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Wastewater-Based Protocols for SARS-CoV-2: Insights into Virus Concentration, Extraction, and Quantitation Methods from Two Years of Public Health Surveillance

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Supplemental Information

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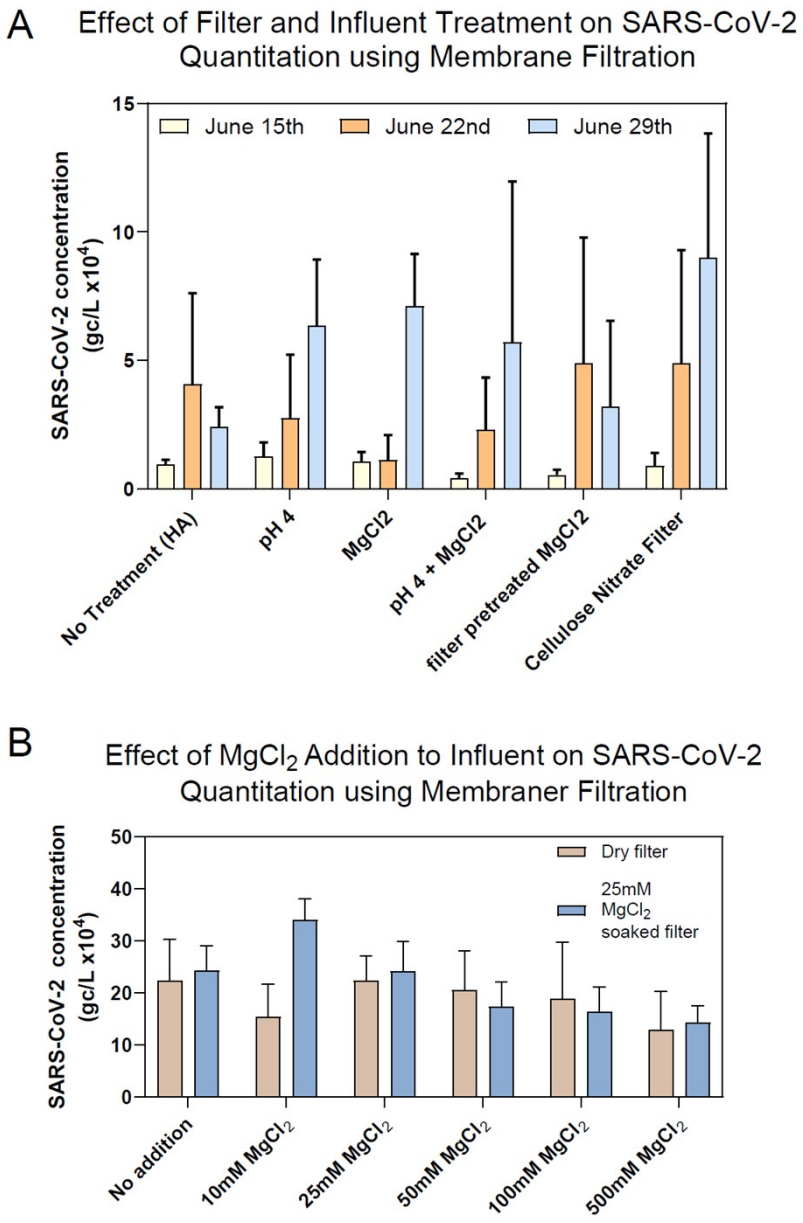
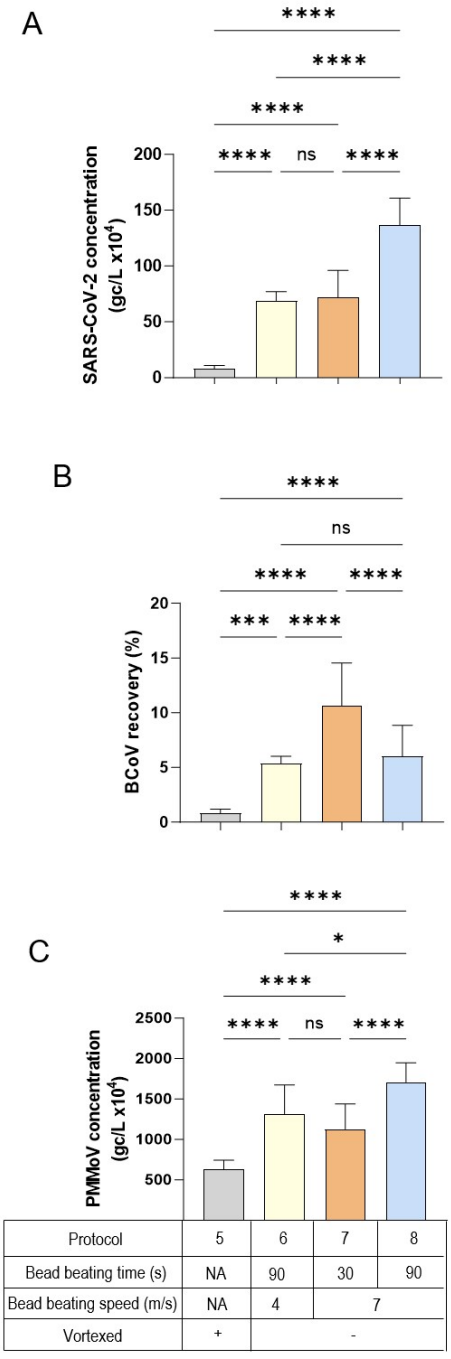


Figure S1. Effect of Filter and Influent Treatment on SARS-CoV-2 Quantitation using HA Membrane Filtration. SARS-CoV-2 average N gene concentration (N1 and N2, gc/L) measured from wastewater samples concentrated by different HA filter or matrix modifications and measured by qPCR. **(A)** Different sample amendments (HCl or $MgCl_2$) compared to HA filter pretreatment (soaking in 25 mM $MgCl_2$) and cellulose nitrate filtration, all using the same POTW influent sample (from June 2020). No significant difference was found between treatments using a two-way ANOVA with multiple comparisons ($F(5, 36) = 0.8041$, $p = 0.55$). **(B)**. $MgCl_2$ titration of the influent sample and its effect on SARS-CoV-2 recovery during HA filtration. Filters were either used untreated (dry) or soaked in 25 mM $MgCl_2$ prior to sample application. No significant difference was found between the two treatments (paired t-test, $p = 0.39$). Error bars are ± 1 SD.



Protocol	5	6	7	8
Bead beating time (s)	NA	90	30	90
Bead beating speed (m/s)	NA	4	7	
Vortexed	+	-		

Figure S2. Influence of Bead Beating Parameters on Viral Target Quantification. The effect of bead beating duration and speed performed prior to centrifugation at 20,627 xg and supernatant extraction. Viral targets SARS-CoV-2, gc/L (A), BCoV % recovery (B), and PMMoV, gc/L (C) were quantified by RT-qPCR. Each protocol tested had six replicate filters except protocol 6, with three replicate filters. Bars show means, while error bars depict the standard deviations. Symbols above groups indicate the significance of the Tukey post-hoc tests (One-way ANOVA): P-values = **** <0.0001, *** < 0.001, ** < 0.01, * <0.05, ns: not significant.

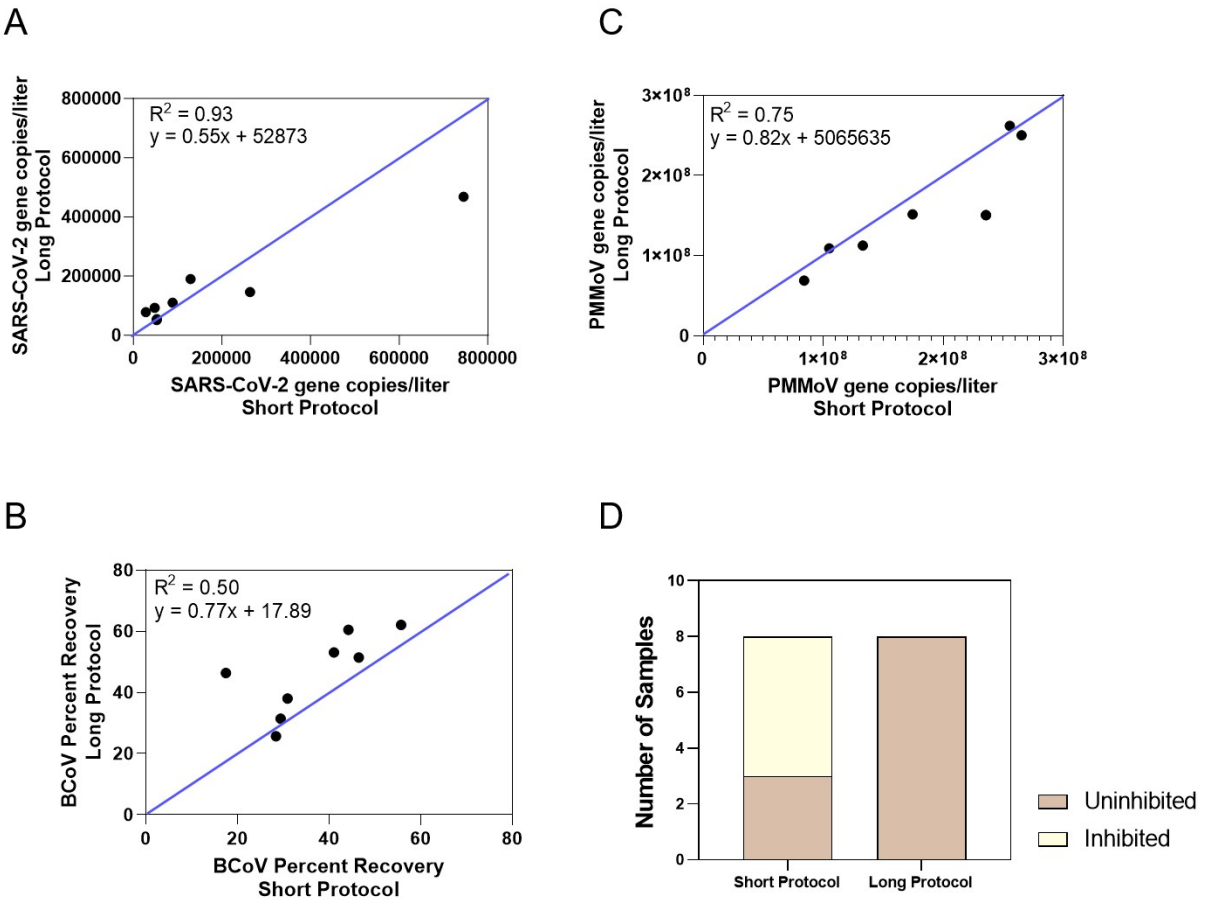


Figure S3. Comparison of Viral Quantification and PCR Inhibition with Two Automated Protocols for Nanotrap® Concentration. (A) SARS-CoV-2 (gc/L) (B) PMMoV (% recovery) and (C) BCoV (gc/L) from eight wastewater influent samples, concentrated by both a short and long Nanotrap® capture method and quantified by RT-dPCR. Blue lines depict the 1:1 ratio. (D) The number of samples inhibited and uninhibited are shown in a bar plot for both Nanotrap® protocols.

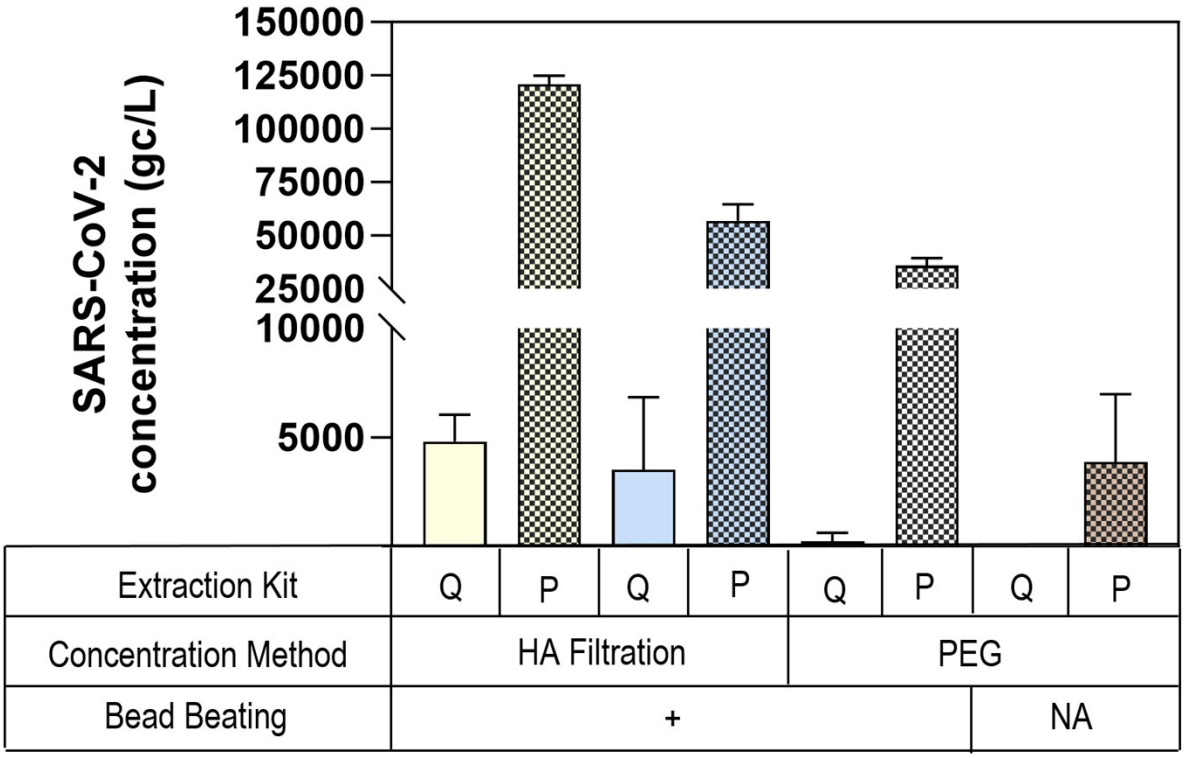


Figure S4. Comparison of Column Based Extraction to Magnetic Bead Based Extraction. SARS-CoV-2 concentration (gc/L) quantified from one influent sample concentrated by HA filtration and PEG method. The resulting filter or pellet underwent additional processes before extraction via either the Power Microbiome column kit, Qiagen (Q) or Promega's Environmental TNA kit, automated on a Kingfisher platform (P). Standard deviation was calculated from two sample-processing replicates and three PCR replicates. A one-way ANOVA with multiple comparisons (Tukey post-hoc analysis) was used to compare the difference of quantification between methods. All extraction kit comparisons were significant (p-value < 0.001) with the exception of the non-bead beated PEG samples, p-value = 0.89.

Table S1. IDT Primers and Probes for qPCR

Oligo Name	Sequence	Concentration in final PCR reaction	Reference
N1 Forward	5’-GAC CCC AAA ATC AGC GAA AT-3'	1µM	CDC assays
N1 Reverse	5’-CAG ATT CAA CTG GCA GTA ACC AGA-3'	1µM	CDC assays
N1 Probe	5’- /FAM/-ACC CCG CAT TAC GTT TGG TGG ACC-3'	250nM	CDC assays
N2 Forward	5’-TTACAAACATTGGCCGCAAA-3'	1µM	CDC assays
N2 Reverse	5’-TTCTTCGGAATGTCGCGC-3'	1µM	CDC assays
N2 Probe	5’-/SUN/-ACAATTTGCCCCCAGCGCTTCAG-3'	250nM	CDC assays
BCoV Forward	5’-CTG GAA GTT GGT GGA GTT-3'	600nM	Decaro et al., 2008
BCoV Reverse	5’-ATT ATC GGC CTA ACA TAC ATC-3'	600nm	Decaro et al., 2008
BCoV Probe	5’-5HEX-CCT TCA TAT /ZEN/CTA TAC ACA TCA AGT TGT T-3IABkFQ-3'	200nM	Decaro et al., 2008
BRSV Forward	5'-GCA ATG CTG CAG GAC TAG GTA TAA T-3'	100nM	Kishimoto et al., 2017
BRSV Reverse	5'-ACA CTG TAA TTG ATG ACC CCA TTC T-3'	100nM	Kishimoto et al., 2017
BRSV Probe (qPCR)	5'-5Cy5-ACC AAG ACT /TAO/ TGT ATG ATG CTG CCA AAG CA-3IAbRQSp-3'	200nM	Kishimoto et al., 2017
BRSV Probe (dPCR)	5’- /5HEX/ACC AAG ACT/ZEN/TGT ATG ATG CTG CCA AAG CA/3IABkFQ/ -3’	200nM	Kishimoto et al., 2017
PMMoV Forward	5’-GAG TGG TTT GAC CTT AAC GTT TGA-3'	400nM	Haramoto et al., 2013
PMMoV Reverse	5’-TTG TCG GTT GCA ATG CAA GT-3'	400nM	Haramoto et al., 2013
PMMoV Probe	5’- /5Cy3/ CCTA+C+CGA+A+GCA+A+ATG /3IAbRQSp/-3’	200nM	Haramoto et al., 2013

Table S2. Positive Control Ultramers

Oligo Name	Sequence
BCoV DNA Ultramer	5'-GTT TAT TAG AAC TGG AAG TTG GTG GAG TTT CAA CCC AGA AAC AAA CAA CTT GAT GTG TAT AGA TAT GAA GGG AAG GAT GTA TGT TAG GCC GAT AAT TGA GGA CTA CCA TA-3'
PMMoV DNA Ultramer	5'-CAT TGG TGG CAG CAA AGG TAA TGG TAG CTG TGG TTT CAA ATG AGA GTG GTT TGA CCT TAA CGT TTG AGA GGC CTA CCG AAG CAA ATG TCG CAC TTG CAT TGC AAC CGA CAA TTA CAT CAA AGG AGG AAG GTT CGT TGA AGA TTG TGT CGT CAG A-3'

Table S3. LOD and LOQ for N1 and N2.

RT-qPCR

Target	*LOD (gc/uL of Rxn)*	*LOQ (gc/uL of Rxn)*
N1	5	15.6
N2	8.2	17.2

RT-dPCR

Target	*LOD (gc/uL of Rxn)*	*LOQ (gc/uL of Rxn)*
N1	0.16	0.38
N2	0.16	0.33

Table S4. qPCR Standard Curve Metrics

	Min				Max			
Target	Y-Intercept	R2	Slope	Efficiency	Y-Intercept	R2	Slope	Efficiency
BCoV	34.10	0.85	-3.62	88.91	43.44	1.00	-2.99	115.98
N1	35.75	0.97	-3.91	80.30	40.96	1.00	-3.12	109.09
N2	35.56	0.99	-3.79	83.55	40.37	1.00	-3.12	109.37
PMMoV	35.82	0.82	-3.55	91.19	40.68	1.00	-3.01	114.84

	Standard Error				Confidence Interval (α = 0.05)			
Target	Y-Intercept	R2	Slope	Efficiency	Y-Intercept	R2	Slope	Efficiency
BCoV	0.12	0.0017	0.01	0.53	0.24	0.003	0.03	1.04
N1	0.09	0.0003	0.02	0.64	0.17	0.001	0.03	1.26
N2	0.09	0.0003	0.02	0.62	0.18	0.001	0.03	1.22
PMMoV	0.21	0.0072	0.03	1.39	0.41	0.014	0.06	2.72

Table S5. dPCR Thermocycling Conditions GT Molecular Kit:

Thermocycling Conditions GT Molecular Kit:			
Step		**Time**	**Temp ºC**
Reverse Transcription		30 min	50
DNA polymerase activation		2 min	95
45 cycles	Denaturation	10 sec	95
	Anneal/Extend	30 sec	55

Target	Channel	Exposure	Gain
N1	Red (ROX)	500	4
N2	Green (FAM)	300	6
BCoV	Yellow (HEX)	300	6
PMMoV	Green (FAM)	300	6
BRSV	Yellow (HEX)	500	6

Table S6. Summary of Variables Evaluated for HA filtration

Conditions tested				Figure	Ranking based on SARS-CoV-2 recovery
Influence of filtration parameters on viral quantification by the HA method					
Pore size: 0.45 vs 0.8 µm HA filter				Data not shown	No significant difference in terms of SARS-CoV-2 recovery, but better throughput with 0.8 µm HA filter
Enhance viral adsorption to HA filters: sample acidification, $MgCl_2$ spike into sample, and soaking of the filter in $MgCl_2$				Fig. S1	No significant difference. Soaking of the filter in $MgCl_2$ offered better throughput than sample amendments
Filtration flow rate: 5.6 vs 22.2 vs 49.9 mL/minute				Fig. 3	SARS-CoV-2 recovery significantly higher using slow flow rate (5.6 mL/min)
Sample volume filtered: 10 vs 25 vs 40 mL				Fig. 3	SARS-CoV-2 recovery significantly higher with large volume vs small volume. However, 25 mL (in-between recovery) offered better throughput
Influence of bead beating parameters on viral quantification					
Protocol	Homogenization speed	Homogenization duration	Centrifugation speed to pellet particles		
1	Vortex: not applicable	15 sec	9 168 $_x$g	Fig. 4	Centrifugation speed had minimal impact on SARS-CoV-2 recoveries compared with bead beating speed and duration. The highest virus concentrations were obtained with the protocols that imparted the greatest energy to the sample (i.e., 4 and 8), while simply vortexing (i.e., 1 and 5) returned the lowest viral concentrations
2	4 m/s*	90 sec	9 168 $_x$g	Fig. 4	
3	7 m/s*	30 sec	9 168 $_x$g	Fig. 4	
4	7 m/s*	90 sec	9 168 $_x$g	Fig. 4	
5	Vortex: not applicable	15 sec	20 627 $_x$g	Fig. S2	
6	4 m/s*	90 sec	20 627 $_x$g	Fig. S2	
7	7 m/s*	30 sec	20 627 $_x$g	Fig. S2	
8	7 m/s*	90 sec	20 627 $_x$g	Fig. S2	

*Homogenized/beat bashed using MPBio FastPrep-24 5G

Table S7. Characteristics of the influent samples used in the different method comparisons

Figure	WWTP ID	Sampling Date (mm/dd/yyyy)	Population Served	pH	Conductivity (µS/cm)	Wastewater Temperature (℃)	% Industrial Input
Fig. 1 A-C	W11	7/13/2021	75,000	7.3	994	18.5	10
	W12	7/13/2021	380,000	7.59	1604	np	np
	W35	7/13/2021	4,000	7.96	955.3	np	1
	W5	7/13/2021	16,000	7.5	1263	np	30
Fig. 1 D-F	W25	10/21/2021	10,800	7.51	np	np	55
	W31	10/23/2021	64,000	7.57	np	np	35
	W57	10/25/2021	4.000	7.5	np	16.8	60*
	W55	10/21/2021	900	7.95	np	10.9	0
	W11	10/22/2021	75,000	7.3	np	20	10
	W2	10/23/2021	75,000	8.2	np	18.33	5
	W28	10/23/2021	42,000	np	np	np	np
	W6	10/20/2021	11,000	np	np	np	10*
Fig. 2 A-C	W12	8/25/2022	380,000	7.7	np	np	np
	W15	8/23/2022	4,000	7.9	np	np	10
	W44	8/23/2022	14,000	0.125	np	np	5
Fig 3 ABC	W12	1/5/2022	380,000	7.61	np	np	np
Fig 3 DEF	W12	5/30/2022	380,000	7.7	np	np	np
Fig. 4/Fig. 6	W12	2/1/2022	38,000	7.73	np	np	np
Fig. 5	W12	9/21/2022	380,000	7.66	np	np	np
	W12	9/22/2022	380,000	7.74	np	np	np
	W66	9/22/2022	83,000	np	np	np	np
	W64	9/22/2022	45,000	np	np	np	np
	W62	9/22/2022	70,000	np	np	np	np
	W63	9/22/2022	107,000	np	np	np	np
	W65	9/22/2022	90,000	np	np	np	np
	W1	9/21/2022	14,000	7.77	np	19.4	10
	W4	9/21/2022	11,000	8.1	np	16.67	14*
	W60	9/21/2022	24,000	np	np	np	15

np: not provided; % combined sewer was zero for all except POTWs marked by *

Supplemental Material and Methods

Bovine Coronavirus (BCoV) preparation and titration

Wastewater was spiked with suspended Bovine Coronavirus (BCoV) obtained from the Calf-Gauard® cattle vaccine (Zoetis, SN439242A). The BCoV spiked into each sample serves as a viral recovery control check for our concentration/isolation processes. To titer (quantify) the BCoV used in calculating viral percent recovery, 12 vials of Calf-Guard® cattle vaccine were suspended in 1 mL 1X-TE Buffer (10 mM tris-HCl and 0.1 mM EDTA) per vial and combined into a 15 mL LoBind conical tube (Eppendorf, 0030122208). The re-suspended Calf-Guard® vaccine was directly extracted using the Maxwell® HT Environmental TNA kit described in the manuscript, except that 125 µL of the re-suspension solution was combined with 125 µL CTAB at the lysing/binding step. To determine the concentration of the titer, the extracted RNA was serially diluted from 1:10 to 1:1,000,000 in 10x increments and quantified in triplicate via RT-qPCR. For dPCR applications, two serial dilutions (5-point) from two distinct RNA extractions were performed by following a 1:2 dilution scheme (1:400 to 1:6,400) and quantified in triplicate via RT-dPCR.

Bovine Respiratory Syncytial Virus (BRSV) preparation

BRSV RNA spiked into the PCR master-mix is used as a method to determine the presence of PCR inhibition. To extract BRSV RNA, one 25-dose vial of INFORCE 3® Respiratory Vaccine (Zoetis, MFR#5263) was suspended in 20 mL of 1X TE buffer and extracted using the Quick-RNA Viral Kit (Zymo, R1034). The extracted BRSV was serially diluted from 1:10 to 1:10,000 following a 1:10 dilution scheme and quantified by both RT-qPCR and RT-dPCR. The BRSV RNA concentration that was quantified and used in the inhibition assay was in the Ct range of 27-30 for RT-qPCR or 400-800 gc/µL for RT-dPCR.

Inhibition assay

For RT-PCR, the master mix was spiked with BRSV RNA that has been diluted to be in the range of Cq value 30-33 for qPCR and 500-900 copies/µL on dPCR to assess for PCR inhibition. BRSV RNA was prepared from the cattle vaccine INFORCE 3® Respiratory Vaccine Zoetis, MFR#5263) as described in the supplemental materials. For qPCR, BRSV Cq values for samples were subtracted from the method blank BRSV Cq value and inhibition was determined to be present when the difference in Cq values is greater than 2. In dPCR, inhibition was determined to be present when the copies/µL of BRSV in a sample was less than 50% of the copies/µL of BRSV in the NTC spiked with BRSV RNA.

LOD/LOQ determination

LOD and LOQ for N1 and N2 assays on RT-qPCR were determined by running replicates of a serial dilution using the SARS-CoV-2 (2019-nCoV) RUO Plasmid Control from IDT of at least 20 replicates per concentration (10000, 1000, 100, 50 and 25 gc/rxn). The Cq values were utilized by an R script previously described (Klymus et al., 2020) to calculate LOD and LOQ for N1 and N2.

For RT-dPCR, the LOD and LOQ were assessed by running a 1:5 serial dilution of the GT Molecular N1-N2-BCoV positive controls using a minimum of six replicates per concentration (~200 to ~10 gc/rnx). The LOQ was defined as the lowest concentration at which the analyte can be reliably and accurately detected (Armbruster and Pry, 2008), that is, with a relative standard deviation between the replicates and with the expected concentration lower than 30%. The LOD (the lowest viral load detectable in 95%) was determined using a probit regression analysis (Stokdyk et al., 2016) between the probability of detection at a given dilution and its corresponding average number of positive partitions.

Assessment of affinity-binding bead automated concentration protocols:

The performance of two standard automated Nanotrap®-based concentration protocols ("short" and "long" Figshare https://doi.org/10.6084/m9.figshare.21538143.v7) was compared. Two 10 mL aliquots of untreated wastewater from each of 8 different POTWs were processed. The first aliquot of wastewater was concentrated on the KingFisher Apex (Thermo Scientific™, Waltham, MA) using the "short" concentration protocol which takes about 39 minutes. In brief, this protocol reduces the time at the binding step for mixing (15 sec mix, 45 sec pause, 3 loops), post-mix (10 sec), collection of beads (3 counts, 3 seconds) and the elution step (10 sec bottom mix, 30 second mix, 90 second pause, loop 5, 10 sec post mix and 3 count, 3 seconds). The second aliquot of wastewater was concentrated using a "long" protocol on the Apex which takes about 78 minutes and primarily increases the time at bead binding/collection steps to reduce bead carry over, a potential artifact of turbid samples and the Promega CLD chemistry. Briefly, the long protocol includes, binding step for mixing (105 sec mix, 45 sec pause, 10 loops), post-mix (15 sec), collection of beads (5 counts, 3 loops) and the elution step (10 sec bottom mix, 15 second mix, 105 second pause, loop 5, 10 sec post mix and 5 count, 5 seconds). The concentrated samples were then extracted as described above using Promega's Maxwell® HT Environmental TNA kit and ran on RT-dPCR for all target assays, including assessment of inhibition.

Effect of filter and influent treatment on SARS-CoV-2 quantification using HA filters:

Wastewater from a south central WWTF in Wisconsin was collected weekly from June 15th through June 29th 2020. Sample amendments and filter types were compared using the three weeks of influent collected. Sample amendments comprised of acidification (pH 4 with HCl)

and/or addition of MgCl2 to the influent (for a final concentration of 25mM, unless indicated otherwise). Filter types included a dry mixed cellulose (MCE) filter (AAWP04700, Millipore) a dry cellulose nitrate filter (7184-004, GE Whatman), and a pretreated, mixed cellulose filter (AAWP04700, Millipore) soaked in 25mM MgCl2. 50 mL of raw influent was used for the sample amendment and filter comparisons. A single filter was concentrated per condition per date of influent. Prior to filtration of influent, sample amendments were added if the sample was being acidified (adding HCL drop by drop to pH of 4 using a pH strip) and/or adding 1 mL of 1000mM MgCl2. For the pretreated, soaked MCE filter, filters were submerged in 25mM MgCl2 for 30 minutes prior to filtering. All filters were stored in 650 µL PM1 (QIAGEN, Hilden, Germany) in Zymo bead bashing tubes (S6003, Zymo Research, Irvine CA, USA), bead tubes that were available to us initially at the time of analysis early in the pandemic. Filters were homogenized by bead bashing and the lysed supernatant from the filters after homogenization were extracted for RNA using the PowerMicrobiome kit (QIAGEN, Hilden, Germany) and eluted in 100 µL RNAse free water. Extracted RNA was quantified for SARS-CoV-2 on RT-qPCR using PerfeCTa qPCR Multiplex Supermix (Quantabio, Beverly, MA, USA) and the 2019-nCoV RUO kit (IDT, Coralville IA, USA) following the protocol.

Use of MgCl2 for virus adsorption from wastewater to MCE filters:

A quart of wastewater from a southcentral WWTF in Wisconsin was collected on 11/23/21 and 150 mL aliquots of influent were made. 4N MgCl2 was added to each aliquot to obtain one of the following concentrations of MgCl2 within the influent aliquot, 10nM, 25nM, 50nM, 100nM, and 500nM. 25 mL of influent from each MgCl2 treated concentration as well as no added MgCl2 to the influent was filtered in triplicates on either a dry 0.8µm MCE filter or a presoaked 0.8µm filter in 25mM MgCl2. Filters were folded in half and rolled before being placed into Lysing Matrix A 2-mL bead-bashing tubes (MP Biomedical, Irvine, CA, USA) containing 400 µL

of cetrimonium bromide buffer (CTAB; Promega, Madison, WI, USA). The tubes were then immediately frozen at -80°C for a minimum of 1 hour before RNA extraction with the Maxwell® HT Environmental TNA kit (Promega, Madison, WI, USA). Filters were thawed at room temperature for 15-20 minutes before being homogenized at a speed setting of 7m/s, for 90 seconds, on a MP FastPrep-24™ 5G homogenizer (MP Biomedical, Irvine, CA, USA). Samples were then spun at 9,168xg for 2 minutes at 4°C in a Microfuge 20R centrifuge (Beckman Coulter, Brea, CA, USA). Without disturbing the pellet, 250 µL of the supernatant was pipetted from each tube and total nucleic acid was extracted in 96 well plates on a KingFisher Flex system (Thermo Fisher, Waltham, MA, USA) following the general procedure described in the Maxwell® HT Environmental TNA Kit (Promega, Madison, WI, USA). Slight adjustments were made to this procedure; 400 µL of isopropanol (after adding the sample and 35 µL of resin) were added to the wells during the first step instead of adding the isopropanol until after an initial mixing step. TNA was eluted in 200 µL of 25mM Tris for RT-qPCR. RT-qPCR reactions were setup in 20 µL using UltraPlex 1 Step ToughMix (Quatabio, Beverly, MA, USA) at a final 1x concentration with 5 µL of template and primers and probe for each assay (Supplemental Table 1). RT-qPCR reactions were performed under the following cycling conditions: 50°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

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