# *Nationwide Trends in COVID-19 Cases and SARS-CoV-2 RNA Wastewater Concentrations in the United States*

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Article

# Nationwide Trends in COVID-19 Cases and SARS-CoV-2 RNA Wastewater Concentrations in the United States

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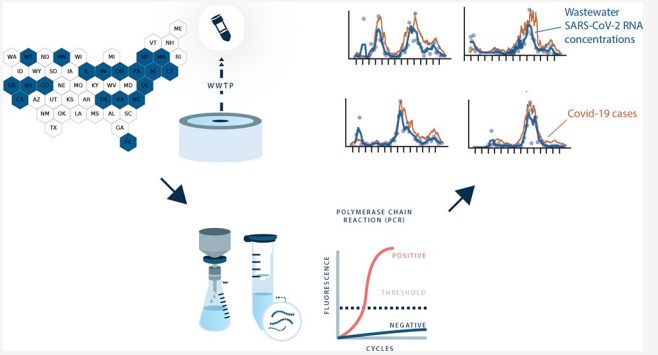
**ABSTRACT:** Wastewater-based epidemiology has emerged as a promising technology for population-level surveillance of COVID-19. In this study, we present results of a large nationwide SARS-CoV-2 wastewater monitoring system in the United States. We profile 55 locations with at least six months of sampling from April 2020 to May 2021. These locations represent more than 12 million individuals across 19 states. Samples were collected approximately weekly by wastewater treatment utilities as part of a regular wastewater surveillance service and analyzed for SARS-CoV-2 RNA concentrations. SARS-CoV-2 RNA concentrations were normalized to pepper mild mottle virus, an indicator of fecal matter in wastewater. We show that wastewater data reflect temporal and geographic trends in clinical COVID-19 cases and investigate the impact of normalization on correlations with case data within and across locations. We also provide key lessons learned from our broad-scale implementation of wastewater-based epidemiology, which can be used to inform wastewater-based epidemiology approaches for future emerging diseases. This work demonstrates that wastewater surveillance is a feasible approach for nationwide population-level monitoring of COVID-19 disease. With an evolving epidemic and effective vaccines against SARS-CoV-2, wastewater-based epidemiology can serve as a passive surveillance approach for detecting changing dynamics or resurgences of the virus.




**KEYWORDS:** *wastewater-based epidemiology, wastewater monitoring, wastewater surveillance*



## INTRODUCTION

The COVID-19 pandemic has galvanized the rapid development of innovative approaches for pandemic preparedness and response. Wastewater-based epidemiology (WBE) for monitoring the SARS-CoV-2 virus in wastewater has emerged as a promising technology for public health surveillance. The SARS-CoV-2 virus is excreted in human feces early in the clinical course of infection and provides a view of COVID-19 that is independent of access to testing, making it an ideal candidate for WBE. WBE has previously been demonstrated in infectious disease monitoring, providing early warnings of polio re-emergence and outbreaks of cholera, norovirus, hepatitis A, and hepatitis B.[1−4] During the SARS epidemic in 2002, traces of SARS coronavirus were detected in wastewater near hospitals in China.[5]

The first successful detection of SARS-CoV-2 in wastewater was reported in The Netherlands in early March 2020,[6] followed by demonstration of SARS-CoV-2 detection in wastewater in the United States.[19,21] Wastewater surveillance for the virus that causes COVID-19 has since been broadly pursued by the scientific community, municipal public health and public works departments, the U.S. Centers for Disease Control and Prevention, and other national organizing bodies in the United States and globally.[6−12,36,37]

Multiple applications of WBE for COVID-19 have been proposed, including as a leading indicator of new COVID-19 cases, an independent confirmation of disease trends, and as an early warning system for COVID-19 re-emergence.[13] Additionally, wastewater has been proposed as an alternative method for estimating the COVID-19 reproductive number[14] or as an indicator of clinical diagnostic testing capacity.[15] Finally,

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wastewater monitoring provides a powerful approach for monitoring existing and emerging variants at the community level.[38,39] In practice, WBE has been applied in multiple ways. In Cambridge, MA, SARS-CoV-2 RNA concentrations were one of three metrics used to evaluate school system reopenings.[16] In Australia, when the number of COVID-19 cases is small, wastewater monitoring serves to warn residents of potential re-emergence.[17] Across the United States, wastewater analysis has been deployed at universities to monitor and mitigate transmission among students.[18]

This study presents results from a nationwide data set of SARS-CoV-2 RNA concentrations in wastewater and represents the largest U.S.-based temporal and geographic WBE data set reported to date. In March 2020, our groups launched a campaign to monitor the presence of SARS-CoV-2, the virus that causes COVID-19, in wastewater across the United States.[19,20] Since then, we have had approximately 100 participating locations regularly monitoring SARS-CoV-2 RNA wastewater concentrations in their communities on a weekly or monthly basis. As a result of this effort, we have generated a wastewater SARS-CoV-2 data set consisting of >15000 samples, allowing us to evaluate the applicability and feasibility of implementing a nationwide wastewater monitoring program for the virus that causes COVID-19. In this study, we report results from a subset of these data, highlighting data from 55 sampling locations with approximately weekly sampling for at least six months. These sampling locations represent 39 counties across 19 states, and we show their data from April 2020 through May 2021. We demonstrate that wastewater data broadly reflect geographic and temporal trends in COVID-19 cases across the United States, confirming the feasibility and applicability of wastewater monitoring for current and future public health COVID-19 surveillance.

## METHODS

**Sample Collection.** The samples in this study were collected by participating wastewater treatment facilities as part of regular wastewater surveillance service provided by our company (Biobot Analytics, Inc.). Because samples were originally collected for nonscientific purposes, we consider this a secondary research study and details related to sample collection were determined by each participating sampling partner. Participating facilities were mailed a sampling kit with instructions to collect a standard 24 h composite sample. Composite samples were shaken to homogenize them, aliquoted into three 50 mL conical tubes, and shipped within 24 h of collection overnight with ice packs to our laboratory (Cambridge, MA). Received samples were immediately pasteurized at 60 °C for 1 h. One of the three sample tubes was used for analysis and processed immediately after pasteurization.

Samples were collected by the participating municipalities, which sampled at wastewater treatment facilities or pump stations. A majority of samples were collected using autosamplers that these facilities already had in house, including both refrigerated and nonrefrigerated models. A majority of samples were collected as 24 h composite samples: 1349 samples were collected as 24 h time-proportional composites, 1084 were collected as 24 h flow-proportional composites, seven samples were grab samples, and the others were another type of sample or did not have a sampling method specified. We encouraged our sampling partners to increase their autosampler's pumping frequency to ensure the representativeness of samples. The sampling frequency was determined by the participating municipal partners, with the majority providing weekly samples. Participating municipalities provided metadata about their sampling locations, including catchment population and average daily flow rate (Table S1).

**Lab Analysis.** Our lab method changed over the sampling time course to improve the sample processing time, throughput, and sensitivity, also accounting for supply chain availability. In both methods, samples for analysis were first filtered to remove large particulate matter using a 0.2 $\mu$m vacuum-driven filter [typically a Steriflip unit (EMD-Millipore SCGP00525), though highly turbid samples that clogged the initial filter were transferred to a secondary bell-style filter unit (Corning 430320), and filtrates were pooled to afford ∼45 mL of filtered wastewater]. Our initial lab method ("PEG-concentrated") used PEG salt precipitation to concentrate viruses from 40 mL of wastewater, as described by Wu et al.[19] The resulting pellets were resuspended in TRIzol (ThermoFisher 15596026), and RNA was purified via phenol/chloroform extraction and ethanol precipitation. The resulting RNA was resuspended in 30 $\mu$L of nuclease-free water. Two-step RT-qPCR was used to quantify the RNA. First, 10 $\mu$L of RNA samples was first reverse transcribed in a 25 $\mu$L reaction (NEB M0368), and then 2 $\mu$L per reaction of cDNA was assayed by qPCR (ThermoFisher 4444557) using a SARS-CoV-2 nucleocapsid (N) gene (N1 and N2 regions), and PMMoV amplicons,[31,32] (Table S4) on CFX96 or CFX-Connect instruments (Bio-Rad).

In June 2020, we switched to our second method ("Amicon-concentrated") that uses Amicon Ultra-15 centrifugal ultrafiltration units (Millipore UFC903096) to concentrate 15 mL of wastewater (filtered to remove solids as described above) approximately 100-fold. Viral particles in this concentrate are immediately lysed by adding AVL buffer containing carrier RNA (Qiagen 19073) to the Amicon unit before transfer and a >10 min incubation in a 96-well 2 mL block. To adjust binding conditions, 100% ethanol was added to the lysate and the entirety of the lysate and ethanol was applied to RNeasy Mini columns or RNeasy 96 cassettes (Qiagen 74106 or 74181), processed per the manufacturer's recommendations, and eluted in a total of 75 $\mu$L of nuclease-free water. For a subset of samples, we processed 30 mL of wastewater across two separate Amicon units, extracted each separately, and pooled the duplicate RNA extracts together prior to analysis. Then, 3 $\mu$L per reaction of RNA sample was subjected to one-step RT-qPCR (ThermoFisher 4444436) analysis in triplicate for N1, N2, and PMMoV amplicons on CFX96 and/or CFX-Connect instruments. Cts were called from raw fluorescence data using the Cy0 algorithm from the qpcR package (version 1.4-1) in R[22] and manually inspected for agreement with the raw traces in the native BioRad Maestro software. Overall, we processed 138 samples with the first PEG concentration method, 2577 samples with the second Amicon concentration method, and eight samples with both (see a discussion of sample reruns below). We confirmed that changing between methods would not introduce a systematic bias by showing that the variability between samples introduced by the method change was comparable to the inherent sample-to-sample variability (Figure S8).

In both methods, we ran a variety of laboratory controls. Positive synthetic SARS-CoV-2 RNA controls were included on every qPCR plate. Ct values for N1/N2 assays outside 31−33 triggered a plate rerun, and Cts on the original plate were discarded. Two no-template controls were included on every qPCR plate; N1/N2 Ct values of >40 and PMMoV Ct values of >35 for these controls triggered a plate rerun. One set of


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extraction blank controls was also run each day. Matrix inhibition was assessed manually by reviewing the raw qPCR curves for gradual slope and failure to reach the same maximum fluorescence as other samples on the plate (Figure S1). Samples with potential inhibition were flagged for more additional downstream data review. Finally, we used PMMoV as a proxy measure for per-plate recovery and flagged any qPCR plates with unusually low PMMoV values (relative to historical PMMoV values for the locations represented on those plates) for further review and potential plate rerun. Only results that passed all quality controls are reported here.

In addition to these laboratory controls, we implemented a thorough data review process, in which results were manually reviewed if they met certain additional criteria. These criteria included PMMoV being below first or above the 99th percentile of previously observed values, SARS-CoV-2 RNA concentrations changing >5-fold since the prior sample, inhibition suspected from manual inspection of qPCR curves, concentrations obtained from N1 and N2 primers not concordant, and pigmentation present in extracted RNA. During the manual review process, we inspected individual replicates of qPCR and timelines of SARS-CoV-2 and PMMoV RNA concentrations for the affected sampling locations. A small fraction of samples that underwent the review process were flagged for a lab rerun. Reruns were performed in duplicate if capacity allowed. If sufficient filtered wastewater from the initially processed tube remained, a second aliquot from that tube was tested. We also always processed an aliquot using a second of the three 50 mL tubes of sewage. Approximately 2% of all samples were rerun through that process. If a rerun differed substantially from the original result (i.e., more than ∼2-fold different), the original result was discarded (approximately 8% of reruns) and the rerun results were reported to the participating facility and included in this analysis; if the rerun recapitulated the original result, the averages of all results were reported to the participating facility and included in this analysis.

**Data Processing.** A standard curve was generated using serial dilutions of Twist Bioscience synthetic SARS-CoV-2 RNA control 2 (MN908947.3) and used to convert Ct values into copies per well (Figure S2). We used pepper mild mottle virus (PMMoV) as a fecal indicator. Because synthetic RNA for PMMoV was not available, we used a DNA gBlock to build a standard curve for PMMoV quantification.

Copies per well measured by qPCR were multiplied by a dilution factor accounting for the volume changes described above (RNA extraction, concentration, etc.) and then divided by the original sewage volume (40, 30, or 15 mL) to convert to a sewage concentration. Nondetected wells were replaced with zero for these calculations. Concentrations of N1 and N2 replicates were averaged first within each primer set and then across primers to obtain the final SARS-CoV-2 RNA concentration; replicates of the PMMoV amplicon were averaged. Samples were required to have at least two detected replicates between N1 and N2 and at least one detected PMMoV replicate to be considered a detection and subsequently quantified. To derive a normalized concentration, SARS-CoV-2 RNA concentrations were divided by the PMMoV RNA concentrations and multiplied by a reference PMMoV concentration derived as the median of our data set comprising samples up to July 31, 2020 ($3.65 \times 10^8$ copies/L). Dividing by the PMMoV concentration of the same sample helps account for fecal content, dilution, and lab processing efficiency, while multiplying by the reference PMMoV concentration converts the ratio back into units of concentration (copies per liter) on a similar scale as the original measured concentration. The reference PMMoV concentration does not have a specific meaning and was primarily used to adjust the normalized concentration ratio back into more interpretable numbers. The results were returned to participating municipalities in the form of a report containing the raw viral copies per liter of sewage and normalized concentrations.

**Data Analysis.** We excluded locations representing fewer than 5000 people in their catchment population to protect participant privacy and exclude small locations that are not expected to reflect community-level COVID-19 case dynamics[34] and locations that did not consent to data sharing. To evaluate the correlations between wastewater results and COVID-19 cases, we analyzed time series for all sampling locations with at least one sample per month for at least six months in the period of June 2020 through May 2021. Sampling location characteristics, including the population and associated county serviced by the catchment, were provided by treatment plant operators from each location (Table S1).

Daily COVID-19 cumulative case data were downloaded from USAFacts.org, which collated daily case counts for each U.S. county from publicly available reports.[23] To prevent negative new cases, any days with nonmonotonically increasing cumulative case numbers (or with missing or non-numeric values) were replaced with the prior valid cumulative case number. New cases were then calculated as the difference in cumulative cases between consecutive days. Seven-day centered moving averages were calculated using the pandas. rolling() function.[24] To derive daily incidence rates per 100 000 people, seven-day averages of reported cases were divided by the reported county populations, which were also downloaded from USAFacts.org.[23] All analyses reported here use these incident case values derived from USAFacts.org. The date associated with each case is the date that is reported by each public health agency as compiled by USAFacts.org.[23]

Monthly averages for the maps were calculated as follows. For the SARS-CoV-2 RNA concentration, we calculated the average normalized concentration per location for each month and then averaged these values across locations within individual states. For the reported cases, we first calculated the per capita new cases by dividing daily reported cases by the county population and multiplying by 100 000. We then selected just the subset of counties represented in our selected sampling locations and averaged daily reported cases per capita over the month within each county and then averaged across counties within the same state per month.

Statistical analyses and visualizations were performed in Python 3.8.

## RESULTS

**Sampling Locations.** We selected sampling locations that had a suitably long time series starting from June 2020, when we started offering COVID-19 wastewater surveillance as a commercial service. Prior to June 2020, sampling locations participated pro-bono as part of an academic collaboration.[20] Fifty-five sampling locations collected at least one monthly sample for at least six months from June 2020 through May 2021. These 55 locations represent approximately 12 500 000 people [mean of 245 000 and median of 55 000 people per location (Table S1 and Figure S3)] distributed across 39 counties in 19 states, with a maximum of five locations in one


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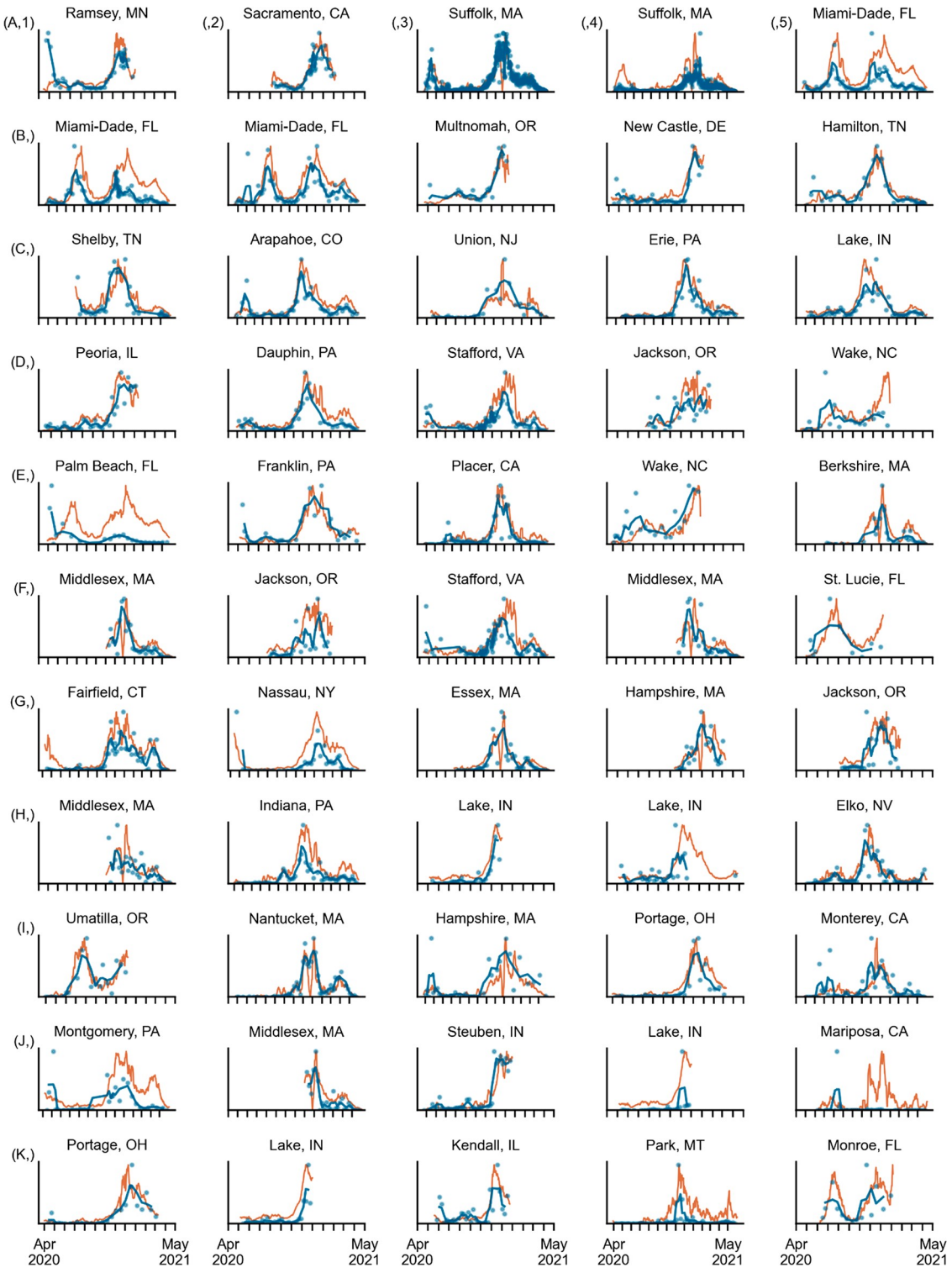


**Figure 1.** Time series of results from sampling locations with at least one monthly sample for at least six months from June 2020 through May 2021 ($n$ = 55): blue line, centered three-sample average of normalized wastewater concentrations (genome copies per liter); blue dots, individual normalized wastewater concentration measurements (genome copies per liter); orange line, centered seven-day average of daily new cases in the respective county (new cases). *Y*-Axes are normalized to the maximum of each time series, and lines are shown without units to emphasize relative trends within each location. *X*-Axes are consistent across plots, with monthly ticks ranging from April 1, 2020, to May 1, 2021. Plots are labeled with county and state names of the associated catchment. Sampling locations are organized in order of decreasing catchment population; i.e., the largest catchment (1 950 000 people) is at the top left (A1), and the smallest catchment (6400 people) is the bottom right most plot (K5). Rows are labeled with letters and columns with numbers for ease of reference. Individual time series for each location, including daily new cases and detailed units for both axes, are provided in the Abstract Graphic.

county. These locations took an average of 4.4 samples per month (Table S1 and Figure S4).

**Temporal Trends.** We compared the trends in wastewater virus concentration with reported cases in the respective



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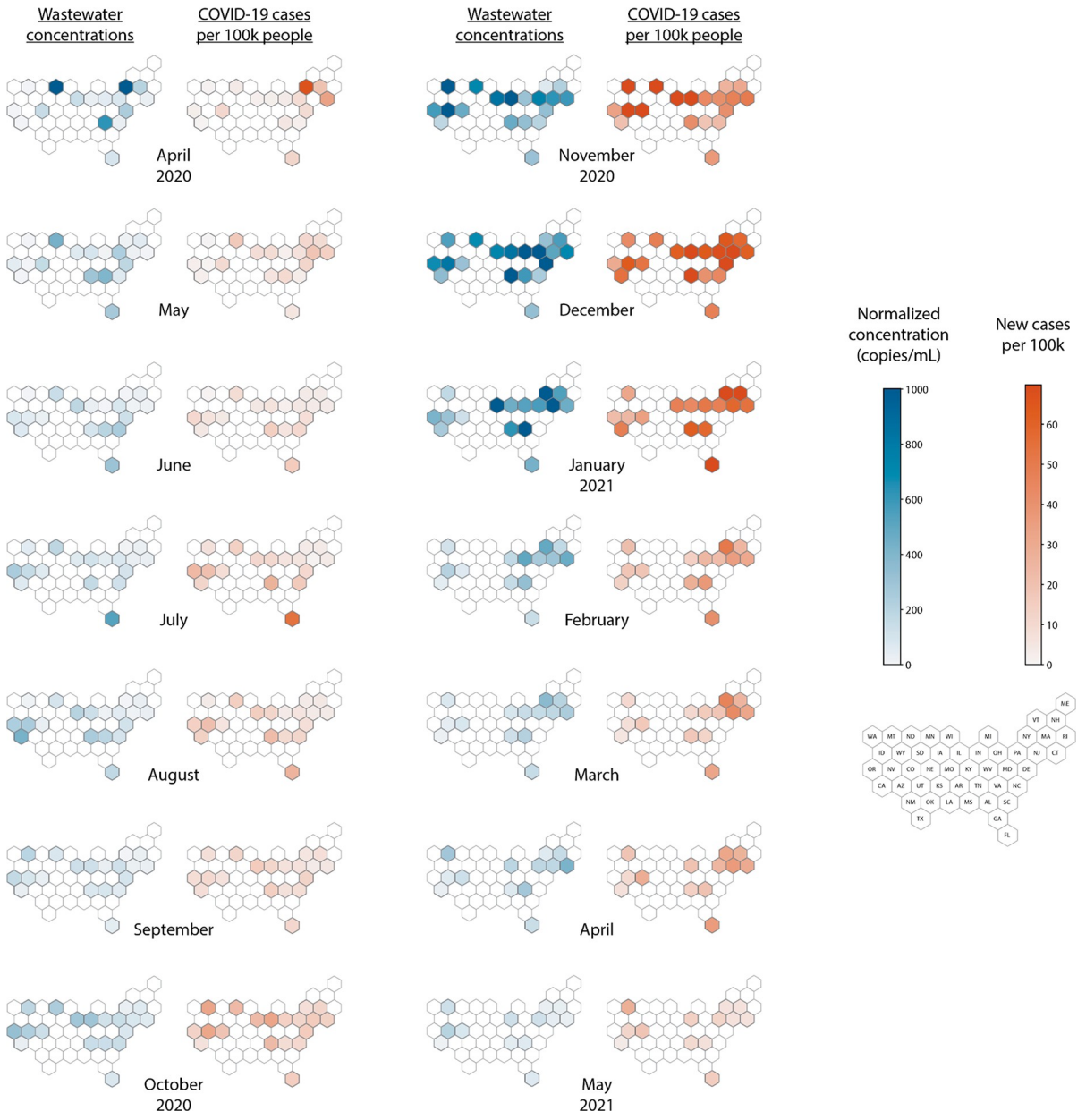


**Figure 2.** Monthly geographic trends in wastewater SARS-CoV-2 RNA concentrations and COVID-19 cases. Blue data (first and third columns from the left): Monthly state-level normalized wastewater SARS-CoV-2 RNA concentrations. The monthly state average is calculated as the average normalized concentration per sampling location per month and then averaged across sampling locations within the same state. Orange data (second and fourth columns from the left): monthly state-level new COVID-19 cases per 100 000 people for counties with at least one wastewater sampling location in that month. The monthly state average is calculated as the monthly average of new daily cases per 100 000 people in counties with a corresponding sampling location and then averaged across counties within the same state. States that did not include any sampling locations are outlined in gray. Color scales apply to all maps and are truncated at the 95th percentile of each data set.

counties for the 55 sampling locations. Wastewater SARS-CoV-2 RNA concentrations closely followed the seven-day average of new clinical cases (Figure 1), correlating well in the majority of locations [median Spearman correlation = 0.75; IQR = 0.65−0.83 (Figure 4)]. Wastewater measurements mirrored a rapid increase in clinical COVID-19 cases in late October through November 2020 that occurred in every county we sampled. It is noteworthy that many locations experienced the highest viral concentrations and highest clinical case counts observed to date in their November 2020 through January 2021 samples, reflecting the COVID-19 winter resurgence experienced nationwide in the United States (Figures 1 and 2). Wastewater also reflected the decrease in the number of clinical cases in the first months of 2021 (Figure 1).

Wastewater also tracked location-specific qualitative trends throughout the year. For example, a subset of counties experienced peaks in COVID-19 cases in the summer (June to August 2020) that were also well-tracked by wastewater data. For example, wastewater concentrations in all three sampling locations in Miami-Dade County, Florida (Figure 1, A5, B1, and B2), reliably reflected the two distinct waves of COVID-19 that Florida experienced during the sampling period. Cases and wastewater concentrations both increased in summer 2020, decreased to a new baseline, increased again in late 2020, and



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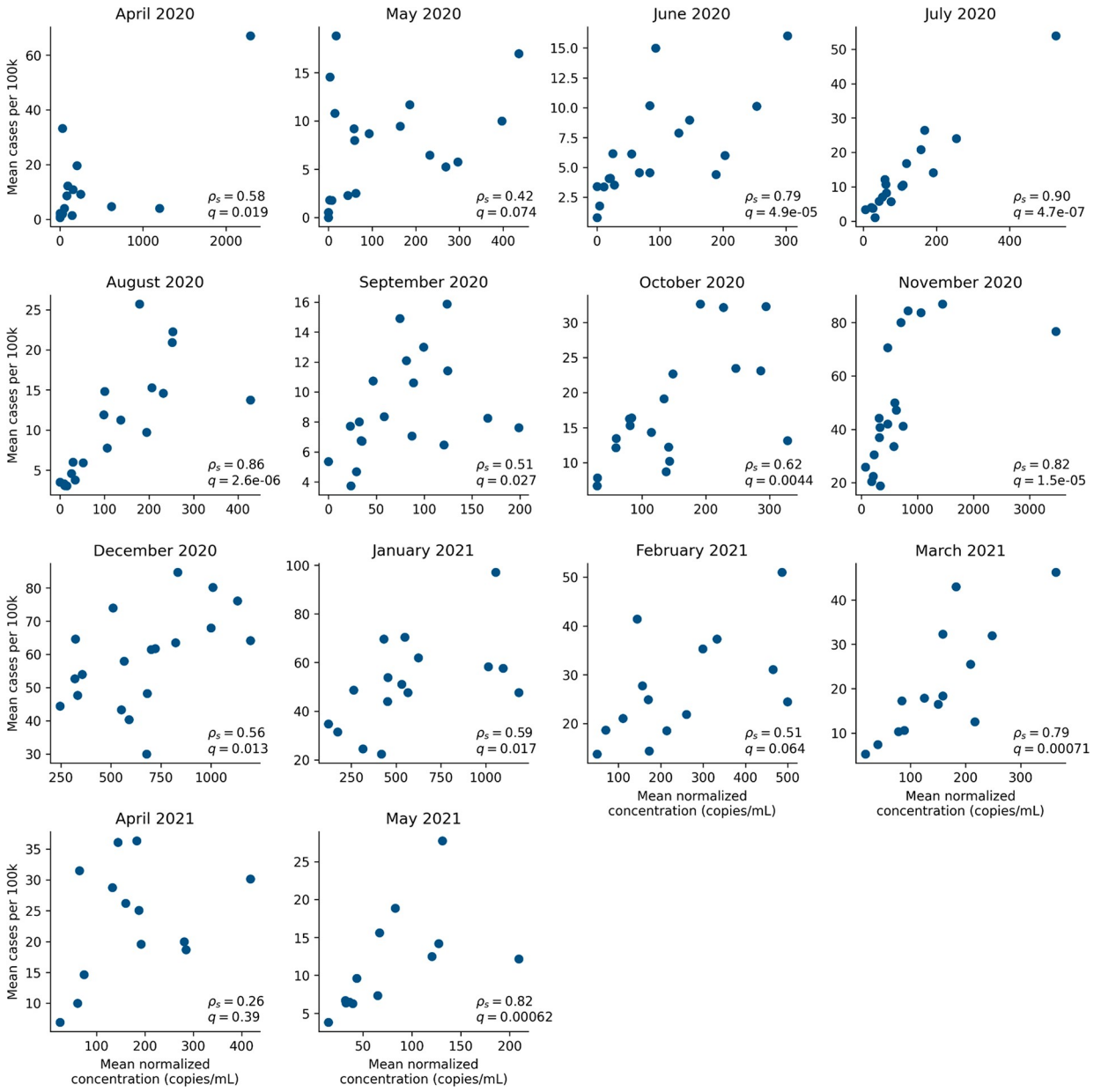


**Figure 3.** Geographic correlations per month. Scatter plots showing normalized concentrations ($X$-axis) vs new cases per 100 000 ($Y$-axis), averaged within each state (points) per month (subplots). Spearman correlations were calculated using scipy.spearmanr; correlations and uncorrected $p$ values are provided on each plot.

decreased once more in early 2021. Similar patterns were observed in the other smaller Florida sampling locations (Figure 1, E1, F5, and K5). Counties in other states experienced COVID-19 peaks earlier in 2020. For example, Suffolk County, Massachusetts, experienced a peak in COVID-19 cases in April 2020, which was reflected in the wastewater data from both associated sampling locations (Figure 1, A3-4). A similar trend was observed in Hampshire County, Massachusetts (Figure 1, I3). Finally, wastewater reflected different dynamics within the decreasing clinical cases early in 2021. For example, in Suffolk County, Massachusetts, wastewater seemed to plateau in February and March 2021 before decreasing again in April, a trend that was also seen in clinical cases. Similarly, many locations [Stafford County, Virginia (D3 and F3); Arapahoe County, Colorado (C2); Dauphin County, Pennsylvania (D2); Erie County, Pennsylvania (C4); and Lake County, Indiana (C5)] had a slight uptick in clinical cases around April 2021, which was reflected by the wastewater measurements. The ability of wastewater to track these more nuanced epidemiological dynamics supports its ability to provide independent confirmation of relative disease burden and trends (Figure 1).

We observed a range of correlations between wastewater measurements and new clinical cases across the 55 sampling locations (Figure 4A and Table S2). We first explored whether the correlation was associated with a sampling location's population, average reported flow, sampling frequency, or county coverage (Figure S5). None of these associations were statistically significant. Notably, wastewater treatment plants that covered more of their respective counties did not yield wastewater virus levels that were better correlated with county-level case counts than plants that served smaller proportions of their counties. These results imply that precise co-location of a


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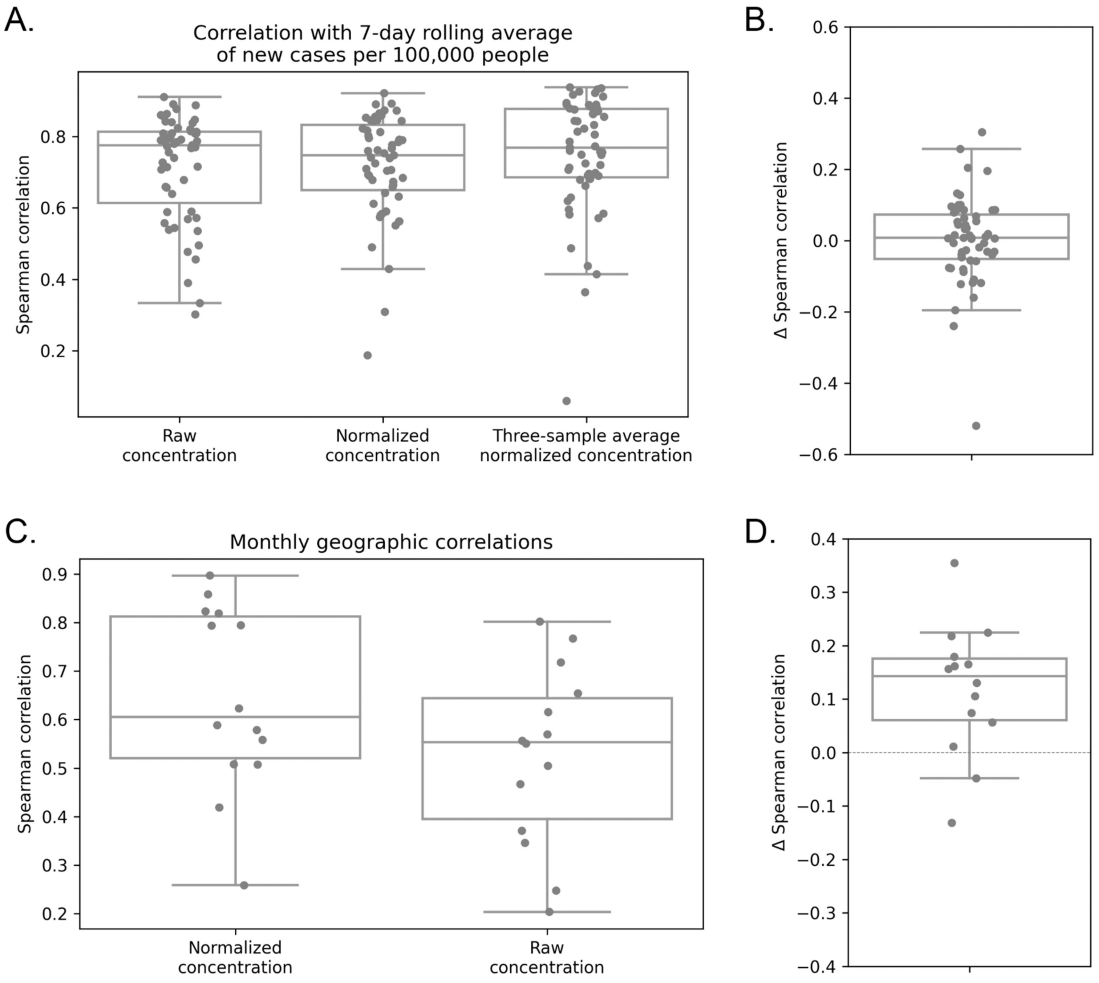


**Figure 4.** Impact of PMMoV normalization on correlations between wastewater SARS-CoV-2 RNA concentrations and COVID-19 cases, within and across locations. The top row shows temporal correlations between wastewater concentrations and new reported COVID-19 cases within locations. (A) A box plot shows the Spearman correlation between wastewater SARS-CoV-2 RNA concentrations (copies per liter) and new reported clinical cases (total cases). Each point is the correlation for one location. Correlations were calculated using three different measures of wastewater concentration: raw, unadjusted SARS-CoV-2 concentrations, SARS-CoV-2 concentrations normalized to a fecal maker (PMMoV), and a three-sample average of the normalized concentrations. (B) Difference in the Spearman correlation when calculated using normalized or raw concentrations. Each point is a sampling location; a positive delta indicates that the Spearman correlation calculated using normalized concentrations is higher than the correlation calculated using raw concentrations. The bottom row shows the geographic correlations with normalized vs raw concentrations. (C) Each point is a month. The *Y*-axis shows the correlation between states during that month. The *X*-axis indicates which concentration measure was used to calculate the correlation. (D) Difference in the Spearman correlation when calculated using normalized or raw concentrations. Each point is a month; a positive delta indicates that the Spearman correlation across states calculated using normalized concentrations is higher than the correlation calculated using raw concentrations.

wastewater catchment and the geographical case reporting area is not critical to wastewater's ability to model COVID-19 burden.

Next, we explored the impact of normalizing raw SARS-CoV-2 RNA concentrations with a human fecal marker (PMMoV) on the observed correlations (Figure 4A,B). We found that normalizing by PMMoV did not always improve the correlation between wastewater measurements and reported clinical cases within individual time series (Figure 4A,B, Figure S6, and Table S2). In some locations, normalization improved the correlation, but in others, raw virus concentrations correlated better with cases (Figure 4B). These results confirm findings by others who have investigated the impact of normalization in multisite studies and found mixed results on the impact of normalization on correlations.[25,26,35] We hypothesize that the broad range of locations profiled leads to a large variability in wastewater matrices and therefore a similarly large variability in the impact of normalization on data quality.

**Geographic Trends.** We next compared geographic trends in average monthly SARS-CoV-2 RNA wastewater concentrations and reported COVID-19 cases across states (Figures 2 and 3 and Table S3). Overall, wastewater concentrations showed similar relative patterns across states as did clinical cases [median Spearman correlation across states = 0.6; IQR = 0.52−0.81 (Figure 3)]. These results indicate that normalized wastewater concentrations can be used to compare the relative COVID-19 burden across different geographies.

The strength of the geographic correlation varied throughout the course of the pandemic (Figure 3). The second-lowest correlation was observed early in the pandemic in May 2020 (Spearman correlation = 0.4), which could be explained in part by different testing capacities across states, leading to geographic differences in the completeness of the case data. Similarly, a


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lower correlation in December 2020 could have been affected by irregularities in case reporting around the American winter holidays. In contrast, some of the highest correlations between wastewater and clinical cases across states were observed in the summer 2020 wave, when COVID-19 was rapidly increasing and clinical testing was well-established nationwide [June 2020, 0.79; July 2020, 0.90; August 2020, 0.86) (Figure 3 and Table S3).

We next investigated the effect of normalization on the ability for wastewater concentrations to reflect patterns in clinical cases across locations (Figure 4C,D). We found that raw wastewater concentrations across states did correlate with cases in most months but that these correlations were weaker than when looking at the normalized concentration (Figure 4C and Table S3). In fact, normalization improved the geographic correlation in all but two months (Figure 4D). These findings align with prior work that has also shown that normalizing wastewater measurements improves comparisons across locations.[35]

## DISCUSSION

Effective and long-term systems will be required to continuously monitor SARS-CoV-2 across the United States. While diagnostic testing and clinical case reporting continue to drive public health recommendations, wastewater assessment of SARS-CoV-2 is a practical and sustainable complement that can be integrated into existing wastewater infrastructure to provide cost-effective population-level monitoring. With effective vaccines against SARS-CoV-2, persistent surveillance to detect resurgences of the virus will become increasingly important. Here, we demonstrate that wastewater data reflect both temporal and geographic trends in COVID-19 disease burden across the United States, suggesting its utility on a national scale as a SARS-CoV-2 surveillance system.

**Lessons Learned from Nationwide Implementation.** Through this work, we identified several key practical considerations for implementing a long-term nationwide wastewater monitoring program. First, the impact of changing laboratory methods on overall data interpretation was minimal. From a scientific standpoint, changing methods throughout the course of a study is usually not recommended due to the possibility of introducing batch effects. However, this secondary research study uses a data set generated through a regular commercial wastewater surveillance service, which required that the laboratory methods changed as our experience improved and reagent supply chains stabilized (Methods). We investigated the impact of these method changes on our data set and found that the overall interpretation of the data remained unaffected (Figures S7 and S8). Importantly, updating methods to reflect current scientific understanding and changing epidemiological contexts will be required for any nationwide WBE monitoring system implemented in practice. For example, as the number of COVID-19 cases decreases substantially, methods will need to be updated to improve sensitivity. Long-term wastewater monitoring studies should evaluate the impact of method changes on data interpretation and ensure that data can be continuously interpreted across methods, or otherwise implement correction factors to adjust data for continuity. Normalization to PMMoV or other fecal markers may aid in this analytical effort. The results presented here demonstrate agreement between wastewater measurements and clinical cases despite our methodological changes, highlighting that while addressing the impact of variations in laboratory methods remains an important area of scientific inquiry, it is not necessary to have a gold standard methodology established for wastewater-based epidemiology to be implemented at scale and to provide reliable reflections of public health trends. From our experience, clearly and transparently communicating about methods was also critical to maintaining trust in our data across academics, wastewater and public health officials, and groups deploying WBE at scale.

Second, we found that identifying best practices for wastewater-based epidemiology requires a holistic approach beyond purely scientific considerations. For example, the rapid turnaround time of the data quickly emerged as a key requirement for our data to be useful to our sampling partners and their public health counterparts. Therefore, our method development efforts prioritized minimizing the operational impact to turnaround time above other considerations. Even at this nationwide scale, we were able to achieve a rapid turnaround time of approximately one to two business days for the majority of the sampling period. In addition to these considerations, any changes to our methods and data interpretation required careful considerations of data continuity and communication to relevant stakeholders. Moreover, we found that a manual data review process was essential for ensuring the quality of the data, development of QC metrics, and understanding of the data trends. As WBE expands in practice, difficult trade-offs will need to be made when improving data quality conflicts with operational requirements.

Finally, logistical considerations also dictate which data sets can be used as epidemiological comparisons for WBE at scale. For example, precise geographic alignment between catchment sewersheds and reported cases was not feasible, as it would have required requesting and coordinating GIS data across a different set of municipal partners for each sampling catchment. We used data provided by USA Facts for our clinical comparison because at the time we launched this work, it was the only data source that provided geographical comparisons more granular than state level and was licensed appropriately for our use. Future WBE efforts implemented at scale will similarly need to leverage systematically collected and curated third-party data sets or expend significant resources to compile them themselves. Importantly, clinical data itself are not necessarily a gold standard for comparing wastewater-based results. In the case of COVID-19, reported cases are used as a benchmark to evaluate how well wastewater is reflecting broad trends but are not necessarily comprehensive due to asymptomatic infections and limitations or inequalities in access to diagnostic testing.[13] In fact, the comprehensiveness of wastewater is one of its key strengths, and deviations between wastewater and clinical data may reflect limitations in the clinical data itself.[15]

**Limitations.** Our study has several limitations. First, wastewater SARS-CoV-2 RNA concentrations are highly variable due to sampling, lab processing, and qPCR analysis. While some variability reflects true fluctuations in COVID-19 incidence and SARS-CoV-2 levels in the wastewater samples, we attempted to reduce the impact of method-related variability through normalization with a fecal virus with biological similarities to SARS-CoV-2[19] and by optimizing our lab protocols (Methods). However, we still observed large spikes in wastewater concentrations in some locations that were not explained by changes in reported COVID-19 cases. Some spikes could be reflective of unreported cases, while others may simply be outlier wastewater measurements. These data challenges are common in the field of wastewater-based epidemiology, and our group and others are actively developing models to correct for


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variability and better interpret spikes. Second, the geographic comparisons between wastewater results and reported cases are not exact, as sampling location catchments may represent a subset or superset of the respective comparison counties (Table S1). However, we found no relationship between a catchment's coverage of its comparison county and its correlation between wastewater and cases, indicating that these wastewater catchments were sufficient to identify broad and general trends in their associated communities (Figure S5). Alternative public health applications of wastewater-based epidemiology like building-level or manhole sampling may require more granular insight, in which geographies may need to be more precisely aligned. Third, wastewater data showed a range of correlation strengths with reported cases (Figures 1 and 4). In addition to technical and geographic factors, these differences could also be due to different testing capacities across space and time. For example, access to testing was extremely limited early in the pandemic, which may in part explain the lower geographic correlation at that time.

**Implications for National Wastewater Surveillance Implementation.** Despite these limitations, wastewater assessment of SARS-CoV-2 reliably reflected trends in clinical COVID-19 cases within and across locations, confirming the feasibility of acquiring useful data from a broad variety of wastewater facilities. The sampling locations profiled in this study were selected in a nonbiased fashion without any preconsideration of the wastewater results; we imposed no exclusion criteria beyond a catchment size cutoff of 5000 people and a suitably long sampling period for the analysis. Furthermore, wastewater data reflected clinical trends across a range of wastewater facility characteristics, including catchment populations ranging from just more than 6000 people to close to 2 million individuals. Results in this study are comparable to prior work that has profiled individual sampling locations and found a range of correlation strengths between wastewater SARS-CoV-2 RNA concentrations and clinical cases.[19,27−29,35] Deploying WBE to this large number of communities demonstrates the generalizability of these results on a large nationwide scale.[12]

This study also provides additional insight into whether and how normalization impacts wastewater data interpretation. When analyzing wastewater concentrations as a reflection of COVID-19 trends within individual sampling locations, we found that normalization improved the correlation between wastewater and reported cases in some sampling locations, but not in others (Figure 4B). In contrast, we found that normalization did improve correlations when comparing across locations, perhaps because different wastewater matrices with differing levels of dilution can be corrected for by a common normalization marker (Figure 4D). Finally, in our experience, fecal normalization had additional practical benefits beyond improving correlations, addressing concerns related to sample quality and potential dilution and serving as an additional endogenous laboratory control for all samples.

This work also demonstrates the practical feasibility of implementing and scaling a national wastewater surveillance system for COVID-19. Wastewater surveillance is straightforward to implement with the participation of wastewater utilities; we achieved broad uptake among different municipalities across the United States.[20] All of our participating municipalities were able to reliably collect samples using standard wastewater sampling devices and often as part of their regular sampling schedules, requiring little extra work on their part. Additionally, samples were sent to us through traditional mail services and the majority passed our internal quality control process (Methods). This confirms that national wastewater surveillance programs like those supported by the U.S. Centers for Disease Control and Prevention are logistically and practically feasible.[12] With sufficient resources, a national WBE dashboard could complement similar population-level mapping of SARS-CoV-2 based on clinical reports,[30,33] lending insights into key operational decisions like phased reopenings, geographic selection of testing locations, and hospital preparedness.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.1c00434.

Figures and tables containing additional analysis results not shown in the text, detailed information about catchment characteristics and experimental methods, and time series of all individual locations with labeled axes (PDF)

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### Notes

The authors declare the following competing financial interest(s): C.D., K.A.M., N.E., M.I., R.F.-O., M.M.P., S.M., S.T.W., F.C., T.M., C.L.S., and S.W.O. are current or former employees of Biobot Analytics, Inc. E.J.A. is scientific advisor to Biobot Analytics, Inc.

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