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Letter

Scaling of SARS-CoV-2 RNA in Settled Solids from Multiple Wastewater Treatment Plants to Compare Incidence Rates of Laboratory-Confirmed COVID-19 in Their Sewersheds

Marlene K. Wolfe, Anand Archana, David Catoe, Mhara M. Coffman, Samuel Dorevich, Katherine E. Graham, Sooyeol Kim, Lorelay Mendoza Grijalva, Laura Roldan-Hernandez, Andrea I. Silverman, Nasa Sinnott-Armstrong, Duc J. Vugia, Alexander T. Yu, Winnie Zambrana, Krista R. Wigginton, and Alexandria B. Boehm*

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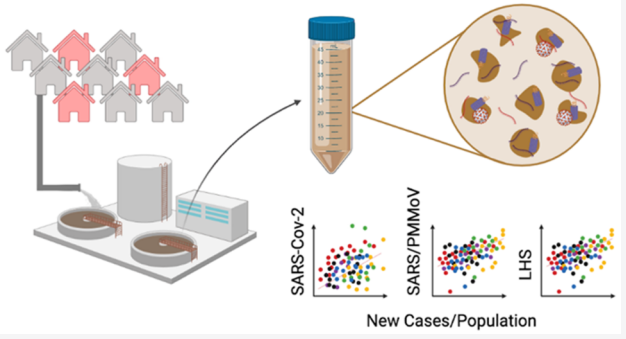
ABSTRACT: Published and unpublished reports show that SARS-CoV-2 RNA in publicly owned treatment work (POTW) wastewater influent and solids is associated with new COVID-19 cases or incidence in associated sewersheds, but methods for comparing data collected from diverse POTWs to infer information about the relative incidence of laboratory-confirmed COVID-19 cases, and scaling to allow such comparisons, have not been previously established. Here, we show that SARS-CoV-2 N1 and N2 concentrations in solids normalized by concentrations of PMMoV RNA in solids can be used to compare incidence of laboratory confirmed new COVID-19 cases across POTWs. Using data collected at seven POTWs along the United States West Coast, Midwest, and East Coast serving ∼3% of the U.S. population (9 million people), we show that a 1 log change in N gene/PMMoV is associated with a 0.24 (range 0.19 to 0.29) $\log_{10}$ change in incidence of laboratory confirmed COVID-19. Scaling of N1 and N2 by PMMoV is consistent, conceptually, with a mass balance model relating SARS-CoV-2 RNA to the number of infected individuals shedding virus in their stool. This information should support the application of wastewater-based epidemiology to inform the response to the COVID-19 pandemic and potentially future viral pandemics.



INTRODUCTION

Municipal wastewater includes biological specimens from community members utilizing the sewage system. Consequently, wastewater can be leveraged for public health use, including for tracking viral[1,2] and bacterial[3,4] diseases. Viral genes of SARS-CoV-2, the causative virus for COVID-19, are detected in the stool of many infected individuals; thus, there has been an increased interest in wastewater-based epidemiology (WBE) during the COVID-19 pandemic.[5−7] Traditional epidemiologic COVID-19 surveillance has relied on laboratory testing, which has been limited in supply,[8−10] varies with test-seeking behavior,[11−13] and includes lag times between symptom onset, obtaining a test, and results reported to the public health system.[14] As a result, local and federal governmental agencies are establishing WBE frameworks to help inform pandemic response.[15]

Previously, we developed methods for measuring SARS-CoV-2 RNA in wastewater settled solids[16] and found that settled solids contain ∼1000 times more SARS-CoV-2 RNA than wastewater influent on a per-mass basis. We quantified SARS-CoV-2 RNA in settled solids from a single publicly owned treatment work (POTW), collected nearly daily from the beginning of the COVID-19 pandemic. Viral RNA concentration in solids was strongly associated with new laboratory-confirmed COVID-19 incident cases (hereafter "COVID-19 cases").[16] Similar trends have been observed around the world using diverse methods and wastewater matrices.[17−23]

The correlative relationship between SARS-CoV-2 RNA in wastewater and COVID-19 cases suggests that WBE may provide useful insight into community-level transmission dynamics. Public health agencies have expressed interest in using wastewater data to estimate incidence over time or

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compare incidence between POTW service areas. However, it is presently unclear whether SARS-CoV-2 RNA concentrations in wastewater might be used to infer quantitative information about COVID-19 cases and, importantly, whether concentrations can be compared across POTWs to infer relative COVID-19 incidence rates (daily incident cases/population).

The goal of this study is to determine how SARS-CoV-2 RNA measurements at different POTW can be used to compare incidence rates of COVID-19 in their sewersheds. We develop a mass balance model that links SARS-CoV-2 RNA concentrations in POTW settled solids to the number of individuals shedding SARS-CoV-2 RNA in stool in the sewershed. We subsequently apply the model to interpret SARS-CoV-2 RNA concentrations in settled solids from seven geographically diverse POTWs (one in New York, one in Illinois, and five in California). We show that POTW with the same ratio of SARS-CoV-2 to PMMoV genes have similar laboratory-confirmed COVID-19 incidence rates in their sewersheds.

MATERIALS AND METHODS

Sample Collection and Storage. Primary settled solids were collected from seven POTWs serving portions of San Jose, CA (SJ), Chicago, IL (Stick), New York, NY (NY), San Francisco, CA (Ocean), Alameda, CA (EB), San Diego, CA (PtL), and Sacramento, CA (SAC) (see Table S1 for POTW details). POTWs NY, Ocean, and SAC treat stormwater as well as wastewater; rainy conditions were absent during the study at Ocean and SAC. POTWs SJ and PtL add $FeCl_3$ to wastewater prior to the headworks for odor control. Between 11 and 88 samples were analyzed per POTW (total 244, not including replicates) from early spring through mid to late summer 2020 (Table S1). The median duration between sample collection was 1 d (SJ), 2 d (PtL), 3 d (SAC), 4 d (Ocean), 5 d (NY), and 7 d (EB and Stick) (Table S2).

Approximately 50 mL of settled solids were collected from POTW primary settling tanks, except at PtL where operators used Imhoff cones to settle solids from an influent grab sample. Table S1 indicates whether samples were grab or 24 h composite samples. Residence times of settled solids in primary clarifiers were typically less than 3 h except at Stick (up to 24 h) (Table S3). Samples were collected in 10% HCl acid-washed plastic containers. Storage conditions are in Table S4.

Analytical Methods. Solids were processed as described by Graham et al.[16] to enumerate SARS-CoV-2 genes N1 and N2 and pepper mild mottle virus RNA (PMMoV) per dry mass of solids using digital RT-PCR. PMMoV is highly abundant in human stool and domestic wastewater globally[24,25] and is used here as an internal recovery and fecal strength control. RNA recovery was also assessed using an exogenous bovine coronavirus (BCoV) control. Negative and positive controls were used, and RT-PCR inhibition was considered. Biological replicates and technical replicates were run for 45 and 2, respectively, of the 244 samples yielding 291 measurements. We omitted data for 28 of the 291 measurements where 0% BCoV recovery was measured and/or the measured PMMoV concentration was less than 10^5 copies/g, leaving 263 measurements. For two measurements, recovery was greater than 100%, and recovery values were lost for one measurement. However, given that other viral measurements were within expected ranges for these samples, they were retained for analysis. Additional details, including MIQE reporting, are in the SI. Wastewater data are publicly available.[26]

Ancillary Wastewater Data. Wastestream influent total suspended solids (TSS) concentrations in mg/L data were obtained from POTW staff. If TSS measurements were not coincident on the day that a sample was taken, the TSS value for that day was estimated using linear interpolation.

COVID-19 Case Data. Counts of laboratory-confirmed COVID-19 incident cases as a function of episode date (earliest of reported symptom onset, laboratory result, or case record create date) for each sewershed were obtained from local or state sources through data-use agreements (SI).

Model. We developed a model linking SARS-CoV-2 RNA in settled solids to inputs from infected individuals (Figure S1). Conceptually, viral RNA is introduced to the wastestream on a per mass basis of feces which, in turn, is assumed to be proportional to wastewater TSS. SARS-CoV-2 RNA concentrations in wastewater (C_{ww}) entering a POTW is

$$C_{WW} = \mathrm{TSS} \times f_{\mathrm{fecal\_solids}} \times f_{\mathrm{shed}} \times C_{\mathrm{feces}} \times \exp(-k_s t) \quad (1)$$

where $f_{\mathrm{fecal\_solids}}$ is the solids fraction that is fecal, f_{shed} is the population fraction shedding SARS-CoV-2 RNA in feces, C_{feces} is the concentration of SARS-CoV-2 RNA in feces, $\exp(-k_s t)$ accounts for first-order decay of SARS-CoV-2 RNA during transport from toilet to sampling location, k_s is the first order decay rate constant, and t is the average time sewage spends in the system, including the primary clarifier, prior to sampling. C_{ww} is the concentration of SARS-CoV-2 per volume of wastewater, containing contributions from liquid (C_w) and solid phases (C_s). k_s is assumed to apply to both liquid and solid-associated SARS-CoV-2 RNA.

We introduce the coefficient $K_d = C_s/C_w$ to describe relative concentrations of SARS-CoV-2 RNA associated with solid and liquid phases of wastewater to express C_s as

$$C_s = K_d(\mathrm{TSS} \times f_{\mathrm{fecal\_solids}} \times f_{\mathrm{shed}} \times C_{\mathrm{feces}} \times \exp(-k_s t))/(1 + K_d \mathrm{TSS}) \quad (2)$$

We assume the fraction of solids that is fecal can be approximated by the concentration of PMMoV RNA in the solids (C_{PMMoV}) normalized by the concentration of PMMoV RNA in feces ($C_{\mathrm{PMMoV\_feces}}$). Taking into account partitioning and decay of PMMoV RNA during transit: $f_{\mathrm{fecal\_solids}} = C_{\mathrm{PMMoV}} \times \exp(k_p t)(1 + K_{dp}\mathrm{TSS})/(C_{\mathrm{PMMoV\_feces}} K_{dp}\mathrm{TSS})$, where k_p and K_{dp} are the PMMoV RNA first-order rate constant and partitioning coefficient, respectively.

C_s/C_w for infectious murine hepatitis virus, an enveloped beta-coronavirus similar to SARS-CoV-2, is approximately 1000 mL/g in wastewater.[27] Previously, we found SARS-CoV-2 RNA was enriched in solids over liquid in wastewater suggestive of similar K_d.[16] We thus set K_d = 1000 mL/g. Coupled measurements of PMMoV RNA in influent and settled solids suggest that K_{dp} = 100 mL/g.[16]

f_{shed} represents the fraction of the sewershed population ($P_{\mathrm{sewershed}}$) contributing SARS-CoV-2 RNA to wastewater. Previous work has found a positive association between SARS-CoV-2 RNA in wastewater solids and COVID-19 incident cases in the sewershed.[16,17,21] We therefore assume $f_{\mathrm{shed}} = A \times \mathrm{cases}/P_{\mathrm{sewershed}}$, where A is a multiplier that relates reported incidence rate to the true number of infected individuals shedding virus in their stool. This formulation assumes newly infected individuals are responsible for contributing the

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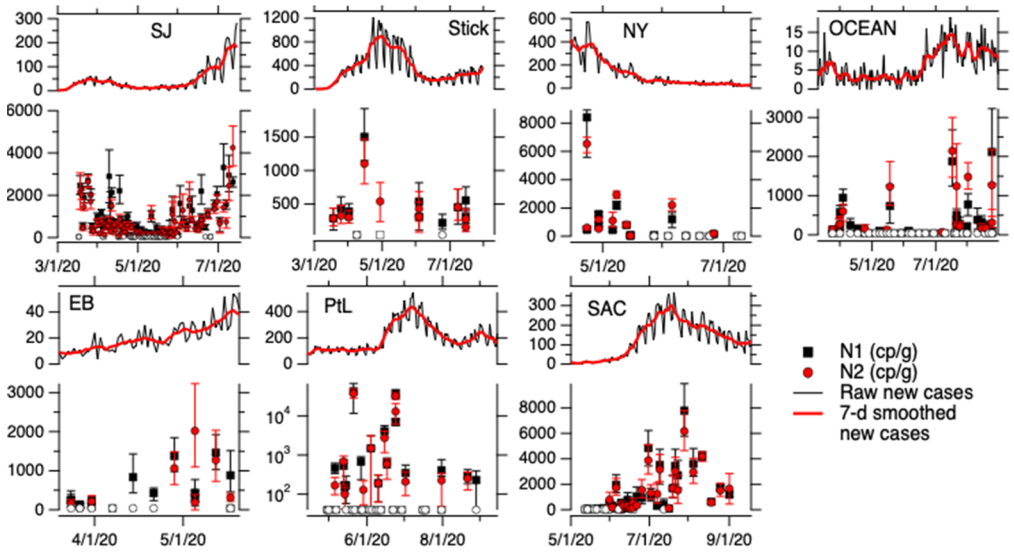

Figure 1. Time series measurements of SARS-CoV-2 N1 and N2 RNA genes in settled solids at seven POTWs. Data for SJ were published by Graham et al.[16] White squares and circles indicate nondetection of N1 or N2, respectively. Absolute new laboratory-confirmed COVID-19 cases for each sewershed as a function of episode date are provided along with the 7 day smoothed case data. Errors on the N1 and N2 measurements represent the total errors reported by the digital PCR instrument as standard deviations. PtL is shown on a log scale to allow visualization of all data.

greatest SARS-CoV-2 RNA.[28] This is supported by reports that newly infected individuals have orders of magnitude higher concentrations of SARS-CoV-2 RNA in feces relative to convalescing cases.[29−34] Case testing data are subject to bias, and A provides information about the magnitude of this bias. It may be a function of time and location, and testing capacity and test seeking behavior are two factors influencing the difference between true and reported incidence.[14]

When the formula for $f_{\text{fecal\_solids}}$ is substituted into eq 1, $k_s - k_p$ appears in the exponent. $k_s - k_p$ represents the difference in the SARS-CoV-2 and PMMoV RNA decay rate constants. SARS-CoV-2 RNA appears persistent over time scales less than 1 day,[35] a time scale relevant to the seven POTWs (Table S3). Although data do not presently exist on PMMoV RNA decay in wastewater, it is persistent through wastewater treatment.[24,36] We neglect the decay term by assuming k_s and k_p are similar in magnitude such that $k_s - k_p \sim 0$. Equation 2 can be written as

$$C_s K_{dp}(1 + K_d \text{TSS})/(C_{\text{PMMoV}} K_d(1 + K_{dp}\text{TSS})) = C_{\text{feces}}/C_{\text{PMMoV\_feces}} \times A \times \text{cases}/P_{\text{sewershed}} \quad (3)$$

The left-hand side (LHS) of eq 3 contains all the wastewater-specific terms. In applying eq 3 to our data, we assume that viral RNA concentrations in settled solids from the primary clarifier are representative of concentrations associated with solids in wastewater, C_s, and that samples represent a temporal composite of inputs. The 24 h composited samples clearly represent a temporal composite (Table S1), and grab samples from the primary clarifier also represent temporally composited solids as they are mixed and raked while they are retained.[16,28]

Statistical Analyses. Statistics were computed using RStudio (version 1.1.463). For ddRT-PCR data, the "total error" from merged wells is reported as standard deviations. Nonparametric Kendall's tau and Kruskal−Wallis methods were used to test hypotheses regarding associations and trends as data were neither normally nor log-normally distributed based on Shapiro−Wilk tests. To account for technical variability of wastewater measurements, Kendall's tau empirical p-values were determined using 1000 bootstrap resamplings that incorporate nondetect measurements and measurement errors[16] (SI); median tau and empirical p are reported. A 7 d smoothed data set of COVID-19 cases was used to represent cases. Analyses were repeated with unsmoothed data, and results were similar (data not shown). Linear regressions were used to assess slopes and intercepts in some cases.

RESULTS

QA/QC. Negative and positive controls were negative and positive, respectively. Recovery and PMMoV concentrations were similar to those reported by us previously using these methods[16] (Figures S2 and S3). BCoV recovery was not significantly different between POTW (Kruskal−Wallis, p = 0.25), but PMMoV was different between POTWs (Kruskal−Wallis, $p < 0.05$) (Figure S4). SJ had the highest PMMoV, and Pt Loma and NY had the lowest. BCoV recovery was positively associated with PMMoV (tau = 0.34, $p < 0.05$).

Time Series from Seven POTWs. Medians N1 and N2 were 166 and 251 cP/g (n = 263, min below detection limit, max 10^4 cp/g), respectively. There were 79 N1 and 106 N2 measurements below the detection limit of approximately 40 cP/g.[16] N1 and N2 were correlated (tau = 0.59, p = 0). Considering all measurements, N1 and N2 were weakly but positively correlated with BCoV recovery (tau = 0.18 and 0.16, p = 0 for N1 and N2, respectively). N1 and N2 normalized by PMMoV were no longer positively associated with BCoV recovery.

Time series of N1 and N2 solids concentrations at the seven POTWs were compared to COVID-19 cases in the sewersheds (Figure 1). N1 and/or N2, N1 and/or N2 normalized by PMMoV, and N1 and/or N2 scaled by the LHS of eq 3 (hereafter "LHS N1" and "LHS N2") were positively associated with COVID-19 cases at all seven POTWs (Figure S5, Table S5, $p < 0.05$). The highest tau estimates are for NY, SJ, and SAC, and the lowest are for Stick, Ocean, and PtL. The

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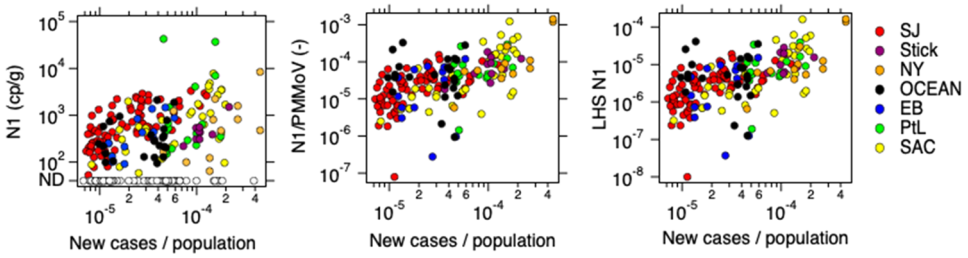

Figure 2. Concentrations of N1 (left), N1/PMMoV (middle), and $C_sK_{dp}(1 + K_dTSS)/(C_{PMMoV}K_d(1 + K_{dp}TSS))$ (right), where C_s is N1 concentration in the solids (see LHS of eq 3, hereafter "LHS N1") versus the COVID-19 incidence rate in the sewersheds. Nondetect (ND) values are provided in the N1 plot as open circles but not in the other plots which show a calculated value on the *y*-axis. Although ND values and errors are not shown in all plots, they were included in all statistical analyses as described in the methods. Results for N2 are similar and can be found in Figure S7.

tau estimates for different wastewater variables (Figure S5) do not show clear trends within POTWs; that is, normalizing or scaling does not consistently improve correlations with COVID-19 cases within sewersheds.

Data Synthesis across POTWs. To explore how SARS-CoV-2 data can best be compared between POTWs, we aggregated data from all seven POTWs and compared N1 and N2 concentrations, N1 and N2 normalized by PMMoV, and LHS N1 and LHS N2 to COVID-19 cases in the sewershed (Figure 2). Considering the data in aggregate, cases are positively and significantly ($p = 0$ for all) associated with each of the scaled and unscaled wastewater measurements. Tau values were smallest when N1 and N2 measurements were employed (0.18 and 0.23, respectively) and larger when normalized by PMMoV or scaled according to eq 3 (tau = 0.33 for N1 and 0.37 for N2 for both normalization and scaling). The differences between tau for unscaled and normalized/scaled wastewater variables were statistically significant (Kruskal–Wallis, $p < 0.05$, Figure S6).

Regressions between incidence rate and N1 and N2 normalized by PMMoV suggest that for a 1 $\log_{10}$ increase in N1/PMMoV or N2/PMMoV, there is a 0.24 (min 0.19, max 0.29) $\log_{10}$ increase in the reported COVID-19 incidence rate. According to eq 3, the slope of a line describing $\log_{10}$-transformed LHS N1 or LHS N2 versus the $\log_{10}$-transformed incidence rate should be 1 and the *y*-intercept equal to $\log_{10}(C_{feces}/C_{PMMoV\_feces} \times A)$. The median bootstrapped slope (and standard error) was near 1 (N1:0.86 ± 0.10, N2:1.1 ± 0.11), and the *y*-intercept was approximately −1 (N1 = −1.8 ± 0.5; N2 = −1.2 ± 0.5). We repeated this analysis varying the partitioning coefficients between 100 and 10^4 mL/g, and results were similar (data not shown). We partitioned data into those collected before and after May 15 (representing time periods when COVID-19 testing was less versus more readily available) to determine if regressions changed substantially; results indicated regressions did not change (data not shown).

DISCUSSION

N1 and N2 concentrations measured in settled solids at seven POTWs are positively associated with COVID-19 cases within seven sewersheds collectively serving 9.2 million people. Associations within POTW are similarly positive and significant when N1 and N2 are normalized by PMMoV RNA concentrations or scaled by a term accounting for TSS and solid–liquid partitioning. The magnitude of associations varied across POTWs, potentially due to differences in the size of sewershed populations, wastewater sampling method, cadence, and duration or reliability of case data. The association between wastewater measurements of SARS-CoV-2 and reported COVID-19 incidence rates corroborates results of studies around the world identifying similar relationships using diverse wastewater matrices with different preanalytical and analytical approaches.[18,22,23,28,37−39] This study did not consider whether wastewater is a leading indicator compared to laboratory-confirmed cases. Such an analysis would need to consider advanced statistical methods (such as ARIMA)[40] that account for autocorrelation of data time series.

POTW- and sewershed-specific attributes modulate the relationship between N1 and N2 concentrations and COVID-19 incidence rates. When data are combined from seven POTWs, there is a positive and statistically significant association between N1 and N2 and COVID-19 incidence, but the association is weak. Modulating attributes might include characteristics of the sewage system such as residence time and fecal strength of wastewater. Direct comparisons of N1 and N2 concentrations between POTW to infer relative COVID-19 incidence rates should be executed with caution.

Accounting for POTW-specific variables may allow for improved comparison of wastewater results across POTWs. When N1 and N2 from the seven POTWs are combined and scaled according to eq 3, the association between scaled N1 and N2 and COVID-19 incidence is strengthened relative to the relationship using unscaled measurements. Conceptually, scaling corrects for POTW-specific attributes that might prevent inter-POTW comparisons and falls from a mass balance model relating concentrations of genes in the wastestream to the fraction of the population shedding SARS-CoV-2 RNA in their stool. The mass balance model is complementary to other approaches that relate wastewater influent SARS-CoV-2 RNA concentrations to infected individuals in the sewershed using wastewater flow rates.[41]

Regression equation coefficients relating scaled N1 and N2 and incidence rates (eq 3) suggest that $A \times C_{feces}/C_{PMMoV\_feces} = 0.1$. There are limited data in the literature on these parameters. Zhang et al.[42] report C_{PMMoV_feces} between 10^6 and 10^9 cp/g. C_{feces} shortly after symptom onset can vary from 10^7 to 10^8 cp/g.[43,44] The *A* is a constant of proportionality that describes the relationship between reported COVID-19 cases in the sewershed and the true fraction of the sewershed population shedding SARS-CoV-2 RNA. Approximately 50% of COVID-19 patients shed SARS-CoV-2 in stool,[7,44] suggesting a minimal possible value for *A* of ∼0.5 assuming reported cases in a sewershed represent true COVID-19 infections. Reported cases may underestimate infections by a factor of 7,[14] so an upper end estimate for *A* is ∼4. The possible values for C_{feces}, C_{PMMoV_feces}, and *A* are not

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inconsistent with the ratio $A \times C_{feces}/C_{PMMoV\_feces} = 0.1$. We made a number of simplifying assumptions in the derivation of the model including that decays of SARS-CoV-2 and PMMoV RNA are similar such that their difference is small and that partitioning coefficients could be approximated from our previous work. Additional work is needed to confirm these assumptions. With further data on partitioning coefficients, C_{feces}, and C_{PMMoV_feces}, the model presented here may be used to better understand differences in testing bias across regions and estimate true COVID-19 cases in a sewershed.

Normalizing N1 and N2 by PMMoV alone improved their association with the COVID-19 incidence rate relative to unscaled measurements with aggregated data from the seven POTW. Given that wastestream TSS does not vary greatly within or between POTW (Table S7) and the same partitioning coefficient values were applied in calculating the scaling factor for each POTW, the additional scaling provided by TSS and partitioning coefficients is similar across the POTWs. Thus, normalizing N1 and N2 by PMMoV alone is conceptually valid and serves two purposes. First, normalizing N1 and N2 by PMMoV corrects for differences in recovery (unnormalized N1 and N2 are correlated to BCoV recovery, but PMMoV-normalized N1 and N2 are not). PMMoV is an indigenous wastewater virus,[24] and thus, recovery of its RNA may better reflect that of SARS-CoV-2 RNA compared to the seeded BCoV. Second, PMMoV controls the "fecal strength" of the wastestream, which may vary between or within POTWs depending on differential and/or intermittent industrial discharges or stormwater that can dilute fecal contributions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.1c00184.

Additional details on methods and results in Tables S1−S8 and Figures S1−S8 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Alexandria B. Boehm − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States;* orcid.org/0000-0002-8162-5090; Email: aboehm@stanford.edu

Authors

Marlene K. Wolfe − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States*

Anand Archana − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States*

David Catoe − *Joint Initiative for Metrology in Biology, SLAC National Accelerator Laboratory, Stanford, California 94305, United States*

Mhara M. Coffman − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States*

Samuel Dorevich − *Division of Environmental and Occupational Health Sciences, School of Public Health, University of Illinois, Chicago, Illinois 60612, United States*

Katherine E. Graham − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States*

Sooyeol Kim − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States*

Lorelay Mendoza Grijalva − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States*

Laura Roldan-Hernandez − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States;* orcid.org/0000-0002-7591-1496

Andrea I. Silverman − *Department of Civil and Urban Engineering, Tandon School of Engineering, New York University, Brooklyn, New York 11201, United States; School of Global Public Health, New York University, New York 10003, United States;* orcid.org/0000-0001-8199-5860

Nasa Sinnott-Armstrong − *Department of Genetics, Stanford University School of Medicine, Stanford, California 94305, United States*

Duc J. Vugia − *Infectious Diseases Branch, California Department of Public Health, Richmond, California 94804, United States*

Alexander T. Yu − *Infectious Diseases Branch, California Department of Public Health, Richmond, California 94804, United States*

Winnie Zambrana − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States*

Krista R. Wigginton − *Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States;* orcid.org/0000-0001-6665-5112

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.estlett.1c00184

Notes

The authors declare no competing financial interest.

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