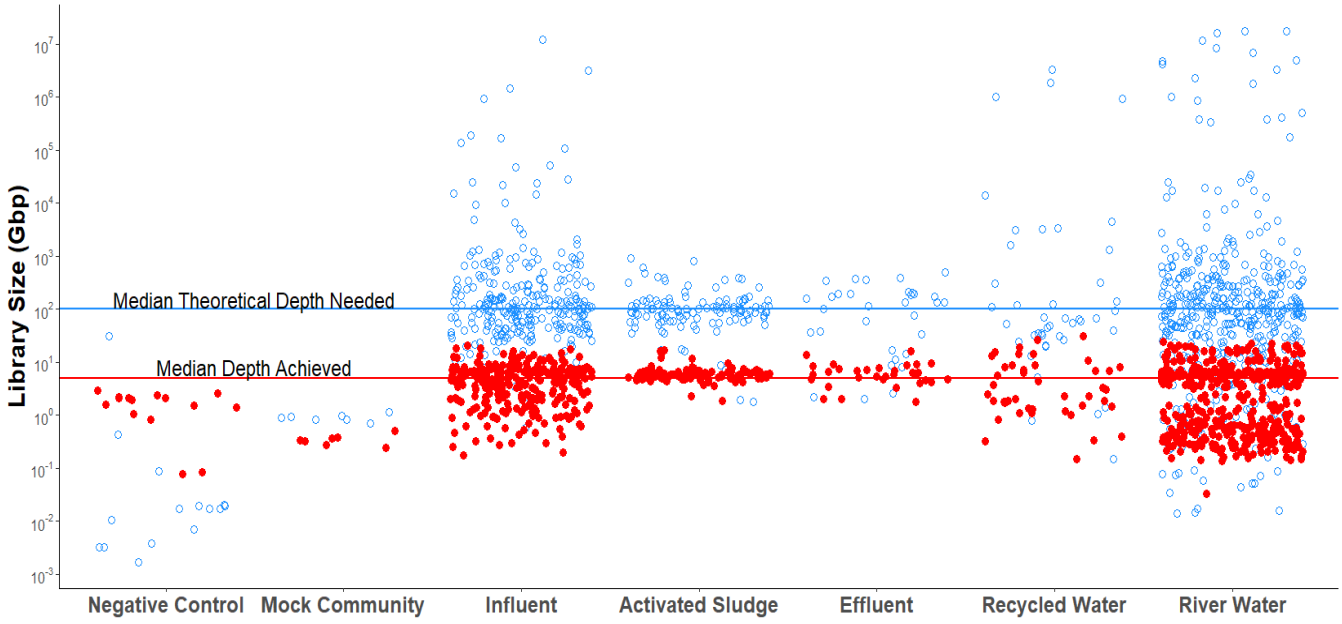

Figure 1. Results of Nonpareil (Rodriguez et al. 2018), an estimator of sequence diversity and depth needed to achieve 100% coverage of metagenomes. Metagenomes (n=946) of diverse water and wastewater matrices were downloaded from the Sequence Read Archive (SRA) from all papers identified in a comprehensive literature review (98 articles). Trends show that an order of magnitude deeper sequencing (measured in giga base pairs (Gbp)) is needed to achieve 100% coverage of the complex microbial communities in many aquatic matrices. Negative Controls = miliq water; Influent = raw sewage entering a wastewater treatment plant; Effluent = treated wastewater.

References:

1. European Commission: *A European One Health Action Plan against Antimicrobial Resistance (AMR)*. 2017.
2. JPIAMR: *Strategic Research and Innovation Agenda on Antimicrobial Resistance*. 2019.
3. Che Y, Xia Y, Liu L, Li AD, Yang Y, Zhang T: **Mobile antibiotic resistome in wastewater treatment plants revealed by Nanopore metagenomic sequencing**. *Microbiome* 2019, **7**:1–13.
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Importance of Validation for Wastewater Surveillance

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Wastewater surveillance has delivered actionable public health data over the course of the COVID-19 pandemic. Because of the urgent nature of the pandemic, the extent of validation for currently used methods varies widely. Many methods were deployed before significant data was gathered on performance, and may not have been widely tested with a diversity of wastewater samples. As wastewater surveillance moves into the mainstream, it is critical that methods be thoroughly validated using real-world wastewater samples, that the validation captures as much natural variation as possible (such as ensuring the validation is performed with samples from many different geographies), and that validation data be made available to laboratories interested in using the method. Such data are critical to evaluate method performance capabilities and limitations, facilitate successful adoption in new laboratories, and ensure consistent and reliable results can be achieved over time.

Key words: method validation, process controls, matrix controls, reference materials, quantification, wastewater, RT-qPCR

Standards and control materials used: PCR positive and negative controls (IDEXX), Extraction and PCR Internal Control (IDEXX), SARS-CoV-2 reference RNA (ATCC), BRSV matrix recovery control, PMMoV and crAssphage human fecal controls

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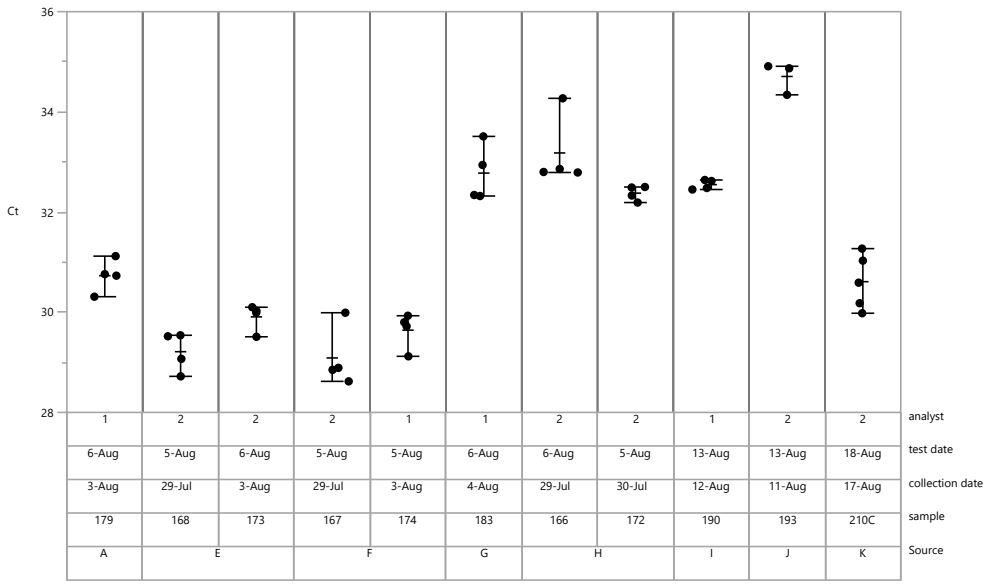

Example validation data demonstrating high Repeatability for detection of SARS-CoV-2 N1 and N2 from a geographically diverse collection of raw wastewaters. Samples were processed using PEG-based concentration, RNA purification with the IDEXX Water DNA/RNA Magnetic Bead Kit, and viral quantification with the IDEXX Water SARS-CoV-2 RT-PCR Test kit following validated protocols for each step.

Early Warnings of COVID-19 Second Wave in Detroit MI

Miyani Brijen[1], Zhao Liang[2], Spooner Maddie[3], Gentry Zachary[4], Mehrotra Anna[5], Norton John[6], Xagoraraki Irene[7*]

[1,2,]PhD candidate, Michigan State University; [3,4]Michigan State University; [5]Environmental Engineering, CDM Smith, Inc; [6]Director of Energy, Research and Innovation, Great Lakes Water Authority; [7]Professor of Environmental Engineering, Michigan State University; [*]corresponding author

Abstract:

This study focuses on using wastewater-based-epidemiology to provide early warnings of the second COVID-19 wave in Detroit metropolitan area in MI, USA. SARS-CoV-2 RNA from untreated wastewater samples was compared to reported public health records. Untreated wastewater samples were collected from the Great Lakes Water Authority (GLWA) Water Resource Recovery Facility (WRRF), located in southeast Michigan, between Sept 6, 2020 and Dec 14, 2020. The WRRF receives wastewater from its service area via three main interceptors: Detroit River Interceptor (DRI), North Interceptor-East Arm (NIEA), and Oakwood-Northwest-Wayne County Interceptor (ONWI). A total of 144 untreated wastewater samples were collected (45, 48, and 51 for ONWI, NIEA and DRI respectively) at the point of intake into the WRRF. Virus-selective sampling was conducted, and viruses were isolated from wastewater using electropositive NanoCeram column filters. For each sample, an average of 33 L of wastewater was passed through NanoCeram electropositive cartridge filters at an average rate of 11 L/m. Viruses were eluted and concentrated and SARS-CoV-2 RNA concentrations were quantified with RT-qPCR. SARS-CoV-2 RNA was detected in 98% of samples and measured concentrations were in the range of 4.45E+04 to 5.30E+06 genomic copies/L. Early warnings of COVID-19 peaks were observed approximately four weeks prior to reported publicly available clinical data. This was confirmed by statistical analysis as well.

Key words: SARS-CoV-2, coronavirus, COVID-19, wastewater, wastewater-based-epidemiology (WBE), Detroit

Standards and control materials used: *SARS-CoV-2 synthetic control as positive control in RT-qPCR.*

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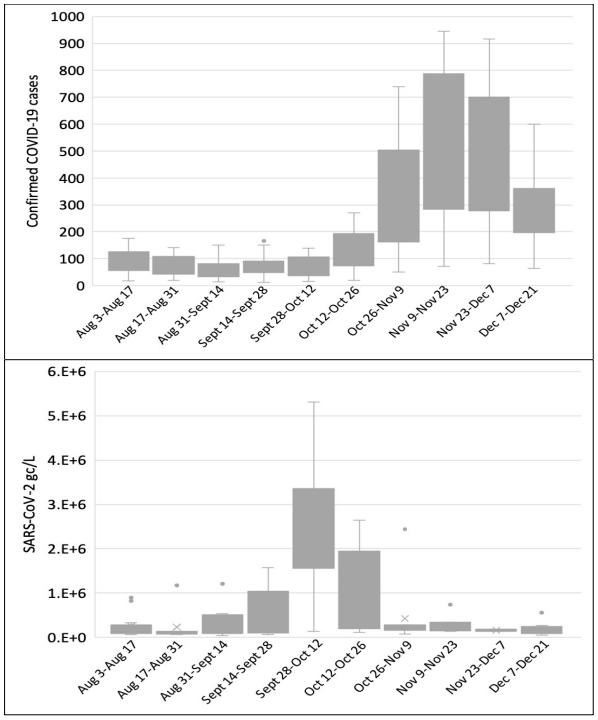

Figure: Biweekly confirmed COVID-19 cases and SARS-CoV-2 RNA concentrations

Wastewater SARS Public Health Environmental Response (W-SPHERE) Global Data Center

Krystin Kadonsky[1], Colleen C. Naughton[1], Gertjan Medema[2], Panagis Katsivelis[3], Vajra Allan[4], Joan B. Rose[5]

[1,2]University of California Merced; [2]KWR Water Research Institute, [3]Venthic Technologies, [4]PATH, [5]Michigan State University

Over a year since the declaration of the global coronavirus disease 2019 (COVID-19) pandemic there have been over 173 million cases and 3.7 million deaths. Using methods to track community spread of other viruses such as poliovirus, environmental virologists and those in the wastewater based epidemiology (WBE) field quickly adapted their existing methods to detect SARS-CoV-2 RNA in wastewater. Unlike COVID-19 case and mortality data, there was not a global dashboard to track wastewater monitoring of SARS-CoV-2 RNA worldwide. We first created COVIDPoops19, a global dashboard for wastewater monitoring of SARS-CoV-2, that has grown into a global data center.

Methods for the COVIDPoops19 ArcGIS online dashboard included google form submission of direct sampling of wastewater for SARS-CoV-2 in several countries and stakeholder engagement, literature review, social media and news key-word searches, and attendance at online wastewater surveillance webinars worldwide. After a year of data tracking, wastewater surveillance for SARS-CoV-2 is conducted in over 55 countries, 2,276 sites, and 263 universities/institutions. A small subset (86) of those monitoring for SARS-CoV-2 in wastewater provide their data publicly and less than 20 provide downloadable data. Of the 55 that are conducting wastewater monitoring: 36 (65%) are in high-income countries, 11 (20%) are upper middle income, 8 (15%) are lower middle income, and 0% are low income countries.

COVIDPoops19 is informing a global data center W-SPHERE global data center (Wastewater SARS Public Health Environmental REsponse) using open data from individual country/city wastewater dashboards and soliciting data submissions and agreements from the research community. The mission of W-SPHERE is to advance environmental surveillance of sewage to inform local and global efforts for monitoring and supporting public health measures to combat COVID-19.

Wastewater surveillance has been a powerful tool to build resilience to the COVID-19 pandemic. However, there is a lack of data standards, limited use in low-income countries, limited data sharing publicly and challenges in analysis of the data to communicate to public health officials for decision making. We will provide a global data center and standards to build resilience beyond COVID-19 in the face of climate change and increased pathogens in the environment.

Key words: Dashboard, COVID-19, Geographic Information System (GIS), Wastewater Based Epidemiology (WBE)

Standards and control materials used: *Various standards and control methods used since data from many sources are aggregated for the data center.*

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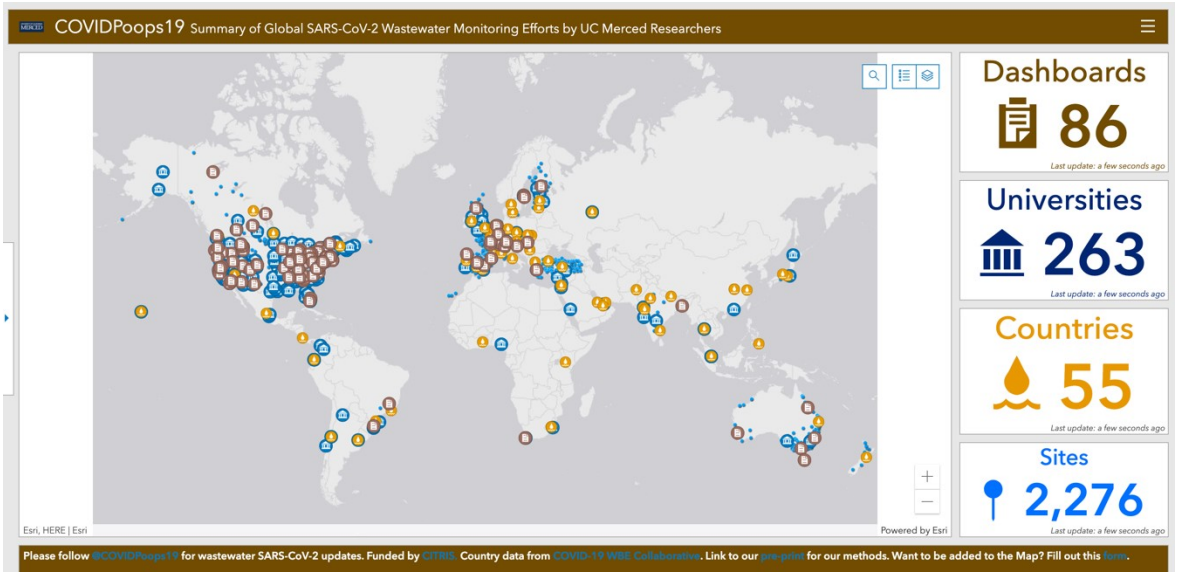

Figure 1. COVIDPoops19 Global Dashboard of Wastewater Monitoring for SARS-CoV-2

Rapid Sample Concentration for Streamlined Workflow in the Wastewater Laboratory

Alburty, David[1], David Goad, PhD[1], and James Brayer[2]

[1]InnovaPrep LLC; [2] Oxford Nanopore

Wastewater-based epidemiology (WBE) is a valuable tool for assessing population dynamics, infection rates, and most recently population-based assessment of vaccine efficacy and detection of variant strains. Collected samples are commonly concentrated for identification of viruses in liquid WW samples via PEG precipitation, ultrafiltration, or electronegative filters. RNA from concentrated samples is extracted and purified by COTS rapid methods such as RT-qPCR or ddRT-PCR and via genetic sequencing. To speed up sample processing through automation while eliminating the potential for sample carryover, an automated bioconcentrator can be used. In the concentrator, samples are aspirated into a single-use, high surface-area hollow fiber filter tip. Particles larger than the filter pore size are retained while the permeate fluid passes through. When the sample has been filtered the device stops and alerts the user to elute the sample. With a button press, a viscous, expanded wet foam is pushed through the retentate tangential to the filter surface recovering the particles in a user-selected volume of clean buffer (wet foam elution) which collapses immediately into a final volume of approximately 200 microliters, providing key benefits of decreased processing time, repeatability, and scalable concentration factor based on the input volume, output volume, and high efficiency to improve the limit of detection. Data are presented from a 3rd party investigation that showed better results for SARS-CoV-2 concentration vs PEG method when analyzed using ddPCR. Published data showed faster processing times and better performance than electronegative filtration[1] and equivalent concentration using centrifugal ultrafiltration.[2, 3] In these studies, bovine coronavirus or MS-2 were used as process controls. Details are presented including summarized materials and methods and comparative data for the complete processes from sample to analysis.

[1] *Juel, A.I. et.al. 2021;* [2] *Rusinol, M. et. al 2021* [3] Forés et. al. 2021.

Key words: wastewater based epidemiology, SARS-CoV-2, sample preparation, viruses

Standards and control materials used: *Bovine Coronavirus as a process control and internal standard for quantification of SARS-CoV-2.*

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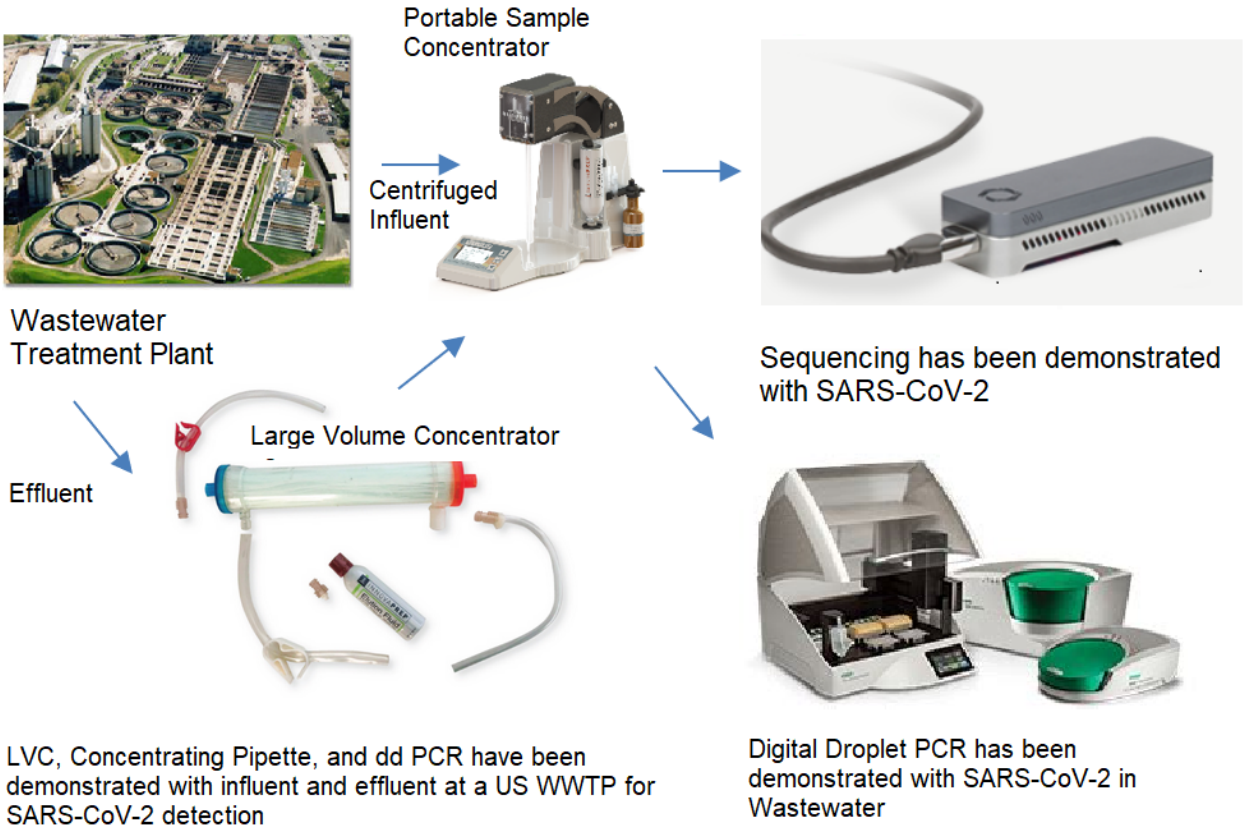

Figure 1. Concentrating Pipette Select for Faster Wastewater Processing.

The Development of a Sensitive and Reliable qRT-PCR-Based SARS-CoV-2 Wastewater Analysis Protocol

Agustin Pierri[1] and Douglas Sieglaff[2]
[1]Weck Laboratories, [2]Agilent Technologies

Wastewater testing can offer valuable insight on community-level occurrence of infectious disease- causing microorganisms, including enveloped ssRNA+ viruses such as SARS-CoV-2. Quantitative reverse transcriptase PCR (qRT-PCR) is well suited for wastewater microbial surveillance due to its sensitivity, specificity, scalability, rapid implementation and cost-effectiveness. Weck Laboratories developed a qRT-PCR-based SARS- CoV-2 wastewater testing procedure that includes sample concentration by ultrafiltration, and nucleic-acid extraction and analysis using Agilent Technologies reagents with the AriaDx Real-Time PCR System. The CDC EUA N1 and N2 primer probes were used to analyze wastewater samples. Each qRT-PCR run batch included wastewater samples analyzed in duplicate, a 5-log dilution standard line, a matrix spike-in process control, a negative template control, no template control, and PCR-inhibition assessment of each wastewater sample tested. The validated procedure reliably interpreted SARS-CoV-2 viral RNA levels within a variety of raw wastewater samples, along with delivering high sensitivity (as low as 8,000 viral genome copies per liter of wastewater). The procedure developed and employed by Weck laboratories is an easily implemented, robust methodology for community-level SARS-CoV-2 surveillance.

Figure

Overview of Weck Laboratories' workflow for extracting and detecting SARS-CoV-2 RNA in wastewater

Review of culture methods for monitoring antibiotic resistant *Acinetobacter*, *Aeromonas*, and *Pseudomonas* in wastewater, recycled and receiving water

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The continued and growing health threat posed by antibiotic-resistant bacterial pathogens has led to increased interest in antibiotic resistance outside of hospital settings, e.g., in wastewater and wastewater-influenced aquatic environments. Environmental dimensions to the antibiotic resistance problem are increasingly being recognized and there has correspondingly been an increase in calls for comparable means to assess antibiotic resistance in environmental samples (Larsson et al., 2018; Smalla et al., 2018; JPIAMR, 2019). Recently, substantial progress has been made in the standardization of methodologies for monitoring antibiotic resistant fecal indicator bacteria in the environment, e.g., extended-spectrum beta lactamase (ESBL)-producing *Escherichia coli* (World Health Organization, 2021) and ESBL-producing Enterobacteriaceae (Marano et al., 2020). However, non-fecal bacteria with environmental niches, especially those that are adept at persisting and growing in aquatic environments, may be a more meaningful target for assessing potential for evolution and dissemination of antibiotic resistance in environmental matrices. For example, the presence of such bacteria in wastewater treatment plants and their versatile ability to survive and grow in receiving environments presents an opportunity to interact with bacteria in multiple niches, where they could potentially acquire and transfer antibiotic resistance genes along the way. Further, such organisms have greater potential than fecal bacteria to regrow in recycled water distribution systems. Human opportunistic pathogens that proliferate in aquatic environments are particularly key targets to consider for monitoring purposes, especially those that have developed a reputation for multi-drug resistance in clinical infections. Studying these organisms in culture provides the advantage of being able to confirm their viability while also being able to further characterize multi-drug resistance, both phenotypically and genotypically. We conducted a systematic literature review and extracted data from studies that quantified antibiotic-resistant *Acinetobacter, Aeromonas,* and *Pseudomonas* by culture methods. The search criteria yielded 50 peer-reviewed articles across 25 countries over the past 20 years. Based on a systematic comparison of the isolation, confirmation, and antibiotic resistance assaying methods reported in these articles, we suggest a path forward for standardizing methodologies for monitoring antibiotic resistant strains of these bacteria in the water environment.

Keywords: antibiotic resistance, wastewater, recycled water, surface water, culture, opportunistic pathogens

Standards and control materials used: *not applicable*

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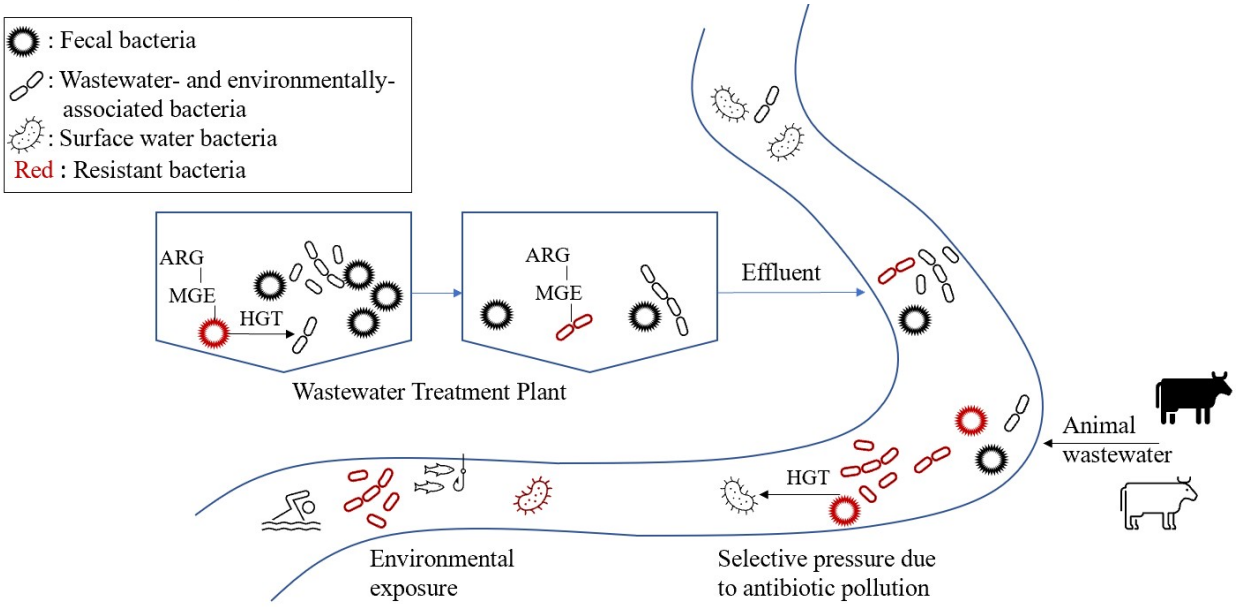

Figure 1. Potential for acquisition of antibiotic resistance genes (ARGs) by environmentally-associated bacteria during wastewater treatment and in affected surface waters.

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Minimizing uncertainties of COVID-19 prevalence by establishing standards for wastewater surveillance

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Wastewater-based epidemiology (WBE) is a promising approach for monitoring population-wide COVID-19 prevalence through detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA in wastewater. However, various methodological challenges associated with WBE affect the accuracy of prevalence estimation. Our previous study investigated the overall uncertainty of WBE and the impact of each step on the prevalence estimation. The uncertainties associated with the different steps in the WBE approach (i.e., virus shedding; in-sewer transportation; sampling and storage; analysis of SARS-CoV-2 RNA concentration in wastewater; back calculation) were quantified through systematic review. The uncertainties of virus shedding and in-sewer transportation are largely uncontrollable, but fortunately not a major contributor to the overall uncertainty in estimated COVID-19 prevalence. The uncertainty for the shedding of SARS-CoV-2 RNA becomes limited when there are more than 10 infected persons in the catchment area. Also, the relative stability of SARS-CoV-2 in wastewater and moderate hydraulic residence time in the sewer system (normally within 12 hr) indicates a mild uncertainty due to in-sewer transportation.

Based on the analysis of different uncertainties, the overall WBE uncertainty is mainly due to sampling and storage; analysis of SARS-CoV-2 RNA concentration in wastewater; back calculation. It is critical to minimize uncertainties of estimated COVID-19 prevalence through establishing and adopting standards for wastewater surveillance. The uncertainty can be reduced mostly by using a high-frequency flow-proportional or time-proportional sampling and estimating the prevalence through actual water usage data. And under such a scenario, the overall uncertainty can be further reduced by improving SARS-CoV-2 RNA detection in wastewater. It is critical to determine the virus recovery efficiency for the various concentration methods being used in different labs. A best-practice RT-qPCR protocol, including choice of primer-probe sets, should be encouraged.

Key words: COVID-19; SARS-CoV-2; Wastewater-based epidemiology; Uncertainty; Prevalence estimation; Standards

Standards and control materials used: *No*

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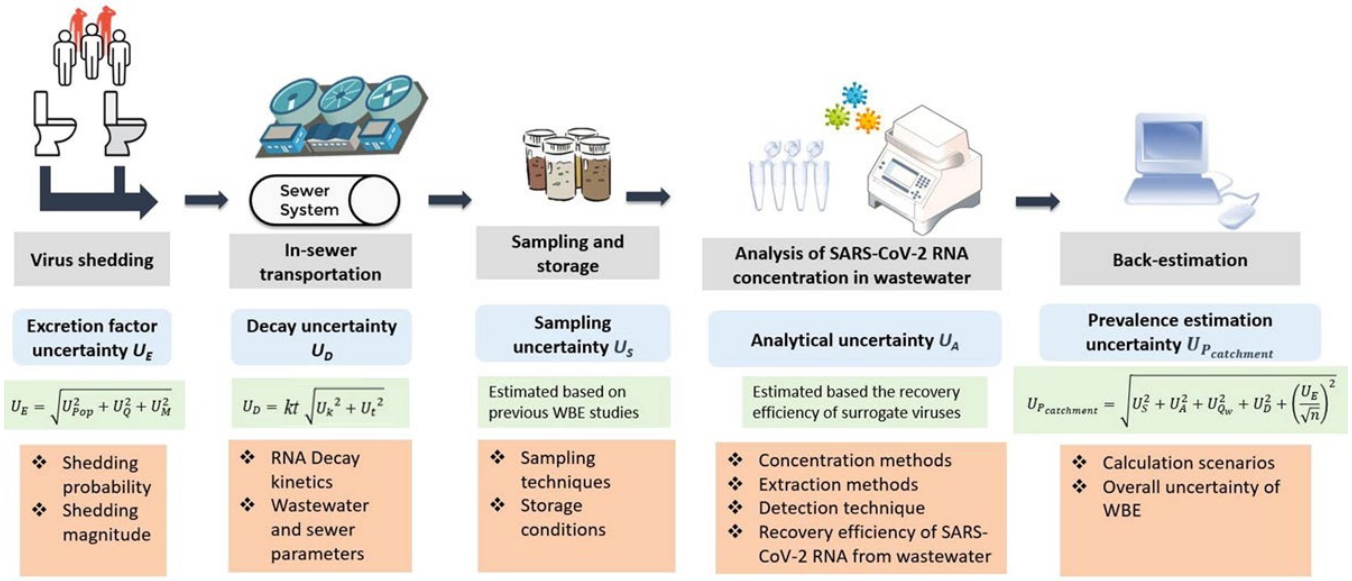

Figure 1. Uncertainty analysis for monitoring COVID-19 community prevalence through wastewater surveillance.

Using a high-resolution sampling in Davis, CA to understand how to best analyze and act on WBE data

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Wastewater-based epidemiology (WBE) is a useful complement to clinical testing for pandemic response. The public-health benefits of WBE depend on the scale at which WBE is carried out. **Samples from building or neighborhood outflows** can support direct and targeted interventions. **Samples isolating different sub-regions of a community sewershed** can help officials decide how to allocate resources (e.g., testing and public messaging) within the community. **Samples from wastewater treatment plant (WWTP) influent** provide information that can reinforce confidence in clinical trends or suggest when clinical testing may be missing key population segments. WBE data can also be incorporated into education and outreach campaigns.

The Healthy Davis Together (HDT) program in Davis, CA applies WBE at all three of the above scales. Since Fall 2020, HDT has been collecting and analyzing levels of SARS-CoV-2 in wastewater collected from the following sites:

- **Building/neighborhood outflow [collected 1–3x/week]:**
  - 22 UC Davis residential buildings or complexes
  - 6 Davis neighborhoods
  - 1 apartment complex
  - 1 elementary school
- **Sewershed sub-regions [3x/week]:**
  - 15 sub-regions of the Davis sewershed
- **WWTP influent [7x/week]:**
  - City of Davis WWTP
  - UC Davis WWTP

We are leveraging the high temporal and spatial resolution of HDT wastewater analysis to understand how to best analyze and act on WBE data. We are pleased to present and discuss early insights related to the following key topics and questions:

- **Treatment of non-detects.** Relatively low concentrations of SARS-CoV-2 in environmental matrices like wastewater mean that qPCR technical replicates of the same sample frequently yield a mix of positive ("detect") and negative ("non-detect") results. Researchers commonly substitute a single constant value (e.g., zero or half the detection limit) for non-detects during data analysis. We are (i) illustrating how this crude approach biases results, and (ii) exploring multiple-imputation methods for more sophisticated handling of non-detects.
- **Sub-regional comparison of wastewater and clinical data.** Many groups have demonstrated strong correlation between trends in wastewater SARS-CoV-2 concentrations and clinically

confirmed COVID-19 cases at the city or college level. We are exploring whether similar relationships exist at the sub-regional level.

Key words: Wastewater-based epidemiology, public health, SARS-CoV-2, COVID-19, data imputation, data correlation

Standards and control materials used: Phi6 bacteriophage (process control), PMMoV (fecal strength indicator), N1/N2 gene regions (target for SARS-CoV-2 quantification), Oregon RNA and N1/N2 plasmids (positive control)

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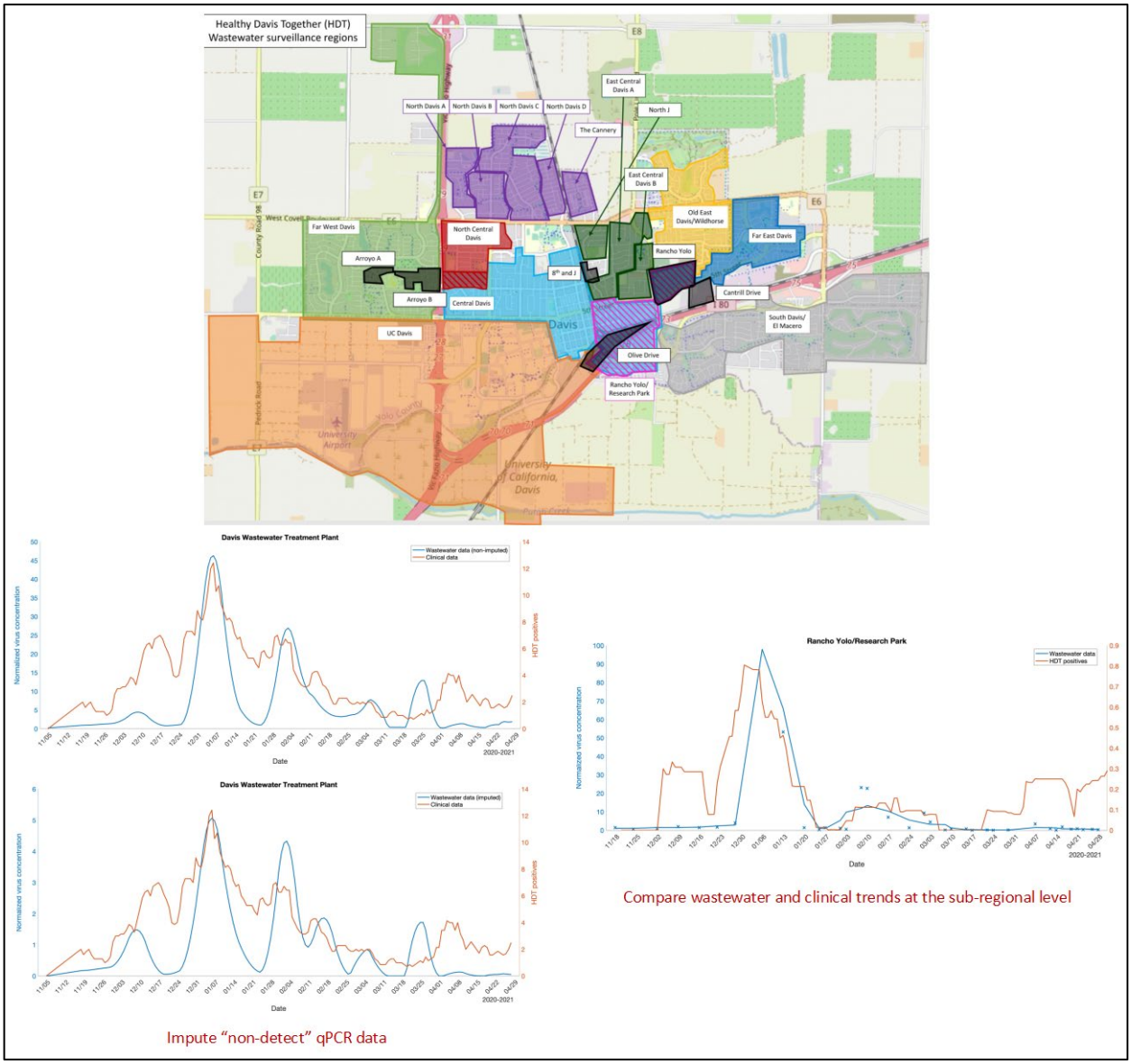

Figure 1. The rich dataset provided by the granular wastewater-sampling campaign and large-scale asymptomatic testing conducted through Healthy Davis Together enables novel insights into key questions such as "How do we handle non-detects in qPCR data collected on wastewater samples?" and "How well do WBE data correlate with clinical test results at the sub-city level?

EzCOVID19: A Cloud-based Bioinformatics Platform for Rapid Detection, Characterization and Epidemiological Sub-typing of SARS-CoV-2

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Background: The COVID-19 pandemic has caused enormous social and economic disruption worldwide. Tracing the evolutionary development and spread of SARS-CoV-2 across the globe requires continuous and exhaustive genomic sequencing. With the development of next generation sequencing (NGS) technology, genomic information is accessible to many researchers. However, the data generated by scientists, globally, comes in varying formats and from different sources. Additionally, despite the need for consensus genome assemblies, there is no single consensus protocol for sequencing SARS-CoV-2. Such information requires standardizing and compiling before it can be accurately analyzed. EzCOVID19, is a cloud-based bioinformatics platform that enables researchers the ability to rapidly detect, standardize, and characterize SARS-CoV-2 genomes from any NGS data suspected of containing SARS-CoV-2.

Method: EzCOVID19 generates consensus genome assembles, locates genetic variations from the SARS-CoV-2 Wuhan-Hu-1 reference (NC_045512.2), utilizes a novel SNV-based classification system for accurate identification, and produces parsimony trees based on public GISAID genomes.

Results: This user-friendly tool provides a platform for scientists to submit samples for rapid detection of SARS-CoV-2, download assembled genomes and profile tables, identify genetic variations, and compare with related genomes in public databases. EzCOVID19 also provides in-depth analyses and visualizations of the data with parsimony and maximum likelihood trees based on novel SNV sites. Its cloud-based system and online support provide immediate results from regularly updated public databases, including monitoring of variants of concern. To analyze your samples, access EzCOVID19 here https://www.ezbiocloud.net/tools/sc2.

Conclusion: Many scientists and public health workers from various research backgrounds have been required to adapt viral genomics into their research as a means of understanding SARS-CoV-2 without prior knowledge of virology or genomics. EzCOVID19 is a cloud-based, user-friendly, robust platform to assist scientists from different disciplines to research SARS-CoV-2. Regardless of method or sequencing instrument used, EzCOVID19 is able to integrate raw metagenomic or isolate sequence data for standardised bioinformatic analyses. Processed data are presented with detailed visuals and relevant information related to concurrently circulated publicly available SARS-CoV-2 genomes.

Key words: COVID19 Surveillance, Bioinformatics, rapid detection, epidemiological sub-typing, mutational analysis.
Standards and control materials used: N/A
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Targeted communications using wastewater monitoring at the sub-sewershed scale in Davis, CA

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Healthy Davis Together (HDT), a joint project between UC Davis and the City of Davis, collects wastewater samples from nodes within the city's sewer system and monitors the City of Davis and the UC Davis Wastewater Treatment Plants. Samples are collected three times per week at 24 sewer nodes in the city and seven days per week from wastewater influent at the City of Davis and UC Davis WWTPs. Sample processing takes about 24 hours, and SARS-CoV-2 concentrations are normalized to pepper mild mottle virus (PMMoV) for presentation and analysis.

HDT formed a Wastewater Action Committee (WAC) to coordinate communications of all wastewater results between HDT scientists and City leadership on a weekly basis and to develop and implement responses to wastewater results. A press release invited community members to opt into the pre-existing Yolo Alert messaging system to receive notifications when elevations are detected.

Virus levels in wastewater that exceed pre-defined action thresholds are reviewed by the WAC with three primary response strategies, together intended to encourage participation in HDT's widely available, free asymptomatic COVID-19 testing program. First, HDT posts results on its website, broken down by sampling region to keep the public informed each week. The slope of a two-week moving average is evaluated to denote on the HDT website if levels in each sampling region are increasing, staying about the same, or decreasing. Second, the highest absolute normalized values of detection are used by HDT and the WAC to identify priority regions within the city and to cross-check participation in asymptomatic individual testing programs within different regions. Third and finally, geo-targeted text, email, and social media messages can be sent to a sub-sewershed region following a sustained elevation of normalized virus concentrations above the limit of detection. Sub-sewershed samples represent smaller populations (hundreds to low thousands of people) and are expected to frequently yield nondetects; an increase in levels above the detection limit represents a significant and easily identifiable change. The city used its opt-in text message alert system as well as Nextdoor to notify residents within the associated sub-sewershed region based on sewer system data collected in March 2021.

Key words: Sub-sewershed wastewater; actionable response strategies; COVID-19

Standards and control materials used: *(short answer)*

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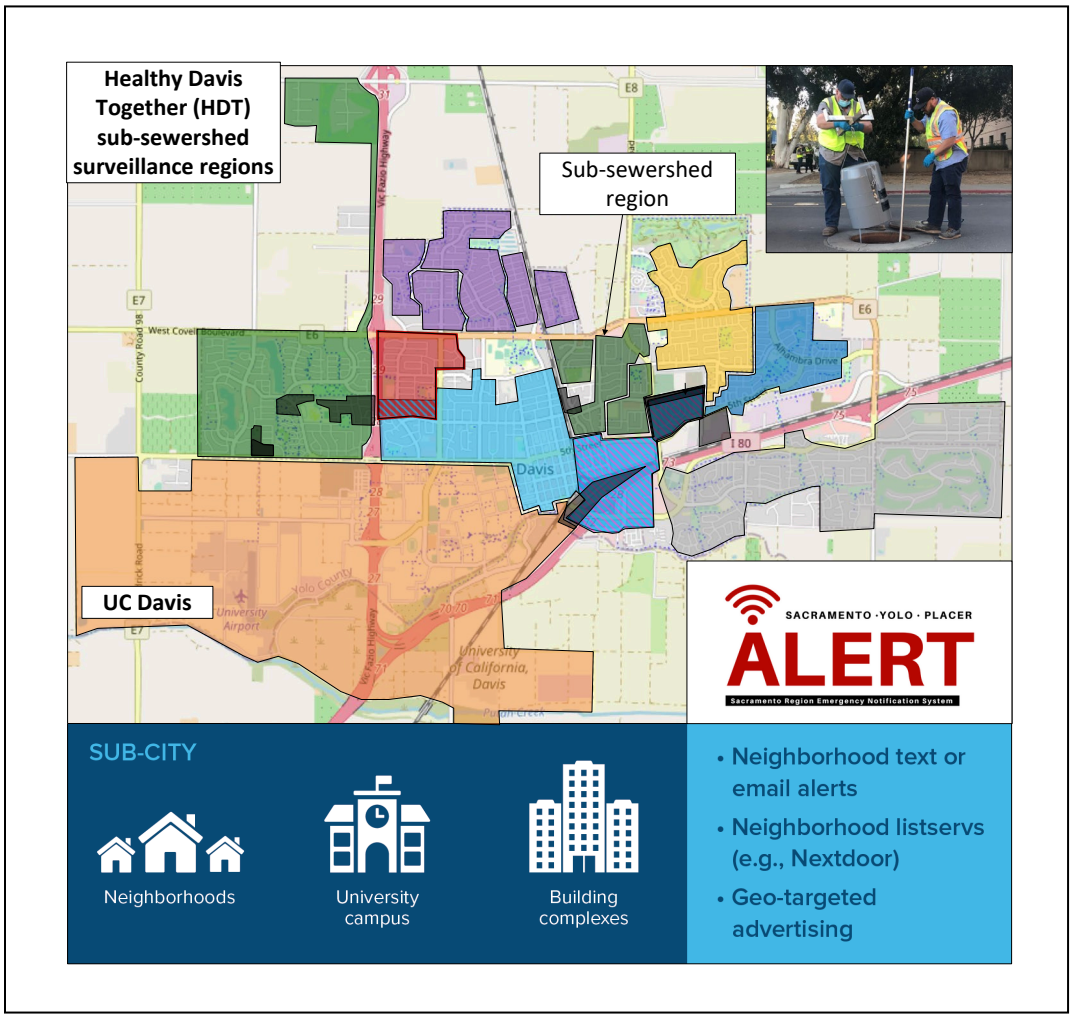

Figure 1. Sub-sewershed regions monitored in the City of Davis and

Assessment of qPCR- targets and protocols for quantifying anthropogenic impacts of antibiotic resistance to the water environment

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There is growing recognition of the need for standardizing monitoring of antibiotic resistance in the aquatic environment. Quantitative polymerase chain reaction (qPCR) is attractive as a quantitative and sensitive means of enumerating antibiotic resistance genes (ARGs) that has been applied broadly over the past two decades to various water matrices. qPCR circumvents challenges and biases associated with culture-based methods, providing a reproducible, quantitative, and highly sensitive measure of specific ARGs carried across a bacterial community. qPCR-based measurements can serve to address key goals for monitoring antibiotic resistance in water environments (Berendonk et al., 2015; Huijbers et al., 2019). Measuring the incidence of specific ARGs can help to predict the potential for the accelerated evolution of antibiotic resistance in pathogens through pollution with selective agents (e.g., antibiotics, heavy metals, personal care products) and bacteria of human or animal origin (Karkman et al., 2018; Kohanski et al., 2010). Additionally, qPCR can be used to estimate the risk of ARB infection in humans by directly measuring the carriage of specific ARGs with clinical implications in a given microbial community.

However, there are thousands of known ARGs that could be targeted, each varying in their relevance to human health and their overall contribution to dissemination of antibiotic resistance. Further, there are various methodological aspects, such as sample concentration, extraction, and PCR inhibition that need to be evaluated to ensure that measurements are representative and comparable across studies. Here we conducted a critical review to identify ARGs and assays that are most commonly measured by qPCR in wastewater, recycled water, and surface water, specifically: *sul1*, *intI1, vanA*, *blaCTX-M*, and *tetA.* We identified 117 peer-reviewed studies meeting the search criteria and systematically assessed the corresponding workflows reported, including sample collection and concentration, DNA extraction, amplification conditions, amplicon length, and level of validation. Resulting concentrations reported for various water matrices were compared across the studies. Based on this evaluation, recommend assays, a standardized workflow, and reporting guidelines for the five genes of interest. Implications for emerging qPCR approaches, such as droplet digital qPCR and high-throughput qPCR, are also discussed and a path forward for standardization is proposed.

Key words: antibiotic resistance, quantitative polymerase chain reaction, standardization, wastewater, surface water, environment

Standards and control materials used: *not applicable.*

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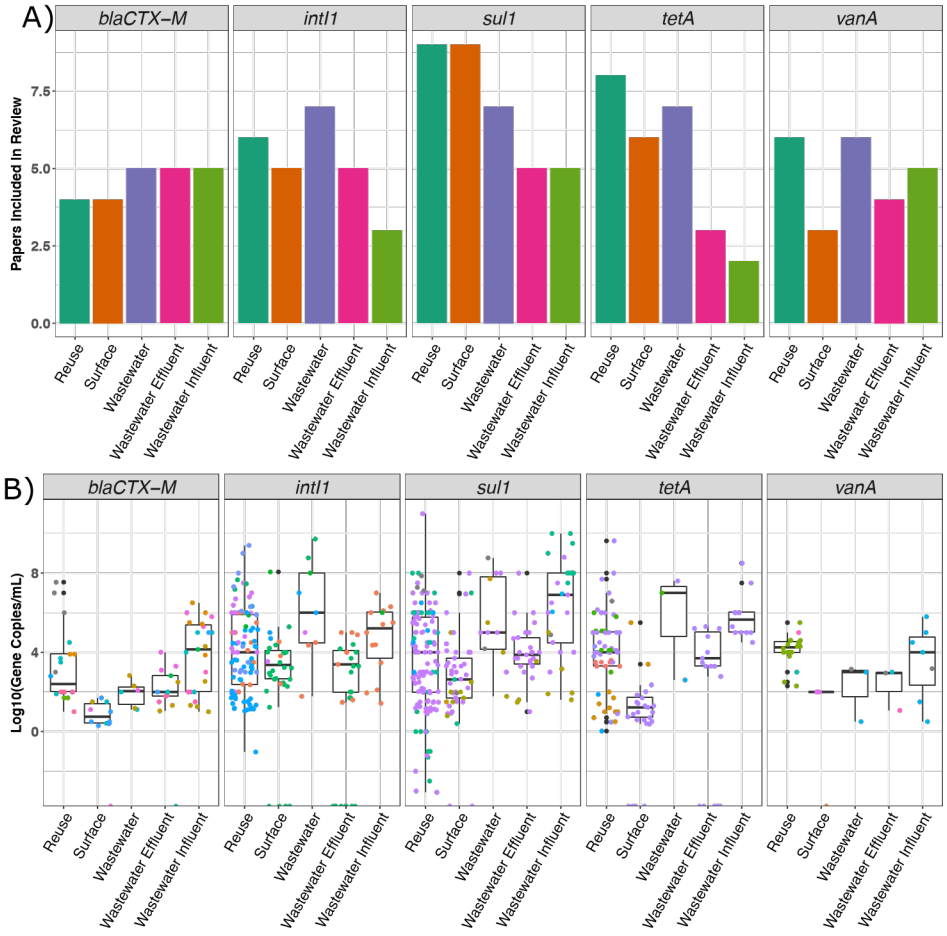

Figure 1**(A)** Number of articles that met the search criteria for each gene by water matrix (recycled/reuse, surface, and wastewater). Articles that examined more than one water matrix are double counted. **(B)** Target gene concentrations measured in each water matrix . Dots are colored by the study the reported data was obtained from**.** Box plots indicate the median, first, and third quartiles and whiskers extend no more than 1.5 times the interquartile range.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317.

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A DNA-based reference material for pathogen detection via metagenomic next-generation sequencing (mNGS)

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NIST has developed a 20-component bacterial DNA-based reference material (RM 8376) to assess the analytical performance of mNGS analyses. Because mNGS includes many steps, each of which contributes bias to the results, it is critical that those biases are identified for optimizing performance. This RM was designed to assess sequencing and informatics. The components include many wastewater-based pathogens (see Table), providing relevant controls for evaluating analytical performance. Further, because these materials are DNA, they may be employed in other diagnostics such as qPCR.

The RM includes a wide range of known pathogens, including Gram positive/negative, high/low G+C content, genome size, and near neighbors. Each chromosome was assembled into a circular contig and is available for use. To quantify the chromosomal copy number concentration, droplet digital PCR assays were developed for each component. The homogeneity and stability of each component assessed over several months, with each genome at approximately 50 ng/μL, or 10^7 copy/μL.

Keywords: bacterial DNA standards, metagenomics, pathogen detection

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Table – NIST RM 8376 components, including source material, identity, assembled chromosome sizes, and copy number concentration with uncertainty.

ATCC ID	Part	Organism	Chr Size(s)	Plasmids	Chromosomal copy number concentration ×10⁶ (copy/μL)[(a)]
43895	A	*Escherichia coli* O157:H7	5564632	2	8.84 ± 0.38
BAA 2309	B	*Escherichia coli* O104:H4	5302905	1	8.89 ± 0.28
700720	C	*Salmonella enterica* enterica	4857492	1	9.72 ± 0.38
12324	D	*Salmonella enterica* arizonae	4482096	0	10.84 ± 0.52
BAA 44	E	*Staphylococcus aureus*	2964115	1	16.49 ± 0.76
12600	F	*Staphylococcus aureus*	2755072	1	17.38 ± 0.68
12228	G	*Staphylococcus epidermidis*	2504458	3	15.99 ± 0.60
BAA 47	H	*Pseudomonas aeruginosa*	6263669	0	8.27 ± 0.34
19606	I	*Acinetobacter baumannii*	3980879	0	12.01 ± 0.56
13077	J	*Neisseria meningitidis*	2181327	0	21.67 ± 0.94
12344	K	*Streptococcus pyogenes*	1914863	0	22.55 ± 0.86
19433	L	*Enterococcus faecalis*	2866948	0	14.75 ± 0.50
27061	M	*Achromobacter xylosoxidans*	6813185	0	7.28 ± 0.36
35654	N	*Aeromonas hydrophila*	4733720	0	9.97 ± 0.34
13883	O	*Klebsiella pneumoniae*	5303036	4	7.68 ± 0.36
25931	P	*Shigella sonnei*	4917056	0	9.67 ± 0.36
35016	Q	*Vibrio furnissii*[(b)]	3275680, 1641536	1	9.70 ± 0.36
19115	R	*Listeria monocytogenes*	2950983	0	17.39 ± 0.64
33152	S	*Legionella pneumophila*	3409194	0	13.63 ± 0.46
GM24385[(c)]	T	*Homo sapiens*			0.0323 ± 0.0015

(a) The values are expressed as $x \pm 2u(x)$, where x is the value and $u(x)$ is the standard uncertainty of x. The standard uncertainty combines reaction volume, unit, and repetition. While the best estimate value lies within the interval $x \pm 2u(x)$, this interval may not include the true value.
(b) Component Q has 2 chromosomes
(c) Sourced from Coriell

An organism-centric approach to performance metrics for metagenomic NGS-based diagnostics

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Complex Microbial Systems Group, NIST

Metagenomic Next-Generation Sequencing (mNGS) analyses applied to wastewater has potential for population-level characterization and tracking. Sequencing is generally agnostic to the DNA/RNA source, allowing the same data to be analyzed for >10k organisms (virus, bacteria, etc.) using different computational tools. The benefits are clear—outbreaks like SARS-CoV-2 could be retrospectively tracked, and health officials could respond quickly to rising levels of potential pathogens such as *Listeria*. However, mNGS-based diagnostics are also prone to false positives and negatives, and those can degrade confidence in the technology and public health administration in general.

We have proposed evaluating mNGS performance on a per organism basis. This *organism-centric* approach has particularly strong potential for wastewater because it allows end users to know the technical limitations and indicate how to address them, especially when distinguishing species or strains; the organism load is low; and the results of an analysis can be actionable. Further, the technology can provide indications of novel pathogens in need of a closer look, and this organism-centric approach can be scaled out to incorporate new taxa as needs evolve.

Keywords: metagenomics, sequencing, pathogen detection

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Table 1 Performance metrics by taxon can reveal fine-grain information, especially since performance varies by organism. Here, 10 hypothetical samples were compared by taxon, revealing poor performance for some taxa. Summarizing performance across all possible taxa (sample-centric) hides those potential deficiencies and overstates performance. When only a subset of the taxa are of interest,

Analysis Results						*Performance Metrics*					
Taxon	TP	FP	TN	FN	n	Sens	Pr	Spec	Acc	F1	DOR
Ak	**3**	**4**	**1**	**2**	10	**0.6**	**0.43**	**0.2**	**0.4**	**0.5**	**0.4**
Bl	**2**	1	4	**3**	10	**0.4**	**0.67**	0.8	**0.6**	**0.5**	2.7
Cm	**3**	1	4	**2**	10	**0.6**	**0.75**	0.8	**0.7**	**0.67**	6.0
Dn	4	1	4	1	10	0.8	0.8	0.8	0.8	0.8	16.0
Eo	5	1	4	0	10	1	0.83	0.8	0.9	0.91	26.7
Fp	5	0	5	0	10	1	1	1	1	1	100
Gq	5	0	5	0	10	1	1	1	1	1	100
Hr	5	0	5	0	10	1	1	1	1	1	100
Is	5	0	5	0	10	1	1	1	1	1	100
Jt	5	0	5	0	10	1	1	1	1	1	100
Sample-centric Results						Sen	Pr	Spec	Acc	F1	DOR
TOTAL	42	8	5042	8		0.84	0.84	1.00	1.00	0.84	100

Targeted NGS for Sensitive Detection of SARS-CoV-2 Genomes

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[1]Integrated DNA Technologies, Ann Arbor, MI

Discovery and detection of pathogenic genomes is of increasing importance. Next Generation Sequencing (NGS) is a sensitive and thorough way to surveil a wide variety of samples. Sequencing also provides insight into the strains that are circulating in a population, including novel strains. Targeted sequencing enriches for sequences of interest from the sample background, increasing sensitivity and saving on sequencing costs.[1] Swift Biosciences (now part of IDT) has developed the Swift Normalase™ Amplicon Panels (SNAP) workflow to enable fast and efficient preparation of targeted libraries for NGS sequencing. The SNAP workflow takes approximately 2.5 hours from cDNA to library and consists of two PCR steps. This one tube workflow is compatible with a wide variety of sample types and capable of sequencing large target regions using overlapping amplicons to achieve continuous coverage. The SNAP SARS-CoV-2 Additional Genome panel covers 99.7% of the SARS-CoV-2 genome using 345 amplicons. The SNAP technology performs well with low levels of target DNA/cDNA and with damaged samples. This panel has been used to determine the lineage of SARS-CoV-2 in wastewater samples.[2] Herein, we demonstrate the efficiency of the SARS-CoV-2 assay at low viral titers and show that this kit is capable of discovery of novel mutations while maintaining high genomic coverage. This technology is not limited to SARS-CoV-2, but also able to target any sequence of interest.

1 Spurbeck et al. Science of the Total Environment. 789 (2021) 147829

2 Fontenele el al. https://doi.org/10.1101/2021.01.22.21250320

NGS: Targeted Sequencing; SARS-CoV-2; wastewater; amplicon

Standards and control materials used: SARS-CoV-2 standards from Twist Biosciences and BEI were used for input titration experiments.

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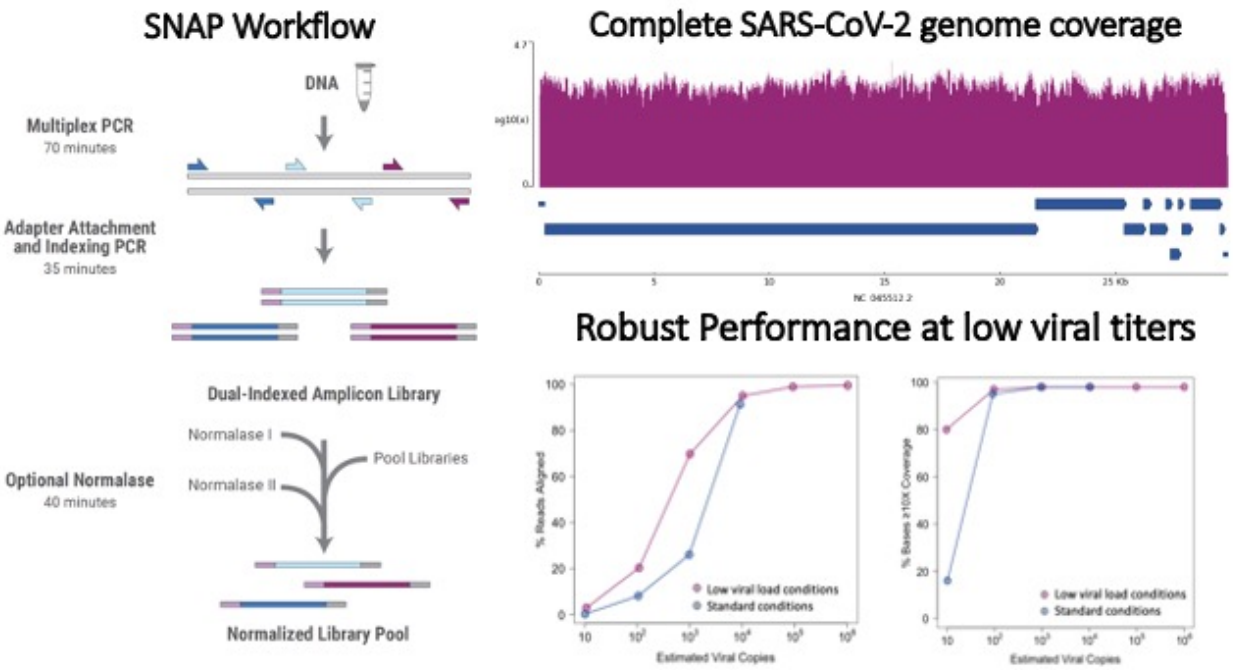

Figure 1. The SNAP Workflow diagram showing the one tube, two step process for library creation. The optional Normalase steps are also show. The SARS-CoV-2 genome coverage attained from the assay is visualized in IGV and show in purple. The performance metrics (% Reads aligned and % Bases >10X coverage) are shown for inputs of 10-10^6 viral copies.

Sources Of Variability In Methods For Processing, Storing, And Concentrating SARS-CoV-2 In Influent From Urban Wastewater Treatment Plants

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During the COVID-19 pandemic, wastewater surveillance of SARS-CoV-2 RNA has emerged as a way to track the virus in a broad population without the drawbacks of testing individuals. Understandably the emphasis has been on rapidly increasing the rate and extent of measuring SARS-CoV-2 RNA in wastewater to gain insight into outbreaks in communities globally. This has resulted in many different methods being used for sample collection, storage, virus capture, and quantification with few studies investigating the variability caused by these methodological differences and the impact of this variability on comparisons of wastewater surveillance data. In this study, controlled experiments were performed to test methods used to store wastewater samples, to inactivate virus in wastewater, to capture and concentrate virus in wastewater, and to extract and measure the viral RNA. We found the highest variability was caused by heat inactivation of the viruses (a 1-3 log decrease) and freezing of influent prior to concentration (1-4 log decrease), with impacts dependent on sample processing method. Sampling frequency, sampling strategy, concentration vs direct extraction, and PCR platform were also minor sources of variability. In contrast, the nucleocapsid gene target had nearly no effects. We found viral capture by membrane adsorption to be robust to changes in SARS-CoV-2 concentration and freeze-thaw variability. Pepper mild-mottle virus was much less sensitive to these methodological differences than was SARS-CoV-2, which challenges its use as a population-level control among studies using different methods. We applied the membrane adsorption method to monitor wastewater from a large wastewater treatment plant in Los Angeles County, California from April 2020-April 2021 and found a high correlation (r=0.78) to SARS-CoV-2 case counts. Wastewater based surveillance holds promise to efficiently measure the prevalence of SARS-CoV-2 in a larger, pooled population sample and has potential to serve as an early warning of future outbreaks. However, the diversity of methods, high variability reported among methods, and a lack of standardization make it difficult for municipalities and public health agencies to be able to interpret the SARS-CoV-2 concentrations from wastewater. Better characterizing the variability associated with methodological choices, in particular the limits of sensitivity of the methods, will aid decision makers in following the effects of vaccination campaigns, early detection of future outbreaks, and, potentially, monitoring the appearance of SARS-CoV-2 variants in the population.

Key words: droplet digital PCR, HA electronegative virus capture, magnetic bead extraction

Standards and control materials used: *Bovine Coronavirus vaccine, Asuragen armored HepG RNA*

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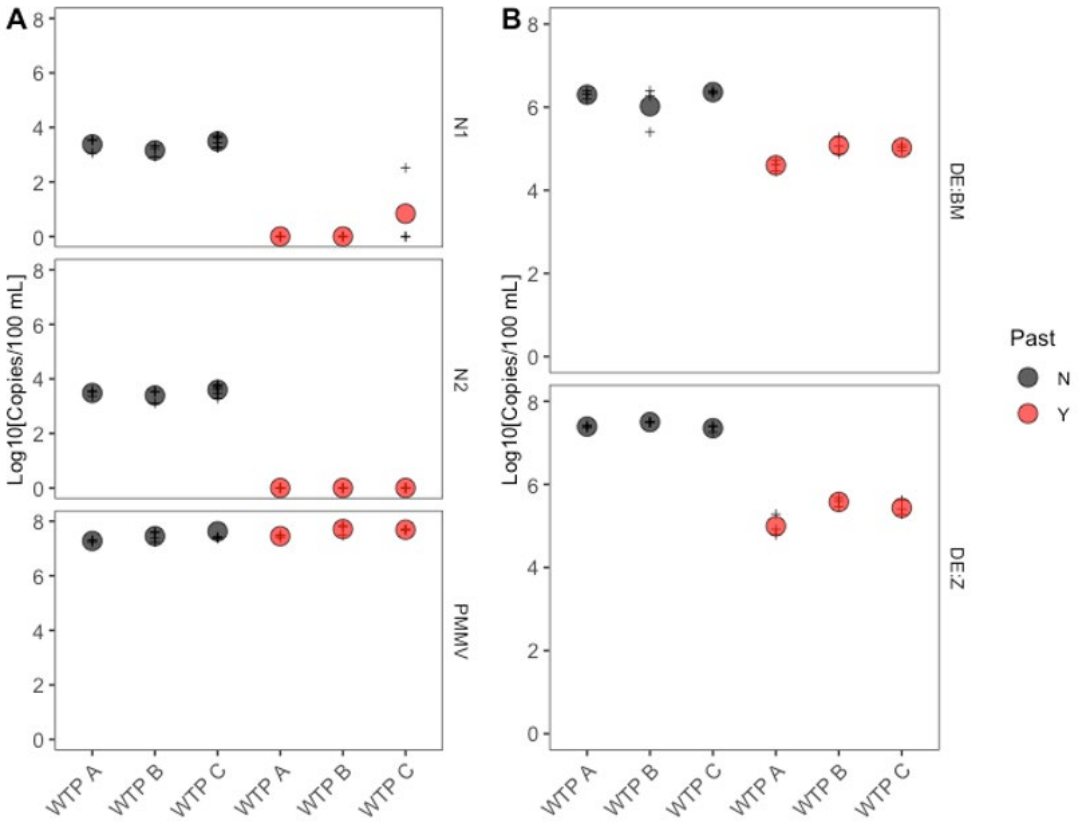

Figure 1. Concentrations measured with and without pasteurization. Circles represent average concentration for the three WTPs and faint crosses represent results from the individual plants. Black circles indicate samples not pasteurized; red circles indicate pasteurized samples; (A) SARS-CoV-2 N1 (top row), SARS-CoV-2 N2 (middle row), and PMMV (bottom row) levels for samples processed by membrane concentration (HA) B) BoCoV levels by direct extraction methods (DE:BM & DE:Z).

Challenges with Standardizing the Measurement of SARS-CoV-2 Recovery Efficiency in Wastewater

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A wide range of methods are currently used to quantify SARS-CoV-2 in wastewater and sludge, involving different concentration and extraction methods and variations on PCR. Quantifying the recovery efficiency of SARS-CoV-2 by different methods would be useful for comparing results, and would ideally offer the ability to correct for different recovery efficiencies. The most common approach to quantify recovery efficiency is to use a proxy virus that is spiked into the sample prior to processing, and is intended to model the behavior of SARS-CoV-2. Proxy viruses that have been used to date include bovine coronavirus, bovine respiratory syncytial virus, murine hepatitis virus, and human coronavirus OC43. However, there are a number of factors that affect the recovery efficiency and may manifest differently depending on sample characteristics, concentration method, and the method used to quantify the initial stock solution. These factors can lead to comparison of "apples and oranges". For example, if the proxy virus and SARS-CoV-2 have different association with solids, the proxy virus will not adequately quantify the differences between methods that capture signal from wastewater solids and those that do. Similarly, if the proxy virus is added as intact virus, and SARS-CoV-2 RNA is present in intact viruses and as free RNA (Wurtzer et al. 2021), the proxy virus will not adequately quantify differences between methods that capture signal from intact viruses and free RNA, and the biases will vary from sample to sample. An early study compared 36 methods used in different laboratories and reported recovery efficiencies varying over seven orders of magnitude (Pecson et al. 2020). A strength of this study is that samples were prepared by a single laboratory and spiked with a proxy virus (betacoronavirus OC43), such that the recovery efficiency could be calculated relative to the same initial concentration and spike-in method. However, in current practice, every laboratory is preparing their own proxy virus and quantifying it differently so that recovery efficiencies cannot be directly compared across methods and labs.

Key words: recovery efficiency, virus proxy

Standards and control materials used: Virus proxy (spike-in) to measure SARS-CoV-2 recovery efficiency

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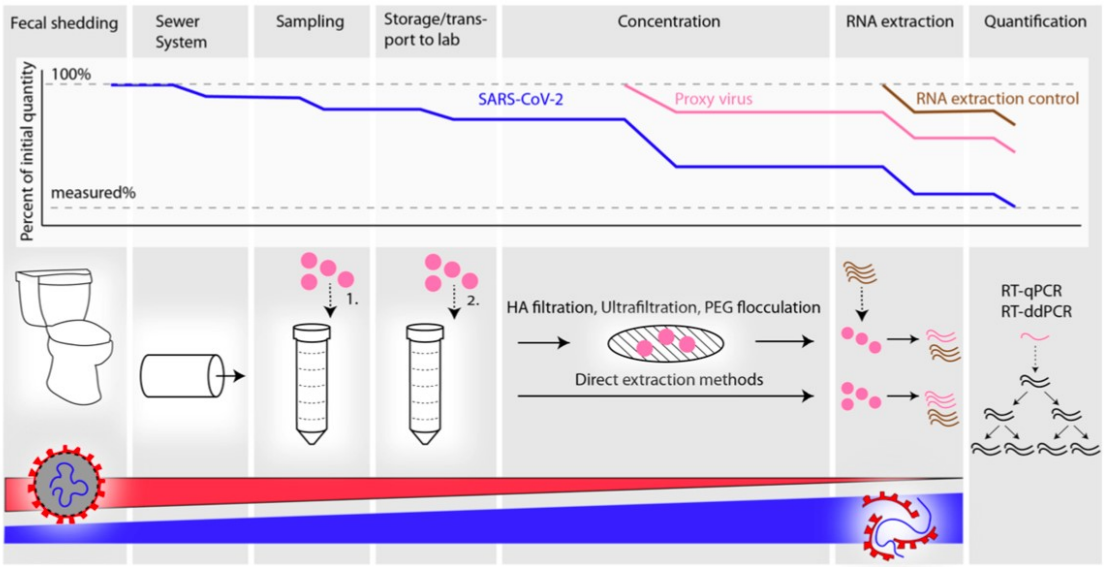

Figure 1. Factors affecting quantification of SARS-CoV-2 from wastewater. SARS-CoV-2 likely exists in wastewater along a continuum of intact (red) and nonintact (blue) viruses, and the ratio of these forms, and their association with solids, changes during transport of the sewage, sampling, and sample processing. For a sludge sample, there may also be loss of signal during primary settling. Spike-in proxy virus controls (pink) can be added 1) at the point of sampling prior to storage or 2) after storage at the beginning of sample processing. Proxy virus controls can account for degradation during storage and loss of signal due to incomplete recovery during concentration (pink line). A second control would be required to independently quantify the loss of signal during RNA extraction (brown line) because the spike control is affected by loss during RNA extraction. (Figure from Kantor et al. 2021).

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SARS-CoV-2 Wastewater Surveillance in a remote municipality in the Aleutians Islands of Alaska

Karie Holtermann, Laboratory Manager, City of Unalaska Water and Wastewater Laboratory

The City of Unalaska is a municipality located in the Aleutian Island chain approximately 800 miles southwest of Anchorage, Alaska. The City owns and operates a chemically enhanced primary treatment sewage facility which screens and disinfects an average of 0.483 million gallons of domestic wastewater per day.
Dutch Harbor is the largest commercial fishing port in the Pacific and is number one in the US for volume of fish. There are approximately 4,768 full time residents on the island and during peak seasons of the year, an additional 5-6,000 people are added to this population. The community's health care services are provided by a local clinic operated by Iliuliuk Family & Health Services, Inc. (IFHS). The clinic obtained two types of "rapid" COVID-19 testing devices at the beginning of the Covid 19 Pandemic, the Abbott IDNow system and the Cepheid GeneXpert IV-2 Molecular system and to date have performed 8,890 local clinical tests.
Wastewater-based epidemiology (WBE) began being explored as a new tool to track the spread of COVID-19 from the onset of the pandemic (Medema et al. 2020). Many studies report viral detection in sewage across the world. Detection of SARS-CoV-2 RNA in wastewater has been shown to be a valuable tool in early detection and informing public health decisions (Wu et al. 2020).
Weekly samples were collected at the Unalaska WWTP Influent and two lift stations from July 2000 until the present. Two methods were used to quantify SARS-CoV-2 in raw sewage. We developed a sensitive, consistent and reliable method to allow for pooled surveillance of wastewater which now serves as an early warning system.

Key words: SARS-CoV-2 Wastewater Surveillance**,** Method Development, Congregate living settings, Remote and rural environments

Standards and control materials used: The primers (N1 and N2) and DNA standards of SARS-CoV-2 nucleocapsid gene were used to quantify the titers of SARS-CoV-2 (Integrated DNA Technologies (IDT) 2019-nCoV CDC EUA qPCR Probe Assay primer/probe mix and 2019-nCoV_N Positive Control plasmid). Two replicates were performed. Positive and negative controls were included in each run as well as internal standardization control Pepper Mild Mottle Virus (PPMoV) and the Seracare AccuPlex SARS-CoV-2 Verification Panel (0505-0168) was used as an extraction control.

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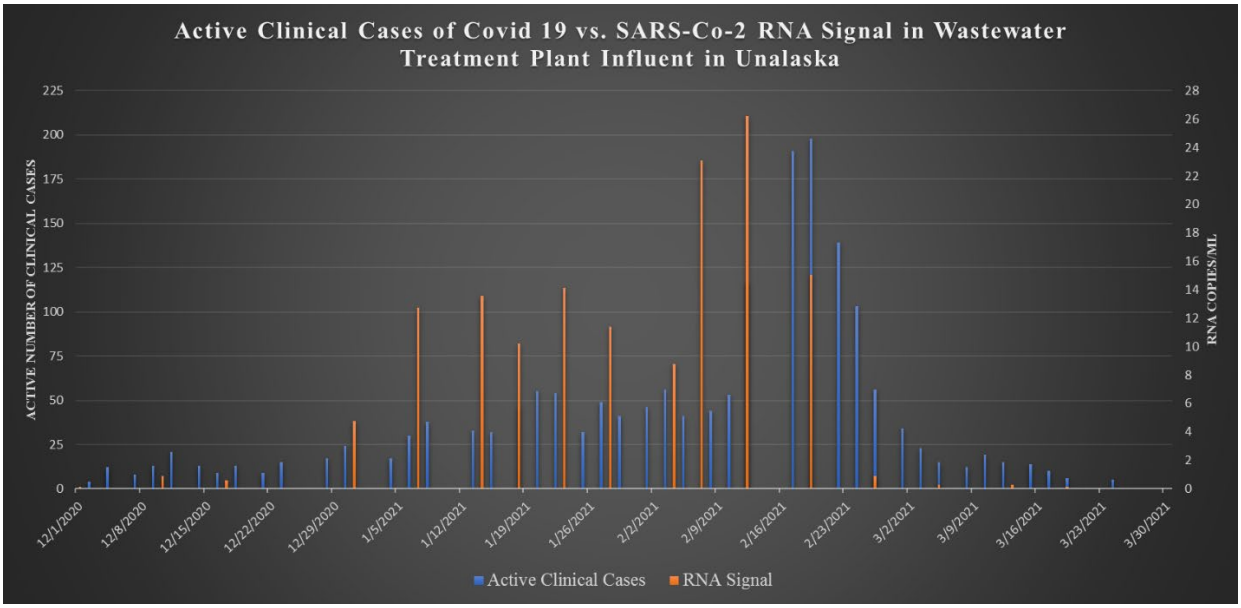

Figure 1. *Isolated cases on vessels kept quarantined and not connected to the city collection system were not included in this graph. The City remained insulated from the pandemic until December of 2020 at which time it experienced two outbreaks, the first at Unisea in Jan. 2021 and the second and more significant at Alyeska in Feb. 2021. Community spread remained low during the year and cases dropped off after vaccinations became widely available. Viral detection began at as low as ten reported active clinical cases. Viral load proceeded clinical cases by several days and followed clinical cases throughout the pandemic.*

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Direct RT-qPCR assay for SARS-CoV-2 variants of concern (B.1.1.7 and B.1.351) detection and quantification in wastewater

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Less than a year following the SARS-CoV-2 outbreak, variants of concern have emerged in the form of variant B.1.1.7 the British variant and B.1.351 the South Africa variant. Due to their high infectivity and morbidity, it has become clear that it is crucial to quickly and effectively detect these and other variants. Here, we report improved primers-probe sets for RT-qPCR for SARS-CoV-2 detection including a rapid, cost-effective, and direct RT-qPCR method for detection of the two variants of concern (B.1.1.7 and B.1.351). All the developed primers-probe sets were fully characterized, demonstrating sensitive and specific detection. These primer-probe sets were also successfully employed on wastewater samples aimed at detecting and even quantifying new variants in a geographical area, even prior to the reports by the medical testing. The novel primers-probe sets developed and presented here have important implications; it will promote proper responses and pandemic containment, and may provide a basis for developing tools for the detection of additional variants of concern.

Key words: Real-Time Polymerase Chain Reaction; Wastewater-Based Epidemiological Monitoring; SARS-CoV-2; Molecular Probes; Variants of concern

Standards and control materials used: This study use synthetic genes for SARS-CoV-2 RT-qPCR detection calibration and wastewater for field proof of concept experiments.

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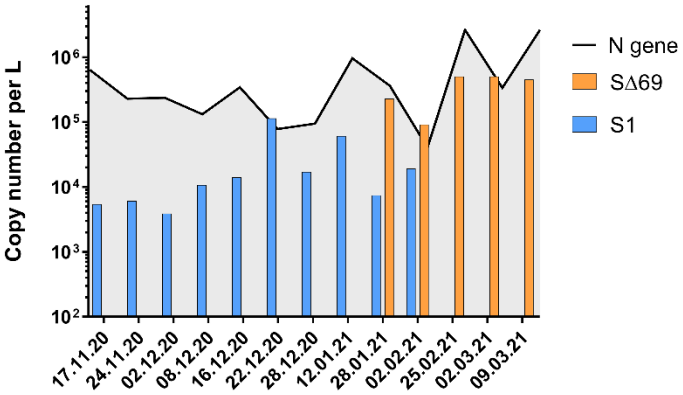

Figure 1. B.1.1.7 variant detection in Beer- Sheva wastewater (WWTP) over time. Samples collected between November 2020 and March 2021 were tested for N gene, S1 (Original lineage) and SΔ69 (British B.1.1.7 lineage) detection.

Assessing the representativeness of Covid-19 testing in different communities through wastewater surveillance

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We highlight the results of wastewater surveillance employed in the following communities to track the prevalence of Covid-19 infections therein.

In a sewershed that covers roughly 1 million residents in the greater New York City region, surveillance of SARS-CoV-2 concentrations is being conducted at different locations including the influent to different wastewater treatment plants. Based on the time-series-trends, concentrations of SARS-CoV-2 in the sewage streams have led the reported clinical case data by up to eleven days and in general have tracked the different waves of Covid-19 in the community.

In smaller residential populations (building-scale) in New York City, where clinical testing has been more frequent, wastewater testing has largely corresponded well with clinical data and has also been used to detect non-compliance with testing as well as the impact of any external interactions with the target community.

Data analysis and interpretation. The equivalence between the SARS-CoV-2 concentrations measured in wastewater and the corresponding fraction of the population infected is site-specific, as expected. Nevertheless, such equivalence calculations can be used to determine the severity of community infections as well as the impact of interventions such as vaccine administration.

Importantly, from a social-perspective, based on sewage surveillance, the progressive non-representativeness of clinical testing within some communities is also revealed.

Key words: wastewater; sewage; surveillance; population equivalence

Standards and control materials used: *(short answer)*: Standards for quantifying target virus biomarkers copy numbers in samples; wastewater flow and load information to attempt virus concentration-population equivalence determination.

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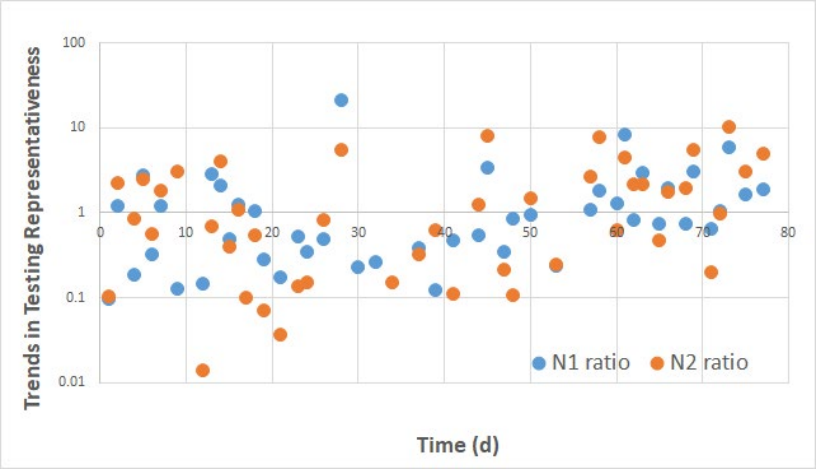

Figure 1. Shifting trends in the representativeness of clinical testing as inferred through wastewater surveillance. Values higher than 1.0 reflect increasing degree of non-representativeness.

2021 Standards for Wastewater Surveillance

Wastewater Surveillance's Role as Part of a Whole-of-University Response in Campus Protection

Jeffrey W. Bethel[1], Benjamin Dalziel[2,3], Roy Haggerty[4], Kathryn A. Higley[5], Katherine R. McLaughlin[6], F. Javier Nieto[7], Justin L. Sanders[8], Brett M. Tyler[9,10], and Tyler S. Radniecki[11].

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During the last year, campuses of higher education have struggled with how to protect their communities in the face of the SARS CoV-2 pandemic. Initial strategies included massive routine testing of students, faculty, and staff to no testing at all[1]. At Oregon State University, selective COVID-19 testing of students, faculty and staff coupled with wastewater sampling and analysis, including RNA sequencing, across multiple campuses was used to inform the university's COVID-19 Continuity Management Team[2]. Health authorities in the relevant counties were also kept advised of the progression of the disease within the campus community, and the presence of variants of concern. While the testing strategy, of necessity, evolved over the course of the pandemic, wastewater remained a consistent, and initially under-appreciated tool for providing insight on locations and pockets of the disease within the college community. Results from routine wastewater sampling across multiple campuses were used by university decision makers in directing increased testing, and subsequently quarantining of on campus residents. The data were also used in crafting website and email messaging for off campus students. The net result was that information from wastewater surveillance informed health staff, underpinned rapid response testing of students, faculty, and staff, and help mitigate the spread of COVID-19 to the larger community.

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[2] https://trace.oregonstate.edu/

Key words: Surveillance; COVID-19; wastewater sequencing; TRACE.

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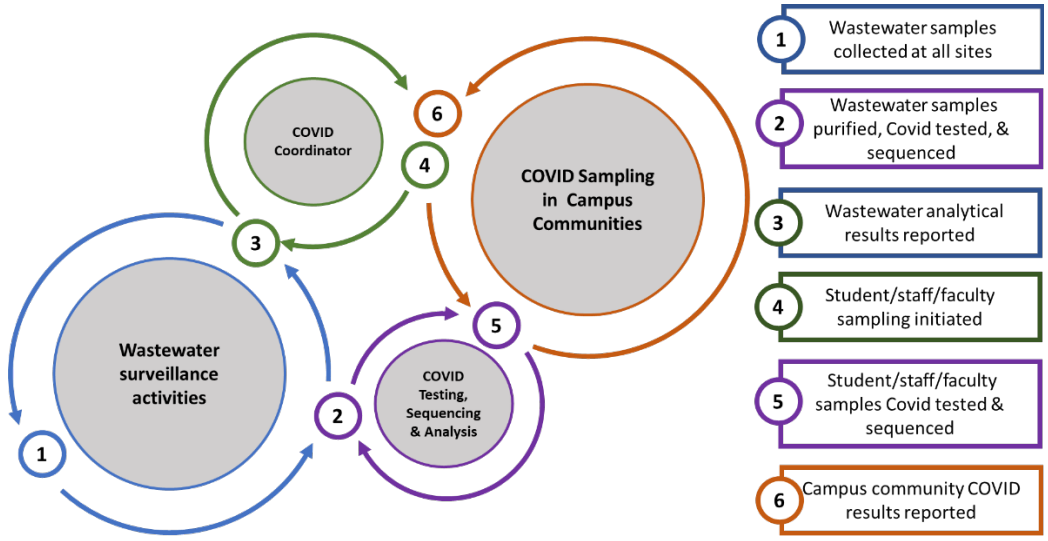

Figure 1. Process followed to collect, analyze, assess results, and direct additional sampling on the OSU campuses.

Towards Standardizing Antimicrobial Resistance Surveillance of Water Systems: An Expert Survey and Workshop

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Monitoring sewage for antibiotic resistant bacteria and antibiotic resistance genes has the potential to inform the status of antimicrobial resistance (AMR) in human populations, while evaluation of treated effluent and recycled water can provide insight into the efficacy of treatment processes for reducing risk of spread in receiving environments (Hendrikson et al., 2019) and monitoring of surface water can reveal concerns for exposure via ingestion or recreation (Nappier et al., 2020). However, standardized methods for monitoring AMR in wastewater, recycled water, and surface water are needed in order to ensure that the data collected are meaningful and comparable across studies and surveillance efforts. While there have been numerous calls for standardization of methods for environmental AMR monitoring (Berendonk et al., 2015; Pruden et al., 2018; Hujibers et al., 2019), a key step towards achieving this goal will be agreement upon the purpose of such monitoring and prioritization of specific monitoring targets for achieving this purpose. To this end, we conducted a survey of 105 experts spanning the fields of academia and research, state and federal government, consulting, and water/wastewater utilities to obtain their recommendations regarding potential AMR targets. Following the online survey, we conducted a 4-day workshop attended by experts to discuss the state of the science, assess existing levels of standardization and feasibility, and obtain a final ranking of priorities for AMR monitoring in the U.S. water industry and regulatory community. Specifically, culture-based, quantitative polymerase chain reaction (qPCR)-based, and metagenomic-based methods were evaluated. Of special consideration was the prioritization of variables that consider overall potential to achieve monitoring objectives and address key research questions, for a wide-variety of stakeholders. A decision-tree framework was developed to assess which methods and targets are most appropriate based on specific monitoring objectives. Without such an effort, AMR research and surveillance will continue in silos and there will be a loss of opportunity to compare spatial and temporal trends, which will be critical to assessing the local and global rates of evolution and spread of AMR, assuring comparability of data and assessing the efficacy of mitigation measures. This perspectives study takes an important step towards identifying suitable targets for AMR monitoring standardization within water and wastewater systems in the U.S. and beyond.

Key words: Antibiotic Resistance; Wastewater Surveillance; Standardized Methods.

Standards and control materials used: *not applicable.*

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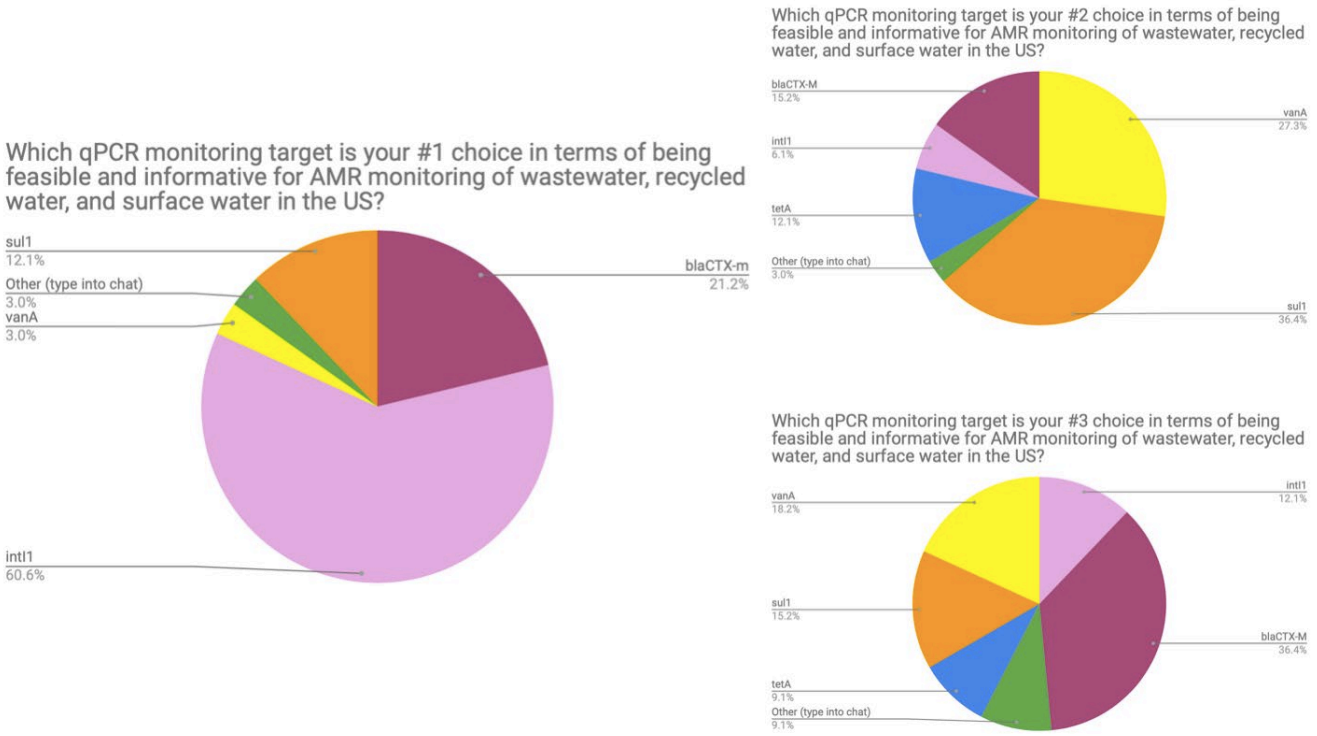

Figure 1. Workshop Experts rank their first, second, and third choices for a feasible and informative qPCR target for AMR monitoring in water for the US water industry.

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First detection of SARS-CoV-2 proteins in wastewater samples by mass spectrometry

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On March 12, 2020, the World Health Organization (WHO) declared COVID-19 as a global pandemic. COVID-19 is produced by a novel β-coronavirus known as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) [1]. Several studies have detected SARS-CoV-2 RNA in urine, feces, and other biofluids from both symptomatic and asymptomatic people with COVID-19 [2], suggesting that SARS-CoV-2 RNA could be detected in human wastewater [3]. Thus, wastewater-based epidemiology (WBE) is now used as an approach to monitor COVID-19 prevalence in many different places around the world [4-10]. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is the most common SARS-CoV-2 detection method in WBE, but there are other methods for viral biomolecule detection that could work as well. The aim of this study was to evaluate the presence of SARSCoV-2 proteins in untreated wastewater (WW) influents collected from two wastewater treatment plants (WWTPs), from Durham Region, Ontario, Canada, using a LC-MS/MS-based proteomics approach. Twenty-four-hour composite influent samples from each of the two wastewater treatment plants (WWTP) were obtained over the course of 15 weeks – for a total of 171 samples across all sampling times and locations. A cross correlation was performed first with all the 160 pairs of data (x: Protein pp1ab 7-day midpoint mean viral signal, y: 7-day midpoint mean in reported new cases of COVID-19 by onset date) and with lags between -15 to 15 as the maximum (cross correlation in time series). The lag with the highest value of r was selected and was used to perform a Pearson correlation analysis (one-tailed with a confident interval of 95%). We identified many SARS-CoV-2 proteins in these wastewater samples, with peptides from pp1ab being the most consistently detected and with consistent abundance (protein related to the COVID-19 infection).

Key words: SARS-CoV-2, COVID-19, liquid-chromatography mass spectrometry, wastewater, proteomics

Standards and control materials used: N/A

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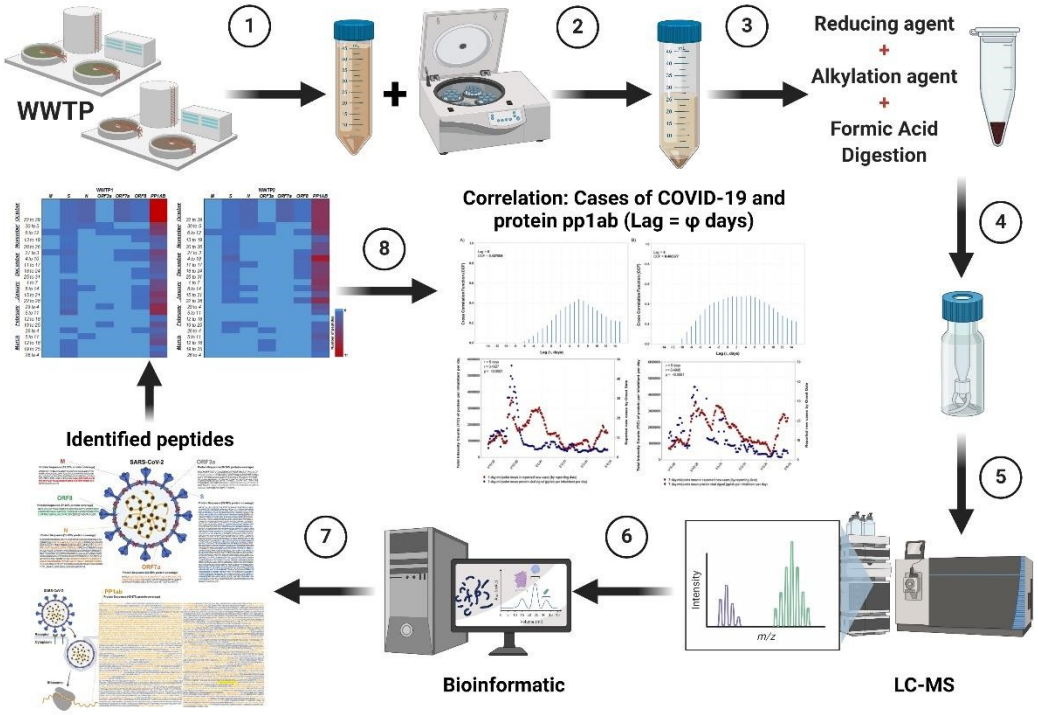

Figure 1. Workflow for protein identification by LC-MS and the correlation of COVID-19 cases vs protein pp1ab.

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WBE Data Sharing, Ethics, and Community Outreach

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Key words: wastewater based epidemiology, COVID-19, SARS-Cov-2, environmental monitoring, research ethics, community engagement

Standards and control materials used: n/a

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Abstract: Issues of privacy or stigmatization pose potential concerns for research, data collection, and analysis of wastewater. However, through the assurance of anonymous results, the protection of identifiable information, and diversity of selected sampling sites, the Co-Immunity Project has incorporated ethical approaches to their work which safeguard the safety and well-being of individual privacy in effective ways. **By developing strategies for transparency around scientific findings and research outcomes**, as well as plans for crafting community agreements that incorporate the input of our community members, **ethical research methods have become a major component of the Co-Immunity wastewater study.** As further knowledge and insights regarding the ethics of wastewater testing continue to emerge and evolve, we are committed to adjusting our own principles and adopting new guidelines into our studies with the intent of promoting equity and inclusivity in the realms of medicine and public health.

Four strategies have emerged that create opportunities to increase transparency and establish bi-directional communication with the public around our wastewater monitoring research:

1. Online dashboard: This dashboard is updated weekly and allows anyone to see current and past levels of SARS-CoV-2 in Louisville's wastewater samples.
2. WBE ethics-centric website: This website hosts facts and frequently asked questions about wastewater work, a history of water monitoring for public health, a link to the online dashboard, and a gallery detailing how wastewater samples are collected.
3. Office Hours: Beginning on Thursday, June 10, 2021 the research team will host an open office hour via Zoom and Facebook Live to make wastewater researchers available to the public, to learn what people think about wastewater monitoring, and hear about possible concerns.
4. Community Survey: The next round of Co-Immunity's Community Testing will run from June 17 through June 23. This round will include a survey about our wastewater monitoring work. This survey will collect the public's level of knowledge about wastewater monitoring, how supportive people are of this type of work, and about possible concerns.

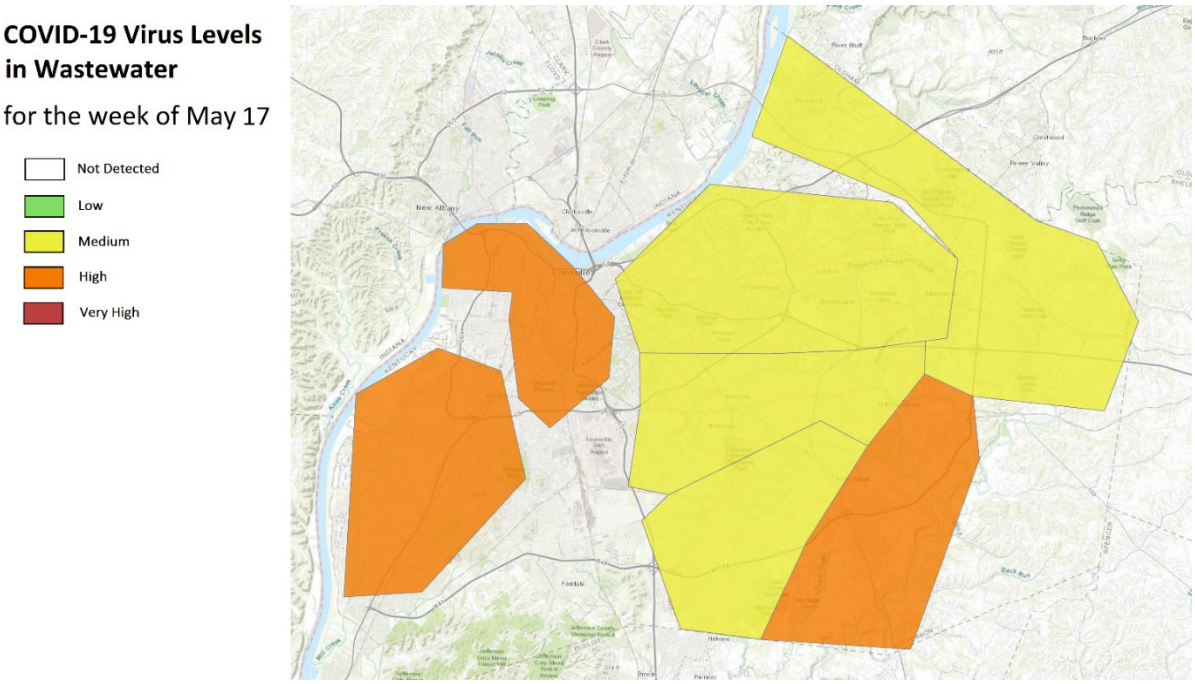

Figure 1. UofL Envirome Institute Wastewater Dashboard Map, May 17, 2021. Access via https://louisville.edu/envirome/thecoimmunityproject/dashboard

Figure 2. Co-Immunity Project's Wastewater Research Office Hour Flyer for June 10, 2021

Systematic review and meta-analysis of correlations for time-lagged COVID-19 cases and viral load in wastewater

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Early detection of cases by the diagnostic testing of symptomatic persons and regular screening, isolation of presumed and confirmed cases and contact tracing are the key to controlling an infectious disease outbreak. However, the standard public health approach to an outbreak requires augmented ancillary surveillance mechanisms to successfully control COVID-19. Amid the pandemic, numerous studies have reported a positive correlation between COVID-19 epidemic indicators and the normalized viral load of SARS-CoV-2 in wastewater. Additionally, studies report that the viral load in wastewater is a leading indicator of confirmed cases within a population by days to weeks. Yet, significant methodological questions remain to robustly trend community cases with the viral signal in wastewater utilizing the technique known as wastewater-based epidemiology (WBE). The aim of this study is to systematically review the literature on WBE for studies trending normalized viral load of SARS-CoV-2 with disease indicators and to compare the effects observed at baseline with those that include a timelag. We also aim to pool results for studies reporting common effect sizes and outcomes to delineate study-level heterogeneity.

Key words: N/A

Standards and control materials used: N/A

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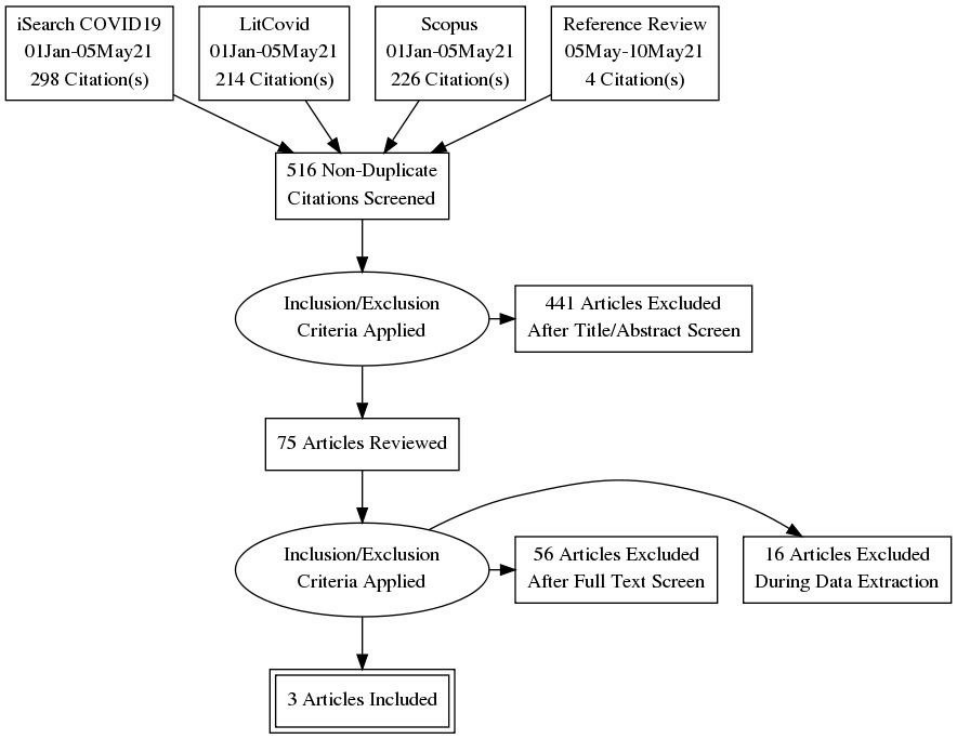
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441 Articles Excluded
After Title/Abstract Screen
75 Articles Reviewed
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Criteria Applied
56 Articles Excluded
After Full Text Screen
16 Articles Excluded
During Data Extraction
3 Articles Included

Nucleic Acid Extraction with Microbubbles from Wastewater Produces Simple and High-Yield Workflows

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The COVID-19 pandemic has highlighted the need for simple but sensitive technologies and simple workflows to enable environmental monitoring for communicable disease agents. Nearly everything about human behavior leaves a signal in urine, stool, or blood – this makes sewage an efficient pooled-sample source to monitor circulating infectious agents in a community. Novel approaches to surveillance will enable sensitive, specific and timely response to infections.

Akadeum's microbubbles are overcoming barriers to rare target capture from complex matrices such as wastewater utilizing an innovative approach: field-deployable, buoyant, functionalized microbubbles. In this presentation we demonstrate the utility and benefits of floatation-based capture reagents and associated workflows. Here proprietary microbubbles are specifically functionalized to capture nucleic acids. Unlike the inherent limitations of magnetic bead-based separation, microbubbles do not have the same volume and equipment restrictions. Using microbubbles for nucleic acid extraction employs a positive selection protocol in which the microbubbles bind to the desired target – in this case, total RNA including SARS-CoV-2 if present – isolating and enriching the analyte for downstream genomic analysis.

First demonstrated in saliva samples, the microbubbles were able to interrogate large fluidic volumes, which enables a pooled sample approach desirable in community surveillance of viral infection. In fact, microbubble extraction delivered 25x higher sensitivity in detecting SARS-CoV-2 RNA based on qPCR detection compared to a magnetic isolation kit. Translating this workflow to wastewater samples, the microbubble nucleic acid extraction can directly interrogate large fluidic volumes of unfiltered sewage samples to increase RNA capture for downstream processing. A sensitivity of at least 100 copies per mL was observed with a protocol that avoids preprocessing steps such as the removal of nucleic acid-rich solids.

Akadeum offers a simplified and cost-effective performance platform for the isolation of cells, proteins and nucleic acids that can be seamlessly integrated into existing workflows. Akadeum is seeking partnerships for applications of the nucleic acid microbubble in diagnostics and community surveillance including cell, protein and nucleic acid-based analyte enrichment for testing.

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	Ct		Copy #	
Condition	N Gene	ORF1ab	N Gene	ORF1ab
Naked RNA	30.7	36.3	2137	46.2

Figure 1. Akadeum microbubble-based RNA extraction technology enables a simplified workflow that avoids preprocessing cleaning steps such as the removal of solids. This increases sensitivity by retaining the RNA that would otherwise be lost by preprocessing. Here we demonstrate detection of covid RNA in a wastewater sample (n = 3) spiked with 100 cp/mL of Sars-CoV-2 RNA.

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Single Particle ICP-MS: A Powerful Tool for Characterization of Nanomaterials in Wastewater Surveillance

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Nanomaterials, defined as having at least one dimension falling in the range from 1 nm to 100 nm, have been identified as contaminants of emerging concern by the Environmental Protection Agency [1]. Similar to traditional chemical contaminants, some synthetic nanomaterials have been shown to be toxic to microbes, plants, and animals. The ever-increasing incorporation of nanomaterials into consumer products results in many routes of environmental exposure, including wastewater.

Analytical methods, critical for the detection and characterization of nanomaterials in wastewater, can address current gaps in knowledge. In particular, single particle inductively coupled plasma mass spectrometry (spICP-MS) is an emerging technique that enables measurements of size distributions and number concentrations of nanoparticles suspended in liquids. Special characteristics of spICP-MS that make it attractive for wastewater surveillance include extremely good nanoparticle detection capability down to truly environmentally relevant levels and extremely rapid analysis. The use of spICP-MS for wastewater surveillance has been outlined in recent reviews [2,3] and demonstrated in publications describing the detection and sizing of TiO_2 nanoparticles in municipal sewage treatment plants [4] and the size characterization of silver nanoparticles in wastewater [5,6]. However, the technique presents challenges and limitations that researchers have highlighted and are striving to overcome, *particularly with respect to the lack of reference materials and standardization of spICP-MS methodologies*. Provision of suitable reference materials and standard methods would enable widespread effective and efficient application of spICP-MS for wastewater surveillance to ensure that human health is not affected detrimentally by nanomaterials.

The goal of our presentation is to highlight a few examples of the work that has been done within the NIST Inorganic Chemical Metrology Group (646.01) toward the advancement of spICP-MS as a mature technique. This work has provided a foundation for developing the standards and reference materials that are necessary to enable routine wastewater surveillance for nanoparticles. In the past five years, our group has engaged in several activities to build a robust nanomaterial spICP-MS measurement infrastructure, including:

- validation of spICP-MS for routine characterization of gold nanoparticles of different sizes, coatings, and surface charge at environmentally relevant concentrations in water;
- analysis of silver and titanium dioxide nanoparticles in water;
- characterization of silicon dioxide food additive materials in aqueous media;
- the characterization of the uptake of gold nanoparticles in *Caenorhabditis elegans*; and
- the potential application of spICP-MS for the characterization of nanoplastics suspended in water.

Key words: Nanomaterials, single particle ICP-MS, environmentally relevant concentration, reference materials, standards.
Standards and control materials used: NIST RM 8012 and 8013 gold nanoparticles; NIST RM 8017 silver nanoparticles; NIST SRM 1898 TiO_2; gold, silver, silicon, and titanium ionic standard solution calibrants

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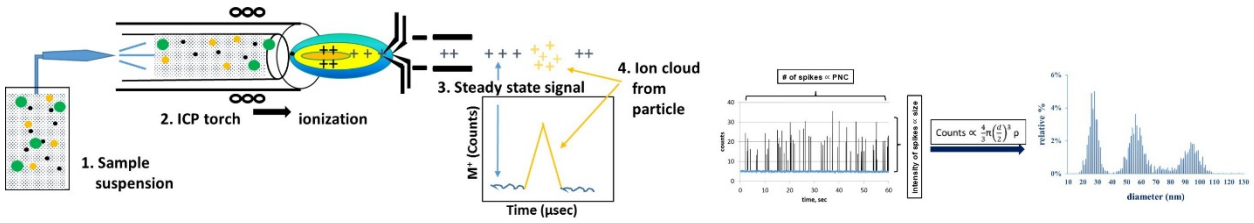

Figure 1. Schematic representation of spICP-MS operation principle and simultaneous information provided.

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[5] https://www.azonano.com/article.aspx?ArticleID=4232. Particle Size Measurements on Silver Nanoparticles in Waste Water with Single Particle ICP-MS.
[6] https://doi.org/10/1016/j.watres.2019.03.031 Incidence and persistence of silver nanoparticles throughout the wastewater treatment process.

A Direct Nucleic Acid Capture Method for Purification and Detection of SARS-CoV-2 Genetic Material from Wastewater

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Early in the COVID-19 pandemic, scientific studies demonstrated that the genetic material of SARS-CoV-2, an enveloped RNA virus, could be detected in wastewater. This finding mobilized investigation of whether wastewater-based epidemiology (WBE) monitoring the genetic signal of SARS-CoV-2 could be used to track the appearance and spread of COVID-19 in communities. The SARS-CoV-2 genetic signal is present at low concentrations in wastewater, making sample concentration a prerequisite for sensitive detection and utility in WBE. We hypothesized that a direct capture method that renders total nucleic acid conducive to binding to affinity resin may be able to overcome cumbersome, non-standardized, and variable viral concentration steps. This led to development of a simple, rapid, and modular alternative to existing purification methods from wastewater. In this approach, chaotropic agents are added to raw sewage allowing nucleic acid binding to a silica matrix. The captured nucleic acid is then washed to remove co-purifying PCR inhibitors, and then eluted with water. The eluted nucleic acid can then be further processed in a second step with either a spin column or using an automated nucleic acid purification system, like the Maxwell RSC®.

In parallel, we formulated RT-qPCR enzyme mixes that demonstrate resistance to PCR inhibitors commonly found in wastewater. RT-qPCR assays were developed to detect N1, N2 (nucleocapsid) and E (envelope) gene fragments of SARS-CoV-2 as part of multiplexed assays. Pepper Mild Mottle Virus (PMMoV), a fecal indicator RNA virus present in wastewater, and an exogenous inhibition control were included in all PCR reactions for quality control. The workflow has a limit of detection of 1.6 GC/ ml with 40 ml of raw wastewater sample. Using this workflow, we monitored wastewater samples from three WWTP in Dane County, Wisconsin, serving a combined population of over 300,000 people, for four months covering the peak of community prevalence. Our analysis was compared to the COVID-19 cases declared by the municipalities, demonstrating strong correlation between the SARS-CoV-2 viral load present in wastewater and clinical cases.

Key words: SARS-CoV-2, Wastewater, RT-qPCR, COVID-19, Direct Capture

Standards and control materials used: *Processing/Testing Methods*

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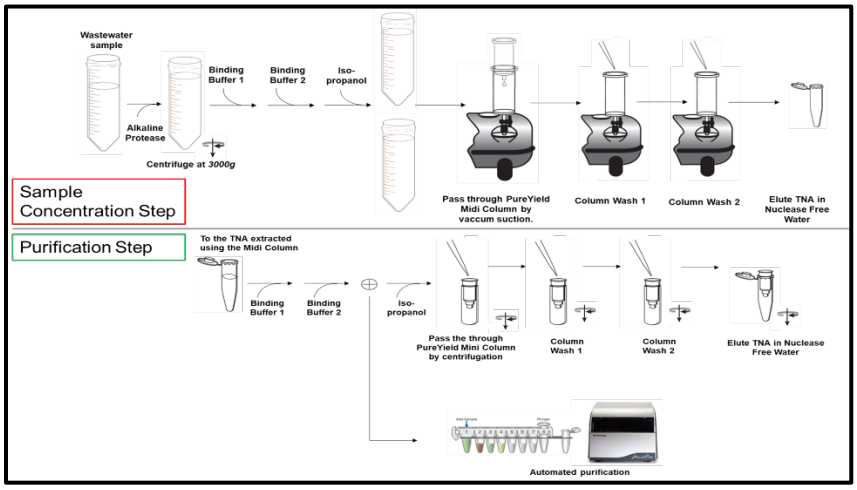

	20X SARS-CoV-2 Primer / Probe
SARS CoV-2	**N1** or **N2** or **E** forward primer
	N1 or **N2** or **E** reverse primer
	N1 or **N2** or **E** Probe (FAM)
Human fecal & Matrix recovery	**PMMoV** forward primer
	PMMoV reverse primer
	PMMoV Probe (Cy5)
Inhibition Assessment	**IAC** control forward primer
	IAC control reverse primer
	IAC Control Probe (HEX)
	IAC 435bp DNA-template
	100X CXR (ROX)

Figure 1. **Workflow of the direct nucleic acid capture method for purification and detection of SARS-CoV-2 genetic material from wastewater**. Image on the left details the direct capture and purification procedure. Table on right demonstrates components of multiplex RT-qPCR reaction.

Using municipal data to develop geographically-precise sampling frame standards

R. Yeager[1,2], R. H. Holm[1], K. Saurabh[3,4], D. Talley[7], A. Bhatnagar[1] and T. Smith[1]

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Wastewater monitoring for SARS-CoV-2 within communities can be utilized as a powerful to surveil and respond to the current COVID-19 pandemic, variants of concern, and future outbreaks with pandemic potential. However, there are currently no evidence-based and widely-accepted standards for urban sampling site identification and assessment. To inform development of such standards, we describe a protocol for developing a geographically-resolved wastewater sampling methodology in Jefferson County, Kentucky, and describe our preliminary results. We utilized this site selection protocol to identify 17 sample locations, based on municipal sewer data, representing distinct wastewater catchment areas. We collected samples (n = 285) at these locations from September 8 to October 30, 2020 from one to four times per week. We then compared SARS-CoV-2 testing results with contemporaneous and geographically-matched clinical testing-based COVID-19 rates. We found that SARS-CoV-2 RNA was consistently present in each catchment area with significant spatial and temporal variation. We also observed substantial differences between trends in wastewater-based detection and clinical-testing. These findings verify that our site selection protocol is an efficacious approach, which may be used to inform development of a standardized best-practice approach of geographically-resolved SARS-CoV-2 wastewater sample site selection in urban areas.

Key words: Wastewater, Geographic, GIS, sewershed, catchment

Standards and control materials used: *Municipal data and preliminary monitoring results*

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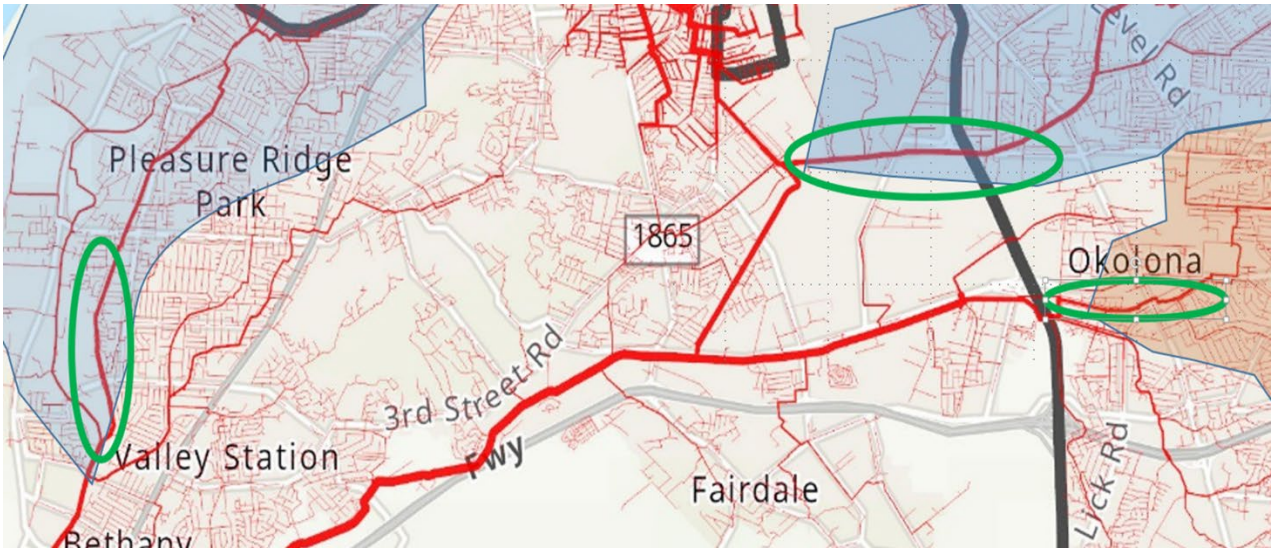

Figure 1. Visualization of geographically-resolved site selection process.

Rapid Capture of SARS-CoV-2 Using Nanotrap® Magnetic Virus Particles

Alex Barclay[1], Smruthi Karthikeyan[2], Ben Lepene[1], Patrick Andersen[1], Daniel Goldfarb[1], Kevin Kolb[1], Joshna Seelam[1], Tara Jones-Roe[1], Rob Knight[2], Robbie Barbero[1]
[1]Ceres Nanosciences, Inc.
[2]University of California San Diego

SARS-CoV-2 is the causative agent of COVID-19, a disease that has caused a worldwide pandemic and claimed over 3 million lives. SARS-CoV-2 is shed in feces, enabling wastewater-based surveillance of the disease, which can allow for monitoring of infection rates in communities as well as to help identify SARS-CoV-2 variants of concern that may occur in an area. Methods currently employed to isolate SARS-CoV-2 from wastewater are long, arduous, and are not suitable for high throughput testing. Here, we offer an alternative method for viral isolation, use of Ceres Nanosciences' Nanotrap® Magnetic Virus Particles, which capture and concentrate whole virus from wastewater. This new method gives comparable results to PEG/ultracentrifugation and HA filtration across a range of viral titers despite using 80% less initial volume than either while also reducing processing time by several hours. Here we demonstrate that this method is compatible with multiple viral RNA extraction kits and that it can readily be automated, allowing for high-throughput testing. Overall, the Nanotrap® Magnetic Virus Particles offer a shorter, more user-friendly workflow than competing virus isolation methods while maintaining comparable sensitivity.

Key words: viral enrichment, viral concentration, wastewater processing
Standards and control materials used: *Heat-inactivated SARS-CoV-2 (ATCC)*
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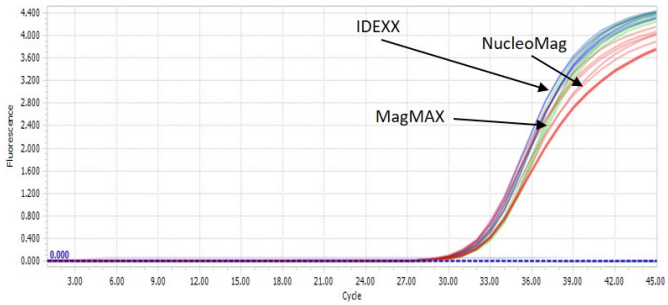

Sample	Ct
NT + IDEXX	29.56
NT+ MagMAX	29.95
NT+ NucleoMag	29.54

Figure 1. Wastewater spiked with heat-inactivated SARS-CoV-2 at 1000 copies/mL. Virus concentration performed using Nanotrap® Magnetic Virus Particles and compatibility with three standard RNA extraction kits demonstrated using the CDC nCoV-2019 N1 RT-PCR assay.

Equity in standardized collection of pooled community stool for pathogen detection: Moving from pit latrines to sewered systems globally

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Sustainable Development Goal (SDG) 3 covers health and 6 covers sanitation, and the COVID-19 pandemic has brought the research community interest in health monitoring using pooled community stool to the forefront. While 41% of the world's population uses a sewered connection, an equal 41% uses a non-sewered sanitation system such as septic tanks, pit latrines and other improved on-site systems (UNICEF and WHO, 2019). Both sewered and non-sewered systems can track pathogens (Capone et al., 2021; Yeager et al., 2021). Yet, sample collection standards for both sewered and non-sewered systems use similar equipment and human capacity; though existing pit latrine sampling utilizing fecal sludge management approaches for pathogen detection are more similar than wastewater sampling approaches for environmental compliance. The pandemic has brought more equitable sharing of sample collection methods globally through avenues such as the National Science Foundation Research Coordination Network webinar series, a wastewater focused Slack channel, and journals waiving publication fees for COVID-19 research. The pandemic has also brought attention to value new professionals entering the sanitation field, where researchers from the global south with experience looking at pathogen detection in pit latrines have unique experience to share with researchers pivoting other research interests (including graduate students, post-docs and early career professionals) in the global north. Post-pandemic, instead of a 'brain drain' of sanitation professionals leaving the global south there could be greater incentive to continue their health monitoring using pooled community stool. The equity in pooled community stools for pathogen detection has uniquely brought about collaboration and cooperation during the pandemic for standardized sample collection that can only help global sanitation capacity building with practical skills across the global south and north to meet the SDGs and support a better post-pandemic world.

Key words: sample collection; capacity building; pit latrines; sewered sanitation systems

Standards and control materials used: Sample collection standards for both sewered and non-sewered systems use similar equipment and human capacity.

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Figure 1. Sample collection methods for pathogens from pit latrines (left) and sewered systems (right).

First detection of SARS-CoV-2 RNA in wastewater in North America: A study in Louisiana, USA

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We investigated the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA in wastewater samples in southern Louisiana, USA. Untreated and treated wastewater samples were collected on five occasions over a four-month period from January to April 2020. The wastewater samples were concentrated via ultrafiltration (Method A), and an adsorption–elution method using electronegative membranes (Method B). SARS-CoV-2 RNA was detected in 2 out of 15 wastewater samples using two reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays (CDC N1 and N2). None of the secondary treated and final effluent samples tested positive for SARS-CoV-2 RNA. To our knowledge, this is the first study reporting the detection of SARS-CoV-2 RNA in wastewater in North America, including the USA. However, concentration methods and RT-qPCR assays need to be refined and validated to increase the sensitivity of SARS-CoV-2 RNA detection in wastewater.

Keywords
Wastewater-based epidemiology
SARS-CoV-2
Surveillance
COVID-19
RT-qPCR
Wastewater

Standards and control materials used: *phi6*

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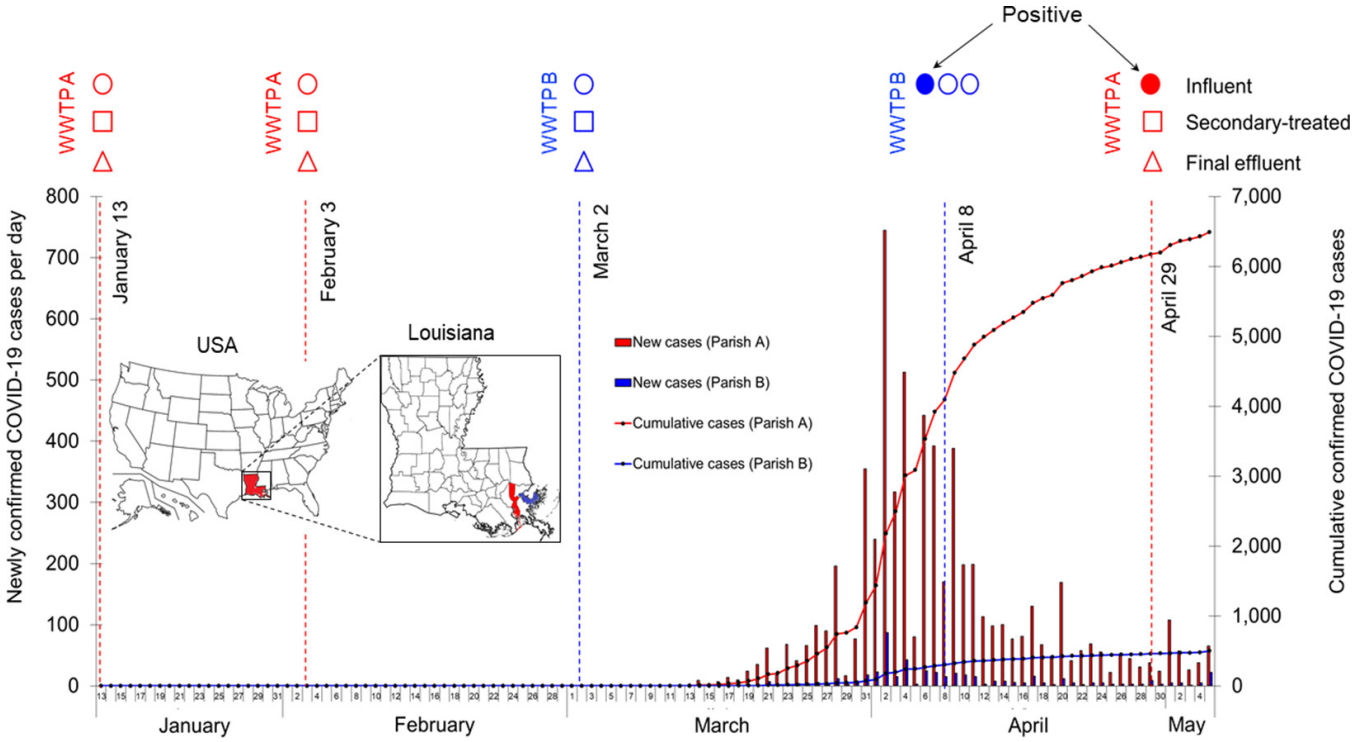

Fig. 1. SARS-CoV-2 RNA detection in wastewater and confirmed COVID-19 cases in southern Louisiana, USA. Circles, squares, and triangles represent sample types, i.e.,influent, secondary-treated, and final effluent, respectively. Red and blue symbols represent samples collected from WWTPs A and B, respectively. Closed and open symbols denote positive and negative SARS-CoV-2 RNA detections, respectively.

Whole Cell Reference Materials to Assess Microbial Detection in Wastewater

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Wastewater surveillance (WWS) holds great promise for monitoring public health concerns such as emerging pathogens (e.g., SARS-CoV-2), microbes (e.g., enteric bacterial pathogens), or antimicrobial resistance (AMR) genes. For example, a global effort is underway to monitor wastewater for AMR genes to assess the extent of AMR in communities.[1] When detecting microbes or their genes in wastewater, it is challenging to compare protocols and assess overall process efficiency due to high variability in samples and workflows across the country. Highly characterized whole cell reference materials (RMs) can normalize analytical workflows, enable method comparison, and challenge the entire process from sample collection to data analysis, ultimately increasing confidence in results. NIST is developing two viable whole cell RMs as surrogates for microbial (bacterial or fungal) targets of interest to assess workflow parameters, such as sampling efficiency, DNA extraction efficiency, limit of detection, and bioinformatics pipelines. The first consists of *Escherichia coli* NIST0056 cells characterized for total and viable cell number and genome copies/cell using flow cytometry and optical microscopy. The second, NIST RM 8230–*Saccharomyces cerevisiae* NE095 (target date Jan 2022), is characterized for total cell number and colony forming units and engineered to contain a noncoding chromosomal DNA target. This target eliminates false positives from near neighbors and can serve as a surrogate for AMR genes in molecular detection methods. These existing RMs could be applied directly to WWS or our capabilities could be translated to develop RMs using other relevant microorganisms. One could envision a whole cell RM based on non-replicating bacteria or fungi to safely spike into any point of the WWS workflow at a known concentration. RMs such as this could serve as internal references to assess the overall process, including operators, technologies, and protocols, or as tools for training, method comparison, or risk management exercises. These current and potential future whole cell microbial RMs could support WWS capabilities and ultimately improve assurance in the results provided to public health decision makers.

Keywords: antimicrobial resistance, *Escherichia coli*, flow cytometry, genome copy number, microbial detection, reference material, *Saccharomyces cerevisiae*, viable

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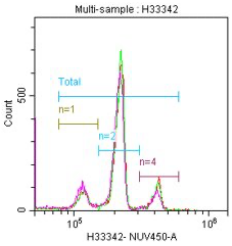

This *E. coli* RM under development will be characterized for total number of cells and genome copies/cell, two parameters that challenge our ability to confidently quantify microbial systems. Viability will also be reported. The characterization is done using flow cytometry (shown, where n is genome copy number) and optical microscopy. The final RM will be lyophilized pellets.

[1] Hendriksen, R.S., Munk, P., Njage, P. *et al.* Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat Commun* **10,** 1124 (2019). https://doi.org/10.1038/s41467-019-08853-3

NIST SRM 2917: A Plasmid DNA Standard for Molecular Recreational Water Quality Testing

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Contamination of waterways with fecal material can lead to the spread of pathogens that can have serious impacts on human and environmental health. The U.S. Environmental Protection Agency (EPA) is responsible for protecting human and environmental health through the safeguard of recreational waters. Traditional monitoring practices involve time consuming cultivation procedures which can delay decision making. To accelerate this process molecular techniques, specifically quantitative polymerase chain reaction (qPCR) methods, for identifying host-associated genetic markers of fecal pollution and quantifying fecal indicator bacteria have been developed at EPA and by other researchers. National implementation of these methods requires rigorous proficiency testing by state and local public health agencies, as well as commercial entities that hope to adopt these methods. In a collaborative effort to support the adoption of these molecular methods, the EPA and NIST developed a plasmid DNA Standard Reference Material (NIST SRM 2917) for the purpose of implementing select qPCR assays for recreational water quality monitoring applications (figure 1). The material consists of 6 Levels of a linearized plasmid DNA containing 13 single-copy PCR targets selected by the EPA. The vector consists of a standard pUC plasmid with an ampicillin resistance gene, an origin of replication, and M13 universal priming sites flanking the target genetic marker construct. Dilution levels span approximately 5 to 500,000 plasmid copies per µL. Each tube of material contains approximately 200 µL of plasmid DNA in TE buffer (pH 8.0) with 10 ng/µL of RNA stabilizer (yeast tRNA) in a 1.5 mL low retention microcentrifuge vial. The material is stored at 4 °C and should not be frozen. Approximately 1,000 units were generated. The material was characterized by NIST using droplet digital PCR to establish homogeneity and stability of the material as well as assign an absolute copy number to each dilution level.

Key words: Reference material, qPCR, ddPCR, molecular detection methods, fecal indicator bacteria

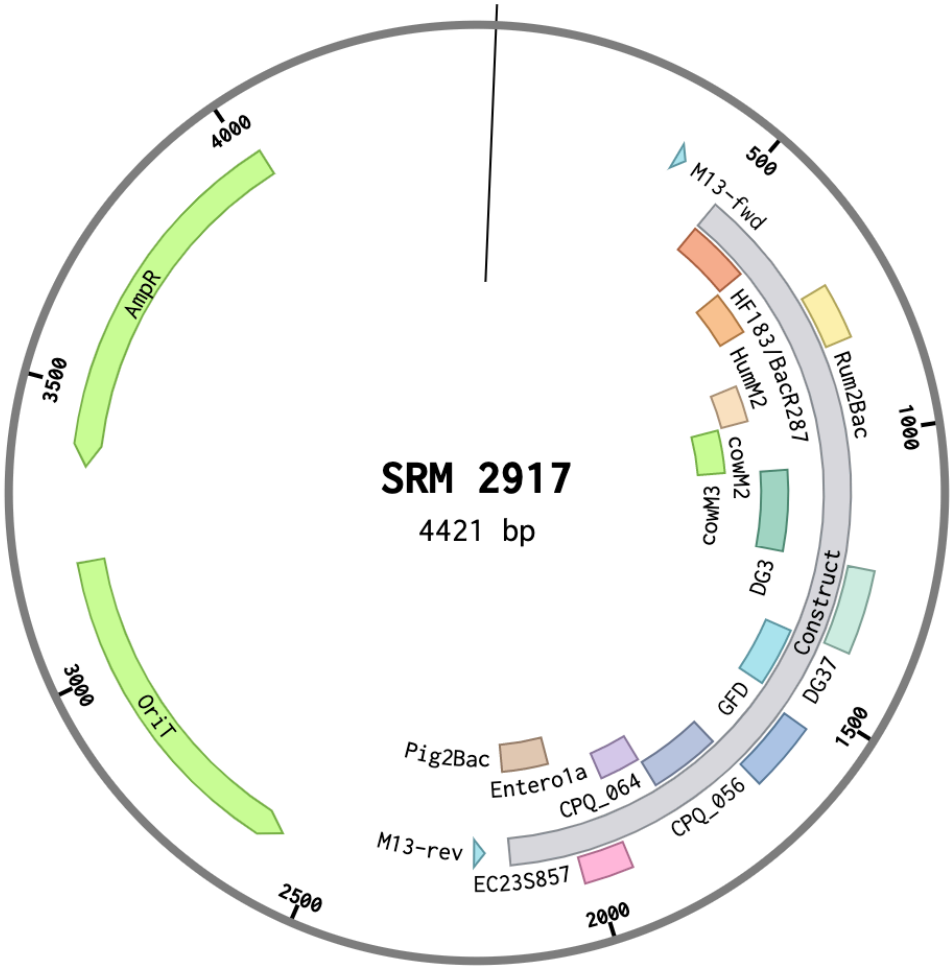

Figure 1. The 1.7 kb construct containing the 13 targets was synthesized *de novo* using the IDT gBlock technology. The 13 targets include genetic markers for human (HF183/BacR287, HumM2, CPQ_056, CPQ_064), ruminant (Rum2Bac), pig (Pig2Bac), cattle (CowM2, CowM3), dog (DG3, DG37), avian (GFD), Enterococcus (Entero1a), and *E. coli* (EC23S857) pollution sources for fecal indicator bacteria. The gBlock was then inserted into a pUCIDT vector (2752 bp) carrying an AMP selection marker (AMP^R) and an origin or replication (ORI) to produce the 4421 bp plasmid (shown circular, but final SRM is linearized).

Development *of RNA Standards for Quantification of SARS-CoV-2 and PMMoV by RT-qPCR for Wastewater Surveillance*

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Over the course of the COVID-19 pandemic, wastewater surveillance has emerged as a promising tool to monitor spread of the virus in a community. Wastewater is a complex matrix, so analytical methods like RT-qPCR needs to be designed with appropriate controls to quantitate and normalize viral levels for trend analysis. In this work we describe methods for designing and manufacture of quantification standards for SARS-CoV-2 and Pepper Mild Mottle Virus (PMMoV) RNA. PMMoV is a plant virus with a ssRNA genome and is a well-documented human fecal indicator. PMMoV RNA also serves as a useful process control and normalization tool for wastewater surveillance.

To generate quantitative standards, we first cloned the Envelope and Nucleocapsid (E/N) genes of SARS-CoV2 in pGEM3z vector. Likewise, a 362bp fragment of the PMMoV genome consisting the RT-qPCR target is also cloned in a pGEM3z vector. The two plasmids are then linearized by XbaI and in-vitro transcribed to generate RNA transcripts. Any remaining DNA in the reaction is eliminated by DNase treatment. RNA was then quantified with a fluorescent RNA dye (QuantiFluor RNA System) and by droplet digital PCR (ddPCR). The relationship of quantity of RNA (in pg) corresponding to copy number as determined by ddPCR is established. The RNA was then diluted at 4 million copies/ul corresponding to 20pg/ul and 7.5pg/ul for the SARS-CoV-2 (E/N) and PMMoV RNA respectively. Both the RNA was then tested with the primes/probe set prescribed by the US Centers for Disease Control (CDC) that target the nucleocapsid (N1 and N2) gene, or the envelope (E) gene of the SARS-CoV2 RNA and primers/probes for PMMoV for the PMMoV RNA. The quantitation standards were stored at -20°C and subjected to ten freeze-thaw cycles. No decrease in Ct values was observed implying that the quantification standards were stable. The standards were used to quantify SARS-CoV-2 and PMMoV RNA from three wastewater treatment plants in Dane county, WI. Normalization of SARS-CoV-2 RNA with PMMoV RNA resulted in better correlation with 7-moving average of new clinical cases a municipality served by a treatment plant.

Key words: RT-qPCR, quantitation standards, in-vitro transcribed RNA

Standards and control materials used: *In-vitro transcribed RNA*

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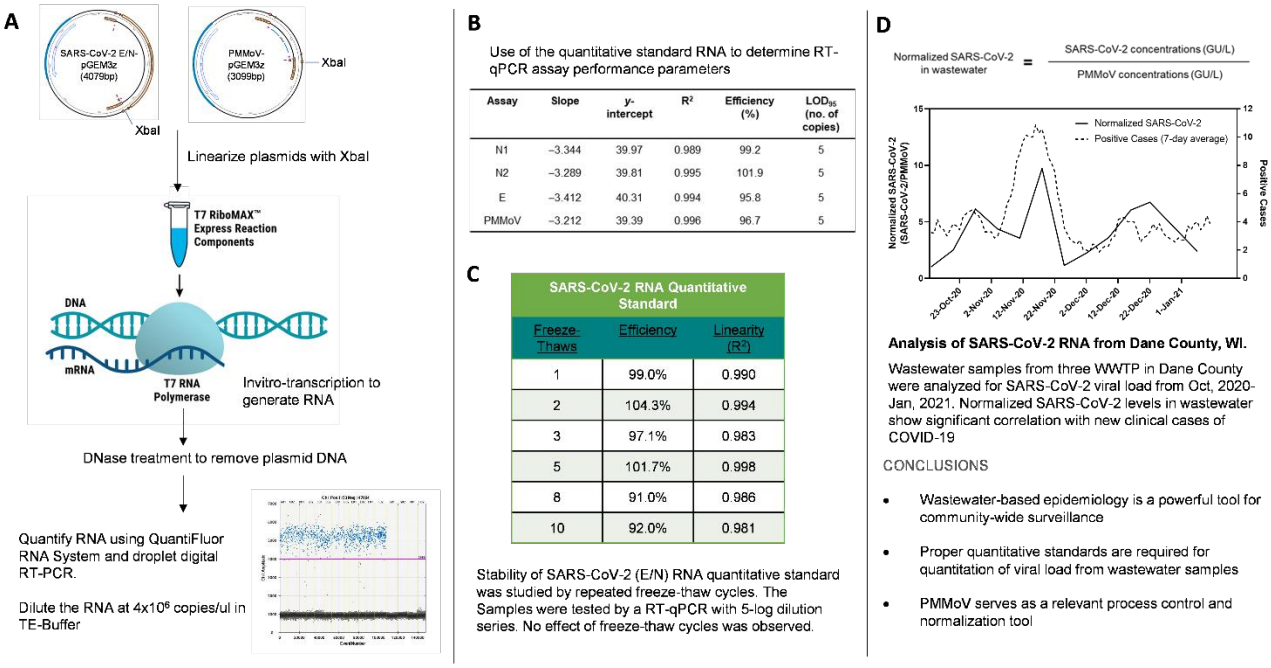

Figure 1. Development of RNA standards for quantification of SARS-CoV-2 and PMMoV by RT-qPCR for Wastewater Surveillance

Assessing Community Acceptance: Wastewater Monitoring Community Survey (WMCS)

Ted Smith[1], Kandi L. Walker[2], Joy L. Hart[2], Rochelle H. Holm[1], Rachel Keith[1], Lauren Anderson[1], Lauren Heberle[3], Dan Riggs[1], and Aruni Bhatnagar[1]

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Community wastewater monitoring can be a powerful early warning tool to protect public health. However, given the wide range of possible implementations, and the situational interpretation of the medical ethics issues present when conducted in a non-regulatory fashion, standards development should include a public perspective. The University of Louisville has developed a straightforward instrument to quickly assess both awareness and acceptance of wastewater monitoring, specifically focused on COVID-19 wastewater surveillance. We propose that standards development and implementation adopt a common feedback approach nationally which could inform regional sensitivities to public health surveillance acceptance as well as strategies for standard approaches to implementation. This seven-item questionnaire includes three items that assess awareness of this type of public health surveillance and three items that focus on key aspects known to affect acceptance of specific implementations. For example, the instrument measures the importance of data sharing with the public and the size of catchment areas that a community member is comfortable having sampled. The instrument is being evaluated as a component of a larger public health community trial which will allow comparison with other data sources and present opportunities to increase its predictive validity.

Key words: Wastewater Surveillance; Ethics; Community Feedback.

Standards and control materials used: *NONE*

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University of Louisville Wastewater Monitoring Community Survey (WMCS)

1) Can the coronavirus that causes COVID-19 be detected in the city sewer system?
   Yes No I Don't know

2) Did you know that the amounts of the COVID virus in sewers reflect the general level of infection in the community ? Yes No

3) Did you know that UofL is working with Louisville Metropolitan Sewer District (MSD) to test whether measurements of coronavirus in wastewater could be used to determine the risk of COVID-19 across Louisville?
   Yes No

4) On a scale of 1 to 7, how much do you support monitoring sewage to better understand COVID infection levels in our community instead of only testing people?

1	2	3	4	5	6	7
Very Supportive	Moderately Supportive	Supportive	Indifferent	Opposed	Moderately Opposed	Very Opposed

5) On a scale of 1 to 7, how important is it to share the results of wastewater testing with the public?

1	2	3	4	5	6	7
Very Important	Moderately Important	Important	Neutral	Unimportant	Moderately Unimportant	Very Unimportant

6) Measuring at different sewer locations can help identify patterns of infection for different sized areas. Measuring coronavirus in samples from manholes or other equipment can help scientist understand what is happening in different areas of the city or even different neighborhoods. Please tell us which statement best describes the smallest number of households you support being measured:

1	2	3	4	5
Support Measuring Largest Areas (>50,000 households)	Support Measuring Smaller Sections (>30,000 households)	Support Measuring Neighborhoods (>5,000 households)	Neither Support or Oppose	Oppose Measuring Any Size

7) Optional: Please share any other information you'd like about your views on monitoring sewers for signs of COVID.

Figure 1. Wastewater Monitoring Community Survey (WMCS)

Comparison of Sampling Frequency and Concentration Methodology for the Detection and Quantification of SARS-CoV-2 in Wastewater

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Wastewater-based epidemiology (WBE) has been implemented across the world to track the COVID-19 disease burden of a community through the quantification of SARS-CoV-2 (the virus responsible for COVID-19) in wastewater samples. Due to the rapid implementation of WBE programs in combination with supply chain issues and varying budget constraints, many different protocols for sampling and analysis have been developed. However, the effects of these various techniques on the interpretation of the reported detection and quantification of SARS-CoV-2 is limited. To that end, studies were conducted to evaluate a variety of sampling and viral concentration techniques. Two of the most common sampling techniques utilized for WBE, grab and composite sampling, were evaluated by comparing the SARS-CoV-2 concentrations in 24 hourly grab samples with their associated 24-h composite sample at four sewershed scales (city, neighborhood, city block and individual building). Additionally, the effects of sampling frequency on reported SARS-CoV-2 composite concentrations were evaluated at a low-flow building site with sampling frequency varying from 5 min to 1 h. The results indicated that composite sampling should be utilized at every sewershed scale. However, at the city scale (*e.g.* wastewater treatment plant influent), grab samples may be acceptable if only attempting to determine the presence or absence of SARS-CoV-2. At the scale of city block and individual buildings, high-frequency composite sampling (*e.g.* 15 min sampling) is required to capture the temporal variation in viral signal. Another critical step to optimize is the concentration of SARS-CoV-2 from wastewater. Six common WBE concentration methods were compared by quantifying the SARS-CoV-2 concentration from the same wastewater treatment plant influent sample. The best relative recovery was found in the methods that utilized electronegative membrane filtration. To supplement this knowledge, the different solid and liquid fractions of an influent were separated into the individual components (*i.e.,* liquid, settleable solids and non-settleable solids) to evaluate where SARS-CoV-2 RNA was partitioned in the wastewater. Of the SARS-CoV-2 captured by the electronegative membrane, roughly 90% was found in the non-settleable solid fraction. Thus, future optimization of concentration methods should focus on retaining the SARS-CoV-2 RNA from the non-settleable solids fraction of wastewater.

Key words: SARS-CoV-2; Wastewater-based Epidemiology; Composite Sample; Grab Sample; Concentration; Frequency; Filtration; Non-settleable Solids

Standards and control materials used: Positive SARS-CoV-2 controls containing the E, N, ORF1ab, RdRP, and S genes and human RNAse P RNA (EDX SARS-CoV-2 Standard, Exact Diagnostics, Fort Worth, TX) and negative controls containing certified SARS-CoV-2-free human RNAse P RNA (EDX SARS-CoV-2 Negative, Exact Diagnostics, Fort Worth, TX) were included in each extraction plate. Extraction blanks of phosphate buffered saline (PBS) were included with every run as an extraction

contamination control. Bovine coronavirus (Bovine Rotavirus-Coronavirus Vaccine from Zoetis, NJ, USA), was selected as a process recovery control due to its morphological and structural similarity to SARS-CoV-2.

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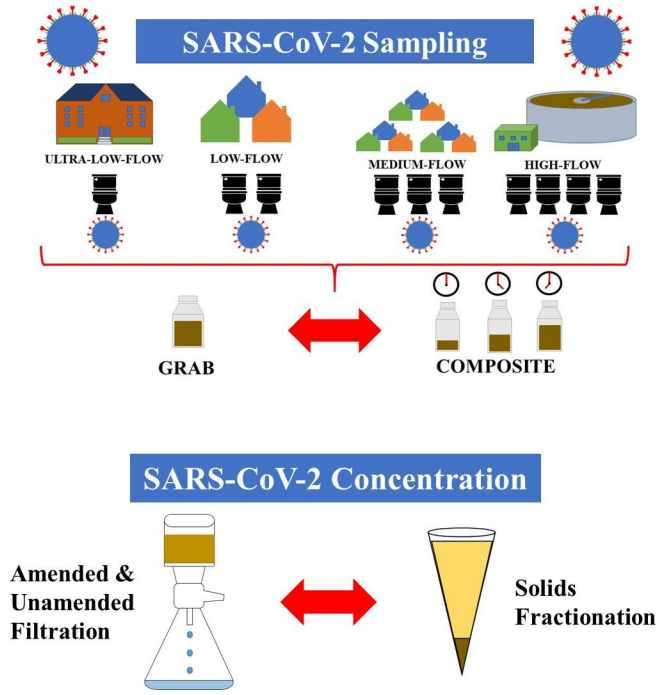

Figure 1. Overview of SARS-CoV-2 sampling frequency and SARS-CoV-2 concentration analyses

PiGx SARS-CoV-2 wastewater: A pipeline for reproducible wastewater sequencing analysis providing comprehensible geo-tagged time series reports

Authors: Altuna Akalin[1], Vic-Fabienne Schumann[1], Ricardo Wurmus[1], Miriam Faxel[1], Jan Dohmen[1], Rafael Cuadrat[1]

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Setting up monitoring systems for the development of SARS-CoV-2 through wastewater sampling has become a crucial mission for many nations all over the world [1],[2] . Developing protocols for comparable sampling and sequencing on the one side but also developing robust and scalable analysis tools on the other side remains an ongoing challenge so far.

We present the **PiGx SARS-CoV-2 wastewater** sequencing pipeline [3] , a Bit by Bit reproducible pipeline using GuixHpC [4] which provides reports that are intuitive to use and easy to interpret. They combine visualisation, access to quality control reports and downloadable data tables for sharing and further processing. They grant geo-tagged visual time series overviews over the dynamics of single mutations and abundances of given variants of concern (VOC) which were derived by deconvolution. Under development right now are features in order to identify untracked mutations that show significant increase over time and also to track the relative abundance of SARS-CoV-2 in wastewater samples.

We aim to collaborate with many groups working with different strategies in order to make this pipeline as easily accessible as possible and applicable for data from various sequencing protocols. With **PiGx SARS-CoV-2 wastewater** we aim to develop a tool to support combined and global research efforts and make wastewater sequencing data comparable, reportable and reproducible.

[1] www.cdc.gov, “Developing a wastewater surveillance sampling strategy” (accessed: 21/06/06)

[2] www.dw.com, “Eu states must monitor sewage systems for covid” (accessed: 21/06/06)

[3] https://github.com/BIMSBbioinfo/pigx_sarscov2_ww (accessed: 21/06/06)

[4] https://hpc.guix.info/, (accessed: 21/06/06)

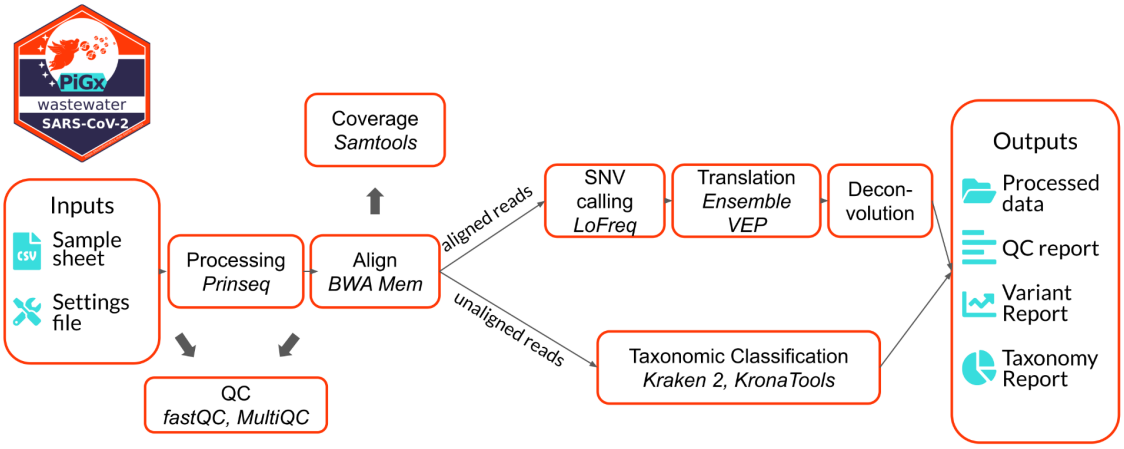

Figure 1: Workflow diagram of the PiGx SARS-CoV-2 wastewater sequencing pipeline