Effect of storage conditions on SARS-CoV-2 RNA quantification in wastewater solids

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SARS-CoV-2 RNA in wastewater settled solids is associated with COVID-19 incidence in sewersheds and therefore, there is a strong interest in using these measurements to augment traditional disease surveillance methods. A wastewater surveillance program should provide rapid turn around for sample measurements (ideally within 24 hours), but storage of samples is necessary for a variety of reasons including biobanking. Here we investigate how storage of wastewater solids at 4°C, -20°C, and -80°C affects measured concentrations of SARS-CoV-2 RNA. We find that short term (7 or 8 d) storage of raw solids at 4°C has little effect on measured concentrations of SARS-CoV-2 RNA, whereas longer term storage at 4°C (35-122 d) or freezing reduces measurements by 60%, on average. We show that normalizing SARS-CoV-2 RNA concentrations by concentrations of pepper mild mottle virus (PMMoV) RNA, an endogenous wastewater virus, can correct for changes during storage as storage can have a similar effect on PMMoV RNA as on SARS-CoV-2 RNA. The reductions in SARS-CoV-2 RNA in solids during freeze thaws is less than those reported for the same target in liquid influent by several authors.

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18 Abstract

19 SARS-CoV-2 RNA concentrations in wastewater settled solids is associated with COVID-19
20 incidence in sewersheds and therefore, there is a strong interest in using these measurements
21 to augment traditional disease surveillance methods. A wastewater surveillance program
22 should provide rapid turn around for sample measurements (ideally within 24 hours), but
23 storage of samples is necessary for a variety of reasons including biobanking. Here we
24 investigate how storage of wastewater solids at 4°C, -20°C, and -80°C affects measured
25 concentrations of SARS-CoV-2 RNA. We find that short term (7 or 8 d) storage of raw solids at
26 4°C has little effect on measured concentrations of SARS-CoV-2 RNA, whereas longer term
27 storage at 4°C (35-122 d) or freezing reduces measurements by 60%, on average. We show
28 that normalizing SARS-CoV-2 RNA concentrations by concentrations of pepper mild mottle virus
29 (PMMoV) RNA, an endogenous wastewater virus, can correct for changes during storage as
30 storage can have a similar effect on PMMoV RNA as on SARS-CoV-2 RNA. The reductions in
31 SARS-CoV-2 RNA in solids during freeze thaws is less than those reported for the same target
32 in liquid influent by several authors.

33

34 Introduction

35 SARS-CoV-2 RNA in settled solids from wastewater treatment plants correlates to COVID-19
36 incidence in the sewershed population [1–5]. As a result, local and federal governmental
37 agencies are establishing wastewater-based epidemiology methods to help inform pandemic
38 response [6]. SARS-CoV-2 RNA concentrations are measured in the wastewater and
39 information about the incidence of COVID-19 can be inferred from the measurement
40 concentrations [1–5].

41

42 Wastewater consists of liquid and solid components. While many wastewater surveillance
43 efforts have focused on measuring SARS-CoV-2 RNA in the liquid component of wastewater
44 [7,8], the solids have 10^3 to 10^4 higher concentrations of SARS-CoV-2 RNA on a per mass basis
45 [2,9]. Settled solids are readily collected from the primary clarifier where they settle as part of
46 the wastewater treatment process (often referred to as sludge), or they can be settled from
47 wastewater influent using standard method SM2540 F [10] if a wastewater treatment plant does
48 not have a primary clarifier unit process.

49

50 In order for wastewater data on SARS-CoV-2 RNA concentrations to be useful for wastewater
51 based epidemiology, real time disease response, samples should be analyzed quickly and
52 results reported as soon as possible to public health officials. In such a scenario, samples are
53 processed within hours of collection. However, sample storage remains essential. Samples may
54 need to be stored for extended periods of time during transport or shipment from wastewater
55 treatment plants to laboratories if the distance between them is far. In cases where a laboratory
56 instrument malfunctions or results do not pass quality control metrics, samples might need to be
57 rerun. Samples therefore need to be stored for at least as long as it takes to obtain laboratory
58 results. Additionally, labs may want to create a biobank of samples; these samples can be used
59 in the future to probe the presence of variants of concerns or other pathogens as needed.
60 However, little is known about how sample storage affects the quantification of SARS-CoV-2
61 RNA in wastewater. If sample storage significantly changes the concentration of SARS-CoV-2
62 RNA relative to fresh, unstored samples, then measurements from stored samples may provide
63 incorrect information on the incidence of COVID-19 in the sewersheds. The goal of this study is
64 to provide data to help fill this knowledge gap.

65

66 Several studies have investigated how storage conditions affect quantification of SARS-CoV-2
67 RNA in wastewater to be used for wastewater based epidemiology, but most have focused on
68 liquid influent [8,11–14] and determined that storage and freeze thaws of the liquid influent can
69 reduce measured concentrations of the viral RNA an order of magnitude or more. Only one
70 study has examined persistence in wastewater solids: Hokajärvi et al. [12] examined
71 degradation of SARS-CoV-2 RNA in solids pelleted from raw influent via centrifugation. The
72 goal of this study is to assess the impact of different realistic storage conditions on the
73 quantification of SARS-CoV-2 RNA and an endogenous viral control (pepper mild mottle virus,
74 PMMoV) in wastewater settled solids (also referred to as primary settled solids or sludge).
75 PMMoV is highly abundant in human stool and domestic wastewater globally [15,16]. The
76 results of this study will inform optimal storage conditions for settled solids for use for
77 wastewater-based epidemiology.

78

Materials & Methods

80

81 **Schematic overview of the experimental approach.**
82 We collected settled solids from wastewater treatment plants and analyzed them immediately
83 within 6 hours to measures SARS-CoV-2 RNA concentrations and PMMoV RNA concentrations.
84 The samples were then stored under different conditions (4°C, -20°C, or -80°C) for different

85 amounts of time and the SARS-CoV-2 RNA was measured again using the same analytical
86 methods. The samples stored at -80°C were first dewatered prior to storage. The
87 measurements obtained immediately from the "fresh" sample (the "control") and measurements
88 obtained from the same sample after storage (called "treatment") were then compared to assess
89 how storage treatments affect the concentration of SARS-CoV-2 and PMMoV RNA. A
90 schematic of the experimental approach is shown in Figure 1, and outlined in Table 1. Full
91 details are provided below.
92
93 **Sample collection.** Eleven (11) 50-ml samples of settled solids were collected from the primary
94 clarifiers at four unique wastewater treatment plants (Table 1) using sterile technique and clean
95 containers. Samples were immediately stored on ice and transported to the lab where they were
96 processed within 6 hours of sample pick up from the plants with high throughput methods [17–
97 19]. These un-stored samples are referred to as "controls". Thereafter, the samples were
98 subjected to different storage treatments in the laboratory (Table 1). Raw samples were either
99 stored at 4°C for 7, 8, 35, 91 or 122 days, or -20°C for 2 or 3 d. Four samples were stored as
100 dewatered solids (described below) at -80°C for 35, 91 or 122 days. The lengths and
101 temperatures of storage were chosen to represent the range of conditions under which we may
102 need to store samples for our own wastewater surveillance project. After storage, refrigerated
103 samples were immediately processed, and frozen samples were removed from freezers and
104 defrosted at 4°C for 24 hours and then processed according to Topol et al. [17–19]. Stored
105 samples are referred to as "treatments". In total, there were 16 samples subjected to a storage
106 treatment.
107
108 **Sample Preparation.** The solids were dewatered by centrifugation at 24,000 x g for 30 minutes
109 at 4°C and aspirating and discarding the supernatant. A 0.5 - 1 g aliquot of the dewatered solids
110 was dried at 110°C for 19-24 hrs to determine its dry weight. Bovine coronavirus (BCoV) was
111 used as a positive recovery control. Each day, attenuated bovine coronavirus vaccine (PBS
112 Animal Health, Calf-Guard Cattle Vaccine) was spiked into DNA/RNA shield solution (Zymo
113 Research, Irvine, CA, Cat # R1100) at a concentration of 1.5 µL /mL. Dewatered solids were
114 resuspended in the BCoV-spiked DNA/RNA shield to a concentration of 75 mg/mL. This
115 concentration of solids was chosen as previous work titrated solutions with varying
116 concentrations of solids to identify a concentration at which inhibition of the SARS-CoV-2
117 assays was minimized. Five to 10 5/32" Stainless Steel Grinding Balls (OPS Diagnostics,
118 Lebanon, NJ, GBSS 156-5000-01) were added to each sample which was subsequently
119 homogenized by shaking with a Geno/Grinder 2010 (Spex SamplePrep, Metuchen, NJ).
120 Samples were then briefly centrifuged to remove air bubbles introduced during the
121 homogenization process, and then vortexed to re-mix the sample.
122
123 **RNA Extraction.** RNA was extracted from 10 replicate aliquots per sample. For each replicate,
124 RNA was extracted from 300 µl of homogenized sample using the Chemagic™ Viral DNA/RNA
125 300 Kit H96 (Perkin Elmer, Akron, OH, Cat # CMG-1033 S T) for the Chemagic 360 (Perkin
126 Elmer, Akron, OH) followed by PCR Inhibitor Removal with the Zymo OneStep-96 PCR Inhibitor
127 Removal Kit (Cat # D6030). Extraction negative controls (water) and extraction positive controls
128 (500 copies of SARS-CoV-2 genomic RNA (ATCC® VR-1986D™)) in the BCoV-spiked

129   DNA/RNA shield solution described above) were extracted using the same protocol as the
130   homogenized samples.

131

132   **Digital PCR.** RNA extracts were used as template in digital droplet RT-PCR assays for SARS-
133   CoV-2 N, S, and ORF1a RNA gene targets in a triplex assay, and BCoV and PMMoV in a
134   duplex assay (see Table 2 for primer and probe sequences, purchased from Integrated DNA
135   Technologies). Undiluted extract was used for the SARS-CoV-2 assay template and a 1:100
136   dilution of the extract was used for the BCoV / PMMoV assay template. Digital RT-PCR was
137   performed on 20 µl samples from a 22 µl reaction volume, prepared using 5.5 µl template,
138   mixed with 5.5 µl of One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, Hercules, CA Cat
139   # 1863021), 2.2 µl Reverse Transcriptase, 1.1 µl DTT and primers and probes at a final
140   concentration of 900nM and 250nM respectively. Droplets were generated using the AutoDG
141   Automated Droplet Generator (Bio-Rad). PCR was performed using Mastercycler Pro with the
142   following protocol: reverse transcription at 50°C for 60 minutes, enzyme activation at 95°C for 5
143   minutes, 40 cycles of denaturation at 95°C for 30 seconds and annealing and extension at
144   either 59°C (for SARS-CoV-2 assay) or 56°C (for PMMoV/BCoV duplex assay) for 30 seconds,
145   enzyme deactivation at 98°C for 10 minutes then an indefinite hold at 4°C. The ramp rate for
146   temperature changes were set to 2°C/second and the final hold at 4°C was performed for a
147   minimum of 30 minutes to allow the droplets to stabilize. Droplets were analyzed using the
148   QX200 Droplet Reader (Bio-Rad). All liquid transfers were performed using the Agilent Bravo
149   (Agilent Technologies, Palo Alto, CA).

150

151   Each sample was run in 10 replicate wells, extraction negative controls were run in 7 wells, and
152   extraction positive controls in 1 well. In addition, PCR positive controls for SARS-CoV-2 RNA,
153   BCoV, and PMMoV were run in 1 well, and NTC were run in 7 wells. Positive controls consisted
154   of BCoV and PMMoV gene block controls (purchased from Integrated DNA Technologies) and
155   gRNA of SARS-CoV-2 (ATCC® VR-1986D™). Results from replicate wells were merged for
156   analysis. Thresholding was done using QuantaSoft™ Analysis Pro Software (Bio-Rad, version
157   1.0.596). Additional details are provided in supporting material per the dMIQE guidelines [20].

158

159

160   **Data analysis.** Concentrations of RNA targets were converted to concentrations per dry weight
161   of solids in units of copies/g dry weight. The total error is reported as 68% confidence intervals
162   and includes the errors associated with the poisson distribution and the variability among the 10
163   replicates. The recovery of BCoV was determined by normalizing the concentration of BCoV by
164   the expected concentration given the value measured in the spiked DNA/RNA shield. If the
165   BCoV recovery was less than 10%, then the sample was rerun.

166

167   PMMoV, N, S, ORF1a, as well as N/PMMoV, S/PMMoV, and ORF1a/PMMoV were compared
168   for each sample control and treatment, 1 by 1, by examining the measurement and the 68%
169   error associated with the measurement. The error associated with the quotients was estimated
170   by propagating errors on the numerator and denominator, as described by Graham et al. [2]. In
171   brief, the relative error of the quotient is approximated by the square root of the sum of the
172   relative errors of the numerator and denominator squared. If the measurement of the treatment

173 condition fell within the error range of the control condition, then the measurement was deemed
174 "not different". This approach is equivalent to a t-test where the null hypothesis (Ho) is the value
175 of the treatment is the same as the control and the alternate hypothesis (Ha) is that the values
176 are different. In this study, we are particularly concerned about type 2 errors (failing to reject Ho
177 when it is false) as we are concerned with whether storage renders different measurements. As
178 such, in order to increase the power of the analysis, we chose to make comparisons using the
179 68% confidence intervals. With the 10 replicates, this gives ~90% power of avoiding a type 2
180 error assuming an effect size equal to the standard deviation. Note that if we had used 95%
181 confidence intervals in our analysis rather than 68% confidence intervals, the likelihood of not
182 detecting a difference in the treatments and controls would be higher as 95% confidence
183 intervals are approximately two times larger than 68% confidence intervals.
184
185 For measurements deemed "different", the percent difference (% diff) was calculated as % diff
186 = 100 x (control-treatment)/control where control and treatment are the associated
187 measurements. A positive % diff indicates that the treatment result is smaller than the control,
188 whereas a negative percent indicates the treatment had higher concentrations than the control.
189 Errors for % diff were propagated from the measurements as standard deviations.
190
191 **Results**
192
193 *Quality control.* All positive extraction and PCR controls were positive, and all negative
194 extraction controls and negative PCR controls were negative indicating no cross contamination
195 between samples. Recovery of spiked BCoV was above 10% for all samples so no samples
196 were rerun owing to unacceptable recovery. SARS-CoV-2 RNA and PMMoV targets were
197 detected in all samples. Data from the experiments is available through Stanford Digital
198 Repository [21].
199
200 *Short-term Storage at 4°C.* Two experiments were carried out to investigate the effects of
201 storage on 8 samples of raw settled solids samples at 4°C (Table 1). First, all samples were
202 processed within 6 hours of collection and the resultant measurements were treated as those of
203 the control condition. Then, in one experiment, 4 samples were stored for 7-8 days at 4°C prior
204 to being processed a second time; and in the other, 4 samples were stored between 35 days
205 and 122 days prior to being processed a second time. The stored conditions are referred to as
206 treatments.
207
208 SARS-CoV-2 RNA measurements made after 7-8 d of storage at 4°C were not different from the
209 control condition for 3 of the 4 samples; PMMoV was not different between treatment and
210 control for all 4 of the samples. When SARS-CoV-2 RNA gene concentrations were normalized
211 by PMMoV gene concentrations, the ratios were not different between treatment and control in
212 any of the 4 samples (Figure 2). For the sample that had lower SARS-CoV-2 RNA gene
213 concentrations in the treatment compared to the control, the concentrations of the N, S, and
214 ORF1 genes differed by 60%, 80%, and 73% respectively (Table 3).
215

216   *Long-term Storage at 4°C.* Longer storage at 4°C (between 35 and 122 days) resulted in
217   significantly lower measurements of SARS-CoV-2 RNA in 3 of the 4 tested samples, and lower
218   PMMoV RNA concentrations in 2 of 4 samples. Normalizing SARS-CoV-2 RNA concentrations
219   by PMMoV concentrations "corrected" the differences observed in 1 sample, but not the other 2.
220   For those 2 samples, ratios were lower and different in the treatments compared to the controls
221   (Figure 2). The difference between the treatment and controls are shown in Table 3. The
222   average   difference in the measurements, when they were different, was 44%.
223

224   *Storage at -20°C.* Four samples were processed within 6 hours of collection to obtain control
225   measurements. The same samples were also frozen at -20°C for 2 or 3 days, and then
226   defrosted and processed to obtain treatment measurement (Table 1).
227

228   SARS-CoV-2 RNA concentrations in treatments were different in 3 of the 4 samples compared
229   to controls (lower in treatment versus control in 1 and higher in 2, average difference 89%).
230   PMMoV concentrations were not different between treatments and controls. Normalizing SARS-
231   CoV-2 RNA concentrations by PMMoV RNA concentrations, "corrected" the differences in
232   SARS-CoV-2 RNA observed in 2 of the 3 samples (Figure 3); for the single sample where the
233   ratio of SARS-CoV-2 RNA to PMMoV RNA concentrations was different, the treatment had 61%
234   (average) lower ratio than the control . Differences between treatments and controls   are
235   summarized   in Table 3.
236

237   *Storage at -80°C.* Four samples were processed within 6 hours of collection to obtain control
238   measurements and also dewatered, frozen at -80°C for between 35 and 122 days, and then
239   defrosted and processed to obtain treatment measurements (Table 1).
240

241   SARS-CoV-2 and PMMoV RNA concentrations were statistically lower in the treatments
242   compared to the controls by 40% to 90% (Figure 3 and Table 3). Normalizing SARS-CoV-2
243   RNA concentrations by PMMoV concentrations "corrected" the differences for 2 of the 4
244   samples. For the other 2, the ratio of SARS-CoV-2 RNA to PMMoV RNA concentrations was
245   49% (on average) lower in the treatment than the control.
246

247   Overview of combined results. Across the treatment/samples where we observed a change in
248   SARS-CoV-2 RNA concentrations relative to the control (n=11 of 16), there was a reduction in
249   measured SARS-CoV-2 RNA concentration in all but 2 sample treatments (mean percent
250   reduction of 63% ±14% standard deviation, n = 27: 9 treatment/samples x 3 SARS-CoV-2 gene
251   measurements different per treatment/sample). In the 2 cases where we observed an increase
252   in SARS-CoV-2 concentrations in the treatment compared to the control, that increase was
253   107% ± 44% on average (n = 4, 2 sample /treatments x 2 SARS-CoV-2 genes were different per
254   sample/treatment). All measurements made in samples receiving a treatment were within an
255   order of magnitude of the measurement in the control.
256

257

258   **Discussion**

259

260   SARS-CoV-2 RNA concentrations in wastewater settled solids correlate to COVID-19 incidence
261   in surrounding sewersheds [1–5]. As a result, SARS-CoV-2 RNA concentrations in wastewater
262   settled solids are being used to inform the response to the COVID-19 pandemic. While
263   immediate processing of samples after they are collected is ideal, it is necessary to store and
264   archive samples in case samples need to be rerun due to failed quality control metrics, or if
265   additional analyses are needed to investigate the presence of other viruses or viral variants, for
266   example. There is limited data on how storage of wastewater samples affects the concentration
267   of SARS-CoV-2 RNA in the samples, so we carried out experiments to help fill this knowledge
268   gap. Our experiments suggest that storage of wastewater settled solids samples, either
269   refrigerated or frozen, may change the concentrations of SARS-CoV-2 RNA measured in the
270   samples, but changes are less than an order of magnitude even for samples stored over 100
271   days.
272
273   Several studies have examined the effect of sample storage on SARS-CoV-2 RNA
274   quantification in wastewater influent which represents a different matrix than that examined
275   herein. Influent consists primarily of liquid wastewater rather than solids. Hokajärvi et al. [12]
276   examined the effect of storage on detection of SARS-CoV-2 RNA in liquid influent and found
277   small differences resulting from storage at freezing temperatures and first order decay of the
278   RNA in influent stored at 4°C with a T_{90} (time until 1 $\log_{10}$ reduction of concentration) of 36 to 52
279   days depending on the genomic target. Ahmed et al. [14] report similar results as Hokajärvi et
280   al. for decay of the SARS-CoV-2 targets in influent during storage at 4°C. Markt et al. [22] found
281   minimal differences in SARS-CoV-2 RNA concentrations measured in influent stored for up to 7
282   days at 4°C compared to concentrations measured with no storage, but found more than an
283   order of magnitude decrease in SARS-CoV-2 RNA concentrations in samples that were stored
284   frozen and subject to a freeze thaw. Fernandez-Cassi et al. [23] report extensive reduction in
285   SARS-CoV-2 RNA concentrations measured in liquid wastewater stored at 4°C and -20°C (1-2
286   orders of magnitude). Our results regarding wastewater solids stored at 4°C are similar to those
287   presented in these influent studies, except for Fernandez-Cassi et al. [23]; overall we saw
288   minimal reduction (less than an order of magnitude) even for samples stored over 100 days.
289   However, the effect of freeze thaw on our measurements with solids is small compared to those
290   reported by Markt et al. [22] and Fernandez-Cassi et al. [23] for infuent. We could identify only
291   one published study on SARS-CoV-2 RNA decay in solids: Hokajärvi et al. [12] report minimal
292   decay (between 0% and 20%) of SARS-CoV-2 RNA solids pelleted rom raw influent by
293   centrifugation during storage at 4°C, -20°C, and -75°C for 84 days, within the range of results
294   reported herein.
295
296   Researchers have used PMMoV RNA as an internal process control in their efforts to monitor
297   SARS-CoV-2 RNA in wastewater [1,24,25]. Assuming endogenous PMMoV RNA is recovered
298   in the sample processing and RNA extraction and purification process in the same manner as
299   SARS-CoV-2 RNA, then normalizing SARS-CoV-2 RNA by PMMoV RNA provides a ratio that
300   does not depend on recovery. Wolfe et al. [1] showed the ratio of SARS-CoV-2 RNA/PMMoV
301   RNA in settled solids is associated with COVID-19 incidence rates empirically, and the
302   relationship between the ratio and COVID-19 incidence rates also falls from a mass balance

303   model that relates wastewater solid concentrations to the number of people shedding SARS-
304   CoV-2 RNA in their stool.

305

306   In this study, we found that normalizing SARS-CoV-2 RNA by PMMoV RNA concentrations
307   corrected for changes in concentration that may result during storage. In 7 of the 11
308   sample/treatments that showed differences between SARS-CoV-2 RNA concentrations and the
309   control, SARS-CoV-2/PMMoV was not different between treatment and control. When there
310   were differences, they were less than an order of magnitude. PMMoV RNA concentration was
311   often affected by storage in a similar manner as SARS-CoV-2 RNA concentrations, thus
312   highlighting an additional benefit of using the internal control to interpret concentrations of the
313   SARS-CoV-2 RNA targets in wastewater-based epidemiology applications. The ability to
314   effectively correct for the impact of storage on samples suggests that the primary concerns for
315   sample storage of wastewater solids are related to times when SARS-CoV-2 concentrations are
316   nearing the limit of detection, and during periods of low incidence immediate sample processing
317   should be a higher priority.

318

319   Additional research should examine a time course of decay of SARS-CoV-2 RNA for a single
320   solids sample stored for various lengths of time, and also investigate the effects of multiple
321   freeze thaws on target quantification. Work with additional viral targets may also be useful to
322   provide guidance on storage for wastewater-based epidemiology applications beyond COVID-
323   19 including using RNA from gastro-intestinal viruses like norovirus and rotavirus to infer
324   incidence of diarrheal illnesses. Finally, our study was not powered to investigate whether
325   storage affected viral quantification in wastewater solids from different wastewater treatment
326   plants in different ways; we used samples from diverse plants to capture potential variations
327   between properties of wastewater solids. Additional work should investigate if storage has
328   differential effects on samples from different wastewater treatment plants. Importantly, studies
329   on the effect of storage on quantification of wastewater based epidemiology targets in
330   wastewater influent and solids should be carried out in the near term, before there is a pressing
331   need to use the measurements for disease outbreak response.

332

333

334   **Conclusions**

335

336   Wastewater based epidemiology is not a new field. It has previously been used to surveil
337   populations for infectious diseases including polio [27], hepatitis [28], and salmonellosis [29]. Its
338   wide-spread use globally during the COVID-19 pandemic to infer trends in COVID-19 incidence,
339   however, is unprecedented. As such, new research is needed to continue to fill research gaps
340   and develop models that link wastewater concentrations of pathogens to disease incidence
341   rates.

342

343   One of those research gaps concerns storage of wastewater samples, and how storage affects
344   measured concentrations of wastewater based epidemiology targets – in this case SARS-CoV-2
345   RNA. Storage of wastewater solids for use in wastewater-based epidemiology applications is
346   essential. Here we examined how storage at 4°C for short (7-8 d) and long durations (35-122 d),

347   -20°C for short (2-3 d), and -80°C for long durations (35-122 d) affects SARS-CoV-2 RNA
348   measurements in wastewater solids, and whether normalizing measurements by concentrations
349   of an internal process control corrects for the effects of storage.
350
351   We found storage at 4°C for short durations of 7-8 days had limited to no effect on measured
352   concentrations, but other storage conditions and durations affected concentrations by reducing
353   them by 61%, on average, and in one case increasing them by up to 170%. However, we found
354   that the normalizing concentrations by the internal process control PMMoV helped to correct for
355   the observed differences.
356
357   Degradation of wastewater based epidemiology targets during storage represents a challenge
358   to application of the tool for public health responses to infectious disease outbreaks. Storage of
359   samples is essential. Identifying matrices and storage conditions where there is limited
360   degradation is essential. With this in mind, we recommend short duration storage at 4°C, and
361   normalizing concentrations of SARS-CoV-2 RNA by concentrations of PMMoV RNA in the
362   sample for use in models that relate trends in wastewater to disease incidence. Even under the
363   longer storage conditions including those that required a freeze/thaw, changes in concentrations
364   observed with the solids were less than one order of magnitude and similar among samples
365   subjected to the same treatment.
366

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370   respects to them and their Elders, past and present, and are grateful for the opportunity to live
371   and work here.

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374

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References

377 1. Wolfe MK, Archana A, Catoe D, Coffman M, Dorevitch S, Graham K, et al. Scaling of
378   SARS-CoV-2 RNA in settled solids from multiple wastewater treatment plants to compare
379   relative incidence of laboratory-confirmed COVID-19 in their sewersheds. Environmental
380   Science & Technology Letters. 2021;In revision.

381 2. Graham KE, Loeb SK, Wolfe MK, Catoe D, Sinnott-Armstrong N, Kim S, et al. SARS-CoV-
382   2 RNA in Wastewater Settled Solids Is Associated with COVID-19 Cases in a Large Urban
383   Sewershed. Environ Sci Technol. 2021;55: 488–498. doi:10.1021/acs.est.0c06191

384 3. Peccia J, Zulli A, Brackney DE, Grubaugh ND, Kaplan EH, Casanovas-Massana A, et al.
385   SARS-CoV-2 RNA concentrations in primary municipal sewage sludge as a leading
386   indicator of COVID-19 outbreak dynamics. Nature Biotechnology. 2020;38: 1164–1167.

387 4. D'Aoust PM, Mercier E, Montpetit D, Jia J-J, Alexandrov I, Neault N, et al. Quantitative
388   analysis of SARS-CoV-2 RNA from wastewater solids in communities with low COVID-19
389   incidence and prevalence. Water Research. 2021;188: 116560.
390   doi:10.1016/j.watres.2020.116560

391 5. Kitamura K, Sadamasu K, Muramatsu M, Yoshida H. Efficient detection of SARS-CoV-2
392   RNA in the solid fraction of wastewater. Science of The Total Environment. 2021;763:
393   144587. doi:10.1016/j.scitotenv.2020.144587

394 6. United States Center for Disease Control. National Wastewater Surveillance System
395   (NWSS). 2020 [cited 24 Aug 2020]. Available: https://www.cdc.gov/coronavirus/2019-
396   ncov/cases-updates/wastewater-surveillance.html

397 7. Medema G, Heijnen L, Elsinga G, Italiaander R, Brouwer A. Presence of SARS-
398   Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the
399   Early Stage of the Epidemic in The Netherlands. Environ Sci Technol Lett. 2020;7: 511–
400   516. doi:10.1021/acs.estlett.0c00357

401 8. Weidhaas J, Aanderud ZT, Roper DK, VanDerslice J, Gaddis EB, Ostermiller J, et al.
402   Correlation of SARS-CoV-2 RNA in wastewater with COVID-19 disease burden in
403   sewersheds. Science of The Total Environment. 2021;775: 145790.
404   doi:10.1016/j.scitotenv.2021.145790

405 9. Li B, Di DYW, Saingam P, Jeon MK, Yan T. Fine-Scale Temporal Dynamics of SARS-CoV-
406   2 RNA Abundance in Wastewater during A COVID-19 Lockdown. Water Research.
407   2021;197: 117093. doi:10.1016/j.watres.2021.117093

408 10. AWWA. Standard Methods for the Examination of Water and Wastewater. 21st ed.
409   Baltimore: American Public Health Association, American Water Works Association, Water
410   Environment Federation; 2005.

411 11. Whitney ON, Kennedy LC, Fan VB, Hinkle A, Kantor R, Greenwald H, et al. Sewage, Salt,
412   Silica, and SARS-CoV-2 (4S): An Economical Kit-Free Method for Direct Capture of SARS-
413   CoV-2 RNA from Wastewater. Environ Sci Technol. 2021;55: 4880–4888.
414   doi:10.1021/acs.est.0c08129

415 12. Hokajärvi A-M, Rytkönen A, Tiwari A, Kauppinen A, Oikarinen S, Lehto K-M, et al. The
416   detection and stability of the SARS-CoV-2 RNA biomarkers in wastewater influent in
417   Helsinki, Finland. Science of The Total Environment. 2021;770: 145274.
418   doi:10.1016/j.scitotenv.2021.145274

419 13. Bivins A, Greaves J, Fischer R, Yinda KC, Ahmed W, Kitajima M, et al. Persistence of
420   SARS-CoV-2 in Water and Wastewater. Environ Sci Technol Lett. 2020;7: 937–942.
421   doi:10.1021/acs.estlett.0c00730

422 14. Ahmed W, Bertsch PM, Bibby K, Haramoto E, Hewitt J, Huygens F, et al. Decay of SARS-
423   CoV-2 and surrogate murine hepatitis virus RNA in untreated wastewater to inform
424   application in wastewater-based epidemiology. Environmental Research. 2020; 110092.

425   doi:10.1016/j.envres.2020.110092
426 15. Colson P, Richet H, Desnues C, Balique F, Moal V, Grob J-J, et al. Pepper Mild Mottle
427   Virus, a Plant Virus Associated with Specific Immune Responses, Fever, Abdominal Pains,
428   and Pruritus in Humans. PLOS ONE. 2010;5: e10041. doi:10.1371/journal.pone.0010041
429 16. Rosario K, Symonds EM, Sinigalliano C, Stewart J, Breitbart M. Pepper mild mottle virus
430   as an indicator of fecal pollution. Applied and Environmental Microbiology. 2009;75: 7261–
431   7.
432 17. Topol A, Wolfe M, White B, Wigginton K, Boehm A. High Throughput pre-analytical
433   processing of wastewater settled solids for SARS-CoV-2 RNA analyses. protocols.io.
434   2021. Available: dx.doi.org/10.17504/protocols.io.btyqnpvw
435 18. Topol A, Wolfe M, White B, Wigginton K, Boehm A. High Throughput SARS-COV-2,
436   PMMOV, and BCoV quantification in settled solids using digital RT-PCR. protocols.io.
437   2021. Available: dx.doi.org/10.17504/protocols.io.btywnpxe
438 19. Topol A, Wolfe M, Wigginton K, White B, Boehm A. High Throughput RNA Extraction and
439   PCR Inhibitor Removal of Settled Solids for Wastewater Surveillance of SARS-CoV-2
440   RNA. protocols.io. 2021. Available: dx.doi.org/10.17504/protocols.io.btyrnpv6
441 20. The dMIQE Group, Huggett JF. The Digital MIQE Guidelines Update: Minimum Information
442   for Publication of Quantitative Digital PCR Experiments for 2020. Clinical Chemistry.
443   2020;66: 1012–1029. doi:10.1093/clinchem/hvaa125
444 21. Simpson A, Boehm AB. Effect of storage on concentrations of SARS-CoV-2 RNA in settled
445   solids of wastewater treatment plants. In: Stanford Digital Repository [Internet]. 2021.
446   Available: purl.stanford.edu/yn042kx5009
447 22. Markt R, Mayr M, Peer E, Wagner AO, Lackner N, Insam H. Detection and stability of
448   SARS-CoV-2 fragments in wastewater: Impact of storage temperature. medRxiv. 2021;
449   2021.02.22.21250768. doi:10.1101/2021.02.22.21250768
450 23. Fernandez-Cassi X, Scheidegger A, Bänziger C, Cariti F, Corzon AT,
451   Ganesanandamoorthy P, Lemaitre, JC, Ort C, Julian TR, Kohn T. Wastewater monitoring
452   outperforms case numbers as a tool to track COVID-19 incidence dynamics when test
453   positivity rates are high. Water Research. 2021; 117252.
454   doi:10.1016/j.watres.2021.117252
455 24. Wu F, Zhang J, Xiao A, Gu X, Lee WL, Armas F, Kauffman K, Hanage W, Matus M, Ghaeli
456   N, Endo N, Duvallet C, Poyet M, Moniz K, Washburne A, Erickson T, Chai P, Thompson J,
457   and Alm, EJ. SARS-CoV-2 Titers in Wastewater Are Higher than Expected from Clinically
458   Confirmed Cases. mSystems. 2020;5: e00614-20. doi:10.1128/mSystems.00614-20
459 25. Feng S, Roguet A, McClary-Gutierrez JS, Newton RJ, Kloczko N, Meiman JG, McLellan,
460   S. Evaluation of sampling frequency and normalization of SARS-CoV-2 wastewater
461   concentrations for capturing COVID-19 burdens in the community. medRxiv. 2021;
462   2021.02.17.21251867. doi:10.1101/2021.02.17.21251867
463 26. Huisman JS, Scire J, Caduff L, Fernandez-Cassi X, Ganesanandamoorthy P, Kull A,
464   Andreas Scheidegger, Elyse Stachler, Alexandria B. Boehm, Bridgette Hughes, Alisha
465   Knudson, Aaron Topol, Krista R. Wigginton, Marlene K. Wolfe, Tamar Kohn, Christoph Ort,
466   Tanja Stadler, Timothy R. Julian. Wastewater-based estimation of the effective
467   reproductive number of SARS-CoV-2. medRxiv. 2021; 2021.04.29.21255961.
468   doi:10.1101/2021.04.29.21255961
469 27. Brouwer, A. F.; Eisenberg, J. N. S.; Pomeroy, C. D.; Shulman, L. M.; Hindiyeh, M.;
470   Manor, Y.; Grotto, I.; Koopman, J. S.; Eisenberg, M. C. Epidemiology of the Silent Polio
471   Outbreak in Rahat, Israel, Based on Modeling of Environmental Surveillance Data. *Proc
472   Natl Acad Sci USA* **2018**, *115* (45), E10625. https://doi.org/10.1073/pnas.1808798115.
473 28. McCall, C.; Wu, H.; Miyani, B.; Xagoraraki, I. Identification of Multiple Potential Viral
474   Diseases in a Large Urban Center Using Wastewater Surveillance. *Water Research*
475   **2020**, *184*, 116160. https://doi.org/10.1016/j.watres.2020.116160.

476   29.   Diemert, S.; Yan, T. Clinically Unreported Salmonellosis Outbreak Detected via
477   Comparative Genomic Analysis of Municipal Wastewater Salmonella</Em>
478   Isolates. *Appl. Environ. Microbiol.* **2019**, *85* (10), e00139-19.
479   https://doi.org/10.1128/AEM.00139-19.
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Figure 1

Figure 1. Schematic of experiment.

We collected settled solids from wastewater treatment plants and analyzed them immediately within 6 hours to measures SARS-CoV-2 RNA concentrations and PMMoV RNA concentrations. The samples were then stored under different conditions (4°C, -20°C, or -80°C) for different amounts of time and the SARS-CoV-2 RNA was measured again using the same analytical methods. The samples stored at -80°C were first dewatered prior to storage. Created with BioRender.com.
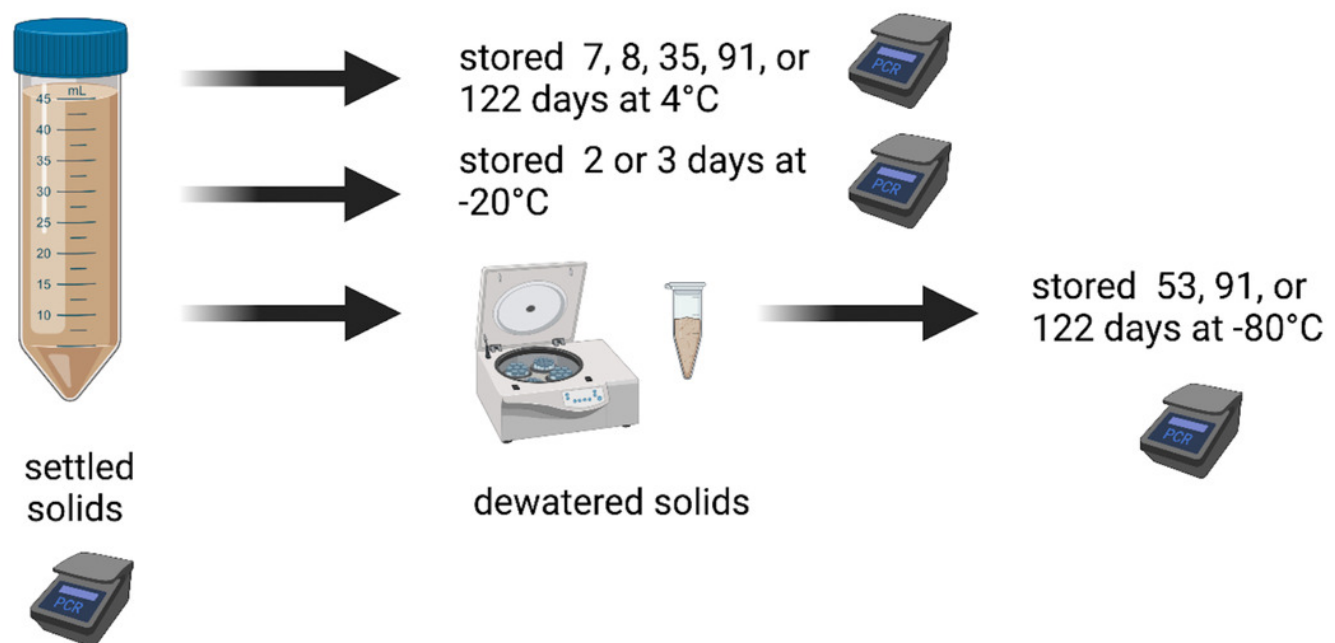
Figure 2

Figure 2. Measurements in 4°C treatments and controls.

Concentrations of SARS-CoV-2 RNA targets (N, S, ORF1a, left column) and PMMoV RNA (middle column), as well as their ratios (right column) as measured in settled solids in controls (darker bars) and treatments (lighter bars) and total errors as reported by the digital PCR instrument, or in the case of the ratios, the errors were propagated. Units are copies per gram dry weight (cp/g) for SARS-CoV-2 and PMMoV RNA targets, and there are no units for the ratio. Asterisks denote measurements or calculated ratios for which the errors on the treatments and controls do not overlap and indicate the measurements were different. Top row shows experiments where solids were stored at 4°C for 7-8 days while the bottom row shows experiments where solids were stored at 4°C for 35-122 days (see Table 1). The sample names on the y-axes consist of a letter indicating the plant location and a random number. The scales are different between plots to properly allow the plotting of the values which vary between samples.
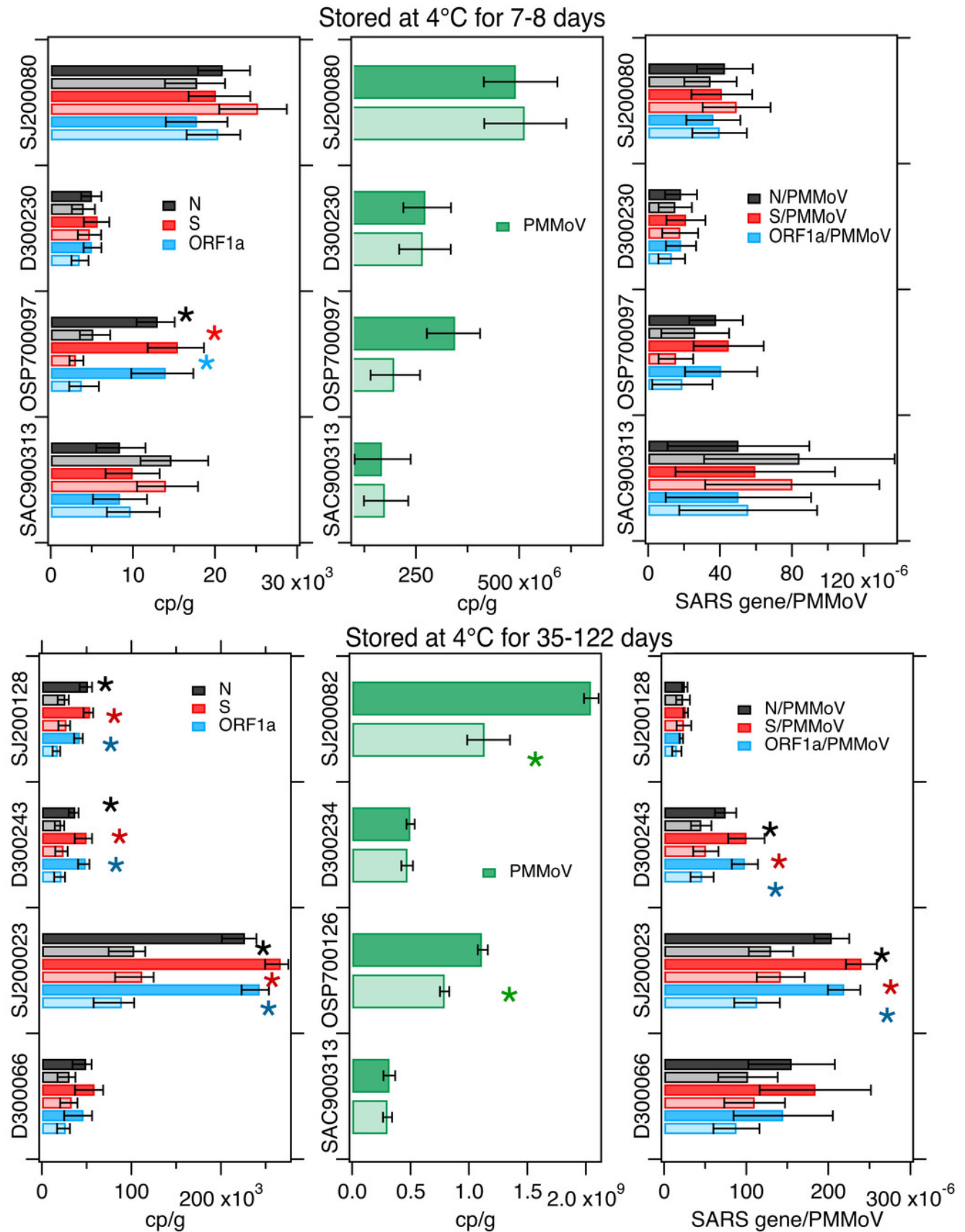
Figure 3

Figure 3. Comparison on measurements in freeze/thaw treatments and controls.

Concentrations of SARS-CoV-2 RNA targets (N, S, ORF1a, left column) and PMMoV RNA (middle column), as well as their ratios (right column) as measured in settled solids in controls (darker bars) and treatments (lighter bars) and total errors as reported by the digital PCR instrument, or in the case of the ratios, the errors were propagated. Units are copies per gram dry weight (cp/g) for SARS-CoV-2 and PMMoV RNA targets, and there are no units for the ratio. Asterisks denote measurements or calculated ratios for which the errors on the treatments and controls do not overlap and indicate the measurements were different. Top row shows experiments where solids were stored at -20°C for 2-3 days while the bottom row shows experiments where dewatered solids were stored at -80°C for 35-122 days (see Table 1).
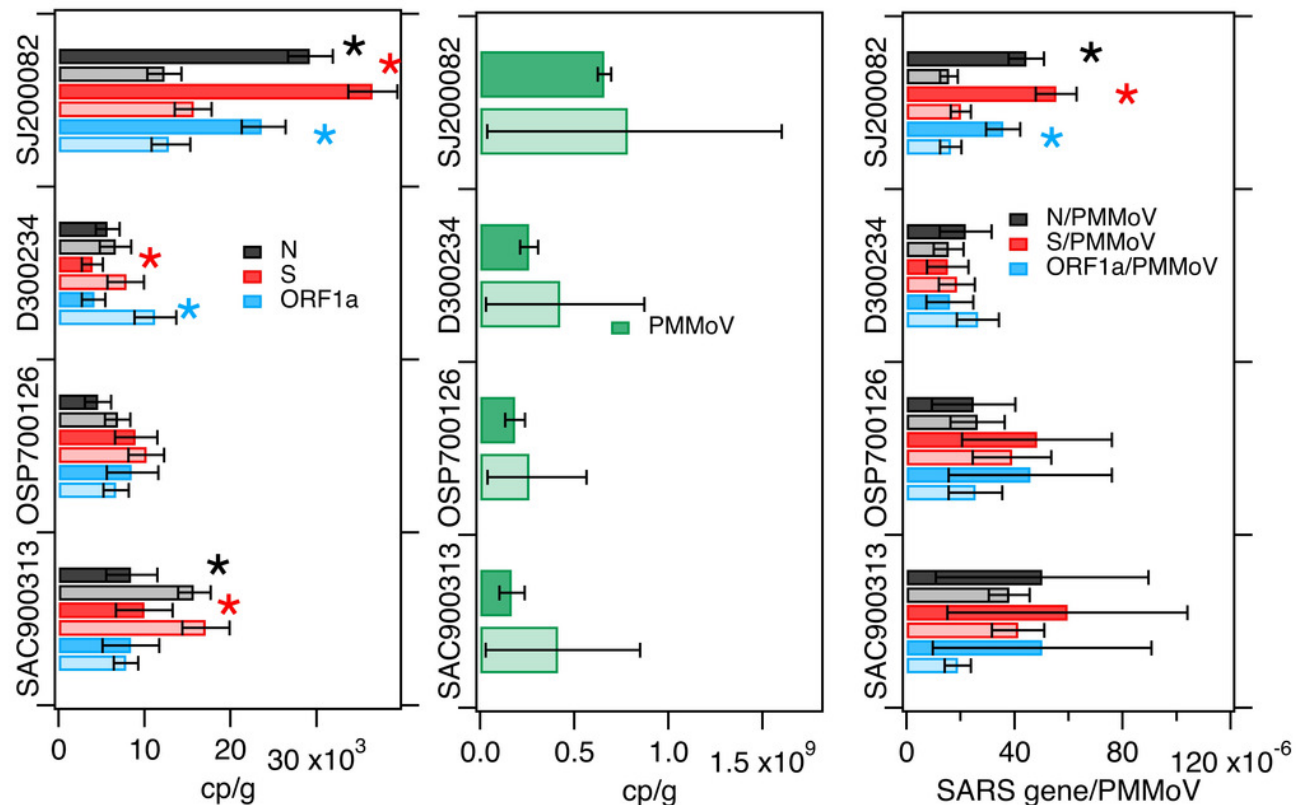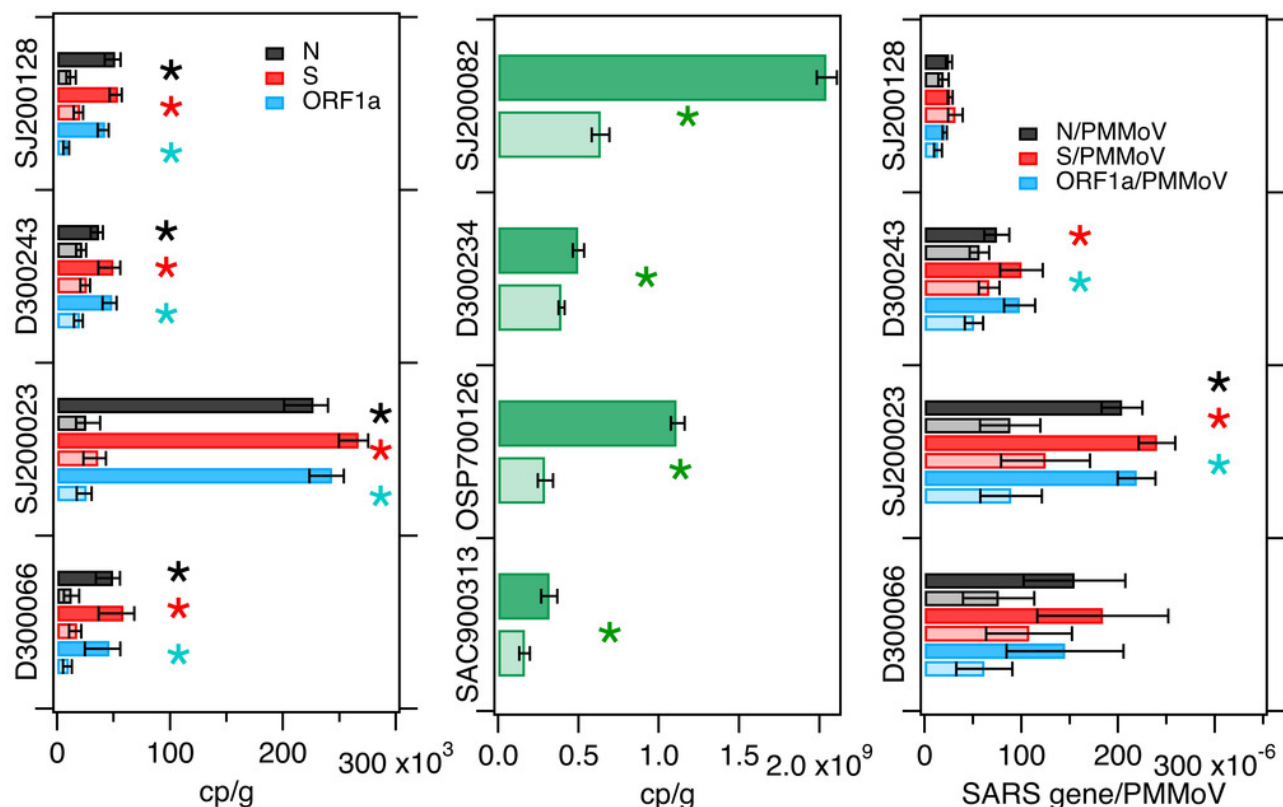
Table 1. Samples used in this study.

Samples used in this study as well as the wastewater plant they were collected from (all located in California) and the date of sample collection, and the storage temperature and duration for the treatment applied to it. In order to protect the privacy of the plants and the inhabitants of their sewersheds, the precise locations are not provided herein.

Sample ID	Plant	Date	Storage condition(s)	duration(s) of storage
SJ200080	SJ	2/28/21	4°C	8 d
D300230	D	3/1/21	4°C	7 d
OSP700097	Ocean	3/1/21	4°C	7 d
SJ200082	SJ	2/23/21	-20°C	3 d
D300234	D	2/24/21	-20°C	2 d
OSP700126	Ocean	2/24/21	-20°C	2 d
SAC900313	SAC	3/1/21	4°C/ -20°C	7 d / 2 d *
SJ200128	SJ	2/25/21	4°C / -80°C	35 d
SJ20023	SJ	11/30/20	4°C / -80°C	122 d
D300243	D	2/25/21	4°C / -80°C	35 d
D300066	D	12/31/20	4°C / -80°C	91 d

1   *The sample stored at -20°C was stored for 2 d and the sample stored at 4°C was stored for 7
2   d.

Table 2. The molecular targets used in this study as well as the primer and probe sequences.

The molecular targets used in this study as well as the primer and probe sequences. The N, S, and ORF1a genes are located within SARS-CoV-2 genome. The BCoV target is for bovine coronavirus, a process control spiked into the sample during processing. PMMoV is for the internal endogenous control which is naturally present in high concentrations in the samples. Additional details of these assays can be found in Huisman et al. [26] .

Target	Primer/Probe	Sequence
N Gene	Forward	CATTACGTTTGGTGGACCCT
	Reverse	CCTTGCCATGTTGAGTGAGA
	Probe	CGCGATCAAAACAACGTCGG (5' FAM/ZEN/3' IBFQ)
S Gene	Forward	CAGACTAATTCTCCTCGGCG
	Reverse	TGCACCAAGTGACATAGTGT
	Probe	AGCTAGTCAATCCATCATTGCCT (5' HEX/ZEN/3' IBFQ)
ORF1a	Forward	CAGAACTGGAACCACCTTGT
	Reverse	TACAGTTGAATTGGCAGGCA
	Probe	TGCCACAGTACGTCTACAAGC (5' FAM or HEX/ZEN/3' IBFQ)
BCoV	Forward	CTGGAAGTTGGTGGAGTT
	Reverse	ATTATCGGCCTAACATACATC
	Probe	CCTTCATATCTATACACATCAAGTTGTT (5' FAM/ZEN/3' IBFQ)
PMMoV	Forward	GAGTGGTTTGACCTTAACGTTTGA
	Reverse	TTGTCGGTTGCAATGCAAGT
	Probe	CCTACCGAAGCAAATG (5' HEX/ZEN/3' IBFQ)

Table 3. Results for comparisons between experimental treatments and controls.

The percent difference (% diff) in SARS-CoV-2 RNA measured in treatments versus their control is shown then the difference was significantly different. "N" indicates that measurements or ratios were not different. The value after the ± is the standard deviation propagated from the measurements used to make the calculation. A positive percent difference indicates the treatment was lower than the control, a negative percent difference indicates the treatment was higher in the control. See methods for more details on the calculations.

Sample	Treatment	% diff N	% diff S	% diff ORF1a	% diff PMMoV	% diff N/PMMoV	% diff S/PMMoV	% diff ORF1a/PMMoV
SJ200080	4°C/8 d	N	N	N	N	N	N	N
D300230	4°C/7 d	N	N	N	N	N	N	N
SAC900313	4°C/7 d	N	N	N	N	N	N	N
OSP700097	4°C/7 d	60±48	80±49	73±67	N	N	N	N
SJ200128	4°C/35 d	49±22	49±19	59±19	45±15	N	N	N
D300243	4°C/35 d	42±23	52±31	55±22	N	40±41	50±48	53±49
SJ200023	4°C/122 d	55±16	58±12	63±14	29±10	36±27	41±23	48±26
D300066	4°C/91 d	N	N	N	N	N	N	N
SJ200082	-20°C/3 d	58±21	57±18	46±28	N	65±32	64±29	54±38
D300234	-20°C/2 d	N	-100±56	-170±35	N	N	N	N
SAC900313	-20°C/2 d	-86±28	-71±38	N	N	N	N	N
OSP700126	-20°C/2 d	N	N	N	N	N	N	N
SJ200128	-80°C/35 d	76±21	62±17	79±18	79±18	N	N	N
D300243	-80°C/35 d	40±21	47±27	59±19	21±13	N	33±40	48±33
SJ200023	-80°C/122 d	89±13	86±9	89±10	74±13	57±32	48±31	59±29
D300066	-80°C/91 d	75±38	70±42	78±51	49±34	N	N	N

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