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Evaluation of intra- and inter-lab variability in quantifying SARS-CoV-2 in a state-wide wastewater monitoring network†

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Abstract

In December 2019, SARS-CoV-2, the virus that causes coronavirus disease 2019, was first reported and subsequently triggered a global pandemic. Wastewater monitoring, a strategy for quantifying viral gene concentrations from wastewater influents within a community, has served as an early warning and management tool for the spread of SARS-CoV-2 in a community. Ohio built a collaborative statewide wastewater monitoring network that is supported by eight labs (university, government, and commercial laboratories) with unique sample processing workflows.

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consequently, we sought to characterize the variability in wastewater monitoring results for network labs. Across seven trials between October 2020 and November 2021, eight participating labs successfully quantified two SARS-CoV-2 RNA targets and human fecal indicator virus targets in wastewater sample aliquots with reproducible results, although recovery efficiencies of spiked surrogates ranged from 3 to 75%. When SARS-CoV-2 gene fragment concentrations were adjusted for recovery efficiency and flow, the proportion of variance between laboratories was minimized, serving as the best model to account for between-lab variance. Another adjustment factor (alone and in different combinations with the above factors) considered to account for sample and measurement variability includes fecal marker normalization. Genetic quantification variability can be attributed to many factors, including the methods, individual samples, and water quality parameters. In addition, statistically significant correlations were observed between SARS-CoV-2 RNA and COVID-19 case numbers, supporting the notion that wastewater surveillance continues to serve as an effective monitoring tool. This study serves as a real-time example of multi-laboratory collaboration for public health preparedness for infectious diseases.

1. Introduction

In December 2019, a virus was reported to cause pneumonia in Wuhan, Hubei Province, China, and has since resulted in a global pandemic, declared by the World Health Organization (WHO).[1] Since this discovery, the virus has been named the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing the coronavirus disease 2019 (COVID-19). As of September 2022, the virus has caused over 600 million infections and caused the death of over 6.5 million people globally.[2] According to the Centers for Disease Control and Prevention (CDC), in the United States alone, there have been more than 95 million cases reported, over 1 million deaths, and the current average 7 day case rate per 100 000 people is 150 (as of September 2022), down from the January 2022 peak of 1700 cases. Some of the highest case peaks over the last two years, particularly witnessed in the U.S., were associated with emerging variants of concern.

Clinical testing has been an important tool to identify infected individuals to isolate them from the uninfected population and therefore limit the spread of SARS-CoV-2. However, implementation of widespread and effective clinical testing strategies continues to be challenging, due to limited access to and availability of tests in some communities. Additionally, a large portion of viral carriers are asymptomatic. Early in the pandemic, it was confirmed that viral fecal shedding occurred in many COVID-19-positive patients.[3] Thus, wastewater became a matrix of interest to understand SARS-CoV-2 prevalence at a population level separate from clinical case testing.[4,5] One commonly applied approach has been wastewater-based surveillance, where samples are analyzed for viral genetic material from wastewater plant influents, upstream in the conveyance system accessed through manholes, or for environmental waters and open sewer areas that lack wastewater infrastructure.[6,7]

Viral RNA signal change in wastewater usually can be seen prior to changes seen in clinically confirmed cases, but can vary by community based on clinical testing capacity or utilization and data reporting turn-around time.[6,8,9] This approach can serve as a

crucial early indicator for COVID-19 trend increases and detection of variants of concern so that state and local agencies, health officials, and stakeholders can prioritize active intervention and invest resources in a timely manner.[10] In addition, wastewater surveillance can provide critical data in locations where medical screening and testing is limited, including smaller communities and during peak case times.[11–13] One study suggests that wastewater surveillance for the current pandemic is not only cost-effective in comparison to clinical testing (0.014% of total clinical testing costs), but is capable of theoretically compiling a health profile consisting of approximately 2.1 billion people over 105 600 operating wastewater treatment plants globally.[11] In addition, this approach was successfully used previously for population monitoring and warning of outbreaks, and/or reduction of illness caused by polioviruses, noroviruses, hepatitis A and E viruses, and many other pathogenic viruses around the world.[14–17]

Ohio's Governor Mike DeWine initiated the development of the Ohio Coronavirus Wastewater Monitoring Network (OCWMN) in May 2020. This network, led by the Ohio Department of Health and Ohio Water Resources Center, consists of various universities, government agencies, and commercial entities, to monitor trends in community viral loads by studying wastewater samples twice a week from over 70 state wastewater treatment plants.[18] To establish statewide wastewater monitoring in a short time frame, the State of Ohio leveraged local researchers' expertise. Two university laboratories (A, D) and the participating government agency (E) were conducting wastewater monitoring for SARS-CoV-2 in Ohio wastewater prior to network initiation, while the other laboratories (B, C, F, G, and H) were recruited for the project based on their location, expertise, and research capacity. The distributed network approach allowed for continued innovation in method development, assured that research results and experts were available to local communities for results interpretation, and shortened turnaround time by locating analytical laboratories close to sampling points. Within this network, researchers perform wastewater monitoring validation trials every two months to assess the variability in results generated by the labs quantifying SARS-CoV-2 genetic targets. These regular performance assessments evaluate the reliability and reproducibility of data resulting from varied analytical protocols used for public health interpretations and actions.[19,20]

For wastewater surveillance to be used successfully as a COVID-19 trend indicator, community monitoring tool, and a source for identifying variants of concern, it is critical to have rapid, practical, and reliable methods in wastewater sample processing (*i.e.,* concentration of viruses), isolation of viral genetic material (*i.e.,* RNA/DNA extraction) and quantification of the target viral genes (*i.e.,* reverse transcription and quantitative polymerase chain reaction (PCR)). More than two years later, there is no standard method available and many researchers around the world have reported various combinations of methods for detecting SARS-CoV-2 genetic signals from wastewater samples.[19–33] For networks (such as the OCWMN) where statewide data contributes to disease monitoring for state public health departments, it is important to minimize between-laboratory variance, as well as sample and measurement variability, wherever possible. Typically seen in clinical studies, one way of assessing interlaboratory differences and determining reproducibility is through simple random effects models.[34–37] These models can then produce interclass correlation coefficients for determining the proportion of variance within the study.[38,39] Here, crossed

random effects model analyses assist in understanding some factors that contribute to the observed variance.

This study describes the results from validation trials implemented within the OCWMN every two months to assess intra- and inter-lab variability within the data among the analytical labs. On seven occasions, each OCWMN laboratory received an aliquot of a composite wastewater influent sample, analyzed the sample using their respective procedures and reported concentrations of SARS-CoV-2 RNA, a viral fecal indicator, an assessment of RT-PCR inhibition, and the recovery efficiency of a spiked coronavirus surrogate. SARS-CoV-2 concentrations were assessed within and among labs *via* variance analysis to assess possible factors contributing to the variability in estimates. SARS-CoV-2 concentrations were also compared to COVID-19 case data retrieved from the corresponding sewersheds, supporting claims that wastewater surveillance is an effective tool for COVID-19 trend tracking. Available water quality parameter and fecal viral indicator data were also correlated to concentrations of SARS-CoV-2 RNA. We discuss the implications of the observed variability and provide recommendations for improvement.

2. Materials and methods

2.1. Participating labs and sample collection

The laboratories participating (hereafter labeled A–H) in the seven trials were university laboratories across the state, one government agency laboratory in Ohio and one commercial lab outside of Ohio. Laboratory participation varied across all trials, with lowest participation of three laboratories to highest participation of all eight. Seven trials were completed from 10/5/2020–9/19/2021. Different wastewater samples from across the state were provided for this study, one sample for each trial (Table 1). Wastewater samples used across the seven trials came from various cities across the State of Ohio, varying in both population size and wastewater treatment plant (WWTP) service size. All samples were collected by WWTP staff as a 24 hour flow or time composite sample from the influent water. Once removed from the composite sampler, the sample was kept at 4 °C and then transported in a sterile 10 L carboy the same day on ice to the local participating host lab within the network. For each trial, the provided sample was divided into 0.5–1.0 L aliquots and shipped on ice overnight to each participating laboratory. Samples were received by each lab the following day by 1:00 PM and processed. The sample aliquot for the participating host lab was kept at 4 °C overnight and processed the following day at 1:00 PM, to simulate similar processing start times for all participating laboratories in the network.

2.2. Sample processing

Each laboratory used different combinations of methods for water sample processing, nucleic acid extraction, and genetic fragment quantification (Fig. 1). Briefly, seven laboratories (excluding F) employed a pre-treatment to the sample, amending the sample with detergents, chemicals or buffers, or adjusted sample pH. Six of these laboratories (A, B, C, D, E, and H) completed a solid separation step *via* a combination of low-speed centrifugation and filtration steps. Subsequently, Lab A conducted viral capture using hollow

fiber concentration methods and laboratories B–E used membrane filtration. The remaining two laboratories (F, G) did not include a solid separation step but completed homogenization and viral capture from the raw water using membrane filtration steps. Lab H did not employ a specific viral capture step following solid separation (adding centrifuged water directly into a Promega kit for nucleic acid extraction). Each lab processed the provided sample in duplicate, with total sample processing volumes ranging from 40 to 225 mL. The detailed description of each method is provided (ESI† Methods).

2.3. Nucleic acid extractions

Following water sample processing, various protocols were used for DNA and RNA extractions. Lab A used the viral eluent as the input to a RNeasy PowerMicrobiome kit (QIAGEN, Germantown, MD). Two laboratories (B, D) processed the pellet and membrane filters from the solid separation and viral capture steps together using either the AllPrep DNA/RNA kit or the RNeasy PowerWater kit (QIAGEN). Lab G processed only a membrane filter using the AllPrep PowerViral DNA/RNA kit (QIAGEN). All QIAGEN extraction protocols that processed a membrane or pellet employed an initial bead beating/rupturing protocol. Lab C combined the pellet and filters from the solid separation step as their input into a TRIzol Plus RNA Purification kit (ThermoFisher Scientific, Waltham, MA). Lab F also processed their membrane filters using a TRIzol extraction protocol. Another lab (E) used a combination of pellet and filters from their solid separation and viral capture steps as the direct input into the RNeasy PowerWater kit (QIAGEN) for Trial 1. Due to supply chain challenges, these same inputs were added into a TRIzol extraction protocol for the remaining trials. Lastly, Lab H used a different total extraction method, combining and adding pellet and eluent fractions into the Wizard Enviro Total Nucleic Acid kit (Promega, Madison, WI). Groups that collected liquid and solid fractions during sample processing combined their fractions during DNA/RNA extraction. For genetic analyses described below, only a single sample of combined nucleic acids were used.

2.4. Target gene quantification

To quantify SARS-CoV-2 gene fragments, reverse transcription (RT) of the RNA into cDNA is required prior to PCR analysis, either in a one-step or two-step process. For gene quantification analyses, six laboratories (B, C, D, F, G, H) employed real-time quantitative PCR (qPCR) and two laboratories (A, E) used droplet digital PCR (ddPCR). RT-PCR was conducted in either duplicate or triplicate using published primers and probes (ESI† Table S1). SARS-CoV-2 concentrations were assessed by targeting either the N1, N2, or both regions of the nucleocapsid (N) gene. Gene quantification details, including limits of detection, limits of quantification, and PCR inhibition status were determined and reported by each individual lab (ESI† Table S2). When PCR inhibition was observed, samples were diluted and remeasured to report uninhibited results or percent inhibition was taken into consideration for calculation when the samples were not re-measured (see ESI† for each lab's method).

During the seven trials, human fecal viral indicators, either the DNA virus cross-assembly phage (crAssphage) or the RNA virus pepper mild mottle virus (PMMoV), were also quantified. These ubiquitous viruses have been used as both a process control and a

normalization tool for SARS-CoV-2 genes in wastewater to account for both dilution (*e.g.* by precipitation) and varying fecal loads.[40–45] Two of the eight labs (F, H) quantified PMMoV for this purpose, and the remaining labs (A–E, G) quantified crAssphage.

Recovery efficiencies were also calculated by each laboratory by spiking a surrogate coronavirus control into the raw wastewater sample prior to water sample processing and gene quantifications. The participating laboratories used a range of matrix spikes available for this test, such as bovine coronavirus (BCoV), a human Betacoronavirus OC43, or murine hepatitis virus (MHV) (ESI† Table S4). Three laboratories (B, C, F) used a bovine coronavirus (BCoV) as their spike, adding an average quantity of 1.34×10^6, 2.1×10^7, and 1.79×10^{10} gene copies per liter (GC/L), respectively. Three laboratories (E, G, and H) used a human Betacoronavirus OC43 as their matrix spike, adding an average quantity of 2.17×10^6, 4.36×10^7, and 2.67×10^8 GC/ L, respectively. Lab A initially used BCoV as their spike, but transitioned to OC43 after the second trial, adding an average quantity of 5.61×10^8 and 3.54×10^8 GC/L, respectively. Lab D used a murine hepatitis virus (MHV) as their matrix spike, adding an average quantity of 3.43×10^6 GC/L. Recovery efficiency percentages were calculated as a proportion of the concentration of directly extracted spiked surrogate prior to sample processing and the final recovered concentration following sample processing and quantification analyses. The SARS-CoV-2 gene fragment concentrations were adjusted using recovery efficiency percentages by dividing the SARS-CoV-2 gene concentration by the percent recovery, multiplied by 100.

2.5. Statistical analyses

Statistical analyses were completed using RStudio (Version 1.2.1335). The following packages were used for initial data manipulation, visualization, and correlation analyses: *dplyr, ggplot2, rcompanion, reshape2, rstatix,* and *tidyverse*.[46–51] PAST (Version 4.05) was employed for mean difference calculations and some figures.[52] A grand mean was determined by calculating the mean of all data points across every laboratory for a specific gene fragment. Statistical comparisons between the quantified genes, water quality parameters, and case numbers were conducted using a nonparametric Spearman's correlation test, following normality tests. A p-value ≤ 0.05 is considered statistically significant for all tests, indicating 95% confidence. COVID-19 case data was downloaded from the Ohio Coronavirus Wastewater Monitoring dashboard (accessed 11/9/2021) and is specific to the sampled sewersheds. Case numbers were calculated as a 7 day average of cases from two days prior to the wastewater sampling date, the wastewater sampling date, and four days after the sampling date during the study period (10/3/2020–9/ 23/2021). Cases per population represents the case 7 day average divided by the community population in that same sewershed, normalized to 100 000 people.

For the evaluation of variability across the data, variation component and intraclass correlation analyses were employed. The following packages were used for further correlation and variability model analyses and statistics: *data. table, lme4, sjstats, performance*, and *stats*.[53–57] The N2 gene fragment data set was exclusively used for all SARS-CoV-2 related analyses, due to early studies highlighting benefits (although contradicted) to targeting the N2 gene fragment *versus* the N1 gene fragment.[33,58]

Laboratories with the ability to also target N1 gene fragments were encouraged to do so. Therefore, the N2 gene fragment data set was more complete compared to the N1 gene fragment data set. For data preparation and clean-up, results were log-transformed then tested for normality and exhibited symmetrical, normal distribution. Random effects regressions were modeled to determine estimated intraclass correlations (of laboratory, trial, and residuals) for each of the SARS-CoV-2 gene targets and the human fecal markers under various conditions. Each model is associated with a specific condition in which the raw data was manipulated. Six individual models were created with differently applied normalization manipulations to the SARS-CoV-2 N2 target gene copy raw data for the purpose of reducing inter-lab variations. Models represented normalization or adjustments using 1) no adjustment, 2) surrogate recovery efficiency, 3) fecal marker concentrations, 4) surrogate recovery efficiency and fecal marker concentrations, 5) flow, or 6) surrogate recovery efficiency and flow.

3. Results and discussion

3.1. Sample characteristics

Across the seven trials, collected wastewater samples varied by wastewater facility characteristics as well as quantified gene fragment results (Tables 1 and 2). Collected samples came from various regions in the state and from a range of WWTPs of varying sizes, from 52 000 to 655 000 people served. The flowrates ranged from 7 to 100 million gallons per day (MGD). The size of the population contributing to the wastewater influent is strongly correlated with the flowrate observed at that treatment plant (p-value < 0.005, Spearman's Rho = 0.95). Other treatment plant characteristics, such as pH, wastewater sample temperature, and TSS, ranged by trial. Although it is known that water parameters can impact viruses, little is known about the relationship between water quality parameters and SARS-CoV-2 in wastewater.[59,60] Further studies would be necessary to better understand these relationships.

3.2. Human fecal marker quantification

Human fecal marker concentrations were quantified in tandem with SARS-CoV-2 gene fragments (Fig. 2). Two labs (F, H) quantified PMMoV, while the remaining labs quantified crAssphage. Lab H initially quantified crAssphage but switched to PMMoV by the end of the study. The average human fecal marker concentration was 6.46×10^8 gene copies per liter and ranged from 4.19×10^6 to 2.45×10^{10} gene copies per liter (Table 2), staying relatively consistent across different locations and different times of the year. Separately, the average concentration for labs who quantified crAssphage was 7.59×10^8 gene copies per liter, while for PMMoV was 7.81×10^7 gene copies per liter. Previous wastewater surveillance studies that also quantified fecal indicator genes (such as crAssphage and PMMoV) saw average gene copies ranging between the levels of ~10^5 and 10^{10}.[40,41,61–64] No correlation was observed between SARS-CoV-2 gene fragment and human fecal marker concentrations, likely due to observed fluctuations in COVID-19 cases as the pandemic progressed (ESI† Fig. S1). Other factors contributing to the observed variability include the use of two different human markers across the labs, direct extraction and quantification from raw wastewater by some laboratories *versus* quantification from processed and extracted

samples, as well as the differing recovery efficiencies associated with RNA or combined RNA and DNA extraction method/kit type. It is recommended to use the same fecal marker and exact protocol for all laboratories, if possible, when comparing concentrations across trials and methods.

Human fecal marker concentrations had no significant correlation to TSS or flow but did have significantly weak negative correlations with pH and temperature across samples (p-value < 0.05, Spearman's Rho = −0.33). It has been reported that some environmental water quality factors, such as pH and temperature, may have an effect on viral particles in various matrices including wastewater.[65–67] However, due to low sample numbers and the limited range of data, it would be appropriate to design a study in a more systematic way to better understand the effects of environmental factors on the human fecal markers in wastewater if needed in the future.

3.3. Quality control

Inhibition related to gene quantification is an important factor when quantifying viruses, including SARS-CoV-2, in wastewater samples. Due to the complexity and differences in wastewater samples, influents may be highly variable across samples and exhibit various levels and types of PCR inhibitors.[20,68,69] Laboratories conducted PCR inhibition assays during most trials, to determine possible inhibition during PCR analyses as a molecular process control. Of the eight laboratories that conducted PCR inhibition tests, only three instances of inhibition were detected during the seven trials (ESI† Table S2). These instances were found by three different labs, each during a different sample trial. Once inhibition was detected, these labs subsequently diluted their samples and re-measured each gene fragment target. Based on these results, inhibition might not be only sample specific, but processing and extraction method specific. Methods should be employed to reduce PCR inhibitors, as they may lead to false negatives.[68,70]

An important part of reducing PCR inhibition is understanding the PCR methods and materials employed. One-step and two-step RT-qPCR have become the most widely used method for gene quantification analysis of SARS-CoV-2, both clinically and for general research purposes.[71,72] During this study, six of the eight laboratories used RT-qPCR (four groups used one-step, one group used two-step, and one group moved from two-step to one-step following trial 2) as their quantification method. RT-ddPCR has proven to also be an efficient method for gene quantification for wastewater analyses and was utilized by two labs (using both one-step and two-step). Coincidentally, the labs during this study that exhibited inhibition happened to employ RT-qPCR methods. Studies comparing methods for SARS-CoV-2 RNA detections concluded that dPCR-based methods exhibited increased sensitivity compared to qPCR and yielded fewer false negative results. Since dPCR is less affected by PCR inhibitors, dPCR is a stronger candidate for environmental samples.[42,69,70,73] Using RT-ddPCR can accurately quantify low viral loads in this complex matrix, while also detecting even the slightest variations across samples – especially at low viral concentrations in wastewater.[74,75] Regardless of method, limited inhibition was detected in three trials and both RT-PCR methods showed good performance for SARS-CoV-2 quantification and is likely not a large source of variability across the data.

Another important component for quantifying SARS-CoV-2 in wastewater is evaluating recovery efficiencies of a matrix spike surrogate in the wastewater samples. Because of the complexity of the wastewater matrix, it is recommended that a coronavirus surrogate be spiked into raw wastewater samples for determining recovery efficiency, as they can vastly differ across wastewater methods and samples.[19,20,22,42,76] Additionally, using spiked surrogates may serve as both a process control and a normalization method for understanding losses of viral RNA during processing.[42,77] In this study, viral spikes were independently chosen (either OC43, BCoV, or MHV) and spiked into the raw wastewater prior to each laboratory's sample processing. The recovery efficiencies ranged across trials and labs, from 3 to 75%, with an average recovery efficiency of 33% across the study. The average recovery efficiency across the seven trials for each chosen surrogate (OC43, BCoV, or MHV) were 31%, 38%, and 23%, respectively. Exhibiting a relatively large range of recovery percentages is not exclusive to this study, as others have shown ranges in recovery based on different surrogates, wastewater samples, concentration methods, and processed fractions.[19,22,68,76–83]

It is important to note that during the initial stages of this study, different methods were utilized for the direct extraction of the viral surrogate, which impacts the calculations for recovery efficiency. The discrepancies lied with some labs quantifying the inoculum concentration before spiking into the raw wastewater sample, while some labs quantified the inoculum concentration once the surrogate was spiked into the wastewater sample, both of which influence the initial concentration. One study mentions how surrogate contact time in wastewater may influence extraction and recovery efficiency, but found no recovery differences – suggesting that other factors in the wastewater matrix are likely influencing recovery efficiency and direct extraction methods.[78] It is possible that the range of recovery efficiencies seen here may have lower if uniform methods were employed early. Recovery efficiencies could also be influenced by downstream processing methods. One instance of improved recovery efficiency within a single method is shown from Lab E, as they switched to a Trizol extraction method after trial 2. Their data suggests that the switch from a QIAGEN kit to a Trizol-chloroform extraction did, in fact, attribute to their increase in recovery efficiency for the remainder of the study. Mean recovery efficiency percentages have been shown to vary per method in other studies, supporting this result.[19,29] For future studies, it is recommended that nucleic acid extraction and calculation methods be standardized across all laboratories and remain consistent throughout the study period. Here, recovery efficiencies were further utilized as a normalization tool for the quantified SARS-CoV-2 gene fragment concentrations and assisted in correcting gene fragment concentrations, as described further in section 3.6. Together, these various tools (human fecal quantifications, PCR inhibition testing, and recovery efficiency determinations) serve as appropriate controls across various methods and samples, and help ensure reproducibility within a single method.[20]

3.4. SARS-CoV-2 gene concentrations in wastewater

The SARS-CoV-2 N1 and N2 gene targets were successfully detected by eight participating laboratories across seven trials (ESI† Table S3). N1 and N2 gene fragments exhibited strong significant correlations to one another across all trials and participating laboratories (*p*-value

< 0.05, Spearman's Rho = 0.77). When the target gene quantifications were adjusted to account for recovery efficiency, this same strong relationship was observed (p-value < 0.05, Spearman's Rho = 0.80). For subsequent analysis on the evaluation of variances using random effects regressions, the N2 gene data was exclusively used, due to a more complete dataset and because of the strong correlation between the two gene fragments. These same SARS-CoV-2 data were also compared to community COVID-19 case counts for the corresponding sewersheds, supporting the notion that wastewater surveillance continues to be a powerful tool for COVID-19 trend tracking (ESI† Results 1).

During the seven trials, SARS-CoV-2 gene targets varied by concentrations (in gene copies per liter of wastewater) (Table 2, Fig. 3). SARS-CoV-2 raw concentrations ranged from 1×10^1 to 1×10^5 gene copies per liter across the seven trials, with averages of 8.74×10^4 and 6.18×10^4 for N1 and N2 gene fragments, respectively. These concentrations and the correlation between the target gene is similar to what was seen of other wastewater surveillance studies in Ohio during the same period of time.[22,79] Because samples were taken sequentially over an 11 month period and from different locations across the state, it is expected to observe varying SARS-CoV-2 target gene concentrations for each trial as COVID-19 prevalence varied by time and location.[84] When the raw data was transformed and adjusted by recovery efficiency of the surrogate spike, mean concentrations of N1 and N2 gene targets were approximately four times higher. Implications of this data transformation using recovery efficiency is described below.

3.5. Intra- and inter-lab variability and normalization

The SARS-CoV-2 $\log_{10}$ transformed gene fragment concentrations exhibit a general spread of approximately 2-$\log_{10}$ within a single trial (Fig. 3). When this same data is compared to the SARS-CoV-2 target gene concentration grand mean by variance model analysis, there appears to be two groups where five labs are below the grand mean (left-shifted group) and three labs are above the grand mean (right-shifted group) (Fig. 4A). Despite the various methodologies used among the labs, it is promising to see these two groups near the grand mean. This indicates that the employed methods are similar enough to produce comparable and consistent results, a result that compares nicely to two interlaboratory method comparison studies from the United States and Canada.[29,85] However, normalization tools during wastewater surveillance are frequently employed to account for wastewater characteristic fluctuations and sample and measurement variability, which allows for improved comparisons across samples.[22,78,79] Many studies have normalized SARS-CoV-2 target gene concentrations using recovery efficiencies of a spiked surrogate, human fecal markers, or flow in order to improve estimates.[22,29,61,78,79,83] These studies found that different normalization tools provided improved SARS-CoV-2 target gene concentration comparisons or estimates while reducing background noise, all at varying degrees of improvement and correlation. To evaluate the potential of various normalization tools to improve gene concentration variations within the dataset, variance component model analyses were performed.

Normalization was explored to account for sample and measurement factors in estimations of SARS-CoV-2 target gene concentration and for promoting reproducibility across the labs

to account for various external factors – particularly the different methods employed by the labs. It is understood that the samples used for each trial were different from one another and carry water quality and wastewater treatment-related differences, which may account for some proportion of variance (absolute amount unknown). The random effects models (Fig. 5) used each modeled dataset to generate interclass correlation coefficients (ICC) for determining proportions of variance that are explained by explicit factors (laboratory, trial, or residual), representing the proportion of variance that is present for each factor. Factors were determined based on the known differences in the study, such as laboratory methods and individual trial samples.

When the SARS-CoV-2 target gene concentrations are normalized by the different models, the proportion of variances exhibited by factor shift (Fig. 5A). For the raw SARS-CoV-2 dataset (model 1), 31% of the variance is observed between laboratories, 45% is between trials, and 24% is residual variance. When the raw data is normalized by human fecal marker (model 3) or by both recovery efficiency percentages and human fecal marker (model 4), the proportion of variance between laboratories is higher (47% and 44%, respectively) than the proportion of variance between trials (23% and 27%, respectively). As mentioned previously, human fecal markers tested included both concentrations of PMMoV and crAssphage, two different viruses with two different relative concentrations in wastewater. Studies have seen a range of effects of human fecal marker normalization on SARS-CoV-2 RNA data, including noise reduction and increased or decreased correlations to clinical case datasets.[22,42,61,78,79,81–83,85] During this study, normalization using human fecal marker concentrations does not reduce variance proportions between the laboratories.

On the contrary, when the raw data is normalized by recovery efficiency (model 2), adjusted for flow (model 5), or adjusted by both (model 6), there is an improved decrease in variance proportion between laboratories (24%, 23%, and 21%, respectively) and an increased proportion of variance between trials (40%, 59%, and 47%, respectively). This is also represented graphically, with the smallest portions shown in blue for laboratories, particularly for models 2, 5, and 6 (Fig. 5B). As mentioned before, recovery efficiencies vary greatly with different viral isolation and concentration methods as seen from other studies.[19,22,68,76–83] Flow is a normalization tool to account for potential fluctuation in temporal samples or varying WWTPs.[30,68,78,79] Ideally, a flow-weighted composite sample helps minimize differences in flow at a single WWTP for obtaining representative samples, and a second choice would be a time-weighted composite sample.[20,42] Of the seven samples collected, six were flow-weighted and help ensure representative samples (Table 1). Utilizing adjustments by both recovery efficiency and flow helps to account for method and environmental sample fluctuations, which may be why we see the strongest improvement in the proportion of variance for laboratories (21% from 31%), the major focus of improvement. In this study, the implementation of normalizing with recovery efficiency and flow served successful in reducing inherent variabilities, particularly compared to the raw N2 gene fragment concentrations (Fig. 4). The raw data shown in model 1 (Fig. 4A) shows two clear groups, one above and one below the grand mean (at 0.0 effect range). In contrast, model 6 (Fig. 4B) provides a more stepwise gradient across the labs while bringing the overall dataset closer to the grand mean. Variance analyses of the remaining models for laboratory and trial factors were also visualized against the grand mean and

exhibited different variance ranges (ESI† Fig. S2). Introducing these factors helps not only explain some of the variability during this study, but also serve as important controls within single laboratories and methods from trial to trial. Further trials may be necessary to better understand how other factors may be influencing overall variability, particularly what makes up the residual factors.

3.6. Method selection considerations

It was determined early on that a variety of methods have been successfully adapted for wastewater surveillance, a reality for both the OCWMN and this field during a global pandemic.[19,29,78,85] Due to various supply limitations, available expertise, and existing equipment, participating OCWMN analysis labs adopted different methods for SARS-CoV-2 quantification in wastewater. Other studies have compared methodologies for wastewater surveillance, and have seen strengths and weaknesses in different methods related to recovery, cost, throughput, and variability.[19,29,86] Taking those variabilities into account, the eight laboratories continued to exhibit similar results between the trials, although a range of data was seen. There is not enough data to draw any method-to-method comparisons for this study. However, there are some known method differences that may have played roles in the proportion of variance caused by laboratory differences.

Laboratory F, on average, produced some of the highest quantifications of SARS-CoV-2 data across all trials. This lab was one of two labs who did not conduct any pre-treatment or solid separation steps prior to viral capture and was the only group that used a non-linearized, double stranded plasmid control during their qPCR standard curve generation. Previous studies have shown that using a nonlinearized plasmid can overestimate and/or exacerbate variations in quantification, and therefore could explain their high variability when compared to the other labs.[85,87,88] In a recent study for SARS-CoV-2 monitoring in wastewater, linearized, double-stranded DNA and linearized, single-stranded RNA positive controls were overestimated by factors of about 2.8 and 9.3, respectively, when quantified using plasmid, double-stranded DNA as standard curve reference material.[89] Thus, it is possible that using a non-linearized plasmid serves as a potential factor in Lab F’s consistent higher SARS-CoV-2 gene quantification results throughout the study.

Another source of method variability may come from the solid removal and viral capture method. Many of the laboratories conducted low-speed centrifugation for large particle solid separation, followed by membrane vacuum filtrations to collect particles larger than the membrane pore size (0.45 μm used by the laboratories), allowing remaining water that may contain smaller than 0.45 μm (*e.g.,* viruses) to pass through. Coronaviruses and many other viruses have the ability to adhere to suspended solids and other particles in water, and wastewater tends to have high solid content.[90–94] It was assumed that membrane filters may collect those suspended solids with bound viral particles that are then included in nucleic acid extractions in downstream analyses. Compared to all other labs where only particle-bound viral fractions were processed after concentrating with 0.45 μm pore-sized membrane, Lab A used an initial step of adding mild surfactant for detaching bound viruses from particles and then employed a bacteria removal step, followed by viral concentration. As the only lab that did not process particle-bound-only viral fractions, Lab

A consistently exhibited quantification close to the grand mean; this step may not have been the sole significant factor influencing their results. Further inter-laboratory analysis of the impact of water quality parameters on the viral interactions in the collected and processed wastewater would be necessary to draw further conclusions. Another consideration for solid content in the water samples is the method for sample collection. This study employed composite samples to gather a more representative sample. Newer studies have highlighted the successful use of passive sampling (with tests of various adsorbent materials) as a more cost effective and simple approach to either grab or composite sampling.[95,96] This method not only serves as an early concentration step, but it also influences the retention of solids onto the chosen adsorbent material, therefore impacting SARS-CoV-2 recoveries. These studies provide yet another opportunity for method and sample collection optimization for future studies and needs.

Wastewater is a complicated matrix and there are many factors that impact surveillance outcomes and results. With limited viral viability understanding and varying viral fecal shedding estimates, this approach can be challenging.[24,29,97–100] It is also important to note that early on, the pandemic brought on many challenges for many of the participating laboratories and for laboratories elsewhere. Firstly, frequent meetings with the network brought on necessary but challenging discussions around method choices, success and failures experienced, as well as critical discussion of data interpretation given the many method differences across the developed network. Secondly, with the increased interest in wastewater surveillance and SARS-CoV-2 over the last two years, supply shortages were frequent. Supply companies who provided materials for the participating laboratories and elsewhere exhibited high demand, slowed shipping, and limited supply. This impacted material availability and therefore method choices, especially for early trials, and has continued to be an observed challenge in this field – particularly in regions with limited resources, funding, and support.[101] It is recommended to conduct recovery efficiency spiking tests regularly to support method consistency within a single lab over time – even when challenges arise.[77] Although the true SARS-CoV-2 concentrations in wastewater are unknown, wastewater surveillance can be used for comparing trends, such as increasing, decreasing, and plateaued concentration levels and should mimic real-world community infection dynamics.[21,22,102–104] In this way, wastewater-based disease surveillance can be a powerful and practical tool in potentially mitigating outbreaks, identifying hotspots, assessing the effectiveness of vaccination and emergence of variants of concern, and serving as one of many important indicators for the WHO to declare the end of the COVID-19 pandemic.[105]

4. Conclusion

This study was a statewide collaboration between local and nationwide government agencies and private companies alongside state universities to evaluate the reproducibility of results when enumerating SARS-CoV-2 in raw wastewater influent. SARS-CoV-2 target gene quantifications across the laboratories and trials were comparable, although many different methods were utilized, and exhibited the ability for these different methods to generate reproducible results. The variability observed between inter-laboratory SARS-CoV-2 concentrations decreased under various normalization adjustments. Utilizing flow

and recovery efficiency adjustments together served most effective for reducing interlab variation, a challenge that statewide monitoring networks may face. It is clear that quantification of human fecal markers and recovery efficiencies, along with collecting wastewater characteristics, together serve as valuable internal controls across and within the different laboratories while accounting for some potential sources of measurement variability. Moreover, this study exemplifies the many possible approaches that laboratories may adopt for conducting wastewater-based surveillance monitoring, to appropriately meet their individual needs and limitations, such as processing simplicity, material availability, or costs, as examples. It is recommended that the same method should be maintained once employed in a monitored location during the surveillance period to ensure minimal target viral quantity variations over time, as changing method will lead to change in monitoring results. If this is not possible, before changing methods, monitoring laboratories should conduct a few weeks of monitoring in parallel, using their current and "future" method, to evaluate the effect of this change on the resulting SARS-CoV-2 concentrations. The OCWMN continues to perform wastewater surveillance across the state, while regularly conducting lab comparisons as the pandemic continues to unfold more than two years later. Studies like this one may help identify the best way to perform between-lab studies for an enveloped pathogenic virus, such as SARS-CoV-2 and other outbreak-causing viruses, for real-time statewide wastewater surveillance monitoring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Water impact

Wastewater can be used as a valuable source for trend tracking of various diseases in various communities. Wastewater-based SARS-CoV-2 monitoring is an effective tool for early management as the pandemic is currently ongoing and variants are continuously emerging. Evaluating methodologies during the height of a global crisis strengthens the field to ensure reproducibility across the state, national, and even global entities.

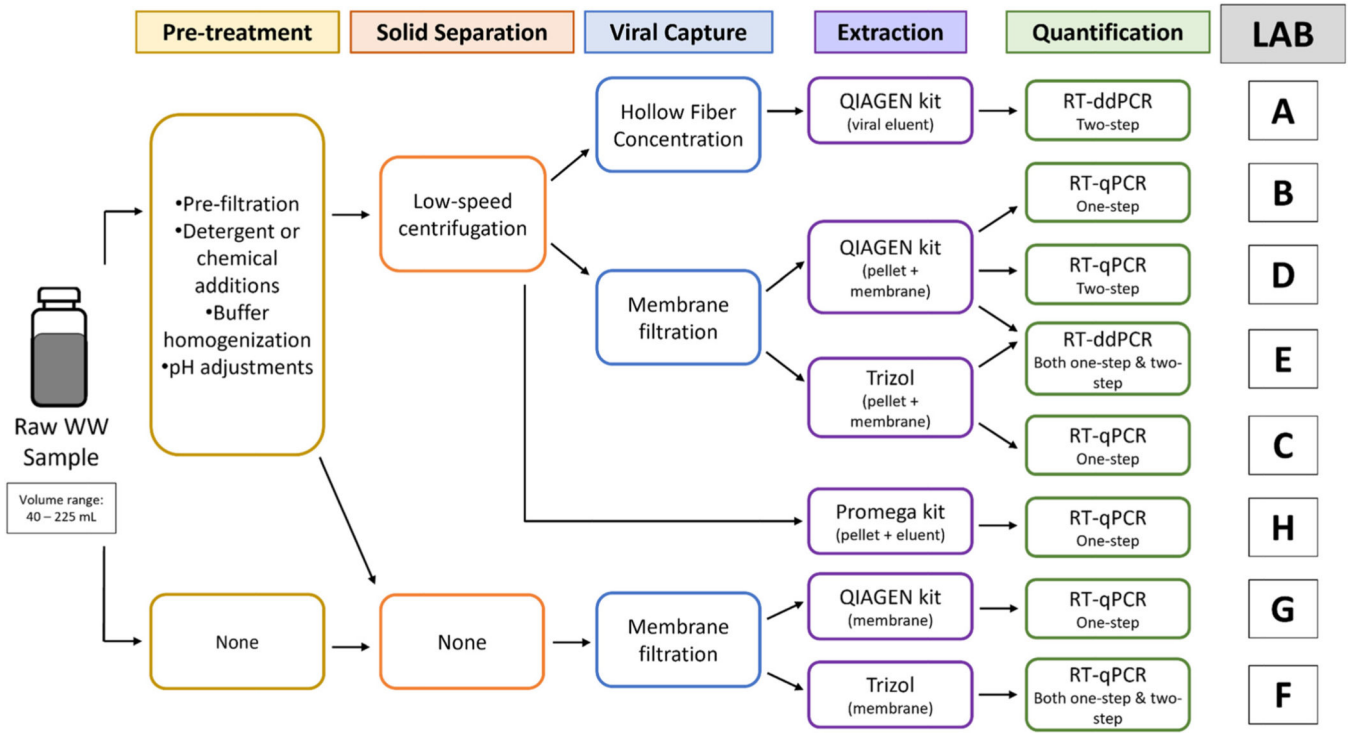


Fig. 1.

Methods employed for SARS-CoV-2 RNA quantification from raw wastewater by each participating lab, based on five main processing categories.

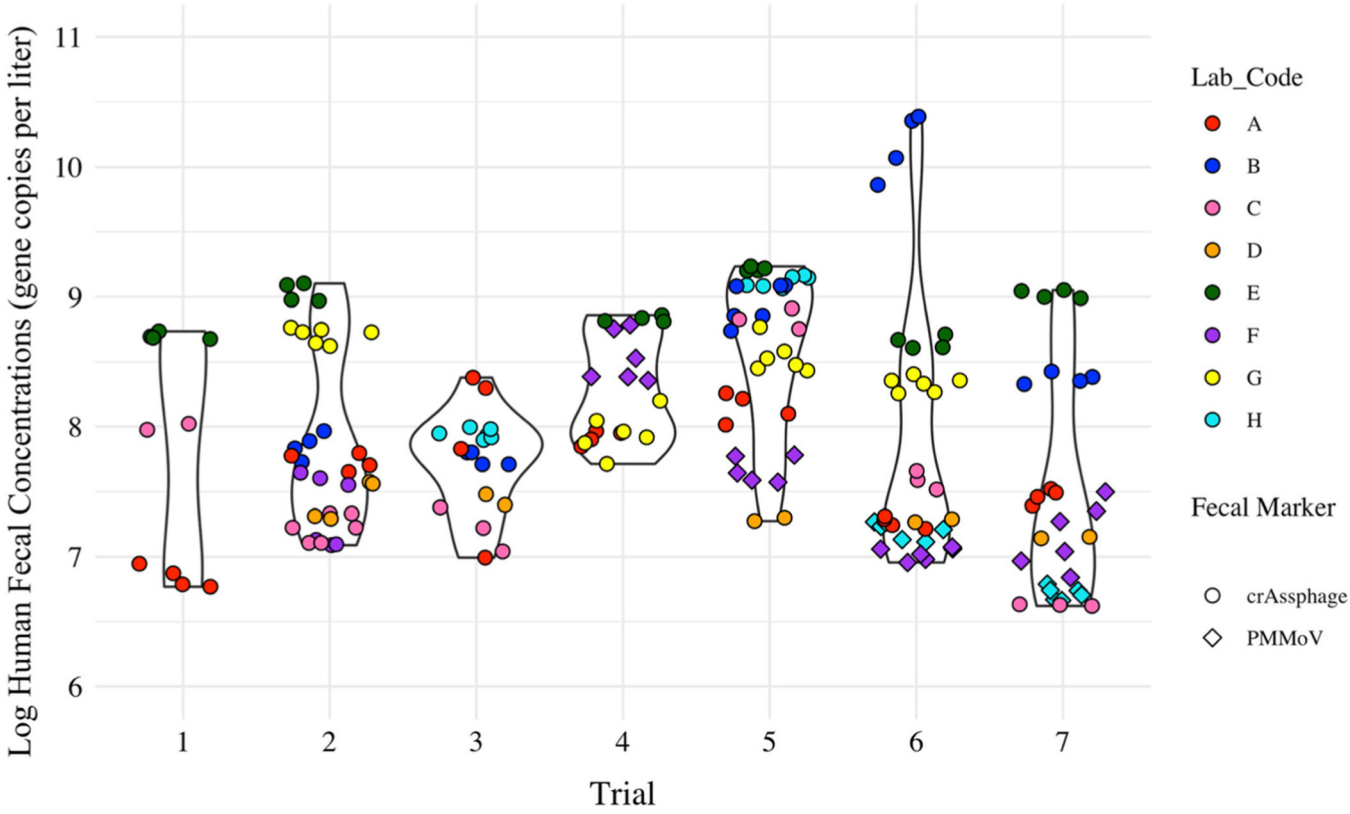


Fig. 2.

Summary of $\log_{10}$-transformed human fecal indicator gene quantification results from each lab across all trials. Single points indicate sample and gene quantification replicates (method specific). Individual laboratories are coded by color, and the fecal marker used is coded by point shape (circles indicate crAssphage, diamonds indicate PMMoV).

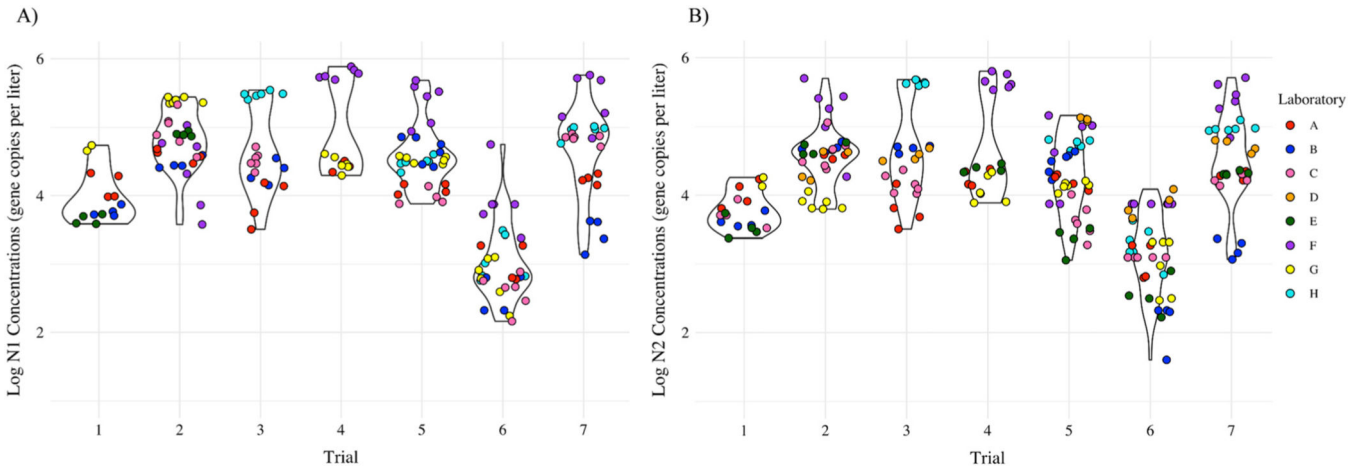


Fig. 3.

Log_{10}-transformed SARS-CoV-2 gene fragment quantification results by laboratory. (A) log_{10} N1 gene copies per liter, (B) log_{10} N2 gene copies per liter. Single points indicate sample and gene quantification replicates (method specific). Laboratories are coded by color.

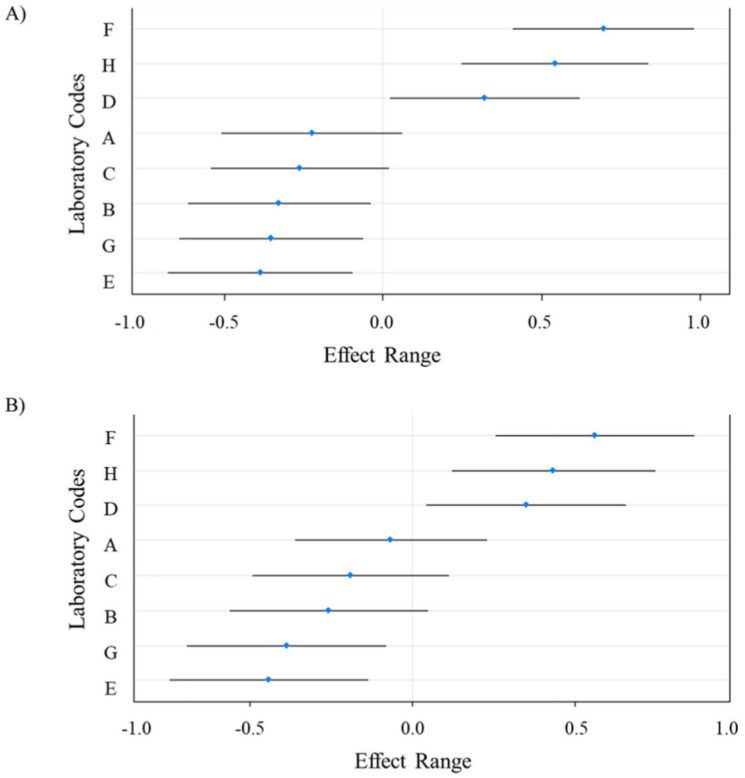


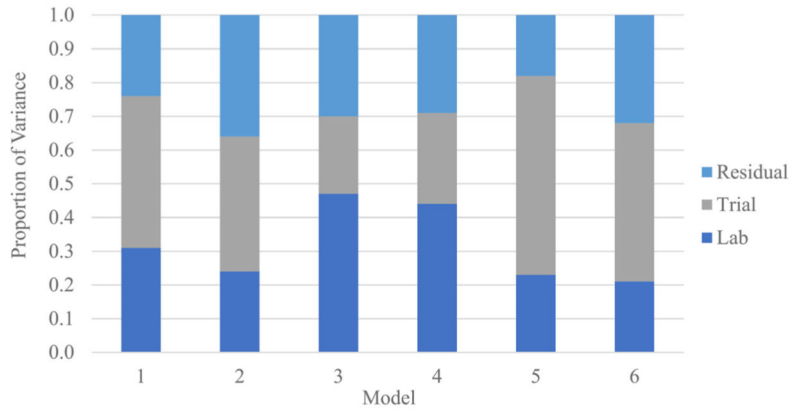
Fig. 4.

Variance model analyses comparing A) raw N2 gene fragment concentration results (model 1) and B) N2 gene fragment concentration results adjusted for recovery efficiency and flow (model 6). The line at 0 represents the average gene fragment quantifications across all labs and all trials. The individual points represent average variance, and the lines stretching from the point represent a 95% confidence interval.

A)

Model		Interclass Correlation Coefficients			
Number	Description	Lab	Trial	Residual	Rank
1	Raw data	0.31	0.45	0.24	4
2	Adjusted by recovery efficiencies	0.24	0.40	0.36	3
3	Normalized by fecal marker	0.47	0.23	0.30	6
4	Adjusted by recovery efficiencies & normalized by fecal marker	0.44	0.27	0.29	5
5	Adjusted for flow	0.23	0.59	0.18	2
6	Adjusted by recovery efficiencies & flow	0.21	0.47	0.32	1

B)



C)

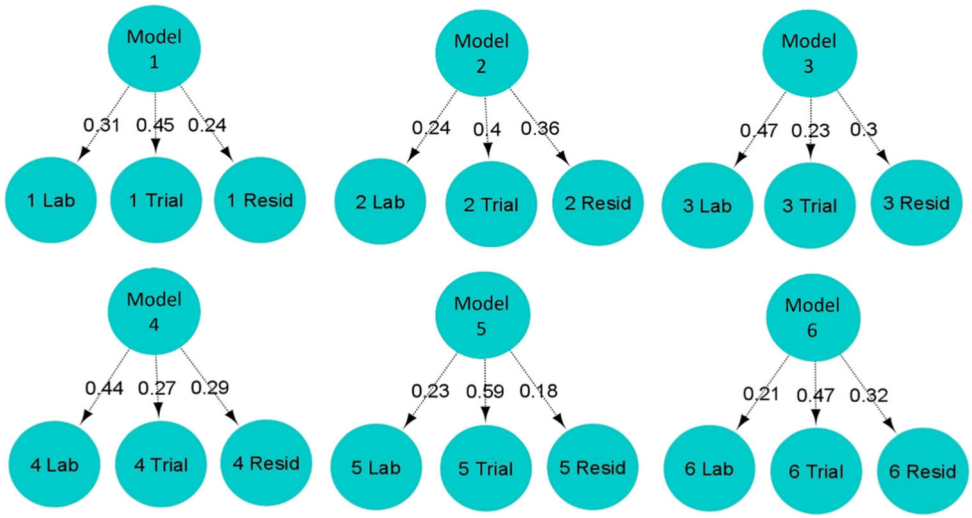


Fig. 5.

Summary of the variance component analyses for models using the N2 gene fragment concentration results, in A) table, B) graphical, and C) visual form. The model description provides the factors that influence the model. The interclass correlation coefficients (ICC) are divided by proportions of lab, trial, and residual variances, and then ranked from lowest (best) to highest (worst) lab variance proportions.

Table 1

Wastewater sample details about composite collection and general influent wastewater details across the seven trials

	24 hour composite sample collection				Influent wastewater				
Trial	**WWTP**	**Start date**	**Start time**	**Type**	**Population served**	**Flowrate (MGD)**	**pH**	**Temperature (°C)**	**TSS (mg L $^{-1}$)**
1	Mansfield	10/4/2020	00:30	Flow-weighted	52000	8.04	8.04	18.8	—
2	Southerly	11/15/2020	00:00	Flow-weighted	654817	101.8	6.95	17.8	192.0
3	Akron	1/31/2021	07:00	Flow-weighted	363897	51.5	7.4	12.2	155.0
4	Jackson Pike	1/31/2021	00:00	Flow-weighted	645940	88.2	7.2	14.0	220.0
5	Lucas County	4/4/2021	07:00	Time-weighted	110000	15.6	6.8	13.7	122.0
6	Southerly	6/13/2021	00:00	Flow-weighted	654817	95.5	7.0	17.2	172.0
7	Tri-cities north regional	9/19/2021	07:00	Flow-weighted	65000	7.6	7.2	22.2	159.0

Dash (—): missing data.

Table 2

Summary statistics for the quantified gene fragments (N1, N2, human fecal markers), recovery efficiencies, and cases per population for all labs across the total seven trials. N1, N2, and human fecal marker concentrations are reported in gene copies per liter (GC/L). Recovery efficiency is reported as a percentage. Cases per population are reported as counts

Gene	Data type	Minimum	Maximum	Median	Mean
N1 gene fragment	Raw	1.45×10^{2}	7.67×10^{5}	2.94×10^{4}	8.74×10^{4}
N1 gene fragment	Recovery efficiency corrected	5.80×10^{2}	4.91×10^{6}	8.08×10^{4}	3.82×10^{5}
N2 gene fragment	Raw	4.00×10^{1}	6.35×10^{5}	1.69×10^{4}	6.18×10^{4}
N2 gene fragment	Recovery efficiency corrected	1.14×10^{2}	3.70×10^{6}	6.80×10^{4}	2.40×10^{5}
Human fecal marker	Raw	4.19×10^{6}	2.45×10^{10}	7.98×10^{7}	6.46×10^{8}
Recovery efficiency percentage	Raw	3	75	30	33
Cases per 10000 people	Raw	18	479	157	217