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

Supplementary Information

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Table S1. Primers and probes used by participating laboratories

Purpose	Target gene	Oligonucleotide	Sequence	Reference	Lab
SARS-CoV-2	Nucleocapsid protein (N) gene	N1_USCDC_F	GACCCCAAAATCAGCGAAAT	(1–4)	All
		N1_USCDC_R	TCTGGTTACTGCCAGTTGAATCTG		
		N1_USCDC_P	ACCCCGCATTACGTTTGGTGGACC		
		N2_USCDC_F	TTACAAACATTGGCCGCAAA		All
		N2_USCDC_R	GCGCGACATTCCGAAGAA		
		N2_USCDC_P	ACAATTTGCCCCCAGCGCTTCAG		
SARS-CoV-2	Envelope protein (E) gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	(5)	A
		E_Sarbeco_R	ATATTGCAGCAGTACGCACACA		
		E_Sarbeco_P	ACACTAGCCATCCTTACTGCGCTTCG		
Fecal indicator virus	CrAssphage	crAss phage_Forward	CAGAAGTACAAACTCCTAAAAAACGTAGAG	(6,7)	All
		crAss phage_Reverse	GATGACCAATAAACAAGCCATTAGC		
		crAss phage_Probe	AATAACGATTTACGTGATGTAAC		
Fecal indicator virus	PMMoV	PMMoV_Forward	GAGTGGTTTGACCTTAACGTTTGA	(8)	F, H
		PMMoV_Reverse	TTGTCGGTTGCAATGCAAGT		
		PMMoV_Probe	CCTACCGAAGCAAATG-FAM		
Matrix spike	Bovine CoV	BCoV_Forward	CTGGAAGTTGGTGGAGTT	(9)	A, F
		BCoV_Reverse	ATTATCGGCCTAACATACATC		
		BCoV_Probe	CCTTCATATCTATACACATCAAGTTGTT		
Matrix spike	MHV	MHV-N-fwd	GCCAAATAATCGCGCTAGAA	(10)	D
		MHV-N-rev	CCGAGCTTAGCCAAAACAAG		

Matrix spike	OC43	OC43_Forward	CGATGAGGCTATTCCGACTAGGT	(11,12)	A, E, G, H
		OC43_Reverse	CCTTCCTGAGCCTTCAATATAGTAACC		
		OC43_Probe	TCCGCCTGGCACGGTACTCCCT		

Table S2. Gene quantification limits of detection (LOD) and quantification (LOQ), as well as quantification inhibition status, by lab. For inhibition, N indicates that none was detected across all trials, and Y indicates that inhibition was detected in one of the total seven trials for that particular lab. *Average, as influenced by processed sample volume per trial and sample.

Lab	Method	Reverse transcription	LOD (GC/L)	LOQ (GC/L)	Inhibition (Y/N)
A	ddPCR	Two-step	667	1,330	N
B	qPCR	One-step	177	537	Y
C	qPCR	One-step	500	1,250	Y
D	qPCR	Two-step	190	407	N
E	ddPCR	Both one- & two-step	231	692	N
F	qPCR	Both one- & two-step	3940*	15,759	Y
G	qPCR	One-step	698*	2,330	N
H	qPCR	One-step	3,000	20,000	N

Table S3: Raw average SARS-CoV-2 gene fragment quantification results, recovery efficiency of spiked surrogate and recovery efficiency-adjusted SARS-CoV-2 concentrations for each and trials. Gene fragment concentrations are reported in gene copies per liter (GC/L). Dash (-) indicates missing data.

Trial	Lab	N1 (GC/L)	N2 (GC/L)	Spike	Recovery (%)	Correct N1 (GC/L)	Correct N2 (GC/L)
1	A	1.50×10^4	1.12×10^4	BCoV	15.90	9.43×10^4	7.07×10^4
	B	5.90×10^3	4.29×10^3	-	-	-	-
	C	-	5.56×10^3	-	-	-	-
	E	4.52×10^3	3.52×10^3	OC43	2.57	1.76×10^5	1.37×10^5
	G	5.00×10^4	1.58×10^4	OC43	29.20	1.71×10^5	5.39×10^4
2	A	3.94×10^4	3.90×10^4	BCoV	53.20	7.41×10^4	7.33×10^4
	B	2.99×10^4	4.97×10^4	BCoV	36.10	8.30×10^4	1.38×10^5
	C	1.05×10^5	4.93×10^4	BCoV	-	-	-
	D	-	3.02×10^4	MHV	17.70	-	1.70×10^5
	E	8.02×10^4	4.82×10^4	OC43	34.03	2.36×10^5	1.42×10^5
	F	4.17×10^4	2.22×10^5	BCoV	13.50	3.09×10^5	1.65×10^6
	G	2.49×10^5	7.77×10^3	OC43	5.68	4.39×10^6	1.37×10^5
3	A	9.49×10^3	7.27×10^3	OC43	24.50	3.87×10^4	2.97×10^4
	B	2.32×10^4	4.80×10^4	BCoV	36.10	6.44×10^4	1.33×10^5
	C	3.45×10^4	1.50×10^4	BCoV	71.84	4.80×10^4	2.09×10^4
	D	-	3.83×10^4	MHV	10.60		3.61×10^5
	H	3.04×10^5	4.36×10^5	OC43	17.65	1.72×10^6	2.47×10^6
4	A	2.74×10^4	1.85×10^4	OC43	18.10	1.51×10^5	1.02×10^5
	E	-	2.46×10^4	OC43	73.16	-	3.36×10^4
	F	6.07×10^5	4.65×10^5	BCoV	-	-	-
	G	2.99×10^4	1.30×10^4	OC43	50.48	5.93×10^4	2.57×10^4
5	A	1.28×10^4	1.62×10^4	OC43	6.30	2.02×10^5	2.56×10^5
	B	4.98×10^4	3.12×10^4	BCoV	29.70	1.68×10^5	1.05×10^5
	C	9.72×10^3	4.91×10^3	BCoV	70.00	1.39×10^4	7.01×10^3
	D	-	1.28×10^5	MHV	56.70	-	2.25×10^5
	E	-	2.40×10^3	OC43	37.19	-	6.45×10^3
	F	2.83×10^5	6.76×10^4	BCoV	65.08	4.35×10^5	1.04×10^5
	G	3.37×10^4	1.37×10^4	OC43	28.25	1.19×10^5	4.86×10^4
	H	3.10×10^4	5.64×10^4	OC43	75.46	4.10×10^4	7.48×10^4
6	A	1.24×10^3	1.26×10^3	OC43	8.10	1.54×10^4	1.55×10^4
	B	4.27×10^2	1.65×10^2	BCoV	35.11	1.22×10^3	4.70×10^2
	C	4.50×10^2	1.25×10^3	BCoV	17.00	2.65×10^3	7.35×10^3
	D	-	7.81×10^3	MHV	17.59	-	4.44×10^4
	E	-	4.06×10^2	OC43	38.93	-	1.04×10^3
	F	1.44×10^4	7.43×10^3	BCoV	32.78	4.39×10^4	2.27×10^4
	G	7.46×10^2	1.30×10^3	OC43	29.96	2.49×10^3	4.34×10^3
	H	1.46×10^3	2.19×10^3	OC43	30.93	4.71×10^3	7.09×10^3
7	A	1.75×10^4	1.89×10^4	OC43	39.80	4.40×10^4	4.76×10^4
	B	3.02×10^3	1.74×10^3	BCoV	36.48	8.27×10^3	4.77×10^3
	C	7.00×10^4	1.81×10^4	BCoV	24.00	2.92×10^5	7.53×10^4
	D	-	5.31×10^4	MHV	13.58	-	3.91×10^5
	E	-	2.09×10^4	OC43	7.37	-	2.84×10^5
	F	3.26×10^5	2.83×10^5	BCoV	26.72	1.22×10^6	1.06×10^6
	H	9.11×10^4	9.66×10^4	OC43	69.62	1.31×10^5	1.39×10^5

Table S4: Average matrix spike surrogate concentrations (in gene copies per liter) by each lab.

Lab	Matrix spike surrogate	Average concentration (GC/L)
A	Trial 1: BCoV Trial 2-7: OC43	5.61×10^8 3.54×10^8
B	BCoV	1.34×10^6
C	BCoV	2.1×10^7
D	MHV	3.43×10^6
E	OC43	2.17×10^6
F	BCoV	1.79×10^{10}
G	OC43	4.36×10^7
H	OC43	2.67×10^8

Figure S1: Log_{10}-transformed SARS-CoV-2 N1 (peach) and N2 (turquoise) gene fragment quantification results across all trials. Black points indicate case numbers at that sampling location (at the time of sample collection). Case numbers within a population were normalized to 100,000.

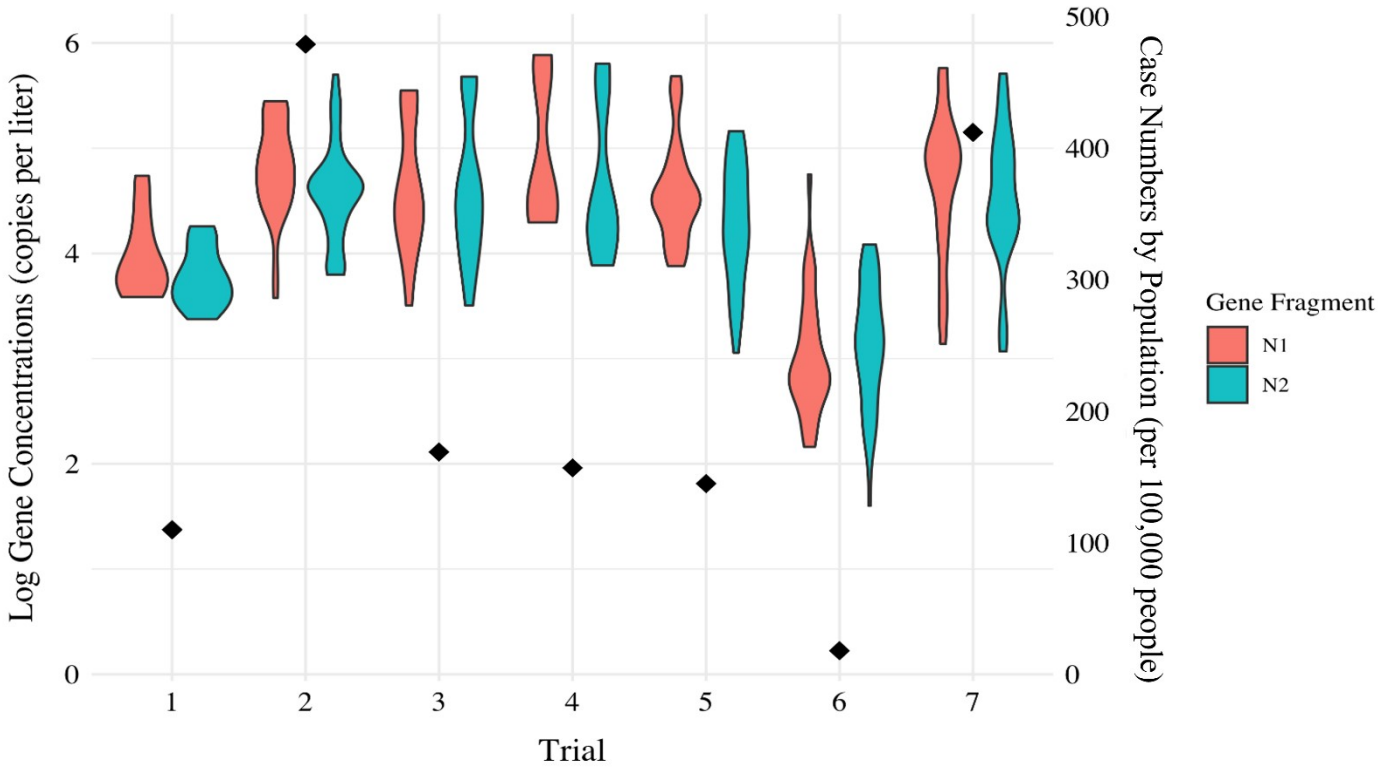
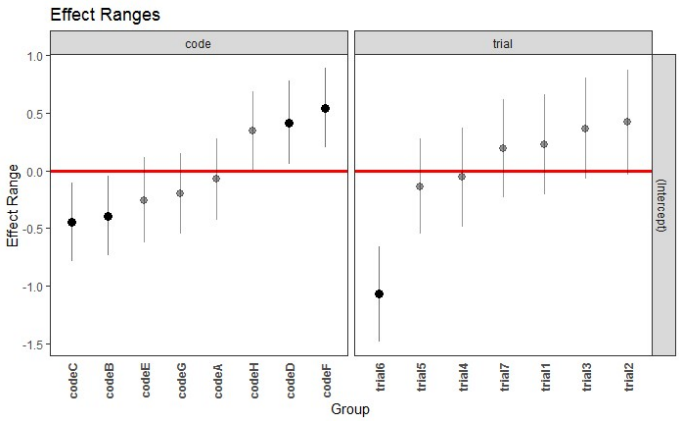
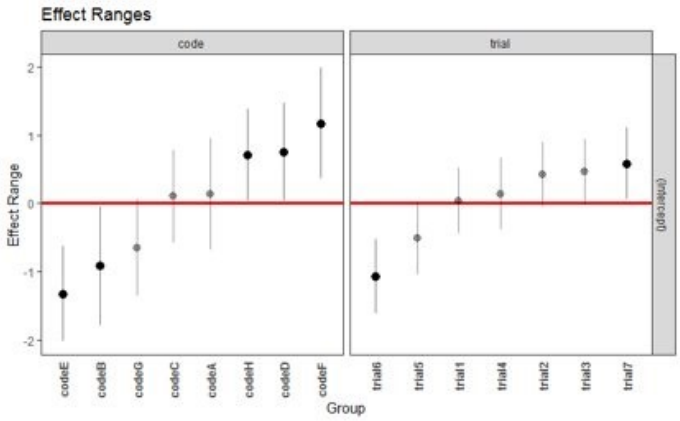


Figure S2: Summary of variance model analyses for N2 gene fragment results across all trials. The "code" graph (left for each model) shows the variance between labs, and the "trial" graph (right for each model) shows the variance between trials. Code indicates the laboratory. The red line at 0 represents the average gene fragment quantifications across all labs and all trials. The individual points represent average variance, and the lines stretching from the point represent a 95% confidence interval.

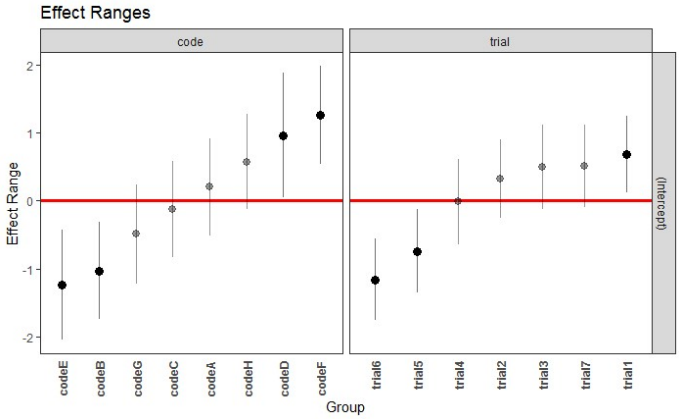
Model 2



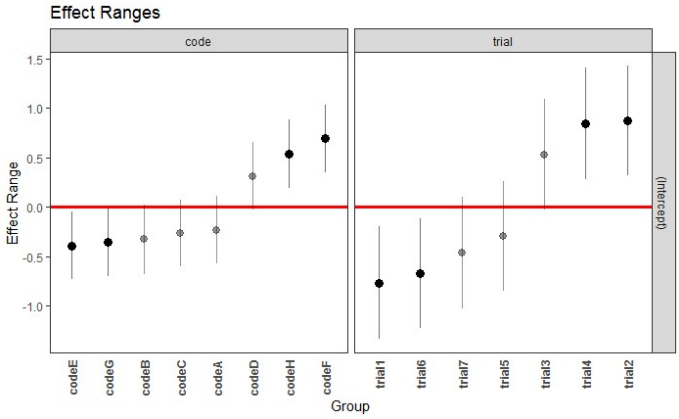
Model 3



Model 4



Model 5



Supplementary Results

SARS-CoV-2 gene concentrations and community case numbers

Cases of COVID-19 within the communities sampled ranged from 18 to 479 cases per 10,000 people, with an average case count of 217 (Table 2). Case numbers do not reflect overall case averages during the entire sampling period but only when samples were taken. The case numbers observed in these individual communities reflected similar proportions of cases as was seen across the state of Ohio during the same sampling periods (13). These communities, although varying greatly in size, followed similar COVID-19 case trends and individually reflect Ohio case trends over this same time. In other words, the sample collected for trial 2 (11/15/20) was a peak for both the individual population served as well as the state, while the sample collected for trial 6 (6/13/2021) reflected a case-low for that individual population served as well as the state (13). Importantly, gene fragment concentrations corresponded with the same relative case numbers by population within the community at individual sites (Supplementary Figure S1). A significant positive correlation was observed between SARS-CoV-2 concentrations and community cases ($p\text{-}value < 0.05$, average Spearman's $Rho=0.59$). This was seen for both raw and recovery efficiency corrected N1 and N2 gene copies. This further supports the notion that wastewater surveillance is a powerful and successful tool for COVID-19 trend tracking and understanding community infections over time (14–16).

Supplementary Methods.

The following methods descriptions detail the exact methods conducted by each participating lab.

Lab A

Methods were adapted from a previous study(16). 1 L of wastewater was collected and immediately stored at 4°C until processing. The viral filtration and concentration protocol employed solid removal and concentration steps. Briefly, 100 mL of raw wastewater was divided into two 50-mL conical tubes (cat. No. 352070, Fisher Scientific, Waltham, MA) and 500 µL 5% Tween-20 was added for increased recovery. To determine recovery efficiency, 50 µL of bovine coronavirus (BCoV) was spiked into each 50 mL sample, a concentration of approximately 2.50 × 10^9 gene copies per mL (9). The spiked surrogate changed from BCoV to *Betacoronavirus* OC43 after the first two trials at the same average concentration and volume (11). The tubes were then briefly inverted and then low-speed centrifuged at 4°C, 2,500 × g for 10 minutes for solid removal. The supernatant was then combined and hand-filtered using a 0.45 µM sterile filter unit (cat. No. SLHPR33RB, Burlington, MA) for bacteria removal. After processing, the sample was concentrated using the Innovaprep Concentrating Pipette Select and a 0.05 µM PS Hollow Fiber Tip (CC08011-200 Unirradiated). The sample was processed with the following recommended protocol settings for wastewater (with some modification): valve open for 600 ms, 1 pulse, foam factor of 10, valve start time of 3.0 seconds, flow end of 10 seconds, flow minimum start time of 40 seconds, ext. delay of 3.0 seconds, pump at 25%, and ext. pump delay time of 1 second(17). Viral concentrate is eluted into a sterile 2.0 mL microcentrifuge tube and stored at -80°C until further processing.

DNA/RNA extraction of the concentrated viral filtrate was conducted using the RNeasy PowerMicrobiome Kit (cat. No. 26000-50, QIAGEN, Germantown, MD) following manufacturer's protocol. 10 µL of total RNA was then reverse transcribed (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, cat No. 4368814). The resulting cDNA was then used for SARS-CoV-2 gene quantification.

Digital droplet PCR assays were implemented for gene quantification of SARS-CoV-2 genes. Three monoplex assays individually targeted the nucleocapsid (N) gene and the envelope (E) gene of SARS-CoV-2 (Table S1). The N gene assays employed two primer and probe sets, both from the U.S. CDC. These sets amplify the N1 and N2 region of N gene(2–4). The RT-ddPCR assay used for the E gene is based on the E_Sarbeco primers and probe set recommended by the WHO(5). Quantifications for BCoV and crAssphage were also conducted(6,9). Gene amplifications were conducted using 20 µL reactions containing RT-ddPCR supermix for probes (cat No. 1863024, Bio-Rad Laboratories, Hercules, CA), DNase- & RNase-free water, 900 nM of forward and reverse primers, 250 nM of probe, and cDNA templates.

Following droplet generation using the QX200 Droplet Generator (Bio-Rad), a C1000 Touch Thermal Cycler (Bio-Rad) was used to amplify the SARS-CoV-2 gene targets as well as BoV with the following conditions: 94°C for 10 minutes, 40 cycles of denaturation and annealing/extension at 94°C for 30 seconds and 60°C for 60 seconds, respectively, followed by 98°C for 10 minutes and then a final hold of 4°C. For crAssphage amplification, 45 cycles of denaturation were employed, with annealing/extension at 94°C for 30 seconds, 53°C for 30 seconds then 60°C for 30 seconds. Following amplification, target gene concentrations were determined using a QX200 droplet reader (Bio-Rad) and QuantaSoft (V 1.7; Bio-Rad).

Lab B

The wastewater sample (225 mL) was mixed with 25 mL 10X PBS, homogenized before being centrifuged at 3000 × g for 15 minutes at 10 ˚C to obtain precipitate large particles and suspended solids. The supernatant was filtered through 0.45 µM pore size membrane filters. The resulting pellet and the filter were transferred into two separate 2 mL tubes that contained RNase inhibitor in 600 µL RLT PLUS buffer (QIAGEN, Chatsworth, CA). The tubes were vortexed using a Mini-Beadbeater-16 (BioSpec Products Inc., Bartlesville, OK) for 30 seconds two times and then centrifuged at 10,000 × g for 3 minutes. The supernatants from pellet and filter samples were then transferred to two separate new sterile 2 mL tubes. DNA and RNA were extracted and purified from each tube using a AllPrep DNA/RNA kit (QIAGEN) following the manufacturer's protocol. For extracted RNA, the genomic DNA was removed using the TURBO DNA-free kit (Life Technologies, Foster City, CA). Extracted DNA served as template for qPCR analysis of crAssphage. Two replicates were processed and extracted from each wastewater sample.

RT-qPCR assays of N1 and N2 genes of SARS-CoV-2 viruses were performed with a Mx3005P Real-Time PCR instrument (Stratagene, CA). The reaction mixtures (20 µL) consisted of 10 µL of Promega GoTag RT-qPCR master mix (Promega, Madison, WI), 0.4 µL Go script RT mix, 1.5 µL of primers and probe mix, 3.1 µL of nuclease-free water, and 5 µL of RNA template(18). The RT-qPCR conditions were as follows: 45 °C for 15 minutes, 95 °C for 2 minutes, and 45 cycles of 95 °C for 3 seconds and 55 °C for 30 seconds(1). Serial dilutions (10, 100, 500, 1000, 5000, 10000 copies/reaction) of the standard plasmid of SARS-CoV-2 were used to produce standard curves.

qPCR assays for crAssphage were performed with the same qPCR instrument. Reaction mixtures (20 µL) consisted of 10 µL of Promga GoTag qPCR master mix (Promega, Madison,

WI), 1.5 µL of primers and probe mix, 6.5 µL of nuclease-free water, and 2 µL of DNA template(7). The qPCR conditions for crAssphage were as follows: 95 °C for 2 minutes, and 40 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds(7). Serial dilutions (10, 100, 500, 1000, 5000, 10000 copies/reaction) of the gBlocks for crAssphage, obtained from the integrated DNA technology (Coralville, IA), were used to produce standard curves. Molecular biology grade water was used as non-template controls and all the qPCR assays were performed in duplicate.

Lab C

Sample concentration and RNA extraction were performed in duplicate, using 100 mL subsamples. The collection bottle was inverted 20 times to mix, then 100 mL of sample was transferred to a 250 mL conical centrifuge tube and amended with 10 mL of 10X phosphate buffered saline (PBS). Samples were centrifuged at max speed (6220 × g) for 10 minutes using an Eppendorf Centrifuge 5810 and A-4-62 rotor. The supernatant was poured from the sample and collected, while the pellet was stored at 4°C until further processing. Particles that remained in the supernatant were collected via vacuum filtration onto a 0.45 µM nitrocellulose filter, replacing the filter when necessary to process the entire sample. Using sterile forceps, the filters were transferred into 2.0 mL screw cap microcentrifuge tubes containing 0.1 mm silica beads, while the pellet was resuspended and transferred via pipet into a separate bead tube. RNA was extracted from the pellet and filters according to the Trizol Plus RNA Purification Kit (Cat. No. 12183555, ThermoFisher Scientific, Waltham, MA), protocol. Briefly, 1 mL Trizol was added to each tube and samples were homogenized using a MiniBeadBeater-8 (BioSpec Products Inc., Bartlesville, OK) for 90 seconds. After centrifugation at 12,000 × g and 4°C, the upper aqueous phase was transferred to a new tube and an equal volume of 70% ethanol was added and mixed

by vortexing. The sample was then centrifuged through a silica membrane spin cartridge and washed with Wash Buffers I and II to remove contaminants and salts. The RNA was eluted using 100 µL of nuclease-free water and stored at -20°C until qPCR analysis.

RT-qPCR assays were performed on a Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA), using CDC diagnostic primers/probes 2019-nCoV_N1 and 2019-nCoV_N2. Reaction mixtures (20 µL) consisted of 2 µL sample RNA, 5 µL TaqPath 1-Step RT-qPCR Master Mix, CG (ThermoFisher Scientific), and 11.5 µL molecular grade H_2O. The qPCR conditions were as described in the CDC Diagnostic Panel instructions: 25°C for 2 minutes, then reverse transcription at 50°C for 15 minutes, RT-deactivation at 95°C for 2 minutes, followed by 45 cycles of 95°C for 7 seconds and annealing for 30 seconds at 55°C. Standard curves were produced using tenfold dilutions of Quantitative Synthetic SARS-CoV-2 RNA: ORF, E, N (ATCC VR-3276SD) from 10^4 to 1 copies/reaction, with additional reactions containing 5, 3, and 2 copies. Molecular grade water was used as the no-template control, and a Luciferase assay was performed as an inhibition control for each sample. All samples were processed in duplicate. Threshold and baseline values were determined using the iCycler default algorithms.

DNA for crAssphage quantification was extracted directly from 200 µL of raw wastewater sample by adding 910 µL EtNa DNA extraction solution (240 nM NaOH, 74% ethanol, 2.7 mM EDTA), heating the mixture at 80°C for 10 minutes, and purifying the raw DNA product using a silica membrane spin cartridge, followed by QIAGEN wash buffer PW and 70% ethanol to remove inhibitory contaminants and salts, as described by Vingataramin and Frost (2015)(19). DNA was eluted into 50 µL of molecular grade water and stored at -20°C until analysis. CrAssphage was quantified by qPCR using CrAssphage primer/probe CrA056

compared to a standard curve of tenfold dilutions of a gBlock standard, from 10^7 to 1 copy/reaction)(6). Reaction mixtures (20 µL) consisted of 5 µL sample DNA, 10 µL Luna Universal Probe qPCR Master Mix (New England BioLabs, Ipswich, MA), 250 nM forward and reverse primers, 125 nM probe, and 3.75 µL molecular grade H_2O. Molecular grade water was used as the no-template control, and all samples were processed in duplicate. Threshold and baseline values were determined using the iCycler default algorithms.

Lab D

The composite wastewater samples were processed in duplicate 180 mL volumes after the addition of 10X phosphate-buffered saline (PBS, Millipore-Sigma, Burlington, MA). The buffered samples were centrifuged at 4,000 × g for 20 minutes, and the supernatants were filtered through 0.45 µM mixed cellulose ester membrane filters (Fisher Scientific). The RNA extraction was carried out by the RNeasy PowerWater Kit (QIAGEN Sciences, Inc., Germantown, MD) using the manufacturer's instructions. The membrane filters were aseptically transferred to PowerWater bead tubes, provided with the kit (QIAGEN). The pellets were resuspended in kit-supplied lysis buffer with β-mercaptoethanol (Millipore-Sigma) and then transferred to the PowerWater bead tubes containing the membrane filters. All PowerWater bead tubes were vortexed for 5 minutes and further processed according to the manufacturer's instructions. The purified RNA was eluted from the spin filters with 100 µL of RNase-free water.

The extracted RNA was reverse transcribed by random hexamer primers using ImProm-II Reverse Transcriptase Kit (Promega). The resultant cDNA library was diluted 1:2 using RNase-free water and qPCR for SARS-CoV-2 N2 gene, crAssphage, and MHV N gene was performed using a Roche LightCycler 96 instrument (Roche). The total reaction mixture (10 µL/well), in

duplicate wells, consisted of 5 µL of the Radiant Green Lo-ROX qPCR mix (Alkali Scientific Inc), 0.4 µL of primers (10 µM), and 4.6 µL of the diluted cDNA. The qPCR conditions were as follows: 95 °C for 2 minutes, and 50 cycles of 95 °C for 5 seconds and 60 °C for 20 seconds and 72 °C for 10s. The serially diluted (20, 200, 2000, 20000, and 200000 copies/reaction) linearized SARS-CoV-2 N plasmid was used to generate the standard curves(20). The SARS-CoV-2 gene copy number per liter was calculated using the standard curve. To estimate the recovery efficiency, murine hepatitis virus (MHV), strain A59 (BEI Resources, NIAID, NIH: Recombinant Murine Coronavirus, icA59, NR-43000) was used as a matrix spike(10). The MHV RNA in the eluted RNA was analyzed by qRT-PCR using MHV N primers, and a gBlock containing the MHV N gene (Integrated DNA Technologies IDT, Research Triangle Park, NC) was used as a standard control. Similarly, the crAssphage gene copy numbers were analyzed by qRT-PCR, as described previously(6). For inhibition test, the extracted RNA as well as the cDNA, at various dilutions (1:5, 1:10, 1:100, and 1:1000), were tested for amplification of unrelated gene fragments (e.g., murine IRF or GFP).

Lab E

Composite samples were processed in duplicate 225 mL volumes after addition of 10X RNase-free phosphate buffered saline (ThermoFisher Scientific, Grand Island, NY) and Betacoronavirus-1, strain OC43 (American Type Culture Collection, Manassas, VA; approximately 10^7 viral particles) as a matrix spike. Mixed samples were centrifuged at 3000 × g for 15 minutes. Pellets were stored for extraction and resulting supernatant volume was split and filtered through two 0.45 µM mixed cellulose ester membrane filters (PALL Corporation, Westborough, MA). Membrane filters were aseptically rolled and transferred to a single

PowerWater DNA bead tube (QIAGEN, Germantown, MD). Centrifuged pellets and membrane filters were stored at -20°C.

Samples collected and processed for Trial 1 were extracted for RNA and DNA using the RNeasy PowerWater Kit (QIAGEN). Thawed pellets were resuspended in kit-supplied lysis buffer with β-mercaptoethanol (Millipore-Sigma, Burlington, MA) and then transferred to a PowerWater DNA bead tube. Lysis buffer with β-mercaptoethanol was added directly PowerWater DNA bead tubes with the membrane filters. All PowerWater bead tubes were vortexed for 5 minutes and further processed according to the manufacturer's instructions. Purified nucleic acids were eluted from silica spin filters with 125 µL of RNase-free water. The 0.2 mL sample volumes removed for direct extraction were processed as described without bead beating.

Due to supply chain issues emerging, the sample products generated for Trial 2 were extracted for RNA using Trizol-chloroform and isopropanol precipitation. Trizol (1.5 mL, ThermoFisher Scientific) was used to resuspend thawed pellets followed by transfer to PowerWater DNA bead tubes. The same volume of trizol was added to bead tubes containing membrane filters. PowerWater bead tubes were vortexed for 5 minutes, centrifuged at 4000 × g for 1 minute and then incubated at room temperature for 5 minutes. After transferring the supernatant to a new sterile bead tube, chloroform (0.3 mL, Millipore-Sigma) was added, samples were vortexed for 1 minute and incubated at room temperature for 5 minutes. After centrifuging samples at 12,000 × g for 15 minutes at 4°C, the aqueous phase was transferred to a new, sterile, RNase-, DNase-free tube. RNase-free glycogen (ThermoFisher Scientific; 5 µg) and 100% isopropanol (750 mL; Millipore-Sigma) was added to the supernatant and samples were mixed. After incubating for 15 minutes at room temperature, samples were centrifuged at 12,000

× g for 10 minutes at 4°C. The supernatant was removed, and the pellet was washed twice by adding 75% ethanol (1.25 mL, Millipore-Sigma), and centrifugation at 7500 × g for 5 minutes. Ethanol was removed, and RNA pellet was air dried for 10 minutes. RNA was resuspended in in 125 µL of DEPC-treated water (ThermoFisher Scientific) supplemented with 0.1 mM RNase-free EDTA (ThermoFisher Scientific) and incubated at 55°C for 10 minutes. RNA was stored at -80°C. For direct extraction of samples, 0.2 mL was transferred to 2 mL bead tubes (Millipore-Sigma). For extraction of RNA, the trizol-chloroform and RNA precipitation method described above was implemented. For extraction of DNA from samples, the DNeasy PowerWater Isolation Kit (QIAGEN) was used as instructed by the manufacturer, with the exception of bead tubes and bead beating time of 5 minutes. DNA was eluted in 125 µL of kit supplied elution buffer and stored at -80°C.

RT-ddPCR and ddPCR were carried out using the QX200 ddPCR System (Bio-Rad Laboratories, Hercules, CA). Each sample extract was run with at least 2 technical replicate reactions for each of the 2 biological sample replicates. For quantification of SARS-CoV-2 RNA, the N1 and N2 primer and probe assays were employed(1). Betacoronavirus-1 OC43 RNA and crAssphage were quantified using a previously described assays(6,12). Each RT-ddPCR or ddPCR reaction was prepared in 22 µL and run as monoplex assays using 900 nM respective primer and 250 nM respective probe. For RT-ddPCR, the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad) was used as instructed by the manufacturer and droplets were generated manually. The thermal profile for RT-ddPCR was 50°C for 60 minutes, 95°C for 10 minutes followed by 50 cycles of 95°C for 30 seconds and 55°C for 1 minute. A final incubation of 98°C for 10 minutes was implemented and reactions were held at 4°C. For ddPCR, ddPCR Supermix for Probes (no dUTP; Bio-Rad) was used as instructed by the manufacturer and droplets were

produced with the Automated Droplet Generator. The thermal profile was 95°C for 5 minutes followed by 50 cycles of 95°C for 30 seconds and 53°C for 1 minute. A final 10-minute incubation occurred at 98°C and then reactions were held at 4°C.

For analysis of ddPCR data, droplet clustering was determined using the AutoAnalyze (combined well) function in the QX200 QuantaSoft software (Bio-Rad). In cases where this function failed, the mean and standard deviation of the amplitude values of the no template control reactions was used to manually apply a threshold. For each sample fraction, the RNA/DNA quantities were calculated using Poisson statistics and sample concentrations (RNA or DNA molecules/L) were calculated using the combined quantities of the sample fractions, the volume of sample processed, the processing concentration factor and the volume of nucleic acid extracts analyzed by RT-ddPCR/ddPCR. RT-ddPCR inhibition was assessed by adding 0.05 pg of Luciferase Control RNA (Promega Corporation, Madison, WI) and comparing concentrations measured in reactions with sample extracts and matrix-free buffer using the primer and probe assay described previously and the RT-ddPCR reaction conditions described above(21).

Lab F

After receipt of the sample, the container was wiped down with a dilute chlorine bleach disinfectant solution (<10%). This lab conducted slightly different methods for their participation in trial 2 vs. their participation in trials 4-7. For both methods, the samples were processed in duplicate, and a negative processing control included extraction of a clean filter. During trial 2, each processing duplicate of 150 mL wastewater sample was spiked with 750 µL Bovine Coronavirus (BCoV) Calf Vaccine (Merck Animal Health, Product No. 1644) RNA virus to calculate process recovery efficiency. Spiked samples were shaken for 10 minutes at 120 rpm at

room temperature. Each 150 mL processing duplicate was vacuum filtered in sterile disposable volumetric filter funnels (VWR International, Product No. 28143-568) containing electronegative 0.45 µM pore size mixed cellulose ester filters (Pall, GN-6 Metricel) to concentrate viral particles, microorganisms, and non-filterable solids on the membranes. After filtration to dryness within 2-3 h, filters were placed in PowerBead Tubes (0.7 mm garnet, QIAGEN) with 1 mL TRIzol™ Reagent (Invitrogen) subjected to 5 minutes bead beating (BioSpec Mini-Beadbeater-16, Bartlesville, OK) before nucleic acid extraction. Extracted nucleic acids were stored at ≤ -70°C until further analysis.

All RNA and DNA separation, precipitation, and washing steps described below were performed according to TRIzol™ User Guide instructions unless stated otherwise. Sample tubes were hand mixed to distribute the chloroform, incubated at room temperature for 5 minutes, and centrifuged at 12,000 × g and 4°C for 30 minutes (Sorvall Legend Micro 21R, ThermoFisher Scientific, Waltham, MA) to separate the solution the upper aqueous phase containing RNA, the middle interphase containing DNA, and the lower pink phase containing beads, filter solids, lipids, proteins, and other organic matter. The aqueous phase was transferred to a new microcentrifuge tube. Normally, this process is only performed once per extraction according to TRIzol™ User Guide instructions. However, we believe that some DNA was transferred along with the aqueous phase, so an additional 1 mL of TRIzol™ was added to the initial aqueous phase of the first extraction process (without bead beating), followed by a 2-minute incubation at room temperature and then centrifugation (12,000 × g, 4°C, 30 minutes) to achieve additional separation of aqueous phase and interphase. From this secondary separation of aqueous phase and interphase, RNA and DNA were extracted, respectively, with elution volumes of 50 µL each in nuclease free water. The interphase from the initial extraction containing solids was stored at ≤

-70°C and then later processed again by bead beating for 2 minutes with 1 mL TRIzol™. The aqueous phase was transferred to a new microcentrifuge tube to perform an additional phase separation step followed by RNA and DNA precipitation. All eluted RNA and DNA from two separate extractions were each combined into one tube for final elution volumes of 100 µL.

Nucleic acids for various targets in samples and controls were quantified in triplicate with QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) or CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). RNA targets included SARS-CoV-2 N1 and N2, BCoV to assess processing recovery, and pepper mild mottle virus (PMMoV) to assess fecal strength of wastewater, and the DNA target was cross-assembly phage (CrAssPhage) to assess the fecal strength of wastewater(6,8–10). Positive controls were the double stranded DNA plasmid for 2019-nCoV_N_Positive Control (Integrated DNA Technologies IDT, Research Triangle Park, NC) (six ten-fold dilutions from 2×10^3 copies/µL) or gBlock synthetic nucleic acids for BCoV (six ten-fold dilutions from 1.37×10^8 copies/µL), PMMoV (six ten-fold dilutions from 1.37×10^8 copies/µL), and CrAssPhage (six ten-fold dilutions from 3.24×10^8 copies/µL). Extraction blanks and triplicate no template controls were included for each instrument run.

Two different reaction mixtures were used for qPCR assays due to separate occasions where validation samples had to be repeated. For water validation assays (N1, N2, crAssphage, BCoV, and PMMoV) and a secondary standard curve assay for crAssphage, the reaction mixture contained 10 µL PrimeTime® Gene Expression 2X Master Mix (Integrated DNA Technologies IDT), 500 nM each of forward and reverse primer, 125 nM probe, and 2 µL extracted nucleic acid from wastewater sample. 20 µL reference dye was added to 5 mL of PrimeTime® Gene Expression Master Mix. Nuclease free distilled water (Invitrogen) was added to bring the total

reaction volume to 20 µL for all assays using 96-well plates. The other reaction mixture, used only to run standard curve assays for wastewater cDNA targets (N1, N2, BCoV, and PMMoV), contained 10 µL qScript XLT 1-Step RT-qPCR ToughMix (QuantaBio, Beverly, MA), 500 nM each of forward and reverse primer, 125 nM probe, and 2 µL template RNA. 96-well plates were centrifuged for 3 minutes at 3200 rpm (Centrifuge 5810 R, Eppendorf) before PCR. Thermal cycling for N1 and N2 consisted of RT at 50°C for 10 minutes, denaturation at 95°C for 3 minutes, and 40 amplification cycles of 95°C for 3 seconds followed immediately by 55°C for 30 seconds. The annealing/extension temperature was increased to 60°C for BCoV and PMMoV assays and the duration was extended to 1 minute. The thermal cycling for crAssphage consisted of RT at 95°C for 3 minutes, and 40 amplification cycles of 95°C for 5 seconds followed immediately by 60°C for 30 seconds. To minimize inhibition, the ten-fold dilutions of each RNA sample for each assay were selected for further analysis.

During trials 4-7, the following methods were used for analysis. Each 1L sample was spiked with 500 µL Bovine Coronavirus (BCoV) Calf Vaccine (Merck Animal Health, Product No. 1644) RNA virus to calculate process recovery efficiency. The sample was processed in duplicate, and a negative processing control included extraction of a clean filter. Spiked samples were shaken for 10 minutes at 180 rpm at room temperature. After shaking, each processing duplicate was vacuum filtered in sterile disposable volumetric filter funnels (VWR International, Product No. 28143-568) containing electronegative 0.45 µM pore size mixed cellulose ester filters (Pall, GN-6 Metricel) to concentrate viral particles, microorganisms, and non-filterable solids on the membranes. After filtration to dryness within 1-2 h, filters were placed in PowerBead Tubes (0.7 mm garnet, QIAGEN) with 1 mL TRIzol™ Reagent (Invitrogen) and 200

μL of chloroform, and subjected to 5 minutes bead beating (BioSpec Mini-Beadbeater-16, Bartlesville, OK) before nucleic acid extraction.

All RNA and DNA separation, precipitation, and washing steps described below were performed according to TRIzol™ User Guide instructions unless stated otherwise. After bead beating samples were centrifuged at 12,000 × g and 4°C for 20 minutes (Sorvall Legend Micro 21R, ThermoFisher Scientific, Waltham, MA) to separate the solution: the upper aqueous phase containing RNA, the middle interphase containing DNA, and the lower pink phase containing beads, filter solids, lipids, proteins, and other organic matter. The aqueous phase was transferred to a new microcentrifuge tube. 10 μg of RNase-free glycogen (SIGMA, Product No. G8751-5G) was added to the aqueous phase. From this, RNA was extracted with elution volumes of 50 μL each in nuclease free water according to the TRIzol™ User Guide.

Nucleic acids for various targets in samples and controls were quantified in triplicate with QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) or CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). As mentioned previously, RNA targets included SARS-CoV-2 N1 and N2, BCoV to assess processing recovery, and pepper mild mottle virus (PMMoV) to assess fecal strength of wastewater. Positive controls were the double stranded DNA plasmid for 2019-nCoV_N_Positive Control (Integrated DNA Technologies IDT, Research Triangle Park, NC) (six ten-fold dilutions from 2×10^5 copies/μL) or gBlock synthetic nucleic acids for BCoV (six ten-fold dilutions from 1.37×10^8 copies/μL), PMMoV (six ten-fold dilutions from 1.37×10^8 copies/μL). Extraction blanks and triplicate no template controls were included for each instrument run.

The reaction mixture contained 10 μL of qScript XLT 1-Step RT-qPCR ToughMix (Quantabio, Beverly, MA), 500 nM each of forward and reverse primer, 125 nM probe, and 2 μL

extracted nucleic acid from wastewater sample. Nuclease free distilled water (Invitrogen) was added to bring the total reaction volume to 20 µL for all assays using 96-well plates. 96-well plates were centrifuged for 3 minutes at 3200 rpm (Centrifuge 5810 R, Eppendorf) before PCR. Thermal cycling for N1 and N2 consisted of RT at 50°C for 10 minutes, denaturation at 95°C for 3 minutes, and 40 amplification cycles of 95°C for 3 seconds followed immediately by 55°C for 30 seconds. The annealing/extension temperature was increased to 60°C for BCoV and PMMoV assays and the duration was extended to 1 minute. To minimize inhibition, the ten-fold dilutions of each RNA sample for each assay were selected for further analysis.

Lab G

Upon arrival, 50-100 mL wastewater sample was processed. 2N HCl was added to the sample to adjust the pH to 3.5. High concentration (about 5×10^7 copies) of heat inactivated human coronavirus HCoV_OC43 (ZeptoMetrix) was spiked into the sample as virus recovery control. After homogenization by vigorous shaking, the sample was filtered through an HA/MCE membrane (0.45 µM pore size, 47mm diameter, PALL). The filter was stored in a 2 mL bead tube with 700 µL of PM1 lysis buffer with beta-mercaptoethanol (both bead tubes and PM1 buffer are from QIAGEN AllPrep PowerViral DNA/RNA Kit) at -20°C freezer until DNA/RNA extraction (no longer than a week at -20°C). Two additional samples were created for each filtration batch: an NPEC filter was created by filtering 50 mL PCR-grade water, and an OC43 positive control was also created by adding the same amount of heat-inactivated OC43 into 400 µL of PM1 lysis buffer with beta-mercaptoethanol for calculating workflow recovery.

To extract DNA/RNA from filter samples, we followed the protocol from AllPrep PowerViral DNA/RNA Kit (QIAGEN, Germantown, MD). Briefly, frozen filter samples were

warmed up in 55°C water bath for 10 minutes to resolve any precipitation in PM1 buffer. Then the tubes with glass bead and filter membrane was lysed on a bead beater (MP FastPrep-24, MP Bio). After collecting all the lysate supernatant from the tube, 5 ng of Mouse Lung Total RNA (Takara Bio Inc., Mountain View, CA) was added into the sample lysate to evaluate extraction efficiency and PCR inhibition. 150 µL of IRS buffer was added for inhibitor removal with incubation the sample at 4°C for 10 minutes. 800 µL or all the supernatant (if the volume is less than 800 µL) was collected after IRS precipitation. Then sample was combined with 600 µL PM3 and PM4 buffer and loaded onto MB Spin columns. The column was washed once with 600 µL PM5 and once with 600 µL PM4. Finally, after drying up the column, DNA/RNA was eluted in 35 µL of nuclease-free water twice for a final volume of 70 µL.

RT-qPCR analysis were carried out on the StepOne Plus system (Applied Biosystems, ThermoFisher Scientific, Waltham, MA), and each sample was analyzed as duplicate reactions. For quantification of SARS-CoV-2 RNA, the N1 and N2 primer and probe assays were employed(1). Primers and probe (RUO kit, Integrated DNA Technologies) and Taqman Fast Virus 1-Step Master Mix (ThermoFisher Scientific) were used. 5-point serial dilution from 20,000 to 2 copies/µL was made with synthetic RNA (ATCC, VR-3276SD) to create the standard curve for N1 and N2. 5 µL of DNA/RNA template was added into a final 20 µL RT-qPCR reaction. The thermo cycling condition for N1 was 50°C for 5 minutes, 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 55°C for 30 seconds. The annealing temperature for N2 as 60°C for 30 seconds. For relative quantification of Mouse ACTB, Mouse ACTB endogenous control primers and probe set (FAM reporter, primers unlimited, ThermoFisher Scientific) was used. 2.5 ng Mouse Lung RNA was run as duplicates with each extraction batch as a reference. For quantification of OC43, published OC43 primers and probe

were used(11). 5-point serial dilution from 20,000 to 2 copies/µL was made with OC43 genomic RNA (ATCC, VR-1558DQ). Both Mouse ACTB and OC43 has the same RT-qPCR reaction setup as described above, and annealing temperature of 60°C. For quantification of human fecal indicator crAssphage, published crAssphage CPQ_056 primers and probe were used with Taqman Environmental Master Mix 2.0 (ThermoFisher Scientific)(6). 6-point serial dilution from 500,000 to 5 copies/µL was made with crAssphage DNA standard (gBlocks, Integrated DNA Technologies). 2 µL DNA/RNA extract was added to a final 20 µL qPCR reaction. The thermo cycling condition was the same as published. After RT-qPCR and qPCR runs, data was processed on StepOne Software (v2.3).

Lab H

Upon receipt of sample, the two collection bottles were mixed well by inversion, and then 41 mL from each bottle was transferred into 50 mL conical tubes. To determine recovery efficiency, 100 µL of Betacoronavirus-1, strain OC43 (American Type Culture Collection, Manassas, VA; VR-1558) was spiked into each duplicate sample. Total nucleic acid extraction was performed using the Wizard Enviro Total Nucleic Acid Kit (Promega, Madison, WI). Samples were lysed using 512 µL of protease followed by a 30-minute incubation at room temperature. After incubation, 1 mL of each duplicate sample was set aside for direct extraction, which was used as a baseline for determining recovery efficiency. The remaining 40 mL of each duplicate sample was centrifuged at 3,000 × g for 10 minutes for solid removal, and the supernatant was decanted into clean tubes. The decanted sample was then extracted following the manufacturer's protocol, including steps to extract the pelleted solids, and total nucleic acid extracts were stored at -80°C until qPCR analysis.

The 1 mL direct extraction duplicate samples were extracted by skipping the initial concentration and cleanup steps of the Wizard Enviro Total Nucleic Acid Kit, performing only the final concentration and cleanup steps: 400 µL of Binding Buffer 1 and 100 µL of Binding Buffer 2 were added to each sample. The samples were then split into two 1.5 mL tubes and 750 µL of isopropanol was added to each tube. The contents of both tubes were then passed through a PureYield Minicolumn by centrifugation and washed with 300 µL of Column Wash 1 and 1 mL of Column Wash 2. Total nucleic acids were eluted using 200 µL of warmed nuclease-free water and stored at -80°C until qPCR analysis.

RT-qPCR assays were performed on a Quantabio Q qPCR machine (Quantabio, Beverly, MA). SARS-CoV-2 N1 and N2 genes and the human marker PMMoV were quantified using the SARS-CoV-2 RT-qPCR Kit for Wastewater (Promega). The reaction mixtures (20 µL), in triplicate wells, consisted of 10 µL of GoTaq WW MasterMix, 0.4 µL of GoScript RT, 1 µL of Primer/Probe/IAC Mix, 3.6 µL of nuclease-free water, and 5 µL of nucleic acid template. The RT-qPCR conditions were as follows: 45°C for 15 minutes, 95°C for 2 minutes, and 40 cycles of 95°C for 3 seconds and 62°C for 30 seconds. The serially diluted (20, 200, 2000, 20000, and 200000 equivalent RNA copies/reaction), linearized dsDNA fragments of SARS-CoV-2 encoding the E and N genes provided with the SARS-CoV-2 RT-qPCR Kit for Wastewater were used to generate the standard curves for N1 and N2 gene quantification. Promega additionally supplied quantified PMMoV RNA, which was serially diluted (20, 200, 2000, 20000, and 200000 copies/reaction) to create standard curves for PMMoV quantification. Nuclease-free water in triplicate wells was used as a no-template control in all RT-qPCR and qPCR assays.

Betacoronavirus-1 OC43 RNA was quantified using the same Quantabio Q qPCR instrument, the qScript 1-Step Virus ToughMix (Quantabio), and published OC43 primers and

probe(11,17). The reaction mixtures (20 µL), in triplicate wells, consisted of 10 µL of qScript Virus 1-Step ToughMix, 1.25 µL of 10µM forward primer, 1.875 µL of 10µM reverse primer, 1.25 µL of 100 µM probe, 0.625 µL of nuclease-free water, and 5 µL of nucleic acid template. The RT-qPCR conditions were as follows: 48°C for 10 minutes, 95°C for 5 minutes, and 45 cycles of 95°C for 15 seconds and 55°C for 1 minute. The serially diluted (21, 210, 2100, 21000, and 210000 copies/reaction) OC43 genomic RNA (ATCC, VR-1558DQ) was used to generate the standard curves.

CrAssphage was quantified as an additional human marker for trials 4 and 6 using the same Quantabio Q qPCR instrument, PerfeCTa SYBR Green SuperMix (Quantabio), and published CrA056 primers(7). The reaction mixtures (25 µL), in triplicate wells, consisted of 12.5 µL of PerfeCTa SYBR Green SuperMix, 2.5 µL of 10µM forward primer, 2.5 µL of 10µM reverse primer, 2.5 µL of nuclease-free water, and 5 µL of nucleic acid template. The qPCR conditions were as follows: 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The serially diluted (200, 2000, 20000, 200000, and 2000000 copies/reaction) gBlocks for crAssphage (Integrated DNA Technologies, Coralville, IA), were used to produce standard curves. All RT-qPCR and qPCR data were processed on Quantabio Q qPCR Instrument Software (v1.0.1).

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