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1 # Sewage, Salt, Silica and SARS-CoV-2 (4S): An economical kit-free method for direct
2 # capture of SARS-CoV-2 RNA from wastewater.

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14 **ABSTRACT:**

15 Wastewater-based epidemiology is an emerging tool to monitor COVID-19 infection
16 levels by measuring the concentration of severe acute respiratory syndrome coronavirus 2
17 (SARS-CoV-2) RNA in wastewater. There remains a need to improve wastewater RNA
18 extraction methods' sensitivity, speed, and reduce reliance on often expensive commercial
19 reagents to make wastewater-based epidemiology more accessible. We present a kit-free
20 wastewater RNA extraction method, titled "Sewage, Salt, Silica and SARS-CoV-2" (4S), that
21 employs the abundant and affordable reagents sodium chloride (NaCl), ethanol and silica RNA
22 capture matrices to recover 6-fold more SARS-CoV-2 RNA from wastewater than an existing

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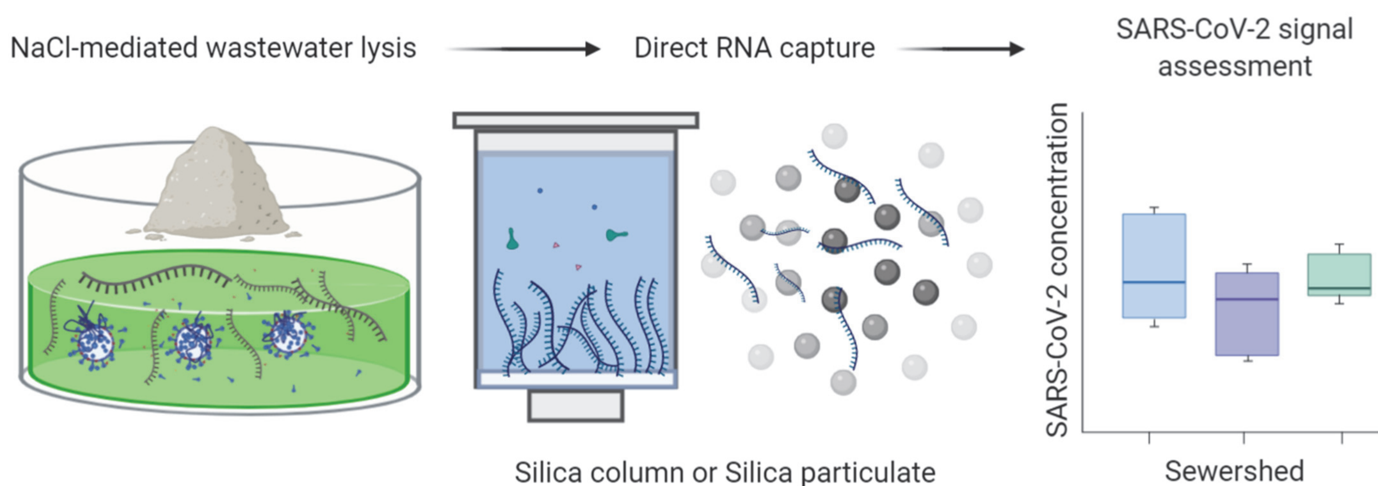
23 ultrafiltration-based method. The 4S method concurrently recovered pepper mild mottle virus
24 (PMMoV) and human 18S ribosomal subunit rRNA, both suitable as fecal concentration
25 controls. The SARS-CoV-2 RNA concentrations measured in three sewersheds corresponded to
26 the relative prevalence of COVID-19 infection determined via clinical testing. Lastly, controlled
27 experiments indicate that the 4S method prevented RNA degradation during storage of
28 wastewater samples, was compatible with heat pasteurization, and could be performed in
29 approximately 3 hours. Overall, the 4S method is promising for effective, economical, and
30 accessible wastewater-based epidemiology for SARS-CoV-2, providing another tool to fight the
31 global pandemic.

32 **KEYWORDS:** Wastewater-based epidemiology, SARS-CoV-2, COVID-19, Wastewater RNA
33 extraction, direct extraction, ultrafiltration, heat pasteurization

34 **SYNOPSIS:** The 4S method for measuring SARS-CoV-2 in wastewater is promising for
35 effective, economical, and accessible wastewater-based epidemiology.

36 **ABSTRACT ART:**



37 **INTRODUCTION:**

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38 Wastewater-based epidemiology (WBE) enables the indirect assessment of viral infection
39 prevalence in populations.[1–3] The quantity of viral nucleic acids shed into wastewater by infected
40 individuals, whether symptomatic or not, serves as a proxy for the relative prevalence of
41 infection.[1] WBE can provide population-level infection information for up to many thousands of
42 individuals in a community to complement individual-level testing and aid public health decision
43 making.[4]

44 WBE is now being applied to monitor and even predict population-level coronavirus disease
45 2019 (COVID-19) outbreaks.[1,5] Local COVID-19 prevalence is difficult to assess due to
46 insufficient individual testing capacity, rendering effective response more challenging.[6]
47 Wastewater can provide insights into COVID-19 prevalence, as COVID-19 patients shed SARS-
48 CoV-2 RNA in their stool and thus into wastewater.[7,8] Emerging studies report wastewater
49 SARS-CoV-2 concentrations that correspond to reported clinical prevalence of COVID-19, with
50 potential for early detection of COVID-19 outbreaks and identification of newly emerging
51 SARS-CoV-2 variants.[9–12] To extract and quantify the concentration of SARS-CoV-2 RNA shed
52 into wastewater, researchers are using size- and charge-based concentration methods that
53 concentrate intact SARS-CoV-2 virus prior to RNA extraction.[13–15] These methods employ a
54 primary concentration step via sieving by particle size, enmeshment of viral particles in
55 precipitates that can be separated by mass, or adsorption via electrostatic interactions, prior to
56 RNA extraction.[15] These methods are relatively time-consuming and inaccessible as they are
57 dependent on reliable supply of commercial reagents, a paucity of which has already hampered
58 clinical SARS-CoV-2 testing efforts.[13–16] Further, the use of primary concentration assumes the
59 recovery of intact virus, and is therefore not geared towards co-capturing RNA from SARS-
60 CoV-2 viruses that have already lysed or capture of non-viral RNAs suitable as fecal

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61 concentration controls.[14] Lastly, current CDC safety guidelines recommend BSL-3 precautions
62 when employing environmental sampling procedures that concentrate viruses presumed to be
63 intact.[17] To mitigate concerns of concentrating potentially infectious virus, heat-based
64 wastewater sample pasteurization and subsequent extraction could allow for easier and safer
65 wastewater processing after collection.

66 We aimed to develop an economical, kit-free method for the direct capture (extraction) of
67 SARS-CoV-2 RNA from wastewater. The method employs lysis of biological particles via
68 sodium chloride (NaCl), heat-based pasteurization, coarse filtration, ethanol precipitation, and
69 RNA capture via silica-based columns (4S-column) or silicon dioxide slurry (4S-Milk-of-Silica).
70 This approach allows recovery of wastewater RNA without mass, size, or charge bias and the co-
71 capture of RNA from lysed SARS-CoV-2 virus alongside RNA from other biological particles in
72 wastewater that are suitable as fecal concentration controls, such as pepper mild mottle virus
73 (present in dietary peppers and shed in feces) and human 18S ribosomal RNA.[18] The 4S method
74 stabilizes the nucleic acids in wastewater via the addition of sodium chloride (NaCl) and
75 ethylenediaminetetraacetic acid (EDTA), and is compatible with heat pasteurization, which
76 makes wastewater samples safer to process. The 4S method's omission of a primary
77 concentration step and kit-free extraction enables lower reliance on commercial reagents and
78 speeds up RNA purification to enable same-day measurement of SARS-CoV-2 wastewater
79 abundance.

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83 ## MATERIALS AND METHODS:

84 ### Sample collection

85 For this study, we obtained composite 24-hour wastewater influent samples from East Bay
86 Municipal Utility District's wastewater treatment plant. These samples represent three discrete
87 sampling areas: North and west Berkeley, El Cerrito, Kensington and Albany (sub-sewershed
88 "N"), Oakland/Piedmont (sub-sewershed "S"), and Berkeley/Oakland Hills (sub-sewershed "A")
89 (interceptor coverage detailed in Fig. S2A). Samples were kept at 4°C on ice during transport
90 and processed within 24 hours or kept at -80°C and processed within two weeks.

91 ### Wastewater RNA extraction

92 Wastewater RNA extraction via the 4S-column and 4S-Milk-of-Silica methods is detailed in
93 depth at https://www.protocols.io/view/v-4-direct-wastewater-rna-capture-and-purification-
94 bpdfmi3n and dx.doi.org/10.17504/protocols.io.biwfkfbn.[28,29]. In brief, for 4S RNA extraction
95 using a silica column, samples were lysed via the addition of Sodium Chloride (NaCl) to a final
96 concentration of 4 M and EDTA to a final concentration of 1 mM and buffered via the addition
97 of 10 mM pH 7.2 tris(hydroxymethyl)aminomethane. Samples were heat inactivated in a water
98 bath (unless indicated otherwise) at 70°C for 45 minutes, filtered using a 5-µM DuraPore PVDF
99 filter membrane (Millipore Sigma) and syringe filter. Ethanol was added to sample filtrate to a
100 final concentration of 35%. Samples were passed through Zymo-IIIP silica columns (Zymo
101 Research) using a vacuum manifold. For all experiments other than the wash buffer tests (Figure
102 4, Supplemental Figure S4), samples were washed with 25 mL of high NaCl (1.5M) and ethanol
103 (20%) containing wash buffer #1 (4S-WB1), and 50 mL of low NaCl (100mM) and ethanol
104 (80%) containing wash buffer #2 (4S-WB2). Washed RNA was eluted from silica columns using

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105 200 µL of ZymoPURE elution buffer (Zymo Research) or pH 8 Tris-EDTA buffer pre-heated to
106 50°C.

107 For 4S-Milk-of-Silica extraction, samples were lysed, heat inactivated and filtered as in the 4S-
108 column extraction. Next, a 1 g/mL silicon dioxide slurry in water was added to the filtered lysate
109 and incubated at room temperature for 10 minutes. The lysate and silica slurry were centrifuged
110 at 4000 x g for 5 minutes, pelleting wastewater RNA bound to silica particulate. The lysate
111 supernatant was decanted, and the silica pellet was washed with 40 mL 4S-WB1 and 40 mL of
112 4S-WB2 via centrifugation and wash buffer decanting. The washed silica pellet was resuspended
113 in 20 mL of pure water pre-heated to 37°C to elute bound RNA. Next, the silicon dioxide
114 particulate was pelleted via centrifugation and the eluted RNA was separated and concentrated
115 via isopropanol precipitation, as previously described.[30,29] 4S-column and 4S-Milk-of-Silica
116 reagent costs are listed in Supplementary Table 6.

117 For sample RNA concentration via ultrafiltration, Amicon 100-kDa ultrafilters (Millipore Sigma)
118 were pretreated to block virus adsorption using 2 mL bovine serum albumen 1% (w/v) in 1x PBS
119 and then washed with PBS. Wastewater samples were divided into 40 mL aliquots and solids
120 were removed via slow centrifugation with a swinging bucket rotor at 4700 x g for 30 min.
121 Supernatant was decanted and passed through a 0.2 µm flat membrane filter (Steriflip, EMD
122 Millipore). Filtrate was loaded onto the ultrafilter in increments of up to 15 mL and ultrafilters
123 were spun for 10 minutes at 4700 xg for each increment. Flow-through was discarded and
124 samples were concentrated until they were reduced to a final volume of ~250 µl. RNA was
125 extracted from the ultrafiltration concentrate using an AllPrep DNA/RNA Mini kit (QIAGEN)
126 following manufacturer instructions.

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127 **RNA detection and quantification via RT-qPCR**

128 This study employed four primer/probe sets: the SARS-CoV-2 N1 assay, Pepper mottle mild
129 virus (PMMoV) coat protein gene assay, bovine coronavirus transmembrane protein gene assay
130 and a newly developed human 18S ribosomal rRNA assay. (Supporting information, Table S3.)
131 RT-qPCR reaction conditions are detailed in Table S1, assay thermocycling conditions are
132 detailed in Table S2, and primer sequence information is in Table S3. RT-qPCR assay
133 performance is detailed in Table S4 (Validation) and Table S5 (Limit of detection). RT-qPCR
134 minimum information for publication of quantitative real-time PCR experiments (MIQE)
135 documentation is detailed in Table S7. RT-qPCR analysis is detailed in the supporting
136 information.

137 **RESULTS AND DISCUSSION:**

138 Many current methods of wastewater viral RNA extraction assume that most viral
139 particles within wastewater are intact and that the concentration of these intact viruses prior to
140 extraction is necessary to achieve sensitive detection of SARS-CoV-2 in wastewater. Given this
141 assumption, these methods typically employ precipitation-, charge-, or size-based viral
142 concentration and subsequent RNA extraction of unpasteurized wastewater to preserve viruses in
143 an intact state.[14,15] Despite concentration, some methods were shown to recover as little as 0-1%
144 of SARS-CoV-1 from wastewater during the SARS-CoV-1 epidemic.[19] We hypothesized that
145 direct extraction could avoid loss of virus during the primary concentration step, and we
146 therefore designed the 4S (Sewage, Salt, Silica and SARS-CoV-2) method to lyse viruses and
147 microorganisms present in wastewater using sodium chloride and subsequently capture the free
148 RNA using a silica RNA binding matrix.

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149 To benchmark the performance of the 4S method, we analyzed a 24-hour composite
150 wastewater sample treated with and without heat pasteurization and compared the recovery of
151 endogenous SARS-CoV-2 to that of an ultrafiltration-based method. In addition, we compared
152 the recovery of indigenous pepper mild mottle virus (PMMoV) RNA, which may be useful to
153 control for variable fecal concentrations in wastewater, and a spiked-in bovine coronavirus
154 vaccine (bCoV), used as an RNA extraction process control (Figure 1). We observed that the
155 4S-column method recovered 6-fold more SARS-CoV-2 RNA than ultrafiltration (Figure 1).

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Figure 1. Comparison of SARS-CoV-2, PMMoV & bCoV spike-in assay signal in gene copies per liter between the 4S-column method with and without heat inactivation, and ultrafiltration. "n" represents the number of wastewater RNA extraction replicates per condition. Bars are plotted at the geometric mean of biological triplicates and error bars represent the variation associated with biological triplicates as quantified by the geometric standard deviation of the biological triplicates.

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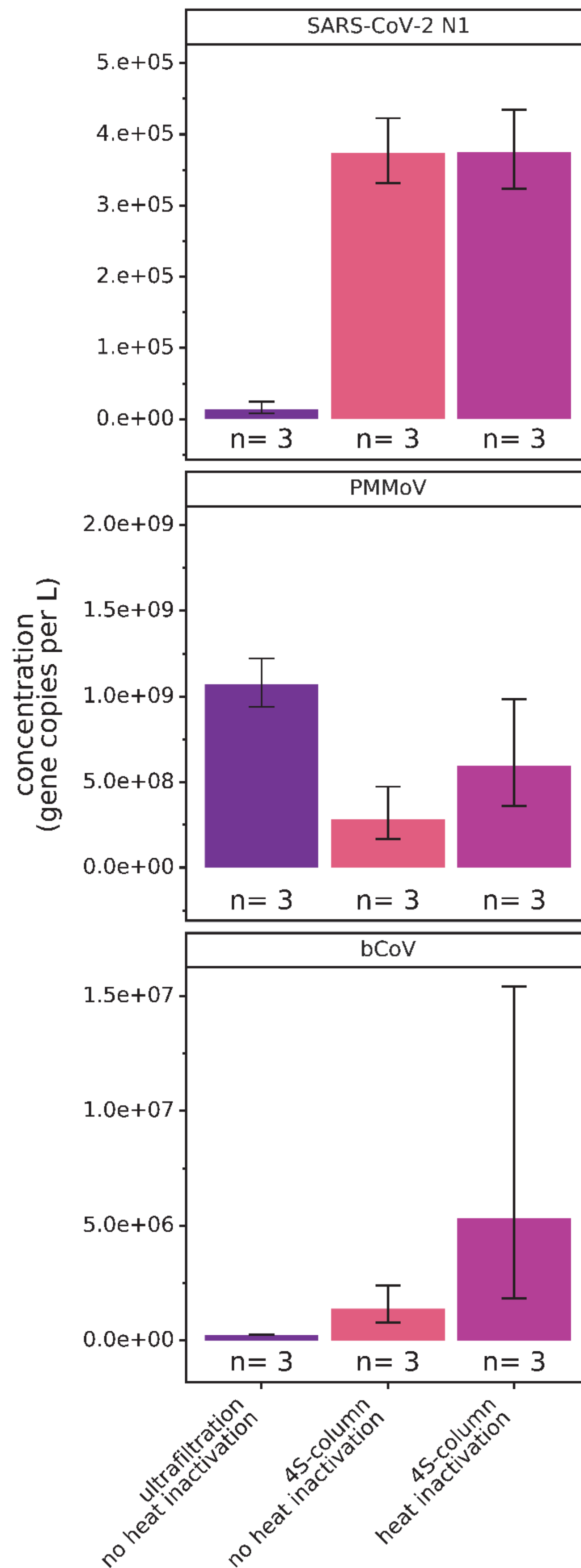

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157 Surprisingly, SARS-CoV-2 recovery by the 4S method was not impacted by heat
158 pasteurization, suggesting that further SARS-CoV-2 virus lysis did not occur. This result may
159 imply that a large fraction of SARS-CoV-2 RNA was not bound to virus particles; this unbound
160 RNA was captured by the 4S method but was not efficiently concentrated by ultrafiltration.

161 The 4S-column method without heat pasteurization also recovered 6-fold more bCoV
162 than ultrafiltration, and 28-fold more bCoV with heat pasteurization. In this case, heat
163 pasteurization may promote additional lysis of encapsidated bCoV, releasing its RNA for
164 subsequent capture. Recovery of PMMoV by 4S was also higher with heat pasteurization (2-fold
165 increase in recovery), but ultrafiltration was more effective in enriching PMMoV (1.6-fold
166 higher than using the 4S-column method with heat pasteurization). Here, ultrafiltration may be
167 effective in concentrating intact virus that is able to persist in wastewater, which is consistent
168 with previous reports on PMMoV.[18,20] Collectively, these results suggest that a significant
169 fraction of SARS-CoV-2 RNA in the analyzed wastewater was not bound to viral particles but
170 was present as free or ribonucleoprotein-bound RNA. This possibility is consistent with reports
171 indicating reduced viability of SARS-CoV-2 and related coronaviruses spiked into
172 wastewater.[13,21]

173 Given that the 4S method is designed to lyse and extract wastewater RNAs without
174 requiring the enrichment of viral particles, we also investigated whether the 4S method could
175 recover human RNAs present in wastewater. Using the 4S method, we were able to recover and
176 detect human ribosomal subunit RNA (18S rRNA) in wastewater influent (Supplementary Figure
177 S1A). 18S rRNA recovery was enhanced 2.5-fold by heat pasteurization, suggesting the lysis of
178 human cells or 18S rRNA bound to ribonucleoprotein complexes present in wastewater
179 (Supplementary Figure S1A). Therefore, the 4S method enabled the recovery and detection of

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180 human RNA, another potential indicator of wastewater fecal concentration, which could allow
181 direct normalization of SARS-CoV-2 RNA quantity to human RNA content of wastewater. As
182 heat pasteurization did not affect 4S recovery of SARS-CoV-2 and improved the recovery of
183 PMMoV, bCoV and 18S rRNA, we recommend integrating this pathogen inactivation step to
184 increase the safety of processing wastewater samples.

185 We sought to adapt the 4S strategy to employ silica powder for RNA capture rather than
186 silica columns to circumvent reliance on commercially manufactured silica columns. In this
187 approach, we added a slurry of silicon dioxide particles to lysed wastewater and used
188 centrifugation to separate particle-bound RNA from the wastewater matrix, an approach we
189 named "4S-Milk-of-Silica". We observed that the 4S-Milk-of-Silica method recovered
190 equivalent SARS-CoV-2 and PMMoV signal to the 4S method using a silica column
191 (Supplementary Figure S1B). Thus, the 4S-Milk-of-Silica method presents an even more cost-
192 effective (~$8 per sample, vs. ~$13 per sample, using the 4S-column extraction method,
193 Supplementary Table 6) and accessible method to extract wastewater RNA without reliance on
194 commercially manufactured silica columns and a vacuum manifold. However, the "Milk of
195 Silica" version of the 4S protocol requires an isopropanol precipitation RNA concentration step,
196 lengthening the protocol time. Therefore, we recommend using the 4S-column method to enable
197 faster sample processing, while "4S-Milk-of-Silica" presents an alternate protocol for use in
198 resource-limited settings.

199 WBE can provide an assessment of different areas' relative COVID-19 infection
200 prevalence, so we assessed whether the 4S-column extraction method could detect differential
201 SARS-CoV-2 RNA levels in wastewaters derived from different subsections of a collection
202 system. We surveyed three wastewater influent interceptors serving North and West Berkeley

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203 and El Cerrito (N), East Berkeley/Berkeley Hills (A) and Oakland (S) (Interceptor area coverage
204 shown in Supplementary Figure S2A). These interceptors served areas exhibiting differential
205 incidence of clinically confirmed COVID-19 cases, ranging from three (A interceptor) to 68 (S
206 interceptor) reported cases per day within the week of our sampling (Figure S2A). To compare
207 clinical COVID-19 case data and wastewater SARS-CoV-2 concentration, we normalized the
208 case data by population, and we normalized the SARS-CoV-2 quantity by PMMoV abundance,
209 to control for fecal concentration in the wastewater. Raw SARS-CoV2 and PMMoV abundance
210 is available in Supplementary Figure 2B. As expected, the normalized wastewater SARS-CoV-2
211 signals trended with the per capita clinical cases per day in the three sub-sewersheds (Figure 2,
212 B). The normalized SARS-CoV-2 RNA concentration was highest in wastewater representing
213 the S interceptor area, where the highest daily per capita new cases also occurred. Normalized
214 SARS-CoV-2 RNA concentrations in wastewaters representing the N interceptor area were only
215 2.3-fold lower than those of S interceptor wastewaters, despite 11.6-fold fewer per capita daily
216 cases being reported in the A interceptor area during the week of our sample collection. One
217 possible reason for this difference could be the presence of undiagnosed infections in the N
218 interceptor service area, in which case wastewater SARS-CoV-2 RNA concentrations may
219 provide a more accurate view of the relative COVID-19 infection prevalence in the week prior to
220 sampling. Alternatively, the variability associated with wastewater measurements may be too
221 large to detect differences of this magnitude.[9,13] Ongoing research seeks to better quantify the
222 measurement variability in wastewater samples over temporal and spatial scales. We emphasize
223 that SARS-CoV-2 RNA levels were quantifiable in the A sub-sewershed despite only 18 cases
224 being reported in an estimated population of 90,000 during the weeklong period of our sampling.

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225 This result implies that the 4S method is highly sensitive and can be used to monitor areas with
226 low COVID-19 prevalence.

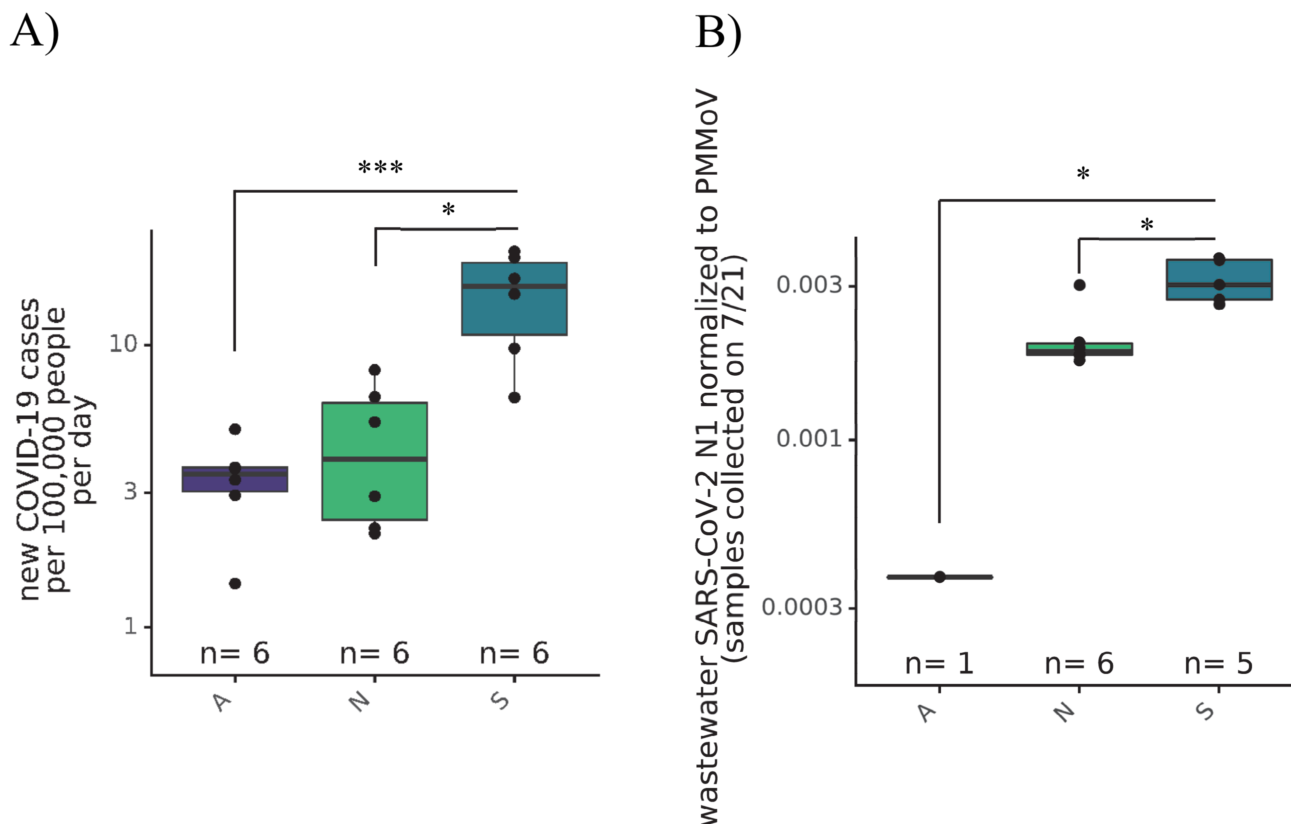

Figure 2. A) New COVID-19 cases per day per 100,000 population in three areas served by the distinct A, N and S wastewater interceptors over 6 days from 7/15 to 7/21. B) Comparison of SARS-CoV-2 N1 assay represented as SARS-CoV-2 gene copies per liter normalized to PMMoV gene copies per liter between interceptors serving the A, N and S East Bay areas. Kruskal-Wallis test followed by Dunn's test was performed to determine significance, where *= $p<0.05$ and ***= $p<0.001$.

227 Wastewater contains many contaminants with the potential to degrade nucleic acids, and
228 it has been previously observed that SARS-CoV-2 RNA in wastewater is degraded during
229 storage.[22–24] Viral detection relying on wastewater RNA extraction methods that concentrate
230 intact viruses may be strongly affected by variable amounts of virus and viral RNA degradation
231 in wastewater. Therefore, we sought to assess whether EDTA and sodium chloride, added to
232 wastewater in the 4S method to promote lysis, could dually act to preserve RNA in wastewater.

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233 Upon receipt of each wastewater sample, we added NaCl to a final concentration of 4 M, added
234 EDTA to a final concentration of 1 mM, and stored the samples either at 4°C for a month, or
235 three days at room temperature (20°C). We observed that salt and EDTA addition prior to
236 storage improved SARS-CoV-2 N1 assay signal after storage at both 4°C for one month (2.6-
237 fold higher signal when stored with salt and EDTA) or at 20°C for three days (22-fold higher
238 signal when stored with salt and EDTA) (Figure 3). Interestingly, the PMMoV assay signal
239 remained similar throughout storage with or without salt, implying that PMMoV remains
240 resistant to RNAses in the wastewater matrix. This observation corroborates previous reports
241 indicating the persistence of PMMoV in wastewater.[18,20] As with SARS-CoV-2 N1 signal, we
242 observed that salt and EDTA addition preserved human 18S rRNA signal at 4°C for one month
243 (126-fold higher) or at 20°C for three days (56-fold higher) (Figure 3). These results again
244 support the conclusion that much of the SARS-CoV-2 in wastewater is not bound by intact
245 capsid, rendering it more susceptible to degradation, unlike PMMoV which may remain
246 encapsidated to protect it from degradation. Overall, the lysis salts added to wastewater as part of
247 the normal 4S method workflow conveniently preserved wastewater RNAs and may mitigate
248 degradation-mediated variation in SARS-CoV-2 and fecal concentration controls caused by RNA
249 degradation during shipping and storage.

250 Given the impact of RNA degradation on SARS-CoV-2 N1 assay signal, we investigated
251 whether bulk RNA yield, representing intact wastewater RNA, could be employed as a
252 normalization measure for SARS-CoV-2 detection. Surprisingly, bulk RNA yield per mL of
253 wastewater input correlated poorly with SARS-CoV-2 and PMMoV detection (Supplementary
254 Figure 3A). These results imply that most bulk wastewater RNA may be contributed by
255 wastewater microorganisms unrelated to human fecal content or viral content, thus weakly

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256 correlating to SARS-CoV-2 N1 assay signal. We also observed that extracting nucleic acids from
257 increasing volumes of wastewater (up to 400 mL) did not strongly increase total RNA yield per
258 extraction past 100 mL of wastewater sample input, implying potential saturation of the RNA
259 capture matrix (Supplementary Figure S3B). From these experiments, we conclude that the RT-
260 qPCR detection of human fecal concentration indicators such as PMMoV and human 18S rRNA,
261 the latter of which is preserved during storage similarly to SARS-CoV-2, are better estimators of
262 wastewater fecal concentration than bulk RNA quantity measurements. Lastly, we observed that
263 the 4S method enriched up to 8 µg of DNA per 100 mL of wastewater, suggesting that the 4S
264 method could be employed for future wastewater surveillance of DNA-based pathogens and NA-
265 sequencing based wastewater surveys (Supplementary Figure S3C).

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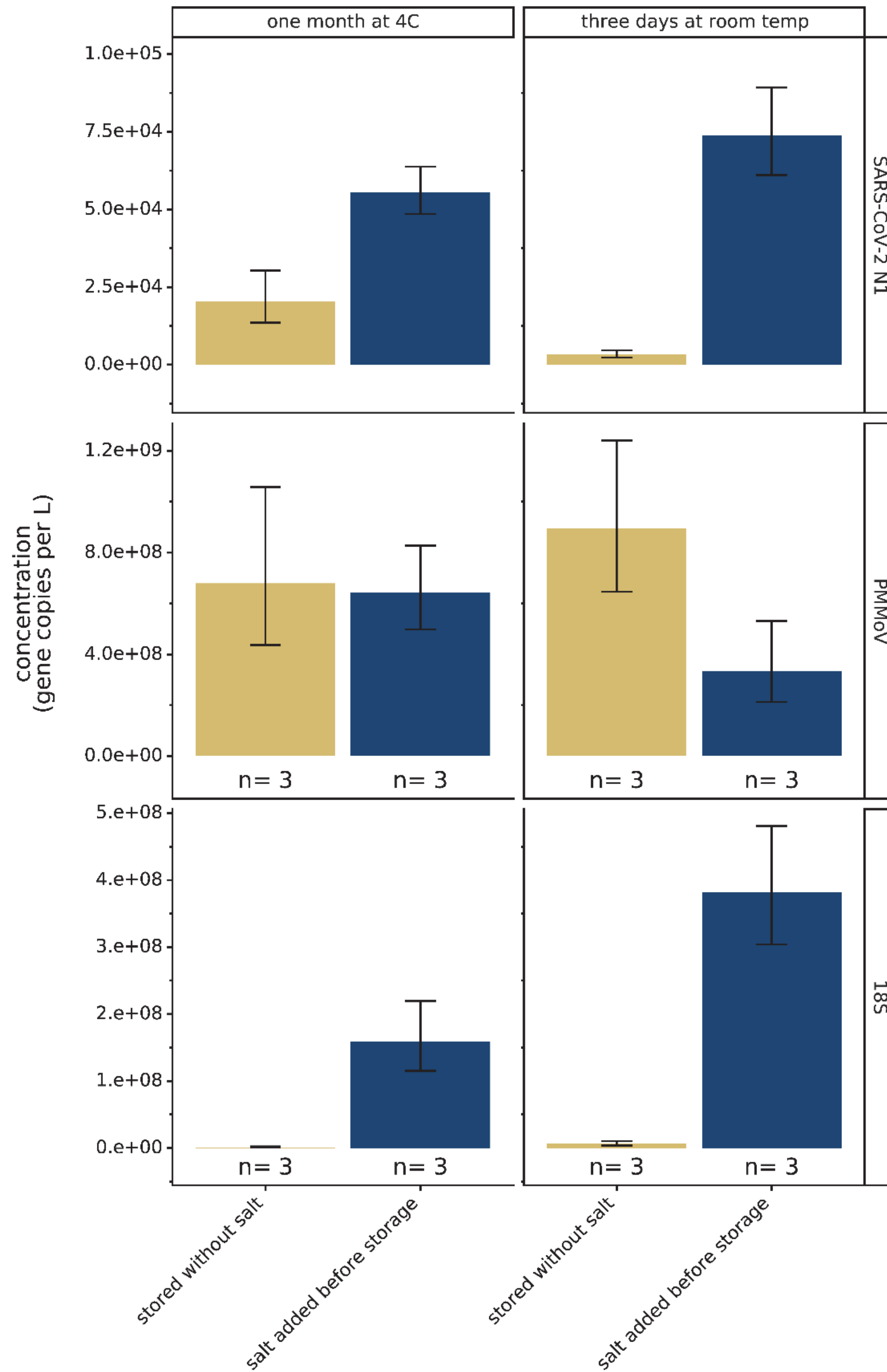

Figure 3. Effect of lysis salt addition prior to wastewater storage on SARS-CoV-2 N1, PMMoV and 18S rRNA assay signal. "n" represents the number of storage and extraction replicates per condition. Bars are plotted at the geometric mean of biological triplicates and error bars represent the variation associated with biological triplicates as quantified by the geometric standard deviation of the biological triplicates.

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267 Wastewater samples contain many contaminants that have previously been reported to
268 inhibit RT-qPCR reactions.[25] Therefore, we sought to assess whether the 4S method could
269 generate purified RNA free of RT-qPCR contaminants by employing the "spike and dilute"
270 method to assess PCR inhibition.[26] Here, we spiked purified wastewater RNA with synthetic
271 RNA standard and sequentially diluted the sample and observed whether SARS-CoV-2 N1,
272 PMMoV, and bCoV detection followed corresponding sample dilutions, indicating an absence of
273 inhibition. We assessed the impact of a range (1-50 mL) of wash buffer volumes during RNA
274 extraction on PCR inhibition and SARS-CoV-2 N1, PMMoV, and bCoV assay signal to identify

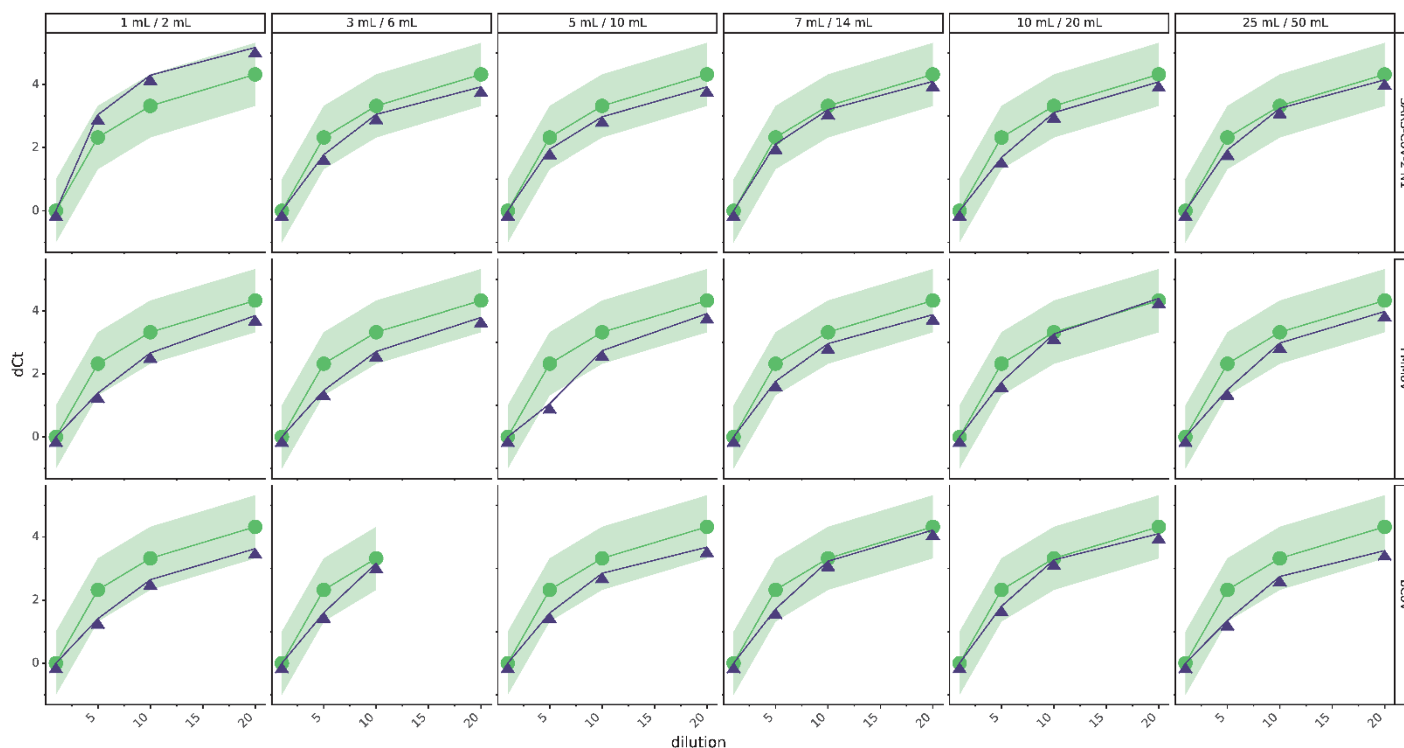

Figure 4. Assessment of RT-qPCR assay inhibition of the SARS-CoV-2 N1, PMMoV and bCoV assays via the "spike and dilute" method for different volumes of 4S-Wash buffer #1 and 4S-Wash buffer #2 (volumes reported at top of each panel). Sample dilutions shown are 1x, 5x, 10x, and 20x. Green line with circular points represents theoretically expected increase in Ct due to sample dilution, blue line with triangular points indicates actual increase in Ct with sample dilution. The green band indicates +/- 1 Ct tolerance range around the expected Ct values, due to variability. An increase in measured Ct that is lower than the expected increase was interpreted as inhibition. RNA sample dilution factor is indicated on x-axis.

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275 the optimal wash buffer volume for RNA purity and recovery. There was no evidence of
276 inhibition for the SARS-CoV-2 N1 assay using the 4S procedure with any wash buffer volume,
277 and slight inhibition of the PMMoV assay when using 5 mL of 4S-Wash buffer #1 (4S-WB1)
278 and 10 mL of 4S-Wash buffer #2 (4S-WB2) (Figure 4). To limit ethanol waste generation, we
279 therefore recommend using at least 7 mL of 4S-WB1 and 14 mL of 4S-WB2 to yield inhibitor-
280 free RNA.

281 Next, we assessed potential assay signal loss due to excess washing of the silica columns.
282 Here, we observed highest SARS-CoV-2, PMMoV and bCoV assay signal using 3 mL of 4S-
283 WB1 and 6mL of Wash 4S-WB2, with minimal losses in signal up until 25 mL of 4S-WB1 and
284 50 mL of 4S-WB2 (Supplementary Figure S4). Using too little wash buffer may not sufficiently
285 wash away lysis salts and contaminants from the silica matrix, reducing RNA recovery and
286 increasing inhibition, whereas too much wash buffer may partially elute bound RNA, decreasing
287 RNA yield. Therefore, we recommend using 7-10 mL of 4S-WB1 and 14-20 mL of 4S-WB2 to
288 extract PCR inhibitor-free RNA while maximizing target RNA recovery.

289 The results presented here are representative of only three wastewater sources which may
290 differ in composition from wastewater collected at other times and from other locations.
291 Different wastewaters may contain different types and quantities of PCR inhibitors, so we
292 recommend assessing PCR inhibition in all sample types, and if necessary, adjusting the wash
293 buffer volumes to effectively remove inhibitors from the purified RNA. Different wastewater
294 samples may also contain varying biological and chemical species influencing RNA stability,
295 potentially impacting the RNA preservation documented here by the 4S method. Furthermore,
296 the 4S method may be less effective in capturing the nucleic acids from wastewater viruses or
297 other microorganisms resistant to the sodium chloride and heat-based lysis evaluated here.

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298 Overall, we demonstrate that the 4S method enabled efficient extraction of SARS-CoV-2,
299 PMMoV, bCoV, and human 18S rRNA. Combined with RT-qPCR, the 4S method allowed
300 monitoring of relative COVID-19 infection prevalence with high sensitivity. These results are
301 consistent with those of a recent inter-laboratory comparison of 36 different wastewater SARS-
302 CoV-2 RNA detection methods. In this comparison, the concentration of SARS-CoV-2
303 measured with the 4S method, identified as "1S.2H", was one of the highest reported (direct
304 measurement, without correcting for recovery efficiency) and the recovery efficiency of a
305 spiked-in OC43 virus efficiency control was the highest reported, among all methods.[27] The 4S
306 method also preserved RNA in wastewater, was compatible with heat pasteurization, and yielded
307 purified RNA free of PCR inhibitors. Given the high efficiency, low cost, and same-day
308 assessment of wastewater SARS-CoV-2 and fecal concentration controls, the 4S method presents
309 an affordable and accessible method for implementing wastewater-based epidemiology for
310 SARS-CoV-2. The method also appears promising for the application of WBE for other RNA-
311 and DNA-based pathogens and facilitating research on the wastewater microbial community
312 more broadly.

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324

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