

RNA in Municipal Wastewater Reveals Magnitudes of COVID-19 Outbreaks across Four Waves Driven by SARS-CoV-2 Variants of Concern

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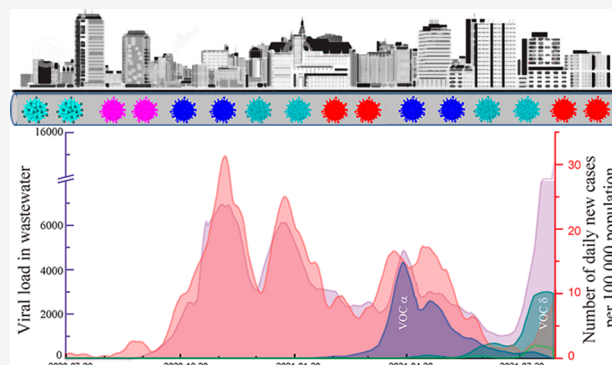
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ABSTRACT: There are no standardized protocols for quantifying severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in wastewater to date, especially for population normalization. Here, a pipeline was developed, applied, and assessed to quantify SARS-CoV-2 and key variants of concern (VOCs) RNA in wastewater at Saskatoon, Canada. Normalization approaches using recovery ratio and extraction efficiency, wastewater parameters, or population indicators were assessed by comparing to daily numbers of new cases. Viral load was positively correlated with daily new cases reported in the sewershed. Wastewater surveillance (WS) had a lead time of approximately 7 days, which indicated surges in the number of new cases. WS revealed the variant α and δ driving the third and fourth wave, respectively. The adjustment with the recovery ratio and extraction efficiency improved the correlation between viral load and daily new cases. Normalization of viral concentration to concentrations of the artificial sweetener acesulfame K improved the trend of viral load during the Christmas and New Year holidays when populations were dynamic and variable. Acesulfame K performed better than pepper mild mottle virus, creatinine, and ammonia for population normalization. Hence, quality controls to characterize recovery ratios and extraction efficiencies and population normalization with acesulfame are promising for precise WS programs supporting decision-making in public health.

KEYWORDS: Population health, Infectious disease, Environmental monitoring, Wastewater-based epidemiology, Population biomarker



INTRODUCTION

Wastewater surveillance (WS) of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that causes coronavirus disease 2019 (COVID-19) has been applied globally to complement traditional clinical testing of individuals.^{1–6} SARS-CoV-2 WS is a promising tool for early warning, trending, and predicting outbreaks and detecting major circulating lineages in sewersheds.^{7–14} Accurate and precise quantification of viral RNA loads in wastewater is critical for the generation of reliable information to help decision-making in public health. Until now, there have been no standardized protocols available for SARS-CoV-2 WS.¹

The optimization of methods is generally conducted at the laboratory level and limited by the availability of the facilities, commercial kits, or authentic standards. Recent interlaboratory comparisons have shown that a variety of methods are suitable for the identification and quantification of viral RNA. However, absolute amounts varied among laboratories.^{15,16} Quality controls (QCs), including but not limited to the

assessment of practical efficiency (recovery ratio and extraction efficiency) and PCR inhibition, are essential for reliable data interpretation in support of public health actions.^{17,18} QCs of key processes have not been fully reported, or possibly not conducted, in studies published to date. Although population normalization is crucial for accurate WS with a dynamic population, there is no consensus about which indicators are the most ideal to normalize viral loads.^{18–22} Viral (pepper mild mottle virus, PMMoV, and cross-assembly phage, crAssphage), human-specific bacterial, and human genetic biomarkers have previously been suggested and validated for SARS-CoV-2 WS.^{7,9,18,23–25} Since volumes and types of wastes discharged to

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a treatment facility vary diurnally and seasonally and can contain various combinations of surface runoff, gray and black domestic sewage, and industrial wastes, the accurate prediction of rates of infections requires not only an accurate quantification of concentrations of viral RNA but also accurate estimates of numbers of persons discharging wastes to the facility so that concentrations of viral RNA can be normalized. However, there were no studies comparing chemical tracers with viral biomarkers for population normalization. Specifically, it would be useful to know the load of feces being discharged in order to normalize the number of viruses. There are significant uncertainties and potential biases in both of these parameters.

Emerging SARS-CoV-2 variants of concern (VOCs) with greater transmissibility and/or immune escape potential pose threats to international public health.^{26,27} Whole genome sequencing is the gold standard to detect mutations and assign variants,²⁸ while allele-specific RT-qPCR-based WS has become available to screen for VOCs with greater throughput and shorter analysis times.^{29,30} Due to the prospect of multiple waves of new VOCs that can spread globally, there are gaps in validation of emerging RT-qPCR assays screening for VOC γ and δ by WS.

To contribute to an accurate and precise standard protocol of SARS-CoV-2 WS, a pipeline was developed to quantify the concentration of fragments of RNA from SARS-CoV-2 virus and key VOCs, including α , γ , and δ , in wastewater from the Saskatoon Wastewater Treatment Plant (SWTP; Saskatoon, Saskatchewan, Canada). The pipeline was applied from July 2020 to August 2021, which captured four waves of COVID-19 and detected the major VOC driving the third and fourth wave. To better present magnitudes of COVID-19 outbreaks, performances of various approaches to the normalization of viral load, including artificial sweetener (acesulfame K; ACE), endogenous human metabolite (creatinine; CRE), wastewater quantity parameters, and PMMoV, were applied. This extensive data set provided a framework for the integration of chemical analyses and biomonitoring to generate reliable information in support of public health decisions. The overall capability of WS to predict and describe surges of COVID-19 was also evaluated.

MATERIALS AND METHODS

Collection of Wastewaters, Clinical Surveillance Data, and Wastewater Quantity Parameters. SWTP (Figure S1) predominantly receives and treats sanitary sewerage of the City of Saskatoon (Saskatchewan, Canada). Inflow of the SWTP was collected from a separate sanitary sewer system. Overall, 145 samples of primary effluent (PE) were collected from primary clarifiers from July 20, 2020 through August 17, 2021. The methods of sampling, transport, and storage can be found in Text S1. Daily reports of new cases, corrected by follow-up information, were provided by the Saskatchewan Health Authority for the SWTP sewershed. The numbers of new cases in 100,000 each day were defined as the clinical incidence to represent the magnitude of the outbreaks. Parameters describing wastewater were obtained from routine monitoring by the SWTP, including average daily inflow rate, pH, temperature, total/volatile suspended solids (TSSs/VSSs), total biological oxygen demand (BOD), and ammonia as N ($\text{NH}_3\text{-N}$), total nitrogen (TN), and total phosphorus (TP) concentrations (Table S1). To determine the population mobility of Saskatoon City, which was normalized to the

baseline of the 5 week period of Jan 3–Feb 6, 2020, residential cellphone mobility was downloaded from Google LLC “Google COVID-19 Community Mobility Reports”.

Sample Preprocessing, Viral Enrichment, and Wastewater Environmental RNA (weRNA) Extraction. To assess the recovery of viral RNA during the whole process, noncontagious, artificial, armored viral particles (AQHRPs; Armored RNA Quant RNase P, Asuragen, TX, USA) were utilized as an internal spiking positive control through the whole process (IPCW). A freshly diluted 30 μL aliquot (3.0×10^3 gene copies, gc) of IPCW was added to 300 mL of PE sample, then incubated for 30 min at 4 $^\circ\text{C}$, and centrifuged at 6500g for 20 min to remove debris. The supernatant was filtered through a 0.45 μm pore size, low protein-binding poly(vinylidene fluoride) membrane filter (Millipore Sigma, Ontario, Canada). Virus in the filtrate was enriched by PEG-8000 precipitation.³¹ After adding 6 g of PEG-8000 and 1.2 g of NaCl to the filtrate, the sample was agitated overnight (12–14 h) at 4 $^\circ\text{C}$ on an orbital shaker. The virus was pelleted by centrifugation at 26 000g and 4 $^\circ\text{C}$ for 1 h. weRNA was directly extracted from the pellet using a Viral RNA Mini Kit (Qiagen, Ontario, Canada). The RNA concentration of AQHRP stock solution was validated by droplet digital PCR (ddPCR) in eight replicates following the manufacturer’s manual (Bio-Rad Laboratories, CA, USA). The details of ddPCR were summarized in Text S2. The sequences of the primers and probes are listed in Table S2. Another freshly diluted 30 μL aliquot of IPCW was extracted as an external positive control (EPC) to estimate the extraction efficiency (EE) for each batch. Quantitative viral load (gc/mL wastewater) was calculated (eq 1).

$$C_{i,j} = \frac{C_{i,j,\text{RNA}} \times V_{\text{RNA-elution},i}}{V_{\text{Template},i} \times V_{\text{WW},i}} \quad (1)$$

where i and j are the sample ID and viral RT-qPCR assay, respectively; $C_{i,j,\text{RNA}}$ is the concentration of viral RNA in the weRNA product (gc/reaction); $V_{\text{RNA-elution},i}$ is the volume of buffer AVE for the weRNA elution (μL); $V_{\text{Template},i}$ is the volume of weRNA added to the RT-qPCR reaction mixture (μL /reaction); $V_{\text{WW},i}$ is the volume of processed wastewater (mL).

The recovery ratio (RR; eq 2) and EE (eq 3) were calculated to assess the practical performance of each batch.

$$\text{RR}_{i,k} = C_{\text{AQHRP},i,k} / C_{\text{AQHRP},\text{EPC},k} \quad (2)$$

$$\text{EE}_k = \frac{C_{\text{AQHRP},\text{EPC},k}}{C_{\text{AQHRP},\text{EPC},\text{ref}}} / \text{DF} \quad (3)$$

where i and k represent the sample and batch IDs, respectively; $\text{RR}_{i,k}$ is the RR estimation of sample i for batch k ; $C_{\text{AQHRP},i,k}$ is the concentration of the IPCW of sample i for batch k ; $C_{\text{AQHRP},\text{EPC},k}$ is the concentration of EPC of batch k ; EE_k is the EE for batch k , $C_{\text{AQHRP},\text{EPC},\text{ref}}$ is the concentration of stock AQHRP solution; DF is the dilution factor for the preparation of the IPCW.

RT-qPCR Assays for the Detection of Virus and VOCs. Concentrations of SARS-CoV-2, PMMoV, F-specific RNA bacteriophages group II (FRNAPH-II), and AQHRP were quantified by use of TaqMan RT-qPCR assays. Quantitative VOC assays, including N.D3L.GAT.CTA,³² S.P681R.CCT.CGT, and S.T20N.ACC.AAC (TaqMan SARS-CoV-2 Mutation Panel, ThermoFisher, CA, USA),

Table 1. Performance of TaqMan RT-qPCR Assays

test	assay	VOC	LOD (gc/ reaction)	Y intercept (mean $\pm$ SD)	R ² (mean $\pm$ SD)	slope (mean $\pm$ SD)	efficiency (mean $\pm$ SD)
SARS-CoV2 N	N1		1.667	33.648 $\pm$ 0.418	0.990 $\pm$ 0.008	−3.284 $\pm$ 0.087	101.756 $\pm$ 3.800
SARS-CoV2 N	N2		1.210	33.362 $\pm$ 0.660	0.994 $\pm$ 0.002	−3.345 $\pm$ 0.103	99.223 $\pm$ 4.301
HRP3	HRP3		1.563	34.057 $\pm$ 0.927	0.990 $\pm$ 0.007	−3.314 $\pm$ 0.167	100.802 $\pm$ 6.771
PMMoV	PMMoV			34.462 $\pm$ 0.594	0.998 $\pm$ 0.002	−3.254 $\pm$ 0.076	103.019 $\pm$ 3.232
IPCpcr	IPCpcr			36.511 $\pm$ 0.371	0.998 $\pm$ 0.002	−3.136 $\pm$ 0.098	101.415 $\pm$ 4.117
N.L3.CTA	N_D3L	α	1.406	33.384 $\pm$ 0.838	0.988 $\pm$ 0.015	−3.277 $\pm$ 0.156	102.353 $\pm$ 6.687
N.L3.A28271del			1.498	33.216 $\pm$ 1.097	0.989 $\pm$ 0.011	−3.227 $\pm$ 0.128	102.868 $\pm$ 5.434
S.P681.CCT	S_P681R	wild type	2.188	35.816 $\pm$ 0.730	0.993 $\pm$ 0.007	−3.303 $\pm$ 0.171	101.273 $\pm$ 6.735
S.R681.CGT		B.1.617	2.916	35.502 $\pm$ 0.215	0.995 $\pm$ 0.003	−3.289 $\pm$ 0.077	101.502 $\pm$ 3.283
S.T20.ACC	S_T20N	wild type	1.456	37.849 $\pm$ 0.507	0.996 $\pm$ 0.002	−3.049 $\pm$ 0.004	102.792 $\pm$ 2.337
S.N20.AAC		γ	1.498	38.078 $\pm$ 0.719	0.993 $\pm$ 0.003	−3.254 $\pm$ 0.135	103.253 $\pm$ 6.198

were adopted for detecting B.1.1.7 (α), P.1 (γ), and B.1.617 (including δ) lineages. Synthetic quantitative RNA standards were aliquoted for single use, and their nominal concentrations were confirmed by ddPCR in eight replicates. Sequences of primers and probes are listed in SI Table S2. RT-qPCR reactions were performed in duplicate. Recipes, quantitative RNA standards, ranges of the standard curve, and settings for threshold and baseline of RT-qPCR assays are listed in Table S2. Thermal cycling condition for RT-qPCR can be found at Text S3.

The wastewater sample was sent to the Division of Enteric Diseases, National Microbiology Laboratory, Public Health Agency of Canada (Winnipeg, MB, Canada) for whole genome sequencing (WGS) of weRNA. cDNA was synthesized using the SuperScript IV First-Strand Synthesis System (Invitrogen, USA).³³ Tiled amplicons were amplified according to the ARTICV3 protocol.³⁴ The tiled amplicons were sequenced with MiSeq 300PE V3 chemistry (Illumina, USA). Mutations were identified on mapping files generated by SAMtools v 1.7³⁵ against a SARS-CoV-2 reference sequence (MN908947.3).³³ SARS-CoV-2 lineage was assigned on the basis of coverage of consensus mutations following the Pango Nomenclature proposal.³⁶

RT-qPCR Inhibition Assay. Novel RNA was designed and synthesized by Integrated DNA Technologies (IDT, USA) as an internal positive control of RT-qPCR (IPCpcr). Random RNA sequences were generated (GC: 45–55%) and checked by discontinuous Mega Blast³⁷ against Nucleotide collection (nr/nt) and env_nt databases (max e-value: 1×10^{-10}). A novel RNA sequence (5'-AACGCACAUAUACGGGUA-GCAUAACUUUCGGAUGCAUCUAGUGACAAUAA-GGUGGUCUAUAGGCGGGAC-3') was selected as an IPCpcr. Primers and probes (Table 1) were designed using Primer3.³⁸ The weRNA template was spiked with a known copy number (2.5×10^3 copies) IPCpcr as well as AVE buffer as a reference to assess inhibition.

Limit of Detection of TaqMan RT-qPCR Assays. The limit of detection (LOD) of RT-qPCR assays is the least concentration of target RNA that can be detected with a 95% detection rate as the standard confidence level.^{39,40} A series of replicate standard curves (20 replicates per standard concentration, Table S2)³⁹ and multiple standard curves from routine monitoring (10–20 batches) were pooled for each quantitative RT-qPCR assay to estimate the LODs. The LODs were determined on the basis of previously described methods.^{39,40}

Sensitivity of the Whole Process. Serially diluted environmental water samples seeded with untreated primary effluent (sampled on Dec. 13, 2020, 307 recent cases in 14 days in 100 000, 18 daily new cases (5 d moving average) in 100 000) were tested by RT-qPCR. 1, 3, and 10 mL of primary effluent sample were added into 299, 297, and 290 mL of river water (South Saskatchewan River; 52°08'10.7" N, 106°38'48.5" W, Saskatoon, Saskatchewan, Canada), respectively. River water was used as a matrix to estimate the process limit of quantification⁴¹ since no negative wastewater was available during the first wave of COVID-19. The supernatant fraction of wastewater is used to concentrate SRA-CoV-2, which can mitigate the major differences between wastewater and river water. A sensitivity assessment of the whole process was undertaken in triplicate.

Chemical Tracer Analysis. ACE, CRE (Sigma-Aldrich, Oakville, ON), and their deuterated internal standards ACE-*d*₄ and CRE-*d*₃ (Toronto Research Chemicals, Toronto, ON) were used for the identification and quantification of the target tracers in wastewater samples. Stock solutions of each standard were made in methanol, and calibration curve standards (10 points, 0.01–500 ng/mL) were made in 90:10 water/methanol. Samples were processed using a direct-injection method. A 3 mL aliquot of a well-mixed PE sample was syringe-filtered through a 0.22 μ m PTFE filter (Pall Life Sciences, Mississauga, ON). Exactly 950 μ L of filtered sample was transferred into an amber LC vial and spiked with 50 μ L of a 1 mg/L internal standard mixture (ACE-*d*₄ and CRE-*d*₃). The analysis was conducted using a Vanquish UHPLC and Q-Exactive HF Quadrupole-Orbitrap mass spectrometer (Thermo-Fisher, Mississauga, ON). Instrument analysis details are summarized in Text S4. All data acquisition and processing were conducted using Xcalibur v. 4.2 (Thermo-Fisher, CA, USA). The target tracer chemicals were not detected in the blank samples. Calibration curve linearity was >0.998 for ACE and CRE across all runs. Limits of detection were 0.11 and 0.54 ng mL^{−1} for ACE and CRE, respectively.

Quality Control. Blank samples for sampling, extraction, and RT-qPCR were completed to account for contamination during the whole process or key procedures. Sample preparation, extraction, PCR setup, and thermal cycling were conducted in separated BSL-2 laboratories to minimize PCR cross-contamination. The UNG technique was applied to avoid potential contamination of DNA from previous RT-qPCR runs. Freshly prepared 10% bleach and 70% isopropanol were applied for disinfection and decontamination of the working area and waste disposal. All blank samples and

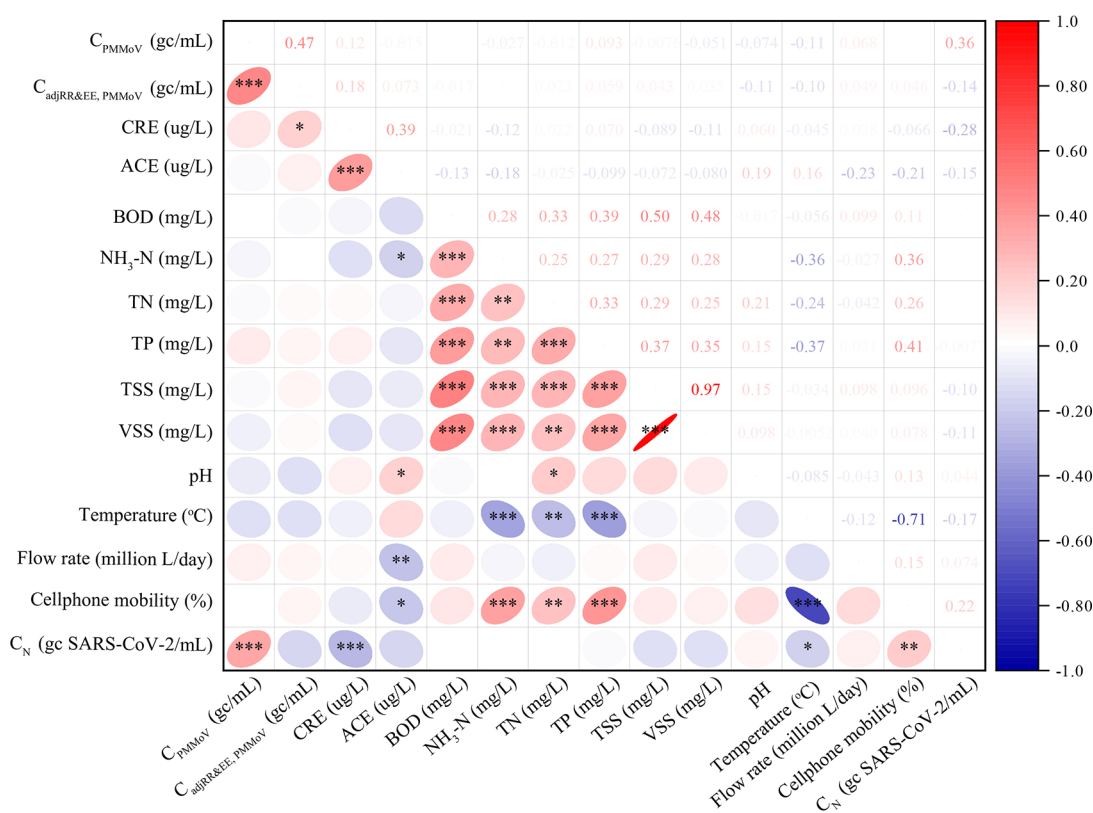


Figure 1. Correlations between concentrations of wastewater parameters, population indicators, and SARS-CoV-2 RNA. Acesulfame (ACE) and creatinine (CRE) are chemical indicators of population size; C_{PMMoV} , concentration of the fecal viral biomarker of population size, PMMoV; $C_{\text{adjRR\&EE, PMMoV}}$, practical efficiency adjusted C_{PMMoV} ; TP, total phosphorus; $\text{NH}_3\text{-N}$, ammonia as N; TN, total nitrogen; BOD, total biological oxygen demand; TSSs, total suspended solids; VSSs, volatile suspended solids. Significant level of Pearson correlation: $P \leq 0.001$, ***; $P \leq 0.01$, **; $P \leq 0.05$, *.

negative controls were negative for all tested targets in this study. During the period of this study, our laboratory participated in two interlaboratory calibrations.¹⁵

Statistics. Statistical analyses were performed using R Statistical Language v. 3.6.1 (R Core Team, 2019). Assumptions of normality and equal variance were assessed; then, depending on whether assumptions of parametric statistics were met, either an analysis of variance (ANOVA) or a Kruskal–Wallis H test was used to compare means between groups. A Type I error (α) of 0.05 was used as the threshold of statistical significance for all tests. Correlations between continuous variables were calculated by the Pearson correlation or linear regression. A locally weighted least-squares (Lowess) smooth was applied for curve fitting of the time-series data with a window size of 15. For the climbing stage of each COVID-19 wave, temporal changes of daily new cases and viral concentration were modeled using nonlinear curve fitting with exponential and polynomial functions. The best model was selected on the basis of the Akaike information criterion and the Bayesian information criterion. β -Coefficient was calculated by finite linear distributed lag models to represent the lag weight.⁴²

When the daily variable performance of the lab processes and dynamics of the wastewater matrix were considered, the viral load of assay j was adjusted with practical efficiencies, following eqs 4 and 5.

$$C_{\text{adjRR}, i, j} = C_{i, j} / \text{RR}_{i, k} \quad (4)$$

$$C_{\text{adjRR\&EE}, i, j} = C_{i, j} / (\text{RR}_{i, k} \times \text{EE}_k) \quad (5)$$

where i , j , and k are the sample id, RT-qPCR assay, and batch id, respectively; $C_{i, j}$ is calculated (eq 1). Adjusted viral loads of overall SARS-CoV-2 and VOCs ($C_{\text{adjRR\&EE}, i, j}$) were normalized by selected population indicators, PMMoV (eq 6), ACE (eq 7), and ammonia (eq 8).

$$\begin{aligned} C_{\text{norPMMoV}, i, j} &= C_{\text{adjRR\&EE}, i, j} / C_{\text{adjRR\&EE}, i, \text{PMMoV}} \\ &= C_{i, j} / C_{i, \text{PMMoV}} \end{aligned} \quad (6)$$

$$C_{\text{norACE}, i, j} = C_{\text{adjRR\&EE}, i, j} / C_{\text{ACE}, i} \quad (7)$$

$$C_{\text{norNH}_3\text{N}, i, j} = C_{\text{adjRR\&EE}, i, j} / C_{\text{NH}_3\text{N}, i} \quad (8)$$

where $C_{\text{PMMoV}, i}$, $C_{\text{ACE}, i}$, and $C_{\text{NH}_3\text{N}, i}$ are the concentrations of PMMoV, ACE, and ammonia as N, respectively.

RESULTS AND DISCUSSION

Analytical Performance Characteristics of SARS-CoV-2 WS. The practical performance of the wet laboratory varied among batches. The mean RR of viral enrichment was $15.4\% \pm 13.7\%$ (mean $\pm$ standard deviation; SD), which is comparable with the previously published PEG-precipitation protocols for coronaviruses.^{31,43} The mean EE was $47.8\% \pm 27.2\%$ (mean $\pm$ SD). The delay of the C_t values across the PCR inhibition assays was 0.15 ± 0.43 cycles (mean $\pm$ SD), which suggests that the inhibition of RT-qPCR was limited. The solid-removal procedure might have contributed to the minimal inhibition of

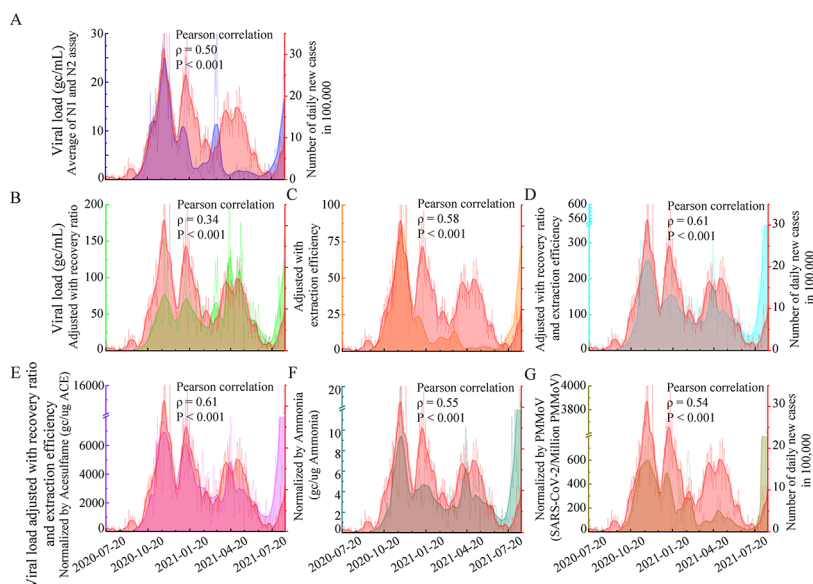


Figure 2. Comparison of viral concentrations adjusted by practical efficiency (A–D) or normalized by selected population indicators (E–G) with daily new cases of COVID-19 in the SWTP sewershed. Longitudinal trend and Lowess smooth of viral load without efficiency adjustment (A), adjusted by recovery ratio (B), adjusted by extraction efficiency (C), and adjusted by both recovery ratio and extraction efficiency (D). Practical efficiencies adjusted viral concentration further normalized by acesulfame (E), ammonia as N (F), and PMMoV (G). Longitudinal trend and Lowess smooth of daily new case numbers per 100 000 population in red. The Pearson correlation was used to determine the associations between viral concentrations and raw case numbers per 100 000. The curve fit of the time-series data was determined using a locally weighted least-squares (Lowess) approach with a window size of 15. The Y axes in red for each line plot are the number of daily new cases in the SWTP sewershed.

PCR observed. The LODs of the RT-qPCR assays and the parameters of standard curves are presented in Table 1. The LODs of the VOC assays (this study) were comparable with a previous publication,²⁹ which is adequate for detecting the trend of the concentrations of RNA for SARS-CoV-2 VOCs in wastewater. C_{N1} was significantly correlated with C_{N2} (linear regression, $R^2 = 0.768$, $P < 0.001$; Figure S2A). The mean of the N1 and N2 assay was applied for further data analysis.

The detection limit of the whole process was approximately 3 recent active cases in 14 days in 100 000, which corresponded to 3 mL of PE in 297 mL of river water. At this dilution, mean concentrations of the N1 and N2 assays were 0.20 ± 0.03 gc/mL diluted wastewater (C_t value: 35.60 ± 0.07) and 0.18 ± 0.01 gc/mL diluted wastewater (C_t value: 35.96 ± 0.19), respectively. However, this WS case detection limit did not consider untested or latent infections in the population, which would lessen the accuracy of the estimated sensitivity. The WS case detection limit (this study) was similar in magnitude to the theoretical limit (5–10 in 100 000),^{44,45} the whole wastewater direct extraction 4S method (2.4 in 100 000),²³ and the ultrafiltration method (2.4 in 100 000).⁴⁶ Compared with the results of previous studies, sampling strategies with large volumes can increase overall sensitivity.

There were no cross-interactions between wild and mutation genotypes for N.D3L.GAT.CTA, S.P681R.CCT.CGT, and S.T20N.ACC.AAC assays. Cross-interaction of the S.P681.CCT assay (wild type) with the Twist synthetic RNA control 15 (VOC α , S.H681.CAT genotype) was observed when concentrations of target viral RNA were greater than 62.5 gc/reaction. Hence, the results of the S.P681.CCT assay were not included in further data analyses. N.L3.A28271del assays can quantify Twist synthetic RNA control 15, although there is one nucleotide base deletion in the forward primer binding region. For the N.D3L assay, concentrations of

N.L3.CTA were significantly correlated with those of N.L3.A28271del (linear regression, $R^2 = 0.876$, $P < 0.0001$; Figure S2B).

Seasonality of Population Indicators. Biological nutrient parameters (BOD, $\text{NH}_3\text{-N}$, TN, and TP) were correlated with TSSs and VSSs (Figure 1). There were no significant differences in pH, average daily inflow rate, TSS, and VSS among seasons (ANOVA, $P > 0.05$, Figure S3A–D). Cross-seasonal differences in wastewater temperature, TP, NH_3 , TN, and BOD were observed (ANOVA, $P < 0.001$, Figure S3E–I). Residential cellphone mobility indicating population mobility was negatively correlated with daily flow rate (Figure 1). Nutrients in wastewater can be affected by seasonal inputs from landfills, road salt and fertilizers during rainy seasons, and snow melting.⁴⁷ Within the nutrient parameters of wastewater, changes of ammonium were associated with weekly and seasonal population dynamics, which could be useful for WS.²¹

To avoid collinearity of wastewater parameters, ACE, $\text{NH}_3\text{-N}$, and PMMoV were selected as representative indicators for population normalization. Observed mean ($\pm$ SD) concentrations of chemical and viral indicators were 542 ± 44.4 μg CRE/L, 35.0 ± 0.61 μg ACE/L, $7.0 \pm 2.0 \times 10^4$ gc PMMoV/mL, and $1.5 \pm 0.17 \times 10^6$ gc $C_{adjRR\&EE,PMMoV}$ /mL. The range of C_t values for PMMoV was 19.1–23.9 cycles, and they were positively correlated with those of FRNAPH-II (Pearson correlation, $\rho = 0.662$, $P < 0.001$); however, C_t values of FRNAPH-II (16.24–27.92) were more variable than those of PMMoV. Concentrations of ACE were positively correlated with CRE and negatively correlated with residential cellphone mobility (Figure 1). Cross-seasonal differences in concentrations of CRE, ACE, and PMMoV were observed (ANOVA, $P < 0.001$, Figure S3J–M). PMMoV levels in SWTP wastewater was comparable to those observed during previous studies conducted in Canada⁷ and the USA.^{23,25,46} Although temporal changes of human bacteroides HF183 and PMMoV

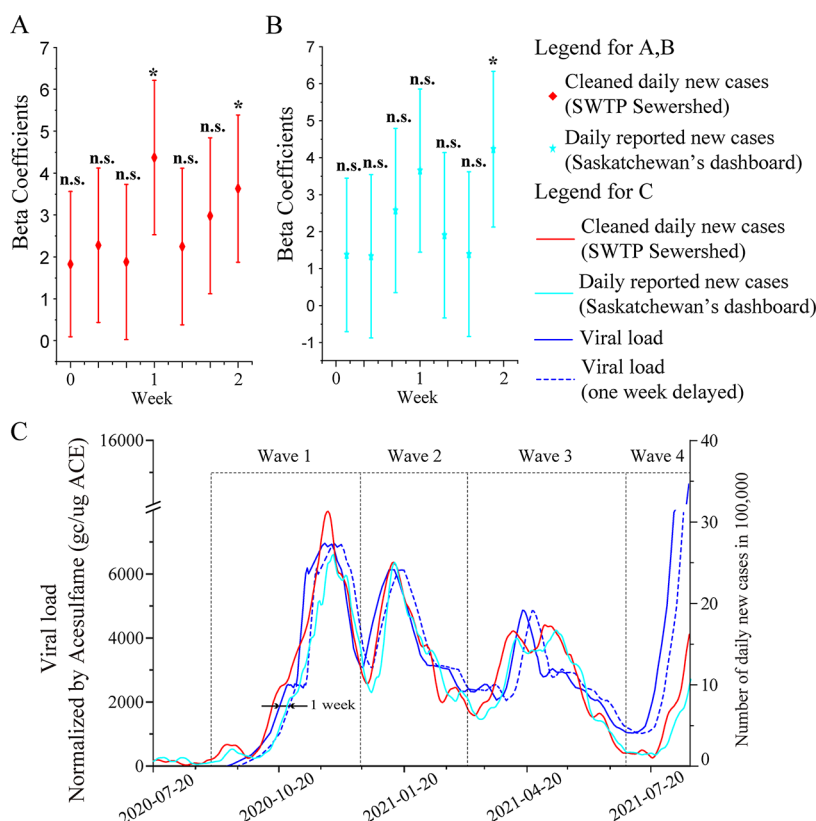


Figure 3. Lead time between wastewater surveillance and surges in numbers of COVID-19 cases expressed as number of new cases per day. Estimation of daily distributed lag parameters describing the association between viral load and daily new cases of the SWTP sewershed (A), daily reported cases from the Saskatchewan dashboard (B). β -Coefficients (mean and standard error) were calculated by finite linear distributed lag models to represent the lag weight. Sample size $N = 121$ during the periods between November 21, 2020 and August 17, 2021. Three samples were analyzed per week. Comparison between longitudinal trends of clinical indices and viral load (C). The curve fit of time-series data was determined using a locally weighted least-squares (Lowess) approach with a window size of 15. Nonlinear models were summarized in Table S5. Viral load was adjusted by practical efficiencies and acesulfame concentration.

were different among studies,^{7,25} crAssphage and PMMoV were the most consistent biomarkers in wastewater.²³

Viral Load Adjusted with Practical Efficiencies Captured the Four Surges in COVID-19 Cases. During the period between October 2020 and August 2021, major spikes in viral load corresponded to the four waves of COVID-19 cases in Saskatoon. The range of C_N without adjustment for practical efficiencies in wastewater varied from 0 (<LOD) to 32.71 gc SARS-CoV-2/mL. Concentrations of virus adjusted by both RR and EE improved performance of WS, closely tracking the magnitude of the outbreak based on daily new cases in the sewershed. The Lowess smooth curve of $C_{adjRR\&EE,N}$ was fit to numbers of new cases better than smoothed plots of C_N , $C_{adjEE,N}$, and $C_{adjRR,N}$ (Figure 2A–D), which was consistent with the greater Pearson correlations between $C_{N,adjRR\&EE}$ and daily new cases (Figure S4). Whole process QC is valuable for longer-term WS programs^{17,18} because wastewater is a dynamic complex matrix, affecting practical efficiency.⁴⁸

Normalization to ACE Corrected Deviation of Population Dynamics During the Holidays. Although normalization of viral loads to ACE did not improve the Pearson correlation with daily new cases (Figure S4), when one compares the profiles of Lowess smoothed curves (Figure 2E–G), ACE outperformed ammonia or PMMoV. Concentrations of ACE decreased during the Christmas and New Year holidays, which was consistent with residential cellphone

mobility (Figure S5). Viral load normalized to the dietary viral indicator, PMMoV, generally followed the trend of clinical incidence but performed poorly during spring and summer. Viral loads adjusted for ammonia were consistent with trends in clinical incidences during the first, third, and fourth waves but not the second wave. Consistent with results of a previous study,⁴⁹ the adjustment with RR of viral enrichment was better than normalization to PMMoV (Figures 2 and S4). Although a few chemical and biomarkers of wastewater and census and cellphone data have been suggested recently,^{18,20–22,47,50,51} there is no agreement on the ideal indicators of population size to normalize viral loads. The sources of wastewater, seasonality of indicators, composition of society, and population dynamics should be considered when selecting appropriate parameters for population normalization and correction for changes in inflow volumes. Because social gatherings during major events are associated with SARS-CoV-2 spread,⁵² multiple indicators might give confidence and overcome some of the biases of individual parameters. On the basis of the results of previous studies,^{47,53,54} a large-scale, long-term study should be conducted to evaluate wastewater indicators relative to census data.

WS Early-Warning Outbreaks of COVID-19. WS had an overall lead time of 1 week ahead of the time series of cleaned daily new cases of the sewershed and 2 weeks ahead of the time series of daily reported new cases from the Saskatchewan dashboard (Figure 3). The first and fourth waves occurred

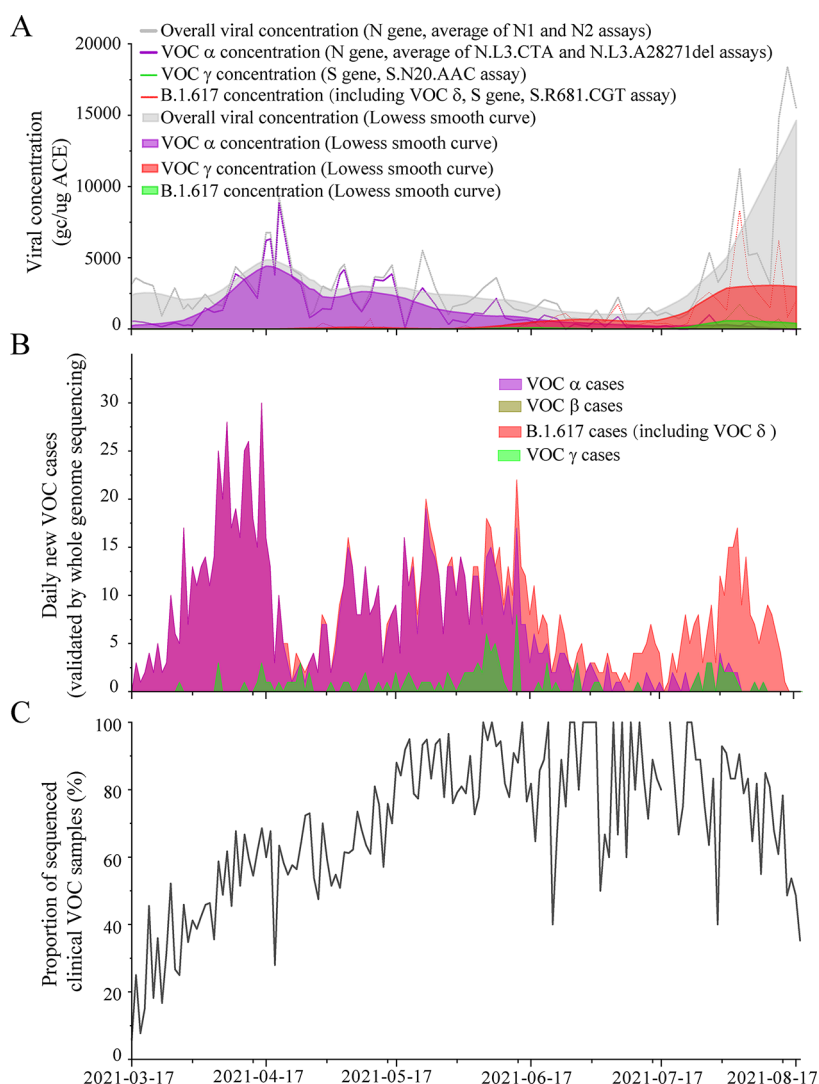


Figure 4. Longitudinal trends of viral loads (A) and daily new cases (B) of variants of concerns, and proportion of sequenced clinical VOC cases (C). The curve fit of time-series data was determined using a locally weighted least-squares (Lowess) approach with a window size of 15. Viral load was adjusted by practical efficiencies and acesulfame concentration. Clinical data was specific for the SWTP sewershed.

after COVID-19 restrictions had been eased, and infections increased due to multiple gathering events. During the climbing stage of the first and fourth waves, nonlinear fitting curves of WS viral load preceded daily reported cases (Figure S6A,D and Table S5). During the climbing stage of the second wave, the Lowess smooth curve of the viral load was also a leading indicator of both cleaned daily new cases and daily reported cases. During the third wave, the lead time of the viral load relative to the number of cases was not clear (Figure S6C), but a small peak of viral load around March 20, 2021 might have been due to the initial infections of the first peak of clinical incidences; additionally, the second peak of viral load in wastewater might have been associated with the second peak of clinical incidences.

The predicted lead time observed during this study was similar to that observed at other locations, where lead time has been estimated to be 6 to 8 days when concentrations of SARS-CoV-2 RNA in primary sludge was used to predict numbers of persons newly testing positive for COVID-19.¹² Regardless of delays in WS data, lag or lead time of WS is determined by the onset, magnitude, and duration of shedding of virus particles, asymptomatic cases that are still actively

shedding virus particles, and false negative and positive determinations, among others.^{49–51} There are also biases and inaccuracies in clinical measures due to the timing or capacities of laboratories and the timing of releases of data, but it is also governed by the timing of the onset of symptoms as well as the willingness of persons to be tested.^{7,20,55–57}

Sampling Frequency Affected the Lead Time of WS.

During the first wave (from October 20 to December 20, 2020), a lesser frequency of sampling of just once per week was insufficient to track the rapid initial onset of outbreaks, but an emergency sampling plan, during which five samples were collected per week, was able to accurately predict the changing profiles of daily new cases in real time (Figure S6A). A sampling frequency of three samples per week, instituted from November 21, 2020 through to the end of the monitoring (August 2021), worked well for tracking trends in numbers of new COVID-19 cases during outbreaks. Those results are consistent with those of previous studies, which suggest at least two samples per week are required to provide a sufficient description of the dynamics of outbreaks of COVID-19.^{9,25}

WS Revealing the COVID-19 Waves Driven Sequentially by VOCs. Allele-specific RT-qPCR assays for screening

VOCs detected dominant lineages in real time, which was consistent with positive clinical determinations of VOCs (Figure 4). The results of allele-specific RT-qPCR were further confirmed by WGS of weRNA (sample size $N = 18$ during the period between February 17 and August 4, 2021, Table S4). During the third and fourth wave, the SARS-CoV-2 load was dominated by VOCs α and δ , respectively. The viral RNA of VOC γ was detected in wastewater at a small concentration, which was consistent with the sporadic small numbers of VOC γ COVID-19 cases in Saskatoon. Allele-specific RT-qPCR is useful for screening circulating VOC lineages at the level of human populations; however, WGS can recover nearly complete genomes from potential degraded SARS-CoV-2 RNA in wastewater, which could be missed by targeted clinical screening assays based on small numbers of mutations.⁵⁸ When one compares VOCs α and γ , VOC δ has a greater ratio between viral load in wastewater to number of daily new cases (Figures 2E and 4B). Viral concentrations of VOC δ in oropharyngeal swabs was about 1000 times greater than those observed for SARS-CoV-2 clade 19A/19B, which makes VOC δ more contagious.⁵⁹ Hence, the pattern of the shedding of virus particles characteristic of circulating lineages should be considered when back-calculating numbers of COVID-19 cases from WS data.

CONCLUSIONS

WS can serve as a leading indicator of COVID-19 outbreaks and reveals the magnitude of outbreaks at an integrated population level that is not contingent upon numbers of tests, which can be determined by testing capacity and the willingness to be tested. Whole progress QCs are critical for precise WS. The adjustment with practical efficiencies improved the correlation between viral concentration and clinical indices. The normalization to concentrations of ACE was useful to improve the detection of trends of viral signals during major events, such as holidays. Cost-effective WS is a complementary tool of clinical testing. It is challenging to estimate the case numbers based on viral load in wastewater, but the overall trends of WS will be useful in predicting the potential numbers of virologically active cases within a population who were infected and are shedding viruses. Accurate and precise WS should consider shedding patterns among age groups and VOCs and uncertainties associated with fate in the sewershed as well as the sampling and analyses. As WS is independent of clinical testing, it represents an additional tool to support public health interventions. The potential lead time provided by WS can be used to make decisions on mitigations and allow for the mobilization and allocation of resources to better manage outbreaks.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsestwater.1c00349>.

Protocol of wastewater sampling, transport, and storage, ddPCR method, thermal cycling conditions, method of chemical tracer analyses, catchment area of the Saskatoon Wastewater Treatment Plant, scatter diagram of the relation of the viral concentration in the N1 and N2 assay and in the N.L3.CTA and N.L3.A28271del assay, characteristics of wastewater, correlations between SARS-CoV-2 concentrations and daily new case

numbers, longitudinal profiles of cell phone mobility and concentrations of acesulfame K, local alignment of the nonlinear curve fitting models for each climbing stage of the COVID-19 waves, raw wastewater characteristics, sequences of primers and probes, RT-qPCR assay parameters, whole genome sequencing data, and nonlinear curve fitting data (PDF)

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Notes

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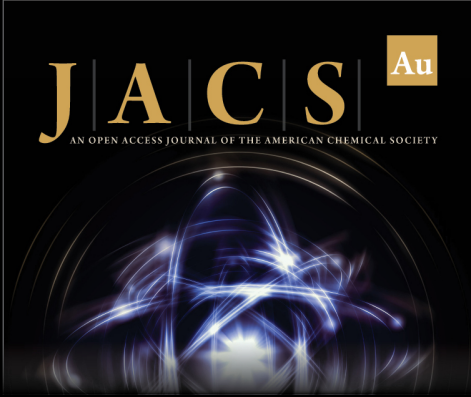
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RNA in Municipal Wastewater Reveals Magnitudes of COVID-19 Outbreaks across Four Waves Driven by SARS-CoV-2 Variants of Concern

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Materials and Methods

Text S1. Wastewater Sampling, Transport, and Storage. Each sampling occasion included one liter of a 24-h composite wastewater sample (about 60 mL every 15 min) collected using an auto-sampler maintained at 4 °C. Samples were transported to the laboratory on ice where it was heat-inactivated at 65 °C for 30 minutes and processed within 24 hours. A 300-mL sub-sample was aliquoted for viral detection, 15 mL for chemical tracer analysis, and the remainder was archiving at -80 °C.

Text S2. ddPCR. Droplet digital PCR (ddPCR) was conducted, by use of a Bio-Rad ddPCR system using One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad Laboratories, CA, USA). Stock solutions of RNA standard were diluted to ~2000 gc/μL base on nominal concentration. The reaction mixture was prepared following the manufacturer's manual: 1x Supermix, 20 U/μL Reverse transcriptase, 15 mM DTT and 2 μL RNA template. Droplet was generated by use of Bio-Rad QX200TM Droplet Generator. Thermal cycling condition consists reverse transcription at 50°C for 60 min, Taq polymerase activation at 95 °C for 10 min, 40 cycles of 95°C (denaturation) for 30 seconds followed by 60 °C for 1 min (annealing, extension, and data collection), and final enzyme deactivation at 98 °C for 10 min. Droplets were counted using Bio-Rad QX200 Droplet Reader and analyzed with Bio-Rad QuantaSoft Analysis Pro software (version: 1.0.596). ddPCR was conducted in eight replicates.

Text S3. Thermal Cycling Conditions. The RT-qPCR assays were performed using a QuantStudioTM 6 Flex thermal cycler (Thermo Fisher Scientific, CA, USA). Thermal cycling conditions consists of Uracil-DNA glycosylase (UNG) incubation at 25 °C for 2

min, reverse transcription at 53 °C for 10 min, denaturation and Taq polymerase activation at 95 °C for 2 min, and 45 cycles of 95 °C (denaturation) for 3 seconds followed by 60 °C for 30 seconds (annealing, extension, and data collection).

Text S4. Method of Chemical Tracer Analyses. Analysis was conducted using a Vanquish UHPLC and Q-ExactiveTM HF Quadrupole-OrbitrapTM mass spectrometer (Thermo-Fisher, Mississauga, ON). LC separation was achieved with a Kinetex 1.7 μ m XB-C18 LC column (100 $\times$ 2.1 mm) (Phenomenex, Torrance, CA) by gradient elution with 95% water + 5% methanol (A) and 100% methanol (B), both containing 0.1% formic acid at a flow rate of 0.2 mL min⁻¹ and column temperature of 40 °C. The gradient method started and held at 5% B to 1 min followed by a linear ramp to 100% B over 3 min, held at 100% B for 2 min to flush, and returned to starting conditions for column re-equilibration at 6 – 8 min.

Samples were ionized by both positive and negative mode heated electrospray ionization (HESI) using a polarity switching method. The Q-Exactive Orbitrap method used the following positive/negative source parameters: sheath gas flow = 35/35; aux gas flow = 10/10; sweep gas flow = 1/1; aux gas heater = 400/300 °C; spray voltage = 3.8/3.0 kV; S-lens RF = 60/60; capillary temperature = 350/350 °C. A full MS/parallel reaction monitoring (PRM) method was used with the following scan settings: 120,000/15,000 resolution, AGC target = $1 \times 10^6/2 \times 10^5$, max injection time = 100 ms/100 ms, full MS scan range of 80-500 m/z and PRM isolation window of 2.0 m/z. The method was operated in positive mode from 0 – 2.5 min for the detection of CRE (1.03 min, [M+H]⁺ = 114.0663) and CRE-d3 (1.03 min, [M+H]⁺ = 117.0851), switching to negative mode to detect ACE (4.20 min, [M+H]⁺ = 161.9859) and ACE-d4 (4.17 min, [M+H]⁺ = 166.0111). No reliable

daughter ions suitable for confirmation were formed during fragmentation so identification and quantification was based upon full MS parent ions (<2 ppm) and retention times.

Batch analyses of samples were conducted by running calibration standards at the beginning of each sample batch along with lab blanks run between replicate treatment sets and single calibration standards (10, 25, or 50 µg/L) every 15-20 samples as a QA/QC protocol.

Table S1. Raw wastewater characteristics measured at the SWTP during July 2020 through August 2021 including minimum, maximum, and average ($\pm$ standard deviation, SD) (Courtesy of SWTP).

Parameter	Minimum	Maximum	Average $\pm$ SD
Inflow rate (ML/D*)	64.05	95.10	77.64 $\pm$ 3.74
pH	7.40	9.30	7.99 $\pm$ 0.43
Temperature (°C)	4.3	22.9	14.6 $\pm$ 3.8
TSS (mg/L)	83.0	688.00	256 $\pm$ 55
VSS (mg/L)	132	374	215 $\pm$ 60
BOD (mg/L)	140	724	296 $\pm$ 73
TN (mg/L)	39.1	79.2	47.8 $\pm$ 4.3
TP (mg/L)	3.96	11.00	5.53 $\pm$ 0.80

*ML/D = million liters per day

Table S2. Sequences of primers and probes.

Assay	Organism	Targeted gene	Primer sets		Ref.
			Name	Sequence (5' – 3')	
N1	SARS-CoV-2	N gene	2019-nCoV_N1-F 2019-nCoV_N1-R 2019-nCoV_N1-P	GACCCCAAAATCAGCGAAAT TCTGGTTACTGCCAGTTGAATCTG 6FAM-ACCCCGCATTACGTTTGGTGGACC-QSY	¹
N2	SARS-CoV-2	N gene	2019-nCoV_N2-F 2019-nCoV_N2-R 2019-nCoV_N2-P	TTACAAACATTGGCCGCAAA GCGCGACATTCCGAAGAA 6FAM-ACAATTTGCCCCCAGCGCTTCAG-QSY	¹
PMMoV	PMMoV ^a	Replication-associated protein gene	PMMV-FP1 PMMV-RP1 PMMV-probe	GAGTGGTTTGACCTTAACGTTTGA TTGTGCGTTGCAATGCAAGT 6FAM-CCTACCGAAGCAAATG-QSY	²
FRNAPH-II	FRNAPH-II ^b	lys gene	VTB4-FphGII ^f VTB4-FphGII ^r VTB4-FphGIIprobe	ACCTATGTTCCGATTCASAGAG GGTAGGCAAGTCCATCAAAGT VIC-CACTCGCGATTGTGCTGTCCGATT-QSY	
AQHRP	AQHRP ^c	HRP3 gene fragment	HRP3-F HRP3-R HRP3-P	AGATTGACCTGCGAGCG GAGCGGCTGTCTCCACAAGT 6FAM-TTCTGACCTGAAGGCTCTGCGCG-QSY	
Inhibition assessment	N.A.	IPC ^{pcr} ^d	IPC-F IPC-R IPC-Probe	ACGCACATATACGGGTAGCA TCCCGCCTATAGACCACCTT VIC-ACTTTCGGATGCATCTAGTGACA-QSY	This study
N.D3L	SARS-CoV-2, B.1.1.7 (alpha) lineages	N gene	D3L_for 2019-nCoV_N1-R 2019-nCoV_N1-P	CATCTAAACGAACAACTAAAATGTCTCTA TCTGGTTACTGCCAGTTGAATCTG 6FAM-ACCCCGCATTACGTTTGGTGGACC-QSY	³
N.D3del			A28271del/D3LF 2019-nCoV_N1-R 2019-nCoV_N1-P	CATCTAAACGAACAACTAAATGTCTCTAA TCTGGTTACTGCCAGTTGAATCTG 6FAM-ACCCCGCATTACGTTTGGTGGACC-QSY	
S.T20N ^e	SARS-CoV-2, P.1 (gamma) lineages	S gene		N.A.	
S.P681R ^e	SARS-CoV-2, B.1.617 lineages (including Delta variant)	S gene		N.A.	

^a Population indication, Pepper mild mottle virus (PMMoV)^b Fecal indication as indigenous processing control, F-Specific RNA Bacteriophages group II (FRNAPH-II)^c Whole processing spike control, Armored RNA Quant RNase P standard (AQHRP)^d Inhibition assessment, internal RT-qPCR positive control (IPC)^e S.T20N.ACC.AAC assay from TaqMan SARS-CoV-2 Mutation Panel (Thermo Fisher Scientific, CA, USA)^f S.P681R.CCT.CGT assay from TaqMan SARS-CoV-2 Mutation Panel (Thermo Fisher Scientific, CA, USA)

Table S3. Recipe, quantitative RNA standard, range of standard curve, and settings for threshold and baseline of RT-qPCR assays.

Assays	Recipe of RT-qPCR assays		Assay/Test	Quantitative RNA standard	Range of standard curve (gc/reaction)	Settings for threshold	Settings for baseline
	Component	Amount (volume) per 10-μL reaction					
N1, N2, PMMoV, D3L, D3L2 (Singleplex)	4X TaqPath™ 1-Step Master Mix (Thermo Fisher Scientific, CA, USA)	2.5 μL	N1	Quantitative Synthetic SARSCoV2 RNA (ATCC, USA)	7.25E4, 7.25E3, 7.25E2, 7.25E1, 7.25E0, 3.63E0, 1.21E0	0.04	auto
	Forward primer	180 nM	N2			0.04	auto
	Reverse primer	180 nM	PMMoV	Synthetic RNA (IDT, USA)	2.0E8, 2.0E7, 2.0E6, 2.0E5, 2.0E4, 2.0E3, 2.0E2	0.04	auto
	Probe	150 nM	AQHRP	Extracted RAN from Armored RNA Quant RNase P (Asuragen, TX, USA)	2.5E4, 2.5E3, 2.5E2, 2.5E1, 6.25E0, 1.56E0	0.04	auto
S.T20N, S.P681R (Duplex)	weRNA/Blank controls/Standards	2.5~ 5 μL					
	4X TaqPath™ 1-Step Master Mix	2.5 μL	N.L3.CTA	Control 15 (Twist Bioscience, CA, USA)	6.25E4, 6.25E3, 6.25E2, 6.25E1, 6.25E0, 2.08E0, 1.04E0	0.04	auto
	40 X Primer & Probe Mix	0.25 μL	N.L3.A28271del			0.04	
FRNAPH-II, AQHRP (Duplex)	weRNA/Blank controls/Standards	2.5 ~ 5 μL	S.T20.ACC	Control 2 (Twist Bioscience, CA, USA)	6.25E4, 6.25E3, 6.25E2, 6.25E1, 6.25E0, 2.08E0, 1.04E0	0.06	auto
	TaqPath™ 1-Step Master Mix (4X)	2.5 μL					
	HRP3-F	180 nM	S.N20.AAC	Control 17 (Twist Bioscience, CA, USA)	6.25E4, 6.25E3, 6.25E2, 6.25E1, 6.25E0, 2.08E0, 1.04E0	0.06	auto
	HRP3-R	180 nM					
	HRP3-P	150 nM	S.P681.CCT	Control 2 (Twist Bioscience, CA, USA)	6.25E4, 6.25E3, 6.25E2, 6.25E1, 6.25E0, 2.08E0, 1.04E0	0.06	auto
	VTB4-FphGIIf	60 nM					
Inhibition assessment	VTB4-FphGIIr	60 nM	S.R681.CGT	Control 18 (Twist Bioscience, CA, USA)	6.25E4, 6.25E3, 6.25E2, 6.25E1, 6.25E0, 2.08E0, 1.04E0	0.06	auto
	VTB4-FphGIProbe	50 nM					
	weRNA/Blank controls/Standards	2.5 ~ 5 μL	IPCpcr	Synthetic RNA (IDT, USA)	5.0E7, 5.0E6, 5.0E5, 5.0E4, 5.0E3, 5.0E2, 5.0E1	0.04	auto
	Samples/Blank controls						
	4X TaqPath™ 1-Step Master Mix	2.5 μL	FRNAPH-II	N.A.	N.A.	0.02	auto
	IPC-F	180 nM					
	IPC-R	180 nM					
	IPC-Probe	150 nM					
	1.0×10 ³ copies/μL IPC RNA	2.5 μL					
	weRNA	2.5 ~ 5 μL					
	Standards/Negative controls						
	4X TaqPath™ 1-Step Master Mix	2.5 μL					
	IPC-F	180 nM					
	IPC-R	180 nM					
	IPC-Probe	150 nM					
	Standards/Negative controls	2.5 μL					

Table S4. Consensus VOC lineage detected by whole genome sequencing (WGS) of wastewater eRNA. WGS data was generated and analyzed by Division of Enteric Diseases, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada. Sequencing library was prepared following ARTIC V3 protocol and sequenced by use of Illumina platform.

Sampling date	% Breadth of coverage ($\geq 5x$ depth)	Average depth of coverage	VOC Detected (consensus)	Number of VOC mutations (consensus)	Frequencies of reads ($>cov 30$) with VOC mutation (consensus)
2021-02-17	99.73%		no	0	n/a
2021-02-28	90.22%	2570.1x	no	0	n/a
2021-03-31	91.71%	1653.19x	B.1.1.7	12/17	0.184-1
2021-04-04	98.83%		B.1.1.7	17/17	0.105-0.966
2021-04-20	99.73%	2694.24	B.1.1.7	17/17	0.018-1
2021-05-05	83.01%	981.25	B.1.1.7	14/17	0.026-1 (avg =0.49)
2021-05-19	91.76%	5469.6	B.1.1.7 (alpha)	16/17	average = 0.8
2021-05-26	71.08%	1474.55	B.1.1.7 (alpha)	13/17	0.6
2021-06-06	98.61%	4612.94	B.1.1.7 (Alpha)	17/17	0.79
2021-06-14	86.50%	3146.36	B.1.1.7 (Alpha)	17/17	0.92
2021-06-20	72.39%	978.32	B.1.1.7 (Alpha)	8/17	0.4
2021-07-04	45.09%	656.88	B.1.1.7 (Alpha)	8/17	0.42
2021-07-11	52.32%	674.51	B.1.1.7 (Alpha)	6/17	0.26
2021-07-18	69.59	1322.27	B.1.1.7 (Alpha)	12/17	0.47
2021-07-25	87.19	1986.88	B.1.617.2 (Delta)	9/13	0.59
2021-07-30	93.94	1861.47	B.1.617.2 (Delta)	8/12	0.58
			Sublineage AY.11	12/12 (cov < 30)	(cov <30 = 0.85)
2021-08-01	93.91	2903.88	B.1.617.2 (Delta)	13/13	0.9
2021-08-04	93.42	2712.42	B.1.617.2 (Delta)	10/12	0.83
				12/12 (cov <30)	cov < 30 = 0.97

Table S5. Summary of nonlinear curve fitting for each climbing stage of COVID-19 wave in Saskatoon (Fig 3. D-G). Model of dependent variable in function of duration of a given climbing stage.

Dependent variable	Y	Model	Parameters
1 st wave (Fig. 3D)	Daily new cases (SWTP Sewershed)	$y = e^{(-A*x)}$ $t_0 = 2020-09-01$ $t_{\max} = 2020-11-24$	$A = -0.04181 \pm 3.5876E-4$ Reduced $\chi^2 = 9.74143$ Adjusted $R^2 = 0.89125$
	Daily new cases (Daily report for Saskatoon zone)	$y = e^{(-A*x)}$ $t_0 = 2020-09-01$ $t_{\max} = 2020-11-24$	$A = -0.03658 \pm 9.90929E-4$ Reduced $\chi^2 = 33.80966$ Adjusted $R^2 = 0.53205$
	Viral load (gc/ug ACE)	$y = a * [1 - e^{(-b*x)}]$ $t_0 = 2020-09-01$ $t_{\max} = 2020-11-24$	$a = -381.71093 \pm 364.78825$ $b = -0.03627 \pm 0.01206$ Reduced $\chi^2 = 4.331E+6$ Adjusted $R^2 = 0.52575$
2 nd wave (Fig. 3E)	Daily new cases (SWTP Sewershed)	$y = y_0 + C * [\frac{\cos(\frac{x-x_c}{S})}{\sin(\frac{x-x_c}{S})} - \frac{S}{x-x_c}]$ $t_0 = 2020-12-18$ $t_{\max} = 2021-01-13$	$y_0 = 17.35161 \pm 0.02818$ $x_c = 14.38229 \pm 0.03667$ $C = 9.67431 \pm 0.06287$ $S = 2.15168 \pm 0.0331$ Reduced $\chi^2 = 0.25802$ Adjusted $R^2 = 0.99307$
	Daily new cases (Daily report for Saskatoon zone)	$y = p1 * e^{(-x/p2)} + p3 + p4 * x$ $t_0 = 2020-12-18$ $t_{\max} = 2021-01-13$	$p1 = 39.49228 \pm 0.20148$ $p2 = 6.92274 \pm 0.05604$ $p3 = -18.34175 \pm 0.23845$ $p4 = 1.72258 \pm 0.00867$ Reduced $\chi^2 = 0.06096$ Adjusted $R^2 = 0.99814$
	Viral load (gc/ug ACE)	$y = a * b^x$ $t_0 = 2020-12-18$ $t_{\max} = 2021-01-13$	$a = 2711.38209 \pm 1232.03574$ $b = 1.04186 \pm 0.02359$ Reduced $\chi^2 = 8.740E+6$ Adjusted $R^2 = 0.20726$
3 rd wave (Fig. 3F)	Daily new cases (SWTP Sewershed)	$y = e^{(a+b*x+c*x^2)}$ $t_0 = 2021-03-09$ $t_{\max} = 2021-04-16$	$a = 1.40068 \pm 0.20691$ $b = 0.06915 \pm 0.01857$ $c = -7.94778E-4 \pm 3.9048E-4$ Reduced $\chi^2 = 6.49479$

			Adjusted R ² = 0.73798
	Daily new cases (Daily report for Saskatoon zone)	$y = e^{(a+b*x+c*x^2)}$ $t_0 = 2021-03-09$ $t_{max} = 2021-04-16$	$a = 1.85492 \pm 0.24694$ $b = -0.013 \pm 0.02476$ $c = 0.00118 \pm 2.5591E-4$ Reduced $\chi^2 = 10.56227$ Adjusted R ² = 0.59152
	Viral load (gc/ug ACE)	$y = A_0 + A_1 * x + A_2 * x^2 + A_3 * x^3 + A_4 * x^4 + A_5 * x^5$ $t_0 = 2021-03-09$ $t_{max} = 2021-04-16$	$A_0 = 1258.33123 \pm 6.85061E-13$ $A_1 = 423.71988 \pm 3.17364E-13$ $A_2 = -31.13223 \pm 4.68023E-14$ $A_3 = 0.59914 \pm 2.91511E-15$ $A_4 = 7.18079E-18 \pm 8.03243E-17$ $A_5 = 6.66479E-21 \pm 8.07243E-19$ Reduced $\chi^2 = 5.33652E-24$ Adjusted R ² = 1.0
4 th wave (Fig. 3G)	Daily new cases (SWTP Sewershed)	$y = a * b^x$ $t_0 = 2021-07-01$ $t_{max} = 2021-08-17$	$a = 0.54451 \pm 0.12884$ $b = 1.07387 \pm 0.00618$ Reduced $\chi^2 = 2.78403$ Adjusted R ² = 0.85553
	Daily new cases (Daily report for Saskatoon zone)	$y = a * b^x$ $t_0 = 2021-07-01$ $t_{max} = 2021-08-17$	$a = 0.42131 \pm 0.12921$ $b = 1.0699 \pm 0.0059$ Reduced $\chi^2 = 10.00046$ Adjusted R ² = 0.83971
	Viral load (gc/ug ACE)	$y = a * e^{b*x}$ $t_0 = 2021-07-01$ $t_{max} = 2021-08-17$	$a = 310.65862 \pm 200.00236$ $b = 0.08506 \pm 0.01504$ Reduced $\chi^2 = 6.727E+6$ Adjusted R ² = 0.77387

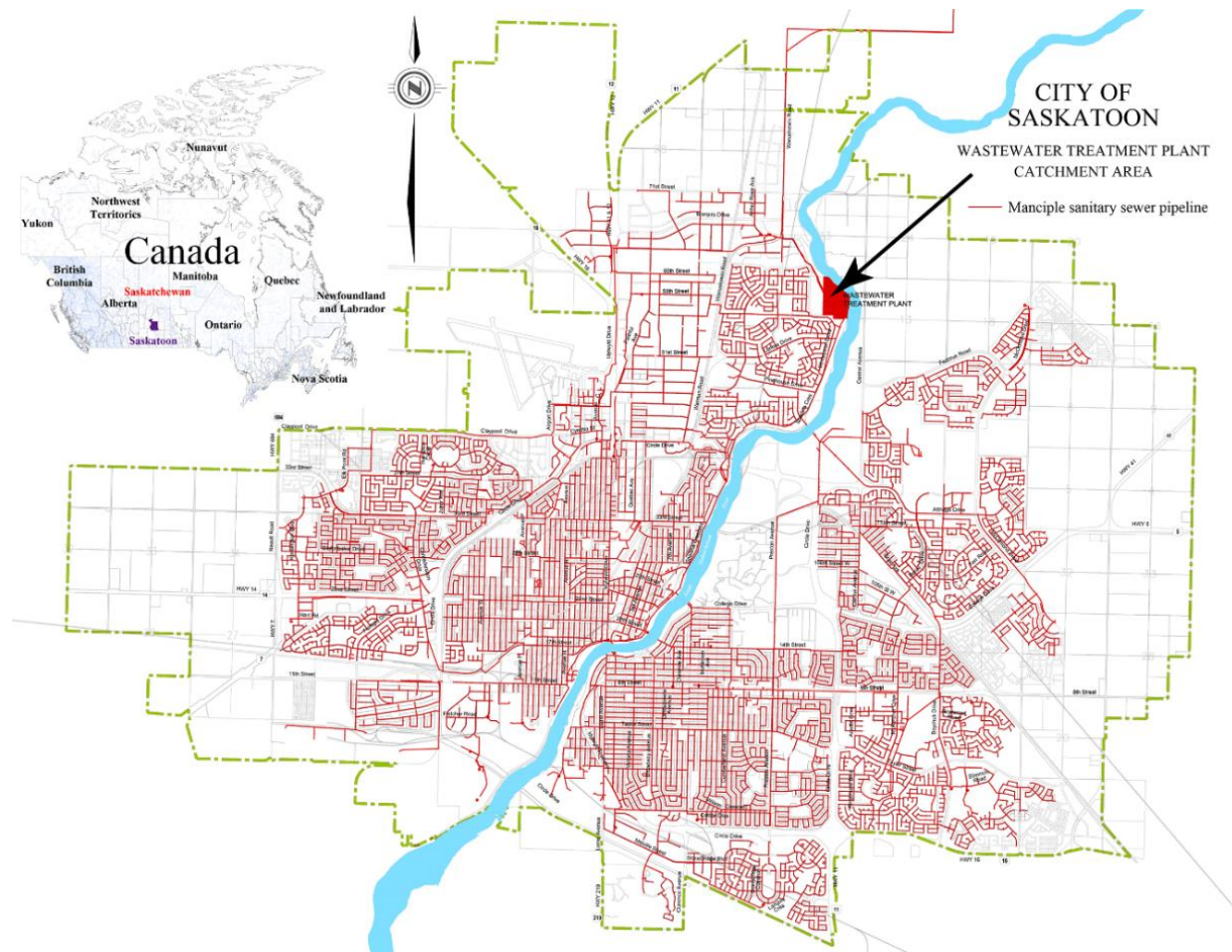


Figure S1. Catchment area of Saskatoon Wastewater Treatment Plant (Saskatoon, Canada). The Saskatoon Wastewater Treatment Plant (SWTP) is an advanced treatment facility that predominantly receives and treats the sanitary sewer of the City of Saskatoon (Saskatchewan, Canada). The mean transit time for sewage from households to the facility is estimated between four to six hours, and the facility's hydraulic retention time is about 24 h.

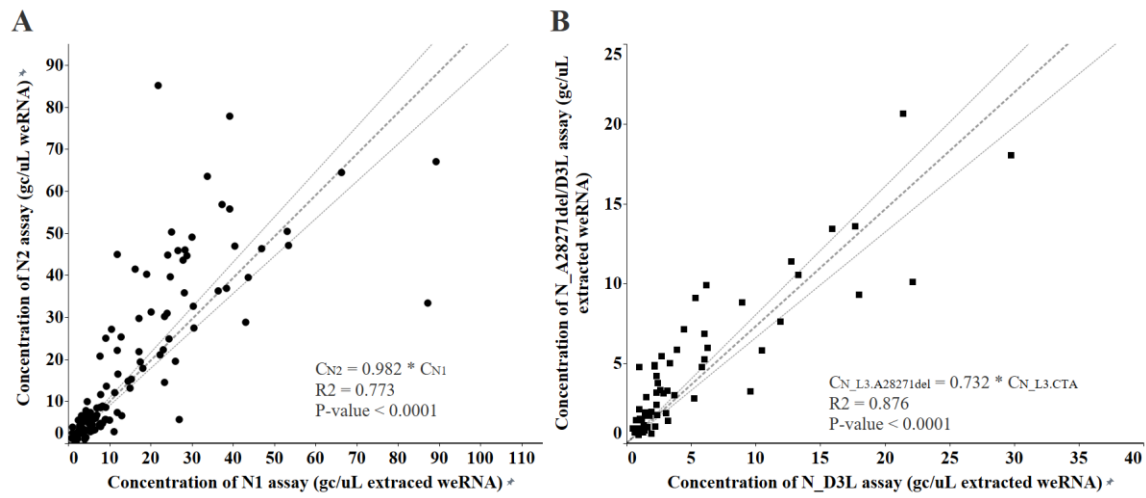


Figure S2. Scatter diagram of relation in viral concentration of SARS-CoV-2 between N1 and N2 assay (A), between N.L3.CTA and N.L3.A28271del (B). The relation was assessed by linear regression with forcing y-intercept to zero option.

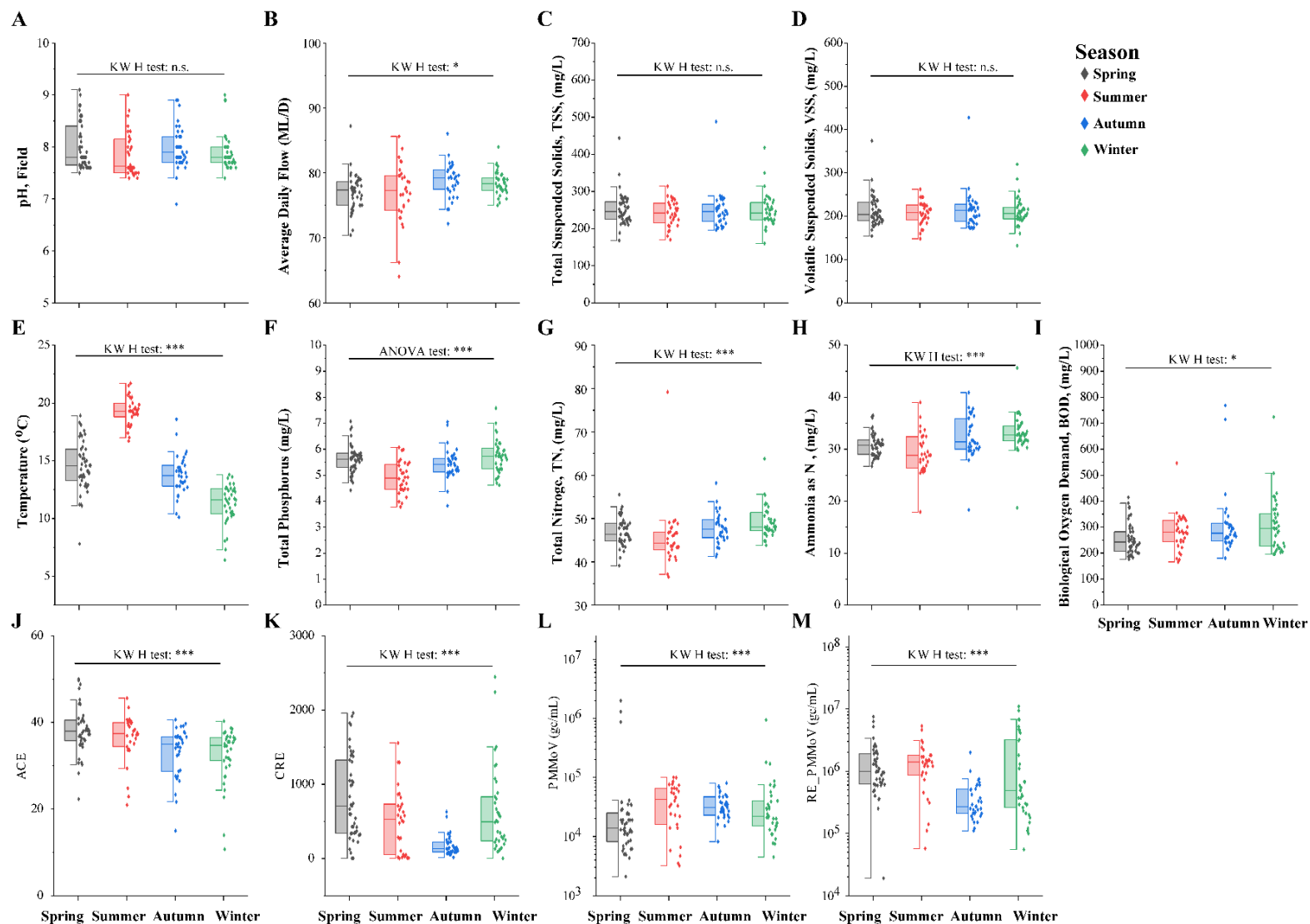


Figure S3. Characteristics of wastewater from the SWTP during the period from July 20, 2020 to August 17, 2021. (A) Filed pH; (B) average daily flow; (C) TSS, total suspended solids; (D) VSSs, volatile suspended solids; (E) wastewater temperature (°C); (F) TP, total phosphorus; (G) TN, total nitrogen; (H) Ammonia as N; (I) BOD, total biological oxygen demand; (J) ACE, acesulfame K; (K) CRE, creatinine; (L) PMMoV, pepper mild mottled virus; (M) PMMoV adjusted by recovery ratio and extraction efficiency. Significant level of One-Way ANOVA or Kruskal–Wallis H test (KW H test): $P \leq 0.001$, ***; $P \leq 0.01$, **; $P \leq 0.05$, *; $P \geq 0.05$, non-significant (n.s.).

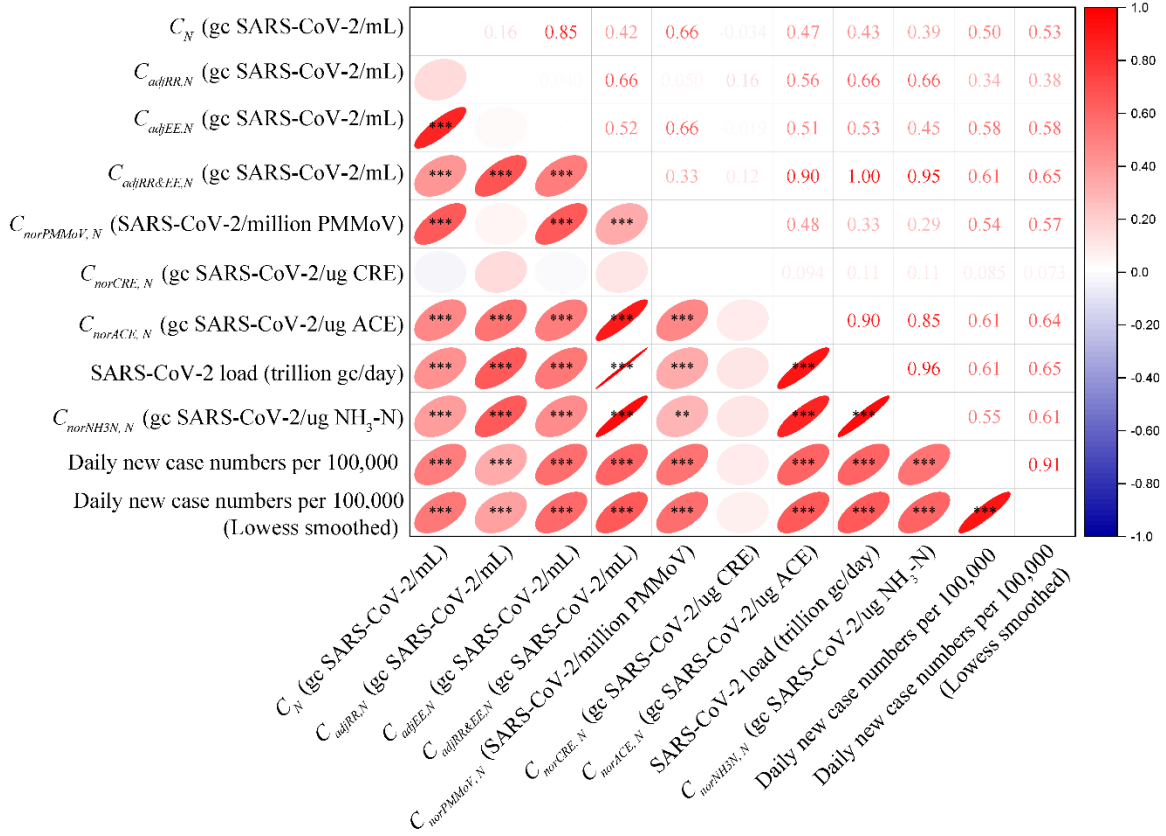


Figure S4. Correlations between SARS-CoV-2 concentrations and daily new case numbers. C_N , average concentration of N1 and N2 assays; $C_{adjRR,N}$, C_N adjusted by recovery ratio; $C_{adjEE,N}$, C_N adjusted by extraction efficiency; $C_{adjRR\&EE,N}$, C_N adjusted by recovery ratio and extraction efficiency; $C_{norACE,N}$, $C_{adjRR\&EE,N}$, normalized by acesulfame; $C_{norCRE,N}$, $C_{adjRR\&EE,N}$, normalized by creatinine; $C_{norNH_3,N}$, $C_{adjRR\&EE,N}$, normalized by ammonia; $C_{norPMMoV,N}$, $C_{adjRR\&EE,N}$, normalized by PMMoV; SARS-CoV-2 load, viral load per day. Curve fit of daily new cases per 100,000 population was determined using locally weighted least squares (Lowess) approach with window size of 15.

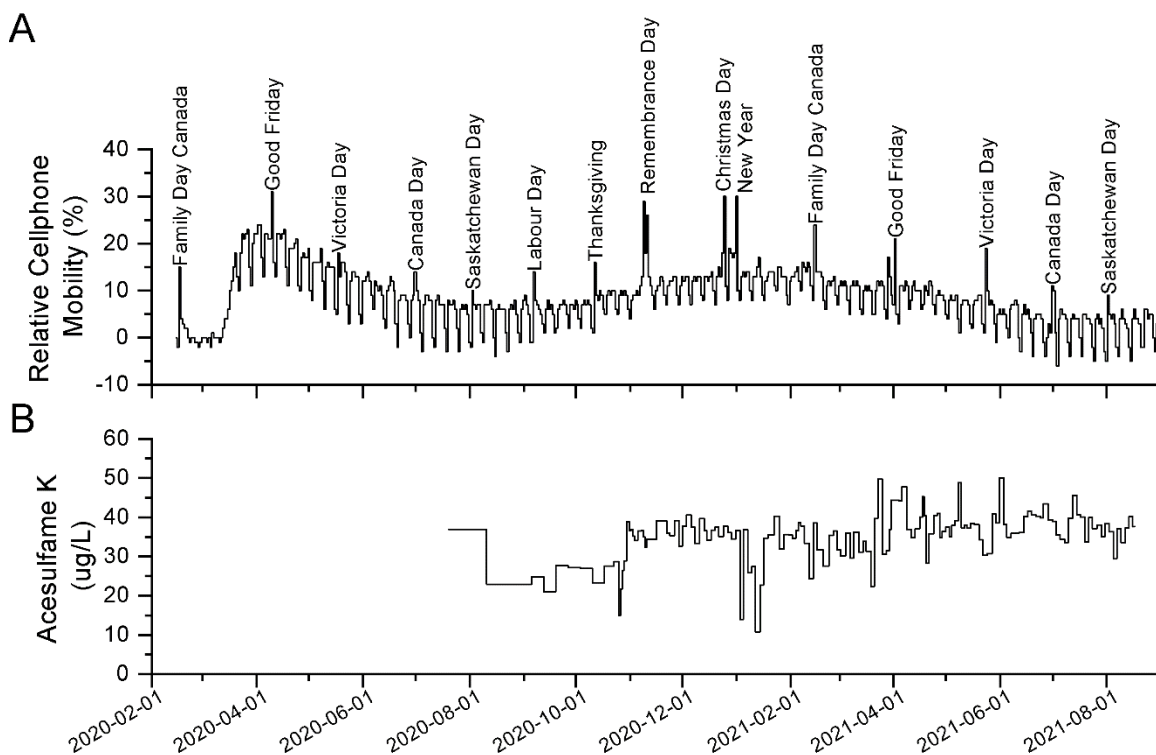


Figure S5. (A) Trend of residential cellphone mobility in Saskatoon city. (B) Longitudinal trend of concentrations of acesulfame K in wastewater. Percentage was calculated from baseline, during the 5-week period Jan 3–Feb 6, 2020. Data was grabbed from Google LLC "Google COVID-19 Community Mobility Reports". <https://www.google.com/covid19/mobility/20210817>.

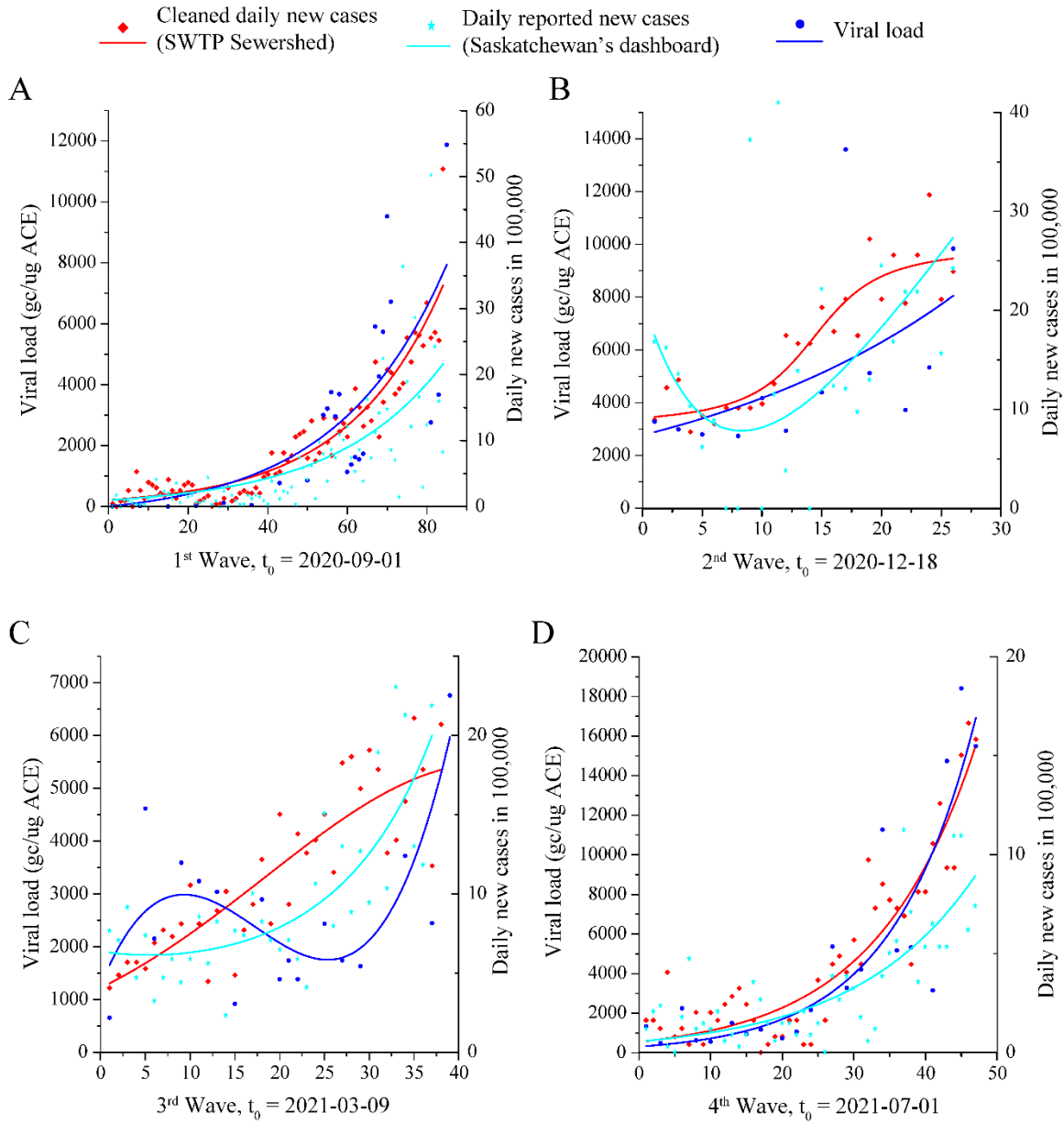


Figure S6. Local alignment of nonlinear curve fitting models for each climbing stage of the first wave (A), second wave (B), third wave (C), and fourth wave (D). Nonlinear models were summarized in SI Table S5. Viral load was adjusted by practical efficiencies and acesulfame concentration.

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