SUPPLEMENTAL MATERIALS

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Design and Validation of Sample Splitting Protocol for Comparison of SARS-CoV-2 Quantification in Wastewater

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S1. Design and operational considerations for wastewater sample splitting

In this work, the following considerations were made in the design and operation of a wastewater sample splitting apparatus and accordant procedure:

Table S1. Design criteria for wastewater sampling splitting apparatus

Design Criteria	Rationale	Description
Effectiveness	SARS-CoV-2 genetic signal should be distributed evenly amongst aliquots split from the same sample.	Validation through evaluation of total suspended solids and SARS-CoV-2 amongst aliquots (see methods and results presented herein).
Ease of use	Set-up time and cross-contamination should be minimized; apparatus should be easy and safe to assemble/prepare/operate/clean.	Quick assembly and replaceable (minimization of cross-contamination), minimizes worker exposure to sample.
Adaptable	Apparatus/protocol should be able to accommodate a range of wastewater sample volumes and aliquot sizes necessary for conducting inter-laboratory method comparisons.	It was anticipated that large sample volume ($\gtrsim$30 L) would typically be required when performing sample splits for the purpose of an inter-laboratory study ($\gtrsim$ 8 participants). Easily adaptable to support greater volumes and/or participants.
Portability	The apparatus should be easily deployed and operated at any location within a wastewater treatment facility (or in a laboratory).	Sizing of the unit should be transportable within facility; accessibility to power may be limited to a regular 120V outlet.
Cost	Materials and equipment that are commonly available to laboratories or easily procured “off-the-shelf”.	Parts are relatively easy and inexpensive to replace if needed.

The key design and operational parameters for the wastewater sample splitting apparatus are summarized below:

Table S2. Summary of design and operational parameters for sample splitting apparatus

Property	***Value***	***Units***	***Comments/References (if applicable)***
Physical properties of liquid			
Density of raw sewage (ρ_l)	1000	kg/m^3	(Xu et al., 2014)
Dynamic viscosity(μ)	1.1	mPa·s	dynamic viscosity of water at 15°C
Kinematic viscosity(υ)	1.0×10^{-6}	m^2/s	kinematic viscosity of water at 15°C
Physical properties of solids			
Particle size (d_p)	0.025	m	maximum anticipated particle size passing through coarse screens (Ministry of Environment, 2008)
Solid density (ρ_s)	1400	kg/m^3	Estimated density of dry sludge (O'Kelly, 2006)
Total suspended solids	200	mg/L	Medium strength domestic sewage (Metcalf & Eddy, Inc. et al., 2013)
Density difference ($\Delta\rho = \rho_s - \rho_l$)	400	kg/m^3	
Process operating conditions			
Liquid depth in vessel (Z)	0.40	m	Maximum liquid depth in pail
Solids concentration (X)	0.02	%	Estimated based on TSS (kg_{solid}/kg_{liquid}x100%)
Geometric parameters			
Vessel diameter (T)	0.285	m	
Bottom head geometry	Flat		
Tank volume when full (V)	0.0255176	m^3	
Impeller type and geometry	Propeller		Mounted at ~10° angle from vertical; ~20° off axis from diameter of vessel
Impeller diameter (D)	0.0762	m	3" diameter propeller
Impeller clearance from bottom (C)	0.1	m	
Liquid coverage above impeller (CV)	0.3	m	Height of wastewater at maximum Z
Operational conditions			
Impeller speed (N)	1000	rpm	(16.7 rps)
Impeller power (P)	4.1	W	Maximum power imparted at maximum Z
Impeller tip speed (v)	4.0	m/s	
Impeller power number (N_p) also known as Newton's number (Ne) as per Zwieterling (1978)	0.34	-	Midpoint average of range of power numbers of propellers with pitch of 1:1 (Crittenden et al., 2012); at turbulent flow, power number is relatively constant
Impeller pumping number (N_q)	0.4	-	Propellers with pitch of 1:1 (Crittenden et al., 2012); a pumping capacity of 3.0 L/s is achieved with a circulation time of 8.6 s
Dimensionless geometric factor (S)	8.2	-	At a T/D = 3.75, and T/C of 2.85, and the assumed Newton's number, Figure 6 from Zwietering (1958) was used to estimate S
Reynold's number ($Re = ND^2\upsilon^{-1}$)	96,630	-	Turbulent regime (>10,000)

Verification of vessel geometry for promoting axial mixing

Table S3. Vessel and impeller geometries for axial mixing: Optimal range and design conditions

Geometric ratio	***Optimal range#***	***Design conditions***
D/T	0.17-0.4	0.27
H/D	2-4	3.16-5.26*
H/T	0.34-1.6	0.84-1.40
B/D	0.7-1.6	1.32

#Ranges as reported in Davis (2020)

**Although the H/D is slightly higher than the ideal range, the impeller diameter and the oversized pail is satisfactory in all other aspects, and therefore is acceptable.*

Determination of operational conditions

Zwieterling's equation (Zwietering, 1958) was used to estimate the minimum speed for complete suspension (N_{js}) of largest particles passing through coarse screens; particles smaller than this particle size will require lower minimum speeds:

$$N_{js} = \frac{S\left(\frac{g\Delta\rho}{\rho_l}\right)^{0.45}(d_p)^{0.2}X^{0.13}v^{0.1}}{D^{0.85}}$$

$$N_{js} = \frac{8.2\left(\frac{g400}{1000}\right)^{0.45}(0.025)^{0.2}0.02^{0.13}(1.1\times10^{-6})^{0.1}}{0.0762^{0.85}}$$

$$N_{js} = 9.8\,rps \;\; OR \;\; 587.9\,rpm$$

Assuming that *complete* suspension can be achieved using this $N_{js,}$ speed ratios (Oldshue, 1983) were used to re-adjust N_{js} to estimate N_{tu}, the minimum speed to achieve *total uniformity* suspension of solids:

$$\frac{N_{js}}{N_{on-bottom\,motion}} = 1.7\,; \qquad \frac{N_{tu}}{N_{on-bottom\,motion}} = 2.9$$

$$\therefore N_{tu} = \frac{2.9\times N_{js}}{1.7} = 16.7\,rps \approx 1000\,rpm$$

Power requirements are estimated using dimensional analysis of the impeller power number:

$$P = \rho_l N^3 D^5 N_p = 1000(16.7)^3(0.076)^5(0.34) = 4.1\,W$$

The manufacturer reports that the selected laboratory stand mixer (RK-50800-00 Caframo – Mfr # BDC1850, Canada) is 0.2 HP (149 W). Assuming 10% losses in power, the selected laboratory stand mixer will provide sufficient mixing power.

The Camp-Stein equation (Camp, 1943) can be used to estimate the velocity gradient (G value) achieved when the reservoir is full.

$$G = \sqrt{\frac{P}{\mu V}} = \sqrt{\frac{4.1}{0.0011(0.026)}} \cong 379\,s^{-1}$$

This calculation was performed to contextualize the velocity gradient that would be achieved as a rule-of-thumb check against the threshold of 300 s^{-1} used in water treatment engineering to adequately disperse solutes in rapid-mixing. As aliquots are dispensed, the velocity gradient is expected to increase from 379 s^{-1} to approximately 490 s^{-1} (approximately 15 L of wastewater remains in the reservoir to reduce potential for impeller to break the surface).

Summary of key rationale and limitations

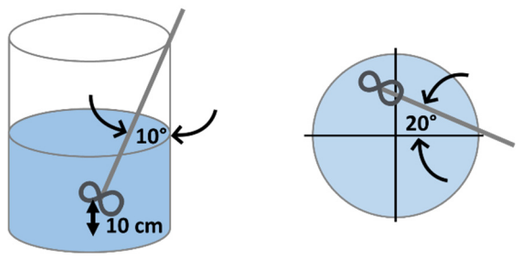
- A batch reactor design, akin to the traditional churn sampler, was selected as the preferred option that best satisfied the design criteria and offered the most flexibility to accommodate a range of aliquot sizes and volumes.
- Existing churn and cone sample splitters available on the market are less than 15 L in size, which are inadequate for total sample volume required to be split in a larger inter-laboratory study with over 20 participants.
- A churn or actively-mixed sample splitter has several limitations, summarized by Gray et al., (2000):
    - Where >0.62 mm particles are present, churn splitters are not recommended for sediment concentration sampling. As the wastewater in this study are collected post-grit removal, this was not deemed an issue.
    - Parameters which can be affected by potential aeration, such as pH and dissolved oxygen are also typically not recommended to by split by churn samplers.
    - Churn splitters were deemed reliable for suspended sediment concentration up to 1000 mg/L where particle diameters are typically less than 250 µm.

Sample splitter configuration

- Reservoir & fittings:
    - The total volume required for the inter-laboratory was expected to be approximately 60 L. Given that handling/lifting/refilling of wastewater sample into the splitting reservoir must be done manually, the largest practical sample container was approximately 30 L. Larger samples would need to be collected in multiple containers. Thus, the batch reactor tank (pail) size was selected to at least be able to receive the entirety of 30 L.
    - Similar to the USGS churn splitter, a fraction of the sample initially present within the submerged tubing would not be well-mixed. This should be primed back into the splitting reservoir to minimize the potential for this initial volume to be subject to different mixing conditions. Out of the bucket height of 0.48 m, aliquots can be distributed when the level of the wastewater sample is between 0.4 m (maximum level to minimize potential for spillage) and 0.2 m (to minimize potential for the propeller to be exposed above the surface of the wastewater) within the pail. These levels can be demarcated on the exterior surface of the semi-translucent plastic bucket.
    - All surfaces in contact the wastewater sample must be inert. This requirement can be achieved through consistent use of materials. The sample reservoir material is high density polyethylene (HDPE). For fittings and tubing, the use of Teflon®, glass, stainless steel are preferred; however, short sections (< 2 m) of surgical grade silicone rubber can be used. Tubing and all other wettable parts should be cleaned or replaced appropriately. The Protocol for the Sampling and Analysis of Industrial/Municipal Wastewater (Ontario Ministry of the Environment, Conservation and Parks, 2016) provides additional guidance on the selection of appropriate materials.
- Mixing:
    - Overhead laboratory mixer was selected; deployed at angle to encourage axial mixing of the batch reactor in the absence of baffles in the cylindrical vessel. Although more inexpensive models of mixers exist, the selected unit had variable speed control that would allow for this apparatus to be adapted to other uses.
    - Calculations have been provided above to determine a starting point for operational conditions.
- Sample dispensing:
    - A plastic pail pump (or stainless steel equivalent) can be used to dispense the sample; plastic pail pumps are designed for one-time use, precluding the need for onerous disinfection/cleaning/verification of decontamination between uses
- Cost:
    - Materials excluding the laboratory mixer costs <$30 USD.

S2. Standard operating procedure

1. Pre-label all pre-cleaned sampling containers and lay out in sample splitting area.
2. Lay out shipping boxes, place wadding pad in the bottom of all boxes. Split ice up and put into bags.
3. Set-up mixer in the sample splitting area. Insert stirring rod and propeller into mixer (all the way up so it is out of the way), tighten using key.
4. * If collecting own sample: collect ~ 60 L of wastewater, post-grit. Ideally this can be done in 3 pails to avoid accidental spilling. Set in area where mixing stand has been set up.
5. Designate one of the pails as the mixing pail and set up pail pump and pre-cut lid on it.
6. Release tightened stirring shaft and lower propeller into bucket until it hits the bottom. Lift propeller up off the bottom by approximately 4" (10 cm) and lock-in using key. Adjust mixer angle to between (a) 5-15 degrees from vertical and (b) 15-30 degrees from horizontal. (see inset figure). This configuration encourages axial mixing of the batch reactor's contents where no baffles are present.



7. Top up the mixing pail with wastewater sample, leave approximately 3 inches of freeboard to avoid spillage.
8. Set mixer to run at 1000 rpm. *This speed may need to be readjusted depending on the wastewater matrix to promote conditions consistent with total uniformity of solids suspension in the pail.
9. Prime the pail pump (and allow for wastewater to return into the pail).
10. Use spreadsheet calculations to determine the number of rounds (R) of sample bottle filling that would be needed based on the total sample volume required. All sample bottles will be filled to approximately 1/R the size of the container. For example, if 2 rounds** are necessary, approximately ½ the volume of the bottle should be filled. **2 rounds would be ideal, but up to 4 rounds can be accommodated.
11. Dispense wastewater into sample bottles based on the calculation performed above. Fill sample bottles for additional desired wastewater quality parameters at the beginning, middle, and end of each round (consistent with the proportion of filling as determined above). This compositing allows for wastewater quality parameters to be volumetrically weighted. Check field parameters at least twice (beginning/end).
12. After each round, re-top up the mixing vessel and allow the additional wastewater to be blended with the remaining liquid (approximately 30 s). Avoid dispensing to the point where the propeller is exposed.
13. For subsequent rounds, work in reverse order to fill up the sample bottles (e.g., Fill order Round 1 = 1,2,3… n, n+1; Round 2 = n+1, n, …3, 2, 1)
14. Repeat Step 12 until all bottles are filled.
15. Place each bottle into a dedicated leak-proof Ziploc™ bag. Multiple aliquots from the same representative sample can be put into the same Ziploc™ bag.
16. Pack Ziploc bags, insert inner letter into separate Ziploc™ bag, top up with ice.
17. Tape up boxes and arrange for courier pick-up.

S3. Details for RT-qPCR analysis of viral targets in wastewater

Every aliquot (Figure S1) was analyzed in duplicate for the genetic signals for each virus. The aliquots were agitated and forty (40) mL of each wastewater aliquot was used for each duplicate. A whole process control recovery surrogate (heat-inactivated human coronavirus strain 229E; HCoV-229E) was administered into one of the duplicates. A mixture of polyethylene glycol (10% w/v) and sodium chloride (2.25% w/v) was administered to all samples to allow for overnight precipitation at 4°C. The sample was centrifuged at 12,000 g for 1 hour (4°C) with no brake. The supernatant was decanted and a shorter spin (30 min) was administered to remove any remaining supernatant. The pellet was weighed and RNA was extracted using the RNeasy® PowerMicrobiome® Kit (Qiagen, USA) following manufacturer's instruction. The final elute volume for the extraction of the RNA was 100 µL. RNA was amplified using a one-step RT-qPCR reaction using Taqpath™ 1-step RT-qPCR Master Mix, CG (A15299, ThermoFisher Scientific, Canada) on a Bio-Rad CFX96™ (Bio-Rad Laboratories Inc., Hercules, CA, USA). The primers and probes sequences for the targets and dedicated thermocycling conditions are summarized in Table S4 below. Concentrations were estimated against a standard curve generated with either EDX standard (SAR-CoV-2 N1 and N2*, Exact Diagnostics*) or gBlocks™ gene fragments (PMMoV, HCoV-229E*, IDT*). RNA extracts were run in triplicate on the PCR plate and the error is expressed as standard deviations. PCR data was analyzed using Bio-Rad CFX Maestro software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Figure S1. Visual check of wastewater aliquots obtained using the sample-splitting apparatus

Table S4. Primer-probe sequences and thermocycling conditions for viral targets of interest

Target	*Sequences*		*Reverse transcription*	*Enzyme activation*	*Denaturation; Annealing/ Extension*	*Standard Curve Characteristics*
SARS-CoV-2 N1	Fwd	GAC CCC AAA ATC AGC GAA AT	25˚C (2 min.); 50˚C (15 min.)	95˚C (2 min)	95˚C (3 sec.); 55˚C (30 sec.) x 45 cycles	Efficiency: 97.5% R^2: 0.98 Range: 100-1.5 gene copies/µL
	Rev	TCT GGT TAC TGC CAG TTG AAT CTG				
	Probe	ACC CCG CAT TAC GTT TGG TGG ACC (6-FAM / BHQ-1)				
SARS-CoV-2 N2	Fwd	TTA CAA ACA TTG GCC GCA AA	25˚C (2 min.); 50˚C (15 min.)	95˚C (2 min)	95˚C (3 sec.); 60˚C (30 sec.) x 45 cycles	Efficiency: 90.5% R^2: 0.99 Range: 100-1.5 gene copies/µL
	Rev	GCG CGA CAT TCC GAA GAA				
	Probe	ACA ATT TGC CCC CAG CGC TTC AG (6-FAM / BHQ-1)				
PMMoV	Fwd	GAG TGG TTT GAC CTT AAC GTT GA	25˚C (2 min.); 50˚C (15 min.)	95˚C (2 min)	95˚C (3 sec.); 55˚C (30 sec.) x 45 cycles	Efficiency: 90.5% R^2: 0.999 Range: 1.8×10^5 - 2.9×10^3 gene copies/µL
	Rev	TTG TCG GTT GCA ATG CAA GT				
	Probe	CCT ACC GAA GCA AAT G (Cy5 / BHQ-3)				
HCoV-229E	Fwd	TTCCGACGTGCTCGAACTTT	25˚C (2 min.); 50˚C (15 min.)	95˚C (2 min)	95˚C (3 sec.); 60˚C (30 sec.) x 45 cycles	Efficiency: 91.5% R^2: 0.998 Range: 1.8×10^6 -18 gene copies/µL
	Rev	CCAACACGGTTGTGACAGTGA				
	Probe	TCCTGAGGTCAATGCA (6-FAM / BHQ-1)				

S4. Additional independent validation of sample-splitting apparatus

A separate sample splitting event was performed on November 29, 2021 (Table S5). Every 10th aliquot was submitted to the same laboratory for analysis. The samples were composited over two rounds of refilling the sample splitting reservoir. Six replicates were processed from one randomly selected aliquot (Aliquot 21). Each replicate was analyzed using triplicate PCR reactions for each viral target. All other aliquots were analyzed once using triplicate PCR reactions for each viral target.

Table S5. Summary of results obtained using RT-qPCR for viral targets estimated in wastewater for a sample collected November 29, 2021. Aliquots 1, 11, 21, 31, 41 were composited over two rounds of refilling the sample splitting reservoir. Six (6) replicates were obtained from a randomly selected sample to characterize the inherent variability associated from processing the same aliquot. The reported Cq values are the arithmetic means of triplicate PCR reactions; concentration estimates are in units of gene copies/mL of wastewater.

Viral target	*SARS-CoV-2*				*PMMoV*	
Target gene	N1		N2			
	Cq	*Conc. est.*	*Cq*	*Conc. est.*	*Cq*	*Conc. est. (× 10^5)*
Aliquot 1	35.6	6.80	35.7	8.23	26.2	2.0
Aliquot 11	35.4	7.56	35.8	7.48	26.4	1.8
Aliquot 21						
Replicate 1	34.5	14.1	34.6	15.8	25.8	2.5
Replicate 2	35.1	9.46	35.6	8.38	25.6	3.0
Replicate 3	34.7	12.2	35.3	10.4	25.8	2.7
Replicate 4	35.1	9.47	35.8	7.48	25.7	2.7
Replicate 5	34.6	12.8	35.2	11.3	25.4	3.5
Replicate 6	34.9	11.1	35.6	8.34	25.9	2.5
Average (across replicates)	*34.8±0.25*	*11.52±1.87*	*35.4±0.42*	*10.28±3.0*	*25.7±0.19*	*2.8 ±0.04*
RSD(%)	-	*16.2%*	-	*29.6%*	-	*13.8%*
Aliquot 31	34.5	13.6	35.1	11.4	26.6	1.5
Aliquot 41	35.7	6.35	35.9	6.95	26.4	1.8
Mean (across aliquots)	*35.2±0.5*	*9.2±3.2*	*35.6±0.3*	*8.9±1.9*	*26.3±0.3*	*2.0 ±0.05*
RSD(%)	-	*35.0%*	-	*21.4%*	-	*26.3%*

The intra- (blue rows) and inter-aliquot (orange rows) variability of *in-situ* viral targets in wastewater were evaluated. These were demonstrated to be comparable and generally within the acceptable range of variability of 25 to 35% RSD expected of reproducible RT-qPCR methods (Forootan et al., 2017; Haugland et al., 2016; Klymus et al., 2020; Kralik & Ricchi, 2017).

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