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Article

Comparing Rates of Change in SARS-CoV-2 Wastewater Load and Clinical Cases in 19 Sewersheds Across Four Major Metropolitan Areas in the United States

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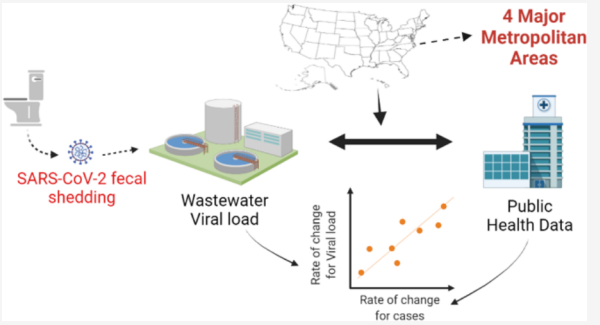
ABSTRACT: There is no standard approach to interoperate the multiple SARS-CoV-2 wastewater surveillance data sets generated during the pandemic. We tested several data processing approaches on wastewater surveillance data sets generated from 19 sewersheds across four major metropolitan areas in the United States from May 2020 through October 2021. First, we explored the effect of different data processing techniques on the correlation between SARS-CoV-2 wastewater RNA load and clinical case counts and found that locally weighted smoothing (LOESS) smoothing applied to multivariate imputation by chain equations (MICE)-imputed wastewater viral load led to the strongest correlations in 16 out of 19 sewersheds (84%). Next, we calculated the rate of change (RC) in wastewater viral load and in clinical cases and found that imputing missing viral load data on a 28-day window produced the strongest correlation (Spearman's ρ = 0.63). Furthermore, we determined an average sensitivity threshold of 2.4 new COVID-19 cases per day resulted in a significant RC in wastewater, but sensitivity varied with the laboratory method used. Our retrospective analysis using RC highlighted certain methodological insights, reduced site-specific impacts, and estimated a wastewater sensitivity threshold—supporting the use of relative, rather than absolute, measures of SARS-CoV-2 wastewater data for more interoperable data sets.



KEYWORDS: *wastewater-based epidemiology, COVID-19, sensitivity, normalization, threshold, imputation, smoothing*

INTRODUCTION

The COVID-19 pandemic has demonstrated the utility of wastewater surveillance as a public health tool. Following early studies that showed that SARS-CoV-2 RNA could be detected in wastewater,[1] there has been a rapid development of laboratory methods for measuring SARS-CoV-2 RNA concentrations in wastewater.[2,3] The application of wastewater for monitoring COVID-19 prevalence has grown. In the United States, there have been investments from the U.S. Centers for Disease Control (CDC) to develop the National Wastewater Surveillance System (NWSS) to centralize local efforts occurring within health departments,[4] and similar investments have occurred around the world. Demonstrations of strong correlations between SARS-CoV-2 RNA concentrations in wastewater and clinical cases in sewersheds[5−7] have helped to provide a proof-of-concept for wastewater surveillance for COVID-19. Comparisons of the multiple wastewater surveillance data sets generated over the course of the pandemic can help build further confidence in wastewater surveillance as a public health tool.

Current wastewater surveillance is marked with disparate sampling and analysis efforts, while health authorities seek to centralize data interpretation. A range of different methods for quantifying SARS-CoV-2 in wastewater has been applied because standard methods have not yet been agreed upon.[8] Yet, interlaboratory comparisons have illustrated method variability of up to 1 log with respect to SARS-CoV-2 RNA concentrations.[9] Even within the same laboratory, researchers have observed site-specific matrix effects that complicate data interpretation.[10] To facilitate public health action, there is a need for a common approach that minimizes the impact of site- and method-specific differences in SARS-CoV-2 recovery and quantification on data interpretation. Comparisons across multiple data sets can help identify points of consensus that will help standardize data interpretation.

Many researchers have focused on using wastewater surveillance for trends analysis. Trends are useful for public

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Table 1. Number of Sampling Events, Number of Sewersheds, Dates Sampled, and Range of Population Served for Each Major Metropolitan Area

city	number of sewersheds	sampling frequency per week	number of samples	dates of sampling	population served
City 1	3	2	39−55	April 2020−Dec 2020	174,260−2,300,000
City 2	10	1	65−67	June 2020−Oct 2021	58,000−420,500
City 3	5	1−2	28−36	May 2020−March 2021	150,000−4,000,000
City 4	1	2−3	101	April 2020−Dec 2020	580,000

health communication, as has been shown in several publicly facing dashboards.[11,12] Currently, the CDC uses a 15-day percent change on a linear regression of the log-transformed SARS-CoV-2 levels to estimate trends with at least two sampling points.[13] However, there is no guidance on how these thresholds correspond to changes in clinical cases. Wastewater viral thresholds largely depend on the sensitivity of the wastewater signal, which can be affected by methodological and sampling choices (e.g., ddPCR vs qPCR,[14] sampling frequency,[15] sample storage,[16,17] and sample matrix[16,17]).

There are additional challenges with conducting trends analysis. There have been several studies showing wastewater can both lead[15,18−20] and lag[21] clinical surveillance data, and the extent to which wastewater leads or lags clinical data likely varied across localities over the course of the pandemic due to SARS-CoV-2 RNA signal decay and turnaround time in clinical surveillance measurements.[22] An additional challenge is the effect of wastewater sampling frequency on trend calculations. Dynamic data of changing conditions on a weekly basis was useful for public health action, but wastewater surveillance efforts occurred at sampling frequencies lower than the daily reporting that is used in clinical case surveillance. In some instances, the NWSS is currently using two-point trends calculations in order to have trend information at a 15-day interval. Imputation approaches may help address this limitation. Further, wastewater surveillance data tends to be noisy, and smoothing is commonly applied,[23] but there is a wealth of smoothing methodologies and no consensus approach. There is no standardized approach on how to manage these challenges when calculating trends.

Toward the development of a standard approach for evaluating the trends between wastewater viral load and clinical cases, we demonstrate the utility of rate of change (RC) analysis on wastewater surveillance data from 19 sewersheds across four major metropolitan areas in the US. The goals were to (1) evaluate the effect of different data processing techniques on raw wastewater viral load and RC in terms of the correlation to clinical cases, (2) estimate an overall sensitivity threshold of wastewater surveillance to detect changes in cases, and (3) leverage RC analysis to develop hypotheses on the relative sensitivity of different laboratory methods that should be tested empirically in future research. Wastewater samples were analyzed for SARS-CoV-2 RNA by four different laboratories, corresponding to the four different metropolitan areas, with varying sampling frequencies and laboratory methods. We expected that RC, a relative marker, rather than absolute values would reduce site- or lab-specific effects such as testing penetration (i.e., the percentage of the population in a locality that is tested for SARS-CoV-2), laboratory method, and RNA decay due to varied sample storage approaches. We show that RC can be useful to develop methodological insights, reduce site-specific impacts, and estimate sensitivity thresholds. This RC analysis can be used to develop hypotheses for laboratory methods that may result in more sensitive wastewater surveillance approaches.

METHODS

Wastewater Sampling and Analysis. Sampling across 19 sewersheds in four major metropolitan areas (City 1, City 2, City 3, and City 4) occurred beginning April 2020 through October 2021. Three of the 19 sewersheds were from City 1 (City 1 S1−S3), 10 of the sewersheds were from City 2 (City 2 S1−S10), 5 sewersheds from City 3 (City 3 S1−S5), and 1 sewershed from City 4 (City 4 S1). Sampling frequency and storage conditions, where applicable, varied across the treatment plants and are summarized in the Supporting Information (SI). All samples were 24 h composite samples of raw influent. Viral concentration, RNA extraction, and quantification methods are summarized in Table S2. Methods for City 1 and City 4 are summarized in Section S1. Methods for City 2 and City 3 have been previously reported.[24,25] Table 1 lists each of the metropolitan areas included in this analysis and the number of sampling events (points of viral load for each sewershed) that were conducted throughout the time period specified in the table. The population served ranged from 58,000 to 4,000,000 people across the sewersheds.

Clinical COVID-19 Case Data. Public health data were collected across the 19 sewersheds. The data for City 1 were obtained from publicly available COVID-19 case data.[26−28] The data for both City1_S1 and City1_S3 were aggregated by adding up zip code level data for all zip codes contributing to the sewershed, and City1_S2 case data were specified at the county level because the sewershed encompasses the entire county. Clinical cases for both City1_S1 and City1_S3 were obtained between May 2020 and December 2020 and for City1_S2 was between April 2020 and August 2020. The case associated with each date is the date when the positive specimen was collected in all the City 1 metropolitan area's sewersheds. For City 2, the number of daily positive cases in each sewershed was obtained from the City 2's Health Department. If the number of daily COVID-19 cases was too low to maintain anonymity in a certain sewershed (fewer than five cases per day), the case data for that day was omitted. For City 3, GIS shapefiles of sewersheds were obtained from utilities. The GIS shapefiles were then overlaid onto the shapefile for Countywide Statistical Areas (CSAs), acquired from the City 3 County GIS Hub. Using QGIS, the distribution of these CSAs within their corresponding sewershed was determined. Next, the proportion of a CSA lying within a given WWTP sewershed was used to quantify the portion of cases that CSA contributed to each sewershed. Using available data from the City 3 County Department of Public Health, new cases per day per CSA were distributed to each of the five sewersheds sampled. For City 4, individual-level, lab-confirmed COVID-19 cases with residential addresses were provided by the state's Department of Health and Human Services. Positive case counts included PCR-

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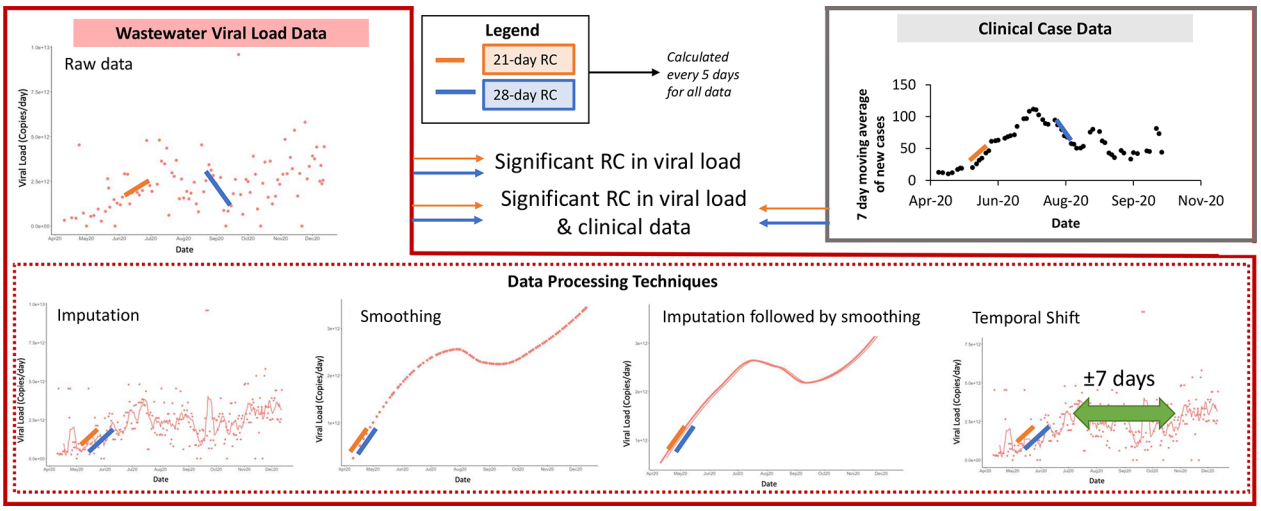

Figure 1. Methods and scenarios used to calculate the rate of change for both viral load and clinical case data.

positive tests, antigen-positive tests, and a small number of PCR-negative tests determined to be positive cases on the basis of physician case notes. Cases in the sewershed were summed to produce daily case counts using specimen collection dates. Across all the cities, the clinical data used were smoothed for the trend analysis using a 7-day moving average.

Trends Calculation. *Wastewater Data Processing.* All data analysis was performed using R and R-Studio.[29] The wastewater analysis data (N1 copies/liters of wastewater) and flow rates were available for each wastewater treatment plant monitored across the four cities. The N1 gene was chosen as the target gene for this analysis due to the better correlations with the clinical case data in comparison to the N2 gene.[15] Viral loads were calculated by multiplying the viral concentration in copies/liter by the flow rate in liter/day. Methods of dealing with replicate samples are described in Section S1.

Data Processing Techniques: Imputation, Smoothing, and Temporal Shift. We tested several data processing techniques for viral loads. The data processing techniques used were the following: (1) imputation, (2) smoothing, (3) combined imputation and smoothing, and (4) applying a temporal shift in the data (Figure 1). We tested the impact of imputation used to develop daily estimates of viral load from the less frequent measurements using the multivariate imputation by chained equations (MICE) algorithm.[30] The MICE framework process is broken down into several steps.[31] First, a regression model (using a random forest model) between the dependent variable (observed values of viral load) and the independent variable (clinical cases) is performed. Next, the missing values in the dependent variable (viral load) are substituted using predictions from the regression model from the first step. The two steps are then repeated for a number of cycles (iterations) that are specified by the researcher. Each time a new cycle starts, the regression model predictions will be based on the previous cycle's imputed values.

To parametrize the imputation, we tested a range of iterations (5, 10, 20, 40, 80, and 160) for each data set. Each of these iterations results in five different imputed data sets. The number of iterations and the number of resulting imputed data sets are kept at the numbers specified above for computational reasons. To determine the best number of iterations for each sewershed, the average Spearman's ρ correlations between viral load and clinical cases from the five imputed data sets were calculated. The number of iterations with the highest average correlation strength was used for subsequent analysis. The highest correlation within the five imputed data sets was used as the imputed data set for each of the sewersheds. This resulted in each sewershed having its own imputation parametrization with a varied range of iterations.

To smooth the data, we evaluated locally weighted smoothing (LOESS), which uses a second-degree polynomial fitting of the viral load and predicts the outcome of the smoothed data. We evaluated the combination of these by first imputing the data, then smoothing the imputed data set. Lastly, we evaluated a temporal shift for each of the 19 sewersheds by shifting the imputed viral load by −7 to +7 days. The correlations between imputed viral load and clinical cases were re-evaluated for each of the shifts using Spearman's rank correlation. The shift that resulted in the highest Spearman's ρ for each sewershed was then used to recalculate rates of change (RC) described below. In some instances, temporally shifting the wastewater data did not result in stronger correlations with case data, in which case the nonshifted data were used for the RC analysis. Spearman's rank correlations were used to test the relationship between viral loads and the 7-day moving average of clinical cases for each data processing technique.

Rate of Change (RC). The rate of change (RC) was calculated for both the viral load and clinical case data for each of the 19 sewersheds monitored using each of the data processing techniques (Figure 1). The RC was found by fitting both viral load and clinical cases into a linear model, where viral load or clinical cases is the dependent variable, day is the independent variable, and RC is the slope of the model. The RC was measured in two different time windows moving every five calendar days starting with the first day of sampling. The first-time window chosen was 21 days and the second was 28 days. The purpose of having the time windows was to evaluate the effect of the time window while ensuring at least three different sampling events for the viral load were captured in the analysis. We made all our sites comparable to one another by normalizing the rate of change to the maximum rate observed in the sewershed.

The p-values values for each of RCs calculated were adjusted to account for multiple comparisons and reduce the false

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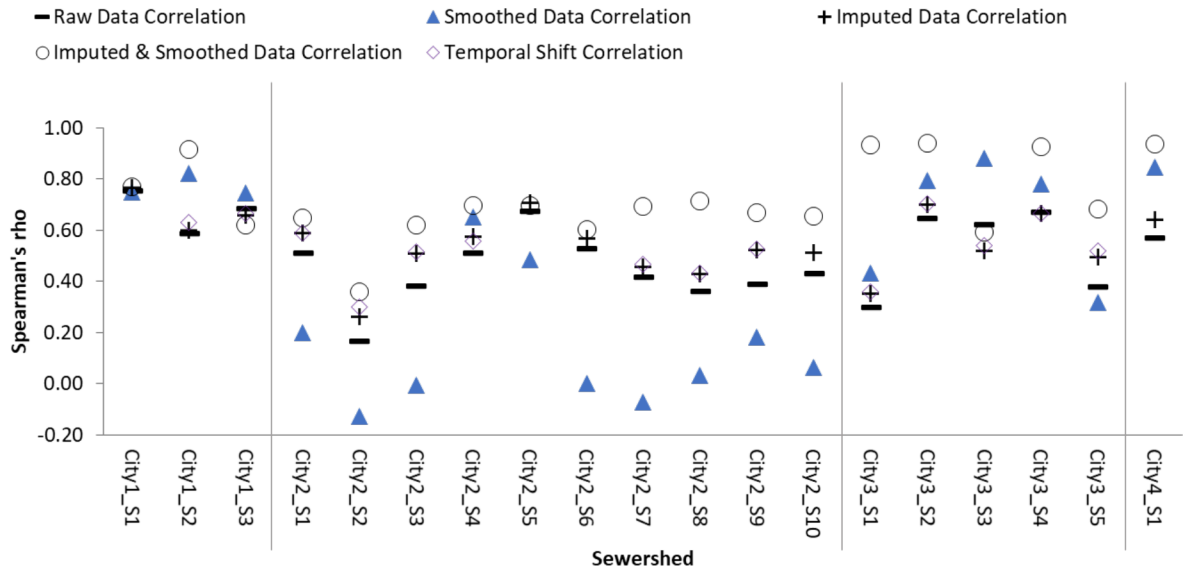

Figure 2. Spearman correlations comparison between the viral load versus the 7-day moving average for clinical cases in each sewershed. Temporal shift correlation corresponds with the temporal shift that had the highest Spearman's ρ. Instances without a temporal shift icon indicate no lead/lag evident in data set.

discovery rate using the Benjamini-Hochberg correction factor. The false discovery rate corresponds to the rate of type I errors in the null hypothesis when doing multiple comparisons. The RCs between viral load and clinical cases only include instances (points in graphs) where rates are significantly different from zero (corrected p-value ≤ 0.05) for viral load or both viral load and clinical cases. The correlations between the RCs of viral load and clinical cases were evaluated by the Spearman's rank correlation.

Sensitivity Threshold Estimation. Sensitivity of the wastewater surveillance efforts was defined in this study as the number of new cases per day required to obtain a significant change in viral load. In other words, it is the slope of the clinical case data that corresponded with instances of significant ($p < 0.05$) changes in viral load on the basis of the linear model. For all 19 sewersheds, we calculated the absolute value of the slopes on the clinical data to not differentiate sensitivity between increasing and decreasing waves. The median slope in the clinical data that corresponded with instances of significant changes in viral load in the wastewater data was estimated using a Wilcoxon Sign-Rank test, because clinical slope data failed a test of normality using the Shapiro Normality Test. A Mann−Whitney U Test was used to compare sensitivity across different laboratory methods.

RESULTS AND DISCUSSION

Compared with all data processing techniques, combined imputation and smoothing viral load led to the strongest correlations between wastewater and clinical cases in over 80% of the sewersheds. We explored the effect of different data processing techniques on the correlation between SARS-CoV-2 wastewater RNA load and clinical case counts in 19 sewersheds. The data processing techniques examined were: (1) smoothing using the LOESS method, (2) imputing using MICE, (3) a combination of imputation and smoothing, and (4) temporally shifting viral load data. Figure 2 shows Spearman correlations between the wastewater viral load (N1 copies/day) and the 7-day moving average for clinical cases for all sewersheds and how the correlations changed with different data processing methods. For each of the sewersheds, at least one of the data processing techniques (i.e., imputation, smoothing, smoothing imputed data, or temporally shifting data) improved the correlations between wastewater and cases, relative to no data processing. However, no single approach resulted in the strongest correlation for all sewersheds.

In two sewersheds, smoothed raw viral loads had the strongest correlation with case data out of all the data processing techniques, but in general smoothing viral loads led to weaker correlations in 11 of 19 sewersheds compared with raw data. However, in some instances, smoothing substantially reduced the strength of the correlations (City 2). This may be related to sewershed size, as sewersheds serving over 1 million people had improved correlations with smoothing and City 2 had 8 out of 10 of the smallest sewersheds analyzed (average population of City 2 sewersheds: 159,000). We posit that there may be higher stochasticity in clinical positive cases of smaller sewersheds that are averaged out with larger populations that affect the correlations with smoothed viral loads. Besides sewershed size, there were no clear patterns on the effect of smoothing the viral load with other lab-specific metrics such as wastewater sampling frequency, methods of RNA extraction (direct capture vs filtration), and methods of SARS-CoV-2 RNA quantification (qPCR vs ddPCR). Smoothing the imputed viral load, rather than smoothing raw viral loads, led to the strongest correlations in 16 out of 19 sewersheds (84%) across all the data processing techniques (including the raw correlations), while imputing the viral load yielded strongest correlation in only one sewershed.

Several studies have applied LOESS smoothing techniques to visualize wastewater SARS-CoV-2 data;[10,32,33] however, a comparison of how LOESS-smoothed versus unsmoothed data correlated with case data has not been reported. From our study, LOESS-smoothing does not consistently improve the correlations to cases, which is in contrast to a study that looked at two sewersheds in Spain where the LOESS quadratic model was best at predicting cases.[34] LOESS smoothing was

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implemented in our study because, compared to other methods, it captures increasing and decreasing trends in our data sets that were due to the multiple COVID-19 waves that occurred across the metropolitan areas. Other researchers have compared different smoothing techniques on wastewater SARS-CoV-2 monitoring data such as Brown's linear exponential model[25] and SPLINES.[35] Those methods were found to improve correlations between viral load and clinical cases. Indeed, SPLINES and Brown's linear exponential model may be more beneficial when doing within-city comparisons where the expected overall trends in infection would be more consistent than in our study. Our study highlights that LOESS smoothing does not provide consistent improvements in correlations across 19 sewersheds. Therefore, a standard approach to data interpretation may not include a requirement for LOESS smoothing—the effect of LOESS smoothing should be evaluated on a case-by-case basis.

Given the different wastewater sampling frequencies across the sewersheds in this study, we tested imputation to fill missing data with less frequent sampling and thereby standardize the data sets. There are several techniques used to fill missing data including simple substitution with mean, minimum, or maximum values, or imputation using models. Few studies have applied imputation in wastewater monitoring studies,[34] and we found only one that used data imputation for interpolation.[32] None of the studies evaluated the effect of imputation on the overall correlations between wastewater data and clinical case counts. For our study, we used the MICE algorithm to impute the missing data for viral load, because it allowed us to use clinical data as an explanatory variable to perform the imputation. Imputation qualitatively did not change the trend of the viral loads for the sewersheds (Figures S3−S6 compared with Figures S11−S14). We expected imputation to improve the correlation for all sewersheds compared with correlations of the raw data since we used clinical case data as an explanatory variable to impute viral load data. Compared, with raw data correlations, imputing the wastewater viral load strengthened the correlation in less than 85% of sewersheds; three sewersheds had stronger raw data correlations than the imputed correlations.

We temporally shifted the imputed wastewater load data to identify if viral load was a leading or lagging indicator in comparison to clinical cases. The correlations with the 7-day moving average of clinical cases were compared when applying a 1−7-day lead and lag offset of the imputed viral load data for each of the 19 sewersheds. Table 2 summarizes the top correlated lead/lag shift for the viral load when compared to the clinical cases. Most of the sewersheds monitored (n = 74%) are either a leading or lagging indicator of clinical cases with various lead times, with equal distributions of leading and lagging indicators (seven each). This is consistent with several other studies that reported wastewater levels as a leading or lagging indicator of clinical cases.[15,18,19,36,37] Five sewersheds had no lead/lag indication, meaning their strongest correlation was without temporally offsetting the data. Although 14 sewersheds had improved correlations when a temporal shift was applied, temporally shifting the imputed data was not the best data processing technique (based on strength of correlation) in any of the sewersheds. The improvement of correlations by temporally shifting the wastewater data could help explain the lower correlations that occurred when just smoothing was applied because the lead/lag analysis was not applied on the raw or smoothed data since several sewersheds had only weekly samples.

Table 2. Lead/Lag Indicated if the Top Correlation Is a Leading or Lagging Indicator[a]

Sewershed	Top lead/lag correlation
City1_S1	No lead/lag
City1_S2	Lead 4 days
City1_S3	Lead 7 days
City2_S1	Lead 1 days
City2_S2	Lag 7 days
City2_S3	Lag 5 days
City2_S4	Lead 1 day
City2_S5	No lead/lag
City2_S6	No lead/lag
City2_S7	Lead 7 days
City2_S8	Lag 1 day
City2_S9	Lag 1 day
City2_S10	No lead/lag
City3_S1	Lag 3 days
City3_S2	Lead 4 days
City3_S3	Lag 6 days
City3_S4	Lag 2 days
City3_S5	Lead 6 days
City4_S1	No lead/lag

[a]The numbers 1−7 indicate the days it leads or lags by. "No lead/lag" indicates that the correlation was strongest when no shift in the data was made. A lead of 4 days means that wastewater trends occurred 4 days prior to the clinical case trends. Sewersheds with leading indicators are highlighted in purple and lagging indicators are indicated with orange.

The RC of SARS-CoV-2 RNA loads in wastewater and clinical COVID-19 cases were correlated with one another. There is no standard approach to interpret COVID-19 wastewater surveillance data. Our analysis of correlations between viral loads and clinical data did not identify a single data processing technique that improved wastewater correlations with clinical cases in all instances. To help further understand the data processing techniques tested, we sought to explore the correlations of RC—rather than absolute values— in viral loads and clinical cases to compare analytical approaches using data from four cities. The resulting correlations from RC data from all analytical approaches are listed in Table S4.

Unlike when imputation was applied to raw data in the prior section, data imputation had a clear benefit when comparing RCs—the strongest correlations are all imputed data for both time windows (21 and 28 days) (Table 3). This analysis underscores that correlations using relative measures can be stronger than correlations using absolute measures; the average correlation between RC of imputed viral load and RC of cases was moderately stronger (Spearman's ρ = 0.59 ± 0.04) than the average correlation that resulted with just imputed viral loads (Spearman's ρ = 0.55 ± 0.13). The two strongest correlations were for the longer time window (28 days). When comparing the relative measure or RC, smoothing generally

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Table 3. Spearman's Rank ρ Correlation for Each of the Criteria Specified for the Rate of Change Analysis[a]

time window = 21 days			time window = 28 days		
rates included	viral load data processing technique	Spearman's ρ	rates included	viral load data processing technique	Spearman's ρ
if load RC significant	imputation	0.559	if load RC significant	imputation	0.634
both load and case RC significant	imputation	0.550	both load and case RC significant	imputation	0.630

[a]The table includes only the four strongest associations; all correlations have $p < 1 \times 10^{-9}$.

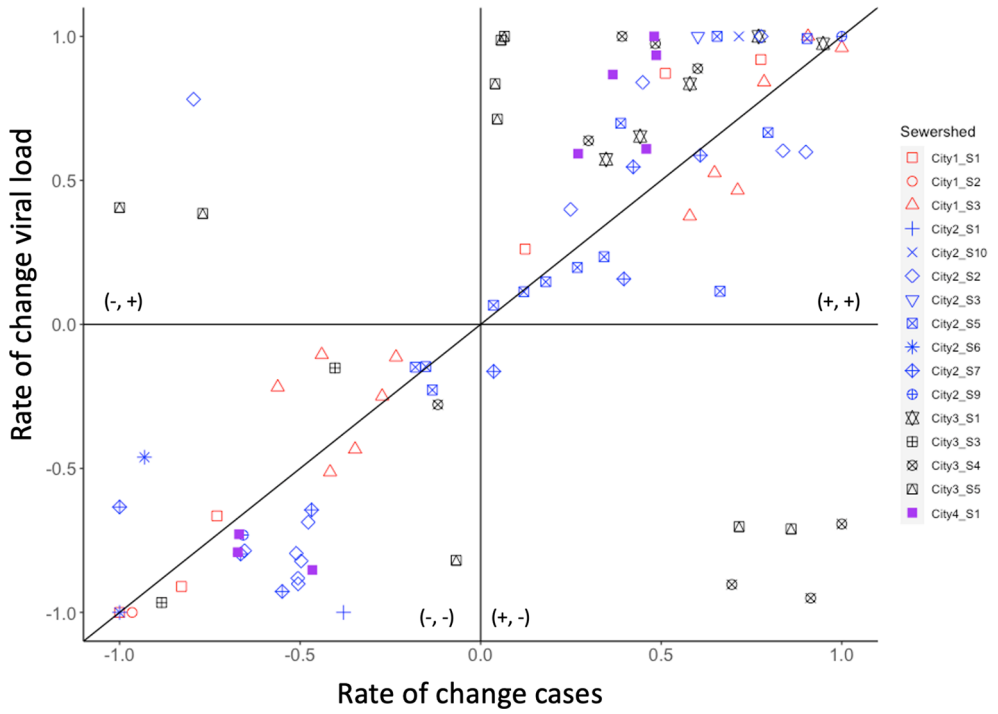

Figure 3. Relationship between imputed rates of change in viral load and clinical cases across different sewersheds. Estimates were across a 28-day moving window, and only significant changes (p value of slope <0.05 after Benjamini-Hochberg correction) in viral load are shown on the figure. Rates of change for clinical estimates and viral load are normalized to the maximum rate of change within that sewershed. The diagonal line represents a 1:1 bisector. Spearman's rank $\rho = 0.634$, p-value = $< 2.2 \times 10^{-16}$ for the correlation between the rates of change in viral load and case surveillance data.

did not improve correlations while imputation improved correlations consistently. Even when combined with imputation, smoothing reduced the strength of the correlation (Table S6). The improvements due to imputation are likely because imputation resulted in more instances where viral load had a significant change; across all sewersheds, the 28-day analysis resulted in 74 instances when the viral load had a significant RC, whereas there were 91 instances where the imputed viral load had a significant RC. This highlights the potential for imputation to increase the sensitivity of wastewater surveillance. Imputation could also be used to decrease the time windows used to calculate RC without being limited by sampling frequency, which is beneficial because shorter time windows may enable faster public health action.

Figure 3 shows the results from all the sewersheds with imputed viral load when only viral load RC was significant in a 28-day window. Additional comparisons of RCs are provided in Figures S19–S24. Around 90% of RCs shown in Figure 3 are either in the first (+, +) or third (−, −) quadrant, indicating agreement in the direction of the trend between wastewater and clinical data. However, these four strongest correlations (Table 3) all had instances where the RCs were not in agreement with respect to the trend direction between wastewater and case data (points that lie in the (+, −) and (−, +) quadrants, Figures S19–S21). The temporal shifts identified in Table 2 were also used to calculate RCs (Figure S22). When compared to the results without implementing lead/lag shifts (Figure 3, Spearman's $\rho = 0.63$), the correlation decreased marginally (Figure S22, Spearman's $\rho = 0.62$).

The RC analysis led to some insights with respect to data processing techniques and the applicability of imputation. Though not widely applied, MICE imputation could be beneficial for analyzing data and normalizing sampling frequency across different sites. Notably, imputing the data allowed us to incorporate the lead/lag analysis that consistently improved correlations in 14 out of the 19 sewersheds. Whereas analysis on raw viral loads/clinical case counts are limited in the types of conclusions that can be drawn due to site-specific impacts (e.g., RT-PCR inhibition, RNA decay), RC can help us determine data processing techniques that can be consistently applied across different sewersheds.

RC analysis could provide insights on the relative sensitivity of methods. We calculated the sensitivity of wastewater surveillance, defined as the change in new COVID-19 cases per day, corresponding to a significant change in wastewater viral load (Figure 4). Across all sewersheds, the

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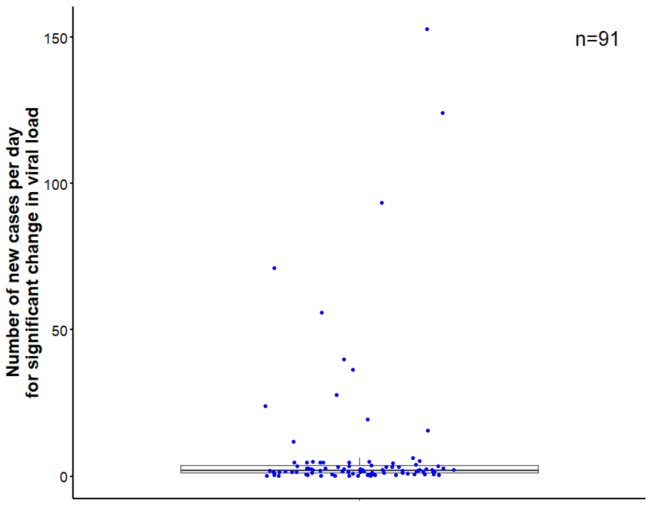

Figure 4. New cases needed per day for a significant change in load aggregated across all sewersheds, 91 instances of significant changes in the load. Blue dots represent threshold calculation across sewersheds, illustrating the long tail in sensitivity threshold.

sensitivity was 2.4 cases (Wilcoxon signed-rank test pseudomedian, 95% CI [1.9, 3.0]). In other words, it takes an increase or decrease of 2.4 new cases per day to result in a significant change in the wastewater viral load over a 28-day period. Unlike other studies with varied sewershed sizes,[38,39] an advantage of comparing RC is that there is no need to normalize by sewershed size because the RC is independent of the absolute number of cases. In other words, on aggregate, each new case should theoretically result in a consistent increase in the viral load across all sewersheds, regardless of size. However, this calculation is dependent on testing penetration as a lower testing penetration may not pick up asymptomatic individuals who are shedding virus; we expect that testing penetration varied throughout the sampling period and across the sewersheds. Our study is relatively underpowered to establish this threshold, and we identified a wide range of sensitivities across the sewersheds (Figure 4). Taking an aggregate approach across many different communities throughout the pandemic could help reduce uncertainty in the sensitivity threshold. This sensitivity calculation can be used with the wealth of wastewater monitoring data that have been generated throughout the pandemic[12] to retrospectively establish sensitivity thresholds of wastewater monitoring efforts, which are important to communicate to public health agencies.

The four cities represented in this study each had their own laboratory and method for processing wastewater samples. With the caveat that this analysis would be improved with data from more laboratories, we conducted a preliminary analysis to investigate how the RC analysis could be used to understand methodological sensitivity between different SARS-CoV-2 RNA concentration, extraction, and quantification methods. The laboratories in City 1 and City 3 used qPCR for quantification, while City 2 and City 4 used ddPCR, which is more resistant to PCR inhibitors,[40] has higher sensitivity, fewer false negatives, and more interlaboratory reproducibility than qPCR.[14,41−43] As previously reported[44] and expected, ddPCR was significantly more sensitive than qPCR (Mann−Whitney U Test, $p = 0.009$). In sewersheds where ddPCR was used, it took a change of 1.8 cases per day over the 28 days to result in a significant change in viral load (Wilcoxon signed rank test pseudomedian, 95% CI [1.1, 1.5]), compared to a RC of 4.7 cases per day needed for sewersheds where qPCR was used. Furthermore, laboratories using ddPCR had stronger RC correlations (Spearman's $\rho = 0.82$) than laboratories using qPCR (Spearman's $\rho = 0.42$) (Figure S25). This RC analysis provides further evidence that ddPCR is more sensitive than qPCR.

Viral concentration methods were also compared between the laboratories. Three of the four laboratories used the same viral concentration method (HA filtration and bead-beating), while one laboratory used a direct capture method. When we evaluated correlations in RCs between concentration methods (Figure S29), direct capture (Spearman's $\rho = 0.94$) had a stronger correlation with clinical cases than filtration (Spearman's $\rho = 0.54$). However, likely due to the underpowered nature of our data set with only one lab using direct capture, there was no statistical difference in the sensitivity of these two methods (Mann−Whitney U Test, $p = 0.6$). A previous study comparing HA filtration + bead beating to four other concentration methods demonstrated high recovery and low variability of the N1 and N2 gene targets with HA filtration + bead beating,[45] but direct capture was not compared in that study. Few laboratories are applying direct capture, and there are limited publications that compare direct capture to other methods. Direct capture for SARS-CoV-2 recovery from wastewater may capture more of the RNA in the wastewater that is in nonintact form,[46] which may otherwise be lost during filtration. Others have shown that direct capture methods show higher recoveries of SARS-CoV-2 because of the inclusion of the nonintact fraction of the virus.[38,46] The higher recoveries (absolute loads) were also evident in this study; the laboratory using direct capture had statistically higher loads than laboratories using filtration methods (t test $p = 5.7 \times 10^{-10}$, Figure S33). It would be important to conduct follow on studies and hold other factors constant (i.e., wastewater source, quantification methods) to draw conclusions regarding the different methods of RNA concentration and extraction between different laboratories.

Over the course of the pandemic, many researchers conducted side-by-side comparisons of methods, but each laboratory had its own resource, budgetary, and biosafety constraints and researchers were attempting to develop their methods at an expedited rate. Consequently, many of these comparisons were on a limited number of samples. Future work could expand our analysis on a larger data set with more varied methods, our analysis could be used at a higher resolution with more advanced statistical models to develop hypotheses on the relative sensitivity of different laboratory methods (e.g., extraction kits, concentration method), harnessing the widely available data that has been generated during the pandemic.

CONCLUSION

A RC analysis was implemented in 19 sewersheds across four metropolitan areas. RC can be applied to reduce site- and method-specific impacts while analyzing data from varied localities. While not widely applied, imputation provides benefits of added sensitivity and the ability to evaluate the leading/lagging nature of wastewater data. Using RC, we estimated a threshold of 2.4 new COVID-19 cases per day over a 28-day period to observe a significant increase/decrease in viral load. RC can also be used to develop hypotheses on the

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relative sensitivity of different laboratory methods being employed for SARS-CoV-2 analysis. Overall, our analysis supports the use of relative, rather than absolute, measures of SARS-CoV-2 wastewater data for more interoperable data sets.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00106.

Discussions of methods used and results of data, tables of summary of concentration, extraction, and quantification methods used for each of the metropolitan areas, qPCR standard curve parameters aggregated across all qPCR plates and LOD, BCoV concentrations and recovery percentages for City 1 samples, and Spearman's rank ρ coefficients, and figures of wastewater concentrations variability of SARS-CoV-2, relationship between rates of change in viral load and clinical cases across different sewersheds, comparison between using qPCR versus ddPCR for the detection of SARS-CoV-2, and comparison between using direct capture versus filtration (PDF)

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Notes

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